NETAJI SUBHAS OPEN UNIVERSITY

STUDY MATERIAL

POST GRADUATE ZOOLOGY

PAPER - 5
GROUP : B

Laboratory Course - III

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 presumption 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 materials. 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 it 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 part 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.

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Third Reprint : June, 2016

Printed in accordance with the regulations and financial assistance of the Distance Education Bureau of the University Grants Commission.

POST GRADUATE ZOOLOGY

[M. Sc.]

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PGZO-5 : B

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Group B

UNIT 1 ❐ HANDLING OF DROSOPHILA, CHROMOSOME MAPPING, GENETIC CROSSES

Structure

1.0 INTRODUCTION

Drosophila melanogaster is commonly referred to as the "fruit fly" because it is so frequently found associated with over ripe fruit such as banana, apple etc. The optimal temperature for breeding *Drosophila melanogaster* is between about 15 and 30°C; the "standard" laboratory temperature is 23-25°C. Several species of *Drosophila* other than *melanogaster* have been used in research, and in recent years, each of the other species has come to occupy a particular research niche. Some are favoured for ecological studies, some for evolution, and some for population biology, but for studies

of genetics, development, and molecular biology, *Drosophila melanogaster* is unequaled. Its primary advantage lies in the reliability of its growth in the laboratory– it is small and grows on simple media, so it is cheap and takes up little space; in addition, it has a short generation time of less than two 'weeks at 25°C and a single female will produce hundreds of offspring during her lifetime. These features make it possible to generate adequate numbers for genetic studies. One can even obtain tile huge numbers of genetically homogeneous individuals at defined stages of their life cycle required for biochemical studies.

Here we will use *Drosophila* to illustrate some of the basic principles of inheritance. Genes, chromosomes, and meiosis are so fundamental to eukaryotic life that the principles we will be observing in tile fruit fly can be directly applied to most eukaryotes, including humans. In order to get the most out of these exercises, it will be important to apply the principles learned in class to the inheritance of the phenotypes we will be following in *Drosophila*.

1.1 LIFE CYCLE OF DROSOPHILA

Drosophila is a holometabolous dipteran insects with two wings. Holometabolous refers to the fact that when it goes through metamorphosis it changes completely in form; butterflies and moths are also holometabolous. An understanding of the life cycle will help us to follow the events occurring in the fly cultures. There are four stages: the embryo, the larva, the pupa, and the adult. The duration of each of these stages is well established, and is generally given for incubation at 25°C unless otherwise indicated. If we consider the moment at which the egg is laid as "time 0", embryonic development lasts for 22 hours. Development begins when the egg is fertilized, but fertilization does not necessarily occur immediately after copulation. The female will store sperm from a single copulation and use it for all her ovulations if necessary. If the female perceives conditions to be optimal, will lay the egg immediately after fertilization. If, however, the temperature is too low, there is no suitable site for laying, or conditions are sub optimal for some other reason, the female will hold the, eggs and development will begin in utero. (Fig. 1, 2 & 3)

The egg has a few characteristics that are visible to the naked eye. It is about 0.4 mm long, oblong, and slightly flatter on its dorsal side than on its ventral side. It has two filaments coming out of the dorsal surface very near the anterior end that are used for gas exchange. At even low power magnification, a tough outer membrane, the chorion, can be seen to be imprinted with a hexagonal cellular pattern. Beneath the chorion is a thin layer of air, then a tough vitelline membrane surrounding the

plasma membrane of the egg itself. At low power, a small protrusion called the micropile can be seen at the anterior end - this is the tube through which the sperm

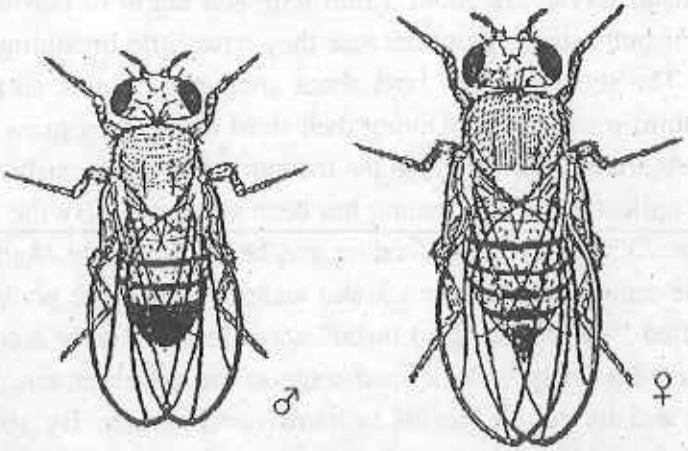


Fig. 1 : Adult *Drosophila* (Male & Female)

enters, After 22 hours of development, the larva emerges from the egg. There are three larval stages called instars separated by moults when the larva sheds its cuticle. Larvae spend their time only by eating; the amount they eat is the major determinant of their size as adults. The first instar larva is about 1 mm long, and stays on the

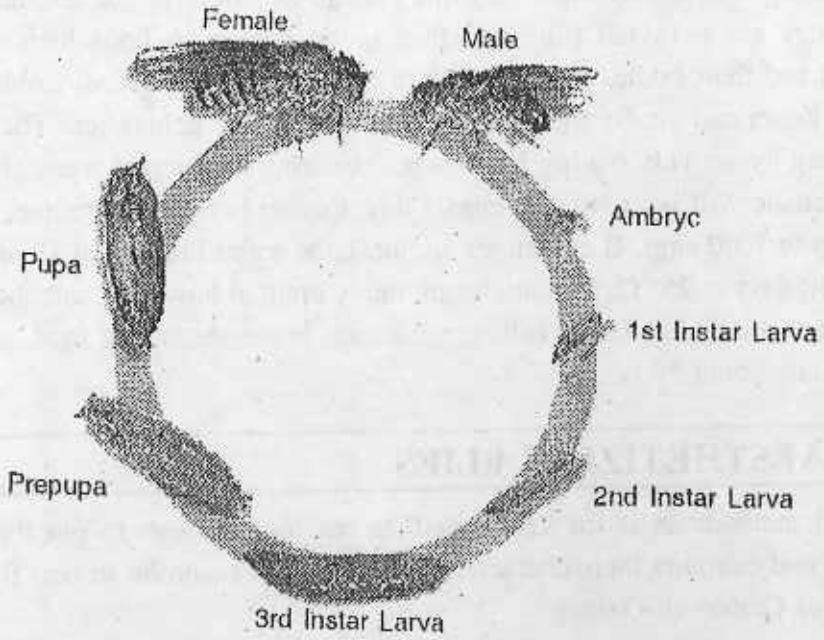


Fig. 2 : Life Cycle of *Drosophila melanogaster*

surface of the medium. The first instar lasts for about 24 hours, so at about 2 days after egg laying it molts to a second instar larva.

The second instar larvae are about 2 mm long and begin to burow into the food, often leaving their butts sticking out because they have little breathing tubes on back, called spiracles. The second instar' lasts about another 24 hours, so at about 3 days they moult to a third instal larvae. During their third instar, they grow to more than 4 mm, if well fed. As they burrow through the medium, they leave easily visible channels that are an early indicator that the mating has been successful. By the end of the fifth day (110 hours at 25°C) they cease feeding and begin to pupate. As they prepare to pupate, the larvae crawl out of the muck and wander around the walls of the culture tube. This so-called "wandering third instar" stage lasts for only a couple of hours, and so is considered a fairly well defined stage in the development. The larva then stops wandering and its cuticle begins to harden and darken. By about 122 hours, the pupa is fully formed and have a dark brown colour; pupation takes about 4.5 days. There is little to be seen during this remarkable stage from outside. However, inside pupal case, inost of the cells that made up the larva are auto-digested and a whole new animal is rebuilt from special sets of cells called imaginal disks. At the end of pupation, the adult emerges from the pupa by splitting it lengthwise and crawling out; this process is called "eclosion". Part of this process involves blowing their heads and bodies up with air, so when they first emerge they have big heads and abdomens. Their wings are wrinkled little wet things, but within an hour their wings have expanded and their bodies develop mature colouration. The sexual maturation takes about 12 hours and the fly then ready to initiate the new generation. The production of offspring by actively mating couples peaks during the second week after ecosion, and the female will lay upto 100 eggs a day. During her entire lifetime, one female can lay up to 1000 eggs. If conditions are ideal, the entire lifespan of *D. melanogaster* is about 50 days at 25° C. Conditions are rarely optimal however, and about a month is more common under normal culture conditions. In the absence of food, an individual will last only about 50 hours.

1.2 ANAESTHETIZING FLIES

Several methods exist for knocking flies out long enough to put them under a binocular and examine their characteristics. The most common in real fly labs is to use ether or Carbon-di-Oxide.

1.2.1 Procedure :

Take a food vial with adults in it, tap it lightly but firmly on the table top a couple times to knock the flies toward the bottom. Then in one smooth motion (by practice!) remove the cotton plug and invert the vial over a second empty vial through a small funnel the base of which is wrapped with cotton where a few drops of ether is applied. Let this setup sit there for 2-5 minutes – watch the flies —when they are all down at the bottom and stand still. Tap them out onto a 3×5 card and place them under the binocular. The flies will stay out for at least 10 minutes, during which time we can sex them, pick virgins, and score phenotypes.

1.2.2 Preparation of Culture medium :

For the preparation of culture medium the following ingredients are used :

Water — 500 ml

Agar powder — 4.1 gm

Maize powder — 45 gm

Brown Sugar — 45 gm

Dried yeast — 13 gm

Nipagin (antifungal) — 500 mg [Na methyl r hydroxybenzoate]

Propionic acid — 0.5 ml

The following procedure may be followed for the preparation of culture medium :

1. Agar in cold water dissolved by boiling.
2. Add maize powder & brown sugar in water.
3. Mix them and stir continuously to avoid clumping. Boil it for 20 minutes.
4. Add dried yeast powder and boil the mixture for another 10 minutes.
5. Add 0.5 ml propionic acid to the mixture & mix it thoroughly.
6. Dissolve nipagin in ethanol and add it to the mixture.
7. Continue boiling until the mixture become viscous.
8. Pour the mixture allow it to settle & plug it with non absorbent cotten.

1.3 SEXING FLIES

A number of sexually dimorphic characters exist in Drosophila, but we will rely on a couple simple ones.

1. The posterior of the abdomen is heavily pigmented in males and more rounded than females.

2. Females have a couple narrow bands of pigment through the region occupied by solid pigmentation in males, and are more pointed at the end. But in these labs you will be expected to confirm the sex of any flies you use by examining the genitalia.
3. The genital arch and anal plate are easily visible in males, whereas in females more discrete.
4. Male have sex comb in left fore leg.

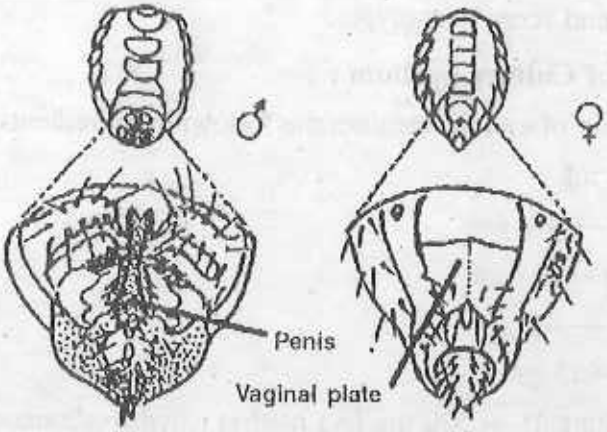


Fig. 3 : Ventral view of genetilia of male (left) and female (right) *Drosophila :* below is enlarged view

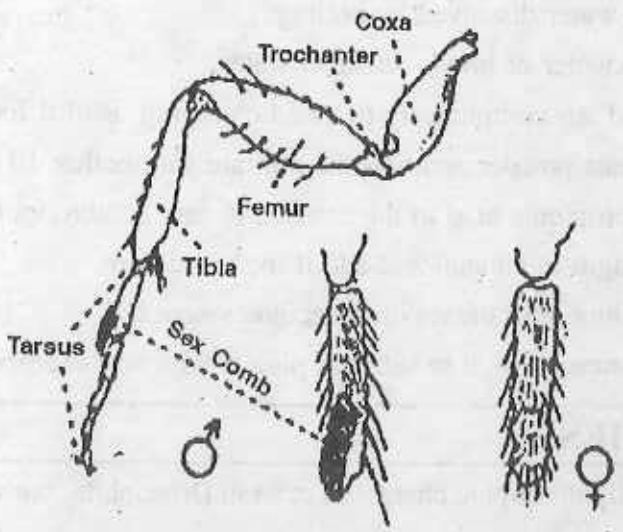


Fig. 4 : Left foreleg of male left and female right *Drosophila; The sex comb on tarsus of male fly, absent in female* (from W. Hewitt).

1.4 COLLECTING VIRGINS

The female stores sperms from a single mating and use it several times during her own ovulations. Therefore, once a fly has mated with one genotype, she cannot be "cleared" and used for matings with other genotypes that will be of any use for genetics. Although the techniques for collecting virgins are straight-forward, it is surprisingly difficult to get through a set of genetic crosses without making a mistake. Therefore, it is essential that the possibility of a non-virgin in the matings be kept in mind when the results of crosses are being interpreted. Typically, the result of a non-virgin mating will be a mixture of the offspring expected from a potential parent present in the vial in which the female eclosed, and the offspring expected from the intended mating. Once a series of crosses has begun, this mixture can be bafflingly complex. It is therefore essential that at each generation, the results we get can be rationalized on the basis of basic genetic principles, and are reproducible.

The simplest way to collect virgin females is to take advantage of the fact that newly eclosed flies will not mate for about 12 hours. Simply clearing the vials of all adults in the morning will ensure that any females collected during at least the next 8-10 hours will be virgins. It is not necessary to use virgin males for mating so, in practice, it is convenient to collect some males into one vial and put them aside to use for mating to virgins females as they are collected. The separated sexes can be kept in vials with food for several days and still are useful for mating.

The other way to collect virgin females is to find newly enclosed individuals based on the characteristics mentioned above : relatively unpigmented bodies, exploding heads, or wet, wrinkled wings. None of these should be considered foolproof however. The only foolproof visible marker of the virgin female is a black mass visible in the gut on the left side between the 2nd and 3rd abdominal segments. This is called the "meconium" (but only by a very few people!) and is actually larval gut tissue. Within a few hours after emerging, this will be excreted, but until it is excreted it blocks mating, and so is a sure sign of a virgin. The males display thee meconium, too, so its presence is not sufficient to ensure that a fly is a virgin female.

1.5 EXPERIMENTS TO BE DONE

Our experiments will involve mating between genetically defined individuals : "genetic crosses". We will work in pairs; each group will do several sets of crosses.

The crosses are designed to illustrate basic genetic principles, such as dominance, segregation, independent assortment, epistasis, sex linkage, linkage, and recombination. There are several different stocks, and several different crosses that can be made. Which cross we do will be determined by lottery. All the information we need for each cross will be given, but the analysis and interpretation will be up to us.

1.6 SETTING UP CROSSES

When handling the flies each week, two things should be done in each session : (1) setting up the genetic crosses, and (2) setting up new cultures of each true- breeding line ("passaging" each "stock") that we are handling so we have flies to work with ill the future.

When setting up genetic crosses, always we will have to think through what we are to do: What stocks will be crossed? Does it matter which is male and which is female? Do we have all the materials we need to proceed? Bring out stocks one at a time so that we will know what we are working with; it is easy to become confused when we have a table full of sleeping flies. Label a vial with the genetic notations for that stock, the date, and whether the flies from each stock will be male and female.

To passage the flies, it is not necessary to use virgin females. Get a vial of food, place a few grains of yeast into it, label it with the stock name, the date, and the name or initials, and place about a dozen males and a dozen females in it; plug it and put it with the other stocks. One week after setting up a cross, clear the adult flies from the vial by tapping them into a morgue so they aren't around to mate with the F_1 when they emerge. Examine the vial for larvae—if there are not any, the cross has to be repeated.

Two weeks after setting up the cross, we should see new adults in the vials. Tap them into an anesthetizing vial, knock the suckers out, and record their phenotypes. Do these phenotypes match our predictions? Let's hope so! Don't discard these flies after scoring them; use them to set up the $P_1 \times P_1$ cross. It will not always be necessary to use virgin females for the $F_1 \times F_1$ cross (why?). If we are setting up a test cross, note that virgins are necessary. If the purpose of the test cross is to measure recombination frequencies, the F_1 heterozygote should be a female because recombination does not occur in males in Drosophila. Remember, each week, clear the adults out of any vials we set up last week. During the fourth week after setting

up our first cross we will begin collecting data on the F_2 generation. It is best to examine flies that are 1-3 days old, because some phenotypes become less clear-cut with age. Score the phenotypic classes that emerge until we have counted at least 100 flies, or the vial has stopped producing. However, do not count flies beyond the third weeks after setting up the vial, because the progeny of the F_1 generation may start to emerge.

1.7 GENERAL INFORMATION AND FLY HUSBANDRY

The fruit fly *Drosophila melanogasterhas* 3 pairs of autosomal chromosomes and an X and Y chromosome. Each autosome has two arms that are simply referred to as left (L) and right (R). Each chromosome ann is numbered as follows : X (1-20), 2L (21-40), 2R (41-60), 3L (61-80), 3R (81-100), and chromosome 4 (101-102). Each chromosome arm is also numbered by recombination units, thus allowing one to know the expected recombination frequency between two genes located on the same chromosome arm. The chromosomal locations of individual genes are identified either by numerical location or by recombination units.

1.8 NOMENCLATURE USED IN GENETICS

The nomenclature used in *Drosophila* genetics is fairly straightforward .The standard rules of nomenclature is followed to properly and clearly describe the complete genotype of a fly stock.

- Chromosomes are written in order, as follows, with a semi-colon separating each chromosome X/Y; 2; 3; 4
- Genotypes are listed only when a mutation is present and are italicized.
- Recessive mutations are written in lower case (e.g. w for white gene),
- Dominant mutations are capitalized (e.g. B for Bar eye).
- Deficiencies : Df(2L)VA = Deficiency of the left (L) arm of chromosome 2 that includes the gene Venea abnonneis (VA).
- Transpositions : Tp (l; 3) HF308 = Transposition involving the X and 3 rd chromosome.
- Inversions : In (2LR) SMC8 = Inversion of the left (L) and right (R) arm of the 2nd chromosome. (SMC–structural maintenanace chromosome)

- Translocations : T(1; 3) Thl = Translocation between the 1st (X) chromosome and 3rd chromosome commas follow rearrangements and indicate mutations present (Th–Transloction heterozygotes)

  e.g. In(2LR)SM1, al^{2}Cy cn^{2}sp$_2$ = Inversion involving the left (L) and right (R) arms of chromosome 2 with the following mutations present: aristaless (al), Curly (Cy), cinnabar (cn), and speck (sp).

1.9 PROBLEMS OF GENETICS CROSS

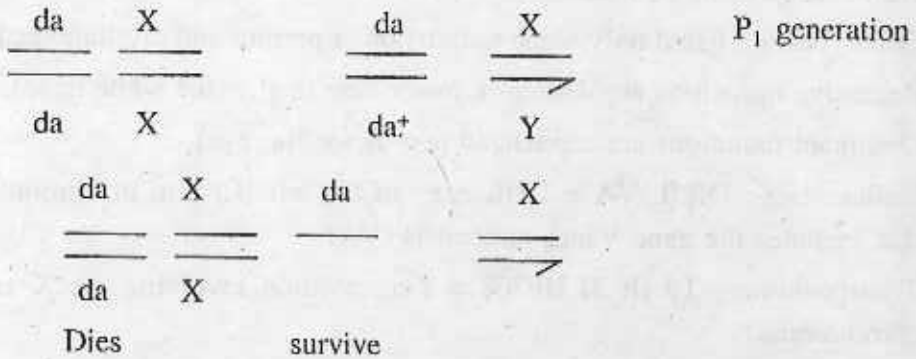
1.9.1 Sample 1

You are provided with a culture vial containing fruit flies. Predict the genotype from the following offspring of the given flies. Test statistically to prove their expected ratio.

Total no flies = 9 Male flies = 9 Female flies =0

Analysis

The observation is that the cross-resulted from the parental progeny yield only male flies. This result indicates that probably a lethality arises in case of female zygote which causes death of all female flies. This may happen if we consider a mutation known as "da" which in homozygous condition causes daughterless progeny. Here probably the female parent was homozygous for the mutation "da" and the male parent mayor may not contain "da" mutation, Daughterless is recessive autosomal mutation located on second chromosome. The probable genotype of the parent is



Statistical Hypothesis

Null Hypothesis : Only male progeny produced (l 00%). Hence the resultant progeny in this case is only 9 male and no females. This is consistent with Null hypothesis. Hence the calculation for chi-square test is not required.

1.9.2 Sample 2

Culture vial containing flies

Total number of flies 20 Curly 14 Normal 6

Analysis

The observation indicates that the appearance of progeny Curly: normal =14 : 6 (approx 2 : 1). In this case probably the parental female & male were heterozygous for the second chromosomal dominant autosomal gene curly. Curly in homozygous condition is lethal.

Cy // Cy$^+$ × Cy // Cy$^+$ Probable genotype of the parent P_1

Cy // Cy Cy // Cy$^+$ Cy // Cy$^+$ Cy$^+$ // Cy$^+$

Dies Curly Curly Normal

Curly: Normal (2: 1)

Statistical analysis

The cross resulted in appearance of progeny curly and normal in the ratio 2 : 1 hence, we have to fill the observed data with 2 : 1 ratio to justify the assumption of the genotype. The evaluation may be made by the χ^2 test for goodness of fit.

Calculation of χ^2

Here the hypothesized ratio for normal : curly is 1 : 2 and tlle formula of χ^2 test is

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

Calculation

	Curly	Normal	Total
Observed (o)	14	6	20
Expected (E)	13.45	6.6	20
O – E	0.6	–0.6	
$(O - E)^2$	0.36	0.36	
$\frac{(O-E)^2}{E}$	0.027	0.055	
$\chi^2 = \sum \frac{(O-E)^2}{E}$		0,027 + 0.055 = 0.082	

Degree of freedom 2-1= 1

Interpretation : At 5% level of significance for df value 1, the tabulated X^2 value (0.082) as obtained here was far below the table value at 5% level of significance which represent a P value greater than 0.05. Hence, the deviation between the observed and expected values may be considered insignificant. Therefore, the hypothesized ratio of curly : normal (2 : 1) was accepted at 5% level of significance. In this condition it could be interpreted that the genotype assumed for the curly and normal flies were very much correct.

UNIT 2 ❐ PREPARATION OF POLYTENE CHROMOSOME

Structure

2.1 MATERIAL REQUIRED

1. Live third instar larvae of *Drosophila*
2. Forceps, needles & needle holders
3. Cavity block or watch glass
4. Slide and cover slip.
5. Binocular dissecting microscope
6. Compound microscope
7. Ringers solution [3.25gm NaCl, 0.17gm KCl, 0.06gm $CaCl_2$, 0.10 gm $NaHCO_3$. 0.005gm Na_2HPO_4 in 500ml of Distilled water, pH 7.2
8. Acetoorcein (2%) [2gm orcein dissolved in 50cc warm acetic acid and finally make it volume with another 50 cc acetic acid. Dissolve it completely & filter]
9. Acetomethauol (fixative) [1 : 3 v/v]
10. Lacto aceto orcein (Mounting medium)[in 2% aceto orcein add equal volume of lactic acid]
11. 45% Acetic acid (Destainer)
12. Nail polish

2.2 PROCEDURE

1. Take out third instar larva from *Drosophila* culture bottle and put them in a cavity block containing Ringers solution
2. Place a clean slide on the stage of a dissecting binocular against a dark background & put a few drop of Ringers solution on it.
3. Hold the back portion of the larva with a forceps. Place the point of a needle just behind the mouth hooks
4. Gradually pull the mouth hooks out of the larval body together with the attached structures.
5. Separate the salivary glands from other tissues and transfer to the Ringer's solution & finally transfer it to another slide.

2.3 STAINING AND FIXATION

1. Fix the salivary gland with a few drops of acetomethanol (1:3v/v)
2. Tilt down the fixative from the salivary gland and put 2-3 drops of acetoorcein stain on the fixed tissue. Cover the slide with petridish and leave in this condition for 10 minutes.
3. Wash the stained tissue in 45% acetic acid for 30-40 sec. Tilt the slide and soak the excess acetic acid with a blotting paper.
4. Pour one drop of acetolacto orcein and place one cover glass over the stained tissue and finally squash the stained salivary gland with the help of thumb or back portion of the rubber headed pencil
5. Some of the mounting reagent will ooze out around the coverslip can be blotted by using blotting paper
6. Seal the edge of the slide with nail polish and the preparation is now ready for observation under microscope.

2.4 OBSERVATION

The polytene chromosome have five radiating arms (Fig 5). These are marked as

X, 2R & 2L, 3R & 3L. The chromosome show alternating dark and light bands. All the Centromere of the chromosome are fused to form chromocenter

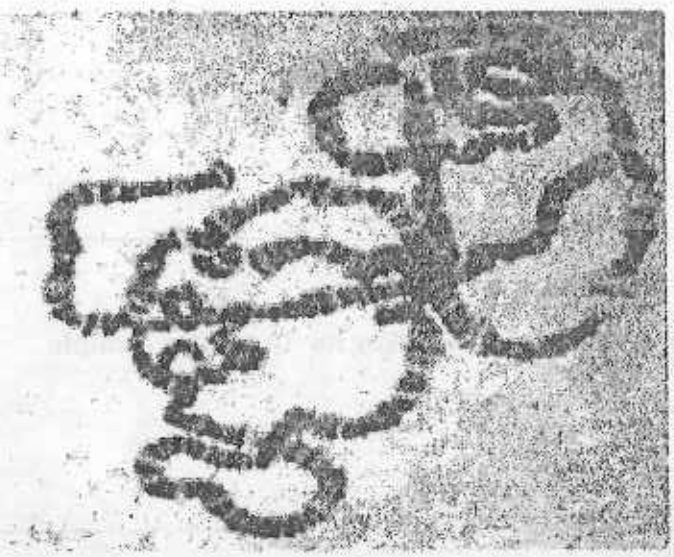


Fig. 5 : Giant polytene chromosome; squash preparation of salivary glands of *Drosophila melanogaster*

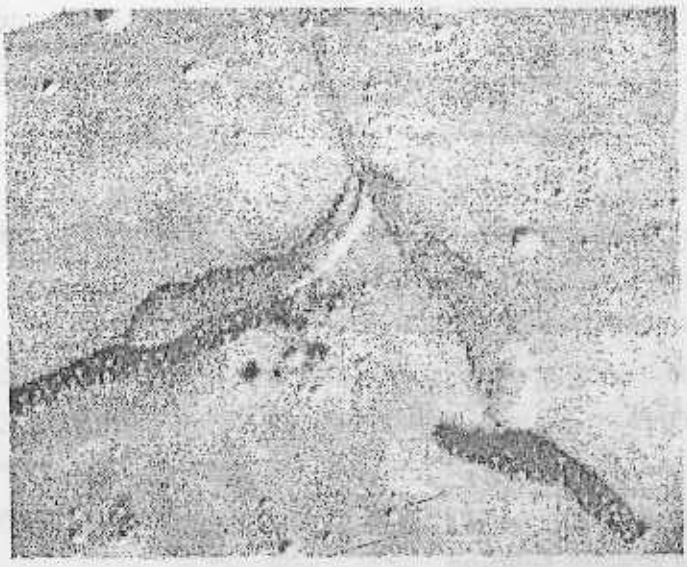


Fig.6 : A pair of dissected salivary gland of *Drosophila sp.*

UNIT 3 ❐ PROTEIN ESTIMATION

Structure

3.1 PRINCIPLE

The method consists of measuring the colour produced by the reaction of peptide bonds with alkaline copper tartrate and between phenolic groups of proteins and the Folin-Ciocalteau reagent. The colour produced has a broad absorbance maximum around 660nm, the wavelength at which this colour is measured.

3.2 REAGENTS

1. ***Folin-Ciocalteau reagent***

Commercially available reagent is freshly diluted (1:1) with equal volume of distilled water before use

2. ***Alkaline copper tartrate solution***

This is prepared fresh by mixing solution A and B in the ratio of 1 : 100

Solution A – 0.3 gm% solution of copper sulphate ($CuSO_4$, $5H_2O$) in 1gm% solution of sodium potassium tartarte (mix in equal volume on the day of use).

Solution B – 2 gm% solution of a sodium carbonate in 0.1N sodium hydroxide.

3. ***Standard protein solution***

This is prepared as a 10-mg/ml solution of Bovine serum albumin

3.3 PROCEDURE

1. 1 ml of protein solution is treated with 5.0 ml of alkaline tartrate solution (Solution 2)and shaken vigorously on a vortex mixer.
2. After exactly 15 min 0.5 ml of the Folin Ciocalteau reagent (solution 1) is added after dilution and the mixture is shaken thoroughly for 30 minutes
3. The optical density of the mixture is measured at 660 nm in a colorimeter,
4. A standard curve is prepared by developing colour in the similar way where solutions containing graded concentration of protein (10 μg to 100 μg).
5. The obtained optical density is then plotted against the protein concentrations determined as above is used to express all values as a function of the protein content.

3.4 ESTIMATION OF KNOWN (STANDARD) PROTEIN

Different concentrations of working solution of BSA were pipetted out into series of test tubes taking at least 3 replica for each concentrations. Then different reagents were mixed step by step as described in the following table :

Conc. of BSA (μg/ml)	*Sample BSA (ml)*	*0.1 N NaOH (ml)*	*Alkaline Copper solution (ml)*	*Folin-Ciocalteu solution (ml)*
0	Blank	1.0	5	0.5
10	0.1	0.9	5	0.5
20	0.2	08	5	0.5
30	0.3	0.7	5	0.5
40	0.4	0.6	5	0.5
50	0.5	0.5	5	0.5
60	0.6	0.4	5	0.5
70	0.7	0.3	5	0.5
80	0.8	0.2	5	0.5
90	0.9	0.1	5	0.5
100	1.0	0	5	0.5

After giving the different concentrations of sample BSA solution in each test tube, 0.1 N NaOH was added so that the total volume of each sample would be 1 ml. After mixing alkaline copper solution and Folin-Ciocalteu solution, the test tubes were shaken well till a bluish colour would appear in the solution. Then the tubes were allowed to incubate at room temperature in dark for at least 30 minutes. The sample contain ing no BSA was taken as the "blank" solution.

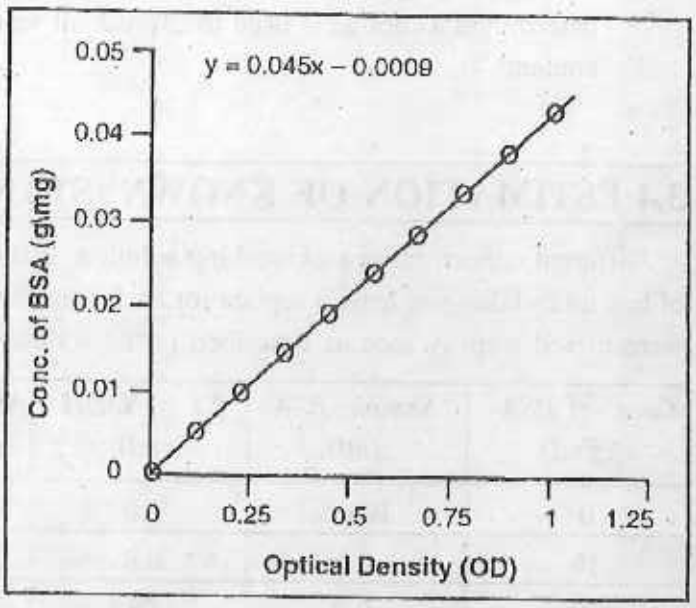
3.5 ESTIMATION OF UNKNOWN PROTEIN

0.1 to 0.5 ml of tissue samples (aliquote) were pipetted out in test tubes keeping at least 3 replica. Then the other steps were followed as described in case of known protein sample.

Reading : The extinction **(Optical Density– OD)** was read at **750 nm** against suitable blank in a spectrophotometer (Double-Beam UV-180 Spectrophotometer, Shimadzu, Japan).

Preparation of standard curve :

At least 3 replicated readings were taken for each concentration of BSA and the mean OD values were plotted against different concentrations of the standard samples prepared (10 µg/ml – 100 µg/ml). A liner curve was obtained which represented the standard curve.



3.6 CALCULATION OF AMOUNT OF PROTEIN FOR UNKNOWN SAMPLE

The concentration (µg/ml) of unknown protein was measured against the standard curve. The amount of protein was calculated as follows :

$$\text{Amount of protein} = \frac{\text{Amount of NaCl} \times \text{Cone. of protein} \times 10}{\text{Sample amount} \times \text{Weight of tissues}} \text{ mg/gm}$$

Sample amount = 0.1-0.5 ml of homogenised tissue solution

UNIT 4 ❐ DETERMINATION OF SPECIFIC ACTIVITY OF ENZYME

Structure

4.0 INTRODUCTION

Enzymes are usually protein that catalyzes a variety of reactions in the biological systems. All the enzymes that are found in living tissue are classified in to major six groups.

A. Oxidoreductase
B. Transferase
C. Hydrolases
D. Lyases
E. Isomerase
F. Ligases

The experiment below describes a few experiments to illustrate the general approach employed for optimizing conditions for assaying activities of enzymes.To study the specific activities of enzyme we select alkaline phosphatase.

4.1 PRINCIPLE

Phosphatase is a broad term used for non-specific phosphomonoestreases, which hydrolyse phosphate ester and thus liberating alcohol derivative of the substrate molecule and inorganic phosphate. Depending on their pH activities this enzymes are of two types, Acid phosphatase (pH 4.0-5.5) and alkaline phosphatase (pH 8-10). For assaying phosphatase enzyme.

Nitrophenyle phosphate can be used as substrate.

4.2 MATERIALS & METHODS

1. Water bath 37°C
2. Colorimeter
3. Glycine NaOH buffer (0.05M, pH 10.5). Dissolved 375 gm glycine in small amount of distilled water then add 42 ml 0.1N NaOH and adjust pH to 10.5
4. NaOH solution (0.085N)
5. $MgCl_2$ solution (10.5mM)
6. ρ nitrophenyle phosphate (35mM). Take 38.8 mg of ρ nitrophenyle phosphate and dissolve it in 5 ml of 0.05 M glycine NaOH buffer, pH 10.5
7. Standard solution of ρ nitrophenyle phosphate (100 mM)
8. Material 5days old germinating seed

4.3 PROCEDURE

1. All the operation must be carried out in cold atmosphere (inside ice bucket). With the help of mortar & pestle grind the germinating seed in presence of chilled glycine NaOH buffer.
2. Centrifuge the homogenate in a refrigerated centrifuge at 10,000 g for 20 min. Decant supernatent and use it as a source of enzyme.
3. Take nine numbered test tubes and add 3.0ml of glycine NaOH buffer, 0.1ml of $MgCl_2$ and 0.3 ml of the enzyme preparation in each tube.
4. Incubate all these tubes in 37°C. After 3 mn start the reaction in seven tubes by adding 0.1 ml ρ nitrophenyle phosphate.
5. Exactly after 5,10,15,20 mn stop the reaction by adding 9.5 ml of 0.085N NaOH.
6. Only one tube receive only P nitrophenyle phosphate and 0.085N NaOH that serves as control and one tube receive 0.1 ml of 0.05M glycine NaOH instead of ρ nitrophenyle phosphate that serves as reagent blank.
7. Adjust colorimeter at 410nm to 100% transmission with the reagent blank and record absorbance of the other tubes.
8. To prepare standard curve take 0-1 ml (0-100mmoles) of ρ nitrophenyle phosphate, Add 3ml of glycine NaOH buffer to all the tubes and make the final volume to 3.5ml with distilled water.
9. Plot a graph of A_{410} vs. μ moles of ρ nitrophenol to obtain a standard curve.
10. From the standard curve determine the amount of ρ nitrophenol present in other tubes.

UNIT 5 ❐ GENOMIC DNA EXTRACTION AND EVALUATION

Structure

5.0 INTRODUCTION

DNA isolation is the basic requirement for any molecular biology work and thus plays a very important role in any molecular biology techniques like PCR, genotyping, sequence analysis etc.

5.1 PRINCIPLE

Blood contains various components, but DNA is present only in the WBC. Firstly all the rest of the components are separated and removed by centrifugation, which is then followed by breaking down the WBC and release of all the various cellular components from the WBC. The proteins are degraded by the enzymatic activity of Proteinase K and DNA purification is facilitated by the treatment of Phenol and chloroform. DNA is alcohol precipated and then is dissolved in the Tris- EDTA (TE) buffer for further analysis.

5.2 REQUIREMENT

- 15 ml and 50 ml plastic tubes
- Lysis buffer :
  320 mM Sucrose
  10 mM Tris- HCl pH 7.5
  5 mM $MgCl_2$
  1 % Triton X-100

- Digestion buffer :
  10 mM Tris-HCI pH 8.0
  110 mM EDTA
  100 mM NaCl
  2% SDS
- Proteinase K (20 mg/ml)
- 1 x TE buffer :
  10 mM Tris-HCI pH 7.5
  10 mM EDTA
- Saturated Phenol
- Chloroform—Isoamyl alcohol (96 : 4)
- Chilled absolute alcohol
- 70% alcohol
- 3M-sodium acetate pH 7.0
- Water bath & centrifuge

5.3 PROCEDURE

1. Take 5 ml blood and add 7ml of lysis buffer to it. Mix it on ice for 2-3 mins.
2. Centrifuge at 1300g for 15 nuns at 4°C.
3. Decant the supernatant
4. Add 10ml of lysis buffer and mix on ice for 2-3 mins.
5. Centrifuge at 1300 g for 15 min at 4°C.
6. Decant the supernatant and add 4ml lysis buffer to it
7. Mix on ice for few mins and then centrifuge at 1300g for 15 min at 4°C
8. Throw away the lysis buffer and add 1200ml digestion buffer
9. Add 20ml Proteinase-K (20mg/ml) and then incubate in water bath at 56°C over night or at least 3-4 hours.
10. Centrifuge for 2 mins to get the precipitate in the bottom of the tube.
11. Add 600 ml phenol and 600 ml chloroform —isoamyl alcohol mixture.
12. Mix gently for about 2 min and centrifuges at 1500g for 5min at 4°C.
13. Remove the supernatant and repeat the above step.
14. Remove the supernatant and add 1200ml chloroform- iso-amylalcohol
15. Mix it gently and centrifuge at 1500g for 5 min at 4°C.

16. Remove the supernatant and add it to a tube containing 10 ml absolute alcohol and 400 ml of 3M sodium acetate pH 7.0.
17. Mix by hand inversion and centrifuge at 8000g for 20 min at 4°C.
18. Remove the alcohol carefully, add 70% alcohol and centrifuge at 8000g for 15 nuns at 4°C.
19. Remove the alcohol and air dry
20. Dissolve in 200ml 1 × TE buffer pH 7.5 overnight
21. Take the photometric readings at 260 nm and 280 nm and check the purity of the sample. Also calculate the amount of DNA present.
22. It is also further subjected to gel electrophoresis for determining the size of the fragments.

5.4 RESULTS AND INTERPRETATION

- For quantitative and qualitative estimation of the DNA, reading should be taken at wavelengths of 260nm and 280nm.
- A solution containing 50 mg/ml of double stranded DNA has an absorbance of 1 at 260nm (A_{260} = 1= 50mg/ml of double stranded DNA)
- The ratio between the reading at 260 nm and 280nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have OD_{260}/OD_{280} value 1.8. If there is contamination with protein or phenol, the OD_{260}/OD_{280} will be significantly less than the given above.

UNIT 6 ❐ ISOLATION OF DNA FROM GOAT LIVER

Structure

6.0 INTRODUCTION

The most common method of DNA isolation is phenol extraction. The fundamental aim of phenol extraction is the deproteinization of an aqueous solution containing the desired nucleic acids. In simple terms the phenol reagent is mixed with the sample under conditions, which favours the dissociation of proteins from nucleic acids.

6.1 SAMPLE COLLECTION

Liver from freshly sacrificed goat should be collected and placed within ice box.

6.2 MATERIAL REQUIRED

1. Centrifuge (10,000 rpm)
2. Tissue homogeniser
3. Autopipette
4. Test tubes
5. Vortex
6. Pasteur pipette

7. Cheesecloth
8. DNA spooling glass rod
9. Incubator
10. Chilled Ethanol, Phenol, isoamyl alcohol

6.3 REAGENT REQUIRED

- Buffer A : Sucrose 0.25M, $CaCl_2$ 0.001IM, Tris 0.05M homozenising buffer
- Buffer B : NaCl 0.15M, Na EDTA0.1M DNA extraction buffer
- SDS 10% solution
- Phenol (distilled pH 8.0)
- Chloroform
- Na Acetate 3M
- Proteinase K (200 µg/ml)

6.4 METHOD

1. Mince the liver tissue in to fine pieces
2. Homogenize gently in 2 volume homogenizing buffer in presence of ice
3. Filter the homogenate through cheesecloth. Take the filtrate in test tube
4. Centrifuge the filtrate at 4000 rpm for 15 min and discard the supernatant.
5. Take the nuclear pellet and resuspende in 10 volume of DNA extraction buffer.
6. Add SDS (1%) and proteinase K (200mg/ml), incubate at 65° C for 15-30 min.
7. After incubation add equal volume of saturated phenol and vortex very gently.
8. Centrifuge at 10,000 rpm for 10 min
9. Collect the aqueous layer and keep it in a test tube.
10. Add equal volume of phenol chloroform isoamyl alcohol (25 : 24 : 1 v/v) and shake gently for 15 min
11. Centrifuge at 10000 rpm for 10 min
12. Collect upper aqueous layer and repeat the process as in 10.
13. Add sodium acetate 0.3M and chilled ethanol
14. DNA will precipitate like a thread

15. Spool out DNA and keep it in micro centrifuge tube in deionized water or suitable buffer medium

6.5 REASONS FOR USING CHEMICALS FOR DNA ISOLATION

The following are the reasons for using chemicals for DNA isolation.

Sucrose–rupturing of cell membrane due to change in osmotic concentration

TRIS–Mgl Acetate- maintain ionic strength

SDS–detergent causes rupturing protein from nucleic acid

Phenol–Precipitate protein. Saturated phenol cause dehydration of DNA by -extracting Water

Chloroform–surface denaturation of protein remove lipid separate two layers aqueous and organic layer

Isoamyl alcohol–Prevent esterification of lipid

EDTA–Chelating agent of divalent cataion like Mg^{++} necessary for inactivation of DNAase

ProteinaseK–cuts two peptide bonds and remove proteins

Chilled ethanol–removes bound water from DNA results precipitation

UNIT 7 ❐ COLORIMETRIC ESTIMATION OF DNA

Structure

7.0 INTRODUCTION

This is the general reaction given by deoxypentoses. The deoxyriboses of DNA in the presence of acid is converted to w hydroxylevulinic aldehyde which reacts with diphenylemaine to form a blue coloured complex with absorbance maxima at 600 mm. Compounds such as furfuryl alcohol and arabinol, which can be converted in to w hydroxylevulinic aldehyde will also give this reaction .. In DNA since only deoxyribose of purine uucleotides is released, the value obtained represents only half of the total deoxyribose in the sample. The reactions leading to the formation of the coloured complex are as follows

$$\text{2-deoxy-D-ribose} \xrightarrow{HClO_4} \omega \text{ hydroxylevulinic aldehyde}$$

$$\text{w hydroxylevulinic aldehyde} \longrightarrow \text{Diphenylamine (Blue colour complex)}$$

7.1 MATERILAS AND REAGENTS

1. **Saturated DNA solution** : Dissolve calf thymus DNA (100mg/ml) in 1N $HClO_4$ by heating at 70°C for 15 min. Make different dilutions of this stock solution ranging from 20-100mg DNA/ml using 0.5 $HClO_4$
2. **1.6% (w/v) acetaldehyde :** Prepare by dissolving lml ice cold acetaldehyde in 50 ml distilled water
3. **Diphenylamine solution :** Dissolve 1.5g Diphenylamine in 100ml glacial acetic acid and 1.5 ml concentration H_2SO_4
4. **Diphenylamine reagent :** Prepare by mixing 0.5ml 1.6% of acetaldehyde and 100ml diphenylamine solutions. This solution must he prepared fresh.
5. Colorimeter or spectrophotometer

7.2 PROCEDURE

1. Take 2.0 ml sample from which DNA has to be estimated in a test tube.
2. In another set of test tubes, pipette 2.0ml of standard DNA solution of different dilutions. In one of the test tubes take 2.0ml of 0.5 N $HClO_4$ as reagent blank.
3 Add 4.0ml diphenyleamine reagent (reagent no 4) to all the tubes, mix the contents properly and keep at room temperature in dark for 16-18hr or overnight. Altematively keep the tubes in boiling water for 10 min and cool them under running tap water.
4. Record the absorbance at 600nm in colorimeter.
5. Draw a standard curve of A_{600} vs DNA concentration. Form absorbance of the sample determines the amount of DNA in it. Express the results as mg of DNA/ gm of tissue.

UNIT 8 ❐ COLORIMETRIC ESTIMATION OF RNA

Structure

8.0 INTRODUCTION

This is the general method for estimation of pentoses. Acid hydrolysis of RNA releases ribose, which in the presence of strong acid undergoes dehydration to yield furfural. Orcinol in the presence of ferric chloride as a catalyst reacts with furfural producing a green coloured compound with absorbance maxima at 665nm. DNA gives a limited positive reaction with orcinol test. The reactions leading to the formation of a green coloured complex are as follows.

$$\text{Ribose} \xrightarrow{\text{Acid}} \text{Furfural} \xrightarrow{\text{Orcinol}} \text{Green coloured compound}$$

8.1 MATERIALS AND REAGENTS

1. Boiling water bath
2. 5% $HClO_4$
3. **Standard RNA solution :** Dissolve yeast RNA (500mgRNA/ml) with 5% $HClO_4$ Make different dilution to obtain solutions containing 100-500mg RNA/ml with 5% $HClO_4$
4. **Orcinol reagent :** Dissolve 100mg of ferric chloride ($FeCl_3$ $6H_2O$) in 100 ml of concentrated HCl and then add 3.5 ml of 6% solution of orcinol prepared in alcohol
5. Colorimeter or spectrophotometer

মানুষের জ্ঞান ও ভাবকে বইয়ের মধ্যে সঞ্চিত করিবার যে একটা প্রচুর সুবিধা আছে, সে কথা কেহই অস্বীকার করিতে পারে না। কিন্তু সেই সুবিধার দ্বারা মনের স্বাভাবিক শক্তিকে একেবারে আচ্ছন্ন করিয়া ফেলিলে বুদ্ধিকে বাবু করিয়া তোলা হয়।

— রবীন্দ্রনাথ ঠাকুর

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— Subhas Chandra Bose

Price : Rs. 150.00
(Not for Sale)

Published by Netaji Subhas Open University, 1, Woodburn Park, Kolkata-700 020 & printed at Printtech, 15A, Ambika Mukherjee Road, Kolkata-700 056