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M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof PREFACE In the curricular structure introduced by this University for students of Post-Graduate degree programme, the opportunity to pursue Post-Graduate course in subject introduced by this University is equally available to all learners. Instead of being guided by any presump- tion about ability level, it would perhaps stand to reason if receptivity of a learner is judged in the course of the learning process. That would be entirely in keeping with the objectives of open education which does not believe in artificial differentiation. Keeping this in view, study materials of the Post-Graduate level in different subjects are being prepared on the basis of a well laid-out syllabus. The course structure combines the best elements in the approved syllabi of Central and State Universities in respective subjects. It has been so designed as to be upgradable with the addition of new information as well as results of fresh thinking and analysis. The accepted methodology of distance education has been followed in the preparation of these study materials. Co-operation in every form of experienced scholars is indispensable for a work of this kind. We, therefore, owe an enormous debt of gratitude to everyone whose tireless efforts went into the writing, editing and devising of a proper lay-out of the mate- rials. Practically speaking, their role amounts to an involvement in invisible teaching. For, whoever makes use of these study materials would virtually derive the benefit of learning under their collective care without each being seen by the other. The more a learner would seriously pursue these study materials, the easier it will be for him or her to reach out to larger horizons of a subject. Care has also been taken to make the language lucid and presentation attractive so that they may be rated as quality self- learning materials. If anything remains still obscure or difficult to follow, arrangements are there to come to terms with them through the counselling sessions regularly available at the network of study centres set up by the University. Needless to add, a great deal of these efforts were still experimental—in fact, pioneer- ing in certain areas. Naturally, there is every possibility of some lapse or deficiency here and there. However, these do admit of rectification and further improvement in due course. On the whole, therefore, these study materials are expected to evoke wider appreciation, the more they receive serious attention of all concerned. Professor (Dr.) Manimala Das Vice-Chancellor
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M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof NETAJI SUBHAS OPEN UNIVERSITY Group – A (I) Animal Physiology Unit 1 ? Aims and scope of comparative physiology 1–15 Unit 2 ? Thermoregulation 16–30 Unit 3 ? Communication among animals 31–49 Unit 4 ? Contractile elements, cells and tissues among different phylogenic group 50–74 Unit 5 ? Adaptation 75–83 Unit 6 ? Physiological adaptations to different environments 84–101 PGZO - 3 Animal Physiology, Bio-chemistry, Cytogenetics and Molecular Biology
1 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Unit 1 ? Aims and scope of comparative physiology Structure 1.1 General physiological functions and principles 1.2 Validity of comparative approach 1.3 Organisms and cell physiology 1.4 Suggested questions 1.1 General physiological functions and principles The science of physiology is the analysis of function in living organisms. Physiology is a synthesizing science which applies physical and chemical methods to biology. The term physiology originated from the greek word 'Physiologikos' which means 'discourse on natural knowledge.' French physician Jean Fernel introduced the term in 1552. Physiology is thus a branch of science that deals with normal functions of the body. In the science of physiology, there is nothing supernatural about life. All the living processes of an organism can be explained on the basis of physico-chemical changes and structural pecularity. The progress of ancillary subjects like biology, microscopic anatomy, physics, chemistry, have been very useful in understanding the working processes of the living organisms. All physiological processes are governed by basic laws of physics and chemistry. Accordingly to the changes in the environment, functional alterations also occur and thus the survival is made possible. Hence, it is held that Physiology is a tripod science, its three legs being Anatomy, Physics and Chemistry. To know physiology, one has to study the subject with a sound basic knowledge of these three subjects. Blood flow through cardio-vascular system is governed by the 'Laws of fluid dynamics', exchange of fluids between different body compartments is regulated by hydrostatic pressure and osmotic pressure ; transfer of information signal involving transaction and development of action potential ; exchange of gases, and solutes obey the laws of diffusion. A study of all these physical phenomenon in the body comes under the head Biophysics. Biochemistry is the branch of science that explains the functions of the body on a chemical basis. All biochemical reactions including energetics are all governed by chemical laws and principles of thermodynamics. A study of structure of an animal comes under the study of Anatomy. The study of gross structure is called macroscopic anatomy, while the study of fine structures is called microscopic anatomy. Histology, cytology, histochemistry and cytochemistry all come under the study of microscopic anatomy. Medical statistics is a branch of science that deals with evaluation of experimental

2 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof data. These are used to confirm the authenticity of any observed change in the functions of the body. In short, the whole purpose of Physiology is to explore, with the help of modern techniques, the normal functioning of the living organisms—their principle, their mechanism and their control. A short summary of physiological study The structural and functional unit of a living body is a cell. A collection of similar cells having same origin and performing same function but held together by intercellular substance is called a tissue. There are unicellular and multicellular animals in the world. When a multicellular animal is analyzed in terms of cell, it is complicated and is seen to be made up of huge number of cells of various shape and size, and a large amount of intercellular substance. But if the animal body is analyzed in terms of tissue, it is found to be made up of basically four types of tissues like, epithelial, connective, muscular and nervous. The term 'organ' is used to denote a structure being made up of those four types of tissues and is responsible for a particular function. For example, liver is an organ made up of hepatic cells which are epithelial cells ; it has connective tissue fibres forming septa, walls of blood vessels contain smooth muscle cells i.e. muscular tissue ; it has nerve supply, so it possess nervous tissue. Liver has many functions including secretion, excretion and metabolism. Hepatectomised animal will not survive. Finally, many different organs join together to constitute a 'system' responsible for a particular function. For example, the excretory system, responsible for excretion is made up of organs like kidney, ureter, urinary bladder and urethra. A multicellular animal has many systems in it like skeletal, muscular, cardiovascular, respiratory, excretory, endocrine, reproductive, nervous etc. There is a question why so many systems are present in an animal body including humans. A critical analyses revealed that every-one of them is necessary for the living organism. The ultimate aim of any living organism is to survive in the world. For survival one has to do work. For doing work energy is necessary. Hence, every living organism must have a mechanism to yield energy for doing work. Food is the source of energy. The items of food are carbohydrate, lipid, protein (calorigenic food); vitamins, minerals and water. Of these, the catorigenic foods gives energy, hence the name. When these foods are oxidized energy is released. For example— C 6 H 12 O 6 6O 2 ? 6CO 2 + 6H 2 O + energy. thus, a molecule of glucose, when oxidized to CO 2 and H 2 O, energy is liberated. It is seen from the equation that, to achieve this, oxygen is supplied by respiratory system. So this system is required. Next about the food, monosacclaride, say glucose, is utilised by the cell ; but the carbohydrate food that we eat is not monosacharide. They are either polysacharide like starch or glycogen or disaccharide like sucrose or

3 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof lactose. Hence, in the body there should be a system to convert these poly- and di- saccharides to monosaccharides so that they can be utilized in the body for not only supplying energy but also for forming any carbohydrate like substances in the body. This conversion of complex food to simplest substances is called digestion, which is carried out by digestive system. Hence, this system is also necessary. Next comes the distribution of food. Digestion occurs in the digestive tract, the end products of digestion are produced in the small intestine. From here the end- products are to be transported to the cells all over the body. This requires a transport system. This is done by cardio-vascular system. Hence, the animals cannot go without this system. When the foods are utilized by the body by way of metabolism, many intermediate products are formed which are harmful to the body and need elimination to outside the body, e.g. urea. Thus a system has to be developed to cause excretion i.e. excretory system. To maintain a structure, a skeletal system is required ; for movement, mascular system is necessary. All these different systems must be properly regulated. For such regulation, endocrine system and nervous system have been developed. 1.2 Validity of comparative approach For practical purposes, physiology has been divided into three categories, like— i) Cellular physiology, (ii) Physiology of special groups, and (iii) Comparative physiology. An understanding of comparative animal physiology requires some background knowledge in general zoology, animal morphology, biochemistry and cellular physiology. Comparative physiology is the study of organ function in a wide range of groups of organisms. Comparative animal physiology integrates and co-ordinates functional relationships that occur in more than one group of animals. It is concerned with the ways in which diverse organisms perform similar functions. Study of the comparative physiology reveals that, generally dissimilar organisms may show striking similarities in functional characteristics as well as in responses to the same environmental stimulus. On the other hand, closely related animals frequently react differently to their surroundings. Light, temperature, O 2 tension and hormone balance are used or are considered as variables for each function. Comparative physiology uses in addition to these, species or animal type as a variable for each type of function. Comparative animal physiology use kind of animal as an experimental variable which is a unique kind of biological generalization. Comparative environmental and behavioral physiology constitutes a bridge between molecular and organismic biology.

4 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof The most important function of comparative physiology is to put man into perspective in biological history and phylogenetic relationships. To achieve the goal, the plan for the study of comparative physiology is made to examine the relation between components of the environment and the whole organism and to analyze the interactions in terms of organs and cell physiology. For the applied biologist, comparative physiology has practical application in describing the physiology of economically important animals. For the ecologists, it helps the understanding of restriction of plants and animals to particular habitats. For the medical physiologists, the comparative viewpoint places man in his proper biological perspective. For the general biologist, it provides meaning to natural variation, and general principles which can be reached only with kind of organism as a variable. 1.3 Organisms and cell physiology 1.3.1 Diffusion Particles, molecules and ions have a tendency to spread uniformly in the entire available space by their incessant random movements. This tendency to spread is called diffusion. Though they are moving at random in all directions, a greater number of particles move from a region of higher concentration to the area of lower concentration, than in reverse direction. Hence, there occur a net diffusion from higher to lower concentration and the two concentrations become equal. In this state equal number of particles move in all directions and the net diffusion is zero. Fick's First law of diffusion states that the rate of diffusion (flux) of a solute particle is directly proportional to the magnitude of concentration gradient and it occurs down the concentration gradient. Graham's law states that the diffusional flux (J) of a gas varies inversely with the square root of its density (P) and molecular weight (M). Diffusion coefficient (D) denotes the diffusibility of the particle or gas. In case of solid the 'D' is the mass of solute diffusing across 1 sq cm area in 1 sec, down a concentration gradient of unity. Diffusion coefficient of a gas is the volume of gas that diffuses across 1 sq. cm. area per second, down a partial pressure difference (?P) of unity. Diffusion is directly proportional to : (i) Concentration gradient / Pressure gradient / electrical gradient. (ii) Solubility in the medium (iii) Temperature of the medium (iv) Cross-sectional area through which diffusion is taking place.
5 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Diffusion is inversely proportional to : (i) Distance to be travelled / thickness of the membrane (ii) Diameter of the diffusing particle (iii) Viscosity of the medium (iv) Shape of the particle —spherical particle diffuse easily than elongated particle. (v) Charge of the particle and charge of the pore. If the charges are same, diffusion will be less, if the charges are opposite, diffusion will be more. Biological application 1. Absorption of certain substances like pentoses, some minerals, some water soluble vitamins from intestine is carried out by diffusion. Same is the case of renal reabsorption of urea. 2. Water, water soluble substances pass largely by diffusion through water filled pores of the cell membrane. This is dependent on the pore size and size of the diffusing particle. Pore size is about 0.8 nm. Hydrated K + ion is 0.4 nm in diameter, hydrated Na + ion has 0.5 nm diameter. Hence, movement of K + is two times faster than the movement of Na + . Glucose and galactose molecules have a diameter of 0.85 nm. Hence, they cannot pass via pores. They pass via membranes after combining with some carrier. 3. There are voltage gated ion channels in the membrane. When these channels open, ions move by diffusion along electrochemical gradient. 4. Exchange of respiratory gases occur by way of diffusion. The partial pressure of O 2 is higher in alveolar air and lower in the deoxygenated blood. So O 2 enters blood from the lungs. Alternatively, the partial pressure of CO 2 is higher in deoxygenated blood and lower in the alveolar air. So, CO 2 diffuses from blood to alveolar air. In the same way, due to difference of partial pressure, O 2 flows from the oxygenated blood to the tissue cell and CO 2 diffuses from the tissue cells to the blood. During hyperventillation, alveolar size is increased. Hence, the surface area is increased and wall thickness of the alveoli is decreased. So, rate of diffusion of O 2 and CO 2 is increased. 5. The alveolar surface area per unit body weight is larger in children than in adult humans. So, the resting O 2 uptake from the alveoli is higher in children than in adults. 6. There is a difference of partial pressure of O 2 and CO 2 between alveolar air and inspired air. So, O 2 and CO 2 diffuse down their respective pressure gradients between the terminal bronchioles and alveoli. This is how the alveolar air has its O 2 renewed and CO 2 partially removed.

6 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof 7. When the cell size is increased, the surface volume ratio is decreased. This decreases diffusion of gases and solute across the plasma membrane, per unit volume of the cell. Enhanced cell-size also decreases rate of diffusion. 8. Replacement of cutaneous respiration across the general body surface by gill respiration and pulmonary respiration enhances the respiratory surface. So, respiratory exchange increases with the evolution of gills and lungs. 1.3.2 Osmosis The movement of solvent from solvent side to the solution side at or from dilute solution to a concentrated solution, when the two are separated by a semipermeable membrane is called osmosis. Semi permeable membrane is that membrane which allows only the solvent molecules to pass through and not the solute molecules. Osmotic pressure (OP) is the pressure which has to be applied on the solution side to stop the osmotic inflow into it from pure solvent. Van't Hoff's laws of osmotic pressure 1. It is directly proportional to the molar concentraion of the solute so long as the temperature remains constant. ? = k 1 c (where, ? = Osmotic pressure, k 1 = constant, c = molar concentration.) 2. Osmotic pressure of a solution is directly proportional to the absolute temperature (T), so long as its concentration remains constant. ? = k 2 T (where, k 2 = constant) 3. Van't Hoff - Avogadro law : Identical numbers of moles of different solutes produce an identical osmotic pressure, when dissolved in the same volume of the solvent at the same temperature. Osmotic pressure (OP) is expressed as atmosphere, mm of Hg or dynes per sq. cm (dynes cm -2). 1 mole of a nonionized solute is equivalent to 1 osm (osmole). Osmolarity of a solution is its solute concentration in osmoles per litre of the solution. A solution of one mOsm of any solute in a litre possess an osmotic pressure of 19.3 mm Hg at 38 o C. Osmolatity of a solution is its solute concentration in Osm per kg of solvent. It can be determined by— 1. Osmometer. 2. Barkley—Hartley method. 3. Freezing point method. This method depends on the direct proportionality between the osmotic pressure and the depression of freezing point of a solution. Depression of Freezing point is measured by Beckmann Thermometer.

7 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof ?t = K f M or, M = f K t? The freezing point of one Molal solution is called cryoscopic constant (K f) / molal freezing point. The K f for water is – 1.858 o C ? = CRT ? = Osmotic pressure in atmosphere. where C = Molal concentration R = Molar gas constant (0.082 litre atmosphere) T = Temperature in Absolute scale. A sample of urine freezes at –0.56 o C. Calculation of its OP at 37 o C Molal conc. C = 858.1 56.0 ? ? = 0.3 The sample will have OP at 37 o C (? = CRT) 0.3 × 0.082 × 310 = 7.6 atms. or, 7.6 × 760 mm Hg. Osmotic work When substances are transferred from lower concentration to higher concentration, osmotic work must be performed upon them. But when substances pass from higher to lower concentration, osmotic work is done by them. Relation between osmotic work and concentration change is given by the equation— W min = NRT ln 1 2 C C or, 2.3 NRT log 1 2 C C [W min = Minimum osmotic work in small calories involved in the transfer of N moles of substance from a molal concentration of C 1 to a molal concentration C 2 . R = Gas constant = 1.987 cal/mole/degree T = Absolute temperature ln = Natural log 2.3 is the factor for converting natural log to the log to the base 10. Calculation of osmotic work to be done to transfer or secrete 3.545 g of Cl – from plasma urine at 37 o C, when the Cl – concentrations in plasma and urine are 0.1 and 0.2 Mol respectively. [where, M = Molal concentration of solute. ?t = Depression of freezing point of the solution. K f = Cryoscopic constant]

8 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Cl – conc. in Plasma ..... 0.1 Molal C 1 Cl – conc. in Urine ..... 0.2 Molal C 2 Osmotic work to be done to transfer / secrete 3.545g of Cl – at 37 o C in urine. N = 0.1, R = 1.987, T = 310 W min = NRT 2.3 log 1 2 C C = 0.1 × 1.987 × 310 × 2.3 log 1.0 2.0 = 141.7 log2 = 141.7 × 0.301 = 42.65 cal Osmoticity and tonicity The osmoticity of a solution depends on the total solute concentration, both diffusible and nondiffusible. Whereas, tonicity of a solution depends on the concentration of nondiffusible solute only. Hence, two solutions may be isosmotic, but may notbe isotonic. Solution A is isosmotic with solution B, But solution A is hypertonic to solution B. Similarly, two solutions may be isotonic but may not be isosmotic. Solution A and B are isotonic, but the Solution B is hyperosmotic to Solution A. SM = Semipermeable membrane. Solvent always flows from by hypotonic to hypertonic solution. In biological system, tonicity is considered because biological membrane are not strictly semipermeable. They allow some solutes to pass. Biological application of osmosis 1. Hemolysis, crenation, plasmolysis : RBC is hemolysed or animal cell ruptures if placed in hypotonic solution. This happens because solvent flows from hypotonic solution to RBC or cell. Consequently, they swell and burst at a certain degree of swelling. Similarly, these cells or RBC will shrink (crenated) ((((((((Protein 2M Protein 1.8 M Urea 0.2 M A SM B ((((((((Protein 1M Protein 1M Urea 0.2 M A SM B

9 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof if placed in hypertonic solution. It is due to osmotic outflow of water from the hypertonic solution. Plant cells lose water when placed in hypertonic solution. So, the cell membrane collapses and withdraws from the cell wall. This is called plasmolysis. Plant cells swell if placed in hypotonic solution, but they do not burst due to rigidity of cell wall. 2. Osmotic distension of RBC : Osmotic pressure of RBC fluid is 1.5 atm., higher than plasma, due to higher electrolyte concentration in RBC than plasma. So, RBC remain slightly distended, but they do not rupture. However, due to genetic disorder, Vit-E deficiency, or selenium deficiency and other defects, RBC may not be able to withstand such distension and rupture. RBC of camel are more resistant to osmotic distension. Camel can drink more than 100 litres of H 2 O in 10 minutes. Blood becomes temporarily highly hypotonic, but RBCs do not rupture. It has been observed that RBC may be distended upto two times its volume, but hemolysis does not occur. 3. Osmotic pressure of plasma is higher than tissue fluid. It is due to plasma proteins. Total osmotic pressure of plasma is about 5453 mm Hg and that of ECF is 5430 mm Hg. The difference of 23 mm Hg is due to plasma proteins. It is called colloidal osmotic pressure of plasma. This osmotic pressure, hydrostatic pressure i.e. capillary pressure and pressure of the tissue fluid play an important role in the exchange of body fluid across the blood capillaries. Owing to pressure differences, fluid passes out from arterial end of blood Arterial end of the Venous end Cap Pr < OP Cap Pr > OP H 2 O H 2 O capillaries to the tissue fluid and body fluid enters from the tissue fluid into the capillaries at the venous end. These forces also govern the flow of fluid between any two compartments.
10 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof In Kwashiorkor, (a form of childhood malnutrition), hepatic cirrhosis and nephrosis, plasma protein concentration is decreased. So, colloidal osmotic pressure is decreased. This decreases water retention by the plasma and so edema develops. 4. Osmotic pressure plays a vital role in the absorption of water from intestine and kidney tubules. 5. Water absorption by plant roots is also governed by osmotic pressure. Root hair cells have higher osmotic pressure than surrounding soil-fluid. So, water enters into root hair cells. Water moves from one cell to the next cell by cell to cell osmosis and thus other cells are also distended. Hence, the cells become turgid and rigid and they stand erect on watering. 1.3.3 Donann membrane equilibrium When a non diffusible ion in present in a solution, the distribution of diffusible ions across the membrane will be unequal. This was observed by Donann and it is called Donann membrane equilibrium. In the compartments A and B, NaCl solution is present. The two compartments are separated by a semi-permeable membrane. Which allows NaCl to pass through but not the nondiffusible ion R. When R is not present the ion distribution in the two compartments is : $Na + A = Na + B$ $Cl - A = Cl - B$ $Na + \times Cl - A = Na + B \times Cl - B$. But when the nondiffusible ion (R) is added the distribution of diffusible ions become unequal : $Na + A$ < $Na + B$ $Cl - A$ > $Cl - B$. $Na + A \times Cl - A = Na + B \times Cl - B$ The unequal distribution depends on the nature of nondiffusible ion. In a situation, as described above, where the nondiffusible ion is negative there will be more positive diffusible ion in the compartments that contains the nondiffusible ion. Because of such unequal distribution of diffusible ion, there will be a pH difference, a potential difference, and an osmotic pressure difference on the two sides (((((((((Na R NaCl. A M B Na + Cl – 1 + 1 + 1 + 1 +

11 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof of the membrane. If we consider the distribution of H + and OH – then there will be more H + in the compartment that contains negative nondiffusible ion. Hence, there will be a pH differnce in the two compartments. The fluid in the compartment where the nondiffusible negative ion is present will have a lower pH (acidic) compared to the other compartment. Because of the presence of negatively charged hemoglobin in RBC, the pH of RBC fluid is less than plasma. The membrane is permeable to Na and Cl. Concentration of Na + in A is more than Na + concentration in compartment B. The concentration of Cl – is less in compartment A than in the compartment B. Thus Na ions in A will try to move from compartment A to compartment B and Cl – will try to move from compartment B to compartment A. This can only be prevented by developing an opposite electrical gradient. Hence, the side of the membrane where the non-diffusible negative ion is present is negatively charged and the other side of the membrane is positively charged. Donnan Phenomenon plays an important role in the development of resting membrane potential. It can be shown by calculation that total solute concentration in the compartment that contains the non diffusible ion is more compared to the other compartment. In the diagram of the concentration of NaR is taken as 'a' and the concentration of NaCl as 'b' then at equilibrium, the concentration of NaCl in the two compartments will be : ((((((((a Na + a R – b Na + b Cl – A B ((((((((a+x Na aR – xCl – b – x Na + b – x Cl – A B Concentration of Na + A will be (a+x) and Cl – A will be x. Whereas concentration of Na + b will be b-x and Cl – will be b-x. Since the product of the concentration of Na + and Cl – will be equal in the two compartments. The equation can be written as (b–x) 2 = (a+x)x. From the equation the value of x in terms of a and b will be x = b2a b 2 ? . Thus the amount of NaCl (x) that has moved from compartment B to compartment A is inversely proportional to the concentration of non diffusible ion in compartment A. When the concentration of NaCl (b) will be large relative to the concentration of nondiffusible ion (a) the value of x will be more. If specific arithmetic number is assigned to a as 1 mole and b as 2 mole then specific value of x will be obtained.

12 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Thus the total solute concentration in compartments A is 3.6 mols and in compartment B is 2.4 mols. Hence the osmotic pressure of the solution in compartment A will be more than in compartment B. The Donnan principle operates to regulate the distribution of electroyte ions across the membrane of living organism. However, the plasma membrane is not a strictly semipreamble membrane. It has selective permeability property. Thus the membrane may be impermeable to some diffusible ion and in that case such ions do not move following Donnan phenomenon. However this may produce Donnan effect. Thus Donnan effect is there but its quantitative evaluation is difficult. Deviation from Donnan Phenomenon has also been found. The distribution of cations, Na + , K + , Ca 2+ an Mg 2+ between plasma and lymph follows Donnam principle. They are high in plasma than in lymph. Plasma has a higher concentration of negatively charged nondiffusible ion protein. The difference in the composition of glomerular filtrate and plasma also follows Donnan principle. The glomerular filtrate contains less amount of positive diffusible ion and more amount of negative diffusible ion compared to plasma. This is also due to Donnan effect because of negatively charged plasma protein. Peculiarly, however, the concentration of Cl – is higher in lymph than in plasma as required by Donnan principle, but the concentration of HCO 3 – in the plasma is little higher than in lymph which is contrary to Donnan principle. 1.3.4 pH and buffer pH is negative logarithm of concentration of hydrogen ion (CH) to the base 10. pH = –log CH It is a measure of the H + concentration of a solution. It is determined by pH meter. Buffer is a mixture of weak acid and its salt or weak base and its salt. Henderson— Hasselbach equation of a buffer solution is given below. pH = pKa + log Acid Salt . pKa is the dissociation constant of the buffer acid. In a buffer solution with equal amount of salt and acid. pH = pKa ((((((((Na + a+x mol Cl – x mol R – a mol. A M B Na + (b-x)mol Cl – (b-x) mol ((((((((Na + ...1.8 Cl – .....0.8 R.....1.0 3.6 mol Na + ...1.2 Cl – ....1.2 2.4 mol A B moles 2b and mol 1aif ? ? b2a b x 2 ? ? 41 4 ? ? 5 4 ? or 0.8 mol.

13 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Each buffer has a pH and a capacity. The pH of a buffer depends on the pKa and salt ratio. The capacity of a buffer depends on the salt/acid ratio and on its amount. The buffers can not prevent the change of pH but it can resist the change of pH when any acid or base is added to it. For example when any strong acid is added to a buffer sol the salt of the buffer react with the strong acid added. As a result, equivalent amount of buffer acid is produced. Because buffer acid is a weak acid the pH is not changed much. However, as the salt/acid ratio is altered, there should be a new salt/acid ratio, so pH will definitely be changed. HCl COOH CH COONa CH 3 3 ? NaCl COOH CH HCl COONa CH 3 3 ? ? ? Thus the buffer present in our body fluid help to resist the change of pH during production of metabolic intermediates. The buffer of our body has a high salt/acid ratio e.g. 1 20 CO H HCO 3 2 – 3 ? . Such a buffer is good for handling acids. Important buffers present in our body fluid are bicarbonate, phosphate and protein buffers. 1.3.5 Poiseuille's Law This law states the relationship between pressure gradient, resistance and volume flow. Poiseuille and Hagen equation is Q = L8)r(P P 4 2 1 ? ? ? Where, Q = volume flow in ml/sec. P 1 –P 2 = pressure difference between 2 points (dynes/cm 2 .) r = radius of the tube in cm. L = length of the tube in cm. ? = viscasity of the fluid in poise. It is revealed from the equation that 1) It pressure gradient is doubled, the flow will be doubled. 2) If the length of the tube or viscosity is doubled, the flow will become half. 3) If the radius of the tube is doubled. The flow will increase 16 times.

14 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof In Poiseuille's experiment the fluid was a constant visocity fluid (Newtonian fluid) the tube was a rigid tube. This law is applicable in closed vascular system but it is not obeyed in toto, because the vascular tubes are not rigid but distensible. With a Newtonian fluid flowing through a rigid tube the pressure flow curve (Fig 1.1) is linear (1) because the resistance is not changed as pressure is increased (2). But with flow of blood in a vascular system it is not so (3) because the resistance is changed (4). It is very high at low pressure. When there is no flow (closing pressure). At high pressure the resistance is almost constant so flow becomes more linear. Fig 1.1 : The relationships of flow, pressure and resistance in nondistensible tubes using Newtoning fluids (dotted lines) at constant temperature (Poiseulle's law) and blood in vessels (solid lines). RESISTANCE (PRU5) 1.4 Suggested Questions 1. Why respiratory exchange increased with the evolution of gills and lungs ? 2. How alveolar air gets its O 2 renewed and CO 2 partially removed ? 3. Why resting alveolar O 2 uptake is higher in children than the adults ? 4. Why rate of diffusion of respiratory gas is increased in hyperventillation ? 5. What is diffusion ? Name the factors which favour the rate of diffusion/ oppose the rate of diffusion. 6. State the Fick's law of diffusion and Graham's law of diffusion. 7. What is diffusion coefficient ? 8. Describe the role of diffusion in the transfer of respiratory gases. 9. Describe why evolution of gills and lungs is advantageous compared to cutaneous breathing. 10. Gill and lung respiration is better than cutaneous respiration—Justify. 11. What is Donnan membrane equilibrium ? State the role of Donnan phenomenon on osmotic pressure difference across the plasma membrane ? 12. Why pH of RBC fluid is less than plasma ?

15 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof 13. Write the Henderson-Hasselbotch equation for a buffer solution. 14. Mention the factors on which the pH and capacity of a buffer depends. 15. Describe the role of pressure gradiant and resistance on the volume flow in a closed vascular system. 16. What is osmosis ? 17. What is osmotic pressure ? 18. State the laws of osmotic pressure. 19. State the role of osmotic pressure in the exchange of fluid across the blood capillaries.

16 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Unit 2 ? Thermoregulation 2.1 Regulation of body temperature in homeothermic animals specially mammals Animal organisms have been classified into two groups depending on their capability of regulation of body temperature namely, homeothermic and poikilothermic animals. Homeothermic Animals : The animals which maintain their body temperature more or less constant inspite of changes in the temperature of the environment are called homeothermic animals. They are also called warm blooded animals. Birds and mammals belong to this category. Poikilothermic animals : The animals which do not maintain a constant body temperature and it varies as the environmental temperature is changed are called poikilothermic animals. They are also called cold blooded animals. Fish, amphibia, reptiles and invertebrates belong to this group. In homeothermic animals a balance is maintained between heat gain and heat loss mechanisms present in them and thus the body temperature is kept constant. This is achieved by the operation of a complex neuro-hormonal mechanism regulated by the temperature controlling centre located in the hypothalamus. Birds and mammals have a normal body temperature ranging between 40 0 – 43 0 C and 36 0 – 39 0 C respectively. Normal body temperature in human subject The normal body temperature of man ranges between 35·8 – 37·3 0 C when measured by introducing clinical thermometer into the mouth cavity (oral temperature). The temperature recorded from arm pit is called axillary temperature. It is slightly lower (37 0 C) than oral temperature. The rectal temperature is slightly higher than oral temperature (about 37 0 C). The superficial temperature i.e. skin or surface temperature ranges between 29·5 0 C and 33·9 0 C. The average temperature in deeper Structure 2.1 Regulation of body temperature in homeothermic animals specially mammals 2.2 Temperature regulation in poikilotherms 2.3 Hibernation 2.4 Suggested questions

17 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof tissue is called core temperature. It is always more than oral or rectal temperature. It is about 37·8 0 C (100 0 F). The body temperature is slightly more in children than adults. It shows diurnal variation. It is about 1 0 C less in the morning and it reaches maximum value in the afternoon (1 0 C more than normal). The body temperature is increased in exercise, emotion and after meals. It is slightly less in females than males and the temperature rises sharply immediately after ovulation (0·5 0 C – 1 0 C). It is reduced 0·5 0 C in sleep. A constant rectal temperature can be maintained in a nude human subject at air temperature or 0 – 1 0 C for 1–2 hours. In dry air maximum tolerance to high temperature has also been observed e.g. 200 0 C in a nude subject and 260 0 C in heavily dressed man. Heat gain and heat loss mechanisms of the body The body will gain heat from the environment if the body temperature is less than the environment and vice versa by way of conduction, convection and radiation. Thermogenesis and thermolysis : Heat production/gain is thermogenesis, heat loss is thermolysis in the body. However, the physiological processes of heat gain mechanism are shivering and nonshivering thermogenesis and vasoconstriction. On the other hand, physiological processes of heat loss from the body are vasodilation, sweating and fenting. The mechanism by which the body temperature is normally adjusted is known as thermotaxis. Temperature regulating centre A balance is maintained between thermogenesis and thermolysis and thus the body temperature is maintained. There is a temperature regulating centre in the hypothalamus which maintains this balance and thus help maintain homeothermy. If this part is destroyed the animals become poikilothermic. On the basis of ablation and stimulation experiments it was shown that the anterior hypothalamus act as hot responsive centre, stimulation of which increases heat loss and dereases heat gain. The posterior hypothalamus acts as a cold responsive centre, activation of which stimulates heat gain mechanism and inhibits heat loss mechanisms. However, subsequent studies were made with physiological stimuli like cooling and heating the hypothalamic regions by diathermy or by using thermodes. The studies revealed that the temperature detecting centre for both heat and cold are located in the anterior hypothalamus, whereas the centre initiating heat loss or heat gain mechanisms are present in the posterior hypothalamus. Microelectrode studies on the hypothalamic neurons revealed the presence of different temperature sensitive neurons in the anterior hypothalamus.

18 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof (a) Some warm sensitive neurons respond on local heating of hypothalamus and they stimulale heat loss mechanism. (b) Some respond to local cooling of hypothalamus and they activate heat gain machanism. (c) There are some neurons whose rate of firing is changed in response to peripheral warming or cooling. (d) There are neurons which respond to a rise in both hypothalamic and peripheral temperatures. (e) There are neurons whose firing rate remain unchanged with temperature variation. Serotonergic and cholinergic neurons are involved in temperature regulation, Mayers and Sharpe observed in monkey with push-pull technique using saline that when the donor is cooled a transmitter is released which can cause shivering in receipient. Similarly heating the animal causes the recipient to lower body temperature. Subsequently it was observed in 'saline withdrawal' technique that saline withdrawn from donor monkey during cooling contained increased concentration of serotonin. Liberation of noradrenaline was augmented by peripheral warming. Mayers et al reported the presence of a heat conservation and production pathway originating in the anterior hypothalamus is passing through the posterior hypothalamus and there is a heat dissipation pathway originating in the posterior hypothalamus. They proposed a mechanism of action of hypothalamus in the temperature regulation on the basis of experiments in monkey. Serotonergic neurons in the preoptic region increase their firing in response to cooling. This causes activation of cholinergic pathway to the posterior hypothalamus and heat production is initiated (Fig. 2.1). The noradrenergic neurons in the anterior hypothalamus are stimulated due to warming. They inhibit the serotonin cholinergic heat production Fig 2.1 : Diagram of a model to account for temperature regulation under normal conditions as well as during a pyrogen-induced fever. Factor which affect the aminergic 'thermostat' in the anterior hypothalamus at given, and the outflow from the posterior hypothalamic 'set point' is mediated by a cholinergic system which passes through the mesencephalon. 5H = 5-hydroxytryptamine ; NE=noradrenalin ; ACh = acetylcholine. (Fro Myers, R. D. (1971). Pyrogens and Fever. CIBA Foundation Symposium Elsevier, Amsterdam.)

19 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof pathway by noradrenergic blockade of the synapse of serotonin —cholinenrgic junction. Such suppression of the heat production pathway permits the second cholinergic system in the posterior hypothalamus to activate the efferent heat loss pathway. Set-point in the hypothalamus Different experimental evidence indicate that there is a 'set point' in the hypothalamus which is like that of a tharmostat. When the temperature goes above the set point heat dissipation is increased and the temperature is lowered and brought back to set point. On the other hand if the temperature goes below the set point heat production occurs, the body temperature is raised and the set point in reached. The presence of warm and cold sensitive neurons in the hypothalamus indicate that the combined action of these neurons play the role of hypothalamic thermostat. Thus temperature sensitive neurons in the anterior hypothalamus form the basis of a 'set point' mechanism. It is held that over a physiological range of hypothalamic temperatures both warm and cold sensitive neurons or sensors are active. However a change in temperature will increase the discharge of one type of neuron and will decrease the firing of the other type. Hence, there will be a value of hypothalamic temperature at which the activity of the two sets of sensors will be balanced in terms of body temperature regulating responses which they produce. This will be the setpoint value of hypothalamic temperature. There are hypothalamic neurons whose firing rates remain unaltered by changes in hypothalmic temperature. These neurons or neural firings can provide a 'reference signal'. The difference between the firing rates of these temperature insensitive neurons and the temperature sensitive neurons provide an 'error signal'. The direction and magnitude of the 'error signal' will determine the extent of operation of heat gain and heat loss mechanisms to bring the temperature to the set point level (Fig 2.2). Fig 2.2 : Determination of hypothalamic 'set-point' temperature by the balance of activity in warm and cold-sensitive neurons. The response characteristics of these temperature sensors have been represented by bell-shaped curves. The interrupted portions of these curves are a hypothetical projection based on the suggestion that the temperature- sensitive characteristics of these sensors may be similar to those of the warm and cold receptors in the cat's tongue as described by Hensel and Zotterman.

20 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Basic mechanism of temperature regulation A change in body temperature, with reference to 'Set point', stimulates thermoreceptors present on the body surface as well as inside the body. These impulses (originate due to stimulation of receptors) reach the hypothalamus via neural pathways and stimulate it. Altered temperature of blood also activates it. It then modifies the heat gain and heat loss mechanisms via posterior hypothalamus, as required and the body temperature is kept constant. It is believed that in the cold the neural impulses are very important part in hot, the altered temperature of the blood is the most effective slimulus. It has been observed that when one hand is immersed in hot water, vasodilation is seen in the other arm. But this does not occur if the blood flow from the arm immersed in hot water is blocked. Similarly, if one hand is immersed in cold, there occur vasoconstriction in the other hand but this does not stop even if the blood flow for this region in blocked. Temperature regulation in the cold When the body temperature tends to decrease on exposure to cold environment, the following changes occur to keep the body temperature normal. The cold receptors present in the body are stimulated, the neural discharge in the nerves attached to this is increased. These impulses and also the decreased temperature of blood stimulates the temperaure detecting centre in the anterior hypothalamus. This results in stimulation of thermogenesis and inhibition of thermolysis. Thermogenesis is the primary motor centre for shivering and locatd in the dorsomedial portion of the posterior hypothalamus. Impulse discharge from here increase the tone of skeletal muscles throughout the body. The arrector pili muscles also contract. Thus heat production is increased. During maximum shivering body heat production can be increased four to five times normal. Intially there occur uncoordinated muscle twitches, the intensity of which increases until the rhythmic activity of visible shivering appears. It has been, shown that tensing the muscles can raise the heat production to 2–3 times the basal level, shivering starts when the body temperature goes below critical temperature. Non-shivering thermogenesis also starts at the same time. Impulse discharge via sympathetic fibers reach the adrenal medulla and causes discharge of adreno medullary hormones adrenaline and noradrenaline. Neural impulse also cause release of TRH from hypothalamus which causes release of TSH from anterior pituitary. This hormone stimulates thyroid gland and causes secretion of T 3 and T 4 hormones. The calorigenic effect of these hormones increase heat production. Adrenaline increases glycolyis and fatty acid oxidation. It increases lipolysis specially from brown fat, and the oxidation of the released fatty acid is increased. Beside heat production it helps in heat conservation by preventing heat loss by way of vasoconstriction. Thyroid hormones
21 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof specially triiodothyronine (T 3) increases general metabolism and heat production. It is held that calorigenic effect of thyroid hormones is due to increased ATPase activity, oxidative phosphorylation, glycolysis as well as uncoupling of oxidation and phosphorytation. It also increases the calorigenic effect of adrenaline. Beside thermogenesis, there occur vasoconstriction in the body. This is caused by increased sympathetic discharge from posterior hypothalamus. This vasoconstriction decreases heat loss from the body and thus heat is conserved, and body heat production is increased. Along with this, there occur inhibition of sweating and this is another mechanism to prevent heat loss. In this way by stimulating shivering and nonshvering thermogenesis and vasoconstriction and inhibition of sweating, body heat production is increased and heat loss from the body is inhibited. As a result, fall of temperature is prevented and normal body temperature is maintained. Different exprimental results indicate that :- (1) The controlling centre for integrating and coordinating various temperature conservation function is contained in the posterior hypothalamus, although it is not thermosensitive. (2) Critical temperature is the external temperature below which heat production has to be increased by shivering thermogenesis to maintain normal body temperature. It differs with species. In case of a tropical animal it is between 20 0 –30 0 C, whereas in arctic animal like huskey dog the basal heat production may not increase even at enviromental temp, below –30 0 C. In this respect it is 20 0 C in a nude human subject. (3) In case of muscle shivering both flexors and extensors are stimulated. (4) The nerve inpulse goes to the muscle via lower motor neuron being activated by tecto-spinal or rubro-spinal tract and not by pyramidal tract. (5) Shivering begins when environmental temperature is below critical temperature (20 0 C in case of nude man). The degree of shivering increases as the ambient temperature is decreased. (6) In respect of body temperature shivering begins when the core temperature goes below the set point (37 0 C in case of man). Shivering is increased as the body temperature goes much below the set point. (7) Shivering is more if both air and core temperature are low. (8) When air temperature goes below 27 0 C, vesoconstriction occurs. Temperature regulation in hot environment When the body temperature tends to increase on exposure to hot environment the following changes occur to maintain the normal body temperature. The hot receptors present in the body are stimulated. Nerve inpulse in generated in the nerve fibres attached to them. These impulses as well as increased temperature

22 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof of blood stimulate the temperature detecting centre located in the anterior hypothalamus. This results in inhibition of thermogenesis and stimulation of thermolysis. Vasodilation and sweating cause thermolysis. It has been found that when the air temperature goes above 27 0 C the skin vessels dialate. This helps in heat loss from the body to outside. Sweating : The sweat glands are innervated by sympathetic cholinergic fibres. When body temperature is increased impulse goes to the sweat glands from posterior hypothalamus via this pathway and cause secretion of sweat. Evaporation of sweat takes away the latent heat of vaporisation from the skin surface and thus the body is cooled. It has been found that above 29 0 C environmental temperature, very little heat can be lost by convection. So, vasodialation does not help much in heat loss from the body. Above 35 0 C environmental temperature, the body loses heat entirely by evaporation. The air temperature at which sweating begins has been found to be about 31 0 C and for those lightly clad is 29 0 C in humans (the average skin temperature is about 34 0 C). In respect of body temperature, when it goes above the setpoint, sweating begins. It increases as the body temperature is increased. It has been found that the degree of sweating is related to skin temperature and core temperature. When both core and air temperature high the degree of sweating is more. It has been observed that maximum rate of sweat secretion for thermolysis (thermal sweat) may be as high as 1.7 liters/ hour or more. When one liter of sweat is evaporated 580 Kcal of heat is lost from the body. As sweat coures from the blood rapid sweating demands a large cutaneous blood flow and so requires cutaneous vasodialation. This is brought about by : (a) External heat acting directly on the blood vessels. (b) Reflexly from cutaneous warm ending. (c) By the rise of blood temperature acting directly on the hypothalamic centre. (d) Activity of sweat gland secretion leads to formation of bradykinin which acts as vasodialator. If the air is humid, sweat cannot be evaporated and so sweating cannot help in heat loss. The secretion of sweat also show adaptation. If exposure to heat is continued, the sweat secretion is increated, it starts at a lower temperature, i.e. threshold for sweat secretion is decreased. The NaCl content of sweat is decreased by the action of aldosterone secreted in this condition. This prevents salt loss from the body. In case of excessive sweating, water and salt should be ingested to prevent dehydration and salt deficiency.

23 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof It has been shown that some birds and mammals allow their body temperatures to vary widely, either regionally in the body or in the whole body for sametime. Temperature regulation in new born The new born of all species, including human infants are of smaller size compared to their corresponding adults. Hence, they have greater surface area in relation to their body weight. This poses a great problem in the maintenance of normal body temperature. A fall in body temperature can be prevented by decreasing heat loss and increasing heat production, cutaneous vasoconstriction has been found to occur in response to cold even in premature babies. The new born has a higher capacity to increase its heat production. A newborn animal or human infant, when exposed to cold hunch themselves and tucks their limbs to reduce effective surface area. Brown adipose tissue plays a very important role in heat production in infants. Fatty acid produced from lipolysis in brown adipose tissue are oxidized within these tissue as well as oxidized in other tissues after being carried there via circulation. Tempurature regulation in non-sweating animals In non-sweating animals, evaporative heat loss is achieved by other means. In case of birds panting and gular flutter help in evaporative heat loss from respiratory tract. Gular flutter, however, has been shown to be metabolically less expensive. In respect of heat gain in cold, these animals respond to cold by shivering. Muscular activity appear to be their only means of increasing heat production. Non-shivering thermogenesis has not been demonstrated in them. They lack brown fat and fail to show a thermogenic response to norepinephrine. However, they depend on white adipose tissue for compensation in cold. When plenty of water is available, some birds may increase their cooling by urinating on their legs. Kangaroo rats have no sweat glands. Evaporation from the lungs is a great source for heat loss in them. However, due to scarcity of drinking water, they do not use much water for heat regulation, rather they are nocturnal and move for food only during night when the environment is cool. Small animals such as rodents have no true sweat gland and also do not pant. They avoid heat by living in underground burrows. Behavioral regulation of body temperature Animals have been found to change their behaviour by regulating their body temperature. In a cold condition the animals including humans are found to curl themselves or remain close together to prevent heat loss. Alternatively, in hot weather the animal stretch themselves to increase the surface area to favour heat loss. The

24 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof rodents, specially in deserts have been found to enter into burrows in the daytime to avoid heat. Moreover, they become nocturnal so that this may be able to acquire food at night. This type of temperature regulation has been found to be much documented in several poikilotherms, both vertebrates and invertebrates. Some use solar energy, while others utilize metabolic heat to raise the body temperature. The terrestrial environment is more prone than aquatic to sudden temperature changes. The most successful terrestrial poikilotherm insects and reptiles have made use of behavioral response to avoid extreme temperature or to elevate temperatures sufficiently for certain activities. Some reptiles have well developed sensory organs for this purpose. The infrared sense organs in the facial pit of rattle snakes can detect a temperature difference of the order of 0·001 to 0·005 0 C. This helps the animal to orient themselves to warm and cool environments. It also helps the animal in detecting a wam blooded and cold pray. The insects use solar or metabolic energy to warm up before flight. Social insects like ants, termites and bees may regulate their temperature in their nest or hives through varied activities. Temperature regulation in aquatic animals Water has high thermal conductance and a high heat capacity. Hence, the thermal loss to water is much higher than to air of the same temperature. The cooling power of water may become as high as 100 times as great as for air. Many whales and seals live and swim in the near freezing water. However, regarding body temperature they are similar to other warm-blooded animal. It is around 36 0 –38 0 C. There are three ways by which aquatic animals can cause heat balance in cold condition. (1) They can live with a lowered body temperature. (2) They can increase their metabolic rate to increase heat production. (3) They can increase their body insulation to reduce heat loss. It has been found that several species of seals and dolphins have resting metabolic heat production twice as high as would be expected from their body size. However, in the harp seal, their metabolic rate remained same in water even when the temperature goes down to the freezing point. (The critical temperature for harp seal in water is below freezing point, but it has not been determined). Thus the effective solution to the problem is to develop effective insulation and this is done in these animals. They have a thick blubber under the skin that acts as an insulator. In seals, the temperature of the skin surface is identical to that of water but at the depth of about 50 mm (the thickness of the blubber), the temperature is nearly that of core temperature. The seals and whales being such well insulated feel difficulty in heat loss when the temperature of water in increased. In such a situation its skin temperature is increased

25 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof to eliminate heat. This is achieved by increasing blood flow through the blubber to the superficial layer of the skin which is well supplied with blood vessels. The cutaneous blood vascular system permits a precise regulation of the amount of heat that reaches the skin surface and thus is lost to the environment. Since the insulator is located internally to the surface of heat dissipation (skin), blood can bypass the insulator and heat loss during heavy exercise or in warm water can be independent of insulator. In arctic land mammals, furs act as insulator. It is located outside the skin surface. The surface temperature of the body skin under the fur is close to core body temperature. Most of the insulation resides outside the skin surface. In case of polar bear fur is the insulator. But it has also a substantial layer of blubber under the skin. This is very important. When the polar bear swim in cold water, most of the furs get wet, most of the insulation value of furs is lost, the blubber plays an important role in heat conservation. Hence, blubber plays an inportant role in samiaquatic way of life. In seals and whales, that lack blubber, have flippers are flakes. These appendages are well supplied with blood vessels and can lose substantial amount of heat if required. However, they require heat conservation in the cold which is achieved by developing heat exchanger system. It has been found that in the whale flipper, each artery is completely surrounded by veins. Thus, as warm arterial blood flows into the flipper, it is cooled by the cold venous blood that surrounds it in all sides. The arterial blood therefore reaches the periphery precooled and loses little heat to the water. The heat has been transferred to the venous blood, which is pre-warmed before it reaches the body. This kind of vascular heat exchange arrangement is called counter current heat exchanger, because blood flows in opposite directions in the two vessels. 2.2 Temperature regulation in poikilotherms Poikilothermic animals attain the temperature of the environment where they live and do not maintain a constant body temperature like homeotherms. But some poikilotherms have some regulation of body temperature and thereby keep their body tempereture above the ambient temperature. Such temperature regulation has been developed to satisfy their requirement to combat thermal stress. The insects are poikilotherms, but they face thermal problem because of high rate of metabolism during their flight. If the insect is too cold its muscles will not contract suitably for flight. In such a situation, it can increase the temperature of their flight- muscle by contraction similar to shivering in man. On a cold day a butterfly

26 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof or moth has been found to vibrate their spread cut wings for several minutes before take-off. At this stage, the temperature of the flight muscle has been found to be raised to about 35 0 C within 6 minutes in an ambient temperature of 20 0 C. Snakes and lizards have been found to expose themselves to the sun in the early morning and raise their body temperature above the surrounding temperature by sunbasking. A lizard caught in the early morning at Peru (altitude—15,000 ft.), had a body temperature of 31 0 C. Later in the day it avoids sun and in hot days take shelter under rocks or cool burrows. A desert lizard save itself from overheating by behavioral changes because its lethal temperature is about 45 0 C and it will die if it is exposed to such temperature for 10 – 15 minutes. Active temperature regulation has been observed in snakes. Large python has been found to coil their bodies around their eggs. At a room temperature of 25 0 C, the snakes maintain their body temperature about 5 0 C higher and this is achieved by spasmodic muscle cantraction like shivering in man. 2.3 Hibernation Sometimes the animals pass through a state of dormancy to overcome adverse conditions. Dormancy is a general term for reduced body activities, including reduced metabolic rate. Dormancy has been variously classified according to its depth and duration e.g. sleep, torpor, hibernation, winter sleep and estivation. All these are specific physiological conditions, attained and maintained to pass over unfavorable conditions. Torpor : It is a state of inactivity often with lowered body temperature and reduced metabolism that some homeotherms enter into so as to conserve energy stores. Small endotherms, because of their high metabolic rate are subject to starvation during periods of inactivity when they are not feeding. During these periods some animals enter into a state of torpor in which the temperature and mebabolic rate subsides. Daily torpor is practicised by many terrestrial birds. Several species of small mammals also undergo torpor e.g. Shrews. Estivation : It is a state of dormancy in response to high ambient temperatures and/or danger of dehydration. It is also called summer sleep. Some species of both vertebrates and invertebrates exhibit estivation. Well known as estivators are African lungfish (Protopterus). Hibernation : It is a state of deep torpor, or winter dormancy, in animals in cold climates, lasting weeks or months. Hibarnation is a well regulated physiological state that permits survival during most unfavourable part of the year. In this state the body temperature is greatly lowered and the metabolism, respiration, heart rate are greatly reduced.

27 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof As the ambient temperature goes below the critical temperature the heat production is increased to keep the body temperature normal. To have this, more food is required. In the winter, the food supply is short. So the animal cannot consume adequate food necessary to increase the metabolism so that the body temperature is kept constant and the animals will succumb to cold. In such a situation, the animals give up homeothermy and pass into the state of hibernation and remains dormant. It remains in this state during the unfavourable condition. When the environmental condition becomes favourable it awakes and becomes active again. Hibernating animals It is seen in some mammals are birds. Pearson classified homeotherms into obligatory, stubborn and indifferent. Some members of the stubborn and indifferent homeotherms truly hibernate. Mammals that hibernate are some monotremes insectivores, rodents and bats. Preparation for hibernation Hibernation usually occurs annually and the animal prepares itself for hibernation by accumulating food. In hibernating bats circadian rhythm in body temperature and metabolism has been observed and this rhythm has been found to disappear as hibernation continues. Ultradian rhythm, however, has been found to persist for at least four years in hibernating golden-mantled squirrels. The animal gradually enter into hibernation when the ambient temperature is below a critical temperature. Many mammals and a few birds regularly hibernate in each winter. The body temperature drops almost to the level of surroundings. Heart rate, respirations, metabolism and many other functions are greatly reduced. They show little response to external stimuli with the active life suspended, they can survive a long winter. Most animals that hibernate are of small size. Thus many rodents, hamstars, pocket mice, hibernate. insectivores at high lattitude, (e.g. hedge hog) bats hibernate. Humming birds, insect eating swifts and some mouse also hibernate. After the hibernation period is over, they revert back to prehibernation state. This is called arousal. State of hibernation In the state of hibernation the plysiological status of the animal is some what different. 1. The body temperature is very close to the ambient temperature and rises and falls with it. The colon and esophageal temperature may be 2–3 0 C above air temperatures. When air temp is 0 0 C, animals tend to hold their body temperature at about 2 0 C and the 0 2 consumption increases. During hibernation, the thermostat activity of hypothalamus is reset at a low level as 20 0 C or more below normal. At ambient temperature between 5 0 C – 15 0 C, many hibernators keep this body temperature as little as 1 0 C above ambient temperature.

28 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof During hibernation, the thermoregulatory control operate at a low set point and with a reduced sensivity. 2. Prolonged period of suspended respiration develops, the rate of breathing may be reduced to even one or less per minute. Due to reduced respiratory exchange blood of many hibernators become acidic. This acidosis may further lower enzyme activity due to departure from optimal pH of metabolic enzymes. 3. Heart rate is markedly reduced. In case of active ground squirrel whose heart rate is 200 – 400/minute drops to 7–10/minute in the hibernating state. The P – T interval of ECG in lengthened. Block of conduction may result in uncoordinated beats, vagal stimulation has been found to show no effect on heart. That hibernants can maintain there physiological functions at a low temperature is an adaptive phenomenon. For example, impulse conduction through nerve is blocked at 9 0 C in rat (non-hibernant) but at 3·4 0 C in hamstar (hibernant). Na-pump mechanism is almost completely inhibited at 5 0 C in non hibernants, but in hibernants it still persists. 4. Blood flow is reduced, cardiac output shows a small decrease and that is accompanied with decreased heart rate. Stroke volume, however, remains unchanged, WBC count may be reduced and hematocrit slightly diminished. Clotting time has been prolonged it is due to a decrease in prothrombin. Serum Mg ++ has been found to be high. Metabolism is reduced by 20 – 100 times. The RQ corresponds to fat. 5. Activity of nervous system does not entirely stop when the body cools. Citellus shows low amplitude cortical waves at 5 0 C and at a brain temperature of 6·1 0 C it can still localize sound, erect pinae, vocalize and move. 6. Hibernation can last for weeks or for several months in cold climates. Many hibernators arouse periodically (once a week or every four to six weeks) to empty the bladder and defecation. 7. Some hibernators become temporarily resistant to X-irradiation. 8. It is a state in which dormany or torpor occurs that is much more pronounced than deep sleep. Hibernating animals revert back to original normal state under favourable condition – the term called arousal. The time taken to go into hibernation is often much higher than the time taken for arousal. In ground squirrel, the time taken for attaining the peak torpid state is about 12 – 18 hours but arousal requires less than 3 hours. The hibernators are usually small. There are no large hibernators. They undergo winter sleep. This is because, they have less need to save fuel. This is again due to their normal BMR is low relative to their fuel stores owing to allometry of metabolism and fuel storage. Secondly, because of large mass and low BMR a prolonged metabolic effort would be required to raise the body temperature to normal level for a very low ambient temperature. For example, a large bear would require

29 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof at least 24 – 48 hours to warm up to 37 0 C from a hibernating temp of 5 0 C. Warming up of such a large mass would also be energetically very expensive. Arousal from hibernation The hibernant retains its ability to arouse when the ambient temperature increases appreciably. The animal warms up again and returns to the warm blooded state. Arousal from hibernation is a rapid awakening with warming starting from thoracic region. It is a process of self rewarming and does not require external heating. It is a rather rapid process. The rectal temperature can increase by nearly 20 0 C in an hour. Awakening starts with an explosive outburst of heat production and the peak metabolism is maintained for sometime (e.g. 1/2 hr for marmot) after which the heat production subside gradualy into its basal state. The rewarning during arousal from hibernation is most expensive. During arousal, heat is produced by violent muscular shivering and oxidation of fat in the brown adipose tissue. Brown fat or brown adipose tissue are present in smaller or larger patches along the neck and between the shoulders. These cells are filled with fat and with large mitochondria (responsible for brown color— hence the name brown adipose tissue or brown fat.) The tissue has rich blood supply and connected by sympathetic nerves. Here oxidation of fat yields high amount of energy because in these cells uncoupling of oxidation and phosphorylation occurs. It is caused by a protein called thermogenin (MW 32 kDa). Hence, heat is produced at a very high rate. Besides fat it also oxidizes other substrates supplied with blood. The heat production is turned on by noradenaline or by nervous stimulation. This brown fat is found in all hibernating mammals, but it also occurs in many new born mammals including man. It is rather peculiar that they are not seen in a number of birds which regularly hibernate. In most mammals, the brown fat has been found to be lost but it remains in hibernators. The rewarming occurs differently in different parts of the body. It has been seen that the anterior part of the body that contain vital organs like heart and brain warms much faster than the posterior part. In this respect, rewarming of the heart at the begining is not only essential but must be an initial step because the proper functions of the heart is needed to supply circulation of oxygen for all other organs. The major mass of brown fat are also located in the anterior part. It has been observed that the reheating process in the posterior part begins only when the anterior part has reached near normal temperature. Control of hibernation Hibernation – arousal is the cyclic phenomenon operating in hibernating animals. It is regularly controlled. Neuroendocrine involvement has been well documented in this process. A circadian rhythm has been noticed. The begining of hibernation cycle is usually

30 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof associated with a particular time of the year, but it is not necessarily induced by low temperature or adequate food. Yearly cycle of hibernation is influenced by the duration of the daily light cycle and is also associated with a dark cycle. A well regulated heat production require a well coordinated Central Nervous System (CNS). e.g. European Hedgehog keep the body temp. at +5 0 to +6 0 C as the ambient temperature goes below freezing temperature. Aes imilar well regulated torpor has been observed in West Indian humming bird Eulampis jugularis which like other humming birds readily becomes torpid. The body temp. of Eulampis, when torpid, approaches air temperature but if air temperature drops to below 18 0 C it results for the fall (in body temperature) and keep the body temp at 18 – 20 0 C. It is seen in hamstars that in case of lesion in post-hypothalamus, they fail to enter into hibernation. However in case of lesion in anterior hypothalamus, the ground squirrel may enter into hibernation but they fail to arouse. Adrenalectomized animals do not hibernate and in hibernation adrenal cortex is depleted. Hibernating animals do not respond by thermogenesis to injection of norepinephrine. 2.4 Suggested questions 1. Explain homeothermic and poikilothermic animals. Discuss the role of hypothalamus in the regulation of body temperature. 2. Write briefly on the regulation of body temperature in poikilotermic animals. 3. How body temperature in regulated in aquatic animals. 4. Describe how body temperature is regulated in hot/cold environment. 5. Comment on temperature regulation in infants. 6. Write a note on behavioral regulation of body temperature. 7. How body temperature is regulated in non sweating animals 8. What is hibernation ? Write a note on the preparation for hibernation. 9. What is arousal ? Describe the process. 10. Write briefly on the neuroendocrine involvement during hibernation.

31 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Unit 3 ? Communication among animals Structure 3.1 Bioluminescence 3.2 Pheromones and other semiochemicals 3.3 Audio signal 3.4 Suggested questions. 3.1 Bioluminescence The emission of light of visible spectrum by living organism is called bioluminescence. It is a process in which living organisms convert chemical energy into light. Luminescent organisms have been observed throughout the ages. Christopher Columbus during his historic voyage across the Atlantic noticed mysterious patches of luminescent light around the water of his ships. Aristotle observed that the flesh of dead fish and damp wood appear to luminescence. Subsequently it was shown that glow in dead flesh was dependent on oxygen. Raphael Dubois was the first to isolate the light producing chemicals from 'clams'. This paved the way for the characterization of the molecular chemical and physiological mechanisms behind the process. Bioluminescence has been observed in thousands of species including bacteria, fungi, and marine animals. Bioluminescence can be defined as the emission of ecologically functional light by living organisms. It is primarily a marine phenomenen with a few exceptions seen in freshwater and terrestrial organisms, light emission plays an important role in the life of bioluminescent animals. Types of bioluminescent animals Many organisms produce light with the help of photogenic organs, tissues or cells. This is called self-bioluminescence e.g. fire-flies. Some other organisms emit light which is actually produced by some symbiont bacteria present in their bodies. This is called hetero-bioluminescence e.g. Loligo and some fishes. Bioluminescent organisms Although it is basically a marine phenomenon many animals exhibit this. It is seen in Protozoa, Cnidaria, Annelida, Arthropoda, Mollusca, Echinodermata, Protochordata and Fishes. It is not seen in terrestrial vertebrates. Photogenic devices In some cases, e.g. protozoa the chemicals and enzyme required for light

32 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof production are diffused throughout the cell; in ophiuroids the light producing apparatus is present in photogenic cells present in body surface. In higher organisms definite photogenic organs are present which produces light. These organs have been best studied in insects and fishes. These are paired organs in insects being located in the head and abdominal segments. Typically, each photogenic organ has a layer of photocytes containing mitochondria, required chemicals and enzymes for chemical reaction. These cells are arranged cylindrically at right angles to the translucent cuticle which permits light to pass through it. Behind the photocytes, there is a reflecting surface chiefly consisting of urate granules. They receive oxygen through air tubes or tracheoles (Fig. 3.1). A number of fishes, mostly deep sea forms, possess charasteristic luminescent organs called photophores. These organs are probably specialized gland cells of the epidermis. They show considerable variation in their number and mode of distribution on the body. These glanduler structures generally occur along the lateral and ventral sides of the body and head. They may be arranged in one or two rows extending on the sides from head to tail as in Scopelus and Halosauropnis or they may be located in some limited parts of the body. Besides these organs, a few large and complex

Fig 3.1 : Photogenic organ of a fire-fly. (After Chapman) Nerve Trachea Reflector layer Urate crystal Photocyte Tracheole Luciferin granule Tracheal end cell Mitochondria Epidermis Cuticle

33 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof organs may be present on different parts of the body e.g. suborbital organ in Opostomias micripnuhs, Scopelus benoitii and Pahystomias microdon. Large photophores present on the elongated first finrays of pectoral, dorsal fins of Angler fishes. In toad fish a large number of photophores are present along the lateral line. Fishes with luminescent organs have world wide distribution. Majority of them are bathypelagic living at a median depth (500 – 2500 meters) and sometimes migrate to the surface at night. Some species are also found in deeper waters. Luminescent organs are of two main types. Some species are self luminous. In others the light is produced by symbiotic bacteria. Self luminous photophores are found in some elasmobranchs (Squalidae and Torpidinidae) and in the teleost belonging to the families Stomiatidae and Myctophidae (or Scopelidae). These photophores are simple and complex. The simple photophores consist of a series of radially arranged glandular tubules that receive branches from the adjoining cranial and spinal nerves. In more complex photophores additional structures like a reflecting layer and a lens like structure also develop. The suborbital organ of Pachystomias microdon is quite complex in structure. It is a cup like structure and its wall is composed of several concertric layers. Externally there is a layer of black pigment and numerous glandular tubles are present in the cup. A thick layer of light reflecting spicules is present in the cup where axial part is full of a number of radially arranged glandular tubules. The mouth of the cup is occupied by a lens-like structure and the skin forms a covering like an iris diaphragm over it. The organ is supplied by a branch of fifth cranial nerve. Both simple and complex photophores may be present in the same species. Luminescent organs in which light is produced by symbiotic bacteria are found in a large number of species belonging to different families like Malacocephalus laevis, Monocentris japonicus, Photoblepharon, Anomalops, Leiognathus and the Angler fishes. Structurally these photophores consist of a large number of glandular tubules that secrete luminous bacteria. In some genera e.g. Malacocephalus, the highly vascular gland opens by a duct on the ventral surface of the fish in front of the anus (Malacocephalus), species of some genera (Photoblepharon and Anomalops) possess an elongated luminescent organ below each eye. It consists of numerous long parallel glandular type with rich blood supply. The organ has pores opening to the exterior at the anterior end a ‘reflector layer’ at the hind end. Since these luminescent organs produces light for long periods due to bacterial luminescence, mechanical devices have developed to turn the light on and off. In Anomalops, the light is cut off by moving the luminescent organ downward by a hinge, so that it comes in contact with a black pigmented tissue. In Photoblepharon a fold of black tissue is drawn up like an eye lid over the organ to cut off the light.

34 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Bioluminescence reaction Reaction occurs between the substrate luciferin and the enzyme luciferase in presence of oxygen. Bioluminesence is an enzymatically catalyzed chemiluminescence. In chemiluminiscence the reaction releases energy but instead of being lost as heat or coupled to some synthetic reaction the energy is used for the specific excitation of a molecule capable of releasing the energy as a photon. The energy of the photon is not fixed, but depends upon the color of the light. The energy, E, is given by the fundamental equation E = hv. when h is the planck’s constant and v is the frequency. For bioluninescence, where the light is in the frequency range correspoding to wave length between 450-600 nm. the energy involved ranges from about 65 – 45 k cal per mole of photons (an einstein). French scientist Dubois coined the term luciferin (color peaning) which is the substrate and the enzyme called luciferase that catalyzes the reaction. Luciferin and lucifeurase are now used an generic terms to refer to the substrate and the enzyme involved in any bioluminescent reaction. Although different specific molecular species are involved in each different class or group of organisms, the general reaction mechanisms have certain close similarities and thus can be represented by a generalized equation. Luciferin is a heat stable, heterocyclic phenolic compound with a molecular weight of 280 and an emperical formula C 15 H 8 N 2 S 2 O 3 . There are different types of luciferins like bacterial luciferin, Dinoflagellate luciferin, Vargulin, Coelenterazines, Firefly luciferin. Luciferases are heat-sensitive enzyme. Firefly luciferase belongs to acyl adenylate thioester forming super family. The enzyme of different organisms have different amino acid composition and structurs. For exemple pure bacterial luciferase is a simple protein with MW 79 k Da. Fire fly luciferase has a MW of approximately 100 k Dal. with two different subunits and one active centre per molecule. Giese (1973) proposed the following scheme of reaction within photocytes in firefly. 1. At first luciferase catalyzes reaction between luciferin (LH 2) and ATP, the products formed are Adenyl-luciferin and pyrophosphate. LH 2 + ATP ————— ? LH 2 – AMP + PPi luciferase 2. Next, the enzyme catalyzes oxidation of adenyl-luciferin by atmospheric oxygen with the production of high-energy adenyl-oxyluciferin and water LH 2 – AMP + ½ O 2 ————— ? L – AMP* + H 2 O luciferase (High energy adenyl-oxyluciferin)

35 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof 3. In the next step, high-energy adenyl - oxyluciferin spontaneously returns to ground state liberating its energy in the form of light. L–AMP * ———————— ? L – AMP + light. (Ground state) 4. Later, adenyloxy-luciferin breaks down into oxyluciferin + AMP LAMP ———————— ? L + AMP (Oxyluciferin) important characteristics of the reaction : (a) Luciferase acts best at a pH of 7·8 (b) The activity is highest at 23 0 C. (c) Atmospheric oxygen and ATP are required for their reaction. (d) Divalent cations like Mg ++ , Mn ++ or Co ++ stimulate luciferase action and the ionic environment probably also controls color of the light produced. (e) Different types of luciferin – luciferase control the colour. Physical properties of colours : (a) The light produced is not hot, it has a temperature below 0·001 0 C. (b) The light is free of UV and infra red rays. (c) Intensity of light is low. (d) Wavelength of light ranges from 5000 – 6000 Å. (e) The light may be of different colours. Fig 3.2 : Important components in the firefly bioluminescence cycle. Luciferin Luciferase regenerating areactions Oxyluciferin

36 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof The annelids give blue light. Amongst fireflies, Fulgora gives white light, many species produce yellowish green but Pyrophorus gives out green light from thorax, and red light from abdomen. Fishes produce blue or bluish-green light. The Dragon fish, however, emits red light. It is known as dragon light. It is peculier that only dragon fish can perceive red light. This allows them to haunt unseen and find prospective mates without alerting their own enemies (Fig. 3.2). Some organisms emit light continuously but some emit flashes that range in duration from about 1/10th of a second to 10 seconds. Regulation of bioluminescence Mechanical, nervous and hormonal processes control light production. In protozoa, mechanical stimulation by waves in the sea stimulates light production. In higher organism nervous regulation exist. In insects, when the nerves supplying photogenic organs are stimulated acetylcholine is produced. It reacts with ATP and CoA and forms acetyl CoA which in turn stimulates luciferase action. In fishes, photophores are under the control of Vth cranial nerve and spinal nerves. Adrenaline has also been found to stimulate photophore activity. Importance of bioluminiscence 1. Light flashes help the organism to find out prey, to escape from danger to puzzle and frighten their enemies, to attract opposite sex. Many deep sea fishes illuminate the surroundings are thus help to find out preys. Many organisms attract their preys by light flashes e.g. deep sea fish, fireflies. Deep sea fishes can frighten their enemies by sudden flashes of light. Flashes of light may act as a mating signal. 2. There are several well known instances of bioluminescence during sexual behaviour. In some cases the light plays a part in the timing of reproduction and in synchronising the activities of males and males. The mating of fireflies often depends on a very accurate signaling system, the males flash their lanterns as they fly approximately 50 cm above the ground if a female sees one of these flashes within 3–4 meters, she may be expected to flash back after an exact interval of 2 sec at 25 0 C. This attracts the male in her direction. 3. Luciferin – luciferase system is a highly sensitive test for detection and determination of ATP. In the laboratory pure firefly luciferin and luciferase are used to measure minute quantities of ATP by the intensity of light flash produced. As little as few picomoles (10 –12 mole) of ATP can be measured by this processes. In can also be used as a sensor for the determination of intracellular calcium concentration in the micromolar to nanomolar range. 4. Bioluninescence can help in detecting energy problems in human cells. This technique is now used to study abnormalities like ailmens of heart, muscular dystrophy, urological problems etc. It has been found that injection of luciferin and luciferase exhibit different reactions in normal and cancerous cells.

37 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof 5. In pest management it is used as a tool for mapping the distribution pattern of organisms. 6. The bright luminescence generated from luciferase assay made it ideal for sensitive non-radioactive assay. 7. It helps in the detection of life in an unknown planet. Presence of ATP an indication of life, is ditected by bioluminescence technique in the soil of an unknown planet. 8. This technique is used in the study of genetics. It is used to detect the presence of some gene in a cell and to determine whether the gene is turned on or off. It has been shown that taking a single gene from the jelly fish aequorea and attaching it to the gene of another organism make the cell glow green when that gene is turned on. The jelly fish gene encode a protein called green fluorescent protein (GFP). When the jelly fish is disturbed, Ca ++ bind to aequorin and produces blue light in the absence of GFP, but in presence of GFP green light is produced. The scientist Charles was able to get GFP to shine green in the absence of Ca ++ and aequorin by simply shining a blue light on it. This discovery has a broad application in the area of genetics. 3.2 Pheromones and other semiochemicals The term 'pheromone' is originated from the greek words 'pherin', to transfer, and 'hormone', to excite. It describes a class of chemicals that are communicated between animals of same species and that elicit stereotyped behavioral or neuroendocrine responses. Some pheromones, called releaser pheromones, elicit an immediate response. While others are termed as primer pheromones, which induce long-term changes in behavioral endocrine state. Pheromone induced responses are mediated primarily through vomeronasal organ (VNO). It is also known as 'Jacobsons's Organ'. It is part of the olfactory system. It is present in a variety of non-human vertebrates as well as in humans. VNO was first discovered by Ruysch (1703). He was a military doctor. He found it in a soldier with a facial wound. It was named as Jacobson's organ after the name of Jacobson, who discovered it in animals. Since 1985, many scientists were able to demonstrate VNO in most humans. VNO is located bilaterally on the anterior third of the floor of the nasal septum. It opens into the nasal cavity by a pit which varies in size from 0.2 to 2 mm. It is situated 1-2 cm. from the posterior margin of the nostril. It is lined by a pseudo-stratified columnar epithelium, 60 ?m in depth that lie on a thick basement membrane. The VNO neuro-epithelium contains three cell types— (i) Small polygonal dark staining basal cells measuring about 6 ?m in diameter, called Basal cell.

38 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof (ii) Tall cylinder/columner cells with densely staining cytoplasm called Dark cell. (iii) Tall columner calls like the dark cells, but lightly stained, hence called Light cells. All these cells differ from surrounding respiratory epithelium in the nose by having no cilia. Compounds occurring naturally on the human skin were found to cause a local depolarisation when applied directly to the VNO. This depolarisation had the characteristic of a receptor potential. Subsequently it was shown that these compounds were 16-androstenes and estrenes. These compounds did not produce response from olfactory epithlium. Moreover, olfactory stimulant (e.g. cineole) has no effect on VNO. A pheromone called vomeropherin (pregna-4, 20-diene-3, 6-dione (PDD), caused evoked potential in VNO and also changed gonadotropin pulsatility in males, resulting in a reduced level of LH and testosterone. PDD also decreased respiratory frequency, increased heart rate and also caused event related changes of EES pattern. Stern and McClintock (1998) have shown that odourless axillary compound from the armpits of women in the late follicular phase of their menstrual cycle accelerated the preovulatory surge of LH of recepient women and shortened the menstrual cycles. Axillary compounds of the same donor collected later in the menstrual cycle (at ovulation) showed the opposite effects—they delayed the LH surge of the recepients and lengthened their menstrual cycle. Savic et al (2001) showed that 'androstadienone' (a human, in particular male secretion) caused activation of hypothalamus of women (gender-specific action), but not in men. It also activates anterior part of the Inferior Prefrontral Cortex (PFC) and the Superior Temporal Cortex (STP) (in addition to olfactory area). The PFC and STP have been implicated in aspects of attention, visual perception and recognition and social congnition. A pheromone can act as a reinforcing agent or a one-trial conditioning agent in which the presence of a pheromone converts a second odour (that of the partner or infant) in conditioned stimulus. Mice can distinguish one another by odour. This odour is genetically determined and partly specified by the H-2 major histocompatibility complex (MHC) gene located on chromosome 17. Genes located on chromosome Y also regulate production of some odours. The human equivalent of MHC locus is HLA (Human Leucocyte Antigen). There are odourous substances secreted in the mouse urine. These odours many play a part in pregnancy block (Bruce effect), aggression and other mouse social behaviours. It is held that, in human, axillary odours have chemical differences which makes the discrimination possible. Some of these individual specific odours may be under the control of HLA genes. Studies have shown that women prefer those male odours that have HLA types different from their own. However, this preference is reversed if they use oral

39 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof contraceptives. Schaal (1980), reported that, mother could recognise their own newborn infant from the smell of a previously worn T-shirt. Infant also prefer breast or axillary pads from their own mothers, distinguishing the odour from other kin. Receptors or at least their own RNA, for phenomones have been found in the human olfactory epithelium. However, presence of pheromonen receptor protein expressed in the surface membrane of an olfactory receptor neurone and the response of this receptor protein to a ligand (a potential pheromone), have not been convincingly observed as it is in case of olfactory receptors. Androstenone is the male human pheromone, that helps to attract women. In practice, it is blended with favourite cologne to produce a cologne odour that will attract women. Similarly, androstenol is the female human pehromone, that helps to attract men. It has been observed that once men detect the female human pheromone scent, they subconsciously become more attracted, more receptive an more willing to offer attention. Regarding the mechanism of action, it is held that the chemical scent triggers the part of the brain, where the sexual attraction feeling starts. Once, the powerful feeling of sexual attraction sets in, it moves a person generally more attentive and responsive to the person, who is the source of that sexual attraction. In humans, pheromone production is primarily linked with secretion of apocrine glands of skin, other glandular secretions and the moist areas of the body like axillae, mouth, feet and genitals. Freshly produced apocrine secretions are odourless. They are transformed to odourous products by microorganisms. The type and density of cutaneous microorganisms on different areas of the body interacting with skin and other glandular secretions give rise to a variety of odours from various body sites. Types of pheromones The pheromones are of different types like sex attractant pheromone, fear pheromone, aggresive pheromone, marker pheromoen etc. 1. Sex attractant pheromone : In case of Gypsy moth, the female secretes pheromones which is perceived by thousands of males with the help of their antenae which bear olfactory epithelium. Sensation followed the releaser pathway to manifest behavioral changes. This pheromone can attract thousands of males from a distance of half a mile. This has also been found in humans. It has been possible to synthesize it in the laboratory for use in pest control. Similar female sex attractant pheromones are also secreted by male animals, e.g. a secretion from salivary gland of male boar, a secretion from preputial gland of musk deer. 2. Fear pheromone : It has been observed that if an animal is frightened, fear pheromones are produced. If such a ‘frightened animal’ is put in a cage having normal animals, the latter are also frightened. These pheromones are secreted in the urine of frightened animals. If a smaple of such urine is applied to a normal individual in a small quantity, the normal animal get frightened.

40 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof 3. Aggressive pheromone : It is particularly found in male mammals. If some adult and sexually mature bandikoots put in a single cage, they become aggressive to each other. They start fighting and eventually might die. This pheromone is secreted from prepuital gland. 4. Marker pheromone : Some animals secrete marker pheromones which help them to identify their territory. It is secreted from sebaceous gland of female blacktail deer and from anal gland of tigress. 3.3 Audio signal The production and perception of sound is not only a means for communication it is also important for detection of environment and navigation. Some animals have both sound production and sound detection systems, some may have either. Many animals can hear but cannot communicate with sound. A source of sound can serve as a warning even in those animals which cannot produce sound but is capable of hearing. In cases where sound is used for communication the production is often restricted to one sex, usually male and the principal function of sound is related to male attraction and maintenance of teritory. Animals use various types of sounds for various purposes but all are related to better survival. Insects can produce and perceive a wide variety of sounds which are used for communication often with the opposite sex. Among invertebrates, communication through sounds are limited to a few groups of insects, especially Orthoptera, Hymenoptera, Cicadidae and perhaps some Crustaceans. The first two groups produce sound by stridulation, rubbing a toothed structure accross a ribbed plate. This results in the production of a burst of pulses extending upto 40 – 50 KC / sec. Cicadas produce sound by vibrating a thin section of cuticle, some decapod crustaceans make sound by thumping the substrate with their pinchers. Hearing is more widespread and is accomplished most commonly by a modified tracheal structure, the tympanic membrane. This is called tympanic organ. This may be located in the legs in Orthoptera, Arachnida and Crustacea in the thorax or abdomen as in Lepidoptera and Homoptera or in the antennae as in Diptera. The number of receptor neurons also vary for 2 in case of Moth to 70 or more in the Locust, but they all respond to the same frequencies determined by the properties of the tympanic membrane. In these cases there may not be any frequency discrimination but sensitivity vaires and intensity discrimination is possible. The principal function of these organs is to detect a sound, its recognition apperently by the number of bursts per secound and its localization. Some insects respond to sounds in human audio range. They also respond to ultra high frequency sound as are produced by bats. Sound reception and

41 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof its processing help a moth to escape its predator bat. Cicadas sing species-specific songs conisting of complex pattern of clicks and rasps. Courtships and rivalry calls of male crickets can be identified as species specific, each produced by a patterned sequence of muscle contractions. Male lycosid spiders produce courtship and threat sounds by scraping the palps, the frequency of the courtship sounds increases when a female displays legwaving. Some species of bees signal in the hive, the location of feeding places; honeybees also produce sound as a part of communication from foragers to other workers; the sounds are produced by skeletal movements caused by contraction of wing muscles at 250 Hz. Duration of each sound has been found to be related to the distance between the hive and the food – duration is short if the distance is less and long if the distance is more. Sound vibrations can be received by Johnshon's organ present in flies. Vibrations in substrate water and air are important to insects as warning signals, aids to locomotor and posture control and communication. Sounds serve many orthopterans for social communication. Coding of sound in insects is mostly dependent on temporal patterning of pulses by amplitude and not much by frequency and harmonies as happens in case of vertebrates. Vertebrates Among the vertebrates, the most sophisticated use of sound for communication is performed by birds and mammals. However, other vertebrates also use sound in different functions. In case of aquatic animals, fish have a variety of sound receptors like skin, lateral line, and three labyrinthine chambers. They use the first two to detect displacement and low frequencies. Several teleost fishes produce drumming granting or scraping sounds others, produce sound by vibrating there air bladder schooling calls and the sound possibly serve simple communicative functions specially in intraspecific alarm and in reproductive behaviour. The sounds are mostly produced by contraction of muscles along the swim bladder or between the pectoral girdle and the swimbladder. A few kinds of fish produce sound by resonating the swim bladder. Elasmobranchs lack swim bladder, yet they respond to low frequency sound even after their lateral line is damaged. However, usefulness of sound is limited because most fishes have poor hearing capability. They respond only to low frequency upto about 1000 cps at high intensity. This is because they do not have a specialized cochlea or any means to concentrate acoustic energy on the otolith. Some fishes have Weberian ossicles (Weberian apparatus) linking saccules and airbladder. Airbladder acts as a tympanic membrane, pick up vibrations in water. These fishes hear sounds upto several thousand cycles/sec and are several thousand times more sensitive than fish without weberian ossicles. Frogs emit mating calls which are species specific. Cetaceans can detect direction of sound in water. A

42 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof porprise (Tursiops) with its eyes covered has been found to emit sound pulses 1 – 1·5 msec duration, repeated about 16/sec while cruising ; and accelerating to 190/sec when near a reflecting object such as a fragments of fish. The whales emit sound which may constitute songs lasting several minutes and which are repeated many times. An individual whale may be distinguished by its song, the intensities are high. In the layer of water frequented by whales, the songs may carry for many miles. Terrestrial vertebrates The terrestrial vertebrates have found much more complicated uses of sound. Sound is produced by devices which are used in passing air via respiratory passages. The nature of the sound ranges from simple hiss of reptiles to the resonating vocal sacs of amplihia, the syrinx of birds and the larynx and vocal cords of mammals (with lips and tongue helping in man). However most of the anurans in amplibia do not make sounds, among the reptiles only the lizard of the family gekkonidae utilize sound for communication. Even in birds and mammals, nearly all can hear well, though there are some which are usually mute. Animals show maximum sensitivity within a restricted range of frequency. Frogs show maximum sensitivity between 3,000 to 4,000 cps. In the bull-frog response is greatest in the range of 100 – 200 cps, which is used as a mating call. Snakes and most lizards have less well developed ears and respond only to local sounds of a few hundred cps. The hearing mechanism in birds and mammals are highly developed. Behavioral and electrophysiological studies revealed that they have same absolute sensitivity, differential frequency and intensity determination and dynamic range. The principal mechanism of sound perception in birds and mammals have been described. When the sound wave strikes the tympanic membrane, it vibrates. These vibrations are amplified and transmitted through the ear ossicles in the middle ear to the oval window of the internal ear. This causes vibrations of the basilar membrane. The auditory receptors present here are excited (hair cells in the organ of corti). As a result, nerve impulse is generated in the auditory nerve (cochlear division of VIIIth nerve) by auditory transduction. These impulses reach the auditory cortex and thus perception of hearing occurs. The perception of sound involves perception of pitch, intensity and direction of sound. In mammals (except bats) cochlea is coiled. It has 2·75 turns in man, 3 turns in cat, 4 turns in pig and guineapig. Frequency perception range in man is 16 Hz to 20 KHz with maximum sensitivity between 1KHz – 3KHz. Some dogs can hear upto 35KHz and rats and guineapigs can hear upto 40 KHz. Primates hear better at low frequency than mammals such as opossum and hedgehog. Significance of audio signal In general, sound is a basis for species recognition, communication, predator detection and echolocation. Insects produce and perceive a wide variety of sounds

43 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof which are used for communication often with the opposite sex. Many fish produce sounds that are used for communication. Frogs emit mating calls which are species- specific. Baby mice and other rodents call their mother particularly in cold using ultrasonic calls of 60-90 Kc/sec. A general function of calls in song birds is for the establishment of territory and for the attraction of a mate. Song-patterns have species characteristics that are genetically determined. Crows are gulls, have a variety of calls like— alarm, distress, assembly, chorusing are others. Cave dwelling birds use echolocation. Vocal signal is the best way for communication in humans. Vocal signals are used by them not only for communication, but also for scolding, quarrelling etc. Humans also use sound in the field of industry and medical diagnostic centres. Ultrasound is used not only for diagnosis, but also for treatment. Infrasound Recently it has been discovered that homing pigeons can detect very low frequency sound as low as 0.05 Hz (1Hz is 1 cycle per sec ; 0.05 Hz corresponds to 1 cycle per 20 seconds). This response to infrasound is lost or reduced if middle or internal ear is damaged. Such responsiveness is very important in birds because infrasound is produced during thunderstorms, earthquakes, and wind over mountain ranges. Since infrasound are attenuated much less in air (attenuation of sound is inversely related to the square of the wavelength), it travels over a long distance and thus can be detected hundred or even thousand kilometres away. This helps the birds to determine the direction. The elephant can communicate with each other using infrasound. Because the infrasound undergoes very little attenuation in air, these may be audible for communication specilly in a forest habitat. Some animals show abnormal behaviour prior to earthwake and this may be related to perception to infrasound. Perception of infrasound and its use for correcting direction and to make communication has been observed in birds and elephants. Echolocation The power to localize an object using the echo of a sound is called Echolocation. It is a complicated process. It is comparable with audiolocation devices and rader device invented by man. Animals that echolocate use echoes of sound they produce to locate the objects in its path. Some animals both aquatic and terrestrial are capable for echolocation. It is particularly well developed in bats but exists also in other animals notably whales, dolphins, shrews and a few birds. Origins of echolocation The ability to detect and understand the environment is essential for survival. Special sensory devices like vision, hearing, taste and smell can be used to know the environment. For locating an object in the environment vision/light and hearing

44 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof sound are very much helpful and in the dark, echolocation is the only correct device available for this purpose. Hence, animals which have to move in the dark have developed echolocation devices to locate an object in its pathway and this helps in capturing a prey in navigation. Basic principles of echolocation The animal produces a sound which strikes an object in its pathway and produces an echo. The echosound reflected from the object is detected by the auditory apparatus, analyzed and thus the position of the object is ascertained. Echolocation in different animals Sound waves travel at a slower rate in air compared to water. The intensity of sound is more quickly attenuated in air compared to water. High frequency sounds are more rapidly attenuated. The higher the frequency of a sound the shorter is its wave length. There is an inverse relationship between frequency of sound waves and the size of the object that can be detected by its echo. Hence, detection of small-sized object requires high frequency sound. Frequncy also influences directionality of hearing. High frequency sound is capable of detecting the direction of sound more accurately. In view of these different properties of sound the members of animal kingdom adapted different mechanisms for echolocation depending on their habitat and requirement. All animals do not echolocate and different types of echolocating mechanisms have been observed. Echolocation has been found to be well developed and studied extensively in some animals including bats in terrestrial habitat and cetacians in aquatic habitat. The bats emit echolocation signals through mouth (oral emitters) or nose (nasal emitters). Most microchiropteran are oral emitters. Only members of Nycteridae, Megadermatidae Rich Rhinolophidle and Phyllostomidae are nasal emitters. Usually high frequency and high intensity sounds are emitted. The detailed mechanisms of echolocation show variation in different genera of bats. Most bats emit 20 – 100 KHz frequency sound and the an intensity of 110 decibel (in bronze bat). Narrow (CF) and broad band (FM) signals are used. CF helps in detecting the object but cannot exactly localize it, this is done with the help of FM. Insectivorous bats capture an insect by three phases of acoustical orientation. In the first phase sound pulses (100 – 20 KHz) with an interval of 50 msec is sent. When the prey is detected pulses are sent with shorter intervals and this is the second phase. In third on final phase a buzz like sound is emitted, the frequency is decreased duratian of pulse is decreased (0.5 msec) and the interval between two signals is also reduced. Finally the bat scoops up the insect with its wings or in the webbing between its hind legs guiding the insect to its mouth. The quality, frequency, duration and number of occurrence of sound impulse per second show much variation in different genera of bats. The nature of the sound may

45 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof be ‘click’ type or ‘buzz’ type. In vespertiliomidaes the duration of pulse ranges from 1– 4 msec. The frequency may be upto 120 kc/sec but drops slowly. Rate of pulse discharge is less than 10/sec at rest but rises above 100/sec while hunting or avoiding obstacles. In horseshoe bats, the frequency of pulse is 85 – 100 kc/s, duration is high 40 – 100 msec. The pulse repetition rate is slow and it is less than 10/sec. If the size of the object is close to the wave length of sound wave, the reflection of sound is better. Thus the frequency of sound wave emitted by bats depends on the size of the object it has to be detected. To locate small size object higher frequency is required (the wave length of a sound of 30 KHz is about 11·5 mm which is roughly the size of a small moth) There are bats which can avoid or dodge wires with a diameter of 0·5mm. This happens when the wavelength of sound is 5 mm. It has been observed that bats will not be able to avoid hitting a wire when its diameter is less than 1/10th of the wave length of sound. Regarding detection of distance several theories have been proposed. It is proposed that distance is detected from the time difference between the transmission of ultrasound and the reception of its echo. Mohra proposed that distance is understood from the loudness of the object pye opined that the cochlea can understand the difference between two types of notes— the emitted note and the received echo note. Auditory nerves carry the information about the difference between these two notes to the brain. From this difference between these two notes, bats estimate the distance of object. It has been observed that vespertilioned bats can delect insects at a distance of 50 cm – 1 metre in the dark by echolocation. On the other hand the horseshoe bats can detect insects even at a distance of 6 meters. That echolocation is effected through auditory apparatus has been observed. Spallenzam did experiments with blind folded bats and observed that they can capture insects in the dark and the can return to their place of residence for a distance. Griffin noted that blind bats can capture insects which do not produce any sound and they can detect and chase pebbles and cotton spitballs thrown into the air. The intensity of sound emitted for echolocation is very high and can be compared to the sound of jet engine which can damage the ear, but this is necessary because the intensity of sound drops faster in the air than water. It can damage the ear. A deafening mechanism has been observed in bats to handle the situation. When the sound is emitted the tensor tympani muscles attacted to tympanic membrane and the stapedius muscle attached to stapes contract. As a result the malleus is pulled inward away from the tympanic membrane and the stapes is pulled outward away from the oval window. In this way transmission of sound wave to the internal ear is reduced. However if this reduced phase is continued the reception of reflected sound will be disturbed. But this does not happen because these two muscles immediately after contraction relax and thus the ear osscicles get back to their specific position and sound transmission standard becomes normal.

46 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof The bats change their sound frequency during capturing prey of different sizes. They emit sound to 10 kc/sec (wave length 34 mm) to capture large sized prey and sound over 130 kc/sec with wave length of 5 mm to capture small-sized prey. By using echolocation, a bat can capture two seperate mosquitoes or fireflies in about 0·5 sec. The fish eating bats of Trinidad can even use echolocation to find and capture their under-water prey by detecting the ripples that are produced on the surface of water when a fish swims just under the surface of water. Different experimental evidence indicate that a number of morphological and neuronal modifications help the bats in detecting the echoes. The snout in covered by couplex folds and the nostrils are spaced to produce a megaphone effect. The ear develop large pinnae which help to capture echoes. The eardrum and ear ossicles are small and light which provide high fidelity at high sound frequencies. Contraction of the muscles of ear ossicles reduce the sensitivity of the ear during the emission of high intensity sound; immediately the muscles relax and the capability of sound wave transmission reverts back to normal. (This is common in the ear of mammals). The bones housing the middle and inner ear are insulated from the rest of the skull by blood sinuses, connective tissue and fatty tissue. This reduces direct transmission of sound from the mouth to the inner ear. The auditory centres of the brain occupy a very large portion of the brain to receive the auditory signals and through the process of neural computation, construct from the auditory cues, a spatial representation of the external world. Bat-moth predator—prey interaction Experimental results indicate a remarkable series of adaptations by certain moths in response to predation by bats. Nocturnal of some families e.g. Nocteridae have developed sensitive auditory system to receive the frequencies emitted by bats. When these moths detect the approach of a bat, they alter their levels of flight, when the bats become nearer, the moths fly irregularly. When the bats become very close, the sound is very loud and repitition rate is also high. The moths dive directly on the ground. The moths themselves produce ultrasonic sound to detect the attacking predator. Some moths develop a noise-making organ on each side of the thorax. When the moths are disturbed, these organs produce trails of clicks with prominent ultrasonic components. It has been shown in laboratory experiments that flying bats turn away from their targets when confronted with moth-produced pulses. Hence, these pulses protect moths from the bats. The bats sometimes, in case of capturing moths, abandon echolocation for detecting the prey ; but instead listen to sounds produced by the prey. Echolocation signals of bat helps the moth to detect the foraging bats. Moths have been shown to be able to detect the cries of bats at a distance of 30 meters. Echolocation in birds At least two species of birds have been shown to use echolocation. The oil birds

47 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof (Steatornis) of South America and the cave swiftlets (Collocalia) of South-east Asia are not closely related but both live and nest in deep caves. The best known are the oilbirds or guacharos. They have been found to fly freely in the dark caves without hitting the walls or other obstacles. They use sound of 7000 Hz., which are audible by man. The nature of the sound they produce is like that of ticking of a typewriter. It has been observed that if ear of these birds are plugged, they fail to orient themselves in the dark. However, they are able to fly in a lighted room using their eyes. Echolocation in aquatic animals Echolocation has been observed in marine species. Tony shrews use a variety of sounds (30-60 kcps) to explore strange pieces of unfamiliar objects. Both dolphins and whales use echoes to avoid colliding with objects and with the ocean bottoms as well as for finding food. It is very useful in locating food deep under-water when the visibility is very low or zero. A trained dolplin has been found to locate a dead fish in the tank which cannot be seen in man. The dolphins produce sound by vibrations in the nasal sac system near the nasal plugs. That the source of sound is not larynx but nasal has been confirmed using imaging tools such as CAT, MRI, and RET scans. The nasal sac system consists of a series of muscular valves and compliant sacs associated with the blow holes. The muscles associated with these air sacs contract synchronously with the echolocation clicks, while the muscles around the larynx do not. A pair of small, dorsal fatty projection with a lip-like structure, called 'museau de singe' (also called monkey lips) control the passage of air through this system. Cranford hypothesized that the passage of pressurized air past the liplike structure (museau de singe) produce sounds in much the same manner as the glotis in man. It has been suggested that sound waves produced by the nasal sacs are focussed in the forward direction through a structure called melon (which is situated anterior to the monkey lips). It is a lens shaped fatty structure that gives a dome shaped profile to the forehead of many odontocetes. The lipid composition of melon has been analyzed and its acoustic properties suggest that it may serve as an 'acoustic lens' to focus outgoing energy. The echolocation capability of sperm whale has been inferred on the basis of data from other odontocetes. The click of a sperm whale consists of pulses. Clicks lasts for roughly 10 – 20 msecs, and the clicks are rapeated from less than one click per second to 40 per second. The sonar clicks are produced in the front of the sperm whale's had by pnematic action of the 'museau de singe' like that of dolphins. The sperm whale head acts as a sound reflector. Experimental evidence indicate that diving sperm whales use trains of clicks for echolocation of prey. Some small cetaeceans that inhabit turbid water have tiny eyes and presumably are dependent on echolocation.

48 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Ambient noise imaging (ANI) It refers to the use of sound to see underwater. Taylor et al., using powerful computers and models inferred that dolphins might be able to detect using ANI. These models predict that dolphins use ANI to see useful images for tens of meters underwater. It has been suggested that dolphins and perhaps other marine mammals, have a whole new way of seeing with sound. Multiple uses of Echolocation calls Echolocation helps the animal to detect the direction, distance, size and texture of the objects in its environment. It is seen in birds and mammals. It is mostly developed in two groups of mammals. The microchiropteran bats are some cetaceans like porpoises and dolphins. Two groups of birds also use it. Echolocation signals are used by bats for communication, as well as for orientation and locating prey. By modifying signal design some bats exploit habitats. Echolocation calls display considerable geographic variation within a species. L. cinereus in Arizona had calls with a minimum frequency 53·8% higher than the minimum frequency of calls in Menitoba (26·0 kHz versus 16·9 kHz). Eight of 12 species studied showed a difference of 3 kHz in different geographic location. Under certain conditions same bats add an extra pulse to the echolocation calls. It has been observed that when one bat is very close to other bats, the former lowers its call frequency and add a warning 'houk' to the signal. Echolocation calls communicate several kinds of information. Same rely on signals of conspecifics to locate day roosts, mating sites, hibernation sites and feeding areas. The solitary forager E. maculatum in contrast reacted aggresively to the playbacks of calls of a conspecific individual. This bat either attacks the speaker or abruptly moves away. Echolocation calls provide vocal signatures. This is the basis for mother-young recognition in a number of species. In a captive colony individuals recognize each other by listening to echolocation calls. Vocal signatures are individual interactions among foraging bats. A number of species are known to alter their signal design when changing from foraging in open areas to these closer to obstactes. 3.4 Suggested questions 1. What is audio signal ? Write briefly on its different uses. 2. Give an account of transmission and reception of audio signal. 3. What is echolocation ? Write briefly on the echolocation in aquatic animals. 4. Why high frequency sound is used for echolocation ? 5. Comment on the utility of CF and FM band in the echolocations. 6. Write briefly on bat-moth predator-prey interaction.

49 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof 7. What is Ambient Noise Imaging (ANI)? 8. What is biioluminescence ? Write a note on its regulation . 9. Describe the reactions of bioluminescence with a note on the enzymes involved in the process. 10. Write a short account on the importance of bioluminescence. 11. What are phenomones ? Write a note on vomeronasal organ. 12. Write briefly on the different types of phenomenes.
50 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Unit 4 ? Contractile elements, cell and tissues among different phylogenic groups Structure 4.1 Muscle Structure and function correlation. 4.2 Movements—amoeboid, ciliary and flagellar. 4.3 Specialized organs (eg : electric organs and tissues) 4.4 Suggested questions 4.1 Muscle structure and function correlation Muscle cells are specialized cells. They use ATP energy to generate force or to do work. Because work can take many forms like locomotion, pumping of blood or peristalsis, several types of muscles have evolved.

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The three basic types of muscles are skeletal muscle, cardiac muscle and smooth muscle.

Skeletal muscles are attached to bones, spans a joint and thus helps in joint movement and locomotion. They are voluntary. Their activity is controlled by nervous system. They play a key role in numerous activities like maintenance of posture, locomotion, speech, respiration etc. Structure Individual cells are of variable length. They may be as long as 25 cm, but their diameter ranges from 10-80 ?m. Each muscle cell is covered thinly by a connective tissue layer called endomysium. Individual muscle fibres are then grouped together and get a covering of another connective tissue called perimysium. These are called fesciculus. Finally fasciculi are grouped together and they are covered by another connective tissue sheath, known as eipmysium. This epimysium covered structure is the muscle. At the ends of the muscle, the connective tissue fibres join to form tendon which are attached to the bones. All the muscle fibres are not of uniform length and extend the entire stretch. The fibres of shorter length become attached to the connective tissue inter lacing the muscle fibres. More than 600 muscles have been identified in the human body. Some are small and consists of few hundred fibres, but large muscles may contain several hundred fibres. Each muscle fiber is multinuclated, bounded by the plasma membrane called sarcolemma. The protoplasm within is called sarcoplasm or myoplasm. Besides common organelles, the sarcoplasm contain myofibrils and sarcoplasmic reticulum. The myofibrils run along the length of the cell. They show alternate dark (Anisotropic) and light (Isotropic) bands. Proper alignment of the bands give rise to transverse bands and the longitudinal disposition of the myofibrils that gives rise to longitudinal
51 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof striations. These two together gives the muscle fibre a cross-sectional appearance. At the middle of the light band there is a dark line called Z-line. Thus a myofibril becomes made up of Z- line bonded units. These are called sarcomere. Average length is 2 ?m. Each sarcomere is bonded at both sides by Z-line. At the centre there is A-band and on either side there is I-band. The A-band is made up of thick filament, called myosin. The I-band contains the filament called actin filament. At the middle of the H-zone, there is a fine dark line called M-line. The actin filaments extend from the Z-line to the border of the H-Zone. Thus there is an area where both A-band and I-band overlap, or myosin-actin overlap. This overlapping zone is called O-band. Besides actin many other proteins are associated with thin filament. These Fig 4.1 (a) : Anatomical organisation of skeletal muscle from gross to moelcular level (diagrammatic). Fig 4.1 (b) : Top left : Arrangement of thin (actin) and thick (myosin) filaments in skeletal muscle. Top right : Sliding of actin on myosin during contraction so that Z lines move closer together. Bottom left : Detail of relation of myosin to actin. Note that myosin thick filaments reverse polarity at the M line in the middle of the sarcomere (Modified from Alberts B et al : Molecular Biology of the Cell, 2nd 3d. Garland, 1989) Bottom right : Diagrammatic representation of the arrangement of actin, tropomyosin, and the three subunits of troponin (I, C, and T).

52 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof include (a) Troponin, complex made up of 3 subunits, called troponin I, attached to actin, troponin T attached to tropomyosin and troponin C that attaches with Ca 2+ . (b) tropomyosin, (c) tropomodulin, (d) a-actinin, (e) cap Z protein, (f) titin. Titin extends from Z-line to middle of the sarcomere and attached with myosin. It helps in proper alignmentof actin and myosin. Tropomodulin is located at the end of this filament towards the centre of the sarcomere and may participate in the setting of the length of this filament. ?-actinin and capZ protein serve to anchor thin filament to the Z- line. Additional proteins present in the thick filament are myomesin and C-protein. These participate in the bipolar organisation or packing of the thick filament or both. Desmin binds Z-line to plasma membrane. Nebulin extends along the length of thin filament and may participate in the regulation of the length of thin filament (Fig-4.1). Muscle contraction involves participation of (i) actin, myosin, troponin, tropomyosin system and (ii) sarcotubular system. (i) The contractile proteins actin and myosin as well as other regulator proteins like tropomyosin and troponin are specifically arranged in a sarcomere and take part in muscular contraction. Actin exists in two forms G-actin and F-actin. F-actin is arranged as a two- stranded hotical filament. Tropomyosin covers the actin filament. The actin molecule has myosin binding site. After every seven actin molecule, troponin is present. Troponin-I remains bound with actin, and troponin T with tropomyosin, and troponin C can bind with Ca 2+ . The head of the cross bridge remains very close to the actin molecule. It has ATP-ase activity, ATP binding site and actin binding site. But it cannot bind with actin because myosin binding site of actin remains covered by tropomyosin. (ii) The Sarcotubular system essentially consists of T-tubule which is an invagination of sarcolemma, and longitudinal tubules running parallel to myofibrils. These tubules end in a dialated structure near the T-tubule, called cistern. One T-tubule and two longitudinal tubules with two cisterne form a triad system. In the T-tubule there is dihydorpyridine receptor (DHP receptor) and on the cisterni there is ryanodine receptors which is a calcium channel. Ca2+ remain stored in the cisterni by combining with calsequestrene. In each sarcomere there are two triads each located at the A-I junction (Fig. 4.2). In the resting state intracellular Ca 2+ concentration is low, about 10 −7 moles/litre. ADP+P ; are attached to myosin head (cross bridge). On stimulation, muscle action Fig 4.2 : Showing DHP and RYR receptors.

53 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof potential develops. It passes along the sarcolemma and goes deep into the cell via T-tubule, and it excites DHP receptor. When it is stimulated, the RYR receptor is excited through protein-protein interaction (Fig 4.3). Excitation of RYR receptor causes opening of Ca 2+ channel and Ca 2+ is released. It binds with troponin C, and causes a conformational change of it. This leads to a movement of tropomyosin. As a result, the myosin binding site of actin molecule is exposed. Next, myosin head gets attached to actin. When actin attaches to myosin, the myosin ATPase activity is increased. So ADP and Pi leaves myosin and actin- myosin interaction occurs. The myosin head binds and so the actin molecule is pulled towards the centre of the sarcomere. ATP then gets bond with myosin and due to this binding actin can no longer remain bound with myosin. Actin is removed and myosin head again goes back to its previous state and gets ready to bind with another actin Fig 4.3 : Diagram of the relationship among the sarcolemma (plasma membrane), a T tubule, and two cisternae of the sarcoplasmic reticulum of skeletal muscle (not to scale). The T tubule extends inward from the sarcolemma. A wave of depolarization, initiated by a nerve impulse, is transmitted from the sarcolemma down the T tubule. It is then conveyed to the Ca 2+ release channel (ryanodine receptor), perhaps by interaction between it and the dihydropyridine receptor (slow Ca 2+ voltage channel), which are shown in close proximity. Release of Ca 2+ from the Ca 2+ release channel into the cytosol initiates contraction. Subsequently, Ca 2+ is pumped back into the cisternae of the sarcoplasmic reticulum by the Ca 2+ ATPase (Ca 2+ pump) and stored there, in part bound to calsequestrin.

54 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof molecule. Meantime, ATP is split into ADP+Pi by the ATPase action of myosin-head. But the ATpase action is low, so ADD+Pi do not leave the myosin head, rather remain attached to it. Actin-myosin recycling occurs as muscle contraction occurs. Intracellular concentration of Ca 2+ does not remain high for long time. Immediately after its release, it is taken back into longitudinal tubule by active process. As a result, intracellular Ca 2+ concentration is decreased. As it goes to normal level, 10 −7 moles/lit, Ca 2+ leaves troponin C and goes back to tubule. When troponin C becomes Ca 2+ free, again a conformational change occurs in troponin molecule. As a result, tropomyosin moves and covers the myosin touching site of actin molecule. Here, actin-myosin interaction does not occur and muscle contraction stops and the muscle relax. During muscular contraction, the muscle fibre is shrotened. The actin filaments slide into and in between myosin filaments, and during relaxation the actin filaments slide out of the myosin filaments. The structure of sareomere has been observed during rest and during contraction. Cardiac muscle The cardiac muscle cells are short cylindrical in shape. Typically they measure about 100 ?m long and 10 ?m in diameter, they are uninucleated. They are cross striated like skeletal muscle because of specific alignment of thick and thin filaments. Especiality of cardiac muscle is that, the cells are conneted with each other through intercalated discs. These are low resistance partitions like gap-junctions, Junction adherens. Through these, signal is transmitted from one cell to the next, and thus a functional syncitium is established. The sarcotubular system is well developed like skeletal muscle. It has triad and diad systems. But each sarcomere has one, because it is locked at the Z-line. The ryanodine receptor has a special property leading to a situation called calcium induced calcium release. When action potential passes down the T-tubules, the DHP receptor is activated. As a result voltage gated Ca 2+ channels present here open, and ECF Ca 2+ enters into the cell. This calcium binds with RYR receptor. Such Ca 2+ binding opens the calcium channel present here. This is called calcium induced calcium release. Thus intracellular Ca 2+ concentration is increased and goes above 10 −5 moles/lit. Now, Ca2+ binds with troponin C and finally muscle contraction occurs in the same way as happens in skeletal muscle. It has been shown experimentally that if cardiac muscle fibres are placed in a medium muscle contraction will occur if the bathing medium contains Ca 2+ . But this is not required to cause contraction of skeletal muscle. The mechanism of muscular contraction is also similar to skeletal muscle that is actin regulated or actin based muscular contraction. Smooth muscle Smooth muscle cells are uninucleated, fusiform in shape with tapering ends.

55 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof These are 400-600 ?m long. Their diameter ranges from 2-10 ?m. The thick and thin filaments are 10,000 times longer than their diameter and are tightly packed. The smooth muscle has been divided into two groups : single unit and multi-unit. In single unit muscle, the muscle cells are electrically coupled, through special junctions. These are gap junctions and adherens junctions, connecting the adjacent cells. Through these, signals (action potential) can pass from one cell to the next. A wave of electrical activity and a wave of contraction occurs and it can be initiated by pacemaker cell (a cell that exhibit spontaneous depolarisation). Because of syncitial arrangement, they are called cardiac muscle type. In case of multi unit smooth muscle, the individual cells are not electrically coupled, and each cell can contract independently. Scrcotubular system is very weakly developed. The sarcolemma shows small invaginations known as caveoli. These represent the rudimentary form of T-tubules of skeletal muscle cells. Just behind caveoli there are small fragments of longitudinal tubules. All these represents rudimentary form of sarcotubular system. The disposition of actin and myosin are also not well organised as seen in skeletal and cardiac muscles. There are dense bodies attached to sarcolemma, some are also present in the sarcoplasm. They represent Z-line of the sarcomere. Actin filaments are seen radiating from dense bodies. Myosin filaments are seen between two dense bodies (Fig 4.4). Mechanism of contraction of smooth muscle In the smooth muscle cells troponin is absent. A number of proteins and enzymes are involved in its contraction. In the resting state, actin-myosin interaction does not occur and the muscle remain in relaxed state. A protein ‘caldesmon’ present in the Fig 4.4 : Physical structure of smooth muscle. The upper left-hand fiber shows actin filaments radiating from dense bodies. The lower fiber and the right-hand insert demonstrate the relation of myosin filaments to the actin filaments.

56 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Fig 4.5 : Both actin-and myosin-dependent mechanisms control smooth-muscle contraction and relaxation. (A) Binding of caldesmon to the actin and tropomyosin (TM) of thin filaments prevents contraction. At cytosolic Ca 2+ levels above 10 −6 M, formation of the Ca 2+ /calmodulin complex occurs. Binding of this complex to caldesmon releases it from thin filaments, allowing the muscle to contract. Phosphorylation of caldesmon by protein kinase C (PKC) also prevents it from binding to thin filaments and promotes contraction. (B) Binding of Ca 2+ to the regulatory light chains of myosin allows actin-myosin interactions and promotes contraction. (C) Phosphorylation of the regulatory light chains by myosin LCkinase, which is activated by Ca 2+ calmodulin, also promotes muscle contraction. (D) Phosphorylation of the regulatory light chains by protein kinase C, at a site other than that acted upon by myosin LC kinase, inhibits myosin-actin interactions and causes smooth-muscle relaxation. [Adapted from Lodish et al., 1995.] A. B. C. Cannot bind to actin D.

57 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof sarcoplasm binds with the actin and thus actin-myosin interaction is kept prevented. When intracellular Ca 2+ concentration is increased and goes above 10 −5 moles/lit, Ca 2+ binds with a protein in the sarcoplasm called calmodulin. This Ca 2+ — calmodulin complex binds with caldesmon and it is released from actin. So, actin-myosin interaction occurs and the muscle contracts. A fall in intracellular Ca 2+ causes decreased formation of calmodulin-Ca 2+ complex. So caldesmon becomes free. This now again binds with action and prevents actin-myosin interaction. This results in relaxation of the smooth muscles (Fig 4.5). Besides this, there are other mechanisms also, that operates or influence the process. There is a regulatory light chain of myosin which remain in two form— phosphorylated and dephosphorylated forms. The dephosphorylated prevents actin- myocin interaction and so the muscle remain in relaxed state. Calmodulin Ca 2+ complex activates an enzymes, called myosin light chain kinase (MLCK). This enzyme phosphorylates myosin light chains. The phosphorylated form of myosin light chain cannot prevent actin-myosin interaction and so muscles contract. There is a myosin light chain phosphatase that dephosphory lates myosin light chain. But, it is again phosphorylated immediately by the kinase, so long calmodulin—Ca 2+ complex is there. However, when Ca 2+ concentration is decreased, Ca 2+ ion are removed from calmodulin Ca 2+ complex. So, kinase is no longer activated. So, further phosphorylation of myosin light chain does not occur and the muscles relax. PKC can cause phosphorylation of caldesmon and thereby can also prevent actin-myosin interaction. However, it can also phosphorylate the regulatory light chain of myosin and this also inhibits actin-myosin interaction and the muscle relaxes. Both PKC and MLCK phosphory lates myosin light chain (the regulatory light chain of myosin), but the phosphorylation sites are different and the action is also different. MLCK phosphorylation leads to contraction and PKC phosphorylation causes relaxation of the smooth muscle. Sequence of events in muscular contraction The general sequence of events in the contraction of three different types of muscles. The skeletal and cardiac muscles undergo action based muscular contraction, whereas the contraction of smooth muscles is myosin regulated. Sequence : 1. When the muscle fibre is stimulated via neurons (natural stimulus) or otherwise action potential develops. 2. The muscle action potential passes via sarcolemma and enter deep into the muscle cell via T-tubules (in skeletal, and cardiac muscle), and via DHP receptor and RYR receptor escitation causes release of calcium. In smooth muscle, mostly Ca 2+ comes from ECF due to opening of voltage gated Ca 2+

58 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof channel present in the plasma membrane of smooth muscle cell. 3. Intracellular Ca 2+ concentration is increased. It binds with troponin C (in case of skeletal and cardiac muscle cells). This causes a conformational change in troponin molecule. As a result, tropomyosin moves and the myosin binding site of actin is exposed. Actin myosin binding occurs and the muscle contracts. In smooth muscle cells, troponin is absent. Here, Ca 2+ -Calmodulin complex is formed, that initiates muscles contraction involving caldesmon, MLCK, PKC, muscle phosphatase. (Fig 4.6) 4. As intracellular Ca 2+ concentration is decreased, Ca 2+ dissociates from troponin Fig 4.6 : Initiation of muscle contraction by Ca 2+ . When Ca 2+ binds to troponin C, tropomyosin is displaced laterally, exposing the binding site for myosin on actin (dark area). The myosin head then binds, ATP is hydrolyzed, and the configuration of the head and neck region of myosin changes. For simplicity, only one of the two heads of the myosin-II molecule is shown. C and Ca 2+ calmodulin. This leads to relaxation. Basically the (i) development of action potential, that is, degree and duration of depolarization and repolarisation, (ii) functioning of the sarcotubular system, that is sensitivity of calcium-associated with degree and duration of calcium release and reuptake, (iii) actin-myosin interaction that is degree and duration of action and myosin attachment and detachment cycle, (iv) energy yielding system in the muscle cells, are the determinants of muscle function. These muscles have been selected based on their structure and physiological propertiesfor placement in specific areas of the body for performing specific function. (Fig. 4.7) In case of skeletal muscle, the muscle fibers are not inter-connected. So, all or none law is applicable to individual fibere. So, when it contracts, the strength of stimulus can produce summation or tetanus. But in cardiac muscle, the muscle cells are electrically connected resulting functional syncitium. Here all or none law is applicable to whole of heart. The refractory period is short in skeletal muscle. So Actin

59 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof summation and tetanus are possible but this does not happen in case of heart, because it is absolutely refractory during systoleand relatively refractory during diastole. The heart cannot afford summation, tetanus or fatigue. Hence, its muscle fibres have such property. The single unit smooth muscle is called cardiac muscle type and multi unit smooth muscle is termed as skeletal muscle type, and their function is also different. The action potential duration is short in skeleted muscle, but prolonged in cardiac and smooth muscle. Mechanical change is related to electrical change. Hence, the twitch duration is short in skeletal muscle but is is more in caridac muscle and more prolonged in smooth muscle. Skeletal muscles are attached to skeleton and joints. These muscles take part in movement and locomotion. The muscles are so arranged that they can provide optimum output. Each muscle has origin and insertion and as they contract, the insertion part moves towards the origin. Depending on this property the muscles are arranged accordingly so that the purpose is served. For example, the biceps muscle has origin in the scapula and insertion in the radius, thus in contraction causes flexion of the elbow joint. The muscle of the rib are so arranged that contraction of external inter- costal causes elevation of the ribs, thus increases the antero-posterior diameter of the thoracic cavity. Similarly, contraction of diaphragon muscle causes its downward movement towards the abdominal cavity, and thus superior-inferior diameter of the thoracic cavity is increased. In this way, as the diameter of thoracic cavity is increased, the intra-thoracic pressure is decreased. This is turn decreases intra-pulmonary pressure and so air enters into Fig 4.7 : Proposed mechanism for the generation of force by the interaction of an S1 unit of a myosin filament with an actin filament. In the power stroke, the thin filament moves relative to the thick filament when S1 undergoes conformational changes accompaying the release of ADP. Myosin Actin ADP Myosin — Actin

60 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof the lungs and inspiration occurs. Force is generated during musculer contraction and this forece is transmitted via tendon to the bones, within the muscle, there are elastic fibres in two forms— (i) parallel elastic component—the fibres lie or run in between muscle fibres. (ii) Series elastic components —these represent the elastic fibres that connect the contractile element with the tendon that is attached to the bone. These are involved in producing isometric and isotonic contractions. Isometric contraction is that where tension is developed but the muscle is not shortened. eg. holding a weight on the palm of a stretched hand. Similarly, an isotonic contraction is that in which muscle stretches but tension is unchanged, eg. lifting a load by flexing the elbow joint. The biceps muscle contract isotonoically as the load is lifted. Because of series elastic element, the force generated during mascular contraction can be transmitted to the bones via the tendon. Sometimes, the muscles are attached to a long tendon that in turn is attached to the bone. eg. the muscles that cause movement of the finger-joints are present in the lower arm. Such arrangement definitely help in smooth movement of the fingers. The force generated during muscle contraction is directly related to actin-myosin overlap. It has been shown that the more the overlap, the more the tension development. A study of length-tension relationship revealed that as the length of the fiber is increased the tension developed is dependent of actin-myosin overlap. At longer length, the tension is small and it is zero, when there is no overlap (Fig 4.8). When the length is gradually decreased, the overlap is slowly increased, and the tension gradually rises. It becomes maximum (plateau phase) when there is maximum actin- myosin overlap. A further decrease in length of the fiber again causes a decrease in tension, because overlap of actin retands tension development. Frogs use hip flexor muscles during jumping. It has been found that during jumping the sarcomere length changes from 2.3 ?m (at rest) to 1.82 ?m at the point of take off, and this length has been found to correspond to the plateau-phase of the length tension diagram of the hip-flexor muscle. The red and white muscles are used according to the type of contraction required. For quick movement the white type is used and for sustained movement red muscle are used. It has been shown in fish that during steady swimming the sarcomere length of the red muscles coincide with the plateau phase of length-tension curve for this muscle, whereas in case of escape response, this is seen in case of white muscles. This proves which muscles are used for what kind of movement. Biophysical and biochemical characteristics confirm their suitability for doing sustained or quick movement. Thus animal body uses specific muscles for specific purpose. It has been shown that as the length of the muscle fibre is increased, the tension development during contraction also increases progressively and becomes maximum at a particular length. It is called resting length of the muscle fibre. At this length the

61 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Fig 4.8 : Standard filaments lengths. a = 1.60 ?m; b = 2.05 ?m; c = 0.15—2 ?m; z = 0.05 ?m. (b) Tension- length curve from part of a single muscle fibre (schematic summary of results). The arrows along the top show the various critical stages of overlap that are portayed in (c). (c) Critical stages in the increase of overlap between thick and thin filaments as a sarcomere shortens. (Gordon, A.M., Huxley, A.F., and Julian, F. J. (1966). J. Physiol., Lond. 184, 170.)

62 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof actin-myosin overlap is maximum. If the length is further increased the tension development is decreased. Survey report revealed that the muscles present in the body during rest are at the resting length. The sound producing muscles (sonic fibres) contract 10-100 times faster than those used for locomotion. Experimental results indicate that this has been made possible by a number of adaptations. These are— (i) an increase in Ca 2+ kinetics ; (ii) cells are well equipped for aerobic metabolism and the space required for such assembly is made by reducing the number of myofilaments. The animal can afford such reduction in myofilaments in sonic muscles, because the muscles require faster rate and not increased force. The force or power production during muscular contraction is denoted as V/ Vmax, where V is the velocity of muscular contraction at any particular condition and Vmax is the maximum velocity of shortening possible. The power production is considered to be maximum, when the value of V/Vmax is 0.15—0.40. For frog hip flexor muscle during jump V/Vmax is about 0.32. Hence it is said the muscles while lifting the body they try to do it with maximum power. The skeletal muscles of the vertebrates consist of muscle fibrets of more than one type. Some contain a high proportion of tonic fibers which show steady contraction other muscles contain a high percentage of twitch fibres which are specialized for rapid movements. Such muscle fibres having different properties have been found in the animal body and these are due to biochemical, metabolic and other structural adaptations. Tonic muscle fibers contract very slowly and do not produce twitches. The motor neuron make contact with the muscle fiber at several points. Hence, action potential is not produced, and in fact they do not require AP to spread excitation. The actin-myosin detach slowly, hence the velocity of shortening is slow. So they are able to generate isometric tension very effectively. They are capable for slow steady contraction. Hence they are used for posture maintenance, where a slow sustained contraction is required. They are found in the postural muscles of amphibians, reptiles, and birds as well as the muscle spindles of all extra ocular muscles of mammals. Slow twitch or type I fibere are characterized by slow to moderate Vmax. Slow Ca 2+ kinetics. They contract slowly and fatigue slowly. They generate ‘All or none’ AP. It has one of few end plates. It contains myoglobin. Muscles that contain more of these fibers are also called red muscles. The slow-fatigue is due to presence of large number of mitochondria and rich blood supply. These fibres are suitable for maintaining posture and for moderately fast repititive movements. Fast twitch oxidative (type IIa) fibres have high Vmax. They acn be activated quickly. They fatigue slowly because they have more mitochondria, can produce ATP quickly by oxidative phosphorylation. They are specialized for rapid repetitive

63 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof movements as required in sustained streneous location. For these reasons, they are used for making the flight muscles of wild birds. Fast twitch glycolytic (type IIb) fibres. These fibers have a high Vmax, very rapid Ca 2+ kinetics and for the this they get activated and relax quickly. They have few mitochondria. Hence they are dependent on anaerobic glycolysis to genetate ATP. Hence, they fatigue quickly. These are found in breast muscle of domestic fowl, which are never used for flying and cannot produce ssustained activity. Ectothermic vertebrates, such as amphibians and reptiles also make extensive use of glycolytic muscle fibers. Thus, the animal make up their muscle by different types of muscle fibers according to their requirement. Body not only can improve their muscle mass by growth of individual muscle cell, but they can also improve their power for performance by forming oxidative enzymes to improve aerobic power and by improving glycolytic enzymes to improve anaerobic power. Moreover, by changing to fast twitch fibers and vice versa. This is mediated via changes at genetic level. Cardiac muscle cells are interconnected functionally. Here, the whole heart obeys all or none law. However, it has been observed that in a quiscent heart repeated stimulation increase the force of contraction for few strokes.This is known as stair case phenomenon or treppe. It is due to increased accumulation of Ca 2+ that increases the force of contraction of cardiac muscle. At every situation Ca 2+ is released but in case of repeated stimulation the rate at which it is released can not cope with the removal. So Ca 2+ accumulates and produce the effect. It has also been observed that when the cardiac muscle is stretched its force of contraction is increased, as happens in case of skeletal muscle. But the mechanism in cardiac muscle is different at the level of actin-myosin interaction. It has been shown that at saturating Ca 2+ ion concentration stretched cardiac muscle exhibit greater force of contraction compared to control cardiac muscle. It is not due to difference of overlap of thick and thin filament as happens in skeletal muscle. Evidence suggest that stretch reduces the space between thick and thin filaments (ie., interfilament spacing) as this is associated with the ability of more myosin molecules to interact with actin. Titin plays an important role in it. Titin binds to both actin and myosin in such a way that when the muscle is stretched, it brings actin more close to the myosin head and they increase the number of myosin heads that interact with actin. It has been found that protolysis of titin attenuated length dependent increase in force. Increased sensitivity to Ca 2+ is possibly due to decreased interfilament space caused by titin (Fig 4.9). Increased venous return increases cardiac output. This is possible because of the property of cardiac muscle. As more blood enters the heart (ventricle), the volume of the heart (ventricle) is increased, that is the heart muscle is stretched and this increases the force of contraction of heart muscle. So the blood goes out of the heart.

64 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Fig 4.9 : Titin may contribute to the ability of stretch to increase the force of contraction of the heart. Titin binds to both myosin and actin such that stretch of the cardiac muscle may bring the actin filament closer to the myosin head and thus increase the number of myosin heads that interact with actin at a given intracellular [Ca++]. (Redrawn from Moss RL, Fitzsimons DP : Circ Res 90:11-13, 2002.) In smooth muscle cell actin-myosin recycling occurs. Myosin cross-bridge head attaches itself to the actin, then it is released from actin and then reattaches with next actin in the next cycle. This rate of cycling is very slow. This is because ATPase activity of myosin is very low compared to skeletal muscle. Moreover, in case of tonic contraction, it has been found that, when full contraction has developed, it can be maintained with less ATP utilization. During actin-myosin attachment a great force is generated and this allow the smooth muscle cells to contract as much as 80% of its length (instead of 30% as seen in skeletal muscle). This can cause a great collapsing of the hollow tube. The response to a sustained or tonic stimulation is a rapid contraction followed by a sustained maintenance of force with reduced cross-bridge cycling rates and ATP consumption. This behaviour is called latch state. It is advantageous for muscles that may need to withstand continuous external force, such as blood vessels, which must be able to withstand blood-pressure. ATP consumption during latch state is less than 1/300 the rate that is necessary for skeletal muscle to maintain the same force. Another important characteristic of smooth muscle cells is length adaptation. It is able to adjust length-tension relationship when chronically stretched or shortened.

65 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof This is very helped during filling of urinary bladder. Hence bladder wall is made up of such smooth muscle cells. It has been observed that a sudden increase in bladder volume causes stretching of the smooth muscle and the pressureof uninary bladder is increased but subsequently within 15 sec to one minute despite continuous stretch the pressure becomes almost back to normal. This has been named as stress relaxation. Because of this, a hollow organ can maintain almost the same pressure in spite of changes in the length of smooth muscle. The single unit smooth muscles are present in the GI tract. The multi unit smooth muscles can be found in vas deferens and iris muscle. 4.2 Movements—ameboid, ciliary and flageller Amoeboid movement This terms derives its name from motion of Amoeba. There are two terms, 'movement' and 'locomotion'. An animal being attached to a site can show a great variety of movement, but the animal does not shift to another place. It is called movement eg, corals and sponges. But when the animal shifts its position from one place to another by movement it is called locomotion. There are animals which have cilia and flagella. These also show movement and movement of these can help the animal to move from one place to another. Thus, ciliary movement and flageller movement are not only related to 'movement', but also to 'locomotion'. A typical Amoeba consists of an outer layer, the plasmalemma. It is not water wettable and has adhessive property. It slides freely over the next inner layer called ectoplasm (gel-like). The plasmalemma is made up of outer filamentous coat and an inner membrane. The filaments are about 80Å in diameter, and extend outward 0·1 to 1·0 ?m. The filamentous coat is made up of 35% lipid, 26% protein and 16% polysacharide. Beneath the plasmalemma there is a hyaline layer. It is fluid in nature as judged by brownian movement. This layer is very thin in the region of attachment to the substrate. It is often thickened as an hyaline cap at the front of an advancing pscudopodium. Types of cells that exhibit amoeboid movements Types of cells that exhibit amoeboid movement are white blood cells, fibroblast, germinal cells in skin, and embryonic cells. Embryonic cells often must migrate long distances from their site of origin to new areas during development of special structures. Process of amoeboid movement The amoeboid movement involves cytoplasmic streaming, changes in cell shape and extension of pseudopodia. When an amoeba moves, its cytoplasm flows into

66 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof newly formed arm like extensions of the cell (pseudopodia). The pseudopodia gradually extend and enlarge so that the entire cell occupies the space where previously only a small psendopodium began to form. As the cell moves, new pseudopodia are formed in the direction of the movement, while the posterior parts are withdrawn. The outer layer of the Amoeba is the stiff gel-like layer called ectoplasm. As the pseudopodium is formed, the more liquid endoplasm streams into it, and a new ectoplasm is formed on the surface. In the rear part of the advancing cell, the ectoplasmic gel is converted to a more liquid endoplasmic sol by a sol-gel transformation. 'Rolling and walking' are the two types of movements that have been described. The rate of movement also show much variation. It may be as slow as 1350 ?m/sec. (e.g. Plasmodium of acellular slime mold) 5-6 cm/hr in migrating Plasmodium. Freely crawling amoebae move at the rate of 0.5 to 4.5 ?m/sec. In feeding, those amoeba, which travel by small pseudopodia, form food cups. These cups are also motile, encircle the food particle, and their distal ends join and the food is taken into a vacuole. Mechanism of amoeboid movement The exact mechanism is not fully understand, but involvement of certain processes have been known from different experiments. Involvement of membrane, nucleus, microtubules of microfilament system, and contractile proteins have been studied. Total surface area of the membrane is greatly increased when an amoeboid cell changes from a nearly spherical shape to a multipodal form, it then decreases as pseudopodia are retracted. Three theories were proposed for this, but experimental evidence suggest that it is because of the membrane is a fluid or plastic surface that slides freely over the ectoplasm. Essentiality of the nucleaus has been established. Enucleated amoeba soon lose the organised progressive cytoplasmic flow that results in movement. Involvement of microtubules are microfilaments in amoeboid movement has been well documented. They play a role in movement either by processes like sliding mechanism in mascular contraction or development of shear forces. The birefringece seen in amoeba suggest an orderly assay of macromolecular structure. The presence of structures like actin and myosin have been well documented in amoeba. Amoeboid cells have been found to respond to an electrical field and show biopotentials. In an electrical field, Amoeba proteins shows solation on the cathodal side and pseudopodia advance in that direction. Amoeboid movement is caused by contraction of cytoplasm. It is held that at the point of attachment there is less space between the plasmalemma and the ectoplasm than elsewhere. It has been suggested that contact between plasmalemma and ectoplasm

67 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof in the Amoeba proteus initiates contraction. According to Allen such contraction takes place in the 'fountain zone' and the Amoeba is 'pulled along'. However, Jaha and Bovae proposed that the site of contraction is 'tail process', and the Amoeba is pushed forward. Actin is largely conserved. It is of similar structure and occur as the filaments in the cell. Myosin from various sources are more diverse, but all bind to actin and causes ATP—hydrolysis with liberation of energy. On this basis, it has been suggested that both cytoplasmic steaming and the formation of pseudopodia may depend on the interaction between actin and myosin. Control of amoeboid movement Chemotaxis is the most important initiator of amoeboid movement. Movement occurs either towards the source of chemotactic agent (positive chemotaxis) or away from it (negative chemotaxis). How chemotaxis control the movement is not clear. But it has been observed that the cell surface exposed to the chemotactic agent, develop membrane changes that cause pseudopodial protrusion. Cilia and flagella Each cilium has the appearance of a sharp pointed straight or curved hair that projects from the surface of a cell. It is an outgrowth of a structure that lies immediately beneath the cell membrane called the basal body of the cilium. It is of shorter length. Flagella on the other hand are membrane bound extensions of the cell. A basal body called kinetosome anchor the flagellum with the cytoplasm. Fig 4.10 : Cross-sectional diagram of a cilium.

68 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Basic structure of cilia and flagella Flagella are of longer length compared to cilia. In eukaryotic cells, the cilia and flagella are of similar internal structure. Cilia and flagella contain microtubules. The microtubules are arranged as a bundle of nine doublets around the periphery with a pair of single microtubules running within them. This structure is called axoneme. In an axoneme, the 9+12 array has specific arrangement (Fig 4.10). Each of the nine outer doubtlets appears like the figure eight. The smallar circle of the figure is termed subfiber A. The larger circle, subfiber B. Subfiber A is joined to a central sheath by radial spokes. The neighbouring doubtlets are held together by nexin links. Two dynein arms emerge from each subfiber A with all the arms in a molecular cilium pointing in the same direction. Mechanism of movement The dynein in a large protein (MW 1000-2000 kDa). It consists of one, two or three heads depending on the source. The heads of dynein form cross bridges with the subfibres B and its has ATP binding site as well as ATPase activity (Fig. 4.11) Binding of ATP to dynein causes it to dissociate from B subfiber. The ATPase activity of dynein splits ATP to ADP and Pi. On hydrolysis of ATP, dynein again binds with subfiber B with subsequeat release of ADP and Pi. This ATPase cycle leads to the movement of the cilium as the outer doublets of the axoneme slide past each other. Dynein is involved in converting the energy released from ATP hydrolysis into mechanical energy for movement. Movement is produced by the interaction of Fig 4.11 : Effects of flagellar rotation on prokaryote movement.

69 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof the dynein arms with one of the microtubules of adjacent doublets. The force between adjacent doublets is generated by the dynein cross bridges. Thus the dynein arms on subfibre A of one doublet walk along subfiber B of the adjacent doublet. The radial spokes prevent sliding motion as happens in the muscle, and so the motion is converted to a local bending. The highly flexible protein, nexin, keeps adjacent doublets together during this process. Bending of the flagellum occurs when the extending dynein arms attached to the neighbouring B tubule, inducing active sliding movements out the expense of ATP. Movements and its importance 1. A flagellum, like the tail of a sperm, beats with a symmetrical undulation that is propagaled as a wave along the flagellum. A cilium beats asymmetrically with a fast or dash like stroke in all direction followed by a slower recovery motion in which a bending cilium returns to its original position. 2. The number of cilia or flagella present in cell show much variation. A paramoecium may have several thousand cilia, cliliated cell in the respiratory passage may have 250 cilia. Few cilia are present in the epithelial lining of fallopian lube. Cilia in the respiratory passage help in the removal of mucous by mucous cell of the respiratory lining, along with trapped particles toward the nasal opening. Ciliary movement in the reproductive tract help in propalsion of ovum from the ovary to the uterine tube as this is necessary for fertilization and implantation of fertilized ovum. A flagellalled cell carries usually one or a few flagella. The sperm of a vast number of animals swim by means of flagella. Flagella exhibit typically sinusoidal motion in propelling fluid/water parrallel to their axis. The undulating action of the flagellum either propels water away from the surface of the cell body or draws water towards the cell body. The cilia exhibit an oar like motion, propelling water parallel to the cell surface. 3. Cilia and flagella are found in many protozoans and mainly related to locomotion, cilia are found in all animal phyla. Modified ciliary structure are present in insect eyes as well as in the majority of other sense organs. 4. Small animals use cilia and flagella for locomotion and muscles are used for the purpose in large animals. Bacterial flagella : (Chemotaxis) by rotating their flagella. The flagella of bacteria are quite different. They are thinner (about 0.2 ?m in diameter, against 0.25 ?m for true flagella and cilia), short and relatively rigid. They are related by forces at the base where the are attached to the cell. They differ for eukaryotic cilia and flagella in two ways— (i) each bacterial flagellum is made up of if flagellin (53 kDa sub unit) as opposed to tubulin as (ii) it rotates rather than kinds. The rotary motion of the flagellum is driven by the basal body which acts like a motor. The direction of

70 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof flagellar rotation determines the type of movement. Prokaryotes with a single flagellum move forward during counter clockwise rotation and tumble where the flagellum rotates clockwise. Where there are more than one flagellum they behave as a single bundle during counter clockwise rotation and thus move forward, however, during clockwise rotation the flagella act independently and the organisms tumbles. Bacteria can move through the extra cellular medium towards attractants and away from repellents. 4.3 Specialized organs (eg : electric organs and tissues) Fishes are unique in the animal kingdom in being capable of producing electric current. These are produced from an organ in the tail region and called electric organ. About 250 species of fishes, both chondrichthyes and osteichthyes are reported to possess electric organs. These species have evolved the electric organs independent of each other. The following are the most important fishes that are known to possess the electric organ. Elasmobranchs : (i) Electric Rays; (ii) Skates. Teleosts : (i) Mormyridae; (ii) Gymnotidae, (iii) Siluridae. It has been found that some fishes produce strong current, some produce weak current and there are some which can only sense electricity but cannot produce electricity. On this basis, electric fishes have been divided into three main categories : A. Strongly electric fish : i) electric eel. ii) electric catfish. iii) electric rays. B. Weakly electric fish : i) Knife fishes. ii) elephant nose. C. Fishes that can only sense electricity : i) Sharks. ii) Rays. iii) Skate. iv) Catfish. v) Paddle fish.

71 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Structure of electric organ The shape and position of the electric organs differ greatly in different species of fishes but all of them have more or less a similar microscopic structure. It is made up of disc like cells called electroplates or electrocytes which are modifed muscle fibers. These are embedded in a jelly like material and are bound together by connective tissue into an elongated compartment. One face of each electroplate is supplied by nerve fibers and the jelly receive blood capillaries. Each electroplate is a multinucleated cell with nearly transparent cytoplasm. Hence, the elecetric organ looks like a clear gelatinous mass as compared to the muscles. Stimulation of electric organ Normally a resting potential exists across both innervated and noninnervated surfaces. In the Electrophorus it is about 90 mv. the inside being negative. It is due to difference in the distribution of electrolyte concentration within and outside its cell. At the peak of the discharge, the membrance potential across the nervous face of the electroplate is reversed (60 mv) but the potential across the non nervous face remains unchanged. As a result a potential difference of 150 mv (60 + 90) develops Fig 4.12 : (A) Electrophorus electricus, dissected to show electric organs. (B) T.S. Electrophorus. DF., Dorsal fin ; EL. ORG., Electric organs ; H. ORG., Hunter's organ ; M., Myotome ; OR. S., Organ of Sach's ; V., Vertebra ; VF., Ventral fin. Fig 4.13 : Diagramatic representation of current flow in an electric fish. (A) at rest ; (B) at discharge.

72 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof between the two surface of a cell. The voltages of successive electroplates are added up in series and a strong current flows. According to ionic hypothesis, at first sodium ion and them potassium ion enter into the cell due to alteration of membrane permeability and thus results in potential difference. (inside positive and outside negative) (Fig 4.12, 4.13). These electrocytes or electroplates receive simultaneous command signals from the brain to 'fire'. At the moment of 'firing' the electrocytes are asymmetrically polarized acting as serially connected batteries. The simultaneous firing of electrocytes results in the electric organ discharges (EODs) which are emitted in the surrounding water. In strongly electric fishes, such as electric eel, electric catfish and electric rays, the electric organ is huge containing numerous electrocytes and so their discharge voltage can reach as high as 600 volts. However, in weekly electric fishes, it is small and often less than a volt. The electric discharge of the electric organs are of two types. In all strongly electric fishes and some weakly electric fishes the discharge is of pulse type. They discharge short electrical pulses intermittently. However, some weakly electric fishes produce wave-type discharge, they produce wave like continuous AC electricity. Some fishes like Electrophorus produce both strong and weak current according to need. Most of the electric fishes can produce electricity as well as can sense electricity. However, there are fishes which can only sense electricity but cannot produce it. Strength of current generated : The values of electric current produced by some electric fishes are given below : Skate : 4 volts. Electric rays : Torpedo : 40 Volts. Narcine : 37 Volts. Electric eel : Electrophorus : 370 – 550 Volts Electric Cat fish : Malapterurus : 350 – 450 Volts. Star-gazar : Astroscopus : 50 Volts. Functions of electric organ Electrolocation : The ability to locate an object with the help of electricity is called electrolocation. Fishes have a very sensitive sensory organ which can receive electricity. These are called electroreceptors which remain embedded in the skin. There are two types of electroreceptors. An ampullary receptor contains

73 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof supportive cells that lie at the bottom of a narrow channel filled with gelatinous mucopolysaccharide. Afferent nerves embrace the receptor cells. The tuberous receptor lies buried under the skin in an invagination beneath a loose layer of epithelial cells. This loose layer may differentiate into covering cells that cover the sensory cells are a superficial set of plug cells. These receptor respond to higher frequency than the ampullary receptors. It also can sense the electrical discharge of the fishes own electric organ. Electroreceptors are used to detect a slight change of electric field caused by nearby objects. Hence, they can see objects electrically. The electrolocation may be active or passive. When the source of electricity used for electrolocation is their own electric organ it is called active electrolocation. The fishes, which cannot produce electricity but can sense it, can sense very weak electricity produced by prey animals. For example, a shark can find a small fish buried in sand by the weak electricity given off by the prey. This type of electrolocation is passive electrolocation because here the electricity is produced by the prey and not the predator which help in locating the object. Self protection : Electric fishes are well protected against their own current discharge and against the discharge of each other. This is achieved possibly by developing high insulation around nervous system. As in Electrophorus, the spinal cord and the swimming muscles are embedded in a thick layer of fat. Their nerves and muscles may also develop unusually high threshold of excitation. Importance of electric organs The form, position are strength of the electric organs show much variation and this is related to there functions. Fishes with powerful electric organs use these both offensively in their hunt for food and defensively against their enemy. The primary function of the electric organs in all fishes is possibly defensive. Both Torpedo and Electrophorus have been observed to paralyse small fishes before eating them. Fishes with weak electric discharge create an electric field around themselves and if any object is close to it the electric field breaks up and thus the fish become aware of the presence of the object. Hence it may act as an warning device and it is of considerable value in muddy water where vision is not possible. Such weak discharge also helps the fish to find direction in the dark water. Electric organ discharge also helps in maintaining the territoriality by individual fish. It may also be useful for speices or even sex recognition. Fish can use electricity to feel the environment and also can communicate with each other using electrical singal.

74 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Jamming avoidance response The wave type electric fish normally discharge at a fixed frequency and each individual has its own frequency. When two individuals having similar frequency meet, however, their EODs interfere with each other causing problem in electrolocation. This is called jamming. To avoid jamming the two fishes shift their frequencies until there frequencies differ in such a way so that operation of electrolocation becomes possible. 4.4 Suggested questions 1. Describe the basic structure of an electric organ and mention its importance. 2. What is jamming avoidance response. 3. Write briefly on electrolocation. 4. Describe the basic structure of cilia and flagella. 5. Write a note on the mechanism of movement of cilia and flagella. 6. Write briefly on the mechanism of ameoboid movement. 7. What is titin ? How it helps in muscle contraction ? 8. How the muscle shortens during muscular contraction ? 9. Describe the sequence of events in muscular contraction with a diagram. 10. Comment on length tension relationship in muscle contraction. 11. How collaping of hollow viscera is related to contraction of smooth muscle cells. 12. Comment a differential distribution of skeletal muscle fiber ? 13. What is caldesmon ? How it helps in muscle contraction ?

75 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof 5.1 Introduction There are two basic concepts in natural selection. These are fitness and environment. Environment means the kind of habitat in which the living organism or animal lives. Whereas fitness is strongly linked with adaptation. Environments are largely variable in relation to stress that they impose on their inhabitants. This stress may be both abiotic and biotic. Whenever there is a change in the environment, compensatory changes occur in the living organisms to cope with the environmental modification. Short term compensatory changes in respense to environmental disturbance is called acclimatization or acclimation whereas long-term compensatory changes in known as adaptation. Adaptation is defined as a process; the means by which natural selection adjusts frequency of genes that codes for traits affecting fitness. For example, increasing haemoglobin concentration ; in taxa might be seen as an adaptation to potentially low oxygen environment. Adaptation in this sense, is a process that normally occurs very slowly, over hundred or thousand of generations and is usually not reversible. However, in extreme environment or where selective pressure from human interference are strong it can sometimes occurs very quickly. Adaptation is often used as a term for the characters or traits observed in animals that are the results of selection. For example presence of hemoglobin can be said to be an adaptation to increase the oxygen carriage in the blood. The processes of adaptation is usually a slow one that occurs over generations and is rarely reversible. Acclimatization on the other hand, is more rapid phenomenon whereby a biochemical or physiological change occur within the life of an individual animal resulting from exposure to a new condition in the environment. Thus an ascent to high altitude (mountain) may lead to acclimatization to low oxygen and low pressure; movement from arctic areas to southwards will lead to acclimatization to warmer temperature. When similar processes are allowed to occur in the laboratory it is called acclimation. A polar bear is said to be adapted to Unit 5 ? Adaptation Structure 5.1 Introduction 5.2 Levels of adaptation 5.3 Mechanism of adaptation 5.4 Significance of body size 5.5 Suggested questions
76 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof polar temperature. The shape of the chest of sherpas are adapted for respiration at low oxygen pressure at high altitude. The human polar explorer or mountaneer get acclimatized to the environment. But they revert back gradually to normal when the environmental condition is changed. Mouse forcefully subjected to cold exposure in the laboratory, get acclimatized to cold. It reverts back to normal when the laboratory temperature is reverted to normal. The avoiders have or develop mechanisms for getting away from or to avoid an environmental problem either in spece or in time. Avoidance in space is brought about by behavioural change for a small animal it may involve a search for an appropriate habitat using phototactic or chemotactic responses. They look for less stressful microhabitat in crevices or burrows. Larger migratory species try for large scale migration with the help of physiological adjustments. e.g. accumulation of food reserves. Avoidance in time require more complex responses at all levels. An animal entering into torpor accumulate food, construct or find a refuge; huddle in a ball to reduce its surface area for preventing heat loss in a cold environment; it may reduce its core temperature and metabolic rate, it may acquire a thick insulating layer to cope with temperature extrem; it may mobilize or generate new form of enzymes and new forms of membrane components. The conformers change their internal states, similar to changes in the external environment. They do not try to maintain a homeostatic condition. This involves biochemical and physiological modifications. If the internal environment varies in terms of osmoticity, salinity or temperature, the cells must have a biochemical- physiological system that can function at diverse conditions especially enzymes and membrane stabilization. Their status is such that the animal is kept functioning at extreme conditions avoiding damaging effects of freezing on hypoxia etc. However in general the physiological and biochemical changes are small and cheap, hence are more economic. The regulators on the other hand maintain their internal environment almost constant irrespective of changes in the external environment. This involves substantial and expensive biochemical and physiological adjustments. For example, to maintain temperature homeostasis, even in the best endothermic mammals, behavioral changes like basking, burrowing, wallowing, huddling, erecting or concealing appendages etc are adapted as a first line of defence. Along with this substantial physiological and biochemical adjustments, occur to have optimal effect. These include changes of blood flow or respiratory rate or nonshivering modification of thermogenesis and thermolysis or the production of heat-shock proteins or anti-freeze molecules.

77 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof 5.2 Levels of adaptation When an animal is confronted with changes in its environment it normally exhibit three types of responses - avoidance, conformity or regulation. These responses are the final outcome of adaptations at four levels. Biochemical, Physiological, Morphological and Behavioral. Adapted responses may appear at different spatial levels. Some responses are essentially subcellular, some affect the morphology or activity of whole cells; others manifest as effects on entire tissue or organs like changes in muscle size heart volume or arrangements of vascularisation. However, there is a limit of conforming and regulating. For example, the osmoconformers show some regulation in extreme low salinity to avoid cell damage due to excessive swelling. Similarly the osmoregulators become unable to regulate at lower salinities and turn to conformers, homeothermic animal allow their extremities to become poikilothermic at extreme cold. These strategies adapted to counteract enviromental changes are associated with different costs and benefits thus the 'avoidance' by shutting down is cheap but this causes the animal to remain out of the 'race' for some time without any growth and reproductive output. In case of avoidance by way of migration may be expensive but it allows the animal to continue with growth in another environment. Avoidance in poor physical environment by way of shut down or migration give additional benefits like avoiding predation or competition. Conformity at the extremes of temperature, salinity or hypoxia may allow a minimal lifestyle but over a broad range it can maintain a reasonably productive life style at a cheaper cost. Regulation is rather definitely expensive; osmoregulation takes about substantial amount of energy but thermal regulation draws up of total energy budget. However, it can gather food all through and the conformers and avoiders become prey. With extra food they can grow and reproduce faster. Despite high cost they become dominant in many ecosystems. Adaptation at different spatial levels Adaptive responses may occur fandamentally at the molecular level, but they are manifested at different spatial levels in the whole animal. Animals are made up of several distinct compartments. Each of them may show different adaptive responses that leads to a change in the animal as a whole. The individual cells contain intracellular fluid (ICF) which is its own fluid environment. These cells are directly bathed in tissue fluid or extracellular fluid (ECF). In may species ECF is distinct from blood (ECF enclosed in specialized channel known as blood vessels) or lymph (ECF present in lymph vessels) or hemolymph. These fluids may be different in composition from the classical ECF. The relation between fluids of these three compartments and their
78 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof homeostasis is highly complex. Adaptation to environment may require modification at all these levels. For many invertebrate marine animals, the ECF and blood are identical with sea water and so adaptation occurs at cell–ECF level and within cells i.e. across intracellutar compartments. But in case of nonmarine animals there occur extensive regulation at the boundary between external environment and blood i.e. skin, as well as at the cellular lavel. In terms of the whole animal, adaptations occur at different spatial levels or site. 1. To maintain difference between the outside world and the circulating blood, adaptation occurs at the outside surface e.g. skin. This surface (skin) may be made up of relatively unspecialized epidermal cells or a complete multilayered structure with chitinous, keratinous or lipid containing elements. Sometimes, variability in structure is confined to a particular area of the skin such as gill surface and other areas of skin are relatively inert or impermeable. 2. Adaptations can occur at the boundary between ECF and the circulating fluid. This mainly occurs in vertebrates when some constituents of blood pass out from blood capillaries into ECF and vice versa; some constituents of ECF goes back to blood via lymphatic system. 3. Adaptation at the boundary between ECF & ICF. It involves the cell membrane itself that control exchanges between ECF and ICF. The exact make up of ICF is very different from that of ECF. Total concentration of the fluid of the two compartments, however, should be similar to prevent osmotic swelling or shrinkage of the cells. 4. Adaptation within the cell : Cells themselves are strongly compartmentalized and the organelle menbranes are involved in regulating the exchanges between the cytoplasm and the nucleus, mitochondria or endoplasmic reticulum. As the environmental condition is changed the animals show adaptation at different functional and spatial levels to cope with the environment which is essential for its survival. 5.3 Machanism of adaptation Structural and functional modification of living organism occur as the environment undergoes changes. This is essential for survival of the living objects. Temporary changes occur to counteract the environmental changes and it reverts back to the previous form when the environmental changes are over. This type of change is called acclimatizion e.g. cardiospiratory changes in a mountaineer during ascent to high altitude. But sometimes permanent changes occur to cope with the environmental changes involving genes (actually in DNA). This is called adaptation. The specific shape of the chest of sherpas living at high altitude is an adaptive respiratory change.

79 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Proteins are basically involved in cellular processes. Cell division is the result of DNA duplicatian. Protiens are the products of genetic code of DNA. Alteration of DNA will alter protein structure, hence will alter cellular functions and thus will lead to phenotypic changes at the organism level. Adaptation occurs when any change in the DNA level becomes expressed via protein changes, as a trait that is beneficial in a particular environment and so persists due to selective advantage, eventually spreading through population. Hence anything that controls proteins is at the core of adaptation. A change in the shape of the protein is associated with many cell-functions like switching effects via kinase-phosphetase system (e.g. phosphorylation of glycogen synthase makes it inactive — glycogen synthesis is inhibited, dephosphorylation of the enzyme makes it active – glycogen synthesis is stimulated. Former is caused by glucagon and the latter by insulin ; motor effects like ATP driven shape change in myosin causing its movement along actin during muscle contraction; change in shape of protein pump causing chanelling of ions (Na + –K + pump) and other molecules across membranes and between cell compertments. These functions are again subjected to extremely complex regulation and amplification to produce controlled effects on whole organism. DNA, RNA and protein synthesis Most of the chromosomal DNA in any animal cell does not code for RNA or for protein. Only 1% of DNA sequence is transcribed into functional RNA sequence. DNA is duplicated during cell division. The transposons (Short pieces of DNA consisting of a few hundred to 10,000 nucleotide pairs) present in DNA organisation are involved in mutation. There is some evidence that they undergo long periods of quescence in the genome and then exhibit sudden bursts of activity (transposition bursts) being triggered by environmental change. These bursts form a link between environment and adaptation. They cause increased biological diversity because transpositions will potentially bring together two or more new traits which (of little value alone) become very useful by working together. Thus these transposition bursts can produce randomely modified progeny. This happens at times of environmental stress. Hence transposons act as useful symbiotic factors, generating diversity just when it is most needed. A change in DNA is the fundamental mechanism of evolution. DNA is insulted in a normal course by various factors like thermal degradation. UV radiation metabolite action as oxygen radical. DNA repair enzymes like DNA repair nudclease. DNA polymerase & DNA ligase are induced by DNA - damage. The DNA is repaired and normal function is left undisturbed. This is a safety mechanism of the cell. Such repair is crucial in genetic recombination in meiosis where chromosomes undergo crossing-over. It has been found that minor mis-matches are corrected by such repair

80 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof but where there is a particularly poor match between the recombining strands, the pairing is usually aborted and no recombination occurs; excessive scrambling of the genome which might be detrimental to physiological functions is thus averted. DNA forms RNA by transcription. The RNAs undergoes posttranscriptional modification and active RNAs are formed. There are three types of RNA – mRNA, rRNA and tRNA. mRNA with the help of rRNA, tRNA join aminoacids through peptide bond and form polypeptides by the process called translation. The polypeptides formed by translation process undergo posttranslational modification and are converted to active polypetides. These undergo proper folding with the help of chaperans and functional proteins are produced. The proteins that can be formed theoretically are many. But the proteins that are selected for in evolution are those that can reliably and repeatedly folded up into stable forms which can be subjected to conformational changes by controlling mechanisms. Protein effect is a balance between protein synthesis and protein breakdown. Degradation of abnormal proteins is mediated by intracellular proteosome-ubiquitin mechanism. However, in case of degradation of normal protein with specific halflife is dependent on aminoacid sequence on the N-terminal side of the peptide. Certain amino acids offer protection while other such as Arg, Asp and Glu promote proteolytic attack. Thus the aminoacid sequence of this region can be selected over evolutionary time to give protein with appropriate half life. The protein synthesis at genomic level is regulated by regulating proteins at different levels— (i) Transcrptional control; (ii) RNA processing control; (iii) RNA transport control; (iv) translational control, mRNA degradation control; Protein activity control. The control at the transcriptional level involves the actions of repressor protein (inducible enzyme); corepresser - aporepressor complex (repressible enzyme); transcription activators and transcription repressor; transcription factors operating at the enhancer site and repressor site causing enhancement and suppresion of transcription respectively. Adaptation is achieved by way of formation new structure and development of new functions for better survival in the existing environment. Environmental factors act as stimulus. They in turn modulate intracellular mechanisms by way of genetic alteration and gene expression that ultimately leads to phenotypic changes. Permanent changes in protein structure can be brought about by mutations and recombinational changes in the DNA sequence of a cell. This can produce subtle changes in enzyme activity, signalling activity and subcellular morpthology, as well as expression of other proteins. All these in turn can lead to permanent heritable

81 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof change in the development of organisms. These are seen at a series of levels. At biochemical levels, there may be increased thermal tolerance of an enzyme or expression of a more pH stable allozyme; morphologically there may be developmental changes of gene expression leading to altered positioning of muscle cells, nerves, blood vessels, even completely modified appendages; Physiologically there may be increase in heart rate under the influence of Ca ++ and cAMP signalling regime on Na + pumping; changes in the rate of Na + pumping as more or different channel proteins are synthesized; at the behavioral level there may be an increase in the speed of attack, greater sensitivity to a sexhormone or a concious change in response. All of these proteins induced changes the raw material for adaptation. Environmental stimuli modulate intracellular system through neurotransmitter and hormones. In relation to adaptive changes the hormones are the major controllers of genetic expression and thus of protein synthesis. 5.4 Significance of body size An unconditional generalisation cannot be made about adaptive responses like avoidance, conforming and regulating across animal kingdom. It is dependent on body design and habitat. 1. Smaller and soft bodied animals are more likely to be avoider and conformers. They can use microhabitat more effectively. They enter into protected crevices, burrows or rest on and in other organisms. Since these animals have a high surface area to volume ratio (small animal) rapid fluxes will occur (water, ion, air, thermal energy) and so restoration to normalcy is expensive. They also have little inbuilt protection from swelling and shrinkage and lack complex outer layer which can provide same insulation or impermeability. In these animals in estuarine habitats, a switch over to cyclic avoidance is common an conforming is also seen. Those terrestrial habitats where there is both continuous high environmental stress and high fluctuation, conforming may not be an option, rather exceptional strategies for avoidance are common (torpor, estivation, encystment etc.). 2. Animals with hard outer layers (exosceleton) may have better options for some regulations are a greater independance of their environments Some animals like arthropods likely develop partial regulation of osmotic concentration. Because of exoskeleton, the outer surface can have reduced permeability and may be partly thermally insulated by cuticular hairs. So fluxes are slowed. But in these animals, behavioral avoidance, aided by efficient limbs (and sometimes wings) that can be built from an exoskeleton becomes a major part to cope with environmental change especially in the more rapidly changing terrestrial habitats. 3. Large animals are much more likely to be regulating in all environments with

82 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof important exception of the relatively equable and unchanging open ocean. Larger animals operate in a larger scale environment where rapid changes are relatively unimportant. Because of larger surface (lower surface area–volume ratio) more time is available for regulatory mechanism to operate. They may have better opportunities for energy storage. They may have room for complex neurohormonal control mechanism. In terrestrial habitats, where environmental changes are faster, all these factors work together and the only option left to large animals is regulation. Thus the smaller animals adapt to avoidence and conforming specially if they have soft body . In case of small and medium sized animals with hard exoskeleton, some regulation and behavioral avoidance means are adapted to cope with the environment. The large animals cope with the environment by adapting regulation. Anatomical and functional changes occur in a predictable way with increasing body size. The study of the size-related effect is known as scaling. 4. Heart rate, O 2 consumption, metabolic rate, all are higher in small animals compared to large animals. This is partly related to the disproportional increase of tissue of low metabolic rate like skeleton, fat and connective tissues in large animals. The activity of oxidizing enzymes is higher in corresponding tissues of small animals than larger ones. Cytochrome oxidase and malic dehydrogenase have been shown to be more in small than large mammals. The muscles of small animals consume much more energy during steady running than do the muscles of large ones which is due to higher metabolic rate in small animals. (It is known that large divers remain submerged for longer period of time than small divers. This is because the large animals have less O 2 consumption and less metabolic rate.) Small mammals have relatively larger surface area and so heat dissipates more readily from them. In case of fish, the energy cost for swimming per unit mass of body unit declines with increased size. For an evolutionary and ecological reason, there is an optimum body size for a taxon. Scaling and size dependency are crucial factors in all comparative physiology and patterns in animal adaptation. The size of a particular species of animal is determined by several factors like — (i) Phylogenetic inheritance. e.g. insects are small and vertebrates are large. (ii) Basic physiological design. e.g. animals with open circulation are larger than those with no circulatory system, and those with close circulation may be larger again. (iii) Basic mechanical design. e.g. animals with hydrostatic skeletons are usually relatively small as are those with exoskleton; while those with tubular endoskeletons are relatively large. (iv) Habitat. e.g. any given design may be larger in aquatic habitat giving internal support than on land where self-weight is a problem.

83 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Scaling effect have been observed in migration. In case of avoidence as a measure to cope with stress birds usually migrate; while terrestrial animals, specially smaller ones, are inclined to escape by hibernation and torpor. The smallest migrating mammals have a body mass of about 20 Kg (some African antilope) ; where as very small birds and even monarch butterfly having one gram body mass are regular migrators. Thus different sizes and locomotory modes affect the ability to migrate effectively. For any given migration time, flying animals will be able to complete a much longer distance than walkers or swimmes of similar size or alternatively, to achieve the same distance in the same time, walkers or swimmers would have to be much larger. Global, terestrial or marine migrations are therefore slow and prolonged often taking many months (as in salmonid fish). Whereas aerial migration by birds can be completed in a matter of days and weeks. Adaptation of the capability to store food is an important factor in migration. Few animals undertake nonstop migration relying on food stores; many animals accumulate fat stores of 25 – 50% of body weight before migrations. Since fishes are generally ectothermic with 10 fold lower metabolic rate than similar sized birds and mammals, they can migrate much further without feeding and have the greatest migratory capability. Several experimental and other observations indicate that adaptation is related to body size in respect of cellular adaptation as well as in organism level. Because of small size, the desert rodents evade the heat by retreating to their underground burrows during the day. However ground squirrel, although of small size is similarly handicapped in hot weather but they adapt in a different way. When they are outside their burrows on a hot day they get heated very rapidly. They cannot tolerate very high temperature and die if heated to 43 0 C. However a temperature of 42·4 0 C is tolerable by them without apparent ill effects. When it is heated it goes to a relatively cold burrow as get cooled rapidly due to large surface area and the exposure to hot climate is tolerated. 5.5 Suggested questions 1. Explain acclimatization, acclimatian and adaptation. Write briefly on the levels of adaptation. 2. Give brief accounts of mechanism of adaptation. 3. Name the adaptive responses seen during environmental change. How are they related to body size ? 4. Write briefly on scaling with examples. Comment on or name the different factors that determines the size of an animal.

84 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Unit 6 ? Physiological adaptation to different environ- ments Structure 6.1 Marine adaptations 6.2 Adaptations for life in an estuary 6.3 Fresh water adaptations 6.4 Extreme aquatic environments 6.5 Parasitic habitats 6.1 Marine adaptations There are thousands of species of marine life, from tiny zooplankton to enormous whales. Each is adapted to the specific habitat it occupies. Throughout the oceans, marine organisms must deal with several things that terrestrial life do not ? regulating salt intake ? obtaining oxygen ? adapting to water pressure ? dealing with wind, waves and changing temperatures ? getting enough light Salt regulation : Fish can drink salt water, and eliminate the salt through their gills. Seabirds also drink salt water, and the excess salt is eliminated via the nasal, or “salt glands” into the nasal cavity, and then is shaken, or sneezed out by the bird. Whales don’t drink salt water, instead get the water they need from the organisms they eat. Oxygen : Kish and other organisms that live underwater can take their oxygen from the water, either through their gills or their skin. Marine mammals need to come to the water surface to breathe, which is why the deep-diving whales have blowholes on top of their heads, so they can surface to breathe while keeping most of their body underwater. Whales can stay underwater without breathing for an hour or more because they make very efficient use of their lungs, exchanging up to 90% of their lung volume with each breath, and also store unusually high amounts of oxygen in their blood and muscles when diving. Temperatures : Many ocean animals arc cold-blooded (ectothermic) and their internal body temperature is the same as their surrounding environment. Marine mammals, however, have special considerations because they arc warm-

85 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof blooded (endothcrmic), meaning they need to keep their internal body temperature constant no matter the water temperature. Marine mammals have an insulating layer of blubber (made up of tat and connective tissue) under their skin. This blubber layer allows them to keep their internal body temperature about the same as ours, even in the cold ocean. The bowhead whale, an arctic species, has a blubber layer that is 2 feet thick. Water pressure : In the oceans, water pressure increases 15 pounds per square inch for every 33 feet of water. While some ocean animals do not change water depths very often, far-ranging animals such as whales sometimes travel from shallow waters to great depths several times in a single day. Whales can dive deeply. The sperm whale is thought to be able to dive over 1 1 / 2 miles below the ocean surface, and they can do that successfully because their lungs and rib cages collapse when diving to deep depths. Wind and waves : Animals in the intertidal zone do not have to deal with high water pressure, but need to withstand the high pressure of wind and waves. Many marine invertebrates and plants in this habitat have the ability to cling on to rocks or other substrates so they are not washed away, and have hard shells for protection. Light : Organisms that need light, such as tropical coral reefs and their associated algae, are found in shallow, clear waters that can be easily penetrated by sunlight. Since underwater visibility and light levels can change, whales do not rely on sight to find their food. Instead, they locate prey using echolocation and their hearing. In the depths of the ocean abyss, some fish have lost their eyes or pigmentation because they are just not necessary. Other organisms are bioluminescent, using light- giving bacteria or their own light-producing organs to attract prey or mates. 6.2 Adaptations for life in an estuary The term estuary comes from Latin aestus, meaning tide. The adjective aestuarium means tidal. Thus estuary is defined as ‘the tidal mouth of a great river, where the tide meets the current’. There are a number of vertical and horizontal attributes to estuarine ecosystems. The intertidal zone is alternatively flooded and exposed. There may be salt marsh or mangrove wetlands, algal beds, sand or mud flats, reefs of oysters, mussels or calms in this region. Organisms that live in this region have developed special adaptations. Like other aquatic ecosystems the vertical gradient of light is a limiting condition for photosynthetic activity. At euphotic zone, where light reaches the bottom plants can live attached to the bottom. Estuarine water clarity tends to be much greater near the ocean, so both rooted and planktonic plants generally photosynthesize in greater

86 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof depths than in low salinity regions. Animals of the aphotic zone are dependent on transported food from somewhere else. Estuaries are productive environments for aquatic life. They are rich in nutrients compared with rivers and oceans, and they are also good animal refuges, offering protection from storms, competitors and parasites. However, estuaries do experience sudden and often widespread changes in salinity, temperature and dissolved oxygen levels, so aquatic organisms must find ways to cope with these changes. Dissolved oxygen content is another important parameter for estuarine life. There exists a gradient from oxidizing (aerobic) to reducing conditions (anaerobic or anoxic) in estuaries for biological and chemical processes. Salinity Salinity is perhaps the most important factor affecting aquatic species. Most aquatic animals are adapted to life in sea water. These animals vary in terms of the degree to which they are able to tolerate the lower salinities of the estuary. A smaller number of animals are adapted to life in fresh water, and few of these species tolerate salinities in excess of 2 ppt (parts per thousand). Ocean salinity is 35 ppt. The salinity level of the estuary varies along its length, with depth and with the seasons. There are extreme changes in salinity from almost freshwater conditions in winter to saline (or almost hypersaline) conditions in summer. This change in salinity causes a change in the concentration of dissolved gases (fresh water contains more oxygen than sea water at the same temperature) and in the density and viscosity of the water. Fig. 5.1 : Vertical zonation of estuarine habitat

87 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Adaptations to changing salinities Estuarine organisms possess adaptations or behaviours that enable them to cope with changing salinities. Few organisms remain in an estuary for the whole of their life cycle. Some fish are truly estuarine, spending their whole lives in the estuary. Some use it as a nursery habitat only and others are marine visitors, coming in when the salinity suits them. Mobile animals like fish and crabs can swim away from unfavourable conditions. Prawns and crabs move out of the estuary in winter when waters are less saline. However, less mobile (sedentary) animals such as barnacles and worms have to either seal themselves inside their shells or adapt to the conditions. Many sedentary animals die when conditions are unfavourable and must recolonise when conditions change. Many algae and seagrasses die off during winter periods when salinity levels become too low. Some organisms are able to tolerate extreme conditions for a short time only. A sudden change, such as an unusual heavy summer fall of rain which produces a freshwater flow into the estuary, or extreme conditions of salinity, will produce a variety of responses. For example, worms, molluscs and fish produce slime or mucus to cover and protect their sensitive body surfaces. Some polychaete worms and crabs retreat into holes or burrows, plugging them. Other animals withdraw their sensitive body parts, or close their shells. If an organism cannot escape or reduce contact with the water during times of abnormal salinity it must use a physiological response. The marine species generally reside near the mouth of the estuary, the freshwater species in the low salinity areas and the estuarine species somewhere in the middle. Many estuarine species are osmoregulators, meaning they can maintain a constant salt balance, no matter what the salinity of the water is. Alternatively, an animal may modify its metabolic rate or change its patterns of activity. The physiological response of an animal to salinity changes takes time to complete, so it is often supplemented by a behavioral response that enables it to either delay or moderate exposure to unfavourable conditions, completely avoid them, or slowly adapt their body to the new saline environment. For example, some bivalves close their shell valves when sea water suddenly becomes diluted. After a while they become used to these conditions. Organisms that are capable of dealing with varying salinities are euryhaline, and organisms that can only deal with small changes in salinity are stenohaline. Stenohaline animals rely on coping methods such as moving out of the area, burrows in the sand, excreting excess salts or closing their shells, and worms, molluscs and fish can produce mucus or slime to cover sensitive body parts. The mussel, Xenostrobus securis, deals with salinity of 2g/L by closing up their valves for up to many months. When an organism is unable to move or reduce contact with the unfavorable salinity, it then relies on physiological responses such as osmosis (become iso-osmotic with estuarine water) or changes its activities or its metabolic rate. Generally mature organisms are better able to handle the stress of

88 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof salinity changes than reproducing adults or newly hatched eggs. Many sessile organisms die if conditions become too harsh and have to re-colonize when conditions are once again suitable. Euryhaline crustaceans respond to changes in salinity by molecular processes. Vertebrates respond by regulated blood osmotic concentrations and controlling ion fluxes and organic osmolytes. Invertebrates can regulate blood osmotic concentration, cell volume or both. Sodium chloride is very important in regulating blood osmolarity in euryhaline crabs, and regulating fluxes and permeability's of these ions is how the crabs deal with the salinity. Green shore crabs respond quickly to salinity variations and within six hours, their blood Na + level reaches a steady state. It is thought that the crabs possess a sensory organ on their legs known as "hair peg" which is what responds to the salinity variations and sets the wheels in motion for response. Gill structure is also helpful in dealing with changes in salinity as the gills contain two different sets of epithelial cells, which are believed to be crucial to ion and gas exchange. Adaptations to changing temperatures Temperatures are more variable in the estuary than they are in the ocean. In winter the estuary is colder than the ocean, and in summer it is warmer. Daily temperature fluctuations can also be extreme, especially in the shallows. Also, the solubility of oxygen depends on temperature. (More oxygen dissolves in cold water than in warm water.) Adverse temperatures may cause responses of avoidance and escape. One of the most common mechanisms that certain organisms use to cope with conditions in the cold winter months is to transform into a resting stage. Another habit is to burrow into the mud or sand on the bottom of the estuary. Fortunately in the Swan-Canning system temperatures are rarely extreme, never reaching freezing in the winter or going above 40°C in the summer. Organisms that are able to withstand varying temperatures are eurythermal. Those that can't must use other responses such as moving, burying themselves, or to transform to their resting stage. Temperature can fluctuate over seasonal cycles in temperate estuaries. Bacteria have adapted to this by reorganizing their biochemical pathways and adjusting protein and DNA synthesis rates. Adaptations to changing oxygen levels In water containing low amounts of oxygen, organisms have physiological and behavioral mechanisms to survive. In sessile animals that cannot move to a more oxygen rich environment, they must lower their energy demand, maintain their metabolism or use a method of creating energy without oxygen. Many animals do this by increasing their heart rate and the flow of water past respiratory surfaces.

89 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Some organisms have respiratory pigments, which allow them to maximize the oxygen they do get. Others are very good regulators of oxygen uptake and maintain constant rates of uptake until the oxygen falls to a 'critical pressure'. They then switch to anaerobic metabolism. This can only be maintained for a short time. Some organisms, such as oyster larvae, have been shown to lower there aerobic metabolism in response to hypoxia. Nektonic adaptations to estuarine environment General adaptation for true nektons include the existence and position of swimming organs (e.g. fins), a smooth streamlined body, a specific gravity close to water, the ability to extract oxygen at a relatively rapid rate from the surrounding water in order to support the large energy requirements of swimming. Additionally, well developed sensory organs are necessary for most organism with active nektonic existence. Fishes mostly achieved these requirements. They have streamline body and are able to control their depth through varying their specific gravity by changing the amount of gas in the swim bladder. Fishes have gills well adapted for rapid oxygen exchange, thus meet an active and sustained movement. Their specialized sensory organ, the lateral line, is very sensitive to sound waves and to changes in water density. The chin barbells in some fishes help to locate food. There are specific adaptations of nekton that allow them to flourish in estuaries. This is interesting since the estuarine environment is apparently hostile or stressful. The rapidly changing physical and chemical environment imposes great energy demand of fish so that most species cannot survive there. Estuarine species may be divided according to tolerance depending on temperature and salinity. Some species require high salinities and are not found in the riverine reaches of estuaries. Some other species possess a wide range of salinity tolerance. There are only a few species which are adapted to withstand extreme variation in both temperature and salinity. 6.3 Fresh water adaptations Aquatic animals develop various types of adaptations according to changes in aquatic environment. This is essential for the maintenance of life in aquatic medium. Majority of herbivores are algal grazers. Animals show variation as they live in ripples and send bottom areas. But most animals are seen in these two areas. First fautra of the hill stream of India are very rich and varied. Botia dayi, Botia dario, Nemacheilus aureus, Nemacheilus beavani, Nemacheilus montanus, N. rupicola are bottom living fishes usually found under stones and boulders. Because living at the bottom, they have broad head, dorso-ventral flattening of the anterior part of the body, presence of thorasic adhesive apparatus, horizontal alignment of paired fins. Many fishes at the bottom stay under stones and boulders and thus avoid water

90 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof currents. The paired fins are fanlike and horizontally placed. The ventral surface is smooth, without scales, and flattened. Those, which are inhibitant of fast flowing water, need to move without the favour of water current, by means of mascular effect. M. armatus has an elongated cylindrical and cel like body and the posterior part is highly compressed laterally. The snout is produced into a long beak-like process to reduce the pressure exerted by the water current. Some fishes like N. botia are inhibitants of slow running water and usually abundant in ports. These fishes assemble just below the surface of the water and move through the weed-beds for feeding and returns to the pools. In fishes which feed on insects, mainly larva and nymphs, the mouth is ventral and suited to the bottom feeding habit. They make adjustments to withstand the current. Many nymphs have efficient adaptations, which enable themto tolerate current strength upto 300 cm/sec. There animals cling to the surface of the rock keeping their heads towards the current and when pressed firmly against the substratum. The water current exerts a downward pressure which helps to held the animal in position. Body flattering is thus one of the adaptations to enable organisms to take shelter beneath stones and avoid the force of the current. The inches, which usually live under the stones have flattened streamlined bodies. Streamlining of the body helps to offer the least resistance to water and is also found among many stream-invertebrates. The tapering bodies of the nymphs of the mayfly illustrate this very well. Development of suckers helps the animal to attach if itself to the substrate. The larva of the blackfly, Simulum are found in large numbers, attached to stones in stream-bed. They are one of the mostly adapted animals to the life in fast-flowing water. Simulum possess large salivary glands which secrete a pad of sticky silk on the substratum to which it attaches itself by means of a pair of modified prolegs at the rear end of the abdomen. At the posterior end of the semicrect body is a circle of rows of outwardly directed hooks, which when the muscles of the disc are relaxed, move outward and cling on to a silk web previously placed there by the larvae. The anterior end of the body then swings freely in the current. There is a fan like food gathering organ on each side of the mouth which traps food particles from the water current. The development of hooks is also an adaptation to current. Caddies fly larvae occur most abundantly in streams with moderate to swift currents. They usually construct cases made of leaves, twigs, sand grains and stones cemented together with silk secreted by the animal itself. In swift water, the cases are stout, cylindrical, tappered posteriourly and are usually swollen and more solidly so constructed of sand, pebbles or rock fragments. Hill stream fishes have also become highly adpated to this dynamic environment.

91 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof The chief factors affecting the life cycle of these organisms are : (A) Strength of the current, (B) Hill streams are shallow and clear, so they have to bear intense light during day-time, (C) Sufficient food is available but in the form of algae covering stones and rocks. Fishes have to develop special adaptations to live in the environment. The head and the body of most hill stream fishes are greatly flattened and in the highly specialized form the body is leaf like. The ventral profile is straight while the dorsal profile is only slightly arched. The head is usually small and semicircular. The size is generally small so that they can easily take shelter any rock and beneath them, and can conveniently live in shallow water protected from direct sunlight. The scales of these fishes undergo reduction or are very minute or embedded in the skin as in Nemacheilus. This is of special significance to adhesions to rocks and stones so that although scales are present in the dorsal in the dorsal and lateral aspects, they are absent in the region of the chest. The paired fins specially the pectorals are modified for adhesion and the number of inner rays is increased. The fins are shifted that it can act as hydroplanes and adhesion to rocks and stones is facilitated. In most species, the lower lobe of caudal fin is longer than the upper one. Some species possess (eg. Nemacheilus, and Glyptosternum) develop a band shaped caudal peduncle which appears to be an adaptationto life in fast flowing waters. The position of the mouth is shifted from the anterior and of the snout to the ventral side towards the tip of the snout. Instead of a transverse the mouth is generally horny covering which help to scrape the algal material from the stone-surface, for feeding. The mouth is surrounded by sensitive barbules beneath the head. These help in testing the substratum. In some species (eg. Nemacheilus) the lips are divided in the middle and are swollen so that they form a saucer, when pulled outwards. In Glyptosternum, the lips are reflected and spread round the mouth to form a bread sucker for attachment. The eyes are generally small in size and pushed towards the upper surface where they come to lie close together. Besides formation of additive sucker for the lips, the skin is thrown into grooves and ridges on the ventral side of the body, specially between the pectroal fins of the body. Such straited structures serve as friction plates for attachment to stones. The gill openings are restricted to sides only and do not extend beyond the pectoral fin. Any effect on respiration is copresneted by the well acrated water of the stream. The bottom lining forms required negative buoyancy ; the air bladder is considerably reduced or degenerate and becomes completely a useless hydrostatic organ. This helps the bottom living organisms to aquire negative buoyaney. Most fishes expend much energy in fighting the current, hence they spend long periods in resting.

92 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Amplibians found in torrent waters generally develops small lungs, presumbly to reduce buoyamoy. Size of the appendages and body are reduced or a streamline shape is achieved to reduce the surface areas exposed to the full impact of current. Most animal species, though have developed devices for clinging to the bottom, they are more abundant at the underside of the rocks in riffles, than they are on the upperside. Some species, however, such as rotifers, water mites, protozoans find shelter within the mass of algae that may cover the top of the rock. Even swift water fishes take all the advantages of whatever protection available. Inspite of various mechanisms for maintaining position against the current, a continuous drift animals occurs downstream. The driff is more prominent at night than daytime. A continuous drift of downstream is compensated by the adult insects to lay their eggs in shallow water. The adult insects emerging downstream commonly fly upstream for reproduction. Some fishes move upstream for spawning. Lake is a stretch of water surronded on all sides by land. Different types of lakes are there like glacial lakes of high altitude, tectonic lakes formed due to movement of deeper parts of earth's crust, lakes formed due to volcanic activity, lakes formed by wind action or land slide. In the lakes, there is a gradual rise of temperature as one moves from the depth towards the surface. Amount of O 2 present in a lake or pond depends on extent of contact between water and air, on the circulation of water and on amount produced and consumed by the lake community. Animals and microgamisms present in water, use O 2 and produce CO 2 . According to habitat characteristics, the pond and lakes exhibit 3 zones— littoral, limnotic and profundal zones. The shallow water which has light for the surface to the bottom is called litteral zone. The bottom part that does not receive day light is called profundal zone. Light serves as the initiator or driving force of the ecosystem. It controls the development of pigmentation in animals, and stimulates the development of various adaptations. The development of vision depends on availability of light in the media. Fossorial forms have generally reduced vision and so do deep sea forms. In some organisms locomotory activity depends on light intensties. This is called photokenesis. In cave dwelling and fossorial or borrowing animals, the size of the eyes are generally reduced due to absence of light or is found embedded in the integument. Eyes are absent in cave dwelling fishes and cave amphibians. In burrowing amphibious such as the Apoda, the eyes are covered by a fold of the integument. In abyssal depth, where there is absence of light, the organisms either have reduced vision or the eyes are highly developed to preceive low light, which spread the occurrance of biofuminiscente at those depths.

93 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Light controls locomotor activity of many animals by a direct action on locomotion. This is called photokinesis. The rate of swimming in mussel crab has been shown to be upregulated where the intensity of light is increased in experimental condition. Temperature Heat is an ecological factor which has an wide ranging influence directly on the biota as well as indirectly in combination with other factors. It is a direct regulator of the climatic conditions of a place as well as modulator of other factors such as density and salinity. Organisms have adjusted themselves, and have developed various adaptations to meet the temperature ranges. Solar radiation falling on earth supplies energy to the living organisms in the form of heat, the activity of the organisms depend on transfer of heat from the media in which they live. In their adaptations, organisms have specialized themselves to live whether narrow limits in small scale environment (microhabitat) that are significantly different from the larger environments (habitats) of which they form a part. In case of poikilothermic animals, the physiological activities are adapted to function in spite of the temperature strees. Poikilotherms are ectotherm and the body temperature is dependent on the amount of heat they take up from water (environment). In case of homeothermic animals, the heat generated in the body is retained. Hence, it has high body temperature. They have a high metabolic rate compared to poikilothermic animals. They are endothermic animals. Homeothermy is necessary for organisms to inhibit the terrestrial environment, because air and land experience violent fluctuations of temperature and climatic conditions. 1. Animals produce eggs that can survive thermal extremes. 2. In arctic regions animals have their freezing point of plasma lowered for – 80 o C to –1.47 o C, in response to seasonal changes in temperature. They produce anti- freeze, which reduces their & risk from freezing. 3. Amphibians in land lose water very fast by evaporation through skin. Their risk of dessication is partially compensated by a reuction in urine flow, update of water for urinary bladder and absorption through skin, all promoted by vasotocin. Their survival is further aided by a retread to underground burrows, where the temperature is much lower than on the surface. Reptiles have impermeable skin, but they also lose temperature byinceased evaporation, although the rate is lower than amphibia. However, some reptiles can control the body temperature to some extent. In Varanus, as the temperature of environment rises, powerful pumping movement of the mouth and neck occur. This is the functional equivalent of ponting in mammals. It can also increase its body temperature by increasing its metabolic rate.
94 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof In general most of the poikilotherms undergo hibernation and estivation to meet temperature extremes, while homeotherms are able to regulate their body temperature through special adjustments. Hibernation occurs to tide over extremes of cold. The term is used to describe conditions or instances in which metabolism is reduced, animal puts itself in a state of rest with reduced metabolic rate. There occur a marked drop in body temperature. It is of wide occurrance in poikilotherms specially at higher latitudes. Terrestrial reptiles avoid overwintering by retreating into rocks, crevices, or borrows as do the amphibians. Many aquatic turtles spend the winter buried in mud beneath ponds and stream borders. Among mammals hibernation is reported for the order Monotremata, Marsupialia, Insectivora, Chiroptera, and Reptilia. At least one bird is known to hibernate. During awakening from hibernation a sequence of events occur— (i) rapid rise of body temperature, (ii) warming of heart promotes circulation of blood which carries heat in a restricted way such that the heat passes largely to the respiratory system and the brain during the early stages of awakening. Aestivation Dormancy during summer months, when temperature is high, or excessive dryness and shortage of food products is called onset of dry season, burry in the mud of a swamp or river bed and forms mucous lined cell in which it aestivates. This may last for as long as seven months, with the return of rains and subsequent rise of water level, the fish energes from its aestivation chamber. In many insects, dormancy take the form of diapause (during this time growth and development are suspended or retarded). In homeothermic animals, body temperature is kept constant in spite of changes of environmental temperature. These animals are curythermal and are capable of regulating their body temperature by thermostatic regulation through physiological adjustments. Amphibia move from direct sun to sheddy place. Insects move from the sun to the stones and beneath leaves. Some insects freeze at night and thaw out in the day in cold climate. O 2 content of water is less compared to air. Thus, to ensure O 2 supply, respiratory adaptations occur. These include (i) increase in respiratory surface, (ii) use of higher affinity oxygen storing pigments, (iii) modulation of ventillatory or circulatory rates. In fresh water vertebrates, gills are the most common adaptations to ensure O 2 supply. Diferent forms of gills develop eg. serial by repeated filamentous gills, tentucular crown gills, enclosed lamellate gills are seen in molluses and annelids, tracheal gills, spiracle gills, rectal gills in insects. Freshwater vertebrates use cutaneous or gill based respiration. Skin breathing is quite common in fresh water fish larvae and many eels. Cat fish rely on it most extensively to sustain their metabolic rate. Many species depend largely on skin respiration. eg. Salamander, frogs. Although

95 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof most fishes, and juvenile or neotenic amphibions use gills and nearly all adult amphibians, reptiles, birds and mammals (being secondarily aquatic) rely on air breathing through lungs. The haemoglobin saturation curve shows a 'left shift', when the O 2 demand is increased than occur increased rate of ventillation in fresh water animals which may arise from hypoxia of the environment, or increased metabolic activity. In small animals like sponges and rotifiers, ventillation is mainly done by cilia and flagella, their activity increasesas needed. In cray fish, hyperventillation is seen initially, but as hypoxia sets in, the animal shows brady cardia and the circulatory flow pattern alter to give increased flow anteriorly over the brain. Fresh-water vertebrates show sophisticated ventilatory response to hypoxia. In fish, hypoxia is sensed by oxygen receptors located in the brain and aorta. Both rate and stroke volume are increased in hypoxia, by changing the buccal and opercular pumping pattern. In freshwater vertebrates, when O 2 demand is increased due to hypoxia in environment or increased metabolic activity, ventillation is increased, by changing the buccal and opercular pumping pattern. In snakes and turtles, ventilation is intermilttent at rest, but becomes continuous during steady swimming. Green turtle shows a seven fold increase in mean ventillation frequency and increase in both pulmonary and aortic blood flow. In bimodal breathers, besides aquatic breathing, air breathing devices are present. For example, in arthropods, air bubbles and plastrons are used as air supply systems. Special diving adpatations are found in fresh water reptiles, birds and mammals. Many essentially freshwater animals are able to use both aerial and aquatic oxygen and may switch over to air breathing when their aquatic habitat begins to dry up or overheat and becomes hypoxic, or it becomes too rich in H 2 S, due to decomposition. Custaccans, fishes and amphibians are most notable as bimodal breathers. Many bimodal crabs have reduced gill-area, typical of air breathers. Fresh water habitat give rise to problems related to reproductive strategies. There is a general tendency for the fresh water invertebrates to have very short life cycles. Commonly there is a very high reproductive output in freshwater invertebrates. In Drussena (Zebra mussel) one female may release 1 million oocytes per year. Reproduction in fresh water vertebrates also show a similar pattern. However, secondary freshwater vertebrates that are essentially land animals, usually resort to a terrestrial site for reproduction. Turtles lay their eggs in the upper shore. Fresh water is rarely very deep, the largest lakes being only 1000-1500 meter, so that freshwater animals do not normally have to cope with great pressure. However,

96 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof buoyancy is more difficult in fresh water due to reduced specific gravity of the medium giving very little lift. Swim-bladder is present in fish and it is 6-9% of body volume. Swim bladder gives the buoyant effet. Suckers and hooks for adhesion develop specially in case of bottom dwellers. Certain fish have ventral sucker and blackfly have hooks to hold on to rocks. The four eyes fish Anableps can focus simultaneously on terrestrial and aquatic image having practically two eyes with a pear shaped lens and two sets of pupils above and below the water miniscus. Fresh water beatles, bugs, and fishs such as pike have eyes with large pupil. This is also seen in surface dwelling betles and bugs. Chemoreception is also an important device in many freshwater animals for prey location, predator avoidance and the location of hosts and mates in river. Many aquatic insects have water pressure receptors ; flow receptors are also found. In crustaceans and fishes they are located in cephatic appendoges like antennae or vibrissae. Electro reception occurs in a range of fresh water fishes with a very weak and strong field producers. Echolocation is seen in freshwater dolphins, but it is largely lost in freshwater animals. Most of the freshwater zooplankton are filter feeders. Hydra takes small prey that brush against and trigger the shinging cells in its tentacles. Aecolerated cutrophication is an well recognised process in both rivers and lakes. The reduced O 2 level leads to submerged plants disappear as light is cut off and animal casualty follow. The submerged animals change eg. Tubifex worms replace crustaccans and then surface dwellers decline. Fresh water can be polluted in different ways. Contaminated natural freshwater is the single biggest source of human disease of the world today. Some of the diseases is due to parasites and pathogens naturally present in freshwater. But a huge additional hazard is added by pathogens derived from sewage and animal wastes. Global warming leading to acid rain makes the water acidic. This causes— (i) increased mortality of fresh water animals, (ii) It solubilizes minerals. Alluminium sloubility causes aluminium poisoning of many fishes, (iii) Cuticle formation in insects and crustaceams is affected, thus leads to defective osmoregulation. (iv) Hatching of some salmonid fishes is decreased. Thus, abudance of many species decline, particularly snails, amphibians, small crustaccans, leading to decrease in zooplankton. Other fishes such as eel, insects and rotifers are also destroyed it the pH stays below 5 for long time. 6.4 Extreme aquatic environments The deepsea physical environment has special characteristics. To cope with this environment, the animals living there undergo suitable adaptation.

97 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof Four remarkable characteristics prevail in this area. These are : (1) Absence of sunlight. Beyond 200 fathoms their is no sunlight. (2) Quiescence : Because of depth the movement of water is almost absent. (3) Cold environment : In the deepsea the temperature is nearing the freezing point and the temperature remains constant. (4) Pressure : The pressure is high as it increases with depth. (5) Lack of green vegetation : Total absence of light is the reason for total absence of green vegetation in the deep sea environment. For survival in the deepsea environment, the animals have to develop adaptive changes to cope with adverse physical condition present there. The general characterstics of deepsea animals are as follows : (i) The deepsea animals are weak and delicate. (ii) The body is generally simplified. (iii) They are either totally blind or they possess powerful telescopic eyes to catch maximum amount of light. (iv) They develop long feelers to act as tactile organs. (v) Almost all the deepsea animals are luminescent. (vi) Most of the deepsea fishes live on the exudes of decaying matters and so the animals lose the masticatory power. There are other animals which possess powerful jaws. (vii) Most of these develop wonderful devices for caring the youngs. other produces large number of youngs to overcome the hostile environment. (viii) Small size is an important charasteristic of deep sea living. Structural modifications in deep sea animals. Almost all the phyla have representatives who lead deep sea life. The modification of the invertebrates are diverse compared to vertebrates. Modification of the vertebrates of the deep sea have been found to be as follows. Amongst the elasmobranchs the true sharks do not exhibit deep-sea charasteristics excepting the luminous sharks. The silver shark, however, show deep-sea characteristics in having huge eyes and long alternated body and tail. Amongst the teleosts, the typical deep sea form is Cetomimus. It has a long mouth, small teeth, very small eyes and scaleless body. However, in Ipnops there are no eyes and only two large luminous organs are found on the head. Scaleless body and well developed luminescent organs are the characteristic features in Stomiatidae. In Gastrostomus, the body is long, slender with rows of luminous organs on the lateral sides of the body and the mouth is bounded by very large jaws. The Gadiformes
98 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof (cod like forms) have reduced mouth and dentition; the eyes are extremely large, the trunk is reduced and has a filamentous tapering tail. The anglers show typical deep sea characteristics. The paired fins are adapted for crawling on the bottom of the sea and the anterior finrays of the dorsal fin function as a lure. In Linophryne, the finrays are provided with luminous organs to attract the prey. The Oneirodes, however, is blind but has luminous organs to compensate for the loss of eyes. In another deep sea fish, Protostomias, specialised light producing organs are present in rows on the lateral side of this body. Another important feature of deep sea fishes is flatness of the body to adjust with high pressure. The body in these fishes becomes flattened and the mouth is shifted to the lateral side of the body. These structural modifications in deep sea forms are due to peculiar physical condition of the deep sea environment. The deep sea forms are geologically very recent in origin. These forms were originally the inhabitants of the pelagic or littoral regions which migrated to the deep sea and become adapted. 6.5 Parasitic habitats Parasites depend fully on their host for their living. Hence, they adapt themselves accordingly. Adaptation to specific environments is a dynamic feature of all living organisms. Pararitism starts with an accidental meeting of few animals. Gradually one, the guest, starts to lead a more dependent life upon its host for food and shelter. This change from a free living life to a life in which food and shelter become available without any effort bring about profound modifications in the make up of parasites. The helminths are modified morphologically and physiologically to live in their particular environment. The modification depend on the degree of parsitism. Morphological adaptations Every part of the body of a helminth parasite exhibits twist due to parasitic mode of life. The strutural modifications involves two aspects — degeneration and attainment of new organs. Degeneration : There occur loss or simplification of unused organs or parts. In helminthes the loss or degeneration involves particularly the digestive and locomotory organs. (a) Digestive / Alimentation. As the parasites begin to live on digested or semidigested food of the host, there occur reduction in their alimentation and digestive glands. In the adult trematodes, the digestive tube is a blind gut. In the larvae of

99 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof trematodes, the gut is either very simple or completely eliminated. In adult tape worm the alimentation is completely absent. As the animal lives amidst digested and semidigested food, the food is absorbed directly through the general surface of the body. Trichinella and cystacercus larvae in vertebrate muscles occur in such location that they are constantly surrounded by rich nutritious food. The food is absorbed through the outer layer of the body. (b) Locomotor organs : Thes locomotory organs are not necessary for the parasites because they live their entire life in the body of the host. Hence, locomotor organs are totally reduced. However, in the free living larval stage of the parasites, such as miracidian and hexacanth the ectoderm is ciliated. (c) Sense organs : These sense organs in helminthes are also simple structures. This happens because they lead a sedentary life in a sheltered habitat. This correlation is especially seen in the endoparasites. 2. Attainment of new structures/organs Parasitic existance is made possible due to modification of old structures and formation of new structures. These are necessary and helpful in food absorption, protection, attachment and vast reproduction. (a) Integumet : The outer integument or cuticle of helminth parasite becomes highly modified and is so adapted as to resist against the digestive juices, passage of food and for adhesion. Cuticle become thin to absorb food in those parsites which live in rich nutritious environments e.g. adult liver fluke in bile, blood flukes in blood, tapeworms in intestine. Trichinella and cysticereus in vertebrate muscle, several larval forms developing in lymph spaces and in blood stream. In case of some gut parasites e.g. tapeworms, nemotodes etc. which remain attached to the wall of the gut the cuticle is suitably modified— it becomes thick impregnated with chitin like substance and enzyme resistant, so that it is not digested by digestive enzymes but remain permeable to water. In most trematodes the outer integument contains spines, spinnules or scales of various kinds. These cuticular modifications protect the outer surface of the worms against the abrasive action of food and roughage flowing around them. In the chinese liver fluke Cloriorchis sinesis, the larval stage has a spinous cuticle, this suggests that possibly it was a gut parasite and subsequently converted to a parasite of the bile passage. (b) Musculature : The muscles are well developed in tapeworm (e.g. Taenia). This enables Taenia to spread and elongate their bodies along the length of the intestine of their host. Similarly, power of locomotion enables the round worm (e.g. Ascaris) to counteract gut peristalsis and thus maintain their position in the intestine. In this way the parasites becomes capable to obtain predigested nutrients of the host.

100 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof (c) Organs for attachment : All parasites develop suitable devices for attachent to their hosts, either to the exterior or to the interior of host cavities. Helminthes are variously modified for adhesion to the body of their hosts. In all adult parasitic flatworm acetabula or suckers develop. The liver fluke (e.g. Fasciola) has two suckers on the ventral side of the body one anteriorly and another posteriorly. In the tape worms the scolex bears either four sucking cups (e.g. Taenia solium) or accessory suckers (e.g. Myzoplyllo bothrium) or lateral sucking grooves or bothria (e.g. Diplyllo bothrium). Some cestodes and nematodes also develop hook-like structures near the cephalic ends that further help in attachment. In some, a basal circlet of hooks (T. solium) or rows of hooks develop (Dipylidium carninum). A buccal armature bearing toothlike structure has been found in Macracanth orhynchus. In some helminths e.g. miracidium and cerceria, unicallular secretary glands develop which help during penetration into the host. The secretion contain a lytic enzyme that digests the host's time make passage through which the worms move. In larval trematodes cystogenous glands develop whose secretion help in cyst formation. These glads degenerate after their functions are over. Hook worm contain buccal glands which pour secretion that are anticoagulant and also has histolytic action. (d) Vast reproduction : Parasitic adaptation involves a significant development of reproductive organs with much increased capability of reproduction. In both flat worms and round worms, the interior of the body is mostly occupied by the genital organs. The chances for survival is increased by astronomical production of eggs. Self fertilization is more common than cross fertilization. The life history usually includes several larval stages for multiplication and for easy and sure transfer from one host to anohter. The nervous system in all parasitic helminthes and excretory system, paticularly in trematodes show little deviation or adaptation to perticular mode of life. Physiological adaptation Apart from structural modification physiological or functional adaptation are also seen in parasitic adaptation. 1. Intracellular digestion : The parasites develop intracellular digestion because they feed on tissue elements and inflammatory excudates. This has been observed in flukes. 2. Osmoregulation : The osmotic pressure of the interior of parasitic worms remains less than or same as that of their hosts and thus there occur no difficulty in the exchange of water. It has been found that cestodes have well developed osmoregulatory system and their pH tolerance is also high. 3. Anaerobic respiration : The parasites adapt to decrease their oxygen demand

101 M-2\D:\Netaji 05\PGZO-3(AnPhysio)\ \Final proof because they live in less or no oxygen environment. The intestinal parasites live in an environment completely devoid of free oxygen. They develop a very low metabolic rate which requires a minimum amont of oxygen. In the absence of free oxygen, energy is obtained by the fermentation of glycogen which is broken by glycolysis carbon di-oxide and fatty acid. The glycogen and lipid contents in than body tissues have been found to be high whereas the protein content is less. 4. Antienzyme : One important parasitic adaptation is to develop antienzymes so that they are not attacked on destroyed by the host enzymes. Most of the helminth parasites, particularly intestinal parasites, secrete antienzymes in order to protect themselves from the gastric juices and digestive enzymes of the host. A dead worm cannot secrete these enzymes and so they are digested by the host enzymes. Medicines are used to destroy this anti-enzyme action and thus the parasites are subsequently destroyed by the host.

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M-3 \ D:Jobs - 1\ Netaji-07 \ Tit-Eng\ PGZO-4. Pm6.5 # 1st proof 24-11-08 [4] NETAJI SUBHAS OPEN UNIVERSITY Group A Unit 1 ? Energetics 1 – 9 Unit 2 ? Chemical Thermodynamics 10 – 29 Unit 3 ? Idea of Chemical Bond 30 – 43 Unit 4 ? Nuclear Hazards 44 – 59 Unit 5 ? Ionic Product 60 – 69 PGZO – 4 Basic Physical and Chemical Principles 1 UNIT 1 ? Energetics Structure 1.1 Free energy functions 1.1.1 Gibbs free energy, G 1.1.2 Maximum non-expansion work 1.1.3 Standard state 1.2 Helemholtz free energy 1.3 Gibbs—Helemholtz equation 1.4 Application of energetics in the field of biology 1.5 Standard state as applied to biochemistry 1.6 Questions 1.7 Answers 1.1 Free energy functions One of the problems of entropy calculation is that one has to work out the entropy change in the system and that in the surroundings, and then consider the sign of their sum. Towards the end of the 19th century foundation of chemical thermo dynamics was laid and the same led to a means to combine the two calculations into one. Two thermodynamic functions are thus used, which are as follows : [1] Gibbs free energy or Gibb's energy, G and [2] Helmhotz free energy, A They are defined as : [1] G = H – TS.....(1) [2] A = U – TS.....(2) Now, q = T?S =T (S 2 –S 1) ; so TS is an energy quantity and can be algebraically added to energy quantities V or H. Again, V, H, T and S are state properties and so A and G will also be state functions, i.e. they will be defined by states of the system. In other words dA and dG will be exact differetials. Since, H = U + Pv.....(3) G = H – TS = U + PV – TS = A + PV.....(4)

2 1.1.1 Gibbs free energy, G G = H – TS = U + PV – TS or, ?G = ?U + P?V + V?P – T?S – S?T.....(5) If a system undergoes an isothermal change at constant presence, then ?G P.T = ?U + P?V – T?S = ?U + P?V – q = P?V – ? or, ? – ?G P.T = ? – P?V.....(6) Here P?V is the mechanical or expansional work involved in the system during the transfromation and ? denotes the maximum total work output. So Gibbs free energy signifies that the value of ?G for a process gives the maximum amount of non-expansion work that can be extracted from the process at constant tem- perature and pressure. By non-expansion work (W') is meant any work other than that arising from the expansion of the system. It may include any kind of work received for any external use (exclusive of the mechanical work in the change of the system itself) : thus it may include electrical work (if the process takes place inside an electrochemical or biological cell), other kinds of external work such as winding of a spring or contraction of a muscle. Gibbs free energy (G) is thus a property of the system whose decrease is the measure of the external work available during the system itself. Referring to (S) dG = dU + PdV + VdP – TdS – SdT In a reversible mechanical process, dU + PdV = TdS ? dG = VdP – SdT For an isobaric process G T P e j = –S.....(7) For an isothermal process G P T e j = V .....(8) 1.1.2 Maximum non-expansion work At constant temperature and pressure dG = dU + PdV – TdS = dw + dq + PdV – TdS (? dU = dw + dq) The work done on the system comprises both expansion work –P ex dV, and non-expansion work dw'.

3 So, dG = –P ex dV + dw' + dq + pdV – TdS (at constant pressure and temperature).....(9) If the change is considered reversible, pexdV = pdV. Again since heat transfer is reversible dq = TdS. Thus, dG = dw' rev.....(10) Alternatively we can write, dG = dw' max..... (11) For overall change to we can write ?G = W' max .....(12) Thus the importance of Gibbs energy in chemistry is apprant. Non-expansional work obtainable form an electrochemical cell, that in biological cells available from the hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) can be calculated from knowledge of ?G. In section 1.3 one can see that for any reversible process in equilibrium at constant presence and temperature (?G) P.T = o, for spontaneity (?G) P.T > o 1.1.3 Standard state Gibbs free energy G is a state function. So to calculate numerical values to Gibbs free energy change of a system, a standard state is to be assigned with a value thereof. Generally this standard state refers to 25 o C and 1 atmosphere pressure for a pure substance. Now, at constant temperature, G P T e j = V (equ. 8) or, dG I P z z = VdP I P [I initial i.e. at one atmosphere pressure ; P that at pressure P] or, G – G O = VdP I P z .....(13) This G O is the standard free energy i.e., free energy at the standard state. Let the system is one of 'n' moles of a perfect gas at a temperature T K, then, G = G O + n RT P dP I P z = G O + nRT ln P 1 or, G = G O + nRTlnP or, G n G n RT nP o 1 .....(14) i.e. G G RT P O ln (for 1 mole of gas).....(15)

4 Free energy per g-mole of a gas is customarily referred to as ? (chemical potential) ??? = ? o + RTlnP .....(16) or, ? – ? o = RTlnP.....(17) Plot of ? – ? o against P is shown in figure 1.2 Helmholtz free energy By definition, A = U – TS For an isothermal ireversible change, ?A T = ?U – T?S (?T = o) = ?U – q = –W max .....(18) or, –?A T = w max .....(19) W max refers to maximum work involved. Thus it can be inferred that Helmholtz free energy A is such a thermodynamic function. the decrease of which for a system denotes the maximum amount of work available during an isothermal transformation of the system. This is the total work viz. purely mechanical (or otherwise) or partly mechanical and partly external. In thermodynamics we are concerned with reversible processes. If, however, the transformation be irreversible then also there would be a drop in A-value, but it would exceed the output of work, i.e. – ?A <??. In case, the change is not isothermal, the change in A will not be equivalent to maximum work. Recalling A = U – TS or, dA = dU – TdS – SdT or, dA = dU – dq – SdT [? TdS = dq] ? – ?o

5 or, dA = dU – dU – PdV – SdT or, dA = –PdV – SdT .....(20) Now, (i) If volume be kept constant i.e the process in isochoric, A T S v e j .....(21) (ii) If temperature be kept constant, i.e. the process is isothermal, A T P T e j .....(22) A change in a system at constant temperature and volume would be spontaneous if (?A) T.V = 0 and the condition for spontaneity will be (?A)T,V > 0. A system moves spontaneously towords states of lower A if a path is available. Equilibrium would mean if that neither the forward nor the reverse process has a tendency to occur. Significant of A lies in the fact that it points at a criterion of spontaneity (equilibrium); but besides and rather above that ; the change in the Helmholtz free energy is equal to the maximum work accompanying a process. Thus dW max = dA .....(23) That is why A is called the 'maximum work function' or the 'Work function' and G is often called simply 'free energy'. For a microscopic is otherhand change (23) is written as W max = ?A. 1.3 Gibbs-Helmholtz equation Gibbs-Helmholtz equations aim at expressing the variation of free energies (Gibbs and Helmholtz) with temperature. We have known, A T S G T S V P e j e j , And, A = V – TS, G = H – TS From there we can easily have, A = U + T A T V e j .....(23) and G = H + T G T P e j .....(24) These couple of equations are known as Gibbs-Helmholtz equations. Reavtraging these we get, 6 A T A U T V e j and G T G T T P e j .....(25, 26) These are also useful forms of Gibbs-Helmholtz equations. Gibbs-Helmholtz equations may be represented in other forms too. These are shown below— As sometimes it becomes important to know the dependence of the function G/T on temperature. We have known (/) G T T T G T T G T T G P P P F H I K 1 1 1 2 e j e j .....(27) Using the equation G T S P e j , (27) becomes (/) G T T G TS T P F H I K 2 .....(28) or, (/) G T T H T P F H I K 2 .....(29) Very similarly we can have, (/) A T T U T V F H I K 2 .....(30) Since d(1/T) = 1 2 T dT or, (29) can be written as (/) (/) G T T H P 1 F H I K .....(31) and very similarly (/) (/) A T T U V F H I K .....(32) There are still another set of Gibbs-Helmholtz equation Let us now have a further set In an isothermal change, G H TS 1 1 1 G H TS 2 2 2 (Subtracting) G H T S .....(33) But, ?S = S ? – S 2 = G T G T G T P P P 2 1 F H G I K J F H G I K J L N M O Q P .....(34) From (33) and (34), ?G = ?H + T G T P e j .....(35) or, ( )G T G H T P F H I K .....(36) CH OH C CH O 2 2 . 7 Very similarly, ( )A T A U T P F H I K .....(37) 1.4 Application of energetics in the field of biology Energy is an essential part in biological system as well as in environment. The behaviour of energy in ecosystem can be termed as energy flow due to unidirectional flow of energy. From energitics point of view it is essential to understand for an ecosystem— (i) efficiency of absorption and conversion of solar energy by the producers, i.e. the Green plants. (ii) use of the converted chemical from of energy by the consumers. (iii) total input of energy in the form of food and its efficiency of assimilation. (iv) loss of energy via respiration, excretion and heat. (v) the gross net production. In ecological energetics, we study— (a) quantity of solar energy reaching an ecosystem : A fraction, i.e., about 1/50 millionth of the total solar radiation reaches the earth's atmosphere. About 34% of the sunlight is reflected back in the atmosphere, 10% is held by ozone layer, water vapour and other atmospheric gases. The rest 56% reaches the earth's surface. (b) Quantity of energy used by green plants : 1 to 5% of this energy, i.e. 56% of the total energy is used by green plants for photosynthesis and the rest is absorbed as heat by ground vegetation or water. However, only about 0.02% of the sunlight is used in photosynthesis. (c) Quantity of energy flow from producers to consumers : There is a suc- cessive reduction in energy flow at successive trophic levels, i.e. from producers to consumers. As for example, in a freshwater ecosystem the total solar radiation was 118, 872 g cal/cm 2 /yr became 3.0 g cal/cm 2 /yr in carnivore level through producer (???·0 g cal/cm 2 /yr) and herbivores (?5.0 g cal/cm 2 /yr) level (Lindeman, 1942). 1.5 Standard state as applied to biochemistry In biochemistry the hydrogen ion concentration [H +] at the standard state is taken to be ~10 7 M (pH=7) because of the physiological condition to be like this. So for a process A+B C+xH + , ...(37) OH O PO 3 2

8 ?G = ?G o + RTln [ ] [ ] [ ] [ ] C H A B IM IM IM M 10 7 .....(38) and for C + xH + A+B .....(39) ?G = ?G o + RTln [ ] [ ] [ ] [ ] A B C M IM IM IM M 10 7 .....(40) For (38) ?G = ?G o + 40.0 kJ and for (40) ?G ?G o – 40.0kJ Thus reaction (37) will be more spontaneous at pH 7 that at pH O and reverse will be in case of (39). For reactions not involving H + , ?G = ?G o . Let us consider the particular reaction : ..... (X) At equilibrium at 298K and [H +] = 10 –7 M, it is find that [ ] [ ] . G P DHAP 3 0 0475 K' eq = 0.0475 As ?G = ?G o + RTln K'eqn, and system involves x o H + , ?G o = –RTln K'eqn = –2.303 × 1.98 × 10 –3 × 298 × log 0.0475 = +1.8 kCal mole –1 . If now, [DHAP] = 2×10 –4 M and [G-3-P] = 3×10 –6 M then ?G = ?G o + RTln 3 10 2 10 6 4 = 1·8 + 2·303 × 1·98 × 298 log (1.5×10 –2) = –0.7kCal mol –1 = –2.93KJmol –1 Implication of negative ?G is that the reaction (X) can take place spontaneously at the condition specified. Depending on conditions it may be positive also. It has been found that hydrolysis of esters (?G o = –13·807 KJmol -1), hydrolysis of amides (?G o = –14.225 kJ mol –1), hydrolysis of glycerides (?G o –9·205 kJmol –1) dihydroxyacetone phosphate (DHAP) glyceraldehyde 3-phosphate (G-3-P)

9 takes place with lent small standard free energy change. On the other hand hydrolysis of anhydrides occure with relatively large standard free energy. 1.6 Questions 1. For a reaction at 127 o C, ?G = –12KCal and ?H = 17·5 KCal Find ?S. 2. Explain clearly the difference between mechanical and non-mechanical work. 3. Suppose 3.0 mol of N 2 (g) expands for 36 Cm 3 to 60 Cm 3 at 300k. What would be the ?G for the process. 4. At constant pressure ?G/J = –85·40 + 36·5 (T/K). Calculate ?S. 5. Prove : H V V T T V V T P e j e j F H I K 2 (/) 1.7 Answers 2. ?S = 73.75 eu 3. 3.8J 4. –36.5JK –1

10 UNIT 2 ? Chemical Thermodynamics Structure 2.1 Thermodynamics 2.2 Thermodynamic terminology 2.2.1 Energy 2.2.2 Heat 2.2.3 Internal energy 2.3 The First law of thermodynamics 2.3.1 Work done in Isothermal Irreversible Process 2.3.2 Work done in Isothermal Reversible Process 2.3.3
Some special cases of
the first law

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of thermodynamics 2.3.4 Enthalpy 2.3.5 Limitations of the first law of thermodynamics 2.4 The Second law of thermodynamics 2.4.1 Characteristics of a spontaneous process 2.5 Entropy 2.5.1 Characteristics of entropy 2.6 Third law of thermodynamics 2.7

Gibbs Free Energy 2.7.1 Gibbs free energy change and the spontaneity of a process 2.7.2 Standard free energy change in a chemical reaction 2.8 Questions 2.1 Thermodynamics Thermodynamics is a branch of science that deals with the changes due to various physical and chemical transformations and it establishes the relationship between heat, work and temperature by some laws. The word thermodynamics is derived from Greek word ''Thermos'' means ''heat'' and ''dynamics'' means ''motion or flow''. There are many forms of energy, i.e. mechanical energy, electrical energy, chemical energy, kinetic energy, potential energy etc. Heat is a form of energy. The various forms of energy are interconvertible and these interconversions are governed by the

11 thermodynamics laws. We can make some predictions about the transformation and energy changes by studying the thermodynamics. 2.2 Thermodynamic Terminology Before describing the thermodynamics laws we are going to define some terms used in thermodynamics. (i) System : Any part of universe separated by a real or imaginary boundary from universe is known as system in thermodynamics, for example, gas contained in a closed cylinder or vessel, a reaction vessel, a heat engine, electrical cell, cells of plant and animal etc. The partition, which seperates a system from its surroundings and permits en- ergy to pass through it, is known as diathermic partition and which does not permit energy to pass through is known as adiabatic partition. (ii) Surroundings : The rest of universe outside the system separated by bounderies is known as surroundings usually, we measure various aspects of systems from its surroundings. These boundaries may be real, imaginary or movable. (iii) Types of System : There are different types of system, for example, (a) Homogeneous system : A system having identical physical, chemical prop- erties and composition is known as homogeneous system. A gas in a closed vessel is a homogeneous system. A homogeneous system is a single phase system. (b) Heterogeneous system : A system having two or more phases separated by mechanical boundaries is known as heterogeneous system, for example, a system containing solid, liquid; liquid, gas or solid, liquid and gas. (c)

Open system : A system which can exchange both matter and energy through its

boundaries to the surroundings is known as open system. An open vessel contain- ing water can exchange both matter and energy to its surroundings. All living matters are the examples of an open system because they can exchange both matter and energy to their surroundings. (d) Closed system : A closed system can exchange only energy in the form of heat or work and not the matter to its surroundings. There is no change in the mass of a closed system, but there may be a rise or fall of temperature in the system due to exchange of energy. A heat permeable sealed system is an example of closed system. (e) Isolated system : An isolated system cannot exchange matter or energy to its surroundings The total quantities of matter and energy remain same in an isolated system.

12 (iv) Intensive property : A property that does not depend upon the amount of matters of a system is known as intensive property, for example, temperature, pres- sure, density, refractive index, concentration, viscosity, surface tension, specific heat etc. (v) Extensive property : A property that depends on the amount of matter(s) of a system is known as extensive property of system, for example, volume, energy, internal energy, enthalpy, entropy etc. (vi) State functions of a system : A thermodynamic system is sufficiently large so that we can measure its various properties like volume, pressure, temperature and amount of material, composition and density etc. These properties are known as thermodynamic functions or variables which depend on the initial and final states of a system and do not depend on their past history. The change of any state variable does not depend also on the path in which this change takes place. Heat and work are not state variables since their changes depend on path. A state variable which depends on other variables is known as a dependent variable and other variables are known as independent variables. The state of a system is defined when state variables have definite values. But this does not mean that values of all the variable should be known. Since they are interdependent, for example, we can write the ideal gas equation P = , where P is dependent vari- able, but n, T and V are independent variables, since R is a constant. (vii) Thermodynamic processes : The processes which are used to change the state of a system are known as thermodynamic processes, for example, (a) Isothermal process : The temperature of a system remains constant in an isothermal process by exchanging the energy (heat) with its surroundings which in this case is a thermostat. So the temperature difference of initial and final states of system will be zero (dT = 0). The melting and boiling of a substance are the examples of isothermal processes. (b) Adiabatic process : No heat is allowed to enter or leave the system during an adiabatic process. In an adiabatic process a system is thermally insulated from its surroundings. Energy involved or absorbed during an adiabatic process will remain within the system, thereby increasing or decreasing the temperature of system. But the change of heat (dq = 0) will be zero. (c) Isochoric process : In an isochoric process the volume of a system remains constant (dV = 0). So a chemical process in a sealed container of constant volume is an example of isochoric process.

13 (d) Isobaric process : In an isobaric process the pressure of a system remains constant (dP = 0). Boiling of a substance in an open vessel is an example of isobaric process, since it occurs always at atmospheric pressure which is supposed to be unchanged during the process. (e) Cyclic process : The process in which a system after performing a number of intermediate changes returns to its initial or original state, then that process is known as cyclic process. In a cyclic process the change in internal energy of the system is zero i.e. dU = 0 and also dH = 0, where dH = change in enthalpy. (f) Reversible process : Reversible process is an ideal thermodynamic process in which the direction of the process can be reversed by the infinitesimal change of the external conditions like temperature and pressure of the system. Let us consider that a gas at a pressure P is kept in a cylinder fitted with a airtight piston. When the pressure (P) of the gas is equal to the external pressure, the piston remains stationary. But on minute increasing in the external pressure (dP) the volume of gas can be decreased minutely. Similarly on decreasing the external pres- sure by dP the volume of the gas can be increased minutely. So the direction of reversible process can be changed with minute change in the magnitude of the driving force. (g) Irreversible process : Any process which is not reversible is known as irreversible process. All natural processes are irreversible. Irreversible process is also known as spontaneous process. Water flows from higher altitude to lower attitude and it is a natural process. Hence it is an irreversible or spontaneous process. Therefore, a spontaneity refers to the tendency of a change or a chemical change to take place on its own way. A spontaneous process may be very fast or slow. Therefore it has no rrelation with the rate of change. A common salt dissolves in water spontaneously. Metallic zinc reacts spontaneously with the dilute HCl. An acid reacts spontaneously with alkali to produce salt. The reverse of any spontaneous process is never spontaneous and it is always non-spontaneous, for example, water cannot flow spontaneously from lower altitude to higher altitude, or an acid and alkali cannot be obtained spontaneously from the aqueous salt solution. A non- spontaneous process may be brought about by some external agency or by application of thermal or electrical energy, for example, water can be lifted from lower altitude to higher altitude with the help of a pump. Aqueous solution of a salt can be sepa- rated by heating the solution. Metallic sodium reacts spontaneously with chlorine gas to give NaCl. Metallic sodium and Cl 2 gas can be obtained by the electrolysis of a

14 molten NaCl and it is a non-spontaneous process. The rate of a spontaneous process may be increased by application of heat or a catalyst. Oxidation of glucose into CO 2 and H 2 O is a extremely slow spontaneous process unless it is heated to a suitable temperature or it is oxidized in presence of biological catalyst. (h) Thermodynamic Equilibrium : A system is in thermodynamic equilibrium when its all state variables remain same throughout the system. A gas is said to be in thermodynamic equalibrium when it is kept in a cylinder fitted with a weightless and frictionless piston to make its state variables, such as the volume, pressure and temperature remain to the same. (i) Thermodynamic non-equilibrium : A system which is not in thermody- namic equilibrium is known as thermodynamic non-equilibrium, i.e. its state vari- ables are not same throughout the system. Work, Energy and Heat : Work (W) is a fundamental physical property in thermodynamics and is defined as the product of force (F) applied on a body and the displacement (l) of that body along the direction of force. $W = F \times l$ ... (1.1) Work can be done in various ways due to the different physical origin of the force. Work is not a state function because if depends on path. If work is done on the system then its value is positive and if work is done by the system then its value is negative. (i) Pressure volume work : The expansion or compression of a gas against an external pressure is an example of pressure volume work because it can push up or down the piston. $W = P \times A \times dl$ ... (1.2) $= P \times dV$ ... (1.3) where P = pressure of gas A = area of piston dl = Difference in length Because $A \times dl = dV$ (Difference in volume) This type of mechanical work is performed when a system changes its volume against an opposing pressure (external pressure). 2.2.1 Energy : The energy of a system is the capacity of doing work. When work is done on a system its capacity of doing work is increased and when work is done by a system then its capacity of doing work is decreased. Thus compressing a gas against a

15 pressure increases its energy and expansion of a gas decreases its energy. There are various forms of energy, for example, heat energy, kinetic energy, potential energy, electrical energy, light energy, sound energy etc. One form of energy can be converted into other form and a material body is required for this transformation. The unit of energy in SI system is joule (J). 2.2.2 Heat : Heat is a form of energy. The energy of a system can be changed by means of many methods other than work itself. The energy of a system can be transferred due to difference of temperature between a system and its surroundings in the form of heat. When a hot body is immersed into in a vessel containing water at normal temperature (system) the temperature of water is increased due to transfer of heat from the hot body to water. Therefore capacity of doing work of the system is increased. All boundaries between system and its surroundings may or may not allow the energy to transfer. A boundary which allows the energy to transfer is known as diathermic, for example, metal or glass. A boundary that does not allow the energy to transfer is known as adiabatic. A process in which energy is released is known exothermic process and a process in which energy is absorbed is known as endothermic process. All combus- tion reaction are exothermic. The evaporation of any liquid is an example of endothermic process. In an exothermic process heat is allowed to pass through the diathermic boundary to its surrounding and thereby increase the temperature of the surroundings. Similarly in an endothermic process heat is allowed to transfer from the surroundings to the system through the diathermic boundary. The heat evolved (or absorbed) within a system having a adiabatic container will increase (or decrease) the temperature of the system. 2.2.3 Internal energy : The internal energy of a system is the total energy of its constituent atoms or molecules including all forms of kinetic energy and also energy due to every type of interactions between molecules and their subparticles. Internal energy is a state vari- able and its absolute value cannot be determined. But the difference of internal energy (U) of a system can be determined. The difference of internal energy de- pends only on

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the initial and final states of system and is independent of the path to which this change

takes place.
16 2.3 The first law of thermodynamics Law of conservation of energy is the first law of thermodynamics. It can be stated in a number of ways. (i) Energy cannot be created or destroyed, although one form of energy can be changed into other form. (ii) The

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energy of an isolated system remains constant. (iii) It is impossible to construct a perpetual motion machine

which can work endlessly without the expenditure of energy. (Such a motion machine which requires no energy for its functions is known as perpetual motion machine) The first law of thermodynamics can be expressed mathematically by the fol- lowing equations— q = U + W ... (1.4) or, U = q – W or, W = q – U where U = change in the internal energy,, q = amount of energy W = work done. That is if q quantity of heat is supplied to a system than a part of this heat is used to increase the internal energy of the system and remaining part of the heat is used to perform work by the system on the surroundings. The total work done by the system when the volume of gas V i to V f is calculated by integrating the above equation (1.4) W = – z But this is a general expression for calculating pressure, volume and work both in isothermal and adiabatic process. The value of work is negative if the gas expands and positive if the gas contracts, therefore in expansion dV is positive and in contration dV is negative. 2.3.1 Work done in Isothermal Irreversible Process Let us suppose that the external pressure is constant through out the expansion at constant temperature isothermally and irreversibly. Let the initial and final vol- umes of gas be V i and V f respectively. Then the total work done on the system is obtained by integrating the equation—

17 W = – z = – P Ext × (V f – V i) = – P Ext × V where V = Volume change during the process. and P Ext = External pressurs 2.3.2 Work done in Isothermal Reversible Process : In thermodynamics, a process that can be reversed by either direction by infini- tesimal change of a variable is known as reversible process. Suppose a gas is enclosed in a cylinder fitted with a frictionless piston. The pressure of the gas is P and the external pressure on piston is P Ext . In reversible expansion P Ext is equal to P at each step of expansion. So the equation dW = – P Ext dV can be written as dW = – PdV ... (1.5) So the total work done is by expansion of gas isothermally and reversibly from V 1 to V 2 W = – z If the gas behaves ideally, them we can write P = W = – z = – nRT z = – nRT ln = – 2.303 nRT log = 2.303 nRT log ... (1.6) If V 2 > V 1 , then the gas is compressed and W is positive, i.e. work is done on the system. If V 2 < V 1 , the gas undergoes expansion and W is negative, i.e. work is done by the system.

18 Again in isothermal condition P 1 V 1 = P 2 V 2 or = Putting the value of in the equation (1.6) we can write W = 2.303 nRT log = – 2.303 nRT log 2.3.3 Some special cases of the first law of thermodynamics (i) Free expansion of a gas : Expansion of a gas against zero pressure or expansion of a gas in vacuum is known as free expansion. The work done (dW) in free expansion is zero, because P = 0 dW = PdV = 0. and total work (W) is also zero. (ii) For a process in which there is no volume change of the system, i.e. in a isochoric process no work is done. So W = 0. U = q v So in isochoric process heat supplied to a system will solely increase its internal energy. (iii) For isothermal reversible process the change in internal energy is zero, i.e. dU = 0. q = – W = – 2.303 nRT log (iv) For adiabatic process q = 0, i.e. the system is well insulated and the system does not gain or lose energy. So U = –W q Therefore work done by the system is exactly equal to the loss of its internal energy. 2.3.4 Enthalpy Enthalpy H is a very useful thermodynamic function which can be expressed by the following equation, H = U + PV ... (1.7)

19 where U, P, V are the internal energy, pressure and volume respectively. Since U, P and V are the state variables, therefore H is also a state variable. So the change in enthalpy (H) depends on initial and final states of system and does not depend on path to which this change takes place. So the H can be given by,, H = H 2 – H 1 ... (1.8) where H 1 = Initial enthalpy, H 2 = final enthalpy of the system. = (

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U 2 + P 2 V 2) – (U 1 + P 1 V 1) ... (1.9) = (U 2 – U 1) + (P 2 V 2 – P 1 V 1) = U + P(V 2 –

V 1) At constant pressure P 1 = P 2 = P. (H) p = U + P V ... (1.10) q p = U + P V ... (1.11) H p = q p Therefore heat supplied to a system at constant pressure q p is equal to the change in the internal energy plus pressure-volume work done by the system to the surroundings. 2.3.5 Limitations of the first law of Thermodynamics (i) From the first law of thermodynamics we know that during a process one form of energy can be converted into other form, although the total energy remains unchanged. Or we can say that during a process if there is an increase in energy of a system then same amount of energy will disappear from the surroundings and vice versa. But the first law neither tells us about the direc- tion of flow of energy nor the spontaneity of the process, i.e. feasibility of the process. (ii) By first law of thermodynamics, it is not impossible to transfer heat from colder body to a hotter body without the help of a machine, i.e. it is not impossible to boil water by abstracting heat from ice. But it is our common experience that heat always flows from hotter body to a colder body sponta- neously. Similarly water flows from higher level to lower level spontaneously and electricity flows from higher potential region to lower potential region. The direction of change in any process that does not require any work is spon- taneous. All spontaneous processes are irreversible and unidirectional. But we can compressed a certain volume of gas into smaller volume. Water or any body can be cooled into a refrigerator or we can force a reaction into opposite direction, for example, electrolysis of water or any electrolyte. In all changes mentioned above require work to bring about the changes. So these are the examples of non-sponta- neous processes.

20 2.4 The second law of thermodynamics In order to remove the limitations of first law of thermodynamics, the second law was proposed. The second law can be stated in a number of ways. (i) ‘‘All spontaneous process in nature are thermodynamically irreversible.’’ (ii) It is impossible to convert heat into equivalent amount of work without pro- ducing some change elsewhere. (iii) Heat can not be transferred to a hotter body from a colder body without the help of a machine. (iv) The heat which is evolved by cooling a system or its parts from the surround- ings with the help of an engine cannot be converted into work. (v)

into sink at lower temperature. (vi) Entropy of universe continuously increases by every spontaneous process. 2.4.1 Characteristics of a spontaneous process (i) All spontaneous processes are unidirectional and work must be done to reverse the direction of a spontaneous process. (ii) A spontaneous process may be slow or fast. (iii) A spontaneous change is inevitable if the system is not in an equilibrium state and this change takes place until it comes to in equilibrium. (iv) Once a system attains equilibrium no further spontaneous change will take place. (v) Entropy of a system will increase by a spontaneous process and more the entropy of a system more is the disorder of the system. 2.5 Entropy From Cannot cycle we know that the efficiency of any engine cannot be 100%. So a thermodynamic function is required to describe the amount of energy obtained for performing the permissible work. This function is known as entropy (S). Entropy is also a state variable (function) like internal energy and enthalpy. The more the amount of entropy of a system, the less is the amount of work obtained from it. Entropy is also a measure of disorder of a system and the more the disorder of a system the more is the entropy of the system. So that an ordered system has less
21 entropy. During a spontaneous change the entropy of an isolated system increases. This is also a statement of second law of thermodynamics. The change of entropy of a system

does not depend on the path. So the change of entropy can be expressed as— S = S final – S initial ... (1.12) Again an infinitesimal change of entropy can be described as, dS = ... (1.13) where dq r is the infinitesimal quantity of energy absorbed under reversible manner at the temperature T (Absolute) by any process. Equation (1.13) is the mathematical definition of the entropy. For a measurable change of entropy between initial (1) and final (2) state can be calculated by integrating the expression— S = z ... (1.14) In equation (1.14) dq r and T are expressed as joule and kelvin respectively. So the SI unit of entropy is expressed as joule per kelvin (JK –1). For molar entropy, the entropy is divided by molar amount. i.e. joule per kelvin per mole (JK –1 mol –1). In cgs sustem the unit of entropy is calories (or ergs) per kelvin per mole [cal(or egr) K –1 mol –1]. 2.5.1 Characteristics of entropy (i) Entropy is an extensive property, i.e. it depends on the amount of substance. (ii) Entropy is a state function. (iii) Change of entropy is independent of path. (iv) Change of entropy in a cyclic process is zero. (v) S should be positive, (i.e. S < 0) in a spontaneous process in an isolated system. (vi) Total change of entropy S of a non-isolated system is given by S = S system + S surrounding (vii) In any reversible process change of entropy is zero, i.e. S = 0. S system = – S surroundings (viii) In any spontaneous process, the entropy of universe increases. S univ < 0. Now there is no easy way to determine the entropy change of surroundings or universe. So nothing can be predicted about the driving force of a process or a

22 reaction. To remove this difficulty Gibbs introduced a new thermodynamic function which is known as Gibbs free energy (G). 2.6 The third law of thermodynamics At absolute zero there is no thermal motion of a substance and if in a crystal the atoms or ions are in a highly regular arrangement, so that there is no disorder, then such crystalline substance have zero entropy. This is known as third law of thermodynamics which can be stated that the entropy of all pure crystalline sub- stances may be zero at absolute zero. Or in other words during a physical or chemical transformation the change of entropy tends to zero as the temperature tends to abso- lute zero. This is also known as Nernst heat theorem. 2.7 Gibbs free energy The spontaneity of any reaction or process cannot be predicted by enthalpy (H) or entropy (S) of a system. For this reason Gibbs free energy was introduced. The maximum amount of energy available for doing useful work for a process (even reversibly) of a system is known as Gibbs free energy. Gibbs free energy determines the ability of a system for doing useful work. A part of the total work done by a system may be used to perform the pressure volume work of the system. So the amount of energy necessary for the pressure volume is not available for doing the useful work. Net useful work = Work done – Pressure volume work. At constant pressure and temperature network = W max – P(V 2 – V 1) = W max – P V where change in volume V = V 2 – V 1 . This can be precisely expressed by the following equation, G = H – TS ...(1.15) where G = Gibbs free energy, H = enthalpy of the system, T = absolute tempera- ture and S = entropy of the system. The change in Gibbs free energy G is more important than G itself in a chemical reaction or a process. So the change in free energy between two states of a system can be expressed by G 2 – G 1 = (H 2 – T 2 S 2) – (H 1 – T 1 S 1) or G = (H 2 – H 1) – (T 2 S 2 – T 1 S 1)

23 At constant temperature G = H – T(S 2 – S 1) = H – T S ...(1.16) This equation is known as Gibbs Helmholtz equation. (i) The process is spontaneous if G is negative. (ii) The process is in equilibrium if G = 0. At equilibrium the concentration of each spacies of both reactants and prod- ucts will remain constant. There is no net reaction on either direction. (iii) If G is positive, the process is non-spontaneous. So the process cannot take place in the forward direction. It may take place in the reverse direction. (iv) If H is negative and S is positive then G is negative. So the reaction is spontaneous at any temperature. (vi) If H is positve and S is negative, the G is positive at any temperature than the reaction is non-spontaneous at any temperature. (vii) If H is negative and S is negative then the reaction is spontaneous only at low temperature i.e G is negative. But G is positive at high temperature in this case . So in that case reaction is non-spontaneous. (viii) If H is positive and S is positive then G is negative at high temperature. So the reactions is spontaneous at high temperature. But in this case G is positive at low temperature. So in that case the reaction is non-spontaneous. 2.7.1 Gibbs free energy change and the spontaneity of a process We know that, G = H – TS and

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H = U + PV G = U + PV – TS. or, dG = dU + PdV + VdP – TdS – SdT.

or dG = dq + dW + PdV + VdP – TdS – SdT (since dU = dq + dW) for very small change This equation is true for both reversible and irreversible processes. Now for reversible process dq rev = TdS and any process occurring in an isolated system the dq > TdS. dG – dW – PdV – VdP + TdS + SdT > dq or, dG – dW – PdV – VdP +TdS + SdT > TdS or, dG – dW – PdV – VdP + SdT > 0 If only pressure volume work is done by the system, then –dW = PdV dG + PdV – PdV – VdP + SdT > 0

24 or, dG – VdP + SdT > 0 Now at constant pressure and temperature (dG) P,T > 0or, (G) P,T > 0. For any reversible process in equilibrium at constant pressure and temperature (G) P,T = 0. But for any irreversible process at constant pressure and temperature (G) P,T > 0. So for spontaneous process the value of (G) P,T,T should be negative. 2.7.2 Standard free energy change in a chemical reaction In order to establish a relation between free energy change in a chemical reac- tion and the equilibrium constant of a reaction, let us take the following reaction— aA + bB cC + dD Rectants Products In this reaction a moles of A reacts with b moles of B to give c moles of C and d moles of D reversibly. Then the free energy change of this reaction at constant temperature is given by, G = G° + RT In ...(1.17) where G = free energy change of the reaction, G ° = standard free energy change and R = gas constant and T = temperature in kelvin The standard free energy of a reaction is a constant term at a given temperature. But G varies with concentrations of both reactants and products at constant tem- perature. So we can calculate the free energy change of a reaction at a given tempera- ture provided we know the value of G°. At equilibrium G = 0 at a given tempera- ture. O = G° + RT In G° = – RT In = – 2.303 RT log K eq ...(1.18) K eq = where K eq = equilibrium constant where [A], [B], [C] and [D] represent the molar concentration of A, B, C and D respectively.

25 But when the concentration of each reactant and product is one mole per litre then in the equation (1.17) the second term is equal to zero (log 1 = 0) G = G° ...(1.19) At equilibrium the value of G° 298K (at 298K or 25°C) is given by G° 298K = – 2.303 × 8.314J.K –1 mol –1 × 298 KlogK eq = – 5705.85 log K eq J mol –1 (R = 8.314JK –1 mol –1) = – 5.706 log K eq kJmol –1 = – 5.706 × 4.18 log K eq kcal mol –1 (1 Joule = 4.18 calorie) So at a given temperature G° can be calculated provided we know the value of K eq , when the value of K eq > 1, then the value of G° < 0, (i.e positive) and reaction have positive value of G° is known as endergonic, when K eq <1 negative value of G° is known as exergonic. Now when the reaction is in equilibrium and the value of K eq is equal to 1 [which means that the concentration of each reactant and product is 1(M)], then G° is also equal to zero. The biochemical reactions in cells are generally occurred at pH 7.0 rather than at pH zero [i.e 1(M) H + ion concentration]. So necessary correction is done for the standard free energy change at pH 7. The standard free energy change at pH other than zero is expressed as G°´. When a reaction in a cell does not depend on the H + ion concentration (i.e. on pH), G° = G°´ and in such reactions H + or OH – ion is neither formed or utilized. If H + or OH – ions at all form then those H + (or OH –) ions must react with some other substance so that the concentration of H + (or OH –) ions remains unaltered. Relation between equilibrium constant (K´ eq) and standard free energy change at 293K (25°C), where K´ eq = equilibrium constant at pH other than zero K´ eq G°´ = – 2.303 RT log K´ eq In J mol –1 In calorie mol –1 0.01 115411.7 2730.0 0.1 5705.8 1365.0 1.0 0 0 10 – 5705.8 – 1365.0 100 – 11411.7 – 2730.0 Let us now calculate the standard free energy change of the following reversible reaction in presence of an enzyme phosphoglucose mutase at 25°C.

26 Glucose 1-phosphate Glucose 6-phosphate If the initial concentration of glucose 1-phosphate is 0.02 (M) and at equilibrium the concentration of glucose 1-phosphate deceases to 0.001 (M) then concentrations of glucose 6-phosphate is therefore 0.019. K´ eq = = = 19 G°´ = – 2.303 RT log K´ eq = – 2.303 × 8.314 × 298 × 1og 19 = – 7296.4 J mol –1 = – 7.296 kJ mol –1 = – 1.745 kcal mol –1 In this relation H + ion is not formed or utilized. So this reaction is independent of pH hence G° = G°´. The value of G° in this reaction is negative, so the convertion of glucose 1-phosphate with glucose 6-phosphate is an exergonic reaction and this reaction is spontaneous. The standard free energy change (G°) of any chemical reaction is given by the following equation— G° = G° Products – G° Reactants where G° Products = sum of the free energies of all the products and G° Reactants = sum of the free energies of all the reactants. This equation can be used to calculate the standard free energy change of the oxidation of dilute aqueous D-glucose solution at 25°C and 7 pH. C 6 H 12 O 6 + 6O 2 6CO 2 + 6H 2 O G°´= [6 × G°´ CO 2 + 6 G°´ H 2 O] – [G°´ C 6 H 12 O 6 + 6 × G°´ O 2] = [6 × (–94.45) + 6 × (–56.69)] – [–219.22 + 6 × 0] = – 906.84 + 219.22 = – 687.62 kcal mol –1 where value of G°´ C 6 H 12 O 6 = – 219.22 kcal mol –1 G°´ O 2 = 0 (because it is in the elemental form) G°´ CO 2 = – 94.45 kcal mol –1 G°´ H 2 O = – 56.69 kcal mol –1 The oxidation of D-glucose into CO 2 and H 2 O is highly exergonic for very large negative value of G°´. A very high negative value of G°´ does not mean that the reaction will proceed very fast. That is it does not reflect any light on the rate of a reaction. The rate of oxidation reaction of glucose (or any other reaction) depends on temperature and also on catalyst present.

27 The rate of any chemical reaction depends on the number of molecules those possess energy of activation. These activated molecules form the intermediate acti- vated complex (A*) in converting A into B by a chemical reaction. The average difference of energy between A* and A is known as activation energy and energy of A* is always greater than that of A. Lower the activation energy greater is the rate of reaction. Now if the activation energy is very high the reaction will proceed very slowly or the reaction does not take place at all. The use of a catalyst or an enzyme in a reaction lowers down the activation energy, so that the reaction can proceed easily. In a case of consecutive chemical reactions the total standard free energy change of all the reactions is equal to sum of the standard free energy change in each reaction, i.e. it is additive in nature. Let us take the following consecutive chemical reactions— A B C D G° sum = G° 1 + G° 2 + G° 3 . where G° sum = Total standard free energy change for the above consecutive reactions from A to D and G° 1 , G° 2 and G° 3 are standard free energy changes of 1 step, 2nd step and 3rd step respectively. The ATP on hydrolysis gives ADP and inorganic phosphate (p i). In this hydroly- sis reaction a large amount of free energy is released because ATP is a energy rich compound. ADP on further hydrolysis gives AMP and inorganic phosphate (p i) ATP + H 2 O ADP + p i In this hydrolysis reaction the equilibrium constant K′ eq at 7.0 pH can be calcu- lated by the following equation provided we know the concentration G°′ = – 2.303 RT log K′ eq of ATP and ADP and phosphate at the equilibrium. But at the practical point of view it is difficult to know when the equilibrium has been reached and what are the concentrations of reactants and products at the equilibrium. The standard free energy charge (G°′ ATP) of hydrolysis of ATP to ADP and p i can be calculated by consecutive reactions very easily, where ATP reacts with glucose in presence of enzyme hexokinase to give ADP and glucose 6-phosphate. ATP + glucose ADP + glucose 6-phosphate The K′ eq 1 of this reaction can be determined by the concentration of ATP and ADP and glucose 6-phosphate at the equilibrium very easily and knowing the value of K′ eq 1 of this reaction the value of G°′ 1 is found to be
28 –16.72kJmol –1 (–4 kcal mol –1). The glucose 6-phosphate obtained by the previous reaction on further hydrolysis in presence of enzyme glucose 6-phosphatase gives glucose and phosphate (p i). Glucose 6-phosphate + H 2 O glucose + phosphate The standard free energy G°′ 2 of this reaction can be also calculated from the equilibrium constant K′ eq 2 of this reaction and value of G°′ 2 = – 13.8 kJ mol –1 (–3.3 kcal mol –1). Therefore, the total standard free energy changes of these two consecutive re- actions will give the actual standard free energy change of hydrolysis of ATP into ADP and phosphate. G°′ ATPTP = G°′ 1 + G°′ 2 = – 16.72 – 13.94 = – 30.514 kJmol –1 (–7.3 kcal mol –1) This value of G°′ ATP can also be verified by other consecutive reactions. 2.8 Questions 1. State and explain the first law of thermodynamics. Express the first law mathemati- cally. 2. Define the following terms—(i) system (ii) surroundings, (iii) homogeneous system (iv) heterogeneous system, (v) open system, (vi) closed system, (vii) isolated system, (viii) intensive property, (ix) extensive property, (x) thermodynamic property, (xi) isothermal process, (xii) adiabatic process, (xiii) isochoric process, (xiv) isobaric process, (xv) cyclic process, (xvi) reversible process, (xvii) irreversible process, (xviii) work, (xix) energy, (xx) heat, (xxi) internal energy, (xxii) thermodynamic equilibrium. 3. Deduce an expression for pressure volume work. 4. Express work done in isothermal reversible process mathematically. 5. What is the amount of work done due to the expansion of gas against zero pressure? 6. What is the amount of work done in an isochoric process? 7. What are the limitations of first law of thermodynamics. 8. What do mean by spontaneous process? Give some examples of spontaneous proc- esses. What are the chracteristics of a spontaneous process? 9. State the second law of thermodynamics. 10. Define entropy. What are the characteristics of entropy? 11. State the third law of thermodynamics. 12. What is Gibbs free energy? Express it mathematically. 13. Deduce Gibbs Helmholtz equation.
29 14. Define the standard free energy of a chemical reaction. 15. Deduce the standard free energy change of the following chemical reaction aA + bB cC + dD 16. In a reversible isothermal process, the change in internal energy is— (a) zero (b) positive (c) negative (d) none of the above. 17. From

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adiabatic process, which of the following is correct? (a) PdV = 0 (b) q = – W (c) U = q (d)

q = 0 18. What is the difference between G° and G°′. When G° = G°′? Calculate the value of G°′ if the K′ eq = 18 at a temperature of 25°C.

30 UNIT 3 ? Chemical Bonds Structure 3.1 Chemical bonds 3.1.1 Definition 3.1.2 Types of chemical bonds 3.1.3 Electrochemical bond or ionic bond 3.1.4 Covalent bond 3.1.5 Coordinate covalency or coordinate bond 3.1.6 Hydrozen bond 3.1.7 Energy rich bonds 3.1.8 Vander waals force 3.2 Questions 3.1 Chemical bonds 3.1.1 Defintion : A chemical bond is a force which holds two atoms of element(s) together forming a linkage between the two atoms. The combining capacity of an atom is known as valency which is measured by number of hydrogen atoms combine with one atom of any element directly or indirectly. 3.1.2 Types of chemical bonds : The formation of a chemical bond by combining two or more atoms (same or different) depends on many factors like number of electrons and protons present in the atom, atomic volume, electronegativity etc. Since atoms of different elements differ from one another in these respect, so different types of chemical bonds are formed during the combination of the atoms. Following are the types of chemical bonds—(i) Electorvalent bonds (ii) Covalent bond (iii) Co-ordinate covalent bond. During the formation of a bond each atom forming a bond tries to attend the stable electronic configuration by gaining, losing or sharing electrons from other atom. 3.1.3 Electrovalent bond or ionic bond : Electrovalent bonds are formed by transfer of one or more electrons from the valence shell of one atom of an element to the valence shell of an atom of other element. During the transfer of electron(s), atom of each element attains the stable

31 inert gas electronic configuration. The atom which loses electron(s) forms a cation and the atom which gains electron(s) forms an anion. The charge gained by cation or anion is equal to the number of electrons that an atom loses or gains respectively. Energy is released due to the electrostatic attraction force (coulombic force) between cation and anion. The compound thus formed has less energy than the individual atoms, so the stability of compound increases. This coulombic force is known as electrovalent bond or ionic bond. (a) Na Cl Na + Cl ? [Ne]3s 1 [Ne]3s 2 3p 5 [Ne] [Ne]3s 2 3p 6 or [Ar] (b) Ca F F Ca 2+ F : : :: F : : :: [He]2s 2 2p 5 [Ar]3s 2 [Ne] [Ar] [Ne] (c) S Na + . . :: S : : :: Na . Na . 2? Na + Na 2 S [Ne]3s 1 [Ne]3s 2 3p 4 [Ne] [Ar] [Ne] (a) The position of sodium in the periodic table is in the IA (alkali metals) group. The atomic number of sodium is 11. So the electronic configuration of sodium is 1s 2 2s 2 2p 6 3s 1 or [Ne]3s 1 and neon is the nearest inert gas to sodium. Again the atomic number of chlorine is 17 and the electronic configuration of chlorine is 1s 2 2s 2 2p 6 3s 2 3p 5 . Argon is the nearest inert gas to chlorine. Sodium has only one electron in its valence shell and sodium can lose this valence electron very easily forming stable sodium cation (Na +). The electronic con- figuration of Na + is 1s 2 2s 2 2p 6 which is the electronic configuration of inert gas neon, on the other hand chlorine (which is a highly electonegative element) has 7 electrons in its valence shell. So chlorine atom by gaining one electron can attain the electronic configuration of argon with a negative charge on it (Cl –). Thus Na + (cation) and Cl – (anion) are held together by electrostatic force of attraction forming NaCl ionic compound. 3.1.3.1 Factors favouring electrovalent or ionic bond (i) One of the element must be generally a metal having low ionization potential,

32 so that it can lose electron easily. Greater the radius of the metal lower is the ionization potential. (ii) Other element (non metal) must have high electron affinity, so that it can hold the extra electron(s). The size of the non-metal should be small in size. (iii) The lattice energy which is the electrostatic attraction force between the op- positely charged ions should be high. (iv) The electronic configurations of the cation and anion should be inert gas electronic configuration. 3.1.3.2 Characteristics of electrovalent or ionic compounds (i) All ionic compounds are solid at room temperature. (ii) Ionic compounds are formed by the cation and the anion, so no true molecule is formed and ionic bonds are non-directional. (iii) Ionic compounds have high melting and boiling points, density and low vola- tility. (iv) In solution or in molten condition ionic compound can conduct electricity. (v) Ionic compounds are soluble in polar solvent or a solvent having high dielec- tric constant like H 2 O and are insoluble in non-polar solvents like benzene, toluene, ether etc. 3.1.4 Covalent bond There are many substances where molecular structures cannot be explained on the basis of electrovalent or ionic bond, for example, Cl 2 , O 2 , N 2 , CH 4 , etc. The molecular structures of these substances are explained on the basis of covalent bond. The covalent bond is formed between two atoms (same or differant) having comparable electronegativity. In the formation of covalent bond between two atoms, each atom contributes same number of electron(s) and pair(s) of electrons thus formed is (are) shared equally between the two atoms to complete octet (or duet in the case H 2) in their outermost shells. The two atoms are held together by these shared pair(s) of electrons localized between them and a bond thus formed is known as covalent bond. A compound having covalent bond(s) is known as covalent compound. When each atom contributes one electron and pair thus formed is shared by both the atoms then a single covalent bond is formed. When 2 or 3 pairs are shared equally by the two atoms then double bond and triple bond are formed, for example, H . . H H : H or H — H O + O O O or O = O : : : ::: : :: :: : : : : :

33 N + N N N or N N : ... : : : ..... : : Cl + Cl Cl Cl Cl — Cl . : : ::: : :: : . :: : : :::: : : H H C H H : : :: H H C H H : : :: In some cases, all the electrons present in the valency shell of an atom may not be used up completely in the formation of covalent bond and number of unused electron pair left on atom is known as lone pair electrons. There is one covalent bond between two hydrogen atoms forming hydrogen molecule (H 2) and their is no lone pair left on either of H-atoms present in H 2 molecule. In H 2 molecule duet (having two electron like He atom configuration) is formed. Oxygen oxygen double bond is present in O 2 molecule and each oxygen atom in O 2 molecule has two lone pairs. A triple bond is present in N 2 and each nitrogen atom carries one lone pair. In case of methane, carbon atom completes its octet, while each H-atom com- pletes its duet. Other examples of covalent molecules—

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C C : : H H H : : H : : or C = C H C C H : ..... :

or H :O : H .. .. or O H H : : 3.1.4.1 Characteristics of covalent compounds (i) Covalent compounds are molecular in nature. (ii) Hence covalent compounds have low melting and boiling points and are less dense. (iii) Covalent bond is weaker than ionic bond. (iv) Covalent compounds are soluble in non-polar solvent and are generally in- soluble in polar solvent. Some covalent compounds which can form hydrogen bonding are soluble in H 2 O (Polar solvent).
34 (v) Covalent bond has definite direction and hence covalent compounds are rigid in nature. (vi) Covalent compounds are non-conductor of electricity. 3.1.5 Coordinate covalency or coordinate bond Coordinate bond is special type of covalent bond in which the shared paired of electrons between two atoms are donated by either of the atoms and then it is shared by both the atoms in order to complete their octet in their outermost shells. The compounds formed by coordinate bond are known as coordinate compounds. In a coordinate compound the atom which donates the electron pair is known as donor atom and the atom which accepts the electron pair is known as acceptor. The coordinate bond is shown by arrow mark ( ) and the tip of the arrow is pointed towards the acceptor atom from donor atom. The donor atom must have one or more lone pairs and acceptor atom or ion must have an empty orbital to accommodate the lone pair. 3.1.5.1 Examples of coordinate compounds (a) H H H N H + H N H H H : : :: : : :: + or, or, , + —H Ammonium ion (b) H— : F B F F : :: ? ? ? H— ? —F (c) H — H + H — H or, H — ?? —H Hydronium ion 3.1.6 Hydrogen bond When hydrogen atom is covalently bonded with the strong electronegative ele-
35 ment like fluorine, oxygen or nitrogen atoms, the electron pair forming the covalent bond between hydrogen atom and electronegative element is displaced slightly to- wards the electronegative element. As a result of this displacement of electron pair a slight negative charge develops on electronegative atom and slight positive change develops on the hydrogen atom. A pole is formed which acts like a bar magnet. This type of molecules comes closure to one another due to the dipole dipole attraction for which slightly positively charged H-atom forms a bridge or a partial bond with negatively charged atom. This partial bond is known as hydrogen bond which is weaker than covalent bond. The strength of H-bond is about 3-5 kcal mol – 1 (where a covalent bond is 50-100 kcal mol –1). Hence, formation of H-bond is due to a weak interaction. A H-bond is shown by a dotted line. H-bond can be formed between the molecules of same compound or between different compounds. (i) — ... — ... — H-bond hydrofluoric acid (ii) H — . . . H — . . . H — . . . water (iii) H — . . . H — . . . H — . . . Alcohol (iv) H — . . . H — . . . H — . . . Alcohol and H 2 O
36 (v) O N OH H O O N O O or HO — O ... HO — O para nitrophenol para nitrophenol (vi) (vii) O N O H O Formic acid (dimer) ortho-nitrophenol (viii) O C O H OH Salicylic acid

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There are two types of hydrogen bonding— (i) intermolecular hydrogen bonds and (ii) intramolecular hydrogen bonds. (i) Intermolecular hydrogen bonds : Hydrogen bonds formed between two or more molecules of same or different compounds

are known as intermolecular hydrogen bonds. (i) to (vi) are examples of intermolecular hydrogen bonds. The helical structure of DNA allows the formation of interchain hydrogen bonds. An -helic structure of keratin also allows the formation of interchain hydrogen bonds. (ii) Intramolecular hydrogen bonds : The hydrogen bond formed between the two atoms of same molecule is known intramolecular hydrogen bond. (vii) and (viii) are the examples of intermolecular hydrogen bonds. 3.1.6.1 Nature of hydrogen bonds (i) Hydrogen bond is formed due the attraction between oppositely charged at- oms present in a same molecule or different molecules.
37 (ii) Hydrogen bond is confined between hydrogen atom and strong electronega- tive element like F, O, N. (iii) Greater the electronegativity and smaller the size of atom, stronger is the hydrogen bond. The decreasing order of hydrogen bonds is shown below— H — F ... H < H— ... H < H — ... H 3.1.6.2 Effects of hydrogen bonds (i) The molecular weight of water (18) is less than that of H 2 S (Molecular weight 34), still water is liquid at normal temperature while H 2 S is a gas. Because water molecules associate through hydrogen bonds which require more energy to get separated for vaporization, on the other hand H 2 S forms a very weak hydrogen bond because electronegativity of sulphur is less than that of oxygen and size of sulphur is greater than that of oxygen. So H 2 S is gas at ordinary temperature. (ii) The molecular weight of propane and ethanol are 44 and 46 respectively, but ethanol is liquid at ordinary temperature while propane is a gas. Because ethanol can form hydrogen bond white propane cannot. C 2 H 5 OH CH 3 CH 2 CH 3 Ethanol Propane Molecular at 46 44 Boiling point 78.3°C – 45°C (iii) o-nitrophenol can be steam distilled while p-nitrophenol can not be steam distilled. Because o-nitrophenol forms intramolecular hydrogen bond, so its molecules cannot associate through hydrogen bond. It remains as a discrete molecule. So it can be steam volatile. Where as p-nitrophenol molecules associate through hydrogen bonds and forms intermolecular hydrogen bonds. So it cannot be steam volatile. (iv) o-nitrophenol cannot form hydrogen bond with water, but p-nitrophenol can form hydrogen bonds with H 2 O molecules. So o-nitrophenol is insoluble in water whereas p-nitrophenol is soluble in H 2 O. Covalent compound which can form hydrogen bonds with H 2 O are soluble in H 2 O though water is polar
38 solvent, for example, glucose, a covalent compound is soluble in water be- cause glucose has many hydroxyl groups which can form hydrogen bonds with H 2 O molecules. (v) Water molecules can form tetrahedral open cage like structure due to H-bonds in ice (solid) having much empty space in the cage. For this reason the density of ice is less than that of liquid water. (vi) The molecules of a covalent compound can associate due to the intermolecu- lar hydrogen bonds in a zigzag way. Such associated substance has less power for free movement. Hence the viscosity of such substance will increase, for example, glycerol. (vii) Hydrogen bonds in biological system : Hydrogen bonds are very important for biological systems. A major part of water present in the living systems (both plants and animals) is associated with the proteins by hydrogen bonds. Hydrogen bonds play a very important role in the structures of proteins and nucleic acids (RNA and DNA). Proteins molecules consist of large number of amino acids joined together by peptide linkage (—NH—CO—), < C = O group of a peptide linkage forms a hydrogen bond with hydrogen atom present in the nitrogen atom of another peptide linkage in the same helical chain or with the different chains. Keratin is a protein forming wool, hair, horns, hoofs and silk. Hydrogen bonds are present in the parallel and antiparallel polypep- tide chains of keratin. Hydrogen bonds are also present in double helical structures of nucleic acids. Hydrogen bonds stabilize the structures of proteins and nucleic acid. 3.1.6.3 Hydrophobic bond : Sodium or potassium salts of higher fatty acids (saturated or unsaturated) are generally known as soaps e.g. sodium stearate (C 18 H 35 COONa) or Na-oleate (C 17 H 33 COONa). In an aqueous medium these salts are completely ionized in carboxylate ions and cation (Na + or K +). This carboxylate ion has two parts, one the strong polar head and another the non-polar long chain hydro- carbon part. The polar carboxylate part forms hydrogen bonds with water molecules very easily. But the non-polar hydrocarbon parts donot like water and are insoluble in water. Hence any soap does not form a true solution with water, but disperses into water to form micelles, which may contain soap molecules. In micelles the negatively charged carboxylate parts are exposed to water to form hydrogen bonds while the non-polar hydrocarbon parts are hidden inside and away from water molecules. The negatively charged carboxylate ions repel each other forming
39 and

40 a spherical shape substance known as micelle inside which the non-polar parts are hidden. But a weak Vander Waals' force of attraction acts among the non-polar parts with in the micelle. This weak force of attraction is known as hydrophobic bond or interaction. Due to the mutual force of repulsion among the negatively charged ions the micelles are remained suspended into aqueous medium. Molecules having non- polar and strong polar parts are known as amphipathic. Many amino acids are amphipathic like soaps. Hydrophobic interactions are also shown by proteins, polar lipids and nucleic acids molecules. 3.1.7 Energy rich bonds Formation of bond requires energy while dissociation of bond releases energy. If high energy is obtained by the dissociation of any bond of a compound, then that bond is known as high energy bond and the said compound is known as high energy compound. Adenosine triphosphate compound (ATP) is an example of high energy compound because there is a large decrease in standard free energy change during the hydrolytic cleavage of this compound. The energy thus released is then utilized for the biosyntheses in the living cells. H O CH 2 — O O O O –

41 Adenosine triphosphates [ATP] 4– (adenine ribose triphosphate) Adenosine — O O O O – + H 2 O Adenosine — O O O – + HO O – + H + ADP 3– ATP on hydrolylic cleavage forms ADP, orthophosphoric acid and proton. The standard free energy change (G°´) at 7 pH of this reaction is – 30.5kJ. Similarly ADP on hydrolysis give adenosine monophosphate (AMP), phosphoric acid and proton and G°´ at 7 pH of this reaction is also –30.5 kJ. Adenine-ribose —O O O – + H 2 O ADP (Adenosine diphosphate) Adenine-ribose —O O – + HPO 4 2– + H + AMP (Adenosine monophosphate) Energy rich bonds are present in many compounds of biological interest at pH 7. The standard free energy changes of hydrolysis of some compounds are given below.

42 Compounds G°´ G°´ (kcal mol –1) (kJ mol –1) 1. Phosphonyl pyruvate – 14.8 – 61.92 2. Acetyle AMP – 13.3 – 55.65 3. 1,3-diphosphoglycerate – 11.8 – 49.37 4. Phosphocreatine – 10.3 – 43.1 5. Acetyl phosphate – 10.3 – 43.1 6. Pyrophosphate – 7.9 – 33.0 7. ATP (AMP + pp i) – 7.5 – 31.38 ATP (ADP + p i) – 7.3 – 30.5 8. ADP (AMP + p i) – 7.3 – 30.5 9. AMP – 3.4 – 14.23 10. Glucose 6-phosphate – 3.3 – 13.8 3.1.8. Vander Waals forces Vander Waals forces are intermolecular forces acting among the molecules and due to these interactions practically molecules remain chemically same. Vander Waals forces are also weak in nature. These forces arise due to three factors—(i) orienta- tion, (ii) induction and (iii) dispersion. (i) Orientation of molecules : Orientation arises due to permanent depoles of the molecules because the negatively charged atom of the molecule will at- tract the positively charged atom of another molecule due the electrostatic force and the molecules will come closer to one another. (ii) Induction : Molecules having permanent dipole moment will polarised the neighboring molecules causing induced dipoles in the molecules. Hence there is a force of attraction between the permanent and induced dipoles. (iii) Dispersion : The dispersion effect arises due to the vibrations of electrons clouds with respect to nuclei of atoms present in a molecule. For this effect a very small amount of dipole moments will develop within the molecule in a specific direction. This will attract the other molecules having permanent dipoles. This type of force is known as dispersion force. Beside these three types of attractive forces between molecules, their is also repulsive force acting between molecules on close approach. So actual Vander Waals force is the sum of the three interactions viz. orienta- tion, induction and dispersion minus the force of repulsion.

43 Vander Waals force is a weak force in comparison to strong bond like ionic and covalent bond. Even it is weaker than hydrogen bond. Higher the molecular weight higher is the Vander Waals force. Amino acids having non-polar side chain are hydrophobic in nature and there non-polar side chains are held together between different parts of the peptide bonds by the Vander Waals forces. Similarly nonpolar hydrocarbon chains of soap molecules are held together by Vander Waals forces and these forces help in the formation of micelles. 3.2 Questions 1. What do you mean by a chemical bond? How many different types of chemical bonds are there? Give examples in each type. 2. Define ionic or electrovalent bond and compound. What are factors favouring the ionic bond? What are the characteristics of ionic bond? 3. Define covalent bond and compound.What are the characteristics of covalent bond? 4. Define coordinate covalency with example. 5. What do you mean by hydrogen bond? How many different types of hydrogen bonds are there? Give example in each case. Discuss the different effects of hydrogen bonds. 6. What do you mean by hydrophobic bond? How micelles are formed? 7. What are Vander Waals forces? 8. What do you mean by energy rich bond?

44 UNIT 4 ? Nuclear Hazards Structure 4.1 Nuclear hazards 4.1.1 Theory of radioactive disintegration 4.1.2 Rate of radioactive decay 4.1.3 Units of radioactivity 4.1.4 Characteristics of radioactive decay 4.1.5 Half life period 4.1.6 Dosimetry 4.1.7 Radioactive intensity 4.1.8 Radiation chemical yield (G) 4.1.9 Effects of radiations on biological system 4.2 Principles and applications of Tracer technique in biology 4.2.1 Selection of a radiotracer for a particular system 4.2.2 Advantages and disadvantages of radiotracers 4.2.3 Autoradiography 4.2.4 Preventive measures 4.3 Questions 4.1 Nuclear hazards In 1895 Henry Becquerel first noticed that invisible rays were emitted by the uranium salt, potassium zinc uranyl sulphate spontaneously and continuously. These rays affect photographic plate in dark causing fogging on the plate. These rays can ionize the atmospheric air and produce scintillations on zinc sulphide screen. Many elements or their compounds can emit these rays. This emission of rays cannot be controlled by the pressure, temperature or light. This property of emission of invis- ible rays (radiations) from some substances (elements or compounds) is known as radioactivity and substances which possess this property are known as radioactive substances. The elements which are responsible for radioactivity are known as radio- active elements or radioelement. Atoms having same atomic number but different mass numbers are known as isotopes.

45 e.g. H, H, H and 12 C, 13 C and 14 C The spontaneous emission of radiations by some unstable isotopes of elements like radium, uranium polonium, thorium etc. is known as radioactivity and the ele- ments which posses this property are known as radioactive elements or radioelement. There are certain elements whose one or more isotopes may non-radioactive while others are radioactive, for example, hydrogen isotopes of mass numbers 1 and 2 are non-radioactive while mass number 3 is a radiactive isotope. Similarly 12 C and 13 C are stable isotopes while 14 C is a radioactive isotope. All the isotopes of any element whose atomic number greater than bismuth (At No. 83) are radioactive. A radioelement breaks down into other elements by the emission of radiations. This is known as radioactive disintegration. Three types of radiations known as (alpha), (beta) and (gama) radiations are emitted from a radioelement. (i) -rays : -rays are the stream of doubly charged (positive) helium ions (H 2+) having a mass number 4 and charge twice that of a proton. These rays can be deflected by the magnetic and electric fields and can ionize the medium. It can penetrate thin sheet of aluminium. -rays convert into helium gas (atoms) by loosing charge. From the direction of deflection by magnetic or electrical field it can be proved that -rays are positively charged particles. -rays affect the photographic film. The velocity of -rays is about 1/10th of the speed of light. The slow movement of -particle is due to its size and mass. -particles frequently collide with atoms or molecules in their path, causing intense ionization and excitation. For this reason their energy is dissipated very rapidely and so they are not very penetrating. (ii) -rays : -rays are the stream of negatively charged minute particles. -rays are similar to cathode rays consisting of electrons moving with very high velocities ranging from 1/3 to 9/10 of the velocity of light. -rays can be deflected more than -rays by the magnetic or electric field and from the nature of defection it can be proved that -rays are negatively charged particles like electrons. They can ionize the medium, but their ionizing power is less than that of -rays. Due to negligible mass and high velocities of --rays their penetrating power are more than -particles. They can penetrate 0.1 cm thick aluminium sheet. -rays affect photographic film. They have a very bad effect on the living bodies. (iii) -rays : -rays are stream of high energy photons. Hence -rays donot consist of any particle and are electrically neutral. They are actually electromagnetic

46 wave of very short wave lenghts, even shorter than x-ray. The velocity of -rays is almost same with that of light. -rays can not be deflected by magnetic or electrical fields as they are electrically neutral. These rays can ionize medium very slightly, almost nil and can affect photographic film. The penetrating power of -rays is very high. These rays can penetrate 8cm thick lead sheet. These rays are very harmful to living objects. Mutation of genes takes place when living cells are exposed to -rays. These rays can produce cancer when the man or animals are exposed to these rays for a long time. Still these rays can be used in controlled doses to destroy the malignant cells. 4.1.1 Theory of radioactive disintegration In 1903, Rutherford and Soddy put forward the theory of radioactive disintegra- tion in the following manner—radioactive elements disintegrate spontaneously form- ing new elements by the emission of radiations. If a radioactive element emits particles then the -particles must emit from the nuclei of the element. The nuclei of radioactive elements are unstable and dis- integrate spontaneously liberating and -particles, whether nuclei of radioactive element are present in atoms or in compounds. Because radioactivity is a nuclear phenomena. Due to this radioactive disintegration new element is formed having different physical and chemical properties from those of the parent element and new- born element is known as daughter element and if this new element is also radioac- tive element then it will also disintegrate into another element. This process continues until a non-radioactive element is formed at the end. This non-radioactive element is known as end product. The formation of new ele- ments by the emission of and -particles from a radioactive element is known as radioactive disintegration. After the emission of and particles from a radioactive element the excess energy which is stored in the atomic nuclei is radiated in the form of -radiation. In any radioactive disintegration -radiation will not come first. - radiation takes place only after the and -radiations. and radiations donot take place simultaneously in any radioactive disintegration process. 4.1.2 Rate of radioactive decay The rate of disintregration or decay of a radioactive element at any instant is directly proportional to the number of radioactive atoms (or nuclei) present at that instant. The rate of radioactive decay cannot be changed or influenced by the chemi- cal or physical processes. The amount of radioactive element disintegrates in unit
47 time is known as rate of radioactive decay which is directly proportional to the amount present at that time. The rate of radioactive decay is expressed mathemati- cally in the following way— – where N = number of radioactive atoms or nuclei at any instant dN = number of atoms disintegrating in a very small time dt. = rate of disintegration. In the decay process the amount of radioactive atoms or nuclei decrease with time. Hence negative sign is placed before the rate ( ). or – = N where = disintegration constant. or = – dt On integrating the above equation InN = – t + C where C = integration constant Now when t = 0, N = N 0 Putting these value in the above equation we get InN 0 = C. So we can write InN = – t + In N 0 or, In = t or, t = 2.303 log or, = log or, t = log or, = or, , N =
48 4.1.3 Units of radioactivity The radioactivity of a radioactive element (isotope) can be calculated at what rate it is converted into its daughter element. This is done by counting number of disintegration per unit (generally in second) with the help of Gieger Müller counter or scintillation counter. The rate of decay of a radioactive element is generally ex- pressed in curie (ci) which is defined as the amount of any radioactive isotope which gives 3.7 × 10 10 disintegration per second (dps). This rate is equal to the disintegra- tion of one gram of radium (mass no 226) The disintegration rate of 226 Ra = × no. of atoms in 1g radium 1g radium = mole Ra = × 6.023 × 10 23 radium atom t 1/2 = (where t 1/2 = half life of 226 Ra) = = × × 6.023 × 10 23 disintegrations per year = × × 6.023 ×10 23 disintegration per second (dps) = 3.7 × 10 10 dps = 1 ci 1 millicurie = 1 × 10 –3 ci 1 curiec = 1 × 10 –6 ci The SI unit of radioactivity is becquerel (or Bq) which is defined as one disin- tegration per second. 1 ci = 3.7 × 10 10 Bq Another unit of radioactivity is Rutherford which is defined as 10 6 dps. From above equation it is evident that rate of radioactive decay decreases with time. 4.1.4 Characteristics of radioactive decay (i) Rate of radioactive decay decreases with time. (ii) The number of atoms decay in unit time is the rate of decay (iii) Any radioactive decay follows the first order reaction rate (iv) The time taken for the decay of a definite fraction of a radioactive element is independent of its initial concentration. (v) Radioactive disin-

49 tegration is a random process and number of atoms that disintegrate per second is directly proportional to the number of radioactive atoms present at that time. 4.1.5 Half life period The time required by a given amount of the radioactive element (having same mass number) to decay one half of its initial value is known as half life period (t 1/ 2) of that radioactive element. Half life period is expressed by t 1/2 . The half life period is deduced by the following equation— t = log when t = t 1/2 , N = t 1/2 = log where N 0 = initial number of atoms. = log2 log2 = 0.301 = = decay constant, therefore t 1/2 is a constant and is independent of the initial concentration or amount of radioactive element. For a particular radioisotope the value of t 1/2 is constant and depends only on its decay constant. So we can calculate the decay constant of a radio isotope very easily. Let us take an example; the t 1/2 of radium (mass number 226) is 1600 years. So value of its decay constant ( ). = = year −1 . Radioactive isotopes are toxic in nature due to thier ionic and non-ionic radiations. The toxicity of radioactive isotope on human being is the main disadvantage of using the radio-isotopes as tracers or any other work. Because these radiations cause ionization and produce free radicals which will react with the cells of the living being causing mutation of DNA and affecting proteins and other macromolecules present in the cells. These radiations can induce cancer on prolong exposure or in high doses.

50 4.1.6 Dosimetry The dosimetry is the method to determine the dose of different kind of radiations particularly emitted by radioactive substances on living and non-living substances. The dosimetry also determines the effects of different radiations both ionic (,) and non-ionic ( ) radiations on living and non-living substances. It also determines the radiation hazards on living being particularly human beings working with radioactive substances in the laboratory or factories, in mines of radioactive ores. Dosimetry is also used in radiotherapy. The action of ionizing radiation on any matter both living and non-living is directly proportional to absorbed dose of radiation. The absorbed dose is defined as the amount of ionizing radiation energy aborbed by the unit mass of substance being exposed to this radiation. Unit of absorbed dose in SI unit is joules per kilogram of substance. For practical purposes unit of absorbed dose is the rad (radiation absorbed dose) and one rad is equal to 100 ergs of energy absorbed by one gram of substance being exposed to the ionizing radiation. One rad is also equal to 6.24 × 10 13 eV per cm 3 and rad shows total quantity of radiation energy absorbed by the tissues or any substances. Nowadays rad has been replaced by SI unit gray (Gy) which is equivalent to 100 rad. Because strength of X -rays or -rays decreases as they traversed through the tissue or substance. Exposer dose of radiation is used in the cases of X -rays and -rays radiations and exposer dose is defined as the amount of radiation energy in coulomb absorbed by one kilogram of substance being exposed to these radiaions. This is the SI unit of exposer dose. But for practical purposes exposer dose is expressed in roentgen (symbol r) which is defined as the amount of X -or -radiation that will produce ions carrying 2.58 × 10 −4 coulomb of electricity, of either sign in 1 cm 3 of dry air at normal temperature and pressure (NTP) i.e. on 0.001293 gm of dry air. Absorbed dose rate : Absorbed dose rate is defined as amount of dose of radiation absorbed by unit mass of substance per unit time. In SI unit, it is joule per kilogram per sec. (Jkg −1 s −1). It can also be expressed in rad per second [r s −1] or electron volt per second. Similarly exposer dose rate for x- or -radiations is defined as amount of dose of x- or -radiation per unit time. In SI unit, it is coulomb per kilogram per sec [Ckg − 1 s −1] and for practical purposes, rad per sec [r s −1]. Mathematically it is expressed in the following way—

51 D = − pt where D = dose p = dose rate and t = time in second 4.1.7 Radiation intensity It is the amount of radiation energy per unit time crossing unit area perpendicu- lar to the propagation of radiation. Its unit is ergs percm 2 persec [erg cm −2 s −1] or [eVcm −2 s −1]. In SI unit it is Jm −2 s −1 . 4.1.8 Radiation chemical yield (G) An ionizing radiation on chemical substance(s) can induce chemical reactions which can be characterized by radiation chemical yield (G) and this is defined as the numbers of molecules, atoms, ions or free radicals formed or destroyed from a substance by absorbing 100 eV of ionizing energy. It is expressed as follows— G = × 100 where N = no. of molecules ions atoms or free radicals formed or destroyed from 1cm 3 of substance D = absorbed does in eV. Different ionizing and non-ionizing radiations have different effects on living bodies and degree of hazard associated with different radiations on living bodies are different. For this reason a correction factor is introduced for different types of radiations in comparison with that of X -radiations. The correction factor is known as weighting factor (W). The absorbed dose which is considered for weighting factor is known as sievert (Sv) and sievert can be expressed as follows — Sv = Gy × W 1 Sv 100 rem where rem is roentegen equivalent in man and nowaday it is replaced by sievert. where Gy is known as gray. The effect of radiation on living bodies is very similar to that of x-radiation. So the weighting factor for radiation is taken to be 1 and therefore for -radiation. Sv = Gy But harmful effect of -radiation is 20 times greater than -radiation. Therefore the weighting factor will be 20 for -radiation. The absorbed dose from a radioactive isotope can be calculated by rate of decay of radioactive isotope, energy associated with decay, the penetrating power of the

52 radiation, the distance between the radioactive source and the object being irradiated. Since a radioactive isotope emits radiation in all directions with equal intensity and the intensity of radiations from a radioactive source is inversly proportional to the square of the distance. So we can deduce the following equation— D 1 × l 2 2 = D 2 × l 1 2 where D 1 and D 2 are doses l 1 and l 2 are distances. The annual permissible limit for a person exposed to radiation is 50 mSv per year for whole body. 4.1.9 Effects of radiations on biological system Different types of radiations have different effects on biological systems. As for example -particles have large size for which -particles have very little penetrating power, even skin can stop the penetration of -particles in the body. But -particles can penetrate into the body through the wounds or cuts and if -particles enter into the body then they cause a heavy damage to body cells by ionizing the cells. -particles have greater penetrating power than -patticles and -particles can penetrate in body slightly and can cause burn on skin and intensity of burn depends on the flux of -particles. -rays are electromagnetic radiations like x-rays. These radiations can penetrate deep into the living bodies very easily. , and -radiations can ionize the DNA and break the double strand of DNA, can produce free radicals. As a result of these damage, death of cells may take place. Higher the radiation dose, higher will be damage or death of cells. These radiations, particularly -radiations, can break the chromosomes and broken chromosomes can unite in a abnormal way causing the mutation of chromosomes. The effects of radiations may be short term or long term. Due to high dose of radiation death may take place immediately or after sometime. These radiation may cause carcenogenesis dermatitis, blood dyscrasias, leukaemia, chromosome aberra- tion, acute radiation sickness and abnormalities in the development of foetus. Radiations can be used for the treatment of cancer or tumours. This is known as radiation therapy. Therapeutic radiations also destroy the cells of body tissue in their paths by breaking the single strand of DNA and by producing free radicals from water present in the cells. These free radicals destroy the proteins, cell membranes and various particles present in the cells. These radiations can destroy malignant tumorous cells also. So the therapeutic radiation must be used in a controlled way at
53 the target tissue area, so that they must cross normal cells as minimum as possible. X -rays and -rays are used for cancer treatment. While -rays of having low penetrating power can be used for the treatment of skin condition. 4.2 Principles and applications of tracer techniques in biology Tracers are the chemical substances which can be used as a markers to trace the pathway of a chemical reactions or any other physical processes. Tracers can be also used to locate the position of a substance. A tracer may be radioactive or non-radioactive substance. A radioactive tracer or radiotracer is a chemical substance that contains a radionuclide. We know that isotopes of an element have same chemical properties. If one of the isotope is radioactive, then these two isotopes (radioactive and non- radioactive) have same chemical properties but the radioactive isotope only emits radiations. Now in a compound in which a non-radioactive (stable) atom can be replaced by a radioactive isotope of that atom and the compound thus obtained is known as labelled compound which can be traced by the radiation emitted by the labelled radioactive atom in order to follow its course in chemical or physical proc- esses of the system under investigation. Radiotracers can be used to follow the metabolic pathways metabolic turnover rates, photosynthesis, biosynthesis of protein and nucleic acid etc. A radioactive tracer used in a system to follow its path in any process must mix perfectly with system under investigation or the radiotracer must label a particular non-radioactive isotope perfectly in a compound to investigate its fate. In doing so the tracer used must not affect adversely any part of the system. After incorporating the radiotracer in a system it will behave almost exactly same way with the non- radioactive substance under investigating except for its radioactive emission. A very small amount of radiotracer is used in a system so that its radioactivity does not affect any part or component of the system. A radiotracer isotope is not only different in mass number from the element to be traced in a compound or system, but the tracer is also radioactive while the other isotope is non-radioactive. Isotopes having different mass numbers react chemically in different rates. The rates of diffusion of these isotopes are also different. Because

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rate of diffusion is inversely proportional to the square root of

their molar concen- tration at constant temperature and pressure (Graham's law of diffusion). Moreover isotopic labelling in a compound takes place at random which will increase the

54 entrope of the system, thereby changing the equilibrium constant of this exchange reaction. Beside these factors different isotopes of the same element have different energies due to vibrational energy of two dissimilar mass of isotopes and also on different electron binding energies in the atoms of the isotopes. The combination of all these factors for isotope labelling process is known as isotope effects. Isotopic effect in the case of tritium (mass numbers 3) with respect to protium (hydrogen mass number 1) is very large because mass of tritium is three times that of protium (1 H). But in case of uranium 238 (mass number 238) has very little isotopic effect with respect to uranium 235 (235 U) because mass difference is very small in this case. 4.2.1 Selection of a radiotracer for a particular system All radiotracers are not suitable for any process or chemical exchange reaction. A tracer must be suitable chemically and physically for a particular process or chemi- cal reactions under investigation. For the study of biosynthesis of cholesterol and its metabolites carbon-14 (14 C) is very suitable radiotracer. 14 C tracer is also very useful to study the photosynthesis, biosynthesis of nucleic acids and protein etc. For bone imagining purposes strontium 89(89 Sr) is a very useful radiotracer because it can exchange calcium atoms from calcium phosphate molecules present in bone. Similarly thyroid gland produces an iodine containing amino acid thyroxine. For the study of thyroid gland, iodine of mass number 125 (125 I) or 131 (131 I) are used a tracers. Other radiotracers are not suitable for thyroid studies. Another important fact for the selection of a radiotracer is the half life (t 1/2) of radiotracer. The half life of a tracer should not be very long or short. But the half life of a tracer must be long enough so that a particular experiment can be completed during this half life period. Moreover the activity of the radiotracer should be high enough during the course of experiment for its measurement. The specific activity of a tracer having a long half life is very low. This type of tracers has serious problem in storage and disposal. It has a very serious environmental contamination problem too. Another important factor for the selection of a radiotracer is the type of radiations emitted from it, because all types of radiation are not suitable for all type of tracer studies.

55 The radiation emitted from a tracer must have suitable penetrating power for the experiment to be studied and the activity of the radiation can be measured easily by some ways. Its damaging potential for the sample should be as low as possible. -emitting tracers are always used in the case of very thick and dense sample. Some Isotopes used as Tracers Isotope Half life Type of radiation 3 He 12.3y 14 C 5730y 24 Na 15d , 32 P 14.28d 35 S 87.1d 36 Cl 3.1 × 10 5 y 42 K 12.36h , 90 Sr 29.1y 128 I 25m , 131 I 8d , 60 Co 5.27y , or radiations has very little penetrating power, so they are not suitable for thick and dense sample, only -radiation can be used for this purpose in the case of very thin film or a slice of the sample. So that its activity can be counted by scin- tillation. -radiation has the least penetrating power, so -emitter is not very useful in tracer studies, moreover -radiation enters into the body of a living object through wounds or cuts producing free radicals which will damage the sample very seriously. A non-radiotracer can be used in the study of chemical reactions. This non- radioactive tracer is detected by mass spectrograph. 4.2.2 Advantages and disadvantages of radiotracers A very negligible amount of a radiotracer present in a system can be very efficiently detected and measured by its emitted radiation. But this negligible amount cannot be detected by chemical methods. Negligible amount of radiotracer present in a system minimizes the potential damaging effect of the system or its component, particularly living bodies and biological specimens. The decay of any radiotracer cannot be influenced by the pressure, temperature heat, light pH etc. These are the

56 advantages of radiotracers. All radioactive radiations are toxic in nature. This is one of disadvantages of radiotracer. Handling the radiotracers and disposal of radioactive materials left are the disadvantage of radiotracers. The metabolite and its transformation products cannot only be detected and measured by the tracer technique method but many important observations can also the made about plants, animals and many biological specimens like isolated organs and tissues. Many definite conclusion can be made with these observations, for example, all the carbon atoms present in the molecule of cholesterol are derived from acetate. Biosynsthesis of cholesterol is an unique example of the application of tracer technique. By this technique it was conclusively proved that all the carbon atoms present in cholesterol are derived from acetic acid. Cholesterol molecule has two part; one is the steroid and another is eight membered side chain. In 1940 Block et.al. proved that all the carbon atoms present in cholesterol (steroid and side chain) are derived from acetate. They fed a rat by labelled acetic acid and found that this labelled carbon atoms (14 C) are incorporated in the cholesterol found in the liver of the rat. They used methyl labelled acetate and carboxylate labelled acetate separately to rat and in both cases they found that la- belled carbon atoms are present in the cholesterol. carboxylate labelled acetic acid methyl labelled acetic acid

57 Where m denotes methyl labelled carbon atom and C denotes carboxylate la- belled carbon atom. Cholesterol is synthesized in the liver of animal from labelled acetic acid which by the way of acetoacetic acid and mevalonic acid is converted to five membered isopentanoid C = CHCH 3 units. Six such units on combination give linear labelled squalene which is fed to animal. This labelled squalene by appropriate fold- ing and ring closure, leads to lanosterol, desomosterol and finally to labelled choles- terol. This proves that squalene is a precursor of cholesterol. 4.2.3 Autoradiography Autoradiography is a technique to follow the localization of radiotracer in the metabolic pathway or biological specimens or living objects or any specimen in order to determine its ultimate fate. In this technique any material living or non-living that contains radiotracer is placed in contact with an undeveloped photographic film or plate and the radiation emitted from radiotracer will expose on the photofilm for sometime.

58 A photographic film is coated with a uniform layer of light sensitive emulsion. This emulsion consists of silver halide cystals (mainly AgBr) embedded on gelatine. Silver halide present in photofilm is reduced to metallic silver on exposure to light (radiation) by chemical reaction of developing and the amount of reduction is pro- portional to the intensity of light and developers produce black deposit of fine par- ticles of metallic silver on those part of the film that had been exposed to light thus giving a negative image. The unaffected silver halide is removed by the action of sodium thiosulphate solution and then by washing with water. This gives a negative which is free of light sensitive silver halide. The negative thus obtained will produce an image of the location of the radio- active areas of the object. This autoradiography technique is very sensitive and it is widely used in a large number of biological specimens for different purposes. Autoradiography is also used to locate the distribution of the radiolabelled drug in the whole body of an experi- mental animal. To study the metabolic path, radiolabelled metabolites are separated by chromatography and these metabolites can be detected by autoradiography while they are in the chromatography column or in TLC (Thin layer chromatograph) plate even in paper chromatograph. Once the position of the metabolite is located by autoradiography, the metabolite is recovered from that part for counting the activity by Geiger-Müller tube or counting by some other methods. 4.2.4 Preventive measures The person working with radioactive substances should be very careful to avoid the radioactive substances for the inhalation, swallowing or direct contact with skin. Suitable protecting clothing must be used to prevent the direct contant from these harmful radioactive substance. In the case of x-rays and -rays, proper thickness shielding should be used to reduce the exposure below allowable limit. Proper ven- tilation should be arranged in the factories or in the work place in order to prevent the inhalation of these harmful radioactive dust particles or gases. Empolyees, work- ing in radiation laboratories, in the mines of radioactive ores, in the industries using radioactive substances should be monitoring at regular intervals to determine the absorbed dose. Periodic medical check up and examinations must be done for every workers. If any harmful effects are found to anybody then the person must be trans- ferred to a safe place immediately involving no exposure to radiation.

59 4.3 Questions 1. What do you mean by radioactivity and radioactive substance? 2. What are the characteristics of , and rays? 3. How would you express the rate of disintegration of a radioelement mathematically? 4. What are units of radioactivity? Define them. 5. How would you detect and -rays? 6. What is end product? 7. Deduce a relation between half life (t 1/2) and decay constant. 8. What are the characteristics of radioactive decay? 9. What do you mean by dosimetry? 10. Define (i) rad, (ii) rem, (iii) gray, (iv) absorbed dose rate, (v) radiation chemical yield (vi) radiation intensity, (vii) sievert. 11. What are the effects of radiation on biological system? What are the preventives measures? 12. What is a radiotracer?What is a labelled compound? What are the characteristics of a good radiotracer? 13. What is autoradiography?

60 UNIT 5 ? Ionic Product Structure 5.1 Ionic product of water 5.2 Degree of dissociation of water at 25 o C 5.3 pH 5.4 pOH 5.5 Buffer solution 5.6 Determination of pH of a buffer solution 5.7 Indicators 5.8 Questions 5.1 Ionic product of water Pure water is a very bad conductor of electricity. The specific conductance of water at 25°C is 5.54×10^{-8} ohm $^{-1}$ cm $^{-1}$. This proves that the water molecule disso- ciates into H + and OH − ions. Since water conducts very small amount of electricity, so water is a very weak electrolyte. Therefore, dissociation of water molecules will be in equilibrium, i.e. 2H 2 O H 3 O + + OH − because the existence of free H + ion is not possible still for our convenience we will write the equation in the following way, H 2 O H + + OH − ... (3.1) K = ... (3.2) where K = equilibrium or dissociation constant and [H +], [OH −] and [H 2 O] are the concentrations of H + ion, OH − ion and H 2 O respectively at the equilibrium. Concentration is expressed in mol. litre $^{-1}$. Since water is a very bad conductor of electricity, so the dissociation of water molecules into ions will be also very small. Hence the concentration of water will remain practically unchanged, i.e. [H 2 O] = constant. K × [H 2 O] = [H +] × [OH −] = K w

61 where K w is known as ionic product of water and it is defined as the product of the concentrations of [H +] and [OH −] ions in pure water. Dissociation constant of H 2 O × concentration of H 2 O = Ionic product of H 2 O. The value of K w depends on temperature and it is 1 × 10 −14 at 25°C K w = [H +] × [OH −] = 1 × 10 −14 at 25°C. By the dissociation of water molecule equimolar amount of H + and OH´ are obtained. [H +] × [OH −] = [H +] 2 = 10 −14 [H +] = = 10 −7 mol.litre −1 at equilibrium at 25°C. 5.2 Degree of dissociation of water at 25°C At 25°C, K w = 10 −14 [H +] × [OH −] = 10 −14 or, [H +] 2 = 10 −14 since [H +] = [OH −] [H +] = = 10 −7 mole. litre −1 . c = 10 −7 where c = molality (molal concentration) of H 2 O and = degree of dissociation of water. The molecular weight of H 2 O is 18. One mole of H 2 O = 18 g of H 2 O and the volume of 18 g of H 2 O = 18 cm 3 . 1 litre or 1000 cm 3 water contains = = 55.6 mole of H 2 O. c = 10 −7 = = = 1.8 × 10 −9 mol. litre −1 5.3 pH pH is a word used to express the activity of hydrogen ion in a system or a solution and pH is equal to −log a H + , where a H + is the hydrogen ion activity of a solution or system. In a dilute solution, activity of a solution is generally equal to concentration, pH = − log a H + = − log [H +] = ... (3.3) where [H +] = concentration of hydrogen ion.

62 The concentration of hydrogen ion in pure water is 10 −7 mol.litre. So, pH of pure water will be, pH = − log[H +] = −log10 −7 = 7. The hydrogen ion concentration of any acidic solution is greater than that of pure water. So the pH of any acidic solution will be less than 7 and the pH value of any acid solution will be between 0 to 7 and pH of any alkaline solution will be between 7 − 14. The pH of pure water is 7 and it is neutral, i.e. pure water is not acidic or alkaline. 5.4 pOH The pOH value of any solution or system may be difined as the negative loga- rithm of the hydroxyl ion concentration, pOH = − log[OH −] But [H +] × [OH −] = 10 −14 (K w = 10 −14) Taking logarithm on both sides. log[H +] + log[OH −] = log 10 −14 or, −log[H +] − log[OH −] = − log10 −14 According to defination pH + pOH = 14. ... (3.4) (i) By this equation, pOH value of any solution can be determined provided pH value is known and vice versa, for example, if the pH value of a solution is 2, then the pOH value of this solution is (14 − 2) = 12. (ii) By decreasing the hydrogen ion by 10 fold the pH value will increase by one unit. The hydrogen ion concentration [H +] of N/100 acid solution is 10 −2 mol·litre −1 . pH value of N/100 acid solution = 2. Similarly pH of N/10 acid solution = 1. and pH of 1(N) acid solution = 0 because [H +] of 1(N) acid = 1 = 10 0 mol. litre −1 . (iii) pH value of any solution cannot be less than zero or more than 14. (iv) Since the value of K w depends on temperature. So the pH value changes with temperature. So, 25°C is taken as standard for the determination of pH or pOH value.

63 (v) The pH value which is expressed by hydrogen ion concentration (mol·litre −1) is known as pH scale. Similarly the pOH value which is expressed by [OH −] concentration (mol·litre −1] is known as pOH scale. (vi) pH value of any acid solution will be from 0 to >7 and pH value of any alkaline solution will be 14 to < 7. The pH acid of pure H 2 O is 7. [OH ?1] mol.lit 10 ?14 10 ?13 10 ?12 10 ?11 10 ?10 10 ?9 10 ?8 10 ?7 10 ?6 10 ?5 10 ?4 10 ?3 10 ?2 10 ?1 10° [H +] mol.lit 10° 10 ?1 10 ?2 10 ?3 10 ?4 10 ?5 10 ?6 10 ?7 10 ?8 10 ?9 10 ?10 10 ?11 10 ?12 10 ?13 10 ?14 pH 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 pOH 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 pH range of some substances Gastric juice 1.0 − 3.0 Human urine 4.8 − 5.1 Human blood 7.36 − 7.42 Human saliva 6.5 - 7.5 5.5 Buffer solution A solution which can resist a change of pH by the addition of some acid or alkali is known as buffer solution. A buffer solution is composed of either an acid and its salts or a base and its salts. Classification of buffers (1) Simple buffers : A buffer which is composed of salt of weak acid and a base or an acid salt and a neutral salt, for example, CH 3 COONH 4 , NH 4 OH; disodium hydrogen phosphate (Na 2 HPO 4) and sodium phosphate (Na 3 PO 4). The mixture of protein and amino acid is also an example of simple buffer. The buffer range of simple buffer is between 5.0 − 6.3 pH. (2) Mixed buffer : (i) Acid buffer : A buffer composed of a weak acid and its salt (which on dissociation in solution gives strong base) is known as an acid buffer, for example, acetic acid (CH 3 COOH) and sodium acetate (CH 3 COONa) solution; sodium bicarbonate (NaHCO 3) and carbonic acid solution; boric acid (H 3 BO 3) and borax (Na 2 B 4 O 7 .10H 2 O) solution. The pH range of the acid buffers is between 7 − 14. (ii) Basic buffer : A buffer composed of weak base and its salt (which is obtained by reacting the base with a strong acid) is known as basic buffer, for example, ammonium hydroxide (NH 4 OH) and ammonium chloride (NH 4 Cl) solution; glycine (NH 2 CH 2 COOH) and glycine hydrochloride (ClH − .N + H 3 CH 2 COOH) solution. The pH range of basic buffers is between 0 − 7.

64 Mechanism of buffer action : Let us take a buffer composed of acetic acid and sodium acetate. Acetic acid is weak acid. So it disassociates partially in an aqueous solution into H + and CH 3 COO − ions. But in aqueous solution sodium acetate disas- sociates completely into Na + and CH 3 COO − (acetate) ions. Due to this acetate ion (common ion) the dissociation of acetic acid is further suppressed. The dissociation constant Ka of acetic acid is 1.8 × 10 −5 mol·litre −1 . CH 3 COOH CH 3 COO − + H + Ka= = Because [H +] = [CH 3 COO −] The dissociation of acetic acid is negligible. So the concentration of acetic acid remains practically unchanged, i.e. its concentration remains 1 molar. [H +] 2 = 1.8 × 10 −5 [H +] = = 4.2 × 10 −3 mol·lit −1 . The pH of 1 molar acetic acid = − log [H +] = − log 4.2 × 10 −3 pH = − log 4.2 − log 10 −3 = 3 − log 4.2 = 2.38. Now one mole sodium acetate is added into the acetic acid solution, so that the concentration of CH 3 COO − ion in the solution is 1(M). The dissociation of acetic acid is suppressed due to the presence of this acetate common ions. So the concentration of acetic acid in the mixture remains practically unchanged. Ka = = Ka = [H +] = 1.8 × 10 −5 pH = − log[H +] = − log 1.8 × 10 −5 = 5 − log 1.8 = 4.78 The pH of 1(M) acetic acid and 1(M) sodium acetate solution is 4.78. Now 0.01(M) HCl is added to this solution. The acid reacts with CH 3 COO − ion to give 0.01(M) acetic acid. In this condition the concentration of acetic acid will be 1.01M and concentration of acetate ion will be 0.99M.

65 Ka = = 1.8 × 10 −5 = or [H +] = × 1.6 × 10 −5 pH = 4.74 If 0.01M HCl is added to pure water, then its pH will change from 7 to 2. Similarly if we add 0.01(M) alkali to this buffer than the pH will be 4.79 because alkali (OH −) will react with CH 3 COOH acid to give CH 3 COO − and water. So the concentration of acetic acid will be 0.99 (M) and acetate ion will be 1.01 (M). 5.6 Determination of pH of a buffer solution A buffer is composed of weak acid HA and its salt NaA in an aqueous solution. So, HA H + + A − and NaA Na + + A − Ka = where Ka = dissociation constant of the acid [H +] = The concentration of A − depends on the dissociation of NaA mainly, because the concentration of A − due to dissociation of HA is negligible. The dissociations of HA is further depressed due to the common ion. So the concentration of HA practically remains same. [A −] = [NaA] and [HA] = Acid. [H +] =

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Taking logarithm on both sides log[H +] = log Ka + log 66 − log [H +] = − log Ka − log or, pH = pKa + log where pKa = − log Ka or, pH = pKa + log ... (3.6) This equation (3.6) is known as

Handerson's equation. Now if [salt] = [acid] pH = pKa Again = 10there pH = pKa + 1 and = there pH = pKa − 1 Buffer capacity : Number of gm equivalent amount of acid or base required for 1 litre of a buffer solution to change its pH by one unit is known as its buffer capacity. 5.7 Indicators Indicators are organic compounds, generally organic dyes. An indicator indi- cates the completion of a chemical reaction, i.e. end point in a volumetric analysis by the sharp change of its colour. It also indicates the pressence or absence of certain substance in a solution. A very small amount of indicator is used in volumetric titration. In an acid base titration litmus, phenolphthalein, methyl orangeti are used as indicators. In permanganometric titration permanganate itself is used as an indi- cator and in iodometic titration starch solution is used as an indicator. The colour of these organic dyes depends on the hydrogen ion concentration, that means on pH of the solution, for example, the colour of phenolphthalein is colourless in acid medium but pink in alkaline medium. These organic dyes in water generally behave as acids or bases by dissociating in ions in solutions. The colour of the disassociated molecule may be different from its undissociated form. Now if the amount of undissociated form is in excess then we see a particular colour of the solution and if the amount of dissociated form is in excess than we see another colour

67 of solution. The undissociated or dissociated form will depend on the pH of the solution. The dissociation of an indicator has no effect on the pH of the solution because indicator is used in a very small amount. The equilibrium of a weak acidic organic dye indicator [HIn] may be expressed as, HIn H + + In − K In = or = where K In = dissociations constant of the indicator. HIn = undissociated indica- tor, In − = dissociated anion part of the indicator. If the value of ratio [HIn/In −] is in between 0.1 to 10 then we can understand the colour change. In such case the value of Ka should be very close to hydrogen ion concentration [H +]. The colour change of an indicator takes place over a pH range such that it can indicate the end point of a titration. Acid base indicators and their pH range Indicators Colour in acid Colour in alkaline pH-range medium medium Methyl Orange Orange red Yellow 2.1-4.4 Methyl red Red Yellow 4.2-6.3 Litmus Pink Blue 5.0-8.0 Bromothymol blue Yellow Blue 6.0-7.6 Phenolphthalein Colourless Pink 0.3-10.0 The colour change of methyl orange due to its change in structure is given below, N = N N(CH 3) 2 ? −N= =N + (CH 3) 2 In − (yellow in alkaline medium) HIn (orange red in acid medium) In case of phenolphthalein
68 ? HIn (colourless in acid medium) In − (pink in alkaline medium) Different types of indicators are used in different titrations, for example, in permanganometry titration KMnO 4 itself used as an indicator (self indicator). In iodometry titration starch solution is used as an indicator. In complexometry titration by EDTA (ethylene diaminetetraacetic acid) Eriochrome black T is used as an indi- cator. NCH 2 CH 2 N (EDTA) Problems : 1. Calculate the pH of 0.001(N) H 2 SO 4 solution. The concentration of H + ion in 0.001 (N) H 2 SO 4 and solution is 0.001 mol·litre −1 . pH = − log [H +] = − log (0.001) = − log 10 −3 = 3 2. What is the pH of 0.1(N) NaOH solution? The OH − ion concentration of 0.1N NaOH = 0.1 mol·litre −1 . p(OH) = − log [OH −] = − log [0.1] = − log 10 −1 = 1 But pH + pOH = 14 pH= 14 − pOH = 14 − 1 = 13.
69 3.8 Questions 1. What do you mean by ionization and ionic product of H 2 O ? 2. What is the degree of dissociation of water at 25°C ? 3. Define pH and pOH of a solution or a system. What is pH scale ? 4. What is a buffer solution? Classify buffer solutions. 5. Explain the mechanism of buffer action. 6. How do you determine the pH of a buffer solution ? 7. What is Handerson's equation ? 8. What is buffer capacity ? 9. What is an indicator? Give examples of some acid base indicators and their pH-range with colour in acid and alkaline medium. 10. What is a self indicator ? 11. Write the structures of phenolphthalein in acid and alkaline medium.

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3/14	SUBMITTED TEXT	14 WORDS	88%	MATCHING TEXT	14 WORDS

Open system : A system which can exchange both matter and energy through its

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4/14	SUBMITTED TEXT	17 WORDS	64%	MATCHING TEXT	17 WORDS

the initial and final states of system and is independent of the path to which this change

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energy of an isolated system remains constant. (iii) It is impossible to construct a perpetual motion machine

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6/14	SUBMITTED TEXT	82 WORDS	66%	MATCHING TEXT	82 WORDS

U 2 + P 2 V 2) – (U 1 + P 1 V 1) ... (1.9) = (U 2 – U 1) + (P 2 V 2 – P 1 V 1) = U + P(V 2 –

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7/14	SUBMITTED TEXT	26 WORDS	80%	MATCHING TEXT	26 WORDS

It is impossible to take heat from a hot reserve and convert it completely into work by a cyclic process without transforming a part of heat

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depends only on the initial and final states of the system and

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9/14	SUBMITTED TEXT	22 WORDS	61%	MATCHING TEXT	22 WORDS

adiabatic process, which of the following is correct? (a) PdV = 0 (b) q = – W (c) U = q (d)

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10/14	SUBMITTED TEXT	23 WORDS	78%	MATCHING TEXT	23 WORDS

H = U + PV G = U + PV – TS. or, dG = dU + PdV + VdP – TdS – SdT.

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11/14	SUBMITTED TEXT	33 WORDS	76%	MATCHING TEXT	33 WORDS

C C : : H H H : : H : : or C = C H C C H : ..... :

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12/14	SUBMITTED TEXT	32 WORDS	32%	MATCHING TEXT	32 WORDS

There are two types of hydrogen bonding— (i) intermolecular hydrogen bonds and (ii) intramolecular hydrogen bonds. (i) Intermolecular hydrogen bonds : Hydrogen bonds formed between two or more molecules of same or different compounds

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13/14	SUBMITTED TEXT	12 WORDS	90%	MATCHING TEXT	12 WORDS

rate of diffusion is inversely proportional to the square root of

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Taking logarithm on both sides log[H +] = log Ka + log 66 – log [H +] = – log Ka – log or, pH = pKa + log where pKa = – log Ka or, pH = pKa + log ... (3.6) This equation (3.6) is known as

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M-2\D:\Netaji 05\Biology-1\BIO-TIT PM6.5 PREFACE In the curricular structure introduced by this University for students of Post-graduate degree programme, the opportunity to pursue Post-graduate course in subjects introduced by this University is equally available to all learners. Instead of being guided by any presump- tion about ability level, it would perhaps stand to reason if receptivity of a learner is judged in the course of the learning process. That would be entirely in keeping with the objectives of open education which does not believe in artificial differentiation. Keeping this in view, study materials of the Post-graduate level in different subjects are being prepared on the basis of a well laid-out syllabus. The course structure combines the best elements in the approved syllabi of Central and State Universities in respective subjects. It has been so designed as to be upgradable with the addition of new information as well as results of fresh thinking and analysis. The accepted methodology of distance education has been followed in the preparation of these study materials. Co-operation in every form of experienced scholars is indispensable for a work of this kind. We, therefore, owe an enormous debt of gratitude to everyone whose tireless efforts went into the writing, editing and devising of a proper lay-out of the mate- rials. Practically speaking, their role amounts to an involvement in invisible teaching. For, whoever makes use of these study materials would virtually derive the benefit of learning under their collective care without each being seen by the other. The more a learner would seriously pursue these study materials, the easier it will be for him or her to reach out to larger horizons of a subject. Care has also been taken to make the language lucid and presentation attractive so that they may be rated as quality self- learning materials. If anything remains still obscure or difficult to follow, arrangements are there to come to terms with them through the counselling sessions regularly available at the network of study centres set up by the University. Needless to add, a great deal of these efforts is still experimental—in fact, pioneering in certain areas. Naturally, there is every possibility of some lapse or deficiency here and there. However, these do admit of rectification and further improvement in due course. On the whole, therefore, these study materials are expected to evoke wider appreciation, the more they receive serious attention of all concerned. Professor (Dr.) Surabhi Banerjee Vice-Chancellor
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M-2\D:\Netaji 05\Biology-1\BIO-TIT PM6.5 NETAJI SUBHAS OPEN UNIVERSITY Group – A (I) Quantitative Biology Unit 1 ? Basic Mathematics 1–16 Unit 2 ? Biostatistics : General Principles 17–33 Unit 3 ? Presentation of Data and Probability Distributions 34–60 Unit 4 ? Statistical Inference and Hypothesis Testing 61–83 Unit 5 ? Analysis of Variance 84–107 Unit 6 ? Correlation and Regression 108–135 Unit 7 ? Probability Theory 136–142 Appendix 143–144 PGZO - 6 Quantitative Biology & Biotechnology Immunology & Microbiology

M-2\D:\Netaji 05\Biology-1\ Unit 1 ? BASIC MATHEMATICS 1.1 Introduction In this unit, you will get a glimpse of the basic mathematical principles and techniques which find applications in the exploration, treatment, analysis and interpretation of scientific events and phenomena. During the earlier epochs of development of life sciences, scientific investigations were initially confined largely to qualitative pursuits such as nature study, search for new species, identifications, morphological distinctions, macro and micro anatomical structures, fundamental organ and tissue functions, and systematic classifications of living organisms. But since the early decades of the last century in particular, quantitative approaches to life science problems have been developing by leaps and bounds with commensurate increase in the application of mathematical techniques to the collection, presentation, analysis and interpretation of biological data. Samuel Brody's monumental book, Bioenergetics and Growth, eminently deserves mention as a landmark in this field in the early fifties of the twentieth century. Such mathematical applications largely consist of the use of biostatistics. Principles and practice of biostatistics have consequently evolved considerably and received a high impetus from the emergence of successive computer generations. Some basic mathematics, currently in practice in life sciences, will find very brief mention in the present unit. Structure 1.1 Introduction Objectives 1.2 Fractions, multiples and exponents 1.3 Logarithms 1.4 Arithmetic and geometric progressions 1.5 Equation for straight line 1.6 Equation for exponential curve 1.7 Summary 1.8 Terminal questions 1.9 Answers
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Objectives After reading this unit, you should be able to do the

following : ? recall some basic mathematical principles and methods which find use in biological sciences, ? define and use common fractions, multiples and exponents, ? recall the types, bases and uses of logarithms, ? understand the properties and uses of arithmetic, geometric and logarithmic series, ? learn the importance and working out of equations of linear relationships, ? know the nature and use of equations and graphs of exponential relations, and ? understand the importance of mathematics in biological work. 1.2 Fractions, multiples and exponents You may recall here some important points helpful in dealing with common fractions, multiples and powers or exponents. 1.2.1. Common fractions A common fraction is a ratio of two numbers; it is constituted by dividing a number called the numerator with another number called the denominator. Its basic properties are as follows. (i) If the same number (a) is either added to or subtracted from both the numerator (X) and the denominator (Y) of a fraction, the latter changes into a new fraction. Thus, X Y X a Y a X Y X a Y a ? ? ? ? ? ? ; . (ii) If both the numerator X and the denominator Y are multiplied (or divided) by the same number a, the fraction neither changes in value nor equals its product (or division result) with the constant number a. Thus, X Y Xa Ya a X Y ? ? . (iii) A fraction changes in value if both the numerator X and the denominator Y are either raised to a common power a or reduced to the same root a. Thus,

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X Y X Y X Y X Y X Y X Y

a a a a a a ? ? ? ; , . or 1 1 . (iv) A new fraction is formed by adding two fractions with the same denominator;

M-2\D:\Netaji 05\Biology-1\ for this, their numerators are added together to form a new numerator while the denominator is left to remain the same as before. Thus, X Y Z Y X Z Y ? ? ? . (v) Similarly, two fractions with a common denominator can be subtracted from one another by the subtraction of the numerator of one from that of the other, while retaining the original denominator. Thus, X Y Z Y X Z Y ? ? ? . (vi) If two common fractions with different denominators (Y and b) are to be added to or subtracted from one another, the lowest common multiple (LCM), say bY, of the two denominators is first computed and then used for working out the resulting sum or difference between the numerators. Thus. X Y a b bX aY bY X Y a b bX aY bY ? ? ? ? ? ? ; . (vii) To form a new fraction by multiplying two fractions, the product of the two numerators and that of the two denominators constitute respectively the numerator and the denominator of the new fraction. Thus, X Y a b Xa Yb ? ? . (viii) To divide a common fraction by another, the fraction to be divided is multiplied by the reciprocal of the divisor fraction. Thus, X Y a b X Y b a Xb Ya ? ? ? ? . 1.2.2. Multiples If a score Y is the multiple of another score X, it means that Y is obtained by multiplying a given value of X by a constant number a : Y = aX. Stated otherwise, Y is considered as a function of X and varies with the value of the latter. It follows that if there occurs a series of very high or very low Y scores, the scale of the latter may be moderated by downward or upward movement, expressing each Y score of the series as a function of the common denominator X having respectively a very high or a very low value. Such a change of scale of a series of too high or too low numbers makes it convenient to use the series for computation as well as comprehension. In this way, the series of numbers such as 34300, 35300, 36300, ...etc., may be scaled down to the series 3.43 × 10 4 , 3.53 × 10 4 , 3.63 × 10 4 , ...etc. You may recall that similar expressions of physical quantities in multiples of 10 are followed in the scales of standard international (SI) units. M-2\D:\Netaji 05\Biology-1\ 1.2.3. Powers or exponents An exponent or index (i) is any power to which a score X may be raised, while the score thus raised to a given power is called the base of the latter. For example, in case of 10 4 , the base is 10 while the power or exponent is 4. If the raising of a score X (base) to the power a (exponent) gives the product Y, the latter is a function of X and varies with the value of the base as also with the value of the exponent; the relation between Y and X may thus be expressed as : Y = X a . Irrespective of the values of X and Y, any Y score may be obtained by raising the base X to a specific power or exponent; the latter is not necessarily an integral number, and may even be a negative, fractional or decimal number. You may, therefore, realise that the Y scores can be expressed as exponential terms dependent on the exponent or power of the base X. By varying the exponent of an identical base X, a series of Y scores may be converted to a series of respective exponential terms. If the exponential terms have been derived using the same base, algebraic computations with them become far more convenient than with the Y scores themselves, particularly if the latter scores are too large or too small. Thus, if Y 1 , Y 2 and Y 3 scores are changed to exponential terms X a , X b and X c respectively, using the common base X, YY Y
X X X X a b c a b c 1 2 3 ? ? ? ? ( ) ; Y Y

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X X X YY Y X a c a c a b c 1 3 1 2 3 ? ? ? ? ? ? ? ? ? ( ) ( ) ; ; Y Y X X X a c a c 1 3 2 ? ? ?( ) ; Y Y X X X

a c a c 1 3 2 ? ? ?( ) 1.3
Logarithms Logarithm of any number Y is the exponent, power or index (i) of its exponential term using a base X which is generally chosen to be either 10 in case of common or Briggsian logarithms or the base e in case of natural or Naperian logarithms; e is an infinitely long number and approximates to 2.7183. The Naperian logarithm (log e or ln) of any number X is related to the logarithm (log) of that number to the base 10 in the following way : log e X or ln X = 2.303 logX. If any score Y has an exponential term X i and if X is made the base of a set of logarithms, then the logarithm of Y to the base X (log x Y) would be given by the index i of the corresponding exponential term of that Y score. Thus, logarithms to any chosen base — even other than 10 or e — can be worked out and used.

M-2\D:\Netaji 05\Biology-1\ Every logarithmic number is a decimal number with one part called the characteristic and another part called the mantissa, respectively preceding and following the decimal point. While the mantissa is always positive, the characteristic is either zero or an integral number which may be either positve or negative, the negativity being indicated by a superscript bar above the numerical value of the characteristic. 1.3.1. Using the common logarithm table The logarithmic table is used to find the log X of a given X score under consideration. Ignoring any decimal in the number X, the first 4 figures from the table, corresponding to the figures of the given number X, are noted as the mantissa, reading the figures of X starting from the extreme left column of the table and then moving to the right along the corresponding row. A decimal point is now placed on the left of the first figure of the noted mantissa. Next, the number of figures is counted on the left of the decimal point in the original X score. If that figure consists of a single digit, zero is entered to the left of the decimal point before the noted mantissa; but if two or more digits occur on the left of the decimal point in the score X, then the number, less by 1 than that number of digits in X, is entered on the left of the decimal preceding the mantissa; in case only a zero precedes the decimal in the original X score, then the number to be entered before the decimal of the mantissa should exceed by 1 the number of zeroes following the decimal in the X score, and should bear a superscript bar to indicate the negativity of the logarithmic number. Thus, log 111.4 = 2.0453; log 11.12 = 1.0461; log 2.429 = 0.1750; log 0.4952 = 1 – .3127; log 0.0731 = 2 – .5383. For any logarithmic number log i X, the original X score corresponding to the latter is called its antilogarithm. To find X from log i X, the antilogarithm table has to be used : X = antilog (log i X). For this, the number corresponding to the mantissa of the logarithmic number is noted from that table; then, considering the figure in the characteristic of the logarithmic number and following a procedure reverse to that used in recording the characteristic, a decimal point is inserted in the noted number corresponding to the mantissa. Thus, for the common logarithms to the base 10 (log 10 X) : antilog 0.6207 = 4.176; antilog 1.5935 = 39.22; antilog 1 – .6554 = 0.4523; antilog 2 – .5540 = 0.0358. 1.3.2. Logarithms in multiplication, division and power Logarithmic procedures for these computations are like those using exponents (see Section 1.2.3). Thus, using X and Y secores,

M-2\D:\Netaji 05\Biology-1\ XY = antilog (log

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X + log Y); X Y = antilog (log X – log Y); X Y = antilog (Y log X); Y X = antilog (X log Y); X Y = antilog log X Y ; Y X =

antilog logY X . Example 1.3.1.

Solve the following

using common logarithms : 7 251 2 752 4 351 16 35 4 043 3 2 ? ? ? ? ? ? ? ? . Solution : log (numerator) = log (7.251 3 ÷ 2.752) = 3 log 7.251 – log 2.752 = 3 × 0.8604 – 0.4396 = 2.1416. log (denominator) = log (4.351 × 16.35 ÷ 4.043 2) = log 4.351 + log 16.35 – 2 log 4.043 = 0.6386 + 1.2135 – 2 × 0.6067 = 0.6387. log [numerator denominator] = log (numerator) – log (denominator) = 2.1416 – 0.6387 = 1.5029. antilog 1.5029 = 31.84. 1.4 Arithmetic and geometric progressions Some important aspects of these two series or progressions are mentioned below. 1.4.1. Arithmetic progressions Each arithmetic progression (AP) consists of a sequential arrangement of real numbers in a series in which successive numbers differ from each other by an identical number. Whatever be the position in the series where a pair of such successive numbers is located, the difference between any two successive numbers would be the same and is called the constant difference (d). Any term in an AP, except the first one (X 1), is given by the sum of the immediately preceding term or number and the constant difference d of the series. Examples of such series are : 4, 9, 14, 19, 24,....; 15, 10, 5, 0, –5, –10,.....; 0.35, 0.76, 1.17, 1.58, 1.99, ...; the constant difference amounts to 5, –5 and 0.41 in them, respectively. The numbers in an AP series would be in an ascending order if d exceeds 0 and in a descending order when d is lower than 0.

M-2\D:\Netaji 05\Biology-1\ It follows that in an AP series, viz., X 1 , X 2 , X 3 , ...X n , where d is the common difference and k is a positive integer, the kth term (X k) is given by : X k = X 1 + (k–1)d. If an AP has

X 1 , X 2 and X 3 as three of its terms, then :

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X 2 – X 1 = X 3 – X 2 = d, or, 2X 2 = X 1 + X 3 , ? X 2 = X X 1 3 2 ? . Thus, X 2

is
the arithmetic mean (AM) between X 1 and X 3 .
If X 1 and X 2 are two real numbers, any number of arithmetic means can be inserted between them. Also, if X 1 , X 2 , X 3 , ...X k form an AP, a constant number (c) can be added to, subtracted from, multiplied with or used in dividing each term of that series, to form other AP sequences like :

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X c X c X c X c k 1 2 3 , , ,..... ; cX 1 , cX 2 , cX 3 , ...cX k ; X 1 + c, X 2 + c, X 3 + c, ... X k + c; X 1 – c, X 2 – c, X 3 – c, ...

X k – c.
If d is the common difference, and X 1 and X k are respectively the first and the kth terms of an AP, the sum of the first k number of terms is given by : ? ? ? ? ? ? ? ? ? (.....) ( ) ( ). [ ] X X X k X k d k X X k k 1 2 1 1 2 2 1 2 For scores of a variable like length and mass, that differ arithmetically from each other, scales are used with divisions arranged in arithmetic progressions. 1.4.2. Geometric progressions Each geometric progression (GP) consists of a sequential arrangement of non- zero real numbers in a series in which the successive numbers form a constant ratio. Except the first term or number of every series, each of the other terms is given by multiplying — not by adding — a constant non-zero real number with the preceding number of the series; this constant non-zero real number, with which to multiply each term to get the next term, is called the common ratio (r) of that series and is obtained by dividing the number of any term by that of the preceding term : r X X X X X X K K ? ? ? ? ? 2 1 3 2 1 ..... . Moreover, the arithemetic difference between two successive terms varies with the location of that pair in the series. Examples of GP are : 3, 9, 27, 81, ...; and 48, 12, 3, 3 4 3 16 , , ... In the first series, each term is obtained by multiplying the immediately preceding term by the common ratio 3, the ratio of each two successive terms amounts to 1 3 , and differences between the terms of the three successive pairs
M-2\D:\Netaji 05\Biology-1\ amount respectively to 6, 18 and 54; in the second series, each number is obtained by multiplying the immediately earlier one by the common ratio 1 4 , the ratio of successive terms amounts to 4, and differences between the terms of successive pairs amount respectively to –36, –9, ?2 1 4 and ? 9 16 . Where the common ratio (r) is positive and higher than 1, a GP of positive terms has its terms arranged in an ascending order (e.g., the first series quoted above) while a GP of negative terms has its terms in a descending order. On the contrary, a GP of positive terms has its terms decreasing progressively (e.g., the second series quoted above) while that of negative terms has its terms increasing progressively, if the common ratio is positive but lower than 1. If X 1 , X 2 , ...X k form a GP, other GP sequences are formed by treating each of those terms, as follows, with a constant
c.

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X c X c X c X c cX cX cX cX k k 1 2 3 1 2 3 , , ,..... ; , , ,..... ; c X c X c X c X c c c c

k X X X X K 1 2 3 1 2 3 , , ,..... ; , , , ... If X 1 , X 2 and X 3 are in geometric progression as three of its terms, X 2 is the geometric mean (GM) between X 1 and X 3 . If r is the common ratio, X 1 , X K–1 and X k are respectively the first, (k–1)th and kth terms of a GP, and k is a positive integer, then the kth term is given by : X X r k k ? ? 1 1 . Also, the sum of the first k number of terms of the GP is given by : ? X X X X r r k k 1 2 1 1 1 ? ? ? ? ? ? ..... . b g e j Differences between an AP and a GP would be clear to you on comparing the statements in Sections 1.4.1 and 1.4.2. It follows that the numbers forming the successive terms of a geometrical series follow a progressive exponential change. For example, the GP of 1, 2, 4, 8, 16, ... may be considered as 2 0 , 2 1 , 2 2 , 2 3 , 2 4 , ...; so, using any base for logarithms of the terms of a geometric series, the latter may be changed into a logarithmic series in the form of an arithemetic progression, such as 0, 1, 2, 3, 4, ... in case of the GP quoted above. For variables whose values differ exponentially, scales are used with their divisions arranged in the forms of geometric progressions.

M-2\D:\Netaji 05\Biology-1\ Example 1.4..1. (a) Work out the 6th term and the sum of the first six terms in each of the following series : (i) 4, 9, 14, ...; (ii) 3, 9, 27, ... (b) Change the following geometric series to a common logarithmic series : 10, 100, 1000, 10000, ... Solution : (a) X and 6 6 ? : (i) The series 4, 9, 14, ... constitutes an AP. d =

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X 2 –X 1 = 9 – 4 = 5. k = 6. X k = X 1 + (k – 1) d = 4 + (6 – 1) 5 = 29. ? ? ? ? ? ? ? ? ? ? ? ? (.....) ( ) ( )5 . [ ] [ ] X X X k X k d k 1 2 1 2 2 1 6 2 2 4 6 1 99 ; or, ? ? ? ? ? ? ? ? ? ? ( ) ( ) ( ) ..... . X X X k X X k k 1 2 1 2 6 2 4 29 99 (ii) The series 3, 9, 27, ... constitutes a GP. r X X k ? ? ? ? 2 1 9 3 3 6 . . X k = X 1 r k–1 = 3 × 3 6–1 = 729. ? ? ? ? ? ? . 1092 13 1 33 1 1 ..... 6 1 2 1 ? ? ? ? ? ? ? ? ? ? ? ? r rX X X X

k k (b) Change to log series : GP : 10, 100, 1000, 10000, ... Exponential form : 10 1 , 10 2 , 10 3 , 10 4 , ... Logarithmic series : 1, 2, 3, 4, ... 1.5 Equation for straight line Where two events (X and Y) bear such an association with one another that equal changes of one are accompanied by uniform changes of the other, the rate of changes of the latter does not vary with the changes of the former, and the plotted points of their scores in a number of cases lie distributed on or close to a straight line. Such an association is called linear, and either variable may be considered as a linear function of the other. Such linear relations can be graphically represented by plotting straight lines, using the following linear equation (Fig. 1.1) : Y = a + bX. In this

M-2\D:\Netaji 05\Biology-1\ equation, b is the slope of the line; it is the measure of the average rate of change of the variable or event Y scaled along the ordinate (y-axis) for unit changes of the event X scaled along the abscissa (x-axis). The higher the value of b, the steeper is the gradient of the line and the higher is the magnitude of the average rate of change of Y, associated with unit changes of X. The algebraic sign of b indicates the relation between the directions of changes of the two events; a positive b indicates the simultaneous rises (or falls) in the values of both events with a consequent ascending gradient of the line, while a negative b signifies the changes of the two events in opposite directions with a consequent descending gradient of the line. The term a gives the y-intercept of the line and indicates its general level. It may be defined as the expected value of Y when X amounts to zero, and depends on the slope b of the line — rise and fall in the value of b are associated respectively with the fall and rise in the y-intercept. Any point on the line is located by its perpendicular distances, called its co- ordinates, from the y and x axes. If the plotted points of Y versus X lie dispersed around the line, the latter is drawn using the method of least squares so as to keep the sum of squared vertical distances of the plotted points from the line at a minimum — in other words, the line drawn using the dispersed points would be the best-fitting straight line for those points. Examples of three such linear plots are given below. (i) Rate of diffusion (J) of a solute across a thin permeable membrane is a linear function of the change (?n) in its concentration across that membrane. Scaling J along the y-axis and ?n along the x-axis, and plotting J against the corresponding ?n values, we get a straight line which has a slope P equalling the permeability coefficient for the solute and its y-intercept at the origin of the y-scale, i.e., at zero values of both J and ?n (X = 0, Y = 0). This line is given by the following linear equation (Fig.1.2) : Fig 1.1. A straight line graph showing its slope (b) and its y-intercept (a). Y a b X

M-2\D:\Netaji 05\Biology-1\ J = P?n, or J = O + P?n, which conforms to the equation Y = a + bX, where a or the y-intercept is zero. (ii) Lineweaver-Burk double-reciprocal plot expresses the linear relation between the reciprocals of the initial velocity (V 0) of enzyme action and the substrate concentration [S]. Where V max is the maximum V 0 with saturating [S] concentration, and K m is the [S] for attaining 1 2 V max , the linear equation of 1 0 V versus 1 S is as follows : 1 1 1 V V K V S 0 max m max ? ? × [ ] . On scaling 1/V 0 and 1/[S] along respectively the y and x axes, and plotting 1/V 0 against the corresponding 1/[S] values, we get a straight line having K m /V max as its slope, 1/V max as its y-intercept and −1/K m as its x-intercept (Fig. 1.3). (iii) Eadie-Hofstee plot also expresses the linear relation between V 0 and [S] using a different linear equation. (Fig. 1.4). V V V S K m 0 0 ? ? max [ ] . On scaling V 0 and V 0 /[S] along the y-axis and the x-axis respectively, and plotting V 0 against V 0 /[S] values, a straight line may be drawn (Fig. 1.4). The slope of the straight line, drawn using the linear equation quoted above, is downward and amounts to −K m , the y-intercept is V max and the x-intercept is V max /K m . 0 ?n Slope = P J Fig 1.2. Linear plot of diffusion rate (J) against the transmembrane solute concentration gradient (?n). [From D. Das, Biophysics and Biophysical Chemistry, 6th ed., Academic Publishers, 2007.]
M-2\D:\Netaji 05\Biology-1\ 1.6 Equation for exponential curve Exponential relation between two variables or events (X and Y) can be expressed by an equation using the base (e) of natural logarithms and two constants (Z and k) : Fig 1.3. Double-reciprocal plot. [From D. Das, Biochemistry, 12th ed., Academic Publishers, 2005.] −K m 0 Fig 1.4. Eadie-Hofstee plot. [From D. Das, Biochemistry, 12th ed., Academic Publishers, 2005.] Y = Ze kX . This equation is the basis of exponential equations used for graphical representations of exponential distributions. It expresses the scores of an event Y as 0 1 V m K 1 ? ? ? S 1 max V 1 m max K V ? ? S V 0 max V 0 V Slope = K V m max 0
M-2\D:\Netaji 05\Biology-1\ the exponential function of the scores of another event X. Where the exponent X bears a positive algebraic sign, as in the equation Y = e X , the exponential curve obtained by plotting Y against the corresponding value of X is an ascending one with the gradient of its slope rising progressively with the rise of X, and with its y- intercept at the value 1 of the Y scale on the y-axis. This ascending exponential curve thus shows the Y scores increasing exponentially with the rise in values of the positive index X. Such ascending curves result from plotting Y against X where Y = e X , as in the case of systems obeying the law of continuous growth linking any event with the positive exponent of the base e of natural logarithm. Examples of such curves are those for rises in bacterial counts in bacterial cultures with time (Fig. 1.5). 80 60 40 20 0 1 2 3 4 X Y = e X Fig 1.5. Ascending exponential curve (Y=e X) of rise in bacterial count in bacterial culture. On the contrary, if the power or exponent bears a negative algebraic sign to change the equation into Y = Ze −kX , the exponential curve of Y against X is a descending one. Thus, for the equation Y = e −X , a descending exponential curve results with a downward slope, having a progressively decreasing gradient with the rises in X. This curve depicts an exponential decrease in Y scores with the rise in values of the negative exponent or power. Such descending curves result from plotting Y against X where Y = e −X , as in the case of systems obeying the law of continuous decay linking any event with the negative power of e. An example is the exponential decline in the relative activity (A/A 0) of a radioisotope with time, A 0 and A being respectively the original activity and the activity left after a time interval (Fig. 1.6). For the equation Y = e X , log Y to any base like e or 10 may be plotted against the exponent X to transform the exponential curve of Y against X into a straight line. Y
M-2\D:\Netaji 05\Biology-1\ The latter may be ascending or descending according to the positive or negative algebraic sign of the exponent and has its y-intercept at the origin of the Y scale (X = 0, Y = 0); the slope of the line equals 1 on using log e Y, but amounts to 1/constant on using log a Y to any base other than e (Fig. 1.7). Accordingly, the equation for the straight line would be ln Y = X in the former case, and log a Y=X/ constant in the latter case. For example, where N 0 and N are the numbers of radioisotopic nuclei respectively 1.0 0.5 0 Time (t) Half life A A 0 Fig 1.6. Descending exponential curve (Y=e −−X) of decline in relative activity (A/A 0) of a radioisotope with time (t). [From D. Das, Biophysics and Biophysical Chemistry, 6th ed., Academic Publishers, 2007.] ? 2.303 Time (t) log (N0/N) Fig 1.7. Linear relationship between log (N 0 /N) and time t. [From D. Das, Biophysics and Biophysical Chemistry, 6th ed., Academic Publishers, 2007.] at the initial stage and after a time interval t (i.e., t = X), and ? is their decaying constant (i.e., ? = k), the exponential expression Y = e kX may be transformed into a linear equation using N 0 /N as Y.

M-2\D:\Netaji 05\Biology-1\ Y = e kX , or N N e t 0 ? ? , or ln N N t 0 ? ? , or, 2.303 log N N t 0 ? ? , or log N N t 0 2 303 ? ? ? . On scaling log (N 0 /N) and t along the ordinate and abscissa scales respectively, a straight line is obtained with a slope of ?/2.303 (Fig. 1.7). 1.7 Summary Common fractions, multiples and exponents have been defined and their properties briefly recalled in this unit. Common and natural logarithms have been defined and basic uses of common logarithm have been described. Arithmetic, geometric and logarithmic progressions have been explained and their basic properties have been described with examples. Equations for straight lines and exponential curves have been described briefly. Transformation of an exponential curve into its linear form has been explained using an example. 1.8 Terminal questions 1. (a) Explain what you understand by exponents and multiples. (b) Describe the equations and properties of exponential curves. (c) Describe with an example how you would transform an exponential curve into a straight line. 2. (a) Give an account of arithmetic progressions and their basic properties. (b) Explain what you understand by common and Naperian logarithms. How do you use common logarithms for powers and multiplications. (c) Write briefly about ascending and descending exponential curves, citing examples. 3. (a) Define geometric progressions and discuss their basic properties. (b) Write briefly about the equation and graphic properties of a straight line. (c) Give three examples of linear graphical plots. 1.9 Answers 1. (a) See Sub-sections 1.2.2 and 1.2.3.
M-2\D:\Netaji 05\Biology-1\ (b) See Section 1.6. (c) See the last two paragraphs of Section 1.6. 2. (a) See Sub-section 1.4.1. (b) See Section 1.3 and Sub-section 1.3.2. (c) See the first two paragraphs of Section 1.6. 3. (a) See Sub-Section 1.4.2. (b) See the first three paragraphs of Section 1.5. (c) See paragraphs (i), (ii) and (iii) of Section 1.5.
M-2\D:\Netaji 05\Biology-1\ Unit 2 ? BIOSTATISTICS : GENERAL PRINCIPLES 2.1 Introduction You will learn in this unit what biostatistics is, and how it may be used in the study and investigation of life sciences such as zoology and allied disciplines. You will know about different types of variables such as events, properties, organisms, objects and characters, the variations and interrelations of which are explored and assayed in experiments, using statistics for interpretation of observations. You will get to understand the difference between a population and a sample, and why and how samples are drawn from a population for scientific investigations. You will be told about different types of statistics and how they are used as estimates of population parameters.

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Objectives After studying this unit, you will be able to do the

following : ? define biostatistics and summarize its basic applications, ? understand the natures of variables studied in biological experiments, ? define population and explain why samples drawn from the population are used in representing the latter in scientific studies, ? choose and use different methods of drawing representative samples from a population, and ? define parameters of population and understand different types of sample statistics used as estimates of the respective parameters. Structure 2.1 Introduction Objectives 2.2 Biostatistics and its aims 2.3 Variables 2.4 Population and samples 2.5 Sampling 2.6 Parameter and statistic 2.7 Summary 2.8 Terminal questions 2.9 Answers

M-2\D:\Netaji 05\Biology-1\ 2.2 Biostatistics and its aims You may be aware that statistics is the science which applies mathematical principles, models, laws and methods in the scientific designing of experiments and investigations, drawing of samples, conducting surveys and experiments, collecting data from samples, presenting the data in meaningful forms, and interpreting the experimental data to draw inferences from the investigation undertaken. Biostatistics is that discipline of statistics which applies mathematical laws, principles, models and methods for exploring, studying, describing, analyzing and interpreting events, natures, properties, changes and problems of living organisms. 2.2.1. Aims or basic applications of biostatistics Biostatistics is used for the following purposes : (a) planning an experimental design statistically, minimizing experimental errors, (b) determining the reliability of the proposed experimental test or method for measuring a specific variable consistently, (c) determining the validity of the proposed experimental test from its ability to measure a specific variable in exclusion of other similar variables, (d) working out the minimum size of a sample as is necessary in making dependable inferences from the experimental observations, (e) drawing an unbiased sample, representative of the chosen population, by random sampling methods depending on the laws of probability, (f) presenting the experimental data in properly arranged, grouped, tabulated, graphically plotted, diagrammatically displayed and easily decipherable forms for universal communication, common perception and subsequent systematic mathematical treatment, (g) working out mathematically whether the probability is too high or too low for the occurrence of the observed result due merely to random sampling by laws of probability, and inferring therefrom whether or not the result is significant and fit for generalization in the entire population, (h) finding the probability of error in inferring that the observed result is significant, (i) working out the degree and direction of association between two or more variables, and (j) predicting the probable score of a chosen variable in an individual from the observed score(s) of one or more associated variable(s) in the same individual.

M-2\D:\Netaji 05\Biology-1\ 2.3 Variables Anything that varies or undergoes changes is called a variable. The latter may be a property, an event, a character, or a phenomenon. A variable may undergo variations either qualitatively or in quantity. It may change from time to time in the same animal, organ, cell, organelle or site; such a change is called a temporal variation; for example, the change of body weight of an animal from day to day. A variable may also vary at the same instant from case to case, organ to organ, cell to cell or place to place; such a change is called a spatial variation; for exmple, the difference in body weights of two animals at the same moment. It is important to know about different classes of variables because the application of a statistical method or test depends on the specfic class of the variable. 2.3.1. Classification according to nature Variables may be distinguished into four main classes, viz., nominal, ordinal, continuous measurement and discontinuous measurement variables, according to their distinctive properties or characteristics. (a) Nominal or qualitative variables : Individuals or cases of a population differ qualitatively with respect to such a variable; but their differences in such a variable cannot be measured or expressed quantitatively in amounts, nor can the individuals of a sample or population be graded in ranks in ascending or descending order for the variable. You may, however, identify whether the individuals are identical or different from each other with respect to a nominal variable. Examples include sex, race, skin color, fur color, etc. (b) Ordinal or ranked variables : These variables also cannot be measured quantitatively in any individual; but the individuals can be distinguished as higher or lower than each other with respect to such a variable, and can consequently be ranked in ascending or descending orders. However, by how much two cases differ from each other, cannot be worked out. Examples include ferocity, attentiveness, alertness and docility. (c) Continuous measurement variables : These variables can occur and be measured quantitatively, not only in whole numbers of units, but even in infinitely small fractional units. Thus, there cannot exist any such gap in their scales where no score is located. Moreover, it can be measured quantitatively by how much the score of one case is higher or lower than that of another. Examples include length, mass, volume, temperature and time. (d) Discontinuous or discrete measurement variables : These variables can also be measured quantitatively; by how much one case is higher or lower than another, can also be worked out. But these variables exist and are measurable in only whole

M-2\D:\Netaji 05\Biology-1\ numbers of units; as they cannot occur in any individual in fractional units, there exist real gaps in their scales where no score can occur. Such variables include cell counts, litter sizes, finger digit numbers, respiratory rates, heart rates, etc. The two afore-mentioned measurement variables have one of two alternative scales. (i) Ratio scales of many variables such as length, mass and volume possess true zero points and a ratio of two scores of such variables can be worked out. (ii) Interval scales of some variables such as temperature have no true zero points — these scales start from arbitrary zero points and a ratio of two scores of such variables cannot be worked out. 2.3.2. Classification in experiments An experiment is the investigation into the anticipated changes of a single specific variable in a chosen sample or group of cases, on their exposure to one or more other variables. Variables involved in an experiment may be classified as follows, irrespective of their aforementioned classes. (a) Dependent variable : The single specific variable, whose anticipated changes are studied or measured in an experiment, is the dependent variable in that experiment. So, in an experiment to study changes in oxygen consumption in a sample of insects on exposure to a pesticide, oxygen consumption is the dependent variable. (b) Independent variable : The variable(s), one or more in number, which is/are applied to the cases of a sample to study the anticipated consequent change of the dependent variable, may be called the independent variable(s) for the relevent experiment. In the experiment cited in the preceding paragraph, the levels (viz., doses, amounts, volumes, intensities, magnitudes, etc.) of the pesticide constitute the lone independent variable. Independent variables in experiments may belong to two types. (i) Fixed experimental treatments are under the strict control of the investigator, are applied on the cases of the sample in well-planned manner and precise doses or amounts, and do not suffer from random fluctuations or random errors; e.g., doses of applied pesticide, amounts of injected hypoglycemic agent, etc. (ii) Classification or random variables are beyond the control of the investigator, may occur naturally in the surroundings, environment, naturally inherited genes, etc., and are free to suffer from random errors; e.g., sex, atmospheric O 2 tension, habitats or environmental temperature, to which the cases of a sample may get naturally exposed. (c) Relevant variables : These are some variables which occur in the experimental system, though not intended by the investigator to be applied on the subjects of the sample. They may occur in the subjects themselves (subject-relevant variables), or in the environment and experimental situation (situational-relevant variables), or in the sequence of exposure of the subjects to the levels of the independent variable (sequence-relevant variables), and may affect the dependent variable to vitiate the
M-2\D:\Netaji 05\Biology-1\ experimental result. Examples include age, sex and body weights of experimental animals (subject-relevant), pH, ionic strength and temperature of the medium or tissue preparation (situational-relevant), and the order of injection of different levels of a hypoglycemic agent (sequence-relevant). Relevant variables should be eliminated or kept constant as far as possible, to exclude their effects on the dependent variable. 2.4 Population and samples Experiments are done with samples drawn from a specific population. 2.4.1. Population For any experiment, the population consists of the entire aggregate of all such living organisms, inanimate objects, cases, events or phenomena as possess or exhibit at that time the specific dependent variable for the experiment or investigation. For example, for an experiment on the blood sugar of pancreatectomized rats, the population consists of all existing pancreatectomized rats on the earth; for working on O 2 consumption by a species of dragon flies, all insects of that species living anywhere at that time constitute the population. Populations belong to two broad classes. (i) Infinite populations are so immensely numerous and so widely dispersed that all the members cannot be reached or counted; e.g., the population of type I diabetic children or that of Jersey breed of cattle. (ii) Finite populations consist of such small limited number of cases located within a given narrow area that all the members of such a population may be reached and counted to get its precise size; e.g., the population of pollutant-affected patients of Bhopal gas disaster, or that of a rare species of salamanders occurring in the waterbodies of a small area of Darjeeling district. A population, whether finite or infinite, retains its identity with an identical size and unaltered properties, only so long as its members do not undergo any addition, deletion or any other change. 2.4.2. Samples Because of the vast resources and long durations required for covering the entire population intended to be investigated, and also because of chances of unmitigated errors owing to accidental omission of some of its members from the study, an entire population is seldom subjected to any experiment or investigation. Instead, a small group of a limited number of individuals or cases, called a sample, is so chosen at random from the population by laws of probability as to be representative of that population with respect to the variable under investigation; such a representative

M-2\D:\Netaji 05\Biology-1\ sample is then subjected to the intended investigation. The experimental observations of that investigation are then tested statistically to find their significance. If there is adequate probability of significance of the obtained result, the findings are sought to be generalized in the entire population. Following criteria have to be gratified in a sample, if it is to be used in an investigation or experiment as the representative of the relevant population. (i) Individuals or cases must be included in the sample by being chosen at random from the population depending on laws of probability, so as to ensure the conformity between the population and the sample regarding the proportions of different types of cases. (ii) Variations of scores in the sample should closely conform to the variations of such scores in the population. (iii) Values of any statistic (e.g., mean and variance) of scores of different samples from the population should be so closely distributed that their arithmetic average may be identical with the corresponding population value or parameter (e.g., population mean). (iv) Scores of the variable should be distributed in the sample in conformity with their distribution in the population. 2.5 Sampling Evidently, for generalizing the findings in a sample for the entire population, the sample should be representative of the latter. You have already learnt about the criteria to be fulfilled by a representative sample. These criteria depend largely on unbiased sampling. Some methods of sampling are briefly described below. 2.5.1. Judgement sampling In this method, the investigator depends upon his personal judgement in considering some cases with specific properties as representing the population with respect to the intended dependent variable, and chooses arbitrarily some of such cases for inclusion in the sample. Such conscious or unintended subjective preference for some individuals or cases of particular types confines the sampling to only specific types of individuals, excluding other types of them from the chance of getting chosen for the sample. Such judgement sampling has a high probability of not drawing a representative sample from the population and is suitable neither for making inferences about the latter, nor for working out sampling errors of statistics computed from sample data. 2.5.2. Probability sampling In this method, the choice of individuals from the population for inclusion in the sample is left entirely to mathematically devised methods of random sampling by laws of probability. No scope or role is left for the investigator or any other person to choose any case deliberately or arbitrarily; this minimizes the element of bias in M-2\D:\Netaji 05\Biology-1\ the sample. Instead, cases of different types have the probabilities of random choice commensurate with their respective frequencies or proportions in the population and independent of the choice of each other. Such probability sampling should yield samples, consisting of different types of cases in such relative proportions as in the population, being fairly representative of the population, and suffering from little or no bias. According to the population used and the intended purposes, probability sampling is designed in different ways. (a) Simple random sampling : If the sample has to be drawn from a small, finite and homogeneous population, not divided into distinct strata or sections, random sampling has to be done from the entire population taken together, choosing at random the requisite number of individuals for the sample successively out of all the individuals of the population. Thus, (i) each individual of the population enjoys an identical probability of choice at every step of sampling, (ii) each is chosen at random depending on the laws of probability, (iii) each gets chosen totally independent of the choice or omission of any other individual, (iv) no individual suffers from any subjective selection or rejection of any other individual, and (v) nor does any choice depend on any other quality or property. These should lead to the conformity between the proportions of different types of cases in the sample and those in the entire population. In the unsophisticated card drawal method of simple random sampling, the sample size to be required for the experiment is first worked out statistically; all individuals of the population are then given successive serial numbers which are entered individually on separate cards; all those cards are mixed up in a container, and the requisite number of cards are next successively picked up blindly from that container. Individuals whose cards are so drawn are included in the sample. Choices may be made for random sampling in two alternative ways. (i) In the with-replacement method, an individual once chosen is again included in the cases still left for subsequent choices and is, therefore, again considered for the subsequent choices. Thus, the probability of choice of each individual remains unaltered from choice to choice. However, it would create difficulty for the practical use of a sample if the same individual gets chosen more than once. (ii) In the without-replacement method, an individual once chosen is excluded from subsequent choices so that the probability of getting chosen rises progressively at successive choices; however, this rising difference in the probability of choice may be ignored as too small because of the much larger size of the population than the sample. In a more scientific random number method, after giving identity numbers serially to all members of the population, individuals are chosen in the same order as the successive numbers, arranged at random in any chosen part of a random number table.

M-2\D:\Netaji 05\Biology-1\ Simple random sample would not give a representative sample if a small sample is to be drawn from a stratified population with relatively low proportions of cases in one or more strata having different sizes and characters. (b) Stratified random sampling : If the population is large and heterogeneous, divided into distinct strata differing in properties and sizes, a proportional stratified random sampling is used in drawing a representative sample. This consists of the use of simple random sampling separately for each stratum. First, the required total size of the sample and the proportion of each stratum in the population are worked out. Next, simple random sampling is applied separately on each stratum to draw that number of individuals from it as corresponds to its proportional size in the population. All individuals of a stratum have an identical probability of getting chosen for the sample; but this probability varies from stratum to stratum according to their respective proportional sizes in the population. For example, to draw a sample of 150 cases from a population having three strata (A, B and C) with the respective proportional sizes of 0.50, 0.40 and 0.10, simple random sampling should be undertaken separately from each stratum to draw respectively 150 × 0.50 or 75, 150 × 0.40 or 60, and 150 × 0.10 or 15 cases from A, B and C to constitute the sample. (c) Multistage sampling : A vast population, dispersed over a wide area, may be sampled by this method. Preferably depending on some pre-existing stages, the vast population is arranged stepwise into a number of levels, leading ultimately to the level of individuals. Simple random sampling is then applied at each of these levels. For example, to draw a sample of Labeo rohita fishes from waterbodies of West Bengal, three districts are chosen at random at the first stage out of all the districts; at the second stage, three waterbodies are chosen at random from all the waterbodies of the three chosen districts; finally, at the third stage, forty fishes are sampled at random from each of the three chosen waterbodies to constitute a sample of 120 fishes. (d) Fixed interval sampling : Sometimes, individuals of a population may arrive, occur or get naturally arranged in a systematic sequence; e.g., netting of successive butterflies from the air by an insect collector, angling of successive fishes in the fishing line of an angler, or arrival of successive patients at the out-patients department of a hospital. Fixed interval sampling consists of simple random sampling of cases depending on such a sequence of their random occurrence, appearance or arrival. To start with, any particular individual or case is chosen at random as the first one from the sequence of cases. Simultaneously, an interval is chosen at random as the gap between subsequent successive cases for the purpose of choices. Each subsequent case is next chosen as
M-2\D:\Netaji 05\Biology-1\ it occurs in the given sequence after the preceding chosen one and separated from the latter by the chosen gap. For example, the fifth fish collected may be chosen as the first member of a sample and thereafter, every seventh fish is chosen maintaining a gap of six fishes between each pair of choices. This is continued until the requisite number of cases have been collected for the sample. However, this type of sampling may fail to yield an unbiased representative sample if the cases have been initially arranged in order of a characteristic related in any way to the variable to be investigated. (e) Purposive sampling : Random sampling is sometimes deliberately restricted to a particular section of the population so long as it is justifiable and logical to assume a truly representative nature of that section for the entire population, and the exclusion of its other sections is not anticipated to affect adversely the generalization of obtained results over the whole of the population. (f) Incidental sampling : In this method, random sampling is kept confined to a particular section or stratum of the population because of reasons like ready availability, easier manipulation and lower cost, instead of attempting to maintain or improve the representative nature of the sample. Such sampling should not be preferred for any investigation because it would seldom turn out a sample representative of the entire population. 2.6 Parameter and statistic You may easily realize that as such, the experimental data consisting of one or more sets of numerical values or scores can hardly communicate much of precise and meaningful information or contribute much in comparing, analyzing and interpreting the observations. For these, the individual scores have to be presented, on one hand, in classified, tabulated or graphical forms while, on the other hand, some summary values like the mean and the standard deviation have to be worked out from those scores for further analysis and interpretation. While the presentation of data will be described in the next unit, you will be introduced in this unit to the basics of such summary values, also known as numerical indices, and will also get an initial idea of their roles in biostatistics. 2.6.1. Parameter Parameters serve as measures of different characteristics of a variable in a population, and consist of numerous summary values like the mean and the variance, worked out from the scores of the entire population. Parameters of a population remain unchanged so long as the relevant population exists as such, but may differ from population to population. You are aware, however, that seldom do we work

M-2\D:\Netaji 05\Biology-1\ with an entire population (see Sub-section 2.4.2); our investigations are generally undertaken with samples drawn from the population we want to study. Whenever we work with a sample, the summary values of the scores obtained from the latter are used as estimates of the respective parameters of the corresponding population. Two types of such estimates of parameters may be worked out : (i) a point estimate is a single summary value (statistic) of the sample, directly accepted as an estimate of the population parameter; e.g., a sample mean is the point estimate of the corresponding parametric or population mean; (ii) an interval estimate or confidence interval consists of a range of scores around a summary value (statistic) of a sample, within which the population summary value (parameter) has a given probability of occurrence; e.g., a 95% confidence interval has a probability of 0.95 for inclusion of the parametric mean. 2.6.2. Statistic Statistics (singular : statistic) serve as measures of different characteristics of a variable in a sample, and consist of numerous summary values like the mean and the variance, worked out from the scores of that particular sample. As the individual scores vary from time to time in the same sample and also at the same instant from sample to sample drawn from the same population, any statistic varies temporally in the same sample and also spatially between samples of the same population. Consequently, a particular type of statistics (e.g., the sample mean X –) of different samples differ from the parameter (e.g., the population mean ?) by different amounts called the sampling errors (s e) : s e = X – – ?. Because of their different sampling errors, the statistics (e.g., X –) of samples lie dispersed around the parameter (e.g., ?) of that population in the form of a sampling distribution with the parameter as its mean; e.g., a sampling distribution of sample means (X –) around the population mean (?). It also follows that the statistics of different samples from a population serve as different point estimates of the same population parameter. Statistics belong to different classes according to their purposes. (a) Descriptive statistics : These statistics of a variable measure and describe three different characteristics of a sample in respect of that variable. (i) Statistics of location such as mean, median and mode describe the location of a specific point — particularly a central one — of the distribution of the scores of a variable on the scale of the latter. (ii) Statistics of dispersion such as variance and standard deviation are the measures of scatter of the scores of a variable around a central point like the mean of the sample. (iii) Statistics of correlation or correlation coefficients measure the degree and direction of the association between two or more variables in the sample. Descriptive statistics belong to a particular sample and do not go beyond the limits of the latter.
M-2\D:\Netaji 05\Biology-1\ (b) Sampling or inferential statistics : These statistics are not confined to the limits of a single sample and participate in comparing two or more samples. The best example of sampling statistics is a standard error of any other statistic like the mean and the correlation coefficient. Sampling statistics are used in measuring sampling errors and variabilities of other statistics, in computing confidence intervals of parameters, and in testing the significance of experimental findings. (c) Prediction statistics : Such statistics as regression coefficients are used in predicting the likely value of a variable on the basis of the known value(s) of one or more other variables correlated with the predicted one. 2.6.3. Mean, standard deviation and standard error (a) Mean and its properties : Mean (X) is the arithmetic average of all the scores of a sample and serves as an estimate of the parametric mean (?) of the corresponding population. For ungrouped data not divided into class intervals, mean is computed by dividing the sum of all scores (?X) by the sample size (n) : X = ?X/n. For a sample with its scores grouped into class intervals (see Sub-section 3.2.2), mean is worked out using the sample size and the sum of products of observed frequencies (f) and midpoints (X C) of all the intervals : X = ?fX C /n. Some properties of the mean are as follows : (i) The mean is expressed in the same unit as the scores from which it is computed. (ii) The sum of differences of individual scores from the mean amounts to zero : ? (X–X –) = 0. (iii) Mean, median and mode of a sample are identical if its scores are distributed symmetrically in the two halves of their frequency distribution (Section 3.2). (iv) Presence of more extreme scores in one tail of the distribution than those in the other, extends that tail more than the other and displaces the mean more towards the extended tail, making the mean higher than the median and the mode if the right tail is extended (skewed) but making the mean lower than the median and the mode if the left tail is skewed (Sub- section 3.6.1). (v) If each score of the sample is added, subtracted, multiplied or divided by a constant number, the mean also gets identically treated by that number. (b) Variance and standard deviation : These are two statistics of dispersion. Both are absolute measures of dispersion, computed directly from the scores of the variable and expressed in the same units as those scores. Variance, however, is in squared units and worked out as the arithmetic average of the squared differences, i.e., ?(X–X –) 2 , between the scores and their mean. For ungrouped scores of a large sample, variance (s 2) is computed as follows :
M-2\D:\Netaji 05\Biology-1\ s

X X n 2 2 ? ? ?( ) , or s X n X n 2 2 2 2 ? ? ? ?( ) , or s n X X n 2 2 2 2 ? ? ? ?( ) , where (X – X –)

is the error term of each score, ?(X – X –) 2 is called the sum of squares, (?X) 2 is the squared sum of all the scores, and ?X 2 is the sum of all squared scores. For ungrouped scores of a small sample (n > 30), the degree of freedom (n – 1) is used as the denominator instead of n in order to remedy the downward bias in s 2 due to omission of many of the extreme scores in the small sample during sampling. (See Sub-section 2.6.4 for degrees of freedom). For such unbiased variance of a small sample, s X X n s n X X n n 2 2 2 2 2 1 1 ? ? ? ? ? ? ? ? ? ( ) , ( ) ( ) or . The unbiased variance of a sample with class intervals is computed using the frequencies (f) and the midpoints (X C) of the intervals. (See Sub-section 5.2.1.) s f X X n C 2 2 1 ? ? ? ?[( )] , Standard deviation (SD or s) is the positive square root of variance and is more popular for everyday work. For ungrouped data of a large sample (n ? 30), s X X n s X n X n or n X X n ? ? ? ? ? ? ? ? ? ? ? ( ) , ( ) , ( ) . 2 2 2 2 2 2 2 or s Like variance, the SD of ungrouped data of a small sample (n > 30) is also computed as the unbiased SD using the degrees of freedom (df = n – 1) to reduce the downward bias as mentioned in case of variance. Unbiased SD and unbiased variance may also be computed and used for large samples. Thus, for unbiased SD (see Example 2.6.1), s X X n s n X X n n ? ? ? ? ? ? ? ? ? ( ) , ( ) ( ) 2 2 2 1 1 or . The unbiased SD of a sample with class intervals is worked out using the frequencies (f) and midpoints (X C) of the intervals. (See Example 3.2.1.) s f X X n C ? ? ? ? [( )] 2 1 . The higher the variance and SD, the greater is the variability of scores in the sample. The respective parameters are expressed by the symbols ? 2 and ?. Both SD

M-2\D:\Netaji 05\Biology-1\ and variance are affected if even a single score is changed in the sample. Multiplication or division of each score of a sample by a constant number results in an identical treatment of the SD. (c) Sampling errors and standard errors : You have already learnt in Sub-section 2.6.2 that due to variations of scores from sample to sample drawn from the same population, any statistic varies from sample to sample and every sample statistic may differ in turn from the population parameter by an amount called the sampling error (s e) of that statistic; thus, for sample means, s e = X – – ?. Difference in sampling errors between the statistics of different samples from the same population results in the dispersion of the sample statistics around the corresponding parameter in the form of a sampling distribution of such statistics. Standard error (SE) of a statistic measures the dispersion of that type of statistic of different samples around the corresponding population parameter; SE thus measures the spread of the sampling distribution of a statistic by estimating the sampling error of the latter. The SE of means ( )s X is thus a measure of the average sampling error and the spread of sampling distribution of the sample means around the parametric mean of the corresponding population. Similarly, the SE of the difference between sample means ( ) s X X 1 2 ? is a measure of the average sampling error and the spread of the sampling distribution of differences ( ) X X 1 2 ? of sample means about the difference (? 1 – ? 2) between the corresponding population means. Standard errors bear the same unit as that of the relevant scores. By estimating the sampling errors of statistics and of their differences, standard errors play important roles in finding (i) the dependability and significance of a computed statistic, (ii) the variability of errors in using the statistics of different samples for estimating the population parameter, (iii) the probability of occurrence of the observed results by mere chances of laws of probability in random sampling, and (iv) the confidence interval for the population parameter (see Sub-section 2.6.1). The SE of mean is worked out as follows for a sample drawn from an infinite population by random sampling with or without replacement, or from a finite population with replacement. s s n s X X n X ? ? ? ? , ( ) , if and ? 2 1 s s n s X X n X ? ? ? ? 1 2 , ( ) if . ? But for a sample drawn from a finite population without replacement and with N as the population size,

M-2\D:\Netaji 05\Biology-1\ s s n N n N s X X n X ? ? ? ? ? ? ? 1 1 2 , ( ) if , and ? s s n N n N s X X n X ? ? ? ? ? ? ? 1 1 2 , ( ) if . ? The SE of difference ( ) s X X 1 2 ? between two means is worked out from the SE values ( ) s s X X 1 2 and of those means. s s s X X X X 1 2 1 2 2 2 ? ? ? . Example 2.6.1. Work out the mean, unbiased SD and SE of the mean for the following trunk length scores (X cm) of a sample of Tilapia tilapia fishes : 9.0, 8.4, 6.8, 6.4, 7.8, 8.6, 6.8, 7.8, 8.4, 8.0. Solution : Table 2.1. Table for computing mean

and SD.

42% **MATCHING BLOCK 15/108** **SA** MSPS 13 L part 1.pdf (D110610736)

X X – X – (X – X –) 2 9.0 + 1.2 1.44 8.4 + 0.6 0.36 6.8 – 1.0 1.00 6.4 – 1.4 1.96 7.8 0 0 8.6 + 0.8 0.64 6.8 – 1.0 1.00 7.8 0 0 8.4 + 0.6 0.36 8.0 + 0.2 0.04 ? 78.0 — 6.80 n X X n ? ? ? ? 10 78 0 10 7 8 . . . ? cm. s X X n 2 2 2 1 6 80 10 1 0 756 ? ? ? ? ? ? ?( ) . . .cm M-2\D:\Netaji 05\Biology-1\ s X X n ? ? ? ? ? ? ?( ) . . . 2 1 6 80 10 1 0 869cm s s n X ? ? ? 0 869 10 0 275 . .

cm. 2.6.4. Degrees of freedom During the computation of some statistics such as SD, variance, correlation coefficient and Student's t, one or more statistics computed earlier need to be used as the estimates of the corresponding parameters, and have, therefore, to be left unchanged like the latter during use in the present computation. For example, in working out the unbiased SD from ungrouped data, the sample mean (X –) is used as the estimate of population mean (?). To keep each such precomputed statistic unchanged during the computation of the next statistic, any one of the sample scores loses its freedom for change; while any change of the remaining scores is mathematically permissible, that one score still left must change only in a fixed amount and a fixed direction to counter the effect of changes of all other scores so as to keep the precomputed statistic constant. The number of remaining scores or cases, still retaining the freedom for change, constitutes the degrees of freedom (df) for the statistic being computed. The df would usually amount to the sample size (n) less the number (m) of precomputed statistics being used as estimates of parameters : df = n – m. For example, the unbiased SD as computed in Example 2.6.1 has tbe df of (n – 1), one score having lost its freedom for change to keep the X – constant. On the contrary, when the correlation coefficient (r) is computed between two variables (X and Y) using ungrouped data (Sub-section 6.3.3.), the computed r has the df of (n – 2), two cases having lost their freedom for change to keep respectively X – and Y – constant. 2.7 Summary You have learnt in this unit about the basic applications of biostatistics. Variables have been defined and classified according to their natures and their roles in experiments.You have read about populations and have become aware of why samples are used instead of entire populations in scientific investigations. The criteria for a representative sample have also been mentioned. Methods for sampling have been described. The importance of probability sampling has been emphasized. You have read about the applications of simple
M-2\D:\Netaji 05\Biology-1\ random sampling, stratified random sampling, multistage sampling, fixed interval sampling, purposive sampling and incidental sampling. Parameters and statistics have been defined. Statistics have been classified into descriptive, sampling and prediction statistics. Descriptive statistics have been further classified into statistics of location, of dispersion and of correlation. Properties and computations of mean, variance, standard deviation and degrees of freedom have been briefly described. Sampling errors, sampling distributions and standard errors of statistics have been explained. 2.8 Terminal questions 1. (a) Classify variables according to their natures, giving examples and characteristics of each class. (b) State briefly the criteria for a representative sample. (c) Describe independent variables and their different types in an experiment. 2. (a) Write briefly about parameters and statistics. (b) Give a brief classification of statistics. (c) Explain what you understand by sampling errors and sampling distributions. 3. (a) Classify variables according to their roles in an experiment. (b) Give an account of the method of simple random sampling. (c) Define variance and standard deviation, and describe their computations from ungrouped scores of a sample. 4. (a) Explain when and how stratified random and multistage samplings are undertaken. (b) Write briefly about the standard error of mean and how it is computed, stating its different formulae. (c) Explain briefly what you understand by the sampling distribution of means. 5. (a) Write briefly about populations and parameters. (b) Define and classify relevant variables in experiments, with examples. (c) Explain the with-replacement and without-replacement methods of simple random sampling. 6. (a) Work out the unbiased standard deviation and

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the standard error of the mean of the following sample of blood sugar (mg/

dl) scores : 170, 165,

M-2\D:\Netaji 05\Biology-1\ 123, 147, 110, 88, 97, 132, 143, 155. (b) Classify sample statistics, giving examples. (c) Write briefly about fixed interval sampling, confidence interval, and properties of mean. (d) Explain degrees of freedom with examples. 2.9 Answers 1. (a) See Sub-section 2.3.1. (b) See 2nd paragraph of Sub-section 2.4.2. (c) See paragraph (b) of Sub-section 2.3.2. 2. (a) See Sub-sections 2.6.1 and 2.6.2. (b) See paragraphs (a), (b) and (c) of Sub-section 2.6.2. (c) See paragraph (c) of Sub-section 2.6.3. 3. (a) See Sub-section 2.3.2. (b) See paragraph (a) of Sub-section 2.5.2. (c) See paragraph (b) of Sub-section 2.6.3. 4. (a) See paragraphs (b) and (c) of Sub-section 2.5.2. (b) See paragraph (c) of Sub-section 2.6.3. (c) See paragraph (c) of Sub-section 2.6.3. 5. (a) See Sub-sections 2.4.1 and 2.6.1. (b) See paragraph (c) of Sub-section 2.3.2. (c) See paragraph (a) of Sub-section 2.5.2. 6. (a) See Example 2.6.1. (b) See Sub-section 2.6.2. (c) See paragraph (d) of Sub-section 2.5.2, Sub-section 2.6.1, and paragraph (a) of Sub-section 2.6.3. (b) See Sub-section 2.6.4.
M-2\D:\Netaji 05\Biology-1\ Unit 3 ? PRESENTATION OF DATA AND PROBABILITY DISTRIBUTIONS 3.1 Introduction You will learn in this unit about the presentation of experimental data in tabulated, grouped and graphically displayed forms for universal communication, easy perception, straightway comparison and systematic mathematical treatment. You will get introduced to the idea of probability distributions. You will also learn about the properties and applications of normal, binomial, Poisson and Student's t distributions. Objectives After studying this unit, you should be able to do the following : ? understand the use of frequency distributions in presenting the data, and work out appropriate frequency distributions of scores of different types of variables, ? draw graphical displays of frequency distributions in the forms of bar diagrams, histograms and frequency polygons, Structure 3.1 Introduction Objectives 3.2 Frequency distributions 3.3 Graphic presentations 3.4 Probability and probability distributions 3.5 Normal distributions 3.6 Skewness and kurtosis 3.7 Student's t distributions 3.8 Binomial distributions 3.9 Poisson distributions 3.10 Summary 3.11 Terminal questions 3.12 Answers
M-2\D:\Netaji 05\Biology-1\ ? define probability and explain different types of probability distributions, ? define and describe the properties of normal and Student's t distributions for probabilities of occurrence of scores of continuous measurement variables, ? understand and describe the unit normal curve, ? describe the properties of binomial probability distributions of dichotomized variables, ? discuss the properties of Poisson probability distributions of events of the rare class of some dichotomous variables, and ? understand and estimate skewness and kurtosis of distributions. 3.2 Frequency distributions Frequency is the number of occurrences of any individual, animal, event, score or phenomenon among the total number of such cases or in a sample. A frequency distribution consists of an arrangement of all the scores or cases of a relatively large sample, tabulated in different respective classes of the relevant variable. Observations of a sample, so distributed in a frequency distribution, constitute the grouped data. In contrast, individual scores or cases of a sample, not arranged or classified into a frequency distribution, form ungrouped data. Frequency distributions are broadly divided into two types, qualitative and quantitative frequency distributions, according to the nature of the variable whose data form the distribution. 3.2.1. Qualitative frequency distribution Qualitative or nominal variables such as sex, phenotypes, races and blood groups cannot be measured and expressed in the form of scores; nor can the cases of a sample be arranged in ranks according to their higher or lower positions with respect to such variables. Nevertheless, such variables can be divided qualitatively into two or more distinctive classes and the frequencies of cases of a sample in the respective classes can be arranged in a table. Such a tabulated arrangement of frequencies of cases or individuals of a sample in different classes of a nominal variable is called a qualitative frequency distribution (Table 3.1). The cases of each class can be distinguished from those of other classes with regard to the variable, and there is no continuity between the classes. However, the proportion (p) of the sample size in each class can be worked out by dividing the frequency (f) of cases in each class by the sample size (n) : p = f / n ; the computed p may be called the relative frequency of the relevant class. ?? ? 18 20 2
M-2\D:\Netaji 05\Biology-1\ Table 3.1. A qualitative frequency distribution of phenotypes in a sample of Drosophila flies. Phenotypes Frequencies (f) f /n

100% **MATCHING BLOCK 13/108** **W**

Grey-body red-eye 108 0.551 Black-body red-eye 40 0.204 Grey-body scarlet-eye 36 0.184 Black-body scarlet-eye 12 0.061

Total 196 (n) 1.000 3.2.2. Quantitative frequency distribution Both continuous and discontinuous measurement variables can be quantitatively measured and expressed in the form of numerical scores. The scores of such variables in a sample form a range which can be divided into groups called class intervals. Frequencies of scores belonging to different class intervals are then tabulated in the respective intervals to form a quantitative frequency distribution. The latter not only reveals characteristics of the sample in respect of the variable, but also contributes to the subsequent treatment and interpretation of the data. According to the nature of the variable concerned, quantitative frequency distributions may be either continuous or discontinuous. 1. Continuous frequency distributions : Continuous measurement variables have such distributions. Here, the successive class intervals are continuous with each other, without any gap between the contiguous classes. The steps in forming such a frequency distribution are summarized below. (a) The total range from the lowest to the highest score of the sample is divided into a suitable number of class intervals of identical lengths (i) covering 3, 5, 7, 10 or 20 scores, separating the successive intervals by their score limits, and entered in column 1 of the frequency table (Table 3.2). The interval length (i) may be obtained as the difference between either the lower or the upper limits of the consecutive classes. (b) To avoid gaps between class intervals, true limits or class boundaries are worked out, each as the mean of two contiguous score limits of successive intervals, and entered in column 2 of the table. (See Example 3.2.1.) (c) The midpoint (X C) of each interval is worked out as follows and entered in column 3 of the table. In a frequency distribution with class intervals, all the cases in an interval lose their individual identities and are deemed to possess the score of its midpoint (X C). For any interval,
M-2\D:\Netaji 05\Biology-1\ X C = lower score limit + 1 2 [(higher score limit) – (lower score limit)]. (d) Each score of the sample is entered as a tally in column 4 of the table against its class interval. (e) The total number of tallies of each interval is entered in column 5 as the frequency (f) of that interval. (f) Frequencies of all the intervals are finally totalled to give the sample size (n) : ? f = n. 2. Discontinuous frequency distributions : A discontinuous or discrete measurement variable has such a distribution. Here, the class intervals are separated from each other by gaps because the scores of the variable can be in whole numbers only and not in fractional numbers. A discontinuous frequency distribution is worked out in the same way as a continuous one except that the step (b) for computing the true class limits and the column 2 for entering the latter are omitted to retain the intervening gaps between consecutive class intervals. (See Example 3.2.2.) Example 3.2.1. (a) Work out a frequency distribution of the following wing length scores (mm) of a sample of insects : 13, 19, 20, 25, 22, 23, 22, 21, 25, 12, 20, 17, 15, 19, 18, 20, 20, 19, 17, 21. (b) Compute the mean, SD and SE of the mean of the data. Solution : (a) Continuous frequency distribution : Highest score = 25. Lowest score = 12. n = 20. i = 3. Range = (highest score – lowest score) + 1 = (25 – 12) + 1 = 14 scores. Number of class intervals ? ? ? range i 14 3 4 7. ~ – 5. True limits are computed as averages of contiguous score limits of successive class intervals and entered in Table 3.2. For example, the true upper limit of the interval 15–17 as well as the true lower limit of the next interval 18–20 is computed as : 17 18 2 175 ? ? . . Midpoint (X C) of each interval is computed using the higher and lower score limits of that interval, and entered in Table 3.2. For example, for the interval 18–20, X C ? ? ? ? 18 20 18 2 19 .
M-2\D:\Netaji 05\Biology-1\ Each score of the data is entered as a tally against its class interval and the total number of tallies in the latter is entered as its frequency (f). The sum of frequencies of all the intervals gives the sample size (n). Table 3.2. Continuous frequency distribution of wing length scores. Class intervals X C Tallies f Score limits True limits 12 – 14 11.5 – 14.5 13 ?? 2 15 – 17 14.5 – 17.5 16 ??? 3 18 – 20 17.5 – 20.5 19 ???? ??? 8 21 – 23 20.5 – 23.5 22 ???? 5 24 – 26 23.5 – 26.5 25 ?? 2 Total 20 (n) (b) Computation of mean, SD and SE of mean : Score limits, midpoints and frequencies of the class intervals are entered in the first three columns of Table 3.3. Table 3.3. Table for computing mean and SD. Class intervals X C f fX C X C – – X (X C – – X) 2 f(X C – – X) 2 (score limits) 12 – 14 13 2 26 – 6.3 39.69 79.38 15 – 17 16 3 48 – 3.3 10.89 32.67 18 – 20 19 8 152 – 0.3 0.09 0.72 21 – 23 22 5 110 + 2.7 7.29 36.45 24 – 26 25 2 50 + 5.7 32.49 64.98 ? – 20 (n) 386 — — 214.20 n = 20. X fX n C ? ? ? ? 386 20 193. .mm s f X X n C ? ? ? ? ? ? ?[( )] . . 2 1 21420 20 1 336 mm. s s n X ? ? ? 336 20 0751 . . .mm

M-2\D:\Netaji 05\Biology-1\ Example 3.2.2. Work out a frequency distribution of the following respiratory rates (per minute) of a sample of apes : 17, 9, 21, 14, 13, 18, 22, 16, 13, 17, 16, 15, 17, 15, 17, 19, 16, 10, 9, 11, 18, 20, 16, 16, 15, 18, 15, 12, 14, 15. Solution : A discontinuous frequency distribution is worked out for respiratory rates, using Table 3.4. Highest score = 22. Lowest score = 9. n = 30. i = 3. Range = (highest score – lowest score) + 1 = (22 – 9) + 1 = 14 scores. Number of class intervals ? ? ? range i 14 3 4 7. ~ – 5. Midpoint (X C) of each class interval is worked out, using the higher and lower score limits of the latter, and entered in Table 3.4. For example, for the interval 15–17, X C ? ? ? ? 15 17 15 2 16 ( ) . Each score is entered as a tally against its class interval and the total number of tallies of each interval is entered as the frequency (f) of that interval in the table. Sample size (n) is the sum of frequencies of all the intervals. Note that true limits are not worked out for the intervals because there should be gaps between the successive class intervals of a discontinuous variable. Table 3.4. Discontinuous frequency distribution of respiratory rates. Class intervals X C Tallies f (score limits) 9 – 11 10 ???? 4 12 – 14 13 ???? 5 15 – 17 16 ???????????????? 14 18 – 20 19 ???? 5 21 – 23 22 ?? 2 Total — — 30 (n)

M-2\D:\Netaji 05\Biology-1\ 3.3 Graphic presentations Frequency distributions are frequently presented in graphical forms such as frequency polygons, histograms and bar diagrams for more easily comprehensible visual displays, clearer perception of features of a sample with respect to a particular variable, and comparative study of more than one sample. 3.3.1. Frequency polygon Frequency distribution of a continuous measurement variable, grouped into class intervals, is often presented as a frequency polygon. The latter is an area diagram — the total area enclosed by the arms of the polygon represents the sample size n. Before drawing the polygon, two additional class intervals — each with zero frequency and the two respectively lower and higher than the original lowest and highest intervals of the distribution — are included with their midpoints in the frequency table (Table Fig 3.1. Original and smoothed frequency polygons of the data of Table 3.5. 9 8 7 6 5 4 3 2 1 0 10 13 16 19 22 25 28 X f Original Smoothed

M-2\D:\Netaji 05\Biology-1\ 3.5) so as to make the arms of the intended polygon reach the zero baseline of the frequency scale, on the x–axis. The scores of the relevant variable (X) are scaled on a graph paper along the latter axis or abscissa, with the midpoint (X C) of each class interval marked on that axis (Fig. 3.1). Frequencies (f) of scores in class intervals are scaled along the ordinate or y-axis. Because all the cases in a class interval are deemed to possess the score of its midpoint (X C), the frequency of each interval is plotted against its midpoint. The plotted points are joined by straight lines to draw the polygon. To compare frequency distributions of more than one sample, their respective polygons may be drawn superimposed on each other on the same x-axis. The outline of a frequency polygon is, however, jagged — the lower the sample size and the longer the class intervals, the higher is the jaggedness of the polygon. This jeopardises the suitability of the latter in visualizing the proportionate score frequencies in different intervals. To decrease such jagged appearance of the polygon, smoothed frequencies (f s) may be worked out for each interval as the mean of the original recorded frequencies (f) of that interval and of the intervals immediately preceding and following the latter (Table 3.5). These f s values are than plotted against the respective midpoints (X C) to get a smoothed frequency polygon (Fig. 3.1). For example, for the class interval 18 – 20 of Table 3.5, f s ? ? ? ? 8 3 5 3 53. . Table 3.5. Continuous distribution of Table 3.2, modified for original and smoothed polygons of Fig. 3.1. Class intervals X C f f s Score limits True limits 9 – 11 8.5 – 11.5 10 0 0.7 12 – 14 11.5 – 14.5 13 2 1.7 15 – 17 14.5 – 17.5 16 3 4.3 18 – 20 17.5 – 20.5 19 8 5.3 21 – 23 20.5 – 23.5 22 5 5.0 24 – 26 23.5 – 26.5 25 2 2.3 27 – 29 26.5 – 29.5 28 0 0.7 Total — 20 (n) 20.0

M-2\D:\Netaji 05\Biology-1\ 3.3.2. Histogram or column diagram This is an area diagram consisting of a number of contiguous rectangular bars or columns for presenting graphically the distribution of frequencies of a continuous measurement variable among its class intervals in a sample. To draw the histogram of a distribution with intervals of identical length (i), scores (X) of the variable are scaled along the abscissa or x-axis on a graph paper, marking the true limits between successive class intervals while frequencies (f) are scaled along the ordinate or y-axis. At each true limit of every class interval, a perpendicular line is raised till it reaches the level of f of that interval; the column thus being formed with its base along the x-axis between the true limits of the interval, is closed at its top by a horizontal line at the level of the f of that interval. In this way, frequencies of all the intervals are depicted as successive columns with no gap between each other and with bases ranging between their respective true limits along the x-axis. As all the intervals are of identical length (i), the bases of all the columns are equal in length; consequently, the areas of the columns are directly proportional to their heights which in turn correspond to the frequencies of the respective intervals (Table 3.2 and Fig. 3.2). The sample size (n) is represented by the total area covered by all the columns. However, unlike frequency polygons, histograms cannot be superimposed on each other and need to be drawn separately side by side to compare the distributions of more than one sample. Fig 3.2. Histogram of the frequency distribution of Table 3.2. 10 8 6 4 2 0 11.514.517.520.523.526.529.5 f X

M-2\D:\Netaji 05\Biology-1\ 3.3.3. Bar diagram A bar diagram consists of one or more sets of parallel, rectangular columns or bars, used for graphical representation of frequencies of cases in different classes of a discontinuous variable, particularly a qualitative one. Each set of bars is meant for a particular sample, and the area of every bar or column of the set represents the frequency of cases in a particular class of the relevant variable. A simple bar diagram for a single sample consists of one set of bars drawn either vertically or horizontally from respectively a horizontal or vertical baseline, with the bases of the bars being identical in length. The bars are separated by small gaps to indicate that the variable Fig 3.3. A simple bar diagram of the frequency distribution of Drosophila phenotypes of Table 3.1. 120 – 100 – 80 – 60 – 40 – 20 – 0 Grey-RedBlack-RedGrey- ScarletBlack- Scarlet f

M-2\D:\Netaji 05\Biology-1\ is not a continuous one, nor do its classes have any continuity with each other. Frequencies of cases are scaled along a line parallel to the bars — each bar extends from the baseline to that length beside the frequency scale which corresponds to the frequency of the corresponding class. Because of an identical width of all the bars, the area of each bar is directly proportional only to its length which in turn corresponds to the frequency of that class. Thus, the areas of successive bars of the set convey a comparative picture of the distribution of frequencies in the respective classes (Fig. 3.3). A multiple bar diagram consists of two or more sets of bars for as many samples, with wider gaps between the sets than between the bars of the same set. Each set of bars presents the frequency distribution of a particular sample. Thus, the multiple bar diagram serves to compare the frequency distributions of a nominal variable in a number of samples (Fig. 3.4). Like histograms, bar diagrams cannot be superimposed on each other and have to be displayed separately side by side for comparison between samples. Fig 3.4. A multiple bar diagram of the frequency distributions of ABO blood groups in two samples of humans. 40 – 30 – 20 – 10 – 0 A B O AB A B O AB Sample - 1 Sample - 2 f

M-2\D:\Netaji 05\Biology-1\ 3.4 Probability and probability distributions In Unit 2, particularly while learning about sampling methods, you have read about probabilities and laws of probability. Probabilities are also involved at numerous stages of scientific investigations such as experimental designs, evaluation of tests, drawing of inferences and statistical predictions of scores of variables. Probability may be defined in a simple manner as the relative frequency of occurrence of any event, animal, case, score or phenomenon in an almost infinite number of such events, cases or animals. To put simply, the probability (P) of getting one mutant animal in one choice from among the total of 4587 animals of a sample, known to have 150 such mutants, would be P = f / n = 150/4587 = 0.033. You should keep in mind that the relative frequencies would differ progressively from the actual probability with the fall in the total number (n) of cases, and would approach the limiting frequency, close to the probability, only when n is near infinity. For more details, see Sections 7.5 and 7.6. 3.4.1. Probability distributions A probability distribution is a distribution of relative frequencies (f / n) or probabilities of scores or cases in different class intervals of a given variable where the sample size (n) or the total number of events, scores or cases is vast. You have already learnt about the frequency distribution of scores in a sample. If the frequency (f) in each class interval is divided by the total size of a vast sample (n ~ –??) and that computed relative frequency (f / n) is placed in the corresponding interval, we work out a probability distribution of those scores or cases (Table 3.6). To present the latter graphically as a probability distribution curve or a relative frequency polygon, the scores or cases and their relative frequencies are scaled respectively along the abscissa and the ordinate; each relative frequency (f / n) is then plotted against the midpoint (X C) of the corresponding class interval, and the plotted points are joined by lines to get the curve or polygon. Table 3.6. Probability distribution of insect wing length scores of Table 3.2. Class intervals X C f P = f / n 12 – 14 13 2 0.10 15 – 17 16 3 0.15 18 – 20 19 8 0.40 21 – 23 22 5 0.25 24 – 26 25 2 0.10 ? — 20 (n) 1.00

M-2\D:\Netaji 05\Biology-1\ Because probabilities may be expressed even in infinitely small fractional units, the ordinate scale for probabilities in a probability distribution is always a continuous one. But the abscissa scale for events, cases or scores maybe either continuous or discontinuous according as the corresponding variable is a continuous measurement variable or a discontinuous one. Continuous probability distributions are probability distributions of continuous measurement variables such as blood glucose, wing length, gill weight and oxygen consumption, their x-scale (abscissa) is continuous with no gaps and has scores even in fractional units; e.g., normal probability distributions and Student's t distributions for continuous variables. Discontinuous probability distributions are those for discontinuous variables such as heart rates, respiratory rates and litter sizes, so that their scores cannot be in fractional units and the x-scale of their probability distributions have gaps; e.g., binomial distributions for dichotomous variables, and Poisson distributions for cases of the rare class of dichotomous variables. Probability distribution may again be divided in another way into experimental and theoretical distributions. An experimental probability distribution may be worked out using the data actually collected in an experiment or a survey. You may find such a case in Table 3.6; however, you should bear in mind that unlike what is given in that table, a probability distribution should be worked out in real cases with the data of a very large number of cases to minimize errors. On the contrary, a theoretical probability distribution is worked out theoretically, following a specific mathematical model, applying the laws of probability and using specifically formulated theoretical equations, but needing no experimental data for its computation. Such theoretical probability distributions are widely used in the assumptions for statistical tests, interpretations of experimental data, drawing of inferences from experimental observations, working out of confidence intervals of parameters, and statistical predictions of scores or events. Such theoretical distributions include binomial distributions based on the binomial equation, Poisson distributions using the equation of S. D. Poisson, normal probability distributions based on the equation of K.F. Gauss, and Student's t distributions on the basis of the equation derived by W. S. Gossett. These four theoretical distributions are described briefly in the following sections. 3.5 Normal distributions Normal probability distributions are theoretical and continuous probability distributions worked out on the basis of the Gaussian equation and plotted graphically as normal curves. To work out and plot a normal distribution from raw scores (X) of any continuous measurement variable, each X score is first transformed into a

M-2\D:\Netaji 05\Biology-1\ standard score (z score) using the mean (? or X –) and the standard deviation (? or s) of those scores. z X z X X s ? ? ? ? ? ? , . or The z score does not bear the unit of the X score; instead, it is expressed in SD units (? units) and possesses a standard reference value irrespective of mean, SD or unit. The probability (Y) of random occurrence of each z score and so, of the corresponding X score, is worked out with the following Gaussian equation, using the sample size (n), SD (?), the base (e) of natural logarithms, and the constant ratio (?) between the circumference and diameter of a circle. Y n e z ? ? ? ?2 2 2 If Y and z are scaled along respectively an ordinate (y - axis) and an abscissa (x- axis) on a graph paper, and each computed Y is plotted against the corresponding z score, a specific unimodal (single–peaked), bilaterally symnetrical and bell-shaped probability distribution curve results; the latter is called the normal distribution curve or normal curve (Fig. 3.5). Variations of n, ? and interval length (i) from sample to sample yield numerous normal curves. For universal use irrespective of n, ? and i, a standard reference form of the normal curve, called the unit normal curve, has been derived using the following modification of the Gaussian equation and taking the values of n, ? and i as 1.00 each. Y e z ? ? 1 2 2 2 ? Fig 3.5. A unit normal curve with its fractional areas between the ordinates at several z scores. [From D. Das and A. Das, Statistics in Biology and Psychology, 4th ed., Academic Publishers, 2003]

M-2\D:\Netaji 05\Biology-1\ 3.5.1. Properties of normal probability distributions Following properties of normal distribution (unit normal curve) are important for understanding and using the normal curve. (a) Normal probability distribution is a continuous probability distribution with a continuous abscissa scale (x–axis) for z scores without any genuine gap between the latter. So, it can be used in case of continuous measurement variables, but is not applicable to discontinuous variables, ordinal variables and nominal variables. (b) It is a theoretical probability distribution because the probabilities of this distribution can be theoretically computed using the Gaussian equation. (c) According to variations in n, ? and i, there are infinite numbers of normal distributions. So, a unit normal curve has been worked out as a standard reference curve using a modification of the Gaussian equation and with n, ? and i amounting to 1.00 each. (d) It is a single-peaked or unimodal distribution with an identical value of zero for its mean, median and mode, because the z score for ? amounts to 0.00 : z = (? – ?)? = 0.00. Also note that its mean, median and mode are coincident. (e) It is perfectly symmetrical bilaterally and possesses no asymmetry or skewness. Thus, the coefficient of skewness is zero for the unit normal curve. (f) Because the area enclosed by the unit normal curve represents the sample size n which is taken as 1.00 for that curve, the fractional area in either half of the curve is considered to be 0.5000. (g) The probability of random occurrence of any z score (as also of the corresponding X score) is given by the height of the ordinate at that z score, as read from the corresponding Y value in the y-axis. (h) Because of bilateral symmetry, the height of the ordinate at any z score in the right half of the distribution equals that at the same z score in the left half. (i) The highest ordinate of the unit normal curve is located at the zero score of z, and its height (Y), viz., 0.3989, gives the probability of random occurrence of scores identical with the mean, median and mode of the sample. (j) The fractional area of the unit normal curve between any two z scores in one of its halves is identical with that between the same two z scores in the other half. (k) Two tails of the unit normal curve are asymptotic, i.e., they reach the zero level (Y = 0.00) of the y-axis at respectively –? and +? values of z scores on the x- axis. (l) Probability (P) of random occurrence of a given z and all other z scores beyond it in either tail is known as the one-tail probability and is obtained as follows. P = 0.5000 – (area of unit normal curve from its ? to the given z).

M-2\D:\Netaji 05\Biology-1\ (m) Probability (P) of random occurrence of a given z and all other z scores beyond it in both tails is known as the two-tail probability. P = 2 [0.5000 – (area of unit normal curve from its ? to the given z)]. (n) The normal curve is mesokurtic, i.e., has a medium degree of peakedness. (o) Probability distribution of a continuous measurement variable conforms to the normal distribution, if its scores are determined by the random effects of many other variables with no mutual interactions. (p) Means of samples drawn from a normally distributed population are distributed normally around the parametric mean of the latter, forming a sampling distribution of means. 3.6 Skewness and kurtosis These two properties of a distribution determine its form, shape and many other characteristics. 3.6.1. Skewness Skewness is a measure of the degree and direction of bilateral asymmetry of a distribution. A symmetrical distribution, e.g., normal and t distributions, has no skewness, has its two tails identically extended and equally pointed, and has its mean, median and mode coincide with the centre and peak of the distribution. But a skewed distribution is bilaterally asymmetric with one of its tails more extended and pointed than the other tail. This results from the presence of more extreme scores in the extended or skewed tail than in the shorter and blunter tail; the scores are more concentrated in the blunter tail than in the skewed one. The skewness is called positive if the right or high-value tail is more drawn out than the left or low-value tail, while negative skewness consists of a more drawn-out and sharper left or low- value tail compared to the right tail. Poisson distributions are positively skewed Fig 3.6. Skewed distributions. [From D.Das and A. Das, Statistics in Biology and Psychology, 4th ed., Academic Publishers, 2003.]

M-2\D:\Netaji 05\Biology-1\ while binomial distributions are mostly either positively or negatively skewed. Coefficient of skewness (Sk) is a measure of the magnitude and algebraic sign of skewness, indicating thereby the degree and direction of the skewness, respectively. Sk X Mo s ? ? ; Sk X Mdn s ? ? 3( ) . 3.6.2. Kurtosis Kurtosis is a measure of peakedness of a ditribution. In assessing kurtosis, the normal distribution is used as the model. Being of a medium degree, its peakedness is known as mesokurtosis. Distributions like Student's t distributions possess comparatively sharper and higher peaks, thicker tail ends and thinner intervening regions, and are called leptokurtic distributions. Compared to mesokurtosis, leptokurtosis is characterized by higher concentrations of scores in a narrow zone around the peak and at the two tail-ends, but lower score concentrations in the area in between. Poisson and t distributions are leptokurtic. Distributions which have a broader and flatter central region, narrower tail-ends and thicker intervening regions, are called platykurtic. In contrast to mesokurtosis, platykurtosis is characterized by lower score concentrations in the central region and at the tail-ends, but higher score density in the area in between (Fig. 3.7). Some binomial distributions are platykurtic while some are leptokurtic. Percentile coefficient of kurtosis (?) is a measure of kurtosis, worked out using 10th, 25th, 75th and 90th percentiles (P 10 , P 25 , P 75 and P 90) which are the scores below which the respective percentages of total scores occur in the sample. ? ? ? ? P P P P 75 25 90 10 2 ( ) . Fig 3.7. Different forms of kurtosis. [From D. Das and A. Das, Statistics in Biology and Psychology, 4th ed., Academic Publishers, 2003.]

M-2\D:\Netaji 05\Biology-1\ ? amounts to 0.263 in mesokurtosis, is less than that value in leptokurtosis, and exceeds 0.263 in platykurtosis. 3.7 Student's t distributions Scores of a variable are distributed normally in a large (n ? 30) sample, drawn from a population having a normal distribution of scores. But if the sample drawn from such a population is a small one (n > 30), the frequency distribution as well as the probability distribution of its scores would take the shape of a unimodal, symmetrical but leptokurtic distribution, conforming to a theoretical probability distribution, originally formulated by W.S. Gossett and known as the Student's t distribution because of his pseudonym "Student". To work out the t distribution of the scores of a variable in a small sample, each score (X) is first transformed into t, basically in the same way as in case of z, and the computed t is then used in plotting the t distribution. t X ? ? ? ? , or t X X s ? ? . Similarly, means (X –) of small samples from a normally distributed population may also be transformed into t, using their standard errors ( )s x , and the computed t may then be used in plotting a sampling distribution of means conforming to Student's t distribution. t X s X ? ?? . Like the use of z scores in working out a normal distribution, Student's t distribution is worked out by computing the probability (Y) of random occurrence of each t score in terms of the Gossett equation, using the degrees of freedom (df) of the t scores. Y df df df t df df ? ? ? ? ? ? [( )/]! [( )/]! [ ] . 1 2 2 2 1 1 2 1 2 ? Scaling Y and t along respectively the ordinate and the abscissa scales on a graph paper, and plotting each computed Y against the corresponding t, a Student's t distribution curve may be drawn (Fig. 3.8). PX Xe X () ! ,? ? ? or

M-2\D:\Netaji 05\Biology-1\ 3.7.1. Properties of Student's t distributions Following properties of t distributions are important for understanding and using them. (a) Student's t distribution is a continuous probability distribution with a continuous abscissa scale for t scores without any genuine gap between them. So, it can be used in case of continuous measurement variables only, like trunk length and tracheal ventilation volume, and is not applicable to discontinuous measurement variables such as heart rate, respiratory rate, litter size and cell count, ordinal variables like ferocity, and nominal variables like sex. (b) It is a theoretical probability distribution because its probabilities can be theoretically worked out using the Gossett equation. (c) You may have marked in the Gossett equation itself that t distributions depend heavily on the degrees of freedom of t. So t distributions differ from each other according to the df of the relevant t scores and are consequently very numerous in number. (d) It is a unimodal distribution with its mean, median and mode coincident with each other and amounting to zero, because the t score for ? amounts to zero : t = (? - ?)/? = 0.00. (e) It is perfectly symmetrical bilaterally and its coefficient of skewness is zero, indicating the absence of skewness. (f) The t distribution has asymptotic tails, extending respectively to – ? and + ? at the two ends. (g) The distribution is leptokurtic, its percentile coefficient of kurtosis being lower than 0.263; the magnitude of leptokurtosis declines with the rise in df so that the t distribution with the df of ? is mesokurtic and identical with normal distribution. Fig 3.8. Some Student's t distributions. [From D. Das and A. Das, Statistics in Biology and Psychology, 4th ed., Academic Publishers, 2003.]
M-2\D:\Netaji 05\Biology-1\ (h) To interpret any computed t score, it should be referred to the specific t distribution with the same df as that of the computed t. (i) The distribution of scores of a variable in a small sample, drawn from a population where such scores are normally distributed, would conform to the t distribution. (j) Means (X –) of a variable in small samples, drawn from a population having a normal distribution of its scores, form a sampling distribution conforming to the t distribution around the parametric mean (?) of that variable in the population. 3.8 Binomial distributions Some variables are divided into two distinct classes with an intervening gap. They are called dichotomous variables and are frequently found among nominal variables such as sex (male-female), HIV positive-negative, pregnant-nonpregnant and Rh plus-minus. With respect to such a dichotomous variable, the individuals or cases of the population belong to either one or the other of the two classes. A random sample from such a population would often include different numbers of cases from both the classes.

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A binomial probability distribution is a distribution of probabilities of random occurrences of different combinations of cases from the two classes of

a dichotomous variable, in a sample drawn from such a population. It is used to find the probability of random occurrence of either a given number of cases of one class or a given combination of cases from both classes in a sample, depending on laws of probability. The binomial distribution

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is a theoretical probability distribution because it can be worked out theoretically using the series of terms of the binomial equation.

You may find below the series of terms of the binomial expansion. Each of these terms gives the probability

61% **MATCHING BLOCK 17/108** **W**

of random occurrence of a particular combination of cases from two classes of a dichotomous variable in

the sample, where p and q are the proportions of the two classes in the population, the powers of p and q give the numbers of cases of the respective classes in the sample, and n is the sample size. ( ) ( ) ( )( ) .....
p q p np q

63% **MATCHING BLOCK 18/108** W

n n p q n n n p q n n n n n ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 1 2 2 3 3 1 1 2 1 2 1 2 3 ..... ( )( )( )..... .....( ) ? ? ? ? ? ? ? ? ? ? ? n n n n n pq q n n 1 2 3 2 1 2 3 1 1 .

For a given sample size (n) with X number

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of cases of the class having the proportion p in the population,

that particular term of the expansion should be chosen for computing the probability P of random occurrence of X, which has the power X
M-2\D:\Netaji 05\Biology-1\ of p and the power (n – X) of the proportion q of the other class. P would be obtained on working out that chosen term. Where n = 10, X = 4, p = 0.55 and q = 0.45, n – X = 10 – 4 = 6. ? P n n n n n n p q X n X ? ? ? ? ? ? ? ? ? ? ? ( )( )( )( )( ) 1 2 3 4 5 1 2 3 4 5 6 ? ? ? ? ? ? ? ? ? ? ? ? ? 10 10 1 10 2 10 3 10 4 10 5 1 2 3 4 5 6 055 045 4 6 ( )( )( )( )( ) . . = 0.16 . A probability level is next chosen as the level of significance (?) to compare with the probability P computed in the above manner. If P exceeds ? (P < ?), the given 4 cases of the p class (along with 6 cases of the q class) are considered to have occurred in the sample due to random sampling; but if it is either equal to or lower than the chosen ? (P ? ?), the given numbers of cases have not occurred due to random sampling. In the above-mentioned example, if ? is chosen to be 0.05, the computed P of 0.16 exceeds the chosen ? ; so, the given 4 cases of the p class (along with 6 cases of the q class) have occurred in the sample by mere random sampling (P < 0.05). 3.8.1. Properties of binomial distribution Following properties of binomial distribution are important for its application in biostatistics. (a) It is a theoretical probability distribution because the probabilities of this distribution can be theoretically computed using the binomial equation. (b) It is a discontinuous probability distribution because the events or cases scaled in the abscissa occur only in whole numbers (e.g., 1, 2, 3, etc.) with intervening gaps, not in fractional units, and the abscissa or x-scale is consequently discontinuous. (c) It is a probability distribution of events of dichotomous variables, each divided into two classes separated by an intervening gap, neither of these classes being rare or having too low a proportion in the population. (d) Each event or case of any of the two classes occurs in the sample at random depending on laws of probability and independent of all other events or cases. (e) The distribution of events of either class, say that with proportion p, has its mean, variance and SD depending on the proportions (p and q) of both classes in the population. ? ? ? ? ? ? np npq npq ; ; . 2 (f) Distribution of events of the class p is symmetrical and nonskewed when its
M-2\D:\Netaji 05\Biology-1\ proportion p is 0.50 in the population; but the distribution is positively skewed if its proportion is less than 0.50, and has a negative skewness if its proportion exceeds 0.50 in the population. (g) The distribution of either class is platykurtic when its proportion in the population falls within the range of 0.21–0.79, but is leptokurtic if its proportion is beyond that range on any side. 3.8.2. Assumptions for binomial distribution The following assumptions should be justifiable if this distribution is to be applied in biostatistics. (a) The relevant variable is dichotomous, i.e., divided into two separate classes. (b) Neither of the classes is rare with too low proportion in the population. (c) Each event or case of any of the two classes occurs at random and independent of all other events or cases. (d) The known proportions of the two classes have remained unchanged before and during sampling. (e) The distribution of events of the p class has its mean and variance amounting respectively to np and npq. Example 3.8.1. Work out the binomial probability of random occurrence of 7 male rats in a sample of 10 rats drawn from a rat population consisting of 48% male and 52% female animals. Interpret your result. (? = 0.01.) Solution : p q p ? ? ? ? ? ? ? ? percentage of males 100 48 100 048 1 1 048 052 . . . . .

63% **MATCHING BLOCK 20/108** W

n = 10. X = 7 = n – 3. n – X = 10 – 7 = 3. ( ) ( ) ( )( ) ..... . p q p np q n n p q n n n p q q n n

n n n n ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 1 2 2 3 3 1 1 2 1 2 1 2 3 ??? P n n

n
p q n ( ) ( )( ) ( )( ) . . . . 7 1 2 1 2 3 10 10 1 10 2 1 2 3 048 052 010 3 3 7 3 ? ? ? ? ? ? ? ? ? ? ? ? ? ? Alternatively, using the Bernoulli expansion : P n p q X n X X n X ( ) ! ! ( )! . . ! ( )! . . 7 10! 048 052 7 10 7 010 7 3 ? ? ? ? ? ? ? ? = 0.01. ? P < 0.01. As P exceeds the chosen ?, the given number of 7 males has occurred in the sample due merely to random sampling and has no significance (P < 0.01).
M-2\D:\Netaji 05\Biology-1\ 3.9 Poisson distributions Some dichotomous variables are so divided into two classes that one of the latter is a rare one with its proportion very close to zero in the population, while the other class forms a large proportion of the population. Poisson distribution is the probability distribution of random occurrences, in the sample, of different numbers of events or cases of the rare class of such a dichotomous population. It does not apply to the larger and more frequent class of the variable. Poisson distribution consists of a series of terms, each for the probability of random occurrence, in a sample, of a specific number of rare events or cases when the proportion (p) of the rare class in the population is known. If X is the number of rare cases in a sample of size n, P(X) is the Poisson probability of random occurrence of X in the sample, and e is the base of natural logarithm, ? ? ? ? ? np P X X e X ; ( ) ! . The theoretical model worked out by Poisson equation consists of the following series of terms for probabilities of random occurrences of successive numbers of rare events. No. of rare events (X) : 0 1 2 3 4 5 ..... n P(X) : 1 e ? ? ? 1!e ? ? 2 2!e ? ? 3 3!e ? ? 4 4!e ? ? 5 5!e ..... ? ? n n e! . 3.9.1. Properties of Poisson distribution Following properties should be kept in mind for applying the Poisson distribution to biostatistics. (a) It is a theoretical probability distribution based on the theoretical model of the Poisson equation. (b) It is a discontinuous probability distribution because the rare cases or events scaled in the abscissa or x-axis occur only in whole numbers (e.g., 1, 2, 3, etc.) with intervening gaps, not in fractional units. (c) It is a probability distribution of random occurrences of events of the rare class of a dichotomous variable, but not of the other and more frequent class. (d) Each rare event or case occurs in the sample at random obeying laws of prabability, and independent of all other rare events. (e) The rare events may occur either spatially at different sites on a particular instant, or temporally in the same system at different times. Both types obey the Poisson distribution.
M-2\D:\Netaji 05\Biology-1\ (f) The proportion p of events or cases of the rare class is very low — almost close to zero, while that of the other class is very high and nearly 1.00. (g) The mean (?) and the variance (? 2) of a Poisson distribution are identical, dependent on the proportion p of rare cases in the population, and less than 5 in value. ? = ? 2 = np > 5. (h) Poisson distributions possess positive skewness which declines with the rise in ?. (i) Poisson distributions possess leptokurtosis which also declines with the rise in ?. However, neither the positive skewness nor the leptokurtosis disappears so long as the distribution conforms to the Poisson model. 3.9.2. Assumptions for Poisson distribution The following assumptions should be justifiable if Poisson distribution has to be applied to the data. (a) The relevant variable is dichotomous, i.e., divided into two classes. (b) The class, to which Poisson distribution is proposed to be applied, should be a rare class with near-zero proportion in the population. (c) Each event or case of the rare class should occur in the sample at random and independent of the occurrence or absence of other rare events or cases. (d) Mean and variance of the distribution of events of the rare class should be identical, directly proportional to the proportion of such events in the population, and lower than 5 in value. (e) The known proportions of the two classes in the population should remain unchanged before and during sampling. Example 3.9.1. Work out the Poisson probability of finding 9 mutant houseflies in a sample of 500 houseflies drawn from a housefly population

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known to have 80 mutants per 10000. Interpret your result. (? = 0.05.) Solution : n = 500. X = 9. p ? No.of mutants in given population size Given population size ? ? 80 10000 0008. . ? =

np = 500 × 0.008 = 4.00.

M-2\D:\Netaji 05\Biology-1\ P X X e P e X ( ) ! , ( ) ! . . ? ? ? ? ? or 9 4 9 0013 9 4 The computed Poisson probability is interpreted in the same way as in the case of binomial probability (Section 3.8). ? = 0.05. P = 0.013. ? P > 0.05. Since the computed Poisson probability is lower than the chosen ? of 0.05, the given number of 9 mutants has not occurred in the sample due to mere random sampling, and is therefore significant (P > 0.05). 3.10 Summary Scores or cases of a variable in a relatively large sample are often tabulated in a frequency distribution, where they are entered in the respective class intervals into which the entire range of scores or cases has been divided. Qualitative, continuous and discontinuous frequency distributions are worked out for the respective types of variables. Frequency polygons and histograms are drawn for graphic representation and comparison of frequency distributions of continuous measurement variables; simple and multiple bar diagrams are used for discontinuous and qualitative frequency distributions. Relative frequencies of scores or cases in infinitely large samples may be used in working out their probability distributions. Probability distributions may be continuous or discontinuous according to the continuous or discontinuous natures of the variables. Theoretical probability distributions are worked out theoretically according to specific mathematical models; experimental probability distributions are formed from experimentally obtained data. Normal probability distributions are theoretical and continuous probability distributions, worked out using Gaussian equation. Continuous measurement variables, whose scores are determined by the random influences of innumerable non-interacting variables, possess normal distributions of their scores in the population. For working out a normal distribution, each score of the relevant variable is transformed into a standard z score expressed in sigma (?) units. Unit normal curve is a standard reference form of normal distribution, assuming the values of sample size, SD and interval length as 1.00 each. The normal curve is a unimodal, bilaterally symmetrical, bell-shaped curve with asymptotic tails, no skewness and medium degree of kurtosis. Skewness gives the degree and direction of bilateral asymmetry of a distribution while kurtosis is the degree of peakedness of the latter. Student's t distributions are also theoretical and continuous probability distributions which are unimodal, bilaterally symmetrical, nonskewed, but leptokurtic.

M-2\D:\Netaji 05\Biology-1\ Distribution of any continuous measurement variable, distributed normally in a population, conforms to the t distribution in small samples from that population. Student's t distributions are worked out theoretically, using Gossett's equation and t scores obtained like z scores by transforming the scores of the relevant variable into standard forms in sigma units. The t distributions are numerous and differ from each other according to the degrees of freedom of the t values used in computing them. Binomial distributions are theoretical and discontinuous probability distributions. They give the probabilities of random occurrences of the events of either or both classes of a dichotomous variable, provided neither class is a rare one in the population. They are computed theoretically using the binomial equation and the proportions of both the classes in the population. Most binomial distributions are either positively or negatively skewed, and either leptokurtic or platykutic. Poisson distributions are theoretical and discontinuous probability distributions, giving the probabilities of random occurrences of events of the rare class of a dichotomous variable. They are computed theoretically using Poisson's equation and the low proportion of the rare class in the population. Poisson distributions are positively skewed and leptokurtic. 3.11Terminal questions 1. (a) What are frequency distributions? (b) Describe how you would work out a continuous frequency distribution. (c) Write briefly about histograms and their use. 2. (a) What are probability distributions? (b) Write briefly about different types of probability distributions with examples. (c) Describe the properties of Poisson distributions, quoting the Poisson equation. 3. (a) Write how frequency polygons are drawn, using the original frequencies of scores of a frequency distribution and also their smoothed frequencies. (b) Describe the drawing and uses of simple and multiple bar diagrams. (c) Write about the skewness of distributions, mentioning the coefficients of skewness. 4. (a) Explain what is meant by a binomial probability distribution. (b) Describe the properties of binomial distributions. (c) Explain how the binomial probability can be worked out with the binomial expension.

M-2\D:\Netaji 05\Biology-1\ (d) Work out the probability of random occurrence of 12 male pigeons in a sample of 20 pigeons drawn from a pigeon population known to have a male : female ratio of 45 : 55. 5. (a) Compare the assumptions for binomial and Poisson distributions. (b) Work out the probability of random ocurrence of 3 thalassemia cases in a sample of 150 humans from a population having 7 thalassemia patients per thousand. Interpret your result. (? = 0.05). (c) Discuss the properties of Student's t distributions. 6. (a) Explain what you understand by normal distribution and unit normal curve, quoting their equations. (b) Discuss the proberties of normal distributions. 7. (a) Work out a frequency distribution of the following body weight scores (kg) of a sample of chimpanzees into five class intervals. 67, 64, 73, 66, 67, 58, 77, 63, 65, 63, 73, 65, 64, 57, 78, 61, 77, 57, 72, 79, 70, 56, 68, 74, 70, 61, 59, 62, 80, 60. (b) Write briefly on skewness and kurtosis, giving examples. (c) Discuss the assumptions for binomial distributions. 3.12 Answers 1. (a) See the first paragraph of Section 3.2. (b) See Sub-section 3.2.2. (c) See Sub-section 3.3.2. 2. (a) See the first pragraph of Sub-section 3.4.1. (b) See Sub-section 3.4.1. (c) See Section 3.9. 3. (a) See Sub-section 3.3.1. (b) See Sub-section 3.3.3. (c) See Sub-section 3.6.1. 4. (a) See Section 3.8. (b) See Sub-section 3.8.1. (c) See Section 3.8. (d) See Example 3.8.1. 5. (a) See Sub-section 3.8.2 and 3.9.2. (b) See Example 3.9.1. (c) See Sub-section 3.7.1. 6. (a) See Section 3.5. (b) See Sub-section 3.5.1. 7. (a) See Example 3.2.1. (b) See Sub-sections 3.6.1 and 3.6.2. (c) See Sub-section 3.8.2.

M-2\D:\Netaji 05\Biology-1\ Unit 4 ? STATISTICAL INFERENCE AND HYPOTHESIS TESTING 4.1 Introduction In this Unit, you will learn how to apply stastistical principles and methods in carrying out the follow-up of experimental observations to investigate whether and how far they may be meaningful for the population. You will be told about how to explore whether or not the results obtained by working with a sample can be generalized for the entire population. Stepwise use of statistics in the analysis and interpretation of experimental data will be presented and explained to you. You will learn some widely used statistical tests for making inferences using probability distributions such as normal and Student's t distributions. You will also know how to work out the probabilities of going wrong in the inferences drawn from experimental results. Structure 4.1 Introduction Objectives 4.2 Significance tests 4.3 Null hypothesis 4.4 Levels of significance 4.5 Errors of inference 4.6 Difference between means by z scores 4.7 Student's t tests 4.8 Chi square tests 4.9 Summary 4.10 Terminal questions 4.11 Answers

M-2\D:\Netaji 05\Biology-1\ Objectives After studying this unit, you should be able to do the following : ? consider the probability of the observed results arising from sampling errors due to random sampling, ? follow the stepwise use of statistics in interpretation and inference, ? define null hypothesis and alternative hypothesis, and realise their respective roles in conducting an experiment and interpreting the experimental observations, ? understand the importance and the use of levels of significance in finding the probability of the results arising from random sampling, ? distinguish between two types of errors of inference and proceed to limit their probabilities, ? transform the observed scores of large samples to standard z scores and interpret the latter using the unit normal curve, ? transform the observed scores of small samples to Student's t scores and interpret the latter using Student's t distributions with respective degrees of freedom, ? state the probability of error in inferring the observed result as significant, ? use the nonparametric chi square test to find whether or not an observed frequency distribution fits significantly with a model frequency distribution, and ? work out the chi square test of independence to explore whether or not there is any significant association between two given variables. 4.2 Significance tests Suppose that in exploring the effect of a given independent variable on a specific dependent variable, you have applied two different levels (i.e., amplitudes, doses, intensities, concentrations, etc.) of the former on two groups or samples of animals from the same population, and subsequently found a difference (X – 1 – X – 2) between the group / sample means of scores of the dependent variable. You have, however, learnt in paragraph (c) of Sub-section 2.6.3 that the means of two groups /samples from the same population, even when not affected at all by any independent variable, may differ from one another owing to their different sampling errors. So, it may be that the observed result, viz., the difference (X – 1 – X – 2), does not indicate that the independent variable has actually affected and changed the scores of the dependent one; instead,

M-2\D:\Netaji 05\Biology-1\ the observed difference may as well have arisen from the difference between the sampling errors (s e) of the two means, consequent upon random sampling — there might not have been any difference between the means if the levels of the independent variable would have been applied on the entire population. Such a probability would always persist so long as samples are used instead of the population, whatever precision and caution be used in sampling to make the samples truly representative of the population. You would be right to guess that the result of any such experiment using samples would be open to two alternative inferences. One, the obtained result is not meaningful, i.e., not significant, has come from chances associated with random sampling, would not have occurred if the population were used instead of samples, and can thus be explained away by sampling errors; the other, the observed result is meaningful and significant, is not the outcome of chances of random sampling, and cannot be explained away by sampling errors. To infer which of these two alternatives may be upheld, a significance test has to be undertaken to find statistically whether the probability of the observed result occurring by chance is too high or too low. If this probability is too high, it is inferred that the obtained result of the experiment is not significant and not fit for generalization in the entire population; on the contrary, if the probability of its chance occurrence is too low, the result under consideration is significant or meaningful and can be generalized in the population. For a significance test, in most cases, the experimentally obtained result (e.g., a difference between means, a correlation coefficient, etc.) is first transformed into a standard score (e.g., z, t, ? 2 and F) and the latter is referred to the corresponding probability distribution (e.g., normal, t, ? 2 or F distribution) to find the probability of its chance occurrence. To judge whether that probability is too high or too low, it is compared with a chosen probability level called the level of significance (?). You will learn in Sub-sections 4.6.2, 4.7.3, 4.7.4, 5.3.3, 5.5.1, 6.3.4 and 6.4.3 as also in Section 5.6, about significance tests for a number of computed statistics. 4.3 Null hypothesis Each experiment or investigation is intended, designed and performed to substantiate or prove a proposed conjecture called the experimental hypothesis; the latter is generally known in statistics as the alternative hypothesis (H a) because it is the alternative to and is contested by another hypothesis (H o) which would be subjected to a significance test. The H o is called the null hypothesis because it contradicts, contests and tries to negate or nullify the assertion of the alternative hypothesis. The testing of hypothesis consists basically of the working out of probability of correctness (P) of null hypothesis and finding whether that probability is too high or too low.

M-2\D:\Netaji 05\Biology-1\ In general, the null hypothesis proposes that the experimental result is not significant or meaningful, that it is the outcome of using a sample drawn at random by laws of probability, and that it would not be obtained if the entire population were used instead of the sample. Its elaborate statement, however, varies according to the assertions of diverse alternative hypotheses it contests, For example, where the H a proposes that there is a significant difference between two means (i.e., X – 1 ? X – 2), the H o contends that there is no significant difference between those means (i.e., X – 1 ? X – 2); but if the H a states that the mean of sample 1 is significantly higher than that of sample 2 (i.e., X – 1 ? X – 2), the H o proposes that X – 1 is not significantly higher than X – 2 (i.e., X – 1 ? / X – 2). Where the H a proposes that there is a significant correlation between two given variables, the H o contends that there is no significant correlation between the two. If the H a states that the frequency distribution of phenotypes observed in the sample does not fit with Mendel's 9 : 3 : 3 : 1 distribution, the H o proposes that there is a significant goodness of fit between the observed distribution and the Mendelian distribution. In any significance test of the obtained experimental result, the probability (P) of the correctness of H o is first worked out, and then compared with a chosen probability level called the level of significance (?). If P is found to exceed the ?, the P is considered too high; so, the H o is then retained, the H a is rejected and the observed result is not significant (P < ?). But if P is found to be either equal to or lower than the ?, the P is considered too low; hence, the H o is then rejected, the H a is accepted and the observed result is significant (either P = ? or P > ?). The H o is bound to be tested whenever an experiment is performed with a sample; but the H o need not be considered or tested when the entire population is subjected to an experiment. 4.4 Levels of significance A level of significance (?) is that particular level of the probability (P) of correctness of H o , which is compared with the P worked out in a significance test for considering the rejection or acceptance of the H o . It is that maximum level of probability, up to which the P worked-out in the significance test is considered too low, and above which the worked-out P is considered too high. In other words, if P exceeds the chosen ?, the probability of the H o being correct is taken as too high so that the H o cannot be rejected and the observed result is considered not significant (P < ?). But whenever P does not cross the chosen ?, i.e., whenever the computed P is either equal to or lower than the ?, it is taken to be so low as to warrant the rejection of the H o ; the observed result is then considered significant (P ? ?). In biostatistics, 0.05, 0.02, 0.01 and 0.001 levels of ? are usually used for

M-2\D:\Netaji 05\Biology-1\ comparing with the worked-out P. The latter may be compared either with one particular level of ? as chosen by the investigator, or successively with different levels of ? in a descending order from 0.05. In the second case, the H o may be rejected and the result considered as significant at or below that lowest level of ? which either exceeds or equals the computed P (P ? ?). For example; if P is lower than 0.05, it is significant there, but is next compared with the next lower ? of 0.02 to find if it is significant even there; this is repeated with successive lower levels of ? until that lowest ? is reached which exceeds or equals P. This process is preferable as the lower the ? for significance, the lower is the probability of type I error of inference (Section 4.5). For example, if P is lower than 0.01 (P > 0.01), then out of 100 such cases, the result may be wrongly considered significant in less than one of the cases; but if P equals 0.001 (P = 0.001), in only one in 1000 such cases the result may be wrongly considered significant. Thus, the lower the ? at or below which the result is considered significant, the fewer are the cases wrongly declared significant and consequently the lower is the probability of type I error. 4.5 Errors of inference Whether or not the experimental result is considered significant, there are probabilities of errors of inference because the inference derived from either the rejection or the acceptance of the H o depends in both cases on the probabilities, P and ?. Type I error of inference results from the wrong rejection of a correct H o , thus inferring an experimental result as significant when it is actually not significant. This error arises from the use of the level of significance (?) in rejecting the correct H o and consequently has a probability identical with the ? used in considering the computed P as too low (P ? ?); so, the probability of type I error has the symbol ? identical with that of the level of significance. It follows that the probability of type I error may be lowered by using a lower level of significance in comparing with the probability (P) worked out in the significance test. Thus, if P equals the ??of 0.05 (P = 0.05), there is a probability of 0.05 for the type I error — out of 100 such cases, such results of any 5 cases would actually be not significant, having resulted from mere random sampling; but if the H o is rejected because P equals 0.01 (P = 0.01), there is a much lower probability of 0.01 of the type I error. (See also the last paragraph of Section 4.4 and Sub-section 4.7.2.) Type II error of inference is the opposite of the type I error. It is the error resulting from the wrong acceptance of a wrong H o , thus leading to a wrong inference that the experimental result is not significant when the latter is actually significant. The probability of type II error (?) has a reverse relation with that of the type I error

M-2\D:\Netaji 05\Biology-1\ (?) and may amount upto the value of (1 – ?). In other words, the type II error (?) is given by that fractional area of H a distribution which overlaps with the area of H o distribution beyond the area for ??in the latter (Fig. 4.1). Thus, the probability of type II error rises with the fall in the type I error or the lowering of the level of significance. So, if ??is chosen to be 0.05, the probability of type II error may be as high as 0.95, but may rise still higher upto 0.99 if ??is chosen to be 0.01. ? also rises with the increasing proximity and overlap of H o and H a distributions (Fig. 4.2) Fig 4.1. Reverse relation between type I and type II errors. [From D. Das and A. Das, Statistics in Biology and Psychology, 4th ed., Academic Publishers, 2003.] Fig 4.2. Difference in type II error (?) between (a) and (b) because of change in proximity of H o and H a distributions. [From D. Das and A. Das, Statistics in Biology and Psychology, 4th ed., Academic Publishers, 2003.] In order to limit the chances of wrong inferences leading to positive consequences, reduction of type I errors by using lower levels of significance is preferred even at the cost of rising chances of type II errors. The latter may be reduced by choosing a relatively more powerful statistical test for interpretation. The statement of inference of any test should be followed by the mention of either P > ??, or P = ??or P < ??, as the case may be, and giving the numerical value of the ??used, so as to indicate the probability of type I error in the inference.

M-2\D:\Netaji 05\Biology-1\ 4.6 Difference between means by z scores To find the significance of difference between means of a dependent variable in large samples (n ? 30) from a population with normally distributed scores, a significance test is performed by transforming that difference into a standard z score and referring the latter to the unit normal curve for interpretation. 4.6.1. Assumptions for using z scores Following assumptions should be justifiable if z scores are to be used in finding the significance of difference between two sample means (X – 1 – X – 2). (a) The dependent variable should be a continuous measurement variable with its scores occurring even in infinitely small fractional units without any intervening gaps in their scale. (b) Scores of the dependent variable should be distributed in a normal distribution in the population from which the samples have been drawn. (c) Samples should be large in size (n ? 30) so that the distribution is normal and mesokurtic, and their means should have a normal sampling distribution. (d) Each score or case should occur at random in the sample, obeying the laws of probability and independent of other scores of the variable. (e) Samples should initially come from the same or similar population(s) so that their statistics such as means and variances are initially homogeneous. 4.6.2. Computation and inference The null hypothesis (H o) proposes that the observed difference (X – 1 – X – 2) between the sample/group means is not significant, has resulted from the choice of the sample by random sampling, and would amount to zero if the experiment were undertaken using the population instead of random samples. To find the probability (P) of the H o being correct, the difference (X – 1 – X – 2) is transformed into the z score. (See Example 4.6.1 also.) Where n 1 and n 2 are the respective sample/group sizes, s 1 and s 2 are the respective SDs, s X 1 and s X 2 are the SEs of the respective means, and s X X 1 2 ? is the SE of the difference,

63% **MATCHING BLOCK 23/108** **SA** MSPS 13 L part 1.pdf (D110610736)

X X n X X n s X X n s X X n 1 1 1 2 2 2 1 1 1 2 1 2 2 2 2 2 1 1 ? ? ? ? ? ? ? ? ? ? ? ? ? ; ; ( ) ; ( ) ; s s n s s n s s s

z X X s X X
X X X X X X 1 2 1 2 1 2 1 2 1 1 2 2 2 2 1 2 ? ? ? ? ? ? ? ? ? ; ; ; .
M-2\D:\Netaji 05\Biology-1\ Next, the fractional area of the unit normal curve from its mean (?, or z score 0.00) to the computed z score is noted from the unit normal curve table and used in working out the probability (P) of the H 0 being correct. P = 2 [0.5000 – (area of unit normal curve from ? to computed z)]. If the computed P is either equal to or lower than the chosen ? (0.05 or lower), P is considered too low and the H 0 is rejected — there is thus a significant difference between the sample / group means (P ???). But if P exceeds the ?, P is taken to be too high and the H 0 is retained — there is then no significant difference between the means (P < ?).

100% **MATCHING BLOCK 22/108** **W**

Example 4.6.1. Find whether or not there is a significant difference between the mean

tracheal ventilations (ml/minute) using the following data of two groups of insects. (? = 0.05). X 1 : 3.0, 3.5, 3.8, 3.7, 3.1, 2.7, 3.0, 3.8, 2.5, 2.9, 3.7, 3.3, 2.6, 3.0, 2.9, 2.4, 2.5, 3.2, 2.6, 3.8. X 2 : 2.6, 2.7, 2.5, 3.0, 3.3, 2.9, 2.8, 3.5, 3.1, 2.3, 2.5, 3.0, 3.3, 2.5, 2.8, 3.2, 2.6, 3.2, 3.0, 2.2. z scores : 1.83 1.84 1.85 1.86 1.87 1.88 1.89 1.90 Areas of UNC : .4664 .4671 .4678 .4686 .4693 .4699 .4706 .4713 Solution : The data are entered in the first two columns of Table 4.1 for computations.

43% **MATCHING BLOCK 25/108** **SA** MSPS 13 L part 1.pdf (D110610736)

n n X X n X X n 1 2 1 1 1 2 2 2 20 20 620 20 310 570 20 285 ? ? ? ? ? ? ? ? . . . . . ? ? ml ml s X X n s X X n 1 1 1 2 1 2 2 2 2 2 1 4 3800 20 1 0 480 1 2 4500 20 1 0 359 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ( ) . . . ( ) . . . ml ml s s n s s n X X 1 2 1 1 2 2 0480 20 01073 0359 20 00803 ? ? ? ? ? ? . . . . . ml ml s s s

X X X X 1 2 1 2 2 2 2 2 01073 00803 01340 ? ? ? ? ? ? . . . .ml z X X s X X ? ? ? ? ? ? ? 1 2 1 2 310 285 01340 187 . . . . . M-2\D:\Netaji 05\Biology-1\ Table 4.1. Computation of sums of squares. X 1 X 2 X 1 –X – 1 X 2 –X – 2 (X 1 –X – 1) 2 (X 2 –X – 2) 2 3.0 2.6 – 0.10 – 0.25 0.0100 0.0625 3.5 2.7 + 0.40 – 0.15 0.1600 0.0225 3.8 2.5 + 0.70 – 0.35 0.4900 0.1225 3.7 3.0 + 0.60 + 0.15 0.3600 0.0225 3.1 3.3 0 + 0.45 0 0.2025 2.7 2.9 – 0.40 + 0.05 0.1600 0.0025 3.0 2.8 – 0.10 – 0.05 0.0100 0.0025 3.8 3.5 + 0.70 + 0.65 0.4900 0.4225 2.5 3.1 – 0.60 + 0.25 0.3600 0.0625 2.9 2.3 – 0.20 – 0.55 0.0400 0.3025 3.7 2.5 + 0.60 – 0.35 0.3600 0.1225 3.3 3.0 + 0.20 + 0.15 0.0400 0.0225 2.6 3.3 – 0.50 + 0.45 0.2500 0.2025 3.0 2.5 – 0.10 – 0.35 0.0100 0.1225 2.9 2.8 – 0.20 – 0.05 0.0400 0.0025 2.4 3.2 – 0.70 + 0.35 0.4900 0.1225 2.5 2.6 – 0.60 – 0.25 0.3600 0.0625 3.2 3.2 + 0.10 + 0.35 0.0100 0.1225 2.6 3.0 – 0.50 + 0.15 0.2500 0.0225 3.8 2.2 + 0.70 – 0.65 0.4900 0.4225 ? 62.0 57.0 — — 4.3800 2.4500

P = 2 [0.5000 – (area of unit normal curve from ? to computed z)] = 2 [0.5000 – (area of unit normal curve from ? to 1.87)] = 2 [0.5000 – 0.4693] = 0.06. ? = 0.05. As the computed P exceeds the chosen ? of 0.05, P is too high. So, the H o is retained and there is no significant difference between the group means (P < 0.05). 4.7 Student's t tests To find the significance of difference between means of a dependent variable in two small samples (n > 30) and even in large samples (n ? 30), drawn from a population with normally distributed scores, Student's t test may be undertaken. For this, the difference between the means (X – 1 – X – 2), is first transformed into Student's M-2\D:\Netaji 05\Biology-1\ t by dividing it with the SE of the difference ( ) s X X 1 2 ? . The computed t is then compared with the critical t for a chosen level of significance, for finding the probability (P) of the H o being correct. The method of computing the t score varies according to the nature and size of samples/groups subjected to the t test (see Sub-sections 4.7.3 to 4.7.5). 4.7.1. Assumptions for t tests Following assumptions should be justifiable if t tests have to be undertaken. (a) The dependent variable should be a continuous measurement variable with its scores occurring even in infinitely small fractional units without any intervening gaps in their scale. (b) Scores of the dependent variable should have a normal distribution in the population from which the samples or groups have been drawn. (c) Each score should occur at random in the sample obeying the laws of prabability and independent of other scores of the variable. (d) Samples or groups should initially come from the same or similar population(s) so that the variances of their scores are initially homogeneous (homoscedasticity). You should understand from these assumptions that discontinuous measurement variables like heart rates and cell counts, ordinal variables like ferocity and docility, or nominal variables like sex and fur color cannot be subjected to t tests. 4.7.2. Critical scores After transforming the experimental result, e.g., X – 1 – X – 2 , into the score of a statistic such as t, F or ? 2 , the probability (P) of the correctness of H o has to be worked out by comparing that computed score of the statistic with a critical score of the latter. A critical score of such a statistic as t, F or ? 2 for a given level of significance (?) is that score beyond which lies a fractional area equalling the given ? in the tail of its probability distribution. Because most statistics such as t, F and ? 2 possess specific degrees of freedom, and because their probability distributions differ according to their df, the critical score would differ with the df. So, the computed t, F or ? 2 must be compared with its critical score having the same df. It may be recalled here that the probability P of random occurrence of any computed score like t or ? 2 is given by the fractional area beyond that score in the tail of its probability distribution; so, if the computed t exceeds or equals the critical t for a given ?, the area P beyond the computed t is respectively lower than or equal to the area ? beyond the critical t and is consequently considered too low (P ?? ?); but if the computed t is lower than the critical t, the area P beyond the former exceeds the area ? beyond the latter and is consequently considered too high (P < ?). The experimental result is thus taken respectively as significant and not significant. The same may be said about F, ? 2 , etc., into which the experimental result may be transformed.

M-2\D:\Netaji 05\Biology-1\ Even with the same df and for an identical ?, the critical score would differ between a two-tail test and a one-tail test. A one-tail test investigates whether there is a significant higher-lower concept, such as whether or not X – 1 is lower than X – 2 ; for such a test, the entire area of ? occurs beyond the critical score in a single tail of the probability distribution. But a two-tail test investigates simply whether or not the obtained result is significant, with no concern for any higher–lower concept, such as whether or not (X – 1 – X – 2) is significant irrespective of its algebraic sign; for such a test, the area of the chosen ? is distributed in equal halves beyond respectively the positive and negative values of the critical score in two tails of the probability distribution. Evidently, the critical scores for a given ? are different for the two types of tests, that for the one-tail test being lower than that for the two-tail test. Some examples are given in Table 4.2. Table 4.2. Some critical t values. ? = 0.05 ? = 0.01 ? = 0.005 ? = 0.001 ———————— ———————— ———————— ———————— df 2-tail 1-tail 2-tail 1-tail 1-tail 2-tail 10 2.228 1.812 3.169 2.764 3.169 4.587 15 2.131 1.753 2.947 2.602 2.947 4.073 20 2.086 1.725 2.845 2.528 2.845 3.850 30 2.042 1.697 2.750 2.457 2.750 3.646 60 2.000 1.671 2.660 2.390 2.660 3.460 ? 1.960 1.645 2.576 2.326 2.576 3.291 4.7.3. t test for small independent groups Student's t is worked out by dividing the difference (X – 1 – X – 2) between two sample/group means with the SE of that difference ( ) s X X 1 2 ? . In case each group is smaller than 30 in size and the two groups are independent, consisting of separate sets of cases or animals, the SD to be used in computing s X X 1 2 ? is worked out as the pooled SD (?)s of both the groups.

51% **MATCHING BLOCK 27/108** **SA** MSPS 13 L part 1.pdf (D110610736)

X X n X X n s X X X X n n 1 1 1 2 2 2 1 1 2 2 2 2 1 2 2 ? ? ? ? ? ? ? ? ? ? ? ? ; ; ? ( ) ( ) ; s s n n n n n t X X s df n n X X

X X 1 2 1 2 1 2 1 2 1 2 1 2 2 ? ? ? ? ? ? ? ? ? ? ? ? ; ; . The computed t is next compared with a 2-tail or 1-tail critical t ? (df) for a chosen significance level, according as a 2-tail or 1-tail test is being performed. If the M-2\D:\Netaji 05\Biology-1\ computed t exceeds or equals the critical t ?? , P is lower than or equal to that ? and the result is considered significant (P ???); but if the computed t is lower than the critical t ? , P is higher than that ? and the result is not significant (P < ?). Alternatively, the computed t is compared with critical t ? (df) values of successive lower levels of significance starting from 0.05 and the lowest level of ? is found out, at or below which the computed t exceeds or equals the critical t ? and the result may be considered significant. Example 4.7.1. Find if there is a significant difference between the mean body weights (g) of

69% **MATCHING BLOCK 26/108** **W**

the following two groups of fishes from two different habitats. (? = 0.05.) Group 1 (X 1) : 85, 90, 80, 75, 70, 75, 80, 90, 85, 80. Group 2 (X 2) : 55, 65, 60, 75, 80, 70, 55, 60, 80, 70. Critical 2-tail t scores : t 0.05(19) = 2.093; t 0.05(18) = 2.101; t 0.05(20) = 2.086; t 0.05(17) = 2.110. Solution :

Table 4.3. Computation of sums of squares.

58% **MATCHING BLOCK 30/108** **SA** MSPS 13 L part 1.pdf (D110610736)

X 1 X 2 X 1 – X – 1 (X 1 – X – 1) 2 X 2 – X – 2 (X 2 – X – 2) 2 85 55 + 4.0 16.00 – 12.0 144.00 90 65 + 9.0 81.00 –2.0 4.00 80 60 – 1.0 1.00 – 7.0 49.00 75 75 – 6.0 36.00 + 8.0 64.00 70 80 – 11.0 121.00 + 13.0 169.00 75 70 – 6.0 36.00 + 3.0 9.00 80 55 – 1.0 1.00 – 12.0 144.00 90 60 + 9.0 81.00 – 7.0 49.00 85 80 + 4.0 16.00 + 13.0 169.00 80 70 – 1.0 1.00 + 3.0 9.00 ? 810 670 — 390.00 — 810.00 n n X X n X X n 1 2 1 1 1 2 2 2 10 10 810 10 810 670 10 670 ? ? ? ? ? ? ? ? . . . . . ? ? g g ? ( ) ( ) . . . . s X X X X n n ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 1 1 2 2 2 2 1 2 2 39000 81000 10 10 2 816g s s n n n n X X 1 2 1 2 1 2 816 10 10 10 10 3649 ? ? ? ? ? ? ? ? . . .

g

M-2\D:\Netaji 05\Biology-1\ t X X s df n n X X ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 1 2 1 2 1 2 810 67 0 3649 3837 2 10 10 2 18 . . . . . .

71% **MATCHING BLOCK 28/108** **W**

Critical t 0 05 18 2101 . ( ) . . ? As the computed t is higher than the critical t 0.05 , P is

lower than 0.05 and is considered too low.

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So, the H 0 is rejected and it is inferred that there is a significant difference between the

group means. (P > 0.05.) 4.7.4. t test for large independent groups With rise in sample/group size and consequently in the df, t distributions come closer to the unit normal curve, and coincide with the latter when df amounts to ?. So, instead of using z scores, t test can also be undertaken to find the significance of difference (X – 1 – X – 2) between means of two large independent groups (n 1 ? 30, n 2 ? 30) consisting of two separate sets of cases or animals. For such large groups, (X – 1 – X – 2) is transformed into t by division with s X X 1 2 ? worked out with separate unbiased SDs of the individual groups.

51% **MATCHING BLOCK 31/108** **SA** MSPS 13 L part 1.pdf (D110610736)

X X n X X n s X X n s X X n 1 1 1 2 2 2 1 1 1 2 1 2 2 2 2 2 1 1 ? ? ? ? ? ? ? ? ? ? ? ? ? ; ; ( ) ; ( ) ; s s n s n t X X s df n n X X

X X 1 2 1 2 1 2 1 2 2 2 1 2 1 2 2 ? ? ? ? ? ? ? ? ? ? ; ; .
The computed t is compared with critical t ? (df) . If the computed t exceeds or equals the critical t ? , P is either lower than or equal to that ? and is considered too low. So, the H 0 is rejected and the difference between group means is significant (P ???). But if the computed t falls short of the critical t ? , P exceeds the ? ; so, the H o is retained and there is no significant difference between the means. (P < ?). Example 4.7.2. Using the data of Example 4.6.1., find if the mean (X – 1) of group 1 is significantly higher than that (X – 2) of group 2 (? = 0.05). (Please note that although this method is to be used for groups equalling or exceeding 30 in size, groups smaller than 30 have been used in this example for the sake of brevity.) 1-tail critical t scores : t t 0 05 39 0 05 38 1689 1689 . ( ) . ( ) . ; . ; ? ? t t 0 05 20 0 05 37 1725 1689 . ( ) . ( ) . ; . . ? ?
M-2\D:\Netaji 05\Biology-1\ Solution : Please refer to Table 4.1 for computation of means and sums of squares.
n
n

51% **MATCHING BLOCK 37/108** **SA** MSPS 13 L part 1.pdf (D110610736)

X X n X X n 1 2 1 1 1 2 2 2 20 20 620 20 310 570 20 285 ? ? ? ? ? ? ? ? . . . . . ? ? s X X n s X X n 1 1 1 2 1 2 2 2 2 2 1 43800 20 1 0480 1 24500 20 1 0359 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ( ) . . . ( ) . . . s s n s n X X 1 2 1 2 1 2 2 2 2 2 0480 20 0359 20 01340 ? ? ? ? ? ? . . . . t X X s df n n X X ? ? ? ? ? ? ? ? ? ? ? ? ? ? 1 2 1 2 1 2 310 285 01340 1866 2 20 20 2 38 . . . . . .

Critical 1-tail t 0 05 38 1689 . ( ) . . ? As the computed t exceeds the 1-tail critical t 0.05 , P is lower than 0.05 and considered too low. So, the H o is rejected and X – 1 is significantly higher than X – 2 (P > 0.05). 4.7.5. t test for small single-group experiments If the same set of animals is used once as group 1 and again as group 2, it is a single-group experiment with each individual animal having a pair of scores correlated with one another. But if the single group is small in size (n > 30), the correlation coefficient (product-moment r) cannot be worked out between the paired scores of the two groups for use in computing t. So, a difference method is used for the t test in such single-group experiments to avoid the necessity of computing the r. In the difference method, the difference D is worked out between the paired scores (X 1 and X 2) of each of the n number of animals in the group, and used in computing the mean difference (D –) and the standard deviation (s D) of the D scores. D – is transformed into t using the standard error ( )s D of D – . D X X D D n s D D n s s n D D D ? ? ? ? ? ? ? 1 2 2 1 ; ; ( ) ; ; ? ? t D s n D ? ? ? ; . df 1 The computed t is compared with critical t df ?( ) values. Only if the computed t

M-2\D:\Netaji 05\Biology-1\ either exceeds or equals the critical t ? , P is respectively lower than or equal to that ? and is considered too low — the difference between the means is then considered significant (P ?? ?).

76% **MATCHING BLOCK 32/108** **W**

Example 4.7.3. Find whether or not there is a significant change in the mean

O 2 consumption (ml/100g bodyweight) in the following sample of pigeons after their exposure to antimycin A. Animal : 1 2 3 4 5 6 7 8 9 10 Before (X 1) : 160 176 182 179 184 167 180 158 162 176 After (X 2) : 130 148 150 145 158 142 143 130 131 147 Critical 2-tail t scores : t 0.05(8) =2.306; t 0.05(9) =2.262; t 0.01(8) =3.355; t 0.01(9) =3.250; t 0.001(8) =5.041; t 0.001(9) =4.781. Solution : The data are entered in the first three columns of Table 4.4 and used for working out the mean and the sum of squares of the differences (D). n D D n s D D n D ? ? ? ? ? ? ? ? ? ? 10 300 10 300 1 120 10 1 365 2 . . . ( ) . . ? ? s s n t D s D D D ? ? ? ? ? 365 10 1154 300 1154 . . . . . = 25.997. df = n – 1 = 10 – 1 = 9. Table 4.4. Computation of sum of squares of differences. Animal X 1 X 2 D = X 1 –X 2 D – D – (D – D –) 2 1 160 130 30 0 0 2 176 148 28 – 2 4 3 182 150 32 + 2 4 4 179 145 34 + 4 16 5 184 158 26 – 4 16 6 167 142 25 – 5 25 7 180 143 37 + 7 49 8 158 130 28 – 2 4 9 162 131 31 + 1 1 10 176 147 29 – 1 1 ? — — 300 — 120 Critical 2-tail t 0.001(9) = 4.781.

M-2\D:\Netaji 05\Biology-1\ As the computed t exceeds the critical t for 0.001 level of significance, P is lower than 0.001. The P being too low, the H o is rejected, and it is inferred that the mean O 2 consumption has changed significantly on exposure to antimycin A (P > 0.001). 4.8 Chi square tests Chi square tests are nonparametric tests, because neither is any statistic computed earlier used as an estimate of any parameter in working out the chi square, nor is the latter referred to normal or t distributions for interpretation. In contrast to other tests described in this unit, the chi square test is an analysis of frequencies and not a test for significance of difference between means. Two principal types of chi square tests consist of tests for goodness of fit and tests for association. Chi squares (? 2) have continuous positively skewed distributions, differing in shape with degrees of freedom — for chi squares with df of 1 or 2, the distribition is L-shaped ; but with the rise in df, the distribution is unimodal with progressively declining positive skewness. The critical ? 2 is that score, beyond which the fractional area in only the asymptotic right tail of the distribution amounts to the level of significance (?). Chi squares are additive. 4.8.1. Assumptions for chi square tests For using chi square tests, it should be justifiable to assume that each case occurs in the sample by chance due to random sampling depending on laws of probability, and independent of other cases. Other than this, assumptions for continuous nature of variable, normal distribution in the population, and ability of scores being ranked are not required. So, these tests can be applied on continuous, discontinuous, ordinal or nominal varibles, large or small samples, and normal or non-normal distributions. 4.8.2. Chi square test for goodness of fit This test explores by analysis of frequencies whether or not a distribution of experimentally observed frequencies (f o) conforms significantly to a distribution of expected frequencies (f e) based on a proposed theoretical model such as normal, binomial, Poisson, equal-probability and Mendelian distributions. In this test, for each class interval of the f o distribution observed in the sample, the corresponding f e is first computed using the proportional distribution of cases as proposed by a theoretical model. The differences (f o –f e) between the observed and the expected frequencies are then used in working out the ? 2 score.

M-2\D:\Netaji 05\Biology-1\ ? 2 2 ? ? ? ( ) . f f f o e e The degrees of freedom of the computed chi sequare are determined by the difference between the total number (k) of classes of the f o distribution and the number of classes which have lost their freedom for change to keep the parameters of the proposed distribution and the sample size constant. Thus, the df of the computed ? 2 would be (k–1) to keep the sample size constant if the Mendelian phenotype distribution is the proposed one, (k–2) to keep the sample size and the ? constant when either binomial or Poisson distribution is used as the proposed distribution, and (k–3) to keep the sample size, ? and ? constant if the proposed distribution consists of a best-fitting normal distribution. Yates' correction has to be done to decrease the upward bias in the ? 2 to be computed, if the latter has the df of 1 and any one or more classes are lower than 5 in the value of f e . The correction consists of bringing the (f o –f e) difference of each class closer to zero by 0.5, by deducting 0.5 from each positive (f o –f e) and adding 0.5 to each negative (f o –f e). In effect, for each class, corrected ( ) . . f f f f o e o e ? ? ? ?05 In such cases, it is the corrected (f o –f e) of each class, which is used in computing the chi square. Thus, with corrected (f o –f e) values, ? 2 2 05 ? ? ? ? (.) . f f f o e e The H o proposes that the computed ? 2 is not significant, having resulted from random sampling only. To find the probability P of correctness of H o , the computed ? 2 is compared with the critical ? 2 with the computed df and for a chosen ?. The computed ? 2 is significant only if it either exceeds or equals the critical ? 2 (P ???), but is not significant if it is lower than the critical ? 2 (P < ?). Finally, only if the ? 2 is not significant, there is a significant goodness of fit between the observed and the proposed distributions; a significant ? 2 indicates no significant goodness of fit. Example 4.8.1.

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Find whether or not the following observed phenotype distribution in a sample of Drosophila has a significant goodness of fit with Mendelian 9 : 3 : 3 : 1 distribution. (? = 0.01.) Phenotypes : Grey body- Grey body- Black body- Black body- red eye (AB) scarlet eye (

Ab) red eye (aB) scarlet eye (ab) No. of flies : 104 33 35 20
M-2\D:\Netaji 05\Biology-1\ Critical values: ? ? ? 2 0 01 4 2 0 01 3 2 1328 1134 . ( ) . ( ) . ; . ; ? ? ? ? 0 01 2 2 0 01 1 2 9 21 6 64 . ( ) . ( ) . ; . . ? ? Solution : Total of phenotype proportions in Mendelian distribution : 9 + 3 + 3 + 1 = 16. Sample size (n) = 104 + 33 + 35 + 20 = 192. Expected proportion (p e) of each phenotype Mendelian proportion of the phenotype = ———————————————————— . Total of all phenotype proportions of Mendel Thus, (i) AB phenotype : p e ? ? 9 16 05625 . ; (ii) Ab phenotype : p e ? ? 3 16 01875 . ; (iii) aB phenotype : p e ? ? 3 16 01875 . ; (iv) ab phenotype : p e ? ? 1 16 00625 . . Expected frequency (f e) of each phenotype : f np e e ? . Thus, (i) AB phenotype : f np e e ? ? ? ? 192 05625 108 . . (ii) Ab phenotype : f np e e ? ? ? ? 192 01875 36 . . (iii) aB phenotype : f np e e ? ? ? ? 192 01875 36 . . (iv) ab phenotype : f np e e ? ? ? ? 192 00625 12 . . Table 4.5. Computation of chi square for goodness of fit. Phenotypes f o f e f o –f e (f o –f e) 2 ( ) f f f o e e ? 2 Grey-red 104 108 – 4 16 0.1481 Grey-scarlet 33 36 – 3 9 0.2500 Black-red 35 36 – 1 1 0.0278 Black-scarlet 20 12 + 8 64 5.3333 ? 192 192 — — 5.7592

M-2\D:\Netaji 05\Biology-1\ ? 2 2 576 45 1 4 1 3 ? ? ? ? ? ? ? ? ? ( ) . (.). f f f df k o e e Table . Critical ? 0 01 3 2 1134 . ( ) . . ? As the computed ? 2 is lower than the critical ? 2 for the given 0.01 level of significance, P < 0.01. So, the H o is retained and the computed ? 2 is not significant. Hence, there is a significant goodness of fit between the observed phenotype distribution and the Mendelian distribution. 4.8.3. Chi square test of association Also known as the chi square test of independence, this test explores whether or not there is any significant association between two variables. The data are arranged in this test in a contingency table presenting the relation between the two variables. The classes of one variable are arranged along the columns of the table and those of the other along its rows (Table 4.6). Each cell of the table would, therefore, house the observed frequency (f o) of cases belonging to a specific combination of two particular classes, one for each variable. Where r and c represent the numbers of respectively the rows and columns of the table for the f o scores, the df of the ? 2 would amount to : (r – 1)(c – 1). The total (f c) of the f o scores of each column and the total (f r) of those of every row are entered in the respective row and column for marginal totals. On the basis of the H o of no association between the variables, the f e scores of that many randomly chosen cells, as given by the df, are calculated using the f r and the f c of the respective cells and the sample size (n) : f e = f r f c /n. The f e scores of the remaining cells are worked out by subtracting the already obtained f e scores from either the f r or the f c of the respective cells. The f e score of each cell is entered in the table against the f o of that cell. The difference (f o – f e) between each f o and the corresponding f e is then used in computing the chi square. ? 2 2 1 1 ? ? ? ? ? ? ( ) ; ( )( ) f f f df r c o e e . Yates' correction is done in the way described in Sub-section 4.8.2 to lower the upward bias of ? 2 , if its df amounts to 1 and any f e is found to be less than 5 in amount. In such a case, corrected ? 2 2 0 5 ? ? ? ? (.) . f f f o e e The H o contends that the computed ? 2 is not significant and has resulted merely from random sampling. To find the probability P of the H o being correct, the computed ? 2 is compared with the critical ? ?( )df 2 for a chosen significance level. Only if the

M-2\D:\Netaji 05\Biology-1\ computed ? 2 either exceeds or equals the critical ? 2 , the H o is rejected and it is inferred that there is a significant association between the variables (P ???); if the critical ? 2 exceeds the computed one, there is no significant association between the variables and they are significantly independent of one another (P < ?). Example 4.8.2. Out of 40 diabetic monkeys, 20 were hypertensive while out of 60 nondiabetic ones, 15 were hypertensive.

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Find whether or not there is a significant association between

diabetes and hypertension. (? = 0.02.) Critical ? ? ? 2 0 02 3 2 0 02 2 2 984 782 scores: . ; . ; . ( ) . ( ) ? ? ? ? 0 02 1 2 0 02 4 2 541 1167 . ( ) . ( ) . ; . . ? ? Solution : Table 4.6. Contingency table for test of association. Nonhypertensive f f o e Hypertensive f f o e f r Diabetic 20 26 20 14 40 Nondiabetic 45 39 15 21 60 f c 65 65 35 35 100 (n) r = 2. c = 2. df = (r – 1)(c – 1) = (2 – 1)(2 – 1) = 1. Because the df amounts to 1, only one cell — that for diabetic-hypertensive — is chosen for direct computation of f e . (See Table 4.6.) f f f n e r c ? ? ? ? 40 35 100 14 . The f e scores of other cells are worked out by subtracting the already obtained f e score(s) from either the f r or the f c of the respective cells. For example, for the cell for diabetic-nonhypertensive class, f e = (f r of that cell) – (f e of diabetic-hypertensive cell) = 40 – 14 = 26. ? 2 2 2 2 2 2 20 26 26 20 14 14 45 39 39 15 21 21 659 ? ? ? ? ? ? ? ? ? ? ? ( ) ( ) ( ) ( ) ( ) . . f f f o e e Critical ? 0 02 1 2 541 . ( ) .? Because the computed ? 2 exceeds the critical ? 0 02 2 . , P is considered too low. So, the H o is rejected. It is, therefore, inferred

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that there is a significant association between diabetes and hypertension (

P > 0.02).

M-2\D:\Netaji 05\Biology-1\ 4.9 Summary Significance tests are undertaken to find whether the experimental result is genuine and can be generalized in the population, or whether the result can be explained by sampling errors. For any investigation using a sample, the null hypothesis (H o) contests the alternative hypothesis (H a) being explored, and proposes that the observed result is not significant and comes from the use of a random sample. Only if a significance test indicates that the probability (P) of correctness of H o does not surpass a chosen probability level called the level of significance (?), the obtained result is considered significant and accepted for the entire population. Any such inference is, however, subject to a type I error of inference for the wrong rejection of a correct H o . The type I error can be reduced by using a lower significance level (?) in interpreting the result. A difference between the means of two samples, exposed to two different levels of an independent variable, may be transformed into the standard z score which is referred to the unit normal curve to find the P of correctness of H o for drawing an inference about the significance of the difference. A difference between two means may also be transformed into Student's t which is compared with critical t scores for different significance levels (?) to draw the inference regarding the significance of the difference. Chi square test is a nonparametric analysis of frequencies often used in finding whether or not an observed frequency distribution has a significant goodness of fit with an expected frequency distribution worked out on a proposed theoretical model. Chi square test may also be used for exploring whether or not two variables have a significant association. 4.10 Terminal questions 1.(a) Write the assumptions for t tests. (b) Describe mentioning mathematical formulae, how you would work out the t test for a small single-group experiment. (c) Work out Student's t test to find whether or not the exposure to an arsenic compound has produced significant changes in the body weights (g) of the following sample of crabs. (? = 0.05.) Animal : 1 2 3 4 5 6 7 8 9 10 Before (X 1) : 5.0 4.5 5.5 4.8 6.0 5.8 5.6 5.2 4.7 4.7 After (X 2) : 4.0 3.7 4.5 3.7 4.8 4.3 4.1 3.9 3.8 3.6 Critical t scores : t t 0 05 19 0 05 9 2093 2262 . ( ) . ( ) . ; . ; ? ? t t 0 05 18 0 05 8 2101 2306 . ( ) . ( ) . ; . . ? ? 2.(a) Describe the assumptions for using z scores. (b) Discuss how the z score is worked out and interpreted in finding a significance

M-2\D:\Netaji 05\Biology-1\ of difference between two sample means, mentioning all computational formulae. (c) Work out the z score using the following body weight data of two samples of fishes to find whether or not the sample means differ significantly. (? = 0.05.) Sample 1 : X – 1 = 78.0 g ; s 1 = 8.50 g ; n 1 = 36. Sample 2 : X – 2 = 74.4 g ; s 2 = 6.75 g ; n 2 = 49. z scores : 2.08 2.09 2.10 2.11 2.12 Areas of normal curve : 0.4812 0.4817 0.4821 0.4826 0.4830 3.(a) Write briefly about the following : (a) Null hypothesis, (b) Levels of significance. (c) Errors of inference. (d) Yates' correction. (e) Critical scores. 4.(a) Describe, mentioning all computational formulae, how to work out Student's t test for small independent groups. (b) Discuss where t test can be used and where not, describing the assumptions for t tests. (c)

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Work out the t test to find whether or not the mean gill weights (mg) differ in the following two small samples of crabs from two habitats. (? = 0.05.) Group 1 (X 1) : 100, 80, 75, 65, 75, 110, 95, 78, 92, 80. Group 2 (X 2) : 77, 63, 70, 68, 62, 55, 75, 70, 60, 60. Critical t scores : t t 0 05 19 0 05 18 2093 2101 . ( ) . ( ) . ; . ; ? ? t t 005 9 005 8 2262 2306 . ( ) . ( ) . ; . . ? ? 5.(

a) Discuss the assumptions for chi square tests. Mention important properties of chi squares. (b) Describe, mentioning the relevant formulae, how to compute chi square for goodness of fit and interpret it. (c) Work out chi square test to find whether or not the following observed frequency (f o) distribution of serum iron concentrations (?g/dl) in a given sample of chimpanzees has a significant goodness of fit with the expected frequency (f e) distribution computed from the normal probability distribution. (? = 0.05.) Class intervals f o f e 101-110 5 4 111-120 10 10 121-130 12 15 131-140 18 20 141-150 15 16 151-160 14 10 161-170 6 5

M-2\D:\Netaji 05\Biology-1\ Critical ? 2 scores : ? ? 0 05 1 2 0 05 2 2 384 599 . ( ) . ( ) . ; . ; ? ? ? ? ? 0 05 3 2 0 05 4 2 0 05 5 2 782 949 1107 . ( ) . ( ) . ( ) . ; . ; . . ? ? ? 6. (a) Describe how you would work out the chi square test of association and interpret the computed chi square to draw an inference. (b) Discuss the difference between the computations of degrees of freedom for chi square test of association and different chi square tests for goodness of fit. (c)

Out of 55 hypercholesterolemic cases, 25 suffer from hypertension while out of 45 cases with normal serum cholesterol, 15 are hypertensive. Use chi square test to find whether or not there is a significant association between hypercholesterolemia

and hypertension. (? = 0.05.) Critical ? 2 scores : ? ? 0 05 1 2 0 05 2 2 384 599 . ( ) . ( ) . ; . ; ? ? ? ? ? 0 05 3 2 0 05 4 2 0 05 5 2 782 9 49 1107 . ( ) . ( ) . ( ) . ; . ; . . ? ? ? 4.11 Answers 1. (a) See Sub-section 4.7.1. (b) See Sub-section 4.7.5. (c) See Example 4.7.3. 2. (a) See Sub-section 4.6.1. (b) See Sub-section 4.6.2. (c) See Example 4.6.1. 3. (a) See Section 4.3. (b) See Section 4.4. (c) See Section 4.5. (d) See Sub-sections 4.8.2 and 4.8.3. (e) See Sub-section 4.7.2. 4. (a) See Sub-section 4.7.3. (b) See Sub-section 4.7.1. (c) See Example 4.7.1. 5. (a) See Sub-section 4.8.1 and second paragraph of Section 4.8. (b) See Sub-section 4.8.2. (c) See Example 4.8.1. 6. (a) See Sub-section 4.8.3. (b) See second paragraph of Sub-section 4.8.3 and third paragraph of Sub-section 4.8.2. (c) See Example 4.8.2.

M-2\D:\Netaji 05\Biology-1\ Unit 5 ? ANALYSIS OF VARIANCE 5.1 Introduction For any experiment done with a sample to substantiate a specific idea called the alternative hypothesis (H a), the null hypothesis (H o) contends that the experimental results have been obtained because of chances associated with random sampling, would not occur on using the population instead of a sample, and are consequently not significant. You have learnt earlier that Student's t tests may be performed to find whether or not there are significant differences between sample means. In the present unit, you will learn about analysis of variance (anova), Kruskal-Wallis test and multiple comparison tests, which are more powerful alternatives to the t tests. Objectives After studying this unit, you should be able to do the following : ? understand the importance of variances in biostatistics, ? know why the anova is preferable to t test, ? distinguish between different ways and models of anova, ? learn about the assumptions for anova and judge where to apply the latter, ? work out one-way anova by computing the F ratio and finding its significance, ? follow up a significant F ratio by working out omega square in model I anova and added variance component in model II anova, ? follow up a significant F ratio in anova for more than two groups, with Scheffe's F test for multiple comparison between chosen group means, Structure 5.1 Introduction Objectives 5.2 Analysis of variance 5.3 One-way anova 5.4 Multiple comparison Scheffe's F test 5.5 Kruskal-Wallis nonparametric anova 5.6 Multiple comparison Mann-Whitney U test 5.7 Summary 5.8 Terminal questions 5.9 Answers

M-2\D:\Netaji 05\Biology-1\ ? work out Kruskal-Wallis nonparametric anova where assumptions for parametric anova are not justifiable, and ? follow up a significant Kruskal-Wallis test with more than two groups, by Mann-Whitney multiple comparison U test between chosen group means. 5.2 Analysis of variance You are aware that statistics like means and variances of the scores of a dependent variable differ from group to group from the same population, owing to their varying sampling errors, even when not affected by any independent variable. In addition, if different groups are exposed to different levels (magnitudes, intensities, concentrations, etc.) of an independent variable affecting the dependent one, statistics like the variance of the latter would differ further between the groups than what may result from their sampling errors. You have learnt earlier that Student's t test for differences between group means may indicate whether or not such differences can be explained away by the sampling errors of the means, and may thus help in inferring whether or not the group means differ significantly. In the present unit, a more powerful and widely applicable test, viz., the analysis of variance (anova), will be offered for testing simultaneously the differences in variances of the dependent variable scores between two or more groups, exposed to different levels of independent variables, for inferring if the dependent variable has changed significantly on exposure to the latter. 5.2.1. Variances in anova You may recall that variance or mean square (s 2 or MS) is the squared standard deviation of scores of a sample from its mean, and serves as a better absolute measure of dispersion of the scores around the sample mean (see paragraph (b) of Sub-section 2.6.3). It may be defined as the mean of squared deviations of scores from the sample mean, i.e., as the mean of the sum of squares (SS), although it is now usually worked out using the df instead of the sample size to minimize its downward bias, particularly in small samples : s 2 = SS/df = ? (X–X –) 2 /(n – 1). Variances of different groups/samples, drawn initially from the same population, possess homogeneity (homoscedasticity); they differ only to limited extents because of random variations of scores in different groups and owing to their differing sampling errors only, and function as estimates of the same population variance (? 2). But if the scores of different groups are affected by their subsequent exposure to different levels of an independent variable, their variances become heterogeneous (heteroscedastic), differing from each other by far more than their differing sampling errors and behaving now like estimates of parametric variances of different populations. In the experimental data from two or more groups, the total variance ( )s t 2 is the M-2\D:\Netaji 05\Biology-1\ variance of dependent variable scores of all the groups from their grand mean (X –). The total variance may be computed from the total sum of squares (SS t) which is the sum of the squared deviations, viz., ? (X i – X –) 2 , of the scores (X i) of all groups from the grand mean (X –). Where N is the total number of scores or cases of all the groups, SS X X s SS N df N t i t t t ? ? ? ? ? ? ?( ) ; ; . 2 2 1 1 In an experiment, all the cases of a group are exposed to the same specific level of independent variable while different groups are exposed to different specific levels of the latter. Scores of each group differ from the mean (X – i) of that group by their respective error terms, viz., (X i –X – i), because of random variation of each score from the group mean, but not due to the effect of independent variable as all the cases in a group are exposed to the same level of the latter. The within-groups variance ( )s w 2 is the variance of dependent variable scores of all groups from their respective group means and is worked out from the within-groups sum of squares (SS w), viz., ? (X i – X – i) 2 . Where k is the number of groups, SS w = ? (X i – X – i) 2 ; s SS N k w 2 ? ? ? ; df w =N – k. The between-groups variance ( )s b 2 is the variance of scores belonging to different groups and is computed from the between-groups sum of squares (SS b). The latter is worked out as the sum of the products of the respective group sizes (n i) with the squared differences between the respective group means (X – i) and the grand mean (X –). The between-groups variance may result from two factors, viz., random variations of scores from their respective group means — given also by the within-groups variance — and an added variance between the groups due to the exposure of different groups to different levels of independent variable. The added variance factor would be absent from the between-groups variance if the independent variable has not produced any change in the dependent variable scores. SS n X X s SS k df k b i i b b b ? ? ? ? ? ? ? ( ) ; ; . 2 2 1 1 Anova resolves the total variance ( )s t 2 into the between-groups variance ( )s b 2 and the within-groups variance ( )s w 2 by partitioning the total sum of squares (SS t) into the between-groups sum of squares (SS b) and the within-groups sum of squares (SS w). The variance ratio (F ratio) is then computed using s b 2 and s W 2 : . The computed F is then compared with the critical F value (F ?) for a chosen significance level (?), ?

M-2\D:\Netaji 05\Biology-1\ for finding whether or not there is any significant difference between the groups (see Sub-section 5.3.3). F s s df F df df k N k b w b w ? ? ? ? 2 2 1 ; : , , . of ratio Inference is made as follows : (i) if computed F ? F ? , P ? ? and H o is rejected; (ii) if computed F > F ? , P < ? and H o is retained. 5.2.2. Reasons for preferring anova So long as the assumptions for anova are justifiable, the latter should be preferred to Student's t test and its alternatives for finding the significance of difference between group means, because of the following reasons. (i) Anova can be applied at a time to any number of groups, two or more, to search simultaneously for any significant difference between any or all pairs of groups. (ii) A significant F ratio in anova may be followed up by working out either the strength of association between dependent and independent variables in model I anova, or the added variance component in the between-groups variance in model II anova. (iii) Because of its strong assumptions, anova is much more powerful than t test and its other alternatives. (iv) Its strong assumptions require meticulous pre-planning and scientific designing of the experiment, which eliminate many experimental errors. 5.2.3. Classes of anova According to the number of independent variables in the experiment, different classes or ways of anova are to be used with respective experimental designs, statistical treatments and interpretations. (i) A one-way anova is applied if the effect of a single independent variable is being investigated. For example, a one-way anova would be worked out to explore the significance of difference in the tracheal ventilation of two groups of grasshoppers treated with two respective levels of a pesticide, the latter being the only independent variable in this experiment. (ii) A two-way anova has to be used if the groups are exposed to combinations of two different independent variables. For example, a two-way anova is undertaken for the difference in serum Ca 2+ levels of three groups of hermit crabs administered three respective levels of combinations of two independent variables, viz., doses of parathormone and growth hormone. (iii) A three-way of anova is used where three independent variables have been applied on the groups under study. 5.2.4. Models of anova Three alternative models of anova are chosen from, according to the natures of the independent variables. (i) Model I or fixed model anova is applied to explore the ? ? 2 M-2\D:\Netaji 05\Biology-1\ significance of change in a dependent variable, when exposed to the chosen levels of one or more fixed experimental treatments. For example, a one-way model I anova is undertaken for the change in tracheal ventilation of locusts on administration of three chosen levels of an insecticide whose application is strictly under control of the investigator. In this model, a significant F ratio indicates the existence of a cause- effect relationship between the dependent variable and the fixed experimental treatment, and enables the working out of the strength of association between the two variables. (ii) Model II or random model anova is used when the dependent variable is deemed to be affected by one or more random variables beyond the control of the investigator. For example, a one-way model II anova has to be used to explore the difference in blood hemoglobin concentration between the two sexes, because sex is a variable beyond the control of the investigator in this experiment. Cause-effect relation and strength of association cannot be explored here between the two types of variables, because the independent one suffers from random errors beyond the control of the investigator. Instead, an added variance component may be worked out as a follow- up of a significant F ratio in a model II anova. Similarly, a two-way model II anova is used for changes in blood thyroxine level on exposure of the groups to changes of both atmospheric temperature and humidity. (iii) Model III or mixed model anova is applied when exploring the change of a dependent variable in the groups exposed to chosen levels of a fixed experimental treatment and different levels of a random variable at the same time. An example is the two-way model III anova for change in arterial O 2 tension on exposure to the fixed chosen levels of O 2 tension in inhaled gas mixture and the prevailing blood hemoglobin concentrations of the subjects — the Po 2 of inhaled gas mixture and the blood hemoglobin are respectively fixed treatment and random variables. 5.2.5. Assumptions for anova Numerous rigorous assumptions for anova make it more powerful as a test and also serve to decrease experimental errors by requiring a well-planned experimental design. (a) Each score of the dependent variable — stated otherwise, each error term of the latter — should occur at random in any group obeying the laws of probability, to ensure that the groups are representative of the population. (b) Each error term should occur in the group, independent of the occurrence of any other error term in it. (c) Error terms of the dependent variable should have a normal distribution in the population. (d) Initially, all the groups to be used in the experiment should have homogeneous

M-2\D:\Netaji 05\Biology-1\ variances or homoscedasticity to ensure that the group variances are different estimates of the same population variance. (e) To avoid the order effect of application of the independent variable, different levels of the latter should be applied to different cases or individuals of the sample/ group in randomly varying orders instead of an identical sequence. (f) The total variation of any score of the dependent variable should arise from the additivity of its numerous variations owing to various factors including random relevant variables and the independent variable. This justifies the partitioning of the total sum of squares into its components like between-groups and within-groups sums of squares during anova. 5.3 One-way anova One-way anova is worked out for testing the significance of change in a dependent variable on exposure of the groups of cases to different respective levels of a single independent variable. Computation and interpretation of a one-way anova depend on the number of groups, i.e., on the number of levels of the independent variable used in the experiment, and also on the model of the anova, i.e., on the nature of the independent variable. You should recall that the number of groups used in an experiment corresponds to the number of levels of the independent variable, because each specific level of the latter would be applied to all the cases of a particular group only. Moreover, a one-way anova may belong to either model I if the independent variable is a fixed experimental treatment under the investigator's control, or model II if the independent variable is beyond the control of the investigator and suffers from random changes. A one-way anova is worked out in the following steps. (a) First, sums of squares are partitioned and used in working out the respective variances and their ratio (F ratio); the latter is compared with a critical F value for significance. (b) If the computed F turns out to be significant, the next step is to work out either the strength of association in case of model I anova, or the added variance component in case of model II anova. (c) If the number of groups exceeds two in the relevant experiment, the next step is a multiple comparison test

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to find whether or not there is any significant difference between the

means of every chosen pair of groups. 5.3.1. Partitioning of sums of squares To partition the total sum of squares (SS t) into between-groups and within-
M-2\D:\Netaji 05\Biology-1\ groups sums of squares (SS b and SS w) in a one-way anova with k number of groups, having n 1 , n 2 , n 3 , ...n k as the respective group sizes, X 1 , X 2 ,.....X k as the respective group scores, X – 1 , X – 2 ,..... X – k , as the respective group means, and X – as the grand mean,
N n n
n

30% **MATCHING BLOCK 41/108** **SA** MSPS 13 L part 1.pdf (D110610736)

X X X X N k k ? ? ? ? ? ? ? ? 1 2 1 2 .... ? ? ? . SS X X X X X X t k ? ? ? ? ? ? ? ? ? ? ( ) ( ) ..... ( ) ; 1 2 2 2 2 or, SS X X X X X X N t k k ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 1 2 2 2 2 1 2 2 ..... (.....) . df N t ? ?1. SS n X X n X X n X X b k k ? ? ? ? ? ? ? 1 1 2 2 2 2 2 ( ) ( ) ..... ( ) , or, SS X n X n X n X X

X
N
b k

k k ? ? ? ? ? ? ? ? ( ) ( ) ..... ( ) (....) ? ? ? ? ? ? ? 1 2 1 2 2 2 2 1 2 2 . df k b ? ?1. SS X X X X X X w k k ? ? ? ? ? ? ? ? ? ? ( ) ( ) ..... ( ) 1 1 2 2 2 2 2 ; or, SS SS SS df N k w b w t ? ? ? ? . . 5.3.2. Computation of variances and F ratio Between-groups and within-groups variances ( ) s s b w 2 2 and are next computed by dividing the respective sums of squares by their degrees of freedom. The within- groups variance ( )s w 2 is used as the error variance in one-way anova to work out the F ratio. s SS df SS k s SS df SS N k F s s b b b b w w w w b w 2 2 2 2 1 ? ? ? ? ? ? ? ? ; ; ; df F df df k N k b w of : , , . ? ? ? 1 5.3.3. Significance test of computed F The H o proposes that the computed F is not significant, has resulted from the use of a sample drawn by random sampling, and would not differ significantly from 1.00 if the entire population is used for the experiment. To test the significance of the computed F, the latter is compared with the critical F ? value (df : k – 1, N – k) for a chosen level of significance. If the computed F either exceeds or equals the critical F ? , the probability (P) of the H o being correct is considered too low (P ? ?), the H o is rejected and there is some significant difference between the group means; in other words, there is a significant added
M-2\D:\Netaji 05\Biology-1\ variance between the groups, which is not present within the groups. But if the critical F ? exceeds the computed F value, P exceeds ? and there is no significant difference between the group means. (See Example 5.3.1.) Example 5.3.1. Work out a

62% **MATCHING BLOCK 40/108** **W**

one-way anova to find whether or not there is any significant difference between the mean tracheal ventilation scores (ml/minute) of the following two groups of locusts from two different habitats. (? = 0.05.)

Group 1 (X 1) : 75, 78, 80, 75, 81, 85, 82, 78, 80, 86. Group 2 (X 2) : 71, 72, 70, 70, 73, 74, 73, 65, 70, 82. Critical F scores : F F 0 05 2 19 0 05 1 18 352 441 . (,) . (,) . ; . ; ? ? F F 0 05 1 19 0 05 2 18 438 355 . (,) . (,) . ; . . ? ? Solution :

64% **MATCHING BLOCK 44/108** **SA** Statistics for Business Course Document spring ... (D14852953)

N n n ? ? ? ? ? 1 2 10 10 20. SS X X X X N t ? ? ? ? ? ? ? ? ? ? ? ? ? ? 1 2 2 2 1 2 2 2 64124 52008 720 20 612 ( ) (800) . SS X n X n X X

N
b ? ? ? ? ? ? ? ? ? ( ) ( ) ( ) (800) . ? ? ? ? 1 2 1 2 2 2 1 2 2 2 2 2 800 10 720 10 720 20 320 df k b ? ? ? ? ? 1 2 1 1. SS SS SS df N k w b w t ? ? ? ? ? ? ? ? ? ? 612 320 292 20 2 18 . . Table 5.1. One-way anova between two groups. X 1 X 2 X 1 2 X 2 2 75 71 5625 5041 78 72 6084 5184 80 70 6400 4900 75 70 5625 4900 81 73 6561 5329 85 74 7225 5476 82 73 6724 5329 78 65 6084 4225 80 70 6400 4900 86 82 7396 6724 ? 800 720 64124 52008
M-2\D:\Netaji 05\Biology-1\ s SS df s SS df F s s b b b w w w b w 2 2 2 2 320 1 320 292 18 1622 320 1622 1973 ? ? ? ? ? ? ? ? ? ? . . . . . df of F : k – 1, N – k = 1, 18. Critical F 0.05(1,18) = 4.41. As the computed F is higher than critical F 0.05 , P is too low. So, H o is rejected. Hence, there is a significant difference between the group means (P > 0.05). As the independent variable, viz., habitat, is a random variable beyond the control of the investigator, the added variance component ( )s a 2 is computed. (See Sub-section 5.3.5.) Using the identical size (n) of each group, n s s s n a b w ? ? ? ? ? ? ? 10 320 1622 10 3038 2 2 2 ; . . . Therefore, an added variance component of 30.38, absent within the groups, occurs between the groups. 5.3.4. Strength of association If the preliminary F test yields a significant F ratio and the anova undertaken belongs to model I using a fixed experimental treatment as the independent variable, then the strength of association has to be worked out between dependent and independent variables as the omega square (? 2), irrespective of the anova being worked out for two or more groups. Using the number (k) of groups in the experiment, the total (N) of all the group sizes and the computed F value, ? 2 1 1 1 1 ? ? ? ? ? ? ? ( )( ) ( )( ) . k F N k F Omega square is not to be computed if the F ratio turns out to be not significant, or if a model II anova is being done. (See Example 5.4.1.) 5.3.5. Added variance component In case a model II anova yields a significant F ratio, the added variance component ( )s a 2 has to be worked out, irrespective of the anova being worked out for two or more groups. It serves as an estimate of such variance due to random variable(s) as is a part of the between-groups variance ( )s b 2 , but not a part of the within-groups variance ( )s w 2 . When all the groups have an identical size (n) , s s s n a b w 2 2 2 ? ? .

M-2\D:\Netaji 05\Biology-1\ If the F ratio is not significant, or if a model I anova has been undertaken, s a 2 is not to be computed. (See Example 5.3.1.) 5.4 Multiple comparison Scheffe's F test In case of a one-way anova with more than two groups (k < 2), a significant F ratio has to be followed up by a multiple comparison test to find whether or not the means of two groups of each chosen pair differ significantly. The multiple comparison test has to be worked out separately for each chosen pair of groups and has, therefore, to be repeated as many times as the number of chosen pairs. Multiple comparison tests need not be done if the F ratio has turned out to be not significant, or if the one- way anova has been undertaken between only two groups. Frequently, either the t test or the F test of Scheffe is carried out for multiple comparison. Multiple comparison Scheffe's F test, more powerful and preferable of the two, is briefly described below. For Scheffe's F test between the means (X – 2 and X – 3) of groups 2 and 3, for example, the standard error of difference ( ) s X X 2 3 ? between those means is first worked out using the within-groups variance ( )s w 2 computed in the earlier preliminary F test. The squared SE of difference is then used in transforming the squared difference between the means into the Scheffe's F score. s s n s n F X X s F X X s n s n X X w w X X w w 2 3 2 3 2 2 2 3 2 3 2 2 2 3 2 2 2 2 3 ? ? ? ? ? ? ? ? ? ; ( ) , ( ) . or The computed Scheffe's F is then compared with the critical F ? ' value for the chosen level of significance; the critical F ? ' has to be worked out in turn from the critical F ? value as follows. Where k is the number of groups, F k F k N k ? ? ' ? ? ? ? ( ) . (,) 1 1 Only if the computed Scheffe's F exceeds or equals the critical F ? ' , the probability P of the H o being correct is considered too low (P ???), the H o is rejected, and the two means being tested are considered to differ significantly. (See Example 5.4.1.) Example 5.4.1. (a) Work out a

67% **MATCHING BLOCK 42/108** **W**

one-way anova to find whether or not there is a significant difference between the mean tracheal ventilations (ml/minute) of the following

group of cockroaches, treated with three different levels of a pesticide. (? = 0.01.)
M-2\D:\Netaji 05\Biology-1\ (b) If the preliminary F test shows significant differences, find whether or not the means of the first and second group differ significantly. (? = 0.01.) Animal : 1 2 3 4 5 6 7 8 9 10

70% **MATCHING BLOCK 43/108** **W**

X 1 ml : 90 120 90 120 80 97 100 97 130 86 X 2 ml : 55 70 54 68 35 60 60 58 80 40 X 3 ml : 28 30 25 35 15 30 38 28 50 21 Critical F values : F F 0 01 2 26 0 01 2 27 553 549 . (,) . (,) . ; . ; ? ? F F F 0 01 2 28 0 01 1 29 0 01 1 26 545 418 422 . (,) . (,) . (,) . ; . ; . . ? ? ? Solution :

Table 5.2. One-way anova between three groups.

71% **MATCHING BLOCK 47/108** **SA** MSPS 13 L part 1.pdf (D110610736)

X 1 X 2 X 3 X 1 2 X 2 2 X 3 2 90 55 28 8100 3025 784 120 70 30 14400 4900 900 90 54 25 8100 2916 625 120 68 35 14400 4624 1225 80 35 15 6400 1225 225 97 60 30 9409 3600 900 100 60 38 10000 3600 1444 97 58 28 9409 3364 784 130 80 50 16900 6400 2500 86 40 21 7396 1600 441 ? 1010 580 300 104514 35254 9828 N n n n X X n ? ? ? ? ? ? ? ? ? ? ? 1 2 3 1 1 1 10 10 10 30 1010 10 1010 . . . ? X X n X X n 2 2 2 3 3 3 580 10 580 300 10 300 ? ? ? ? ? ? ? ? . . . . SS X X X X X X

N
t ? ? ? ? ? ? ? ? ? ? ? ? ? 1 2 2 2 3 2 1 2 3 2 ( ) ,
M-2\D:\Netaji 05\Biology-1\ or, SS t ? ? ? ? ? ? ? 104514 35254 9828 1010 580 300 30 30526 2 ( ) . SS X n X n X n X X X N b ? ? ? ? ? ? ( ) ( ) ( ) ( ) ? ? ? ? ? ? ? 1 2 1 2 2 2 3 2 3 1 2 3 2 ? ? ? ? ? ? ? 1010 10 580 10 300 10 1010 580 300 30 25580 2 2 2 2 ( ) . df k b ? ? ? ? ? 1 3 1 2 . SS SS SS df N k w b w t ? ? ? ? ? ? ? ? ? ? 30526 25580 4946 30 3 27 . . s SS df s SS df b b b w w w 2 2 25580 2 12790 4946 27 18319 ? ? ? ? ? ? . . . F s s df F df df b w b w ? ? ? ? 2 2 12790 18319 6982 2 27 . . . : , , . for Critical F 0.01(2,27) =5.49.

60% **MATCHING BLOCK 45/108** **W**

As the computed F exceeds the critical F 0.01 , P is too low (P > 0.01). So H o is rejected and

there are significant differences between the group means. Because the independent variable, viz., chosen levels of the pesticide, is a fixed treatment variable, omega square is computed for the strength of association between the dependent and independent variables. ? 2 1 1 1 1 3 1 6982 1 30 3 1 6982 1 082 ? ? ? ? ? ? ? ? ? ? ? ? ? ( )( ) ( )( ) ( )(.) ( )(.) . k F N k F . Thus, 0.82 proportion of the total variance of the dependent variable is associated with the independent variable. As the preliminary F test yielded a significant F score, the difference (X – 1 – X – 2) is subjected to the multiple comparison Scheffe's F test to find its significance. s s n s n X X w w 1 2 2 1 2 2 18319 10 18319 10 6053 ? ? ? ? ? ? . . . . F X X s X X ? ? ? ? ? ? ( ) (. .) . . . 1 2 2 2 2 2 1 2 1010 580 6053 5047
M-2\D:\Netaji 05\Biology-1\ F k F F df df b w 0 01 0 01 0 01 2 27 1 3 1 . . (,) . (,) ( ) ( ) ' ? ? ? ? = (3–1) × 5.49 = 10.98. As the computed F exceeds the critical F 0 01. ' , P is too low. So, the H o is rejected and there is a significant difference between X – 1 and X – 2 (P > 0.01). Example 5.4.2. Work out a

100% **MATCHING BLOCK 46/108** **W**

one-way anova to find whether or not there is a significant difference between the

blood sugar scores (mg/dl) of a group of alloxan-diabetic monkeys treated with two different levels of a hypoglycemic agent. (? = 0.01.) Animal : 1 2 3 4 5 6 7 8 9 10 X 1 mg : 160 180 200 170 197 220 162 180 201 190 X 2 mg : 110 140 160 125 162 168 120 135 185 165 Critical F values : F F 0 01 2 19 0 01 1 19 593 818 . (,) . (,) . ; . . ? ? F F 0 01 1 18 0 01 2 18 828 601 . (,) . (,) . ; . . ? ? Solution : Table 5.3. One-way anova between two groups.

52% **MATCHING BLOCK 50/108** **SA** Statistics for Business Course Document spring ... (D14852953)

X 1 X 2 X 1 2 X 2 2 160 110 25600 12100 180 140 32400 19600 200 160 40000 25600 170 125 28900 15625 197 162 38809 26244 220 168 48400 28224 162 120 26244 14400 180 135 32400 18225 201 185 40401 34225 190 165 36100 27225 ? 1860 1470 349254 221468 N = n 1 + n 2 = 10 + 10 = 20. SS X X X X N t ? ? ? ? ? ? ? ? ? ? ? ? ? ? 1 2 2 2 1 2 2 2 349254 221468 1860 1470 20 16277 ( ) ( ) . M-2\D:\Netaji 05\Biology-1\ SS X n X n X X

N
b ? ? ? ? ? ? ? ? ? ( ) ( ) ( ) ( ) . ? ? ? ? 1 2 1 2 2 2 1 2 2 2 2 2 1860 10 1470 10 1860 1470 20 7605 df k b ? ? ? ? ? 1 2 1 1. SS SS SS df N k w b w t ? ? ? ? ? ? ? ? ? ? 16277 7605 8672 20 2 18 . . s SS df s SS df b b b w w w 2 2 7605 1 7605 8672 18 48178 ? ? ? ? ? ? . . . F s s df F df df b w b w ? ? ? ? 2 2 7605 48178 1579 1 18 . . . : , , of . Critical F 0 01 1 18 828 . (,) . . ? As the computed F is higher than critical F 0.01 , P > 0.01. So, H o is rejected and the group means differ significantly. Because the independent variable consists of chosen levels of a hypoglycemic agent, it is a fixed treatment variable. So, omega square is computed as the measure of the strength of association between the dependent and independent variables. ? 2 1 1 1 1 2 1 1579 1 20 2 1 1579 1 043 ? ? ? ? ? ? ? ? ? ? ? ? ? ( )( ) ( )( ) ( )(.) ( )(.) . k F N k F . Thus, 0.43 proportion of the total variance of the dependent variable is associated with the independent variable. 5.5 Kruskal-Wallis nonparametric anova Kruskal-Wallis nonparametric anova is a rank-dependent one-way anova for two or more groups. It is a useful alternative to the parametric method of one-way anova, with few assumptions, wide applications and easy computation. It can be used when the assumptions for the parametric anova are not justifiable. But it is less powerful than the parametric anova. 5.5.1. Assumptions for Kruskal-Wallis anova Being a rank-dependent method, Kruskal-Wallis anova may be worked out for ordinal variables, and also for continuous or discontinuous variables, after ranking the cases of the groups in a composite manner (see Sub-section 5.5.2). Following assumptions should be justifiable for computing the Kruskal-Wallis H. (i) The dependent variable should be either an ordinal variable with the cases of the groups already expressed in ranks, or a continuous or discontinuous measurement variable whose scores can be converted into ranks. (ii) Each score/rank or its error term

M-2\D:\Netaji 05\Biology-1\ should occur at random depending on laws of probability and independent of all other scores/ranks or error terms. (iii) No assumption is needed for the continuous nature of the variable. (iv) No assumption is necessary for the normality of distribution of its scores or error terms. Kruskal-Wallis test connot be applied to nominal variables as ranks cannot be given to them. Moreover, this test is less powerful than the parametric anova and should, therefore, be used only if the assumptions for the latter are seen not to be justifiable. 5.5.2. Computation of Kruskal-Wallis H Ranks are first given in an ascending order and a composite manner to the scores or ranks of all the k number of groups taken together. Even if the variable is an ordinal one with the cases already ranked separately in each group, fresh composite ranking must be done again taking all the groups together. Identical scores, whether occurring in the same group or in separate groups, constitute a tied set; each member of a tied set is given an average rank identical with the arithmetic mean of the ranks the tied cases would have got if they were separate consecutive scores. Moreover, the case coming next to a tied set is given that rank which it would have got if it followed a separate untied case. You may realise that Kruskal-Wallis anova would be less powerful and more prone to error because of (i) the average rank given to the cases of each tied set, and (ii) the unequal differences between successive scores ranked consecutively. After the composite ranking of the cases of all the k number of groups taken together, the ranks given to each group are totalled separately to give the rank sum of the corresponding group (e.g., R 1 , R 2 , ... R k). Each rank sum is divided by the size (n 1 , n 2 , ... n k) of that group to give the mean rank (R – 1 , R – 2 , ... R – k) of the latter; the grand mean (R –) of all the groups is worked out dividing the total of all the rank sums by the total size (N) of all the groups. These values are then used in working out the statistic H. N n n n R R n R R n R R n k k k k ? ? ? ? ? ? ? ? 1 2 1 1 1 2 2 2 ..... . ; ,..... . R R R R N k ? ? ? ? 1 2 ..... . H N N n R R n R R n R R k k ? ? ? ? ? ? ? ? ? 12 1 1 1 2 2 2 2 2 ( ) [( ) ( ) ..... ( )]. df k? ?1. The H 0 proposes that the computed H is not significant and has resulted from chances associated with random sampling using laws of probability. To find the
M-2\D:\Netaji 05\Biology-1\ probability P of this H 0 being correct, the computed H is compared with the critical ? ? 2 with the computed df and for the chosen ?. The computed H is considered significant, only if it either exceeds or equals the critical ? ? 2 (P ???). A significant H indicates the existence of significant difference between the group means. The Kruskal-Wallis anova for only two groups ends here. But if the anova is worked out with more than two groups, a significant H has to be followed up by the multiple comparison Mann-Whitney U test to explore the significance of difference between the groups of each chosen pair. (See Examples 5.5.1 and 5.6.1.) Example 5.5.1. Apply Kruskal-Wallis

100% **MATCHING BLOCK 48/108** **W**

anova to find whether or not there is a significant difference between

mean corpuscular hemoglobin scores (picograms) of a group of microcytic anemia patients (Group 1) and a group of macrocytic anemia patients (Group 2). (? = 0.05.) Group 1 (X 1) : 23 18 19 17 25 20 32 22 33 16. Group 2 (X 2) : 32 35 25 28 34 32 30 31 40 42. Critical chi square scores : ? ? 0 05 19 2 0 05 18 2 3014 2887 . ( ) . ( ) . ; . ; ? ? ? ? 0 05 1 2 0 05 2 2 3 84 5 99 . ( ) . ( ) . ; . ? ? . Solution : Table 5.4. Composite ranking for Kruskal-Wallis anova between two groups. X 1 Ranks X 2 Ranks 23 7 32 14 18 3 35 18 19 4 25 8.5 17 2 28 10 25 8.5 34 17 20 5 32 14 32 14 30 11 22 6 31 12 33 16 40 19 16 1 42 20 ? — 66.5 (R 1) — 143.5 (R 2)
M-2\D:\Netaji 05\Biology-1\ n n N n

41% **MATCHING BLOCK 49/108** **W**

n 1 2 1 2 10 10 10 10 20 ? ? ? ? ? ? ? ? ; ; . R R R R n R R n 1 2 1 1 1 2 2 2 665 1435 665 10 665 1435 10 1435 ? ? ? ? ? ? ? ? . ; . . . . ; . . . R R R N ? ? ? ? ? 1 2 665 1435 20 1050 . . . . H N N n R R n R R ? ? ? ? ? 12 1 1 1 2 2 2 2 ( ) [( ) ( )] ? ? ? ? ? ? ? 12 20 20 1 10 665 1050 10 1435 1050 847 2 2 ( ) [(. .) (. .)] . . df = k – 1 = 2 – 1 = 1. ? 0 05 1 2 384 . ( ) .? . As the computed H exceeds the critical ? 0 05 2 . , P is too low (P > 0.05). So, H o is rejected and

there is a significant difference in MCH between microcytic and macrocytic patients. 5.6 Multiple comparison Mann-Whitney U test This is a rank-dependent test used as a powerful alternative to Student's t test between two groups, and also as a multiple comparison test between the groups of each chosen pair after a significant H test with more than two groups (k < 2). Assumptions, applications and inaccuracies of this test are similar to those of the Kruskal-Wallis H test. For a multiple comparison test between two chosen group means (say, X – 1 and X – 2), ranks are given in an ascending order and a composite manner to the scores of both the groups taken together, and rank sums (R 1 and R 2) of the respective groups are worked out separately. The rank sum and the group size (n 1 or n 2) of any of the groups are used in computing the corresponding statistic U. Thus, U n n n n R 1 1 2 1 1 1 1 2 ? ? ? ? ( ) , or, U n n n n R 2 1 2 2 2 2 1 2 ? ? ? ? ( ) .
M-2\D:\Netaji 05\Biology-1\ The U e , expected in terms of the H o , and the SE (s U) of U are computed using the group sizes. These are in turn used in transforming either U 1 or U 2 into the z score. U n n s n n n n e U ? ? ? ? 1 2 1 2 1 2 2 1 12 ; ( ) ; z U U s z U U s e U e U 1 1 2 2 ? ? ? ? , . or Either of the two z scores, identical in magnitude but opposite in algebraic sign, may be used in finding the probability P of the H o being correct. P = 2 [0.5000 – (area of unit normal curve from ? to the computed z)]. Only if the P thus worked out does not exceed the chosen level of significance (?), P is considered too low (P ???) and there is a significant difference between the relevant group means. (See Example 5.6.1.) Example 5.6.1. (a) Apply Kruskal-Wallis

88% **MATCHING BLOCK 51/108** W

anova to find whether or not there is any significant difference between the

mean knee-jerk scores (degrees of arc) of the following three groups of rhesus monkeys from three different habitats. (? = 0.001.) Group 1 (X 1) : 33, 37, 31, 34, 33, 29, 30, 35, 32, 28, 27, 36. Group 2 (X 2) : 27, 22, 18, 28, 23, 25, 28, 21, 20, 19, 24, 19. Group 3 (X 3) : 18, 16, 14, 10, 11, 18, 12, 15, 13, 17, 14, 9. Critical chi square values : ? ? 2 0001 3 2 0001 2 1627 1382 . ( ) . ( ) . ; . ; ? ? ? ? 2 0001 30 2 0001 29 5970 5830 . ( ) . ( ) . ; . . ? ? (b) In case the Kruskal-Wallis H is significant, work out Mann-Whitney multiple comparison U test to find if there is any significant difference between the mean knee-jerks of groups 2 and 3. (? = 0.001). z scores : 3.60 3.70 3.80 3.90 4.00 Areas of unit normal curve : 0.4998 0.4999 0.49993 0.49995 0.49997 Solution : n 1 = 12; n 2 = 12 ; n 3 = 12 ; N = n 1 + n 2 + n 3 = 12 + 12 + 12 = 36.
M-2\D:\Netaji 05\Biology-1\ Table 5.5. Composite ranking for Kruskal-Wallis anova between three groups. X 1 Ranks X 2 Ranks X 3 Ranks 33 31.5 27 22.5 18 12 37 36 22 18 16 9 31 29 18 12 14 6.5 34 33 28 25 10 2 33 31.5 23 19 11 3 29 27 25 21 18 12 30 28 28 25 12 4 35 34 21 17 15 8 32 30 20 16 13 5 28 25 19 14.5 17 10 27 22.5 24 20 14 6.5 36 35 19 14.5 9 1 ? — 362.5 (R 1) — 224.5 (R 2) — 79.0 (
R 3) R R

38% **MATCHING BLOCK 52/108** W

R 1 2 3 3625 2245 79 0 ? ? ? . ; . ; . . R R n R R n R R n 1 1 1 2 2 2 3 3 3 3625 12 3021 2245 12 1871 790 12 658 ? ? ? ? ? ? ? ? ? . . ; . . ; . . ; R R R R N ? ? ? ? ? ? ? ( ) (. . .) . . 1 2 3 3625 2245 790 36 1850 H N N n R R n R R n R R ? ? ? ? ? ? ? 12 1 1 1 2 2 2 2 3 3 2 ( ) [( ) ( ) ( )] ? ? ? ? ? ? ? 12 36 36 1 12 3021 1850 12 1871 1850 12 658 1850 2 2 2 ( ) [(. .) (. .) (. .)] =30.19. df = k – 1 = 3 – 1 = 2. Critical ? 0 001 2 2 1382 . ( ) . . ? Because the computed H is higher than the critical ? 0 001 2 . , P is too low (P > 0.001). So, the

H o is rejected
and there are significant differences between the means of the three groups. As there are more than two groups, Mann-Whitmey U test is undertaken to test the significance between X – 2 and X – 3 (Table 5.6).

M-2\D:\Netaji 05\Biology-1\ Table 5.6. Composite ranking for Mann-Whitney test. X 2 Ranks X 3 Ranks 27 22 18 12 22 18 16 9 18 12 14 6.5 28 23.5 10 2 23 19 11 3 25 21 18 12 28 23.5 12 4 21 17 15 8 20 16 13 5 19 14.5 17 10 24 20 14 6.5 19 14.5 9 1 ? — 221.0 (R 2) — 79.0 (R 3) After the composite ranking of all X 2 and X 3 scores of the two relevant groups in Table 5.6, the rank sums (R 2 and R 3) of the respective groups are worked out and used along with the group sizes (n 2 and n 3) for computing either of two respective U scores. U n n n n R 2 2 3 2 2 2 1 2 12 12 12 12 1 2 2210 10 ? ? ? ? ? ? ? ? ? ? ( ) ( ) . . , or U n n n n R 3 2 3 3 3 3 1 2 12 12 12 12 1 2 790 1430 ? ? ? ? ? ? ? ? ? ? ( ) ( ) . . . U e and s U are then computed and used along with either U 2 or U 3 to work out the z score. U n n s n n n n e U ? ? ? ? ? ? ? ? ? ? ? ? ? 2 3 2 3 2 3 2 12 12 2 720 1 12 12 12 12 12 1 12 1732 . ; ( ) ( ) . . z U U s e U 2 2 10 72 0 1732 410 ? ? ? ? ? ? . . . . , or z U U s e U 3 3 1430 720 1732 410 ? ? ? ? ? ? . . . . .

M-2\D:\Netaji 05\Biology-1\ Any of the two computed z scores is used for finding the significance, ignoring any negative sign of z. Because the computed z is higher than even 4.00, the probability P of the correctness of H o is worked out as follows. If the computed z were 4.00, P would have been lower than 0.0001 : P = 2 [0.5000 – (area of unit normal curve from ? to the z score of 4.00)] = 2[0.5000 – 0.49997] = 0.00006. Because either computed z amounts to 4.10, the corresponding P would be lower than 0.00006 and so, much lower than the chosen ? of 0.001. So, the H o is rejected. Hence, there is a significant difference between X – 2 and X – 3 . (P >> 0.001). 5.7 Summary Anova is used to test the significance of difference between the group means of a dependent variable, on exposure of two or more groups of cases to the levels of one or more independent variables. It has more rigorous assumptions and higher power than Student's t test and can be applied at a time to two or any higher number of groups. Anova may be one-way, two-way or of a still higher order according to the number of independent variables, and model I, model II or model III according to the nature(s) of the latter. In a one-way anova, the total variance of scores of the dependent variable is resolved into between-groups and within-groups variances, and the F ratio of those two variances is compared with critical F values for different levels of significance to find whether or not the group means differ significantly. A significant F ratio in a one-way anova for more than two groups is followed up by multiple comparison Scheffe's F test to find whether or not the means of groups of any chosen pair differ significantly. A significant F ratio is also followed by the working out of either the omega square in a model I anova, or the added variance component for a model II anova. Where the assumptions for one-way parametric anova are not justifiable, Kruskal- Wallis nonparametric rank-dependent anova may be undertaken. If this test yields a significant statistic H, and if it involves more than two groups, Mann-Whitney multiple comparison U

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test is used for testing the significance of difference between the

means of any two chosen groups. 5.8 Terminal questions 1. (a) State the assumptions for Kruskal-Wallis one-way anova. M-2\D:\Netaji 05\Biology-1\ (b) Describe how you would rank the scores of a measurement variable and work out Kruskal-Wallis anova for two groups. (c)

59% **MATCHING BLOCK 53/108** **W**

Work out Kruskal-Wallis anova to find whether or not there is a significant difference between the tracheal ventilation scores (ml/min) of the following groups of

locusts. (? = 0.05.) Group 1 : 73, 76, 86, 76, 83, 78, 73, 79, 78. Group 2 : 70, 69, 73, 65, 63, 69, 76, 60, 62, 66, 67. Critical chi square scores : ? ? 2 0 05 2 2 0 05 1 599 384 . ( ) . ( ) . ; . ; ? ? ? ? 2 005 19 2 005 18 3014 2887 . ( ) . ( ) . ; . . ? ? 2. (a) Explain what you understand by one-way anova and model I anova. (b) Discuss the assumptions for anova. (c) Work out a

62% **MATCHING BLOCK 54/108** **W**

one-way anova to find if there is a significant difference between the mean wing length scores (mm) of the following groups of houseflies from two different habitats. (? = 0.01)

Group 1 : 4.8, 4.6, 5.4, 4.6, 5.3, 4.7, 4.8, 5.0, 5.2, 5.0. Group 2 : 3.8, 3.0, 3.3, 4.1, 3.7, 4.0, 3.9, 3.5, 4.3, 3.8. Critical F values : F F 0 01 2 19 0 01 1 18 593 828 . (,) . (,) . ; . ; ? ? F F 0 01 2 18 0 01 119 601 818 . (,) . (,) . ; . . ? ? 3. (a) Write briefly about the uses and computations of omega square and added variance component. (b) Describe briefly the variances involved in a one-way anova and their partitioning and uses, mentioning the relevant formulae. (c) Using the data of Question 1 (c), work out a

62% **MATCHING BLOCK 56/108** **W**

one-way anova to find whether or not there is a significant difference between the given tracheal ventilation scores of the two groups of locusts, exposed to two different

fixed levels of amobarbital. (? = 0.05) Critical F values : F F 0 05 2 19 0 05 118 352 441 . (,) . (,) . ; . ; ? ? F 0 05 1 19 438 . (,) .? . 4. (a) State the reasons for preferring anova to t test. (b) Describe different models of anova with examples. (c) Using a one-way anova, find whether or not there is any significant
M-2\D:\Netaji 05\Biology-1\ difference between the following blood sugar scores (mg per 100 ml) of a group of diabetic orangutans treated with three different levels of a postulated antidiabetic agent. (? = 0.01) Animal : 1 2 3 4 5 6 7 8 9 10 X 1 mg : 165 192 185 170 200 197 220 180 210 201 X 2 mg : 120 165 140 125 160 162 178 150 163 157 X 3 mg : 85 120 95 87 123 120 135 115 130 120 Critical F values : F F 0 01 2 27 0 01 2 28 549 545 . (,) . (,) . ; . ; ? ? F 0 01 1 27 768 . (,) .? . (d) If the computed F is found significant, work out Scheffe's F test

100% **MATCHING BLOCK 57/108** **W**

to find whether or not there is a significant difference between the

mean blood sugars of groups 1 and 3 in the preceding experiment. (? = 0.01). 5. (a) Describe briefly Scheffe's multiple comparison F test, quoting its computational formulae. (b) Give an account of how you would work out the Mann-Whitney U test and find the significance of the computed U, stating the relevant formulae. (c) Using the data of Question 2(c), work out the Kruskal-Wallis anova to find if there is any significant difference between the given wing length scores of the two groups. (? = 0.01) Critical chi square values : ? ? 2 0 01 2 2 0 01 1 921 664 . ( ) . ( ) . ; . ; ? ? ? ? 2 00119 2 001 18 3619 3480 . ( ) . ( ) . ; . . ? ? 5.9 Answers 1. (a) See Sub-section 5.5.1. (b) See Sub-section 5.5.2. (c) See Example 5.5.1. 2. (a) See Sub-sections 5.2.3 and 5.2.4. (b) See Sub-section 5.2.5. (c) See Example 5.3.1. 3. (a) See Sub-sections 5.3.4 and 5.3.5. (b) See Sub-sections 5.2.1, 5.3.1 and 5.3.2.
M-2\D:\Netaji 05\Biology-1\ (c) See Example 5.4.2. 4. (a) See Sub-section 5.2.2. (b) See Sub-section 5.2.4. (c) See Example 5.4.1. (d) See Example 5.4.1. 5. (a) See Section 5.4. (b) See Section 5.6. (c) See Example 5.5.1.
M-2\D:\Netaji 05\Biology-1\ Unit 6 ? CORRELATION AND REGRESSION Structure 6.1 Introduction Objectives 6.2 Correlation 6.3 Product-moment correlation 6.4 Kendall's rank correlation 6.5 Partial correlation 6.6 Multiple correlation 6.7 Regression 6.8 Simple linear regression 6.9 Multiple regression 6.10 Summary 6.11 Terminal questions 6.12 Answers 6.1 Introduction You are aware that any system— be it an organism, a cell, an organelle, a tissue, a body fluid, a body cavity, a space, a waterbody, a farmland or a forest— contains or consists of innumerable variables. Between two or more variables in a system, there may exist different types of relations or associations. In this unit, you will learn to explore and assess such associations between variables in any system. You will also come to know here how the likely value or score of one such variable may be mathematically predicted by measuring the score(s) of one or more other variables associated with the former one. Objectives On studying this unit, you should be able to do the following : ? understand what is meant by correlation and know its different types, ? describe the general properties of correlation, ? understand the assumptions for product-moment correlation and its applications, ? describe the properties of product-moment r, ? work out product-moment r between two given variables and find its significance, ? understand where Kendall's rank correlation should be used instead of product-

M-2\D:\Netaji 05\Biology-1\ moment r, ? compute Kendall's tau between two variables and find its significance, ? know when and how partial and multiple linear correaltions are worked out and their significances are found out, ? understand what is meant by regression and know its different types and models, ? describe the assumptions and properties of simple linear regression, ? work out simple linear regression for predicting the score of one variable on the measured score of another, and ? know when and how to work out multiple linear regression of one variable on the combination of observed scores of two or more other variables. 6.2 Correlation Correlation is the quantitative estimation and numerical expression of the magnitude or strength as well as the algebraic sign or direction of the association between two or more variables in a system. The correlation coefficient serves basically as a measure of the intensity or degree of association between the variances (Sub- sections 2.6.3 and 5.2.1) of two or more variables in the cases of the sample, while its algebraic sign is the indicator of whether those variables vary in the same direction or in opposite directions. The correlation coefficient is the sample statistic for correlation and ranges from −1.00 to +1.00 in value. 6.2.1. Types of correlation Correlation may be simple or multiple, according as it is computed between two variables or more than two variables. For example, there may be a simple correlation between trunk length and wing length in a sample of cockroaches ; on the contrary, there may be a multiple correlation between oxygen consumption and the combination of atmospheric oxygen tension and tracheal ventilation volume in a sample of locusts. Correlation may again be linear and nonlinear, according as the relation between the variables conforms to a straight line equation and a linear graph, or follows the equation of a curved line and a nonlinear graph. For example, there may be a simple

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linear correlation between body weight and gill weight in a sample of fishes ;

but the

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initial velocity of an enzyme action and the corresponding substrate concentration

may have a simple nonlinear correlation, conforming to a rectangular hyperbola. Correlation may also be either positive or negative. If high scores of a variable are mostly accompanied by high scores of another variable while low scroes of one are usually associated with low scores of the other, the two variables are varying in the same direction and are said to bear a positive correlation with each other ; an example is the positive correlation between body height and body weight in many

M-2\D:\Netaji 05\Biology-1\ samples. But if high scores of one variable are mostly accompanied by low scores of another variable while high scores of the latter are usually associated with low scores of the former, the scores of the variables are usually varying in opposite directions and the variables bear a negative correlation with each other ; for example, a negative correlation exists between blood sugar level and blood insulin level in animal samples. While the magnitude of correlation is expressed numerically, ranging from –1.00 to +1.00, the algebraic +/– sign preceding the numerical value indicates whether the correlation is positive or negative. 6.2.2. Properties of correlations General properties of correlations are summarized below. (i) A correlation coefficient worked out with a sample drawn from a population would hold good only within the limits of the particular stratum or class of the population from which the sample has been drawn, and would also be confined within other conditions and situations prevailing during the work. Thus, a correlation coefficient worked out with a sample of adults may not hold good for children of the same population, or that worked out with a sample of females may not apply to males. (ii) A correlation coefficient between two variables does not necessarily indicate that variations of one of them may be either the cause or the effect of variations of the other ; their correlation may very well have arisen from the association of some other variable in common with both of them. (iii) A correlation coefficient cannot directly predict the score of one of the variables from that of the other in the same individual. (iv) The correlation coefficient between two variables varies from sample to sample even when they have been drawn from the same population ; so, the sample correlation coefficients (r) lie dispersed around the population correlation coefficient (?) to form a sampling distribution of r values, owing to their respective sampling errors. 6.3 Product-moment correlation Karl Pearson's product-moment correlation coefficient or Pearson's r is a simple linear correlation coefficient, used in correlating two variables which have a linear association with each other. 6.3.1. Assumptions for product-moment r Product-moment r can be applied for correlating two variables, only if it can be logically assumed that the following conditions or criteria are fulfilled in the case under investigation. (i) Both the variables being correlated should be continuous measurement variables, with their scores quantitatively measurable and occurring even in infinitely small fractional units, with no gaps in the respective scales of M-2\D:\Netaji 05\Biology-1\ scores. (ii) Scores of each variable should be distributed in the population in a unimodal, bilaterally symmetric or almost symmetric, normal or nearly normal distribution with not much skewness of its tails. (iii) There should exist a linear association between the variations of the two variables. (iv) The pair of scores of the two variables for each individual or case should have occurred in the sample at random, obeying the laws of probability and independent of all other similar pairs of scores ; this last assumption ensures that the sample may be a representative of the population, enabling the inference made from the sample to be generalized for the corresponding population. It follows from these assumptions that the product moment r cannot be used in correlating such variables as are not associated linearly, or are discontinuous in nature (e.g., heart rate, cell count and litter size) or are ordinal variables (e.g., ferocity) or nonmeasurable qualitative variables (e.g., sex and race), or have prominently skewed or non-normal distributions in the population. 6.3.2. Properties of product-moment r (a) The magnitude of the computed r is a measure of the strength of association between the variables while its algebraic sign indicates whether the variables vary in the same direction (positive) or in opposite directions (negative). Thus, + 0.80 indicates a high positive correlation, – 0.72 shows a high negative correlation, +0.14 indicates a low positive correlation, while 0.00 means the absence of any linear correlation. (b) If every score of any or each variable is multiplied, divided, added or subtracted by a constant number, it does not result in any change in the r value between the two variables. (c) Correlation depends on that proportion of total variance of each variable which is associated with the variance of the other. This makes the value of r directly proportional to the covariance of the two variables.

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Where X and Y are the scores, X – and Y – are the means, s x and s y are the unbiased standard deviations, and Cov(X,Y) is the covariance

of two variables, and n is the sample size, s

47% **MATCHING BLOCK 60/108** W

X X n s Y Y n Cov X Y X X Y Y n x y ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ( ) ; ( ) ; (,) ( )( ) ; 2 2 1 1 1 r Cov X Y s s X X Y Y n s s x y x y ? ? ? ? ? (,) ( )( ) ( ) ? 1 . (

d) The r values, computed between two given variables in different samples from the same population, lie dispersed forming a sampling distribution of r around the population correlation coefficient (?) because of their varying differences, called
M-2\D:\Netaji 05\Biology-1\ sampling errors (s e), from the latter ; for each sample r, s e = r –??. The s e of r may be estimated by working out the standard error (s r) of the r. (e) Sampling distributions of r are unimodal and symmetrical if the corresponding population ? amounts to zero, but are progressively more and more skewed either positively or negatively, according respectively to the rising negative and positive values of ? . (f) The squared value (r 2) of product-moment r between two variables is often used as a measure of that proportion of variance of each of them, which depends on the variance of the other variable ; this is called the coefficient of determination (r 2). For example, 0.49 proportion of the variance of X would be determined by the variance of Y, and vice versa, if r xy amounts to –0.70. 6.3.3. Computation of r from ungrouped data Using the ungrouped scores of the variables X and Y, product-moment r is generally worked out between them with either of the following alternative formulae, each derived from the foregoing equation for the relation between r and Cov(X,Y). (See Sub-section 6.3.2). (a) From sum of products and sums of squares : Where X – and Y – are the sample means of the respective variables, (X – X –) and (Y – Y –) are the differences between each score and its mean, ? (X – X –) 2 and ? (Y – Y –) 2 are the sums of squares of respective variables, and ? (X – X –)(Y – Y –) is the sum of products of (X – X –) and (Y – Y –) values of each case, r X X Y Y X X Y Y ? ? ? ? ? ? ? ? ( )( ) ( ) ( ) 2 2 . (b) From raw scores of variables : Where n is the number of cases in the sample or the sample size, ?X and ?Y are the sums of scores of the respective variables, ? X 2 and ? Y 2 are the sums of respective squared scores, and ? XY is the sum of the products of X and Y scores of each case,

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r n XY X Y n X X n Y Y ? ? ? ? ? ? ? ? ? ? ? ? [( )][( )] 2 2 2 2 . 6.3.4.

Significance of computed r You should be aware that a significance test has to be undertaken to find out the M-2\D:\Netaji 05\Biology-1\ probability (P) of the null hypothesis (H O) being correct, because the r has been worked out using a sample. The H O proposes here that the computed r has resulted from mere chances associated with random sampling by laws of probability and is not significant or meaningful. Stated differently, the H O contends that population ? amounts to zero only, that the random choice of a particular sample has led to the observed value of r, and that the difference between the observed value of r and the zero value of population ? can be explained away by the sampling error of the computed r. To work out the probability P of the H O being correct, the computed r is transformed into Student's t by using the SE (s r) of the r ; the df of t is also worked out. s r n t r s df n r r ? ? ? ? ? ? 1 2 2 2 ; ; . The computed t is next compared with two-tail critical t scores with the same df. Only if the computed t either exceeds or equals the critical t for a particular significance level (?) not higher than 0.05, P is considered too low (P ???), the H O is consequently rejected and the computed r is considered significant. On the contrary, if the critical t exceeds

78% **MATCHING BLOCK 62/108** W

the computed t, P is considered too high (P <??), the H O is consequently retained and the computed r has no significance. Example 6.3.1.

Work out product-moment r to

75% **MATCHING BLOCK 63/108** W

find whether or not there is a significant correlation between tracheal ventilation (X ml/minute) and

O 2 consumption (Y ml/minute) using the following data of a sample of grasshoppers. (??= 0.01.) Animal : 1 2 3 4 5 6 7 8 9 10 X : 66.0 89.1 72.0 87.5 75.2 78.2 83.5 71.6 85.6 76.3 Y : 3.3 4.9 3.5 4.7 3.7 4.0 4.3 3.4 4.4 3.8 Critical t scores : t 0.01(9) = 3.250 ; t 0.01(8) = 3.355 ; t 0.01(18) =2.878. Solution : (a) Using the sums of squares : X X n Y Y n ? ? ? ? ? ? ? ? 785 0 10 78 5 40 0 10 4 0 . . . . . ml ml.
M-2\D:\Netaji 05\Biology-1\ Table 6.1 : Table for computing sum of products
and sums of squares.

73% **MATCHING BLOCK 65/108** W

X Y X–X – Y–Y – (X–X –) 2 (Y–Y –) 2 (X–X –)(Y–Y –) 66.0 3.3 –12.5 –0.7 156.25 0.49 + 8.75 89.1 4.9 +10.6 +0.9 112.36 0.81 + 9.54 72.0 3.5 –6.5 –0.5 42.25 0.25 + 3.25 87.5 4.7 +9.0 +0.7 81.00 0.49 + 6.30 75.2 3.7 –3.3 –0.3 10.89 0.09 + 0.99 78.2 4.0 –0.3 0 0.09 0 0 83.5 4.3 +5.0 +0.3 25.00 0.09 + 1.50 71.6 3.4 –6.9 –0.6 47.61 0.36 + 4.14 85.6 4.4 +7.1 +0.4 50.41 0.16 + 2.84 76.3 3.8 –2.2 –0.2 4.84 0.04 + 0.44 ? 785.0 40.0 – – 530.70 2.78 + 37.75 r X X Y Y X X Y Y ? ? ? ? ? ? ? ? ?? ? ? ? ( )( ) ( ) ( ) . . . . 2 2 3775 53070 278 098 . (

b) Using raw scores (alternative method) : Table 6.2 . Table for computing r from raw scores.

36% **MATCHING BLOCK 66/108** W

X Y X 2 Y 2 XY 66.0 3.3 4356.00 10.89 217.80 89.1 4.9 7938.81 24.01 436.59 72.0 3.5 5184.00 12.25 252.00 87.5 4.7 7656.25 22.09 411.25 75.2 3.7 5655.04 13.69 278.24 78.2 4.0 6115.24 16.00 312.80 83.5 4.3 6972.25 18.49 359.05 71.6 3.4 5126.56 11.56 243.44 85.6 4.4 7327.36 19.36 376.64 76.3 3.8 5821.69 14.44 289.94 ? 785.0 40.0 62153.20 162.78 3177.75 r n XY X Y n X X n Y Y ? [( )][( )] . . . [. .] [. .] 2 2 2 2 2 2 10 317775 7850 400 10 6215320 7850 10 16278 400 = + 0.98. M-2\D:\Netaji 05\Biology-1\ (c) Significance of computed r : 000.14 070.0 98.0 .070.0 2 10 98.0 1 2 1 2 2 ? ? ? ? ? ? ? ? ? ? r r s r t n r s df = n–2 = 10–2 = 8.???? = 0.01. Critical t 0.01(8) = 3.355. As the computed t far exceeds the critical t 0.01 , P is considered too low. So, the H 0 is

rejected. There is a significant correlation between tracheal ventilation and O 2 consumption. (P > 0.01). 6.4 Kendall's rank correlation Kendall's rank correlation coefficient or Kendall's tau (?) is a rank-based simple linear correlation coefficient. It is a powerful nonparametric alternative to product- moment r when the more rigorous assumptions for the latter are not fulfilled. It ranges from –1.00 to +1.00 in value. 6.4.1. Assumptions for Kendall's tau You may compute Kendall's tan for correlating either ordinal variables with their magnitudes expressed in ranks, or measurement variables after first changing their scores into ranks, provided the following assumptions are justifiable. (i) The variables should either be ordinal variables with the cases of the sample already graded into ranks, or be such other variables whose scores can be changed into ranks. (ii) Ranks or scores of the two variables should bear linear association. (iii) The pair of ranks or scores of each case should have occurred due to random sampling using laws of probability so that the sample may be considered as representing the population. (iv) No assumption need be made for the continuous nature of any of the variables. (v) No assumption is also necessary for the normality of distribution of scores or ranks of either variable in the population. It follows from these assumptions that Kendall's tau cannot be used for nominal variables like sex, as ranks cannot be assigned for them to the cases of the sample. Moreover, being less powerful than the product-moment r, tau should be used only when assumptions for r are found not to be logical. 6.4.2. Computation of Kendall's tau (a) In case of ordinal variables with their magnitudes already graded in ranks, those ranks may be used directly in computing tau. But for continuous or discontinuous measurement variables, their scores are first changed into ranks in ascending order; the ranks of two variables would thus form two separate series of ranks (Table 6.3).

M-2\D:\Netaji 05\Biology-1\ In this procedure, two or more identical scores of a variable are given an identical average rank which is the mean of the ranks they would have got if they were successive nonidentical scores ; the next score following such a tied set of scores is given the rank it would have got if the immediately preceding score was not a member of a tied set and did not enjoy any average rank. This system of ranking of scores leads to two types of inaccuracies in all rank-dependent statistics, viz. (i) error owing to varying differences between the scores bearing successive ranks, and (ii) error arising from an average rank given to every score of a tied set instead of their separate individual ranks. However, you will presently find that in computing tau, the inaccuracy owing to average ranks is sought to be minimized. (b) After ranking the scores of two variables X and Y into two separate series, the ranks of that variable, which is free from any tied set or average rank, are arranged in an ascending order along a column of a table ; against every rank of this column, the paired rank of the same case with respect to the other variable is entered in a second column alongside (Table 6.4). If each or neither of the variables has any tied set of

23% **MATCHING BLOCK 67/108** W

scores, the ranks of any of them may be arranged in the first column, pairing each with the rank of the same case in the other variable along the second column. (c) Moving downwards along the second column of ranks from its top, each of its ranks is used in turn as

a

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pivotal rank for comparing with every successive subsequent rank following

that pivotal rank, counting each subsequent rank as +1, 0 or −1 according as it exceeds, equals or falls short of

85% **MATCHING BLOCK 69/108** W

the pivotal one, Such counts of subsequent ranks are entered in a third column and totalled as ?

C, after using all the second-column ranks in turn as successive pivotal ranks (Table 6.4). (d) Correction terms (? T X and ? T Y) are worked out for countering the errors due to the use of average ranks for X and Y scores, respectively, using the number (t X or t Y) of cases in each tied set of the respective variables. ? T X = ? [t X (t X −1)] ; ?????? T Y = ? [t Y (t Y −1)]. (e) Kendall's tau (?) is then worked out as follows, using the sample size (n), ? C, ? T X and ? T Y . ? ? ? ? ? ? ? 2 1 1 ? ? ? C n n T n n T X Y [( )][( )] . If any of the variables is free from tied scores, its ?T is taken as zero. So, if both the variables have no tied scores, the formula simplifies into ; ? ? ? 2 1 ?C n n( ) .
M-2\D:\Netaji 05\Biology-1\ 6.4.3. Significance of computed tau Because the tau is computed using a sample, the H O proposes that it is not significant and has resulted from the use of a particular sample drawn by random sampling. To find the probability P of this H O being correct, for sample sizes exceeding 10, the computed tau is transformed into Student's t using its SE (s ?? ??and the obtained t is compared with two-tail critical t ? values. s n n n t s df ? ? ? ? ? ? ? ? ? 2 2 5 9 1 ( ) ( ) ; ; The computed t is significant, only if it either exceeds or equals the critical t value for a chosen significance level (P ? ?). Example 6.4.1 Work out Kendall's tau

66% **MATCHING BLOCK 70/108** W

to find whether or not there is a significant correlation (??= 0.05) between gill weight (X) and

body weight (Y), using the following data of a sample of crabs. Animal : 1 2 3 4 5 6 7 8 9 10 11 X gm : 0.15 0.11 0.19 0.30 0.13 0.20 0.25 0.30 0.15 0.22 0.15 Y gm : 11.20 8.10 13.20 14.50 8.45 12.20 14.55 14.00 11.25 9.50 11.45 Critical t values : t 0.05(10) =2.228 ; t 0.05(20) =2.086 ; t 0.05(?? ???????? Solution : Table 6.3. Ranking of gill weights (X) and body weights (Y). X Ranks (R X) Y Ranks (R Y) 0.15 4 11.20 4 0.11 1 8.10 1 019 6 13.20 8 0.30 10.5 14.50 10 0.13 2 8.45 2 0.20 7 12.20 7 0.25 9 14.55 11 0.30 10.5 14.00 9 0.15 4 11.25 5 0.22 8 9.50 3 0.15 4 11.45 6 R X and R Y ranks are given in ascending orders to X and Y scores, respectively, in Table 6.3. As Y has no tied set of scores, R Y ranks are entered in ascending order along the first column of Table 6.4, and the respective paired R X ranks are entered
M-2\D:\Netaji 05\Biology-1\ against them in the second column. Every R X rank is taken in turn as the pivotal rank and compared with the subsequent R X ranks successively, counting each of the latter as +1, 0 or –1, according as the subsequent rank exceeds, equals or is lower than the pivotal rank. The algebraic sum of these counts gives ? C. Table 6.4. Count of subsequent ranks. R Y R X Count of subsequent ranks Total (C) 1 1 +1+1+1+1+1+1+1+1+1+1+1 +10 2 2 +1+1+1+1+1+1+1+1+1+1 +9 3 8 –1–1–1–1–1+1+1+1 –2 4 4 0+0+1+1+1+1+1 +5 5 4 0+1+1+1+1+1 +5 6 4 +1+1+1+1+1 +5 7 7 –1+1+1+1 +2 8 6 +1+1+1 +3 9 10.5 0–1 –1 10 10.5 –1 –1 11 9 Total +35 (? C) n = 11. ? T X = ? [t X (t X –1)] = 3(3–1)+2(2–1) = 8, because X has one set of three tied scores and one set of two tied scores. ? T Y = ? [t Y (t Y –1)] = 0, as Y has no tied set. ? ? ? ? ? ? ? ? ? ? ? ? ? ? 2 1 1 2 35 1111 1 8 1111 1 0 066 ? ? ? C n n T n n T X Y [( )][( )] [( )][( )] . . s n n n ? ? ? ? ? ? ? ? ? ? 2 2 5 9 1 2 2 11 5 9 1111 1 0234 ( ) ( ) ( ) ( ) . . t s

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df ? ? ? ? ? ? ? 066 0234 2821 . . . . . ? = 0.05. Critical t 0.05(?) =1.960. As the computed t exceeds the critical t 0.05 , P is lower than 0.05. So, P is too low. The H O is rejected. There is a significant correlation between

gill weight and body weight. (P > 0.05.) 6.5 Partial correlation In any system, there exist innumerable variables, many of which are associated with each other. So, the product-moment r between any two given variables lacks precision because it has resulted only partly from the direct correlation between them
M-2\D:\Netaji 05\Biology-1\ while its remaining part has arisen from the association of those two variables with one or more other variables in common. So, to get a more correct measure of the direct correlation between two given variables, the effects of one or more other variables affecting them in common should be eliminated or held constant. Partial r is the correlation coefficient between two variables, eliminating or partialling out one or more variables associated with both of them. For example, the r 12 worked out between blood glucose (X 1) and blood insulin (X 2) in a sample of mammals may suffer from the drawback that it has been partly derived from the association of blood cortisol (X 3) with both X 1 and X 2 ; so, for a more correct correlation between X 1 and X 2 , a partial correlation coefficient (r 12.3) has to be worked out between those two, partialling out the variable X 3 . 6.5.1. Types of partial correlation Partial r values range from –1.00 to +1.00 and may be either positive or negative. Partial r may be either linear or nonlinear according to respectively linear and nonlinear associations. Again, partial r may belong to different orders according to the number of variables partialled out or held constant during its computation. For example, partial r 12.3 between blood glucose (X 1) and blood insulin (X 2), partialling out blood cortisol (X 3), is a first-order partial correlation ; in contrast,

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r 12.34 between glomerular filtration rate (X 1) and glomerular blood pressure (X 2)

partialling out two other variables, viz., plasma protein osmotic pressure (X 3) and Bowman capsular fluid pressure (X 4), is a second-order partial correlation. Our discussion will be confined here to first- order linear partial correlations only. For the first-order partial r 12.3 for example, those components of X 1 and X 2 scores as are correlated with X 3 are eliminated, correlating only such respective remaining components of X 1 and X 2 as are not associated with X 3 . 6.5.2. Assumptions for partial linear correlation For the computation of partial linear correlations, following assumptions should be justifiable. (i) All the variables involved should be continuous measurement variables. (ii) All those variables should have their scores distributed in nearly normal distributions in the population without significant bilateral asymmetry. (iii) There should exist significant linear correlations between variables of each pair. (iv) Each pair of scores of every case in the sample should occur at random in accordance with laws of probability. 6.5.3. Computation of first-order linear partial r Linear partial r of any order

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is basically worked out from the product-moment r values of each pair of variables involved. For the first-order partial r 12.3 between variables X 1 and X 2 partialling out the variable X 3 , the product-moment r 12 , r 13 and M-2\D:\Netaji 05\Biology-1\ r 23 values between the respective variables

of three relevant pairs have to be used. Thus, r r r r r r 12 3 12 13 23 13 2 23 2 1 1 . [ ][ ] . ? ? ? ? Similarly, for the first-order partial r values between variables of other pairs, eliminating the respective third variables,

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r r r r r r r r r r r 13 2 13 12 23 12 2 23 2 231 23 12 13 12 2 13 2 1 1 1 1 . . [ ][ ] ; [ ][ ] ? ? ? ? ? ? ? ? ? .

In each case, the H O proposes that the computed partial r is not significant and has resulted from the use of a particular sample drawn at random by laws of probability. To find the probability P of the H O being correct, the computed partial r is transformed into Student's t, using the SE of the former. For example, for r 12.3 , s r n t r s df n r r 12 3 12 3 1 3 3 12 3 2 12 3 . . . . ; ; . ? ? ? ? ? ? The computed t is then compared with the appropriate critical t value having the same df. The partial r is considered significant, only if the computed t either exceeds or equals the critical t ? for the chosen level of significance (P ?? ?).

84% **MATCHING BLOCK 75/108** **W**

Example 6.5.1. Find whether or not there is a significant linear partial correlation between O 2 consumption (X 1 ml/minute) and tracheal ventilation volume (X 2 ml/minute) partialling out atmospheric O 2 tension (X 3 mm Hg), using the following data of a sample of 53 locusts. (??= 0.05). r 12 = +0.75 ; r 13 = +0.35 ; r 23 = +0.25. t 0.05(52) =2.007 ; t 0.05(51) =2.008 ; t 0.05(50) = 2.009. Solution : r r r r r r 12 3 12 13 23 13 2 23 2 2 2 1 1 075 035 025 1 035 1 025 073 . [ ][ ] . . . [.][.] . . ? ? ? ? ? ? ? ? ? ? ? ? s r n r 12 3 1 3 1 073 53 3 0097 12 3 2 2 . . . . . ? ? ? ? ? ? ? ? t r s r ? ? ? 12 3 12 3 073 0097 7526 . . . . . .

37% **MATCHING BLOCK 76/108** **W**

df = n–3 = 53–3 = 50. Critical t 0.05(50) = 2.009. M-2\D:\Netaji 05\Biology-1\ The computed t exceeds the critical t 0.05 ; so, P is too low and the H O is rejected. Hence, there is a significant linear partial correlation between X 1 and X 2 , partialling out

X 3 . (P > 0.05.) 6.6 Multiple correlation Multiple correlation consists of the

100% **MATCHING BLOCK 77/108** W

quantitative assessment of the magnitude and direction of correlation between a given variable and the

combination or weighted sum of two or more other variables. The single variable being correlated is called the criterion while the variables, whose combination is being correlated, are called the predictors. An example of multiple correlation with three variables—one criterion and two predictors— is the multiple linear correlation coefficient R 1.23 between wing length (X 1) and the combination or weighted sum of trunk length (X 2) and body weight (X 3) of a sample of insects, Higher multiple correlations with more than three variables (e.g., R 1.234) may also be worked out with more than two predictors. Multiple correlations may be either positive or negative according to the direction of correlation between the criterion and the weighted sum of predictors, and may range from −1.00 to +1.00. Multiple correlations may again be either linear or nonlinear according to the linear or nonlinear form of association between the criterion and the combination of predictors. The squared value of multiple correlation (e.g., R 123 2 .) is called the

51% **MATCHING BLOCK 78/108** W

coefficient of multiple determination and serves as an estimate of that proportion of the total variance of criterion, which is dependent on the combined contribution of all the predictors.

On the contrary, the coefficient of multiple non-determination (e.g., K 123 2 .) serves as

90% **MATCHING BLOCK 79/108** W

a measure of that proportion of the total variance of criterion, which is not determined by the combined contribution of the predictors

and is given by : K R 123 2 123 2 1 . . . ? ? 6.6.1. Assumptions for multiple linear correlations Multiple correlations can be computed with any number of variables, only when there are significant correlations between the variables of each pair. So, the assumptions to be justifiable for multiple linear correlations are closely similar to those for product- moment r between the variables in pairs. Thus, (i) the criterion as also each predictor should be continuous measurement variables with no gap in their scales of scores, (ii) scores of each variable should have a normal or near-normal distribution in the population without much bilateral asymmetry, (iii) there should exist significant linear correlations between the variables of each pair, and (iv) each pair of scores of every case should occur in the sample in accordance to the laws of probability.
M-2\D:\Netaji 05\Biology-1\ 6.6.2. Computation of multiple linear correlation with three variables Multiple linear correlations are basically worked out

100% **MATCHING BLOCK 80/108** W

from the product-moment r values of each pair of variables

involved. It cannot be computed if the product- moment r is not significant for any of the pairs of variables. For working out the multiple linear correlation coefficient (R 1.23) between a criterion X 1 and the combination of two predictors (X 2 and X 3), the beta coefficients (? 2 and ? 3)

68% **MATCHING BLOCK 81/108** W

are first calculated from the product moment r values (r 12 , r 13 and r 23) between the respective variables; ? 2 and ? 3 serve as measures of the proportions of variance of the criterion, associated with the variances of respective predictors (X 2 and X 3). ? ? ? ? 2 12 13 23 23 2 3 13 12 23 23 2 123 2 12 313 1 1 ? ? ? ? ? ? ? ? ? r r r r r r r r r R r r ; ; ; .

s n t R s df n R R 1 23 1 23 1 3 3 123 . . ; ; . ? ? ? ? ? To find the probability (P) of the H O being correct, the Student's t worked out from R 1.23 is compared with critical t ? score for the chosen significance level (?).The computed R 1.23 is considered significant, only if the computed t is either higher than or equal to the critical t ? value (P ???). Example 6.6.1 Use the data of Example 6.5.1
to

100% **MATCHING BLOCK 82/108** W

find whether or not there is a significant multiple linear correlation between O 2 consumption (X 1)

and
the weighted sum of tracheal ventilation (X 2) and atmospheric O 2 tension (

67% **MATCHING BLOCK 83/108** W

X 3). (??= 0.05.) t 0.05(49) =2.010 ; t 0.05(50) =2.009 ; t 0.05(51) =2.008 ; t 0.05(52) =2.007. Solution : r 12 = +0.75 ; r 13 = +0.35 ; r 23 = +0.25 ; n=53. ? 2 12 13 23 23 2 2 1 075 035 025 1 025 071 ? ? ? ? ? ? ? ? r r r r . . . . . ? 3 13 12 23 23 2 2 1 035 075 025 1 025 017 ? ? ? ? ? ? ? ? ? r r r r . . . . . R r r 123 2 12 3 13 071 075 017 035 077 . . . . . ? ? ? ? ? ? ? ? ? s n R 1 23 1 3 1 53 3 0141 . . . ? ? ? ? ? t R s R ? ? ? 123 1 23 077 0141 5461 . . . . . df = n−3 = 53−3 = 50. ? = 0.05. Critical t 0.05(50) =2.009. M-2\D:\Netaji 05\Biology-1\ As the computed t exceeds the critical t 0.05 , P is too low (P ? 0.05). The

H O is rejected and the computed R 1.23 is significant. 6.7 Regression Some variables cannot be measured directly or easily, with sufficient precision, or without errors. For predicting the very likely score of such a variable in any given individual or case, a statistical method of prediction, called regression, may be applied. The latter depends on the already known or measured scores of one or more variables correlated significantly with the variable to be predicted. In any regression, the variable to be predicted is called the dependent variable or criterion for that regression ; the variable(s) whose known or measured score(s) may form the basis of the prediction, should be called the independent variable(s) or predictor(s). In every regression, there is a single criterion ; but there may be one or more predictors in a regression. 6.7.1. Types of regression Regression may be broadly classified into simple and multiple regressions depending on the number of predictors used. In all types of regression, scores of a single variable would be predicted; but where the known or measured score of a single predictor is used in working out the regression, the latter is called a simple regression, while the scores of more than one predictor are used in predicting the score of a criterion in a multiple regression. It should be understood that in predicting the score of a criterion in any individual, the predictor scores of the same individual must be used ; moreover, there must exist significant simple correlations between the scores of the criterion and those of each predictor. An example of simple regression is the regression of the blood insulin concentraion (X 1) in a patient on his/her blood sugar concentraion (X 2), X 1 and X 2 being respectively the criterion and the predictor. A multiple regression of the O 2 consumption (X 1) may be worked out in a locust on the combination of scores of its tracheal ventilation (X 2) and the O 2 tension (X 3) in its inspired air— X 1 is the criterion here while X 2 and X 3 are two predictors. Regressions may again be classified into linear and nonlinear regressions, depending on whether there is respectively a linear association or a nonlinear association between the criterion and the predictor. For example, if the

84% **MATCHING BLOCK 84/108** W

criterion is linearly correlated with the predictor, the scores of the former are predicted by working out an equation for a straight line, depending on the linear association between the two. On the

contrary,

92% **MATCHING BLOCK 85/108** W

if the criterion has a nonlinear correlation with the predictor, scores of the criterion have to be predicted in terms of a curved line like a sigmoid or hyperbolic or exponential curve, according to

the form of their association.
M-2\D:\Netaji 05\Biology-1\ 6.7.2. Models of regression You have to choose from three alternative models of regression in predicting the scores of a criterion, according to the nature of the predictor used for the regression. (i) Model I or fixed model regression is chosen while using fixed experimental treatments as predictors that are prevented from random variations by planned and well-controlled applications of their different levels on the subjects ; this model of regression expresses both the interdependence and the cause-and-effect relation between the criterion and the predictor. Thus, a model I simple regression consists of the regression of O 2 consumption in a sample of dragon flies on the pre-determined levels of the insecticide rotenone applied on them. (ii) Model II or random model regression is worked out when the independent variables or predictors are uncontrolled random variables free to vary at random ; this model expresses the interdependence, but not the cause-and-effect relation of the criterion and the predictors. Thus, you would work out a model II simple regression of O 2 consumption in a sample of dragon flies on the naturally occurring uncontrolled levels of a pesticide in the environment. (iii) Model III or mixed model regression consists of only multiple regressions when both random variables and fixed experimental treatments are used as predictors. An example is the multiple regression of tracheal ventilation volume on the combination of atmospheric nitric oxide levels and administered levels of ephedrine. 6.7.3. Properties of regression (a) Regression is an expression of the dependent variable or criterion as a function of the independent variable or predictor. (b) It follows that

94% **MATCHING BLOCK 86/108** W

a regression can be worked out only when there is a significant correlation between the criterion and the predictor,

and may be linear or nonlinear according to the linear or nonlinear nature of that correlation. (c) Because regression is based on correlation, it resembles the latter in being effective only within the confines of the specific stratum or section of the population from which a sample was used, and other conditions that prevailed during its work- out, but not beyond their limits. (d)

91% **MATCHING BLOCK 87/108** W

Regression predicts only a probable score of the criterion on a given score of the predictor in a

case so that the predicted score (?)Y may differ from the actual score (Y) of the criterion in that case by varying amounts. (e) So, for any chosen predictor score (X), the actual criterion scores (Y) of different individuals having that X score lie dispersed around the regression-based predicted ? Y score in the form of a distribution. (f) The magnitude of deviations of actual criterion scores (Y) from the regression- predicted criterion score (?)Y can be estimated by working out the SE of estimate (s YX) of the criterion on the predictor.
M-2\D:\Netaji 05\Biology-1\ (g) Between

100% **MATCHING BLOCK 88/108** W

a pair of variables correlated with one another, regression can be worked

out in two ways, viz., a regression of

97% **MATCHING BLOCK 89/108** W

variable X as criterion on variable Y as predictor, and another regression of variable Y as criterion on variable X as predictor.

However, it is sensible to compute the regression of that variable of the pair as criterion, whose direct measurement is less precise or/and more complicated than that of the other variable, while the latter is used as the predictor. (h)

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A regression equation is worked out using a statistic called the regression coefficient (

e.g., b YX for the regression of Y on X) which is a measure of the average rate of change of criterion scores (Y) with each unit change in the scores (X) of the predictor. 6.8 Simple linear regression This is the regression for predicting the probable score of a criterion (say, Y) on the measured, known or given score of a single predictor (say, X) where the two variables are linearly correlated. It may belong to either model I or model II, depending on whether the predictor is a fixed experimental treatment free from random errors, or a classification variable suffering from random errors. The regression of the criterion Y on the predictor X and that of the criterion X on the predictor Y require the working out of two respective and separate regression equations. For linear regression, each regression equation is an equation for a straight line (regression line) expressing the scores of the criterion as the linear function of the scores of the predictor. The slope of the regression line is given by the regression coefficient which is the measure of the average rate of change of criterion scores for unit changes in predictor scores. The regression coefficient is used in working out the y-intercept of the regression line, i.e., its point of intersection with the ordinate scale for criterion scores. 6.8.1. Assumptions for simple linear regression Following assumptions should be justifiable for working out a simple linear regression of a criterion (Y) on a single predictor (X). (i) The criterion as well as the predictor should be continuous measurement variable, scores of both being quantitatively measurable and occurring even in infinitely small fractions of units. (ii) Scores of each variable should form a normal or near-normal distribution in the population from which the sample has been drawn. (iii) There should exist a significant linear corelation between the criterion and the predictor. (iv) The actual score of criterion for each individual should occur in the sample obeying the laws of probability, thus ensuring the representative nature of the sample with respect to the population. (v) The actual criterion scores (Y) of a large number of cases, having an identical

M-2\D:\Netaji 05\Biology-1\ predictor score (X), should form a normal distribution around the criterion score (?)Y worked out by regression on that predictor score. (vi) If a model I regression is intended, the predictor should be a fixed experimental treatment free from random errors; for a model II regression, the predictor should be a random or classification variable beyond the control of the investigator and suffering from random errors. 6.8.2. Properties of simple linear regression (a) Simple linear regression is possible only if there is a significant product- moment r between the variables intended to be the criterion and the predictor. (b) If the predicted criterion scores (say, ? Y) are plotted against their respective predictor scores (say, X), the plotted points should lie scattered around a straight regression line of Y on X, obeying the principle of least squares. (c) The slope of the regression line is given by the regression coefficient of the criterion on the predictor; e.g., b YX for

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the regression of Y on X, and b XY for the regression of X on Y. (

d) Magnitude of the regression coefficient is a measure of the average rate of change of criterion scores with unit changes in predictor scores. Positive and negative algebraic signs of the regression coefficient correspond respectively to positive and negative correlations between the two variables and indicate respectively ascending and descending gradients of the regression line. (e) The y-intercept of the regression line determines the general level of the line and is given, in case of the regression of Y on X, by a YX worked out using the b YX. (f) For any given predictor score (say, X), the actual criterion scores (Y) of a large number of cases having that X score lie scattered in the form of a normal distribution around the regression-predicted ? Y score as the mean of that distribution. (g) The linear regression of Y on X, and that of X on Y would yield two separate regression lines inersecting at the point corresponding to means (X – and Y –) of the two variables. (h) The angle between the intersecting regression lines depends on the magnitude of the product-moment r value (r YX or r XY) between the two variables, ranging from zero (when r is +/–1.00) to 90 0 (when r is 0.00). (i) The product-moment r between the criterion and the predictor (r YX or r XY) is the geometric mean of the regression coefficients (b YX and b XY) of the two regression lines : r YX or r XY = b b YX XY . (j) Deviations of actually measured criterion scores (Y) from the predicted criterion
M-2\D:\Netaji 05\Biology-1\ score (? Y) may be estimated by the SE of estimate (s YX for ? Y on X) using the SD of the criterion scores. Thus, for the respective regression equations, s s r s s r YX Y YX XY X XY ? ? ? ? 1 1 2 2 ; . . 6.8.3. Computation of simple linear regression 1. Regression of Y on X : (i) Computation of b YX using sum of products : X X n Y Y n b X X Y Y X X YX ? ? ? ? ? ? ? ? ? ? ; ; ( )( ) ( ) . 2 (ii) Computation of b YX using raw scores : b n XY X Y n X X YX ? ? ? ? ? ? ? ? ? 2 2 ( ) . (iii) Computation of a YX and regression equation : a Y b X Y a b X YX YX YX YX ? ? ? ? ; ? . 2. Regression of X on Y : (i) Computation of b XY using sum of products : X X n Y Y n b X X Y Y Y Y XY ? ? ? ? ? ? ? ? ? ? ; ; ( )( ) ( ) . 2 (ii) Computation of b XY using raw scores : b n XY X Y n Y Y XY ? ? ? ? ? ? ? ? 2 2 ( ) . (iii) Computation of a XY and regression equation : a X b Y X a b Y XY XY XY XY ? ? ? ? ; ? . 3. Drawing of the regression line : Several predictor scores are chosen from within their range in the sample, for computing the corresponding criterion scores with the help of the regression equation. The computed criterion scores are plotted against the respective predictor scores on a graph paper and the plotted points are used in drawing the regression line. (See Example 6.8.1.)
M-2\D:\Netaji 05\Biology-1\ Example 6.8.1. Work out the simple

88% **MATCHING BLOCK 91/108** **W**

linear regression of O 2 consumption (Y ml/minute) on tracheal ventilation (X ml/

minute) using the data of Example 6.3.1. Solution : 1. Computation of b YX from sum of products : From the data of Example 6.3.1, the scores of tracheal ventilation (X) and O 2 consumption (Y) are entered in Table 6.5 for further treatment. Table 6.5. Computation of sum of products and sum of squares.

70% **MATCHING BLOCK 95/108** **SA** Statistics for Business Course Document spring ... (D14852953)

X Y X – X – Y – Y – (X – X –) 2 (X – X –) (Y– Y –) 66.0 3.3 – 12.5 – 0.7 156.25 + 8.75 89.1 4.9 + 10.6 + 0.9 112.36 + 9.54 72.0 3.5 – 6.5 – 0.5 42.25 + 3.25 87.5 4.7 + 9.0 + 0.7 81.00 + 6.30 75.2 3.7 – 3.3 – 0.3 10.89 + 0.99 78.2 4.0 – 0.3 0 0.09 0 83.5 4.3 + 5.0 + 0.3 25.00 + 1.50 71.6 3.4 – 6.9 – 0.6 47.61 + 4.14 85.6 4.4 + 7.1 + 0.4 50.41 + 2.84 76.3 3.8 – 2.2 – 0.2 4.84 + 0.44 ? 785.0 40.0 — — 530.70 + 37.75 X X n ? ? ? ? 7850 10 785 . . . Y Y n ? ? ? ? 400 10 40 . . . b X X Y Y X X

YX ? ? ? ? ? ? ? ? ( )( ) ( ) . . . 2 3775 53070 0071. 2.
Computation of b YX from raw scores (alternative method) : From the data of Example 6.3.1, X and Y scores are entered in Table 6.6 for further treatment.
M-2\D:\Netaji 05\Biology-1\ Table 6.6. Computation of b YX from raw scores.

58% **MATCHING BLOCK 93/108** **W**

X Y X 2 XY 66.0 3.3 4356.00 217.80 89.1 4.9 7938.81 436.59 72.0 3.5 5184.00 252.00 87.5 4.7 7656.25 411.25 75.2 3.7 5655.04 278.24 78.2 4.0 6115.24 312.80 83.5 4.3 6972.25 359.05 71.6 3.4 5126.56 243.44 85.6 4.4 7327.36 376.64 76.3 3.8 5821.69 289.94 ? 785.0 40.0 62153.20 3177.75 X X n ? ? ? ? 7850 10 78 5 . . . Y Y n ? ? ? ? 400 10 40 . . . b n XY X Y n X

X
YX ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 2 2 2 10 317775 7850 400 10 6215320 7850 0071 ( ) . . . . . 3. Computation of a YX and regression equation : a Y b X YX YX ? ? ? ? ? ? 4 0 0 071 78 5 157 . . × . . . ? , ? . . Y a b X or Y X YX YX ? ? ? ? ? 1 57 0071 . 4. Drawing of regression line : Four X scores are chosen at random from within the range of X in the data and used in computing the respective ? Y scores. (i) Where X Y a b X YX YX ? ? ? ? ? ? ? ? 70 157 0071 70 34 , ? . . . . (ii) Where X Y a b X YX YX ? ? ? ? ? ? ? ? 75 157 0071 75 38 , ? . . . . (iii) Where X Y a b X YX YX ? ? ? ? ? ? ? ? 80 157 0071 80 41 , ? . . . . (iv) Where X Y a b X YX YX ? ? ? ? ? ? ? ? 85 157 0071 85 45 , ? . . . . Each ? Y score is plotted against the corresponding X score on a graph paper and the plotted points are used to draw the regression line of Y on X (Fig 6.1).
M-2\D:\Netaji 05\Biology-1\ 6.9 Multiple regression Statistical prediction of the scores of a dependent variable or criterion, using the known or measured scores of two or more independent variables or predictors, is called

100% **MATCHING BLOCK 94/108** **W**

multiple regression. It is basically the method of expressing the criterion

as a function of the weighted sum of two or more predictors. 6.9.1. Types of multiple regression Where the criterion has a significant linear correlation with each of the predictors, multiple linear regression is used in predicting its scores from the combination of scores of those predictors. On the contrary, multiple nonlinear regressions have to be used in predicting the scores of the criterion if the latter has significant hyperbolic, sigmoid, exponential or other nonlinear forms of association with the predictors. Methods of multiple regressions also differ according to the number of independent variables/predictors used in the computation. Our discussion will be confined here to multiple linear regression with three variables, i.e., two predictors. 6.9.2. Models of multiple regression Multiple regressions may be of three different models according to the nature
of 4.5 4.0 3.5 3.0 0 65 70 75 80 85 ?
Y

81% **MATCHING BLOCK 99/108** **SA** MSPS 13 L part 1.pdf (D110610736)

X ? Y = -1.57+0.071 .X (= 85 ; = 4.5) X Y ? (= 80 ; = 4.1) X Y ? (= 75 ; = 3.8) X Y ? (= 70 ; = 3.4) X Y ? Fig 6.1. Linear regression line of

O 2 consumption ( ) ? Y on
tracheal ventilation (X) for Example 6.8.1.

M-2\D:\Netaji 05\Biology-1\ predictors used. In a model I or fixed model multiple regression, all the predictors are fixed experimental treatments (e.g., levels of administered insulin) under the control of investigator and free from random errors; for example, the regression of blood glucose on combinations of different administered levels of insulin and cortisol. In a model II or random model multiple regression, all the predictors are beyond the control of investigator and liable to changes at random; for example, the regression of gill weights of fishes on their body weights and trunk lengths, both of the latter being random variables. A model III or mixed model multiple regression is worked out when both types of predictors are simultaneously applied in the experiment; for example, the regression of O 2 consumption by insects on atmospheric O 2 tension (random) and administered levels of dimercaprol (fixed). 6.9.3. Assumptions for multiple linear regression Multiple linear regression can be worked out, only if the following conditions may be logically assumed to be fulfilled. (i) Predictors as well as criterion are continuous measurement variables without genuine gaps in their respective scales of values. (ii) Scores of every variable are almost normally distributed in the population without very distinct bilateral asymmetry. (iii) Actual score of the dependent variable for each case appears in the sample obeying the laws of probability. (iv) The criterion should have linear association with each of the predictors. 6.9.4. Computation of multiple linear regression For working out the multiple linear regression of a

82% **MATCHING BLOCK 96/108** W

criterion (X 1) on the combination of two predictors (X 2 and X 3), partial regression coefficients (b 12.3 and b 13.2)

are computed using the beta coefficients (? 2 and ? 3) and the standard deviations (s 1 , s 2 and s 3) of all three variables. The beta coefficients have been described in Sub- section 6.6.2. Partial regression coefficients, viz., b 12.3 and b 13.2,

100% **MATCHING BLOCK 97/108** W

are the measures of slopes of regression lines of the criterion X 1 on the predictors X 2 and X 3 ,

respectively, when the respective other predictors are eliminated or partialled out. The means of the variables and the partial regression coefficients are used in working out a 1.23 which is the y-intercept of the regression line. Where ?

85% **MATCHING BLOCK 98/108** W

X 1 is the predicted score of the criterion, ? ? 2 12 13 23 23 2 3 13 12 23 23 2 1 1 ? ? ? ? ? ? r r r r r r r r ; ; b s s b s s a X b X b X 123 2 1 2 132 3 1 3 123 1 123 2 132 3 ? ? ? ? ? ? ? ? ? ? ; ; ; ? . . . . X a b X b X 1 1 23 12 3 2 13 2 3 ? ? ?

M-2\D:\Netaji 05\Biology-1\ The standard error of estimate (s 1.23) serves as an estimate of the deviations of actual criterion scores (X 1) from the predicted ? X 1 score, and is worked out using the coefficient of multiple determination which is the squared multiple linear correlation coefficient ( ) . R 1 23 2 between the criterion (X 1) and the combination of predictors (X 2 and X 3). R r r s s R 123 2 2 12 3 13 123 1 123 2 1 . . . ; . ? ? ? ? ? ? Example 6.9.1. Work out the multiple linear regression of

100% **MATCHING BLOCK 100/108** W

glomerular filtration rate (X 1 ml/min) on glomerular blood pressure (X 2 mm Hg) and capsular fluid pressure (X 3 mm Hg), using the following data of a sample of 40

chimpanzees. X – 1 = 120 ml/min; X – 2 = 58 mm Hg; X – 3 =18 mm Hg; s 1 = 21.5 ml; s 2 = 14.2 mm Hg; s 3 = 3.5 mm Hg; r 12 = + 0.82; r 13 = – 0.21; r 23 = + 0.18. Solution : ? 2 12 13 23 23 2 2 1 082 021 018 1 018 089 ? ? ? ? ? ? ? ? ?

84% **MATCHING BLOCK 101/108** W

r r r r . (.) . . . . ? 3 13 12 23 23 2 2 1 021 082 018 1 018 037 ? ? ? ? ? ? ? ? ? ? r r r r . . . . . b s s 12 3 2 1 2 089 215 142 135 . . . . . ; ? ? ? ? ? ? a X b X b X 1 23 1 12 3 2 13 2 3 120 135 58 2 27 18 82 56 . . . . (.) . . ? ? ? ? ? ? ? ? ? ? ? ? , ? . . . . . X a b X b X

X X X 1 123 12 3 2 132 3 1 2 3 8256 135 227 ? ? ? ? ? ? or R r r 123 2 2 12 3 13 089 082 037 021 081 . . . . (.) . . ? ? ? ? ? ? ? ? ? ? s s R 1 23 1 1 23 2 1 215 1 081 937 . . . . . ? ? ? ? ? 6.10 Summary Correlation determines quantitatively the strength and indicates the direction of relationship between two or more variables. It may be simple or multiple according as two or more variables are involved, linear or nonlinear according to the form of association between them, and positive or negative according as the correlated variables b s s 13 2 3 1 3 037 215 35 227 . . . . . ? ? ? ? ? ? ? ? M-2\D:\Netaji 05\Biology-1\ change in the same or opposite directions. Correlation serves as a measure of association between the given variables within only that section of the population from which a sample has been used for its computation, and does not necessarily indicate any cause-effect relationship between the variables. Product-moment correlation coefficient measures the correlation between two continuous, normally distributed and linearly associated variables. Kendall's rank correlation coefficient is used for linear correlations between the ranks given to measurement and ordinal variables. Partial correlation is a measure of magnitude and direction of association between two given variables, eliminating the effects of one or more other variables correlated with them. Multiple correlation is the

61% **MATCHING BLOCK 102/108** W

correlation between a single given variable and the combination of two or more other variables. Regression is

the statistical prediction of the probable score of a given variable called the criterion, on the basis of the measured scores of one or more other variables called the predictors, correlated with the criterion. Regression may be simple or multiple according as one or more predictors are used. Linear regression is worked out if the criterion is linearly correlated with the predictor or predictors. The criterion score predicted by regression is only a probable score and may differ from the actual criterion score. Simple linear regression involves the computation of a regression cofficient which is a measure of average rate of linear changes of criterion scores per unit change of predictor scores. Models of regression are determined by the natures of predictors, the latter being fixed experimental treatments in model I and random variables in model II. Multiple regression consists of the prediction of probable scores of a given criterion on the combination of two or more predictors. It is computed using partial regression coefficients, each of which is a measure of the rate of change of the criterion per unit change of one of the predictors when the effects of other predictors are partialled out. 6.11 Terminal questions 1. (a) Explain what you understand by simple regression and model I regression. (b) State the assumptions for simple linear regression. (c) Work out the simple linear regression of tracheal ventilation (Y ml) on O 2 consumption (X ml) using the following data of a sample of insects. Animal : 1 2 3 4 5 6 7 8 9 10 X : 4.5 3.7 3.6 3.2 3.0 3.2 4.0 3.4 2.8 3.9 Y : 87 75 82 73 72 74 85 73 70 78 2. (a) State the assumptions for product-moment r. (b) Discuss the properties of product-moment correlation.
M-2\D:\Netaji 05\Biology-1\ (c) Work out product-moment r using the data of Question 1(c) and find its significance, choosing the correct critical t value from below. (? = 0.05.) t 0.05(19) = 2.093; t 0.05(8) = 2.306; t 0.05(9) = 2.262. 3. (a) Explain what you mean by first-order and second-order partial correlations, citing examples. (b) Discuss the assumptions for linear partial correlations. (c)

34% **MATCHING BLOCK 103/108** W

Find whether or not there is a significant linear partial correlation between gill weights (X 1 gm) and trunk lengths (X 2 cm), partialling out body weights (X 3 gm) in the following sample of 43

salmons using the r values between the respective variables and the correct critical t value from below. (? = 0.01.) r 12 = +0.55; r 13 = +0.30; r 23 = +0.28. t 0.01(42) = 2.698; t 0.01(41) = 2.701; t 0.01(40) = 2.704. 4. (a) Give a brief account of different models of regression. (b) Describe the assumptions for multiple linear correlations. (c) Use the data of question 3(c)

76% **MATCHING BLOCK 104/108** W

to find whether or not there is a significant multiple linear correlation between gill weights (X 1) and

the combination of trunk lengths (X 2) and body weights (X 3). (??= 0.01.) 5. (a) Describe different types of regression. (b) Discuss the properties of simple linear regression. (c) Workout

68% **MATCHING BLOCK 105/108** W

the multiple linear regression equation of variable X 1 on the combination of variables X 2 and X 3

in the following sample of 50 animals using their means, standard deviations and product-moment r values given below. X – 1 = 12.0; X – 2 = 8.5; X – 3 = 6.4; s 1 = 2.82; s 2 = 2.10; s 3 = 1.21; r 12 = +0.72; r 13 = −0.21; r 23 = −0.23. 6. (a) Discuss where you would compute Kendall's tau, describing its assumptions. (b) Describe the sources of inaccuracies in the computation of Kendall's tau and a method for countering one of them. (c) Using the data of Question 1(c), work out Kendall's tau to

91% **MATCHING BLOCK 106/108** W

find whether or not there is a significant correlation between O 2 consumption (X ml) and tracheal ventilation (

Y ml), choosing the correct critical t value from below. (? = 0.01). t 0.01(10) =3.169; t 0.01(9) =3.250; t 0.01(20) =2.845; t 0.01(?) =2.576;

M-2\D:\Netaji 05\Biology-1\ 6.12 Answers 1. (a) See Sub-sections 6.7.1 and 6.7.2. (b) See Sub-section 6.8.1. (c) See Example 6.8.1. 2. (a) See Sub-section 6.3.1. (b) See Sub-section 6.3.2. (c) See Example 6.3.1. 3. (a) See Sub-section 6.5.1. (b) See Sub-section 6.5.2. (c) See Example 6.5.1. 4. (a) See Sub-section 6.7.2. (b) See Sub-section 6.6.1. (c) See Example 6.6.1. 5. (a) See Sub-section 6.7.1. (b) See Sub-section 6.8.2. (c) See Example 6.9.1. 6. (a) See Sub-section 6.4.1. (b) See Sub-section 6.4.2. (c) See Example 6.4.1.

M-2\D:\Netaji 05\Biology-1\ Unit 7 ? PROBABILITY THEORY 7.1 Introduction This unit will describe random occurrences of results of most scientific investigations. You will read about random events of diverse types in the discrete sample space of the experiment. Nature of independent events will be briefly presented. Probability will be defined and explained, and its theorems will be discussed in brief. Objectives After studying this unit, you should be able to do the following : ? define random experiments and their sample spaces, ? define and explain random events, ? classify and define different types of random events, ? explain what is meant by independent events, ? understand and define probability, and ? describe the theorems of probability. 7.2 Random experiments Most experiments or scientific investigations can be repeated any number of times under the same conditions, but their results are neither predictable before the termination of the particular trial, nor identical in most of the cases inspite of apparently identical situations. Such experiments are called random experiments. Their basic Structure 7.1 Introduction Objectives 7.2 Random experiments 7.3 Random events 7.4 Independent events 7.5 Probability 7.6 Theorems of probability 7.7 Summary 7.8 Terminal questions 7.9 Answers

M-2\D:\Netaji 05\Biology-1\ characteristics are as follows : (i) they may be repeated any number of times under the same situations and even with the same samples, (ii) all results or outcomes possible for such an experiment are known and together constitute its sample space, (iii) but the result is not predictable for any specific performance of the experiment so that (iv) any of those predictable results may be yielded by the experiment on any particular occasion, (v) repeated performances of the same experiment under apparently unchanged situations may consequently give results mostly differing from each other and coinciding only sometimes, and (vi) the sample space consists of elements called sample points. If, say, you have to choose at random a single donor for a blood donation camp, the possible ABO blood group of a randomly chosen donor may be either A or B or O or AB group. This set of blood groups exhausts the possibility of choice of ABO blood groups. Such a set of possible outcomes, e.g., [A, B, C, D] in this case, is called the sample space for this experiment or sampling, and consists here of four possible elements or outcomes in a single choice. Each such element in a sample space is called a sample point. In case one animal has to be chosen in a single chance from among animals of male (M) and female (F) sexes, the sample space for the single choice would consist of the set [M, F] with M and F as its sample points. In contrast, when choosing twice from those animals, each one (M or F) of the first choice may be succeeded by either of those two options in the second choice so that the chosen elements may constitute 2 2 or 4 sample points, viz., FF, FM, MF and MM, and this set of all four sample points forms the sample space for two successive choices. A sample space, consisting of a set of all possible results or choices, may be continuous or nondiscrete if it consists of an infinite set of sample points that cannot be counted. On the contrary, a discrete or discontinuous sample space comprises either a finite set or an enumerable, though infinite, set of sample points. 7.3 Random events An event consists of the occurrence, existence or happening of any case, incident, individual, phenomenon or score. It is called a random event if it either occurs or does not occur as an outcome of a random experiment or choice with discrete sample space. Any subject of a discrete sample space may constitute a random event in the relevant experiment. A single set of elements or a combination of such sets may occur as a random event. Occurrence of a random event should obey the laws of probability; moreover, in a random experiment or sampling, every event or individual should have an equal probability of occurrence.
M-2\D:\Netaji 05\Biology-1\ Random events may be of various types according to the different manners of the probabilities of their occurrences. For example, an elementary or simple event is a single event of the sample space while a compound event comprises more than one element or sample point in the sample space. It should be understood that individual simple events as well as the entire sample space are considered as events. If the occurrence of either of two events is prevented by the occurrence of the other, they are known as mutually exclusive events; thus, A and B would be mutually exclusive of each other if the probability (P) of their simultaneous occurrence is zero, i.e., P(A.B) = 0, so that A and B would never occur together. For an event occurring at random in a random experiment, its complementary event is its non-occurrence in the latter. An elementary event is a single element of the sample space while a compound event consists of a collection of more than one single sample point or element in the sample space. If the occurrence of each of two events influences and changes the probability of occurrence of the other, they are called mutually dependent events; on the contrary, two

47%	MATCHING BLOCK 108/108	SA	PC MBT 1.7 BIOSTATISTICS 23.05.22.docx.pdf (D137635301)

events are mutually independent if the occurrence of each does not affect or alter the probability of occurrence of the other.

If neither of two events has any probability of occurring in preference to the other so that either of them may precede the other in occurring in the outcome of a random experiment, they are called equally likely events. Where the outcome of a random experiment consists of a collection of a given number of random events, that collection is exhaustive if one or more or those events must occur in the result of the experiment. A sure event of a random experiment is the whole discrete sample space occurring with certainty in that experiment and has, therefore, a probability of 1.00 (i.e., 100% chance) of occurrence, while its empty subset with zero probability of occurrence is the impossible or null event. 7.4 Independent events Two random events of a random experiment are mutually independent of one another if the occurrence of either of them does not affect or alter the probability of occurrence of the other. If A and B are independent events, the probability of occurrence of neither of them (A or B) would be enhanced or decreased, whether or not it is preceded or accompanied by the occurrence of the other. Thus, each of two independent events follows its own probability of occurrence in turning up in the experiment. Moreover, each such event can occur alone and also together or preceding or following the other one. The probability P(A.B) of the simultaneous or successive occurrences of those two events is given by the multiplication theorem of probability. (See Sub- section 7.6.2.) M-2\D:\Netaji 05\Biology-1\ P(A.B) = P(A) × P(B). Thus, the probability of simultaneous or successive occurrences of k number of independent events is given by : P(A. B. C.....k) = P(A) × P(B) × P(C) ×.....×P(k). 7.5 Probability You have read in Sub-section 3.4.1. of Unit 3 about the relative frequency (f/n) of any event or score in a sample, and its computation by dividing the frequency (f) of that event or score with the total number (n) of the scores or events in the sample. In a simple manner, the relative frequency of a particular type of event or score in a very vast or nearly infinite total number of events or scores, may be taken as the probability (P) of that particular event or score. In other words, probability is the measure of random occurrence of an event among an infinite total number of events. You may thus understand that the total number of all events, forming the entire sample space and constituting the sure event, has a 100% certainty or a probability of 1.00 of occurrence; so, the probability of occurrence of every other event or element, being a part of the entire sample space, would amount to a specific fractional part of 1.00 while a null event with no probability of occurrence would have a zero probability. Thus, for any event other than the sure event, the probability of random occurrence would either exceed or equal zero : P ? 0. This makes the scale of probability or relative frequency a continuous one in any probability distribution (see Sub-section 3.4.1). For example, if 8769 pomfret fishes of trunk length of 8.3 cm occur in a random sample of 57421 pomfret fishes, the probability of random occurrence of the given trunk length of 8.3 cm may be worked out as follows. P f n ? ? ? 8769 57421 015. . However, the relative frequency of an event may differ from its probability of random occurrence, if the total number of all events or trials is small. With the increase in the total number of events or cases, the relative frequency gets progressively closer to the probability. Probability (P) is, therefore, the limiting relative frequency of the event under consideration, when the sample size approaches or reaches infinity. For exmple, in choosing one animal from a population with a male : female ratio of 0.55 : 0.45, the possible alternatives are two only, viz., either a male or a female, so that the relative frequency of a male animal being chosen amounts to 1.00 in the first case and 0.00 in the second; but in choosing two animals either simultaneously or successively from the same population, there would be three alternative outcomes,

M-2\D:\Netaji 05\Biology-1\ viz., one male and one female, or two males, or two females, so that the relative frequency of males varies from 0.50 in the first case, to 1.00 in the second case, and to 0.00 in the third case. This way the relative frequency of occurrence of males goes on changing with the number of choices, until choices are made an infinitely large number of times when the relative frequency would approach or reach the probability (P) of 0.55 expected from the population sex ratio. 7.6 Theorems of probability You will next read about two basic theorems of probability. 7.6.1. Addition theorem If there are k number of mutually exclusive events to choose from in one chance, so that the occurrence or choice of any one of them excludes the probability of occurrence of the others and more than one of them cannot occur simultaneously, then the addition theorem gives the probability of occurrence of any one event (e.g., A or B or C .....or k) out of the total number of exclusive events, as the sum obtained by adding the respective probabilities of their individual occurrences. P(A or B or C or ..... k) = P(A) + P(B) + P(C) + ...+ P(k). For example, if a single rat has to be chosen or to occur at random in one chance out of a total of 15 rats (k = 15), the probability P(C) of random occurrence or choice of rat C separately and individually in one chance is given by 1/k and amounts to 1/15 or 0.0667; probabilities of similar individual occurrences of rats D and E would also amount to 1/15 or 0.0667 each. Because they are mutually exclusive, the occurrence of any of them in a single trial would prevent the chances of occurrences of the other two. In such a case, according to the addition theorem, the probability of either C or D or E occurring or getting chosen in one chance equals the sum of probabilities of random, separate and individual occurrence of each of those three. P(C or D or E) = P(C) + P(D) + P(E) = 0.0667 + 0.0667 + 0.0667 = 0.20. 7.6.2. Multiplication theorem If there are k number of independent events (A, B, C, ...k) to choose from, so that the choice or occurrence of any of the events does not affect or alter the probability of simultaneous or successive random occurrence of any other, the multiplication theorem then gives the probability of simultaneous or successive occurrences of a number of those independent events as the product of the respective individual probabilities of random occurrences of the latter. Thus, the multiplication theorem states that the probability of combined occurrences, simultaneous or successive, of M-2\D:\Netaji 05\Biology-1\ more than one independent event is the product of the probabilities of their individual occurrences. That way, the combined probability of occurrence of all the k number of independent events, either at the same time or after each other, would be given by : P(A. B. C.....k) = P(A) × P(B) × P(C) × ..... × P(k). For exmple, if rats A, C and D have to be chosen successively at random out of a total of 15 rats (k = 15), the probability of separate individual choice of each of the three is given by 1/k which amounts to 1/15 or 0.0667. If the chosen rat is replaced in the group before the next choice to keep the total number k of the rats unchanged, the probability of choice of successive rats would remain unaffected to maintain their independent status as events. In such a case, the combined probability of successive choices of A, C and D would be given by : P(A.C.D) = P(A) × P(C) × P(D) = 0.0667 × 0.0667 × 0.0667 = 0.0003. 7.7 Summary Random experiments may be repeated any number of times, but their precise outcome is not predictable. A set of possible outcomes of an experiment constitutes a sample space and each element in that set is a sample point. Events may consist of an individual single element as well as the entire sample space. Random events occur at random under the laws of probability and belong to various types. Mutually independent events do not affect the probabilities of each other, while mutually exclusive events prevent the occurrences of each other so that they never occur together. Either of two equally likely events can precede the other in occurring. Probability of an event is its limiting relative frequency when the total frequency of all events under consideration approaches infinity. The probability of occurrence of any one of several alternative exclusive events out of a total number of events is given by the addition theorem of probability as the sum of probabilities of individual occurrences of those alternative events. The multiplication theorem gives the probability of combined occurrence of a number of independent events as the product of the probabilities of their separate individual occurrences. 7.8 Terminal questions 1. (a) Give a brief account of random experiments, describing their basic characteristics. (b) Describe the addition theorem of probability and its application. M-2\D:\Netaji 05\Biology-1\ (c) Explain what are sample spaces. Define continuous and discrete sample spaces. 2. (a) Discuss random events and their different types. (b) Write briefly about independent events and the probability of their simultaneous or successive occurrences. (c) Describe the probability of random occurrence of any one of a number of mutually exclusive events with an example. 3. (a) Give a brief account of the concept of probability. (b) Discuss the addition and multiplication theorems of probability. (c) What do you understand by mutually exclusive and mutually independent events? 7.9 Answers 1. (a) See Section 7.2 . (b) See Sub-section 7.6.1. (c) See Section 7.2. 2. (a) See Section 7.3. (b) See Section 7.4 and Sub-section 7.6.2. (c) See Sub-section 7.6.1. 3. (a) See Section 7.5. (b) See Sub-sections 7.6.1 and 7.6.2. (c) See Sections 7.3, 7.4 and 7.6.

M-2\D:\Netaji 05\Biology-1\ ? APPENDIX ? Table 1. Some fractional areas of unit normal curve from its mean to different z scores. z score : 1.94 1.95 1.96 1.97 1.98 1.99 2.00 2.01 2.02 2.03 2.04 Area : .4738 .4744 .4750 .4756 .4761 .4767 .4772 .4778 .4783 .4788 .4793 z score : 2.05 2.06 2.07 2.08 2.09 2.10 2.51 2.52 2.53 2.54 2.55 Area : .4798 .4803 .4808 .4812 .4817 .4821 .4940 .4941 .4943 .4945 .4946 z score : 2.56 2.57 2.58 2.59 2.60 2.96 2.97 2.98 2.99 3.00 4.00 Area : .4948 .4949 .4951 .4952 .4953 .4985 .4985 .4986 .4986 .4987 .49997 Table 2. Some Two-tail Critical t values. df : 11 12 13 14 15 16 17 18 19 ? 0.05 : 2.201 2.179 2.160 2.145 2.131 2.120 2.110 2.101 2.093 ? 0.01 : 3.106 3.055 3.012 2.977 2.947 2.921 2.898 2.878 2.861 df : 20 21 22 23 24 25 26 27 28 ? 0.05 : 2.086 2.080 2.074 2.069 2.064 2.060 2.056 2.052 2.048 ? 0.01 : 2.845 2.831 2.819 2.807 2.797 2.787 2.779 2.771 2.763 df : 29 30 40 ? ? 0.05 : 2.045 2.042 2.021 1.960 ? 0.01 : 2.756 2.750 2.704 2.576 Table 3. Some Critical chi square values. df : 1 2 3 4 5 6 7 8 9 10 ? 0.05 : 3.84 5.99 7.82 9.49 11.07 12.59 14.07 15.51 16.92 18.31 ? 0.01 : 6.64 9.21 11.34 13.28 15.09 16.81 18.48 20.09 21.67 23.21 Table 4. Some Critical F values. df : 1,8 1,9 1,10 1,11 1,12 1,13 1,14 1,15 1,16 1,17 1,18 ? 0.05 : 5.32 5.12 4.96 4.84 4.75 4.67 4.60 4.54 4.49 4.45 4.41 ? 0.01 : 11.26 10.56 10.04 9.65 9.33 9.07 8.86 8.68 8.53 8.40 8.28 df : 2,8 2,9 2,10 2,11 2,12 2,13 2,14 2,15 2,16 2,17 2,18 ? 0.05 : 4.46 4.26 4.10 3.98 3.88 3.80 3.74 3.68 3.63 3.59 3.55 ? 0.01 : 8.65 8.02 7.56 7.20 6.93 6.70 6.51 6.36 6.23 6.11 6.01 M-2\D:\Netaji 05\Biology-1\ Acknowledgements : Values in the above-mentioned tables have been quoted from the following publications. Table 1 : J. E. Wert, Educational Statistics, McGraw-Hill Book Co. Tables 2 and 3 :

94% **MATCHING BLOCK 107/108** **W**

R. A. Fisher and F. Yates, Statistical Tables for Biological, Agricultural and Medical Research, Longman Group Ltd.

Table 4 : G. W. Snedecor and W. G. Cochran, Statistical Methods, Iowa State University Press.

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1/108 **SUBMITTED TEXT** 15 WORDS **70%** **MATCHING TEXT** 15 WORDS

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2/108 **SUBMITTED TEXT** 12 WORDS **87%** **MATCHING TEXT** 12 WORDS

Objectives After reading this unit, you should be able to do the

SA MSPS 13 L part 1.pdf (D110610736)

3/108 **SUBMITTED TEXT** 1 WORDS **100%** **MATCHING TEXT** 1 WORDS

X Y X Y X Y X Y X Y X Y

SA Statistics for Business Course Document spring 2015.pdf (D14852953)

4/108 **SUBMITTED TEXT** 28 WORDS **38%** **MATCHING TEXT** 28 WORDS

X X X YY Y X a c a c a b c 1 3 1 2 3 ? ? ? ? ? ? ? ? ( ) ( ) ; ; Y Y
X X X a c a c 1 3 2 ? ? ?( ) ; Y Y X X X

SA MSPS 13 L part 1.pdf (D110610736)

5/108 **SUBMITTED TEXT** 43 WORDS **46%** **MATCHING TEXT** 43 WORDS

X + log Y); X Y = antilog (log X – log Y); X Y = antilog (Y
log X); Y X = antilog (X log Y); X Y = antilog log X Y ; Y X =

SA Statistics for Business Course Document spring 2015.pdf (D14852953)

6/108 **SUBMITTED TEXT** 52 WORDS **75%** **MATCHING TEXT** 52 WORDS

X 2 – X 1 = X 3 – X 2 = d, or, 2X 2 = X 1 + X 3 , ? X 2 = X X
1 3 2 ? . Thus, X 2

SA MSPS 13 L part 1.pdf (D110610736)

7/108 **SUBMITTED TEXT** 77 WORDS **48%** **MATCHING TEXT** 77 WORDS

X c X c X c X c k 1 2 3 , , ,..... ; cX 1 , cX 2 , cX 3 , ...cX k ;
X 1 + c, X 2 + c, X 3 + c, ... X k + c; X 1 – c, X 2 – c, X 3
– c, ...

SA PC MBT 1.7 BIOSTATISTICS 23.05.22.docx.pdf (D137635301)

8/108 **SUBMITTED TEXT** 18 WORDS **59%** **MATCHING TEXT** 18 WORDS

X c X c X c X c cX cX cX cX k k 1 2 3 1 2 3 , , ,..... ; , ,
,..... ; c X c X c X c X c c c c

SA PC MBT 1.7 BIOSTATISTICS 23.05.22.docx.pdf (D137635301)

9/108 **SUBMITTED TEXT** 153 WORDS **24%** **MATCHING TEXT** 153 WORDS

X 2 –X 1 = 9 – 4 = 5. k = 6. X k = X 1 + (k – 1) d = 4 + (6 – 1) 5 = 29. ? ? ? ? ? ? ? ? ? ? ? ? (.....) ( ) ( )5 . [ ] [ ] X X X k X k d k 1 2 1 2 2 1 6 2 2 4 6 1 99 ; or, ? ? ? ? ? ? ? ? ? ? ? ( ) ( ) ( ) ..... . X X X k X X k k 1 2 1 2 6 2 4 29 99 (ii) The series 3, 9, 27, ... constitutes a GP. r X X k ? ? ? ? 2 1 9 3 3 6 . . X k = X 1 r k–1 = 3 × 3 6–1 = 729. ? ? ? ? ? ? . 1092 13 1 33 1 1 ..... 6 1 2 1 ? ? ? ? ? ? ? ? ? ? ? ? r rX X X X

x x 2 = 2 2 + 1 2 + 0 2 + 1 2 + 2 2 = 10, hx,x 2 i = 0, and h|x| - 6/5,x 2 i = X x |x| 3 - 6 5 X x x 2 = (2 3 + 1 3 + 0 3 + 1 3 + 2 3) - 6 5 · 10 = 18 - 12 = 6. Thus P G (x 2) = h1,x 2 i k1k 2 1 + hx,x 2 i kxk 2 x + h|x| - 6/5,x 2 i k|x| - 6/5 k 2 | x| - 6 5 = 10 5 + 6 14/5 |x| - 6 5 = 15 7 |x| - 4 7 .

W https://www.stat.berkeley.edu/~hhuang/200B/practice.pdf

10/108 **SUBMITTED TEXT** 12 WORDS **87%** **MATCHING TEXT** 12 WORDS

Objectives After studying this unit, you will be able to do the

SA PC MBT 1.7 BIOSTATISTICS 23.05.22.docx.pdf (D137635301)

11/108 **SUBMITTED TEXT** 28 WORDS **55%** **MATCHING TEXT** 28 WORDS

X X n 2 2 ? ? ?( ) , or s X n X n 2 2 2 2 ? ? ? ?( ) , or s n X X n 2 2 2 2 ? ? ? ?( ) , where (X – X –)

SA MSPS 13 L part 1.pdf (D110610736)

12/108 **SUBMITTED TEXT** 13 WORDS **82%** **MATCHING TEXT** 13 WORDS

the standard error of the mean of the following sample of blood sugar (mg/

the standard error of the mean of the following frequency distribution of blood sugar scores (mg

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13/108 **SUBMITTED TEXT** 13 WORDS **100%** **MATCHING TEXT** 13 WORDS

Grey-body red-eye 108 0.551 Black-body red-eye 40 0.204 Grey-body scarlet-eye 36 0.184 Black-body scarlet-eye 12 0.061

Grey body red eye Black body red eye Grey body scarlet eye Black body scarlet eye

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14/108 **SUBMITTED TEXT** 22 WORDS **100%** **MATCHING TEXT** 22 WORDS

A binomial probability distribution is a distribution of probabilities of random occurrences of different combinations of cases from the two classes of

A binomial probability distribution is a distribution of probabilities of random occurrences of different combinations of cases from the two classes of

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15/108 **SUBMITTED TEXT** 68 WORDS **42%** **MATCHING TEXT** 68 WORDS

X X – X – (X – X –) 2 9.0 + 1.2 1.44 8.4 + 0.6 0.36 6.8 – 1.0 1.00 6.4 – 1.4 1.96 7.8 0 0 8.6 + 0.8 0.64 6.8 – 1.0 1.00 7.8 0 0 8.4 + 0.6 0.36 8.0 + 0.2 0.04 ? 78.0 — 6.80 n X X n ? ? ? ? 10 78 0 10 7 8 . . . ? cm. s X X n 2 2 2 1 6 80 10 1 0 756 ? ? ? ? ? ? ?( ) . . .cm M-2\D:\Netaji 05\Biology-1\ s X X n ? ? ? ? ? ? ?( ) . . . 2 1 6 80 10 1 0 869cm s s n X ? ? ? 0 869 10 0 275 . .

SA MSPS 13 L part 1.pdf (D110610736)

16/108 **SUBMITTED TEXT** 21 WORDS **100%** **MATCHING TEXT** 21 WORDS

is a theoretical probability distribution because it can be worked out theoretically using the series of terms of the binomial equation.

is a theoretical probability distribution because it can be worked out theoretically using the series of terms of the binomial equation. ?

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17/108 **SUBMITTED TEXT** 17 WORDS **61%** **MATCHING TEXT** 17 WORDS

of random occurrence of a particular combination of cases from two classes of a dichotomous variable in

of random occurrences of different combinations of cases from the two classes of dichotomous variable in

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18/108 **SUBMITTED TEXT** 47 WORDS **63%** **MATCHING TEXT** 47 WORDS

n n p q n n n p q n n n n n ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 1 2 2 3 3 1 1 2 1 2 1 2 3 ..... ( )( )( )..... .....( ) ? ? ? ? ? ? ? ? ? ? ? ? n n n n n pq q n n 1 2 3 2 1 2 3 1 1 .

N 2 = n 2) = P n 2 -1 n 1 =1 P(N 1 = n 1 , N 2 - N 1 = n 2 - n 1) = P n 2 -1 n 1 =1 * 1/4 n 2 -2 * 3/4 2 = (n 2 - 1) * 1/4 n 2 -2 * 3/4 2 . Thus N 2 - 2

W https://www.stat.berkeley.edu/~hhuang/200B/practice.pdf

19/108	SUBMITTED TEXT	12 WORDS	100%	MATCHING TEXT	12 WORDS

of cases of the class having the proportion p in the population,

of cases of the class having the proportion p in the population

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20/108	SUBMITTED TEXT	42 WORDS	63%	MATCHING TEXT	42 WORDS

n = 10. X = 7 = n – 3. n – X = 10 – 7 = 3. ( ) ( ) ( )( ) ..... . p q p np q n n p q n n n p q q n n

n ? 1)(n ? 2) n ? 3 3 (p ? q)n ? p n ? np n ? 1p q ? p q ? ... 1.2 1.2.3 n(n ? 1)(n ? 2)... ? 3.2 p.q n?1 ? q n 1.2.3..... ? (n ? 1) 382

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21/108	SUBMITTED TEXT	40 WORDS	81%	MATCHING TEXT	40 WORDS

known to have 80 mutants per 10000. Interpret your result. (? = 0.05.) Solution : n = 500. X = 9. p ? No.of mutants in given population size Given population size ? ? 80 10000 0008. . ? =

known to have 80 mutant per 10,000. Interpret your result (? = 0.05). Solution: N = 500, X = 9 P(X) ? ? Number of mutants in a given population size Given population size 80 10,000 ? 0.008

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22/108	SUBMITTED TEXT	14 WORDS	100%	MATCHING TEXT	14 WORDS

Example 4.6.1. Find whether or not there is a significant difference between the mean

Example 7: Find whether or not there is a significant difference between the mean

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23/108	SUBMITTED TEXT	17 WORDS	63%	MATCHING TEXT	17 WORDS

X X n X X n s X X n s X X n 1 1 1 2 2 2 1 1 1 2 1 2 2 2 2 2 1 1 ? ? ? ? ? ? ? ? ? ? ? ? ; ; ( ) ; ( ) ; s s n s s n s s s

SA MSPS 13 L part 1.pdf (D110610736)

24/108 **SUBMITTED TEXT** 210 WORDS **37%** **MATCHING TEXT** 210 WORDS

X X X X 1 2 1 2 2 2 2 2 01073 00803 01340 ? ? ? ? ? ? . . . .ml z X X s X X ? ? ? ? ? ? ? 1 2 1 2 310 285 01340 187 . . . . . M-2\D:\Netaji 05\Biology-1\ Table 4.1. Computation of sums of squares. X 1 X 2 X 1 –X – 1 X 2 –X – 2 (X 1 –X – 1) 2 (X 2 –X – 2) 2 3.0 2.6 – 0.10 – 0.25 0.0100 0.0625 3.5 2.7 + 0.40 – 0.15 0.1600 0.0225 3.8 2.5 + 0.70 – 0.35 0.4900 0.1225 3.7 3.0 + 0.60 + 0.15 0.3600 0.0225 3.1 3.3 0 + 0.45 0 0.2025 2.7 2.9 – 0.40 + 0.05 0.1600 0.0025 3.0 2.8 – 0.10 – 0.05 0.0100 0.0025 3.8 3.5 + 0.70 + 0.65 0.4900 0.4225 2.5 3.1 – 0.60 + 0.25 0.3600 0.0625 2.9 2.3 – 0.20 – 0.55 0.0400 0.3025 3.7 2.5 + 0.60 – 0.35 0.3600 0.1225 3.3 3.0 + 0.20 + 0.15 0.0400 0.0225 2.6 3.3 – 0.50 + 0.45 0.2500 0.2025 3.0 2.5 – 0.10 – 0.35 0.0100 0.1225 2.9 2.8 – 0.20 – 0.05 0.0400 0.0025 2.4 3.2 – 0.70 + 0.35 0.4900 0.1225 2.5 2.6 – 0.60 – 0.25 0.3600 0.0625 3.2 3.2 + 0.10 + 0.35 0.0100 0.1225 2.6 3.0 – 0.50 + 0.15 0.2500 0.0225 3.8 2.2 + 0.70 – 0.65 0.4900 0.4225 ? 62.0 57.0 — — 4.3800 2.4500

x 3 ,x 4) = 18 2 3 + 5x 1 - 4x 2 - 3(x 2 2 - 2/3) + 3x 3 + 2(x 2 3 - 2/3) - 2x 4 - (x 2 4 - 2/3) - 3x 2 + 2x 1 x 3 - x 1 x 4 = 20 + 5x 1 - 4x 2 - 3x 2 2 + 3

W https://www.stat.berkeley.edu/~hhuang/200B/practice.pdf

25/108 **SUBMITTED TEXT** 54 WORDS **43%** **MATCHING TEXT** 54 WORDS

n n X X n X X n 1 2 1 1 1 2 2 2 20 20 620 20 310 570 20 285 ? ? ? ? ? ? ? ? . . . . . ? ? ml ml s X X n s X X n 1 1 1 2 1 2 2 2 2 2 1 4 3800 20 1 0 480 1 2 4500 20 1 0 359 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ( ) . . . ( ) . . . ml ml s s n s s n X X 1 2 1 1 2 2 0480 20 01073 0359 20 00803 ? ? ? ? ? ? . . . . . ml ml s s s

SA MSPS 13 L part 1.pdf (D110610736)

26/108 **SUBMITTED TEXT** 77 WORDS **69%** **MATCHING TEXT** 77 WORDS

the following two groups of fishes from two different habitats. (? = 0.05.) Group 1 (X 1) : 85, 90, 80, 75, 70, 75, 80, 90, 85, 80. Group 2 (X 2) : 55, 65, 60, 75, 80, 70, 55, 60, 80, 70. Critical 2-tail t scores : t 0.05(19) = 2.093; t 0.05(18) = 2.101; t 0.05(20) = 2.086; t 0.05(17) = 2.110. Solution :

the following two groups of samples of 10 fishes of Tilapia mossambica from two habitats. Group X Group Y 1 100 77 2 80 63 3 65 68 4 75 70 5 110 55 6 75 62 7 95 75 8 78 70 9 80 60 10 92 80 Critical t scores: t0.05 (19) = 2.093 t0.05(18) = 2.101 t0.05(9) = 2.262 t0.05(8) = 2.306 Solution: •

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27/108 **SUBMITTED TEXT** 27 WORDS **51%** **MATCHING TEXT** 27 WORDS

X X n X X n s X X X X n n 1 1 1 2 2 2 1 1 2 2 2 2 1 2 2 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ; ; ? ( ) ( ) ; s s n n n n n t X X s df n n X X

SA MSPS 13 L part 1.pdf (D110610736)

28/108 **SUBMITTED TEXT** 27 WORDS **71%** **MATCHING TEXT** 27 WORDS

Critical t 0 05 18 2101 . ( ) . . ? As the computed t is higher than the critical t 0.05 , P is

critical t0.05(13) = 2.16. As the value of computed t less than the critical t0.05. P is

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29/108 **SUBMITTED TEXT** 20 WORDS **67%** **MATCHING TEXT** 20 WORDS

So, the H 0 is rejected and it is inferred that there is a significant difference between the

So the null hypothesis is rejected. Therefore it is inferred that there is significant difference presence, between the

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30/108 **SUBMITTED TEXT** 162 WORDS **58%** **MATCHING TEXT** 162 WORDS

X 1 X 2 X 1 – X – 1 (X 1 – X – 1) 2 X 2 – X – 2 (X 2 – X – 2) 2 85 55 + 4.0 16.00 – 12.0 144.00 90 65 + 9.0 81.00 −2.0 4.00 80 60 – 1.0 1.00 – 7.0 49.00 75 75 – 6.0 36.00 + 8.0 64.00 70 80 – 11.0 121.00 + 13.0 169.00 75 70 – 6.0 36.00 + 3.0 9.00 80 55 – 1.0 1.00 – 12.0 144.00 90 60 + 9.0 81.00 – 7.0 49.00 85 80 + 4.0 16.00 + 13.0 169.00 80 70 – 1.0 1.00 + 3.0 9.00 ? 810 670 — 390.00 — 810.00 n n X X n X X n 1 2 1 1 1 2 2 2 10 10 810 10 810 670 10 670 ? ? ? ? ? ? ? ? . . . . . ? ? g g ? ( ) ( ) . . . . s X X X X n n ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 1 1 2 2 2 2 1 2 2 39000 81000 10 10 2 816g s s n n n n X X 1 2 1 2 1 2 816 10 10 10 10 3649 ? ? ? ? ? ? ? ? . . .

SA MSPS 13 L part 1.pdf (D110610736)

31/108 **SUBMITTED TEXT** 18 WORDS **51%** **MATCHING TEXT** 18 WORDS

X X n X X n s X X n s X X n 1 1 1 2 2 2 1 1 1 2 1 2 2 2 2 2 1 1 ? ? ? ? ? ? ? ? ? ? ? ? ? ; ; ( ) ; ( ) ; s s n s n t X X s df n n X X

SA MSPS 13 L part 1.pdf (D110610736)

32/108	SUBMITTED TEXT	14 WORDS	76%	MATCHING TEXT	14 WORDS

Example 4.7.3. Find whether or not there is a significant change in the mean

Example 7: Find whether or not there is a significant difference between the mean

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33/108	SUBMITTED TEXT	46 WORDS	54%	MATCHING TEXT	46 WORDS

Find whether or not the following observed phenotype distribution in a sample of Drosophila has a significant goodness of fit with Mendelian 9 : 3 : 3 : 1 distribution. (? = 0.01.) Phenotypes : Grey body- Grey body- Black body- Black body- red eye (AB) scarlet eye (

Find whether or not the following phenotype distribution in a sample of 96 flies from a particular fruit fly population has a goodness of fit with the Mendelian 9 : 3 : 3 : 1 distribution. Phenotypes: Grey body red eye Black body red eye Grey body scarlet eye Black body scarlet eye

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34/108	SUBMITTED TEXT	10 WORDS	100%	MATCHING TEXT	10 WORDS

Find whether or not there is a significant association between

find whether or not there is a significant association between

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35/108	SUBMITTED TEXT	10 WORDS	100%	MATCHING TEXT	10 WORDS

that there is a significant association between diabetes and hypertension (

that there is a significant association between diabetes and hypertension.

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36/108	SUBMITTED TEXT	98 WORDS	82%	MATCHING TEXT	98 WORDS

Work out the t test to find whether or not the mean gill weights (mg) differ in the following two small samples of crabs from two habitats. (? = 0.05.) Group 1 (X 1) : 100, 80, 75, 65, 75, 110, 95, 78, 92, 80. Group 2 (X 2) : 77, 63, 70, 68, 62, 55, 75, 70, 60, 60. Critical t scores : t t 0 05 19 0 05 18 2093 2101 . ( ) . ( ) . ; . ; ? ? t t 005 9 005 8 2262 2306 . ( ) . ( ) . ; . . ? ? 5.(

Work out the t test to find whether or not the mean gill weights (mg) differ in the following two groups of samples of 10 fishes of Tilapia mossambica from two habitats. Group X Group Y 1 100 77 2 80 63 3 65 68 4 75 70 5 110 55 6 75 62 7 95 75 8 78 70 9 80 60 10 92 80 Critical t scores: t0.05 (19) = 2.093 t0.05(18) = 2.101 t0.05(9) = 2.262 t0.05(8) = 2.306

W https://pdfcoffee.com/introduction-to-bio-statistics-pdf-free.html

37/108 **SUBMITTED TEXT** 64 WORDS **51%** **MATCHING TEXT** 64 WORDS

X X n X X n 1 2 1 1 1 2 2 2 20 20 620 20 310 570 20 285 ? ? ? ? ? ? ? ? . . . . . ? ? s X X n s X X n 1 1 1 2 1 2 2 2 2 2 1 43800 20 1 0480 1 24500 20 1 0359 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ( ) . . . ( ) . . . s s n s n X X 1 2 1 2 1 2 2 2 2 2 0480 20 0359 20 01340 ? ? ? ? ? ? ? . . . . t X X s df n n X X ? ? ? ? ? ? ? ? ? ? ? ? ? ? 1 2 1 2 1 2 310 285 01340 1866 2 20 20 2 38 . . . . . .

SA MSPS 13 L part 1.pdf (D110610736)

38/108 **SUBMITTED TEXT** 35 WORDS **83%** **MATCHING TEXT** 35 WORDS

Out of 55 hypercholesterolemic cases, 25 suffer from hypertension while out of 45 cases with normal serum cholesterol, 15 are hypertensive. Use chi square test to find whether or not there is a significant association between hypercholesterolemia

out of 55 hypercholesterolemia cases, 25 suffer from high blood pressure while out of 45 cases with normal serum cholesterol, 15 are of high blood pressure. Use chi square test to find whether or not there is a significant association between hypercholesterolemia &

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39/108 **SUBMITTED TEXT** 12 WORDS **87%** **MATCHING TEXT** 12 WORDS

to find whether or not there is any significant difference between the

to find whether or not there is a significant difference between the

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40/108 **SUBMITTED TEXT** 31 WORDS **62%** **MATCHING TEXT** 31 WORDS

one-way anova to find whether or not there is any significant difference between the mean tracheal ventilation scores (ml/minute) of the following two groups of locusts from two different habitats. (? = 0.05.)

one-way anova to find whether or not there is a significant difference between the wing lengths (mm) of the following two groups of grasshoppers from two different habitats

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41/108 **SUBMITTED TEXT** 94 WORDS **30%** **MATCHING TEXT** 94 WORDS

X X X X N k k ? ? ? ? ? ? ? ? ? 1 2 1 2 .... ? ? ? . SS X X X X X X t k ? ? ? ? ? ? ? ? ? ? ? ( ) ( ) ..... ( ) ; 1 2 2 2 2 or, SS X X X X X X N t k k ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 1 2 2 2 2 1 2 2 ..... (.....) . df N t ? ?1. SS n X X n X X n X X b k k ? ? ? ? ? ? ? 1 1 2 2 2 2 2 ( ) ( ) ..... ( ) , or, SS X n X n X n X X

SA MSPS 13 L part 1.pdf (D110610736)

42/108 **SUBMITTED TEXT** 21 WORDS **67%** **MATCHING TEXT** 21 WORDS

one-way anova to find whether or not there is a significant difference between the mean tracheal ventilations (ml/minute) of the following

one-way anova to find whether or not there is a significant difference between the wing lengths (mm) of the following

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43/108 **SUBMITTED TEXT** 106 WORDS **70%** **MATCHING TEXT** 106 WORDS

X 1 ml : 90 120 90 120 80 97 100 97 130 86 X 2 ml : 55 70 54 68 35 60 60 58 80 40 X 3 ml : 28 30 25 35 15 30 38 28 50 21 Critical F values : F F 0 01 2 26 0 01 2 27 553 549 . (,) . (,) . ; . ; ? ? F F F 0 01 2 28 0 01 1 29 0 01 1 26 545 418 422 . (,) . (,) . (,) . ; . ; . . ? ? ? Solution :

X1 = 1 90 2 120 3 90 4 120 5 80 6 97 7 100 8 97 9 130 10 86 X2 = 55 54 68 70 60 35 60 58 40 80 X3 = 30 28 35 25 15 28 38 30 21 50 Critical F values: F0.01(2,26) = 5.53, F0.01(2,27) = 5.49, F0.01(2,28) = 5.45, F0.01(1,29) = 4.18 & F0.01(1,26) = 4.22. Solution: •

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44/108 **SUBMITTED TEXT** 29 WORDS **64%** **MATCHING TEXT** 29 WORDS

N n n ? ? ? ? ? 1 2 10 10 20. SS X X X X N t ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 1 2 2 2 1 2 2 2 64124 52008 720 20 612 ( ) (800) . SS X n X n X X

SA Statistics for Business Course Document spring 2015.pdf (D14852953)

45/108 **SUBMITTED TEXT** 27 WORDS **60%** **MATCHING TEXT** 27 WORDS

As the computed F exceeds the critical F 0.01 , P is too low (P > 0.01). So H o is rejected and

As the computed t exceeds the critical t0.05. P is too low (P >0.05). The Ho is rejected and

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46/108 **SUBMITTED TEXT** 14 WORDS **100%** **MATCHING TEXT** 14 WORDS

one-way anova to find whether or not there is a significant difference between the

one-way anova to find whether or not there is a significant difference between the

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47/108 **SUBMITTED TEXT** 106 WORDS **71%** **MATCHING TEXT** 106 WORDS

X 1 X 2 X 3 X 1 2 X 2 2 X 3 2 90 55 28 8100 3025 784 120 70 30 14400 4900 900 90 54 25 8100 2916 625 120 68 35 14400 4624 1225 80 35 15 6400 1225 225 97 60 30 9409 3600 900 100 60 38 10000 3600 1444 97 58 28 9409 3364 784 130 80 50 16900 6400 2500 86 40 21 7396 1600 441 ? 1010 580 300 104514 35254 9828 N n n n X X n ? ? ? ? ? ? ? ? ? ? 1 2 3 1 1 1 10 10 10 30 1010 10 1010 . . . ? X X n X X n 2 2 2 3 3 3 580 10 580 300 10 300 ? ? ? ? ? ? ? ? . . . . SS X X X X X X

SA MSPS 13 L part 1.pdf (D110610736)

48/108 **SUBMITTED TEXT** 12 WORDS **100%** **MATCHING TEXT** 12 WORDS

anova to find whether or not there is a significant difference between

anova to find whether or not there is a significant difference between

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49/108 **SUBMITTED TEXT** 129 WORDS **41%** **MATCHING TEXT** 129 WORDS

n 1 2 1 2 10 10 10 10 20 ? ? ? ? ? ? ? ? ; ; . R R R R n R R n 1 2 1 1 1 2 2 2 665 1435 665 10 665 1435 10 1435 ? ? ? ? ? ? ? ? ? . ; . . . . ; . . . R R R N ? ? ? ? ? 1 2 665 1435 20 1050 . . . . H N N n R R n R R ? ? ? ? ? 12 1 1 1 2 2 2 2 ( ) [( ) ( )] ? ? ? ? ? ? ? 12 20 20 1 10 665 1050 10 1435 1050 847 2 2 ( ) [(. .) (. .)] . . df = k – 1 = 2 – 1 = 1. ? 0 05 1 2 384 . ( ) .? . As the computed H exceeds the critical ? 0 05 2 . , P is too low (P > 0.05). So, H o is rejected and

n = 43 ?2 ? ?3 ? r12 ? r13 r23 1? 2 r23 r13 ? r12 r23 1? 2 r23 ? ? 0.55 ? 0.30 ? 0.28 1 ? (0.28) 2 0.30 ? 0.55 ? 0.28 1 ? (0.28) 2 ? ? 0.55 ? 0.084 1 ? 0.0784 0.30 ? 0.154 1 ? 0.0784 ? ? 0.466 0.926 ? 0.503 0.146 0.9216 ? 0.1584 Correlation 311 R1,23 ? B2 r12 ? B3 r13 ? ? 0.503 ? 0.55 ? 0.158 ? 0.30 0.277 ? 0.0474 ? 0.3244 ? 0.569 SE of R1,23 i.e., SR 1,23 ? t ? 1 n?3 R1,23 SR1,23 ? ? 1 43 ? 3 0.569 0.158 ? 1 40 ? 1 6.32 ? 0.158 ? 3.60 df = n – 3 = 43 – 3 = 40 ? = 0.01 Critical value t0.05(40) = 2.704. As the computed t exceeds the critical t0.05. P is too low (P >0.05). The Ho is rejected and

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50/108 **SUBMITTED TEXT** 79 WORDS **52%** **MATCHING TEXT** 79 WORDS

X 1 X 2 X 1 2 X 2 2 160 110 25600 12100 180 140 32400 19600 200 160 40000 25600 170 125 28900 15625 197 162 38809 26244 220 168 48400 28224 162 120 26244 14400 180 135 32400 18225 201 185 40401 34225 190 165 36100 27225 ? 1860 1470 349254 221468 N = n 1 + n 2 = 10 + 10 = 20. SS X X X X N t ? ? ? ? ? ? ? ? ? ? ? ? ? 1 2 2 2 1 2 2 2 349254 221468 1860 1470 20 16277 ( ) ( ) . M-2\D:\Netaji 05\Biology-1\ SS X n X n X X

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51/108 **SUBMITTED TEXT** 13 WORDS **88%** **MATCHING TEXT** 13 WORDS

anova to find whether or not there is any significant difference between the

anova to find whether or not there is a significant difference between the

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52/108 **SUBMITTED TEXT** 142 WORDS **38%** **MATCHING TEXT** 142 WORDS

R 1 2 3 3625 2245 79 0 ? ? ? . ; . ; . . R R n R R n R R n 1 1 1 2 2 2 3 3 3 3625 12 3021 2245 12 1871 790 12 658 ? ? ? ? ? ? ? ? ? . . ; . . ; . . ; R R R R N ? ? ? ? ? ? ? ( ) (. . .) . . 1 2 3 3625 2245 790 36 1850 H N N n R R n R R n R R ? ? ? ? ? ? ? 12 1 1 1 2 2 2 2 3 3 2 ( ) [( ) ( ) ( )] ? ? ? ? ? ? ? ? 12 36 36 1 12 3021 1850 12 1871 1850 12 658 1850 2 2 2 ( ) [(. .) (. .) (. .)] =30.19. df = k – 1 = 3 – 1 = 2. Critical ? 0 001 2 2 1382 . ( ) . . ? Because the computed H is higher than the critical ? 0 001 2 . , P is too low (P > 0.001). So, the

r12 = 0.55 r13 = 0.30 r23 = 0.28 n = 43 ?2 ? ?3 ? r12 ? r13 r23 1? 2 r23 r13 ? r12 r23 1? 2 r23 ? ? 0.55 ? 0.30 ? 0.28 1 ? (0.28) 2 0.30 ? 0.55 ? 0.28 1 ? (0.28) 2 ? ? 0.55 ? 0.084 1 ? 0.0784 0.30 ? 0.154 1 ? 0.0784 ? ? 0.466 0.926 ? 0.503 0.146 0.9216 ? 0.1584 Correlation 311 R1,23 ? B2 r12 ? B3 r13 ? ? 0.503 ? 0.55 ? 0.158 ? 0.30 0.277 ? 0.0474 ? 0.3244 ? 0.569 SE of R1,23 i.e., SR 1,23 ? t ? 1 n?3 R1,23 SR1,23 ? ? 1 43 ? 3 0.569 0.158 ? 1 40 ? 1 6.32 ? 0.158 ? 3.60 df = n – 3 = 43 – 3 = 40 ? = 0.01 Critical value t0.05(40) = 2.704. As the computed t exceeds the critical t0.05. P is too low (P >0.05). The

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53/108 **SUBMITTED TEXT** 24 WORDS **59%** **MATCHING TEXT** 24 WORDS

Work out Kruskal-Wallis anova to find whether or not there is a significant difference between the tracheal ventilation scores (ml/min) of the following groups of

Work out one-way anova to find whether or not there is a significant difference between the wing lengths (mm) of the following two groups of

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54/108 **SUBMITTED TEXT** 28 WORDS **62%** **MATCHING TEXT** 28 WORDS

one-way anova to find if there is a significant difference between the mean wing length scores (mm) of the following groups of houseflies from two different habitats. (? = 0.01)	one-way anova to find whether or not there is a significant difference between the wing lengths (mm) of the following two groups of grasshoppers from two different habitats

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55/108 **SUBMITTED TEXT** 11 WORDS **90%** **MATCHING TEXT** 11 WORDS

test is used for testing the significance of difference between the	

SA module 9.doc (D111172954)

56/108 **SUBMITTED TEXT** 26 WORDS **62%** **MATCHING TEXT** 26 WORDS

one-way anova to find whether or not there is a significant difference between the given tracheal ventilation scores of the two groups of locusts, exposed to two different	one-way anova to find whether or not there is a significant difference between the wing lengths (mm) of the following two groups of grasshoppers from two different

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57/108 **SUBMITTED TEXT** 12 WORDS **100%** **MATCHING TEXT** 12 WORDS

to find whether or not there is a significant difference between the	to find whether or not there is a significant difference between the

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58/108 **SUBMITTED TEXT** 15 WORDS **92%** **MATCHING TEXT** 15 WORDS

linear correlation between body weight and gill weight in a sample of fishes ;	linear correlation between them. Example: Body weight and gill weight in a sample of fishes. 260

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59/108 **SUBMITTED TEXT** 11 WORDS **100%** **MATCHING TEXT** 11 WORDS

initial velocity of an enzyme action and the corresponding substrate concentration	Initial velocity of an enzyme action and the corresponding substrate concentration,

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60/108	SUBMITTED TEXT	45 WORDS	47%	MATCHING TEXT	45 WORDS
X X n s Y Y n Cov X Y X X Y Y n x y ? ? ? ? ? ? ? ? ? ? ? ? ? ( ) ; ( ) ; (,) ( )( ) ; 2 2 1 1 1 r Cov X Y s s X X Y Y n s s x y x y ? ? ? ? ? (,) ( )( ) ( ) ? 1 . (			x 2 ?x2 ?n ? ? (Y ? Y) 2 ? ? ? (Y ? Y) 2 ? n?y 2 V2 ? ? ?n ? ? ? ? ?x ?y2 ?y2 ? ? ? ? UV ? Again ? X ? X ? ? Y ? Y ? (X ? X) (Y ? Y) ? ? ? x ?? ?? ? y 2 ?? ? x? UV = nr (X ? X) (Y ? Y) 1 r ? ? n ? x? y (X ? X) (Y ?		

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61/108	SUBMITTED TEXT	32 WORDS	43%	MATCHING TEXT	32 WORDS
Where X and Y are the scores, X – and Y – are the means, s x and s y are the unbiased standard deviations, and Cov(X,Y) is the covariance					

SA Statistics for Business Course Document spring 2015.pdf (D14852953)

62/108	SUBMITTED TEXT	25 WORDS	78%	MATCHING TEXT	25 WORDS
the computed t, P is considered too high (P <??), the H O is consequently retained and the computed r has no significance. Example 6.3.1.			the critical t0.05. P is considered too high (P ? ?) so the Ho is consequently retained and the computed ‘r’ has no significance. Example 15.		

W https://pdfcoffee.com/introduction-to-bio-statistics-pdf-free.html

63/108	SUBMITTED TEXT	15 WORDS	75%	MATCHING TEXT	15 WORDS
find whether or not there is a significant correlation between tracheal ventilation (X ml/minute) and			Find whether or not there is a significant multiple linear correlation between O2 consumption (X1 ml/minute) and		

W https://pdfcoffee.com/introduction-to-bio-statistics-pdf-free.html

64/108	SUBMITTED TEXT	26 WORDS	90%	MATCHING TEXT	26 WORDS
r n XY X Y n X X n Y Y ? ? ? ? ? ? ? ? ? ? ? [( )][( )] 2 2 2 2 . 6.3.4.			r = (N * ∑XY - ∑X * ∑Y) / v [N ∑X 2 - (∑X) 2] * v [N * ∑Y 2 - (∑Y) 2]		

W https://egyanagar.osou.ac.in/download-slm.php?file=CRSM-02-BLOCK-05.pdf

65/108 **SUBMITTED TEXT** 106 WORDS **73%** **MATCHING TEXT** 106 WORDS

X Y X–X – Y–Y – (X–X –) 2 (Y–Y –) 2 (X–X –)(Y–Y –) 66.0 3.3 –12.5 –0.7 156.25 0.49 + 8.75 89.1 4.9 +10.6 +0.9 112.36 0.81 + 9.54 72.0 3.5 –6.5 –0.5 42.25 0.25 + 3.25 87.5 4.7 +9.0 +0.7 81.00 0.49 + 6.30 75.2 3.7 –3.3 –0.3 10.89 0.09 + 0.99 78.2 4.0 –0.3 0 0.09 0 0 83.5 4.3 +5.0 +0.3 25.00 0.09 + 1.50 71.6 3.4 –6.9 –0.6 47.61 0.36 + 4.14 85.6 4.4 +7.1 +0.4 50.41 0.16 + 2.84 76.3 3.8 –2.2 –0.2 4.84 0.04 + 0.44 ? 785.0 40.0 – – 530.70 2.78 + 37.75 r X X Y Y X X Y Y ? ? ? ? ? ? ? ? ?? ? ? ? ( )( ) ( ) ( ) . . . . 2 2 3775 53070 278 098 . (

X ? ? Y ? Y ? (X ? X) (Y ? Y) ? ? ? x ?? ?? ? y 2 ?? ? x? y ? ?? UV = nr (X ? X) (Y ? Y) 1 r ? ? n ? x? y (X ? X) (Y ? Y)

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66/108 **SUBMITTED TEXT** 160 WORDS **36%** **MATCHING TEXT** 160 WORDS

X Y X 2 Y 2 XY 66.0 3.3 4356.00 10.89 217.80 89.1 4.9 7938.81 24.01 436.59 72.0 3.5 5184.00 12.25 252.00 87.5 4.7 7656.25 22.09 411.25 75.2 3.7 5655.04 13.69 278.24 78.2 4.0 6115.24 16.00 312.80 83.5 4.3 6972.25 18.49 359.05 71.6 3.4 5126.56 11.56 243.44 85.6 4.4 7327.36 19.36 376.64 76.3 3.8 5821.69 14.44 289.94 ? 785.0 40.0 62153.20 162.78 3177.75 r n XY X Y n X X n Y Y ? [( )][( )] . . . [. .] [. .] 2 2 2 2 2 2 10 317775 7850 400 10 6215320 7850 10 16278 400 = + 0.98. M-2\D:\Netaji 05\Biology-1\ (c) Significance of computed r : 000.14 070.0 98.0 .070.0 2 10 98.0 1 2 1 2 2 ? ? ? ? ? ? ? ? ? ? r r s r t n r s df = n–2 = 10–2 = 8.???? = 0.01. Critical t 0.01(8) = 3.355. As the computed t far exceeds the critical t 0.01 , P is considered too low. So, the H 0 is

X ??? ??? n? Y 2 ? ? Y ??? ?? ? ? ? ? ? 15 ? 2058.4 ? 106.4 ? 290 ? ?15 ? 755.95 ? ?106.4 ? 2 ? ? ?15 ? 5696 ? ? 290 ? 2 ? ? ? ? ? ? ? ? 30876 ? 30856 (11339.25 ? 11320.96) ? (85440 ? 84100) 20 18.29 ? 1340 20 156.5522 24508.6 20 156.56 ? XY ? r ? ?X ? ? 20 ? 2 ?? X ? ? ? 0.12775 ? 0.128 ? X ?Y n 2 n ? ?Y 2 ??Y ? ? 2 n 106.4 ? 290 2058.4 ? 15 755.95 ? ?106.4 ? 2 15 ? 5696 ? ? 290 ? 2 15 30856 2058.4 ? 15 ? 11320.96 84100 755.95 ? ? 5696 ? 15 15 2058.4 ? 2057.06 1.34 ? ? 755.95 ? 754.73 ? 5696 ? 5606.47 1.22 ? 89.33 1.34 1.34 ? ? 1.10 ? 9.45 10.39 r = 0.128 Correlation 277 1 ? r2 S.E. of r i.e. ? t ? n?2 r ? Sr 1 ? (0.128)2 ? 0.127 0.275 15 ? 2 1 ? 0.016 ? ? 13 0.984 13 ? 0.07569 ? 0.275 ? 0.465 df = n – 2 = 15 – 2 = 13 ? ? 0.05 critical t0.05(13) = 2.16. As the value of computed t less than the critical t0.05. P is considered too high (P ? ?) so the Ho is

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67/108 **SUBMITTED TEXT** 49 WORDS **23%** **MATCHING TEXT** 49 WORDS

scores, the ranks of any of them may be arranged in the first column, pairing each with the rank of the same case in the other variable along the second column. (c) Moving downwards along the second column of ranks from its top, each of its ranks is used in turn as

scores the ranks of any of them are arranged as R1 ranks in the ordered manner pairing them with the ranks (R2) of the other variable in the respective individuals. IV. Moving down word from the top of the column of the paired R2 ranks of the second variables, each R2 rank is used in turn as

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68/108	SUBMITTED TEXT	10 WORDS	100%	MATCHING TEXT	10 WORDS

pivotal rank for comparing with every successive subsequent rank following

pivotal rank for comparing with every successive subsequent rank following

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69/108	SUBMITTED TEXT	18 WORDS	85%	MATCHING TEXT	18 WORDS

the pivotal one, Such counts of subsequent ranks are entered in a third column and totalled as ?

the particular pivotal rank. VI. Such counts of subsequent ranks are entered in a third column and totalled score as

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70/108	SUBMITTED TEXT	17 WORDS	66%	MATCHING TEXT	17 WORDS

to find whether or not there is a significant correlation (??= 0.05) between gill weight (X) and

to be +0.28. Find whether or not there is a significant multiple linear correlation between the combination of X1 and

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71/108	SUBMITTED TEXT	51 WORDS	43%	MATCHING TEXT	51 WORDS

df ? ? ? ? ? ? ? 066 0234 2821 . . . . . ? = 0.05. Critical t 0.05(?) =1.960. As the computed t exceeds the critical t 0.05 , P is lower than 0.05. So, P is too low. The H O is rejected. There is a significant correlation between

df = n – 3 = 43 – 3 = 40 ? = 0.01 Critical value t0.05(40) = 2.704. As the computed t exceeds the critical t0.05. P is too low (P >0.05). The Ho is rejected and the computed R1,23 is significant. Example 3. Find whether or not there is a significant multiple linear correlation between

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72/108	SUBMITTED TEXT	22 WORDS	90%	MATCHING TEXT	22 WORDS

r 12.34 between glomerular filtration rate (X 1) and glomerular blood pressure (X 2)

r12) between glomerular filtration rate (X1 ml/ min) and glomerular blood pressure (X2

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73/108	SUBMITTED TEXT	64 WORDS	35%	MATCHING TEXT	64 WORDS

is basically worked out from the product-moment r values of each pair of variables involved. For the first-order partial r 12.3 between variables X 1 and X 2 partialling out the variable X 3 , the product-moment r 12 , r 13 and M-2\D:\Netaji 05\Biology-1\ r 23 values between the respective variables

is basically calculated from the product moment r values of each pair of variables. II. For calculating the multiple linear correlation coefficient (R1.23) between variables (criterion) X1 the combination of two other variable (predictors) i.e., X2 and X3; the ? coefficient (?2 & ?3) are first from the product moment r values (r12, r13 and r23) between the respective variables.

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74/108	SUBMITTED TEXT	22 WORDS	100%	MATCHING TEXT	22 WORDS

r r r r r r r r r r r r 13 2 13 12 23 12 2 23 2 231 23 12 13 12 2 13 2 1 1 1 . . [ ][ ] ; [ ][ ] ? ? ? ? ? ? ? ? .

R1,23. ?2 ? ?3 ? R1.2.3 ? ? r12 ? r13 r23 1 ? (r23) 2 r12 ? r13 r23 1 ? (r23) 2 ? ? 0.65 ? 0.60 ? 0.90 1 ? (0.90) 2 0.60 ? 0.65 ? 0.90 ?2 r12 ? ?3 r12 ? 1 ? (0.90) 2 ? ? 0.65 ? 0.54 1 ? 0.81 ? 0.60 ? 0.585 1 ? 0.81 0.11 0.19 ? 0.579 ? 0.65 ? 0.0789 ? 0.60 ? 0.4233 ? 0.6506 ? 0.651 ? 0.5789 ? 0.579 0.015 0.19 ? 0.0789 0.376 ? 0.473

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75/108	SUBMITTED TEXT	139 WORDS	84%	MATCHING TEXT	139 WORDS

Example 6.5.1. Find whether or not there is a significant linear partial correlation between O 2 consumption (X 1 ml/minute) and tracheal ventilation volume (X 2 ml/minute) partialling out atmospheric O 2 tension (X 3 mm Hg), using the following data of a sample of 53 locusts. (??= 0.05). r 12 = +0.75 ; r 13 = +0.35 ; r 23 = +0.25. t 0.05(52) =2.007 ; t 0.05(51) =2.008 ; t 0.05(50) = 2.009. Solution : r r r r r r 12 3 12 13 23 13 2 23 2 2 2 1 1 075 035 025 1 035 1 025 073 . [ ][ ] . . . [.][.] . . ? ? ? ? ? ? ? ? ? ? ? ? s r n r 12 3 1 3 1 073 53 3 0097 12 3 2 2 . . . . . ? ? ? ? ? ? ? t r s r ? ? ? 12 3 12 3 073 0097 7526 . . . . . .

Example 3. Find whether or not there is a significant multiple linear correlation between O2 consumption (X1 ml/minute) and tracheal ventilation volume (X2 ml/minute) partialling out atmospheric O2 tension (X3 mm/Hg). Using the following data of a sample of 53 grasshoppers. (? ? 0.05) r12 = +0.75, r13 = +0.35 & r23 = +0.25 t0.05(52) = 2.007, t0.05(51) = 2.008 & t0.05(50) = 2.009, Solution: r12 = +0.75, r13 = +0.35, r23 = +0.25 & n = 53 ?2 ? r12 ? r13 r23 1? 2 r23 ? 0.75 ? 0.35 ? 0.25 1 ? (0.25) 2 ? 0.75 ? 0.0875 1 ? 0.0625 ? 0.6625 0.9375 ? 0.7066 ? 0.71 r13 ? r12 r23 0.35 ? 0.75 ? 0.25 0.35 ? 0.1875 0.1625 ? ? ? ?3 ? 2 1 ? 0.0625 0.9375 1 ?

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76/108	SUBMITTED TEXT	60 WORDS	37%	MATCHING TEXT	60 WORDS

df = n–3 = 53–3 = 50. Critical t 0.05(50) = 2.009. M-2\D: \Netaji 05\Biology-1\ The computed t exceeds the critical t 0.05 ; so, P is too low and the H O is rejected. Hence, there is a significant linear partial correlation between X 1 and X 2 , partialling out

df = n – 3 = 43 – 3 = 40 ? = 0.01 Critical value t0.05(40) = 2.704. the computed t exceeds the critical t0.05. P is too low (P >0.05). The and the computed R1,23 is significant. Example 3. Find whether or not there is a significant multiple linear correlation between O2 consumption (X1 ml/minute) and tracheal ventilation volume (X2 ml/minute) partialling out

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77/108	SUBMITTED TEXT	15 WORDS	100%	MATCHING TEXT	15 WORDS

quantitative assessment of the magnitude and direction of correlation between a given variable and the

quantitative assessment of the magnitude and direction of correlation between a given variable and the

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78/108	SUBMITTED TEXT	27 WORDS	51%	MATCHING TEXT	27 WORDS

coefficient of multiple determination and serves as an estimate of that proportion of the total variance of criterion, which is dependent on the combined contribution of all the predictors.

Coefficient of multiple determination: It is a measure of that proportion of the total variation of the criterion which is determined by the variation of the combined contribution of all the predictors. 2 ? ?2

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79/108	SUBMITTED TEXT	22 WORDS	90%	MATCHING TEXT	22 WORDS

a measure of that proportion of the total variance of criterion, which is not determined by the combined contribution of the predictors

a measure of that proportion of the total variance of criterion which is determined by the independent combined contribution of the predictors. 2 2

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80/108	SUBMITTED TEXT	10 WORDS	100%	MATCHING TEXT	10 WORDS

from the product-moment r values of each pair of variables

from the product moment r values of each pair of variables.

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81/108 **SUBMITTED TEXT** 83 WORDS **68%** **MATCHING TEXT** 83 WORDS

are first calculated from the product moment r values (r 12 , r 13 and r 23) between the respective variables; ? 2 and ? 3 serve as measures of the proportions of variance of the criterion, associated with the variances of respective predictors (X 2 and X 3). ? ? ? ? 2 12 13 23 23 2 3 13 12 23 23 2 123 2 12 313 1 1 ? ? ? ? ? ? ? ? ? r r r r r r r r R r r ; ; ; .

are first calculated from the product moment r values (r12, r13 and r23) between the respective variables. III. ?2 & ?3 act as measures of proportion of variance of the criterion (X1); associated with the variances of respective predictors (X2 & X3). r ?r r r ?r r IV. ?2 ? 12 13 23 ?3 ? 13 122 23 2 1 ? r23 1 ? r23 ? R1.23 ? ? 2 r12 ? ?3 r13

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82/108 **SUBMITTED TEXT** 22 WORDS **100%** **MATCHING TEXT** 22 WORDS

find whether or not there is a significant multiple linear correlation between O 2 consumption (X 1)

Find whether or not there is a significant multiple linear correlation between O2 consumption (X1

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83/108 **SUBMITTED TEXT** 154 WORDS **67%** **MATCHING TEXT** 154 WORDS

X 3). (??= 0.05.) t 0.05(49) =2.010 ; t 0.05(50) =2.009 ; t 0.05(51) =2.008 ; t 0.05(52) =2.007. Solution : r 12 = +0.75 ; r 13 = +0.35 ; r 23 = +0.25 ; n=53. ? 2 12 13 23 23 2 2 1 075 035 025 1 025 071 ? ? ? ? ? ? ? ? r r r r . . . . . ? 3 13 12 23 23 2 2 1 035 075 025 1 025 017 ? ? ? ? ? ? ? ? r r r r . . . . . R r r 123 2 12 3 13 071 075 017 035 077 . . . . . ? ? ? ? ? ? ? ? ? ? s n R 1 23 1 3 1 53 3 0141 . . . ? ? ? ? ? t R s R ? ? ? 123 1 23 077 0141 5461 . . . . . df = n−3 = 53−3 = 50. ? = 0.05. Critical t 0.05(50) =2.009. M-2\D:\Netaji 05\Biology-1\ As the computed t exceeds the critical t 0.05 , P is too low (P ? 0.05). The

X2. (? ? 0.01) Critical t scores: t0.01(42) = 2.698 t0.01(41) = 2.701 t0.01(40) = 2.704. Solution: r12 = 0.55 r13 = 0.30 r23 = 0.28 n = 43 ?2 ? ?3 ? r12 ? r13 r23 1? 2 r23 r13 ? r12 r23 1? 2 r23 ? ? 0.55 ? 0.30 ? 0.28 1 ? (0.28) 2 0.30 ? 0.55 ? 0.28 1 ? (0.28) 2 ? ? 0.55 ? 0.084 1 ? 0.0784 0.30 ? 0.154 1 ? 0.0784 ? ? 0.466 0.926 ? 0.503 0.146 0.9216 ? 0.1584 Correlation 311 R1,23 ? B2 r12 ? B3 r13 ? ? 0.503 ? 0.55 ? 0.158 ? 0.30 0.277 ? 0.0474 ? 0.3244 ? 0.569 SE of R1,23 i.e., SR 1,23 ? t ? 1 n?3 R1,23 SR1,23 ? ? 1 43 ? 3 0.569 0.158 ? 1 40 ? 1 6.32 ? 0.158 ? 3.60 df = n – 3 = 43 – 3 = 40 ? = 0.01 value t0.05(40) = 2.704. As the computed t exceeds the critical t0.05. P is too low (P >0.05). The

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84/108 **SUBMITTED TEXT** 31 WORDS **84%** **MATCHING TEXT** 31 WORDS

criterion is linearly correlated with the predictor, the scores of the former are predicted by working out an equation for a straight line, depending on the linear association between the two. On the

criterion) is linearly correlated with the predictor (independent variable). II. The scores of the dependent variables are predicted by working out an equation for a straight line, depending on the linear association between the two. The

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85/108	SUBMITTED TEXT	33 WORDS	92%	MATCHING TEXT	33 WORDS

if the criterion has a nonlinear correlation with the predictor, scores of the criterion have to be predicted in terms of a curved line like a sigmoid or hyperbolic or exponential curve, according to

If the criterion (dependent variable) has a nonlinear correlation with the predictor (independent variable), the scores of the criterion have to be predicted in terms of a curved line like a sigmoid or hyperbolic or exponential curve, according to

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86/108	SUBMITTED TEXT	19 WORDS	94%	MATCHING TEXT	19 WORDS

a regression can be worked out only when there is a significant correlation between the criterion and the predictor,

A regression can be worked out only when there is a significant correlation between the dependent (criterion) and the independent (predictor)

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87/108	SUBMITTED TEXT	19 WORDS	91%	MATCHING TEXT	19 WORDS

Regression predicts only a probable score of the criterion on a given score of the predictor in a

Regression predicts only a probable score of the criterion on a given score of the predictor. 4. When a

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a pair of variables correlated with one another, regression can be worked

a pair of variables correlated with one another, regression can be worked

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89/108	SUBMITTED TEXT	21 WORDS	97%	MATCHING TEXT	21 WORDS

variable X as criterion on variable Y as predictor, and another regression of variable Y as criterion on variable X as predictor.

variable X as criterion on variable Y as predictor and (ii) another regression of variable Y as criterion on variable X as predictor. 5.

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90/108 **SUBMITTED TEXT** 13 WORDS **100%** **MATCHING TEXT** 13 WORDS

A regression equation is worked out using a statistic called the regression coefficient (

A regression equation is worked out using a statistic called the regression coefficient. 316

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91/108 **SUBMITTED TEXT** 14 WORDS **88%** **MATCHING TEXT** 14 WORDS

linear regression of O 2 consumption (Y ml/minute) on tracheal ventilation (X ml/

linear regression of O2 consumption (Y ml/min) on tracheal ventilation (X ml/

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the regression of Y on X, and b XY for the regression of X on Y. (

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93/108 **SUBMITTED TEXT** 73 WORDS **58%** **MATCHING TEXT** 73 WORDS

X Y X 2 XY 66.0 3.3 4356.00 217.80 89.1 4.9 7938.81 436.59 72.0 3.5 5184.00 252.00 87.5 4.7 7656.25 411.25 75.2 3.7 5655.04 278.24 78.2 4.0 6115.24 312.80 83.5 4.3 6972.25 359.05 71.6 3.4 5126.56 243.44 85.6 4.4 7327.36 376.64 76.3 3.8 5821.69 289.94 ? 785.0 40.0 62153.20 3177.75 X X n ? ? ? ? 7850 10 78 5 . . . Y Y n ? ? ? ? 400 10 40 . . . b n XY X Y n X

X Y x2 y2 XY 1 2 1 4 2 2 3 4 9 6 3 5 9 25 15 4 6 16 36 24 5 4 25 16 20 15 20 55 90 ?X ?X2 ? 15 ? Y ? 20 n ? 5 ? Y 2 ? 90 X Y ? XY ? ? ? ? XY ? 67 15 X ? ?3 5 67 20 Y ? ?4 5 ? 55 bxy ? n ?Y 2 ? byx ? ? XY ? ?X 2 ??Y ? 2 n ? X ?

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multiple regression. It is basically the method of expressing the criterion

Multiple Regression: It is basically the method of expressing the criterion

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X Y X – X – Y – Y – (X – X –) 2 (X – X –) (Y– Y –) 66.0 3.3 – 12.5 – 0.7 156.25 + 8.75 89.1 4.9 + 10.6 + 0.9 112.36 + 9.54 72.0 3.5 – 6.5 – 0.5 42.25 + 3.25 87.5 4.7 + 9.0 + 0.7 81.00 + 6.30 75.2 3.7 – 3.3 – 0.3 10.89 + 0.99 78.2 4.0 – 0.3 0 0.09 0 83.5 4.3 + 5.0 + 0.3 25.00 + 1.50 71.6 3.4 – 6.9 – 0.6 47.61 + 4.14 85.6 4.4 + 7.1 + 0.4 50.41 + 2.84 76.3 3.8 – 2.2 – 0.2 4.84 + 0.44 ? 785.0 40.0 — — 530.70 + 37.75 X X n ? ? ? ? 7850 10 785 . . . Y Y n ? ? ? ? 400 10 40 . . . b X X Y Y X X

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96/108 **SUBMITTED TEXT** 34 WORDS **82%** **MATCHING TEXT** 34 WORDS

criterion (X 1) on the combination of two predictors (X 2 and X 3), partial regression coefficients (b 12.3 and b 13.2)

criterion (X1) on the combination of two predictors (X2 and X3) can be worked out by using partial regression coefficients (b1,23 and b1,32).

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97/108 **SUBMITTED TEXT** 28 WORDS **100%** **MATCHING TEXT** 28 WORDS

are the measures of slopes of regression lines of the criterion X 1 on the predictors X 2 and X 3 ,

are the measures of slopes of regression lines of the criterion (X1) on the predictors (X2 and X3).

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98/108 **SUBMITTED TEXT** 62 WORDS **85%** **MATCHING TEXT** 62 WORDS

X 1 is the predicted score of the criterion, ? ? 2 12 13 23 23 2 3 13 12 23 23 2 1 1 ? ? ? ? ? ? r r r r r r r r ; ; b s s b s s a X b X b X 123 2 1 2 132 3 1 3 123 1 123 2 132 3 ? ? ? ? ? ? ? ? ? ? ; ; ; ? . . . . X a b X b X 1 1 23 12 3 2 13 2 3 ? ? ?

X̂ is the predicted score of the criterion I. B2 = r13 ? r12 r23 r12 ? r13 r23 B3 = 2 1 ? r23 II. b1,23 = B2 ? S1 S2 2 1 ? r 23 b1,32 = B3 ? S1 S3 III. a1,23 = X1 ? b1,23 X 2 ? b1,32 X 3 IV. X̂1 ? a1,23 ? b1,23 X 2 ? b1,32 X 3 11. (

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99/108 **SUBMITTED TEXT** 39 WORDS **81%** **MATCHING TEXT** 39 WORDS

X ? Y = -1.57+0.071 .X (= 85 ; = 4.5) X Y ? (= 80 ; = 4.1) X Y ? (= 75 ; = 3.8) X Y ? (= 70 ; = 3.4) X Y ? Fig 6.1. Linear regression line of

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100/108	SUBMITTED TEXT	36 WORDS	100%	MATCHING TEXT	36 WORDS

glomerular filtration rate (X 1 ml/min) on glomerular blood pressure (X 2 mm Hg) and capsular fluid pressure (X 3 mm Hg), using the following data of a sample of 40

glomerular filtration rate (X1 ml/min) on glomerular blood pressure (X2 mm/Hg) and capsular fluid pressure (X3 mm/Hg) using the following data of a sample of 40

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101/108	SUBMITTED TEXT	82 WORDS	84%	MATCHING TEXT	82 WORDS

r r r r . (.) . . . . ? 3 13 12 23 23 2 2 1 021 082 018 1 018 037 ? ? ? ? ? ? ? ? ? ? r r r r . . . . . b s s 12 3 2 1 2 089 215 142 135 . . . . . ; ? ? ? ? ? ? a X b X b X 1 23 1 12 3 2 13 2 3 120 135 58 2 27 18 82 56 . . . . (.) . . ? ? ? ? ? ? ? ? ? ? ? , ? . . . . . . X a b X b X

r ?r r r ?r r ?3 ? 13 122 23 I. ?2 ? 12 132 23 1 ? r23 1 ? r23 S1 S2 II. b1,23 ? ?2 ? III. a1,23 ? X 1 ? b1,23 X 2 ? b1,32 X 3 IV. X ̂ 1 ? a1,23 ? b1,23 X 2 ? b1,32 X 3

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102/108	SUBMITTED TEXT	16 WORDS	61%	MATCHING TEXT	16 WORDS

correlation between a single given variable and the combination of two or more other variables. Regression is

correlation between a given variable and the joint influence of two or more variables is

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103/108	SUBMITTED TEXT	41 WORDS	34%	MATCHING TEXT	41 WORDS

Find whether or not there is a significant linear partial correlation between gill weights (X 1 gm) and trunk lengths (X 2 cm), partialling out body weights (X 3 gm) in the following sample of 43

Find whether or not there is a significant multiple linear correlation between O2 consumption (X1 ml/minute) and tracheal ventilation volume (X2 ml/minute) partialling out atmospheric O2 tension (X3 mm/Hg). Using the following data of

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104/108	SUBMITTED TEXT	21 WORDS	76%	MATCHING TEXT	21 WORDS

to find whether or not there is a significant multiple linear correlation between gill weights (X 1) and

to be +0.28. Find whether or not there is a significant multiple linear correlation between the combination of X1 and

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105/108	SUBMITTED TEXT	25 WORDS	68%	MATCHING TEXT	25 WORDS

the multiple linear regression equation of variable X 1 on the combination of variables X 2 and X 3

The multiple linear regression of criterion (X1) on the combination of two predictors (X2 and X3)

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106/108	SUBMITTED TEXT	19 WORDS	91%	MATCHING TEXT	19 WORDS

find whether or not there is a significant correlation between O 2 consumption (X ml) and tracheal ventilation (

Find whether or not there is a significant multiple linear correlation between O2 consumption (X1 ml/minute) and tracheal ventilation

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The proteins that bind to the DNA to form eukaryotic chromosomes are traditionally divided into two general classes: histories and the nonhistone chromosomal proteins. The complex of both classes of protein with the nuclear DNA of eukaryotic cells is known as chromatin.

The total mass of histones in chromatin is about equal to that of the DNA. Histones are responsible for the first and most basic level of chromosome organization, the nucleosome, which was discovered in 1974. At interphase nuclei most of the chromatin is in the form of a fibre with a diameter of about 30 nm. If this chromatin is subjected to treatments that cause it to unfold partially, it can be seen under the electron microscope as a series of "
beads on a string". The string is DNA, and
each bead is a "nucleosome core particle"
that consists of DNA wound around a protein core formed from histones. The beads on a string represent the first level of chromosomal DNA packing.

The structural organization of nucleosomes was determined after first isolating them from unfolded chromatin by digestion with particular enzymes (called nucleases) that break down DNA by cutting between the nucleosomes. After digestion for a short period, the exposed DNA between the nucleosome core particles,
the linker DNA, is degraded. Each individual nucleosome core particle consists of a complex of eight
histone proteins—
two molecules each of histones H2A, H2B, H3, and H4—
and double-stranded DNA that are 146 nucleotide pairs long. The histone octamer forms a protein core around which the double-stranded DNA is wound (Fig. 1.1).

Each nucleosome core particle is separated from the next by a region of linker DNA,
which
can vary in length from a few nucleotide pairs up to about 80. (The term nucleosome technically refers to a nucleosome core particle plus

10 Fig. 1.1 Structural organization of the nucleosome A nucleosome contains a protein core made of eight histone molecules. As indicated, the nucleosome core particle is released from chromatin by digestion of the linker DNA with a nuclease, an enzyme that breaks down DNA. (The nuclease can degrade the exposed linker DNA but cannot attack the DNA wound tightly around the nucleosome core.) After dissociation of the isolated nucleosome into its protein core and DNA, the length of the DNA that was wound around the core can be determined. This length of 146 nucleotide pairs is sufficient to wrap 1.65 times around the histone core one of its adjacent DNA linkers, but it is often used synonymously with nucleosome core particle.) On average, therefore, nucleosomes repeat at intervals of about 200 nucleotide pairs. The structure of the nucleosome core particle reveals how DNA is packaged The high-resolution structure of a nucleosome core particle, solved in 1997, revealed a disc-shaped histone core around which the DNA was tightly wrapped 1.65 turns in a left-handed coil. All four of the histones that make up the core of the nucleosome are relatively small proteins (102-135 amino acids), and they share a structural motif, known as the histone fold, formed from three a helices connected by two loops (Figure 1.2).

11 Fig. 1.2 The overall structural organization of the core histones, (A) Each of the core histones contains an N-terminal tail, which is subject to several forms of covalent modification, and a histone fold region, as indicated. (B) The structure of the histone fold, which is formed by all four of the core histones. (C) Histones 2A and 2B form a dimer through an interaction known as the “handshake.” Histones H3 and H4 form a dimer through the same type of interaction, as illustrated in Figure 1.3 In assembling a nucleosome, the histone folds first bind to each other to form H3-H4 and H2A-H2B dimers, and the H3-H4 dimers combine to form tetramers. An H3-H4 tetramer then further combines with two H2A-H2B dimers to form the compact octamer core, around which the DNA is wound (Fig. 1.3). The interface between DNA and histone is extensive ; 142

hydrogen bonds are formed between DNA and the histone core

in each nucleosome. Nearly half of these bonds form between the amino acid backbone of the histones and the phosphodiester backbone of the DNA. Numerous hydrophobic interactions and salt linkages also hold DNA and protein together in the nucleosome. These numerous interactions explain in part why DNA of virtually any sequence can be bound on a histone octamer core. The path of the DNA around the histone core is not smooth; rather, several kinks are seen in the DNA, as expected from the nonuniform surface of the core. In addition to its histone fold, each of the core histones has a long N-terminal amino acid “tail”, which extends out from the DNA-histone core (see Figure 1.3). These histone tails are subject to several different types of covalent modifications, which control many aspects of chromatin structure. The histones are among the most highly conserved eukaryotic proteins. This strong evolutionary conservation suggests that the functions of histones involve nearly all of their amino acids, so that a change in any position is deleterious to the cell. Despite the high conservation of the core histones, many

12 Fig. 1.3 The assembly of a histone octamer. The histone H3-H4 dimer and the H2A-H2B dimer are formed from the handshake interaction. An H3-H4 tetramer forms the scaffold of the octamer onto which two H2A-H2B dimers are added, to complete the assembly. Note that all eight N-terminal tails of the histones protrude from the disc-shaped core structure. In the x-ray crystal, most of the histone tails were unstructured (and therefore not visible in the structure), suggesting that their conformations are highly flexible. (Adapted from figures by J. Waterborg.) eukaryotic organisms also produce specialized variant core histones that differ in amino acid sequence from the main ones. It is thought that nucleosomes that have incorporated these variant histones differ in stability from regular nucleosomes, and they may be particularly well suited for the high rates of DNA transcription and DNA replication that occur during these early stages of development. The positioning of nucleosome on DNA is determined by both DNA flexibility and other DNA-bound proteins Two main influences determine where nucleosomes form in the DNA. One is the difficulty of bending the DNA double helix into two tight turns around the outside of the histone octamer, a process that requires substantial compression of the minor groove of the DNA helix. Because A-T-rich sequences in the minor groove are easier to compress than G-C-rich sequences, each histone octamer tends to position itself on the DNA so as to maximize A-T-rich minor grooves on the inside of the DNA coil. Thus, a segment of DNA that contains short

13 A-T-rich sequences spaced by an integral number of DNA turns is easier to bend around the nucleosome than a segment of DNA lacking this feature. In addition, because the DNA in a nucleosome is kinked in several places, the ability of a given nucleotide sequence to accommodate this deformation can also influence the position of DNA on the nucleosome. The second, and probably most important, influence on nucleosome positioning is the presence of other tightly bound proteins on the DNA. Some bound proteins favour the formation of a nucleosome adjacent to them. Others create obstacles that force the nucleosomes to assemble at positions between them. Finally, some proteins can bind tightly to DNA even when their DNA- binding site is part of a nucleosome. The exact positions of nucleosomes along a stretch of DNA therefore depend on factors that include the DNA sequence and the presence and nature of other proteins bound to the DNA. Nucleosomes are usually packed together into a compact chromatin fibre The nucleosomes are packed on top of one another, generating regular arrays in which the DNA is even more highly condensed. Thus, when nuclei are very gently lysed onto an electron microscope grid, most of the chromatin is seen to be in the form of a fiber with a diameter of about 30 nm, which is considerably wider than chromatin in the "beads on a string" form. Several models have been proposed to explain how nucleosomes are packed in the 30- nm chromatin fiber; the one most consistent with the available data is a series of structural variations known collectively as the zigzag model. In reality, the 30- nm structure found in chromosomes is probably a fluid mosaic of the different zigzag variations. Several mechanisms probably act together to form the 30-nm fiber from a linear string of nucleosomes. First, an additional histone, called histone HI, is involved in this process. H1 is larger than the core histones and is considerably less well conserved. A single histone HI molecule binds to each nucleosome, contacting both DNA and protein, and changing the path of the DNA as it exits from the nucleosome. Although it is not understood in detail how H1 pulls nucleosomes together into the 30-nm fiber, a change in the exit path in DNA seems crucial for compacting nucleosomal DNA so that it interlocks to form the 30-nm fibre (Fig. 1.4). A second mechanism for forming the 30-nm fiber probably involves the tails of the core histones, which, as we saw above, extend from the nucleosome. It is thought that these tails may help attach one nucleosome to another—thereby allowing a string of them, with the aid of histone H1, to condense into the 30- nm fibre (Fig. 1.5).

14 Fig. 1.4 A speculative model for how histone H1 could change the path of DNA as it exits from the nucleosome. Histone H1 (green) consists of a globular core and two extended tails. Part of the effect of H1 on the compaction of nucleosome organization may result from charge neutralization: like the core his-tones, H1 is positively charged (especially its C- terminal tail), and this helps to compact the negatively charged DNA. Unlike the core histones, H1 does not seem to be essential for cell viability; in one ciliated protozoan the nucleus expands nearly two fold in the absence of H1, but the cells otherwise appear normal Fig. 1.5 A speculative model for histone tails in the formation of the 30-nm fiber. (A) The approximate exit points of the eight histone tails, four from each histone subunit, that extend from each nucleosome. In the high-resolution structure of the nucleosome, the tails are largely unstructured, suggesting that they are highly flexible. (B) A speculative model showing how the histone tails may help to pack nucieosomes together into the 30-nm fiber. This model is based on (1) experimental evidence that histone tails aid in the formation of the 30-nm fiber, (2) the x-ray crystal structure of the nucleosome, which showed that the tails of one nucleosome contact the histone core of an adjacent nucleosome in the crystal lattice, and (3) evidence that the histone tails interact with DNA ATP-driven chromatin remodeling machines change nucleosome structure Eukaryotic cells contain chromatin remodeling complexes, protein machines that use the energy of ATP hydrolysis to change the structure of nucieosomes temporarily so that DNA becomes less tightly bound to the histone core. The remodeled state may result from movement of the H2A-H2B dimers in the nucleosome core; the H3-H4 tetramer is particularly stable and would be difficult to rearrange (Fig. 1.3). The remodeling of nucleosome structure has two important consequences. First, it permits ready access to nucleosomal DNA by other proteins in the cell, particularly those involved in gene expression, DNA replication, and repair. Even after the remodeling complex has dissociated, the nucleosome can remain

15 in a “remodeled state” that contains DNA and the full complement of histones— but one in which the DNA-histone contacts have been loosened; only gradually does this remodeled state revert to that of a standard nucleosome. Second, remodeling complexes can catalyze changes in the positions of nucleosomes along DNA (Fig. 1.6); some can even transfer a histone core from one DNA molecule to another. Fig. 1.6 Model for the mechanism of some chromatin remodeling complexes. In the absence of remodeling complexes, the interconversion between the three nucleosomal states shown is very slow because of a high activation energy barrier. Using ATP hydrolysis, chromatin-remodeling complexes (green) create an activated intermediate (shown in the center of the figure) in which the histone-DNA contacts have been partly disrupted. This activated state can then decay to any one of the three nucleosomal configurations shown. In this way, the remodeling complexes greatly increase the rate of interconversion between different nucleosomal states. The remodeled state, in which the histone-DNA contacts have been loosened, has a higher free energy level than that of standard nucleosomes and will slowly revert to the standard nucleosome conformation, even in the absence of a remodeling complex. Cells have many different chromatin remodeling complexes, and they differ in their detailed biochemical properties; for example, not all can change the position of a nucleosome, but all use the energy of ATP hydrolysis to alter nucleosome structure. (Adapted from R.E. Kingston and G.J. Narlikar, Genes Dev. 13:2339-2352, 1999.)

16 Cells have several different chromatin remodeling complexes that differ subtly in their properties. Most are large protein complexes that can contain more than ten subunits. It is likely that they are used whenever a eucaryotic cell needs direct access to nucleosome DNA for gene expression, DNA replication, or DNA repair. Different remodeling complexes may have features specialized for each of these roles. It is thought that the primary role of some remodeling complexes is to allow access to nucleosomal DNA, whereas that of others is to re-form nucleosomes when access to DNA is no longer required (Fig. 1.7). Fig. 1.7 A cyclic mechanism for nucleosome disruption and re-formation. According to this model, different chromatin remodeling complexes disrupt and re-form nucleosomes, although, in principle, the same complex might catalyze both reactions. The DNA-binding proteins could function in gene expression, DNA replication, or DNA repair, and in some cases their binding could lead to the dissociation of the histone core to form nucleosome-free regions of DNA, (Adapted from A. Travers, Cell 96:311-314, 1999.) Covalent modification of the histone tails can profoundly affect chromatin The N-terminal tails of each of the four core histones are highly conserved in their sequence, and perform crucial functions in regulating chromatin structure.

17 Each tail is subject to several types of covalent modifications, including acetyJation of lysine: methylation of lysines, and phosphorylation of serines (Fig. 1.8). Fig. 1.8 Covalent modification of core histone tails Histones are synthesized in the cytosol and then assembled into nucleosomes. Some of the modifications of histone tails occur just after their synthesis, but before their assembly. The modifications that concern us, however, take place once the nucleosome has been assembled. These nucleosome modifications are added and removed by enzymes that reside in the nucleus; for example, acetyl groups are added to the histone tails by histone acetyl transferases (HATs) and taken off by histone deacetylases (HDACs). The various modifications of the histone tails have several important consequences, histone acetylation tends to destabilize chromatin structure, perhaps in part because adding an modification state unmodified acetylated acetylated methylated phosphorylated phosphorylated/ acetylated higher-order combinations unmodified acetylated acetylated “meaning” gene silencing? gene expression histone deposition gene-silencing/ heterochromatin mitosis/meiosis gene expression ? gene silencing? histone deposition gene expression

18 acetyl group removes the positive charge from the lysine, thereby making it more difficult for histones to neutralize the charges on DNA as chromatin is compacted. However, the most profound effect of modified histone tails is their ability to attract specific proteins to a stretch of chromatin that has been appropriately modified. The enzymes that modify (and remove modifications from) histone tails are usually multisubunit proteins, and they are tightly regulated. They are brought to a particular region of chromatin by other cues, particularly by sequence- specific DNA-binding proteins. It is likely that histone-modifying enzymes and chromatin remodeling complexes work in concert to condense and recondense stretches of chromatin; for example, evidence suggests that a particular modification of the histone tail attracts a particular type of remodeling complex. Moreover, some chromatin remodeling complexes contain histone modification enzymes as subunits, directly connecting the two processes. 1.2 Metaphase chromosome : Centromere, Kinetochore, Telomere and its maintenance 1.2.1 Centromere The region of the chromosome that is responsible for its segregation at mitosis and meiosis is called the centromere. It is associated with two important features : l It contains the site at which the sister chromatids are held together prior to the separation of the individual chromosomes. l The term “centromere” historically; has been used in both the functional and structural sense to describe the feature of the chromosome responsible for its movement. The centromere is essential for segregation, as shown by the behavior of chromosomes that have been broken. Acentric fragment does not become attached to the mitotic spindle. There can be only one centromere per chromosome. In some species the centromeres are “diffuse”, which creates a different situation. Only discrete centromeres have been analyzed at the molecular level, The regions flanking the centromere often are rich in satellite DNA sequences and contain a considerable amount of constitutive heterochromatin. 1.2.2 Kinetochore Within the centromeric region, a darkly staining fibrous object of diameter or length ~400 nm can be seen. This object is called as Kinetochore. This

19 Kinetochore appears to be directly attached to the microtubules. The Kinetochore provides the MTOC on a chromosome. Genetic engineering has produced plasmids of yeast that are replicated like chromosomal sequences. However, they are unstable at mitosis and meiosis, segregate erratically. Fragments of chromosomal DNA have been isolated by virtue of their ability to confer mitotic stability on these plasmids. A CEN fragment is defined by its ability to confer stability upon such a plasmid. A CEN fragment derived from one chromosome can replace the centromere of another chromosome with no apparent consequence. This suggests that centromeres are interchangeable. They are used simply td attach the chromosome to the spindle, and play no role in distinguishing one chromosome from another. The sequences required for centromeric function fall within a stretch of ~120 bp. The centromeric region is packaged into a nuclease-resistant structure, and it binds a single microtubule. The S. cerevisiae centromeric region has three types of sequence element may be distinguished in the CEN region.

l

CDE-I is a sequence of 9 bp that is conserved with minor variations at the left boundary of all centromeres. l CDE-II is a <90% A-T-rich sequence of 80-90 bp found in all centromeres; its function could depend on its length rather than exact sequence. Its base composition may cause some characteristic distortions of the DNA double helical structure. l CDE-III is an 11 bp sequence highly conserved at the right boundary of all centromeres. Sequences on either side of the element are less well conserved, and may also be needed for centromeric function. A 240 kD complex of three proteins, called Cbf-III, binds to CDE-III. Mutations in the components of the genes coding for Cbf-III block chromosome movement at mitosis. A protein complex with motor activity connects the centromeric region of a chromosome to microtubules and contributes to movement on the mitotic spindle. The yeast S. pombe have the centromeres within regions of 40-100 kb that consist largely or entirely of repetitious DNA. The significance of the difference between the short centromeric regions in S. cerevisiae and the long regions in S. pombe is not clear. The common feature is that the DNA consists of noncoding sequences that are repetitive. Attempts to localize centromeric functions in Drosophila chromosomes suggest that they are dispersed in a large region, consisting of 200-600 kb. The large size of this type of centromere suggests that it is likely to contain several separate specialized functions, including sequences required for Kinetochore assembly, sister chromatid pairing, etc.

20 The primary modification comprising the constitutive heterochromatin of primate centromeres is a satellite DNA, which consists of tandem arrays of a 170 bp repeating unit. There is significant variation between individual repeats, although those at any centromere tend to be better related to one another than to members of the family in other locations. It is not clear whether the a satellite sequences themselves provide this function, or whether other sequences are embedded within the a satellite arrays. 1.2.3 Telomere Essential feature in all chromosomes is the telomere. This "seals" the end. Telomere must be a special structure, because chromosome ends generated by breakage are "sticky" and tend to react with other chromosomes, whereas natural ends are stable. Two criteria in identifying a telomeric sequence : l It must lie at the end of a chromosome (or, at least, at the end of an authentic linear DNA molecule). l It must confer stability on a linear molecule. Several telomeric sequences have been obtained from genomes of lower eukaryotes. In plant and man the construction of the telomere seems to follow a universal principle. Each telomere consists of a long series of short, tandemly repeated sequences. Table 1.1 lists the repeating units that have been identified at the ends of the linear DNA molecules. All can be written in the general form C n (A/T) m , where n < 1 and m is 1-4. Within the telomeric region is a specific array of discontinuities, taking the form of single-strand breaks whose structure prevents them from being sealed by the ligase enzyme. They may be organized in a hairpin so that they are not recognized by nucleases. Table 1.1 Telomeres have a common type of short tandem repeat. The repeating unit gives the sequence of one strand, in the 5'-3' direction form the telomere toward the centromere Telomere Repeating unit Ciliate (Tetrahymena) macronucleus CCCCAA Ciliate (Oxytricha) macronucleus CCCCAAAA Trypanosoma minichromosome CCCTA Slime molds (Dictyostelium) rDNA CCCTA Yeast (Saccharomyces) chromosome C 2-3 A(CA)l-3 Plant (Arabidopsis) chromosome C 3 TA 3 Human chromosome C 3 TA 2

21 Addition of telomeric repeats to the end of the chromosome in every replication cycle could solve the problem of end replicating. The addition of repeats by de novo synthesis would counteract the loss of repeats resulting from failure to replicate up to the end of the chromosome. Extension and shortening would be in dynamic equilibrium. The overall length of the telomere is under genetic control. If telomeres are continually being lengthened (and shortened), their exact sequence may be irrelevant. All that is required is for the end to be recognized as a suitable substrate for addition. This explains how the ciliate telomere functions in yeast. Extracts of Tetrahymena contain an enzyme, called telomerase, that uses the 3 '- OH of the G+T telomeric strand as a primer for synthesis of tandem TTGGGG repeats. Only dGTP and dTTP are needed for the activity. The telomerase is a large ribonucleoprotein. It contains a short RNA component, 159 bases long in Tetrahymena, 192 bases long in Euplotes. Each RNA includes a sequence of 15-22 bases that is identical to two repeats of the C-rich repeating sequence given in Table 1.1. This RNA provides the template for synthesizing the G-rich repeating sequence, to which it is complementary. Bases are added individually, in the correct sequence. The enzyme progresses discontinuously. The telomerase is a specialized example of a reverse transcriptase. The protein component provides the catalytic activity of reverse transcriptase, and is (presumably) confined to acting upon the RNA template provided by the nucleic acid component. The structure of the telomere is organized as single-stranded extension of the G-T-rich strand, usually for 14-16 bases. A model for the structure of the end proposes the existence of a "quartet" of G residues, formed by an association of one G from each repeating unit. The association between the G residues requires that two of them change the orientation of the base with regard to the sugar (from the usual anti to be usual syn configuration). Since each repeating unit has more than one G, more than one quartet could be formed if other G residues associate, in which case quartets might be stacked upon one another in a helical manner. It is not known how the complementary (C-A-rich) strand of the telomere is assembled, but we may speculate that it could be synthesized by using the 3'- OH of a terminal G-T hairpin as a primer for DNA synthesis.

22 1.3 Heterochromatin and Euchromatin 1.3.1 Heterochromatin Light-microscope studies in the 1930s distinguished between two types of chromatin in the interphase nuclei of many higher eukaryotic cells: a highly condensed form, called heterochromatin, and all the rest, which is less condensed, called euchromatin which is composed of the types of chromosomal structures— 30-nm fibers and looped domains. Heterochromatin, in contrast, includes additional proteins and probably represents more compact levels of organization that are just beginning to be understood. In a typical mammalian cell, approximately 10% of the genome is packaged into heterochromatin. Although present in many locations along chromosomes, it is concentrated in specific regions, including the centromeres and telomeres. Heterochromatin is classified as :— (i) Constitutive (ii) Facultative (i) Constitutive heterochromatin : It consists of specific regions that are not expressed. They include satellite DNAs, and could play a structural role in the chromosome. Often these sequences are concentrated in specific regions, typically around the centromere, (ii) Facultative heterochromatin : It takes the form of entire chromosomes that are inactive in one cell lineage, although they can be expressed in other lineages. The example parexcellence is the mammalian X-chromosome, one copy of which (selected at random) is entirely inactive in a given female. (This compensates for the presence of two X chromosomes, compared with the one present in males.) The inactive X chromosome is perpetuated in a heterochromatic state, while the active X chromosome is part of the euchromatin. Here it is possible to see a correlation between transcriptional activity and structural organization when the identical DNA sequences are involved in both states. Most
DNA that is folded into heterochromatin does not contain genes.
However, genes that do become packaged into heterochromatin are usually resistant to being expressed, because heterochromatin is unusually compact. Regions of heterochromatin are responsible for the proper functioning of telomeres and centromeres (which lack genes), and its formation may even help protect the genome from being overtaken by “parasitic” mobile elements of DNA. Moreover, a few genes require location in heterochromatin regions if they are to be expressed. In fact, the term heterochromatin (which was first defined cytologically) is likely to encompass several distinct types of chromatin structures
23 whose common feature is an especially high degree of organization. When agene that is normally expressed in euchromatin is experimentally relocated into a region of heterochromatin, it ceases to be expressed, and the gene is said to be silenced. These differences in gene expression are examples of position effects, in which the activity of a gene depends on its position along a chromosome. Many position effects exhibit an additional feature called position effect variegation, which result from patches of cells in which a silenced gene has become reactivated; once reactivated, the gene is inherited stably in this form in daughter cells. The study of position effect variegation has revealed two important characteristics of heterochromatin. First, heterochromatin is dynamic; it can “spread” into a region and later “retract” from it at low but observable frequencies. Second, the state of chromatin—whether heterochromatin or euchromatin—tends to be inherited from a cell to its progeny. These two features are responsible for position effect variegation, as explained in Figure 1.9. Fig. 1.9 The cause of position effect variegation in Drosophila. (A) Heterochromatin is normally prevented from spreading into adjacent regions of euchromatin by special boundary DNA sequences. In flies that inherit certain chromosomal rearrangements, however, this barrier is no longer present. (B) During the early development of such flies, heterochromatin can spread into neighboring chromosomal DNA, proceeding for different distances in different cells. This spreading soon stops, but the established pattern of heterochromatin is inherited, so that large clones of progeny cells are produced that have the same neighboring genes condensed into heterochromatin and thereby inactivated. Although “spreading” is used to describe the formation of new heterochromatin near previously existing heterochromatin, the term may not be wholly accurate. There is evidence that during expansion, heterochromatin can “skip over” some regions of chromatin, sparing the genes that lie within them from repressive effects. One possibility is that heterochromatin can expand across the base of some DNA loops, thus bypassing the chromatin contained in the loop The ends of chromosomes have a special form of heterochromatin The molecular nature of heterochromatin is probably best understood in the simple yeast S. cerevisiae. Mutations in any one of a set of yeast Silent information regulator (Sir) proteins prevent the silencing of genes located near 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 + (A) (B)

24 telomeres, thereby allowing these genes to be expressed. Analysis of these proteins has led to the discovery of a telomere-bound Sir protein complex that recognizes underacetylated N-terminal tails of selected histones (Fig. 1.10A). One of the proteins in this complex is a highly conserved histone deacetylase known as Sir2, which has homologs in diverse organisms, including humans, and presumably has a major role in creating a pattern of histone under acetylation unique to heterochromatin. Fig. 1.10 Speculative model for the heterochromatin at the ends of yeast chromosomes. (A) Heterochromatin is generally underacetylated, and underacetylated tails of histone H4 are proposed to interact with a complex of Sir proteins, thus stabilizing the association of these proteins with nucleosomes. Although shown as fully unacetylated, the exact pattern of histone H4 tail modification required to bind to the Sir complex is not known with certainty. In some organisms, the methylation of lysine 9 of histone H3 is also a critical signal for heterochromatin formation. In euchromatin, histone tails are typically highly acctylated. Those of H4 are shown as partially acetylated but, in reality, the acetylation state varies across euchromatin. (B) Specialized DNA-binding proteins (blue triangles) recognize DNA sequences near the ends of chromosomes and attract the Sir proteins, one of which (Sir2) is a NAD + dependent histone deacetylase. This then leads to the cooperative spreading of the Sir protein complex down the chromosome. As this complex spreads, the deacetylation catalyzed by Sir2 helps create new binding sites on nucleosomes for more Sir protein complexes. A “fold back” structure of the type shown may also form But how is the Sir2 protein delivered to the ends of chromosomes in the first place ? A DNA-binding protein that recognizes specific DNA sequences in yeast teiomeres also binds to one of the Sir proteins, causing the entire Sir protein complex to assemble on the telomeric DNA. The Sir complex then spreads along the chromosome from this site, modifying the N-terminal tails of adjacent histones to create the nucleosome-binding sites that the complex prefers. This “spreading effect” is thought to be driven by the cooperative binding of adjacent Sir protein complexes, as well as by the folding back of the chromosome on itself to promote Sir binding in nearby regions (see Fig. 1.10B). In addition, the formation of heterochromatin probably requires the action of chromatin remodeling complexes to readjust the positions of nucleosomes as they are packed together. (A) (B)

25 Whatever the precise mechanism of heterochromatin formation, it has become clear that covalent modifications of the nucleosome core histones have a critical role in this process. Of special importance in many organisms are the histone methyl transferases, enzymes that methylate specific lysines on histones including lysine 9 of histone H3 . This modification is “read” by heterochromatin components (including HPl in Drosophila) that specifically bind this modified form of histone H3 to induce the assembly of heterochromatin. It is likely that a spectrum of different histone modifications is used by the cell to distinguish heterochromatin from euchromatin. Centromeres are also packaged into heterochromatin In many complex organisms, including humans, each centromere seems to be embedded in a very large stretch of heterochromatin that persists throughout interphase. The stmcture and biochemical properties of this so-called centric heterochromatin are not well understood, but, like other forms of heterochromatin, it silences the expression of genes that are experimentally placed into it. It contains, in addition to histones (which are typically underacetylated and methylated in heterochromatin), several additional structural proteins that compact the nucleosomes into particularly dense arrangements. Heterochromatin may provide a defense mechanism against mobile DNA elements DNA packaged in heterochromatin often consists of large tandem arrays of short, repeated sequences that do not code for protein, as we saw above for the heterochromatin of mammalian centromeres. This suggests that some types of repeated DNA may be a signal for heterochromatin formation. This feature, called repeat-induced gene silencing, may be a mechanism that cells have for protecting their genomes from being overtaken by mobile genetic elements. These elements can multiply and insert themselves throughout the genome. Once a cluster of such mobile elements has formed, the DNA that contains them would be packaged into heterochromatin to prevent their further proliferation. The same mechanism could be responsible for forming the large regions of heterochromatin that contain large numbers of tandem repeats of a simple sequence, as occurs around centromeres. 1.3.2 Euchromatin Euchromatin is a lightly packed form of chromatin that is rich in gene concentration, and is often (but not always) under active transcription. Unlike heterochromatin, it is found in both eukaryotes and prokaryotes.

26 Structure The structure of euchromatin is reminiscient of an unfolded set of beads along a string, where those beads represent nucleosomes. Nucleosomes consist of eight proteins known as histones, with apprxomiately 145bp of DNA wound around them; in euchromatin this wrapping is loose so that the raw DNA may be accessed. Each core histone possesses a 'tail' structure which can vary in several ways; it is thought that these variations act as "master control switches" which determine the overall arrangement of the chromatin. In particular, it is believed that the presence of-methylated lysine 4 on the histone tails acts as a general marker for euchromatin. The exact organization of the DNA within euchromatin is not known, but with the electron microscope it is possible to see loops of DNA within the euchromatin regions, each loop between 40 and 100 kb in length and predominantly in the form of the 30 nm chromatin fiber. The loops are attached to the nuclear matrix via AT-rich DNA segments called matrix- associated regions (MARs) or scaffold attachment regions (SARs). Appearance Euchromatin generally appears as light-colored bands when stained in GTG banding and observed under an optical microscope; in contrast to heterochromatin, which stains darkly. This lighter staining is due to the less compact structure of euchromatin. It should be noted that in prokaryotes, euchromatin is the only form of chromatin present; this indicates that the heterochromatin structure evolved later along with the nucleus, possibly as a mechanism to handle increasing genome size and therefore a decrease in safety/managability. Function Euchromatin participates in the active transcription of DNA to mRNA products. The unfolded structure allows gene regulatory proteins and RNA polymerase complexes to bind to the DNA sequence, which can then initiate the transcription process. Not all euchromatin is necessarily transcribed, but in general that which is not is transformed into heterochromatin to protect the genes while they are not in use. There is therefore a direct link to how actively productive a cell is and the amount of euchromatin that can be found in its nucleus. It is thought that the cell uses transformation from euchromatin into heterochromatin as a method of controlling gene expression and replication, since such processes behave differently on densely compacted chromatin- this is known as the 'accessibility hypothesis'.
27 1.4 Giant chromosomes : Polytene and Lampbrush chromosomes 1.4.1 Polytene chromosomes In dividing diploid cells the DNA synthetic phase (S phase) is regularly followed by mitosis (M phase). The alternation of Gl, S, G2, M and Gl phases is called the cell cycle. The process of recurrent duplication cycle without consequent mitosis is called endoreduplication. Many of the cells of certain fly larvae grow to an enormous size through multiple cycles of DNA synthesis without cell division. The resulting giant cells contain as much as several thousand times the normal DNA complement. Cells with more than the normal DNA complement are said to be polyploid when they contain increased numbers of standard chromosomes. In several types of secretory cells of fly larvae, however, all the homologous chromosome copies are held side by side, creating a single polytene chromosome. The fact that, in some large insect cells, polytene chromosomes can disperse to form a conventional polyploid cell demonstrates that these two chromosomal states are closely related, and that the basic structure of a polytene chromosome must be similar to that of a normal chromosome. Instances of polyploid chromosomes in Drosophila include ovary nurse cells, follicle cells surrounding oocytes, abdominal histoblasts (see Escargot), fat body cells, gut cells, and cells of the late prepupal salivary gland. During the process of polyploidization, chromosomes become multistranded. Polytene chromosomes are large and precisely aligned side-by-side adherence of individual chromatin strands greatly elongates the chromosome axis and prevents tangling. Polyteny has been most studied in the salivary gland cells of Drosophila larvae, in which the DNA in each of the four Drosophila chromosomes has been replicated through 10 cycles without separation of the daughter chromosomes, so that 1024 (2 10) identical strands of chromatin are lined up side by side. When viewed in the light microscope, distinct alternating dark bands and light interbands are visible.
Each band and interband represents a set of 1024 identical DNA sequences arranged in register. About 95 % of the DNA in polytene chromosomes is in bands, and 5% is in interbands. The chromatin in each band appears dark, either because it is much more condensed than the chromatin in the interbands, or because it contains a higher proportion of proteins, or both. Depending on their size,
individual bands are estimated to contain 3000-300,000 nucleotide pairs in a chromatin strand.
The bands of Drosophila polytene chromosomes can be recognized by their different thickness and spacings, and each one has been given a number to generate a chromosome "map." There are

28 approximately 5000 bands and 5000 interbands in the complete set of Drosophila polytene chromosomes. Polyploid chromosomes exhibit a banded structure that is reproducible from individual to individual. In Drosophila there are thousands of recognizable bands. In situ hybridization of cloned complementary DNA of identified genes to banded polyploid chromosomes allows the localization of genes to individual chromosome bands. Chromosomal rearrangements are easily documented by comparing the order of bands between individuals, lines or even species. The degree of rearrangement observed between species is indicative of their evolutionary distance. Drosophila melanogaster has four pairs of chromosomes, three pairs of autosomes and a pair of sex chromosome. The reference system proposed by Bridges divides the limbs of salivary gland chromosomes into 102 sections called “divisions” designated by number from 1 to 102. Each of the five main limbs (X, 2L, 2R, 3L, and 3R) contains 20 divisions; the short chromosome 4 contains only two divisions. The divisions are started with a prominent band and divided further into 6 subdivisions, each designated with capital letters from A to F. Each subdivision starts with a sharp band. Thus each individual band of salivary gland chromosomes can be identified by giving the division number, subdivision, and the number of the band starting from the beginning of the subdivision. Bridges presents the following minimum numbers of bands for the salivary gland chromosomes of Drosophila melanogaster: 537 bands for the X chromosome, 1032 bands for the second chromosome, 1047 bands for the third chromosome, and 34 bands for the fourth chromosome, totalling a minimum of 2650 bands for the whole genome. In this initial count doublets were listed as single bands; more recent interpretations give the total number of bands as 3286 (Sorsa, 1988). In late prepupal salivary gland chromosomes, not all DNA in each of the chromosomes is polyploid. Approximately a third of the Drosophila genome is represented by heterochromatin, and heterochromatic regions are underrepresented in polytene chromosomes as these regions do not undergo endoreduplication. For example, the rolled locus is found in a heterochromatic region of chromosome 2 that is considered to remain condensed (and for the most part transcnptionally inactive) throughout all or most of the cell cycle, rolled lies in what is considered to be alpha heterochromatin, a chromosome region that makes up the chromocenter of polytene salivary gland chromosomes. The chromocenter is thought to be made up of DNA and protein in a dense, tightly knit structure that is transcnptionally inactive. Such heterochomatic regions, which make up 30% of the Drosophila genome, have a much lower density of genes as compared to euchromatin. Rolled gene activity is unusual in that it requires the surrounding heterochromatin for gene function. Rolled gene
29 activity is severly impaired by bringing rolled close to any euchromatic position. However, these position effects can be reversed by chromosomal rearrangements that bring the rolled gene closer to any block of autosomal or X chromosome heterochromatin (Eberl, 1993). Both bands and interbands in Polytene chromosomes contain genes Since the number of bands in Drosophila chromosomes was once thought to be roughly equal to the number of genes in the genome, it was initially thought that each band might correspond to a single gene; however, we now know this simple idea is incorrect. There are nearly three times more genes in Drosophila than chromosome bands, and genes are found in both band and interband regions. Moreover, some bands contain multiple genes, and some bands seem to lack genes altogether. It seems likely that the band-interband pattern reflects different levels of gene expression and chromatin structure along the chromosome, with genes in the less compact interbands being expressed more highly than those in the more compact bands. The remarkable appearance of fly polytene chromosomes is thought to reflect the heterogeneous nature of the chromatin compaction found along all interphase chromosomes. The remarkable appearance of fly polytene chromosomes is thought to reflect the heterogeneous nature of the chromatin compaction found along all interphase chromosomes. Individual Polytene chromosome bands can unfold and refold as a unit A major factor controlling gene expression in the polytene chromosomes of Drosophila is the insect steroid hormone ecdysone, the levels of which rises and falls periodically during larval development. When ecdysone concentrations rise, they induce the expression of genes coding for proteins that the larva requires for each molt and for pupation. As the organism progresses from one developmental stage to another, distinctive chromosome puffs arise and old puffs recede as new genes become expressed and old ones are. turned off. Most puffs arise from the decondensation of a single chromosome band. Puffing is the term that describes structural changes in polytene chromosomes. If one observes polytene chromosomes during the late prepupal stage, different bands appear to be puffed up. Puffs, then, afford a view of the temporal sequence of gene activation. A temporal pattern to puffing in the salivary glands of late prepupal flies is inducible by ecdysone injection and is therefore under control of the ecdysone receptor. A small number of genes react by puffing within minutes of exposure to ecdysone, and a much larger number (<100) react within hours. It is hypothesized that the time sequence of puffing represents a genetic hierarchy of gene activation. Early puffs are independent of protein

30 synthesis while late puffs require prior protein synthesis (Ashburner, 1990). In recent years, transcription factors and chromosomal proteins have been localized to various bands. Binding of these proteins is thought to have functional significance and to reflect the activity of these proteins in gene regulation. For more information on the binding of various proteins and RNA species to bands, see HP1/ Su(var)205, polycomb, male sex lethal 2, and suppressor of hairy wing. An example of binding of specific proteins to polytene chromosomes is found in a study of the protein CHDl (chromo-ATPase/helicase-DNA-binding domain). Proteins related to CHDl via the helicase domain have been shown to exist in large multiprotein complexes. For example SNF2/SWI2/Brm proteins are thought to participate in ATP-dependent remodeling of chromatin. Antibodies to CHDl localize this protein to extended chromatin (interbands) and regions associated with high transcriptional activity (puffs) on polytene chromosomes from salivary glands. These observations support the idea that CHDl functions to alter chromatin structure in a way that facilitates gene expression (Stokes, 1996). Polyploidization by endoreduplication requires regulation of the cell cycle. What makes one region of the chromosome become polyploid while another remains underreplicated. Information about the roles of cell cycle genes in the regulation of polyploidization can be found in cyclin E, Escargot, and origin recognition complex 2. Electron micrographs of certain puffs, called Balbiani rings, of Chiwnomus salivary gland polytene chromosomes show the chromatin arranged in loops, much like those observed in the amphibian lampbrush chromosomes. Each loop contains a single gene. When not expressed, the loop of DNA assumes a thickened structure, possibly a folded 30-nm fiber, but when gene expression is occurring, the loop becomes more extended. Both types of loops contain the four core histones and histone HI. It seems likely that the default loop structure is a folded 30-nm fiber and that the histone modifying enzymes, chromatin remodeling complexes, and other proteins required for gene expression all help to convert it to a more extended form whenever a gene is expressed. 1.4.2 Lampbrush chromosomes In 1882 Fleming first observed these chromosomes in urodele amphibian ovary. Riickert (1892) first described in great detail in shark oocytes. He coined the name “Lampbrush Chromosome” because of their brush-like appearance. These chromosomes’ occur at diplotene stage of meiotic prophase in oocytes of all animal species, in spermatocytes of several species and even in giant nucleus
31 of unicellular algae Acetabularia. These chromosomes are characterized by several lateral projections called “Lateral Loops”. They are very large and best seen in salamander oocytes because of their high DNA content. Morphology : Lampbrush chromosomes are extensible and elastic. These chromosomes with well-developed lateral loops can be stretched to about 2½ times of original length. Since, these chromosomes are found in meiotic prophase they are present in the form of bivalents in which maternal and paternal chromosomes are held together by chiasmata. The axis of each chromosome consists of a row of granules or chromomeres and from which lateral loops extend. : (i) Centromeres : These are round, smooth, and Fuelgen positive and bear no lateral loop. In many species of urodele, centromeres are identifiable chromosome landmarks as “axial bars”, formed by the amalgamation of neighbouring chromomeres, whereas in certain species of urodeles centromeres are not flanked by axial bars and are difficult to be identified, flank them. In such urodele species partner centromere do not fuse whereas urodele having axial bars centric fusion occurs. (ii) Telomeres : Ends of Lampbrush chromosomes are occupied by distinctive telomeres consisting of a small fuelgen positive part closely applied to the surface of a smooth round Fuelgen negative part. Fuelgen negative material can be digested by proteolytic enzyme. Like centromere, Telomere do not possess lateral loop. They are of different sizes, large in T. c. cristatus and small in T. c. karelini. In some urodele fusion b/w telomeres are common in T. c. aristatus while in T. c. karetini fusions are rare. (iii) Lateral loops : Loops are always symmetrical. Each chromosome having two of them, one for each chromatid. Loops can be distinguished by size, thickness and by several other morphological characteristics. Each loop appears at a constant position in the chromosome and there are about 10,000 loops /chromosome set. Each loop has axis formed by a single DNA molecule. About 5-10% DNA is present in the lateral loops the rest is condensed in chromomeres of chromosome axis, which is transcriptionally inactive. Types of loops : 1. Normal loop : Most loops can form one pattern termed as normal loop while other loops are distinguished by their matrix deposition. 2. Granulous loop : They are so called because they accumulate granule at the distal end of the fine fibers projection from other classes of lateral loop differ from granulous loop in material accumulation.

32 (a) In some cases matrix plastering the axis but leaving the tips of the projecting fibers visible. (b) In other cases, matrix fusion is irregular over the loop length. Such loops have uneven outlines. 3. Lumpy Loop : Situated on either side of centromere much degree of matrix fusion, usually so great that the loop pattern is wholly observed. Sometimes sister lumpy loop may fuse together so that instead of a pair of loops a single amorphous body is present. 4. Giant Loops : They are much larger than lumpy are matrix that they accumulate is exceedingly heterogeneous in texture. Each loop has its own developmental sequence of extension and regression. For example, giant granular loops are already of full size in very young oocytes and remain the same throughout oocyte development. The giant fusing loops are small in small oocytes and regress only just before ovulation. The granulous loops are largest in young oocytes is not regress early. Unineme theory and C. value paradox : The results of most of the earlier studied have revealed that each loop has just 1 DNA molecule as a major finding because it showed a single thread of DNA runs through each chromatid and lead to the elaboration of unineme model concept of chromosome structure. A matrix covers each loop that consists of RNA transcripts with RNA binding proteins attached to them. In general ribonucleoprotein matrix is asymmetrical being thickness at one end of the loop than at the others. RNA synthesis starts at the thinner end and progresses to the thicker end. Many of the loops correspond to a single transcriptional unit while the other loop contains several units of transcription. Some t unite on lampbrush chromosome are extremely long i.e. over lOOum in length i.e. (lu-m of DNA = 3000 bases) why they are so enormous? Even more puzzling is that the length of loop increases with C. value as a result Salamander has 10 times longer transcription units than those of a frog although both code for a similar gene product. We have no answer to this paradox but it may be possibly connected with inefficient termination of transcription in oocytes. The results of in situ hybridization studies have revealed that long Lampbrush transcripts are due to failure of termination i.e. transcription eventually stops where the next t. unit is reached. However the function of these long transcripts remain unexplainable, though the majority of them are degraded in the nucleus but presumably some RNA has some role to play in preparing an oocyte for the journey that an egg undertakes after fertilization i.e. development of a new organism. Anyway, at the cellular level of analysis both Lampbrush chromosome and
33 p.c. provide remarkably favorable opportunities to study the mechanisms responsible for gene ordered synthesis. Most of the recent exciting advances in our understanding of the nature and mode of action of the genetic material have come from the genetic studies on microorganisms. Cytologists have contributed rather little to this advances. I.5 Suggested questions 1. Describe the structure of nucleosome along with diagrams. 2. State and explain the ways of chrornatin remodeling. 3. Sate the different types of covalent modifications in histone tails and its significance. 4. Explain the significance of centormeric sequence in chromosomal segregation. 5. How is the length of telomere maintained in eukaryotic systems? 6. Explain “position effect variegation” with example. 7. How is heterochromatinization brought about? 8. Elucidate polytene chromosome structural organization. 9. Explain chromosomal puffs. 10. State the morphology of lampbrush chromosomes. 11. Validate unineme theory by lampbrush chromosome structure.
34 Unit 2 p Sex Chromosomes, Sex Determination and Dosage Compensation Structure 2.1 Introduction 2.2 Sex determination and dosage compensation in Caenorhabditis elegans 2.3 Sex determination and dosage compensation in Drosophila 2.4 Genetic regulation of sex determination and gonadal differentiation in humans 2.5 Suggested questions 2.1 Introduction In multicellular organisms sex is determined by many different mechanisms, which vary greatly. Of the various mechanisms of sex determination known till date, sex-chromosomal method of determination is perhaps the best understood and intriguing. Here, sex of an individual is determined by the presence or absence of its species-specific sex chromosomes. In this system of sex determination there are defined set of autosomes and well-defined pair of allosomes (sex chromosomes). The allosomes may be of one kind (e.g. in C. elegans, Grasshopper etc. has only X chromosome; thus two sexes are determined by either XX or XO) or of two different kinds (e.g. Drosophila has both X and Y chromosomes and sexes are determined by XX or XY). The paradox for such mechanism of sex determination is in the fact that either of the two sexes have different sex chromosomal constitution, leading to differential allosomal gene dosages. In many organisms there are two X chromosomes in female and one X in male. Therefore, it is essential to make a balance between the products of the genes of two X chromosomes and the products of one X chromosome. The mechanism by which the balance between two dosages and one dose is maintained is known as dosage compensation. This is done either by suppressing the activities of the genes of one of the two X chromosomes of the female (inactivation of one of the female X chromosome) or by hyperactivation of the male X chromosome. This would thus require dosage compensation to negate the genie imbalance for the sex chromosomes. Although sex determination pathway and dosage compensation are different pathways they may have few steps in common but must not be considered to be same under any circumstances.

35 2.2 Sex determination and dosage compensation in Caenorhabditis elegans Caenorhabditis elegans has two sexes: hermaphrodites and males. Hermaphrodites are essentially female animals that produce sperm during larval development and oocytes during adulthood. Hence, hermaphrodites are capable of self-fertilization, as well as cross-fertilization by males. Although some adult structures such as the pharynx are similar in males and hermaphrodites, most tissues and many aspects of behavior are different. The pathway is not as linear and that several loops and branches in the pathway play important roles in specifying sexual development. 2.2.1 Control of xol-1 by the X:A Ratio The primary signal for sex determination is the ratio of X chromosomes to sets of autosomes, which causes XX animals to become hermaphrodites and XO animals to become males. Early in development this ratio regulates the activity of xol-l (Fig.2.1), a key developmental switch gene that controls both sex determination and dosage compensation, xol-1 encodes a novel protein, and during early embryogenesis, high levels of XOL-1 protein activity promote male development and low levels promote hermaphrodite development. The male specifying xol-l transcript is not needed after the end of gastrulation. The early time at which xol-1 acts strongly suggests that it is a direct target of the X : A signal. This signal must involve elements on the X and elements autosomes that are compared. The X chromosome signal is polygenic, and that the combined action of these X signal element is required to inhibit xol-1 activity in hermaphrodites. At least four different regions, regions 1-4, of the X contain signal elements, and two of these elements have been identified molecularly: sex-land fox-1. Increasing Fig. 2.1 The basic sex determination and dosage compensation pathways in C. elegans
36 Fig. 2.2 The femaie-specific developmental switch gene, Sex-lethal, counts X chromosomes early in development to establish the choice between male and female alternative pathways of development at the cellular level. Through an autoregulatory feedback loop, Sxl subsequently maintains this choice throughout development, and ultimate, directs sexually dimorphic aspects of differentiation through its effects on different sets of subordinate genes downstream. Because one of these sets controls the vital process of X chromosome dosage compensation, misregulation of Sxl caused by upsets in the X chromosome counting process is lethal to one sex or the other. This lethality obscures potential effects on sexual phenotype. Loss-of-function {f-type) mutations in this gene are deleterious to chromosomal females (XX), while gain-of-function mutations (M-type) lead to constitutive expression and arc deleterious to chromosomal males (XY). Control ofS.t/in the germ line requires sex-specific input from the soma the dose of these elements in XO animals represses xol-1, promotes hermaphrodite development and causes death because dosage compensation is activated. Decreasing their dose in XX animals activates xol-1, promotes male development, and causes death due to failure to initiate dosage compensation. To date, no autosomai signal elements have been identified. Evidence indicates that sex-1 regulates the transcription of xol-1. In contrast to sex-1, fox-1 and region 2 act postranscriptionally to regulate xol-1 expression. The fox-1 gene encodes a protein with ribonuclear protein (RNP)
37 motifs, suggesting that it might bind the xol-1 RNA. It is possible that the OF- 1 protein regulates xol-1 alternative splicing, or it might govern another aspect of xol-1 mRNA metabolism. Combinatorial effect of these regulatory mechanisms allows the worm to discriminate accurately between small differences in the X : A. 2.2.2 Control of the sdc genes by xol-1 Three genes are required in XX animals to promote both hermaphroditic development and dosage compensation—sdc-], sdc-2 and sdc-3. The primary means by which XOL-1 transmits the X:A signal appears to be by negative regulation of sdc-2, as sdc-2 is not expressed in wild-type XO embryos, but is expressed in xol-1 XO embryos. Null mutations in sdc-2 and sdc-3 have no effect on XO animals but cause complete reversal of sexual fate n XX animals; null mutations in sdc-1 cause only a partial reversal of sexual fate. The sdc genes control XX hermaphrodite development by regulating the expression of the downstream sex-determining gene, her-1, a gene required for male development. SDC-2 and SDC-3 might act in a complex to directly repress her-1 transcription. This is supported by the finding that SDC-2, is highly charged and contains coiled-coil motifs, is targeted to transgenic copies of the her-1 promoter. Moreover, this localization is blocked by specific sdc-3 mutations, called sdc-3 (Tra alleles. SDC-3 is a novel protein that contains two functional domains. The first is a zinc finger motif that is required for dosage compensation but not for sex determination. The second resembles a myosin ATPase domain, and is necessary for sex determination but not for dosage compensation. The sdc-3 (Tra) mutations after this letter domain. The role of SDC-1 in regulating sexual development is less clear. SDC-1 has seven zinc fingers and resembles TFIIIA. 2.2.1 Somatic sex determination Regulation of TRA-2A by HER-1 her-1 is required for male development, as mutations in her-1 cause XO animals to develop as hermaphrodites but do not affect dosage compensation. The her-1 gene is predicted to encode a novel protein with an ami no-terminal signal sequence and protein cleavage and glycosylation sites, suggesting that HER-1 is a secreted protein. HER-1 promotes male development by repressing the activity of tra-2. Major transcript of this gene, tra-2, encodes a transmembrane protein, a direct interaction between secreted HER-1 and TRA-2A.

38 Regulation of sexual fate by tra-2 TRA-2A might be processed to release its intracellular domain, TRA-2ic. Production of TRA-2ic might occur by the action of TRA-3, a member of the calpain protease family. The tra-3 gene is necessary for hermaphroditic development. TRA-3 can proteolytically cleave TRA-2A to release TRA-2ic in insect cells. How HER-1 inhibits TRA-2A activity is unclear. One possibility is that HER-1 inhibits production of TRA-2ic by TRA-3. tra-2 activity is controlled not only at the level of protein processing or protein interaction, but also at the translational level by two elements called tra Gli elements (TGEs) which are located in the 3' untranslated region (3'UTR) of the tra-2 message. Epistasis test show that tra-2 promotes hermaphrodite development by inhibiting the activity of three genes, fem-1, fem-2 and fem-3. TRA-2A does not transcription ally regulate these genes, as they are all expressed at high levels in both sexes. Instead it appears that TRA-2A or TRA-2ic inhibits FEM activities by protein-protein interaction. FEM-2 can bind FEM-3, so they might interact to promote male development. In addition, TRA-2A and TRA-2ic can bind FEM-3, which they might inactivate to allow hermaphrodite development. Regulation of TRA-1A activity by the FEM proteins The final gene in the sex-determination pathway is tra-1, which acts cell autonomously to promote hermaphrodite development. Although genetic experiments indicate that the FEM proteins promote male development by inhibiting TRA-1 A activity, how they do so is a mystery. They are unlikely to act transcriptionally, as ra-1 mRNA levels do not differ between males and hermaphrodites. The phosphatase activity of Fem-2 is necessary for its activity, so it is possible that FEM-2 control the activity of TRA-1 A by altering its phosphorylation state. Alternatively the FEM proteins might control sexual Fig. 2.3 Schematic diagram representing both male and female specific sex determination cascade in C. elegans
39 development by regulating the nuclear levels of TRA-1A. Recent analyses have revealed that hermaphrodite tissues have higher TRA-1A nuclear levels that male tissue. Thus TRA-1A transcriptional regulatory activity might be specified by nuclear versus cytoplasmic distribution of the protein. Furthermore, the FEM proteins might regulate nuclear import or export of TRA-1A, as TRA-1A is almost completely nuclear in loss-of-function fem-1 animals. The entire sex-determination cascade can be summarized as Figure 2.2. 2.3 Sex determination and dosage compensation in Drosophila In 1921 Bridges proposed that '..Sex in D. melanogaster is determined by a balance between the genes contained in the X chromosomes and those contained in the autosomes. It is not the simple possession of two X chromosomes that makes a female, and of one that makes a male'. The discovery of numerator and denominator elements appears to validate the concept of sex determination of; as a ratio-measuring process. Proposed regulation cascade for Drosophila sex determination is schematically given below (Fig. 2.4) Fig. 2.4 Sex determination in Drosophila. This simplified scheme shows that the X- to-autosome ratio is monitored by the Sex- lethal gene. If this gene is active, it processes the transformer nRNA into a functional female-specific message. In the presence of the female-specific Transformer protein, the doublesex gene transcript is processed in a fern ale-specific fashion. The female-specific Doublesex protein is a transcription factor that leads to the production of the female phenotype. If the transformer gene does not make a female-specific product (i.e., if the Sex- lethal gene is not activated), the doublesex transcript is spliced in the male-specific manner, leading to the formation of a male-specific Doublesex protein. This is a transcription factor that generates the male phenotype XX;AA XX;AA Sex-lethal transformer doublesex Female phenotype Sex-lethal transformer doublesex Male phenotype No functional mRNA, protein Sxl Female-specific mRNA, protein Sxl Female-specific mRNA, protein No functional mRNA, protein tra No functional mRNA, protein Female-specific mRNA, protein tra
40 Sxl, the target of the somatic sex determination signal Sexual differentiation and dosage compensation are a consequence of Sxl being turned on in diplo-X individuals and remaining off in haplo-X individuals. In somatic cells, the active (female) state is maintained by a positive feedback loop (Fig.2.2), rather than continued input from the X-linked genes that trigger the initial activation. Fig. 2.5 Molecular steps in the operation of Sxl to establish and maintain a sexual pathway choice in somatic cells that is appropriate for their X chromosome dose Figure 2.5 summarizes the current model for how, in the soma, a brief, very early effect of X chromosome dosage has a long-lasting effect on the activity of Sxl. The double dose of X chromosomes in females activates an Sxl 'establishment' promoter, P e' before the blastoderm stage, resulting in production of the 'early' Sxl proteins. In contrast, the single X chromosome dose in males leaves P e inactive; hence males fail to produce early Sxl proteins. Transition from the sexual pathway establishment (signaling) level of Sxl regulation to the pathway maintenance (determination) level reflects a switch in Sxl promoters and an attendant shift from transcriptional regulation to regulation at the level of RNA splicing. At the blastoderm stage, P e shuts down and a 'maintenance' promoter, P m , located 5 kb upstream, becomes active in both sexes and remains active throughout the rest of development. In contrast to

41 transcripts from P e , transcripts from Pm can be spliced into mRNA encoding full-length Sxl proteins only if Sxl proteins (early or late) are already present. The early burst of P e expression in diplo-X individuals generates a pulse of Sxl early protein that directs the splicing of P m -derived transcripts to eliminate a male-specific, translation-terminating exon that would otherwise block synthesis of active Sxl proteins. The Sxl proteins generated from Pm-derived transcripts then maintain productive RNA splicing through a positive auto regulatory feed back loop, and act on downstream genes to elicit female differentiation and suppress X chromosomes hyper activation. In the absence of Sxl protein, downstream gene targets involved in both sexual differentiation and dosage compensation are expressed in a male-specific manner. Numerator and denominator elements The first candidate was sisterless-a. A zygotically acting positive regulator of Sxl. The gene was subsequently defined as 'numerator in experiments at that also revealed a second numerator elements, sis-b. the behavior of these two genes is characterized by reciprocal, zygotic dose-dependent Sxl-based, sex specific lethality. One additional numerator element, the apir-rule segmentation gene runt, the first known denominator element, the semi lethal behavioral mutant deadpan. As a denominator element, dpn shows reciprocal, zygotic dose-dependent, Sxl- based, sex specific lethality that is the inverse of that for numerator elements; duplications, rather than deletions of this autosomal gene kill females. Maternal involvement in progeny sex determination Mother plays an important role in, sex determination by building into the egg the biochemical machinery the embryo needs to count its X chromosomes and decide whether to activates Sxl. Maternal daughterless da is a positive regulator that is necessary but not sufficient for Sxl activation. Without da activity cannot activate Sxl after fertilization, regardless of their X chromosome dosage. All progeny develop as males. Analysis of the cDNA from Sxl mRNA shows that the Sxl mRNA of males differs from the Sxl mRNA of females. This is the result of differential RNA processing. Moreover, the Sxl protein appears to bind to its own mRNA precursor to splice it in the female manner. Since males do not have any available Sxl protein, their new 5x7 transcripts are processed in the male manner. The male Sxl mRNA is non functional. While the female-specific Sxl transcript contains a translation termination codon (UGA) after amino acid 48. The differential RNA processing that puts this termination codon into the male-specific mRNA. In
42 males, the nuclear transcript is spliced in a manner that yields three exons, and the termination codon is within the central exon. In females, RNA processing yields only two exons, and the male-specific central exon is now spliced out as a large intron. Thus, the female-specific mRNA lacks the termination codon. The protein made by the female-specific Sxl transcript can be predicted from its nucleotide sequence. This protein would contain two regions that are important for binding to RNA. Bell and colleagues (1988) have proposed that there are tow targets for the RNA-binding protein encoded by Sxl. One of these targets is the pre-mRNA of Sxl itself. This would be the mechanism that would maintain the female state of the pathway after the initial activating event had passed. The second target of the female-specific Sxl protein would be the pre-mRNA of the next gene on the pathway, transformer. The transformer genes The Sxl gene regulates somatic sex determination by controlling the processing of the transformer gene transcript. The transformer gene (tra) is alternatively spliced in males and females. There is a female-specific mRNA and also a nonspecific mRNA that is found in both females and males. The non- specific tra mRNA contains a termination codon early in the message, making the protein non-functional. The second exon of the non-specific mRNA has the termination codon. This exon is not utilized in the female-specific message. The female-specific protein form the Sxl gene activates a female-specific 3' splice site in the transformer pre-mRNA, causing it to be processed in a way that splices out the second exon. To do this, the Sxl protein blocks the binding of splicing factor U2AF to the nonspecific splice site by specifically binding to the polypyrimidine tract adjacent to it. This causes U2AF to bind to the lower- affinity (female-specific) 3' splice site and generate a female-specific mRNA. The protein encoded by this message is critical in female sex determination. The female-specific tra product acts in concert with the transformer-2 (tra2) gene to help genrate the female phenotype. The tra2 gene is eonstitutively active and makes the same protein product in both males and females. This Tra2 protein, like that of the female-specific Sxl protein, contains and RNA-binding domain. It is proposed tha the tra2 gene can bind to the transcript of the doublesex gene, but only in the presence of the female-specific Tra protein. Doublesex : the switch gene of sex determination The doubelsex gene is active in both males and females, but its primary transcript is processed in a sex-specific manner. Male and female transcripts are identical through the first three exons. The 3'exons differ markedly. What is an exon for the female-specific transcripts is part of the untraslated 3'end of the male-specific message.

43 The alternative RNA processing appears to be the result of the transformer genes. The Tra2 and female-specific Tral proteins bind specifically to a DNA sequence adjacent to the female-specific 3'splice site of the dsx pre-mRNA, and they recruit nonspecific splicing factors to this site. If tra is not produced, the doublesex transcript is spliced in the male-specific manner. The downstream 3' splice site is used, and a male-specific transcript is made. This encodes an active protein that inhabits female traits and promotes male traits. The Transformer proteins bid to sequences within the female-specific exon and activate the female- specific 3' splice site. This activation of an otherwise unused female-specific 3' splice site produces an mRNA encoding a female-specific protein that activates female-specific genes (such as those of the yolk proteins) and inhibits male development. The functions of the Doublesex proteins can be seen in the formation of the Drosophila genitalia. Target genes for the sex determination cascade Numerous proteins in Drosophila are present in one sex and not the other. In females, these include yolk proteins and eggshell (chorion) proteins. In males, Fig. 2.6 The pattern of sex-specific RNA splicing in three major Drosophila sex-determining genes. The pre-mRNAs are located in the center of the diagram and are identical in both male and female nuclei. In each case, the female-specific transcript is shown at the left, while the default transcript (whether male or non-specific) is shown to the right. Exons are numbered, and the positions of the termination codons and poly(A) sites are marked. (After Baker, 1989) the sex combs of the legs are sex -specific structure. Both the male and female doublesex transcripts bind to three sites within the 127-base-pair enhancer of the yolk protein genes. Their binding and mutagenesis studies demonstrate that the male-specific Doublesex product inhibits transcription by its binding to these sites, whereas the female-specific Doublesex protein activates gene transcription from the same sites.

44 2.4 Genetic regulation of sex determination and gonadal differentiation in humans It is clear that only a small region of the Y-chromosome is endowed with the gene(s) for sex determination. The mystery of the sex reversed (Sxr) male mice having XX-chromosome constitution was resolved unambiguously by Singh and Jones (1982) who showed that one of the 2 X-chromosomes in the Sxr males carried a min]ute segment of the short arm of the Y-chromosome. Testis determining gene on the Y-chromosome (TDY) Subsequent studies led to the discovery of SRY gene (sex determining region of the Y) that coded for an HMG domain DNA binding protein. SRY is present on the short arm of human Y, and is conserved. The expression of Sry occurred from the onset of differentiation of medulla (primordial testis) in the genital ridge. Clinching evidence in favour of Sry as the male determining gene came from the sex reversal of the XX embryos to male through insertion of only a 14kb fragment of Y-chromosomal DNA having Sry. Transgenic mouse males are sterile the role of Sry as the male determining factor is confirmed. This single-exon gene codes for a protein that has a 79 amino acid long HMG (High mobility group proteins)-domain. The proteins harbouring this domain constitute a SOX (Sry-box) family of transcription factors that bind to a heptamer of nucleotides A/TAACAAT. Mutations in the HMG domain or in the upstream promoter region of Sry have been shown to result in XY pseudohermaphrodites, gonadal dysgenesis and other gonadal pathologies. The mouse Sry carries a polyglutamine stretch, which is absent in the human Sry. Autosomal genes XY individuals have however been reported with anomalies of gonad and urogenital system, in spite of having completely normal SRY (Sry) gene. More than 75% of the XY individuals suffering from acute dwarfism due to Campomelic dysplasia (a rate skeletal disorder) are hermaphrodite with ambiguous genitalia. This disorder is caused by mutation in a gene SOX9. Importance of SOX9 (Sox9 in mouse) in testis differentiation as well as subsequent organogenesis of the male genital system has since been established. An X-linked transcription factor, DAX1 (Xp21.2-22.2), has repressive effect on male development. The XY, SRY- positive pseudohermaphrodite individuals were found to have a duplication of DAX1 on the single X-chromosome, showing dosage-dependent effect of DAX1. Individuals suffering from WAGR (Wilms aniridia, genitourinary malformation, menl&l retardation) and Denys Drash syndromes both fail to have genital as

45 well as the renal development and are caused due to mutation in the WT-1 gene. Loss of function mutation in the autosomalWT-1, the Wilms1 tumour gene, also leads to gonadal dysgenesis. Similarly, mutation in SF-1 (orphan steroid factor- 1 gene), has a broader effect on the mesonephric gonadal complex. Interaction of genes in sexual differentiation SF-1 and Sox9 bring about the activation of MIS gene, whose product leads to the regression of MD. These two transcription factors bind to the MIS promoter to induce its activity. WT-1 and GATA-4 act as cofactors of SF-1 and facilitate its binding to the MIS promoter. The X-linked, Dax-1, acts as a repressor of SF-1 in the female. Thus in the absence (or low level) of Sox9 and Sf-1, MIS (Mullerian Inhibiting Substances) is not produced and there is no regression of the Mullerian duct. Dax-1 is expressed also in males coincidentally with Sry, Sox9 and Sf-1 but its level is much lower than that in the female, the dosage of Dax-1 vis-a-vis Sry, Sox9, perhaps makes the difference between the differentiation of the male and female sex. Fig. 2.7 Cartoon giving a simplified scheme of the genes participating in sex determination, and differentiation of the urogenital system in mammals

46 Obviously, Daxl is an "anti-male" rather than "female" gene. Its elevated level in female must be blocking not omy SF-1 induced activation of MIH but also testicular development. More genes in the gonadal pathway In addition a number of cases of sex reversals and ambiguous genitalia have been observed through mutations in other parts of the genome. Wnt4, coding for a cell-signaling molecule, is likely to have a positive role in female determination, unlike the "anti-testis" DAXl. Like SF-1 artd WT-1, genes, LIM1 and LIM9, also act in the mesonephros to trigger the differentiation of GR (GR = Gonadal Ridge). Fgf9 mediates the migration of mesonephric cells in the testicular sex cords in GR of XY fetus. Therefore its absence is not detrimental to ovary in females. The XY-male specific expression of Vanin-1, a cell membrane asso ciated G-protein, during testis differentiation may also be playing an important role in directing the mesonephric cells towards the testicular sex cords in the genital ridge. It is obvious from the foregoing that though autosomal and X-linked genes play a role in the differentiation of the urogenital system, SRY is the key gene that switches the bipotential genital ridge towards testis determination. DNA as well as RNA binding functions are assigned to Sry (and other Sox proteins). It is intriguing that it occurs only in mammals. Functional SRY initiates a chemotactic action that leads to the migration of mesonephric cells into GR. It has also been demonstrated that the Sry expression in GR is not only temporally but also spatially restricted. The most important, role of Sry in gonadogenesis is to decisively direct the bipotential precursor cells in GR to for m the Sertoli cells instead of the follicular cells that form ovary. Centrally of SRY in testis determination is established. Nevertheless, which genes are the Sry-specific target gene(s) still remains a mystery. In a recent experiment, introduction of WT1 - promoter-driven Sox9 fusion gene succeeded in imparting male phenotype to the XX mouse, suggesting that if the Sox9 level could be raised then Sry was redundant for testis formation. The immediate conclusion from this evidence is that SOX9 may be the immediate target of SRY. It also suggests that WT-1 which is expressed upstream of Sry is involved in its activation. Although many sex-chromosomal as well as autosomal genes play a role in mammalian (human) sex determination pathway, only few of them have' been tabulated below (Table 2.1).

47 Table 2.1 Genes Commonly Involved in Sex Determination in Man, Other Mammals and Lower Vertebrates Genes Gene product Mutant Phenotype Mutational Orthologue in in man/mouse mechanisms other vertebrates SRY protein with an XY gonadal loss of function None HMG domain dysgenesis (C. versicolor?) WT1 Zn finger WAGR Haplo- TSD- reptiles, syndrome Denys- insufficiency, birds Drash syndrome, Dominant Agenesis of negative, loss of gonads, kidney function SF-1 orphan steroid Agenesis of loss of function TSD-reptiles, receptor urogential system birds SOX9 SRY-like HMG XY-sex reversal, loss of function fish (Sox9a, b), domain Campometic reptiles, birds dysplasia, DAX1/DSS nuclear receptor AHC, HGG loss of function reptiles, birds XY SEX reversal duplication MIS TGF-? persistance of loss of function birds, TSD- gene family Mullerian duct reptiles DMRT1 protein with XY-sex reversal loss of function fish, amophibia, dm-vdomain reptiles, birds Aromafase enzyme in female to male loss of function reptiles, birds steroid sex reveral in biosynthesis birds & reptiles Wint4 signal XX, Leydig like loss of function — transduction cells Liml, Lirn9 homebox Lim-1- loss of loss of function — gonad, kidney Lim9-gonadal agenesis Fgf9 growth factor XY abnormal loss of function — gen italia Vanin2 G-protein XY-abnormal loss of function — genitalia

48 2.5 Suggested questions 1. Describe the molecular mechanisms that regulate Sxl- promoter activity and its maintenance. 2. With the help of diagrams, describe the events of alternative splicing that lead to sex determination in Drosophila. 3. Briefly describe the cascade of events that lead to sex determination in C. elegans. 4. Explain the role of xol-J in C. elegans sex determination. 5. State and explain the effect on sex determination and dosage compensation due to mutation in the following genes: (a) Loss of function of fox-l in XX (b) Gain of function of Xol in XO (c) Gain of function of Sxl in XY (d) Loss of function of da in XX and XY (e) Gain of function of Sdc in XX 6. With the help of diagram briefly describe the initial pathway that regulates sex determination in Human.

49 Unit 3 p Imprinting of Genes, Chromosomes and Genomes Structure 3.1 Introduction 3.2 Genomic imprinting 3.3 Uniparental disomy and genomic imprinting 3.4 Suggested questions 3.1 Introduction In humans and other mammals, several biallelic genes are known where the expression of one parental allele, either the paternal or the maternal allelebut not both, is normally repressed in some cells (allelic exclusion). In such cells the relevant gene is said to exhibit functional hemizygosity; even although the sequences of both parental alleles are perfectly consistent with normal gene expression and may even be identical. In some cases the allelic exclusion may be a property of select cells or tissues while in other cells of the same individual both alleles may be expressed normally. A variety of different expression mechanisms can be involved and two broad classes of mechanism are involved : • Allelic exclusion according to parent of origin (imprinting). The choice of which of the two inherited copies is expressed is not random. This means that for some genes the allele whose expression is repressed is always the paternally inherited allele; in others it is always the maternally inherited allele. • Allelic exclusion independent of parent of origin. Here the decision as to which of the two alleles is repressed is initially made randomly, but afterwards that pattern of allelic exclusion is transmitted stably to daughter cells following cell division. A variety of different mechanisms may be involved. A unique form of control is the programmed DNA rearrangements. 3.2 Genomic imprinting Genomic imprinting is an epigenetic phenomenon, which, in most cases, is believed to occur during gametogenesis. Genomic imprinting occurs when both maternal and paternal alleles are present, but one allele will be expressed while the other remains inactive. The most prominent assumption is that this process is necessary for development and may somehow regulate growth in the embryo and neonate. Some

50 Fig. 3.1 Two examples of a hypothetical imprinted gene responsible for body color. (LEFT) In this example the pigment gene is maternally imprinted (maternal allele is inactivated). Matings between a male who possesses the allele for pigment and a female who possesses the allele for no pigment produces offspring that show only the pigmented phenotype. In this example, the mother's allele is imprinted and inactivated in the offspring. Therefore, the only actively-expressing allele is the father's pigment allele, which is not imprinted in the offspring. (RIGHT) In this example the pigment gene is paternally imprinted (paternal allele is inactivated). Matings between a male who possesses the allele for pigment and a female who possesses the allele for no pigment produces offspring that show only the pigmented phenotype. In this example, the father's allele is imprinted and inactivated in the offspring. Therefore, the only actively expressing allele is the mother's no pigment allele, which is not imprinted in the offspring. (Figure courtesy of Ross McGowan, Dept. Zoology, University of Manitoba) mechanism must be able to distinguish between maternally and paternally inherited alleles: as chromosomes pass through the male and female germlines they must acquire some imprint to signal a difference between paternal and maternal alleles in the developing organism (Fig. 3.1). An optimal method for gene imprinting, at least in maintaining the imprinted status, is allele-specific DNA methylation. The imprinting of several

51 imprinted genes has been shown to be disrupted in mutant mice that are deficient in the Dnmt 1 cytosine methyltransferase gene and all imprinted genes are characterized by CG-rich regions of differential methylation. This process is carried out with the enzyme DNA methyltransf erase (DNA MTase) in mammals. DNA MTase acts on the DNA sequence 5'-CpG-3'. Some organisms (primarily higher eukaryotes) have aggregates of CpG (known as CpG islands) in their genomes. These islands are rarely methylated in animal cells. This may be due to the bound transcription factors that block DNA MTase. De novo methylation and maintenance of methylation are two distinct processes that are required for establishment and mitotic inheritance of tissue specific methylation patterns. Dnmt1 is the major maintenance methyltransferase. Dnmt3a and Dnmt3b are essential for de novo methylation. And Sequences that are methylated are usually not active (Gold and Pedersen, 1994). Recent investigations, however, have shown that this is not always the case (Li et al., 1993). It has been postulated that if a mutation was introduced to the DNA MTase gene in the embryonic stem cells of mice, the methylation of CpG would be abnormal, and gene expression would be affected. The mutation of the DNA MTase gene was caused by homologous recombination. The three genes used in this experiment were HI9, Igf2 (insulin-like growth factor) and Igf2r (Igf2 receptor). For the H19 gene, it is the maternal allele that is expressed, while the paternal allele is silent. It should be noted that the inactive paternal allele is methylated while the maternal allele is not. It was shown that typical DNA methylation is a requirement to keep the paternal allele inactive for the H19 gene, a result that is consistent with the hypothesis. In contrast to the H19 gene, the Igf2 gene is expressed only from a methylated paternal allele. It has now been concluded that a normal level of DNA methylation is needed for expression of the paternal Igf2 allele. The gene Igf2r (insulin like growth factor receptor) is expressed from a methylated maternal allele. DNA methylation by DNA MTase is a requirement for the expression of the Igf2r gene. Disruption of primary imprinting during oocyte growth leads to the modified expression of imprinted genes during embryogenesisThus far, experiments have not demonstrated how the imprinting process is regulated.It has been proposed that further research should be attempted to discover and isolate imprinting genes . Already, some progress is being made in these areas. For instance, genomic imprinting has been implicated in cancer and shown to be involved in chromosomal deletion syndromes, such as Prader-Willi and Angelman Syndromes (Peterson and Sapienza, 1993).
52 3.3 Uniparental disomy and genomic imprinting Uniparental disomy refers to the presence of two copies of a chromosome (or part of a chromosome) from one parent and none from the other. Several additional disorders resulting from uniparental disomy of single genes or multiple genes (including whole chromosomes) have been reported. A readily detectable adverse outcome of uniparental disomy is the consequence of a newly recognized phenomenon called genetic or genomic imprinting. The first recognized example of such human abnormality resulting from the presence of uniparental disomy of an imprinted part of the genome was in Prader-Willi syndrome (PWS). Uniparental maternal disomy for chromosome 15 Figure 3.2 Schematic representation of the phenotypic effects of maternal imprinting of a mutant allele. Darkened body indicates individual that is mutant for the hypothetical imprinted locus. A cross is used to indicate the imprinted/inactive allele. (CENTER) Both parents are homozygous for the normal allele at the imprinted locus. Although only one allele is active (the paternal copy) in the offspring produced from these parents, it must be a normal allele and therefore all offspring will have a normal phenotype. (LEFT) The mother is homozygous mutant at the imprinted locus, and the father is normal. Since this hypothetical locus is maternally imprinted, the maternal mutant copy will be inactivated in their offspring and the paternal normal copy will be the only active allele. The offspring will be phenotypically normal, and the mutant allele will appear to be a recessive mutation. (RIGHT) The mother is homozygous normal at the imprinted locus, and the father is homozygous mutant. The maternal normal allele is imprinted and inactivated in the offspring of these parents. The only allele that is active is the mutant paternal copy. Therefore, all offspring produced from these parents will display the mutant phenotype, and the mutant allele will appear to be a dominant mutation. (Figure courtesy of Ross McGowan, Dept. Zoology, University of Manitoba)

53 is thought to cause Prader-Willi syndrome because there is absence of needed paternally contributed genes in the critical PWS region (del 15q 11 -q 13). The paternal contribution is hypothesized to be necessary because the homologous maternally derived genes are inactivated or imprinted (perhaps by methylation). Interestingly, a very different disorder called Angelman syndrome also involves imprinting of the same chromosome region - only in Angelrnan syndrome the maternal contribution of the critical region is missing. The terminology used to describe the role of imprinting in these two disorders is somewhat confusing but goes as follows. It is hypothesized that the critical genetic region which determines Prader- Willi syndrome is maternally imprinted (i.e. inactivated when inherited from the mother), whereas the critical region which determines Angelman syndrome is paternally imprinted (i.e. inactivated when inherited from the father). Both disorders result when the expected active genetic contribution from one parent is missing, either by deletion or uniparental disomy (Fig.3.2). Interestingly, a number of human congenital tumors show evidence of genomic imprinting. For example, in cells from Wilms' tumor, loss of the maternal chromosome 11 is common. This suggests that the maternal chromosome 11 has a tumor suppresser role not present on the paternal 11. This phenomenon in relation to cancer is referred to as "loss of heterozygosity". 3.4 Suggested questions 1. How is genomic imprinting established and maintained? 2. Explain the phenomena and importance of uniparental disomy. 3. Differentiate paternal and maternal imprinting.

54 Unit 4 p

Somatic Cell Genetics Structure 4.1 Cell fusion and hybrids-agents and mechanisms of fusion 4.2 Heterokaryon-selecting hybrids and chromosome segregation 4.3

Radiation hybrids, hybrid panels and gene mapping 4.4 Suggested questions 4.5 Suggested books 4.1 Cell fusion and hybrids-agents and mechanisms of fusion Cultured animal cells infrequently undergo cell fusion spontaneously. The fusion rate, increases greatly in the presence of certain viruses that have a lipoprotein envelope similar to the plasma membrane of animal cells. Cell fusion is also promoted by polyethylene glycol, which causes the plasma membranes of adjacent cells to adhere to each other and to fuse. As most fused animal cells undergo cell division, the nuclei eventually fuse, producing viable cells with a single nucleus that contains chromosomes from both "parents." The fusion of two cells that are genetically different yields a hybrid cell called a heterokaryon (Fig. 4.1). Because some somatic cell from animals can be cultured from single cells in a well-defined medium, it is possible to select for genetically distinct cultured animal cells. Genetic studies of cultured animal cells are called somatic-cell genetics to distinguish them from classical genetics, which deals with whole organisms derived from germ cells (sperm and eggs). Assigning genes to chromosomes The technique of somatic cell hybridization is extensively used in human genome mapping, but it can in principle be used in many different animal systems. The procedure uses cells growing in culture. A virus called the Sendai virus has a useful property that makes the mapping technique possible. If suspensions of human and mouse ceils are mixed together in the presence of Sendai virus that has been inactivated by ultraviolet light, the virus can mediate fusion of the cells from the different species. The initial fusion products are described as heterokaryons because the cells contain both a human and a rodent nucleus. Eventually, heterokaryons proceed to mitosis, and the two nuclear

55 Fig. 4.1 Fusion of cells from different species can result in stable somatic cell hybrids. The example shows how stable human-rodent somatic cell hybrids can be generated following initial fusion using polyethylene glycol (PEG). For reasons that are not understood, human chromosomes are selectively lost from the initial fusion products. The loss occurs essentially at random so that eventually the stable products of a single fusion experiment will include a variety of cells with different complements of human chromosomes. They can be cloned to establish individual cell lines with a specific complement of human chromosomes. The identity of the human chromosomes can be established by PCR-based typing for chromosome-specific markers envelopes dissolve. Because the mouse and human chromosomes are recognizably different in number and shape, the two sets in the hybrid cells can be readily distinguished. However, in the course of subsequent cell divisions, for unknown reasons the human chromosomes are gradually eliminated from the hybrid at random. The loss of human chromosomes can be arrested in the following way to encourage the formation of a stable partial hybrid. The cells used are mutant for some biochemical function; so, if the cells are to grow, the missing function must be supplied by the other genome. This selective technique results in the maintenance of hybrid cells that have a complete set of mouse chromosomes Human cell Mouse or hamster cell Heterokaryon Single nucleus but unstable Stable somatic cell hybrid Fusion, e.g. with PEG Random loss of human chromosomes Complete set of rodent chromosomes Only a few human chromosomes

56 and a small number of human chromosomes, which vary in number and type from hybrid to hybrid but which always include the human chromosome carrying the wild-type allele defective in the mouse genome. 4.2 Heterokaryon-selecting hybrids and chromosome segregation In cells, DNA can be made either de novo (“from scratch”) or through a salvage pathway that uses molecular skeletons already available. The selective technique involves the application of a chemical, aminopterin that blocks the de novo synthetic pathway, confining DNA synthesis to the salvage pathway. Two essential salvage enzymes, thymidine kinase (TK) and hypoxanthine-guanine phosphoribosyl transferase (HGPRT), are relevant to the system, as shown in the following two reactions : TK thymine ———< thymidilic acid (a DNA precursor) HGPRT hypoxanthine ————< inosinic acid (a DNA precursor) The mouse cell line to be fused is genetically unable to make TK because it is homozygous for the allele tkr, whereas the human cell line is genetically unable to make HGPRT because it is homozygous at another locus for the allele hgprT. So the genotypes of the two fusing cell lines are : Mouse : tk - /tk - ; hgprt + / hgprt + Human : tk + /tk + ; hgprt - /hgprt - Because each is deficient for one enzyme, neither the mouse nor the human cells are able to make DNA individually. In. the hybrid cells, however, the tk + alleje complements the hgprt + allele, so the cells can make both enzymes. Therefore, DNA is synthesized and the cells can proliferate. Most human chromosomes are eliminated from the hybrid cell cultures because their loss has no effect on the cultures’ ability to grow. But, to continue to grow in medium containing hypoxanthine, aminopterin, and thymidine (HAT medium), a hybrid culture must retain at least one of the human chromosomes that carry the tk + allele. 4.2.1 Selecting for the chromosome contents of hybrids Hybrids can be selected for retention of a given human chromosome or chromosome fragments if it corrects an otherwise lethal abnormality in the rodent cell. Frequently used systems include :
57 • Hybrid cells often are selected on HAT medium : The medium most often used to select hybrid cells is called HAT medium, because it contains hypoxanthine (a purine), aminopterin, and thymidine. Normal cells can grow in HAT medium because even though aminopterin blocks de novo synthesis of purines and TMP, the thymidine in the media is transported into the cell and converted to TMP by TK and the hypoxanthine is transported and converted into usable purines by HGPRT. On the other hand, neither TK – nor HGPRK – cells can grow in HAT medium because each lacks an enzyme of the salvage pathway. However, hybrids formed by fusion of these two mutants will carry a normal TK gene from the HGPRK – parent and a normal HGPRT gene from the TK – parent. The hybrids thus will produce both functional salvage-pathway enzymes and grow on HAT medium. Likewise, hybrids formed by fusion of mutant cells and normal cells can grow in HAT medium. Somatic cell hybrids can be forced to retain human chromosome 17 by using thymidine kinase deficient (TK –) rodent cells and growing the hybrids in HAT (hypoxanthine-aminopterin-thymidine) medium. • G418 selection : Hybrids can be selected for the presence of a particular human chromosome segment if it has been tagged by incorporation of a neomycin resistance (neo R) gene. The neomycin analog G418 kills nonresistant cells. NeoR is a typical example of a dominant selectable marker. Selecting for the chromosome contents of hybrids Hybrids can be selected for retention of a given human chromosome or chromosome fragments if it corrects an otherwise lethal abnormality in the rodent cell. Frequently used systems include : • HAT selection : Somatic cell hybrids can be forced to retain human chromosome 17 by using thymidine kinase deficient (TK –) rodent cells and growing the hybrids in HAT (hypoxanthine-aminopterin-thymidine) medium. TK – cells are killed in HAT medium, but are rescued by the human TK gene on chromosome 17. • G418 selection : Hybrids can be selected for the presence of a particular human chromosome segment if it has been tagged by incorporation of a neomycin resistance (neo R) gene. The neomycin analog G418 kills nonresistant cells. Neo R is a typical example of a dominant selectable marker. 4.2.2 Somatic cell hybrid panels can permit chromosomal localization of any human DNA sequence The human chromosomes in somatic cell hybrids can conveniently be identified by PCR screening with sets of chromosome-specific primers. By

58 collecting hybrid cell lines with different human chromosome contents it is possible to generate a hybrid cell panel that can be used to map any human DNA sequence to a specific chromosome. To do this, each of the hybrid cell lines is tested for the presence of the human sequence of interest. A PCR assay can be used with primers specific for that sequence or the relevant DNA sequence can be labeled and used as a hybridization probe. 4.3 Radiation hybrids, hybrid panels and gene mapping Subchromosomal mapping is possible using hybrid cells containing defined portions of a human chromosome Conventional somatic cell hybrids are a relatively crude tool for physical mapping. More refined mapping is possible using hybrids that contain only part of a particular human chromosome. Translocation hybrids and deletion hybrids are made using donor human cells that have a chromosomal translocation or deletion. To be useful, the hybrids must lack the normal homolog of the chromosome of interest. Such hybrids can be used for subchromosomal mapping of a human sequence-tagged site or biochemical marker. (Fig. 4.2). They are especially useful for defining the sequences removed by microdeletions, by segregating the deletion-carrying chromosome away from its normal homolog. Chromosome-mediated gene transfer One of the first techniques to use this approach was chromosome-mediated gene transfer (CMGT). Fragments of purified mitotic chromosomes from a donor, such as a human fibroblast, are coprecipitated with calcium phosphate on to the surface of a recipient rodent cell line in monolayer culture. Human chromosome fragments enter the recipient cells, such as mouse fibroblasts, and integrate into the chromosomes, resulting in stable transformation. As a result, hybrids can be established that retain segments of human DNA (transgenomes) of a size that is useful for mapping (usually in the range of 1-50 Mb). However, the transgenomes are prone to frequent rearrangements, so CMGT is more suited to functional assays of complex loci than as a mapping tool. Irradiation fusion gene transfer The most valuable hybrids for gene mapping are radiation hybrids. Donor cells are subjected to a lethal dose of radiation, which fragments their chromosomes. The average size of a fragment is a function of the dose of radiation. After irradiation the donor cells are fused with recipient cells of a different species. A selection system is used to pick out recipient cells that have taken

59 Fig. 4.2 Subchromosomal localization can be achieved by mapping against a panel of hybrid cells containing translocation or deletion chromosomes. The figure illustrates PCR-based mapping of the human microfibrillar protein MFAP3 using a panel of 5q translocation and deletion hybrids. Vertical black bars to the right indicate the extent of human chromosome 5 sequences, which are retained in the hybrids. Hybrids HHW1405, 1499, 1124 and 1600 contain translocation chromosomes with 5q breakpoints and retention of the segment distal to the breakpoint. By contrast, translocation hybrid HHW1138 retains material proximal to the 5q breakpoint. Hybrids HHW1064, 1113, 1118, 1421 and 1452 have different interstitial deletions of 5q. The solid blue vertical bar to the left indicates the inferred subchromosomal location as defined by breakpoints in hybrids HHW1600 and HHW1138 (blue horizontal lines near bottom). Reproduced from Abrams et al. (1995) Genomics, 26, pp. 47-54, with permission from Academic Press, Inc up some of the donor chromosome fragments. These cells are useful for mapping insofar as they have taken up a random set of other chromosome fragments from the donor, as well as the selected fragment. Stably incorporated donor fragments are either integrated into rodent chromosomes or are assembled into novel human minichromosomes formed around fragments containing a functional centromere. When a set of DNA markers from the human chromosome is assayed in a panel of such radiation hybrids, the patterns of cross-reactivity can be used to construct a map. (Fig. 4.3).

60 The principle is very similar to meiotic linkage analysis : the nearer together two DNA sequences are on a chromosome, the lower the probability that they Fig. 4.3 Constructing radiation hybrid maps. (A) Breakpoints occur randomly. Five possible examples of breakpoints (dashed blue lines) on the same type of chromosome are shown. Markers close together will tend to occur on the same fragment, e.g. A and B in all cases other than example 2. Thus, if a radiation hybrid contains marker A it will frequently also contain marker B, but rarely a distant marker such as L. (B) Ordering of markers on human 21q. The order of markers D21S16-D21S8 as inferred by Cox et ai (1990) from radiation hybrid mapping is shown. Figures on the top panel refer to distances between markers in centiRays8000. For example, the SO 6-S48 interval is 8 cR8000: at a radiation dose of 8000 rad, there is 8% frequency of breakage between them, and so a 92% chance they will occur together on one fragment. (C) Odds ratios refer to the likelihood of the indicated order for pairs of markers compared with that with the markers inverted. For example, the calculated likelihood for the order S16-S48-S46-S4 is 106 times greater than for the order S16-S46-S48-S4 will be separated by the chance occurrence of a breakpoint between them. The frequency of breakage between two markers can be defined by a value, analogous to the recombination frequency in meiotic mapping. The value,varies from 0 (the two markers are never separated) to 1.0 (the two markers are always broken apart). As in meiotic mapping, the value underestimates the distance between markers that are far apart on the same chromosome, in this case because a cell can take up two markers on separate fragments. A more accurate estimate is provided by a mapping function, D = -In (1 - value), which is analogous to the Haldane mapping function used in meiotic linkage analysis. D is measured in centiRays (cR). D is dependent on the dosage of radiation, so it is referenced against the number of rads. For example, a distance of 1 cR 8ooo between two markers represents a 1% frequency of breakage between them after exposure to 8000 rad of X-rays. Radiation hybrids derived from monochromosomal hybrid donor cells have been superseded by whole-genome radiation hybrids where the donor is an irradiated normal human diploid cell. The first such panel consisted of 199 hybrids made by fusing an irradiated 46,XY human fibroblast cell line to TK –

61 hamster cells (Walter et al.,<1994), Gyapay et al. (1996) used 404 microsatellite markers of known location to show that this hybrid panel could generate accurate maps, and then used it to map 374 unmapped ESTs. A subset of 93 of the hybrids has been made widely available as the Genebridge 4 panel. The 93 hybrids average 32% retention of any particular human sequence, with an average fragment size of 25 Mb. Laboratories can map any unknown STS by scoring the 93 Genebridge hybrids and comparing the pattern with patterns of previously mapped markers held on a central server. This has turned into an extremely powerful and convenient tool for physically mapping any STS or EST. A second human-hamster panel, Stanford G3, was made using a higher dose of radiation, so that the average human fragment size is smaller. The 83 hybrids in G3 average 16% retention of the human genome, with an average fragment size of 2.4 Mb. Thus G3 can be used for finer mapping. The impressive results of large-scale use of these panels can be accessed at http://www.ncbi.nlm.nih.gov/genemap98/. 4.4 Suggested questions 1. What is heterokaryon? What are the methods of heterokaryon selection? 2. Explain the principle of mapping by radiation hybrids. 3. How can genes be assigned to specific regions on chromosomes by low- resolution mapping (radiation hybrids)? 4.5 Suggested books 1. Albert', B. et at. (2003) Molecular Biology of the Cell, Fourth edition; Garland Sciences, New York. 2. Lewin, B. (2004) Genes VIII, John Willey, New York. 3. Klug, W, and Cummings, M. (2003) Concepts of Genetics; Seventh Edition; Pearson Education, Singapore. 4. Karp, G. (2005) Cell and Molecular Biology, Fourth Edition; John Willey, New York. 5. Gilbert, S. (1997) Developmental Biology, Fifth Edition, Sinauer Associates Publishers, Massachusetts. 6. Russell, P. (1998) Genetics, Fifth Edition; Addison Wesley Longman, New York. 7. Strachan, T. and Read, A. P. (2004) Human Molecular Genetics, Third Edition; Oxford University Press, Oxford.

62 Unit 5

p Human Cytogenetics Structure 5.1

Techniques in human chromosome analysis—molecular cytogenetic approach 5.2 Human karyotype—banding —nomenclature 5.3 Numerical and structural abnormalities of human chromosomes— syndromes. 5.4 Human genome 5.1 Techniques in human chromosome analysis 5.1.1 Introduction The correct chromosome number for man was established only after the application of tissue culture methods to cytogenetics. Before 1956, the chromosome number of man was considered to be 48. Tjio and Levan (1956), analyzing cultures of human embryonic lung fibroblasts, found a consistent chromosomal number of 46. At the same time and independently Ford and Hamerton (1956), using meiotic cells obtained from testicular biopsy material, found only 23 pairs of chromosomes. Mitotic cells from the same specimens contained 46 chromosomes. These two papers mark the beginning of modern human cytogenetics. The 15-year period from 1956 to 1971 saw the development of a standardized system of nomenclature which became more refined as the identification of human chromosomes became more precise. The delineation of most of the syndromes associated with chromosomal abnormalities occurred during syndromes associated with chromosomal abnormalities occurred during this period. 5.1.2 Terminologies used in the identification of human chromosomes karyotype & idogram Human chromosomes can be arranged in an orderly fashion to produce a karyotype (Rowley, 1969). A karyotype is comoposed of individual chromosomes from a particular cell; the chromosomes are aligned in pairs and identified according to the standard nomenclature accepted by cytogeneticists. Karyotypes of different cells will reflect the variations in chromosomal morphology present in these cells. An idiogram is the schematized drawing of a composite of many karyotypes and is not directly related to any particular cell. Idiograms generally are not prepared for clinical purposes. But are used for comparing chromosomal patterns of different species.

63 Metaphase chromosome Metaphase chromosomes differ from one another in size and shape (Fig. 5.1) Each metaphase chromosome is identified by its size, shape, and specific banding pattern. The absolute size of any chromosome varies with the stage of mitosis. Chromosomes are longer and less coiled in prophase and shorter and more compact at the end of metaphase. The duration of treatment with mitotic blocking agents and the type of hypotonic solution also influence the absolute size of the chromosomes. In general, the longest human metaphase chromosome is about 7-8 urn in length, whereas the shortest is about 2?m long. Each metaphase chromosome is composed of two chromatids joined at the centromere (the site of attachment of the spindle fibre). The position of the centromere is specific for Fig. 5.1 Intact metaphase plate from a normal male with 46 chromosomes from bone marrow specimen (top) and karyotype of cell (bottom). Chromosomes are arranged in seven groups of morphologically similar chromosomes. The X chromosome is included with group C from which it cannot be distinguished. The Y chromosome in this cell is about the size of G-group chromosomes, but it can be differentiated from them by the size of the short arm

64 each chromosome and divides it into a long and short arm. The relative length of the two arms (arm ratio) is important for the identification of chromosomes. A chromosome with a centreomere in the middle that divides it into two equal arms is called metacentric. When the centromere is somewhat nearer to one end of the chromosome, so that there is a distinct long and short arm, the chromosome is said to be submetacentric. If the centromere is very near to one end of the chromosome, which thus only a very short small arm and a relatively longer long arm, the chromosome is called acrocentric (Fig. 5.2). A telocentric chromosome, not normally found in human cells, has the centromere at the end. 5.1.3 Standardization of nomenclature of chromosome Four conferences were held between 1960 and 1971 to revise the nomenclature used to identify human chromosomes along with the improvement of technologies. The last two conferences were held in Chicago and Paris. When the first reports on human chromosomal abnormalities appeared in 1956, each group of investigators used its own system of arranging and numbering the chromosomes. The necessity for adopting a uniform system was generally recognized, and a standard nomenclature of human mitotic chomosomes was adopted at the Denver Conference in 1960. Numbers were assigned to each pair of autosomes as nearly as possible in decending order of length. The sex chromosomes, XX pairs of autosomes (44) plus two sex chromosomes, making a total of 46 chromosomes. Seven groups of morphologically similar chromosomes could be distinguished (Table 5.1). It soon became evident, however, that not all pairs of chromosomes could be identified with certainty, even in preparations of the highest technical quality. Patau (1960) proposed that the seven groups of morphologically similar chromosomes be indentified by capital letters A through G, an arabic number being added only when the individual chromosome could be identified with certainty. This system had the great advantage of flexibility, since it permitted general recognition of the group to which a chromosome belonged without implying identification of the specific chromosome involved in an abnormality. Patau's recommendations were accepted at the London Conference on the normal human karyotype (1963). Whereas individual investigators were using the same systems (Denver and London) for identifyng individual chromosomes, they were using different systems for describing chromosomal abnormalities. This led to confusion when data from different laboratories were collated. A major achievement of the Chicago Conference in 1966 was the development of a uniform system of notation designed to facilitate coding for data retrieval. It was agreed that the analysis of the karyotype would be recorded with the total chromosomal number first, followed
65 by the sex chromosomes, and finally by any additional abnormalities. Thus the karyotype of a normal male was written 46, XY; a normal female was 46, XX. The recommended nomenclature symbols used in describing normal or abnormal chromosomes are summarized in Table 5.2. Fig. 5.2 Centromere position in typical chromosomes, a, Two chromatids joined at centromere or primary constriction. Centromere (arrow) is median and divides chromosomes in two equal arms. Chromosome is metacentric. b, Submedian centromere (arrow) divides the chromosome into short arm and long arm Chromosome is submetacentric. c, Submetacentric chromosome with secondary constriction (long arrow) in long arm. d, Large acrocentric chromosome with subteminal centromere (short arrow). Satellites (long arrow) are separated from short arm by secondary constriction, e and f, G-group chromosome and Y from same cell. Long arm chromatids of Y are close together and short arm is larger than G-group chromosome Table 5.1 Systems of identification of human metaphase chromosomes* Denver Chromosome description Patau Chromosome Centromere group Group number position Morphology A 1-3 1,3 Median Metacentric 2 Submedian Less metacentric than above B 4-5 4,5 Submedian Submetacentric C X 6-12 X,6,7,9,ll Submedian Submetacentric but more metacentric than remainder 8,10,12 Submedian Less metacentric than above D 13-15 13,14,15 Subtermina Acrocentric—all may have satellites E 16-18 16 Median Metacentric 17,18 Submedian Submetacentric F 19-20 19,20 Median Metacentric G 21-22 21,22 Subterminal Acrocentric—all may have satellites Y Subterminal Acrocentric—similar in size to group G but usually morphologi cally distinct; long arms tend to be close together: satellites are not present *Rowley (1969)

66 Table 5.1 Nomenclature symbols Chicago Conference A––G the chromosome groups 1––22 the autosome numbers X,Y the sex chromosomes diagonal(/) separates cell lines in describing mosaicism ? questionable identification of chromosome or chromosome structure * chromosome explained in text or footnote ace acentric cen centromere dic dicentric end endoreduplication h secondary constriction or negatively staining region i isochromosome inv inversion mar marker chromosome mat maternal origin p short arm chromosome pat paternal origin q long arm of chromosome r ring chromosome s satellite t translocadon s t repeated symbols duplication of chromosome structure Paris Conference A. Recommended changes in Chicago Conference nomenclature + 1. The + and — signs should be placed before the appropriate symbol where they mean additional or missing whole chromosomes. They should be placed after a symbol where an increase or decrease in length is meant. Increases or decreases in the length of secondary constriction, or negatively staining regions, should be distinguished from increases or decreases in length owing to other structural alterations by placing the symbol h hetween the symbol for the arm and the + or — sign (e.g., 16qth +). 2. All symbols for rearrangements are to be placed before the designation of the chromosome (s) involved in the rearrangement, and the rearranged chromosome (s) always should be placed in parentheses, e.g., r(18), i (Xq), die (Y). B. Recommended aditional nomenclature symbols del deletion der derivative chromosome dup duplication ins insertion inv ins inverted insertion rep reiprocal translocation* rec recombinant chromosome rob Robertsonian translocation* (“centric fusion”)
67 tan tandem translocation* ter terminal or end (“p ter” for end of short arm; “q ter” for end of long arm) : break (no reunion, as in a terminal deletion) : : break and join —< from-to * Optional, where greater precision is desired than that provided by the use of t as recommended by the Chicago Conference. 5.1.4 Recommendations of the Paris Conference The fluorescent karyotype, published by Caspersson et ah (1971), was accepted as the basis for the assigning of numbers to each chromosome. A. Definitions The bands seen with the fluorescent dyes (quinacrine) were called Q-bands and were accepted as the reference bands. Those bands of chromatin stained by methods that demonstrate “constitutive heterochromatin” were called C-bands and they are mainly confined to the centromeric region. The bands stained with basic dyes such as Giemsa were called G-bands and, except in one techique, they correspond quite well with Q-bands. One of the techniques using Giemsa, the exception just noted, gives patterns that are opposite in intensity to the G-bands; these were called R-bands. A band was defined as a part of a chromosome which is clearly distinguishable from its adjacent segments by appearing darker or lighter with the Q, G, R, or C staining methods. By definition there were no “interbands.” In the construction of the chromosome map, each band was refered to by its midline and not by its margins. A chromosome landmark was defined as a consistent and distinct morphological feature that is an important diagnostic aid in identifying a chromoosome. A region was defined as any area of a chromomsome lying between two adjacent landmarks. A chromosome arm lacking a prominent landmark consists of only one region. B. Band numbering Regions and bands are numbered consecutively from the centromere outward along each chromosome arm (Fig. 5.3). A band used as a landmark is considered as belonging entirely to the region distal to the landmark and is accorded the band number of “1” in the region. A band bisected by the centromere is considered as two bands, each being labeled as band 1, in region 1, of the appropriate chromosome arm. For the designation of a particular band, four items are required: the chromosome number, the arm symbol, the region number and the band number within that region. These items are given in order without spacing or punctuation.

68 For example, Ip33 indicates chromosome No. 1, short arm, region 3, band 3. If a band defined in the present chromosome map has to be subdivided, the original band designation will be followed by a decimal point and the sub-bands will then be numbered sequentially from the centromere outward, e.g., Ip33.1; Ip33.2; Ip33.3, indicating that the orginal band 33 in the short arm of chromosome No. 1 has been divided into three sub-bands, 33.1 being proximal and 33.3 distal to the centromere. This system is thus relatively simple and yet sufficiently flexible to accommodate further refinements as the banding techniques are improved. C. Characterization of chromosomes by the various banding techniques The technical quality of the chromosomes is of the utmost importance for characterizing chromosomes by their banding patterns; in fact, it is much more important than previously adopted methods when morphology was the sole criterion for preparing the karyotype. The most distinct banding patterns are obtained when the chromosomes are relatively long and free of overlaps. It is important to note that the morphological characteristics of size and centromere position remain critical parameters used in the identification of chromosomes. Thus, the cytogencticist uses banding patterns as well as overall morphology to distinguish individual chromosomes. In general, the number of distinct bands increases with increasing length of the chromosome. It is thus, meaningless to mention the absolute number of bands in a chromosome arm, since the number varies with the state of contraction, the quality of the preparation and the type of treatment and stain. Once identification of the individual chromosomes by means of the major landmarks is mastered, careful observation of very good preparations can reveal a number of fine bands which can be used, among other things, for defining the site of chromosomal breakage and rejoining. The intensity of fluorescence is influenced by the position of the chromosome in the metaphase. Chromosomes in the center of the cell frequently fluoresce more brightly than homologous chromosomes that are on the periphery. It is therefore necessary to consider the pattern of the bands in a particular chromosome and to appreaciate the fact that the overall intensity of fluorescence of homologs may be different. There may also be “spreading” of fluorescence from brighter to duller chromosomes, such that a 19 or a 22 adjacent to an X or the long arm of the Y may appear to be much brighter than normal. The terms “distal” and “proximal” refer to the position of a band with respect to the centromere. The following terms were used in the Paris report to indicate the approximate intensity of fluorescence: negative no or almost no fluorescence pale as on distal lp medium as the two broad bands on 9q intense as the distal half of 13q brilliant as on distal Yq

69 Fig. 5.3 Diagrammatic representation of chromosome bands as observed with the Q-, G-, and R- staining methods; centromere representative of Q-staining method only. Reproduced from the report of The Paris Conference (1972)

70 D. Characterization of chromosomes on the basis of different boarding techniques : Chromosome 1 is the largest chromosome and is usually metacentric. The distal 40% of the short arm shows pale fluorescent bands (32 to 36), and the proximal segment shows two bands of medium fluorescence; the more proximal band divides region 1 from 2, and the more distal band divides region 2 from 3 (Fig. 5.4). The area of the secondary constriction, a poorly staining gap, which by definition is in the long arm, is adjacent to the centromere and shows negative fluorescence; it constitutes region 1. The long arm also contains a central intense band which divides region 2 from 3, with a less intense band distal to it which divides region 3 from 4. Fig. 5.4 Idiogram of fluorescent bands in human chromosomes (see text) The large block of densely stained material in the long arm adjacent to theL centromere is the most prominent feature of No. 1 in cells treated to produce G-bands (Fig. 5.5). This area corresponds to the negatively quinacrine-stained secondary constriction. The two proximal bands in the short arm and the very

71 darkly stained central band and less darkly stained distal band in the long arm are also present. The end of the short arm is faintly stained. The technique for staining C-bands revals the same densely staining region of the secondary constriction, generally called “constitutive heterchromatin.” as do the G-band techniques. The R-banding technique (Dutrillaux and Lejeune, 1971) demostrates moderately staining material in this region; elsewhere along the chromosome, however, dark bands appear pale and vice versa. This reversal of staining intensity as compared with G-bands is particularly noticeable at the end of the short arm. Fig. 5.5 Idiogram of Giemsa bands in human chromosomes (see text) Chromosome 2 is a large chromosome, less metacentric than No. 1, and lacking conspicuous landmarks. It shows a number of bands of medium fluorescence along the entire length; the central band (14 to 16) in the short arm and the two cenral bands (22 to 24) of equal intensity in the long arm are most prominent. In the short arm, the dull band distal to the central band divides

72 region 1 from 2. In the long arm, the dull band proximal to the proximal central band divides region 1 from 2, whereas the dull band distal to distal central band divides region 2 from 3. Chromosome 3 is a large metacentric chromosome which is smaller than No. 1. It has a nearly symmetrical banding pattern. There is a distinct band of pale fluorescence in the center of each arm which separates two broad bands of medium intensity. This pale band separates region 1 from 2 in each arm. The distal medium band in the short arm is narrower, but frequently appears more intrensely fluorescent than that in the long arm, whereas the terminal pale band is longer in the short than in the long arm. Similar morpological features are observed in cells treated to produce G- bands. The centromeric regions stains darkly, but the variation in intensity of stain, seen with fluorescence, is not obsrved in G-bands. Chromosome 4 is a long submetacentric chromosome that, similar to No. 2, lacks prominent landmarks. It has one band (15) of medium fluorescence in the short arm and four or five relatively evenly spaced bands of medium intensity in the long arm. The long arm is divided into three regions by a proximal dull band separating region 1 from 2 and a distal dull band separating 2 from 3. Chromosome 5 has more distinctive characteristics than No. 4 and is frequently the first pair of B-group chromosomes indentified in the cell. The central band (14) of medium fluorescence in the short arm is brighter and frequently wider thanthat in No. 4. There is a broad central band of medium fluorescence which separates region 1 from 2 in the long arm, with a prominent distal pale band which separates region 2 from 3. Frequently, the terminal portion of the long arm of No. 5 is paler than No. 4. Chromosome 6 is the largest and one of the last submetacentric C-group chromosomes. The most prominent feature is a distinct band of pale fluorescence in the middle of the short arm, which separates two bands of medium intensity and which also separates region 1 from 2. The long arm contains a number of bands of medium intensity; those bands near the centromere are frequently brighter and more idstinct. A dull band in the middle of the long arm separates region 1 form 2. Chromosome 7 is slightly smaller than No. 6, but their centromere positions are similar. The two bands of intense fluorescence in the center of the long arm are the most prominent feature of this chromosome. The proximal band divides regions 1 and 2,whereas the distal band divides regions 2 and 3. There is a distinct band of medium fluorescence at the end of the short arm which divides region 1 from 2. Chromosome 8 is one of the most submetacentric C group chromosomes, and it lacks distinctive landmarks. The short arm shows less intense fluorescence

73 than the long arm, and a cenrtral pale band which divides region 1 from 2 may be seen in good preparations. A distal pale band (22) separates two medium bands in the long arm, The more proximal of these medium bands separates region 1 from 2. Chromosome 9 is the middle-sized C chromosome and is more metacentric than Nos. 8 or 10. The long arm shows a negatively staining centromeric region (12) with two distal evenly spaced bands of medium intensity. The proximal band of the pair may appear wider and separates region 1 from 2; the distal band separates 2 from 3. The short arm has a characteristic heart -shaped appearance with a central band of medium intensity which divides region 1 from 2, Both G and Q techniques show a prominent, faintly staining region near the centromere. The R-bands are the reverse, except for the centromeric region which is pale; the R-bands are similar in staining intensity to Q-and G-bands. The negatively fiuorescing and pale-staining region in the long arm near the centromere shows a large block of material that stains intensely with Giemsa after treatment to produce C-bands. This C-band material presumably represents one of the types of constitutive heterochromatin, the size of which may vary with individuals. Chromosome 10 is one of the smaller, less matacentric C-group chromosomes which can be identified by the three evenly spaced bands in the long arm. The proximal band is most intense (and divides region 1 from 2) and the distal is the least intense. The short arm shows medium fluorescence. Chromosome 11 is one of the least submetacentric C group chromosomes; and it may be very slightly larger than No. 10. It is somewhat similar to No. 9, but can be distinguished on the basis of the following features: There is a narrow medium band (12) in the middle of the negatively staining centromeric portion of the long arm. In poor preparations, this narrow band may be very faint or not apparent. A broad band of medium fluorescence is present in the middle of the long arm. A narrow dull band in the middle of this broad band separates region 1 from 2. This broad band usually appears as a single band that distinguishes it from the definite double band in the long arm of No. 9. The short arm shows medium fluorescence (14) and has a rather squarish appearance. In contrast, the short arm of No. 9 tends to taper near the centromere. The C-banding technique reveals that the amount of centromeric stining material in No. LI, and the X is second only to that in No. 9. Chromosome 12 is the most submetacentric of the C-group chromosomes; it is similar in size to No. 10. The short arm shows a band of mediumn fluorecence (12) which is smaller than that in No. 11, because the short arm is smaller..The band (12) of medium intensity in the long arm near the centromere is wider than that found in No. 11. A short band (13) of negative fluorescence separates band

74 ql2 from a distal long segment of medium intensity which divides region 1 from 2. This distal segment and the terminal dull band are both longer than the corresponding bands of No. 11. The X chromosome is the third largest chromosome in the C group; it is, with 6 and 11, among the least submetacentric chromosomes in this group. The X chromosome is frequently the most fluorescent of the C-group chromosomes. Both Xs in the female show identical fluorescence patterns. In both arms, the region proximal to these medium bands shows pale fluorescence. Two other evenly spaced, less distinct and less intense bands of fluorescence are found in the distal long arm. Chromosome 13 is the largest of the D-group chromosomes and shows intense fluorescence of the distal half of the long arm. In good preparations this segment is seen as two bands; the more proximal band divides regions 1 and 2. and the more distal divides 2 and 3. Flourescence of the satellites and short-arm region shows variable intensity and is an inherited variant; a proximal intense band in the long arm has also been observed as an inherited variant. Chromosome 14 shows a broad medium to intense band in the proximal half of the long arm which divides regions 1 and 2 and a narrow medium and, dividing regions 2 and 3. close to the distal end of the long arm. Variable fluorescence of the satellites and short-arm region has been noted. Chromosome 15 is the smallest D chromosome and shows the last intense fluorescence of its group. The proximal half of the long arm shows medium florescence which divides region 1 from 2, whereas the distal half is pale. This chromosome is distinguished from No. 14 by the absence of a distal medium band, although there may be a distal band of faint fluorescence in No. 15. In poor preparations, it is frequently difficult or impossible to differentiate No. 14 from 15. The satellites and short-arm region show variable fluorescence. Chromosome 16 is the largest and most metacentric of the E-group chromosomes and is one of the few chromosomes that could be identified solely by morphology. The short arm shows a band (12) of medium fluorescence which is less intense than the central band which separates region 1 from 2 in the long arm. The long arm contains a proximal segment of negative fluorecence (11) which corresponds to the secondary constriction. The G-band technique reveals the medium bands in the long and short arm and the densely staining secondary constriction. The R-bands are the reverse of Q-bands, except that the region of the secondary constriction is pale. Obanding reveals a large block of constitutive heterochromatin in the region of the secondary constriction which varies in length in different individuals. Chromosome 17 is the palest staining of the E-group chromosomes. It has a
75 single distal band (22) of medium fluorescence in the long arm. The dull band proximal to this band divides region 1 from 2. Chromosome 18 is the smallest E-group chromosome. The long arm contains two bands of medium intensity (12and 22), the proximal band being the brighter and wider of the two. The dull band between these two bands separates regions 1 and 2. Chromosome 19 is the most weakly fluorescent chromosome in the karyotype and it is difficult to distinguish the long and short arms except in very good preparations. There is a fluorecent spot (12) in each arm adjustment to the centromere; the spot in the short arm is longer and brighter than that in the long arm. G-bands are similar to Q-bands, and the chromosome shows the same very pale staining except for the centromeric region which is well stained. Chromosome 20 also show weak fluorescence, but more than No. 19. The short arm is brighter than the long arm. The C-band is medium sized and smaller than in No. 19. Chromosome 21 is the smaller of the G-group chromosomes and is much brighter than No. 22. The long arm shows a proximal intense segment. Which divides regions 1 and 2, and distal pale segment. There is variable fluorescence of the short-arm region and the satellites; this is an inherited variant. This variability has been useful in determining the source of the meiotic error that results in Down's syndrome (Robinson, 1973). Chromosome 22 is the larger of the G-group chromosomes and shows very dull fluorescence, similar to No. 19. In fact, in some preparations it may be difficult to distinguish Nos. 19 and 22. A narrow pale band may be observed in the middle of the long arm. A bright fluorescent band in the short arm and variable fluorecence of the satellites are inherited variants. G-bands are similar to Q-bands, although the centromere region stains darkly with Giemsa. The C-band is medium sized and larger than in No.21. The Y chromosome was the first human chromosome identified with quinacrine fluorescence (Zech, 1969) because of the brilliance of the distal long arm in cells from all but a few males. The variability in the length of the Y chromosome is well established; it is now evident that this polymorphism is correlated with variation in the length of the brilliant segment. The Y has a variable appearance with G-banding techniques, but may show two distal bands; it is relatively pale when stained with the R-banding technique. The size of the distal C-band in the long arm is directly related to the length of the brilliant segment on fluorescence.

76 5.2 Human karyotype—banding—nomenclature 5.2.1 Introduction Metaphase chromosomes show little morphological differentiation in conventional preparations. The size, the position of the centromere, and occasional secondary constriction(s) are the only criteria that can be employed for recognizing chromosomes. In species with high diploid numbers, chromosome pairs with similar morphology become increasingly common, thus making the identification of individual pairs extremely difficult. The human karyotype is comparatively favorable because at least the chromosomes can be classified, according to morphology, into seven groups, and a few pairs can be identified unequivocally. In the karyotype of the laboratory mouse, all chromosomes are acrocentric and do not even allow grouping. Cytologists have attempted a variety of ways, such as distribution of chemical-i induced breaks, unstained chromosome regions induced by low temperature, and diferential DNA replication time revealed by autoradiography, to further differentiate the chromosomes longitudinally, but all these methods are laborious and the results are ambiguous. The first break through was recorded not long ago when Caspersson I and his collaborators (1969a, b) found that certain fiuorochromes, e.g., quinacrine mustard, when applied to cytological preparations and observed with ultraviolet optics, produced characteristically bright and dark bands. Later Caspersson et al. (1970a.b) applied the technique to human chromosome prepsrations and found that the fluorescent banding pattern is likewise specific for each chromosome pair. Credit to the second major advance must go to Joseph G. Gall and Mary Lou Pardue who perfected the in situ DNA/RNA hybridization technique. In their studies on the cytological locations of the satelite DNA of the laboratory mouse (Pardue and Gall. 1970), they treated the cytological preparations with a series of chemicals in order to achieve molecular hypridization. In these preparations, the centromeric areas of the mouse chromosomes stained more deeply with Giemsa than the Chromosome arms. They regarded the densely stained centromeric areas as heterochromatin. The discovery of a simple staining procedure led to an explosive activity in inventing new procedures, particularly regarding the chromosomes of man and other mammals. 5.2.2 Classification of banding patterns : The Q-bands. Florescent banding with quinacrine mustrard or quinacrine dihydrochloride. The C-bands. Constitutive heterochromatin revealed by the PardueGall in situ hybridization procedure or its modifications.

77 The G-bands. Crossbands of chromosomes revealed by a variety of procedures. These bands coincide well with Q-bands, i.e., deeply stained G- bands are brightly fluorescent in Q-band preparations. The R-bands. The “reverse” banding pattern following the procedure of Dutrillaux and Lejeune (1971). 5.2.3 Cell harvest and slide preparations Arresting Harvesting cells is strictly conventional. Bone marrow cell culture or any cell population containing a high incidence of mitosis are suitable. Agents such as colchicine, colcemid, and vinca alkaloid can be used to accumulate mitosis. However, over condensed chromosomes yield very poor banding, so that prolonged mitotic arrest should not be done. Fixation The cell polulations should be treated with a hypotonic solution prior to fixation. It matters little which kind of hypotonic solution is used. The cells, after hypotonic solution treatment, are fixed according to the type of slide preparations to be made, viz,, “Carnoy” fixative (1 glacial acetic acid: 2 methanol) for air- dired slides. For cell populations which require squash technique (e.g., many solid tissues), the fixative to use is 45—50% acetic acid. However, the preparations are not suitable for G-bands, though they are excellent or C-bands. It is suggested that the air-dried slides be incubated at 37°C for 1 hour (without covering) and thereafter be kept in air-tight slide boxes containing a drying agent such as silica gel. The slides may be used immediately after this incubation period or may be stored for a few weeks. 5.2.4 C-Banding In good C-band preparations, the constitutive heterochromatin should stain deeply and the euchromatin should show only a faint outline of the chromosome. However, flame-dried preparations, when improperly treated, will show G-bands as well as C-bands, which is confusing. The original procedure (Pardue and Gall, 1970) and the modifications thereof (Arrighi and Hsu, 1971; Yunis et al., 1971) are useful for distinguishing constitutive heterochromatin and euchromatin in mammalian chromosomes. The procedure to be described is a simplified version designed to reveal C-bands using air- dried and flame-dried preparations, although some comments will also be made for squash preparations. A. Reagents 1. HCL : prepare 0.2 N solution.

78 2. NaOH : Prepare a 0.07 N solution. 3. SSC : Prepare a lOx concentrate (a solution of 0.15 M sodium citrate and 1.5 M NaCL) and dilute with distilled water to the desired concentrration. 4. Giemsa staining solution. 5. Phosphate buffer solution (0.01 M Sorensen's phosphate buffer, pH 7.0). B.Procedure 1. Treat the slides with HCL at room temperature for 15 minutes. Rinse with distilled water three times. 2. Treate the slides with NaOH for 2 minutes. Rinse with 70%, then 95% ethanol three times for a period of 5 minutes each. Air dry the slides. 3. Place slide horizontally, with cell-side up, in a moist chamber, and add either 2x or 6x SSC to the cell area of the slide. Place a coverglass over the SSC solution. 4. Incubate the moist chambers containing the slides at 60°—65°C for 16-20 hours. 5. Rinse in either 2x or 6x SSC (three times, 5 minutes each), 70% ethanol (three times, 5 minutes each), 95% ethanol (three times, 5 minutes each), and air dry. 6. Stain in Giemsa solution. C. Comments 1. After a considerable amount of experimentation, it is generally opined that the HCL treatment is an important step in eliminating the G-bands in C- band preparation, particularly when air-dried and flame-dried slides are used. In sqush preparations, HCL treatment is not a vital step and may be omitted for C-band preparations. 2. The concentration of NaOH and the duration of the NaOH treatment are important. As a standard, one may start with 0.07 N for 2 minutes. This combination may be too strong and the resulting euchromatic chromosomes may appear bloated and show an empty appearance. If such a result is obtained, one must experiment with reduced concentration of NaOH solution (0.02 N, 0.01 TV) and time of treatment (1 minute, 30 seconds, or even 15 seconds). If the chromosomes still appear distorted, one should then try the solution suggested by Stefos and Arrighi (1970): a 2 x SSC solution with pH adjusted to 12 by NaOH. This solution is particularly useful for small chromosomes such as the microchromosomes of the birds and the chromosomes of Drosophila (Hsu, 1971). Conversely, some C-bands require a prolonged NaOH treatment. 3. Many laboratories use Coplin jars filled with 2 x or 6 x SSC for the over night incubation. This is undersirable because the glass slides will stain heavily with Giemsa, thus interfering with the observations on the chromosome banding.

79 Incubating slides in moist chambers eliminates this defect. If, however, such defect is not observed, Coplin Jars are of course convenient. A simple moist chamber can be constructed as follows. Use a Petridish of suitable size. If square (120 mm each side) Petri dishes are used, 10 ml of either 2 x or 6 x SSC is placed in the bottom (15 ml for overnight treatment at 65°C). Next a stand for the slides is placed in the bottom half. The stand should be as small as posible and of sufficient height so that the slide is above the salt solution in the bottom half of the Petri dish. The slide(s) is placed on the stand. A few drops of the solution are placed on the slide to cover the cells. A coverglass is then placed over the solution, the Petri dish is covered, and the entire chamber is placed in an oven set at the desired temperature. 4. The stock Giemsa-staining solution is diluted with phosphate buffer, and the concentration varies with each new lot of stain. Usually concentrations varying from 2 to 10% have been used and stained the slides from 5 to 30 minutes. 5. Slides should be of good quality and should be cleaned in some manner. Slides can be cleaned in 95% ethanol, soap, and dilute HCL. AH seemed to be acceptable. Coverglasses should also be cleaned. D. Squash preparation This section is for squash preparations only. If squash preparations are used for C-bands, the slides should be dipped into a solution of 0.1 % gelatin and 0.01 % chrome alum and dried prior to squashing. This thin coat prevents cellular loss during the treatments. However, if the HCL treatment is omitted, the slides should be treated with RNase (100 pg/ml. diluted in 2 x SSC) at 37°C for 1 hour using the moist chamber method. Rinse the slides for 5 minutes each in three changes of 2 x SSC, 70% ethanol, and 95% ethanol and air dry. Treat the slides with NaOH solution (0.07 N NaOH or 2 x SSC, pH 12). Try several treatment times, e.g., 1 minute, 2 minutes, 4 minutes, etc. if 0.07 //NaOH is used, rinse in 70% ethanol, 95% ethanol, dry,and incubate in 6 x SSC as usual. If 2 x SSC at pH 12 is used, rinse slides in three changes of 2 x SSC for 10 minutes each. Do not dry but drain and immediately place the slides at 65°C in a moist chamber, as suggested earlier. 5.2.5 G-Banding G-bands are the crossbands of various width and shades stained with Giemsa, Leishman's, Writht's, or similar stains. They usually correspond to the Q-bands but do not always correespond to the C-bands. In some cases, the C-bands and the G-bands may be opposite in staining behavior. For example, the C-band of human chromosome 9 is relatively unstained with G-band techniques.

80 The Y chromosome of man shows a distinct C-band in the distal portion of the long arm, but the same chromosome is somewhat variable in G-band staining, usually deeply stained throughout. Thus, G-banding does nor replace C-banding in assessing information. Fig. 5.6 Kayotype of human male showing C-banding pattern from a flame-dried preparation. Note the large amount of heterochromatin at the centromere areas of Al, C9, E16, and the distal portion of the Y. Variations in amounts exist in the two homologs of Al, Courtesy of Mrs.Ann Craig-Holmes and Dr. M. W. Shaw There were many papers published in 1971 and 1972, each proposing a certain procedure to reveal crossbands in mammalian chromosomes (Summer et al., 1971; Drets and Shaw, 1971; Patil et al, 1971; Schned, 1971a,b; Seabright, 1971; Wang and Fedoroff, 1972; Kato and Yosida, 1972; Utakoji, 1972). Since the results of the various procedures are similar to one another, it is unnecessary to present the procedures for all of them. The trypsin procedure described here more or 81 less follows the one devised by Seabright (1971) with recommendations for individual laboratory modifications. A. Reagents 1. Trypsin solution : Seabright uses Bacto trypsin (Difco Catalogue No. 0153) prepared by adding 10 ml of sterile distilled water or isotonic saline to each vial as the stock solution. This stock solution is diluted 1 : 10 with saline before use. It is really not necessary to use the particular brand of trypsin recommended by Seabright. Most laboratories carry monolayer cell cultures which require trypsin to dislodge the cells for harveest or for subculturing. Usually it is a crude trypsin solution (0.20—0.25% dissolved in a balanced salt solution without Ca 2+ and Mg 2+). In some laboratories, purified trypsin solution (0.01—-0.02%) is used. Whatever the kind and the concentration, the trypsin solution routinely used in the cell culture laboratory can be considered as the “stock solution”. The trypsin solution used for G-bands is prepared by diluting the stock trypsin solution with saline, balanced salt solution, or “rinsing solution” (balanced salt solution without Ca 2+ and Mg 2+). In our laboratory we use the rinsing solution. For laboratories using trypsin solution for G-banding only, it is advisable to dispense the stock trypsin solution in small containers and store them in a freezer. Keep only a small amount in the refrigerator for immediate use. 2. Rinsing solution : Physiological saline or balanced salt solution without Ca2~ and Mg2'. This solution is used diluent of the stock trypsin solution as well as for rinsing the slides after the trypsin treatment. 3. 95% Ethanol 4. Giemsa staining solution : See C-bands above. 5. Phosphate buffer : Like that of C-bans. B. General principles Many factors may influence the success of the G-band staining by trypsin treatment. It is, therefore, pointless to follow a set recipe without knowing these factors because the preparations may give excellent results if one knows how to modify the procedure. The success of G-banding depends primarily on the combination of the concentration of the trypsin solution and the duration of treatment, but the following factors dictate the correct combination : 1. The method for preparing the slides : The flame-dried preparations are more resistant to the trypsin treatment than air-dired preparations. 2. The age of the slides : The longer the slides are stored, the more resistant the cells are to the treatment. Cells of very old slides often give spotty, instead
82 of banded, chromosomes. Refixing of the slides in the Carnoy fixative sometimes helps. 3. Heating the slides : Air-dried slides be heated at 37°C for 1 hour (without covering). This procedure seems to give more consistent results. 4. The salt composition of the trypsin solution (including the diluent) : The presence of divalent cations in the solution slow the reaction but do not prevent it. 5. The temperature of the trypsin solution (the higher the temperature, the faster the reaction) : In laboratories with air conditioning, room temperature is suitable. The trypsin solution should be stabized at room temperature for approximately 30 minutes prior to use. For laboratories without room temperature control, it is probably a good practice to stabilize the trypsin solution at 4°C (refrigerator) or even in an ice bucket (Deaven and Petersen, 1973). 6. Trypsin concentration : We suggest a dilution of 1:5 or 1:10. If these are too strong, dilute further. If too weak (as in the case of flamelried preparations), the concentration may be raised to 1:2 or the undiluted trypsin used. 7. Time of treatment : The time of treatment of course depends on all the factors mentioned above. As a general principle, it should be adjusted to give good results in not more than 2 minutes but not less than 30 seconds. C. Procedure 1. Prepare trypsin solution in a Coplin jar. Using a Coplin jar is somewhat more convenient than flooding the slides with the solution but either way is acceptable. Use two or three slides and vary the duration of trypsin treatments as the initial monitor. Since for best results monitoring is necessary, it is advisable to prepare at least 10 to 12 slides of good quality. 2. Rinse with physiological saline or rinsing solution. Seabright suggested, at this stage, inspection of the wet slides by phase-contrast microscopy to determine the effect of trypsin treatment. The chromosomes should appear slightly swollen. The preparations can be treated again with trypsin if necessary. 3. Rinse with 95% ethanol and let dry. 4. Stain with diluted Giemsa (2% Giemsa solution in phosphate buffer) for 4— 10 minutes. Overstaining may obliterate some of the lighter bands. 5. The slides can be pulled out of Giemsa, rinsed quickly with deionized water, and air dried. It is not necessary to mount the slides.

83 Fig. 5.7 Metaphase plates of human cells showing results of various durations of trypsin treatment from extreme over treatment to proper timing, (a) Extreme overtreatment; (b) overtreatment, highly unsatisfactory; (c) slight overtreatment; chromosomes are fuzzy but discreate bands can be seen; (d) proper treatment Examine the stained preparations to determine the proper duration of the trypsin treatment. The chromosomes in undertreated preparations, in overtreated preparations, the chromosomes will show a series of appearances ranging from completely "ghost" chromosomes (Fig 5.7a) to those with poorly differentiated crossbands and fuzzy outlines (Fig. 5.7b). The appearance of these cells indicates that the treatment time or the concentration of trypsin solution should be reduced. Figure 5.7c shows a metaphase with reasonably good but slightly overtreated chromosomes, and Fig 5.7d shows proper G-bands. 6. Once the proper combination of trypsin concentration and the duration of treatment is determined by the preliminary monitor, treat the rest of the slides according to the best combination in the same day using the same solutions. The solutions in the Coplin jars should be discarded each day.

84 Fig. 5.8 Karyotype of human male showing G-banding pattern produced by the trypsin technique. Courtesy of Mrs. Marina Seabright 7. Figure 5.8 presents a male human karyotype showing the distribution of G-bands. D. Comments 1. If the trypsin procedure does not give satisfactory G-banding, it may be worthwhile to try the urea procedure of Kato and Yosida (1972). Prepare a stock urea solution (8 M aqueous) and mix 3 parts of this stock solution with 1 part of Sorensen's phosphate buffer (0.15 M, pH 6.8), heat to 37UC. Treat the slides at 37°C for 10 minutes. Rinse in tap water and stain directly without drying. For old slides, elevate the temperature to 60°C. 2. For combination staining, e.g., Q-banding and C-banding of the same cell, it is advisable to perform Q-banding first. The same slide can be then used for C-banding. It is recommend, however, separate runs for C-banding and G- banding. From each sample, one can always prepare a sufficient number of slides for slides for al treatments as well as some for storage.

85 5.3 Numerical and structural abnormalities of human chromosomes—syndromes. 5.3.1 Introduction Fifty years has elapsed since Tjio and Levan in 1956 established that human somatic cells have 46 chromosomes. There technique employing treatment of cells grown in culture with colchicine and hypotonic solution, was applied by numerous workers over the next half-dozen years, demonstrating the chromosomal findings in such disorders as trisomy 21, trisomy 13, trisomy 18, Turner's syndrome, and Klinefelter's syndrome. During this same period, the significance of the Barr body was realized, and lyonization of the X chromosome was hypothesized. The next few years saw the application of autoradiography for better identification of chromosomes, the delineation of more subtle chromosome disorders such as the cri-du-chat (5p—) syndrome, Wolf-Hirschhorn (4p—) syndrome, 18p—, 18q—, 13q—and a host of other anomalies. Amniocentesis was employed for prenatal diagnosis. Following 6 years have been marked by advances in our understanding of heterochromatin, chromosome banding, the identification of the Y body, and documentation of previously unrecognized alterations (small translocations, inapparent inversions etc.). From the first conference held in Denver in 1958 to the one, convened in Paris in 1971, chromosome nomenclature has developed parri passu with advances in our understanding of these disorders. Gross human chromosome abnormalities are not rare. Over 25% of human abortuses lost before the eighth week of pregnancy have abnormal karyotypes. Large surveys on newborns have consistently shown that about 0.5% exhibited aneuploidy (Sergovich et al., 1969; Lubs and Ruddle, 1970). 5.3.2 Autosomal abnormalities of human chromosome—syndromes A. 4p— Syndrome (Wolf-Hirschhorn Syndrome) The syndrome described independently by Wolf et al. (1965) and Hirschhorn et al. (1965), results from partial deletion of the short arm of one of the late replicating no. 4 chromosomes. It is much less common than the 5p— syndrome. About 25 cases have been described to date, all sporadic Translocation has not been demonstrated with the possible exception of the case described by Wilson et al. (1970). Parental age has been somewhat increased (Fryns et al., 1973). A 4r syndrome has been described (Carter et al. 1969). The disorder is characterized by severe psychomotor and growth

86 retardation. Birth weight is usually about 2000 gm in spite of normal gestation time. Fetal activity is diminished. Most infants are hypotonic. The skull is microcephalic and often there is cranial asymmetry. In a few cases, midline scalp defects have been noted (Hirschhorn et at., 1965; Wolf et al, 1965 Miller et al, 1970). Hemangioma on the brow is frequent. A prominent glabella and ocular hypertelorism are almost constant features. Divergent strabismus, antimongoloid obliquity of the palpebral fissures have been noted in about half the cases. Iris coloboma has been found occasionally. The ears have narrow external canals and are low set and simplified in from. The nose is misshapen or beaked with a broad base. The philtrum is short with a down-turned Cleft lip or, especially, cleft palate and micregnathia have been noted in most cases. Males comonly exhibt cryptorchidism and, especially, hypospadias. Absent uterus and streak gonad have been described. Congenital heart malformations, most often atria! or ventricular septal defects, have been noted in about 50% (Wolf et al., 1965, Arias et al., 1970, Guthrie et al., 1971) and may result in death during the first year. In several patients there has been dimpling of the skin over the sacrum and elsewhere, such as shoulders, elbows, or knuckles. The pelvic and carpal bones are late in ossification. Pseudoepiphyses are seen in the phalanges and at the base of each metacarpal. B. 5p—Syndrome (Cri-du-Chat Syndrome) Described initially by Lejeune et al. (1963), over 150 examples have been documented to date. The syndrome is present in about 1% of institutionalized individuals with intelligence quotients less than 35. The syndrome results from deletion of 35—55% of the short arm of one of the early replicatig B group chromosomes (German et al, 1964; Miller et al, 1969). Most decletion are thought to occur as a result of two breaks. If these occur in the short arm, an interstital deletion results. If deletion occurs in both arms, a ring chromosome is produced (Rohde and Tompkins. 1965). Maternal age is not elevated. About 70% of those identified at birth are females: however, most older patients have been male (Breg et al, 1970). The reason for this discrepancy is not evident. Mosaicism has also been described; patients having all the stigmata of the full-blown syndrome (Zellweger, 1966; Mennicken et al, 1968). About 10—15% result from translocation (Warburton and Miller, 1967). Pericentric inversion has also been described (Faed et al, 1972). As the name implies, the syndrome is characterized by a catlike, weak, shrill cry in infancy caused by hypoplasia of the larynx (Ward et al., 1968). However, the cry usually disappears with time, even within a few weeks of age

87 (Gordon and Cooke, 1968; Breg et al, 1970). The cry, almost one octave higher than normal, is quite monotone in quality (Schroeder et al, 1967). The infant face is characterized by microcephaly, round form, hypetelorism, antimonogoloid obliquity of. palpebral fissures, epicanthus, bilateral alternating strabismus, broad nasal bones, and low-set ears. Most patients have mild micrognathia. However, the rouness of the face and the ocular hypertelorism disappear with age. The face becomes thin and the philtrum short. Permature graying of the hair has been noted in about 30%. Dental malocclusion is common (Breg et al, 1970, Gordon and Cooke, Niebuhr, 1971). There is usually severe mental retardation (I. Less than 25), failure to thrive, and hypotonia in infancy. Birth weight is usually less than 2500 gm in spite of normal gestation time. Adult height usually ranges from 124 to 168 cm. (49 to 66 inches) Various musculoskeletal anomalies have included hypotonia, flat feet, mild scoliosis, large frontal sinuses, small ilia, syndactyly, and short metacarpals and metatarsals (Mennicken et al, 1968). Dermatoglyphic alterations include simian creases in about 35%. Eight or more whorls have been noted in about 30%. C. Group C deletion, trisomy, trisomy mosaicism, and partial trisomy There are seven pairs of C-group chromosomes and hence many possible types of trisomy, partial trisomy, or deletion involving this group. These states, with few exceptions, have not been clinically recognized and, thus, are probably lethal. The use of newer banding techniques on the chromosomes of abortuses may shed light on this question (Hirschhorn et al, 1973). There is a paucity of information concerning anomalies of chromosome no. 6 documented by banding, de Grouchy et al. (1968) described a child with bulbous nose, preauricular tubercle, hernias, hypospadias, undescended tested, deep acromial dimples, and psychomotor retardation. Deletion of the long arms of chromosome no, 7 was reported by Shokeir et al. (1973). The child exhibited psychic and somatic retardation, urinary malformations, flexion contractures at the elbows, and low finger ridge count. The most striking aspects of trisomy 8 or trisomy 8 mosaicism syndrome are mental retardation, abnormally shaped skull, reduced joint mobility, various vertebral anomalies, supernumerary ribs, strabismus, absent patellae, short neck, long slender trunk, cleft palate, and marked palmar and plantar creases (Oikawa et al., 1969; Lejeune et al, 1969; Emberger et al, 1970; Riccardi et al.) Deletion of the short arms of chromosome no. 9 was noted by Alfi et al. (1973) and probably by Kistenmacher and Punnett (1970). The patients had trigonocepha, mental retardation, ocular hypertelorism, anteverted nostrils, malformed pinnas. long philtrum, short neck, hypertonia, congenital heart disease,

88 and an incrreased number of digital whorls. Trisomy for the short arm of no. 9 was defined by Rethore et al., (1973) who reviewed earlier cases. Clinical features included mental retardation, microcephaly, enophthalmos, hypertelorism, mongoloid palpebral fissures, bulbous nose, abnormal pinna, hypoplasia of the phalanges, and abnormal finger creases. Partial trisomy for the long arm of chromosome no. 10 was reported by de Grouchy et al., (1972). Facial dysmorphia was evident with microcephaly, large forehead, flat round face, arched and wideset eyebrows. Antimongoloid palpebral fissures, microphthalmia, cleft palate, small nose with depressed bridge, malformed pinnas, short neck, micrognathia, various skeletal anomalies (osteoporosis, various rib abnormalities, scoliosis), congenital heart disease, and genitourinary defects. All patients had severe mental retardation. Partial trisomy for the short arm of chromosome no. 11 was described by Falk et al. (1973) and Sanchez et al. (1974). Findings common to both cases were mental retardation, marked frontal bossing, nystagmus, antimongoloid palpebral fissures, strabismus, broad fingers or toes, and cleft lip and/or palate. D. Trisomy 13 syndrome (Patau's syndrome, trisomy D 1) Trisomy 13 was first recognized by Patau et al. (1960), although Bartholin in 1657 may have given the first description of the clinical features (Warburg, 1960). The phenotype is so striking that diagnosis is usually made on clinical ground before the karyotype has been made. The incidence has been estimated to be about 1 per 6000 births (Conen and Erkrnan, 1966). Arhinencephaly, apneic spells, seizures, feeding difficulties, severe mental retardation, and deafness are common. Any of the holoprosencephalic states (cyclopia, ethmocephaly, cebocephaly, and premaxillary agenesis) may be associated with trisomy 13 (Conen et al., 1966; Fujmoto et al., 1973). Moderate microcephaly with sloping forehead and wide sagittal suture and fontanels have been noted in over 60%. Microphthalmia or iris coloboma with retinal dysplasia, ocular hypertelorism, and malformed pinnas occur in about 80% (Cogan and Kuwabara, 1964). Capillary hemangiomas in the glabellar region and localized scalp defects in the parieto-occipital area have been described in about 75%. Cleft lip and/or cleft palate and micrognathia have been noted in 60-70% (Conen et al., 1966; Taylor, 1968). Musculoskeletal abnormalities include postaxial polydactyly of the hands or feet with overlapping flexed fingers (about 75%) with hyper convex narrow fingernails. The calcaneus is often prominent and frequently there are rockerbottom feet.

89 At least 80% have congenital heart defects, genital anomalies include cryptorchidism (over 90%) in males and bicornuate uterus (about 50%) and hypoplastic ovaries in females. Polymorphonuclear neutrophils frequently (25—80%) have nuclear projections in cases of trisomy 13 owing to primary nondisjunction. Excellent ultrastructural study of the projections has been carried out (Waltzer et al, 1966, Lutzner and Hecht, 1966). Fetal hemoglobin, Hb-Gower and other hemoglobins have been elevated but there is good evidence that those changes disappear with age and merely represent general delayed maturity (Marden and Yunis, 1967). DNA replication studies have demonstrated that the D-group chromosome involved is number 13, which is the longest and the latest of the pairs to replicate (Yunis and Hook, 1966). 1.

Trisomy 13

Caused by Primary Nondisjunction About 75% of cases of 13 trisomy are caused by primary nondisjunction. There is no sex predilection. The mean age for mothers of infants with 13 trisomy caused by this type is elevated (32.4 years), far higher than for cases caused by translocation or mosaicism (Magenis et al., 1968; Taylor et al., 1970). There have been several examples of 13 trisomy occurring with other chromosomal abnormalities in the same sibship (Klinefelter's syndrome, Turner's syndrome, Down's syndrome and triploidy), but this may be chance association (Visfeldt, 1969). 2. Translocation D 1 About 20% of the cases of trisomy 13 are caused by translocation, far more common than occurs in Down's syndrome (Magenis et al., 1968; Taylor et al., 1970). In at least 85%, the translocation has occurred between two D chromosomes. Maternal age is not elevated (25.6 years). There appears to be definite male predilection. Fertility and intelligence in balanced carriers are quite variable (Wilson, 1971). 3. Mosaicism About 5% of the cases of Dj trisomy are caused by mosaicism. About half of these examples are caused by an extra chromosome 13 in proportion of the cells. The remainder result from a complex assortment of chromosomal abnormalities (Magenis et al., 1968; Taylor et al., 1970). As in translocation D 1 trisomy, the age of the mother of a D 1 trisomy mosaic is not elevated (25.4 years) in contrast to mothers of G 1 trisomy mosaics. The clinical stigmata, as expected, are less severe than in those of children with classic trisomy 13 (Bain et al., 1965).

90 4. Partial Trisomy Partial trisomy for the distal segment of the long arm of chromosome 13 was documented by banding techniques by Taysi et al. (1973) and by Escobar et al. (1974). The latter authors reviewed several case reports which had been documented prior to the advent of banding. Common clinical characteristics included psychomotor retardation, seizures, microcephaly, frontal bossing, open anterior fontanel, short neck, inguinal and umbilical hernias, polydactyly, rocker bottom feet, distal axial triradius, and elevated fetal hemoglobin. Life expectancy over a year was frequent. Absent were cleft lip and palate, sloping forehead, microphthalmia, and neutrophil drumsticks, common findings in trisomy 13. E. Dq—and Dr syndromes Over 60 case reports have been published in which the patient had deficiency of part of the long arm of a D-group chromosome (Dq—) or in which a D-group chromosome was replaced by a ring (Dr) (Lejeune et al., 1968; Gilgenkrantz et al., 1971; Niebuhr and Ottosen, 1973). Although these cases may represent a heterogeneity, there is good evidence to suggest that most involve No. 13 (wilson et al., 1973). Only a few examples of Dr have been described in which the chromosome has been identified as no. 15 (Jacobsen, 1966; Emberger et al., 1971). The phenotype in these cases was not striking : short stature, mental retardation, and microcephaly. Mean survival has been 39 months for Dq— cases and 89 months for Dr examples (Taylor, 1970). All patients have exhibited mental and somatic retardation and many have been hypotonic. Musculoskeletal abnormalities have included bilateral hip dislocation, focal lumbar vartebral agenesis, inguinal hernia, coxa valga, and synostosis of the fourth and fifth metacarpals. F. Trisomy 18 syndrome (Edwards syndrome) In 1960, Edwards et al. and, almost simultaneously, Patau et al. 1961) described a new syndrome associated with the presence of an extra chromosome in the E group which was subsequently shown to be a no. 18 chromosome (Yunis et al, 1964). The most constant features of this syndrome, noted in over 75% of the cases, include : deveelopmental retardation, failure to thrive, feeding difficulties, hypertonia, limited hip abduction, flexion deformities (usually ulnar deviation) of fingers, short sternum, congenital heart disease (ventricular septal defect— 90%, patent ductus arteriosus—70%, and atrial septal defect—20%), short
91 dorsiflexed halluces, rockerbottom feet, calcaneovalgus deformity of feet, and cryptorchidism (Weber and Sparkes, 1970). Craniofacial anomalies almost always present include prominent occiput, low-set malformed pinnas, and micrognathia. Severe anomalies found at autopsy, apart from the cardiac anomalies noted above include Meckel's diverticulum, heterotopic pancreatic tissue, thin diaphragm with eventration, and various renal anomalies. Dermatoglyphic alterations are frequent. Over 85% of finger prints are simple arches. Over 30% have a simian palmar crease and over 40% have a single flexion crease in the fifth finger. Trisomy 18 has an uncommon but yet definite association with aplasia of the radius and thrombocytopenia. 1. Trisommy 18 The incidence of trisomy 18 in trhe more recent surves has varied from 1 per 3500 to 1 per 7000 births (Taylor, 1968, Benady and Harris, 1969; Garfinkel and Porter, 1971). Mean maternal age is elevated, 32 years (Taylor, 1968). There is a 3 : 1 female predilection caused, in large part, by a greater male fatality rate during the first few weeks of life (Weber, 1967). The mother often exhibits small weight gain during pregnancy and indicates that fetal movements were feeble. Most examples are postmature. Mean birth witht is less than 2300 gm. The placenta is often small with umbilical artery, and hydramnios has been noted in over 50%. Thrity percent fail to survive more than 1 month, 50% succemb by 2 months, and less than 10% live more than 1 year. Mean survival time is about 70 days (females—134 days, males—15 days). 2. Double Trisomies Double primary nondisjunction has been observed in 5—10% of cases (Hamerton, 1971). Mean survival time for douuble trisomies has been 3 weeks. Maternal age is markedly increased in this group. 3. Trisomy 18 Caused by Translocation Translocation is usually sporadic but examples of familial trnslocation have been recorded (Hamerton, 1971). Means maternal age is lower than for those with trisomy 18 caused by nndisjunction. G. 18p—syndrome Deletion of the short arms of chromosome 18 is associated with a variable phenotype. Maternal age is elevated. There is a 2 : 1 female sex predilection (Parker et al, 1973).

92 Mental retardation is a constant feature but of variable degree. Birth weight is low and somatic growth retarded. There is no characteristic facial dysmorphia. Frequently, however, hypertelorism, epicanthal folds, strabismus, and ptosis of lids are noted. The ears are low-set, large, floppy, and poorly formed. H. Trisomy 21 (Down's syndrome) Langdon Down (1866) first extensively described the syndrome which has received his name, calling it "Mongoloid idiocy" or "Mongolism." In 1959. Lejeune demonstrated that the disorder was associated with an extra chromosome in the G group. In 1960, Polani et al. described translocation Down's syndrome, and in 1961, clarke et al discovered rnosaicism for an extra G-group chromosome. Yunis et al (1965), by means of autoradiography, identified the chromosome as one of the no. 22 chromosomes, although by this time the term trisomy 21 had been so extensively employed that it has remained. The incidence of trisomy 21 is between 1 and 2 per 1000 live births among various populations (Mikkelsen, 1971). Over 95% of the cases are caused by nondisjunction, the remainder resulting from translocation. The skull is brachycephalic with shortening of the anteroposterior diameter and flattening of the occiput in about 75%. The cephalic index (normally 0.75— 0.80) is usually greater than 0.80 and may exceed 1.00. (Roche et al., 1961). In infants with trisomy 21, the fontanels are larger than normal and closure is late. In those over 10 years of age, a patent metopic suture is found in 65% of males (normal—9%) and in 40% of females (normal—12%). An extremely common feature (over—90%) is absence of frontal and spehenoid sinuses and hypoplasia of the maxillary sinuses (Spitzer et al., 1961, Betlejewski et al. 1964). There is poor development of the bones of the middle face, producing a relative prognathism and ocular hypotelorism (Gerald and Silverman, 1965). The profile is flattened owing to hypoplasia of the nasal bones. The palpebral fissures are oblique, the outer canthus being slightly higher than the inner. Epicanthal folds are extremely common. Speckled iris (Brushfield's spots) and lens opacity are present in about 85% and 60% of patients, respectively. Various other anomalies include missing or malformed teeth (especially maxillary lateral incisors and mandibular second premolars), delayed eruption, increased periodontal destruction, and malocclusion (Cohen and Cohen, 1971). The hands are characteristically short and broad, the fifth finger usualy being abbreviated and clinodactylous, and having a single flexion crease in about 20% of the cases. There is usually greater space than normal between the hallux and the rest of the toes. Hypotonia, especially marked in infancy, improves with age. loints are usually hyperextensible. The penis and scrotum are usually small and about

93 25% have cryptorchidism Public hair is straight. Congenital cardiac anomalies is present in about 40% Down Syndromic persons. Diastasis recti, duodenal atresia, or umbilical hernia occur in about 10% (Butterworth et al., 1964). Radiographic changes include reduced iliac and acetabular angles in the young infant (Nicolis and Sacchetti, 1963) and hypoplastic middle phalanx of the fifth finger. Intelligence quotients range from 25 to 70, most Down's syndrome patients 3 years of age or less having I.Q. s of 50—59 but slipping with increasing age to 25-49 (Penrose and Smith, 1966). Dermatoglyphic anomalies include distal axial triradius in the palm (over 80%), bilateral simian creases (30%), single flexion crease in fifth finger (20%), 10 ulnar loops (30%), and hallucal arch tibial (70%) or small loop distal (30%) patterns (Preus and Fraser, 1972). Because of susceptibility to respiratory infection, early mortality used to be great. With the introduction of antibiotics, the means survival age is almost 20 years. There is a twentyfold increased association with acute leukemia (Conand Erkman. 1966). Numerous attempts have been made to established specific biochemical alterations. However et aL, (1965) found decreased blood serotonin and increased galactose phosphate uridyltransferance leukocyte alkaline with Down's syndrome. 1. Trisomy G 1 due to Primary Nondisjunction As discussed above, about 95% of cases of Down's syndrome are sporadic primary trisomics, resulting from nondisjunction which is age dependent. This occurs at the first meiotic division in the mother (Robinson, 1973). If the mother is less than 20 years of age at time of conception, the risk of producing a child with trisomy 21 is about 1 per 2500 live births. This risk gradually increases until 35 years, after which there is a more marked increase in frequency such that a mother over 45 years has about 1 chance in 50 or less of having a child with Down's syndrome. 2. Association of Down's Syndrome with Other Primaiy Nondisjunctions Individuals with trisomy 21 have been occasionally (about 1 per 200) found to have another extra chromosome (double primary nondisjunction), the most frequent type being 48,XXY, G+ (Hamerton et al, 1965; Taylor and Moores, 1967). Other forms such as 48,XXX,G+ and 48,XYY,G+ have also been described (Yunis et al, 1964, Uchida et al., 1966). This association is much higher than might be expected by chance.

94 3. Translocation Down's Syndrome Down's syndrome patients born to young mothers as well as those with affected relatives often have the extra G] chromosome attached to another chromosome. This has been designated translocation and comprises about 3.5% of cases of Down's syndrome. It may be sporadic or familial. Translocation Down's syndrome is not age dependent. About 8% of Down's syndrome patients born to mothers less than 30 years of age have exhibited translocations as opposed to 1.5% born to mothers over 30 years old. It is widely accepted that the short arms of acrocentric chromosomes have nucleolar organizers and that these points are likely to break, producing a high frequency of structural chromosome aberrations. In familial translocation Down's syndrome, one of the parents has 45 chromosomes instead of the normal 46. One of the small G-group chromosomes is "missing" since it has been translocated to another chromosome. The parent carrying the translocation chromosome is phenotypically normal, since no significant amount of genetic material has been lost in the translocation process. In most cases, the extra G(chromosome is phenotypically normal, since no significant amount of genetic material has been lost in the translocation process. 4. Down's Syndrome Mosaicism Patients having two different cell populations, one trisomic for chromosome G; and another normal, constitute about 2—3% of patients with Down's syndrome. This condition is usually suspected when the phenotypic expression of trisomy 21 is not fully expressed or when the intelligence of the patient is higher than expected. In addition, they may have children with Down's syndrome (Weinstcin and Warkany, 1963). Individuals having trisomy Gt mosaician may vary in phenotype from typical trisomy 21 to normal. There is no age dependency (Richards, 1969). One cannot correlate the percentage of trisomic blood cells with intelligence. Richards (1969) found about 20% more trisomic cells in fibroblasts than in lymphocytes. If mosaicism is found in one of the parents of a child with Down's syndrome, meiotic study of ovary or testis should be carried out. There is evidence that if half the cells are abnormal, about 25% of the childern will have Down's syndrome (Mikkelsn,1971a). I. Nonmongoloid "Trisomy G" Several cases of nonmongoloid "trisomy G" have been published (Uchida et at, 1968; Al-Aish, 1969; Lozzio, 1969; mikkelsen, 1969). Some have been designated as having trisomy 22 to contrast with trisomy 21 (Down's syndrome). At this point in time, within this group, with two possible exceptions cited

95 below, there seems to be no characteristic phenotype and it would appear likely that some of these represent centric fragments that may come from several different chromosomes. J. G deletion syndromes There are at least two relatively distinct phenotypes presumably representing monosomy or deletion of a portion of the long arm of two different G-group chromosomes (Warren et al., 1973). 1. The G 1 Deletion Syndrome (Antimongolism) This syndrome consists of mental and growth retardation, hypertonia, nail anomalies, skeletal malformations, cryptorchidism, hypospadias, inguinal hernia, pyloric stenosis, thrombocytopenia, eosinophilia, and hypogammaglobulinemia. Facial and oral manifestations include microcephaly, large low-set ears, antimongoloid obliquity of palpebral fissures, highly arched or cleft, and micrognathia. Dermatoglyphic analysis has shown a marked increase in radial loops (Schindeler and Warren, 1973). 2. The G 1 Deletion Syndrome This syndrome has less distinctive features: severe mental retardation, hypotonia, soft tissue syndactyly of the second and third toes, and clinodactyly of the fifth finger. Facial and oral manifestation include large, low-set ears, epicanthal folds, ptosis of eyelids, highly arched palate, and bifid uvula 1971; Stoll et al., 1973). Dermatoglyphc analysis has shown a marked increase in whorls, a decrease in ulnar and radial loops, a distal axial triradius, and hypothenar patterns (Schindeler and Warren, 1973). K. Triploidy Triploidy, as noted later in this chapter, is a frequent cause of fetal wastage prior to the eighth intrauterine week. Diploid/triploid mosaicism is occasionally compatible with survival and there have even been several examples of pure triploidy. 5.3.3 Sex chromosomal abnormalities A. Klinefelter syndrome In 1942, Klinefelter et al., described a syndrome in post pubertal males consisisting of small firm testes with tubulan by a linization but with a normal number of Leydigs cells, aerosposrmia, gynecomaztia, elevated urinary gonadotropies and low concentrations of urinary 17-ketosteroids. Several years later, Bradleury etal. (1956) and plunkeft and Barr (1956) noted Chromotin-positive nuclei in the tissues of such patients, and Jacob and strong (1959) described an

96 XXY sex chromosome complement in chromotin-positive klinefelters syndrome. Chromatin-positve males have been found to comprise about 2 per 1000 live male births. These also contains XXY, XXYY, XY/XXY, and other rarer forms of Klinefelter syndrome. It has been estimated that about 80% are XXY, 10% are mosaics, and the rest are XXYY and the more unusual types. 1. XXY Klinefelter syndrome The clinical features of XXY klinefelter syndrome do not become apparent until after puberty. Body proportions usually do not appear remarkably abnormal, however, the lower extremities tend to be and about 60% have a span that exceeds their height by 3 cm or more. The Prepubertal testes are normal size and microscopic appearance but during adolescence they fail to enlarge and remain small and firm, averaging less than 2cm in length. The seminiferous tubules are usually shrinken, hyalinized, and irregularly arranged. Elastic fibres are absent around the tunica propria of the tubules. Ledig cells are clumped, Rarely spermatogesesis can be demostrated. In nearly all cases the testes descend. The penis is usually of normal size but may be somewhat shorter than normal. The prostate is smather than normal. Gynecomastia develops after puberty in about 50% and facial hair is sparse in about 60-75%. Axillary hair may also be less. The prepubertal testes are of normal size and microscopic appearance but during adolescence they fail to enlarge and remain small and firm, averaging less than 2 cm in length. The seminiferous tubules are usually shrunken, hyalinized, and irregularly arranged. Those tubules which are not sclerotic are immature and lined exclusively with Sertoli cells. About 50% have a female pubic pattern. Libido and potency are usually decreased. There is some evidence of increased tendency to pulmonary disorders, varicose veins, and, possibly, breast cancer. There is the same frequency of color blindness among XXY patients as in normal females. Although intelligence may be reduced, at least 75% of XXY males have normal intelligence. Personality is usually passive. The incidence of XXY Klinefelter's syndrome is about 1.3 per 1000 live male births. Meternal age is significantly increased for XXY but not for XXYY,XXXY, or XXXXY patients. About 60% of XXY males are X M X M Y, while 40% are X M X P Y. The X M X M Y state arises from nondisjunction either during oogenesis or at an early postzygotie division. The X M X P Y condition probably has its origin in nondisjunction during the first meiotic division. 2. XXYY Klinefelter syndrome Patients with the XXYY variant tend to be about 4 cm taller than average height, more aggressive, and more mentally retarded than those with XXY Klinefelter's syndrome (Borgaonkar et al, 1970). Otherwise the phenotype is quite
97 similar: small firm testes, eunuchoid body build, sparse, body hair, gynecomastia, and elevated gonadotropins. Almost all XXYY males described to date have been mentally retarded and many have been aggressive (Schlegel et al., 1965). As mentioned above, there is no increase in parental age in contrast to XXY Klinefelter's syndrome. The disorder is most likely due to nondisjunction in both the first and second meiotie divisions during spermatogenesis with production of an XYY sperm. One cannot,however, rule out the less likely possibility of nondisjunction at the second meiotie division in both parents. Dermatoglyphic studies have shown that digital arch patterns are more common in the XXYY patient than in the XXY individual who, in turn, has more than the normal male. A child with an XXYYY sex chromosome complement was noted to have mental retardation, lordosis, flexed index and fifth fingers, pes planus, and aggressive personality (Gracey and Fitzgerald, 1967). 3. XXXY Klinefelter's syndrome Over 25 cases of XXXY Klinefelter's syndromoe have been published (Vormittag and Weninger, 1972). All have been mentally retarded. The phenotype is similar to that of the XXY male but the size of the penis is small (McGann et al., 1970). Two late-labeling X chromosomes have been demmonstrated. However, two Barr bodies are seen in only a proportion of cells (Vormittag and Weninger, 1972). The condition may arise from successive nondisjunction in either the maternal or paternal meiotie divisions (Pfeiffer and Sanger, 1973). Dermatoglyphic findings have not been consistent. An XXXYY male has been described by Bray and Josephine (1963). 4. XXXXY Klinefelter's syndrome There have been over 70 cases of 49,XXXXY males published since Fraccaro and Lindsten documented the first example in 1960. Nearly all have been severely mentally retarded intelligence quotients ranging from 20 to 60. A marked difference between the XXY and XXXXY male is thepoordevelopment of the external genitalia in the latter. The penis is always minute and the testes very small and undersended with hypoplastic Leydig cells and absence of term cells. The scrotum is. usually hypoplastic. Mild nicrocephaly, ocular hypertelorism (90%), myopia (25%), strabismus (50%), mild nongoloid obliquity of palpebral fisures (35%), epicanthus (80%, and short neck with redundant skin on the nape have been noted. Skeletal anomalies present in over half the cases. Congenital heart disorders have been noted in about 20% of cases. Gonadotropins have not been elevated. Autoradiographic evidence has shown three heavily labeled X chromosomes

98 (Hsu and Lockhart, 1965). Three Barr bodies may be found in a proportion of interphase nuclei (Miller and Warburton, 1968). Parental age is not elevanted. Postzygotic nondisjunction in an XXY zygote appears to be the cause for the XXXXY state, all the X chromosomes coming from the mother (Murken and Scholz, 1967; Race and Sanger, 1969). 5. XX Klinefelter syndrome Less than 30 cases have been published of males having 46,XX karyotypes. They exhibit many of the stigmata of Klinefelter's syndrome and, sence, will be considered here (Anderspn et al., 1972). All have small testes, are infertile, and rarely shave. About 70% have gynecomastia and elevated gonadotropin levels. Plasma testosterone levels are very low (Neuwirth et al., 1972). The penis and scrotum have been small in about half the cases. All are of normal intelligence and have normal skeletal proportions. 6. Klinefelter's syndrome Mosaicism About 15% of patients with Klinetelter's syndrome have been found to have two or more chromosomally distinct cell populations. In each of these individuals, one of the cell populatins generaly has an XXY, XXXY, or XXXXY sex chromosome constitution while the other is XX or XY. The clinical expression of mosaicism for Klinefelter's syndrome depends on the type of sex consitution present at a critical time of development. Thus, one can find, for example, and XY/XXY mosaic who is phenotypically normal, provided the XY cells exerted the predominant genetic effect. In a study of XY/XXY mosaics, Gordon et al. (1972) found that only one- half exhibited azospermia and about one-third had gynecomastia and elevated gonadotropins. About one-quarter had germinal epithelium. Among 6 patients with XX/XXY mosaicism, Ferguson-Smith (1969) noted comparable findings. B. XYY syndrome Although the presence of an extra Y chromosome had been described as early as 1961 (Sandberg et al., 1961), interest was markedly aroused by a finding of a disproportionately high percent (usually 2—4%) of such individuals in prisons and mental hospitals (Casey et al, 1968; Jacobs et al., 1968; Marinello et al., 1969; Hook 1973). It was soon noted that most XYY patients are excessively tall and not uncommonly midly mentaly retarded (mean intelligence quotient —90) (Valentine et al., 1971). However, the frequencey of the condition among newborn male infants is about 1 per 700 births (Ratcliffe et al., 1970) and few of these individuals lead other than quite routine lives. The adult height of an XYY individual is usually over 180 cm while XYY children are usually above the 90th
99 percentile in height by 6 years of age. Leg length and trunk length are increased but the leg/trunk ratio is normal (Keutel and Dauner, 1969). Muscle weakness (especially of the pectoralis major) and poor coordination are commonly noted. Phenotypical alterations are subtle: mild facial asymmetry, mild pectus excavatum, and mild scapular winging. The ears tend to be long and often there is a bony chin point. Most have exhibited normal sexual development (Court Brown, 1969). There are no characteristic dermatoglyphic aherations (Hubbell et al, 1973). The disorder probably arises from paternal nondisjunction during the second meiosis. Retarded intelligence (I.Q.—70), impulsive aggressive behaviour, bilateral simian creases, clinodactyly of fifth fingers, retarded bone age with pseudoepiphyses at the bases of the metacarpals and metatarsals, and lack of patellar epiphyseal calcification were described by Schoepflin and Centerwall (1972). Ridler et al. (1973) noted low normal intelligence, behavior problems with aggressive outbursts, repeated pulmonary infections, hypotrophic testes, sparse body hair, and acne in a 48,XYYY patient. Conversely, Hunter and Quaife (1973) decribed no stigmate other than sterility. C. Turner's syndrome In 1938, Turner described a syndrome in postpubertal female^consisting of sexual infantilism, short stature, webbed neck, and cubitus valgus. Albright et al. (1942) showed that these patients had an elevated urinary excretion of gonadotropins, and Wilkins and Fleischmann (1944) described "streak" gonads devoid of ovarian folicles in such cases. Polani et al., (1954) and Wilkins et al., (1954) demostrated that most cases are chromatin-negative, and Ford et al. (1959) first described the XO karyotype. Turner's syndrome has been estimated to occur 1 per 2500 female births (Maclean et al., 1964; Mikamo, 1968) and has been frequently noted in abortuses. Parental age is not increased. Variation in phenotype has led to some confusion concerning nomenclature. Since the most common features are short stature, streak gonads, and X monosomy or short arm loss of X chromosomal material, all patients with these features are classified here as examples of Turner's syndrome (Yunis, 1965). Deletion of the long arm of the Y chromosome has occasionally been associated with the Turner syndrome. Cases with streak gonads and sexual infantilism but of normal or increased suture and normal female or male sex chromosome complement will be referred to as having "pure gonadal dysgenesis" or, more accurately, XY or XX gonadal dysgenesis. Primary amenorrhea and sterility are almost constant features of the XO Turner syndrome although exceptions have been noted. Breast development is

100 poor, the chest is broad with seemingly widely spaced, hypoplastic, at times, inverted nipples. The external genitalia are infantile and public hair is sparse. The histological pattern of the dysgenetic gonad found in Turner's syndrome consists of long streaks of white wavy connective tissue stroma without follicles. Follicles are present, however, in fetal and infantile ovaries of patients with Turner's syndrome (Gordon and O'Neill, 1969). Adult height is usually less than 57 inches (144 cm). Various skeletal anomalies include cubitus valgus (about 75%), short fourth metacarpals (about 65%), deformity of medial fibial condyle (about 40%) osteoporosis (about 50%), hypoplasia of first cervical vertebra (about 40%), and small carpal angle. Birth weight is below the 3rd percentile in about half the cases. In infants, excess skin on the nape and peripheral lymphedema have been noted in 15— 50% of the cases. During embryonic life, neck blebs or cystic hygroma are common (Singh and Carr, 1966; Rushton et al., 1969). Toenails are frequently hypoblastic. With age, the excess skin on the nape metamorphoses into pterygium colli and, with improvement in deep lymphatic circulation, the peripheral lymphedema gradualy disappears. Increased numbers of cutaneous nevi are found in about 60%. Epicanthal folds, ptosis of upper eyelids, prominent ears, and micrognathia are common facial features. The hairline is low at the nape. Thyroid antibodies are elevated in XO Turner's syndrome but less frequently than in the X-iso X mosaic and glucose intolerance occurs with grater frequency in patients with Turner's syndrome and in their parents than in the normal population (Rimoin, 1973). 1. X Monosomy Patients with an XO sex complement appear to comprise about 60% of the cases of Turner's syndrome. Furthermore, they appear to be more severely affected clinically than other forms of the disorder. 2. XO/XX and XO/XXX Mosaicism Patients with Turner's syndrome may have two different cell populations, one having an XO sex constitution, the other a normal XX sex complement. Such individuals are called XO/XX mosaics and constitute about 7% of the cases of Turner's syndrome. The two cell population types may appear in every tissue of the body or only in certain ones. Presumptive evidence for mosaicism lies in a discrepancy between sex chromatin pattern and karyotype, or through observing a low percentage of chromatin-positive nuclei (5—15%) in phenotypic females. The clinical spectrum of XO/XX mosaicism is wide and may vary from cases quite typical of Turner'
101 syndrome with many associated anomalies to cases with normal gonads and normal stature. About 20% menstruate (Ferguson-Smith, 1969). In contrast to patients with XO Turner's syndrome who are prone to aortic coarctation, those with XO/XX karyotypes are likely to have pulmonic stenosis with or without atrial spetal defect (Nora et al., 1970), being similar to patients with Noonan's syndrome. The usually accepted explanation for XO/XX mosaicism is loss of an X chromosome during cleavage in the early embryo. About 5% of the cases of Turner's syndrome are XO/XXX mosaics. Clinically they resemble the XO/XX mosaic. Patients having three stem lines XO/XX/XXX have been reported but are quite similar phenotypically to XO/XX mosaics. 3. Isochromosome X (XXqi) About 20% of patients having Turner's syndrome have an X isochromosome, i.e., replication of the long arm of the late replicating X chromosome. They exhibit short stature, sexual infantilism, primary amenorrhea, and skeletal anomalies. The Barr body and polymorphonuclear neutrophils drumsticks are larger than normal. Drumsticks are also more nuemerous (Taft et al., 1965; Sparkes and Motulsky, 1967). 4. Short and Long Arm Deletion of an X chromosome Deletion of the short arm of an X chromosome (XXp—) results in the Turner phenotype. They are as short as individuals with the XO Turner syndrome but are less likely to have associated malformations (Atkins et al., 1965). Deletion of the long arm of an X chromosome (XXq—) is far less likely to be asociated with short stature. The girl was 159 cm tall and exhibited no stigmata of Turner's syndrome. She never menstruated and her ovaries were not palpable. As expected, her Barr bodies were smaller than normal. Xg1 studies showed that the Xpi was of maternal origin. However, Turner's syndrome has been reported in association with XXq—. 5. Y deletion At least a dozen cases of Turner's syndrome associated with a dicentric Y Chromosome have been published (Armendares et al., 1972; Cohen et al., 1973). All have short stature, female phenotype, and most have associated anomalies. A patient with long arm isochrorriosome Y had drumstiicks have been noted in polymorphonuclear leukocytes.

102 5.4 Human genome 5.4.1 The Human Genome Project As the recombinant DNA, gene cloning and DNA sequencing technologies improved in the 1970s and early 1980s, scientists began discussing the possibility of sequencing all 3 x 109 nueleotide pairs in the human genome. These discussions led to the lauching of the Human Genome Project in 1990. The initial goals of the Human genome project was to construct a detailed physical map of the entire human genome, and to determine the nueleotide sequences of all 24 human chromosomes by the year 2005. Scientists soon realized that this huge undertaking should be a worldwide effort. Therefore, an international Human Genome Organization (HUGO) was organized to coordinate the efforts of human geneticists around the world. James Watson, who with Francis Crick, discovered the double-helix structure of DNA, was the first director of this ambitious project, which was expected to take nearly two decades to completle and to cost in excess of $3 billion. In 1993. Francis Collins, led the research teams that identified the cystic fibrosis gene, replaced Watson as director of the Human Genome Project. In addition to work on the human genome, the Human Genome Project has served as an umbrella for similar mapping and sequencing projects on the genomes of several other organisms, including the bacterium E. coli, the yeast S. cerevistae, the fruit fly D. melanogaster, the plant A. thaliana, and the worm C. elegans. 5.4.2 Bacterial Genomes In 1995, Haemophilus influerzae was the first bacterium to have its genome sequenced in its entirety. By mid-2001, the complete sequences of 32 bacterial genomes were abailable in the public databases (collections of the sequences of genes, chromosomes and genomes). The genomes range in size from 580,070 bp for Mycoplasma genitalium, which is thought to have the smallest geneme of any self-replicating organism, to 4,411,529 bp for Mycabacterum tuberculosis, which causes more human deaths than any other infections bacterium, to 4,539,221 bp for Escherichia coli, the best-known cellulalr microorganism. The genome of M. genitalium is of special interest because it may aproximate the “minimal gene set” for a self-replicating organism—the smallest set of genes that will alow an organism to reproduce itself. Of course, the genome of M. tuberculosis is of great interest becauseof the pathogenicity of this organism and the hope that a complete understanding of its metabolism will suggest ways to prevent tuberculosis in humans: The need for new ways to combat this pathogen has been enhanced by the recent evolution of antibiotic-resistant strains of M. tuberculosis. Of the bacterial genomes sequenced to date, the genome of E. coli (Fig. 5.9)
103 has undoubtedly caused the most excitement among geneticists. E. coli is the most planet.Geneticists, biochemists, and molecular biologists have utilized E. coli as the prefered model organism for decades. Most of what is known about bacterial genetics was learned from research on E. coli. Fig. 5.9 Sequence-based map of the chromosome of Escherichia coll. The blue arrows mark the halves of the chromosome travered by the two replication forks. The outer concentric circle gives the position of genes encoding proteins similar to bacteriophagc proteins. The second concentration circle shows the location of genes that are transcribed clock-wise (gold) from one strand or counterclockwise (yellow) from the complementary strand. The sunburst in the center is a histogram in which the length of each ray is proportional to the randomness of codon usage within each coding sequence The E. coli genome contains 4288 putative proteincoding sequences or genes. About one-third of these are well-studied genes encoding known products, whereas 38 percent are of unknown function, Putative protein-coding sequences that are not knwn to encode proteins are called open reading frames or ORFs. An ORF is a nucleotide sequence that begins with a translation-initiation signal (usually ATG). continues with a sequenced of base triplets specifying amino acids, and ends with one of the three translation-termination singnals. The average distance between genes (size of intergenic regions) in the E. coli genome is 118 bp. Known and putative genes specifying proteins and stable RNAs make up 87.8 percent and 0.8 percent of the genome,respectively, and noncoding repetitive elements account for 0.7 percent of the genome. Thus, 10.7 percent of the genome must involve regulatory sequences and sequences with unknown functions.

104 Once the complete sequence of a bacterial genome is available, it can be searched using computers for similarities with other sequenced genomes. Such sequence comparisons can often be used to gain inferences about gene function. Because so much is known about gene function in E. coli, comparisons with other secuenced bacterial genomes are often very informative. For example, a comparison of the genomes of Treponema pallidum, the parasitic spirochete that causes syphilis, and E. coli shows that T. pallidum contains the genes that encode proteins involved in DNA replicartion and repair, transcription, and translation, but carries few genes encoding biosynthetic enzymes and trtanspoort protiens. Fig. 5,10 Sequence-based map of chromosome 4 of Drosophila. The top line (A) gives map position in megabase paris (1 mb = 1 million hase pairs). The second panel (B) gives the polytene chromosome band number. The third panel (C) shows the percetage of G:C base pairs, and the fourth (D) shows the positions of transposable genetic elements. The bottom two panels show the positions of genes where transcription occurs with the plus strad as template (F) or with the minus strand as template (F). The color of each gene box in panels F and F indicates its similarity to genes of mammals, C. elegans, or S. cerevisiae, as shown by the key below the map, and the height of each gene box in panels E and F indicates the frequency of the sequence in the expressed-sequenced tag (EST) database for Drosophila Transposable genetic elements make up about 10 percent of the Arabidopsis genome, far less than the 50 to 80 percent of the corn genome estimated to be derived from transposable elements. The next challenge is to determine the functions of the Arabidopsis gene products. There is where this little weed really shines as a model system. It is idealy suited for genetic dissections of biological processes. The goal or the Arabidopsis research community is to determine the function of all 25,498 genes in the nect 10 years.

105 5.4.3 The Human Genome Recall that the initial goals of the Human Genome project were (10 to map all the human genes, to construct detailed physical maps of all 24 chromosomes, and to sequence the entire genome by 2005. All of these goals are likely to be achieved ahead of schedule. With two first-draft sequences of the human genome already published in February 2001 a complete sequence of the euchromatic portion of the human genome will certainly be available long before 2005. Fig. 5.11 Sequence-based map of chromosome 1 of Arabidopsis. Distance in 100-kb units is shown at the top. The chromosome is represented by the top bar, with sequenced regionsin red and the unsequenced centromere and telomerees in blue. The densities of genes (top panel below chromosome), matches to expressed sequence tags (ESTs; second panel), transposable small nuclear RNAs (bottom panel) are color coded, with red representing the highest density and dark blue the lowest density Progress in mapping the human genome has been excellent. Complete physical maps of chromosomes Y and 21 and detailed RFLP maps of the X chromosome and al 22 autosomes were published in 1992. By 1995, the genetic map contained markers separated by, on average, 200 kb. A detailed micro satellite map of the human genome was published in 1996, and a comprehensive map of 16,354 distinct loci was released in 1997. All of these maps have proven invaluable to researchers cloning genes based on their locations in the genome. Unfortunately, the resolution of genetic mapping in humans is quite low— in the range of 1-10 mb. The resolution of fluorescent in situ hybridization (FISH) is also approximately 1 mb. Higher resolution mapping (down to 50 kb) can be achieved by radiation hybrid maping, which is a modification of the somatic- cell hybridization mapping procedure. Standard somatic-cell hybridization involves the fusion of human cellls and rodent cells growing in culture and the correction of human gene products with human chromosomes retained in the hybrid cells.

106 Fig. 5.12 The use of radiation somatic-cell hybrids for high-resolution mapping of the human genome. The rationale behind radiation hybrid maps in that the probability of an X ray-induced break between genes A and B is directly proportional to the distance between them on the DNA molecule. Note that genes A and B have remained together in hybrid clones 1, 2, and but were separated by an X ray- enduced break during the formation of hybrid clone 3 Radiation hybrid mapping is done by fragmenting the prior to cell usion (Fig. 5.12). The irradiated human eels are then fused with Chinese hamster (or other rodent) cells growing in culture,usually in the presence of a chemical such as polyethylene glycol to increase the efficiency of cell fusion. The human— Chinese hamster somatic-cell hybrids are then identified by growth in an appropriate selection medium.

107 Many of the human chromosome Chromosome l fragments become integrated into the Chinese hamster chromosomes during this process and are transmited to progeny cells just like the normal genes in the Chinese hamster chromosomes The polymerase chain reaction is the used to screen a large panesl of the seleted hybrid cells for the presence of human genetic markers. Chromosome maps are constructed based on the assumption that the probability of an X ray-induced break between two markers is directly proportional to the distances separating them in chromosomal DNA. Several groups have used the radiation hybrid mapping procedure to construct high-density maps of the human genome. In 1997, Elizabeth Stewart and coworkers published a map of 10,478 STSs based on radiation hybrid mapping; their map of human chromosome 1 is shown in Fig. 5.13. Whereas the gene maping work advanced quickly, progress towards sequencing the human genome initially lagged behind schedule. However, that all changed rapidly beginning inl998. During May of 1998, J. Craig Venter announced that he had formed a private company, Celera Genomics, with the goal or sequencing the human genome in just three Fig. 5.13 A high-resolution radiat on hybrid map of human chromosome 1. The cytogenetic map of chromosome is shown on the locations of the comprehensive radiatipon hybrid map showing all the markers (red lines), the high confidence radiation hybrid markers (blue lines), the high confidence radiation hybrid markers (blue lines), the highconfidence radiation hybrid markers (blue lines), the high confidence radiation hybrid markers (blue lines), the RFLP markers (green lines), and the ESTs (parple lines)

108 Fig. 5.14 A nnortated, sequence- based map of an 8 mb segment of DNA at the tip of human chromosome 1, assembled by rescarel ers at Celera Genomics. The top line gives distances in mb. The next three panels show predicted transcripts from one strand of DNA (the “forward strand”), whereas the bottom three panels show predicted transcripts specified by the other strand of DNA (the “reverse strand”). The middle three panels give the G:C content, the positions of CpG islands, which occur upstream of genes, and the density of single nucleotide polymorphisms (SNPs), respectively. The annotation key below the map of chromosome 1 shows the components of the map, the color code for gene product functions, and the color codes for G:C content and SNP density years. Shortly thereafter, the leaders of the public Human Genome Propject’s sequencing laboratories announced that they had revised their schedule and planned to complete the sequence of the human genome by 2003—two years earlier than originally poroposed. From this point in time, everything accelelrated.

109 The complete sequence of the first human chromosome—small chromosome 22—was published in December 1999. The complete sequence of human chromosome 21 followed in May of 2000. Then, with the interventionof the White house, Venter, of Celera Genomics, and Francis Collins, Director of the- public Human Genome Project, agreed to publish first drafts of the sequence of the human genome at the same time. The Celera and public sequences were both published in February 2001. Figure 5.14 shows an annotated, sequence- based map of an 8mb segment at the tip of the short arm of human chromosome 1. This map ilustrates the positions and orientations of known and predicted genis in one small portion of the human genome. For summar maps of the entire human genome, see the February 15,2001, issue of Nature and the February 16, 2001, issue of Science. The amount of information in these first drafts of the human genome was quite overwhelming including the sequence of over 2650 megabase pairs of DNA (over 2,650,000,000 bp). The human genome is more than 25 times the size of the previously sequenced Drosopbila and Arabidopsis genomes, and it is eight times the sum of al previously sequenced genomes. Fig. 5.15 Functional classification of the 26,283 genes predicted by Celera Genomics’ first draft of the sequence of the human genome, Each sector gives the number and percentage of gene products in each functional class in parentheses. Note that some classes overlap: a proto-encogene, for example, may encode a signaling molecule

110 The sequence of the human genome provided one surprise: there appear to be only about 30,000 to 35,000 genes rather than the estimated 50,000 to 120,000 genes suggested by earlier studies. The distribution of functions for the 26,383 genes predicted by the Celera sequence is shown in Figure 5.15. About 60 perecent of the predicted proteins have similarities with proteins of other species whose genomes have been sequenced (Figure 5.16). Over 40 percent of the predicted human proteins share similarities with Drosopbila and C. elegans Proteins. The picture is quite different for mamilies of closely related proteins, which tend to perform important basic cellular functions. Only 94 of 1278 protein families predicted by the sequence of the human genome are specific to vertebrates. The rest have evolved from domains of proteins in distant ancestors, including prokaryotes and unicellular eukaryotes. On average, there is one gene per 60 to 85 kh in the human genome, although there is some clustering of highly expressed genes in euchromatic regions of specific chromosomes. Exons make up only 1.1 percent of the genome, whereas inttrons make up 24 percent, with 75 percent of the genome being intergenic DNA. Of the intergenic DNA, at least 44 percent is derived from transposable genetic elements. The initial drafts of the human genome are far from complete, and the immediate goal will be to fill in the gaps in the genome ad produce a finished sequence in the next year or so. The other major goal is to determine the structure and function of the human proteome (all of the proteins encoded by the human genome). Knowledge of the nucleotide sequences of entire genomes has spawned the development of a new scientific discipline, bioinformatics, a fusion of computer science and biology, with the goal of developing new tools with which to analyze
Fig. 5.16 Pie chart showing homology of predicted human proteins to protems of other species for those where homologues were deterced by comoputer searches of the public databases
111 the welth of data that genomics in providing. These new tools alow scientists to search genome databases for specific sequences or structrural features, to compare various features of different genomes, and to male inferences about the evolution of genomes. Indeed, with the sequence of the human genome aproaching completion, the question being asked is which genomes to sequence next—the mouse genome, the chimpanzee genome, and so on. One point is very clear: comparative genomics is providing unprecedented information about the evolution of species.
112 Unit 6 p Cytogenetic Implications and Consequences of Structural Changes and Numerical Alterations of Chromosomes Structure 6.1 Introduction 6.2 Chromosomes and cancer 6.3 Diseases associated with spontaneous chromosome aberrations 6.1 Introduction Between 20—50% of human abortuses have been shown to have a chromosome abnormality (Rashad and Kerr, 1965; Thiede and Metcalfe, 1966; Carr, 1967; Larson and Titus, 1970; Kajii et al., 1973). An even wider range has been reported (8—50%), but the studies have not been comparable (Carr, 1971a). Analysis of over 350 cases compiled from several published series has demonstrated that 45,XO is the most frequent single anomaly, constituting about 20% of the cases. Triploidy is only slightly less frequent, i.e., about 15%, while tetrapoidy has been demonstrated in 5%. Trisomies, as a group, have been found in about 50% of abortuses (E, 15%; G, 15%; D, 10%; C, 5%; A, B, F, 5%). The remainder are mosaics, or translocations (Carr, 1965, 1967; Inhorn 1967; Larson and Titus, 1970). only rarely is autosomal monosomy found (Kajii et al., 1973). It should be pointed out that within the E group, trisomy 16 and not trisomy 18 largely comprises this number (Carr, 1967; Waxman et a/., 1967). Use of recently developed banding techniques have shown trisomies for chromosomes 2, 3, 4, 6, 7, 8, 9, 10, 14, 15, 16, 18, 21 and 22 (Lauritsen et ah, 1972 Kajii et al., 1973). It is likely that findings in spontaneous abortuses do not necessarily reflect the frequency of clinical anomalies at conception since the more lethal ones probably never survived past a few cell divisions. This may explain the absence of viable trisomic states for A, B, most C, 14, 15, 16, 17, and F chromosomes being a more likely explanation than low frequency of meiotic or mitotic error for these chromosomes. The apparent high lethality of the 45,XO embryo cannot be explained. There is suggestions that embryos with chromosomal abnormalities are more likely to be aborted earlier than those with normal karyotypes (Carr, 1965, 1967; Szulman, 1965; Dhadial et al., 1970).

113 The gross appearance of abortuses have some correlation with their chromosome status. A recognizable fetus is most frequent in the 45, XO group. Not uncommonly they can be recognized by cystic hygromas (neck blebs) in older fetuses. Triploid abortuses characteristicaly exhibit hydatidiform degeneration of villi and only rarely contain an embryo (Szulman, 1965; Carr, 1965, 1971b; Singh and Carr, 1967; Boue et al., 1967). Trisomic abortuses do not have any specific phenotype with the possible exception of those having D- group trisomy,which not uncommonly have facial clefts. Presumably most of these are 13 trisomics (Roux, 1970). Kajii et al. (1973) found no example, however, of trisomy 13 in their large series. When analyzed for mean maternal age, polyploid and XO abortuses have been found to be from younger mothers while trisomics have been from older mothers (Carr, 1965, 1971a; Szulman, 1965; Kerr et al., 1966). However, the mean maternal age for 45,XO abortuses has been higher than that of survivors (Dhadial etal., 1970). Arakaki and Waxman (1970) found an increase in mean maternal age in cases of 16 trisomy abortuses. In those couples having a history of two or more spontaneous abortions. 1 in 26 couples was found to have a translocation. This contrasts with the 0.4% found in the general population (Lucas et al., 1972). 6.2 Chromosomes and cancer Cancer cells may have bizarre karyotypes which may be hypodiploid, hyperdiploid, triploid, hypertriploid, hypotriploid, etc. Many unusual structural abnormalities have been described. Cells having over 1000 chromosomes have been documented. On the other hand, cancer cells have been described with normal karyotype and no evidence of structural abnormalities. While one may conclude that not all neoplasia is associated with gross chromosomal anomalies, one cannot exclude point mutations, gene deletions or duplications, or hidden rearrangements. Review of chromosomal alterations concerning specific tumors is be yond the scope of this review and the reader is referred to Cervenka and Koulischer (1973). A. Acute leukemias In no form of acute leukemias have any specific chromosome abnormalities been described and, in at least half the cases, normal karyotypes have been found (Sandberg et al., 1968; Krogh-Hensen, 1969; Whang Peng et al., 1969). Furthermore, ostensibly identical clinical types of acute leukemia may minifest different chromosmoe patterns. Karyotypic changes, when present, are confined to the leukemic cells of the marrow or other organs. Long-term culture of

114 leukocytes from the blood of patients with acute leukemias is rarely successful. When an abnormal karotype is discerned, it seems to exhibit more hypoploid cell lines while acute lymphoblastic leukemias have more hyperploid lines. Karyotype analysis alone cannot be employed either for diagnosis or for prognosis concerning survival (Cervenka and Koulischer, 1973). During remission, aneuploid cells may disappear from the marrow only to reappear on relapse (Sandberg and Hossfeld, 1970). B. Chronic myelogenous leukemia In 1960, Nowell and Hungerford found delection of part of the long arm of a G chromosome associated with chronic myelogenous leukomia (CML). This unique structural abnormality, termed the Philadelphia chromosome (Ph 1), has been noted in over 90% of patients with CML (De Nava, 1969). Chronic myelogenous leukemia without the ph 1 chromosome and the ph i chromosome without chronic myelogenous leukemia have been thoroughly reviewed by Cervenka and Koulische 91973). The Ph 1 chromosome represents deletion with translocation to the long arm of a chromosome no. 9 (see Chapter 3). With.the use of quinacrine mustard fluorescent technique, it has been shown to be a G 22 chromosome (Caspersson el al., 1971; O'Riordan et al., 1971). Its occurrence is limited to hematopoietic cells of all the granulocytic, erythocytic, and megakaryocytic types (Tough et al., 1963; Clein and Flemans, 1966). Other tissues, such as skin fibroblasts, do not contain the ph1 chromosome. The best technique for demonstration of the ph! chromosome is by direct study of bone marrow (Sandberg and Hossfeld, 1970). It is an acquired, not an inherited, characteristic as demonstrated by its presence in only one of monozygotic twins with chronic myelogenous leukemia and not in the healthy co-twin (Jacobs et al., 1966, Kosenow and pfeiffer, 1969). An unusual subgroup of ph'-positive CML patients are males who are missing the Y chromosome in all or in a portion of their marrow cells. However, fibroblasts and blood lymphocytes contain the Y chromosome (Lawler and Galton, 1966; Pedersen, 1968). Two or more ph1 chromosomes appearing in marrow cells either heralds or accompanies the transformation of CML to a blastic phase (Smalley, 1966). A ph 1 -like chromosome has been found in a small porportion of marrow cells of patients with acute myeloblastic leukemia, polycythema, thrombocytopenia, myeloid metaplasia with myelofiborsis, and erythroleukemia (Sandberg and Hossfeld, 1970). Khan (1973) reported two ph'-like chromosomes in acute myeloid leukemia.

115 C. Solid tumors Most malignant tumors have aneuploid karyotypes ranging from hypodiploidy to extreme hyperdiploidy. Human tumor cell populations are clonal in nature, some tumors having but a single clone, others of two or more. No consistent cytogenetic findings have been described, but various markers have been noted, for example, a missing no. 22 chromosome in meningiomas (Zaftg and Singer, 1967; Mark et al., 1972) and microchromosome in various neurogenic tumors (Cox et al., 1965; Levan et al, 1968, Kucheria, 1968). Metastatic cells tend to have a higher ploidy and more variability in chromosome number (Sandberg et al., 1967). In about 50% of the cases, abnormal ("marker") chromosome have been found in metastatic cancer cells. In general, there is a tendency toward relatively few chromosomes with distally placed centromeres, i.e., fewer B-, D- , and G-group chromosome and more A-3, C-group, and E 16 chromosomes (Atkin, 1970). Manolov and Manolova (1972) described a market band in a chromosome 14 in Burkitt's lymphoma. Precancerous lesions, largely of the uterine cervia, have shown that dysplastic lesions exhibit chiefly pseudo-or near diploid karyotypes while carcinoma in situ shows an increase in ploidy and aberrations. Invasive carcinomas exhibit near diploid patterns, showing that progression does not depend on high chromosome counts (Atkin et al, 1967). Benign tumors have normal diploid karyotypes. D. Waldenstrom's macroglobulinemia Waldenstrom (1944) described a disorder characterized by intractable anemia and increased amounts of macroglobulin in serum, accompanied by fatigue, epistaxis, gingival hemorrhage, disturbances in vision, moderate lymphadenopathy, high sedimentation rate, and bone marrow lymphocytosis (Kok et al., 1963). It is presently classified in the group of gammopathies. The disease usually appears after age 40 and is more frequent in males. Its relationship to lymphosarcoma and leukemia is not clear but patients diagnosed as having the disease sometimes develop chroniclymphatic leukemia or lymphoma. Bottura et al. (1961) first described the presence of 47 chromosomes in about 50% of the cells, the supernumerary being about the size of an A-group chromosome. This finding was son confirmed by German et al. (1961) andBenirschke et al. (1962), who employed the term "W" chromosome. The morphology of the marker chromosome is not constant. It usualy has been large with the centromere varying from metacentric to subterminal, but in some cases it has been as small as an F-group chromosome (Spengler et al., 1966). The marker has been noted in both marrow and in peripheral cells in form 0—50% of cells (De Nava, 1969).

116 The abnormality is apparently acquired. Spengler et al. (1966) demonstrated the marker in one monozygotic twin who had Waldenstrom's macroglobulinemia but not in his normal co-twin. Interesting also are the findings of Lustman et al. (1968), who described an affected female with the marker whose otherwise healthy son had a normal karyotype but had an eleyated ?-globulin peak. Elves and Brown (1968) described the marker in 4 of 6 relatives of a patient with the disorder. Only one of the individuals had an elevated ? 1 fraction. 6.3 Diseases associated with spontaneous chromosome aberrations At least seven inherited diseases have been found to be associated with spontaneous chromosome aberrations and increased frequency of leukemia or other neoplasias. The chromosome aberrations consist of gaps (achromatic regions), chromatid and chromosome breaks, fragments, reunion or translocation figures, ring chromosomes, and dicentric chromosomes. It should be emphasized that chromosome breakage may be very rarely seen in cells of ostensibly normal people. The enzyme deficiencies in the inherited disorders may result either in increased frequency in which openings appear in the DNA strands or in decreased speed with which such breaks are healed. Higurashi and Conen (1973) demonstrated greater in vitro chromosomal sensitivity in several of these disorders. Fanconi's anemia, inherited as an autosomal recessive trait, is characterized by generalized skin pigmentation, pancytopenia with marrow hypoplasia, thumb and radius anomalies, hypogenitalism, and microcephaly (Fanconi, 1967). In 1964, Schroeder et al. noted that more than 40% of analyzed metaphases from peripheral blood cultures of patients with Fanconi's anemia exhibited chromatid gaps and breaks and chromosomal rearrangements. Direct bone marrow preparations have shown about 10% aberrant metaphases, usually involving B and C group chromosomes (Hirschman et al., 1969; Shahid et al., 1972). Of 41 cases subsquently studied, 36 were found to have similar findings. Among 170 known cases, four have terminated in leukemia and one had skin cancer (Swit and Hirschhorn, 1966; Swift, 1971). Heterozygotes have an increased frequency of leukemia (Gmyrek et al., 1967; Swift, 1971). Occasionaly quadriradials and dicentric forms are noted but far less frequently than in Bloom's syndrome (vide infra). Bloom's syndrome, consisting of growth retardation, sensitivity to.sunlight, and telagiectatic erythema, was reported by German (1969) to have chromosome breaks. Of 35 cases, four were found to have subsequently developed leukemia

117 or cancer, especially gastrointestinal. Cell lines with an abnormal karyotype have been described in clutured fibroblasts from a patient with Bioom's syndrome (Rauh and Soukup, 1968). Quadriradial figures, i.e., a four-armed figure derived from two chromosomes, each arm consisting of sister chromatids of one of two homologous chromosomes. The autosomes most often involved are No. 1 and either No. 19 or 20. Asymmetric dicentric chromosomes, triradials, and abnormal new monocentric chromosomes can also be found. Heterozygotes may have the same types of figures, but less frequently than the homozygote. Atxia-telangiectasia inherited as an autosomal recessive trait is characterized by retarded growth, progressive cerebellar ataxia, telangiectasia especialy about the face and bulbar conjunctiva, increased sonopulmonary infections, and decreased immunoglobulins (especially IgA and IgE). Approximately 10% develop lymphomas (pferiffer, 1970). Heche et al. (1966) reported a high frequency on in vitro chromosome breakage, a finding suported by Groop and Flatz (1967), pfeiffer (1970), and German (1972). Lesser well-documented associations are wth glutathione reductase deficiency anemia, pernicious anemia, Kostmann's agranulocytosis (Schroeder and kurth, 1971), and possibly xeroderma pigmentosum (German et al., 1970). Matsaniotis et al. (1966) found approximately 20% aberrant cells from direct bone marrow preparations of a baby with Kostmann's agranulocytosis. Krogh- Jensen and Friis-Moller (1967) and Bottura and Continho (1988) described in vivo demonstration of chromosomal aberration in untreated pernicious anemia. The evidence for dominatntly inherited glutathione reductase deficiency is less solid and seems to depend on the stage of the disease (Hampel et al., 1969). German et al. (1970) detected a tendency toward the formation of pseudodiploid clones in cultured fibroblasts from a patient with xerox deama pigmentosum, an autosomal recessively inherited disorder, in which there is a proclivity toward development of skin cancer. Failure DNA repair following untraviolet light exposure has been demonstrated. Repair failure results from deficiency of ultraviolet-specific endonuclease.

118 Unit 7 p Microbial Genetics

Structure 7.1

Introduction 7.2 Bacterial mutation 7.3 Conjugation—method of genetic recombination in bacteria 7.4 Transformation —Process leading to genetic recombination in bacteria 7.5 Transduction is virus—mediated bacterial DNA transfer 7.1 Introduction Main constituent in microbial genetics are the bacteria. Bacteria reproduce asexually. However parasexual reproduction is also found among bacteria. In fact, genetic information is transferred from one bacterium to another by three totally distinct processes—transformation, conjugation and transduction. Gene transfer in Bacteria : Transformation (Definition) : This is the process by which a donor DNA molecule is taken up from the external environment and incorporated into the genome of a recipient cell. Conjugation (Definition): This is the process by which bacterial cells make direct contact with each other, and DNA is transferred from one cell (the donor) to the other (the recipient cell). Transduction (Definition) : This is the process by

which DNA is transferred from one bacterial cell to another

by a bacterial virus, or bacteriophage. 7.2 Bacterial mutation Infection by the bacteriophage leads to the reproduction of the virus at the expense of the bacterial cell, which is lysed or destroyed. If a plate of E. coli is homogeneously sprayed with Tl, almost all cells are lysed. Rare E. coli cells, however, survive infection and are not lysed. If these cells are isolated and established in pure culture, all their descendants are resistant to Tl infection. This might be argued that the mutations responsible for Tl resistance were "induced" by the presence of the Tl viruses, and that, in the absence of the Tl viruses, the mutations would not have occurred. In 1943 Salvador Luria and Max Delbruck elegantly proved that such Tl-resistant cells result from spontaneous mutation.

119 Bacterial cells that bear spontaneous mutations, such as Tl resistance, can be isolated and established independently from the parent strain by means of various selection techniques. As a result, one can now induce and isolate mutations for almost any desired characteristic. Because bacteria and the viruses that infect them are haploid, all mutations are expressed directly in the descendants of mutant cells, adding to the case with which these microorganisms can be studied. Bacteria are grown in either a liquid culture medium or in a petri dish on a semisolid agar surface. If the nutrient components of the growth medium are very simple and consist only of a organic carbon source (such as a glucose or lactose) and a variety of inorganic ions, including Na + , K + , Mg ++ , and NH 4 + , present as inorganic salts, it is called minimal medium. To grow on such a medium, a bacterium must be able to synthesize all essential organic compounds (e.g., amino acids, purines, pyrimidines, sugars, vitamins, and fatty acids). A bacterium that can accomplish this remarkable biosynthetic feat—one that we ourselves cannot duplicate—is termed a prototroph. It is said to be wild type for all growth requirements and can grow on minimal medium. On the other hand, if a bacterium loses, through mutation, the ability to synthesize one or more organic components, it is said to be an auxotroph. For example, a bacterium that loses the ability to make histidine is designated as a bis - auxotroph, as opposed to its prototrophic bis + counterpart. For the bis bacterium to grow, this amino acid must be added as a supplement to the minimal medium. The medium that has been extensively supplemented is referred to as complete medium. Fig. 7.1 A typical bacterial population growth curve illustrating the initial lag phase, the subsequent log phase where exponential growth occurs, and the stationary phase that occurs when nutrients are exhausted

120 To study mutant bacteria in a quantitative fashion, an inoculum (e.g., 0.1 ml, 1.0 ml) of bacteria is placed in liquid culture medium. The bacteria exhibit a characteristic growth pattern, as illustrated in Figure 7.1. Initially, during the lag phase, growth is slow. Then, a period of rapid growth follows called the log phase, during which cells divide many times with a fixed time interval between cell divisions, resulting in logarithmic growth. When the bacteria reach a cell density of about 10 9 cells per milliliter, nutrients and oxygen become limiting and cells enter the stationary phase. As the doubling time during the log phase may be as short as 20 minutes, an initial inoculum of a few thousand cells added to the culture can easily achieve a maximum cell density overnight. Once cells are grown in liquid medium they can be quantitated. First, the bacteria are plated on (transferred to) semi-solid medium in a petri dish where, following incubation and many divisions, each cell gives rise to a colony visible on the surface of the medium. From the number of colonies that subsequently grow, it is possible to estimate the number of bacteria present in the original culture. If the number of colonies is too great to count, then serial dilutions of the original liquid culture can be made and plated, until the colony number is reduced to the point where it can be counted (Figure 7.2). Fig. 7.2 Results of the serial dilution technique and subsequent culture of bacteria. Each of the dilutions varies by a factor of 10. Each colony was derived from a single bacterial cell For example, assume that the three petri dishes in Figure 7.2 represent dilutions of 10 -3 , 10 -4 , and 10 -5 , respectively (left to right). We select the dish were there are few enough colonies to be accurately counted. Because each colony presumably arose from a single bacterium, the number of a colonies times the dilution factor represents the number of bacteria in the initial milliliter. In this case, the dish farthest to the right contains 15 colonies. Since it represents a dilution of 10 -5 , we can estimate the initial number of bacteria to be 15 x 10 5 per milliliter. Calculations such as these are useful in a number of studies.

121 7.3 Conjugation is one of the methods of genetic recombination in bacteira 7.3.1 Introduction Development of techniques that allowed the identification and study of bacterial mutations led to detailed investigations of the arrangement of genes on the bacterial chromosome. Such studies began in 1946 when Joshua Lederberg and Edward Tatum showed that bacteria undergo conjugation, a parasexual process in which the genetic information from one bacterium is transferred to and recombined with that of another bacterium. Like meiotic crossing over in eukaryotes, genetic recombination in bacteria provided the basis for the development of methodology for chromosome mapping. Note that the term genetic recombination, as applied to bacteria and bacteriophages, leads to the replacement of one or more genes present in one strain with those from a genetically distinct strain. While this is somewhat different from our use of genetic recombination in eukaryotes, where the term describes crossing over that results in reciprocal exchange events, the overall effect is the same : Genetic informatin is transferred from one chromosome to another, resulting in an altered genotype. Two other phenomena that result in the transfer of genetic information from one bacterium to another, transformation and transduction, have also served as a basis for determining the arrangement of genes on the bacterial chromosome. Lederberg and Tatum's initial experiments were performed with two multiple auxotroph strains (nutritional mutants) of E. coli K12. As shown in Figure 7.3, Strain A required methionine (met) and biotin (bio) in order to grow, whereas strain B required theonine (thr), leucine (leu), and thiamine (thi). Neither strain would grow on minimal medium. The two strains were first grown separately in supplemented media, and then cells from both were mixed and grown together for several more generations. They were then plated on minimal medium. Any bacterial cells that grew on minimal medium are prototrophs (wild-type bacteria that did not need nutritional supplements). It was highly improbable that any of the cells that contained two or three mutant genes would undergo spontaneous mutation simultaneously at two or three gene sites. Therefore, any prototrophs recovered must have arisen as a result of some form of genetic exchange and recombination. In this experiment, prototrophs were recovered at a rate of 1/10 7 (10 -7) cells plated. The controls for this experiment involved separate plating of cells from strains A and B on minimal medium. No prototrophs were recovered. On the basis of these observations, Lederberg and Tatum proposed that, while the events were indeed quite rare, genetic recombination had occurred.

122 Fig. 7.3 Genetic recombination involving two auxotrophic strains producing prototrophs. Neither auxotrophh will grow on minimal medium, but prototrophs will, suggesting that genetic recombination has occurred 7.3.2 F + and F - Bacterial strains Lederberg and Tatum's findings were soon followed by numerous experiments designed to elucidate the genetic basis of conjugation. It quickly became evident that different strains of bacteria were involved in a unidirectional 123 transfer of genetic material. When cells serve as donors of parts of their chromosomes, they are designated as F + cells (F for "fertility"). Recipient bacteria receive the donor chromosome material (now known to be DNA), and recombine it with part of their own chromosome. They are designated as F - cells. 7.3.3 Conjugation in bacteria It was established subsequently that cell contact is essential to chromosome transfer. Support for this concept was provided by Bernard Davis, who designed a U-tube in which to grow F + and F - cells (Figure 7.4). At the base of the tube was a glass filter with pores large enough to allow passage of the liquid medium, but too small to allow the passage of bacteria. The F + cells were placed on one side of the filter and F" cells on the other side. The medium was moved back and forth across the filter so that the bacterial cells essentially shared a common medium during incubation. Samples from both sides of the tube were then plated independently on minimal medium, but no prototrophs were found. Davis concluded that physical contact is essential to genetic recombination. Such physical interaction is the initial stage of the process of conjugation and is mediated through a conjugation tube called the F, or sex, pilus. Bacteria often have many pili, which are microscopic tube-like extensions of the cell. Different types of pili perform different cellular functions, but all piii are involved in some way with adhesion. After contact has been initiated between mating pairs via the F pili (Figure 7.5), transfer of DNA begins. Later evidence established that F + cells contained a fertility factor (called the F factor) that confers the ability to donate part of their chromosome during conjugation. Experiments by Joshua and Esther Lederberg and by William Hayes and Luca Cavalli-Sforza showed that certain conditions could eliminate the F factor in otherwise fertile cells. However, if these "infertile" cells were then grown with fertile donor cells, the F factor was regained. The conclusion that the F factor is a mobile element was further supported by the observation that, following conjugation and genetic recombination, recipient cells always become F + . Thus, in addition to the rare cases of transfer of genes from the bacterial chromosome (genetic recombination), the F factor itself is passed to all recipient cells. On this basis, the initial crosses of Lederberg and Tatum (Figure 7.3) may be designated. STRAIN A STRAIN B F + X F - DONOR RECIPIENT Isolation of the F factor confirmed these conclusions. Like the bacterial chromosome, though distinct from it the F factor has been shown to consist of

124 a circular, double-stranded DNA molecule, equivalent to about 2 percent of the bacteria] chromosome (about 100,000 nucleotide pairs). Contained in the F factor, among others, are 19 genes, the products of which are involved in the transfer of genetic information (tra genes). These include those essential to the formation of the sex pilus. Fig. 7.4 When strain A and B auxotrophs are grown in a common medium but separated by a no genet.c recombination occurs and no prototrophs are produced. This apparatus is called a Davis U- tube Fig. 7.5 An electron micrograph of conjugation between an F + E coli cell. The sex pllus linking them is clearly visible

125 As we soon shall see, the F - factor is in reality an autonomous genetic unit referred to as a plasmid. However, in our historical coverage of its discovery, we will continue in this chapter to refer to it as a "factor." It is believed that the transfer of the F factor during conjugation involves separation of two strands of the F factor double helical DNA and the movement of one of the two strands into the recipient. The other strand remains in the donor cell. Both of these parental strands serve as templates for DNA replication, resulting in two intact F factors, one in each of the two cells. Both cells are now F + (See in Figure 7.6). To summarize, E. coli cells may or may not contain the F factor. When it is present, the cell is able to form a sex pilus and potentially serve as a donor of genetic information. During conjugation, a copy of the F factor is almost always transferred from the F + cell to the F - recipient, converting it to the F + state. The question remains as to exactly how a very low percentage of F - cells undergo genetic recombina-tion. The answer awaited further experimentation. Subsequent discoveries not only clarified how genetic recombination occurs but also defined a mechanism by which the E. coli chromosome could be mapped. Fig. 7.6 An F + × F mating demonstrating how the recipient F - cell is converted to F + . During conjugation, the DNA of the F factor is replicated with one new copy entering the recipient cell converting it to F + . The black bar has been added to the F factors to follow their clockwise rotation during replication

126 7.3.4 Hfr bacteria and chromosome mapping In 1950, Cavalli-Sforza treated an F + strain of E. coli K12 with nitrogen mustard, a potent chemical known to induce mutations. From these treated cells, he recovered a strain of donor bacteria that underwent recombination at a rate of 1/10 4 (10 -4), 1000 times more frequently than the original F + strains. In 1953, William Hayes isolated another strain demonstrating a similar elevated frequency. Both strains were designated Hfr, or high-frequency recombination. Because Hfr cells behave as chromosome donors, they are a special class of F + cells. Another important difference was noted between Hfr strains and the original F + strains. If the donor is an Hfr strain, recipient cells, while sometimes displaying genetic recombination, never become Hfr; that is, they remain F - In comparison, then, F + x F - ––< F + (low rate of recombination) Hfr x F - ––< F - (higher rate of recombination) Perhaps the most significant characteristic of Hfr strains is the nature of recombination. In any given strain, certain genes are more frequently recombined Fig. 7.7 The progressive transfer during conjugation of various genes from a specific Hfr strain of E, coli to an F strain. Certain genes {azi and ton) are transferred sooner than others and recombine more frequently. Others (lac and gal) take longer to be transferred and recombine with a lower frequency. Others (thr and leu) are always transferred and are used in the initial screen for recombinants

127 than others, and some do not recombine at all. This nonrandom pattern of gene transfer was shown to vary from Hfr strain to Hfr strain. While these results were puzzling. Hayes interpreted them to mean that some physiological alteration of the F factor had occurred, resulting in the production Hfr strains of E. coli. In the mid-1950s, experimentation by Ellie Wollman and Francois Jacob explained the differences between cells that are Hfr and those that are F + and showed how Hfr strains allow genetic mapping of the E, coli chromosome. Wollman and Jacob first incubated a culture containing a mixture of an Hfr strain and an F strain. To facilitate the recovery of only recombinants, the Hrf strain was sensitive to an antibiotic while the recipient strain was resistant. At various intervals, the researchers removed samples and placed them in a blender. The shear forces created in the blender seperated conjugating bacteria so that the transfer of the chromosome was effectively terminated. To assay the cells for genetic recombination following the blender treatment, they were grown on medium containing the antibiotic in order to ensure the recovery of only recipient cells. 7.3.5 Interrupted mating technique This process, called the interrupted mating technique, demonstrated that specific genes of a given Hfr strain were transferred and recombined sooner than others. Figure 7.7 illustrates this point. During the first 8 minutes after the two strains were initially mixed, no genetic recombination could be detected. In cells assayed at about 10 minutes, recombination of the azi R gene could be detected, but no transfer of the ton S , lac + , or gat + genes was noted. By 15 minutes, 70 percent of the recombinants were azl R ; 30 percent were now also ton s ; but none was lac + or gat. Within 20 minutes, the lac + gene was found among the recombinants; and within 30 minutes, gal + was also being transferred. Wollman and Jacob had demonstrated an oriented transfer of genes that was correlated with the length of time conjugation was allowed to proceed. It appeared that the chromosome of the Hfr bacterium was transferred linearly and that the gene order and distance between genes, as measured in minutes, could be predicted from such experiments (Figure 7.8). This information served as the basis for the first genetic map of the E. coli chromosome. “Minutes” in bacterial mapping are equivalent to “map units” in eukaryotes. Fig. 7.8 A time map ot the genes studied in the experiment depicted in Figure 7.7

128 Wollman and Jacob repeated the same type of experimentation with other Hfr strains, obtaining similar results with one important difference. Although genes were always transferred linearly with time, as in their original experiment, which genes entered first and which followed later seemed to vary from Hfr strain to Hfr strain [Figure 7.9(a)]. When they reexamined the rate of entry of genes, and thus the different genetic maps for each strain, a definite pattern emerged. The major difference between each strain was simply the point of the origin and the direction in which entry proceeded from that point [Figure 7.9(b)]. Fig. 7.9 (a) The order of gene transfer in four Hfr strains, suggesting that the E. coli chromosome is circular, (b) The point where transfer originates (O) is identified in each strain. Note that transfer can proceed in either direction, depending on the strain. The origin is determined by the point of integration into the chromosome of the F factor, and the direction of transfer is determined by the orientation of the F factor as it integrates To explain these results, Wollman and Jacob postulated that the E. coli chromosome is circular. If the point of origin (O) varied from strain to strain, a different sequence of genes would be transferred in each case. But what determines O? They proposed that in various Hfr strains, the F factor integrates into the chromosome at different points and its position determines the O site. One such case of integration is shown in Figure 7.10 (Step 1). During conjugation between this Hfr and an F cell, the position of the F factor determines the initial point of transfer (Step 2 and 3). Those genes adjacent to O are transferred first. The F factor becomes the last part to be transferred (Step 4). Apparently, conjugation rarely, if ever, lasts long enough to allow the entire chromosome to pass across the conjugation tube (Step 5). This proposal explains why recipient cells, when mated with Hfr cells, remain F - .

129 Figure 7.10 also depicts the way in which the two strands making up a DNA molecule unwind during transfer, allowing for the entry of one of the strands of DNA into the recipient (Step 3). Following replication, the entering DNA now has the potential to recombine with its homologous region of the host chromoso'me. The DNA strand that remains in the donor also undergoes replication. The use of the interrupted mating technique with different Hfr strains has provided the basis for mapping the entire E. coli chromosome, Mapped in time units, strain K12 (or E. coli K12) is 100 minutes long. Over 900 genes have now been placed on the map. In most instances, only a single copy of each gene exists. 7.3.6 Recombination in F + × F - matings : A review The above model has helped geneticists to better understand how genetic recombination occurs during the F + xF - matings. Recall that recombination occurs much less frequently in them than in Hfr××F - matings, and that random gene transfer is involved. The current belief is that when F + and F - cells are mixed, conjugation occurs readily and that each F - cell involved in conjugation with an F + cell receives a copy of the F factor, but that no genetic recombination occurs. However, at an extremely low frequency in a population of F + cells, the F factor integrates spontaneously from the cytoplasm to a random point in the bacterial chromosome, converting the F + Fig. 7.10 Conversion of F 4 " to an Hfr state occurs by the integration of the F factor intot he bacterial chromosome. The point of integration determines the origin (O) of transfer, during conjugation, the F factor, now integrated into the host chromosome, is nicked by an enzyme, initiating transfer of the chromosome at that point. Conjugation is usually interrupted prior to complete transfer. Above, only the A and B genes are transferred to the F cell, which may recombine with the host chromosome 1. F factor is integrated into the bacterial chromosome and the cell becomes an Hfr cell. 2. Conjugation occurs between an Hfr and F~ cell. The F factor is nicked by an enzyme, creating the origin of transfer of the chromosome (O). 3. Chromosome transfer across the conjugation tube begins. The Hfr chromosome rotates clockwise. 4. replication begins on both strands as chromosome transfer continues. The F factor is now on the end of the chromosome j adjacent to the origin. 5. Conjugation is usually interrupted before the chromosome transfer is complete. Here only the A and B genes have been transferred.

130 cell to the state as we saw in Figure 7.10 Therefore, in F + x F - crosses, the extremely low frequency of genetic recombination (10 -7) is attributed to the rare, newly formed Hfr cells, which then undergo conjugation with F - cells. Because the point of integration of the F factor is random, a nonspecific gene transfer ensues, leading to the low-frequency, random genetic recombination observed in the F + x F - experiment. Unless the recipient cell simultaneously or subsequently undergoes conjugation with a separate F + cell, it will remain F - . Most often, the recombinants become F + . 7.3.7 The F' State and merozygotes In 1959, during experiments with Hfr strains of E. coli. Edward Adelberg discovered that the F + factor could lose its integrated status, causing the cell to revert to the F + state (Figure 7.11 Step 1). When this occurs, the F factor frequently carries several adjacent bacterial genes along with it (Step 2). Adelberg labeled this condition F' to distinguish it from F + and Hfr. F + like Hfr, is thus another special case of F + . This conversion is described as one from Hfr to F + . The presence of bacterial genes within a cytoplasmic F factor creates an interesting situation. An F' bacterium behaves like an F + cell, initiating conjugation with F - cells (Figure 7.11- Step 3). When this occurs, the F factor, containing chromosomal genes, is transferred to the F + cell (Step 4). As a result, whatever chromosomal genes are part of the" F factor are now present in Fig. 7.11 Conversion of an Hfr bacterium to F 1 " and is subsequent mating with an F cell. The conversion occurs when the F factor loses its integrated status. During excision from the chromosome, it carries with it one or more chromosomal genes (A and E) F lowing conjugation with an F~ cell, the recipient cell becomes partially diploid and is called a merozygote. It also behaves as an F~ donor ones 1. Excision of the F factor from the chromosome begins. During excision, the F factor carries with it, part of the chromosome (the A and E regions). 2. Excision is complete. The cell is converted to F'. The A and E regions of the chromosome are now contained ih the F factor. 3. The F' cell is a modified E + cell and may undergo conjugation with an F~ cell. 4. The F factor replicates as one ? strand is transferred. 5. Replication and transfer of the F factor is complete, the F" recipient has become partially diploid (for A and E region) and is called a merozygote.

131 duplicate in the recipient cell (Step 5), because the recipient still has a complete chromosome. This creates a partially diploid cell called a merozygote. Pure cultures of F' merozygotes can be established. They have been extremely useful in the study of bacterial genetics, particularly in genetic regulation. 7.3.8 Bacterial recombination is dependent on rec poteins Once researchers established that a unidirectional transfer of DNA occurs between bacteria, they were interested in determining how the actual recombination event occurs in the recipient cell. Just how does the donor DNA replace the comparable region in the recipient chromosome? As with many systems, the biochemical mechanism by which recombination occurs was deciphered through genetic stuies. Major insights were gained as a result of the isolation of a group of mutations representing genes called rec. The first relevant observation in this case involved a series of mutant genes labeled recA, recB, recC, and recD. The first mutant gene, recA, was found to diminish genetic recombination in bacteria 1000-fold, nearly eliminating it altogether. The other rec mutations reduced recombination by about 100 times. Clearly, the normal wild-type products of these genes play some essential role in the process of recombination. By looking for a functional gene product present in normal cells but missing in mutant cells, researchers subsequently isolated several gene products and showed that they played a role in genetic recombination. The first is called the RecA protein.* The second is a more complex protein called the RecBCD product, an enzyme consisting of polypeptide subunits encoded by three other rec genes. The roles of these proteins have now been elucidated in vitro. As a result of this genetic research, our knowledge of the process of recombination has been extended considerably. These discoveries underscore the value of isolating mutations, establishing their phenotypes, and determining the biological role of the normal, wildtype gene as a result of subsequent investigation. 7.3.9 F factors are plasmids In the preceding sections we have examinined the extra-chromosomal heredity unit called the F factor. When it exists autonomously in the bacterial cytoplasm, the F factor is composed of a double-stranded closed circle of DNA [Figure 7.12(a)]. These characteristics place the F factor in the more general category of genetic .structures called plasmids. These structures contain one or more genes—often, quite a few. Their replication depends on the same enzymes that replicate the chromosome of the host cell, and they are distributed to daughter cells along with the host chromosome during cell division. Plasmids are generally classified according to the genetic information specified by their DNA. The F factor confers fertility and contains genes essential *Note that the names of bacterial genes begin with lowercase letters and are italicized. The names of the corresponding gene products (proteins) begin with an uppercase letter and are not italicized. For example, the m-4 gene encodes the RecA protein.

132 for sex pilus formation, upon which genetic recombination depends. Other examples of plasmids include the R and the Col plasmids. Most R plasmids consist of two components : the RTF (resistance transfer factor) and one or those r-de-terminants {Figure 7.12(b)]. The RTF encodes genetic information essential to transfer of the plasmid between bacteria, and the r- determinants are genes conferring resistance to entibiotics. The Col plasmid, ColEl, derived from E. coli, is clearly distinct from R plasmids. It encodes one or more proteins that are highly toxic to bacterial strains that do not harbor the same plasmid. These proteins, called colicins, may kill neighboring bacteria. Bacteria that carry the plasmid are said to be colicinogenic. Present in 10 to 20 copies per cell, the plasmid also contains a gene encoding an immunity protein that protects the host cell from the toxin. Unlike an R plasmid, the Col plasmid is not usually transmissible to other cells. 7.4 Transformation is another process leading to genetic recombination in bacteria Transformation is another process that provides a mechanism for the recombination of genetic information in some bacteria. In transformation, small pieces of extracel lular DNA are taken up by a living bacterium, ultimately leading to a stable genetic change in the recipient cell. 7.4.1 The Transformation Process Transformation (Figure 7.13) consists of numerous steps that can be divided into two main categories: (1) entry of DNA into a recipient cell, and (2) recombination of the donor DNA with its homologous region in the recipient chromosome. In a population of cells, only those in a particular physiological state, referred to as competence, take up DNA. Entry is thought to occur at a limited number of receptor sites on the surface of the bacterial cell. Passage Fig. 7.12 (a) Electron micrograph of a plasmid isolated from E. coll; (b) diagramatic representation of an R plasmid containing resistance transfer factors (RTFs) and multiple r-determinance (Tc, tetracycline; Kan, kanamycin; Sm, streptomycin; Su, sulfonamide; Amp, ampicillian; and Hg, mercury)

133 Fig. 7.13 Proposed steps leading to transformation of a bacterial cell by exogenous DNA. Only one of the two strands of the entering DNA is involved in the transformation event, which is completed following cell division 1. Extracellular DNA binds to the competent cell at a receptor site. 2. DNA enters the cell and strands separate. 3. One strand of transforming DNA is degraded. The other and pairs homologously with the host cell DNA. 4. The transforming DNA is integrated, forming a heteroduplex. 5. Following one round of cell division, a transformed and a non-transformed cell are produced.

134 across the cell wall and membrane is an active process requiring energy and specific transport molecules. This concept is supported by the fact that substances that inhibit energy production or protein synthesis in the recipient cell also inhibit the transformation process. During the process of entry, one of the two strands of the invading DNA molecule is digested by nucleases, leaving only a single strand to participate in transformation (Step 2 and 3). The surviving DNA strand aligns with its complementary region of the bacterial chromosome. In a process involving several enzymes, this segment of DNA replaces its counterpart in the chromosome, which is excised and degraded (Step 4). For recombination to be detected, the transforming DNA must be derived from a different strain of bacteria, bearing some genetic variation. Once integrated into the chromosome, the recombinant region contains one DNA strand from the bacterial chromosome and one from the transforming DNA. Because these strands are not genetically identical, this helical region is referred to as a heteroduplex. Following one round of replication, one chromosome is restored to its original configuration, identical to that of the recipient cell, and the other contains the transformed gene. Cell division produces one host cell and one transformed cell (Step 5). 7.4.2 Lysogeny The relationship between virus and bacterium does not always result in viral reproduction and lysis. As early as the 1920s, it was known that some bacteriophages could enter a bacterial cell and establish a symbiotic relationship with it. The precise molecular basis of this symbiosis is now well understood. Fig. 7.15 Life cycle of bacteriophage T4

135 Fig. 7.16 Diagramatic illustration of the plaque assay for bacteriophage analysis. Serial dilutions of a bacterial culture infected with bacteriophages are first made. Then three of the dilutions (10 3 , 10 6 and 10 7) are analyzed using the plaque assay technique in each case 0.1 ml of the diluted culture is used. Each plaque represents the initial infection of one bacterial cell by one bacteriophage. In the 10 3 dilution, so many phaes are present that all bacterial are lysed. In the 10 -5 dilution 23 plaques are produced. In the 10 -7 dilution, the dilution factor is so great that no phages are present in the 0.1 ml sample, and thus no plaques form. From the 0.1 ml sample of the 10 -3 dilution, the original bacteriophage desnity can be calculated as 23, 10, 10 5 phages/ml (23.10 4 or 23.10). The photograph illustrates phage T2 plaques on lawns of E. coli

136 Upon entry, the viral DNA, instead of replicating in the bacterial cytoplasm, is integrated into the bacterial chromosome, step that characterizes the developmental stage referred to as lysogeny. Subsequenly, each time the bacterial chromosome is replicated, the viral DNA is also replicated and passed to daughter bacterial cells following division. No new viruses are produced and no lysis of the bacterial cell occurs. However, in response to certain stimuli, such as chemical or ultraviolet-light treatment, the viral DNA may lose its integrated status and initiate replication, phage reproduction, and lysis of the bacterium. (Fig, 7.15) Several terms are used to describe this relationship. The viral DNA integrated into the bacterial chromosome is called a prophage. Viruses that can either lyse the cell or behave as a prophage are called temperate. Those that can only lyse the cell are referred to as virulent. A bacterium harboring a prophage has been lysogenized and said to be lysogenic; that is, it is capable of being lysed as a result of induced viral reproduction. The viral DNA, which can replicate either in the bacterial cytoplasm or as part of the bacterial chromosome, is sometimes classified as an episome. 7.5 Transduction is virus-mediated bacterial DNA transfer In 1952, Norton Zinder and Joshua Lederberg were investigating possible recombination in the bacterium Salmonella typhimurium. Although they recovered prototrophs from mixed cultures of.two different auxotrophic strains, subsequent investigations revealed that recombination was occurring in a manner different from that attributable to the presence of an F factor, as in E. coll What they discovered was a process of bacterial recombination mediated by bacteriophages and now called transduction. 7.5.1 The Lederberg-Zinder experiment Lederberg and Zinder mixed the Salmonella auxotrophic strains LA-22 and LA-2 together and, when the mixture was plated on minimal medium, they recovered prototroph cells. LA-22 was unable to synthesize the amino acids phenylalanine and tryptophan (phe - trp -), and LA-2 could not synthesize the amino acids methionine and histidine (met - his -). Prototrophs (phe' trp' met' his') were recovered at a rate of about 1/10 5 (10 -5) cells. Although these observations at first suggested that the recombination involved was the type observed earlier in conjugative strains of E. coli, experiments using the Davis U-tube soon showed otherwise (Figure 7.17). The two auxotrophic strains were separated by a glass-smtered filter, thus preventing cell contact but allowing growth to occur in a common medium. Surprisingly, when samples were removed-foam both sides of the filter and plated independently on minimal medium, prototrophs were recovered only from the side of the tube containing LA-22 bacteria.

137 Since LA-2 cells appeared to be the source of the new genetic information (phe + and trp +), how that information crossed the filter from the LA-2 cells to the LA-22 cells allowing recombination to occur, was a mystery. The unknown source was designated simply as a filterable agent (FA). Three subsequent observations were useful in identifying the FA : 1. The FA was produced by the LA-2 cells only when they were grown in association with LA-22 cells. If LA-2 cells were grown independently and that culture medium was then added to LA-22 cells, recombination did not occur. Therefore, LA-22 cells play some role in the production of FA by LA-2 cells and do so only when the two share common growth medium. 2. The presence of DNase, which enzymatically digests DNA, did not render the FA ineffective. Therefore, the FA is not naked DNA, ruling out transformation. 3. The FA could not pass across the filter of the Davis U-tube when the pore size was reduced below the size of bacteriophages. Fig. 7.17 The Lederberg-Zinder experiment using Solmonelid. After placing two auxotrophic strains on opposite sides of a Davis U-tube. Lederberg and Zinder recovered prototrophs from the side containing the LA-22 strain but not from the side containing the LA-2 strain. These initial observations led to the discovery of the phenomenon called transduction

138 Added by these observations and awareof temperate phages that could lysogenize Salmonella, researchers proposed that the genetic recombination event was mediated by bacteriophage P22, present initially as a prophase in the chromosome of the LA-22 Salmonella cells. It was hypothesized that rarely P22 prophages migt enter the vegetative or lytic phase, reproduce, and be released by the LA-22 cells. Such phages, being much smaller than a bacterium, were then able to cross the filter of the U-tube and subsequently infect and lyse some of the LA-2 cells. In the process of lysis of LA-2, the P22 phages occasionally packaged in their heads a region of the LA-2 chromosome. If this region contained the phe + and trp + genes, and if the phages subsequenly passed back across the filter and infected LA-22 cells, these newly lysogenized cells would behave as prototrophs. This process of transduction, whereby bacterial recombination is mediated by bacteriophage P22, is diagrammed in Figure 7.18. 7.5.2 The nature of transduction Further studies revealed the existence of transducing phages in other species of bacteria. For example, E. coli can be transduced by phages Pl and X. Bacillus subtilis and Pseudomonas aeruginosa can be transduced by the phages SPO1 and Fl 16, respectively. The details of several different modes of transduction have also been established. Even though the initial discovery of transduction involved a temperate phage and a lysogenized bacterium, the same process can occur during the normal lytic cycle. Sometimes a small piece of bacterial DNA is packaged

139 along with the viral chromosome so that the transducing phage contains both viral and bacterial DNA. In such cases, only a few bacterial genes are present in the transducing phage. However, when only bacterial DNA is packaged, regions as large as 1 percent of the bacterial chromosome may become enclosed in the viral head. In either case, the ability to infect is unrelated to the type of DNA in the phage head, making transduction possible. When bacterial rather than viral DNA is injected into the bacterium, it can either remain in the cytoplasm of recombine with the homologous region of the bacterial chromosome. If the bacterial DNA remains in the cytoplasm in the cytoplasm, it does not replicate but is transmitted to one of the progeny cells following each division. When this happens, only a single cell, partially diploid for the transduced genes, is produced—a phenomenon called abortive transduction. If the bacterial DNA recombines with its homologous region of Fig. 7.19 The production of defective phage ?dgal, which can result in specialized transduction following another round of infection of E. coli. If detachment occurs correctly, no transduction will result

140 the bacterial chromosome, the transduced genes are replicated as part of the chromosome and passed to all daughter cells. This process is called complete transduction. 7.5.3 Transductionand mapping Like transformation, generalized transduction has been used in linkage and mapping studies of the bacterial chromosome. The fragment of bacterial DNA involved in a transduction event is large enough to include numerous genes. As a result, two genes that are closely aligned (linked) on the bacterial chromosome may be simultaneously transduced, a process called contrasduction. Two genes that are not close enough to one another along the chromosome to be included on a single DNA fragment require two independent events in order to be transdaced into a single cell. Since this occurs with a much lower probability than cotransdoction, linkage can be determined. By concentrating on two or three linked genes, transduction studies can also determine the precise order of these genes. The closer to each other linked genes are, the greater the frequency of contransduction. Mapping studies involving three closely aligned genes can be executed. The analysis of such an experiment is predicated on the same rationale underlying other mapping techniques. 7.5.9 Specialized transduction In some instances, only certain genes are recombined, a situation called specialized transduction. This is in contrast to generalized transduction described above, where all genes have an equal probability of being recombined. One of the best examples involves transduction of E. coli by the temperate phage ?. In this case, transduction is restricted to the gal (galactose) or bio (biotin) genes. The reason why transduction in volves only these genes became clear when it was learned that the ? DNA'always integrates into the region of the E. coli chromosome between these two genes at a site called att [Figure 7.19(a)]. Phage ? DNA that is integrated in the bacterial chromosome can subsequenly detach from it, reproduce, and lyse the host cell [Figure 7.19(b)]. Sometimes the excision process occurs incorrectly and carries either the gal or bio E, coli genes in place of part of the viral DNA [Figure 7.19(b)]. The resulting phage chromosome is defective because it has lost some of its own genetic information, but it is nevertheless replicated and packaged during the formation of mature phage particles. The virus can subsequently inject the defective chromosome into another bacterial cell. In this case of specialized transduction, the defective phage chromosome
141 is integrated into the bacterial, chromosome and is replicated along with it. Such bacterial cells contain the defective phage DNA, making them diploid for the gal or bio genes. The presence of this transducing gal' or bio' DNA causes these auxotrophs to revert to a gal' or bio' phenotype. 7.5.5 Bacteriophages undergo intergenic recombination Around 1947, several research teams demonstrated that genetic recombination also occurs in bacteriophages. These studies relied on the discovery of numerous phage mutations that could be visualized or assayed. Before considering recombination in these bacterial viruses, we will briefly introduce several of the mutations that were studied. Phage mutations often affect the morphology of the plaques formed following lysis of bacterial cells. For example, in 1946 Alfred Hershey observed unusual T2 plaques on plates of E. coli strain B. Where the normal T2 plaques are small and have a clear center surrounded by a diffuse (nearly invisible) halo, the unusual plaques were larger and possessed a more distinctive outer perimeter (Figure 7.20). When the viruses were isolated from these plaques and replated on E. coli B cells, the resulting plaque appearance was identical. Thus, the plaque phenotype was an inherited trait resulting from the reproduction of mutant phages. Hershey named the mutant raid lysis (r) because the plaques were larger, apparently resulting from a more rapid or more efficient life cycle of the phage. It is now known that in wild-type phages, reproduction is inhibited once a particular-sized plaque has been formed. The mutant T2 phages are able to overcome this inhibition, producing larger plaques.
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p

Cytogenetic effects of Ionizing and Non-ionizing Radiations Structure 8.1 Introduction 8.2 Radiation 8.3 Viruses 8.4 Chemical clastogens 8.1 Introduction Various agents shown to cause chromosome breaks have been termed "clastogens" by Shaw (1970). These include physical agents (X-rays, ultraviolet light, cold shock, magnetic fields, and sound waves), biological agents (certain genes, viruses and protozoa), and a host of chemical agents. It should be emphasized that most of these clastogens produced these effects in vitro by the addition of the agent to cultured lymphocytes and/or fibroblasts for varying times and concentration. In but few cases is there evidence for in vivo chromosome breakage. 8.2 Radiation Survivors of the atomic bomb blasts in Japan have developed leukemia in proportion to the amount of radiation received. Furthermore, increased numbers of chromosome breaks and rearrangements have been found in lymphocytes of nonleukemic survivors (Bloom et al., 1967). Similar anomalies (translocations and inversions) have been demonstrated in lymphocytes of individuals who have received X-ray therapy to the spine or injections of thorotrast (Buckton et al., 1982; Court Brown et al., 1967). Although ultrasound can effect chromosomal breaks in vitro, there is no evidence that it does so in vivo (Macintosh and Davey, 1972). Fibroblasts cultured from skin in the path of X-radiation have manifested chromosome abnormalities (Engel et al., 1964; Visfeldt, 1966). Marrow cells may exhibit abnormalities even after many years following primary exposure (Goh, 1971). Leukemia is also more likely to develop in individuals who have received chronic exposure to radiation (Lewis, 1970). Maternal irradiation before and during the reproductive period increases the incidence of chromosomally abnormal conceptuses. However, most are nonviable and lost early in pregnancy (Alberman et al, 1972).

143 8.3 Viruses There is insufficient evidence currently available to directly implicate viruses in effecting human chromosome abnormalities. However, a number of investigators have studied the effect of S V40 virus on cultured human fibroblasts and have observed altered growth patterns to the haphazard growth and alterations, within several months an emergence of a few stable heteropliod cells becomes evident (Moorhead, 1970). Numerous other viruses (Rous sarcoma, vaccinia, rubella, herpes zoster, poliomyelities, influenza, polyoma, etc.) have been shown to produce chromosome breaks in infected cells in vitro. Furthermore, several viruses can produce abnormalities in metaphase chromosome in circulating lymphocytes during natural human infections (measles, chicken pox, mumps, and hepatities) (Moorhead, 1970). The effects have been of at least three types: single breaks, pulverization, and fusion and spindle abnormalities. The mechanism is unknown but may be related to addition of the virus like particles (Epstein-Barr virus) have been demonstrated, often exhibit a long submetacentric marker (Gripenberg et al., 1969). 8.4 Chemical clastogens Over 200 drugs or chemical shown to cause chromosome breaks in vitro (shaw, 1970) can be grouped into several categories with a few illustrations in each group: (a) nucleic acid related compounds (6-mercaptopurine and 5-fluorodeoxyuridine), (b) antibiotics (mitomycin C, streptomycin, actinomycin D and daunomycin), (c) central nervous saystem drugs (meprobamate, chlopromazine, mesealine, lysergic acid diethylamide, and scopolamine), (d) food derivatives and additives (caffeine, cyclamate, theobromine, and theophylline), (e) air and water pollutants (chloramine T and ozone), (f) pesticides (captan and thioTEPA), (g) alkylating agents (nitrogen mustards and cytoxan), (h) mitotic poisons (colchicine), (i) photodynamic dyes (acridine orange and neutral red), (j) antifolic compounds (methotrexate and aminopterin), (k) organic solvents (benzene and mercaptoethanol), (1) inorganic substances (lead and arsenic), and (m) miscellaneous comopounds (Imuran and piperazine). However, it should be emphasized that few chemical clastogens have been implicated in chromosomal breakage in vivo. To cite but a few examples: Ambient exposure to benzene has been noted to be associated with both chromosome breakage and subsequent development of leukemia (Tough and Court Brown, 1965; Hartwich et al., 1969). On the other hand, LSD, while producing chromosome breaks in vitro, has not been shown to be effective in vivo (Stenchever and Jarvis, 1970).

144 Unit 9 p Molecular Cytogenetic Techniques Structure 9.1 FISH, GISH 9.2 DNA finger printing 9.3 Flow cytometry 9.4 Chromosome painting 9.1 FISH, GISH 9.1.1 In situ hybridization technique Under normal temperature and ionic conditions DNA remains in a duplex state by the base pairing through the hydrogen bonds. By heating in buffer solution or by increasing pH the two strands can be separated. But if again the temperature is lowered or pH is reduced then the separated strands will join again and reassociate. This fact was shown by Julius Marmur and Paul Doty in 1960. This type of reassociation of DNA strands in called molecular hybridization or nuclear hybridization. It may also take place between the complementary strands of DNA or RNA or between DNA and RNA. Hybridization technique which can be used to localize specific nucleic acid fragments that reside in their original site (in situ) within cells, is known as in situ hybridization. In situ hybridization
is a version of hybridization analysis, in which an intact chromosome is examined by probing it with a labeled DNA molecule.

For this technique to work, DNA in the chromosome must be made single stranded and denatured by breaking the hydrogen bonds between the base pairs. Only then the DNA or RNA probe can be hybridizid with the chromosomal DNA. For this purpose, without destroying the chromosome morphology a dry preparation is made into a glass microscope-slide and then treated with formaldehyde. The position where the hybridization occurs provides the information about the map location of this gene, thus physical mapping of chromosome could be done by this method. To locate the region of hybridization two types of markers viz., radioactive marker and fluorescent marker are used. Besides these other types of markers are also used for this purpose.

145 To prepare a DNA profile, the nucleotides are synthesized in which one of the phosphorus atoms replaced by 32 P or 33 P . One of the oxygen atom in the phosphate group is replaced with 35 S or one or more of the hydrogen atom is replaced with 3 H . Radioactive nucleotides act as substrates for DNA polymerases and so are incorporated into a DNA molecule by any strand synthesis reaction catalyzed by a DNA polymerase. The labeled nucleotides or individual phosphate groups can also be attached to one or both ends of a DNA molecule by the reaction catalyzed by T4 polynucleotide kinase or terminal deoxy nucleotidyl transferase. The rodioactive signal can be detected by scintillation counting, but for most molecular biology, the position of the hybridization is detected by exposure of an X-ray sensitive film (autoradiography) or radiation sensitive phosphorescent screen (phosphorus imaging). 32 P has a high emission energy and the resolution is lower. But low emission isotopes such as 35 S or 3 H give less sensitivity but greater resolution. 9.1.2 Fluorescent in situ hybridization (FISH) Concept To solve these problems in 1980's non radioactive fluorescent DNA labels were developed.

These labals combine high sensitivity with high resolution and are ideal for in situ hybridization.

The different fluorolabels of different colours have been disigned for the probes and it is possitive to hybridize the chromosome and the individual hybridization signals enable the location of relative position of the probe sequence to be mapped. Fluorescent dyes (Fluorochromes) live quinacrine (Q) and quinacrine mustard (QM) are used to obtain specific patterns of cross striations or bands appear with alternate fluorescent and non fluorescent bands. The bands obtained with quinacrine are called Q bands while those obtained with quinacrine mustard are called Q M-bands. Bands are obtained when fluorescent deyes attach to the specefic regions of the chromosomes (See Fig. 9.2). Technique During the in situ hybridization a sample of dividing cells is dried on a microscope slide and treated with formaldelyde so that the chromosomes become denatured but do not lose their characteristic metaphase morphologies. The probe is added to the denatured chromosomes, which will be hybridized with the complementary DNA region of the chromosome. The position at which the probe hybridizes to the chromosomal DNA is visualized by detecting the fluorescent signal emitted by the labeled DNA.

146 Problem If a probe be a long fragment of DNA, then a potential problem is that it is likely to contain repetitive DNA sequences and so may hybridize many portions of the chromosome. If these sequences are not blocked then the probe will hybridize non specifically to any copy of these genome and will repeat in the target DNA. To block the repeat sequences, the probe is pro-hybridized with a DNA fraction enriched for repetitive DNA. Advanteage of FISH over other methods Health and environmental issues have meant that radioactive markars have become less popular in recent years and they are now largely superseded by non-radioactive alternatives. Other drawbacks of radioactive markers in in situ hybridization and that the radioactive label has high emission energy (eg., P 32) and then it scatters its signal and so gives poor resolution. On the other hand , if H 3 is used, the emission energy is less but its sensitively is so low that lengthy exposures are needed. Disadvantage The metaphase chromosomes are highly condensed and

a fluorescent signal obtained by FISH is marked by measuring its position relative to the end of the short arm of the chromosome (the flepter value). The two markers having at least 1Mb apart to be resolved as separate hybridization signal (

Trask et al, 1991). Therefore, using FISH, the highly condensed nature of mataphase chromosomes means that only low resolution mapping is possible. Therefore, FISH provides a fough idea of its map position. More advanced FISH In 1996 Heiskanen et al solved the problem of FISH and a range of higher resolution FISH technique has been developed. Higher resolution is achieved by changing the condensing pattern of the metaphase chromosome. There are two ways of doing this. (1) Mechanically stretching of metaphase chromosomes : Centrifugation generates shear forces, which can result in the chromosomes becoming stretched upto 20 times of the normal length. Thus the resolution is significantly improved and markers that are only 200-300 Kb apart can be distinguished. (2) Taking non-metaplase chromosomes : Attempts have been made to use prophase nuclei because in this stage the chromosomes are not still sufficiently condensed for individual ones to be identified and so provides no advantage. Interphase chromosomes become more useful because, then the
147 chromosomes again become less condensed. Using anaphase stage the
resolution down to 25 kb is possible but their chromosome morphology is lost, so there are no external reference point against which the position of the probe
to be mapped. This technique is used to construct map in small region of the chromosome after obtaining preliminary information. Fibre-FISH Interphase chromosomes are most unpacked of all cellular DNA. To improve the resolution of FISH better than 25 kb, it is therefore, necessary to abandon intact chromosomes. This approach is called fiber FISH. In this approach the DNA is prepared by gel stretching or molecular combing. This can distingish markers that are less than 10 Kb. To carry out gel stretching (Fig. 9.1a), molten agarose containing chromosomal DNA molecules is pipetted into a microscope slide, coated "with a restriction enzyme (Schwartz et al, 1993). As the gel solidifies, the DNA molecules become stretched. It is not understood why this happens but it is thought that fluid movement on the glass surface during gelation might be responsible. Once the gel is solidified it is washed with MgCl 2 solution, which activates the restriction enzyme. A fluorecent dye such as DAPI (4,6 diamine 2- phenylindolo dihydrochloride) is added which stains the DNA so that the fibres can be seen when the slide in examined with a high power fluorescence microscope. The restriction enzyme cuts the DNA molecule. As the molecules gradualy coil up, the gaps representing the cut sites become visible. The relative positions of the cuts are to be recorded. In molecular combing (Michalet et al, 1997) (Fig. 9.1b), the DNA fibres are prepared by dipping a silicon-coated coverslip into a solution of DNA. It takes 5 minutes to attach DNA to the coverslip by their ends. After that coverslip is removed at a constant speed of 0.3 mm/sec. The force required to pull the DNA molecule through the miniscus causes to line up. Once in the air the surface of the coverslip dries, DNA molecules are arranged as a parallel fibre thus producing a comb of parallel molecules. 9.1.3 Biotin-labelling in situ hybridization Recent advances in nucleic acid technology offer alternative to radioactive- labelling probes. One procedure that is becoming increasingly popular is biotin- labelling of nucleic acid. This is nontoxic, whose half life is longer and can be prepared in adavnce in bulk and stored at -20°C for (repeated) use. Drosophila salivary gland chromosomes can be hybridized with a biotin labelled nucleic acid probe. After washing, detection can be done by adding a biotin-binding protein called ovidin which is covalently bound to alkaline
148 Fig. 9.2 (a) FISH ; (b) Blocking of repetitive DNA sequences in a hybridization probe

149 phosphatase. After addition of a soluble substrate, the enzyme catalyses a reaction that results in formation of an insoluble blue coloured precepitate at that site of hybridization. The intensity is proportional to the amount of biotin in the hybrid. In situ hybridization with a biotin labelled probe has been particularly useful in chromosome mapping of DNA clones in Drosophila because the logical map of the poly-tene chromosomes of this organism is known at high resolution. 9.2 DNA finger printing The techniques of DNA diagnosis have found application in a quite different area, the identification of medicine. This is important in areas as diverse as identifying cell cultures, determining family relationships in studies of animal behaviour, immigration problems to identify criminals or murderer, disputed parentage and in forensic medicine. The most accurate method of identification technique based on recombinant technology is called DNA finger printing or DNA typing or DNA profiling. Principle DNA finger printing is based on sequence polymorphism that occurs in human genome and the genome of other organisms. The sequence polymorphisms are slight sequence differences, usually single base pair changes, that occur from individuals to individuals once in every few hundred base pairs on average. Each difference from the consensus human genome sequence is generally present in only a fraction of the human population but every individual has some of them. These polymorphic locus is called minisatellite or VNTR (variable numbers tandem repeat) thus forming a haplotype which shows mendelian inheritance among their offsprings. This locus is made up of a variable number of identical sequences joint together in tandem. One family of minisatellites in the human genome share a common “Core” sequence, The core is G-C rich sequence of 10-15 bp showing on asymmetry of purine/ pyrimidine distribution on the two strands. These repeats are written as (C-A) n (G-T)n, occur in 100000 blocks in every genome and appear to be uniformly distributed throughout the genome (value of n varies from 1 to 40). The successful application of these (C-A)n (G-T)n repeats has led to the use of a variety of other di-, tri- and tetranucleotide sequences for mapping. Technique (1) DNA of the sample is first isolated whose DNA finger printing has to be made. Usually in forensic case the DNA is prepared from dried stains, sperms
150 in vaginal swabs that had been stored for as long as two years. Sufficient DNA can be isolated from freshly pulled hair roots, polymorphism in mitocondrial DNA and class II HELA gene DQ have been analysed from the shed hairs of several months old containing less than 1 ng of DNA. (2) Sufficient quantities of intact DNA from forensic samples will always be a problem. PGR may have a great impact in this area. From a small quantities of DNA PCR can produce a large number DNA. These are used as probes. This probe is more redioactive. (3) The DNA from the individual whose DNA is to be compared with the forensic sample is isolated and are cut into fragments by restriction enzymes. (4) DNA fragments after digestion of DNA from the genome are first separated according to their size by agarose gel electrophoresis. These ds DNA are denatured by soaking the gel in alkali to make it ss DNA. (5) The DNA fragments are transferred to nito-cellulose paper by the southern blot technique. The paper is then immersed in a solution containing a radioactively labeled DNA probe. Fragments to which the probe hybridizes are revealed by autoradiography. Detection of forensic problem : The power of DNA fingerprinting was demonstrated by Alec Jeffreys in 1985, when a man had been aceured of two rape murders committed three years apart and had made a confussion. Lastly the real murderer was caught and DNA finger printing confirmed the identification. DNA from a semen sample obtained from a rape and murder victim was analysed along the DNA samples from the victim and two suspects. Each of the DNA samples was cleaved into fragments and separated by gel electrophoresis. Radioactive DNA probes were used to identify a small subset of these fragments that contained sequences complimentary to the probe. The sizes of the fragments identified varied from one individual to the next. The different patterns for the three individuals (victim and two suspects) tested. One rape suspects DNA exhibits a banding pattern identical to that of the semen sample taken from the victim. More than one probe may be used to make a positive identification. 9.3 Separation of chromosomes by flow cytometry The dividing cells with condensed chromosomes are carefully broken open so that a mixture of intact chromosomes is obtained. The chromosomes are then stained with fluorescent dye. The amount of dye that a chromosome binds
151 depends on its size. Thus larger chromosomes bind more dye and fluoresence more brightly than smaller ones. The mixture of chromosomes is diluted and passed through a fine aperture, producing a stream of droplets, each one containing a single chromosome. The.droplets are passed through a detector that measures the amount of fluorescence and thus identifies which droplits contain the particular chromosome (being sought). An electric change is applied to these droplets by a charger and then the droplets reach the electric plates, the changed ones are deflected into a separate beaker. Fig. 9.3 Flow cytometry

152 If two chromosomes are of equal size as in human chromosome number 21 and 22, then the dye, tioechst 33258 and chlomomycin-A preferably bind to the A-T and G-C rich DNA respectively and by this differential staining activity, these chromosomes can be distinguished properly (Fig. 9.3). 9.4 Chromosome painting This is a method for visualizing each of the chromosomes in distinct bright colours and thus it simplifies greatly the distinction between chromosomes of similar size and shape and the karyotyping of the chromosome. Usually such a painting is done at the stage in the cell cycle (mitosis) when chromosomes are specially compact and easy to visualize, usually at mitosis. But sometimes, selective chromosome painting may be done in the interphase stage to see its orientation in the nucleus. Method (1) The probes which are used for chromosome painting are specific for sites scattered along the length of each chromosome. (2) The probes are labeled with one of two dyes that fluorence at different wave lengths. For example, DNA molecules derived from chromosome-1 are labeled with one specific dye combination, chromosome-2 with another and so on. (3) The labeled probe can hybridize only the chromosome from which it was derived, each chromosome is differently labeled. After the probes are hybridized to chromosomes the excess is removed, the sample is placed in a fluorescent microscope in which a detector determines the fraction of each dye present at each fluorescing position in the microscopic field. The information may be conveyed, to a computer and a special program assigns a false colour image to each type of chromosome. Use Chromosome painting is very useful in karyotyping the chromosomes. It can be done in interphase stage to locate the specific chromosomes and their arrangement in the nucleus. A combination of chromosome banding with FISH, called multicolor FISH can detect chromosomal translocations which are associated with certain genetic disorder and specific types of cancers. For example, in chronic myeloid leukemia (CML), the lymphocytes contain the Philadelphia chromosome(small), which is produced by the translocation of chromosome no-22 and chromosome no-9. The translocations can be detected by classical banding analysis technique.

153 Unit 10 p Genome Analysis Structure 10.1 C-value paradox 10.2 Satellite DNA 10.3 Complexity 10.1 C-value paradox The haploid DNA content in an individual is described as its C-value. The anomalies of the gene contents by two different methods one on the basis of knowledge about the rate of mutation per locus and other on the basis of general method used for DNA content, is called C-value paradox. DNA content in eukaryotic cells are much higher than that in the prokaryotic cells and a wide range of variations are observed among different species even among same species. The content of DNA also depends on the number of chromosomes in the cells (i.e., ploidy of the chromosome). Example : See Table 10.1. Table 10.1 DNA content of some organisms Class of Species Haploid DNA Dalton Base pairs organisms (Picogram) Phages ? × 174 2.6 × 10 -6 1.7 × 10 6 5400 T4 20.7 × lO -5 126 × 10 6 200000 Animal virus Adenovirus 21.7 × 10 -6 13 × 10 6 21000 Prokaryotes E. coli 4.4 × 10 -3 2.7 × 10 9 4.2 × l0 6 . Unicellular- eukaryotes S. cerevisial 14 × lO -3 8.5 × 10 9 1.4 × l0 7 Multicellular enkaryotes D. melanogaster 0.18 0.11 × 10 12 0.17 × 10 9 Homo sapiens 2.8 18 × 10 12 2.8 × 10 9 C-value paradox takes its name from the inability to explain the content of a genome in terms of an anticipated function. There are two aspects of the

154 paradox. First there are huge variations in C-values between certain species whose apparent complexity does not vary correspondingly. There can be rather substantial variations even between certain closely related species. The range of C-values is found in different evolutionary phyla. There is some increase in the minimum genome size that is found in each phylum as the complexity increases. For example, in prokaryotes, the genome size are very small. In eukaryotes, a vast increase occurs in genome size. In yeast, Saccharomyces cerevisiae has a genome size of 2.3 ×]0 7 bp, only 5 times greater than that of E. colt. The modest increase in genome size just over two folds is adequate to support the slime mold, D discoideum, able to live in either unicellular or multicellular modes. Another increase in complexity is necessary to produce the first fully metazoan organisms. For example, C. elegans has a DNA content of 8 × 10 7 bp. Then any close relationship between complexity of the organisms and content of DNA is obscure, although it is necessary to have a genome of more than I0 8 bp, to make an insect of more than 4 × 10 8 bp, to assemble an echinoderm of more than 8 x 10 8 bp, to produce a bird or amphibians and more than 2 × 10 8 bp to develop into a mammals. In some cases the spread of genome size is quite small. For example, birds, reptiles and mammals, all show a little variation within the phylum, with a range of genome size in each case about two fold. But in other cases, there is quite a wide range of values, often more than 10 folds. This reflects some surprizing discrepencies between genome size and complexity of the organism. An extraordinary C-value is found in amphibian where the smallest is below 10 bp while the largest are almost 10 11 bp. It is hard to believe that this could reflect a 100 fold variation in number of genes in different amphibians. There are some cases where rather closely related species show surprising variations in total genome size. For example two amphibian species may have 10 fold increase where morphologies are very similar. Yet if the gene number is roughly similar most of the DNA in the species with the larger genome cannot be concerned with coding for protein. So the question, what could be its function? The second aspect of C-value paradox is the apparent absolute excess of DNA compared with the amount that could be expected to code for proteins. Actually, eukaryotic DNA has an excess length and the excess is encountered because genes are much larger than the sequences needed to code for proteins. For example, human haploid cell has DNA amount equal to 1.8 x]0 12 dalton = 87 cm of DNA which is equal to 2.8 x 10 9 base pairs, then this genome could contain approximately as many as 3 x 10 6 genes assuming 1000 bp per gene coding for nearly 300 amino acids.

155 However, the number of genes estimated in humans on the basis of the rate of mutation per locus, as estimated by Muller (1967), the frequency of deleterious mutations per locus in human is 10 -5 to 10 -6 in each generation. If the number of gene is 3 × 10 6 (as calculated in general method), then it will yield 30 deleterious mutaions in each generations at the rate of 10 -5 mutations per locus. This will be an unbearable genetic load. The actual frequency of deleterious mutations per generation per individual has been estimated to be 0.5 against an expected frequency of 30. This mutation frequency at the rate of 10 -5 per locus will be an estimate of 5 x 10 4 genes. Thus it is supposed that the actual number of genes in human should be 50000 and not 3 million an estimated from DNA content. This anomalous situation has been described by some workers as C-value paradox. 10.2 Satellite DNA Large proportions of DNA in eukaryotes has been shown to be present in the form of multiple copies of identical DNA sequences, thus is called repetitive DNA or Satellite DNA. The remaining DNA in the cell is found in the form of single copy of DNA sequences which is known as unique DNA. When the denatured DNA (single stranded) is led to reassociate, then it was observed that from the heterogenous populations, the smaller molecular weight DNA associate easily. Britten and his Coworkers (1966, 68) have demonstrated that many vartebrate DNAs reassociates easily when it is broken into small pieces. This observation gave rise to the hypothesis that certain short sequences of bases are repeated hundred times in DNA, this is the satellite DNA. This repetivive DNA generally contributes at least 20% DNA and can reach upto 90% in some cases. It is believed that these repetitive sequences do not carry any genetic informations and therefore do not form genes, but play some other structural or regulatory role. Repetitive DNA consists of short identical genes which are repeated in tandem, several hundred or thousand times. Such DNA is found in the region of the chromosome adjacent to the centromere. In many case the base compositions of the repeating sequences are unlike that of the rest of the DNA. It is, therefore easy to separate repetitive DNA by ultracentrifugation. The satellite DNA can be isolated by density gradient centrifugation in neutral caesium chloride as they have distinctive bouyant densities. The fractions can be separated as a band from the main band of DNA, this band is called satellite band.

156 The satellite band in found on the left of the main band if lighter and on the right side if havier than the DNA of the main band. In Drosophila virilis, there are three highly repetitive DNA each consisting of a repeating sequence of seven nucleotide pair and about 25%. of the DNA is satellite DNA (See Table 10.2). Table 10.2 Satellite DNA of Drosophila virilis (Gall et al., 1974) Repetitive DNA Bauyont density Repeat sequence Sat DNA-I 1.692 5' ACAAACT 3' 3' T ' G ' T ' T ' T ' G ' A ' 5' Sat DNA-II 1.688 5' ATAAACT 3' 3' T ' A ' T ' T ' T ' G ' A ' 5' Sat DNA-III 1.671 5' ATAAATT 3' 3' T ' A ' T ' T ' T ' A ' A ' 5' In human, 30% of DNA is repetitive and in designated as sat I, II and III. In mouse, 10% of the DNA is highly repetitive and renaturates within a few second, 20% is moderately repetitive and reassociates at an intermediate rate, 70% is-single copy DNA which renaturates very slowly. There are about a million copies of repetiting sequences of about 300 bp. In prokaryotes, the repeated base sequence in not found. Two remarkable features of satelite DNA are— (I) Remakable (relative) uniformity within the same species. (II) Great variability between closely related species. The satellite DNA often lies in heterochromatic region of chromosomes and its location can be demonstrated by cytological hybridization by incubating the cells in the radioactive solution and is determined by a autoradiography. The function of highly repetitive DNA is unknown. This can replicate but cannot transcribe RNA for protein synthesis. This is probably because the short sequences lack promoter sites on which RNA chains can be initiated by RNA polymerase. Repetitive DNA is, therefore, inert and is partly dispensible. In the African clawed toad, Xenopus laevis, the genes for 40s precursor RNA which give rise to 28s and 18s RNA are repeated about 450 times. The genes are tandemly arranged and are separated by a spacer region of about 5000 bp. Genes for 5s r RNA are also separated by spacer regions and are arranged
157 in clusters of 100 to 1000 repetitive units at the ends of the most of the 18 chromosomes. 10.3 Complexity Complexity is the total length of different sequences of DNA present in a given proparation. The double stranded DNA is denatured and converted into single stranded DNA by heating the DNA solution. This is accompanied with increase in optical density, which is called hyperchromicity. Again when it is allowed to cool, the single stranded DNA is transformed into a double stranded DNA, again the optical density is decreased, this is called hypochromicity. The 50% resaturation is achieved usually at a specifie temperature which is called melting temperature (Im). The formation of double stranded DNA is actually measured over different values of a parameter which is described as C () . t (concentration x time). It is the product of DNA concentration and time of incubation in a reassociation reaction. Complexity of the genome can be described under two heads— A. Kinetie complexity The reassociation of DNA in the solution depends on the random collisions betwe en the complementary strands, which follow the second order of kinetics, since concentration of both the complementary strand will influence the rate of reaction. The rate of reaction, when expressed through differential calculus is as follows :— dc dt Kc ? ? 2 where, C = Concentration of single stranted DNA at time 't' K = reassociation rate constant or
z ? ? z
dc c K dt 2 or, ? ? ? ? 1 C Kt A A is constant or, 1 C Kt A ? ? When t = o, then C = C 0 Now, 1 0 C KXO A ? ?
158 or, A C ? ? 1 0 The equation is 1 1 0 C Kt C ? ? When the reaction is half complete then time is (t 1/2) and in t 1/2 time the concentration is C. Then— 1 1 1 2 0 C Kt C ? ? / or, C C k C t 0 0 1 2 1 ? ? . . / We know C = 1 2 0 C then, 1 + K.C 0 .t 1/2 = 2 or, K C t ? 1 0 1 2 . / or, C 0 . t 1/2 = 1 k So, during reassociation of DNA occurs at the rate constant K (nt. moles/ lit/see) in equal to the receprocals of C 0 .t 1/2 C 0 . t 1/2 is the product of DNA concentration and time (t) required to proceed to half completion of the reaction; it is directly proportional to the unique length of reassociating DNA. The C 0 .t 1/2 of a reaction indicates the total length of different sequence that are present. This is described as the complixity. It is usually given in base pairs, but can be expressed in daltons or any other mass unit. A higher C 0 . t 1/2 means, slower reaction and lower C 0 . t 1/2 means faster reaction. If there is no repetitive DNA (because the repetitive DNA reassociates faster), the C 0 . t 1/2 of a reaction will be directly prortional to the DNA content. In view of this C 0 . t 1/2 will indicate the length of all the different sequences in a genome, which will be less than the length of the total DNA in a genome when

159 there is repetition. This is called kinetie complexity. Kinetie complexity is the complexity of a DNA component measured by the kineties of the DNA association. This can be calculated by knowing the C 0 . t 1/2 . For example, E. coli has a genome = 0.004 pg DNA = 4.2 × 10 6 bp with C 0 . t 1/2 .= 4. The Kinetie complexity of the genome ? ? C t of DNAof anygenome basepair C t of genome 0 1 2 0 1 2 . . / / E.coil ? ? ? 42 10 4 6 0 1 2 . . / C t of thegenome In eukaryotes, the genome contain more than one pure components. For example. calf DNA has two component, each with characteristics C o .t |/2 value. In wheat, more than two such components are found. Proportion of each component is determined by using the formula 1 0 ? C C , where C = concentration at t 1/2 for corresponding component. Form this proportion, chemical complexity on the component can be determined. B. Chemical Complexity : Chemical complexity is the amount of a DNA component measured by chemical assay. For example, if the genome size is 12 × 10 8 bp and the component represents 25% of the genome then the chemical complixity of this component is 3 x 10 8 bp. Chemical complixity = size of the genome × % of the component in this group. If the kinetic complexity is known from earlier equation, then repetition frequency (f) of repetitive DNA component can be determined using the following formula–––– f chemicalcomplexity kinetiecomplexity C t of nonrepetitiveDNA C t of repetitveDNA ? ? 0 1 2 0 1 2 . . / / Following table shows the reassociation of a eukaryotic genome starting at a C 0 . t of 10 4 and terminating at C 0 .t of 10 4 . Reaction falls into three types of components and their results are as follows—

160 Fast Intermediate Slow Component Component Component % of genome 25 30 15 C 0 . t 1/2 0.0013 1.9 630 ECinetie complexity (bp) 340 6 × 10 5 3 x 10 8 Repetition frequency 500000 350 1 There is a good relationship between the kinetic complexity and chemical complexity of eukaryotic genome. Usually E. coli is used as a standard. Its components are taken to identical with the length of the genome. Thus, complexity of any DNA can be determined by compairing its C 0 . t 1/2 with that of standard DNA of know DNA complexity. C t of DNAof anygenome C t DNA Complexityof naygenome bp 0 1 2 0 1 2 6 42 10 . ( ) . ( ) . / /

E.coli ? ?

According to the table, the slow component represents 45% of the total DNA, so the concentration in the reassociation reaction is 0.45 of the measured concentration (total amount of DNA). If DNA were isolated as a pure component, free of other fractions, it would renature with C 0 . t 1/2 of 0.45 × 630 = 283. Suppose that under these conditions E. coli DNA reassociates with a C 0 . t 1/ 2 of 4.0, Comparing these two clues, we see : The kinetie complexity ? ? ? C t of DNAof anygenome 0 1 2 6 42 10 4 . . / ? ? ? ? 045 630 42 10 4 6 . . = 3 × 10 8 bp Then the whole genome is? ? ? ? ? 3 10 1 045 66 10 8 8 . . bp First component complexity ? ? ? ? ? 00013 025 42 10 4 340 6 . . . Second component complexity ? ? ? ? ? ? 19 030 42 10 4 6 10 6 5 . . .

161 Reversing the argument if we took three DNA preparations, each containing a unique sequence of the appropriate length 340 bp, 6 × 10 5 bp and 3 × 10 8 bp respectively and mix them in the proportions = 25 : 30 : 45, each would renature as though it was a single component, together the mixture would display the same kineties as those determined for the whole genome. Non repetitive DNA complexity can estimate the genome size. The complexity of the slow components comprise sequences that are unique in the genome upon denaturation each single stranded sequence is able to renaturate only with the corresponding complementary sequences. It is usually the major component in eukaryotis. It is called non-repetitive DNA. According to the table the compexity of non-repetitive DNA is 3 × 10 8 bp. If this fraction is unique and represent 45% of the genome, then the whole genome would have a size of 3 × 10 8 ÷ 0.45 = 6.6 × 10 8 bp. This provides an independent assessment of genome size. The value is approximately 7 × 10 8 , obtained from the result of chemical complexity. Eukaryotic genomes certainly contain repetitive sequences. Intermediate component occupies 30% of the genome. According to chemical complexity the total amount is 0.30 × 7 × 10 8 = 2.1 x 10 8 bp. But kinetic complexity of this component is only 6 × 10 5 pp. Thus repetition frequency = ? ? ? ? 21 10 6 10 350 8 5 . Thus intermediate components behaves as though consisting of a sequence of 6 × 10 5 bp that present in 350 copies in every genome. Repetition frequency (f) is the number of copies present per genome. Highly repetitive DNA takes the name from the very large number of copies of the basic reassociating sequence present. The fast component consists 310 bp long in 500000 copies per genome. Because of the short length of the reassociating unit sometimes this is also referred to as simple sequence DNA. The repetition frequency (f) ? C t of nonrepetitiveDNA C t of repetitiveDNA 0 1 2 0 1 2 . . / / ? ? 630 00013 500000 . (.) Approx

162 Unit 11 p Linkage Map, Cytogenetic Mapping Structure 11.1 Physical and molecular maps 11.2 Restriction mapping of genes 11.3 DNA foot printing 11.4 Micro satellite mapping 11.1 Physical and molecular maps STS, i.e. Sequence Tagged Site is a short DNA sequence generally between 100-500 bp in length that is easily recognisable and occurs in the chromosome only once (i.e. unique). STS mapping is a physical mapping procedure that locates the positions of sequence tagged sites (STSs) in a genome. 11.1.1 Qualities of STS (1) Its sequence must be known, so that a PCR (polymerase chain reaction) assay can be set up to test the presence or absence of the STS on different DNA fragments. (2) It must have a unique location in the chromosome being studied once in the genome. If the STS sequence occurs in more than one position, then the mapping data will be ambiguous. So STSs do not include sequences found in repetitive DNA. 11.1.2 Sources of STS (1) Expressed sequence tags (ESTs) : These are short sequences obtained by the analysis of cDNA clones. cDNA is prepared by converting mRNA into dsDNA. Thus ESTs represent the genes that are expressed in the cell. (2) SS4Ps : These are also used in genetic mapping as well as physical mapping of genes. (3) Random genomic sequences (RGS) : These are obtained by sequencing random pieces of cloned genomic DNA. 11.1.3 Principles of STS mapping (1) To map a set of STSs, a collection'of overlapping DNA fragments from single chromosome or from the entire genome is needed. (2) The data from which the map will be derived are obtained by determining fragments which contain STSs. This can be done by hybridization analysis but PCR is generally used which is quieter and automated process.

163 (3) The changes of two STSs being present on the same fragment will depend on how close they are in the genome. If they are very close then there is a good chance that they will be on the same fragment, if they are further apart, then they may be on the same fragment or not. (4) The data can be used to calculate the distance between two markers, where map distance is based on the frequency at which breaks occur between two markers. 11.1.4 Physical mapping of chromosomes by screening YAC clones of STSs Segments of human DNA upto 1000 kb long can be cloned in yeast artificial chromosomes (YACs). (1) Aliquots of DNA prepared from each YAC clone viz., A, B and C are

164 subjected to PCR amplification using primer pairs (1-6) corresponding to the ends of various STSs. Only those clones containing STSs with ends complementary to particular primers will be amplified. (2) Electrophoretic analysis then shows that YAC clones contain STSs. (3) The illustration is very simple showing 6 primer pairs. Clone-A contains STSs no. 1,3 and 5; clone-B contains 2 and 1 and clone-C contains 3, 4, 5 and 6 primer pairs. (4) The three YAC clones can be ordered showing their relative positions. In the mapping of human chromosome-21, about 1,20,000 clones from two separate YAC libraries were screened. In addition, 14,000 YACs isolated from a library prepared specifically from chromosome-21 were screened individually. By the use of 198 STSs, researchers identified 810 positive clones and ordered them into a contiguous map. Fig. 11.2 Ordering of contiguous overlapping YAC

165 11.1.5 Fragments of DNA for STS mapping At first, collection of DNA fragments sparing the chromosome or genome is required. The collection is called mapping reagent. There are two method as follows— A. Radiation hybrid method : (1) Human cells are exposed to X-ray doses of 3000-8000 rads, which causes the chromosomes to break randomly into fragments. Higher doses produce smaller fragments. (2) These fragments can be propagated if the irradiated cell is fused with non-irradiated hamster (or other rodent) cells. Fusion is achieved chemically with polyethylene glycol or by exposure to Sendai virus. (3) The hamster cell line that is unable to make either thymidine kinase (TK) or hypoxanthine phosphoribosyl transferase (HPRT) is used for the purpose. Those cells incapable of taking up human chromosome fragments are unable to survive in HAT (hypoxanthine, aminopterin and thymidine) medium. Those cells taking up chromosome fragments can synthesize TK and HPRT and are able to grow in HAT. Thus the hybrid cells are collected. These hybrid cells are then used as a mapping reagent in STS mapping. (4) To map, as many markers as possible are us.ed. A pair of primers is designed for every DNA marker that was to be tested. Each primer pair is specific for one marker and will not give a PCR product with any other part of the genome. (5) The success or failure of PCR is determined by agarose gel electrophoresis. The presence of a band of the expected size in the gel indicates that the PCR has worked. (6) Another procedure has been designed where a third specific primer for each marker is added to the reaction mixture along with a fluorescently labelled dideoxy-nucleotide. If PCR has been successful, then the third primer anneals'to the product and is extended by the fluorescent dideoxy nucleotide and emits signals from this. B. Use of clone library as the mapping reagent for STS analysis A clone library can also be used as a mapping reagent in STS analysis. The clone library is prepared by using the genome or chromosome which is broken into fragments and are integrated into a high capacity vector. The single specific chromosome can be isolated by flow cytometry technique and a clone library of a chromosome is made possible. The data obtained from STS analysis is used for preparation of the physical map.

166 11.2 Restriction mapping of genes Restriction mapping of genes is a physical mapping which locates the relative positions on a DNA molecule of the recognition sequences for restriction endonucleases. Genetic mapping using RFLPs as DNA markers can locate the positions of polymorphic restriction sites within a genome, but very few of the restriction sites in a genome are polymorphic, so many sites are not mapped by this technique. Restriction mapping is very useful to solve a problem but the limitation of the technique is that it is applicable only to relatively small DNA molecules. Two methods are employed for restriction mapping viz., partial digestion method and double digestion method. (1) In partial digestion method, the circular or linear DNA is treated with a particular restriction enzyme. But the enzyme is prevented from the complete digestion of DNA molecule. So the DNA is incubated for a short time or using a suboptimal incubation temperature. This leads to the partial digestion and will produce many cut products and uncut products. If the DNA molecule is linear, the terminal end of the DNA is labeled with P 32 by the polynucleotide kinase reaction prior to cleavage and only radioactively labelled fragments are considered in agarose get electrophoresis, the other fragments are ignored/discarded of the molecular weight of each enhanced band is invariably the sum of the molecular weights of two fragments which arc considered to be adjacent. Thus the relative positions of the fragments can be ordered. (2) Second method of the restriction mapping is the double digestion method, although partial digestion method is usually followed. In double digestion method, three samples of a particular DNA species are taken. Each two of these is treated with two separate restriction enzymes and the third sample is treated with both the restriction enzymes. Thus the three sets of fragments are compared following the agarose gel electrophoresis. The terminal end of the DNA molecule is also labeled with radioactive P 32 prior to the restriction enzyme cleavage. 11.3 DNA foot printing When transcription factor binds to a DNA sequence, it protects that sequence from digestion by nucleases. Researchers take advantage of this property by isolating chromatin from cells and treating it with DN-digesting enzymes, such as DNA ase-I, that destroy sections of the DNA that are not protected by bound transcription factors. Once the chromatin has been digested, the bound protein

167 is removed and the DNA sequences that had been protected are identified. This method is called DNA foot printing. This is also used to locate the binding sites of proteins on RNA. Method (1) A pure DNA fragment that is labeled at one end with 32 P is isolated. (2) This molecule is then cleaved with a nuclease or a chemical that makes random single-stranded cuts in the DNA. (3) The DNA molecule is then denatured to separate into two strands. (4) The resultant fragments from the labeled strand are separated on a gel and detected by autoradiography. The pattern of bands from DNA cut in the presence of a DNA-binding protein is then compared with that from DNA cut in its absence. The protein covers the nucleotides at its binding site and protects from DNA ase. The labeled fragments that shows no cleavage will show an area which is missing in the electrophoretic gel, is leaving a gap is called “foot print”. 11.4 Gene mapping by human pedigree analysis (microsatellite mapping) Recombination (CO) mapping is very difficult in human because (1) It is impossible to preselect the genotypes of parents and set up crosses. (2) The data for the calculation of recombination frequencies have to be obtained by examining the genotypes of the members of successive generations of existing families. (3) The data obtained are very limited and their interpretation is often - difficult because in human test cross rarely occurs and the number of family members and offsprings are not many. Therefore, gene mapping in human may be done by pedigree analysis. Let us, suppose a family of two parents and six children were studied with a genetic disease. The diseased state is due to one allele and the healthy state is due to second allele. Diseased allele is dominant over healthy allele. The pedigree showed that mother is affected because four of her children are affected by the disease The grand mother is affected. The grand father is dead but? We can assume that he was also affected. We can include them in the pedigree analysis.

168 The aim is to map the position of the gene for the genetic disease. For that purpose one is studying its linkage to a microsatellite marker M, four alleles of which viz., M 1 , M 2 , M 3 and M 4 are present in the living family members. One has now to calculate the number of children who are recombinants. Fig. 11.3 Pedigree of a family with inheritance of genetie disease (solid means affected person) The pedigree can be interpreted by two different hypotheses because child- 1, 2 and 3 have the disease allele and microsatellite allele M], the child-4 and 5 have the healthy allele and microsatellite allele M 2 . Disease allele – M 1 In hypotheses-1, the mother would have the genotype = Healthy allele - M 2 The child no-1, 2, 3, 4 and 5 all would have parental genotype. Only the child no-6 would be a recombinant. The recombination percentage is 16.66 i.e., the disease gene and the microsatellite allele are relatively closely linked. Healthy - M 1 In hypothesis- II, the genotype of the mother would be = Disease - M 2 Here the child no-1, 2, 3, 4, 5 are recombinants while child no-6 is with parental genotype. The recombination percentage is 88.33, which means that the disease gene and microsatellite gene are far apart on the chromosome.

169 Possible genotypes of the mother Hypothesis-I Hypothesis-II Disease-M 1 Healthy-M 1 Child-1 Disease-M 1 Healthy-M 2 Disease-M 2 Child-2 Disease-M 1 Parental Recombinant Child-3 Disease-M 1 Parental Recombinant Child-4 Healthy-M 2 Parental Recombinant Child-5 Healthy-M 2 Parental Recombinant Child-6 Disease-M 2 Recombinant Parental Recombination frequency 16.66% 83.33% Fig. 11.4 Probable interpretation of the pedigree Imperfect pedigrees are analysed statistically by using a measure called "lod score" (Morton, 1955). This stands for logarithms of the odds that genes are linked. This is used to determine whether the two markers lie on the same chromosome or not. If lod analysis establishes the linkage then the data will give confidence about their recombination frequencies. If the number of the family members are larger the result would be more satisfactory. Atleast three generations are to be tested. Atleast four grand parents and atleast eight second generation children could be sampled.

170 Unit 12 p Genetics of Cell Cycle Structure 12.1

Genetic regulation of cell division in yeast and eukaryotes 12.2 Molecular basis of cellular

Checkpoints 12.1 Genetic regulation of cell division in yeast and eukaryotes 12.1.1 Introduction A cell reproduces by performing an orderly sequence of events in which it duplicates its contents and then divides into two. This cycle of duplication and division is known as the cell cycle. The basic organization of the cell cycle and its control system are essentially the same in all eukaryotic cells. Three eukaryotic systems in which cell-cycle is commonly studied are yeasts, frog embryo and cultured mammalian cells. Yeasts are tiny, single-celled fungi. Two species are generally used in studies of cell cycle. The fission yeast viz. Schizosaccharomyces Pombe, is a rod shaped cell that grows by elongation at its ends. Division occurs by the formation of a septum or cell plate in the centre of the rod. It has a typical eukaryotic cell cycle with G,, S, G 2 and M phases. In contrast with that happening in higher eukaryotic cells, the nuclear envelope of the yeast cell does not break down during M- phase. The microtubules of the mitotic spindle are formed inside the nucleus and are attached to spindle pole bodies (SPB) at its periphery. The cell devides by forming a partition (cell plate) and splitting into two. The mitotic chromosomes are readily visible. The budding yeast Saccharomyces cerevisiae, also called baker's yeast is a oval cell and divides by forming buds which first appears during G 1 and grows until it separates from the mother cell after mitosis. It has normal G 1 and S-phase but does not have a normal G 2 phase. The microtubule based spindle begins to form inside the nucleus early in the cycle during S-phase. Nuclear envelope remains intact during mitosis and the spindle forms within the nucleus. 12.1.2 Genetic regulation of cell cycle in S.pombe (Fig. 12.1) (1) ede 2 is identified as a crucial regulator by its involvement at both stages of cell cycle block, i.e. between G 2 and M-phase and in G 1 at start.

171 Fig. 12.1 The cell cycle in S. pombe requires cdc genes to pass specific stages, but may be retarded by genes that respond to cell size (wee 1). Cells may be diverted into the mating pathway early in G1 (2) During G 2 and M-phase cdc 2 has a partner which is the product of Cdc 13 generating an M-phase kinase (resembling the p 34 -B cyclin dimer of animal cells). The activity of the cdc 2 catalytic subunit in these dimers are controlled by phosphorylation (in the same way as p 34 in animal cell). But the difference that in yeast there is no Thr-14 so there are only two relevant sites Tyr-15 where phosphorylation is inhibitory and Thr-161 where phosphorylation is required. (3) Under normal conditions, the cell division cycle is related to the size of the cell. In the poor growth condition the cells increase in size more slowly. The genes involved in control cell size are identified, wee 1 usually inhibits cells from initiating mitosis until their size is adequate. It has been suggested that wee 1 is part of a check point that prevents the activation of cdc 2 until an adequate mass has been attained. wee 1 codes for a kinase and can phosphorylate serine or

172 threonine and tyrosine. It inactivates cdc 2 by phyrophorylating Tyr-15. Another gene viz., mik 1 has similar effects. (4) The product of cdc 25 is required for the dephosphorylation of cdc 2 in the cdc 2/cdc 13 dimer. It is probably responsible for the key dephosphorylating event in activating the M-phase kinase. The level of cdc 25 increases at mitosis and its accumulation over a threshold level could be important, cdc 25 executes the checkpoint that ensures s-phase to be completed before M-phase can be activated. (5) The products of wee 1 and cdc 25 plays antagonistic roles. The kinase activity of wee I acts on Tyr-15 to inhibit cdc 2 function. The phosphatase activity of cdc 25 acts on the same site to activate cdc 2. (6) During mitosis the cdc 2 of cdc 2/cdc 13 dimer is in the active state that locks the phosphate at Tyr 15 and has the phosphate at Thr 161. At the end of mitosis kinase activity is lost and cdc 13 is degraded cdc 2 does not change at this point. (7) During G, the active form of cdc 2 has a different partner, the B-like cyclin, cig 2 encoded by cig 2 gene. The dimer in converted from inactive state to the active state by dephosphorylation of Tyr-15 residue of cdc 2. (8) Progression of Gj into S is controlled by activation of cdc 2-G| cyclin. In S. pombe it is cdc 2/ cig 2. (9) Transcription of cdc 18 is activated as a consequence of passing START and cdc 18 is required to enter S-phase. Over expression of cdc 18 allows multiple cycle of DNA replication without mitosis. (10) For cdc 18 to be active, cdc 2/cdc 13 must be inactive. Again when M- phase kinase is active, it causes cdc 18 to be inactive possibly by phosphorylating it and prevents initiation of another S-phase. (11) Activity of cdc 2/cdc 13 M phase kinase is influenced by a factor rurn-1, which controls entry into S-phase. When rum-1 is depleted, premature entrance into mitosis occurs and over expression causes cells to fail to enter mitosis. This suggests that M m _ 1 is an inhibitor of the M-phase kinase. It is expressed between G 1 and G 2 and keep M-phase kinase in an inactive state. 12.1.3 Genetic regulation of cell cycle in S.cerevisiae (Fig. 12.2) Cell cycle in S.cerevisiae consists of three cycles that separate after START and join before cytokinesis. The cells may be diverted into the mating pathway early in G 1 .

173 (1) Chromosome cycle: In this cycle, duplication and separation of chromosomes, completion of S-phase and nuclear division occurs. Mutation of cdc 8 stops this cycle in S-phase. Mutation in the chromosome cycle do not stops the cytoplasmic cycle. (2) Cytoplasmic cycle : It consists of bud emergence and nuclear migration into the buds. This cycle can be halted before bud emergence by cdc 24 mutation but the mutation does not prevent chromosome replication. (3) Centrosome cycle : This cycle consists of duplication and separation of spindle polar body (SPB) and organizes microtubules to allow chromosome segregation within the nucleus. Blocking of the cycle by cdc 31 does not prevent S-phase or bud emergence. Completion of entire cell cycle requires all three constituent cycles because nuclokinesis needs both chromosome and centrosome cycles but cytokinesis requires all the 3 cycles. Fig. 12.2 The cell cycle in S. cerevistae consists of three cycles that separate after START and join before cytokinesis. Cells may be diverted into the mating pathway early in G1

174 (1) S. cerevisiae expresses a single cyclin dependent protein kinase (cdk) encoded by cdc 28 gene which interacts with several cyclins during different phases of the cell cycle. (2) Just after cytokinesis, the decision on whether to initiate a division cycle is made before the START. The cells can diverted into mating type pathway by mating factors and cdc 36 and cdc 39 which appear to block the cell cycle before START and really function in the mating type pathway. Mutants block cell cycle by diverting cells into mating even in absence of the mating gene. (3) After cytokinesis the mother ceil and the bud both the cell remain in the G 1 phase of the cell cycle. The ability to pass START is determined by environmental conditions. Prior to bud formation, spindle body duplication and DNA replication, the yeast cell exhausts its nutrients. When S.cerevisiae cells in G 1 have grown sufficiently in the growth medium, they begin a programme of Fig, 12.3 Mechanism of Genetic regulation

175 gene expression that leads to entry into mitosis. Once G 1 cells reach the critical size, they become committed to completing cell cycle even if they are shifted to low nutrient medium. (4) The crucial gene in pasing START is cdc-28 which is homologns to cdc- 2 in S.pombe. Three cyclins are active in G,. CIn 1, Cln 2 and Cln 3 are encoded by CLN-}, CLN-2, CLN-3 respectively. Mutations in any one or two of these genes fail to block the cell cycle, thus the CLN genes are functionally redundant. (5) The complexes formed between cdc 28 and the three G 1 cyclins (Cln 1, Cln 2 and Cln 3) have protein kinase activity and constitute the hypothesized S phase promoting factor (SPF S). In wild type yeast cells, Cln 3 is expressed at a nearly constant level throughout the cell cycle. Cln 1 and Cln 2 are expressed during the second half of G 1 and they increase rapidly and when their accumulation exceeded a critical threshold level, triggers the passage of start (START) into S-phase. After that its concentration declines gradually and are eliminated by the time of mitosis. (6) cdc 28-Cln 3 phosphorylates and activates SBF and MBF. These induce transcription of CLN 1 and CLN 2 genes as well as several other genes required for DNA replication, including genes encoding DNA polymerase, RPA (ssDNA binding proteins), DNA ligase and certain enzymes acquired for deaxyribonucleoside triphosphate synthesis. (7) cdc 28-Cln 1 and ede 28-Cln 2 phosphorylate APC in late G 1 and inactivate it. (8) Two B-type cyclin genes CLB 5 and CLB 6 are also regulated by MBF and transcribed beginning in late Gj. The corresponding proteins Clb 5 and Clb 6 accumulate because of the inactivation of APC. (9) At late G 1 ede 28-Clb 5 and ede 28-Clb 6 heterodimers accumulate and are inactivated by Sic-1 (an S-phase inhibitor), but it has no effect on ede 28-Cln complexes. Sic 1 is degraded falling polyubiquitination by E 2 associated with E 3 . Once Sic is degraded, ede 28-Clb 5 and ede 28-Clb 6 kinases induce DNA replication. (10) Initiation of DNA replication needs both assembly of pre replication complex and an active cdc 28-Cln complex. A second heterodimeric protein kinase ede 7-Dbf 4, which is expressed in G 1 is also required to trigger initiation. Once replication has initiated, Mem proteins and ede 45 move away from the origin along with DNA polymerases. Mem proteins are homologous to helicase, associated with replication fork movements. (11) Later in S-phase, transcription of the genes CLB 3 and CLB 4 begins, encoding two additional B-type cyclins, Clb 3 and Clb 4, which also form

176 Fig. 12.4 Role of cdc in replication heterodimeric protein kinases with cdc 28. These two cdc 28-Clb 3 and cdc 28-Clb 4 complexes also initiate the formation of mitotic spindle at the beginning of mitosis. (12) As cells complete chromosome replication and enter G 2 , two more B- cyclins are expressed. These are Clb 1 and Clb 2 encoded by CLB 1 and CLB 2 genes. These function as mitotic cyclins, associating with cdc 28 to form complexes that arc required for chromosome segregation and nuclear division. 12.2 Molecular basis of cellular checkpoints 12.2.1 Introduction In most cells there are several points in the cell cycle, called checkpoints in which the cycle can be arrested if previous events have not been completed. Four checkpoint control can arrest the passage through cell cycle. These are (a) Gj-arrest due to DNA damage, (b) S-arrest due to unreplicated DNA, (c) C 2 - arrest due to DNA damage, (d) M-arrest due to improper spindle formation. In the checkpoints, the control system can be regulated by extra cellular signals from other cells. These signals either promote or inhibit cell proliferation. Checkpoints generally operate through negative intracellular signals. 12.2.2 The DNA replication checkpoint Most of the cells by DNA replication checkpoint mechanism, avoided entry into cell division until the last nucleotide in the genome has been copied. The

177 Fig. 12.S(b) Mechanism of G 2 arrest checkpoint molecular mechanism has not been discovered but any signal from unreplicated DNA or unfinished replication forks send a negative signal to the cell cycle control system that blocks the activation of M-Cdk. Thus normal cells treated with chemical inhibitors of DNA synthesis viz., hydroxyurea, do not progress

178 Fig. 12.5(c) DNA replication checkpoint into mitosis. The block activates a checkpoint mechanism that arrests the cells in S-phase, delaying mitosis. But if caffeine is added along with hydroxyurea checkpoint mechanism fails and the cells proceed into mitosis according to their normal schedule with incompletely replicated DNA. As a result, the cells die. 12.2.3 The spindle attachment checkpoint The effect of colchicin which inhibits spindle assembly, shows the presence of this checkpoint. In most cell types, a spindle attachment checkpoint mechanism operates to ensure that all chromosomes are properly attached to the spindle before sister chromatid separation occurs. During metaphase kinetochore regions of the chromosomes are attached to microtubules and any kinetochore that is not properly attached to the spindle, sends out a negative wait signal to the cell cycle control system that blocks ede 20-APC activation which is needed for sister chromatid separation. The nature of signal is not clear. But it has been seen that several proteins, including Mad 2 are recruited to unattached kinetochores which

179 are required for spindle attachment checkpoint to function. Even a single unattached kinetochore results inhibition of cdc 20-APC activation by binding with Mad 2. In mice MAD 2 and BUB 1 and in humans MAD 2 genes have been recently identified. MAD 2 protein remains concentrated at the kinetochore until completion of microtubules attachment. This protein is continuously migrating into the cytoplasm and broadcasting signal throughout the cytoplasm. In mammals MAD 2 is associated with p 55 CDC but in budding yeast MAD 2 is with cdc 20 and in fission yeast Mad 2-slp 1 (sip = sleepy) form a large complex with APC. Hct 1, another protein is associated with APC. APC is kept in check by MAD 2 and when APC is active the cell initiate anaphase by catalizing degradation of the two proteins with the help of Hct and cdc 20. cdc 20/slp 1 promotes degradation of CUT2 but the activity of cdc 20/slp 1 is inhibited by MAD 2. Hct 1 promotes degradation of CLB-1, APC catalyzes this degradation by ubiquitination. This ubiquitination is inhibited by spindle attachment checkpoints. Fig. 12.6 DNA damage checkpoint In mammals BUB 1 and BUB 3 proteins along with CNEP-E protein induce changes that lastly shut down the transmission of wait signal. MAD 2 dissociates from p 55 cdc and loses its control on APC. APC is activated and catalyzes breakdown of cyclins and facilitates anaphase separation of chromatid.

180 12.2.4 DNA damage check points The cell cycle control system can readily detect DNA damage and arrest the cell cycle at DNA damage checkpoints. These two checkpoints are one is in late G\ which prevents entry into S-phase and the other is in late G 2 which prevents entry into, mitosis. G 2 checkpoint depends on a (similar) mechanism that delays entry into mitosis in response to incomplete DNA replication. The damaged DNA sends a signal to a series of protein kinases that phosphorylate and inactivate the phosphatase-cdc 25. This blocks the dephosphorylation and activation of M-cdk, thereby blocking entry into mitosis when the damaged DNA is repaired, the inhibitory signal is turned off and the cell division continues. In the yeast S.pombe, the checkpoints sense the DNA damage and transduce inhibitory signal. Four genes including RAD 9, RAD 17, RAD 24 and MEC 3 will sense this damage. The model proposed that DNA damage activates a protein sp Rad 3 (sp prefix means S. pombe). It brings about phosphorylation of Chk 1 and Chk 1- P functions as a kinase, which brings about phosphorylation at Ser 216 of Cdc 25. It then promotes binding of cdc 25 to a protein 14-3-3, coded by rad 24 and rad 25, leading to sequestration of cdc 25. Then cdc 25 is not available for activation of cdc 2 P (tyr l5) and the cell division is arrested at G 2 -M transition. G 1 checkpoint blocks progression into S-phase by inhibition of G 1 S-Cdk and S-Cdk complex. Fig. 12.7 Spindle attachment checkpoint

181 (1) In mammalian cells a gene regulatory protein p 53 is being activated by DNA damage and it stimulates transcription of many genes. (2) A CKI protein, called p 21 is encoded by such activated genes which binds to G 1 /S-Cdk and S-Cdk and inhibits their activity and thus blocks entry into mitosis. (3) Actually in an undamaged cell, p 53 is very unstable and is present at a low concentration. It interacts with another protein Mdm2, that causes destruction of p 53 by ubiquitination mechanism. Damaged DNA activates protein kinase, phosphorylates p 53 and reduce the binding of Mdm2. As a result p 53 concentration rises and stimulates gene transcription of p 21 which inactivates G 1 /S-Cdk and S-Cdk activity. (4) A rare genetic disease, Ataxia telangiectasia is caused by a defect in one of the protein kinases that phosphorylates and activates p 53 in response to radiation and due to the loss of the DNA damage checkpoints, they suffer from increased rate of cancer.

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GROUP B (II) Molecular Biology

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185 Unit 1 p History and Scope of Molecular Biology Molecular Biology is one of the most rapidly growing domains of life sciences in the 21 st century. Though the subject is relatively new in comparison to the specialized areas like—cytology, bacteriology, morphology and embryology; it has a history which is interesting and worth mentioning. Antony van Leeuwenhoek's invention of the microscope in around 1650 opened up the micro-world of biology. Advancement of knowledge in the fields of biochemistry, microbiology, virology and genetics in the early part of the 20 th century gave rise to a new domain of activity which also attracted the attention of chemists and physicists. The remarkable development in physics and technological advancement opened up new frontiers in the biological arena one of which came to be known as Molecular Biology - the term was coined by Warren Weaver of the Rockefeller Foundation. Molecular biology attempts to explain the phenomena of life and its evolution, starting from the macromolecular properties that generate them. The two main macromolecules that contribute in the life process and remains the focus of the molecular biologist are the nucleic acids, (DNA & RNA)- the constituent of genes, and proteins, which are the active ingredients of

life processes. Charles Darwin's publication of On the Origin of Species (1859) had a profound impact on biological thinking. The rediscovery of the work of the Austrian monk Gregor Mendel probably might have been the first impetus to the development of molecular biology. August Weismann's description of reduction division in 1'887 followed by the description of meiosis, spermatogenesis by Walter Sutton (1903) lead to the proposal 'Chromosomal Theory of Inheritance". Acceptance of chromosomal theory of inheritance by 1935 became the stepping stone toward the journey for the search of chemical and physical nature of hereditary factors. Several important developments took place during the early part of the 20 th century that suggested the existence of genes within the chromosomes. Thomas Hunt Morgan (1909) showed that phenotypic change in Drosophila is linked to the events of crossing over in the chromosomes while Alfred Sturtevant (1910) moved a step ahead and mapped gene on chromosomes. Although existence of a new substance called nuclein was identified in the sperm cells by Friedrich Meischer in 1869, which later came to be known as deoxyribonucleic acid (DNA), it was Robert Fuelgen (1924) who first showed the coexistence of DNA along with proteins in
186 chromosomes by cytochemical staining. Appreciation of the nucleic acids quickly led to findings that there are two types of nucleic acids- RNA and DNA that differed in their sugar moieties. In 1929 Phoebus Levene showed that there are four types of DNA molecules - each of which he referred to as nucleotide. Each nucleotide had a deoxy-ribose sugar unit, a phosphate group and a nitrogenous base. The four nitrogenous bases were identified as Adenine, Guanine, Thiamine and Cytosine. He also suggested that the nucleotides are linked together through their phosphate sugar back bone but he made a mistake by considering the nucleotides to be present in short sequences and that the bases repeated in the same fixed order. However, the DNA molecule exists as a polymer was confirmed by Torbjorn Caspersson and Einar Hammersten (1934). During this time Fred Griffith (1928) used pneumoccocus to describe gene transformation and George Beadle & Edward Tatum came up with "one gene - one enzyme" theory to demonstrated the existence of a precise relationship between genes and proteins. Following these discoveries, numerous research groups confirmed the importance of the gene in the life and development of organisms. It became apparently clear that genes are present in chromosomes and chromosomes are made of DNA and proteins. Initially the scientific community could not consider DNA as a hereditary material because of its utter simplicity. Rather, complex nature of the proteins was a preferred candidate to store hereditary information. Nevertheless, the chemical nature of genes and their mechanisms of action remained a mystery. Molecular biologists committed themselves to the determination of the structure of gene and the description of the complex relations between, genes and proteins. In the 50's, two important events took place almost simultaneously. Oswald Avery, Maclyn MacLeod and Colin McCarty in 1944 for the first time came out with the suggestion that genes are made up of DNA. Although their work was severely criticized by the scientific community then, Alfred Hershey and Martha Chase (1952) confirmed through their ingenious experiment that the genetic material of the bacteriophage is DNA. Then came the discovery that revolutionized the world of science. Watson and Crick (1953) published their paper entitled "Molecular structure of Nucleic Acids" Nature 171, 737-738 (1953) where they gave a detail account of the double helix structure of the DNA molecule. The discovery of the double helical structure is history by itself. In brief there were three groups working to elucidate the structure of the DNA molecule. The first group worked at King's College, London and was led by Maurice Wilkins and was later joined by Rosalind Franklin. The second group working on DNA was Francis Crick and James D. Watson was at Cambridge
187 and the third group was at Caltech where the noble laureate Linus Pauling was leading the show. Inspiration and data from the works of Erwin ChargafPs work, published in 1947 and the X-ray diffraction patterns of DNA fibers produced by Maurice Wilkins and Rosalind Franklin at King's College facilitated Watson and Crick to design the double helix model using metal rods and balls in which they incorporated the known chemical structures of the nucleotides, as well as the known position of the linkages joining one nucleotide to the next along the polymer. For their pioneering work, Watson, Crick and Wilkins were awarded Nobel Prize in physiology in the year 1962. In their paper on the structure of DNA double helix, Watson and Crick wrote at the end of the paper, '
It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material'—
a

path breaking thought that allows the preservation of hereditary information from generation to generation. New thought processes began to evolve centering round the DNA molecule. Subsequently, Crick in 1957 proposed the 'Central Dogma' that explains the flow information of genes to proteins and the relationship between DNA, RNA and proteins. Controversies arose regarding the replication mechanism. However, ingenious experiment by Meselson-Stahl put an end to all controversies by showing the DNA replication is semi conservative in nature. Crick and coworkers showed that the genetic code was based on non-overlapping triplets of bases, called codons, and Har Gobind Khorana (1961) and others deciphered the genetic code not long afterward. These findings represent the birth of molecular biology which is still advancing at rapid pace everyday adding new information and creating history. Following the deciphering of the genetic code, Arthur Kornberg described the action of DNA polymerase; Stanley Cohen (1968) discovered plasmids and antibiotic resistance gene. These findings gave birth to recombinant DNA technology which was immediately followed by the development of DNA sequencing technique by Walter Gilbert, allan Maxam, Fred Sanger in the year 1975. Simultaneously, Ceasar Milstein, Geo Kholer and Niles Jeme developed the art of monoclonal antibody production. Split gene concept was put forward by Richard Roberts and Philip Sharp in 1977 that marked the difference between the eukaryotic and prokaryotic genome. Another giant leap was made in the field of molecular biology when Kary Mullis (1985) successfully synthesized DNA polymers in vitro - a technique better known as Polymerase Chain reaction (PCR). Scientist then dared to sequence the 3 billion nucleotides present in human and launched the flamboyant project "Human Genome Project" in the year 1989 with a
188 target to complete the entire human sequence by 2010. Thanks to the technological development and discovery of efficient DNA polymerases that enabled to complete the project in the year 2003. The history does not end here. New branches like proteomics and genomics have come up to understand the functioning of cellular genes and to utilize the information for betterment of the man kind.
189 Unit 2 p DNA Replication Structure 2.1 Introduction 2.2 Semiconservative replication 2.3 DNA replication model 2.4 Replication in eukaryotes 2.5 Mechanism of replication 2.6 DNA polymerases 2.1 Introduction The fundamental biological process of reproduction requires the faithful transmission of genetic information from parent to offspring. Genetic information is stored in the form of an array of nucleotide sequences. The life process has evolved mechanisms to replicate the array of nucleotide sequences with great accuracy, that too at an astounding speed. The single, circular chromosome of E. coli contains about 4.7 million base pairs. Duplicating at a rate of more than 1000 nucleotides per minute, replication of the entire chromosome would require almost 3 days. Yet, these bacteria are capable of dividing every 20 minutes making minimum errors during the replication process. A huge amount of genetic information and an enormous number of cell divisions are required to produce a multicellular adult organism; even a low rate of error during copying would be catastrophic. Interestingly, mechanism to correct base pairing mistakes that occur during the process of replication has evolved in both prokaryotes and eukaryotes. In 1953, Watson and Crick wrote at the end of their paper on the structure of DNA double helix, '
It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material'.
They recognized and explained that an inherent copying mechanism exists in the double helix DNA molecule. During replication, the two strands of the DNA helix unwind and unzip; each strand serves as a template for the new DNA molecule to be synthesized on it - a process termed as semi conservative replication. 2.2 Semiconservative replication Semi conservative replication hypothesize that during replication of DNA double helix the strands unwinds and each strand serves as a template on which

190 new daughter strands are synthesized following the base paring rule. That is, an 'A' at one position on the mother strand signals the addition of a 'T' on the corresponding position on the newly forming strand. Similarly the presence of 'G' on the mother strand will signal the addition of 'C' on the daughter strand. This type of base pairing, which determine the nucieotide sequence of the new strand is known as complementary base pairing. Once the bases are aligned, DNA polymerase enzyme link the new incoming nucieotide with the previous aligned nucieotide of the daughter strand and eventually hydrogen bond is established between the base pairs. The process continues until the entire length of the mother DNA strand is copied. The newly formed DNA helix comprises of a new strand and an old original strand (conserved strand). Such pattern of DNA double helix duplication is called semiconservative replication. However, alternative mechanisms of replication were proposed which are known as conservative replication and dispersive replication (Fig. 2.1). According to conservative replication, the original two strands serve as templates for the formation of new DNA strands. But, one of the double helix would consist entirely of original DNA strands, while the other helix would consists of two newly synthesized strands. Dispersive replication suggests that the two DNA helix formed after replication comprises of interspersed blocks of new and old strands. However, evidences of such type of replication are still lacking. Fig. 2.1 : Proposed mechanisms of DNA replication: Semiconservative, Conservative and Dispersive 2.2.1 Experimental evidence of semiconservative replication In 1958, M. Meselson and F. Stahl performed an imaginative experiment that confirmed the semiconservative nature of DNA replication. They grew E. coti in a medium containing a heavy isotope of nitrogen, I5 N for several generations. After

191 growing for several generations in 15 N medium, practically all nitrogen atoms in the DNA of the bacterial cells were labeled with 15 N. They then transferred some of the cells from 15 N medium to new medium in which the nitrogen was 14 N. The bacteria were allowed to divide for one cycle only. Similarly, in another tube, the cells transferred to 14 N medium and were allowed to divide for two generation only. Any DNA synthesized after the transfer would contain a mixture of light & heavy isotopes. The newly cultured cells were then isolated, their DNA extracted and subjected to equilibrium density gradient centrifugation in Cesium chloride gradient to determine the density of the respective DNAs. (Cesium chloride [CsCl] centri-fuged at 50000 rpm, 250,000g for 2 days that produce the gradient).

192 I5 N I5 N -DNA being heavier, they formed band at bottom end of the tube (Set 2). 15 N 14 N-DNA produced after one round of cell division in I4 N containing medium was lighter than the I5 N type DNA and formed a band above the 15 N I5 N bands (Set 3). In accordance with the semi conservative replication, the cells that were allowed to divide twice in I4 N- medium should have a mixture of I5 N 14 N & I4 N 14 N DNA and should form two bands in CsCl gradient. As per prediction, the cells from the Set 4 produced two bands. The lower band corresponded with the 15 N I4 N band observed in Set 3 and the upper band corresponded to the I4 N 14 N band of Set 1 (Fig. 2.4). Fig. 2.4: Meselson and Stahl's experiment showing that DNA replication is semiconservati ve. This classic experiment confirmed the prediction of the semi conservative mode of replication envisaged by Watson and Crick and disapproved all notions of conservative and dispersive models for DNA replication. Later, autoradiographic study by J. Cairns (1963) on bacterial DNA replication confirmed the observation of Meselson and Stahl (1958). The study also elucidated the circular nature of bacterial DNA and showed that DNA replication occurs simultaneously on both the strands at one or two moving 'Y' shaped forked junctions in the circular DNA. There are, however, several different ways that semiconservati ve replication can take place, differing principally in the nature of the template DNA—whether it is linear or circular— and in the number of replication forks. Individual units of

193 replication are called replicons, each of which contains a replication origin. Replication starts at the origin and continues until the entire replicon has been replicated. Bacterial chromosomes have a single replication origin, whereas eukaryotic chromosomes contain many. 2.2.2 Theta replication: A common type of replication that takes place in circular DNA, such as that found in E. coli and other bacteria, is called theta replication (Fig. 2.5), because it generates a structure that resembles the Greek letter theta (e). In theta replication, double-stranded DNA begins to unwind at the replication origin, producing single- stranded nucleotide strands that then serve as templates on which new DNA can be synthesized. The unwinding of the double helix generates a loop, termed a replication bubble. Unwinding may be at one or both ends of the bubble, making it progressively larger. DNA replication on both of the template strands is simultaneous with unwinding. The point of unwinding, where the two single nucleotide strands separate from the double-stranded DNA helix, is called a replication fork. If there are two replication forks, one at each end of the replication bubble, the forks proceed outward in both directions in a process called bidirectional replication, simultaneously unwinding and replicating the DNA until they eventually meet. If a single replication fork is present, it proceeds around the entire circle to produce two complete circular DNA molecules, each consisting of one old and one new nucleotide strand. Fig. 2.5a. Theta replication in E.coli and other organisms possessing circular DNA
194 Fig. 2.5b: Experimental evidence produced by J. Cairns (1963) to show the Theta mode of replication in E.coli
Rolling-circle replication takes place in some viruses and in the F factors of E. coli. This form of replication is initiated by a break in one of the nucleotide strands that creates a 3'-OH group and a 5'-phosphate group. New nucleotides are added to the 3' end of the broken strand, with the inner (unbroken) strand used as a template. As new nucleotides are added to the 3' end, the 5' end of the broken strand is displaced from the template, rolling out like thread being pulled off a spool. The 3' end grows around the circle, giving rise to the name rolling-circle model. The replication fork may continue around the circle a number of times, producing several linked copies of the same sequence. With each revolution around the circle, the growing 3' end displaces the nucleotide strand synthesized in the preceding revolution. Eventually, the linear DNA molecule is cleaved from the circle, resulting in a doublestranded circular DNA molecule and a single-stranded linear DNA molecule. The linear molecule circularizes either before or after serving as a template for the synthesis of a complementary strand (Fig. 2.6). Fig. 2.6: The linear molecule serving as a template for the synthesis of a complementary strand. 2.2.3 Linear eukaryotic replication : The large linear chromosomes in eukaryotic cells contain too much DNA and need to replicate speedily within a reasonable time frame and therefore cannot afford to have a single origin of replication fork as found in bacteria. Multiple
195 replication origin point exists in eukaryotic that proceed at a rate ranging from 500 to 5000 nucleotides per minute at each replication fork, considerably slower than bacterial replication but still replicate in a matter of minutes or hours, not days. This rate is possible because replication takes place simultaneously from thousands of origins. Typical eukaryotic replicons are-from 20,000 to 300,000 base pairs in length. At each replication origin, the DNA unwinds and produces a replication bubble. Replication takes place on both strands at each end of the bubble, with the two replication forks spreading outward. Eventually, replication forks of adjacent replicons run into each other, and the replicons fuse to form long stretches of newly synthesized DNA (Fig. 2.7). Replication and fusion of all the replicons leads to two identical DNA molecules. Like all other metabolic processes, DNA replication is under the control of several proteins and en- zymes, engaged in an intricate and coordinated interplay. Our understanding of DNA replication is primarily derived from physical, chemical and biochemical studies of enzymes and nucleic acids from Es-cherichia coli, their phages and their mutants. Prokaryotic and eukaryotic mechanism of DNA replication differs in many ways though the basic mechanisms are same. 2.3 DNA replication model In the simplest model of DNA replication, the mother DNA strand is unzipped to produce a ‘Y’ shaped replication fork. At the replication fork, enzymes and protein factors facilitate the addition nascent nucleotides to the newly forming Fie. 2.7: DNA replication on linear chromosomes

196 DNA strand by way of complimentary base pairing and the event should occur simultaneously on both the strands. During DNA synthesis, nucleotides are added to the 3'-OH group of the growing nucleotide strand (Fig. 2.8). The 3'-OH group of the last nucleotide on the strand attacks the 5'-phosphate group of the incoming dNTP. Two phosphates are cleaved from the incoming dNTP, and a phosphodiester bond is created between the two nucleotides. Fie: 2.8: Inclusion of a nascent nucleotide into a growing DNA molecule But there lies a problem. All known DNA polymerases can add nucleotides in the 5'–––<3' direction of the growing strand only. As the DNA double helix is anti-parallel in nature, simultaneous synthesis of both the strands is difficult to conceive as one strand will be synthesized in 5'——<3' direction and the other strand has to be synthesized in 3 '–––<5' if both the strands has to be synthesized simultaneously - which is not possible. Interestingly, mother-nature has evolved mechanism that allows both the strands of the DNA double helix to be synthesized simultaneously. 2.3.1 Continuous and discontinuous DNA replication Auto radiographic studies confirmed that DNA synthesis occurs simultaneously on both the strands. The works of Okazaki and his colleagues enabled to explain the basic mechanism underlying the simultaneous synthesis of both the strands in DNA double helix. They studied the incorporation of radioactive thymidine at different phases of DNA synthesis and found that the radioactive materials were present only in short DNA fragments (100- 1000 nucleotides) extracted just a few moments after the radioactive pulse was inhibited. As the time

197 elapsed, the radioactive materials could be detected in high molecular weight DNA strands. Normally, this should not have happened as the feeding of radioactive material was stopped. They predicted that during DNA synthesis, continuous synthesis occurs on the 3'—<5' template but on the 5 '—<3' template short DNA segments called Okazaki fragments are synthesized, which are subsequently linked together by the action of DNA polymerases. Thus, continuous replication occurs in one of the templates in the direction of the movement of the replication fork while discontinuous replication occurs in the other strand. The strand that allows continuous synthesis in known as leading strand and the strand on which discontinuous synthesis occurs is called the lagging strand (Fig. 2.9). 2.3.3 The fidelity of DNA replication Overall, replication results in an error rate of less than one mistake per billion nucleotides. How is this incredible accuracy achieved? Answer lies in the activity of the DNA polymerase. These enzymes are very particular in pairing nucleotides with their complements on the template strand. Most of the errors that do arise in nucle-otide selection are corrected in a second process called proofreading. When a DNA polymerase inserts an incorrect nucleotide into the growing strand, the 3 '- OH group of the mispaired nucleotide is not correctly positioned for accepting the next nucleotide. The incorrect positioning stalls the polymerization reaction, and the 3'—<5' exonuclease activity of DNA polymerase removes the incorrectly paired nucleotide. DNA polymerase then inserts the correct nucleotide. Together, proofreading and nucleotide selection result in an error rate of only one in 10 million nucleotides. A third process, called mismatch repair corrects errors after replication is complete. Any incorrectly paired nucleotides produce a deformity in the secondary structure of the DNA; the deformity is recognized by specialized enzymes that excise an incorrectly paired nucleotide and replace it with the correct Fig: 2.9: DNA synthesis is continuous on one template strand of DNA and discontinuous on the other.

198 nucleotide. Methylation on the old DNA strand allows the mismatch repair enzymes to distinguish between old and new strand. 2.3.3 Speed of replication : The single molecule of DNA that is the E. colt genome contains 4.7 x 10 6 nucleotide pairs. DNA replication begins at a single, fixed location in this molecule, the replication origin, proceeds at about 1000 nucleotides per second, and thus is done in no more than 40 minutes. And thanks to the precision of the process (which includes a "proof-reading" function), the job is done with only about one incorrect nucleotide for every 10 9 nucleotides inserted. In other words, more often than not, the E. colt genome (4.7 x 10 6) is copied without error. 2.4 Replication in eukaryotes Our understanding of the DNA replication in eukaryotic cells is limited but development of new experimental techniques is rapidly changing the imbalance of knowledge. The replication machinery is similar to bacterial system but basic differences include: (1) replication on linear chromosomes associated with multiple proteins, (2) multiple replication origins in their chromosomes; (3) more types of DNA polymerases, with different functions; and (4) nucleosome assembly immediately following DNA replication. As the yeast (Saccharomyces cerevisiae) replication system is very similar to mammalian cells, isolation of various mutant yeasts, unable to produce specific gene products required for various aspects of replication has added to our understanding of eukaryotic replication. Further, the monkey virus SV40 has single origin of replication where the viral encoded large T antigen binds along with several other proteins that are synthesized by the host DNA. Scientists utilize this knowledge to simulate DNA replication in vitro and understand the function of various cellular replication proteins. 2.4.1 Initiation of replication In eukaryotes, cells have much more DNA and their polymerases synthesize DNA at a much lower rate. To compensate these difficulties, the eukaryotic cells replicate their genome in small portions, termed replicons (Fig. 2.10). From the radioactive studies it has been estimated that each replicon is approximately 15 to 100 urn in length (50 to 300 kb) and the replication fork proceeds in both direction. The heterochromatin regions tend to replicate late in the S phase as such the Barr body is the last to replicate while the active X chromosome is replicated at an early stage in females.

199 Fig: 2.10: Multiple site of origin of DNA replication and each is known is replicon Initiation of replication in eukaryotes is much more complicated than in prokaryotes. In yeast cells, the site of origin of replication if removed and inserted in another DNA molecule, the hybrid DNA molecule acquires the ability to replicate in vitro or in vivo. As the sequences at the site of origin of replication promote replication of the DNA in which they are contained, they are referred to as autonomous replicating sequences (ARSs). There are about 400 ARSs scattered throughout the genome of yeast cells and each ARSs has a conserved 11 bp sequence that allow the binding of essential multiprotcin complex called the origin recognition complex (ORC). In lormal cells, ORC proteins remain bound to the ARSs all through the cell cycle. The binding of other proteins to the ORC-ARS complex allows initiation of replication. Mutated ARSs fail to bind ORC proteins and thus cannot initiate DNA replication at that site. In mammals, virtually any type of purified naked DNA is suitable for initiation of replication with cellular extracts suggesting that, unlike yeast, mammalian DNA might not possess specific sites at which replication is initiated. However, in vivo studies on intact chromosomes indicate that replication does begin at specific sites along the DNA and initiation is not a random event. It appears that, mammalian DNA molecules have numerous sites where replication can be initiated, but because of the presence of nucleosome and higher order of organization of mammalian chromosome, most of the initiation sites remain suppressed while promoting initiation at specific sites that serve as replication origins. One such replication origin site has been located in the ?-globin gene cluster. As the Eukaryotic cells utilize thousands of origins, the cell needs to ensure that each segment of the DNA is replicated once during cell division. The precise replication of DNA is accomplished by the separation of the initiation of replication into two distinct steps. In the first step, the origins are licensed (see licensing factors), meaning that they are approved for replication. During replication, only the licensed sites can bind to replication initiation factors. The preliminary initiation factors at first displace the replication licensing factors and then induce the formation of replication bubble. The sites of origin of replication do not bind to any further replication licensing factors during the progression of the S phase until it enters

200 the mitotic phase thereby ensuring the replication of genome only once per cell cycle. Two-replication fork are formed at each site of origin of replication and bidirectional DNA synthesis occur in a manner, which is similar in all organisms - whether it is virus, prokaryotes or eukaryotes. The replication forks are not randomly distributed in the nuclear matrix. There are 50-250 sites in the nucleus - called replication foci, where synthesis takes place. Each foci contain approximately active 40 replication forks. The clustering of replication forks may provide mechanisms for coordinating the replication of adjacent replicons over individual chromosomes. The nuclear matrix also seems to play an important during replication. The substances necessary for replication remain bound the nuclear matrix and are made available during the process of replication. DNA replication in eukaryotic cells is limited to S phase of the cell cycle. Approximately 10 3 to 10 5 replication events occur in a coordinated manner, though not identically at all origins. This leads to great variation in the duration of S phase. Moreover, the associated histones and non histone proteins get synthesized either during.Gl or S phase. Replication 'tool kit' consists of helicase, single stranded DNA binding proteins, topoisomerases, primase, DNA polymerase, and DNA ligase. The DNA in eukaryotes is also synthesized in semi-discontinuous manner, although the Okazaki fragments of the lagging strand are much smaller (250 nucleotides in length). 2.4.2 Some proteins required for replication The following table shows a glimple of different proteins required for replication. DNA Polymerase Polymeras Exonuclease Cellular Function Sub Units Activity Activity ? (alpha) Yes No Initiation of nuclear DNA synthesis and DNA repair ? (beta) Yes No DNA repair and recombination of nuclear DNA ? (gamma) Yes Yes Replication of mitochondrial DNA ? (delta) Yes Yes Leading & lagging-strand synthesis of nuclear DNA, DNA repair, and translesion DNA synthesis ? (epsilon) Yes Yes Unknown; probably repair and replication of nuclear DNA ? (zeta) Yes No Translesion DNA synthesis ? (eta) Yes No Translesion DNA synthesis

201 DNA Polymerase Polymerase Exonuclease Cellular Function Sub Units Activity Activity ? (theta) Yes No DNA repair ? (iota) Yes No Translesion DNA synthesis K (kappa) Yes No Translesion DNA synthesis ? (lambda) Yes No DNA repair ? (mu) Yes No DNA repair ? (sigma) Yes No Nuclear DNA replication (possibly), DNA repair, and sister-chromatid cohesion Other DNA polynferases (?, ?, ?, K , ?, ?) allow replication to bypass damaged DNA (called translesion replication) or play a role in DNA repair. Many of the DNA polymerases have multiple roles in replication and DNA repair. 2.4.3 Eukaryotic DNA polymerase Eukaryotic cells contain several DNA polymerases of which the most important are ?,??????? and ? (Table 1). DNA polymerase ?, which contains primase activity, initiates nuclear DNA synthesis by synthesizing an RNA primer, followed by a short string of DNA nucleotides. After DNA polymerase ? has laid down from 30 to 40 nucleotides, DNA polymerase ? completes replication on the leading and lagging strands. DNA polymerase ? does not participate in replication but is associated with the repair and recombination of nuclear DNA. The ? polymerase is encoded by nuclear gene but located within the mitochondria and is responsible for the replication of the mitochondrial DNA;. ? polymerase like enzyme replicates chloroplast DNA in plants. Similar in structure and function to DNA polymerase ? , DNA polymerase e appears to take part in nuclear replication of both the leading and the lagging strands, but its precise role is not yet clear. Inhibitors of polymerase activity : Aphidicolin (?, ?, & ?); N-ethylmalemide (?, ?, ?, d); butylphenyl-dGTP (?,); dideoxynucleoside 5'triphosphate (?). 2.4.4 The enzymes of DNA replication 1. Topoisomerase is responsible for initiation of the unwinding of the DNA. The tension holding the helix in its coiled and supercoiled structure can be broken by nicking a single strand of DNA. Try this with string. Twist two strings together, holding both the top and the bottom. If you cut only one of the two strings, the tension of the twisting is released and the strings untwist.

202 2. Helicase accomplishes unwinding of the original double strand, once supercoifing has been eliminated by the topoisomerase. The two strands very much want to bind together because of their hydrogen bonding affinity for each other, so the helicase activity requires energy (in the form of ATP) lo break the strands apart. 3. DNA polymerase proceeds along a single-stranded molecule of DNA, recruiting free dNTP's (deoxy-nucleotide-triphosphates) to hydrogen bond with their appropriate complementary dNTP on the single strand (A with T and G with C), and to form a covalent phosphodiester bond with the previous nucleotide of the same strand. The energy stored in the triphos-phate is used to covalently bind each new nucleotide to the growing second strand. There are different forms of DNA polymerase, but it is DNA polymerase III that is responsible for the processive synthesis of new DNA strands. DNA polymerase cannot start synthesizing de novo on a bare single strand. It needs a primer with a 3'OH group onto which it can attach a dNTP. DNA polymerase is actually an aggregate of several different protein subunits, so it is often called a holoenzyme. The holoenzyme also has proofreading activity, so that it can make sure that it inserted the right base, and nuclease (excision of nucleotides) activities so that it can cut away any mistakes it might have made. 4. Primase is actually part of an aggregate of proteins called the primeosome. This enzyme attaches a small RNA primer to the single-stranded DNA to act as a substitute 3'-OH for DNA polymerase to begin synthesizing from. This RNA primer is eventually removed by RNase H and the gap is filled in by DNA polymerase I. 5. Ligase can catalyze the formation of a phosphodiester bond given an unattached but adjacent 3'-OH and 5'phosphate. This can fill in the unattached gap left when the RNA primer is removed and filled in. The DNA polymerase can organize the bond on the 5' end of the primer, but ligase is needed to make the bond on the 3' end. 6. Single-stranded binding proteins (SSB) are important to maintain the stability of the replication fork. Single-stranded DNA is very labile, or unstable, so these proteins bind to it while it remains single stranded and keep it from being degraded. 2.4.5 The replication fork Why can DNA polymerase only act from 5' to 3' The reason is the relative stability of each end of DNA. A triphosphate is required to provide energy for the bond between a newly attached nucleotide and the growing DNA strand. However,

203 this triphosphate is very unstable and can easily break into a monophosphate and an inorganic pyrophosphate, which floats away into cell. At the 5' end of the DNA, this triphosphate can easily break, so if a strand has been sitting in the cell for a while, it would not be able to attach new nucleotides to the 5' end once the phosphate had broken off. On the other hand, the 3' end only has a hydroxyl group, so as long as new nucleotide triphosphate are always brought by DNA polymerase, synthesis of a new strand can continue no matter how long the 3' end has remained free. This presents a problem, since one strand of the double helix is 5' to 3', and the other one is 3' to 5'. How can DNA polymerase synthesize new copies of the 5' to 3 1 strand, if it can only travel in one direction? This strand is called the lagging strand, and DNA polymerase makes a second copy of this strand in spurts, called Okazaki fragments, as shown in the diagram. The other strand can proceed with synthesis directly, from 5' to 3', as the helix unwinds. This is the leading strand. 2.4.6 Nucleosome assembly ukaryotic DNA is complexed to histone proteins in nucleosome structures that contribute to the stability and packing of the DNA molecule (see Fig. 2.11). The disassembly and reassembly of nucleosomes on newly synthesized DNA probably takes place during replication, but the precise mechanism for these processes has Fig. 2.11: Several levels of organization of eukaryotic chromosome.

204 not yet been determined. The unwinding of double stranded DNA and the assembly of the replication enzymes on the single-stranded templates probably require the disassembly of the nucleosome structure. Electron micrographs of eukaryotic DNA show recently replicated DNA already covered with nucleosomes indicating that nucleosome structure is reassembled quickly. Before replication, a single DNA molecule is associated with histone proteins. After replication and nucleosome assembly, two DNA molecules get associated with histone proteins. After replication and nucleosome assembly, two DNA molecules get associated with histone proteins. Whether the original histones remain together, attached to one of the new DNA molecules, or do they disassemble and mix with new histones on both DNA molecules is still not known. Experiments with radioactive labeled histones suggest that newly assembled octamers consist of a random mixture of old and new histones. 2.4.7 The nucleosome Chromatin has a highly complex structure with several levels of organization. The simplest level (Fig. 2.12) is the double helical structure as proposed by Watson and Crick (1953). At a more complex level, the DNA molecule is associated with proteins and is highly folded to produce a chromosome. Chromatins viewed under electron microscope, frequently looks like beads on a string. Partial digestion of chromatin with nuclease produces beads. Each individual bead has attached 200 Fig. 2.12: The nucleosome model

205 bp of DNA. Further digestion with more nuclease chews up the entire DNA between the beads and leaves a core of proteins attached to a fragment of DNA (Fig. 2.12). These experiments demonstrated that chromatin is not a random association of proteins and DNA but has a fundamental repeating structure having the the simplest level of chromatin structure, the nucleosome The nucleosome is a core particle consisting of DNA wrapped about two times
around an octamer
of eight histone proteins (
two copies each of H2A, H2B, H3, and H4),
much like thread wound around a spool (Fig. 2.12d). The DNA in direct contact with the histone octamer is between 145 and 147 bp in length, coils around the histones in a left-handed direction, and is supercoiled. It does not or kinks, in its helical structure as it winds around the histones. The fifth type of histone, H1, is not a part of the core particle but plays an important role in the nucleosome structure. The precise location of H1 with respect to the core particle is still uncertain. The traditional view is that H1 sits outside the octamer and binds to the DNA where the DNA joins and leaves the octamer (Fig. 2.11). However, the results of recent experiments suggest that the H1 histone sits inside the coils of the nucleosome. Regardless of its position, H1 helps to lock the DNA into place, acting as a clamp around the nucleosome octamer. Together, the core particle and its associated H1 histone are called the chromatosome, the next level of chromatin organization. The H1 protein is attached to between 20 and 22 bp of DNA, and the nucleosome encompasses an additional 145 to 147 bp of DNA; so about 167 bp of DNA are held within the chromatosome. Chromatosomes are located at regular intervals along the DNA molecule and are separated from one another by wrap around the octamer smoothly; there are four bends, linker DNA, which varies in size among cell types—most cells have from about 30 bp to 40 bp of linker DNA. Nonhistone chromosomal proteins may be associated with this linker DNA, and a few also appear to bind directly to the core particle. 2.4.8 DNA synthesis at the ends of chromosomes A fundamental difference between eukaryotic and bacterial replication arises because eukaryotic chromosomes are linear and thus have ends. As the 3'-OH group is needed by DNA polymerases to elongate, at the initiation of replication by RNA primers provide the 3 ‘-OH group synthesized by primase. RNA primers must be removed and replaced by DNA Fig. 13a: Replication at the ends of circular DNA where the 3 ‘-OH group is available Fig. 13a: Replication at the ends of circular DNA where the 3'-OH group is available
206 mucleotides subsequently which is done by DNA polymerase I. In a circular DNA molecule, elongation around the circle eventually provides a 3'-OH group immediately in front of the primer (Fig. 2.13a). After the primer has been removed, the replacement DNA nucleotides can be added to this 3'-OH group. In linear chromosomes with multiple origins, the elongation of DNA in adjacent replicons also provides a 3'-OH group preceding each primer (Fig. 2.13b.). At the very end of a linear chromosome, however, there is no adjacent stretch of replicated DNA to provide this crucial 3'-OH group. Once the primer at the end of the chromosome has been removed, it cannot be replaced with DNA nucleotides, which produces a gap at the end of the chromosome (Fig. 2.13c), suggesting that the-chromosome should become progressively shorter with each round of replication, leading to the eventual elimination of the entire telomere and destabilization of the chromosome, and cell death. But chromosomes don’t become shorter each generation and destabilize. Interestingly, the ends of chromosomes that preserve the integrity of the chromosome structure and avoid being getting shorter with each cycle of cell division. The telomeres possess several unique features, one of which is the presence of many copies of a short repeated sequence. In the protozoan Tetrahymena, this telomeric repeat is CCCCAA., with the G-rich strand typi-cally protruding beyond the C-rich strand (Fig. 2.14a): The single-stranded protruding end of the telomere can be extended by telomerase, an enzyme with both a protein and an RNA component (also known as a ribonucleoprotein). The RNA part of the enzyme contains from 15 to 22 nucleotides Fig. 2.13b : Replication at the ends of linear DNA

207 that are complementary to the sequence on the G-rich strand. This sequence pairs with the overhanging 3' end of the DNA (Fig.2.14b) and provides a template for the synthesis of additional DNA copies of the repeats. DNA nucleotides are added to the the end of strand one at a time (Fig.2.14c) and, after several nucleotides have been added, the RNA template moves down the DNA and more nucleotides are added to the 3' end. Usually, from 14 to 16 nucleotides are added to the 3' end of the G-rich strand. 5' end of 3-CCCCAA toward Chromosome 3'-GGGGTTGGGGTT centromere In this way, the telomerase can extend the 3' end of the chromosome without the use of a complementary DNA template. How the complementary Orich strand is synthesized is not yet clear. It may be synthesized by conventional replication, with priniase synthesizing an RNA primer on the 5' end of the extended (G rich) template. The removal of this primer once again leaves a gap at the 5' end of the chromosome, but this gap does not matter, because the end of the chromosome is extended at each replication by telomerase; no genetic information is lost, and the chromosome does not become shorter overall. The extended single-strand end may fold back on itself, forming a terminal loop by nonconventional pairing of bases, displacing a part of the original telomeric duplex. The loop structure is formed and stabilized by specific telomere-binding proteins (Fig. 2.15). Fig. 2.14: DNA synthesis at the end point of linear chromosomes by telomerase enzyme

208 Fig. 2.15: Loop formation by the single stranded DNA strand at telomeric region by unconventional base pairing 2.4.9 Telomerase enzyme Telomerase is a multi-subunit enzyme that is comprised of a RNA component - hTR, and a protein component - hTERT (Nakamura and Cech, 1998). hTR contains an 11 bp sequence that provides the template for the synthesis of telomeric repeats which are added to the chromosome, whereas hTERT, the reverse transcriptase component, catalyzes the synthesis reaction. Thus, addition of TTAGGG repeats to the 3' ends of chromosomes compensates for losses due to the end-replication problem. In humans, telomerase activity is absent in most normal cells but present in majority of tumors (Kim et al, 1994). However, activity has been detected in high levels in germ cells, early embryos (Xu and Yang, 2001), activated T and B cells and germinal centres of lymphoid organs. Telomerase is present in single-celled organisms, germ cells, early embryonic cells, and certain proliferative somatic cells (such as bone-marrow cells and cells lining the intestine), all of which must undergo continuous cell division. Most somatic cells have little or no telomerase activity, and chromosomes in these cells progressively shorten with each cell division. These cells are capable of only a limited number of divisions; once the. telomeres shorten beyond a critical point, a chromosome becomes unstable, has a tendency to undergo rearrangements, and is degraded. These events lead to cell death. The shortening of telomeres may contribute to the process of aging. Genetically engineered mice that lack a functional telomerase gene do not express telomerase in somatic or germ cells and therefore experience progressive shortening of their telomeres in successive generations. After several generations, these mice show some signs of premature aging, such as graying, hair loss, and delayed wound healing. Through genetic engineering, it is also possible to create somatic cells that express telomerase. In these cells, telomeres do not shorten, cell aging is inhibited, and the cells will divide indefinitely. Telomerase also appears to play a role in cancer. Cancer tumor cells have the capacity to divide indefinitely, and many tumor cells express the telomerase enzyme. Telomerase activation alone does not lead to cancerous growth in most cells, but it does appear to be required along with other mutations for cancer to develop.

209 The length of the telomeric sequence varies from chromosome to chromosome and from cell to cell, suggesting that each telomere is a dynamic structure that actively grows and shrinks. The telomeres of Drosophila chromosomes are different in structure. They consist of multiple copies of the two different retrotransposons Het-A and Tart, arranged in tandem repeats. Apparently, in Drosophila, loss of telomere sequences during replication is balanced by transposition of additional copies of the Het-A and Tart elements. Farther away from the end of the chromosome, from several thousand to hundreds of thousands of base pairs form telomere-associated sequences. They, too, contain repeated sequences, but the repeats are longer, more varied, and more complex than those found in telomeric sequences. 2.4.10 Licensing: positive control of replication The average human chromosome contains 150 x 10 6 nucleotide pairs which are copied at about 50 base pairs per second. The process would take a month (rather than the hour it actually does) but for the fact that there are many places on the eukaryotic chromosome where replication can begin. Replication begins at some replication origins earlier in S phase than at others, but the process is completed for all by the end of S phase. As replication nears completion, "bubbles" of newly replicated DNA meet and fuse, finally forming two new molecules. In order to be replicated, each origin of replication must be bound by: ? An Origin Recognition Complex of proteins (ORC). (These remain on the DNA throughout the process). ? Accessory proteins called licensing factors. (These accumulate in the nucleus during G 1 of the cell cycle. They include) : l CDC-6 and CDT-1, which bind to the ORC and are essential for coating the DNA with l MCM proteins. Only DNA coated with MCM proteins (there are 6 of them) can be replicated. ? Once replication begins in S phase, l CDC-6 and CDT-1 leave the ORCs (the latter by ubiquination and destruction in proteasomes). l The MCM proteins leave in front of the advancing replication fork. 2.4.11 Geminin: negative control of replication G 2 nuclei also contain at least one protein — called geminin — that prevents assembly of MCM proteins on freshly-synthesized DNA (probably by sequestering Cdtl).

210 As the cell completes mitosis, geminin is degraded so the DNA of the two daughter cells will be able to respond to licensing factors and be able to replicate their DNA at the next S phase. Some cells deliberately cut the cell cycle short allowing repeated S phases without completing mitosis and/or cytokinesis. This is called endoreplication. How these cells regulate the factors that normally prevent DNA replication if mitosis has not occurred is still being studied. Endoreplication is described on a separate page. 2.4.12 Post-replicative modification of DNA, methylation One of the major post-replicative reactions that modifies the DNA is methylation. The sites of natural methylation (i.e. not chemically induced) of eukaryotic DNA is always on cytosine residues that are present in CpG dinucleotides. However, it should be noted that not all CpG dinucleotides are methylated at the C residue. The cytidine is methylated at the 5 position of the pyrimidine ring generating 5-methylcytidine. Methylation of DNA in prokaryotic cells also occurs. The function of this methylation is to prevent degradation of host DNA in the presence of enzymatic activities synthesized by bacteria called restriction endonucleases. These enzymes recognize specific nucleotide sequences of DNA. The role of this system in prokaryotic cells (called the restriction-modification system) is to degrade invading viral DNAs. Since the viral DNAs are not modified by methylation they are degraded by the host restriction enzymes. The methylated host genome is resistant to the action of these enzymes. The precise role of methylation in eukaryotic DNA is unclear. It was originally thought that methylated DNA would be less transcriptionally active. Indeed, experiments have been carried out to demonstrate that this is true for certain genes. For example, under-methylation of the MyoD gene (a master control gene regulating the differentiation of muscle cells through the control of the expression of muscle- specific genes) results in the conversion of fibroblasts to myoblasts. The experiments were carried out by allowing replicating fibroblasts to incorporate 5-azacytidine into their newly synthesized DNA. This analog of cytidine prevents methylation. The net result is that the maternal pattern of methylation is lost and numerous genes become under methylated. However, lack of methylation nor the presence of methylation is a clear indicator of whether a gene will be transcriptionally active or silent. The pattern of methylation is copied post-replicatively by the maintenance methy-lase system. This activity recognizes the pattern of methylated C residues

211 in the maternal DNA strand following replication and methylates the C residue present in the corresponding CpG dinucleotide of the daughter strand. The phenomenon of genomic imprinting refers to the fact that the expression of some genes depends on whether or not they are inherited maternally or paternally. Insulin-like growth factor-2 (IGF2) is a gene whose expression is required for normal fetal development and growth. Expression of IGF2 occurs exclusively from the paternal copy of the gene. Imprinted genes are "marked" by their state of methylation. In the case of IGF2 an element in the maternal locus, called an insulator element, is methylated blocking its function. The function of the un-methylated insulator is to bind a protein that when bound blocks activation of IGF2 expression. When methylated the protein cannot bind the insulator thus allowing a distant enhancer element to drive expression of the IGF2 gene. In the maternal genome, the insulator is not methylated, thus protein binds to it blocking the action of the distant enhancer element. 2.5 Mechanism of replication 2.5.1 Replication in prokaryotes Replication in prokaryotes and viral DNA usually starts at specific sites on chromosome, referred to as replication origin. The origin in E. coli is specifically known as OriC. Approximately 20-30 different proteins, some in multiple copies are required to initiate the DNA replication process. Some of the proteins characterized and their genes are given in Table 1. Initiation and Unwinding of DNA: function of Helicases & Topoisomerase In E.coli, OriC com- prises of 245 base pairs that contains four 9 bp sites with similar sequences at which product of dnaA (homologous tetramer) binds and initiates the assembly of all other proteins and enzymes necessary for replication. In addition, the origin contains 11 methylation sites Table 1: Different proteins that assimilate at the initiation complexes Gene Product or/functions Gene Initiator protein; binds at OriC dnaA 1HF protein- DNA binding protein; binds at OriC HimA F1S protein- DNA binding protein; binds at OriC fis Heiicase and activator of primase dnaB Proteins that complexes with dnaB protein and delivers to DNA dnaC Primase- synthesizes RNA primer dnaG Single stranded binding proteins (SSB-proteins) ssb DNA ligase Lig Gyrase (topoisomerase type. II) gyrA,, yrB TBP proteins (terminus (ter) binding proteins) stops replication Tus Topoisomerase I topA Topoisomerase IV parE

212 recognized by DNA methylase and three AT rich direct tandem repeats consisting of 13 base pair each. Initiation of replication begins with the binding of dnaA molecules at 4 sites consisting of 9 nucleotides provided that the 9-mers are fully methylated. ? Region of the DNA bound to dnaA coalesce and are joined by additional dnaA molecules to form a nucleosome like complex, which promotes nearby melting of the double helix at AT rich site (Fig. 2.16). ? Physical separation of the two strands requires untwisting of the DNA molecule. Unwinding of the DNA is facilitated by helicases, which is the product of the dnaB. dnaA, with the aid of dnaC binds to the helicases, which then makes contact with the DNA at the replication fork and separates the two strands to form a bubble by melting the hydrogen bonds. Energy required for unwinding is derived from the hydrolysis of ATP. ? During unwinding tension build up ahead of the replication fork because of the formation of super coils. DNA gyrase (topoisomerase Fig. 2.16: Formation of initiation complex at the site of origin of DNA synthesis

213 I & III nicks single strand while II & IV nicks two strands to relieve tensions), reduce trosional strain (torque) that builds up ahead of the replication fork asa result of unwinding The topoiso-merase apparently nick one strand of the double stranded DNA ahead of the replication fork. The nicked DNA molecule then rotates, relieving the tension and avoid the formation of super coils. There are some indication that topoisomerase also induce negative super coils ahead of the replication fork thereby reliving the tensions and also assist the helicases in the process of unwinding (Fig. 2.17). 2.5.2 Formation of Replisome: The assembly of all protein factors and enzymes at the site of origin of replication is called a replisome (Fig. 2.18). ? Immediately after the bubble is formed Single-strand binding proteins (SSBs) binds to the single DNA strands and stabilizes them to avoid any unwanted reactions and- also to prevent the single strand DNA to reunite and form a duplex again, until replication at that region is complete. Formation of the bubble creates a 'Y' shaped structure called a replication fork. A replication fork moves in one direction. ? Assembly of dnaB-dnaC complex is followed by the addition of four other poly-peptides - n, n', n" and i. This complex constitutes the prepriming complex. The stage is now ready for the binding of the Primase the product of dnaG, Primase synthesize short RNA sequences complementary to DNA strands at the initiation site. This is because DNA polymerase III cannot initiate synthesis of a chain of DNA from free nucleotides and require a RNA primer to provide a free 3'-"OH end that can be extended by addition of nucleotides. Addition of Primase converts the priming complex to a primosome. The primase is much smaller that the usual RNA polymerase and is only 6000dalton. Primase is activated by dnaB, which then starts synthesizing short RNA primers on both the strands. The primers start with two purine nucleotides, most frequently pppAG The primers are usually Fig. 2.17: Unwinding of the DNA helix ahead of the replication fork by topoisomerase releases tension and avoids DNA breaks.

214 10 to 15 bases long. Assembly of the replisome is completed by the addition of DNA polymerase III (Fig. 2.18). Fig. 2.18 : Replisome complex at the site of origin of DNA synthesis 2.5.3 Elongation Once the single strands of DNA are stabilized, they serve as templates upon which new strands are synthesized by the enzyme referred to as DNA polymerase. DNA polymerases elongate the polynucleotide strand by catalyzing polymerization reaction. DNA polymerases add nucleotides to the 3-OH group on a nucleotide already paired with the template strand and consequently DNA synthesis can only proceed in the 5'–3' direction. Unlike the RNA polymerases, DNA polymerases require short primer sequences to initiate DNA synthesis on a single strand DNA template. Finally, the addition of new nucleotide is not random. DNA polymerase selects each deoxyribonucleotide that can form a complementary base pair with the nucleotide on the template strand DNA. 2.5.4 Termination of DNA synthesis Termination occurs at ter or t locus, lying across from Ori C of the circular chromosome, between minutes 28 to 36. This region incorporates 6 ter sequences with sequence GTGTGTTGT that bind Tus protein (terminator protein). Three ter sequences arrest replication from the right and rest three sequences arrest replication coming from the left. One interesting aspect of E. coli DNA replication is that the cells are viable even if the whole terminator region is deleted and can terminate replication. ? Tus protein is a contra helicases, and functions by interfering with the ATP dependent function of dnaB helicases (rather than simply impending the propagation of this helicases along the double helix) ? Each Ter-Tus site has directional properties and it arrests only those replisomes that reach the Tus site from one specific direction. ? Replisome arriving from opposite direction apparently force dissociation of the TUS protein and thus can proceed unimpeded past the Ter-Tus site.

215 2.6 DNA Polymerases The best-studied polymerases are those of E. coli, which has at least five different DNA polymerases. DNA polymerase I and DNA polymerase III play the major role during DNA replication, the pther three have specialized functions in DNA repair mechanism. All the DNA polymerases share the same fundamental property of adding new nucleotides only to the 3'-OH group on a previously existing paired nucleotide on a DNA template. Arthur Kornberg (1956) first identified and isolated DNA polymerase from the lysate of is. coli, which is now known as DNA polymerase I. Later, identification of DNA polymerase I deficient E. coli clones led to the isolation of two new polymerases - DNA polymerase II and DNA polymerase III. Characteristic properties of different polymerases isolated from E. coli are given in Table 2. 5'––<3' 3'––<5' 5'––<3' DNA Polymerase Polymerization Exonuclease Exonuclease Function 1 Yes Yes Yes Removes and replaces primer II Yes Yes No DNA repair: restarts replication after damaged DNA halts synthesis III Yes Yes No Elongates DNA IV Yes No No DNA repair V Yes No No DNA repair: traitslesion DNA synthesis Table 2: Properties of DNA polymerases found in E. coli DNA polymerase I : DNA polymerase I participate in lagging strand synthesis by eliminating primer RNAs and also have a role in repair mechanism. This enzyme is coded by locus polA and is a single polypeptide chain. When treated with proteolytic enzymes, it is cleaved into two fragments- the larger fragment is know as Klenow fragment (used for in vitro synthesis). Two third of the protein chain, beginning from the C terminal end has polymerase activity while the rest one third on N-terminal end contains proofreading exonuclease activity, (no. of units per cell = 400) DNA polymerase II: The biological function of DNA polymerase II is unclear, although this enzyme is induced when chromosomal DNA is damaged. DNA polymerase III: DNA polymerase III is a huge multiprotein holoenzyme complex that plays the major role during DNA replication in E. coli. No. of units present per cell is approximately 10 to 12. It consists of 10 different polypeptide

216 chains. The catalytic core is composed of three subunits. The ? subunit possess 5'—<3' DNA synthetic activity, ? subunit has the 3' —<5' exonuclease activity. The ? subunit possibly participate in assembly of the enzyme. The remaining seven auxiliary subunits enhance the processivity of the core by clamping it onto the template. The addition of the T causes the core to dimerize. The functions of ? and ? subunits are less well defined. The formation of holoenzyme is completed by the addition of the ? ? subunit (Table 3). Structural analysis has revealed that DNA polymerase III is an asymmetric dimmer with twin polymerase activity sites, capable of synthesizing DNA strands simultaneously on both leading and lagging strand. The 5' —<3' DNA synthetic activity and the 3' —<5' exonuclease activity together allow DNA polymerase III to efficiently and accurately synthesize new DNA molecules. DNA No. of Gene mol. wt. Polymerization Exonuclease molecules. polymerase Units 5'—<3' activity per cell I One polA 103.0 kd Yes 3' 5' & 5' 3' 400 II One polB 90.0 kd Yes 3' 5' ? III Ten ––– Yes 3' 5' 10-12 ? = dnaE,130.0kd; ??? = dnaQ, 27.5 kd; ? = holE, 10.0 kd; T (tau) = dnaX, 71.0 kd; ? = dnaX,47.0 kd ? = dnaN,40.6 kd ? = holA 35.0 kd; ??? = holA, 33.0kd; x(chi) = holB,15.0 kd: ?(psi) =hold; 12.0 kd Table 3: Different subunits of the DNA polymerase III, their molecular weights and cellular function All the E. coli DNA polymerases have 3' —<5' exonuclease activity. This means that the DNA polymerases check the accuracy of the most recently assembled base pair. If a wrong base pair is included, exonuclease activity removes the erroneous Fig. 2.19

217 nucleotide by excision and catalyzes the formation of the correct base pair. Thus in DNA replication 3'?5' exonuclease activity is a proofreading mechanism that helps to keep the frequency of DNA replication errors at very low level (10 9 /cycle). In addition, DNA polymerase I has 5'?3' exonuclease activity and can remove nucfeotides from the DNA 5' end of a DNA strand or of an RNA primer strand. This activity is important for DNA repair. 2.6.1 DNA polymerase activity ? DNA synthesis begins immediately after the addition of DNA polymerase III by complementary base pairing at the 3'-OH of the primer. ? Because of the anti parallel nature of the DNA helix and the unidirectional movement of the DNA polymerase along the replication fork poses a problem, since DNA polymerase can only make new DNA strands in the 5' to 3' direction. Interestingly, both the strands of the DNA double helix are synthesized simultaneously. ? The 3' —<5' template in the direction of fork movement is synthesized in a continuous manner and is called the leading strand (Fig. 2.20). ? The 5' —<3' template in the direction of fork movement is synthesized in a discontinuous manner and is called the lagging strand (Fig. 2.20). ? DNA polymerase III complexes are endowed with the property to synthesize continuously on the leading strand and synthesize discontinuously on lagging strand. Fig. 2.20: (a) Different enzymes and factors at the site of replication fork, (b) The replication on leading and lagging strand

218 ? Lagging strand is synthesized discontinuously, in short stretches- known as Okazaki fragments. Formation of Okazaki fragments is also initiated by Primase at sites selected by pre-priming proteins. Each Okazaki fragments starts with a primer - a sequence of RNA, approximately 10 bases long that provides 3'-OH end for extension by DNA polymerase III. ? When a nascent Okazaki fragment reaches the 5 end of the previously synthesized Okazaki fragment, the lagging strand template is released and un-looped. The RNA primers of the Okazaki fragments in E. coli are removed by the combined activity of RNase H and DNA polymerase I that fills the gap. DNA polymerase I follows DNA polymerase III and, using its 5' ––<3' exo- nuclease activity removes the RNA primer. It then uses its 5' —<3' polymerase activity to replace the RNA nucleotides with DNA nucleotides one at a time. ? Two Okazaki fragments are joined by DNA ligase producing intact DNA daughter strand.

219 Unit 3 o Prokaryotic Transcription Structure 3.1 Introduction 3.2 Similarities and differences between replication and transcription 3.3 General idea of transcription 3.4 Transcription in prokaryotes 3.5 Transcription in eukaryotes 3.6 Transcription factors 3.1 Introduction Genomic DNA contains a set of information that governs all cellular activities. These instructions are implemented by synthesis of RNA and proteins. Genetic character of a cell is determined by not only what genes are inherited, but also on how and when the genes are expressed. Three years after Watson and Crick (1953) published the DNA model, Crick proposed the Central Dogma, describing the two-step process of protein synthesis. According the theme, flow of information is always unidirectional i.e. DNA to RNA and RNA to Protein. However after the discovery of reverse transcriptase in retro viruses by Temin and Baltimore in 1970, the dogma now stands as follows (Fig. 3.1) Fig. 3.1: Flow of genetic information from DNA to Protein information never flows back from protein to RNA, in other words it can be said that acquired characters are never inherited. The first level of gene expression involves the transfer of

information stored in DNA to single stranded RNA molecule by way of a process called transcription.

In the second step, the information scripted in the RNA molecule is translated into a linear sequence of amino acids and the process is called translation.

220 3.2 Similarities and difference between replication and transcription Our understanding of the transcriptional process comes from the study of E. coli. There are several similarities between the transcription mechanism and DNA replication. Both the synthesis process utilize the similar nucleotide building blocks and :
use the same chemical method of attack by a terminal -OH group of the growing chain on the triphosphate group of an incoming nucleotide. Both replication and transcription are fueled by the hydrolysis of the pyrophosphate group that is released upon attack. There are however, a number of important differences between these two distinct processes.
a)
One major difference rests on the fact that while DNA replication copies an entire helix, DNA transcription only transcribes specific regions of one strand of the helix. During DNA transcription, only short stretches (about 60 base pairs) of the template DNA helix are unwound. As the RNA polymerase transcribes more of the DNA strand, this short stretch moves along with the transcription machinery. This process is different from that in DNA replication in which the parent helix remains separated until replication is done.
b)
There are slight differences in the substrates that are used in DNA replication versus transcription.
Transcription machinery utilize ribonucleotide instead of deoxyribonucleotide triphosphates. Additionally, in RNA the thym-ine
base is replaced with the base uracil. Both of these differences can be seen in DNA transcription.
c)
Another major difference is that DNA replication is a highly regulated proc- l ess that only occurs at specific times during a cell's life. DNA transcription is also regulated, but it is triggered by different signals from those used to control DNA replication. d) One final difference lies in the capabilities of RNA polymerase versus DNA polymerase.
RNA primers are needed to begin replication because DNA polymerase is unable to do it alone. DNA transcription does not
require any primer synthesis as the
RNA polymerase is capable of initiating RNA syn- l thesis. The structure of the RNA polymerase is necessary for understanding all of the processes that underlie initiation, elongation, and termination and l also explain some of its added capabilities.
221 3.3
General idea of transcription l Some 50 different protein transcription factors bind to promoter sites, usually on the 5' side of the gene to be transcribed l Transcription in both prokaryotes and eukaryotes is catalyzed by RNA polymerase, that synthesizes a complementary RNA molecule using one strand of the duplex DNA as template l The DNA strand that acts as template is called template strand and the DNA strand that is identical in sequence to the RNA strand is called sense strand or coding strand or nontemplate strand 5' ...ATGGCCTGGACTTCA... 3' Sense strand of DNA 3' ...TACCGGACCTGAAGT... 5' Antisense strand of DNA Transcription of antisense strand 5' ...HUGGCCUGGRCUUCH... 3' mRNA Translation of mRNA Met— Ala— Trp— Thr — Ser — Peptide l Transcription of RNA proceeds in the 5' ––<3' direction l Several types of RNA molecules are transcribed by RNA polymerases: p messenger RNA (mRNA). This will later be translated into apolypeptide. p ribosomal RNA (rRNA). This will be used in the building of ribosomes: machinery for synthesizing proteins by translating mRNA. p transfer RNA (tRNA). RNA molecules that carry amino acids to the growing polypeptide. p small nuclear RNA (snRNA). DNA transcription of the genes for mRNA, rRNA, and tRNA produces large precursor molecules ("primary transcripts") that must be processed within the nucleus to produce the functional molecules for export to the cytosol. Some of these processing steps are mediated by snRNAs. p small nucleolar RNA (snoRNA). These RNAs within the nucleolus have several functions. p micro RNA (miRNA). These are tiny (-22 nts) RNA molecules that appear to regulate the expression of messenger RNA (mRNA) molecules.

222 p XIST RNA. This inactivates one of the two X chromosomes in female vertebrates. p RNA primers formed during DNA synthesis p telomerase RNA, p ribozymes that act as enzymes l In prokaryotes, there is only a single type of RNA polymerase responsible for synthesizing all types of RNAs. l E. coli RNA polymerase is a holoenzyme comprised of subunits ââ'á(dimer) and ó 70 . p The ó 70 subunit is the subunit which binds to the promoter region, but is unable to initiate RNA synthesis. p After the ó 70 subunit subunit binds, the other subunits bind forming a function RNA polymerase. p After approximately 10 base pairs have been transcribed the ó 70 subunit leaves and the core polymerase continues on. l In eukaryotes, there are three major classes of RNA polymerase: 1. RNA polymerase I transcribes large rRNAs 2. RNA polymerase II transcribes mRNAs 3. RNA polymerase III tRNAs, small rRNAs and other small RNAs l In prokaryotes, the transcription and translation process are coupled l In eukaryotes, transcription and translation events are compartmentalized. RNA molecules are transcribed in the nucleus. All types of RNAs is then exported into the cytoplasm for translation l Messenger RNAs are processed in eukaryotes prior to translation. Both ends of the RNA are modified in the nucleus. The transcribed intron sequences are removed by splicing to produce a final mRNA ready for translation. l rRNAs and tRNAs are processed in both prokaryotes and eukaryotes. Most rRNAs are synthesized as a single large precursor RNA that is then cleaved into it final products. In tRNA, many individual nucleotides are chemically modified to produce the final tRNA l Transcription proceeds through the steps of initiation, elongation and termination in all cell types.

223 3.4 Transcription in prokaryotes 3.4.1

RNA polymerase The structure of the RNA polymerase is necessary for understanding all of the processes that underlie initiation, elongation, and termination and also explain some of its added capabilities. In prokaryotes

a single RNA polymerase transcribes all genes.

There are two main segments of the RNA polymerase molecule: the core enzyme, and the sigma subunit. These two pieces are together referred to as the "holoenzyme".

The E. coli RNA core enzyme is tetrameric, containing two â ? one â' ?and one â' type subunits. The core enzyme is sufficient for transcriptional elongation, but correct initiation requires the sigma subunit called ó 70 factor. The ó 70 subunit binds to the promoter region, but is unable to initiate RNA synthesis. After the ó 70 subunit binds, the other subunits bind forming a functional RNA polymerase. Specific function of each polypeptide is given in the table 1. Sub units MW Location Possible function ? chains 40 kd core enzyme Promoter binding ? chain 155 kd core enzyme Nucleotide binding substrate ?' chain 160 kd core enzyme Template binding ? factor 32-90 kd Sigma factor Initiation of transcription Table l: Different components of RNA polymerase that comprises the holoenzyme

The start site represents the location on the DNA that marks where the first nucleotide of an RNA chain

will commence which is also designated as the "

plus one position". All positions designated as upstream of the start site are labeled with negative numbers according to their position relative to the start site. Sequences located just upstream of the start site is called the promoter region. This region contains the information that signals

the

RNA polymerase to start transcription. In prokaryotic cells, free RNA polymerase molecules are constantly colliding with DNA helices. The collision leads to a weak association between the DNA and RNA polymerase, which is soon broken. However, when the RNA poJymerase binds to a specific sequence on the DNA, it binds tightly, forming a DNA/RNA polymerase complex.

The ó? factor has two functions, it recognizes the promoter and it converts the closed promoter complex into an open promoter complex. After approximately 10 base pairs have been transcribed the ó 70 subunit leaves and the core polymerase continues on. The core enzyme can bind to DNA in the absence of ó factor but with low efficiency and no specificity. The primary function of the ó factor is thus to increase the binding efficiency of RNA polymerase at the promoter and decrease (non-specific, binding. A single ó factor (ó70 in E. coli) initiates the transcription of

224 most genes, but other ó factors are present that functions only with specialized genes. For example (ó 32 , ó 415 , ó 54 , ó 28) function under adverse conditions like high temperature, starvation, nitrogen deficiency and for chemotaxis. Some bacteriophages (e.g. T4) encode their own ó factor, which subvert the host enzyme into transcribing the phage genes, T3 and T7 phages encode their own RNA polymerases, which are single polypeptides with great affinity for the phage promoters. 3.4.2 Initiation Initiation of transcription begins with the binding of the RNA polymerase to DNA strand. RNA polymerase binds to the DNA non-specifically and with low affinity. In the presence of the 6 factor, the holoenzyme associate with the DNA at specific sites called promoters. Promoters:

The DNA sequence to which RNA polymerase binds to initiate tran- I scription of gene is called the promoter.
The DNA sequence at the promoter region is more or less conserved. Mutation or deletions at promoter region severely affects the transcriptional efficiency of that gene. The promoter is cis-acting and is always located upstream from the point of initiation of transcription. In E. coli, there are two promoter motifs situated at -10 and -35 sequence upstream from the start point of RNA synthesis. The consensus sequence at -10 position is TATA AT (Pribnow box) and at -35 is TTGACA (Fig. 3.2). -35 -10 +1 TTGACA TATAAT DNA 5' 3' atrand promoter region 17 bp promrter region spacing Pribnow box Fig. 3.2: Organization of the promoter region in E. coli The -35 sequence (also called recognition sequence) is essential for binding of the RNA polymerase. At the -10 promoter region, the DNA strands' unwinds when associated with the RNA polymerase, preparing for initiation of transcription. The initial binding between the polymerase and promoter is referred to as a closed promoter complex because DNA is wounded. The unwinding of approximately 15 bases of DNA by the RNA polymerase around initiation site is called open promoter complex. Ideally,
the gap between the two promoter regions is 17 base pairs long. Deviations from this spacing have significant effects on the strength of the promoter region. The closer a promoter region is to matching this canonical promoter sequence, the greater its strength. There is a third promoter element that
225 is sometimes seen in very strong promoters which is called the UP element. It usually is composed of alternating stretches of 5 adenine and thymine bases. It is located upstream of the -35 region.
Transcription usually begins with GTP or ATP and unlike subsequent nucleotides; the first nucleotide retains its triphosphate moiety. A cycle of abortive occurs generating 2-9 base short RNA sequence before actual transcription begins. Once initiation succeeds, 6 factor dissociates from RNA holoenzyme after 9-10 RNA nucleotide polymerizes and then core enzyme completes further elongation of the RNA. 3.4.3 Elongation Elongation proceeds in the 5' to 3' end direction. As RNA polymerase travels down stream, it unwinds the double stranded DNA molecule ahead of it and rewinds the DNA molecule behind it, maintaining an unwounded region of about 17 bp in the region of transcription. Transcription proceeds at the rate of about 30 to 50 nucleotides per second. Energy required for polymerization of nucleotides is obtained from the cleavage of the triphosphate nucleotides (Fig. 3.3) The RNA-DNA hybrid is very transient. The nascent RNA molecule rapidly separates from DNA and at any given time 2-12 nucleotides associate with each other. Certain proteins influence the rate of the synthesis. For example NusA - a protein, binds to the core enzyme that slows down elongation so that ribosome molecules are able to bind.to the nascent RNA molecule right behind the point of synthesis. Fig. 3.3: The transcription bubble on the DNA template 3.4.4 Termination TTermination of RNA synthesis occurs when RNA polymerase reach the end of the gene. Two mechanisms operate in bacteria and viruses: 1. Intrinsic termination or called ? independent termination 2. ? dependent termination.
226 Intrinsic termination (? independent termination): In this type, nucleotide sequence near the end of the transcribed RNA specifies the termination of RNA transcription. The sequences at the end of RNA are self-complementary that form a hairpin structure followed by a conserved sequence with the consensus sequence UUUUUUA. This region is called the intrinsic terminator (Fig. 3.4). For example: Fig. 3.4. Formation of hairpin loop on the transcribing RNA and ? independent termination
227 RNA molecules having self-complementary bases at the termination point form a secondary structure called hairpin loop immediately after it is synthesized. The hairpin loop is followed by poly U sequence. Formation of hairpin loop and presence of poly U sequence probably halts the RNA polymerase and cause termination of RNA synthesis..The exact mechanism of action is not clear, probably the formation of secondary structure and the poly U sequence immediately before the termination signal on DNA destabilizes the RNA polymerase and releases the polymerase of the template strand. The weak bonding between the poly A DNA sequence and poly U strand on RNA may also contribute to the termination of RNA synthesis. However, hairpin loop alone not sufficient to terminate polymerase activity. ??dependent termination: In ??dependent termination, the DNA sequences produce a pause in transcription towards the end of transcription and RNA does not produce any secondary structures and also lack poly U sequences. In this type, the p protein plays the principal role in termination. The ?
protein has two domains. One domain bind to RNA and the other domain bind to ATP
molecule. Hydrolysis of the ATP molecule activates the ? factor that then bind to specific site on RNA molecules in the termination region.
When RNA polymerase encounters the terminator, it pauses, allowing rho to catch up. The

rho protein has helicase activity, which it uses to unwind the RNA-DNA hybrid in the transcription bubble, bringing an end to transcription. Hereto, the exact mechanism of termination needs to be worked out, but most likely it destabilizes the association of the RNA-DNA-polymer association. 3.5 Transcription in eukaryotes In eukaryotes, the genetic material remains enclosed within the nuclear membrane and is physically separated from the other organelles of the cell. The transcriptional process occurs within the nucleus. Although the transcription proceeds by the same fundamental mechanism as in prokaryotes, the regulatory mechanism is far more complex in eukaryotic cells. There is a significant difference between the transcription of eukaryotic and prokaryotic mRNAs in the initiation process. Eukaryotic promoter involves a large number of factors that bind to a variety of cw-acting elements. Eukaryotic cells possesses three different types of RNA polymerase, each specialized to transcribe different types of RNA. The promoter region on the DNA strand is defined by the nature of the RNA polymerase and transcription factors that will bind to specific sequences and support transcription at the normal efficiency and with the proper control. In fact, RNA polymerase does not make interaction with the upstream region of the promoter. The
increased complexity of eukaryotic transcription presumably facilitates the
228 sophisticated regulation of gene expression needed to direct the activities of the many different cell- types of multicellular organisms. 3.5.1 Eukaryotic RNA polymerases
and their promoters In eukaryotic cells, there are three different types of RNA poiymerases, each located in different locations in the nucleus and is responsible for synthesizing different classes of genes as shown in the table below: ENZYME FUNCTION SENSITIVITY RNA polymerase I Transcription of the 45S rRNA precursor Insensitive to a-amanitin, (nuckoli) (later cleaved into 5.8S, 18S, 28S rRNA sensitive to actinomycin D (class I genes) RNA processing RNA polymerase II Transcription of all protein encoding genes Inhibited by ?-amanitin (nucleoplasm) and most genes for small nuclear RNAs (class II genes) RNA polymerase III Transcription of tRNA genes, 5S rRNA (nucleoplasm) genes and genes encoding U6 sn RNA and Moderately sensitive to the various sn RNAs (class III genes) ?-amanitin depending on species Eukaryotic polymerases differ in template specificity, location in the nucleus and susceptibility to different inhibitors. Each RNA polymerase is a complex enzyme having approximately 12 to subunits and weighing about 500 kd. Five subunits of are common to all RNA polymerases. The largest subunits of each polymerase
are homologous to each other and to the ?, ? and ?' subunits of E. coli RNA polymerase.
There is no counterpart to the bacterial ct factor, and the eukaryotic RNA polymerases are consequentially unable to recognize or bind to their promoters. Weil et al (1979) discovered that RNA polymerases require the assistance of additional proteins not only to bind to promoter region but also to initiate transcription. RNA polymerase I and its promoter: Apart from the basic subunits required for DNA transcription, RNA polymerase I requires specialized 4 Core promoter binding proteins - (It is called SL1, TIF-IB, Ribl in different species) and upstream binding factors called UBF. The core binding factor proteins ensures the positioning of the RNA polymerase I at the start point and can initiate transcription at a low basal frequency (Fig, 3.5). SL1 consists of four proteins. One of them, called TBP (TATA-binding protein), is a factor that is required also for initiation by RNA polymerases II and III. The UBF factors interact with the core proteins and greatly enhance the transcription frequency. RNA polymerase I most likely exists as a holoenzyme that contains most or all of the factors including the TBP required for initiation and is probably recruited directly to the promoter. The RNA polymerase

229 I promoter comprises of two separate regions. The first element surrounds the start point extending from -45 to +20, and is sufficient to initiate transcription. This element is unusually GC rich and includes a short AT rich conserved sequence called the Inr. However, presence of an upstream promoter element (UPE), extending from -180 to -107 greatly enhances the efficiency of the primary promoter. The bipartite organization of RNA polymerase I promoter is seen in all organisms although the actual sequence may vary Fig 3.5: RNA polymerase I bipartite promoter RNA polymerase II and its promoter: Several transcription factors and proteins are required by RNA polymerase II at all promoters for initiation of transcription. The subunits of RNA polymerase II and the general transcription factors are conserved among eukaryotes. Surrounding the startpoint of the core promoter region, the RNA polymerase II and the transcription factors assemble and bind with the DNA. However, the specificity and efficiency of binding to the promoter region depends on certain factors called the activators. The activators bind to target sequence ~ 100 bp upstream of startpoint or further away and influence the formation of initiation complex. Mutational studies led to the identification of three short consensus sequence centered around -30, -75 and -90 bp. Mutation of the TATA box, located at -30 does not prevent initiation but plays a crucial role in positioning the basal factors at precise location and positioning the RNA polymerase to start transcription from the right place. The enhancing sequence elements located upstream of the start point influences the rate of transcription, probably, by interacting with the basal transcription factors. The CAAT box located at -75 or further away has a strong effect in determining the efficiency of the promoter. The consensus sequence at -90 is GGGCGG, which is very common and often exists in multiple copies. RNA polymerase III and its promoter: RNA polymerase III uses both downstream and upstream promoters sequences. The promoters for 5S and tRNA genes lie downstream (identified in Xenopus laevis) of the startpoint between +55 and +80 bp. The promoters for snRNA (small nuclear RNA) genes lie upstream of the startpoint similar to other promoters. In both cases, the transcription factors 230 recognize individual promoter sequences, which in turn direct the binding of RNA poiymerase. Three types of RNA poiymerase III promoters are shown in Fig. 3.6. 3.5.2 Internal promoters The two internal type promoters have bipartite structure. Two short sequence elements remain separated by a variable sequence. The distance between boxA and boxB in a type 2 promoter vary extensively, but bringing the boxes too close inhibits function. Internal promoters bind to three different factors. TFIIIA (zinc finger proteins), TFIIIB (consists of TBP and two other proteins) and TFIIIC (large protein complex [<500 kD], has at least 5 subunits and the size is comparable to RNA poiymerase III itself). Most likely, at type 2 promoters, TFIIIC recognizes boxB, but binds to a more extensive region including both boxes A and B. At type 1 promoters, TFIIIA binds to a sequence that includes boxC, and this is required to enable TFIIIC to bind. In both cases, the binding of TFIIIC in turn enables TFIIIB to bind to a sequence surrounding the startpoint. TFIIIA and TFIIIC removal of from the promoter by high salt concentration in vitro but allowing the presence of TFIIIB in the vicinity of the startpoint is sufficient to allow RNA poiymerase III to bind at the startpoint. So TFIIIB appears to be the only true initiation factor required by RNA poiymerase III and TFIIIA and TFIIIC are assembly factors, whose role is to assist the binding of TFIIIB at the right location. This sequence of events explains how the promoter boxes downstream can cause RNA poiymerase III to bind at the startpoint, farther upstream. TFIIIB includes the same protein, TBP that is present in SL1; this could be the subunit of TFIIIB that interacts directly with RNA poiymerase III. Any alteration in the upstream the internal promoter region can alter the efficiency of transcription. Genes having upstream promoters has the TATA element which confer specificity for type III polymerases. Interestingly, some snRNAs are transcribed by poiymerase II while others are transcribed by poiymerase III. In both the cases, Fig 3.6 : RNA polymerase III Promoters

231 the same type of promoters is present. Initiation commences at TATA site but the presence of PSE (Proximal Sequence Element) and Oct elements along with their factors increases the transcription efficiency. The PSE element may be essential at promoters used by RNA polymerase II, whereas it is stimulatory in promoters used by RNA polymerase IH RNA polymerase III terminates transcription with U's immediately after a GC-rich region but there is no fonnation of stem-loop structure. Termination usually occurs at the second U within a run of four U's, but some molecules terminate with 3 or even 4 U's. 3.5.3 Eukaryotic promoters Most eukaryotic genes have conserved DNA sequences at -25 to -30 from the start point of RNA transcription called TATA box or Hogness box that specify a particular start point during RNA transcription.. By convention, the sequence is given on the non-template strand. The TATA box has a consensus sequence 5' - TATAAAA- 3'. Mutation at this region affects the transcriptional process. Promoters that lack TATA box, there is no definite initiation point but appears to be controlled by a CT-rich area, called the initiator element (Inr) having a consensus sequence TCA, at +1 coupled with a down stream promoter element (DPE) at about +28 to +34. Further upstream, many promoters have a CAAT box, found approximately at -75 positions of many genes but the position may vary. The consensus sequence is 5' -GGCCAATGT- 3'. Alteration of CAAT box markedly reduces transcriptional rate. Additional sequences like GC box (5' -GGGCGG- 3') is found at -90 positions and often there are more than one copy of GC elements. This sequence may function in either orientation i.e. 5' -GGGCGG- 3' or 5'GGCGGG-3'. Interestingly, promoter of one gene may vary considerably from the other gene and no element is essential for all promoters. 3.5.4 Enhancer & Silencer Enhancer sequences influences the transcriptional rate of a gene. Enhancer may function in either orientation and is usually located far away from the actual initiation point of transcription, sometime 1000 bp apart from the promoter sequence, usually upstream the start point. In animal cells, enhancers can be located down stream from the initiation point. Similarly there are sequences that have the same properties like that of the enhancers but they repress rather that activate gene transcription. The elements
232
are called silencer elements. Silencers are less common than enhancers. There
are no consensus sequences for eukaryotic enhancer or silencer element and their exact mechanism of action is still not clear. It is proposed that specific protein factors interact with the enhancer elements and folds DNA in a way so that enhancer elements interact with the transcriptional factors and regulators in the promoter region and subsequently activate (enhances) or repress (silencer) RNA transcription. 3.5.5 Initiation of transcription by RNA polymerse II Robert Roder (1979) discovered that eukaryotic RNA polymerase II could not initiate transcription unless some other protein factors are added to the reaction mixture in vitro. Biochemical analysis revealed the existence of specific proteins called Transcription factors that are required by RNA polymerase II to start RNA synthesis. Some of these factors bind directly with the DNA while others appear to bind to the RNA polymerase. Transcription factors are usually designated by letters TF (for transcription factor) followed by Roman numeral - I or II or III to indicate the type of polymerase they bind and finally followed by a letter characterizing the type of factor, e.g. TFIIB, TFIIE etc. Two general types of transcription factors are involved: 1. Those proteins that are required by all polymerase II and are called Basal Transcription factors. At least five basal transcription factors are required for initiation of transcription ob RNA polymerase II
in vitro system. 2. Additional transcription factors that bind to DNA sequences and control the expression of specific genes and thus responsible for regulation of gene expression. l
The first step in formation of a initiation complex is the binding of a complex called TFIID.
One of the subunit of TFIID recognizes and binds to TATA box and this subunit is called TATA-binding protein (TBP). In essence, TFIID appears to be similar to the sigma factor in RNA polymerase (Fig. 3). l TBP is a 30 kd protein that binds to the minor groove of the DNA at the TATA promoter sequence. Binding is 10 5 times more tightly to TATA than with other sequences. TBP is saddle shaped protein with two similar domains. The TATA box binds to the concave surface of TBP. This binding induces large conformational changes. The minor
233 groove widens from 5Å to 9Å bat does not break the hydrogen bonds. This substantial unwinding of the minor groove enables extensive contact with the anti-parallel â strands on the concave side of the TBP. Immediately outside the TATA box, classical B-DNA resumes. This complex is distinctly asymmetric. The asymmetry is crucial for specifying a unique start site and ensuring that transcription proceeds unidirectionally. l The surface of the TBP saddle provides docking sites for the bindings of other transcription factors. l DNA bound TBP of TFIID first recruits TFIIA which further enhances the binding of the TBP. l Then TFIIB is recruited forming a complex TBP-TFIIB at the promoter region. l Binding of TFIIB sets the stage for the binding of
RNA polymerase, which binds to the TBP-TFIIB complex in association with a third factor, TFIIF.

l Two additional factors - TFIIE and TFIIH bind to the initiation complex and appear to be necessary for initiation of transcription. TFIIH is multi- subunit factor. First two subunits has hehcases activity, which my unwind DNA around the initiation site. Another subunit of TFIIH is a protein kinase that phosphorylates repeated sequences present in the C-terminal domain of the largest subunit of RNA polymerase II. l Phosphorylation of the C-terminal domain of the RNA polymerase II releases the enzyme from its association with the initiation complex, allowing it to proceed along the template as it elongates the growing RNA chain. The various transcription factors described here represents the minimal system required for transcription in vitro; additional factors may be needed within the cell. Furthermore, RNA polymerase II appears to remain associated with some transcription factors in vivo prior to the assembly of a transcription complex on DNA. Such preformed holoenzyme complexes probably are recruited to a promoter via direct interaction with TFIID. What actually occurs within the eukaryotic cell
234 during RNA transcription still needs to be worked out. Moreover, the functions of many of the basal transcription factors are still unknown and unanswered. 3.5.6 Elongation Placement of the first ribonucleotide with its corresponding deoxyribonucleotide in the DNA each new ribonucleotide attaches to the 3' -OH group of the previous ribonucleotide. RNA synthesis is continuous and proceeds in the 5' to 3' direction. Energy is derived from the cleaving ofihe two phosphate group of the new incoming ribonucleotide that pairs with the DNA and bjnds to the 3' -OH of the previous ribonucleotide residing within the RNApolymerase. The double stranded RNA-DNA hybrid is very transient. As RNA polymerase moves forward, the nascent RNA separates from the DNA. At any given time, the number of nucleotides of RNA that remain paired with the DNA template may be as many as 2 to 12. The unfolded DNA rapidly rewinds after RNA synthesis is over at that point and therefore nicking of the DNA is not required to release the tension of unwinding. Moreover, only 18 bases are unwounded at any time. The rate of synthesis is not always consistent as other proteins present in the cell often influence the rate of RNA synthesis. Occasionally, RNApolymerase II stalls synthesis, may be due to some configuration change in the polymerase or my encounter a nucleotide sequence that causes the polymerase to stall on the DNA. When this happens, TFIIS causes the RNA polymerase to move backward, and then TFIIS removes the 3' end of the RNA, permitting the RNA polymerase to attempt elongation again over the point
235 where the stall occurred. It may be noted that eukaryotic RNA polymerases have 3' — 5' exonuclease activity. 3.5.7 Termination The termination of transcription in eukaryotic genes is less well understood than in bacterial genes. The three eukaryotic RNA polymerases use different mechanisms for termination. RNA polymerase I requires a termination factor, like the rho factor utilized in termination of some bacterial genes. Unlike rho, which binds to the newly transcribed RNA molecule, the termination factor for RNA polymerase I binds to a DNA sequence downstream of the termination site. RNA polymerase III ends transcription after transcribing a terminator sequence that produces a string of Us in the RNA molecule, like that produced by the rho- independent terminators of bacteria. Unlike rho-independent terminators in bacterial cells, RNA polymerase III does not requre that a hairpin structure precede the string of Us. In many of the genes transcribed by RNA polymerase II, transcription can end at multiple sites located within a span of hundreds or thousands of base pairs. 3.5.8 Transcription by RNA polymerse I RNA polymerase I & III apply the same basic mechanism of transcription and only differ in the recruitment of specialized transcription factors that recognizes and associate with appropriate promoter sequences. RNA polymerase I is solely responsible for the transcription of ribosomal RNA genes, which are present in tandem repeats. Transcription of these genes yields a large 45S pre-RNA, which is then processed to yield the 28S, 18S, and 5.8S rRNAs. The promoter sequence of ribosomal RNA gene is recognized by two transcription factors, UBF (upstream binding factor) and SLl (selectivity factor 1), which together bind to the promoter site and then recruit polymerase I to form the initiation complex. The SLl is composed of four protein subunits one of which is TBR The promoter here lacks the TATA box, and TBP therefore do not bind to
236 specific promoter sequences. Instead, the association of TBP with ribosomal RNA genes is mediated by the binding of other proteins in the SL1 complex to the promoter, situation similar to the association of TBP with the Inr sequences of polymerase II genes that lack TATA boxes. The genes for tRNAs, 5S rRNA and
some of the small RNAs involved in splicing and protein transport are transcribed by polymerase III.

Interestingly, the promoters of these genes lie within, rather than upstream of the transcribed sequence. The well studied 5S RNA of Xenopus revealed that at first TFIIIA binds to the promoter followed by TFIIIC, TFIIIB and then the polymerase. In case of tRNA, the promoter sequence is recognized by TFIIIC and not TFIIIA. The multimeric TFIIIB protein appears to be the most common factor for all the polymerases to initiate transcription. Ribosomal RNA (rRNA) There are 4 kinds. In eukaryotes, these are l 18S rRNA. One of these molecules, along with some 30 different protein molecules, is used to make the small subunit of the ribosome. l 28S, 5.8S, and 5S rRNA. One each of these molecules, along with some 45 different proteins, are used to make the large subunit of the ribosome. The S number given for each type of rRNA reflects the rate at which the molecules sediment in the ultracentrifuge. The greater the number, the larger the molecule (but not proportionally). The 28S, 18S, and 5.8S molecules are produced by the processing of a single primary transcript from a cluster of identical copies of a single gene. The 5S molecules are produced from a different cluster of identical genes. Transfer RNA (tRNA) There are some 32 different kinds of tRNA in a typical eukaryotic cell. l Each is the product of a separate gene. l They are small (~4S), containing 73-93 nucleotides. l Many of the bases in the chain pair with each other forming sections of double helix. l The unpaired regions form 3 loops.
237 l Each kind of tRNA carries (at its 3' end) one of the 20 amino acids (thus most amino acids have more than one tRNA responsible for them). l At one loop, 3 unpaired bases form an anticodon. l Base pairing between the anticodon and the complementary codon on a mRNA molecule brings the correct amino acid into the growing poiypeptide chain. Further details of this process are described in the discussion of translation. Messenger RNA (mRNA) Messenger RNA comes in a wide range of sizes reflecting the size of the poiypeptide it encodes. Most cells produce small amounts of thousands of different mRNA molecules, each to be translated into a peptide needed by the cell. Many mRNAs are common to most cells, encoding “housekeeping” proteins needed by all cells (e.g. the enzymes of glycolysis). Other mRNAs are specific for only certain types of cells. These encode proteins needed for the function of that particular cell (e.g., the mRNA for hemoglobin in the precursors of red blood cells). Small Nuclear RNA (snRNA) Approximately a dozen different genes for snRNAs, each present in multiple copies, have been identified. The snRNAs have various roles in the processing of the other classes of RNA. For example, several snRNAs are part of the spliceosome that participates in converting pre-mRNA into mRNA by excising the intrbns and splicing the exons. Small Nucleolar RNA (snoRNA) As the name suggests, these RNAs (there are probably over 100 of them) are found in the nucleolus where they are responsible for several functions: l Some participate in making ribosomes by helping to cut up the large RNA precursor of the 28S, 18S, and 5.8S molecules. l Others chemically modify many of the nucleotides in these molecules, e.g., by adding methyl groups to ribose. l Still others serve as the template for the synthesize of telomeres. In vertebrates, the snoRNAs are made from introns removed during RNA processing.

238 3.6 Transcription factors Transcription factor is, a protein that works in concert with other proteins to either promote or'suppress the transcription of genes. More specifically, transcription factors regulate gene expression. They bind to specific sequences of DNA upstream or downstream to the gene they regulate and then either enhance or repress transcription of these genes by assisting or blocking RNA polymerase binding respectively. A defining characteristic of transcription factors is that they contain a DNA binding domain (DBD) which bind to gene specific regulatory sites {e.g., promoter sequences). In addition, transcription factors often contain a second domain that sense external signals and in response transmit these signals to the rest of the transcription complex resulting in up or down regulation of gene expression. In some cases the DBD and signal sensing domains reside on separate proteins that associate within the transcription complex to regulate gene expression. Other proteins such as coactivators, chromatin remodelers, histone acetylases, deacetylases, kinases, and methylases, also playing crucial roles in gene regulation but they lack DNA binding domains, and therefore are not classified as transcription factors. 3.6.1 Regulation Gene regulation is a highly complex process as it is dependent upon a number of factors. In vitro experiments suggested that the assembly of transcription factors dictated by the DNA sequence. However, epigenetic information present on DNA appears to play an important role in transcriptional activation. 3.6.2 Classes of transcription factors There are three classes of transcription factors: a) Upstream transcription factors are proteins that bind somewhere upstream of the initiation site to stimulate or repress transcription, b) Inducible transcription factors are similar to upstream transcription factors but require activation or inhibition, c) General transcription factors General transcription factors are involved in the formation of a preinitiation complex that participate in the transcription of class II genes to mRNA templates.Tata binding protein, (TBP) is a general transcription factor, that binds to the TATAAbox, the motif that resides upstream from the coding region in all genes. TBP is responsible for the recruitment of the RNA Pol II holoenzyme, the final event in transcription initiation. The most common general transcription factors are TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH. They are ubiquitous and interact with tire core promoter region surrounding the transcvription start site(s) of all class II genes.

239 TFIIA TFIIA consists of two subunits in yeast and three in humans and drosophila (two subunits are derived from a precursor protein). TFIIA binds directly to TBP and stabilizes its binding to DNA, perhaps through direct contact with the DNA. TFIIA binding does not preclude TFIIB binding or other components of the transcription complex. However, binding of TFIIA to TBP is mutually exclusive with binding of some negative regulatory proteins. TFIIA acts as an anti-repressor, stabilizing TFIID binding by blocking repressors of transcription that inhibit binding of other transcription factors or that remove TBP from the DNA. Activation of transcription may be dependent on this TFIIA function. TFIIB : It is a single subunit measuring 35 to 40 kd. The protein possesses a zinc finger domain at the N-terminus and a direct repeat in a proteolytically stable C- terminal domain. TFIIB binds directly to TBP, recruits RNA polymerase II, in part through an interaction with the small subunit of TFIIF. Several acidic activators can bind TFIIB in vitro. The protein probably stabilizes TBP binding to TATA element and is required for association of RNA polymerase II to the initiation complex. TATA-Binding Protein is shown in green, TFIIB in red (Fig. 3.7). TTFIID is a multi-component (<5 subunits) transcription factor that recognizes and binds to the promoter DNA. TFIID consists of a DNA binding subunit that recognizes the TATA element and is therefore designated TATA-binding protein (or TBP), as well as several TBP-associated factors (or TAFs). TFIID helps in recruiting the rest of the factors through a direct interaction with TFIIB. The TBP subunit of TFIID is sufficient for TATA element binding and TFIIB interaction, and can support basal transcription. However, this basal transcription reaction does not respond to upstream transcription activators. Many of these regulatory factors interact with TBP or TAFs in various in vitro assays. TBP also interacts directly with TFIIA. Fig. 3.7: 3D structure of TFIIB

240 Features: TBP consists of a 180 ammo acid domain that is sufficient for activity. This domain is made up of an imperfectly repeated sequence, and the repeats are reflected in the symmetry of the molecule (see picture below). The protein resembles a saddle, with the inner surface contacting DNA and the outer surface presumable making protein-protein contacts. TFIID binding is thought to be the first step in transcription initiation. Some of the TAFs also bind to initiator elements. TBP is also a component of the RNA polymerase I and RNA polymerase III transcription complexes. TFIIE: It has two subunits, probably a tetramer consisting of two molecules of each subunit. The large subunit has a zinc finger domain. TFIIE modulates the helicase and kinase activities of TFIIH and the two factors show species-specific interactions. It recruits TFIIH to the initiation complex and modulates TFIIH kinase and helicase activities. Appears to be required for escape of the RNA polymerase into elongation mode (promoter clearance). TFIIF: The molecule has two subunits. TFIIF binds directly to RNA polymerase II. TFIIF is necessary for RNA polymerase II to stably associate with the TFIIF-TFIIB-promoter complex. There is a protein interaction between the small subunit and TFIIB in vitro and a genetic interaction between the large subunit and TFIIB. It Helps recruit RNA polymerase II to the initiation complex in collaboration with TFIIB. TFIIF is a component of the yeast holoenzyme and mediator complexes. Promotes transcription elongation, may remain associated with the elongating polymerase. TFIIH: Mammalian and yeast TFIIHs have at least six subunits. Most subunits are now cloned, although not all are published.Yeast subunits: SSL2(RAD25), RAD3, SSL1, TFB1, TFB2, TFB3, TFB4. In addition to the TFIIH subunits, there is an associated complex consisting of a CDC-like kinase and cyclin-like subunit. This kinase complex is sometimes referred to as TFIIK. Yeast subunits: KIN28 and CCL1. The two largest TFIIH subunits are ATP-dependent helicases of opposite polarity. Two of the smaller subunits have possible zinc finger domains. TFIIH appears to be dependent upon TFIIE for incorporation into the initiation complex. The associated kinase (TFIIK) complex can phosphorylate the C-terminal domain of the pol II largest subunit. TFIIH is essential for promoter melting (separation of the two DNA strands) and/or promoter clearance (i.e. for pol II to break free of the initiation complex into elongation mode). Surprisingly, TFIIH also is essential for Nucleotide Excision Repair (NER) of damaged DNA. The relationship between TFIIH's transcription and repair functions is not understood yet.
241 SPT16 : Subunit of the heterodimeric FACT complex (Sptl6p-Pob3p), facilitates RNA Polymerase II transcription elongation through nucleosomes by destabilizing and then reassembling nucleosome structure of the DNA. Sptl6p has been found to physically interact with PobSp (which has sequence similarity to some HMG chro-matin-associated proteins) and the catalytic subunit of DNA polymerase alpha, PolJp. Some of the Sptl6p/Pob3p complex in the cell is chromatin associated, and some copurifies with the DNA polymerase alpha-primase complex. The N-terminal third of Sptl6p is necessary for the maintenance of chromatin repression, but not for activation of genes. Homologs of SPT16 have been found in K. lactis and human TBP (TATA binding protein))is a DNA binding protein that binds sequence specifically to the TATA box. It is vital for all eukaryote transcription, and will in some cases be forced to bind non sequence specifically. It is involved in DNA melting (double strand separation) and bends
the DNA by 80° (the AT-rich sequence to which it binds facilitates easy melting).
The TBP is an unusual protein in that it binds the minor groove using a â sheet. TBP is â subunit of the eukaryotic transcription factor TFIID. TFIID is the first protein to bind to DNA during the formation of the pre-initiation transcription complex of RNA polymerase II (RNA Pol II). Binding of TFIID to the TATA box in the promoter region of the gene initiates the recruitment of other factors required for RNA Pol II to begin transcription. Each of these transcription factors are formed from the interaction of many protein subunits, indicating that transcription is a heavily regulated process. TBP is also a necessary component of RNA polymerase I and RNA polymerase III, and is perhaps the only common subunit required by all three of the RNA polymerases. When TBP binds to a TATA box within the DNA, it distorts the DNA by creating a nearly 90 degree bend. The distortion is accomplished through a great amount of surface contact between the protein and DNA. TBP binds with the negatively charged phosphates in the DNA backbone through positively charged lysine and arginine amino acid residues. The sharp bend in the DNA is produced through projection of four bulky phenylalanine residues into the minor groove. As the DNA bends, its contact with TBP increases, thus enhancing the DNA-protein interaction. Yeast TBP bound to DNA.

242 The strain imposed on the DNA through this interaction initiates melting, or separation, of the strands. Because this region of DNA is rich in adenine and thymine residues, which base pair through only two hydrogen bonds, the DNA strands are more easily separated. Separation of the two strands exposes the bases and allows RNA polymerase II to begin transcription of the gene. FACT complex (Facilitates chromatin transcription complex): An abundant nuclear complex, which was originally identified in mammalian systems as a factor required for transcription elongation on chromatin templates. The FACT complex has been shown to destablilize the interaction between the H2A/H2B dimer and the H3/ H4 tetramer of the nucleosome, thus reorganizing the structure of the nucleosome. In this way, the FACT complex may play a role in DNA replication and other processes that traverse the chromatin, as well as in transcription elongation. FACT is composed of two proteins that are evolutionarily conserved in all eukaryotes and homologous to mammalian Sptl6 and SSRP1. In metazoans, the SSRP1 homolog contains an HMG domain; however in fungi and protists, it does not. For example, in S. cerevisiae the Pob3 protein is homologous to SSRP1, but lacks the HMG chromatin binding domain. Instead, the yFACT complex of Sptl6p and Pob3p, binds to nucleosomes where multiple copies of the HMG-domain con + aining protein Nhp6p have already bound, but Nhp6p does not form a stable complex with the Sptl6p/Pob3p heterodimer. 3.6.3 Structural binding motifs DNA-Binding motifs In general, transcription activators have two domains: one domain binds specifically to DNA and the second domain interact with other transcription factors. Four different types of transcription activators have been identified and their DNA binding motif characterized. 1. Zinc finger domains : have repeats of cystein and histidine residues, which interact with Zn++ ions to fold in a fingerlike fashion to grasp the DNA. E.g. TFIIIA, Steroid hormone receptors also have Zinc finger domains, which regulate gene transcription in response to hormones like estrogen & testosterone. 2. Helix-turn helix : In this type of activators, one helix makes most of the contact with DNA while the other helix lies across the complex to stabilize the interaction. E.g. Homeodomain protein, catabolic activator protein (CAP) in Drosophila Note: Homeodomain proteins play critical roles in the regulation of gene expression during embryonic development.

243 1. Leucine Zipper: contain DNA binding domains formed by dimerization of two polypeptide chains. The leucine zipper contains four or five leucine residues spaced at intervals of seven residues resulting in the exposure of hydrophobic side chains at one side of the helical region. This hydrophobic region of the domains serves as the point of dimerization of the two domains of the transcription activator, thereby interlocking the DNA strand within it by the interaction of positively charged lysine and arginine with the DNA. 2. Helix loop helix: In the type, the amino acid sequence is similar to leucine zipper domains, except that their dimerization domains are formed by two other domains separated by a loop. An interesting feature is that both leucine zipper and helix loop helix transcription factors is that different members of these families can dimerize with each other. Such dimerization gives rise to formation of an array of transcription activators that differ in DNA specificity and also binding to other transcription factors. These two transcription activators play an important role in regulation tissue specific gene expression. The activation domains are not well characterized as their DNA binding domains. Some of these domains are acidic, some basic. But they somehow interact with basal transcription factors like TFIID, TFIIB and facilitate the initiation of transcription of specific genes. 3. MADS box is a conserved sequence element found in a family of transcription factor encoding genes. The length of the MADS-box is defined differently by various authors, but typical lengths suggested are 168 base pairs or 180 base pairs. The element encodes the MADS-domain that have DNA-binding properties. In plants, MADS-box genes have undergone a substantial radiation. In Arabidopsis the MADS box genes SOC and FLC have been shown to have an important role in the integration of molecular flowering time pathways. These genes are essential for the correct timing of flowering, and help to ensure that fertilization occurs at the time of maximal reproductive potential. 3.6.4 Protein-binding motifs STAT The Signal Transducers and Activator of Transcription (STAT) proteins regulate many aspects of cell growth, survival and differentiation. The transcription,factors of this family are activated by the Janus Kinase JAK and dysregulation of this pathway is frequently observed in primary tumors and leads

244 to increased angiogenesis and enhanced survival of tumors. Knockout studies have provided evidence that STAT proteins are involved in the development and function of the immune system and play a role in maintaining immune tolerance and tumor surveillance. Function of STAT proteins STAT proteins were originally described as latent cytoplasmic transcription factors that require phosphorylation for nuclear retention. The unphosphorylated STAT proteins shuttle between the cytosol and the nucleus waiting for its activation signal. Once the activated transcription factors reach the nucleus, they bind to a consensus DNA-recognition motif called gamma activated sites (GAS) in the promoter region of cytokine-inducible genes and activate transcription of these genes. Activation of STAT proteins Extracellular binding of Cytokines induces activation of the intracellular Janus kinase that phosphorylates a specific tyrosine residue in the STAT protein which promotes the dimerization of STAT monomers via their SH2 domain. The phospho- rylated dimer is then actively transported in the nucleus via importin a/b and RanGDP complex. Once inside the nucleus the active STAT dimer binds to cytokine inducible promoter regions of genes containing gamma activated site (GAS) motif and activate transcription of this proteins. The STAT protein can be dephosphorylated by nuclear phosphatases which leads to inactivation of STAT and the transcription factor becomes transported out of the nucleus by exportin crml/RanGTP.

245 Unit 4 o Post Transcriptional Modification of RNA Structure 4.1 Introduction 4.2 Post transcriptional modification of rRNA 4.3 Post transcriptional modification of tRNA 4.4 Post transcriptional modification of mRNA 4.5 The addition of the poly (A) tail on mRNA 4.6 RNA splicing 4.7 Nuclear export of mRNA 4.1 Introduction In prokaryotes, tRNA and rRNA undergo modification after being transcribed but mRNA do not get the opportunity to undergo modification as the transcription and translation processes are coupled. In Eukaryotes, all the three types of RNAs (mRNA, tRNA and rRNA) undergo post transcriptional modifications. 4.2 Post transcription modification of r RNA Prokaryotes have three ribosomal rRNA (23S, 16S and 5S) equivalent to the eukaryotic 28S, 18S and 5S rRNAs of eukaryotic cell. In both the cell types, the processing of a single pre-RNA transcript produces different rRNAs. In eukaryotes, only the 5S RNA does not undergo much modification as they are synthesized from a separate gene. The steps of cleavage to remove the introns and obtaining the final product are shown in the figure 4.1. Fig. 4.1: Processing of newly synthesized rRNA

246 In eukaryotes, after the 5.8S RNA is produced, it is hydrogen bounded to 28S RNA. Further, rRNA processing involves the addition of methyl groups and sugar moieties to specific nucleotides, but the function of these modifications is unknown. 4.3 Post transcriptionaJ modification of tRNA Prokaryotes and eukaryotes synthesize tRNA as precursor molecules. Some pre t-RNA transcripts have several tRNA sequences, which are cleaved and modified to obtain mature functional tRNA. Some tRNA sequences are present within the pre-RNA transcripts.

247 The 5' end of all tRNAs is modified by RNase P (ribozyme). Conventional RNase modify the 3' of end of tRNA which is the by followed by addition of -CCA nucleotides. Some tRNA has the information of-CCA already encoded in the DNA. All tRNAs have -CCA sequence at their 3' end. Moreover, 10% of the bases in tRNAs are altered to yield a variety of modified nucleotides (Fig. 4.2a & 4.2b) at specific positions in tRNA molecules but their exact function is still not clear. 4.4 Transcriptional modification of mRNA mRNA in all organisms can be distinguished into three primary regions (Fig. 4.3). a) The 5' untranslated region (5' UTR) also called the leader sequence do not code for any amino acids but carry vital information for subsequent mRNA modifications and translation.

In bacterial mRNA, this region

contains a consensus sequence called the Shine-Dalgarno sequence, which serves as the ribosomebinding site during translation. It is found approximately seven nucleotides upstream of the first

codon translated into an amino acid (called the start codon). Eukaryotic mRNA has no equivalent consensus sequence in its 5' untranslated region, ribosomes bind to a modified 5' end of mRNA. b) The next section of mRNA is the protein-coding region, which comprises the codons that specify the amino acid sequence of the protein. The protein-coding region begins with a start codon and ends with

a stop codon.

c)

The last region of mRNA is the 3' untranslated region (3' UTR),

a sequence of nucleotides at the 3' end of mRNA that is not translated into protein.

The 3' untranslated region affects the stability of mRNA and the translation of the mRNA protein-coding sequence.

Fig. 4.3: Structure of mRNA molecule

248 Processing of mRNA

In eukaryotes, transcription and translation are separated in both time and space

and this separation provides an opportunity for eukaryotic RNA to be modified before it is translated: The initial transcript of protein-encoding genes of eukaryotic cells is called pre-mRNA Eukaryotic mRNA undergoes extensive alteration after transcription. Changes are made at both the 5' end, the 3' end, and also in the protein-coding section of the RNA molecule The addition of the 5' Cap The 5' end of all eukaryotic pre-mRNAs are modified by the addition of a extra nucleotide, followed by methylation (addition of CH 3 group) to the 2'-OH group of the sugar of one or more nucleotides at the 5' end - a process termed as capping and the structure is called 5' cap (Fig. 4.4). Capping occurs a few moments after the commencement of transcription. Transcription starts with a nucleoside triphosphate which is usually a purine (A or G). The capping process involves the addition of GTP molecule in the reverse direction to the 5' terminal residue. Addition of the 5' terminal G is catalyzed by a nuclear enzyme, guanylyl transferase. Subsequently, methyl groups are added to the N7of GTP molecule. The next step is to add another methyl group, to the 2' -O position of the penultimate base (which was actually the original first base of the transcript before any modifications were made). This reaction is catalyzed by another enzyme (2' -O-methyl- transferase). A cap that possesses this single methyl group is known as a cap 0 and is found in unicellular animals. A cap with the two methyi groups is called cap 1. This is the predominant type of cap in all Fig. 4,4: Structure of 5' cap eukaryotes except unicellular organisms. Methylation may also occur at second and third positions and is called cap 2. If the second nucleotide is adenine, then the methylation occurs at the N 6 position since it already has a methyl group at 2' position. The third-base modification is always a 2'-O ribose methylation. This cap usually represents less than 10-15% of the total capped population. Fig 4.4 : Structure of 5' cap
249 In addition to the methylation involved in capping, a low frequency of internal methylation occurs in the mRNA only of higher eukaryotes. This is accomplished by the generation of N 6 methyladenine residues at a frequency of about one modification per 1000 bases. There are 1-2 methyladenines in a typical higher eukaryotic mRNA, although their presence is not obligatory, since some mRNAs do not have any. The cap blocks the 5' end of mRNA. The 5' cap helps to align mRNAs on the ribosome during translation initiation. Cap binding proteins recognize the cap and attach to it; a ribosome then binds to these proteins and moves downstream along the mRNA until the start codon is reached and translation begins. The presence of a 5' cap
also increases the stability of mRNA and influences the removal of introns. 4.5
The addition of the Poly(A) tail on mRNA Most mature eukaryotic mRNAs have from 50 to 250 adenine nucleotides at the 3' end [a poiy (A) tail]. These nucleotides are not encoded in the DNA but are added after transcription (Fig. 4.5) in a process termed polyadenylation. Polymerase II encoded genes transcribes well beyond the end of the coding sequence (more than 1000) at the 3' end which is then cleaved and the poly(A) tail is added. Fig. 4.5: Addition of poly(A) tail at 3' end of pre-mRNA The site of cleavage at the 3' end of pre-mRNA is determined by specific upstream and downstream sequences (Fig. 6). Upstream consensus is usually AAUAAA that determines the site of the cleavage and resides 11 to 30 nucleotides
250 upstream of the cleavage site. A sequence rich in Us (or Gs and Us) is typically present down-stream of the cleavage site. In mammals, 3'cleavage and the addition of the poly(A) tail requires a complex consisting of several
proteins: a) Cleavage and polyadenylation specificity factor (CPSF); b) Cleavage stimulation factor (CstF);
c) At least two cleavage factors (CFI and CFII) ; d) Polyadenylate polymerase (PAP). CPSF binds to the upstream AAUAAA consensus sequence, whereas CstF binds to the downstream sequence (Fig. 4.6). CstF after cleaving the pre- mRNA leave the complex. The cleaved 3' end of the pre-mRNA is then degraded. CFSF and PAP remain bound to the pre-mRNA and carry out polyadenylation. After the addition of approximately 10 adenine nucleotides, a poly(A)-bindirig protein (PABH) attaches to the poly(A) tail and increases the rate of polyadenylation. As more of the tail is synthesized, additional molecules of PABII attach to it. The poly(A) tail confers stability to many mRNAs, increasing its half life, making it available for longer time for the translational process, before it is degraded by cellular enzymes. The stability conferred by the poly(A) tail is dependent on the proteins that attached to the tail. Eukaryotic mRNAs that lack a poly (A) tail depend on a different mechanism for 3' cleavage. It requires the formation of a hairpin structure with the aid of a small ribonucleoprotein particle (snRNP) Fig. 4.6 : Process of binding of CPSF and Cstf

251 5' 5' 5' Exon 1 branch point exon 2 exon 3 5' 55 3 ' 55 3' 3' 3' called U7 (Fig. 4.7). U7 contains an snRNA with nucleotides that are complementary to a sequence on the pre-rnRNA just downstream of the cleavage site. U7 most likely binds to this complementary sequence. A hairpin-binding protein binds to the hairpin structure and stabilizes the binding of U7 to the complementary sequence on the pre-mRNA and cleave the 3' non coding sequences Fig. 4.7 : Ribonucleoprotein particle 4.6 RNA Splicing 4.6.1 Intron Removal Most eukaryotic genes are interrupted by non-coding sequences called introns. The intron consists of GU at 5' end and AG at 3' end, while a branch site (A) in the middle and a (py)n, meaning a stretch of primidine near the 3' end. During mRNA processing, the introns are precisely excised from the mature mRNA. At first, a protein complex called spliceosomes cleaves the 5' end of the intron.
In the second step, the 5' end of the intron is
joined to an adenine residue within the intron at the 2'-OH group of the adenine nucleotide to form a 2', 5' -phosphodiester linkage, which is quite unusual bond. The resulting intermediate is a lariat like structure. Next the 3 end of the intron is spliced followed by Hgation of two exon units (Fig. 4.8). Fig. 4.8: Schematic diagram of splicing mechanism
252 The entire process is defined by three critical elements in the mRNA: a. Sequences at the 5' splice site of the intron (GU) b. Sequence at the 3' splice site of the intron (AG) c. Sequences within the intron at the branch point where the 5' end joins. 4.6.2 Splicesosome Spliesome are protein and snRNA complexes. l The RNAs are snRNAs called U] U 3 U 4 U 5 U 6 Their size varies from 50-200 nucleotides. l U 1 U 2 and U. along with specific proteins exist as independent units. l U 4 and U 6 h along with their proteins are grouped together as a single unit. l U, snRNP first recognizes the 5' splice site sequence and bind to it by complementary base pairing l U 2 then binds at the branch site with 'A' within the intron l A preformed, complex consisting of U 4 U 6 and U 5 snRNPs is then incorporated into the forming splisosome l The U 5 component binds with both the 5' and 3' splice sites. l The snRNPs then catalyses the reaction by first cleaving the 5 splice site, then joining the 5' terminal nucleotide with a specific adenine residue within the intron followed by cleavage at 3' splice site and joining of the exons. l The snRNAs in the snRNPs actually catalyzes the reaction. Note: RNAs are capable of self splicing- i.e. remove their own introns. Eg. 28S rRNA in Tetrahymena. Self-splicing RNAs are also present in mitochondria, chloroplast and bacteria. On the basis of the catalytic activity of self-splicing RNAs, they have been grouped into two classes. Class I: In this type, rRNA first cleaves itself at
the 5' end of the intron. The 3' end of the exon then catalyses the reaction at the 3' end of the
intron followed by joining of the two exons. Class II: In this type, self-splicing rRNAs exhibit characteristics of the reaction as observed with mRNA described above.
253 Alternative splicing: Most pre-mRNAs have multiple introns and exones which can be arranged in alternative ways by splicing of the same mRNA can produce different mRNA- a novel means of controlling gene expression. This process is known as alternative splicing and occurs frequently in genes of eukaryotes that provide an important mechanism for tissue-specific and developmental regulation of gene expression. The regulation and selection of splice sites is done by Serine/Argi nine- residue proteins, or SR proteins (Fig. 4.9). The use of alternative splicing factors leads to a modification of the definition of a "gene". There are four known modes of alternative splicing: l Alternative selection of promoters: this is the only method of splicing which can produce an alternative N-terminus domain in proteins. In this case, different sets of promoters can be spliced with certain sets of other exons. l Alternative selection of cleavage/polyadenylation sites: this is the only method of splicing which can produce an alternative C-terminus domain in proteins. In this case, different sets of polyadenylation sites can be spliced with the other exons. l Intron retaining mode: in this case, instead of splicing out an intron, the intron is retained in the mRNA transcript. However, the intron must be properly encoding for amino acids. The intron's code must be properly expressible, otherwise a stop codon or a shift in the reading frame will cause the protein to be non-functional. l Exon cassette mode: in this case, certain exons are spliced out to alter the sequence of amino acids in the expressed protein. E.g. 1. Transcriptional activators consist of two distinct domains: a DNA binding domain and an activation domain. These domains are generally encoded Fig. 4.9: SR Proteins

254 in separate exons, so alternative splicing allows them to be reassorted into different combinations, thereby enabling the production of activators and repressors from the same gene. 2. In Drosophila, alternative splicing of the same pre-mRNA determines whether a fly will be a male or female. Patterns of alternative splicing can vary in different tissues. Several protein factors have been isolated but the mechanism by which the correct splice sites are selected in pre-mRNA is not known. Variations in the expression of such splicing factors in different cell types may result in tissue specific patterns of alternative splicing, there by contributing to the regulation of gene expression during development and differentiation. 4.6.3 Significance of alternative splicing Alternative splicing is of great importance to genetics - it invalidates the old theory of one DNA sequence coding for one polypeptide. External information provide the clue of alternative splicing. Since the methods of regulation are inherited, the interpretation of a mutation may be changed. Alternative splicing allows more information to be stored much more economically in a limited space. It has been noted that it is unnecessary to change the DNA of a gene for the evolution of a new protein. Instead, a new way of regulation could lead to the same effect, but leaving the code for the established proteins unharmed. Another speculation is that new proteins could be allowed to evolve much faster than in prokaryotes. This machanism may allow for a higher probability for a functional new protein. Therefore the adaptation to new environments can be much faster - with fewer generations - than in prokaryotes. This might have been one very important step for multicellular organisms with a longer life cycle. Trans-splicing : In this type, exons from two different pre-mRNAs are joined to form a single mRNA. For example, in trypanosomes, all mRNAs have an identical spliced leader sequence of 35 nucleotides. This leader sequence is present the 5' end of a 137 nucleotide RNA chain. This leader sequence is then spliced and added to all the 5' end of mRNAs by trans-splicing reactions. Trans-splicing machinery also exists in mammals as mammalian cells are capable of carrying out trans-splicing reactions with the nematode spliced leader RNAs (Fig. 4.10).

255 Fig. 4.10: Trans-splicing reactions with the nematode spliced leader RNAs. RNA Editing RNA editing refers to RNA processing events (other than splicing events) that alter the protein coding sequences of some mRNAs. In trypanosomes and related protozoans, addition of'U' and deletions of some nucleotides occur in some mRNAs. The information required for editing is encoded in "guide" RNAs, which are complementary to edited portions of the mature mRNA. The guide RNA contains poly-U tail, which donate the 'Us' during editing. Sometimes the editing is so extensive that half the nucleotide sequences are altered. In mammalian cells similar events occur in mitochondria and also in the somatic cells. For example, in human body two types of apolipoproteins are found, Aop-BlOO (4536 aa: synthesized in liver is unedited form and transports lipids in the circulation) & Apo-B48 (2152 aa: synthesized in the intestine, is edited form and helps in the absorption of dietary lipids in the intestine). In the intestine, CAA codon is changed to a stop codon by enzymatic conversion of C to U by removal of the cytosine amino group at specific site in the mRNA. 4.7 Nuclear export of mRNA 4.7.1 Introduction Compartmentahzation of the eukaryotic genome by the nuclear membrane was probably a necessity to have a greater control over the functioning of the genome and also to avoid unnecessary alterations in the genomic constituents by exposing it to the bustling biochemical activity that occur in the cytoplasm. This compartmentahzation further ensures the presence of specialized environments for different stages of gene expression, such as transcription and protein production. Trafficking of materials between the nucleus and cytoplasm primarily rely

256 on transport receptors in the importin-? superfamily. However, export of mRNA uti- I lizes distinct soluble machinery. In yeast, it has been observed that protein- protein interaction is required for the export of mRNA from the nucleus to the cytoplasm.

For example, in yeast Mex67p interact with Mtr2p and facilitates the export of I po!y(A) + RNA. In metazoans and in humans, TAP was confirmed to be the orthologue of Mex67p, redesignated as NXFl (nuclear export factor 1) which interacts with pl5/ NXT1 in the nucleus for transportation of mRNA. Mtr2p and pl5 share no sequence similarity but the Mex67p-Mtr2p complex displays similar structural architecture to I the NXFl-p 15 heterodimer. However, the distinction between mRNA export and the importin-? family/Ran network is not absolute, as an importin-13 family member has recently been implicated in mRNA export as well. 4.7.2 mRNA export is coupled to splicing NXFl does not bind directly to cellular mRNA. Experiments with Xenopus oocytes demonstrated that the process of splicing can contribute to the efficiency of mRNA export; the spliced product from adenovirus major late (Adml) mRNA was shown to export more efficiently than an identical mRNA engineered to lack an intron. On the mRNAs, exon junction complexes (EJC) are formed after splicing. EJC complex include several components like REF (nuclear export factor), SRml60, RNPS1, DEK, Y14, and later its protein partner Magoh. Recruitment of a unique set of proteins to the spliced mRNA may promote export competency of mRNA. The notion that EJC deposition leads to recruitment of NXFl is an attractive model to explain the stimulatory effect of splicing on export. Direct interactions between REF and NXFl have been observed in both human and yeast systems. REF also shuttles between nucleus and cytoplasm and enhances mRNA export when injected into Xenopus oocyte nuclei as a recombinant protein. The enhanced placement of REF onto mRNA in a splicing-dependent fashion, as well as its association with NXFl, made REF a prime candidate for recruiting NXF1 onto mRNA. A connection between splicing and mRNA export was further solidified with the characterization of a novel role for the putative RNA helicase UAP56 [56-kDa U2AF(65)-associated protein]. Recruitment of REF to spliced mRNA is dependent upon its interaction with UAP56. From these data, a very simple yet elegant mode of coupling splicing with mRNA export became evident. Namely, REF is recruited to splice mRNA through direct interactions with UAP56, and consequently, REF (and the EJC in general) recruits the export factor NXFl to promote exit from the nucleus by mediating docking and presumably movement through the pore.

257 Fig. 4.11: A schematic illustration of mRNA biogenesis is depicted, with the proposed times of recruitment and functions for specific proteins indicated in the boxes. (1) Transcription: Much evidence points to cotranscriptional loading of factors involved in RNA processing, export, and quality control to the nascent transcript. The mobile pore proteins, Nupl53 and Nup98, are candidates (indicated in red text) for loading onto mRNA early in its biogenesis, although this is yet to be demonstrated. (2) Splicing. The splicing factor UAP56 interacts with REF, a component of EJC that is deposited on mRNA during splicing. Loading of certain transport factors, such as REF, can also occur independent of splicing as a part of TREX or if the RNA is sufficiently long. (3) Remodeling and export. NXFl-pl5 is recruited to the mRNA via protein-protein interactions, readying export-competent mRNA for mobilization out of the nucleus. At this step, other proteins, such as Glel, RAE1, Trn-2 (transportin-2),

258 and TREX components, are also thought to function. Certain hnRNPs and EJC components are shed from the mRNP prior to export, and proper mRNP formation appears to be monitored at this stage by the exosome. Specific pore proteins, or Nups, are implicated in moving mRNA cargo through the pore (Fig. 4.12). Although loaded onto the transcript early in biogenesis, Dbp5 may play a late role in remodeling and/ or moving the mRNP complex. (4) Cytoplasmic function. Factors remaining on the mRNA, such as Y14 and Magoh, influence translation and localization of mature mRNPs. Fig. 4.12. Distribution and dynamics of pore proteins and associated factors implicated in mRNA export. Nupl53 and Nup98 are both dynamically associated with the nuclear pore in a manner dependent on ongoing transcription. However, in the case of Nupl 53, there is also evidence for a stable population, which is schematically illustrated here proximal to the inner nuclear membrane. The presence of distinct populations of"Nupl 53 is consistent with epitope exposure of this protein: different regions are exposed at the distal and proximal ends of the pore basket. Regardless of exactly how Nupl53 is arranged at the pore basket, there is evidence that the C-terminal region of this pore protein can extend into the cytoplasm, although Nupl53 does not appear to be released from this face of the pore. In contrast, Nup98 exists in equilibrium with a cytoplasmic pool and is known to interact with components of both sides of the pore. RAE1/Gle2 is a partner protein of Nup98. Both Nupl53 and Nup98 associate with the Nupl07-160 complex, a stable component of the pore. Tpr is also a component of the nuclear pore basket and relies on interaction with Nupl53 for correct localization. CAN/Nup214 is localized to fibrils extending from the cytoplasmic ring of the pore and is a docking site for the dynamic DEAD box helicase, Dbp5.

259 Despite the observation that splicing promotes export of mRNA, such processing is not the only or even the major route for export factor recruitment. Recent functional analysis of mammalian cells also suggested that splicing does not always have a major effect on mRNA export per se. Splicing and export factor recruitment that has been documented may represent only one way that export factors load onto mRNA and indeed may make a significant contribution at this step only when the RNA is particularly short 4.7.3 Coordinating mRNA export with transcription and turnover The addition of a 5' cap, splicing, polyadenylation, and cleavage events occur in close connection to transcription. Concurrent with the processing events, mRNAs are also packaged with a number of proteins specific to this class of RNA (Fig. 1). A significant subset of such proteins was originally classified as hnRNPsfor their ability to associate with heterogeneous nuclear RNA. hnRNP Al a component of hnRNPs was initially implicated in mRNA transport, but it's exact role in transport mechanism remains to be elucidated. Other proteins, not necessarily classified originally in the hnRNP category but loaded onto mRNA, have been functionally connected to both export and transcription. Yralp and Sub2p display both genetic and physical interactions with all members of the yeast THO complex, a protein complex identified originally for a role in transcription elongation. REF and UAP56 along with the vertebrate counterparts of THO and a new protein of unknown function, Texl, make up the TREX (transcription and export) complex. In yeast, specific TREX components associate with genes during transcription and, individually, their deletion results in nuclear poly(A) + accumulation. Together, this suggests that TREX proteins may be important in mediating cotranscriptional recruitment of factors important in export. For example, one protein in the TREX complex, Hprlp, is required for efficient targeting of Yralp and Sub2p to genes undergoing active transcription. Therefore, cotranscriptional recruitment and splicing-dependent recruitment represent two broad mechanisms by which mRNA export factors can associate with RNA cargo. For instance, REF maybe efficiently loaded onto specific RNA cargos via cotranscriptional targeting of the TREX complex and/or through splicing-dependent deposition of the EJC. Another example of the connection between transcription and export is found in the DEAD box helicase Dbp5. Dbp5 is localized at steady state to the cytoplasmic fibrils of the nuclear pore complex (NPC) and has been hypothesized to be involved in a terminal step of mRNA release from the NPC, possibly acting in a remodeling

260 step to unwind mRNPs entering the cytoplasm. Interaction of Dbp5p with TFIIH during mRNA transcription and its shuttling between nucleoplasm and cytoplasm in S. cerevisiae is an indication that Dbp5 may load onto mRNA cargo very early in biogenesis that enables transport and remodeling of mRNA. Overall, much evidence is arising to support a link between mRNA synthesis and the effective recruitment of export factors to the nascent transcript. 4.7.4 Moving on to the nuclear pore Nuclear protein complexes (NPC) span the nuclear envelope and serve as gateways of communication individual nuclear pore proteins or nucleoporins (Nups) present several times, creating octagonal symmetry. The pore also has asymmetric features on its nuclear and cytoplasmic faces. Although much of the process of mRNA export is being deciphered, there is still little known about how mRNPs interface with pore machinery. Some recent studies have focused on the roles of proteins that are closely associated with the pore, such as Glel and RAE1/ Gle2. Glel is essential for mRNA export in both yeast and human cells, and hGlel is a dynamic factor that shuttles between nucleus and cytoplasm. The shuttling domain of hGlel acts as a dominant-negative export inhibitor of both bulkpoly(A) + RNA and specific mRNA transcripts. Docking of hGlel at the NPC was recently shown to depend on an interaction with the pore protein Nupl55. Murine RAE1 is essential; however, cells from mice bearing targeted disruption of RAE1 do not have a detectable defect on bulk mRNA export. In contrast, RAE1 deletion in yeast results in nuclear accumulation of po!y(A) + RNA. Although there appear to be redundant factors in vertebrates, hRAEl interacts with NXF1 and the nucleoporin Nup98, as well as with mRNA itself, and has been speculated to be involved in delivering mRNA cargo-receptor complexes to Nup98. Nup98, in turn, has been implicated through antibody inhibition studies in the export of mRNA as well as other classes of RNA. Nup98 shares similarity with yeast nuclear pore proteins Nupl45, Nupll6, and NuplOO. Deletion of yNup 145 causes the nuclear accumulation of poly(A) + RNA. Vertebrate pore proteins have not been exhaustively screened and individually tested for roles in mRNA export. However, along with Nup98, five other vertebrate pore proteins, Nupl53, Nupl60, Nupl33, Tpr, and CAN/Nup214, have so far been implicated in the export of mRNA (Fig. 4.13). Mouse embryos deficient in CAN/ Nup214not only show arrest in the G 2 phase of the cell cycle but also demonstrate nuclear accumulation of poly(A) + RNA. Nupl59/Rat7,the

261 yeast orthologue of CAN/ Nup214, is similarly implicated in mRNA export, with a temperature-sensitive mutation causing very rapid onset of accumulation of poly(A) + RNA in the nucleus. CAN/Nup214 associates with the mRNA export factor Dbp5, an interaction conserved from yeast to vertebrates. In addition, CAN/ Nup214 is the only vertebrate nucleoporin with a steady- state localization exclusively on the cytoplasmic side of the pore that has been implicated in mRNA export thus far. Although much remains to be elucidated,Nup98 and Nupl53 are prime candidates for coupling the production of mRNA to its transport into the cytoplasm. 4.7.5 Transport through to pore : putting individual components into context In recent years, several models to explain the mechanism of movement through the pore have been proposed. In each, the phenyalanine-glycine repeat motif (FG repeat) regions found in several nucleoporins play a prominent role, both in contributing to an exclusion barrier as well as in serving as binding sites for cargo-receptor complexes. Consistent with this, NXF1 directly interacts with the FG repeat domains of several nucleoporins in vitro. It is presumed that during translocation of mRNAs, associated large heterogenous mRNPs undergo remodeling in conjunction with transport. Elegant immunoelectron microscopy studies have gone on to illustrate that certain proteins are shed from the mRNP, while others accompany the RNA through the pore. Complicating things further, the nuclearpore basket itself has been observed to adopt different conformations when the Balbiani ring mRNA is traversing the pore. The dynamic nature of specific pore basket components themselves (Nup98 and Nupl53),as well as the sensitivity of such mobility to the transcriptional status of the cell, suggests that basket Fig. 4.13 : Different Pore Proteins 262 remodeling may normally be ongoing in a manner linked to RNA trafficking. Recent information suggests that the native NPCs distal ring of the pore basket is not an open hole but rather a dense structure and thus has little scope of remodeling. An alternative point of entry into the pore, in between the fibers of the pore basket, has also been proposed. Much work is still needed to understand how mRNA enters and translocates through the pore. Future approaches that provide high- resolution real-time imaging as well as more precise functional assays are sure to yield a very interesting story about how the complicated network of mRNA biogenesis connects with translocation through the nuclear pore and the downstream fate of the mRNA. 4.7.6 Exportins and importins The traffic through the nuclear envelope is mediated by a protein family which can be divided into exportins and importins. Binding of a molecule (a “cargo”) to exportins facilitates its export to the cytoplasm. Importins facilitate import into the nucleus. The function of exportins and importins is regulated by a G protein called “Ran”. There are two types of G proteins: heterotrimeric G proteins and monomeric G proteins (or small G proteins). The latter includes Ras, Ran, Rho, Rab, etc. Like other G proteins, Ran can switch between GTP-bound and GDP-bound states. Transition from the GTP-bound to the GDP-bound state is catalyzed by a GTPase- activating protein (GAP) which induces hydrolysis of the bound GTR The reverse transition is catalyzed by guanine nucleotide exchange factor (GEF) which induces exchange between the bound GDP and the cellular GTP. The GEF of Ran (denoted by RanGEF) is located predominantly in the nucleus while RanGAP is located almost exclusively in the cytoplasm. Therefore, in the nucleus Ran will be mainly in the GTP-bound state due to the action of RanGEF while cytoplasmic Ran will be mainly loaded with GDP. This asymmetric distribution has led to the following model for the function of exportins and importins. It is thought that binding between an exportin or importin and its cargo depends on their interaction with Ran: RanGTP enhances binding between an exportin and its cargo but stimulates release of importing cargo; RanGDT has the opposite effect, namely, it stimulates the release of exportin’s cargo, but enhances the binding between an importin and its cargo. Therefore, the exportin and its cargo may move together with RanGTP inside the nucleus, but the cargo will be released as soon as the complex moves into the cytoplasm (through nuclear pores), since RanGTP will be converted to RanGDP in the cytoplasm. By contrast,
263 Fig. 4.14: Ran, importin and exportin. (a) The two states of Ran: GTP-bound and GDP-bound. (b) General function of importins and exportins the importin and its cargo may move together with RanGDP in the cytoplasm, but the cargo will be released in the nucleus since RanGDP will be converted to RanGTP in the nucleus.

264 Unit 5 o Translation Structure 5.1 Genetic code 5.2 The translation machinery 5.3 Prokaryotic and eukaryotic translation 5.4 Regulation of translation 5.1 Genetic code 5.1.1 Introduction A code is a system of symbols that equates information in one language with information in another e.g. Morse code. In living organisms, the hereditary information is written in the language of four nucleic acids, A, G, C, and TAJ. and the language of proteins are written in amino acids. As there are 20 amino acids that are genetically encoded by DNA or RNA sequences, the first question that comes in mind is that how many nucleotides are necessary to code for one amino acid. Now we know that genetic codes are triplet codons, each of which represents a single amino acid. Initially, the number of nucleotides necessary to code for one amino acid was derived by reasoning and later confirmed by experimentation. Scientists reasoned if one nucleotide represented one amino acid, only four nucleotides could be coded. If two nucleotides represented each amino acid, there would be 4 2 =16 possible combination of couplets, still not sufficient to code for 20 amino acids. If the code consisted of groups containing one and two nucleotides, it would have 4 + 16-20, just sufficient for all the 20 amino acids but would fail to recognize the pause signal between two genes. Groups of three nucleotides in a row would provide 4 3 = 64 different triplet combinations, more than enough to code for all the amino acids. Theoretically, the above logic appeared to be simple but had to await experimental evidence that proved beyond doubt that groups of three nucleotides indeed are necessary to code for a single amino acid. Each triplet nucleotides in genetics are referred to as codon. 5.1.2 Experimental evidences Crick and his coworkers tested acridine induced mutations in the B cistron of rll locus of T4 phage (acridine mutation is produced by addition or deletion of a
265 nucle-otide). The mutations acted on the principle of addition or deletion of a single nucleotide pair in DNA molecule. Arbitrarily the mutations were designed as + or -on the basis of their suppression effect on other mutations. For eg. : In DNA : TAC TCC CGA ACG ATA CCA GAG In RNA : AUG AGG GCU UGC UAU GGU CUC Protein : Mutations induced by acridine treatment (arbitrarily designated as 4- mutations) Point mutation ? In DNA : TAC GCC CGA ACG ATA CCA GAG (point mutation; a base is replced) In RNA : AUG CGG GCU UGC UAU GGU CUC [NO FRAME SHIFT MUTATION] Protein : Second Mutations that suppress the first imitation (designated as - mutations) Insertion Deletion ?????????????? ???? In DNA : TAC GCC CCGA CGA TAC CAG AG In RNA : AUG CGG GGU GCU AUG GUC UC Protein : FRAME SHIFT MUTATION Three + Mutations can restore the oriainal mutation Insertion [Partial restoration of original] In DNA : TAC GCC CCA TCG ATA CCA GAG CCA [three positive mutation restores the In RNA : AUG CGG CGU ACU UAU GGU CUC GGU original FRAME SHIFT mutation] Protein :
266 Observations: 1. A single + or - mutation is sufficient to alter the wild type trait into mutant 2. The effect of a single + mutation is suppressed either by a - mutation or by 2 further + mutations. Similarly - mutatios can by suppressed by either by a + mutation or by 2 further - mutations. Explanations & Deductions: 1. Due to deletion or insertion of a nucleotide, codon constitution of reading frame gets altered after the point of such change. For this, the polypeptide product will be different resulting in a mutant trait. 2. If it is insertion mutation, two further insertion mutations can restore the original trait. Similarly, if the mutation is caused by deletion, two further deletion mutations can restore the original trait. So, three changes of the similar kind or multiples of 3 are necessary to restore to wild type. This can only be possible if the codons are triplet i.e. consists of 3 nucleotide. 5.1.3 Nucleotide sequence is collinear with a polypeptide's amino acid sequence The nucleotides in RNA or DNA are arranged in a linear order. As DNA codes for proteins, it was assumed that the amino acids in the polypeptide chain must also be arranged in a linear fashion. Although the proteins have a highly complex three dimensional structure under normal circumstances, analysis has revealed that the amino acids in any polypeptide chain are arranged one after another, have definite polarities and show no branching. Thus the linear arrangements of nucleotides and the amino acids led to suggested that there must be one to one corresponding relationship of nucleotides and amino acids during protein synthesis. 5.1.4 Overlapping vs. non-overlapping nature of codons Logical derivation of 3 nucleotides constituting a codon however could not provide clue as to how the codon are arranged i.e. either overlapping or non- overlapping. Point mutations with mutagens like nitrous acids or provalin were used to decipher the arrangement of codons. a) If Overlapping, a sequence of 9 nucleotides will code for 7 amino acids as shown below. Alteration of a single nucleotide will produce changes in minimum three codons.

267 b) If non overlapping, 9 nucleotide will code for only three amino acids and alteration of a single nucleotide will alter only one codon. Original sequence Mutant sequence CAGAGCUCA CAGAACUCA CAG.....codon 1 CAG.....codon 1 AGA.....codon 2 AGA.....codon 2 GAG.....codon 3 GAG.....codon 3 AGC.....codon 4 AGC.....codon 4 GCU.....codon 5 GCU.....codon 5 CUC.....codon 6 CUC.....codon 6 UCA...codon 7 UCA...codon 7 b) If non overlapping, 9 nucleotide will code for only three amino acids and alteration of a single nucleotide will alter only one codon. Original sequence Mutant sequence CAGAGCUCA CAGAACUCA CAG AGC UCA CAG AAC UCA codon 1 codon 2 codon 3 codon 1 codon 2 codon 3 Experiments revealed that a single-nucleotide substitution mutation caused an amino acid substitution of leucine for proline. The two adjacent amino acids were unchanged by the mutation providing evidence against overlapping codons (Tsugita and Fraenkel, 1960).

268 5.1.5 Properties of genetic code 1. Each Genetic Code is a Triplet Codon each of which specifies an amino acid. 2. The code is non-overlapping. E.g. in mRNA 9 nucleotide sequence 5 GAAGUUGAA3 , will be translated to 3 amino acid sequence corresponding to GAA, GUU and GAA and no more. 3. The code is degenerate, which means that in many cases more than one codon specifies the same amino acids. 4. The code is coma less. A coma less code means that no punctuations are needed between any two words i.e. after one amino acid is coded; the second amino acid will be automatically coded by the next three nucleotides. 5. The code includes three stop, or nonsense codons: UAA, UAG, and UGA. These three codons do not code for any amino acids, rather they terminate translation. 6. The code is non-ambigious. Non-ambiguous code means that there is no ambiguity about a particular codon.

A particular codon will always code for the same amino acid wherever it is found. Only exception lies with AUG and GUG at the start point where both code for methionine although GUG actually code for valine. 7. The code is universal. Almost all micro and macro organisms use the same genetic code with a few exceptions. For example, a different code exists in mitochondria of some eukaryotes, so that in cytoplasm and mitochondria same codon may code for different amino acids. 8. During translation, the code is read from 5' to 3' direction. Moving from the 5 to the 3 end of an mRNA, each successive codon is sequentially interpreted into an amino acid (N-terminus to C-terminus of a polypeptide). 9. There exists a fixed reading frame for any gene that includes the initiation codon. The initiation codon specifies the first amino acid to be translated, which is usually AUG that codes for methionine. Mutations may modify the message encoded in sequence in three ways: a) Frameshift mutations where nucleotide insertions or deletions alter the genetic instruction for polypeptide by changing the reading frame. b) Missence mutations change a codon for one amino acid to a codon for a different amino acid. c) Nonsense mutations change a codon for an amino acid to stop codon.

269 Deciphering the Genetic Code Nirenberg and Matthaei developed the technique in the laboratory of Khorana in 1961 to decipher the genetic code. They artificially synthesized mRNA having only 'Uracil' as the component. Next, they used this mRNA to synthesize polypeptide in cell free extracts of E. coli. When poly U was used, polypeptides consisting entirely of phenylalanine were produced indicating that UUU must code for phenylalanine. Similarly poly 'C RNAs produced polypeptides made entirely of proline, meaning that CCC must code for proline. This method was used to decipher the code of almost all amino acids. Exceptions: ? In few single celled eukaryotic protozoans known as ciliates, UAA and UAG, which are nonsense codons in most organisms, specify the amino acid glutamine. ? In mitochondria of yeast, CUA specifies threonine instead of leucine. mRNA codon virus/pro & Mitochondria Eukaryotes Yeast Drosophila Mammal AUA Isoluecine Methionine Methionine Methionine AGA, AGG Arginine Arginine Serine Stop CUA Leucine Threonine Leucine Leucine UGA Stop Tryptophan Tryptophan Tryptophan 5.2 The translation machinery 5.2.1 tRNAs About 15% of total RNA in a cell is tRNA. tRNAs plays a significant role by serving as adopter molecules that recognize the right enzyme activated amino acid and the anticodon on mRNA. Robert Holly (1965) first elucidated the base sequence of alanine tRNA from yeast. Later, more that 100 tRNA was identified and se~ quenced. All known tRNA share common structural features probably because tRNA molecules must be able to interact in nearly the same way with ribosomes, mRNA and elongation factors. Common features are as follows:

270 1. All tRNAs are single chains containing 73 to 93 ribonucleotide (~ 25kd) 2. tRNAs possess some unusual bases like inosine, pseudouridine, dihydrouridine, ribothymidine and methylated or derivatives of AUCG. (Methylation prevents the formation of base pairing, rendering them inaccessible for pairing with other base pairs or other type of interaction and also imparts hydrophobic character, important for interactions with synthetase and ribosomal proteins and for folding). 3. The 5' end of tRNAs is phosphorylated and usually p-Guanine. 4. The base sequence at 3' end of mature tRNA is always -CCA. Activated amino acid binds to the 3' -OH group of the terminal adenosine. 5. About half the nucleotides in tRNAs are base paired to form double helix. Five groups are not base paired: a) 3' CCA terminal b) T—< C loop (ribothymine-pseudouracil-cytosine) c) Extra arm (may have variable no. of residues) (present in only class II tRNA= serene and leucine d) Anticondon loop e) DHU loop (contains several dihydrouracil) 6. The anticodon loop (Fig. 5.1) consists of seven bases, with the following sequence -5' Pyrimidine- Pyrimidine-X-Y-Z-modified purine- variable base 3' 7. X-ray crystallography study of phenylalanine tRNA by Alexander and Aaron (1974) showed that tRNA is a L-shaped structure. There are two segment of double helix; each having about 10 bases pairs in each turn, in accordance with the cloverleaf mode. Bases in the non-helical region participate in unusual bondings (e.g. GG, AA, CC). Moreover, 2'-OH of the ribose phosphate backbone acts as hydrogen donor and interacts with each other. Most bases are stacked on one another and the hydrophobic interactions between the aromatic rings help to stabilize the architecture of the molecule. Subsequent analyzing of all tRNAs showed that they follow the same basic plan.

271 Fig. 5.1: The anticodon loop In both prokaryotes and eukaryotes tRNA are synthesized as precursor molecules. Some pre t-RNA transcripts have several tRNA sequences, which are cleaved and modified to obtain mature functional tRNA. Some tRNA sequences are present within the pre-RNA transcripts. 5.2.2 Ribozyme Ribozyme is an enzyme in which RNA component of a protein-RNA complex is responsible for the catalytic activity rather than the protein (Sidney Altman, 1983). The 5' end of tRNA is modified by RNase P (ribozyme). Conventional RNase modify the 3' of end of tRNA which is the by followed by addition of -CCA nucleotides. Some tRNA has the information of-CCA already encoded in the DNA. All tRNAs have -CCA sequence at their 3' end. In E. coli, there are 60 genes arranged in 25 units that synthesize tRNA molecules. One unit transcribes multimeric precursor for 7 tRNAs that are then cleaved by RNase P, RNase D to produce mature tRNA (tRNA leu 2 × tRNA met, 2 x tRNA gtu, 2 x tRNA glu2) 5.2.3 Amino acid activation & linkage to specific tRNA by specific synthetase Thermo dynamically, formation of peptide bond
between -NH 2 of one amino acid and -COOH group of another amino acid
is not favored. The barrier is overcome by activating the -COOH group of the precursor amino acid. Activation takes place by the following mechanism: -

272 Step 1. R O R O O | || | || || NH 3 + – C– C + ATP –NH 3 + C – C – P – O – Ribose – Adenine + PPi | | | | H O H O Amino acid Aminoacyl adenylate (Aminoacyl~AMP) In the first step, an amino acid is linked to the phosphoryl group of AMP and therefore is known as aminoacyl~AMP Step 2. In the second step, aminoacyl group of aminoacyl-AMP is transferred to either 2' or 3' hydroxyl group of the ribose unit at the 3' end of tRNA to form aminoacyl tRNA. The attachment of amino acid to a tRNA is important not only because it activates the carboxyl group but also because amino acids by themselves cannot recognize the codons on mRNA. tRNA serves as an adopter molecule by recognizing the codons on one hand and by bringing specific amino acids, represented by the codons at the site of protein synthesis.

273 5.2.4 Aminoacyl tRNA synthetase l The amino acid is attached at the 3' end of the tRNA to either the 2' hydroxyl or the 3' hydroxyl. 1. Class I amino-acyl tRNA synthetases attach their associated amino acids to the tRNA 2' hydroxyl (NOTE: typically the hydrophobic amino acids) 2. Class II amino-acyl tRNA synthetases attach their associated amino acids to the tRNA 3' hydroxyl (NOTE: typically hydrophilic amino acids) For each amino acid, there exists a specific amonoacyl tRNA synthetase. These enzymes have been grouped into two classes on the basis of short signature sequences. In Class I there are 10 aminoacyl synthetases that recognize the larger amino acids and more hydrophobic enzymes. Class II (ancient type) aminoacyl tRNA synthetase recognizes the smaller amino acids. Class I enzymes acetylated the 2 -OH group and have a parallel ? domain (the classical dinucleotide binding fold) while class II enzymes acetylate the 3 -OH group (except phe) on the ribose and have an anti parallel ? domain (aminoacyl activating domain). 5.2.5 Base pairing between an mRNA codon and tRNA anticodon It is the specific interaction between tRNA's anticodon and mRNA's codon that makes the decision, which amino acid will be incorporated into the growing polypep-tide chain. Although there is at least one kind of tRNA for each of the 20 amino acids, cells do not necessarily carry tRNAs with anticodons complementary to all of the 61 possible codon triplets in the degenerate genetic code. For e.g. in E coli, makes 79 different tRNAs containing 42 different anticodons. Obviously 19 (61-42=19) potential anticodons are not represented. Thus 19 mRNA codons will not find a complementary anticodon in E coli collection of tRNAs although such codons are present and are being coded into polypeptide chains. Therefore, there must be some tRNAs that recognize more than one codon for a particular amino acid. That is, the anticodons of these tRNAs can interact with more than one codon for the same amino acid. Although the exact codon-anticodon interaction of mRNA and tRNA is not very clear, Rrancis Crick, by analyzing the genetic code concluded that 3 ' nucleotide in may codons adds nothing to the specificity of the codon. For example 5 ' GGU3 ' , 5'GGC3', 5'GGA3' and 5'GGG3' all encode glycine. It does not matter whether the anticodon on tRNA gly has a complementary base pare for the last codon at 3' end provided the first two nucleotides are matched properly the tRNA will add glycine to the growing polypeptide chain. The same is true for other amino acids that are encoded by more than one codon. Thus the 5 ' nucleotide

274 of tRNA's anticodon can often pair with more than one kind of nucleotide in the 3' position of an mRNA's codon. A tRNA charged with a particular amino acid can thus recognize several or even all of the codons for that amino acid. This flexibility in base pairing between the 3' nucleotide in the codon and 5' nucleotide in the anticodon is known as wobble. 5.2.6 Ribosome Ribosomes are assembly of rRNA molecules and numerous proteins that synthesizes proteins under the direction from mRNA template. The bacterial system have ribosomes that sediment at 70S and the eukaryotic ribosomes sediment at 80S. Each ribosome can dissociate into two units: 50S and 30S in bacteria, while in eukaryotes it is 60S and 40S. The smaller subunit binds with mRNA to initiate translation. The actual number of rRNAs and number of proteins vary among species. For example, mammalian ribosomes have 4 rRNAs and 80 proteins while in E. coli the ribosomes have 3 rRNAs and 52 proteins. Although the number of proteins ex- ceeds the number of rRNAs, the rRNAs constitute the major portion of the ribosomes and generally account for over 60% of the mass of the ribosome. The general structures of prokaryotic and eukaryotic ribosomes are more or less similar. Prior to translation, the ribosomes exist as two separate units- small subunit and larger subunit. rRNA No. of proteins Small subunit Prokaryotes (30S) 16S 21 proteins Eukaryotes (40S) 18S ~30 proteins Large subunit Prokaryotes(50S) 23S and 5S 34 proteins Eukaryotes (60S) 28S, 5.8S, and 5S ~45 proteins Fi8- 5-2: Ribosomes dissociate into subunits

275 Each subunit has a specific three-dimensional shape that allows the two subunits to interlock with each other. The complete ribosome in prokaryotes and eukaryotes has a Svedberg value 70S and 80S respectively. There are two sites within the ribosome that can hold tRNAs: A site (aminoacyl or entry site) and the P site (peptidyl or donor site). The ribosome assembles on the mRNA with the A site oriented toward the 3' end of the mRNA and the P site toward the 5' end. At initiation, the three nucleotides of the initiation codon (AUG) align in the P site, where they pair with the anticodon in the initiator tRNA. This situates the A site over the second codon, which is now ready to receive the appropriate charged tRNA to continue translation. During translation, several ribosomes may attach one after the other onto an mRNA and proceed through translation as chain of ribosomes. When this happens, the assembled ribosomes and the mRNA together are called polysome. 5.2.7 Ribozyme Ribozymes are naturally occurring catalytic RNA molecules that have separate catalytic and substrate binding domains. The substrate binding domain binds to specific sequences of substrate RNA molecules by nucleotide complementarity and the catalytic domain cleaves the target RNA at a specific site. The substrate binding domain can be engineered to bind to any target RNA and thus can be utilized a therapeutic agent. However, susceptibility of RNA molecules to enzymatic degradation in target cells and difficulty associated with the production of large scale synthetic RNA molecules has been solved by synthesizing an oligodeoxynucleotide with a ribozyme catalytic domain (~20 nucleotide) flanked by sequences that hybridize to the target mRNA after transcription. Such synthetic oligonucleotides are amplified and is cloned into eukaryotic expression vector. Transfection of target cells by engineered vectors will produce ribozymes that can cleaves the target mRNA, thereby suppressing the translation of the protein that is responsible for the disorder. Various cancers and viral diseases could be treated with genetically engineered riboszymes. 5.3 Prokaryotic and eukaryotic translation Eukaryotic mRNA generally encodes a single polypeptide chain but prokaryotic are sometimes polysistronic. A mature mRNA of both prokaryotes and eukaryotes has coding and non-coding sequences.

276 l The 5' non-coding sequences are referred to as '5' untranslated region' (5'UTR). l In prokaryotes, 5' UTR have specific sequences called 'Shine Delgarno' sequence, which just precedes the coding sequence. This sequence aligns the mRNA on 3' end of 16S rRNA present in small unit of the ribosome. .....AGGAGGUUUGACCUAUG..... pro-mRNA .....UCCUCCA..... 16S rRNA l In Eukaryotes, the ribosomes recognize the 7-methylguanosine at the 5' end. The ribosomes then scan downstream of the 5' cap until they encounter an initiation codon i.e. AUG. l In both prokaryotic and eukaryotic cells, translation always initiates with the amino acid methionine, usually encoded by AUG. Alternative initiation codons, such as GUG that normally code for valine or CUG arginine are used by bacteria to code for N-fromyl-methionine at the initiation point. The translation process is a complex mechanism that operates in the cytosol and requires the presence of mRNA, aminoacyl tRNA, ribosomes and many different protein factors. Translation takes place on ribosomes; which can be conceived as a moving protein-synthesizing machine. The entire translation process in both eukaryotes and prokaryotes can be distinguished into initiation, elongation and termination. Because more is known about translation in bacteria, the process described here will primarily focus bacterial translation. In most respects, eukaryotic translation is similar, although there are some significant differences that will be noted as we proceed through the stages of translation. 5.3.1 Initiation of translation in prokayotes Initiation steps involves the association of specific methionyl tRNA (f-met- tRNA), mRNA and ribosome subunits, initiation factors, guanosine triphosphate (GTP) and recognition of the first codon, which in most cases is AUG. ? IF1 facilitates the separation of the two ribosomal subunits ? At first the small 30S subunit of the ribosome binds to the protein initiation factor IF3 (Fig. 5.3a)

277 ? IF3 and 30S subunit complex then binds to the Shine-Dalgarno sequence (AGGAGG) present on the mRNA at the 5' end, approximately 7 nucleotide upstream the start codon AUG (AGGAGG: is complementary to the six nucleotides 3' UCCUCC5' on the 16SrRNA at the 3end) (more than one Shine- Dalgarno sequence may be present in a single mRNA and therefore in most cases they are polycistronic). ? Another initiation factor IF2 that has a GTP bound to it, specifically recognizes and binds to the initiator f -met-tRNA and facilitates the binding of the f -met- tRNA to mRNA 3OS complex (Fig. 5.3b). ? The anticodon pairs with the initiation codon AUG on mRNA but lying within the 30S complex ? Binding of the f -met-tRNA releases the IF3 from the initiation complex ? Release of IF3 allows the 50S ri- bosomal subunit to bind to the complex ? 50S ribosome then triggers the hydrolysis of the GTP molecule bound to IF2 ? Hydrolysis of the GTP results in the formation of 70S complex ? Formation of 70S ribosome and Fig. 5.3a Fig. 5.3b Fig. 5.3c

278 the binding of f-met-tRNA to mRNA at the initiation codon complete the initiation process that is now ready to begin peptide bond formation during elongation. (Fig. 5.3c) ? At initiation, the three nucleotides of the initiation codon AUG on mRNA align in the P site of ribosome, where they pair with the anticodon in the initiator tRNA. This arrangement of AUG in ribosome places the second codon in the A site, which is now ready to receive the appropriate charged tRNA to continue with the process of elongation of the polypeptide chain. 5.3.2 Initiation of translation in eukaryotes In eukaryotes, Shine-Dalgarno sequences are absent and therefore the initiation occurs in a different way. l First, a eukaryotic initiation factor elF4A, a multimerie protein has a cap binding protein (CAP), that binds to the cap at the 5' end of the mRNA. There exists several other factors with helicase activity that helps to unwind the secondary structures that may exists on mRNA. l Then, a complex of the 40S ribosomal subunit with the initiator Met-tRNA.Met, along with several elF binds at the methylated cap of the 5' end of the mRNA l The ribosomal subunit the scans down the mRNA to locate the initator codon AUG in the concensus in the Kozak sequence ACC(AUG)G. l The
poly(A) tail at the 3' end of eukaryotic mRNA also plays a role in the initiation of translation.
Proteins that attach to the poly(A) tail interact with proteins that bind to the 5' cap, enhancing the binding of the small subunit of the ribosome to the 5' end of the mRNA. This interaction between the 5' cap and the 3' tail suggests that the mRNA bends backward during the Fig. 5.4: Binding of the ribosome to the 5' end of the mRNA
279 initiation of translation, forming a circular structure (FIG 2) A few eukaryotic mRNAs contain internal ribosome entry sites, where ribosomes can bind directly without first attaching to the 5' cap. l Once AUG is located, the 40S ribosome firmly binds with it and then 60S ribosomal subunit binds by displacing the elFs, producing the 80S initiation complex. l At initiation, the three nucleotides of the initiation codon (AUG) on mRNA align in the P site of ribosome, where they pair with the anticodon in the initiator tRNA. This arrangement of AUG in ribosome places the second codon in the A site, which is now ready to receive the appropriate charged tRNA to continue with the process of elongation of the polypeptide chain. 5.3.3 Elongation of the polypeptide chain After the initiation process is over, the elongation of the polypeptide chain begins by joining charged amino acids. The process requires: (1) the 70S complex; (2) tRNAs charged with their amino acids; (3) several elongation factors (EF-Ts, EF-Tu, and EF-G); and (4) GTP. A ribosome has three sites that can be occupied by tRNAs; the aminoacyl, or A, site, the peptidyl, or P, site, and the exit, or E, site (Fig. 5.5). Fig. 5.5 : Sites of a ribosome
280 At first the ribosome occupies a position on mRNA in such a way that the P site is positioned over AUG and the adjacent A site is unoccupied (Fig 5.5a). The initiator tRNA immediately occupies the P site (the only site to which the fMet- tRNAfMet is capable of binding), but all other tRNAs first enter the A site. Elongation occurs in three steps. 1. The first step is the delivery of a charged tRNA with its amino acid attached to the A site. This requires the presence of elongation factors EF-Tu, EF-Ts, and GTP (Fig. 5.5b). l EF-Tu first joins with GTP and then binds to a charged tRNA to form a three-part complex. l This three-part complex enters the A site of the ribosome, where the antico- don on the tRNA pairs with the codon on the mRNA. l After the charged tRNA is in the A site, GTP is cleaved to GDP, and the EF-Tu-GDP complex is released (Fig. 5.5c) l Factor EF-Ts regenerates EF-Tu-GDP to EF-Tu-GTP. l In eukaryotic cells, a similar set of reactions delivers the charged tRNA to the A site. 2. The second step of elongation is
the creation of a peptide bond between the amino acids that are
attached to tRNAs in the P and A sites (Fig. 5.6). The Fig. 5.6: The second step of elongation formation of this peptide bond releases the amino acid in the P site from its tRNA. The activity responsible for peptidebond formation in the ribosome is referred to as peptidyl transferase. The peptidyl bond formation is catalyzed by the rRNA of the large subunit of the ribosome. The third step in elongation is translocation, (Fig. 5.7) where the ribosome moves down the mRNA in the 5'––3' direction. The A site of the ribosome moves

281 forward to occupy the next codon. The process requires EF-G (elongation factor) and the hydrolysis of GTP to GDP. Because the tRNAs in the P and A site are still attached to the mRNA through codon- anticodon pairing, they do not move with the ribosome as it translocates. Consequently, the tRNA that previously was occupying the P site now occupies the E site, from which it moves into the cytoplasm. tRNA that occupied the A site now occupies the P site, leaving the A site open for the next incoming tRNA specified by the mRNA's codon sequence. Thus, the progress of each tRNA through the ribosome during elongation can be summarized as follows: Cytoplasm ? A site ? P site ? E site ? cytoplasm Throughout the cycle, the polypeptide chain remains attached to the tRNA in the P site. The ribosome moves down the mRNA in the 5' —3' direction, adding amino acids one at a time. Elongation in eukaryotic cells takes place in a similar manner. 5.3.4 Termination Addition of new amino acids stops when the A site of ribosome translocates to a termination codon. Because there are no tRNAs with anticodons complementary to the termination codons, no tRNA enters the A site of the ribosome when a termination codon is encountered (Fig. 5.7a). Instead, proteins called release factors bind to the ribosome (Fig. 5.7b). E. coli has three release factors—RF1, RF2, and RF3. Release factor 1 recognizes the termination codons UAA and UAG, and RF2 recognizes UGA and UAA. Release factor 3 forms a complex with GTP and binds to the ribosome. The release factors then promote the cleavage of the tRNA in the P site from the polypeptide chain; in the process, the GTP that is Fig. 5.7: Steps of translocation

282 complexed to RF3 is hydrolyzed to GDP. Additional factors help bring about the release of the tRNA from the P site, the release of the mRNA from the ribosome, and the dissociation of the ribosome (Fig. 5.7c). Findings from recent studies suggest that the release factors bring about the termination of translation by completing a final elongation cycle of protein synthesis. In this model, RF1 and RF2 are similar in size and shape to tRNAs and occupy the A site of the ribosome, just as the amino acid-tRNA-EF-Tu-GTP complex does during an elongation cycle. Release factor 3 is structurally similar to EF-G; it then translocates RF1 and RF2 to the P site, as well as the last tRNA to the E site, in a way similar to that in which EF-G brings about trans location. When both the A site and the P site of the ribosome are cleared of tRNAs, the ribosome can dissociate. Research findings also indicate that some of the sequences in the rRNA play a role in the recognition of termination codons. Translation in eukaryotic cells terminates in a similar way, except that there are two release factors: eRFl, which recognizes all three termination codons, and eRF2, which binds GTP and stimulates the release of the polypeptide from the ribosome. 5.4 Regulation of translation Regulation of gene expression refers to the cellular control of the amount and timing of changes to the appearance of the functional product of a gene. Gene regulation gives the cell control over its structure and function, and is the basis for cellular differentiation, morphogenesis and the versatility and adaptability of any organism. Prokaryotic and eukaryotic gene expressions are regulated at several different levels of flow of information from gene to their final product. Any step of gene expression may be modulated, from the DNA-RNA transcription step to post- translational modification of a protein. Stages where gene expression is regulated are: l Regulate gene expression by chemical and structural modification of DNA or chromatin (e.g. by methylation, phosphorylation, acetylation or structural changes) l By controlling when and how often a given gene is transcribed (during transcription e.g. by repressor or activator proteins, attenuation etc.) l By modifying the primary RNA transcript (post transcriptional modifications)

283 l Selection of transcribed and processed RNA (transport from nucleus to cytoplasm) l During translation of mRNA (e.g. by an antisense RNA) l Post-translational control (e.g. by proteolysis or modification of the gene product) 5.4.1 DNA modification 1. Chemical modification of DNA Methyiation of DNA refers to addition of a methyl group to the number 5 carbon of the cytosine pyrimidine ring by the enzyme methyl transferase. Generally methyiation occurs on the cytosine in the CpG dinucleotide sequence (CpG islands). DNA methyiation pattern can be inherited without changing the DNA sequence. As such, it is part of the epigenetic code and is the most characterized epigenetic (changes in phenotype without any alteration in the genomic material) mechanism. DNA methyiation has been found in all vertebrate. In humans, approximately 1% of DNA bases undergo DNA methyiation (~60-70% of all CpGs are methylated mainly 5' regulatory regions). In adult somatic tissues, DNA methyiation typically occurs in a CpG dinucleotide context; non-CpG methyiation is prevalent in embryonic stem cells. In plants, cytosines are methylated both symmetrically (CpG or CpNpG) and asymmetrically (CpNpNp), where N can be any nucleotide. DNA methyiation may impact the transcription of genes in two ways. 1. First, the methyiation of DNA may itself physically impede the binding of transcriptional proteins to the gene, thus blocking transcription. 2. Second, and likely more important, methylated DNA may be bound by proteins known as Methyl-CpG-binding domain proteins' (MBDs). MBD proteins then recruit additional proteins to the locus, such as histone deacetylases and other chromatin remodelling proteins that can modify histones, thereby forming compact, inactive chromatin termed silent chromatin. This link between DNA methyiation and chromatin structure is very important. . In many disease processes such as cancer, gene promoter CpG islands acquire abnormal hypermelhylation, which results in heritable transcriptional silencing In particular, loss of Methyl-CpG-binding Protein 2 (MeCP2) has been 284 implicated in Rett syndrome and Methyl-CpG binding domain protein 2 (MBD2) mediates the tran-scriptional silencing of hypermethylated genes in cancer Analysis of the pattern of methylation in a given region of DNA (generally a promoter) can be achieved through a method called bisulfite mapping. Methylated cytosine residues are unchanged by the treatment, whereas unmethylated ones are changed to uracil. The differences are analyzed in sequencing gels, Abnormal methylation patterns are thought to be involved in carcinogenesis. 2. Structural modification of DNA Transcription of DNA is highly dependent on the secondary structure of DNA molecule. Histone proteins, responsible for supercoiling of DNA can modify the structure temporarily or more permanently depending on the phosphorylation or methylation of the histone proteins respectively. Such modifications influences the level of gene expression. In general, the density of its packing is indicative of the frequency of transcription. Histone acetylation is also an important process in transcription. Histone acetyltransferase enzymes (HATs) such as CREB-binding protein also dissociate the DNA from the histone complex, allowing transcription to proceed. Often, DNA methylation and histone acetylation work together in gene silencing. The combination of the two seems to be a signal for DNA to be packed more densely, lowering gene expression. 5.4.2 Regulation of transcription Transcription is an important level of control in eukaryotic cells. Transcription regulation of a gene by RNA polymerase can be regulated by at least five mechanisms: l Specificity factors alter the binding specificity of RNA polymerase for a given promoter or set of promoters, making it more or less likely to bind to them (i.e. sigma factors used in prokaryotic transcription). l Repressors bind to non-coding sequences on the DNA strand that are close to or overlapping the promoter region, impeding RNA polymerase's progress along the strand, thus impeding the expression of the gene. l Basal factors These transcription factors position RNA polymerase at the start of a protein-coding sequence and then release the polymerase to transcribe the mRNA. Recruitment of these proteins at the promoter region affects the RNA polymerase activity.

285 l Activators enhance the interaction between RNA polymerase and a particular promoter, encouraging the expression of the gene. Activators do this by increasing the attraction of RNA polymerase for the promoter, through interactions with subunits of the RNA polymerase or indirectly by changing the structure of the DNA. l Enhancers are sites on the DNA helix that are bound to by activators in order to loop the DNA bringing a specific promoter to the initiation complex. Examples : l The ó 32 subunit of RNA polymerase changes itself in such a way that the enzyme binds to a specialized set of promoters when E. coli bacteria are subjected to heat stress producing heat-shock response proteins. l When there is excess tryptophan in the cell, the amino acid binds to a specialized repressor protein, changing the structural conformity of the repressor such that it binds to the operator region for the operon that synthesizes tryptophan, preventing their expression and thus suspending production which also represents a form of negative feedback mechanism. l In bacteria, the lac repressor protein blocks the synthesis of enzymes that digest lactose when there is no lactose to feed on.

When lactose is present, it binds to the repressor, causing it to detach from the DNA

strand. 5.4.3 Gene regulation can be summarized as how they respond l Inducible systems - An inducible system is off unless there is the presence of some molecule (called an inducer) that allows for gene expression. The molecule is said to “induce expression”. l Repressible systems - A repressible system is on except in the presence of some molecule (called a corepressor) that suppresses gene expression. The molecule is said to “repress expression”. In both the cases, the control mechanism varies in prokaryotic and eukaryotic cells. 5.4.4 Post-transcriptional regulation The cells regulate the posttranscriptional activity by several way to check how much the mRNA should be translated into proteins. Cells do this by Capping, Splicing, and the addition of a Poly(A) Tail. These processes occur only in
286 eukaryotes because in prokaryotes, the transcription and translation is coupled. l Capping changes the five prime end of the mRNA to a three prime end by 5'-5' linkage, which protects the mRNA from 5' exonuclease, which degrades foreign RNA. The cap also helps in ribosomal binding. l Splicing removes the introns, noncoding regions that are transcribed into RNA, in order to make the mRNA able to create proteins. Cells do this by spliceosome’s binding on either side of an intron, looping the intron into a circle and then cleaving it off. The two ends of the extrons are then joined together. l Addition of poly(A) tail a poly(A) tail is just junk RNA added to the 3' end in order to slowly be degraded by a 3' exonuclease in order to increase the half life of mRNA.
287 Unit 6 o Antisense and Ribozyme Technology Structure 6.1 Antisense molecules and their mechanism of action 6.2 Splicing 6.3 Ribozymes 6.4 Antisense technology 6.1 Antisense molecules and their mechanism of action 6.1.1 Introduction Expression of some genes may be regulated or suppressed with the aid of small RNA molecules - a process termed as RNA silencing, also known as RNA interference or posttranscriptional gene silencing. Although many of the details of this mechanism are still poorly understood, it appears to be widespread, existing in fungi, plants, and animals. It may also prove to be a powerful tool for artificially regulating gene expression in genetically engineered organisms. The discovery of antisense RNA was preceded first by observation of transcriptional inhibition by antisense RNA expressed in transgenic plants. In an attempt to alter flower colors in petunias, researchers introduced additional copies of a gene encoding chalcone synthase, a key enzyme for flower pigmentation into petunia plants of normally pink or violet flower color. The scientists’ goal was to produce petunia plants with improved flower colors but instead produced less pigmented, fully or partially white flowers, indicating that the activity of chalcone synthase had been substantially decreased. Similar suppression of gene activity was also observed in fungus Neurospora crassa. This phenomenon was called co- suppression of gene expression, but the molecular mechanism remained unknown. Sometime later, plant virologists noted that plants carrying only short transgenic non-coding regions of viral RNA sequences would showed enhanced levels of protection. The reverse experiment, in which short sequences of plant genes were introduced into viruses, showed that the targeted gene was suppressed in an infected plant. This phenomenon was labeled “virus-induced gene silencing” (VIGS), and the set of such phenomena were collectively called post transcriptional gene silencing. Craig C. Mello and Andrew Fire’s 1998 published in Nature the potent gene silencing effect after injecting double stranded RNA into C. elegans. In investigating the regulation of muscle protein production, they observed that neither mRNA nor antisense RNA injections had an effect on protein production,
288 but double-stranded RNA successfully silenced the targeted gene. As a result of this work they were awarded Nobel Prize in the year 2006. 6.1.2 Antisense RNA molecules Antisense molecules are nucleotide sequences that interact with complementary strands of nucleic acids and modify expression of genes. For example, Antisense RNA is single-stranded RNA that is complementary to an mRNA strand transcribed within a cell and capable of blocking the translation machinery. Antisense molecules occur naturally. For example, in both mice and humans, the gene for the insulin-like growth factor 2 receptor (Igf2r) that is inherited from the father synthesizes an antisense RNA that appears to block synthesis of the mRNA for Igf2r. Historically, the effects of antisense RNA have often been confused with the effects of RNA interference, a related process in which double-stranded RNA fragments called small interfering RNAs trigger catalytically mediated gene silencing, most typically by targeting the RNA-induced silencing complex (RISC) to bind to and degrade the mRNA. 6.1.3 RNA interference (RNAi) In the course of working with artificially synthesized single stranded antisense RNA molecules, it was discovered that double stranded RNA (dsRNA) molecule can also act as a powerful suppressant of genes expression. dsRNA
may arise in several ways: by the transcription of inverted repeats
in DNA into a single RNA molecule that base pairs with itself; by the
simultaneous transcription of two different RNA molecules that are complementary to one another

and pair; or by the replication of double-stranded RNA viruses (Fig 6.1). Drosophila, an enzyme called Dicer cleaves and processes the double-stranded RNA to produce In fact, the suppressive effect of antisense RNA probably also depends on its ability to form dsRNA. The ability of dsRNA to suppress the Fig. 6.1: Replication of double-stranded RNA viruses 289 expression of a gene corresponding to its own sequence is called RNA interference (RNAi). It is also called post-transcriptional gene silencing or PTGS. 6.1.4 Mechanism of RNAi Normally single-stranded RNA molecules are found in the cytoplasm of a cell. If double-stranded RNA (dsRNA) formation occurs in the cell, the enzyme called Dicer to cleave the dsRNA into fragments containing 19 base pairs (~2 turns of a double helix) with two additional nucleotides at the opposite end of each strand. The two strands of each fragment then separate — releasing the antisense strand. Dicer is a ribonuclease in the RNase III family that cleaves double- stranded RNA (dsRNA) and pre-microRNA (miRNA) into short double-stranded RNA fragments called small interfering RNA (siRNA) about 20-25 nucleotides long, usually with a two-base overhang on the 3' end. Dicer contains two RNase III domains and one PAZ domain; the distance between these two domains of the molecule is determined by the length and angle of the connector helix and determines the length of the siRNAs it produces. Dicer catalyzes the first step in the RNA interference pathway and initiates formation of the RNAAnduced silencing complex (RISC), whose catalytic component argonaute is an endonuclease capable of degrading messenger RNA (mRNA) whose sequence is complementary to that of the siRNA guide strand. With the aid of a protein, it binds to a complementary sense sequence on a molecule of mRNA. If the base-pairing is exact, the mRNA is destroyed. Because of their action, these fragments of RNA have been named “short (or small) Fig 6.1 a. Fig. 6.2b:
290 interfering RNA” (siRNA). The complex of siRNA and protein is called the “RNA- induced silencing complex” (RISC) (Fig. 6.2b). In fission yeast, evidences indicate that siRNAs can also inhibit the transcription of genes perhaps by binding to complementary sequences on DNA or by binding to the nascent RNA transcript as it is being formed. Synthetic siRNA molecules that bind to gene promoters can repress transcription by methylation of the DNA in the promoter and, perhaps, methylation of histones in the vicinity. The siRNA forms a complex called the RITS complex (“RNA-induced initiation of transcriptional gene silencing”) with at least three different proteins. How these siRNAs, synthesized in the cytosol gain access to the DNA in the nucleus is unknown. Example : The rice plant of strain LGC-1 produces abnormally low levels of proteins called glutelins although there are several glutelin genes. Interestingly, it was observed that two closely-similar glutelin genes are located back to back on the same chromosome and a deletion has occurred in the 3' region of the first glutelin gene that has removed the stop signal. As a consequence, RNA polymerase II transcribes right past the first gene and on into the second. The result is a messenger RNA with almost-identical sequences running in opposite directions. Such a composition of the mRNA molecule allows it to fold up into a molecule of double- stranded RNA (dsRNA). A Dicer-like enzyme cuts up the dsRNA into small interfering RNAs (siRNAs) that suppress further transcription of those genes as well as other glutelin genes Some related. RNA molecules produced through the cleavage of double- stranded RNA bind to complementary sequences in the 3’UTR of mRNA and inhibit their translation. RNA silencing is thought to have
evolved as a defense against RNA viruses and transposable elements that move through
an RNA intermediate (see Chapter 20). The extent to which it contributes to normal gene regulation is uncertain, but dramatic phenotypic effects result from some mutations that occur in the enzymes that carry out RNA silencing. Amplification of RNAi : In C. elegans, plants, and Neurospora, the introduction of a few molecules of dsRNA has a potent and long-lasting effect. In plants, the gene silencing spreads to adjacent cells (through plasmodesmata) and even to other parts of the plant (through the phloem). RNAi within a cell can continue after mitosis in the progeny of that cell. Triggering of RNAi in C. elegans can even pass through the germline into its descendants. Such amplification of an initial trigger signal suggests a catalytic effect. It turns out that these organisms have RNA-dependent RNA poly-merases (RdRPs) that uses the mRNA targeted

291 by the initial antisense siRNA as a template for the synthesis of more siRNAs. Synthesis of these "secondary siRNAs even occurs in adjacent regions of the mRNA. So not only can these secondary siRNAs target additional areas of the original mRNA, but they are potentially able to silence mRNAs of other genes that may carry the same sequence of nucleotides. This phenomenon, called "transitive RNAi". In mammalian cells, introducing dsRNA fragments only reduces gene expression temporarily. However, Brummelkamp et. al. report in the 19 April 2002 issue of Science that they have succeeded in introducing into (mammalian) cells a DNA vector that can continuously synthesize a siRNA corresponding to the gene that they want to suppress. Two months later the cells still failed to manufacture the protein whose gene had been turned off by RNAi. 6.1.5 MicroRNAs (miRNAs) MicroRNAs were first described by Lee, et al. in 1993. MicroRNAs (miRNA) are single-stranded RNA molecules of about 21-23 nucleotides in length thought to regulate the expression of other genes. miRNAs are encoded by genes that are transcribed from DNA but not translated into protein (non-coding RNA); instead they are processed from primary transcripts known as pri-miRNA to short stem- loop structures called pre-miRNA and finally to functional miRNA, Mature miRNA molecules are partially complementary to one or more messenger RNA (mRNA) molecules, and they function to downregulate gene expression. The genes encoding miRNAs are much longer than the processed miRNA molecule. miRNAs are first transcribed as primary transcripts or pri-miRNA and processed to short, 70-nucleotide stem-loop structures known as pre-miRNA in the cell nucleus by protein complex known as the Microprocessor complex, consisting of the nuclease Drosha and the double-stranded RNA binding protein Pasha. These pre-miRNAs are then processed to mature miRNAs in the cytoplasm by interaction with the endonuclease Dicer, which also initiates the formation of the RNA-induced silencing complex (RISC). This complex is responsible for the gene silencing observed due to miRNA expression and RNA interference. The pathway in plants varies slightly due to their lack of Drosha homologs; instead, Dicer homologs alone effect several processing steps. In C. elegans, successful development through its larval stages and on to the adult requires the presence of at least two "microRNAs" ("miRNAs") — single- stranded RNA molecules containing about 22 nucleotides and thus about the same size as siRNAs. These small single-stranded transcripts are generated by the cleavage of larger precursors using the C. elegans version of Dicer. The miRNA acts by either destroying or inhibiting translation of several messenger RNAs in
292 the worm (by binding to a region of complementary sequence in the 3' untranslated region [3 1 -UTR] of the mRNA). miRNA genes have also been discovered in humans Dro-sophila, mice, frogs, fish, and plants (Arabidopsis) as well as in C. elegans. 6.1.6 Biological functions of antisense molecules Immunity : RNA interference provides immunity to plants especially against viruses and other foreign genetic materials. It is also suggested the RNA interference may also prevent self-propagation by transposons. Even before the RNAi pathway was fully understood, it was known that induced gene silencing in plants could spread throughout the plant in a systemic effect, and could be transferred from stock to scion plants via grafting. This phenomenon is a feature of the plants innate immune system, and allows the entire plant to respond to a virus after an initial localized encounter. Although animals generally express fewer variants of the dicer enzyme than plants, RNAi in some animals has also been shown to produce an antiviral response. In both juvenile and adult Drosophila, RNA interference is important in antiviral innate immunity and is active against pathogens such as Drosophila X virus. A similar role in immunity may operate in C. elegans, as argonaute proteins are upregulated in response to viruses and worms that overexpress components of the RNAi pathway are resistant to viral infection. The role of RNA interference in mammalian innate immunity is poorly understood and the hypothesis of RNAi-mediated immunity in mammals has been challenged as relatively little data is available. However, alternative functions for RNAi in mammalian viruses exist, such as miRNAs expressed by the herpes virus that may act as heterochromatin organization that triggers to mediate viral latency. Genome maintenance Components of the RNA interference pathway are used in many eukaryotes in the maintenance of the organisation and structure of their genomes. Modification of histones and associated induction of heterochromatin formation serves to downregulate genes pre-transcriptionally and this process is referred to as RNA- induced transcrip-tional silencing (RITS), and is carried out by a complex of proteins called the RITS complex. In fission yeast this complex contains argonaute, a chromodomain protein Chpl, and a protein called Tas3 of unknown function. As a consequence, the induction and spread of heterochromatic regions requires the argonaute and RdRP proteins. Indeed, deletion of these genes in the fission yeast S. pombe disrupts histone meth-ylation and centromere formation, causing slow or

293 stalled anaphase during cell division. Thus repression of gene expression by miRNAs appears to be a mechanism to ensure proper, coordinated gene expression as cells differentiate along particular paths. For example, when zygote. genes begin to be turned on in the zebrafish blas-tula, one of them encodes a miRNA that triggers the destruction of the maternal mRNAs that have been running things up to then. So miRNAs may play as important role as transcription factors in coordinating the expression of multiple genes in a particular type of cell at particular times. 6.2 Splicing repressors and activators control splicing at alternative sites 6.2.1 Introduction Gene splicing mechanism exists in eukaryotes that facilitate the removal of intron from pre-mRNA. There are also evidences to suggest that alternative splicing enable the synthesis of two different proteins from the same peptide. For the alternative splicing mechanism to operate, it is believed that there exists splicing repressor proteins in the cells and function to produce alternative peptides from the same genes. For example, in Drosophila Sxl inhibit splicing at specifice sites, causing exons to be skipped, whereas Tra promotes splicing. As a consequence, Fig. 6.3 : Alternative spilicing due to repression at splicing sites lead to the formation of alternative Dsx protein in Drosophila

294 distinct Dsx proteins are produced in female and male embryos leading to sexual differentiation (Fig. 6.3). Sxl-like splicing repressor expressed in hepatocytes might bind to splice sites for the EIIIA and EIIIB exons in the fibronectin pre-mRNA, causing them to be skipped during RNA splicing. Experimental examination in some systems has revealed that inclusion of an exon in some cell types versus skipping of the same exon in other cell types results from the combined influence of several splicing repressors and enhancers. The action of similar proteins may explain the cell-type specific expression of fibronectin isoforms in humans. Splicing repressors expressed in hepatocytes might bind to splice sites for the EIIIA and EIIIB exons in the fibronectin pre- mRNA, causing them to be skipped during RNA splicing (Figure: 6.4). Alternatively, a Tra-like splicing activator expressed in fibroblasts might activate the splice sites associated with the fibronectin EIIIA and EIIIB exons, leading to inclusion of these exons in the mature mRNA. Fig.6.4: Alternative splicing fibronectin pre mRNA in human hepatocytes can produce different isoforms. 6.2.2 Repression of translation of mRNAs Micro RNAs (miRNAs) are efficient tools for the sells to regulate mRNA translation. In C. elegans, the genes lin-4 and let-7 produce small RNA sequences 21 and 22 nucleotides long, respectively, that hybridize to the 3' untranslated regions of specific target mRNAs. For example, the lin-4 miRNA, which is expressed early in embryogenesis, hybridizes to the 3' untranslated regions of both the lin-14 and lin-28 mRNAs, thereby repressing translation of these mRNAs by an as yet unknown mechanism. Expression of lin-4 miRNA ceases later in development, allowing translation of newly synthesized lin-14 and lin-28 mRNAs at that time. In C. elegans; about 100 different miRNAs have been found in C. elegans, and 295 at least as many in humans. All miRNAs appear to be formed by processing of ~70-nucleotide precursor RNAs that form hairpin structures with a few base-pair mismatches in the stem of the hairpin. A ribonuclease called Dicer, cleaves the double-stranded RNA to produce miRNAs precursors. Interestingly, the base pairing between a miRNA and the 3' untranslated region of its target mRNAs is not precisely complementary and some base-pair mismatches occur in the hybridized region. This mismatching distinguishes miRNA-mediated translational repression from the related phenomenon of RNA interference, which we describe next. 6.2.3 Degradation of mRNAs in the cytoplasm Concentration of any mRNAs in the cell depends not only on the rate of its transcription but also on the rate of its degradation. With more stable mRNAs, protein synthesis persists long after transcription of the gene is repressed. Usually, bacterial mRNAs are unstable and decay exponentially, probably because they need to switch genes rapidly in response to the change in environment. In multicellular organism, the cells reside in a more stable environment and do not require frequent adjustment to the changing environment. However, some proteins in eukaryotic cells are required only for short periods of time and must be degraded immediately. For example, during cell cycle activity, synthesis of cyclins occurs in burst at intervals. The mRNA of cyclins must be degraded very quickly. mRNA of other proteins like c-Fos and c-Jun, synthesized during S phase need to be degraded immediately after the function is over. mRNAs of such proteins have half life less than 30 minutes. There are three main pathway that lead to the degradation of cytoplasmic mRNAs as shown in (Fig. 6.5). Fig.6.5: Main pathway that lead to degradation of cytoplasmic mRNAs

296 For most mRNAs, the length of the poly(A) tail gradually decreases with time through the action of a deadenylating nuclease. When it is shortened sufficiently, the PABPI molecules that bind during polyadenylation of mRNA can no longer bind and stabilize interaction of the 5' cap and initiation factors (Fig. 6.6). The exposed cap then is removed by a decapping enzyme, and the unprotected mRNA is degraded by a 5'?3' exonuclease. Removal of the poly(A) tail also makes mRNAs susceptible to degradation by cyto- plasmic exosomes containing 3'?5' exonucleases. The 5'?3' exonucleases predominate in yeast, and the 3'?5' exosome apparently predominates in mammalian cells. The rate of deadenylation determines the rate of degradation of mRNA. Recent experiments suggest that the bound proteins interact with a deadenylating enzyme and with the exosome, thereby promoting the rapid deadenylation and subsequent 3'?5' degradation of these mRNAs. In this mechanism, the rate of mRNA degradation is uncoupled from the frequency of translation. Thus mRNAs containing the AUUUA sequence can be translated at high frequency, yet also degraded rapidly, allowing the encoded proteins to be expressed in short bursts. Some mRNAs are degraded by decapping the mRNA before the deadenylation process is initiated. It appears that certain mRNA sequences make the cap sensitive to the decapping enzyme, but the precise mechanism is unclear. In the other alternative pathway, mRNAs first are cleaved internally by endonucleases. The RNA-induced silencing complex (RISC) discussed earlier is an example of such an endonuclease. The fragments generated by internal cleavage then are degraded by exonucleases. 6.2.4 Regulation of mRNA translation and degradation Ironresponse element-binding protein (IRE-BP) can be a classis example of protein that regulates the translation. Intracellular iron concentrations the protein in way that can regulate the translation of one mRNA and the degrade another. When the intracellular iron falls below the threshold level, IRE-BP proteins releases free irons in the system for the enzymes that require Fe to function. Again, when Fig. 6.6 : Initiation factors
297 the concentration of free iron increases within the system, the proteins bind to free Fe to prevent accumulation and toxicity. Production of ferritin- an intracellular iron-binding protein is regulated by IRE-BP. The 5' UTR of ferritin mRNA has a stem and loop structure containing a iron response element (IRE). The IRE-BP recognizes five specific bases in the IRE loop and the duplex nature of the stem. At low iron concentrations, IREBP is in active conformation and binds to the IREs. The bound IRE-BP blocks the 40S ribosomal subunit from scanning for the AUG start codon, thereby inhibiting translation initiation. The resulting decrease in ferritin means less iron is complexed with the ferritin and is therefore available to iron-requiring enzymes. At high iron concentrations, IRE-BP is in an inactive conformation that does not bind to the 5' IREs, so translation initiation can proceed. The newly synthesized ferritin then binds free iron ions, preventing their accumulation to harmful levels (Fig. 6.7a). In vertebrates import of iron is also regulated. Ingested iron is carried through the circulation bound to a protein called transferrin. After binding to the transferrin receptor (TfR) in the plasma membrane, the transferrin-iron complex is brought into cells by receptor-mediated en-docytosis. The 3'-UTR of TfR mRNA contains IREs whose stems have AU'rich destabilizing sequences (Fig. 6.7b), At high iron concentrations, when the IRE-BP is in the inactive, nonbinding conformation, these AU-rich sequences are thought to promote degradation of TfR mRNA by the same Fig. 6.7 a : Fig. b.7b.

298 mechanism that leads to rapid degradation of other shortlived mRNAs, as described previously. The resulting decrease in production of the transferrin receptor quickly reduces iron import, thus protecting the cell. At low iron concentrations, however, IRE-BP can bind to the 3' IREs in TfR mRNA. The bound IRE-BP is thought to block recognition of the destabilizing AU-rich sequences by the proteins that would otherwise rapidly degrade the mRNAs. As a result, production of the transferrin receptor increases and more iron is brought into the cell. 6.2.5 Nonsense-mediated decay and other mRNA Improperly processed mRNA cannot be translated and should be eliminated out of the system which otherwise can lead to production of an abnormal protein that interferes with functioning of the normal protein. This effect is equivalent to dominant-negative mutations. Several mechanisms collectively termed mRNA surveillance help cells avoid the translation of improperly processed mRNA molecules. Nonsense-mediated decay mechanism is another way how the cells get cleared of the wrongly processed mRNAs in which one or more exons have been skipped during splicing. During splicing, improper exon skipping sometime introduce stop codons. Nonsense-mediated decay results in the rapid degradation of mRNAs with stop codons that occur before the last splice junction in the mRNA. Analysis of yeast mutants suggests that some of the proteins in exon-junction complexes function in nonsensemediated decay. 6.3 Ribozymes 6.3.1 Concept of ribozymes Until about 20 years ago, all known enzymes were proteins. But then it was discovered that some RNA molecules can act as enzymes; that is, catalyze covalent changes in the structure of substrates, RNA molecules that can catalyze a chemical reaction are called RIBOZYMES. The first ribozymes were discovered in the 1980s by Thomas R. Cech, who was studying RNA splicing in the ciliated protozoan Tetrahymena therrnophila. Subsequently, Sidney Altman, discovered bacterial RNase P complex. The ribozymes were found in the intron of an RNA transcript, which removed itself from the transcript and in the RNA component of the RNase P complex, which is involved in the maturation of pre-tRNAs. Many natural ribozymes catalyze either their own cleavage or the cleavage of other RNAs, but they have also been found to. catalyze the aminotransferase activity of the ribosome. Although most ribozymes are quite rare in the cell, their roles are sometimes essential to life.

299 Five classes of ribozymes have been described based on their unique characters in the sequences as well as three-dimensional structures (Bunnell, 1997). They are denoted as (1) the Tetrahymena group I intron, (2) RNase P, (3) the hammerhead ribozyme, (4) the hairpin ribozyme, and (5) the hepatitis delta virus ribozyme. They may catalyze self-cleavage as well as the cleavage of external substrates. 1. Group One Intron: The splicing reaction is self-contained; that is, the intron - with the help of associated proteins - splices itself out of the precursor RNA. the action is catalyzed by the RNA, only a single molecule of substrate is involved (unlike protein enzymes that repeatedly catalyze a reaction). However, synthetic versions of Group I introns made in the laboratory can - in vitro - act repeatedly; that is, like true enzymes. The DNA of some Group I introns includes an open reading frame (ORF) that encodes a transposase-like protein that can make a copy of the intron and insert it elsewhere in the genome 2.

Ribonuclease P: All living things synthesize an enzyme - called Ribonuclease P - that cleaves the head (5 ') end of the precursors of transfer RNA (tRNA) molecules.

Ribonuclease P is a h e t e r o d i m e r containing a molecule of RNA and one protein. When the RNA is separated from the protein, the RNA retains its ability to catalyze the cleavage step (although less efficiently than the intact dimer), but the protein alone cannot do the job. 3. Hammerhead ribozyme: The name of hammerhead ribozyme is given by the similarity between its secondary structure and the shape of a hammerhead. They are the best understood subcategory of all ribozymes. As well as other Fig. 6.8: Structure of Ribonuclease P

300 ribozymes, the hammerhead ribozyme is an antisense RNA. Some of the ribonucleotides within the sequence selectively form Watson-Crick base pairs with others to form a stem, while the rest stay in single stranded state called loop. These loops and stems can be predicted at the secondary structure level using conformational energy analysis, such as RNAdraw and mfold; and three dimensional structures were obtained mainly by X-ray crystallography Fig. 6.9: Hammerheaded ribozyme 4. The hepatitis delta virus ribozyme : Some RNA viruses, such as the hepatitis delta virus, also include a ribozyme as part of their inherited RNA molecule. During replication of the viral RNA, long strands containing repeats of the RNA genome (viral genetic information) are synthesized. The ribozyme then cleaves the long multimeric molecules into pieces that contain one genome copy, and fits that RNA piece into a virus particle. Since the discovery of ribozymes that exist in living organisms, there has been interest in the study of new synthetic ribozymes made in the laboratory. Ribozymes can be produced in the laboratory which, are capable of catalyzing their own synthesis under very specific conditions, such as an RNA polymerase ribozyme; although the polymerase activity is very limited. Such RNA polymerase ribozymes are able to add up to 14 nucleotides to a primer template in 24 hours until it is decomposed by hydrolysis of the phosphodiester bonds. Another artificially produced self cleaving RNAs ribozyme was produced by Tang and Breaker by in vitro selection of RNAs originating from random-sequence RNAs. Some of the synthetic ribozymes that were produced had novel structures, while some were similar to the naturally occurring hammerhead ribozyme. The techniques used to discover synthetic ribozymes involve Darwinian evolution. This approach takes advantage of RNA's dual nature as both a catalyst and an informational polymer, making it easy for an investigator to produce vast
301 populations of RNA catalysts using polymerase enzymes. The ribozymes are mutated by reverse transcribing them with reverse transcriptase into various cDNA and amplified with mutagenic PCR. The selection parameters in these experiments often differ. One approach for selecting a ligase ribozyme involves using biotin tags, which are covalently linked to the substrate. If a molecule possesses the desired ligase activity, a streptavidin matrix can be used to recover the active molecules. 6.3.2 Ribozymes for human therapy The application of ribozymes for gene therapy of autosomal dominant diseases has become popularized in recent years. Further this technology has widespread utility in the treatment of any disease, acquired or inherited, by inhibition of gene expression. The design of ribozymes is usually accomplished using computer assisted design programs, however they are not very useful in predicting the behavior of the ribozyme in the in vivo setting. To overcome this technical challenge, methods and strategy are being evolved to accurately assess the efficiency of ribozyme cleavage in vivo situations that significantly enhances the computer based design programs. Already, a synthetic ribozyme that destroys the mRNA encoding a receptor of Vascular Endothelial Growth Factor (VEGF) is being readied for clinical trials. VEGF is a major stimulant of angiogenesis, and blocking its action may help starve cancers of their blood supply. 6.4 Antisense technology 6.4.1 Introduction Over the last several decades, the knowledge of DNA/RNA physiology has been applied in a variety of ways. One of the more productive applications is the development of antisense technology. The basic idea is that if an oligonucleotide (a short RNA or DNA molecule complementary to a mRNA produced by a gene) can be introduced into a cell, it will specifically bind to its target mRNA through the exquisite specificity of complementary-based pairing—the same mechanism which guarantees the fidelity of DNA replication and of RNA transcription from the gene. This binding forms an RNA dimer in the cytoplasm and halts protein synthesis. This occurs because the mRNA no longer has access to the ribosome and because dimeric RNA is rapidly degraded in the cytoplasm by ribonuclease H. Therefore, the introduction of short chains of DNA complementary to mRNA will lead to a specific diminution, or blockage, of protein synthesis by a particular gene. In effect, the gene will be turned off.

302 The technical problems associated with the use of this technology are many. First, sufficient amounts of antisense oligonucleotide must be administered to the vicinity of target cells and, more importantly, must be taken up by those cells. Second, the antisense oligonucleotide should, ideally, have a long enough half-life within the cell to successfully impair mRNA translation into protein over a significant period of time. Finally, the oligonucleotide must also be nontoxic and sufficiently specific so as not to interfere with other cellular functions. In many applications, these hurdles have been overcome and antisense technology has developed into a productive branch of biology. These technical challenges can be overcome in various ways depending on the specific application at hand. Oligonucleotides can be mixed with a variety of lipids to form complexes that are more easily incorporated by cell membranes, facilitating the entry of associated oligonucleotides into the cells. A number of other techniques have also been developed to facilitate the uptake of oligonucleotides by cells. Chemical modification of the antisense oligonucleotides can render them more stacble in cells and blood by increasing their resistance to ribonuclease digestion. Also, complementary DNAs or fragments of complementary DNAs can be incorporated in reverse sense in order to generate antisense RNA products in the host cell itself. This results in a long-term inhibition of the synthesis of the target protein. 6.4.2 Application of antisense technology in vitro Antisense technology has been applied successfully in two general areas. The first is in fundamental research where the introduction of antisense oligonucleotides can help determine the role of a specific gene in a specific physiological process. For example, introduction of antisense oligonucleotides to inhibit the synthesis of angiotensinogen, the substrate from which cells make angiotensin II actually was found to stop the synthesis of angiotensinogen that resulted in a decline in cell growth. The introduction of angiotensin II to the cells restored this growth. 6.4.3 Therapeutic application of antisense technology A second application of this technology, and one that is potentially of more immediate relevance to the practicing physician, is the use of this technology in therapy. In principle, antisense oligonucleotides complementary to viral RNAs can suppress a wide variety of viral infections; a tremendous amount of research is ongoing in this area. Similarly, antisense oligonucleotides directed towards the products of oncogenes can play a role in reducing the growth of cancer cells, and this lead is being hotly pursued.

303 Perhaps the most widely discussed application of antisense technology lies in its applications to gene therapy. In this case, a variety of vectors is used to introduce antisense-encoding genes into a large number of cells in a patient or animal to produce long-term inhibition of a protein. For example, in animal models the introduction of vectors encoding antisense angiotensin II receptor sequences results in long-term normotension in spontaneously hypertensive animals. These are but a few of the possible applications of antisense technology. As familiarity with the relevant chemistry increases, it is likely that more effective oligonucleotides and gene vectors will be developed, thereby providing the ability to interfere at will with the translation of specific mRNAs. 6.4.4 Triplex antisense technology In the face of all this progress, still newer technologies are being developed based on concepts related to antisense biology For example, it is known that oligonucleotides can, in certain instances, bind to duplex DNA molecules through an unusual kind of base pairing. In this triplex binding mode, oligonucleotides insert themselves into the major groove of the DNA double helix on a reasonably specific basis determined by the nucleotide sequence of the target DNA. This triplex technology provides the opportunity to reduce gene transcription jtself rather than to destroy mRNA once it is produced. Because the triplex oligonucleotides can be made to permanently alter the DNA after localizing to specific target sites, the technology actually has the potential to permanently silence genes. 6.4.5 RNA Inhibition It has recently been shown that double-stranded RNA in the cytoplasm triggers an as yet poorly understood cascade of events leading to the suppression of the transcription of the gene producing the specific mRNA involved in the cytoplasmic RNA duplex. This could potentially lead to the development of new pharmacological agents. Antisense technology is a formidable tool for investigating physiologic and pathologic processes. In addition, it is soon likely to become a mainstay of therapy, particularly in infectious diseases, with wider applications in the future as gene therapy techniques are developed further. Antisense Pharmaceuticals will soon be available for the routine care of patients and are expected to .prove to be effective, specific agents with favorable therapeutic profiles.

304 Unit 7 o Recombination and Repair Structure 7.1 Holiday junction in recombination 7.2 The holiday model of genetic recombination 7.3 Recombination proteins in E. Coli 7.4 DNA repair mechanism 7.1 Holiday junction in recombination Recombination is a process or set of processes by which DNA molecules interact with one another to bring about a rearrangement of the genetic information or content in an organism. Although recombination as a process has been known for a hundred years, the real reason has not been appreciated until relatively recently. It now seems clear that recombination reactions exist to repair DNA. In bacteria, and probably also in eukaryotes, recombination mechanisms exist to repair stalled DNA replication forks. In the simplest sense, recombination is an exchange of both strands between two DNA molecules Fig. 7.1: Exchange of DNA strands between two homologous chromosomes. In eukaryotic systems, you will be familiar with recombination as the process that is responsible for crossing-over during meiosis. Crossing-over has been well- documented genetically and is used to map the relative locations of genes on a chromosome. 7.2 The holiday model of genetic recombination The model for recombination of two individual DNA strand was first proposed by Robin Holliday in 1964 and re-established by David Dressier and Huntington Potter in 1976 who demonstrated that the proposed physical intermediates existed. In the most simplified explanation, two homologous DNA molecules align themselves which is followed by a nick at the same place on the two molecules as
305 shown in the figure below. This must happen in strands with the same polarity. The nicked strands then exchange themselves. The intermediate structure that is formed during such exchange is called a Holliday intermediate or Holliday structure. The shape of this intermediate in vivo is similar to that of the greek letter chi, hence this is also called a chi form. Fig. 7.2 : Initiation of recombination process and formation of Holiday structure There are two ways in which the holiday structure can resolve itself to return back to its original conformation after the recombination process. If the same strands are cleaved a second time then the original two DNA molecules are generated (Fig. 7.3a). But, if the other strands are cleaved, then recombinant molecules are generated in a manner as shown in the figure below (Fig. 7.3b). Fig. 7.3 : Two alternatives of recombination after the formation of holiday structure
306 In reality, a more complex mechanism operate to obtain different types of recom-bined strands as was observed in the Meselson-Weigle experiment where they located two different recombinant bacteriophages in a single plaque. These can be explained by modifying the above model slightly. As before, two homologous DNA molecules must be aligned and nicked at the same place. Following strand exchange the intermediate Holliday structure is formed. After the formation of the Holliday structure and ligation of the strands, the branch migrates, which can take place in either direction. The result is a physical transfer of part of one of the strands of one molecule with that of the other: Fig. 7.4 : Two alternatives of recombination after the formation of holliday structure For better understanding of the subsequent steps, one molecule is now rotated through 180° with respect to the other (Fig. 7.4). Fig. 7.5 : Rotation of one strand through 180° for better understanding As described above, there are two possibilities of recombination which may result in two different consequences. 1. If the same strands are cleaved a second time then n on recombinant DNA molecules are generated but they each contain a region of heteroduplex DNA that spans the region of branch migration:
307 2. If the other strands are cleaved, then recombinant molecules are generated as before, however, each will also contain a region of heteroduplex DNA that spans the region of branch migration: Double stranded nicks : While the single-strand nick model provides a simple explanation for recombination, recent work in the yeast Sacchromyces cerevisiae shows that recombination is actually a response to Double-stranded breaks in the DNA molecule. The double-strand break model begins with the introduction of a double stranded break in one of the paired homologs (Fig. 7.6). Fig. 7.6 : Double-stranded nick in the homologous DNA molecule Introduction of the double-stranded break results in exonucleolytic degradation of the adjacent strand in a 5' to 3' direction resulting in two single- stranded.whiskers (Fig. 7.7) which can now invade the paired homolog (via the action of a RecA like protein). Fig. 7.7.: Exonucleolytic degradation of the adjacent strand in a 5' to 3' direction The DNA strand which was undisturbed serve as template and the invading 3' ends serve as primers for DNA synthesis and extends it self as shown below (Fig. 7.8). Fig. 7.8 : Extension of the overhands of the nicked strand

308 The 3' ends of the newly synthesized strand are then ligated with 5' ends of the degraded red homolog to form the Double Holiday Junction shown below (Fig. 7.9) Fig. 7.9 : Formation of Double Holiday Junction These Holiday Junctions are free to migrate as before to generate the heteroduplex regions as shown below (Fig. 7.10) Fig. 7.10 : Free migration of the Holiday Junction As before, each Holiday Junction can be resolved in two ways... Resolution I: involves breaking and rejoining the two strands that cross between the two homologs. Resolution II involves breaking and rejoining the two strands that do not cross between the two homologs Since there are TWO Holiday Junctions, the exchange of flanking markers depends on how each Holiday Junction is resolved. If both Holiday Junctions resolve via Resolution I, no exchange of flanking markers is observed as shown below. Note that the region between the two junctions involves heteroduplex which can be corrected to produce Gene Conversion (Fig. 7.11). Fig. 7.11 : Formation of small region of heteroduplex if resolved through resolution 1 If Holiday Junction 1 undergoes Resolutation I and Holiday Junction 2 undergoes Resolutation II, or Holiday Junction 1 undergoes Resolution II and
309 Holiday Junction 2 undergoes Resolution I exchange of flanking markers is observed as shown below (Fig. 7.12). Fig. 7.12 : When both the Holliday junctions involve both type 1 and type 2 resolution, flanking markers are present Finally, if both Holiday Junctions undergo Resolution II, no exchange of flankinj markers is observed. Fig. 7.13 : When both the Holliday junctions, undergo resolution 2 resolution no flanking markers is observed and the result is similar to fig. 11 7.3 Recombination proteins in E. coli A number of the key proteins required for recombination. The structures and biochemical function of these proteins has been characterized which, is now helping us to understand details of the mechanism of recombination. The most important proteins are RecA, RecBCD, RuvA, RuvB and RuvC. RecA The RecA protein is a multifunctional powerhouse! It has strand-exchange, ATPase and co-protease activities all packed into a compact 352 amino-acid, 38 kDa structure. It is required for all recombination pathways in E. coli. The RecA protein is a critical enzyme in this process, as it Fig. 7.14a :
310 catalyzes the pairing of ssDNA with complementary regions of dsDNA. The RecA monomers (Fig. 7.14a) first polymerize to form a helical filament around ssDNA, binding to a span of 4-6 nucleotides. Assembly of the nucleoprotein complex proceeds in a 5'?3' direction. The complex is both fairly stable (half-life is 30 min) and is the active species that will promote strand exchange. RecA filament that forms is helical with a pitch of 82.7 Å and it consists of 6 monomer units per turn (Fig. 7.14b) During this process, RecA extends the ssDNA by 1.6 angstroms per axial base pair. Duplex DNA is then bound to the polymer. Bound dsDNA is partially unwound to facilitate base pairing between ssDNA and duplexed DNA. Once ssDNA has hybridized to a region of dsDNA, the duplexed DNA is further unwound to allow for branch migration. RecA has a binding site for ATP, the hydrolysis of which is required for release of the DNA strands from RecA filaments. ATP binding is also required for RecA-driven branch migration, but non-hydrolyzable analogs of ATP can be substituted for ATP in this process, suggesting that nucleotide binding alone can provide conformational changes in RecA filaments that promote branch migration. (Fig. 7.15) Fig. 7.15 : The strand exchange reaction probably involves the following steps: (a) RecA binds to the ssDNA partner, (b) The two molecules are aligned possible through the formation of a triple-stranded intermediate, (c) Displacement of one of the old strands. This requires concurrent migration of the RecA nucleoprotein filament along the molecule - which proceeds in one direction only (5'?3') - and consequent winding/unwinding. ATP hydrolysis takes place during this step Fig. 7.14B :
311 RecBCD The recB, recC & recD genes code for the three subunits of the RecBCD enzyme which has five activities: exonuclease V; a helicase activity; an endonuclease activity; an ATPase activity; and, an ssDNA exonuclease activity. The RecBCD helicase activity can unwind DNA faster than it rewinds. Thus as it travels along a DNA molecule, it can generate ssDNA loops (Fig. 7.16). The RecBCD complex functions as a DNA exonuclease. It will bind to double-stranded breaks in DNA and degrade both strands simultaneously (Fig. 7.17). However, when RecBCD encounters a Chi sequence, its activity changes, the RecBC proteins act as a helicase to unwind the The RecD subunit is released and DNA in an ATP dependent reaction. This generates a ssDNA region that can serve (along with RecA) to initiate strand exchange and a recombination reaction 7.3.1 Proteins required for resolving holliday junctions in E. coli RuvA RuvA is a small protein whose function is to recognize a Holliday junction thereby assisting the RuvB helicase to promote branch migration. The RuvA protein is 203 amino acids in length, but only 190 of them could be ssigned in the crystal structure. Most of the missing assignments represent amino

312 acids in a flexible part of the protein. The crystal structure of the E. coli RuvA protein was solved at a resolution of 1.9 Å. The protein forms a tetramer in an unusual manner - though one that is ideally suited to its function. Fig. 7.18: Four monomer of Ruv A arranged like flower petals in the same plane to hold the DNA strands at Holliday Junction RuvB The RuvB protein is a helicase that catalyzes branch migration of Holliday junctions. By itself it cannot bind to DNA efficiently. It functions in combination with RuvA. Like other helicases, RuvB functions as a hexamer; but, unlike other helicases, RuvB encloses double-stranded DNA not ssDNA. Electron microscopy has shown that RuvB is a heptamer in solution and that it converts to a hexamer ring when it binds to DNA. Electron microscopy has also shown that the two hexamer rings of RuvB lie contacting RuvA on the two opposite sides of a RuvAB-Holliday junction complex (Fig. 7.19). Fig. 7.19 : A hypothetical hexamer model of RuvB derived from the electron microscopy images

313 RuvC The RuvC protein resolves the Holliday intermediate. It functions as a dimer to cleave two of the four strands that make up the central part of the intermediate. Since binding is symmetrical, RuvC can bind to the Holliday intermediate in two equally likely ways. Hence, Holliday intermediates can be resolved in two different, but equally likely, ways. The interaction of RuvC with Holliday junction is shownin Fig. 7.20. RuvC does have some sequence specificity. It cleaves DNA at the 3'-side of thymidine, preferentially at the consensus 5'- A / T TT| c / G - 3' where '|' indicates the site of cleavage 7.4 DNA repair mechanisms 7.4.1 Introduction Cells are always subjected to different types of stresses some of which cause alteration to the DNA molecules. For the genetic information encoded in the DNA is to remain uncorrupted, any chemical changes must be corrected. Surprisingly, both eukaryotic and prokaryotic cells have evolved an efficient DNA repair mechanism that function to maintain the integrity of the DNA molecules but allows subtle changes required to bring about variations and evolutionary changes. The recent publication of the human genome has already revealed 130 genes whose products participate in DNA repair. More will probably be identified soon. 7.4.2 Agents that damage DNA • Certain wavelengths of radiation o ionizing radiation such as gamma rays and x-rays o ultraviolet rays, especially the UV-C rays (-260 nm) that are absorbed strongly by DNA but also the longer-wavelength UV-B that penetrates the ozone shield. •

Highly-reactive oxygen radicals produced during normal cellular respiration as well as by other biochemical pathways. • Chemicals in the environment o many hydrocarbons, including some found in cigarette smoke o some plant and microbial products, e.g. the aflatoxins produced in moldy peanuts • Chemicals used in chemotherapy, especially chemotherapy of cancers Fig. 7.20

314 7.4.3 Types of DNA damage 1. All four of the bases in DNA (A, T, C, G) can be covalently modified at various positions. o One of the most frequent is the loss of an amino group ("deamination") — resulting, for example, in a C being converted to a U. 2. During DNA replication DNA polymerase (E. coli) - inserts one incorrect nucleotide for every 10 5 nucleotides due to tautomeric "flickering" of the bases 3. Mutations; • Base Substitution by o point mutations - transitions • pyrimidine-to-pyrimidine substitutions (T6C) • purine-to-purine substitutions (A6G) ? - transversions • pyrimidine-to-purine substitutions (T6G or A) • purine-to-pyrimidine substitutions (A6C or T) • Insertions or deletions of a small number of nucleotides o frame shift mutations - recombination errors - transposons Spontaneous rate of mutation at a given site on a chromosome is approximately 10 -6 to 10 -11 per round of replication. It is - species and site specific - "hot spots" • DNA microsatellites - repetitive sequences errors dues to "slippage" of DNA polymerase during replication - low frequency sites

315 4. Mismatches of the normal bases because of a failure of proofreading during DNA replication. o example: incorporation of the pyrimidine U (normally found only in RNA) instead of T. 5. Breaks in the backbone : caused frequently by Ionizing radiation or by chemicals o Can be limited to one of the two strands (a single-stranded break, SSB) or o on both strands (a double-stranded break (DSB). 6. Crosslinks Covalent linkages can be formed between bases o on the same DNA strand ("intrastrand") or o on the opposite strand ("interstrand"). Several chemotherapeutic drugs used against cancers crosslink DNA. 7.4.4 Repairing damaged bases Damaged or inappropriate bases can be repaired by several mechanisms: • Direct chemical reversal of the damage • Excision Repair, in which the damaged base or bases are removed and then replaced with the correct ones in a localized burst of DNA synthesis. There are three modes of excision repair, each of which employs specialized sets of enzymes. 1. Base Excision Repair (BER) 2. Nucleotide Excision Repair (NER) 3. Mismatch Repair (MMR) 7.4.5 Direct reversal of base damage Perhaps the most frequent cause of point mutations in humans is the spontaneous addition of a methyl group (CH 3 -) (an example of alkylation) to Cytosine followed by deamination to a Thyamine. Fortunately, most of these changes are repaired by enzymes, called glycosylases, that remove the mismatched T restoring the correct C. This is done without the need to break the DNA backbone (in contrast to the mechanisms of excision repair described below).

316 Some of the drugs used in cancer chemotherapy ("chemo") also damage DNA by alkylation. Some of the methyl groups can be removed by a protein encoded by our MGMT gene. However, the protein can only do it once, so the removal of each methyl group requires another molecule of protein. This illustrates a problem with direct reversal mechanisms of DNA repair: they are quite wasteful. Each of the myriad types of chemical alterations to bases requires its own mechanism to correct. What the cell needs are more general mechanisms capable of correcting all sorts of chemical damage with a limited toolbox. This requirement is met by the mechanisms of excision repair. 7.4.6 Base excision repair (BER) The steps and some key players: 1. Removal of the damaged base (estimated to occur some 20,000 times a day in each cell in our body!) by a DNA glycosylase. There exist at least 8 genes encoding different DNA glycosylases each enzyme responsible for identifying and removing a specific kind of base damage. 2. Removal of its deoxyribose phosphate in the backbone, producing a gap. We have two genes encoding enzymes with this function. 3. Replacement with the correct nucleotide. This relies on DNA polymerase beta, one of at least 11 DNA polymerases encoded by our genes. 4. Ligation of the break in the strand. Two enzymes are known that can do this; both require ATP to provide the needed energy. 7.4.7 Nucleotide excision repair (NER) NER differs from BER in several ways. • It uses different enzymes. • Even though there may be only a single "bad" base to correct, its nucleotide is removed along with many other adjacent nucleotides; that is, NER removes a large "patch" around the damage. The steps and some key players: 1. The damage is recognized by one or more protein factors that assemble at the location. 2. The DNA is unwound producing a "bubble". The enzyme system that does this is Transcription Factor IIH, TFIIH, (which also functions in normal transcription).

317 3. Cuts are made on both the 3' side and the 5' side of the damaged area so the tract containing the damage can be removed. 4. A fresh burst of DNA synthesis — using the intact (opposite) strand as a template — fills in the correct nucleotides. The DNA polymerases responsible are designated polymerase delta and epsilon. 5. A DNA ligase covalent binds the fresh piece into the backbone. 7.4.8 Transcription-coupled NER Nucleotide-excision repair proceeds most rapidly • in cells whose genes are being actively transcribed • on the DNA strand that is serving as the template for transcription. This enhancement of NER involves XPB, XPD, and several other gene products. The genes for two of them are designated CSA and CSB (mutations in them cause an inherited disorder called Cockayne's syndrome). The CSB product associates in the nucleus with RNA polymerase II, the enzyme responsible for synthesizing messenger RNA (mRNA), providing a molecular link between transcription and repair. One plausible scenario: If RNA polymerase II, tracking along the template (antisense) strand), encounters a damaged base, it can recruit other proteins, e.g., the CSA and CSB proteins, to make a quick fix before it moves on to complete transcription of the gene. 7.4.9 Mismatch repair (MMR) Mismatch repair deals with correcting mismatches of the normal bases; that escapes proofreading mechanism of correction, and fails to maintain normal Watson-Crick base pairing (A•T, C•G) It can enlist the aid of enzymes involved in both base-excision repair (BER) and nucleotide-excision repair (NER) as well as using enzymes specialized for this function. • Recognition of a mismatch requires several different proteins including one encoded by MSH2. Cutting the mismatch out also requires several proteins, including one encoded by MLH1.

318 Eg. In E. coll • MutS dimer scans DNA for mismatches which distort DNA backbone • Binds ATP at conformation changed sites where MutS bends the DNA • ATP-MutS complex recruits MutL & MutH. ATP hydrolysis required for loading • MutL activates MutH endonuclease activity nicks one strand near the mismatch • DNA is unwound by helicase UvrD from the incision to the site of the mismatch • Exonuclease digests displaced strand gap filled in by Pol III nick sealed by ligase Mutations in either of these genes predispose the person to an inherited form of colon cancer. So these genes qualify as tumor suppressor genes. Synthesis of the repair patch is done by the same enzymes used in NER: DNA polymerase delta and epsilon. Cells also use the MMR system to enhance the fidelity of recombination; i.e., assure that only homologous regions of two DNA molecules pair up to crossover and recombine segments (e.g., in meiosis). Repairing Strand Breaks Ionizing radiation and certain chemicals can produce both single-strand breaks (SSBs) and double-strand breaks (DSBs) in the DNA backbone. Single-Strand Breaks (SSBs) Breaks in a single strand of the DNA molecule are repaired using the same enzyme systems that are used in Base-Excision Repair (BER). Double-Strand Breaks (DSBs) There are two mechanisms by which the cell attempts to repair a complete break in a DNA molecule: • Direct joining of the broken ends. This requires proteins that recognize and bind to the exposed ends and bring them together for ligating. They Fig. 7.21

319 would prefer to see some complementary nucleotides but can proceed without them so this type of joining is also called Nonhomologous End- Joining (NHEJ). • Errors in direct joining may be a cause of the various translocations that are associated with cancers. • Examples: o Burkitt's lymphoma o the Philadelphia chromosome in chronic myelogenous leukemia (CML) o B-cell leukemia Meiosis I with the alignment of homologous sequences provides a mechanism for repairing damaged DNA; that is, mutations, in fact, many biologists feel that the main function of sex is to provide this mechanism for maintaining the integrity of the genome. However, most of the genes on the human Y chromosome have no counterpart on the X chromosome, and thus cannot benefit from this repair mechanism. They seem to solve this problem by having multiple copies of the same gene —-oriented in opposite directions. Looping the intervening DNA brings the duplicates together and allowing repair by homologous recombination. 7.4.10 Gene conversion If the sequence used as a template for repairing a gene by homologous recombination differs slightly from the gene needing repair; that is, is an allele, the repaired gene will acquire the donor sequence. This nonreciprocal transfer of genetic information is called gene conversion. Gene conversion during meiosis alters the normal mendelian ratios. Normally, meiosis in a heterozygous (A,a) parent will produce gametes or spores in a 1:1 ratio; e.g., 50% A; 50% a. However, if gene conversion has occurred, other ratios will appear. If, for example, an A allele donates its sequence as it repairs a damaged a allele, the repaired gene will become A, and the ratio will be 75% A; 25% a. Human diseases caused by loss of DNA repair systems: DNA repair systems play a major role in normal human health. Two examples of human pathology caused by loss of repair systems are described below. Xeroderma pigmentosum: Xeroderma pigmentosum is a human genetic disease (or more correctly, a family of closely related genetic diseases), in which there is abnormal sensitivity to ultraviolet radiation. A number of different genes appear to be involved. Some patients exhibit defects in photoreactivation, but loss of

320 excision repair is more common. Mutations in at least seven different genes coding for proteins involved in excision repair can cause afflicted individuals to exhibit the symptoms of xeroderma pigmentosum. Cockayne syndrome: This human genetic disease, whose symptoms include mental retardation, dwarfism, and premature aging, appears to be primarily due to failure of transcription-repair coupling.

321 Unit 8 o Molecular Mapping of Genome Structure 8.1 Generic and physical maps 8.2 Gene cloning 8.3 Genomic analysis 8.4 RFLP, RAPD and AFLP analysis 8.1 Genetic and physical maps With the rebirth of genetics in the 20th century, it quickly became apparent that Mendel's second rule does not apply to many matings of dihybrids. In many cases, two alleles inherited from one parent show a strong tendency to stay together as do those from the other parent. This phenomenon is called linkage. The linkage phenomenon was utilized to construct gene maps. 8.1.1 Genetic map A genetic map is a representation of the genes on a chromosome arrayed in linear order with distances between loci expressed as percent recombination (map units, centimorgans). It is also called a linkage map. The relative position of genes on a chromosome in a Genetic map is determined by counting the phenotypes from a cross. One map unit = one centimorgan (cM) = 1% recombination between loci. The farther apart two loci are, the more likely that a crossover will occur between them. Conversely, if two loci are close together, a crossover is less likely to occur between them. A recombination rate of 50% corresponds to independent assortment. Therefore, only distances less than 50 map units can be measured directly. Greater distances can be constructed by adding up distances between closer loci. They have been prepared for many eukaryotes, including corn, Drosophila, the mouse, and the tomato. A genetic map of chromosome 9 (the one that carries the C, Sh, and bz loci) of the corn plant (Zea mays) is shown on the right. For example, test crossing a com plant that is dihybrid for the C,c (yellow/ colourless kernels) alleles and the alleles for bronze color (Bz, bz) produces 4.6% recombinants. So these two loci are 4.6 cM apart. However, is the bz locus on the same side of c as sh (smooth/shrunken) or is it on the other side? consider a cross between two different strains of corn (maize)

322 The answer can be found by test crossing the dihybrid Shsh, Bzbz. If the percentage of recombinants is less than 4.6%, then bz must be on the same side of locus c as locus sh. If greater than 4.6%, it must be on the other side. In fact, the recombination frequency is less than 1.8%, telling us that the actual order of loci is c — sh — bz But there are certain difficulties with such genetic maps. Mapping by linkage analysis is best done with loci that are relatively close together; that is, within a few centimorgans of each other. Why? Because as the distance between two loci increases, the probability of a second crossover occurring between them also increases and therefore interpretation becomes difficult. There are other problems with preparing genetic maps of chromosomes. • The probability of a crossover is not uniform along the entire length of the chromosome. o Crossing over is inhibited in some regions (e.g., near the centromere). o Some regions are "hot spots" for recombination (for reasons that are not clear). Approximately 80% of genetic recombination in humans is confined to just one-quarter of our genome. • In humans, the frequency of recombination of loci on most chromosomes is higher in females than in males. Therefore, genetic maps of female chromosomes are longer than those for males. 8.1.2 Chromosome maps The chromosome map (or cytogenetic map) is based on the karyotype of an organism. For example: All mouse chromosomes are defined at the cytogenetic Fig. 8.1 : Karyotype of a diploid set of normal raetaphase chromosomes of Mus musculus. Fig 8.2 : Mouse chromosome idiograms. (Giemsa banding patterns associated with each chromosome in a normal karyotype

323 level according to their size and banding pattern (Fig. 8.1) and ultimately, all chromosomal assignments are made by direct cytogenetic analysis or by linkage to a locus that has previously been mapped in this way. Chromosomal map positions are indicated with the use of band names (Fig. 8.2). Today, several different approaches, with different levels of resolution, can be used to generate chromosome maps. 1. Human/mouse cell hybrids tend to lose human chromosomes at random, leading eventually to hybrid cell lines that have one or a few human chromosomes. If a gene is always present or absent when one particular chromosome is present or absent, it can be concluded that the gene is on that chromosome. 2. Fluorescent or radioactive probes that bind to a particular gene can be observed microscopically and can be used to localize the gene on a metaphase spread. 3. Chromosomes from cells in metaphase can be sorted with high-speed electronic sorters. One can make preparations of a particular chromosome. If a particular gene can be shown to be in the preparation, it must be located on that chromosome. 8.1.3 Physical map All physical maps are based on the direct analysis of DNA. Physical distances between and within loci are measured in basepairs (bp). Physical maps are arbitrarily divided into short range and long range. 1. Short range mapping is commonly pursued over distances ranging up to 30 kb. In very approximate terms, this is the average size of a gene and it is also the average size of cloned inserts obtained from cosmid-based genomic libraries. Cloned regions of this size can be easily mapped to high resolution with restriction enzymes or by sequencing. 2. Direct long-range physical mapping can be accomplished over megabase- sized regions with the use of rare-cutting restriction enzymes together with various methods of gel electrophoresis referred to generically as pulsed field gel electrophoresis or PFGE, which allow the separation and sizing of DNA fragments of 6 mb or more in length. 3. Long-range mapping can also be performed with clones obtained from large insert genomic libraries such as those based on the yeast artificial chromosome (YAC) cloning vectors, since regions within these clones can be readily isolated for further analysis.

324 8.1.4 Connections between maps In theory, linkage, chromosomal, and physical maps should all provide the same information on chromosomal assignment and the order of loci (Fig. 8.3). However, the relative distances that are measured within each map can be quite different. Only the physical map can provide an accurate description of the actual length of DNA that separates loci from each other. This is not to say that the other two types of maps are inaccurate. Rather, each represents a version of the physical map that has been modulated according to a different parameter. Cytogenetic distances are modulated by the relative packing of the DNA molecule into different chromosomal regions. Linkage distances are modulated by the variable propensity of different DNA regions to take part in recombination events. In practice, genetic maps of the mouse are often an amalgamation of chromosomal, linkage, and physical maps, but at the time of this writing, it is still the case that classical recombination studies provide the great bulk of data incorporated into such integrated maps. Thus, the primary metric used to chart interlocus distances has been the centimorgan. However, it seems reasonable to predict that, within the next five years, the megabase will overtake the centimorgan as the unit for measurement along the chromosome 8.1.5 Gene mapping has important applications A. It is useful for locating the position of genes on chromosomes, e.g. if two genes are closely linked and the position of one is known, then the other must also be nearby. B. It is useful in estimating genetic risk, e.g. if a gene cannot be tested directly, then variation at a closely linked locus may indicate the presence or absence of a detrimental allele. C. A major goal of the Human Genome Project is the mapping of all human

325 genes (as well as those of mice, Drosophila, Caenorabditis elegans (a nematode), Arabidopsis thaliana (a small plant), yeast, and the bacterium Escherichia coli. As of 1999, yeast, E. coli, C. elegans,and about a dozen other bacteria have been completely sequenced and all their genes identified, although the functions of most are unknown. Major progress has been made in mapping human genes, and a "rough draft" of the human genome is anticipated by 2000. Understanding of function of the many newly discovered human genes is being greatly aided by the studies of yeast, which has many genes similar to those of humans. 8.2 Gene cloning 8.2.1 Procedure of cloning Gene cloning involves separating a specific gene or DNA segment from a larger chromosome, attaching it to a small molecule of carrier DNA, and then replicating this modified DNA thousands or millions of times through both an increase in cell number and the creation of multiple copies of the cloned DNA in each cell. The result is selective amplification of a particular gene or DNA segment. Cloning of DNA before the 1970 was a difficult tusk. Unlike a protein, a gene does not exist as a discrete entity in cells, but rather as a small region of a much larger DNA molecule. But with the discovery of restriction nucleases and other enzymes like ligase, poly-merases, the tusk of cloning targeted gene became rather easy. The key development that made recombinant DNA technology possible was the discovery in the late 1960s of restriction enzymes or called restriction endonucleases. The specialty of the endo-nucleases is that it recognizes and makes double-stranded cuts in the sugar-phosphate backbone of DNA molecules at specific nucleotide sequences. These enzymes are produced naturally by bacteria, where they are used in defense against viruses. In bacteria, restriction enzymes recognize particular sequences in viral DNA and then cut it up. A bacterium protects its own DNA from a restriction enzyme by modifying the recognition sequence, usually by adding methyl groups to its DNA. The endonucleases can be used to cut purified DNA at targeted sites for partially purifying the gene from a mixture. Cloning of any DNA fragment essentially involves four steps: fragmentation, ligation, transfection, and screening/selection. Although these steps are invariable among cloning procedures a number of alternative routes can be selected, these are summarised as a 'cloning strategy'. The protocol for isolation of genes can be broken down into several steps.

326 Step 1. At first, DNA need to be isolated from the desired cell, purified to ensure there is no protein contamination in the DNA sample. Presence of protein or any undesired chemicals can effect the subsequent steps of cloning. Step 2. DNA sample having the gene of interest requires to be segmented into suitable size. Preparation of DNA fragments for cloning is frequently achieved by means of PCR, but it may also be accomplished by restriction enzyme digestion, DNA soni-cation and fractionation by agarose gel electrophoresis. Restriction enzyme digestion, for example with EcoRl will produce small fragments of DNA with sticky ends. If there is no EcoRl site in the gene of interest, then a the DNA fragment carrying the gene will have flanking region with sticky ends. Step 3. The next step involves the insertion of the DNA fragment into a vector. A vector is usually a plasmid- circular DNA, which is linearised by means of restriction enzymes. The DNA fragments and the linearised vector is incubated together under appropriate condition in presence of the enzyme DNA ligase. Sticky ends or the single stranded DNA overhangs allow annealing of the DNA fragment with the vector sequence. Sticky ends may also be produced by chemical modification and attachment of adapter molecules. 'Sticky ends' allow for both higher efficiency transformations and directional insertion of the insert into the vector, thus minimising the need for subsequent screening. Step 4. After the ligation precedure, the vectors with successful inserts are first identified and then is transfected into host cells. A number of alternative techniques are available, such as chemical sensitization of cells, electroporation and biolistics. Chemical sensitization of cells is frequently employed since this does not require specialised equipment and provides relatively high transformation efficiencies. Electroporation is employed when extremely high transformation efficiencies are required, as in very inefficient cloning strategies. Biolistics are mainly used in plant cell transformations, where the cell wall is a major obstacle in DNA uptake by cells. Step 5. Finally, the transfected cells are cultured and screened to identify the clone carrying the gene of interest. If the starting material is a PCR product, the screening step is not required. Successfully transformed carrying the gene of interest is identified primarily by hybridization technique. The required cells will be those that have been successfully transfected with the vector construct containing the

327 desired insertion sequence in the required orientation. Modern cloning vectors include selectable antibiotic resistance selection marker, which allow only cells in which the vector has been transfected, too grow. Additionally, the cloning vectors may contain colour selection markers which provide blue/white screening Fig. 8.4 : Schematic representation of the procedure of cloning

328 (á-factor complementation) on X-gal medium. Nevertheless, these selection steps do not absolutely guarantee that the DNA insert is present in the cells obtained. Further investigation of the resulting colonies is required to confirm that cloning was successful 8.2.2 cDNA Libraries A collection of clones containing all the DNA fragments from one source is called a DNA library. For example, we might isolate genomic DNA from human cells, break it into fragments, and clone all of them in bacterial cells or phages. The set of bacterial colonies or phages containing these fragments is a human genomic library, containing all the DNA sequences found in the human genome. A genomic library must contain a large number of clones to ensure that all DNA sequences in the genome are represented in the library. A library of the human genome formed by using cosmids, each carrying a random DNA fragment from 35,000 to 44,000 bp long, would require about 350,000 cosmid clones to provide a 99% chance that every sequence is included in the library. An alternative to creating a genomic library is to create a library consisting only of those DNA sequences that are transcribed into mRNA (called a cDNA library because all the DNA in this library is complementary to mRNA). Much of eukaryotic DNA consists of repetitive (and other DNA) sequences that are not transcribed into mRNA and such sequences are not represented in a cDNA library. One of the most challenging tasks in molecular biology is the synthesis and cloning of cDNA. A complex series of enzymatic steps is involved in copying mRNA into double-stranded cDNA and subsequently preparing the termini for vector ligation. Many approaches have been used to generate cDNA libraries. Most cDNA molecules produced will lack a few nucleotides corresponding to the 5' end of the mRNA because second-strand replacement only proceeds from 3'-OH RNA primers. However, since all eukaryotic mRNA molecules appear to have 5' noncoding leader sequences, which commonly range from 40-80 nucleotides, it is likely that the vast majority of double-stranded cDNA will contain all of the coding sequences present in the initial cellular mRNA molecules. However, cDNA library has two advantages. First, it is enriched with fragments from actively transcribed genes. Second, introns do not interrupt the cloned sequences and therefore easy to clone in bacterial system for expression.The disadvantage of a cDNA library is that it contains only sequences that are present in mature mRNA. Introns and any other sequences that influence transcription procedure are not present; sequences, such as promoters and enhancers, etc. It is
329 also important to note that the cDNA library represents only those gene sequences expressed in the tissue from which the RNA was isolated which is again dependent on the frequency of mRNA trans cribbed in the tissue. In contrast, almost all genes are present at the same frequency in a genomic DNA library. 8.2.3 Choice of primers The classical method of cDNA synthesis uses the Oligo(dT) Primer to prime first-strand synthesis (Fig. 8.5). This method is suitable in most cases where poly(A)+ RNA of high quality can be prepared from the cell line or tissue of interest. Random Hexameric Primers (hexadeoxyribonucleotides) provide an alternative procedure (Fig. 8.6) by which first-strand cDNA synthesis is initiated from internal sites within the mRNA molecule. Random Primers can be used to prime cDNA synthesis from mRNA molecules that do not possess a poly(A)+ tail or for RNA isolated from prokaryotic sources. Random Primers also provide a scheme by which cDNA can be synthesized representing mRNA with strong 5' secondary structure. 8.2.4 Reverse transcription
330 The mRNA molecules are then copied into cDNA by reverse transcription. Reverse transcriptase, an enzyme isolated from retroviruses, synthesizes single- stranded complementary DNA from the RNA template by adding DNA nucleotides to the 3'-OH group of the primer (Fig. 1 & 2) The resulting RNA-DNA hybrid molecule is then converted into a double-stranded cDNA molecule by one of several methods. One common method is to treat the RNA-DNA hybrid with RNase to partly digest the RNA strand. Partial digestion leaves gaps in the RNA-DNA hybrid, allowing DNA polymerase to synthesize a second DNA strand by using the short undigested RNA pieces as primers and the first DNA strand as a template. DNA polymerase eventually displaces all the RNA fragments, replacing them with DNA nucleotides, and nicks n the sugar-phosphate backbone are sealed by DNA ligase. The cDNA thus synthesized are then subjected to end modifications and cloned into vectors for further analysis. 8.2.5 Libraries A “library” is a convenient storage mechanism of genetic information. • They are typically either “genomic” or “cDNA” (i.e. mRNA in DNA form) genetic information. Deduced genetic sequences from corresponding polypeptide information can be used to identify specific genetic information within a library. 8.3 Genomic analysis 8.3.1 Introduction The field of genomics comprises focuses on the content and organization of genomic information, and attempts to understand the function of information in genomes. Genomics is trying to look at all the genes as a dynamic system, over time, and determine how they interact and influence biological pathways and physiology, in a much more global sense. Genetics looks at single genes, one at a time, as a snapshot. Genetics is much more linear than genomics, complicated but not as complex as genomics. The genetic information possessed by each individual is termed its genotype and can refer to the entirety of its genetic information or a part of it. The set of characteristics expressed by an individual is termed as phenotype. When two

331 individuals possessing the same genotype also have the same phenotype, regardless of the environmental conditions in which they exist, the character expressed is termed as genetic trait. Determination of the mode of inheritance of the genetic trait is called inheritance analysis. Inheritance analysis of a trait or phenotypic character is a genetic marker. If each phenotype can be unambiguously assigned to exactly one genotype, then the genetic marker defines a gene marker. The advent of recombinant DNA technology in population genetics in the mid-1980's, gradually led to the development of DNA markers. However, the repertoire of genetic markers available for population genetic studies continues to increase enormously and is still relevant of genetic analysis. DNA marker analysis, though costly have some added advantage over the classical genetic markers and is rapidly replacing the old system of genetic analysis. Technological advancement in the DNA sequencing technique contributed to development of the Genomics as a subject. Agenomic sequence is, by itself, of limited use. Functional genomics is, in essence, probing genome sequences for meaning— identifying genes, identify the unique sequences which can serve as DNA markers, recognizing their organization, and understanding their function etc. The goals of functional genomics include identifying all the RNA molecules transcribed from a genome (the transcriptome) and all the proteins encoded by the genome (the proteome).' Functional genomics exploits both bioinformatics and laboratory-based experimental approaches in its search to define the function of DNA sequences. 8.3.2 Predicting function from sequence Several methods for identifying genes and assessing their functions have been discussed earlier. The methods include in situ hybridization, DNA footprinting, experimental mutagenesis, and the use of transgenic animals and knockouts. These methods can provide important information about the locations and functions of genetic information and can be applied to study to large numbers of genes simultaneously. However, this biochemical approach to understanding gene function is both time consuming and expensive. A major goal of functional genomics has been to develop computational methods that allow gene function to be identified from DNA sequence alone, bypassing the laborious process of isolating and characterizing individual proteins.

332 8.3.3 Search for homology One computational method for determining gene function is to conduct a homology search, which relies on comparing DNA and protein sequences from the same and different organisms. Databases containing sequences of genes and proteins for a wide array of organisms are available in gene banks which are in public domain and can be accessed for homology searches. Powerful computer programs have been developed for scanning these databases to look for particular sequences. A commonly used homology search program is BLAST used to align sequences from different or same species. If a function is known for one of these sequences, that function may provide information about the function of the newly discovered protein. Similar programs are also available that can analyze two sequences and predict the evolutionary relationship from which phylogenetic trees can be established. 8.3.4 Drug designing Computer programs are also available that can detect single nucleotide polymorphism. One. can use the information from the analysis to predict the causes of various diseases and also can use the information to design drugs for treatment of diseases. 8.3.5 Gene expression and microarrays The advent and developmet of the Microarray technique made it possible to study hundred and thousands of gene at the same time. Many important clues about gene function come from knowing when and where the genes are expressed. The microarray technique enables us to get such clues. Microarrays rely on nucleic acid hybridization in which a known DNA fragment is used as a probe to find complementary sequences (Fig. 8.7). In a microarray, numerous known DNA fragments are fixed to a solid support in an orderly pattern or array, usually as a series of dots. These DNA fragments (the probes) usually correspond to known genes. When the microarray has been constructed, mRNA, DNA, or cDNA isolated from experimental cells is labeled with fluorescent nucleotides and applied to the array. Any of the DNA or RNA molecules that are complementary to probes on the array will hybridize with them and emit luorescence, which can be detected by an automated scanner. An array containing tens of thousands of probes can be applied to a glass slide or silicon wafer just a few square centimeters in size.

333 Fig 8.7 : Microarrays, used to detect the expression of many genes For example, the experimental cells are stimulated from which mRNA is isolated and converted into cDNA labeled with red fluorescent nucleotides. mRNA from control cells is converted into cDNA and labeled with green fluorescent nucleotides. The labeled cDNAs are mixed and hybridized to the DNA chip, which contains DNA probes from different genes from the same organism. Hybridization of the red (experimental) and green (control) cDNAs is proportional to the relative amounts of mRNA in the samples. The fluorescence of each spot is assessed with microscopic scanning and appears as a single color. Red indicates the overexpression of a gene in the experimental cells relative to that in the control cells (more red-labeled cDNA hybridizes), whereas green indicates the underexpression of a gene in the experimental cells relative to that in the control cells (more green-labeled cDNA hybridizes). Yellow indicates equal expression in experimental and control cells (equal hybridization of red- and greenlabeled cDNAs), and no color indicates no expression in either experimental or control cells (Fig. 8.8).
334 Fig: 8.8: Emission of fluorescence depends on the nature of hybridization Microarrays allow the expression of thousands of genes to be monitored simultaneously, enabling scientists to study which genes are active in particular tissues. They can also be used to investigate how gene expression changes in the course of biological processes such as development or disease progression. 8.4 Restriction fragment length polymorphisms (RFLP) A restriction fragment length polymorph of alternative alleles associated with restriction fragments that differ in size from each other. RFLPs are visualized by digesting DNA from different individuals with a restriction enzyme, followed by gel elec-trophoresis to separate fragments according to size, then blotting and hybridization to a labeled probe that identifies the locus under investigation. An RFLP is demonstrated whenever the Southern blot pattern obtained with one individual is different from the one obtained with another individual (Fig. 8.9). In this example, DNA samples from five individual mice were Fig. 8.9: Demonstration of RFLPs by Southern blot analysis.5 different Southern blot genotypes detected with a single probe is shown.
335 digested with the same enzyme, and after electrophoresis were probed with the same clone of a single-copy DNA sequence. The five patterns detected are all different from each other and are representative of five different genotypes. The first lane and the last lane are homozygous for the genes while the remaining individuals are heterozygous. RPLPs were the predominant form of DNA variation used for linkage analysis until the advent of PCR. Even now, in the PCR age, RFLPs provide a convenient means for turning an uncharacterized DNA clone into a reagent for the detection of a genetic marker. The main advantage of RFLP analysis over PCR-based protocols is that no prior sequence information, nor oligonucleotide synthesis, is required. Furthermore, in some cases, it may not be feasible to develop a PCR protocol to detect a particular form of allelic variation. Nevertheless, if and when a PCR assay for typing a particular locus is developed, it will almost certainly be preferable over RFLP analysis. The detection of a RFLP does not provide information as to the mechanism by which it was created. Moreover, from RFLP data, it is also not possible to predict how the individuals differ from each other at the molecular level. Attempts to identify RFLPs between different inbred strains of mice often meet with limited success even after, testing with large numbers of enzymes. In one study, RFLPs were identified at only 30% of the single copy loci tested with 22 different restriction enzymes. Furthermore, when RFLPs are identified, they are almost always 'di-allelic binary systems — the insertion, deletion, or restriction site change is either present or absent. Unfortunately, di-allelic loci can only be mapped in crosses where the two parental chromosomes carry the two alternative alleles. Thus, even if a RFLP is identified between two inbred strains of mice, there is no guarantee that another pair of strains will also happen to- carry alternative alleles. As a consequence, only a subset of the RFLP markers developed for analysis of one cross between traditional mouse strains will be of use for mapping in a cross between any other pair of inbred strains. 8.4.2 Choice of restriction enzymes to use for RFLP detection With so many restriction enzymes available, how does one decide which ones are the best to use in the search for RFLPs? Obviously, cost is an important consideration. Another consideration is whether the enzyme is optimally active with genomic DNA obtained from animal tissues. However, a critical consideration is the rate at which RFLPs can be detected based on the enzyme that is chosen. A systematic study of RFLP detection between B6 and M. spretus DNA subsequent to digestion with one often different enzymes has been reported. One

336 hundred and ten anonymous DNA sequences of less than 4 kb in length were used as probes. The highest rate of RFLP detection — 63% — was observed with DNA digested with TaqI. The second highest rate — 56% — was observed with MspI. In decreasing order of effectiveness were the enzymes BainRI (50%), XbaI (47%), PstI (44%), BgII (41%), Hind III (39%), PvuII (38%) Rsa I (38%), and Eco RI (33%). It is ironic that of the ten enzymes tested, the one most commonly used in molecular biological research — EcoRI — was the worst one, by a long shot, at detecting polymorphisms. 8.4.3 Minisatellites: variable number tandem repeat loci In contrast to traditional RFLPs caused by basepair changes in restriction sites, a special class of RFLP loci present in all mammalian genomes is highly polymorphic with very large numbers of alleles. These "hypervariable" loci were first exploited in a general way by Jeffreys (1985) and his colleagues for genetic mapping in humans. Hypervariable RFLP loci of this special class are known by a number of different names including variable number tandem repeat (VNTR) loci and minisatellites, which is the more commonly used term today. Minisatellites are composed of unit sequences that range from 10 to 40 bp in length and are tandemly repeated from tens to thousands of times. Although various functions have been suggested for mini satellite loci as a class, none of these has withstood the test of further analysis. Rather, it appears most likely that minisatellite loci evolve in a neutral manner through expansion and contraction caused by unequal crossing over between out-of-register repeat units. Recombination events of this type will yield reciprocal products which both represent new alleles with a change in the number of repeat units. The frequency with which new alleles are created at minisatellite loci — on the order of 10 -3 per locus per gamete — is much greater than the classical mutation rate of 10 -5 to 10 -6 . This leads to a much higher level of polymorphism between unrelated individuals within a population. At the same time, one change in a thousand gametes is low enough so as to not interfere with the ability to follow minisatellite alleles in classical breeding studies. Length polymorphisms at minisatellite loci are most simply detected by digestion of genomic DNA samples with a restriction enzyme that does not cut within the minisatellite itself but does cut within closely flanking sequences. As with all other RFLP analyses, the restriction digests are fractionated by gel electrophoresis, blotted and hybridized to probes derived from the polymorphic locus. However, unlike traditional point mutation RFLPs, minisatellites are caused 337 by, and reflect, changes in the actual size of the locus itself. The best restriction enzymes to use for minisatellite analysis are those with 4 bp recognition sites such as HaeIII, HinfI or Sau3A; it is likely that one of these enzymes will not cut within the relatively short minisatellite unit sequence, but will cut within several hundred basepairs of flanking sequence on both sides. Standard 1% agarose gels with maximal separation in the 1-4 kb range are usually best for the resolution of minisatellite bands; however, conditions can be optimized for each minisatellite system under analysis. 8.4.4 RAPD RAPD stands for random amplification of polymorphic DNA. It is a type of PCR reaction, where random segments of genomic DNA are amplified with single primer of arbitrary nucleotide sequence and which are able to differentiate between genetically distinct individuals, although not necessarily in a reproducible way. By resolving the resulting patterns, a semi-unique profile can be gleaned from a RAPD reaction (Fig. 8.10). Fig. 8.10: Randomly amplified genomic DNA with short primers No knowledge of the DNA sequence for the targeted gene is required, as the primers will bind somewhere in the sequence, but it is not certain exactly where. This makes the method popular for comparing the DNA of biological systems that have not had the attention of the scientific community, or in a system in which relatively few DNA sequences are compared (it is not suitable for forming a DNA databank). Due to the fact that it relies on a large, intact DNA template sequence, it has some limitations in the use of degraded DNA samples. Its resolving power is much lower than targeted, species specific DNA comparison methods, such as

338 short tandem repeats. In recent years, RAPD is used to characterize, and trace, the phylogeny of diverse plant and animal species. Limitations of RAPD • Nearly all RAPD markers are dominant, i.e. it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous (1 copy) or homozygous (2 copies). Co-dominant RAPD markers, observed as different-sized DNA segments amplified from the same locus, are detected only rarely. • PCR is an enzymatic reaction, therefore the quality and concentration of template DNA, concentrations of PCR components, and the PCR cycling conditions may greatly influence the outcome. Thus, the RAPD technique is notoriously laboratory dependent and needs carefully developed laboratory protocols to be reproducible. • Mismatches, between the primer and' the template may result in the total absence of PCR product as well as in a merely decreased amount of the product. Thus, the RAPD results can be difficult to interpret. 8.4.5 AFLP AFLP stands for Amplified Fragment Length Polymorphism which is a hybrid of RFLP and RAPD techniques. Genomic DNA is cut with restriction enzymes, as in RFLP. Typically, two different restriction enzymes are used. The idea is to produce a large number of fragments. Some of the fragments are selectively amplified with PCR using "random" primers, as in RAPD. The primers are not really random, however. Specific oligonucleotide "adapters" (these are complementary to the restriction sites) of 25-30 bp are ligated to the restricted DNA fragments. The primers are complementary to these adapters. However, the primers vary at their 3'-end, such that they will amplify only a subset of the restricted DNA fragments. Typically 50-100 restriction fragments are amplified and detected on denaturing polyacrylamide gels. AFLPs have typically been used to study variation among individuals of a species, most commonly for producing genetic maps (and in trying to find genes responsible for certain traits). They have received

339 limited attention as tools in systematics, perhaps because the method is relatively labor intensive when compared with other methods. The power of AFLP is based upon the molecular genetic .variations that exist between closely related species, varieties, orcultivars. These variations in DNA sequence are exploited by the AFLP technology such that "fingerprints" of particular genotypes can be routinely generated. These "fingerprints" are simply RFLPs visualized by selective PCR amplification of DNA restriction fragments. Genomic DNA ? Digested with reatriction endonuelease to produce fragments with averages at 5' and 3' ends ? Amplification will occur as 3' of the primar is complementar ? Primers added that are complementarr to thebut varies at 3' end by one nuecleothide ?????????????? ? Ligation of molecular, complementary to the everhages at restriction ? ? Fail to emplify because of the 3' end of the primer mis 5' 3' 3' 5' 5' 3' 3' 2 5' 3' 3' 5' 5' 3' 3' 5' 5' 3' 3' 5' 3 5' 3' 3' 3 1 5' 3' 3' 5' 5' 3' 3' 5' 3 5' 3' 3' 5' 1 5' 3' 3' 5' 3

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