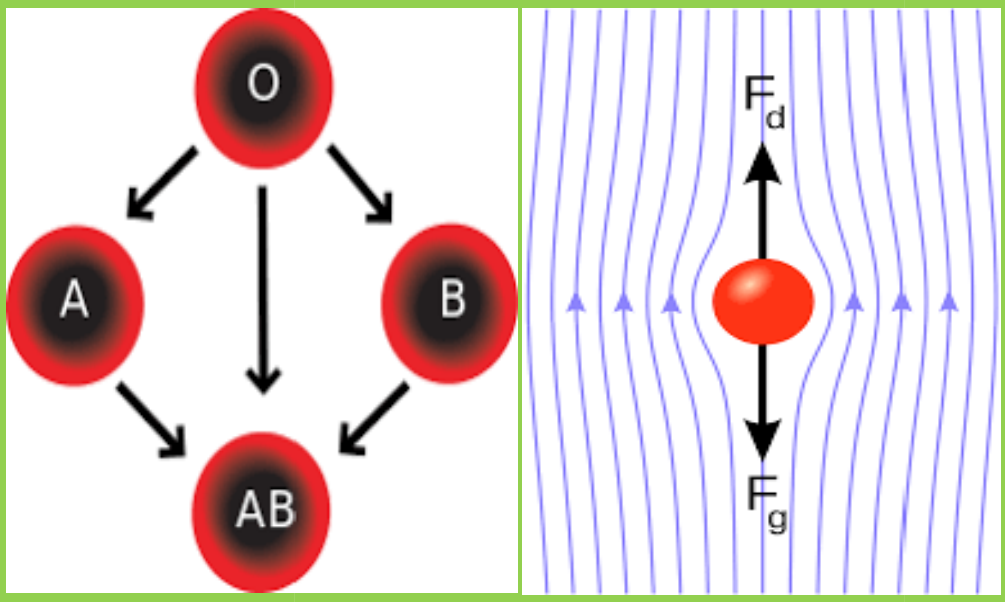
BSCZO-304

B. Sc. III YEAR
LABORATORY COURSE



DEPARTMENT OF ZOOLOGY
SCHOOL OF SCIENCES
UTTARAKHAND OPEN UNIVERSIT

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SCHOOL OF SCIENCES

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CONTENTS

COURSE 12: LABORATORY COURSE

COURSE CODE: BSCZO304 **CREDIT: 3**

UNIT 1: HEMATOLOGY EXERCISE

Content

1.1 Estimation of hemoglobin, % RBC & WBC and preparation of Haematin crystal.

1.2 Estimation of presence of sugar and albumin in a sample of human urine.

EXPERIMENT NO. – 01

Objective To estimate the amount of hemoglobin in own blood group.

Requirements

a) Haemometer (Haldane's hemoglobin meter)

b) Decinormal (N/10) HCL (1.2 ml of conc. HCl dissolved in 100 ml of distilled water)

c) Micropipette (20 µcm)

d) Small glass rod stirrer

e) Small bottle brush and a dropper

f) N/10 HCL solution

g) Distilled water.

Apparatus

The haemometer consists of two sealed lateral comparison tubes containing a suspension of acid haematin. These are held in a black frame against a white ground glass. Besides, a graduated test –tube of the same diameter is also provided which can fit in the haemometer in between the two side tubes for comparison. A micropipette of 20 µcm is also provided. The other things provided are a small glass rod stirrer, a small bottle brush and a dropper and also a small bottle to contain the decinormal acid solution.

PRINCIPLE

The method to estimate the hemoglobin content of blood is based on the principle of making an acid haematin solution of blood under experimentation in the graduated tube and then comparing it with the sealed comparison tubes containing the standard acid haematin.

PROCEDURE

- The graduated tube is first cleaned with distilled water and then with methylated spirit or 90% alcohol. It is thoroughly dried up before being used.
- Now with the help of a dropper the N/10 HCL solution is filled in the graduated tube up to 2 gm mark.
- The micropipette is now filled up by sucking fresh blood of the vertebrate under experimentation up to the mark of 20µcm.
- The small amount of blood adhering to the outside of micropipette should be wiped off by sterilized cotton.
- The blood of micropipette is now added to the N/10 Hcl solution in the graduated tube.
- The micropipette should be introduced carefully into the tube and its lower mouth should pass right up to the bottom into HCL solution.
- When blood has been expelled the micropipette is rinsed twice or thrice by distilled water
- Every time the contents of micropipette should be expelled into the graduated tube.
- The acid haematin solution is now thoroughly stirred with the help of a glass rod and then allowed to stand at least for 10minutes.
- Afterwards the acid haematin solution is gradually diluted by adding distilled water by drop wise manner.
- With the addition of each drop of distilled water the solution should be stirred and its colour matched with that of the standard sealed tubes.
- This should be continued till the colour of the acid heamatin solution just fades away as compared to that of the standard comparison tubes.
- The reading before the colour just fades is taken as the correct and final reading.

Observations

The readings are tabulated in the following manner:

Readings	Human blood
1. 2. 3.	12.40 12.60 12.50
Final	12.50

Results

The blood of man contains 12.50gm. Of hemoglobin in 100ml. of blood

Precautions

The followings precautions are to be taken:

1. In case human blood is being tested, give a light prick to the finger tip of the person providing blood.
2. The finger and the prick (needle) should be disinfected first with methylated sprit or alcohol (90% or absolute).
3. Avoid uncleaned tubes and pipettes, etc.
4. Avoid incorrect filling up of micropipettes.
5. Avoid inclusion of blood sticking at the outer surface of the mouth of pipette.
6. Reading should be taken always of the upper meniscus.
7. Experiment should be performed quickly without waste of time so that fresh blood is not allowed to coagulate before transfer to decinormal HCl.

Experiment No. – 02

Objective

To determine the number of Red Blood Cells (R.B.C.) present in one cubic mm volume of blood.

Requirements

a) Haemocytometer,
b) Hayem's solution,
c) Microscope
d) Blood.

Apparatus

The haemocytometer includes two graduated micropipettes in which dilution of blood is done. One micropipette with a red bead is used for counting R.B.C.s, while the other with a white bead for counting W.B.Cs, Each micropipette bears at least 3 graduations; those on the micropipette for R.B.Cs are 0.5,1 and 101. The graduations on the micropipette for W.B.Cs are 0.5, 1 and 11. Besides these micropipettes the haemocytometer includes a glass slides; 7.5cm long by 3.75cm wide. It bears two counting chambers with a cover slip. One side of the slide bears two central platforms bordered by an edged-shaped groove .Beyond this groove on either side is a lateral platform further demarcated by a lateral groove. Each central platform bears one counting chamber, each counting chamber is formed by a several straight perpendicular and horizontal lines enclosing squares of various measurements; the biggest square is 3x3 mm with an area of 9sq.mm. It is divisible into 9sq.mm. It is divisible into 9squares of 1x1mm each, the central squares is again divisible into 25 smaller squares. Each squares measures 1/5x1/5=1/25sq.mm. Each of these squares is again divisible into 16 smaller squares so that there are 400 smallest squares in all. Each with an area of 1/400 sq.mm.

Hayem's solution has the followings composition:

1. Mercuric chloride (HgC_{l2}) = 0.5gm
2. Sodium chloride (NaCl) = 1.0gm
3. Sodium sulphate (Na_2So_4) = 5.0gm
4. Distilled water(H_2O) = 200ml
5. The mercuric chloride acts as corrosive sublimate and fixes the R.B.Cs, present in the blood. The other ingredients act isotonically so that the R.B.C.s may not burst due to haemolysis. The Hayem's solution also serves to dilute the blood.

Procedure

- Blood is obtained either directly from the heart of a frog or from some body part externally in case of man. Human blood is generally taken out from a finger (avoid thumb and first finger) which is thoroughly washed and cleaned by spirit or alcohol (absolute or 90%).
- It is now pricked by an ordinary injection needle which is also sterilized with sprit and dried before use. The finger is pricked quickly and effectively .
- It should not be pressed hard to let out blood so as to avoid other body juices also oozing out.
- For this reason also the first one or two drops of oozing blood should be avoided and wiped off
- Now take the micropipette meant for R.B.C.s which is already rinsed with alcohol or spirit or either and thoroughly dried.
- Suck the blood in the micropipette up to 0.5mark taking care that air bubbles are not included. The excess of blood, if any, may be run out by touching the mouth of micropipette to the palm.
- The blood which is sticking to the outer side of micropipette should also be carefully cleaned.
- The micropipette should now be transferred to the container of Hayem's solution which is carefully sucked up to 101mark.

- The micropipette is now held horizontally between the fore-finger and thumb or palm surfaces of the hand and rotated several times so that blood thoroughly mixes with Hayem's fluid.
- The red bead in the micropipette also helps in mixing. In this way dilution of blood becomes 200 times. Before starting to count R.B.C.s in this diluted blood, place the cover-slip on the counting chambers.
- The cover-slip is supported upon the side platform but remains separated from the central platforms by a distance of 0.1 mm.
- First reject 3 or 4 drops of mixture from the micropipette. Now apply the tip of the micropipette between the cover –slip and the counting chambers.
- If necessary both chambers may be filled in this manner.
- Blood mixture remains filled up between the cover-slip and the counting chambers because of capillary action.
- Air should not be taken into and also pouring excess blood mixture so that the H-shaped groove remains free from it.
- When the counting chambers are properly flooded the slide may be kept aside for a few minutes so that the R.B.Cs settled down on the bottom floor of the two counting chambers.
- Now transfer the slide gently and carefully under the microscope without disturbing the settled R.B.Cs and starts counting them.

Counting

It is not necessary to count the R.B.Cs in all the 400 smallest squares or even in the 25 smaller squares, count them only in five smaller squares' i.e., in the 1^{st}, 5^{th}, 13^{th}, 21^{st}, 25^{th}. The R.B.Cs lying on the lower and right sides of a square are to be added in the total, while those lying on the upper and left sides are to be rejected.

Calculations

The calculations may be done by the following methods:

- Number of R.B.C.s per cubic mm= Number of cells counted x dilution x 4000/Number of small squares counted.
- Suppose total R.B.Cs for 5 smaller squares are equal to A+B+C+D+E.
  Therefore, Total R.B.Cs in 80 smallest squares = A+B+C+D+E.

Since, R.B.Cs in one smallest squares = A+B+C+D+E/80

Since, R.B.Cs in 400 smallest squares = 5(A+B+C+D+E)

But the height of blood film = 0.1

Dilution of blood = 200 times

So that one cubic mm of blood will contain = 200x10x5(A+B+C+D+E) R.B.Cs

= 10,000(A+B+C+D+E) R.B.Cs.

Thus, in order to avoid the detailed calculations to get quick results four zeros are added to the total R.B.C.s, of 5 small squares (80 smallest squares).

The readings are tabulated in the following table

Frog's blood

Smaller squares	R.B.C.s in 1 chamber	R.B.C.s in 2 chamber
A	**25**	**15**
B	**30**	**20**
C	**15**	**18**
D	**20**	**25**
E	**22**	**20**

Human Blood

Smaller squares	R.B.C.s in 1 chamber	R.B.C.s in 2 chamber
A	**83**	**105**
B	**87**	**97**
C	**74**	**79**
D	**96**	**86**
E	**98**	**83**

	Frog	Human
Total R.B.C.s	**112+98**	**438+450**
Average	**112+98/2=105**	**438+450/2=444**

Results

The results are given as follows:

1cubic mm of frog's blood contains 10, 50,000 R.B.C.s.

1cubic mm of human blood contains 44, 40,000 R.B.C.s.

Precautions

The followings precautions are to be taken:

1. In case human blood is being tested, give a light prick to the finger tip of the person providing blood.
2. The finger and the prick (needle) should be disinfected first with methylated sprit or alcohol (90% or absolute).
3. Avoid un cleaned tubes and pipettes, etc.
4. Avoid incorrect filling up of micropipettes.
5. Avoid inclusion of blood sticking at the outer surface of the mouth of pipette.

Experiment No. – 03

Objective

To determine the number of White Blood Cells (W.B.Cs.) per cubic mm in the human blood.

Requirements

a) Haemocytometer,

b) Glacial acetic acid

c) Solution of gentian violet in water or distilled water.

d) The glacial acetic acid haemolyses the red cells,

e) While the gentian violet slightly stains the nuclei of white blood corpuscles (W.B.Cs), the W.B.Cs may therefore be easily recognized.

Procedure

The procedure of counting of W.B.Cs is the same as that of the R.B.Cs.

The W.B.Cs is counted in the four corners of 1 square millimeter in the central ruled area on both the sides of the counting chambers of the haemoytometer.

The W.B.Cs is recognized by the retractile appearance and by the slight colour given to them by the cells touching the boundary lines are not counted.

Calculations - The calculations may be done by the following method:

Number of W.B.Cs per cubic mm = Number of cells counted × dilution ×10 / Number of 1sq. mm counted

Since the dilution is 20 times and the cubic capacity of the area of counted is 1/10 cubic millimeter, the total volume is 1/200 cubic millimeter. Say for instance, the number of W. B.Cs in 4 outer squares is (10+12+13+10) = 45.

In other words number of W.B.Cs in 1/200 cubic millimeter = 45.

Therefore, the number of W.B.C.s in 1 cubic millimeter = 45 × 200 × 1/2

= 4500W.B.Cs

Result

1 cubic mm of human blood contains 4500W.B.Cs

Precautions

The followings precautions are to be taken:

1. In case human blood is being tested, give a light prick to the finger tip of the person providing blood.
2. The finger and the prick (needle) should be disinfected first with methylated sprit or alcohol (90% or absolute).
3. Avoid uncleaned tubes and pipettes, etc.
4. Avoid incorrect filling up of micropipettes.
5. Avoid inclusion of blood sticking at the outer surface of the mouth of pipette.

EXPERIMENT NO. – 04

Objective

To prepare Haematin crystals.

Requirements

a) Sterilized needle

b) Glass slide

c) Cover glass

d) Acetic acid (glacial)

e) Spirit lamp

Principle

Hemoglobin imparts a red colour to blood, it is a conjugated protein made of a non- protein pigment **haematin** or haeme, and a colourless protein known as **Globin.** The haematin is made of a pigment called porphyrin which is combined with iron. A hydrochloride of haematin forms **haemin.**

Procedure

- The some dried blood or a few drops of fresh blood , add a little glacial acetic acid and heat gently , on cooling dark brown haemin crystals (also known as Teichmann's crystals) are formed.
- Examine haemin crystals under a microscope; they appear as shining rhombic plates, prisms and star – shaped clusters.
- These crystals contain haematin and iron.
- Haematin crystals test is used in medico- legal work to differentiate fresh or dried blood marks from any other red colouring material.

Precaution

In the case of old dried blood it is necessary to boil with glacial acetic acid , and at boiling to add a crystals of sodium chloride .

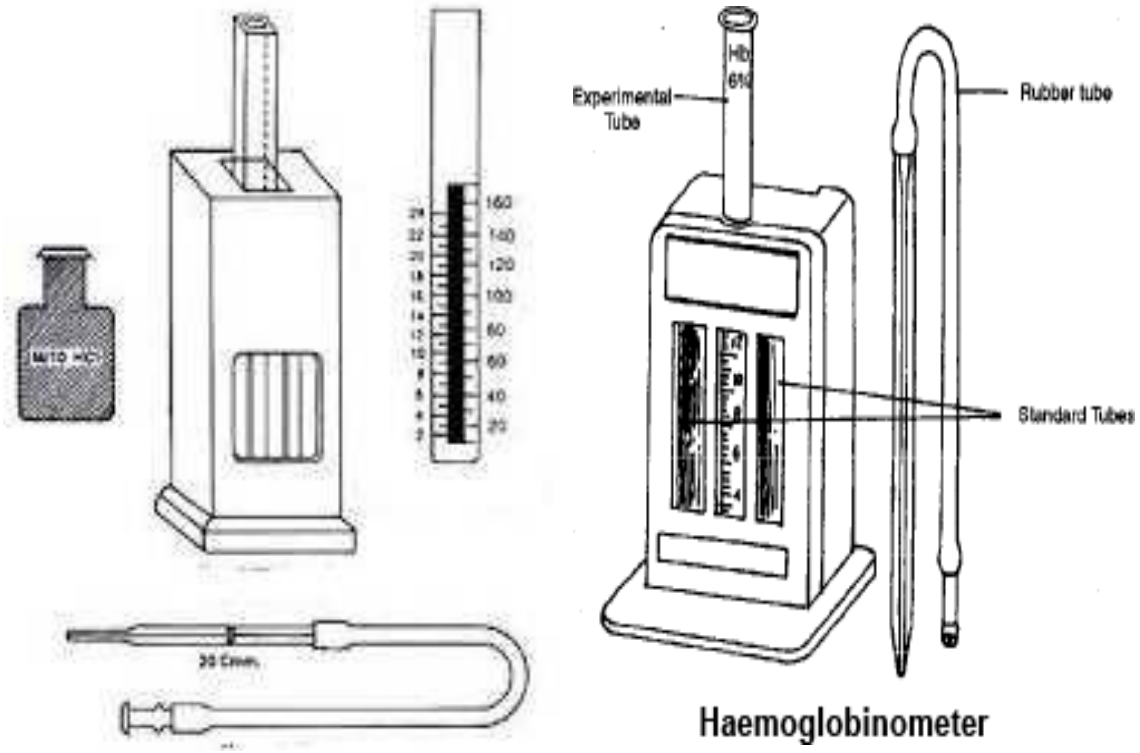

Figure 1.1 Haemoglobinometer

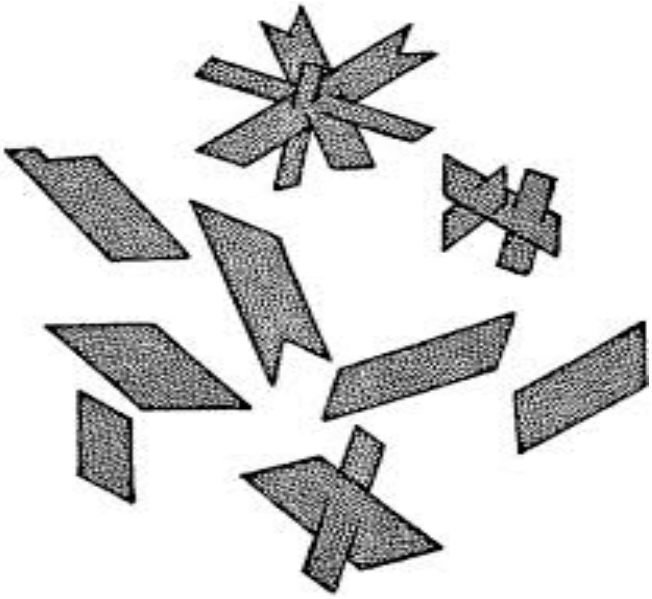

Figure 1.2 Hemin Crystals

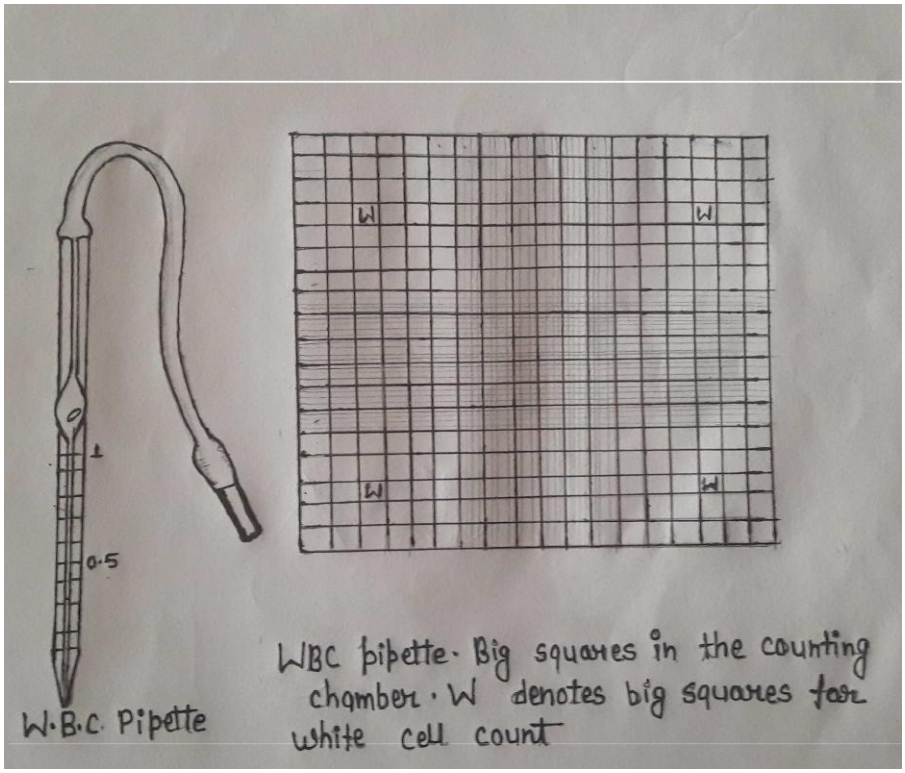

Figure 1.3 WBC Pipette

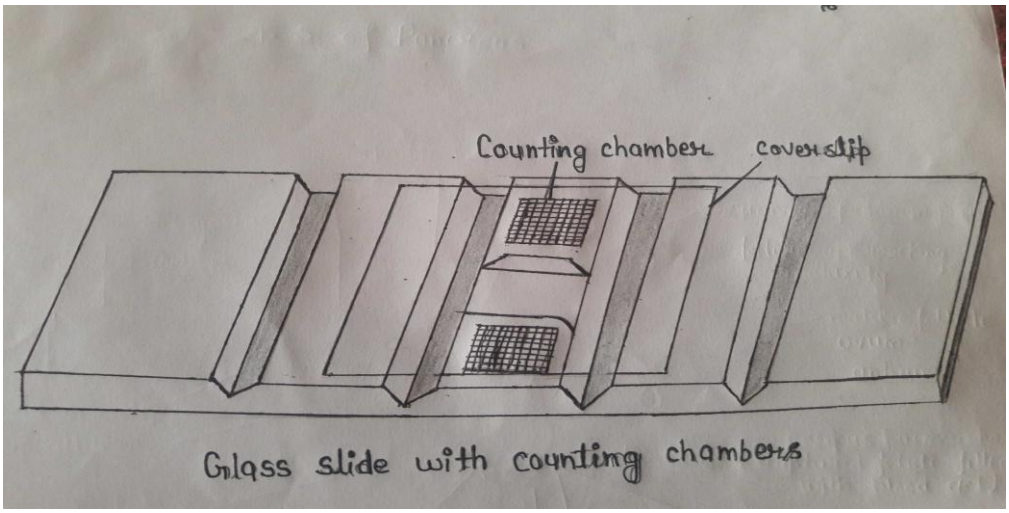

Figure 1.4 Counting Chamber

Questions & Answer related Experiment:

Q1**. What is a hemoglobin test?**

Answer - A hemoglobin test measures the levels of hemoglobin in your blood. Hemoglobin is a protein in your red blood cells that carries oxygen from your lungs to the rest of your body. If your hemoglobin levels are abnormal, it may be a sign that you have a blood disorder.

Q2. **What is it used for?**

Answer - A hemoglobin test is often used to check for anemia, a condition in which your body has fewer red blood cells than normal.

Q3. **Why do I need a hemoglobin test?**

Answer - Your health care provider may have ordered the test as part of a routine exam, or if you have:

a- Symptoms of anemia, which include weakness, dizziness, pale skin, and cold hands and feet.
b- A family history of thalassemia, sickle cell anemia or other inherited blood disorder.
c- A diet low in iron and minerals.

Q4. **Are there any risks to the test?**

Answer- There is very little risk to having a blood test. You may have slight pain or bruising at the spot where the needle was put in, but most symptoms usually go away quickly.

Q5. **What do the results mean?**

Answer- There are many reasons your hemoglobin levels may be outside the normal range.

Low hemoglobin levels may be a sign of:

- Different types of anemia
- Thalassemia
- Iron deficiency
- Liver disease
- Cancer and other diseases

High hemoglobin levels may be a sign of

- Lung disease
- Heart disease
- Polycythemia vera, a disorder in which your body makes too many red blood cells. It can cause headaches, fatigue, and shortness of breath.

If any of your levels are abnormal, it does not necessarily indicate a medical problem needing treatment. Diet, activity level, medications, a women's menstrual cycle, and other considerations can affect the results. In addition, you may have higher than normal hemoglobin if you live in a high altitude area. Talk to your health care provider to learn what your results mean.

REFERENCE

U.S. National Library of medicine (Medline Plus**)**

SUGGESTED READINGS

- Lal, S. S. (1998). A text book of practical zoology. vol. 3:1-250.
- U.S. National Library of medicine (Medline Plus)

Unit 2 Physiology Experiment

Content

2.1 Study of Microscopic structure of endocrine glands, thyroid, pancreas, ovary, testis, adrenal and pituitary.

2.2 Study of estrous cycle in mice/ rat.

Experiment No. 01

A) OBJECTIVE

Study of Microscopic structure of endocrine glands V.L.S. of anterior lobe of pituitary gland of a mammal.

Comments:

1) The pituitary gland is an endocrine gland of utmost important to organisms.
2) The pituitary gland is more or less glandular in shape and occurs at the base of brain in the region of diencephalon.
3) It is composed of 3 lobes they are- anterior lobe, intermediate lobe and posterior lobe.
4) The anterior lobe forms the largest part of pituitary gland
5) Usually on the outer side are the basophile cells which cells are stained by basic stains.
6) It is formed of three distinct kinds of cells differing in their staining reactions.
7) In the centre Acidophilus or oxyphil cells are found which taken stain with acid stain.
8) The third type of cells called Chomophode cells which are indifferent to either basic or acid stains. These cells are found scattered throughout the anterior lobe.
9) The anterior lobe produces many hormones which are somato tropic hormones, Thyroid hormone, Adrenocortico tropic hormones, gonadotrophic hormones and thus controls growth, development of sex glands as well as activities of thyroid, adrenal and parathyroid glands.
10) The intermediate lobe is composed of neuroglial cells and connected tissue fibres and blood vessels. It produces piyuitrin, vasopressin and oxytocin hormones.

B) OBJECTIVE

Study of microscopic structure of endocrine glands vs. of thyroid gland of a mammal

Comments:

1) Thyroid is an endocrine gland. The thyroid gland of mammals lies on the ventro – lateral surface of the larynx and the posterior portion of trachea.
2) It secretes thyroxin hormone which controls the entire metabolism of animals.
3) It is composed of two lobes which are connected by an isthumus.
4) Histologicaly it consists of an **outer fibrous capsule** and **thyroid follicles** which separated by **interfollicular tissue.**
5) The **fibrous capsule** is composed of **fibrous connective tissue** containing large blood vessels and surrounds the thyroid gland.
6) Each thyroid follicle is lined with simple cuboidal epithelium and the cuboidal epithelium cells contain large nuclei and pour their secretion into the cavity of the follicle.
7) The cavity of each follicle is filled with **colloid**.

C) T.S. OF ADRENAL GLAND OF MAMMALS

Comments:

1) The adrenal gland is a endocrine gland and located on the top of the kidneys.
2) Each adrenal gland has two parts **External adrenal cortex** and **internal adrenal medulla.**
3) The adrenal cortex is developed from the **mesoderm** of the embryo.
4) The adrenal cortex is sub divided into three zones which are

I. **Zona glomerulosa -** this is outer zone that lies just below the capsule.
II. **Zona fasciculate –**this is the middle zone which is widest of the three zones. The cells of this zone secrete mainly **glucocorticoids.**
III. **Zona reticularis** –this is the inner zone that constitiutes about 7% of the glands. This secretes **gonadocorticoids.**

5) All hormones of adrenal cortex are synthesized from cholesterol.
6) Adrenal medulla develops from the **neuroectoderm** of the embryo.

7) The medulla secretes two hormones – Norepinephrine (noradrenaline) and epinephrine (adrenaline) and both are derived from tyrosin amino acid.
8) Adrenaline is responsible for maintaining the blood pressure, dilation of vessels and muscles, increasing the general metabolic rate and also for hastening the coagulation of blood.

D) T.S. OF PANCREAS OF A MAMMAL

Comments:

1) The pancreas consists of two portion namely **exocrine portion** and **endocrine portion.**
2) The exocrine portion produces pancreatic juice which contains trypsin, amylase and lipase enzymes.
3) The endocrine portion is composed of **islets of langerhans** which found between the acini or lobules.
4) The exocrine portion consists of a series of lobules or acini.
5) The lobules are bound together by loose connective tissue containing blood vessels, nervous and lymph vessels.
6) Each lobule made up of few pyramidal pancreatic cells having granular cytoplasm and prominent nuclei.
7) The lobules open into the small ductules which join large ducts and eventuallt the main pancreatic nuclei.
8) The isolates of langerhans are compact masses of cells and secrets two hormones.
9) It reduces the sugar contents of the blood.
10) Pancrease acts both as an exocrine as well as endocrine glands.

E) T.S. OF TESTIS OF A MAMMAL

Comments:

1) The mammalian testis is covered with a thick fibrous tissue called tunica albuginea.
2) The testis consists of a many seminiferous tubules embedded in the intertubular tissues.
3) The intertubular tissue is consist of connective tissue which holds the tubules together and contains blood vessels and interstitial cells.

4) The interstitial cells secrete a hormones testosterone which is responsible for male secondary sexual characters.
5) Various type of cells are present from outside towards the lunar in the following order-
6) Spermatogonia (2n) ⟶ spermatocytes(1n) ⟶ spermatids ⟶ spermatozoan
7) A spermatozoon or sperm has an elongated head and long delicate tail.
8) Its nucleus lies in the head.
9) The group of spermatocytes, spermatids and separated from each other by sertoli cells.

E) T.S. OF OVARY OF A MAMMAL

Comments:

1) The ovary is lined by germinal epithelium which is bounded by th connective tissue, the tunica albuginea.
2) It consists of a **mass of connective tissue** and **spindle shaped cells** the two together forming the stroma .
3) Lying in thestroma are eggs cells in the various stages of development each surrounded bt a nourishing epithelial layer,the follicle and blood vessels.
4) The primary follicles arise from ingrowth of th eferminal eoithilium into the stroma.
5) One group of cells enlarges to from a developing ovum, while the other forming a single layer of cells the follicle around it.
6) The follicle and ovum slowly move deeper into the stroma and become larger.
7) Later ovum and cells around it become separated by fluid - filled space from rest of the follicle cells except at one point forming the antrum.
8) Furthur the enlargement of the ovum and follicle results in the production of a mature follicle or Graffian follicle
9) A mature follicle (graffian follicle) is made up of three layers, an outer theca externa, middle layer theca interna and the inner layer membrana granulose.
10) The mature graffian follicle then migrates to the surface of the ovary and ruptures releasing the ovum into the Fallopian tube.

11) After discharging of ovum , the follicle cells undergo proliferation and change in structure to form corpus luteum which secrets hormone , progesterone.

EXPERIMENT NO. 2

OBJECTIVE

Study of Estrous Cycle in Mice / Rat.

INTRODUCTION

- The reproductive cycle in rodents called the ***Estrous cycle*** it is short and precise .It undergoes four phases which are **Proestrus, Estrus, Metestrus, and Diestrus** and last for 4 to 5 days.
- Estimation of the estrous cycle is crucial to assess the functioning status of the female reproductive system in mice when used as an experimental model in reproductive biology.
- Ever since the method evolved t estimate the estrous cycle from vaginal smear in guinea pig by stockard and Papanicolaou and visual method by Allens in his classical research on the satrous cycle in mice there has not been a single gold strandardmethod for its precise estimation.
- Vaginal smear technique in guinea pig by Stockard and Papanicolaou has been the accepeted standard method for other mammalian species. The Vaginal smear technique is not without disadvantages; however it ensures estimation of the estrous stage.
- The reproductive period of mice begins with the opening of vagina at round 26^{th} day of birth.
- Vaginal opening is an apoptosis mediated event and is an important secondary sexual character in mice. It is used as an external indicator for the puberty onset which can be identified by simple visual inspection.
- The proestrus stage corresponds to the human follicular phase in which there is increase in 17-β- Estradiol levels and small surge in Prolactin.
- Lutenizing hormones and follicle stimulating hormones (FSH) are released from anterior pituitary into the circulation in the response to the elevated Estradiol hormones.

In Estradiol stage there is sharp decline of 17-β- Estradiol levels and Prolactin levels peak. It is the period of heat of sexual receptivity, in which progesterone levels peak. The physiological changes occurring in the estrous cycle in mice can be assessed by different methods like vaginal cytology ,electrical impedance method ,biochemical analysis of urine and visual examination of vagina .All the four stages of estrous cycle can be estimated using vagina smear method ,but it is labour intensive and time consuming .this method is relatively quicker , easier than vaginal cytology method and accurate .

Aim - Estimation of estrous cycle by visual method and vaginal lavage method.

MATERIALS AND METHOD

1) A cross – sectional study was done on 60 healthy female swiss albino mice from the central animal facility of Sri Ramachandra University with the approval of Institutinal Animal Ethics Committee.
2) The experiment was conducted between April and May 2016 in compliane with the ethical guidelines of Committee for the purpose of control and supervision of experiments on animals (CPCSEA), Government of India , New Delhi.
3) All the animal were housed in polypropylene cages and maintained in 12 hours light dark cycle with relative humidity of 25 +2 ℃ and were fed normal pellet diet with free access to water and libitum.

Assessment of Vaginak Opening in Mice – Vaginal opening occurs due to increase In the estradiol levels in mice. unlike rats , where vaginal opening occurs during first ovulation , in mice vaginal opening occurs 10 days prior to vaginal cornification and the onset of estrous cycle .in the present study the mice were monitored for the vaginal opening from 24 to 30 days after birth by vaginal inspection. The vaginal opening occurred normally within this duration in the mice in the present study. After the vaginal opening the mice were kept in adaptation period for a week followed by which estrous cycle was estimated by both visual and vaginal lavage method.

Visual Method- In this method vagina of each animal was examined carefully to avoid misinteroretation due to cursory observation .the investigation room was provided with adequate illumination because light sourse is extremely important for visual examination. LED light was

avoided as it has a purple hue which will make the setection difficult as observation by Shannon Byers et al. Each mouse was held the non dominant hand and laid in the restraint with the forepaws. The tail of the mouse was lilfted gently and the vulva was examined by the method described by Champion et al, Photographs were taken in Sony cyber shot digital camera (16.2 megapixels) for the purpose of documentation .

Vaginal Lavage Method – In this method the vaginal cells were flushed by introducing small amount of distilled water oe saline through pipette and placing few drops of cell suspension in a glass slide for microscope examination. The vaginal secretion is normally composed of three types of cells namelt Leucocytes, cornified epithelium cells and nucleated epithelium cells. The estrous cycle is estimated by thr proportion of these cells in this method .in thepresent study, vaginal smears were collected by non invasive method proposed by Ashleigh C. Mclean .for cytology assessment , 0.1 crysta lviolet strain was used . it was prepared by adding 0.1g of crystal violet powder in 100ml of double in the present study for sterile aginal lavage.

PROCEDURE

- A 100u of ddH20 was taken in a sterile latex bulb. The mouse was kept in a restrainer with its forepaws.
- The tail was elevated to visualize the vagina .The end of the latexbuld containg 100ul of ddH20 was placed at the entrance of the vaginal canal.
- Care was taken to avoid penetration into the vaginal orifice.
- The bulb was gently pressed and ddH20 was expelled into the vaginal canal.
- The pressure on the bulb was slowly released and the water was drawn back unto the tip.
- The step was repeated 4-5 times in the same bulb. The fluid was then placed in a glass slide, air dried and stained with 0.1 % crystal violet stain.
- The slide was overlaid with a cover slip and examined under light microscope.

RESULT

Visual method – In proestrus, the vaginal opening was wide , moist and the tissues appeared pink Striations were seen in both the dorsal and the ventral lips of the vulva . In estrus phase , the vagina appeared similar to proestrus, but it was less pink , less moist but striation were more pronounced at this stage. Metestrus phase was characterized by pale , dry vaginal opening which was sloughed with white cellular debris .In diestrus , vaginal opening was very moist , too small and closed in some mice with no tissue swelling.

Vaginal lavage method

1) **Proestrus –** It is the short phase of estrous cycle and refers to the preovulatory day. In the present study, it was characterized by the presence of nucleated epithelial cells which were seen in clusters or individually. A few anucleated cells and cornified cells were also present.
2) **Estrus** – It was chaacterised by the presence of cornified epithelilal cells which were abundant and often none nucleated. The cytoplasm is granular and shape of the cells is irregular.
3) **Metestrus –** It is a brief stage which consisted of large number of leucocytes and small number of large, non leucocytes formed small, tighty packed paked clumps of cells.
4) **Diestrus –** Dietrus was identified was with the large number of leucocytes. Some degree of epithelial and cornified cells was also present .The cells at ths stage did not form tightly packed clumps. In the pewsent study, most of the animal exhibited regular cycles. Cycle length of less than 4 days and more than 6 or 7 days were considered irregular. Five animals in the present study showed, continuous estrus throughout the smearing period. Acyclic smear is reported in two animals in the present study showed, continuous estrus throughout the smear for 12 to 16 days which is due to the increased sensitivity of the animals showed more amount of mucus .Such changes were monitored by visual examination method also.

DISCUSSION

The endocrine milieu of the experiment animal can be ascertained by the estimation of estrous cycle especially for mating studies. The proportion of cells present in smears is indicative of the estrous cycle stage of the animal in which the change of the number of cells showing characteristic of two stages is high. Such transition stage samples are common if smears are taken very early or late in the day. Likewise, short phases like poestrus can be missed if samples are taken early morning because in some females proestrus does not appear until mid morning .So, it would be advisable to take samples between 10.00 and 13.00. The occurrence of ectended estrous cycle increases with age or due to increase period of light illumination in the animal facility. A cyclic smear is largely due to the stimulation of cervix during smearing and it occurs more commonly in vaginal swab and vaginal pipette methods. However, in some hypersensitive animals non invasive methods can also lead to acyclicity as in the present study.

Emmens CW recommended gentle scraping of vaginal wall for better vaginal smears. Epithelial cells can be obtained from the vaginal walls in scraping method rather than the sloughed cells n lavage method. But, vaginal scraping method may not be applicable for long term study of estrous cycle in smaller species like mice because of its potential stress. The smears obtained by lavage method are clearly indicative of the sedtous stage and so undue stree can be avoided. The vaginall smears can be examined unstained in microscopes in studies where the general cyclic pattern is to be estabilished. But when estrous cycle is to be evaluated as an endpoint measure, vaginal smears need to be fixed with 95% ethanol, dried and stained.

The housing of animals and atmoshpheric control in animal facility has an impact in the cyclic pattern in mice. Female mice housed with male mice in the same room showed more regular cycles than in all female environments where peolonged anestrus periods were observed. But in the present study , most of the animals exhibited regular cycles in an all female environment .Estrous cycle can also be affected by continuous illumination in which case there will be persistent estrus .A complete estrous cycle can be defined by the period from one proestrus to another . Some authors conclude metestrus as a transition period during the early part of diestrus and its smear consists of leucocytes, confirmed and round epithelial cells. In the present study, the animals were not restrained for a longer period in vaginal lavage method to avoid stress

which may affect the estrous cycle. The animals initially were restless but after the adaptation period the animals co-operated for the procedure. The estimation of the estrous cycle was done at the same period of the day at 10.00 am and vaginal lavage yielded higher cellularity samples for microscopic examination in the present study.

In visual method of examination, initial assessments may be difficult and take time. According to Shannon L. Byers et al., if a person is trained well, approximately 100 female mice can be evaluated in 10 to 15minutes, no equipment is required. In visual detection, proestrus and estrus can be identified clearly, but other stages will be difficult to identify with this method alone [10]. In the present study, the evaluation of estrous cycle by visual method coincides with the evaluation by vaginal lavage method. The main disadvantage in visual method is the bias and cursory observation of the examiner. The risk of pseudo pregnancy and mechanical trauma in vaginal swab method makes it inapplicable in long term estrous cycle estimation. Hence, for accurate estimation of estrous cycle non invasive method as an identification tool is recommended.

Limitation - The present study is done in a specific period of time in a limited number of mice. Though, the findings are conclusive of the precise estimation of the estrous cycle, a larger number of animals for a considerably longer duration of experiment is necessary. The present study is done in mice, however the non-invasive method can be replicated in other species like rats, guinea pigs and rabbits so that a standard protocol for the estimation of estrous cycle in experimental animals can be determined.

CONCLUSION

The visual method provides a rapid means of assessing the estrous cycle in mice through practice and careful observations. But with visual method alone, it is difficult to assess the transitional stages of estrous cycle. The non invasive method of estrous cycle estimation is ideal for the precise estimation of the estrous cycle and this method can be applied for a long term as it is non invasive and there is no risk of pseudo pregnancy as in vaginal smear method.

REFERENCE

U.S. National Library of medicine (Medline Plus**)**

SUGGESTED READINGS

- Lal, S. S. (1998). A text book of practical zoology. vol. 3:1-250.
- U.S. National Library of medicine (Medline Plus)

UNIT 3 BIO-CHEMISTRY EXPERIMENTS

Content:

3.1 Chemical test of urine for the presence of urea, sugar, proteins and ketone bodies.

3.2 Color tests for carbohydrates, proteins and lipids.

3.3 Action of amylase on its respective substrates.

Experiment No – 1

OBJECTIVE

Biochemical estimation of carbohydrate for given sample.

REQUIREMENTS

a) Test Tube

b) Given Sample

c) Molisch's Reagent

d) Con. H_2SO_4.

PRINCIPLE

Before proceeding with the specific tests, check the solubility of given sample of water if it is in crystalline or powered form.

For this, dissolve a little of the sample in water in a test tube.

If it dissolves completely, the given sample is water soluble. Now proceed with the specific test given below.

PROCEDURE

Molisch's Test –

- Take two test tube, mark them A and B.
- Now take solution of the given sample test tube A.
- Add 2 drops of Molisch's reagent in it and shake the test tube so that the two solutions mix up thoroughly.
- Then take conc. H_2SO_4 in test tube B and pour it gently in test tube A along its side so that the two solutions do not mix.

OBSERVATION

A pink or purple ring appears at the junction of two solutions.

Chemistry involved – H_2SO_4 when reacts with carbohydrates ,it forms **furfural derivatives,** which on reaction with Napthol of molisch's reagent gives pink or purple ring at the surface because acid is heavier and does not mix with solution until stirred **.**

RESULT

The given sample is a **Carbohydrate.**

EXPERIMENT NO. – 2

OBJECTIVE

General color test for fructose with given sample.

REQUIREMENTS

a) Test tube

b) Given sample

c) Foulger's reagent

d) Sprit lamp.

Foulger's Test

PROCEDURE

- Take 0.5 ml test solution (Test solution is the sample provided for detection of substances or self prepared solution from given sample in crystalline or powder form) in a test tube.
- Add 3ml of Foulger's reagent in it boil and shake the test tube gently.

OBSERVATION

Blue color is appears.

Inference – Test solution contains **Fructose.**

EXPERIMENT NO. – 3

OBJECTIVE

General color test for glucose**.**

REQUIREMENTS

a) Test Tube

b) Given Sample

c) Molisch's Reagent

d) Con. H_2SO_4.

Processor

1) Moor's Test –

PROCEDURE

- Take 4ml of test tube solution in attest tube.
- Add an equal volume of 2% sodium hydroxide solution in it and boil.

OBSERVATION

Solution turns yellow in the beginning and reddish brown later. It is due to the formation of **Carmel**, a condensation product of glucose.

Inference – The test solution contain **glucose.**

EXPERIMENT NO. -4

OBJECTIVE

General colour test for protein.

REQUIREMENTS

a) Test tube

b) Given sample

c) NaOH sol.

d) $CuSO_4$ sol.

e) Sprit lamp.

Processor – Here are two colour test described for glucose.

1). Biuret Test –

PROCEDURE

- Take 3ml of test tube solution in a test tube.
- Add 1ml of 40% NaOH solution in a test tube to make solution alkaline and stir it thoroughly.
- Then add 2 drops of 1% $CuSO_4$ solution.

OBSERVATION

Violet or pink colour appears which disappears it excess of $CuSO_4$ solution is added.

Inference – Presence of **Protein** confirmed in the test solution.

2) Millon's Test

PROCEDURE

- Take 5ml of test solution in a test tube. Add 3 or 4 drops' of Millon's reagent in the test tube.
- Mix and heat the mixture till it boils.

OBSERVATION

White precipitate appears which turns yellow on heating.

Inference – Presence of **Protein** confirmed in the test solution.

EXPERIMENT NO. – 5

OBJECTIVE

Colour Test for lipid with Sudan (3).

Requirements

a) Test tube

b) Chloroform

c) Sudan (3) reagent.

Procedure

- Take a test tube containing 0.5ml of chloroform and add to it 0.5ml of test sample drop by drop.
- When fully dissolved add one drop of Sudan (3) reagent in the test tube.

Observation

Red colour appears.

Inference – The test sample contain f**at.**

EXPERIMENT NO. - 6

Objective

Test for salivary Amylase.

Requirements

a) Incubation tube

b) Starch solution

c) Toluene

d) Benedict sol.

e) Test tube.

Principal

The enzyme **salivary amylase** is present in the **saliva**. It is also known as **ptyalin**. It changes starch into **glucose** and **maltose**. Salivary amylase acts steadily at body temperature but it acts optimally between 40 -50 degree C. The optimum pH for activity of amylase is between 6 **to 7** and that of salivary amylase of man is **6.6**.

Preparation of enzyme extract – For the preparation of extract of salivary glands, 5 to 10 salivary glands are taken out from the cockroaches. The solution is diluted with 50% glycerol to make 10% solution of salivary gland extract. The extract is stored in a bottle with toluene.

Procedure

- Take 5 drops of extract in an incubation tube and add 5 drops of 1% starch solution , mix It and then cover the mixture with a layer of toluene
- In incubation tube take 5 drops of boils extract, add 5 drops of starch solution. This will serve as the control experiment; incubate both the tubes in an oven at 37^0C for about an hour.
- After one hour take a small part of the incubated mixture and add iodine solution. No change in colour of iodine solution will indicate the presence of amylase enzyme.

- Take another part of extract, add few drops of Benedict's solution and heat it till it boils.
- A brick red precipitate will confirm the presence of amylase enzyme in the extract.
- Repeat the above two tests with the control mixture. In the first test violet colour will confirm the presence of starch as such which has not been digested.
- Starch always gives **bluish – violet colour** with iodine solution. In the experimental solution the enzyme extract is mixed with iodine solution, the amylase present in the enzyme extract hydrolyses the starch into maltose and glucose; therefore, there are no changes in colour of iodine.
- In the control experiment amylase is destroyed by boiling the enzyme extract, the starch is not hydrolyzed into maltose and glucose, therefore , it gives bluish-violet colour with the iodine solution .
- In the second test with Benedict's solution a red precipitate of cuprous oxide appears. In this glucose is formed.
- Since **Glucose** is reducing sugar, it reduces **copper sulphate** into **cuprous oxide.**

Inference – Saliva contains **Amylase.**

REFERENCE

U.S. National Library of medicine (Medline Plus**)**

SUGGESTED READINGS

Lal, S. S. (1998). A text book of practical zoology. vol. 3:1-250.

Unit 4: Immunology Experiment/study

4.1 DLC (Differentiation Leucocytes count)

A white blood cell (WBC) count measures the number of white blood cells in our blood, and a WBC differential determines the percentage of each type of white blood cell present in our blood. A WBC count can also be called a leukocyte count, and a WBC differential can also be called a leukocyte differential count.

Blood (A.S., *blod,* blood) is defined as a specialized connective tissue which is an opaque, thick, rather viscid (viscosity of blood ranges from 4.5 to 5.5) liquid of red colour, salty to taste (contains about 0.85-0.90% NaCl) and alkaline (pH 7.4) in reaction. It flows through the circulatory system, helps in the transport of nutrients, oxygen (O_2), carbon dioxide (CO_2), waste products (metabolic), hormones, etc. and performs different important physiological functions including immunity. Blood is composed of liquid intercellular substance known as **plasma** (Gk. *Plasma,* form) and formed elements such as **erythrocytes** Gk. *Erythros,* ed; *kytos,* hollow(RBC), **leucocytes** Gk. *Leukos,* white; *kytos,* hollowWBC and **platelets** (F. *plat,* Gk. *Platys,* flat) that remain suspended in the plasma.

A mature human red blood corpuscle (RBC) or erythrocytes is defined as a formed element which is a circular, biconcave and non-nucleated cell which has haemoglobin in its cytoplasm, and helps in the transport of O_2 and CO_2.

In adults, red bone marrow proerythrobloast cells or **proerythrocytes** (Gk. *Pro,* before; *erythros,* red; *blastos,* bud) is the site of origin of RBCs (erythropoiesis). The cell membrane of a red blood cell becomes fragile and the cells are rendered non-functional in about 120 days. Under normal circumstances, the average count of RBCs in the peripheral blood of male adults is around 5 million per cubic millimetre of blood. In adult females, however, it is around 4.5 million. Different clinical conditions (anaemia, polycythaemia, etc.) and physiological states (time of the day, muscular exercise, attitude etc.) are responsible for the variation and deviation from the normal RBC count of an individual. Average life span of an RBC is about 120 days in the blood. Destruction and disintegration of old and senile RBCs takes place in the **reticulo-**

endothelial (L. *reticulum,* small net; Gk. *Endon,* within; *thele,* nipple) cells of spleen and liver. To maintain normal quantities of erythrocytes, the body produces new mature RBCs at the astonishing rate of 2 million/second.

White blood corpuscles or leucocytes are defined as formed elements of blood which are nearly circular in shape, larger than RBCs, possess indefinite sized nucleus, carries no haemoglobin like pigment in its granulated or agranulated cytoplasm and helps in the defence mechanisms (immunity) of our body.

While granulocytic WBCs originate **(leucopoiesis)** from **myeloblast**or **promyelocytes** of the bone narrow, agranulocytic WBCs such as **lymphocytes** and **monocytes** originate mainly from **lymphoblast** and **monoblast** cells of **lymph nodes.** The average total number of WBC is 6000-8000/cubic-millimeter (range 5000-10000). The average ratio of WBC

Haematological values in percent								
Study items	**Male**				**Female**			
	6 weeks		**10-12 weeks**		**6 weeks**		**10-12 weeks**	
Complete blood count (CBC)	WBC	6.80	WBC	5.24	WBC	4.80	WBC	6.71
	RBC	7.10	RBC	7.82	RBC	6.90	RBC	8.10
	Hb	15.50	Hb	16.40	Hb	14.60	Hb	18.63
Differential Count (DC)	N	8.20	N	11.33	N	9.70	N	13.88
	L	91.60	L	88.11	L	89.80	L	85.75
	M	00.20	M	00.44	M	00.40	M	00.25
	E	00.00	E	00.11	E	00.10	E	00.13
	B	00.00	B	00.00	B	00.00	B	00.00

N = Neutrophil, L = Lymphocyte, M = Monocyte, E = Eosinophil, B = Basophil

to RBC total count is 1:700. This count varies under different clinical conditions and altered physiological states. The life span of WBCs is variable, starting from 12-15 days of basophils to 2-3 days of lymphocytes. Old and senile WBCs undergo destruction and are removed from the system by different mechanisms.

To examine the total count of RBC and/or WBC, a definite volume of blood is diluted with a known amount of diluents and the number of corpuscles is counted in the known volume of this mixture under microscope with the help of an instrument called **haemocytometer.** The date gathered by this method is used for enumerating the total number of corpuscles in the sample. However, such enumeration is done mathematically.

AREAS OF APPLICATION

In normal human beings, total leucocytes' count range from 5,000 to 10,000 per µl of blood. A count below this range, known as leukopenia, can be observed in case of heavy metal poisoning, radiation sickness or infections (measles, mumps, chicken pox, poliomyelitis, influenza, typhoid fever, and AIDS). It can also be produced by glucocorticoids, anti-cancer drugs and immunosuppressant drugs.

A leucocytes' count on the contrary, above 10.000 white blood cells per µl of blood, called exocytosys usually indicates infection, allergy, varied diseases, dehydration and emotional disturbances. Since different types of leucocytes increase or decrease in response to an enormous variety of ailments, given below is a short tabular representation that describes normal and abnormal distribution of leucocytes with possible correlation of the type of ailments that the leucocytes respond to.

Total 4.3b Normal and abnormal distribution of leucocytes

Name of leucocytes	Percentage in normal subjects	Clinical applications vis-à-vis functional aspects
Neutrophils (_____span 7 hours in blood)	60%-70-%; Mean count: 4150 cells/µl of blood	Involved in immune defence. Increases in response to bacterial infection.
Eosinophil's (life span: unknown)	2%-4% Mean count: 165 cells/µl of blood	Increases in parasitic infections, allergies, collagen associated diseases, and the diseases of spleen and central nervous system.
Basophils (life span: unknown)	<0.5%-1%; mean count: 44 cells/µl of blood	Involved in inflammatory responses. Relatively stable, although elevated during allergic reactions.
Lymphocytes (life span: unknown)	25%-33%; mean count: 2185 cells/µl of blood	Involved in antibody production and cellular immune responses. Increases in diverse infections and immune responses.
Monocytes (life span: 3 days in blood)	3%-8%; Mean count: 456 cells/µl of blood	Involved in immune surveillance. Increases in viral infection, and inflammations. Elevated

		count of monocytes generally indicates chronic infections.

THEORETICAL FUNDAMENTALS

Differential count involves determination of the number of each kind of white blood cells (collectively called leucocytes) in a sample of 100 cells for clinical diagnostic purposes. Altogether, there are five different kinds of leucocytes in man-**neutrophils, eosinophils, basophils, lymphocytes,** and **monocytes.** The first three are considered to be the members of a group, named as **granulocytes,** while the rest two belong to another group, known as **granulocytes.**

Three important criteria are considered most useful for the purpose of identifying the leucocytes.

1. Cytoplasmic granules
2. Cell size
3. Nuclear shapes of the leucocytes

All these characteristics for each kind of leucocytes are presented in Table 4.3.

EXPERIMENTAL METHODOLOGY

Materials required

(i) Glass slides, (ii) 70% alcohol, (iii) sterilized needles, (iv) Leishman's stain, (v) double distilled water, (vi) Pasteur pipette and (vii) compound microscope.

Table 4.3 Identifying characters and other relevant information's with respect to white blood cells in man

	Diameter (µm)	Cytoplasmic granules	Nuclear shape	Specialities
Neutrophils	09-12	Fine granules (violet)	Divided into 3 to 4 lobes with fine strands of cytoplasm.	Young cells sometimes possess undivided nucleus.
Eosinophil's	10-14	Course granules (rosy)	Bilobed nucleus.	Bilobed nucleus appears as if two ballons are tied together
Basophils	08-10	Course granules (purple	S or U-shaped nucleus.	Nucleus is made obscure from view by course granules in cytoplasm.
Lymphocytes	5-8 (small) 10-12 (medium) 14-17 (large)	-	Large nucleus; almost fills the cell leaving only a narrow rim of cytoplasm.	Many intermediate forms are possible; light microscopic study appears difficult

Monocytes	12-15	-	Round/oval/lobed/kidney shaped/C-shaped; cytoplasm is abundant and clear	Largest of all leucocytes; usually twice the size of erythrocyte.

4. Gently mix the bacteria, and spread the bacteria over the slide covering an area not more than 1 cm^2.

Fixation

For fixation (and also drying of cells), hold the slide horizontally with forceps and quickly pass it just over the yellow flame of Bunsen burner two to three times. This step is to be done (For beginners) under direct supervision of your instructor, because overheating may spoil the entire endeavour. Fixing cells in such a way kills the bacteria (by coagulating the cytoplasm) and makes them stick to the slide.

Staining

1. Flood the slide with 0.5% crystal violet stain, and leave for about 30 seconds. Then pour off the excess stain (Crystal violet makes all bacteria violet).
2. Flood the slide with Lugol's iodine, leave it for 30 seconds and then wash off the iodine with distilled water (iodine fixes the stain more permanently onto the cells).
3. Wash with acetone-alcohol for not more than 2-5 seconds. Immediately wash with water to prevent excessive decolourization
4. Counterstain with freshly filtered neutral red for 1-2 minutes/safranin for 1 minute. Counter staining imparts red colour on the Gram-negative bacteria.
5. Wash with water and blot dry (hold the slide between sheets of clean blotting paper, and finally allow drying in air).

OBSERVATIONS

Examine the slide under oil immersion lens of a good quality compound microscope. The results will ideally be as follows:

1. Gram-positive organisms (bacteria) will appear violet or blue-black (e.g., *Lactobacillus* sp.). Gram-negative bacteria will appear red (e.g., *Escherichia coli, Azotobacter* sp.).

AREAS OF APPLICATION

Gram staining technique is considered the most basic tool in classifying bacteria as well as in different pathological diagnostics.

Materials required

(i) Improved Neubauer haemocytometer, (ii) a thick square cover glass, (iii) two pipettes for drawing of blood-RBC pipette and WBC pipette, (iv) RBC diluting fluid, (v) WBC diluting fluid, (vi) lancet or needle, (vii) cotton (non-absorbent), (viii) rectified spirit (90% ethanol) and (ix) compound microscope with halogen light source.

Description of the apparatus

Haemocytometer

The improved Neubauer haemocytometer is a thick glass slide on which there are three flat platforms extending across the slide. These platforms are separated from each other by deep grooves. The middle platform is 0.1 mm lower than the other two platforms. On the surface of the middle platform there are two sets (duplicate) of ruled area. The area consists of 9 large squares, each having 1 square millimetre area and is elaborately ruled. The four corner squares are divided into 16 equal sized squares. The central square is ruled into 25 groups of 16 small squares, each group is separated by triple lines. The ruled surface is 1/10 mm below from the inner surface of the cover glass placed over the-middle platform, so each smallest square of the corner has the volume of ¼ X ¼ X 1/10 or 1/160 cubic millimetre, while the smallest square of the canter has the volume of 1/20 X 1/20 X 1/10 or 1/4000 cubic millimetre.

RBC pipette

It bears three graduations-0.5 and 1 is on the stem of the pipette, and the 101 mark is placed just above the bulb of the pipette. There is a red bead kept inside the bulb. The pipette is fitted to a rubber tube.

WBC pipette

This is similar to the RBC pipette in structure except for the graduations which are 0.5, 1 and 11 instead. There is one white bead kept inside the bulb.

Preparation of solutions/reagents

RBC diluting fluid

It is an isotonic solution which prevents coagulation, haemolysis, rouleaux formation, and bacterial growth. Hayem's diluting fluid contains the following:

Sodium sulphate (Na_2SO_4)	-	5.0 g
Sodium chloride (NaCl)	-	1.0 g
Mercuric chloride ($HgCl_2$)	-	5.0 g

In distilled water (final volume: 200 ml)

WBC diluting fluid

It contains a weak acid (to lyses RBCs) and a dye (for staining the nucleus of white blood cells) in the following composition-Glacial acetic acid (1.5 to 3 ml) and distilled water (96.5 to 97 ml). To the acetic acid solution of this specified strength, a few drops of an aqueous solution of methylene blue or gentian violet are added for obtaining WBC diluting solution.

Total count of RBCs

1. Puncture the fingertip of volunteers/patients/subjects aseptically by a lancet or needle. Wipe off the first drop of blood by cotton and allow the blood to flow freely.
2. Draw blood into the RBC pipette up to 0.5 or 1 mark.

3. Wipe off extra blood from the outer surface of the pipette and immediately draw RBC fluid immediately up to 101 marks.

4. Mix the contents of the bulb thoroughly by using the red coloured bead present inside the bulb of the pipette. Rotate the pipette well for about a minute holding in a horizontal position and finally shake sidewise; perform mixing carefully and avoid any leakage).

5. When blood is drawn up to 1 mark then dilution becomes 100 fold, but when blood is drawn up to 0.5 then 200 fold VC dilutions is achieved (because last part of the fluid remains locked up in the stem and is not available for dilution).

6. Place the cover glass properly in position over the ruled area of haemocytometer.
7. Expel the clear fluid from the stem of the pipette which does not contain blood. Quickly put a drop of diluted blood at the edge of cover glass placed over the counting chamber, and allow the very drop to flow under the cover glass by capillary action. Overflow of fluid or introduction of air bubble inside the chamber should strictly be avoided.

8. Allow the cells to settle at the bottom of the chamber for 2-3 minutes, but avoid drying up of the material, (For this take a petridish, place a wet cotton or filter paper at its bottom and then place the charged counting chamber on to it. Finally cover up the dish and keep in this condition for about 2-3 minutes.)

9. Place the haemocytometer on the stage of the microscope. Focus the light and reduce its intensity by lowering the condenser and partially closing the diaphragm (examine under 40X or 20X objective lens of the microscope).

10. Count the RBCs at the five groups of 16 smallest squares of the central square in ruled area. Stated otherwise, count the number of RBC present in 80 smallest squares.

11. When properly focused, the red corpuscles are sharply defined and the rulings appear as well-defined black lines.

12. Calculate the total count of RBC using the following procedure:

If the number of RBC present in 80 smallest squares = *x*,

Then one smallest square contains *x*/80 RBCs, or 1/4000 cubic millimeter of the diluted blood contains *x*/80 RBCs.

Hence, one cubic millimeter of undiluted peripheral blood of the individual (volunteer/patient/subject), contains *x*/80 x 4000 x dilution = Z of RBCs.

Total count of WBCs

1. Following the same procedure (as described for TC of RBC) draw blood into the WBC pipette up to 0.5 or 1 mark, and then quickly draw WBC diluting fluid up to 11 mark.

2. Following the procedure described in steps 6-10 with respect to RBCs' count the number of WBCs under 10X objective lens of the microscope.

3. Count the WBCs present in the four corner squares of the ruled area. Each of these four squares is subdivided into 16 smaller squares. Stated otherwise, count the number of WBCs present in (16 x 4) or 64 small squares.

4. Calculate the total count of WBCs using the following procedure:

   If the number of WBC in 64 small squares = *x*,

   Then one small square contains *x*/64 WBCs, or 1/160 cubic millimetre of the diluted blood contains *x*/64 WBCs.

   Hence, one cubic millimetre of undiluted peripheral blood of the individual (volunteer/patient/subject), contains *x*/64 x 160 x dilution = Y number of WBCs.

AREAS OF APPLICATION

Total count of RBCs and WBCs is included in the list of routine tests in most of the clinical pathology laboratories. Results of these tests are used for differential diagnosis of different clinical conditions.

EXPERIMENTAL METHODOLOGY

Materials required

(i) Aqueous solution (0.5%) of crystal violet, (ii) Lugol's iodine (3 g iodine and 6 g potassium iodine in 900 ml of distilled water), (iii) acetone alcohol (1:1 v/v), (iv) safranin (1% aqueous solution), (v) wire loop, (vi) Bunsen burner, (vii) glass slide (wiped with alcohol), (viii) forceps, (ix) petri dishes, (x) distilled water in wash bottle, (xi) blotting paper, (xii) compound microscope and (xiii) bacterial culture on plate.

Preparation of bacterial smear

1. Take a wire loop, flame the same on Bunsen burner and allow it to cool.
2. Take two loopful of distilled water on the centre of a clean of a clean glass slide.
3. Bring the wire loop in touch with the bacterial colony of interest (or provided) very quickly by opening the lid of the plate (for the reasons of safety against contamination) and transfer the bacteria in the loop to the glass slide.

Method/Protocol

1. Clean the slides with absolute alcohol.

2. Clean the tip of middle finger of the subject (individual) and prick with a sterilized needle.

3. Wipe away the first drop of blood that appears on the fingertip with sterilized cotton, soaked in alcohol. Take the second drop of **free flowing** blood on the clean slide about 1 cm from the right narrow edge of it.

4. Place the narrow edge of the second slide making an angle of 45^0 to the first slide just left to the drop of blood to be examined.

5. Slowly pull the second slide, holding in position, to the right until the second slide touches the blood on the first. Immediately afterwards, push the second slide to the left at uniform speed. Such leftward movement of the second slide produces a thin blood film on the first slide. Make 3-4 such blood films.

6. Place the slide with blood smear in a dust-free open space for 10 minutes to get the smear air-dried properly.

7. Put a scratch mark on the first slide on one corner on the surface containing the blood smear.

8. Put appropriate quantity of Leishman's stain on the air-dried blood smear, and leave for 5-10 minutes. After this stage put an equal amount of buffered water (composition-3.76 g $Na_2HPO_42H_2O$ + 2.10 g anhydrous KH_2PO_4 with a maximum volume of 1000 ml with double distilled water) on the dye, and leave for another 3-5 minutes. Now drain off the dye and rinse the slide with distilled water for once or twice. Finally, dry up the stained slide in air. Now the slide is ready for microscopic observation.

4.2 BLOOD GROUPS AND RH FACTORS

The ABO blood group system involves two antigens and two antibodies found in human blood. The two antigens are antigen A and antigen B. The two antibodies are antibody A and antibody B. The antigens are present on the red blood cells and the antibodies in the serum. Regarding the antigen property of the blood all human beings can be classified into 4 groups, those with antigen A (group A), those with antigen B (group B), those with both antigen A and B (group AB) and those with neither antigen (group O). The antibodies present together with the antigens are found as follows:

1. Antigen A with antibody B
2. Antigen B with antibody A
3. Antigen AB has no antibodies
4. Antigen nil (group O) with antibody A and B.

There is an agglutination reaction between similar antigen and antibody (for example, antigen A agglutinates the antibody A and antigen B agglutinates the antibody B). Thus, transfusion can be considered safe as long as the serum of the recipient does not contain antibodies for the blood cell antigens of the donor.

The *ABO system* is the most important blood-group system in human-blood transfusion. The associated anti-A and anti-B antibodies are usually *immunoglobulin M*, abbreviated IgM, antibodies. It has been hypothesized that ABO IgM antibodies are produced in the first years of life by sensitization to environmental substances such as food, bacteria, and viruses, although blood group compatibility rules are applied to newborn and infants as a matter of practice.[11] The original terminology used by Karl Landsteiner in 1901 for the classification was A/B/C; in later publications "C" became "O". Type O is often called *0* (*zero*, or *null*) in other.

Phenotype	Genotype
A	AA or AI
B	BB or BI
AB	AB
O	II

Rh blood group system

The Rh system (Rh meaning *Rhesus*) is the second most significant blood-group system in human-blood transfusion with currently 50 antigens. The most significant Rh antigen is the D antigen, because it is the most likely to provoke an immune system response of the five main Rh antigens. It is common for D-negative individuals not to have any anti-D Ig G or Ig M antibodies, because anti-D antibodies are not usually produced by sensitization against environmental substances. However, D-negative individuals can produce IgG anti-D antibodies following a sensitizing event: possibly a fetomaternal transfusion of blood from a fetus in pregnancy or occasionally a blood transfusion with D positive RBCs. Rh disease can develop in these cases. Rh negative blood types are much less common in Asian populations (0.3%) than they are in European populations (15%).The presence or absence of the Rh(D) antigen is

signified by the + or − sign, so that, for example, the A− group is ABO type A and does not have the Rh (D) antigen.

4.3WIDAL TEST FOR THE IDENTIFICATION OF TYPHOID

In 1896 and named after its inventor, Georges-Fernand Widal, is a presumptive serological test for enteric fever or undulant fever whereby bacteria causing typhoid fever is mixed with a serum containing specific antibodies obtained from an infected individual. In cases of *Salmonella* infection, it is a demonstration of the presence of O-soma false-positive result. Test results need to be interpreted carefully to account for any history of enteric fever, typhoid vaccination, and the general level of antibodies in the populations in endemic areas of the world. Typhidot is the other test used to ascertain the diagnosis of typhoid fever. As with all serological tests, the rise in antibody levels needed to perform the diagnosis takes 7–14 days, which limits its applicability in early diagnosis. Other means of diagnosing *Salmonella typhi* (and *paratyphi*) include cultures of blood, urine and faeces. These organisms produce H_2S from thiosulfate and can be identified easily on differential media such as bismuth sulfite agar.
Widal agglutination. **Widal** agglutination was introduced as a serologic technique to aid in diagnosis of **typhoid** fever. The **test** was based on demonstrating the presence of agglutinin (antibody) in the serum of an infected patient, against the H (flagellar) and O (somatic) antigens ofSalmonella typhi.

The Widal test is one method that may be used to help make a presumptive diagnosis of enteric fever, also known as typhoid fever. Although the test is no longer commonly performed in the United States or other developed countries, it is still in use in many emerging nations where enteric fever is endemic and limited resources require the use of rapid, affordable testing alternatives. While the method is easy to perform, concerns remain about the reliability of the Widal test. It is not specific for typhoid fever and can be positive when a person does not have the infection.

Enteric fever is a life-threatening illness caused by infection with the bacterium *Salmonella enterica* serotype Typhi (*S. typhi*), usually transmitted through food and drinks contaminated

with fecal matter. It is associated with symptoms that include high fever, fatigue, headache, abdominal pain, diarrhea or constipation, weight loss, and a rash known as "rose spots." Early diagnosis and treatment are important because serious complications, including severe intestinal bleeding or perforation, can develop within a few weeks.

The infection is rare in the U.S. and other industrialized nations but is more common in developing countries, including India, parts of South, East and Southeast Asia, and countries in Africa, the Caribbean, Central and South America, and Eastern Europe. Cases of enteric fever in the U.S. are usually attributed to travelers to these endemic areas.

In the U.S. and other developed nations, testing for enteric fever usually involves a blood culture to detect the bacteria during the first week of fever. A stool, urine or bone marrow culture may also be performed. A blood culture, however, can be labor- and time-intensive in areas of the world that lack the resources for automated equipment. In developing countries, such as those in Africa, the Widal test continues to be used instead of cultures because it is quicker, simpler, and less costly to perform.

The World Health Organization (WHO) has said that due to the various factors that can influence the results of a Widal test, it is best not to rely too much on this test. WHO instead recommends the use of cultures, whenever possible? Until another simple, inexpensive, and reliable option becomes available, however, use of the Widal test will probably persist in those countries with limited resources. There are newer rapid antibody tests for typhoid fever commercially available, several of which have been included in comparative studies of their reliability, for example in India and Africa. Findings seem to vary as to whether any are as reliable as blood culture for diagnosing this infection.

4.4 VDRL TEST FOR SYPHILIS

The venereal disease research laboratory (VDRL) test is designed to assess whether you have syphilis, a sexually transmitted infection (STI). Syphilis is caused by the bacterium *Treponema pallidum*. The bacterium infects by penetrating into the lining of the mouth or genital area.

The VDRL test doesn't look for the bacteria that cause syphilis. Instead, it checks for the antibodies your body makes in response to antigens produced by cells damaged by the bacteria. Antibodies are a type of protein produced by your immune system to fight off invaders like bacteria or toxins. we don't need to have the symptoms of syphilis for this test to be accurate. Because it checks for antibodies produced as a result of a syphilis infection, the VDRL test can be used regardless of whether you currently have any symptoms.

Early symptoms that may prompt your doctor to order this test include:

- one small, painless sore
- swelling in lymph nodes near the sore
- a skin rash that doesn't itch

In other cases, doctor may screen for syphilis even if patient don't have any symptoms. For example, doctor will screen for syphilis as a routine part of your care if you're pregnant. This is a standard procedure, and it doesn't mean your doctor thinks you have syphilis.

If patient being treated for another STI such as gonorrhea, if patient infected with HIV, or if patient engaged in high-risk sexual activity. If patient already been treated for syphilis, the Centers for Disease Control and Prevention (CDC) Trusted Source recommend follow-up testing to be sure that the treatment worked and the infection has been cured.

Usually, all we need to do for the VDRL test is allow a healthcare professional to draw our blood. Blood is generally drawn from a vein at the crease of the elbow or the back of the hand. This blood sample will then be sent to a laboratory and tested for the antibodies produced as a result of syphilis.

The VDRL test doesn't require you to fast or stop taking any medications. If doctor wants you to make an exception, they'll let you know before your test. If your doctor suspects that the syphilis infection has spread to your brain, doctor may choose to test our spinal fluid in addition to our blood.

REFERENCE

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Unit 5: Microbiology

5.1 PREPARATION OF CULTURE MEDIA, STERILIZATION

Bacteria (Gk. *Bacterion,* little rod) are the most primitive and simplest organisms known. They are included under the Kingdom Monera in the five Kingdom system of classification (Whittaker, 1959). Although about 2,500 species of bacteria have so far been described and identified (and have been given names), there exist a huge body of unidentified species of bacterial Most bacterial species are single-celled organism, although a few are multicellular and filamentous. Bacteria appear as spherical, rod-shaped, or spiral-shaped organisms. Bacteria are broadly classified as **eubacteria** and **archaebacteria**. Eubacteria include those that are commonly found in living organisms, soil and water. The bacterial species belonging to Archaebacteria are, however, found in more hostile surroundings like hot springs, salty marshes, methane rich marshes, or in the ocean depths.

Bacteria vary greatly in size. The mean diameter can range from as small as 100 mm to as long as 10 µm. The extreme variation in size is reflected by the fact that there exists a bacterium, *Epulopiscium fishelsoni* by name that is a million times larger than *Escherichia coli*-the rod-shaped bacterium that is extensively used in the field of molecular biology, gene technology, molecular genetics and microbial pathology. Most eubacteria are encased by a strong cell wall (a general term that refers to the rigid, outermost layer of cells of plants, some protists, and most bacteria; the cell wall surrounds the cell membrane/plasma membrane), in which a carbohydrate matrix is cross-linked by short polypeptide chains. The characteristic composition of cell wall is often used to distinguish bacteria into Gram-positive and Gram-negative categories depending on their ability to retain or not to retain a stain, called Gram stain. The name ‘Gram’ refers to the Danish microbiologist and physician Hans Christian Gram (1853-1938), who developed a staining procedure in 1884, to distinguish two classes of bacteria as a way to detect the presence of certain disease-causing bacteria. In general, Gram-positive bacteria are those that possess a single, thick cell wall that is able to retain the Gram stain. In the case of Gram-negative bacteria, however, the cell wall is thinner which is unable to retain Gram-stain, when subjected to it.

PREPARATION OF MEDIA AND CULTURE

CULTURE MEDIA

The method for the preparation of basic microbiology media is given below. In situations where preparation is uneconomic in time, prepared, sterilized media (liquid and solid) are available from the major school science equipment suppliers. Sterilization is at 121 °C (15 lb in $^{-2}$) for 15 minutes. pH values are 7.0 unless stated otherwise.

Note: Allow 15 cm³ of agar for each Petri dish and 5-10 cm³ of broth for each McCartney bottle. All cotton wool plugs should be made of non-absorbent cotton wool. Plastic or metal caps may also be used.

NUTRIENT AGAR

Suspend 28 g of nutrient agar powder in 1 litre of distilled water. Bring to the boil to dissolve completely. Dispense as required and sterilize.

NUTRIENT BROTH

Add 13 g of nutrient broth powder to 1 litre of distilled water. Mix well. Dispense as required and sterilize.

MALT EXTRACT AGAR

Suspend 18g agar powder in 1 litre of distilled water. Bring to the boil to dissolve completely. Add 15g malt extract per litre. Mix well. Dispense as required and sterilize.

MANNITOL YEAST EXTRACT AGAR

Suspend 10 g agar in 1 litre of distilled water. Heat to dissolve. Add 0.5 g K_2HPO_4, 0.2g $MgSO_4.7H_2O$, 0.2 g NaCl, 0.2 g $CaCl_2.6H_2O$, 10 g mannitol and 0.4 g yeast extract. Dispense as required and sterilize.

GLUCOSE NUTRIENT BROTH

Make up nutrient broth as already directed and add 10 g per liter of glucose.

SUGAR PEPTONE WATER

Add 10 g of peptone, 5 g of NaCl, 5 g of sugar and 20 cm^3 of Universal indicator to 1 litre of distilled water; pH should be 7.4. Dispense as required and sterilize.

TRIBUTYRIN AGAR

Supplied ready for use. Heat to melt and dispense aseptically. May be prepared by adding 1% tributyrin to nutrient agar.

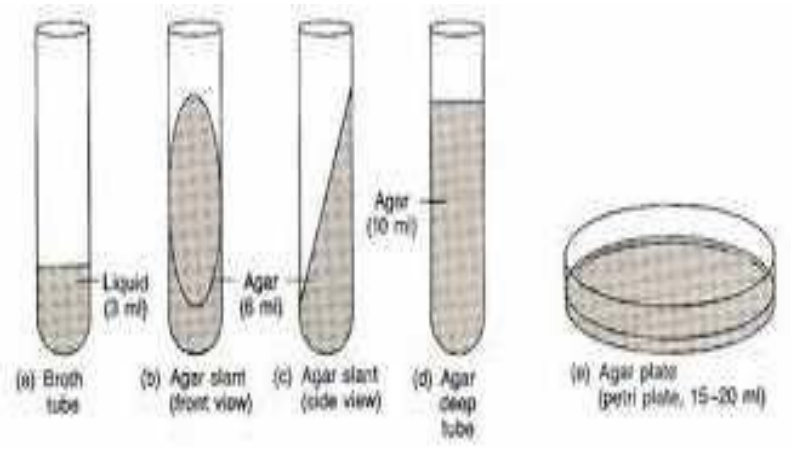

Fig .Preparation of Culture Media

GLUCOSE YEAST EXTRACT BROTH

Add 10 g of peptone, 5 g of NaCl, and 3 g of yeast extract to 1 liter of distilled water. Dispense as required and sterilize.

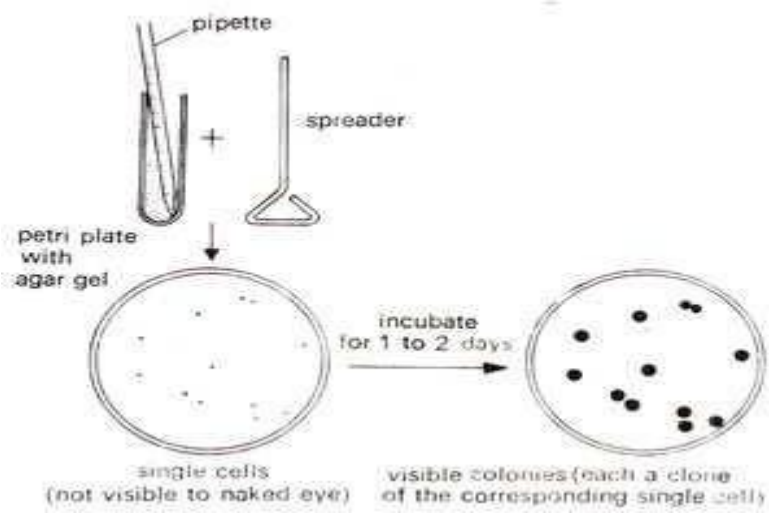

Fig: Culture media and Mutant Strains

GLUCOSE YEAST EXTRACT LEMCO BROTH

Add 10 g of Lemco (meat extract) to glucose yeast extract broth.

MILK AGAR

Make up nutrient agar as above but using only 900 cm^3 of distilled water. Dissolve 20 g of dried skimmed milk in 100 cm^3 of distilled water. Sterilize separately. Transfer the milk to the agar aseptically after cooling to 45-50 °C. Dispense aseptically.

STARCH AGAR

Suspend 15 g of nutrient agar in 100 cm^3 distilled water. Bring to the boil to dissolve completely. Heat 40 g of soluble starch in 100 cm^3 of distilled water to form a suspension. Allow to cool and then mix with the nutrient agar solution. Dispense and sterilize.

IODINE SOLUTION

Dissolve 1 g of iodine crystals and 2 g of potassium iodine in 300 cm^3 of distilled water.

Cellulose broth (for *Trichoderma reesei*)

- 800 cm^3 distilled water
- 0.1 g $CaCl_2$
- 0.5 g $(NH_4)_2SO_4$
- 0.5 g yeast extract powder
- 0.5 g asparagines
- 10 g carboxymethylcellulose
- 1.0 g KH_2PO_4
- pH6.2

Mix ingredients, heat gently, and stir until dissolved.

5.2 GRAM STAINING

Gram stain or **Gram staining**, also called **Gram's method**, is a method of staining used to distinguish and classify bacterial species into two large groups (Gram-positive and Gram-negative). The name comes from the Danish bacteriologist Hans Christian Gram, who developed the technique.

Gram staining differentiates bacteria by the chemical and physical properties of their cell walls. Gram-positive cells have a thick layer of peptidoglycan in the cell wall that retains the primary stain, crystal violet. Gram-negative cells have a thinner peptidoglycan layer that allows the crystal violet to wash out. They are stained pink by the counterstain, commonly safranin or fuchsine.

The Gram stain is almost always the first step in the preliminary identification of a bacterial organism. While Gram staining is a valuable diagnostic tool in both clinical and research settings, not all bacteria can be definitively classified by this technique. This gives rise to *Gram-variable* and *Gram-indeterminate* groups.

Gram staining is a bacteriological laboratory technique used to differentiate bacterial species into two large groups (Gram-positive and Gram-negative) based on the physical properties of their cell walls. Gram staining is not used to classify archaea, formerly archaeabacteria, since these microorganisms yield widely varying responses that do not follow their phylogenetic groups.

The Gram stain is not an infallible tool for diagnosis, identification, or phylogeny, and it is of extremely limited use in environmental microbiology. It is used mainly to make a preliminary morphologic identification or to establish that there are significant numbers of bacteria in a clinical specimen. It cannot identify bacteria to the species level, and for most medical conditions, it should not be used as the sole method of bacterial identification. In clinical microbiology laboratories, it is used in combination with other traditional and molecular techniques to identify bacteria. Some organisms are Gram-variable (meaning they may stain either negative or positive); some are not stained with either dye used in the Gram technique and are not seen. In a modern environmental or molecular microbiology lab, most identification is

done using genetic sequences and other molecular techniques, which are far more specific and informative than differential staining.

STAINING MECHANISM

Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50–90% of cell envelope), and as a result are stained purple by crystal violet, whereas Gram-negative bacteria have a thinner layer (10% of cell envelope), so do not retain the purple stain and are counter-stained pink by safranin. There are four basic steps of the Gram stain:

- Applying a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture. Heat fixation kills some bacteria but is mostly used to affix the bacteria to the slide so that they don't rinse out during the staining procedure.
- The addition of iodide, which binds to crystal violet and traps it in the cell
- Rapid decolorization with ethanol or acetone
- *Counterstaining* with safranin. Carbol fuchsin is sometimes substituted for safranin since it more intensely stains anaerobic bacteria, but it is less commonly used as a counterstain.

GRAM-POSITIVE BACTERIA

Gram-positive bacteria generally have a single membrane (*monoderm*) surrounded by a thick peptidoglycan. This rule is followed by two phyla: *Firmicutes* (except for the classes Mollicutes and Negativicutes) and the *Actinobacteria*. In contrast, members of the Chloroflexi (green non-sulfur bacteria) are monoderms but possess a thin or absent (class Dehalococcoidetes) peptidoglycan and can stain negative, positive or indeterminate; members of the *Deinococcus–Thermus* group stain positive but are diderms with a thick peptidoglycan.

Historically, the Gram-positive forms made up the phylum *Firmicutes*, a name now used for the largest group. It includes many well-known genera such as *Lactobacillus*, *Bacillus*, *Listeria*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Clostridium*. It has also been expanded to include the *Mollicutes*; bacteria like *Mycoplasma*, *Thermoplasma* that lack cell walls and so cannot be Gram-stained, but are derived from such forms.

Some bacteria have cell walls which are particularly adept at retaining stains. These will appear positive by Gram stain even though they are not closely related to other Gram-positive bacteria. These are called acid-fast bacteria, and can only be differentiated from other Gram-positive bacteria by special staining procedures.

GRAM-NEGATIVE BACTERIA

Gram-negative bacteria generally possess a thin layer of peptidoglycan between two membranes (*diderms*). Most bacterial phyla are Gram-negative, including the cyanobacteria, green sulfur bacteria, and most *Proteobacteria* (exceptions being some members of the *Rickettsiales* and the insect-endosymbionts of the *Enterobacteriales*).

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Unit 6 Biotechniques (exercise based on chart/ picture or sample instrument)

CONTENT

6.1 Determination of pH using pH meter.

6.2 Demonstration of functioning of spectrophotometer.

6.3 Demonstration of use of bright field, phase contrast, dark field, fluorescence, confocal and electron microscopes (on photograph basis).

EXPERIMENT NO-01

OBJECTIVE: To demonstrate the pH using by pH meter.

INTRODUCTION

A **pH Meter** is a scientific instrument that measures the hydrogen-ion concentration (or pH) in a solution, indicating its acidity or alkalinity.

The pH meter measures the difference in electrical potential between a pH electrode and a reference electrode. It usually has a glass electrode plus a calomel reference electrode, or a combination electrode.

In addition to measuring the pH of liquids, a special probe is sometimes used to measure the pH of a **pH Meter** is a scientific instrument that measures the hydrogen-ion concentration (or pH) in a solution, indicating its acidity or alkalinity.

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In addition to measuring the pH of liquids, a special probe is sometimes used to measure the pH of semi-solid substances.

Uses

- Knowledge of pH to greater or lesser accuracy is useful or critical in a great many situations, including of course chemical laboratory work.
- pH meters of various types and quality can be used for soil measurements in agriculture; water quality for water supply systems, swimming pools, etc.; brewing, industrially or

domestically; healthcare, to ensure that solutions are safe when applied to patients or lethal as sterilants and disinfectants; and many other applications.

Circuit and operation

- Simple potentiometric pH meters simply measure the voltage between two electrodes and display the result converted into the corresponding pH value.
- They comprise a simple electronic amplifier and a pair of probes, or a combination probe, and some form of display calibrated in pH.
- The probe is the key part: it is a rod-like structure usually made of glass, with a bulb containing the sensor at the bottom.
- Frequent calibration with solutions of known pH, perhaps before each use, ensures the best accuracy. To measure the pH of a solution, the probe is dipped into it.

Probe care and cleaning

- Probes need to be kept clean of contamination as far as possible, and not touched by hand.
- Probes are best kept moist with a medium appropriate for the particular probe (distilled water, which can encourage diffusion out of the electrode, is undesirable) when not in use.
- If the bulb becomes contaminated with use it can be cleaned in the manner recommended by the manufacturer; a quick rinse in distilled water immediately after use, blotted (not wiped) off may be sufficient.
- One maker of laboratory-grade equipment gives different cleaning instructions for general cleaning (15' soak in a solution of bleach and detergent), salt (hydrochloric acid solution followed by sodium hydroxide and water), grease (detergent or methanol), clogged reference junction (KCl solution), protein deposits (pepsin and HCl, 1% solution), and air bubbles.

Calibration and use

- For very precise work the pH meter should be calibrated before each measurement. For normal use calibration should be performed at the beginning of each day.
- The reason for this is that the glass electrode does not give a reproducible e.m.f. over longer periods of time
- Calibration should be performed with at least two standard buffer solutions that span the range of pH values to be measured.
- For general purposes buffers at pH 4.00 and pH 10.00 are acceptable.
- The pH meter has one control (calibrate) to set the meter reading equal to the value of the first standard buffer and a second control which is used to adjust the meter reading to the value of the second buffer.
- A third control allows the temperature to be set.
- Standard buffer sachets, which can be obtained from a variety of suppliers, usually state how the buffer value changes with temperature.
- For more precise measurements, a three buffer solution calibration is preferred. As pH 7 is essentially, a "zero point" calibration (akin to zeroing or taring a scale or balance), calibrating at pH 7 first, calibrating at the pH closest to the point of interest (e.g. either 4 or 10) second and checking the third point will provide a more linear accuracy to what is essentially a non-linear problem.
- Some meters will allow a three-point calibration and that is the preferred scheme for the most accurate work. Higher quality meters will have a provision to account for temperature coefficient correction, and high-end pH probes have temperature probes built in.
- The calibration process correlates the voltage produced by the probe (approximately 0.06 volts per pH unit) with the pH scale.
- After each single measurement, the probe is rinsed with distilled water or deionized water to remove any traces of the solution being measured, blotted with a scientific wipe to absorb any remaining water which could dilute the sample and thus alter the reading, and then quickly immersed in a solution suitable for storage of the particular probe type.

TYPES OF PH METERS

A simple pH meter

pH meters range from simple and inexpensive pen-like devices to complex and expensive laboratory instruments with computer interfaces and several inputs for indicator and temperature measurements to be entered to adjust for the variation in pH caused by temperature. Specialty meters and probes are available for use in special applications, harsh environments, etc.

There are also holographic pH sensors, which allow pH measurement calorimetrically.

Figure 6.1 a simple pH meter

Figure 6.2 a Digital pH meter

HISTORY

The concept of pH was defined in 1909 by S. P. L. Sorensen, and electrodes were used for pH measurement in the 1920s.

In October 1934 Arnold Orville Beckman registered the first patent for a complete chemical instrument for the measurement of pH, U.S. Patent No. 2,058,761, for his "acidometer", later renamed the pH meter.

Beckman developed the prototype as an assistant professor of chemistry at the California Institute of Technology, when asked to devise a quick and accurate method for measuring the acidity of lemon juice for the California Fruit Growers Exchange (Sunkist). On April 8, 1935, Beckman's renamed National Technical Laboratories focused on the making of scientific instruments, with the Arthur H.

Thomas Company as a distributor for its pH meter.131–135. In its first full year of sales, 1936, the company sold 444 pH meters for $60,000 in sales. In years to come, it would bring in millions.

Radiometer in Denmark was founded in 1935, and began marketing a pH meter for medical use around 1936, but "the development of automatic pH-meters for industrial purposes was neglected.

Instead American instrument makers successfully developed industrial pH-meters with a wide variety of applications, such as in breweries, paper works, alum works, and water treatment systems. In 2004 the Beckman pH meter was designated an ACS National Historic Chemical Landmark in recognition of its significance as the first commercially successful electronic pH meter.

In the 1970s Jenco Electronics of Taiwan designed and manufactured the first portable digital pH meter. This meter was sold under Cole-Parmer's label.

Building a pH meter

A basic pH meter essentially measures the potential difference between two electrodes and displays the result calibrated in pH; the electronic circuit is very simple and easily built with a few cheap standard electronic components, plus the specialized pH probe.

1. SCOPE

- This test method is the procedure for determining the pH of water or soil samples by use of a pH meter. However, if the pH of any industrial by-product material (e.g.: cinders, flyash, etc.) is required, the procedure under 3.B. will be followed.

2. APPARATUS AND MATERIALS

- A 0.1 pt. (50 mL), wide-mouth glass beaker with a watch glass for cover. If lightweight material is to be tested, it may be necessary to increase beaker size up to a maximum of 0.5 pt. (250 mL).
- A pH meter, suitable for laboratory or field analysis, with either one or two electrodes.
- Standard buffer solutions of known pH values - standards to be used are pH of 4.0, 7.0, and 10.0.
- Distilled water
- A teaspoon or small scoop
- A thermometer capable of reading 77±18°F (25±10°C) to the nearest 0.1°C.
- A ¼ in. (6.3 mm) sieve conforming to the requirements of AASHTO Designation M-92-91 and a pan.
- A glass stirring rod
- A scale, minimum capacity of 1.1 lb. (500 g). It shall be accurate to 0.1% and be readable to 0.1 g.

3. PROCEDURE

A. Water pH Determination

- Stir the water sample vigorously using a clean glass stirring rod.
- Pour a 40 mL ± 5 mL sample into the glass beaker using the watch glass for a cover.
- Let the sample stand for a minimum of one hour to allow the temperature to stabilize, stirring it occasionally while waiting. Measure the temperature of the sample and adjust the temperature controller of the pH meter to that of the sample temperature. This adjustment should be done just prior to testing.
- On meters with an automatic temperature control, follow the manufacturer's instructions.
- Standardize the pH meter by means of the standard solutions provided. Temperature and adjustments must be performed as stated.

- Immerse the electrode(s) of the pH meter into the water sample and turn the beaker slightly to obtain good contact between the water and the electrode(s).
- The electrode(s) require immersion 30 seconds or longer in the sample before reading to allow the meter to stabilize. If the meter has an auto read system, it will automatically signal when stabilized.
- Read and record the pH value to the nearest tenth of a whole number. If the pH meter reads to the hundredth place, a round off rule will apply as follows: If the hundredth place digit is less than 5, leave the tenth place digit as is. If it is greater than 5, round the tenth place digit up one unit. If the hundredth place digit equals 5, round the tenth place digit to the nearest even number.
- Rinse the electrode(s) well with distilled water, then dab lightly with tissues to remove any film formed on the electrode(s). Caution: Do not wipe the electrodes, as this may result in polarization of the electrode and consequent slow response.

B. SOIL pH DETERMINATION

The material must be separated on the ¼ in. (6.3 mm) sieve. Only the minus ¼ in. (6.3 mm) material is to be used for testing.

- Weigh and place 30±0.1 g of soil into the glass beaker.
- Add 30±0.1 g of distilled water to the soil sample. Stir to obtain soil slurry and then cover with watch glass.
- The sample must stand for a minimum of one hour, stirring every 10 to 15 minutes. This is to allow the pH of the soil slurry to stabilize.
- After one hour, the temperature of the sample should be stabilized. Measure the temperature of the sample and adjust the temperature controller of the pH meter to that of the sample temperature. This adjustment should be done just prior to testing. On meters with an automatic temperature control, follow the manufacturer's instructions.
- Standardize the pH meter by means of the standard solutions provided. Temperature and adjustments must be performed as stated.
- Immediately before immersing the electrode(s) into the sample, stir the sample well with a glass rod. Place the electrode(s) into the soil slurry solution and gently turn beaker to

make good contact between the solution and the electrode(s). DO NOT place electrode(s) into the soil; only into the soil slurry solution.

- The electrode(s) require immersion 30 seconds or longer in the sample before reading to allow the meter to stabilize. If the meter has an auto read system, it will automatically signal when stabilized.
- Read and record the pH value to the nearest tenth of a whole number. If the pH meter reads to the hundredth place, a round off rule will apply as follows: If the hundredth place digit is less than 5, leave the tenth place digit as is. If it is greater than 5, round the tenth place digit up one unit. If the hundredth place digit equals 5, round the tenth place digit to the nearest even number.
- Rinse the electrode(s) well with distilled water, then dab lightly with tissues to remove any film formed on the electrode(s). Caution: Do not wipe the electrodes as this may result in polarization of the electrode(s) and consequent slow response.

NOTE 1 - To standardize the pH meter, use the 7.0 pH buffer standard solutions plus the other standard solution which is nearest the estimated pH value of the sample to be tested. If the manufacturer's instructions indicate a method other than that noted above, then those instructions must be followed.

NOTE 2 - When immersing electrode(s) into the glass beaker, care should be taken not to hit the bottom or side, causing damage to electrode(s).

NOTE 3 - If polarization does occur, as indicated by a slow response, rinse the electrode(s) and dab lightly again.

4. PRECAUTIONS

- Periodically check for damage to electrode(s).
- Electrode tips should be kept moist during storage. Follow manufacturer's instructions.

EXPERIMENT NO-02

***OBJECTIVE*:** To demonstrate the functioning of Spectrophotometer.

INTRODUCTION

In addition to the light microscope, the spectrophotometer is usually found in the biological laboratory. This instrument allows the investigator to identify compounds of biological interest and quantify them. Identification is determined by producing an absorption spectrum of a compound. Quantifying the amount of the material present, usually in solution, is done:

(a) directly if the substance is a strong absorber of a measurable wavelength ( ) of light; (b) indirectly by chemically modifying the compound so that it is a strong absorber at a measurable wavelength of light; or

(c) Indirectly by stoichiometrically coupling its reaction to the formation of another light absorbing compound.

MATERIALS

2,6-dichlorophenol indophenol, graduated cylinders, flasks, stir plates, stir bars, micropipettors, spectrophotometer tubes, rulers, spectrophotometer.

A BEER-LAMBERT LAW

Spectrophotometry, or spectrophotometric analysis, refers to the quantitative determination of the radiant energy ratio of incident to transmitted light beams at a given wavelength.

Spectrophotometers are instruments that allow for the determination of this ratio. Spectrophotometers are designed to make these measurements over a given range of wavelengths of the electromagnetic spectrum.

Usually, in the biological laboratory a spectrophotometer will allow for measurements in the UV and visible wavelengths.

If $\mathbf{P_i}$ is the intensity of the incident beam of light and $\mathbf{P_t}$ is the intensity of the transmitted light, then, by definition, the ratio P_t/P_i equals the **transmittance**, **T**, and log P_i/P_t equals the **absorbance, A**. Thus:

$A = - \log T$ .....(1)

A fundamental law of spectrophotometry is the Beer-Lambert law, or simply Beer's Law that states that the amount of radiant energy absorbed ($\log P_i/P_t$) by a compound in solution is proportional to its concentration and the length of the path that the light beam passes through the solution:

$\log P_i / P_t = A = acl$ .....(2)

Where:

c = concentration

l = path length

a = proportionality constant called **absorptivity**.

The absorptivity at a given wavelength is dependent upon the chemical structure of the compound, which determines the probability that the wavelength of light will be absorbed. The absorptivity constant is sometimes referred to as the **extinction coefficient.**

If the concentration is expressed in molarity, then **a** is called molar absorptivity or **molar extinction coefficient (e)**. Remember that **a** or **e** is dependent upon the wavelength. Conventionally, the path length used is 1 cm, and thus, the units for **e** are cm^{-1} M^{-1}. Note that **A**, therefore, is a unit less value, which it should be, based on its definition above (i.e., a ratio).

A plot of the absorbance of a substance in solution at a given wavelength, as a function of the molar amount of the substance present, is called a calibration plot or **standard curve.** If you examine equation #2, you will see that the slope of a standard curve is **e**. Having determined **e**, and knowing l (the path length is 1 cm), the concentration of the substance in any other similar sample can be determined using equation #2, Beer's Law.

An **absorbance spectrum** of a given compound is a graphical representation of the absorbance at each wavelength over a given wavelength range (usually UV - visible), that is, **A** vs. 8. Chemically different compounds have their own distinctive absorbance spectrum. You will be determining an absorbance spectrum for the electron acceptor 2, 6-dichlorophenol inophenol (DCPIP) and will also plot absorbance spectra in a future lab exercise on plant pigments.

B. The Instrument

There are four essential parts to a spectrophotometer:

1. Light source
2. Monochromator or filter
3. Sample cell with holder
4. Detector

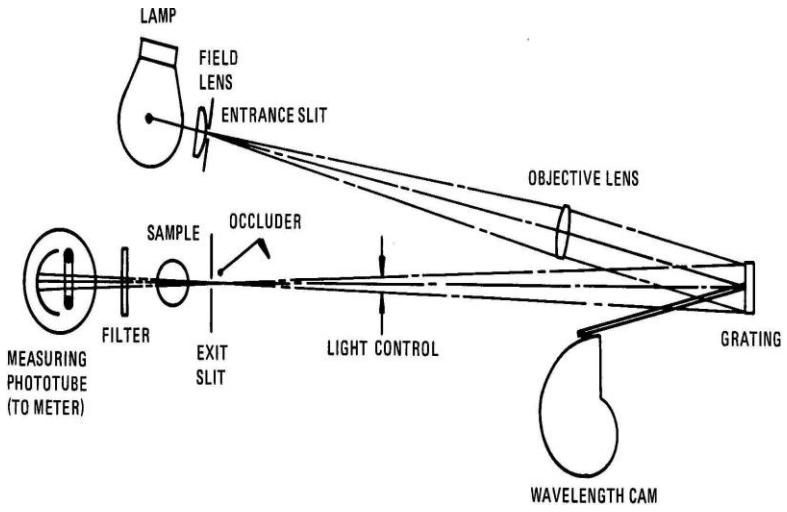

Figure 6.3.Essential parts of the spectrophotometer

The detector converts the radiant energy of the transmitted light into an electrical signal, the strength of which is proportional to the intensity of light transmitted (actually the number of photons striking its surface). A phototube is often used for this purpose. The electronics of the spectrophotometer convert this signal into a reading of transmittance or absorbance.

- Light sources do not emit the same light intensity over the entire spectrum; indeed, each source has its own inherent **emission spectrum.**
- Therefore, when determining an absorbance spectrum, it is important to adjust for the difference in light intensity at each wavelength of incident light.
- This can be done manually (using a "*ZERO CONTROL*" knob) or automatically, depending on the sophistication of the instrument.

C. Directions for Spectronic 20 Genesys ™ Spectrophotometer

1. Choose **A** from the **A/T/C** button to select for absorbance mode.
2. Press nm to select for correct wavelength.
3. Insert your blank into the cell holder and close the sample door.
4. Press the **0 ABS/100 %T** button to zero the absorbance.
5. Remove blank and insert your sample into the cell holder. Close door, and read absorbance from the LED display.

D. Preparing a standard curve and calculating the molar extinction coefficient

1. Prepare 100 ml of 1 mM 2, 6-dichlorophenol indophenol (DCPIP) in distilled water (DW) using the glassware provided. Make sure all the DCPIP goes into solution. *(The molecular weight of DCPIP is 290.08. g mole^{-1})*

2. Dilute the solution 1:10 by adding 10 ml of 1 mM DCPIP to 90 ml of DW to form a 100 M stock solution of DCPIP.

3. Prepare a serial dilution series of different concentrations of DCPIP in the spectrophotometer tubes provided. Take care to label each tube with a marker

pen to indicate the appropriate concentration. Remember to use a new pipette tip for each manipulation.

4. Check that the dilution curve is correct by eye. Tube #9 should be the deepest blue in color.

5. Adjust setting to 500 nm. Zero the spectrophotometer using tube #1.

6. Remove tube 1 and place tube 9 in the spectrophotometer and record the absorbance value.

7. Repeat steps 5 and 6 at 10-nm increments from 500-700 nm. Zero at each wavelength before taking a measurement. Record values in Table. Plot these data (absorbance: y-axis; : x-axis) on the graph paper provided and determine the absorbance maximum, the wavelength at which DCPIP reaches its highest absorbance.

8. Set the wavelength to the absorbance maximum and readjust the zero control at this wavelength.

9. Determine the absorbance of tubes #1-9 at this wavelength. Record values in Table and construct a standard curve on the graph paper provided.

10. Determine the molar extinction coefficient for the dye from your standard curve. Enter this value below, for it will be used in a later laboratory exercise.

Micromolar Extinction Coefficient DCPIP @_____nm = _____

Wavelength (nm)	Absorbance
500	
510	
520	
530	
540	
550	
560	
570	
580	
590	
600	
610	
620	
630	
640	
650	
660	
670	
680	
690	
700	

Table: 1 Absorbance of Tube 9 as a Function of Wavelength

Tube	Absorbance (at $_{max}$)	ml of stock solution	ml distilled water	Final concentration of DCPIP (µM)
1		0.0	5.0	0
2		0.05	4.95	1.0
3		0.25	4.75	5.0
4		0.5	4.50	10.0
5		1.0	4.0	20.0
6		1.5	3.5	30.0
7		2.5	2.5	50.0
8		4.0	1.0	80.0
9		5.0	0.0	100.0

Table 1.1 Absorbance at $_{max}$ as a Function of DCPIP Concentration

11. You are provided with an additional sample containing an unknown concentration of DCPIP. Using your standard curve from part 9, determine the concentration of your unknown.

Unknown _____ Unknown absorbance _____

Unknown Concentration _____

12. knowing the molar extinction coefficient for DCPIP, Determine the concentration of your unknown Using Beer's law. Recall that A = acl

Calculated "unknown" concentration using Beer's law _____

- In chemistry, spectrophotometry is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength.
- It is more specific than the general term electromagnetic spectroscopy in that spectrophotometry deals with visible light, near-ultraviolet, and near-infrared, but does not cover time-resolved spectroscopic techniques.
- Spectrophotometry uses photometers that can measure a light beam's intensity as a function of its color (wavelength) known as spectrophotometers.
- Important features of spectrophotometers are spectral bandwidth (the range of colors it can transmit through the test sample), the percentage of sample-transmission, the logarithmic range of sample-absorption, and sometimes a percentage of reflectance measurement.
- A spectrophotometer is commonly used for the measurement of transmittance or reflectance of solutions, transparent or opaque solids, such as polished glass, or gases.
- However they can also be designed to measure the diffusivity on any of the listed light ranges that usually cover around 200 nm - 2500 nm using different controls and calibrations.
- Within these ranges of light, calibrations are needed on the machine using standards that vary in type depending on the wavelength of the **photometric determination.**

An example of an experiment in which spectrophotometry is used is the determination of the equilibrium constant of a solution. A certain chemical reaction within a solution may occur in a forward and reverse direction where reactants form products and products break down into reactants. At some point, this chemical reaction will reach a point of balance called an equilibrium point. In order to determine the respective concentrations of reactants and products at this point, the light transmittance of the solution can be tested using spectrophotometry. The amount of light that passes through the solution is indicative of the concentration of certain chemicals that do not allow light to pass through.

- The use of spectrophotometers spans various scientific fields, such as physics, materials science, chemistry, biochemistry, and molecular biology.

- They are widely used in many industries including semiconductors, laser and optical manufacturing, printing and forensic examination, and as well in laboratories for the study of chemical substances.
- Ultimately, a spectrophotometer is able to determine, depending on the control or calibration, what substances are present in a target and exactly how much through calculations of observed wavelengths.

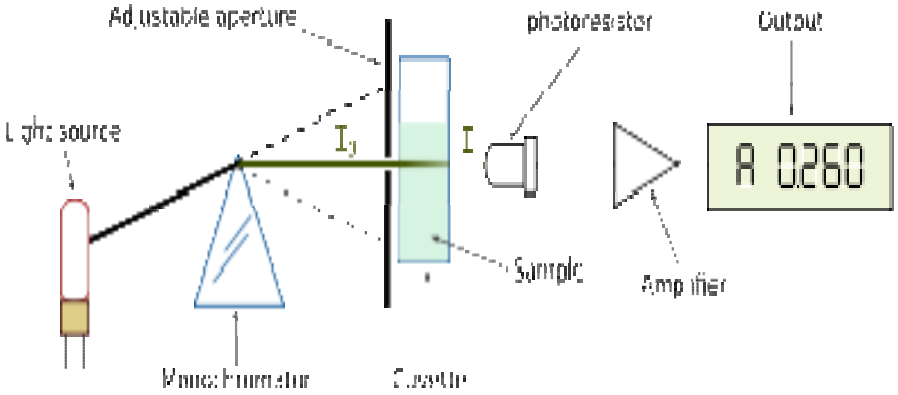

Figure 6.4 Single beam spectrophotometer

There are two major classes of devices: single beam and double beam.

- A double beam spectrophotometer compares the light intensity between two light paths, one path containing a reference sample and the other the test sample.
- A single-beam spectrophotometer measures the relative light intensity of the beam before and after a test sample is inserted.
- Although comparison measurements from double-beam instruments are easier and more stable, single-beam instruments can have a larger dynamic range and are optically simpler and more compact.
- Additionally, some specialized instruments, such as spectrophotometers built onto microscopes or telescopes, are single-beam instruments due to practicality.

Historically, spectrophotometers use a monochromator containing a diffraction grating to produce the analytical spectrum.

The grating can either be movable or fixed. If a single detector, such as a photomultiplier tube or photodiode is used, the grating can be scanned stepwise so that the detector can measure the light intensity at each wavelength (which will correspond to each "step"). Arrays of detectors, such as charge coupled devices (CCD) or photodiode arrays (PDA) can also be used.

In such systems, the grating is fixed and the intensity of each wavelength of light is measured by a different detector in the array. Additionally, most modern mid-infrared spectrophotometers use a Fourier transform technique to acquire the spectral information. The technique is called Fourier transform infrared spectroscopy.

When making transmission measurements, the spectrophotometer quantitatively compares the fraction of light that passes through a reference solution and a test solution, then electronically compares the intensities of the two signals and computes the percentage of transmission of the sample compared to the reference standard. For reflectance measurements, the spectrophotometer quantitatively compares the fraction of light that reflects from the reference and test samples.

Light from the source lamp is passed through a monochromator, which diffracts the light into a "rainbow" of wavelengths and outputs narrow bandwidths of this diffracted spectrum through a mechanical slit on the output side of the monochromator. These bandwidths are transmitted through the test sample.

Then the photon flux density (watts per metre squared usually) of the transmitted or reflected light is measured with a photodiode, charge coupled device or other light sensor. The transmittance or reflectance value for each wavelength of the test sample is then compared with the transmission or reflectance values from the reference sample. Most instruments will apply a logarithmic function to the linear transmittance ratio to calculate the 'absorbency' of the sample, a value which is proportional to the 'concentration' of the chemical being measured.

In short, the sequence of events in a modern spectrophotometer is as follows:

1. The light source is shone into a monochromator, diffracted into a rainbow, and split into two beams. It is then scanned through the sample and the reference solutions.
2. Fractions of the incident wavelengths are transmitted through, or reflected from, the sample and the reference.
3. The resultant light strikes the photodetector device, which compares the relative intensity of the two beams.
4. Electronic circuits convert the relative currents into linear transmission percentages and/or absorbance/concentration values.

Many older spectrophotometers must be calibrated by a procedure known as "zeroing", to balance the null current output of the two beams at the detector. The transmission of a reference substance is set as a baseline (datum) value, so the transmissions of all other substances are recorded relative to the initial "zeroed" substance. The spectrophotometer then converts the transmission ratio into 'absorbency', the concentration of specific components of the test sample relative to the initial substance.

APPLICATIONS IN BIOCHEMISTRY

Spectrophotometry is an important technique used in many biochemical experiments that involve DNA, RNA, and protein isolation, enzyme kinetics and biochemical analyses.

A brief explanation of the procedure of spectrophotometry includes comparing the absorbency of a blank sample that does not contain a colored compound to a sample that contains a colored compound.

The spectrophotometer is used to measure colored compounds in the visible region of light (between 350 nm and 800 nm), thus it can be used to find more information about the substance being studied.

In biochemical experiments, a chemical and/or physical property is chosen and the procedure that is used is specific to that property in order to derive more information about the sample, such as the quantity, purity, enzyme activity, etc.

Spectrophotometry is also a helpful procedure for protein purification and can also be used as a method to create optical assays of a compound. Because a spectrophotometer measures the wavelength of a compound through its color, a dye binding substance can be added so that it can undergo a color change and be measured.

Spectrophotometers have been developed and improved over decades and have been widely used among chemists. It is considered to be a highly accurate instrument that is also very sensitive and therefore extremely precise, especially in determining color change.

This method is also convenient for use in laboratory experiments because it is an inexpensive and relatively simple process.

UV-visible spectrophotometry

The most common spectrophotometers are used in the UV and visible regions of the spectrum, and some of these instruments also operate into the near-infrared region as well.

Visible region 400–700 nm spectrophotometry is used extensively in colorimetry science. It is a known fact that it operates best at the range of 0.2-0.8 O.D.

Ink manufacturers, printing companies, textiles vendors, and many more, need the data provided through colorimetry. They take readings in the region of every 5–20 nanometers along the visible region, and produce a spectral reflectance curve or a data stream for alternative presentations.

These curves can be used to test a new batch of colorant to check if it makes a match to specifications, e.g., ISO printing standards.

Traditional visible region spectrophotometers cannot detect if a colorant or the base material has fluorescence. This can make it difficult to manage color issues if for example one or more of the printing inks is fluorescent.

Where a colorant contains fluorescence, a bi-spectral fluorescent spectrophotometer is used. There are two major setups for visual spectrum spectrophotometers, d/8 (spherical) and 0/45.

The names are due to the geometry of the light source, observer and interior of the measurement chamber. Scientists use this instrument to measure the amount of compounds in a sample.

If the compound is more concentrated more light will be absorbed by the sample; within small ranges, the Beer-Lambert law holds and the absorbance between samples vary with concentration linearly.

In the case of printing measurements two alternative settings are commonly used- without/with UV filter to control better the effect of UV brighteners within the paper stock.

Samples are usually prepared in cuvettes; depending on the region of interest, they may be constructed of glass, plastic (visible spectrum region of interest), or quartz (Far UV spectrum region of interest).

APPLICATIONS

- Estimating dissolved organic carbon concentration
- Specific Ultraviolet Absorption for metric of aromaticity
- Bial's Test for concentration of pentoses

Infrared Spectrophotometry

Spectrophotometers designed for the infrared region are quite different because of the technical requirements of measurement in that region.

One major factor is the type of photo sensors that are available for different spectral regions, but infrared measurement is also challenging because virtually everything emits IR light as thermal radiation, especially at wavelengths beyond about 5 μm.

Another complication is that quite a few materials such as glass and plastic absorb infrared light, making it incompatible as an optical medium. Ideal optical materials are salts, which do not absorb strongly.

Samples for IR spectrophotometry may be smeared between two discs of potassium bromide or ground with potassium bromide and pressed into a pellet. Where aqueous solutions are to be measured, insoluble silver chloride is used to construct the cell.

Spectroradiometers

Spectroradiometers, which operate almost like the visible region spectrophotometers, are designed to measure the spectral density of illuminants.

Applications may include evaluation and categorization of lighting for sales by the manufacturer, or for the customers to confirm the lamp they decided to purchase is within their specifications.

COMPONENTS

1. The light source shines onto or through the sample.
2. The sample transmits or reflects light.
3. The detector detects how much light was reflected from or transmitted through the sample.
4. The detector then converts how much light the sample transmitted or reflected into a number.

EXPERIMENT NO - 03

OBJECTIVE: To demonstrate of use of bright-field microscopy

INTRODUCTION

Bright-field microscopy is the simplest of all the optical microscopy illumination techniques. Sample illumination is transmitted (i.e., illuminated from below and observed from above) white light and contrast in the sample is caused by absorbance of some of the transmitted light in dense areas of the sample.

Bright-field microscopy is the simplest of a range of techniques used for illumination of samples in light microscopes and its simplicity makes it a popular technique. The typical appearance of a bright-field microscopy image is a dark sample on a bright background, hence the name.

Light path

The light path of a bright-field microscope is extremely simple; no additional components are required beyond the normal light microscope setup. The light path therefore consists of:

- A transillumination light source, commonly a halogen lamp in the microscope stand;
- A condenser lens which focuses light from the light source onto the sample; and
- Objective lens which collects light from the sample and magnifies the image.
- Oculars and/or a camera to view the sample image

Bright field microscopy may use critical or Köhler illumination to illuminate the sample.

Performance

Bright-field microscopy typically has low contrast with most biological samples as few absorb light to a great extent. Staining is often required to increase contrast, which prevents use on live cells in many situations. Bright field illumination is useful for samples which have an intrinsic colour, for example chloroplasts in plant cells.Comparison of transillumination techniques used to generate contrast in a sample of tissue paper. 1.559 µm/pixel. Bright field illumination, sample contrast comes from absorbance of light in the sample.

Cross-polarized light illumination, sample contrast comes from the rotation of polarized light through the sample. Dark field illumination, sample contrast comes from light scattered by the sample.

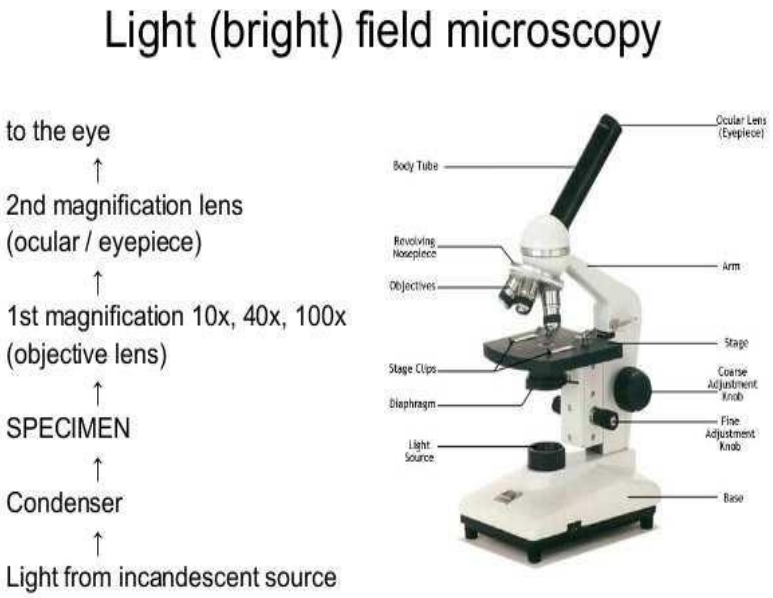

Figure 6.5 Light field microscopy

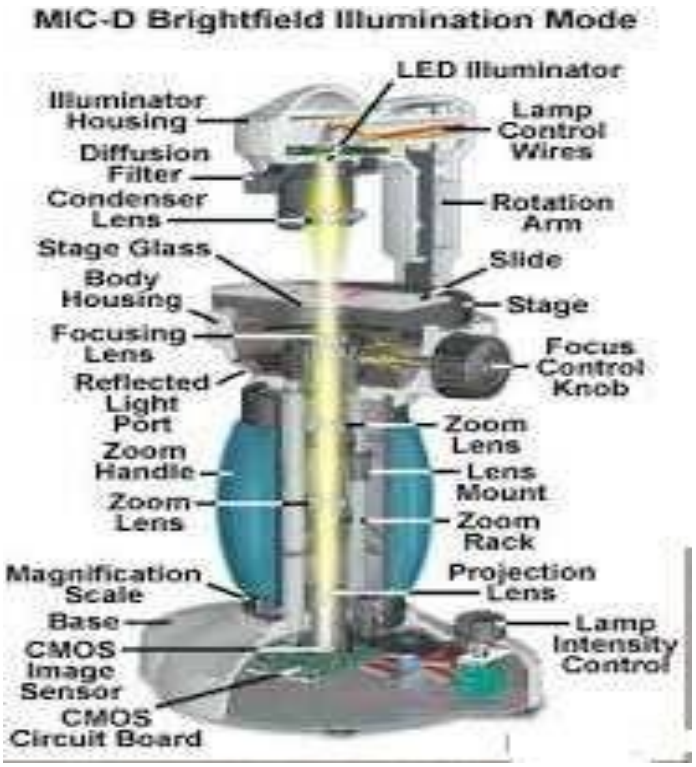

Figure 6.6 Bright field Illumination Mode

Phase contrast illumination, sample contrast comes from interference of different path lengths of light through the sample. Bright-field microscopy is a standard light microscopy technique, and therefore magnification is limited by the resolving power possible with the wavelength of visible light.

ADVANTAGES

- Simplicity of setup with only basic equipment required.

Limitations

- Very low contrast of most biological samples.
- Low apparent optical resolution due to the blur of out of focus material.

- Samples that are naturally colorless and transparent cannot be seen well, e.g. many types of mammalian cells.
- These samples often have to be stained before viewing. Samples that do have their own colour can be seen without preparation, e.g. the observation of cytoplasmic streaming in Chara cells.

Enhancements

- Reducing or increasing the amount of the light source via the iris diaphragm.
- Use of an oil immersion objective lens and special immersion oil placed on a glass cover over the specimen. Immersion oil has the same refraction as glass and improves the resolution of the observed specimen.
- Use of sample staining methods for use in microbiology, such as simple stains (Methylene blue, Safranin, Crystal violet) and differential stains (Negative stains, flagellar stains, endospore stains).
- Use of a colored (usually blue) or polarizing filter on the light source to highlight features not visible under white light. The use of filters is especially useful with mineral samples

EXPERIMENT NO – 3.1

OBJECTIVE: To demonstrate of use of Phase contrast microscopy

INTRODUCTION

Phase contrast microscopy, first described in 1934 by Dutch physicist Frits Zernike, is a contrast-enhancing optical technique that can be utilized to produce high-contrast images of transparent specimens, such as living cells (usually in culture), microorganisms, thin tissue slices, lithographic patterns, fibers, latex dispersions, glass fragments, and subcellular particles (including nuclei and other organelles).

In effect, the phase contrast technique employs an optical mechanism to translate minute variations in phase into corresponding changes in amplitude, which can be visualized as differences in image contrast.

One of the major advantages of phase contrast microscopy is that living cells can be examined in their natural state without previously being killed, fixed, and stained.

As a result, the dynamics of ongoing biological processes can be observed and recorded in high contrast with sharp clarity of minute specimen detail.

In **f**igure **1** is a cut-away diagram of a modern upright phase contrast microscope, including a schematic illustration of the phase contrast optical train.

Partially coherent illumination produced by the tungsten-halogen lamp is directed through a collector lens and focused on a specialized annulus (labeled **condenser annulus**) positioned in the sub stage condenser front focal plane.

Wave fronts passing through the annulus illuminate the specimen and either passes through undeviated or is diffracted and retarded in phase by structures and phase gradients present in the specimen.

Undeviated and diffracted light collected by the objective is segregated at the rear focal plane by a **phase plate** and focused at the intermediate image plane to form the final phase contrast image observed in the eyepieces.

Prior to the invention of phase contrast techniques, transmitted bright field illumination was one of the most commonly utilized observation modes in optical microscopy, especially for fixed, stained specimens or other types of samples having high natural absorption of visible light.

Collectively, specimens readily imaged with bright field illumination are termed **amplitude objects** (or specimens) because the amplitude or intensity of the illuminating wave fronts is reduced when light passes through the specimen.

The addition of phase contrast optical accessories to a standard bright field microscope can be employed as a technique to render a contrast-enhancing effect in transparent specimens that is reminiscent of optical staining.

Light waves that are diffracted and shifted in phase by the specimen (termed a **phase object**) can be transformed by phase contrast into amplitude differences that are observable in the eyepieces.

Large, extended specimens are also easily visualized with phase contrast optics due to diffraction and scattering phenomena that occur at the edges of these objects.

The performance of modern phase contrast microscopes is so refined that it enables specimens containing very small internal structures, or even just a few protein molecules, to be detected when the technology is coupled to electronic enhancement and post-acquisition image processing.

In figure is a comparison of living cells in culture imaged in both bright field and phase contrast illumination.

The cells are human glial brain tissue grown in monolayer culture bathed with a nutrient medium containing amino acids, vitamins, mineral salts, and fetal calf serum.

In bright field illumination, the cells appear semi-transparent with only highly refractive regions, such as the membrane, nucleus, and unattached cells (rounded or spherical), being visible.

When observed using phase contrast optical accessories, the same field of view reveals significantly more structural detail. Cellular attachments become discernable, as does much of the internal structure. In addition, the contrast range is dramatically improved.

Interaction of Light Waves with Phase Specimens

An incident wave front present in an illuminating beam of light becomes divided into two components upon passing through a phase specimen.

The primary component is an undeviated (or undiffracted; **zeroth-order**) planar wave front, commonly referred to as the **surround** (**S**) wave, which passes through and around the specimen, but does not interact with it.

In addition, a deviated or **diffracted** spherical wave front (**D**-wave) is also produced, which becomes scattered over a wide arc (in many directions) that passes through the full aperture of the objective.

After leaving the specimen plane, surround and diffracted light waves enter the objective front lens element and are subsequently focused at the intermediate image plane where they combine through interference to produce a resultant **particle** wave (often referred to as a **P**-wave). The mathematical relationship between the various light waves generated in phase contrast microscopy can be described simply as:

Formula 1 - Relationship between Various Light Waves Generated in Phase Contrast Microscopy $P = S + D$

Detection of the specimen image depends on the relative intensity differences, and therefore on the amplitudes, of the particle and surround (**P** and **S**) waves.

If the amplitudes of the particle and surround waves are significantly different in the intermediate image plane, then the specimen acquires a considerable amount of contrast and is easily visualized in the microscope eyepieces.

Otherwise, the specimen remains transparent and appears as it would under ordinary bright field conditions (in the absence of phase contrast or other contrast-enhancing techniques).

In terms of optical path variations between the specimen and its surrounding medium, the portion of the incident light wave front that traverses the specimen (**D**-wave), but does not pass through the surrounding medium (**S**-wave), is slightly retarded.

For arguments in phase contrast microscopy, the role of the specimen in altering the optical path length (in effect, the relative phase shift) of waves passing through is of paramount importance.

In classical optics, the optical path length (**OPL**) through an object or space is the product of the refractive index (**n**) and the thickness (**t**) of the object or intervening medium as described by the relationship:

Formula 2 - Optical Path Length

Optical Path Length (OPL) = $n \times t$

When light passes from one medium into another, the velocity is altered proportionally to the refractive index differences between the two media.

Thus, when a coherent light wave emitted by the focused microscope filament passes through a phase specimen having a specific thickness (**t**) and refractive index (**n**), the wave is either increased or decreased in velocity.

If the refractive index of the specimen is greater than that of the surrounding medium, the wave is reduced in velocity while passing through the specimen and is subsequently retarded in relative phase when it emerges from the specimen.

In contrast, when the refractive index of the surrounding medium exceeds that of the specimen, the wave is advanced in phase upon exiting the specimen. The difference in location of an emergent wave front between the specimen and surrounding medium is termed the **phase shift** (δ) and is defined in radians as:

Formula 3 - Phase Shift $\delta = 2\pi\Delta/\lambda$

In the equation above, the term **D** is referred to as the **optical path difference**, which is similar to the optical path length:

Formula 4 - Optical Path Difference

Optical Path Difference (OPD) = $\Delta = (n_2 - n_1) \times t$

Where **n(2)** is the refractive index of the specimen and **n(1)** is the refractive index of the surrounding medium.

The optical path difference results from the product of two terms: the thickness of the specimen, and its difference in refractive index with the surrounding medium.

In many cases, the optical path difference can be quite large even though the thickness of the specimen is small.

On the other hand, when the refractive index of the specimen equals that of the surrounding medium, the optical path difference is zero regardless of whether the specimen thickness is large or small.

Wave Interactions in Phase Contrast Microscopy

Phase relationships between the surround, diffracted, and particle (**S**, **D**, and **P**) waves in the region of the specimen at the image plane for bright field microscopy (in the absence of phase contrast optical accessories) are presented in figure.

The surround and particle waves, whose relative amplitudes determine the amount of specimen contrast, are illustrated as red and green lines (respectively).

The wave produced by diffraction from the specimen, which is never directly observed, is depicted as a blue wave of lower amplitude.

The surround and diffracted waves recombine through interference to generate the resultant particle wave in the image plane of the microscope.

The amplitude of each wave illustrated in figure represents the sum of the electric vectors of the individual component waves.

Interpretation of Phase Contrast Images

Images produced by phase contrast microscopy are relatively simple to interpret when the specimen is thin and distributed evenly on the substrate (as is the case with living cells grown in monolayer tissue culture).

When thin specimens are examined using positive phase contrast optics, which is the traditional form produced by most manufacturers, they appear darker than the surrounding medium when the refractive index of the specimen exceeds that of the medium.

Phase contrast optics differentially enhance the contrast near the edges surrounding extended specimens, such as the boundary between a cellular membrane and the bathing nutrient medium, and produce overall high-contrast images that can be roughly interpreted as density maps.

Because the amplitude and intensity of a specimen image in phase contrast is related to refractive index and optical path length, image density can be utilized as a gauge for approximating relationships between various structures.

In effect, a series of internal cellular organelles having increasing density, such as vacuoles, cytoplasm, the inter phase nucleus, and the nucleolus (or mitotic chromosomes), are typically visualized as progressively darker objects relative to a fixed reference, such as the background.

It should also be noted that numerous optical artifacts are present in all phase contrast images, and large extended specimens often present significant fluctuations in contrast and image intensity.

Symmetry can also be an important factor in determining how both large and small specimens appear in the phase contrast microscope.

Sensible interpretation of phase contrast images requires careful scrutiny and examination to ensure that artifacts are not incorrectly assigned to important structural features.

For example, some internal cellular organelles and components often have a lower refractive index than that of the surrounding cytoplasm, while others have a higher refractive index.

Because of the varying refractive indices exhibited by these numerous intracellular structures, the interior of living cells, when viewed in a positive phase contrast microscope, can reveal an array of intensities ranging from very bright to extremely dark.

For example, pinocytotic vesicles, lipid droplets, and air vacuoles present in plants and single cell protozoan's have a lower refractive index than the cytoplasm, and thus appear brighter than other components. In contrast, as discussed above, organelles that have high refractive indices (nuclei, ribosome, mitochondria, and the nucleolus) appear dark in the microscope. If the phase retardation introduced by the specimen is large enough (a phase shift of the diffracted wave by approximately a half-wavelength), interference between the diffracted waves and the surround waves becomes constructive, rendering these specimens brighter than the surrounding background.

In order to avoid confusion regarding bright and dark contrast in phase contrast images, the optical path differences occurring within the specimen preparation should be carefully considered.

As discussed above, the optical path difference is derived from the product of the refractive index and the specimen (object) thickness, and is related to the relative phase shift between the specimen and background (diffracted and surround) waves.

It is impossible to distinguish between high and low refractive index components in a phase contrast image without information pertaining to the relative thickness of the components.

For example, a small specimen having a high refractive index can display an identical optical path difference to a larger specimen having a lower refractive index. The two specimens will have approximately the same intensity when viewed through a phase contrast optical system.

In many biological experiments, conditions that produce a shrinking or swelling of cells or organelles can result in significant contrast variations. The external medium can also be replaced with another having either a higher or lower refractive index to generate changes in specimen image contrast.

In fact, the effect on image contrast of refractive index variations in the surrounding medium forms the basis.

Fig 6.8 *Dark field and phase contrast microcopies operating principle*

Fig.6.9 A phase-contrast microscope

WORKING PRINCIPLE

The basic principle to making phase changes visible in phase-contrast microscopy is to separate the illuminating (background) light from the specimen-scattered light (which makes up the foreground details) and to manipulate these differently.

The ring-shaped illuminating light (green) that passes the condenser annulus is focused on the specimen by the condenser. Some of the illuminating light is scattered by the specimen (yellow).

The remaining light is unaffected by the specimen and forms the background light (red). When observing an unstained biological specimen, the scattered light is weak and typically phase-shifted by −90° (due to both the typical thickness of specimens and the refractive index difference between biological tissue and the surrounding medium) relative to the background light.

This leads to the foreground (blue vector) and background (red vector) having nearly the same intensity, resulting in low image contrast.

In a phase-contrast microscope, image contrast is increased in two ways: by generating constructive interference between scattered and background light rays in regions of the field of view that contain the specimen, and by reducing the amount of background light that reaches the image plane.

First, the background light is phase-shifted by −90° by passing it through a phase-shift ring, which eliminates the phase difference between the background and the scattered light rays.

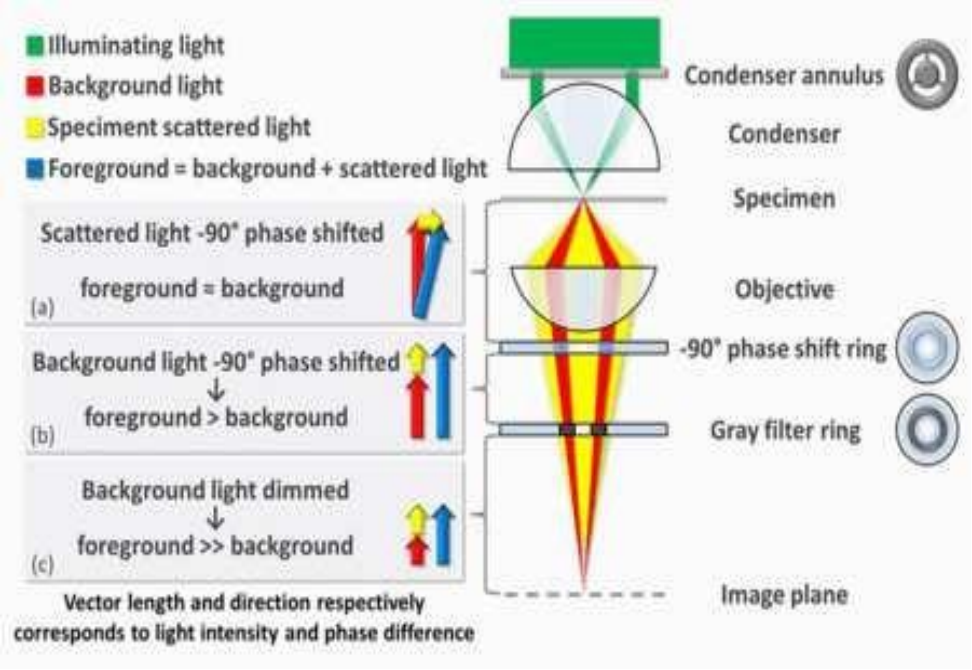

Fig 6.10 Operating principle dark field and phase contrast microcopies

EXPERIMENT NO- 3.2

OBJECTIVE: To demonstrate of use of dark field microscopy

INTRODUCTION

Have you ever heard of a dark field microscope? While such a name may sound like a sci-fi gadget used to measure black holes, in reality it's just a handy tool used to view certain types of translucent samples.

The average microscope user may not know about the concept of dark field microscopy, yet it can shed new light on the old way of viewing specimens.

Most people who have survived a biology class know what a light field microscope is. This type of scope uses bright field illumination, meaning it floods the specimen with white light from the condenser without any interference.

Thus the specimen shows up as a dark image on a light background (or white field if you will).

This type of unit works best with specimens that have natural color pigments. The samples need to be thick enough to absorb the incoming light; so staining is usually paired with this type of microscope.

Yet what if the specimen is light colored or translucent, like the plankton on the right? It certainly won't stand out against a strong white background. Additionally, some specimens are just too thin.

They cannot absorb any of the light that passes through them, so they appear invisible to the user. This is where the concept of dark field illumination comes in!

Rather than using direct light from the condenser, one uses an opaque disk to block the light into just a few scattered beams.

Now the background is dark, and the sample reflects the light of the beams only. This results in a light colored specimen against a dark background (dark field), perfect for viewing clear or translucent details.

On a grand scale, the same thing happens every day when you look up at the sky. Do the stars disappear when it's light out? Of course not! They're still there, their brilliance blotted out by the mid-day sun.

If you're still having a hard time visualizing this concept, think of a dusty room with the light on and the door open. You may feel the dust affecting your breathing, but you probably won't see it flying through the air.

Now turn off the light and close the door to just a sliver, while leaving the light on in the adjacent room. If you look at that sliver of light coming through the door, you'll see all sorts of dust motes suspended in it. You're employing a similar principle when you use dark field illumination!

Use

Dark field microscopes are used in a number of different ways to view a variety of specimens that are hard to see in a light field unit.

Live bacteria, for example, are best viewed with this type of microscope, as these organisms are very transparent when unstained.

There are multitudes of other ways to use dark field illumination, often when the specimen is clear or translucent. Some examples:

- Living or lightly stained transparent specimens
- Single-celled organisms
- Live blood samples
- Aquatic environment samples (from seawater to pond water)
- Living bacteria
- Hay or soil samples
- Pollen samples
- Certain molecules such as caffeine crystals (right)

Dark field microscopy makes many invisible specimens appear visible. Most of the time the specimens invisible to bright field illumination are living, so you can see how important it is to bring them into view!

ADVANTAGES AND DISADVANTAGES

No one system is perfect and dark field microscopy may or may not appeal to you depending on your needs. Some advantages of using a dark field microscope are:

- Extremely simple to use
- Inexpensive to set up (instructions on how to make your own dark field microscope are below)
- Very effective in showing the details of live and unstained samples

Some of the disadvantages are:

- Limited colors (certain colors will appear, but they're less accurate and most images will be just black and white)
- Images can be difficult to interpret to those unfamiliar with dark field microscopy
- Although surface details can be very apparent, the internal details of a specimen often don't stand out as much with a dark field setup.

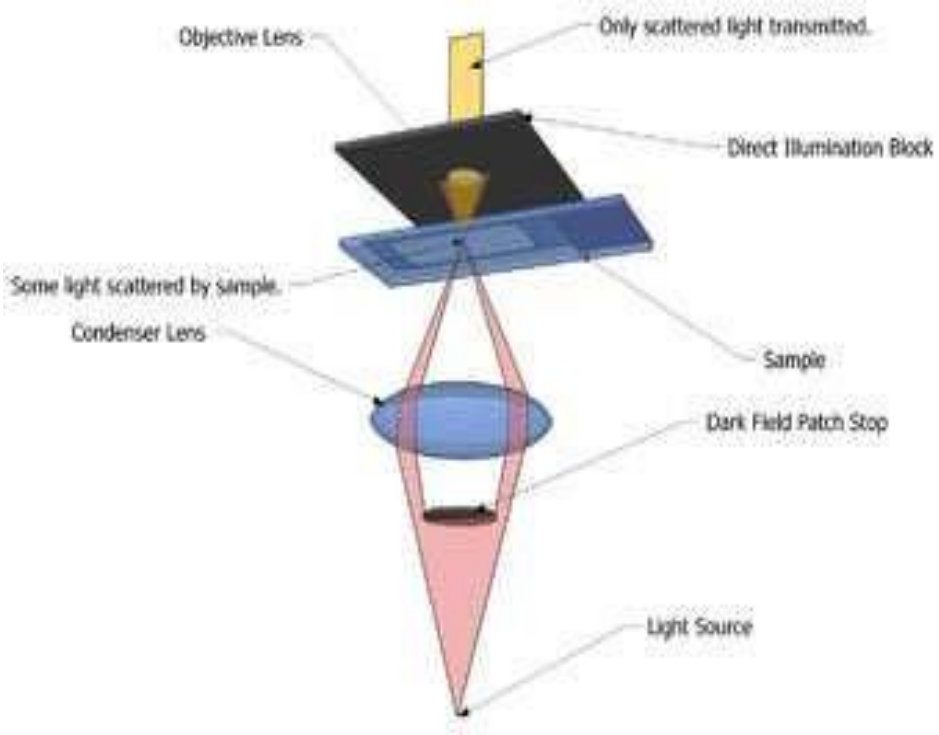

Fig 6.11Contrasting examples of dark field illumination

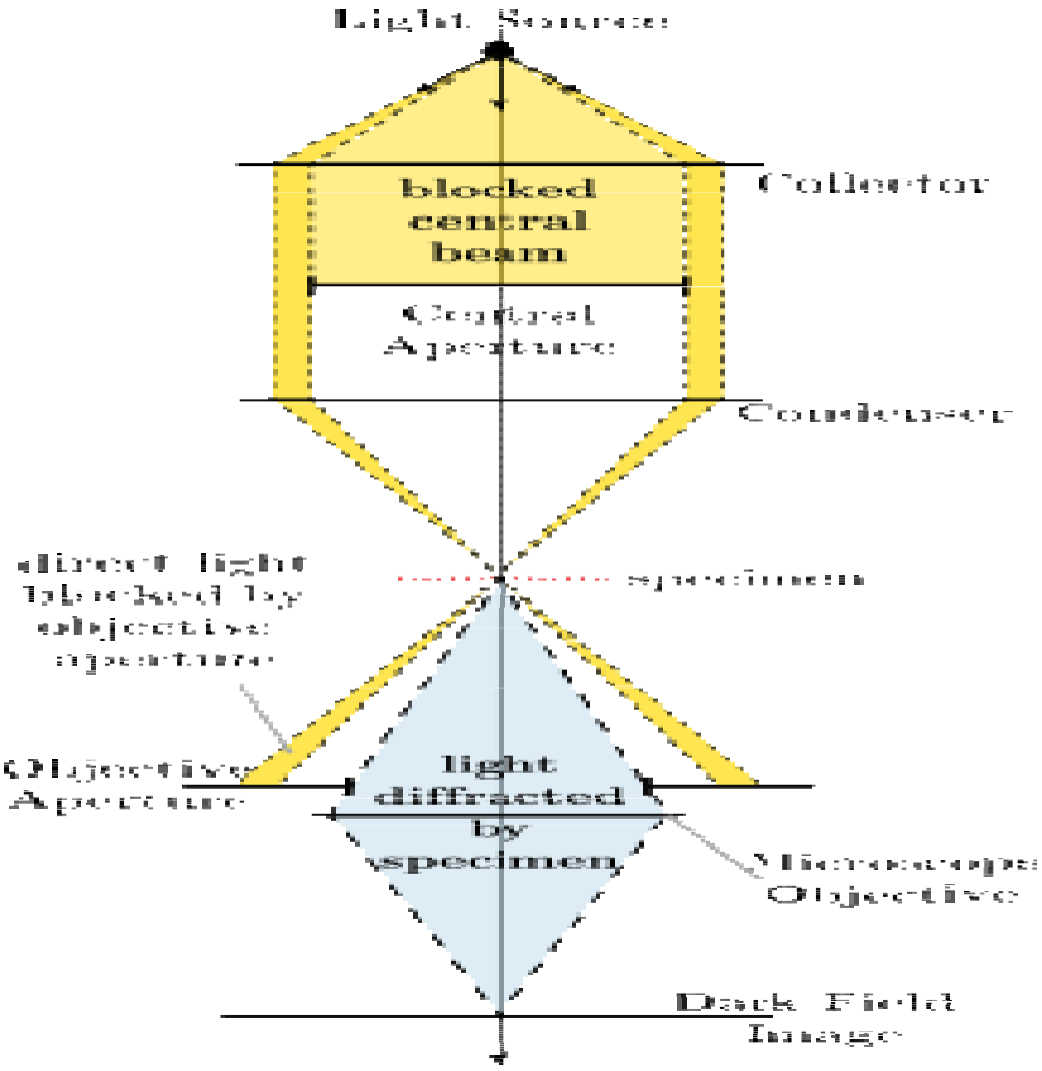

Fig 6.12 Diagram illustrating the light path through a dark field microscope

Digital dark field analysis

This a mathematical technique intermediate between direct and reciprocal (Fourier-transform) space for exploring images with well-defined periodicities, like electron microscope lattice-fringe images.

As with analog dark field imaging in a transmission electron microscope,it allows one to "light up" those objects in the field of view where periodicities of interest reside.

Unlike analog dark field imaging it may also allow one to map the Fourier-phase of periodicities, and hence phase-gradients which provide quantitative information on vector lattice-strain.

Experiment No- 3.3

OBJECTIVE: To demonstrate of use of Fluorescent microscopy

INTRODUCTION

The absorption and subsequent re-radiation of light by organic and inorganic specimens is typically the result of well-established physical phenomena described as being either **fluorescence** or **phosphorescence**.

The emission of light through the fluorescence process is nearly simultaneous with the absorption of the excitation light due to a relatively short time delay between photon absorption and emission, ranging usually less than a microsecond in duration.

When emission persists longer after the excitation light has been extinguished, the phenomenon is referred to as phosphorescence.

A fluorescence microscope is much the same as a conventional light microscope with added features to enhance its capabilities.

- The conventional microscope uses visible light (400-700 nanometers) to illuminate and produce a magnified image of a sample.
- A fluorescence microscope, on the other hand, uses a much higher intensity light source which excites a fluorescent species in a sample of interest. This fluorescent species in turn emits a lower energy light of a longer wavelength that produces the magnified image instead of the original light source.

Fluorescent microscopy is often used to image specific features of small specimens such as microbes. It is also used to visually enhance 3-D features at small scales.

This can be accomplished by attaching fluorescent tags to anti-bodies that in turn attach to targeted features, or by staining in a less specific manner.

When the reflected light and background fluorescence is filtered in this type of microscopy the targeted parts of a given sample can be imaged.

This gives an investigator the ability to visualize desired organelles or unique surface features of a sample of interest. Confocal fluorescent microscopy is most often used to accentuate the 3-D nature of samples.

This is achieved by using powerful light sources, such as lasers, that can be focused to a pinpoint. This focusing is done repeatedly throughout one level of a specimen after another.

Most often an image reconstruction program pieces the multi level image data together into a 3-D reconstruction of the targeted sample.

Fig 6.13Fluorescent Microscopy

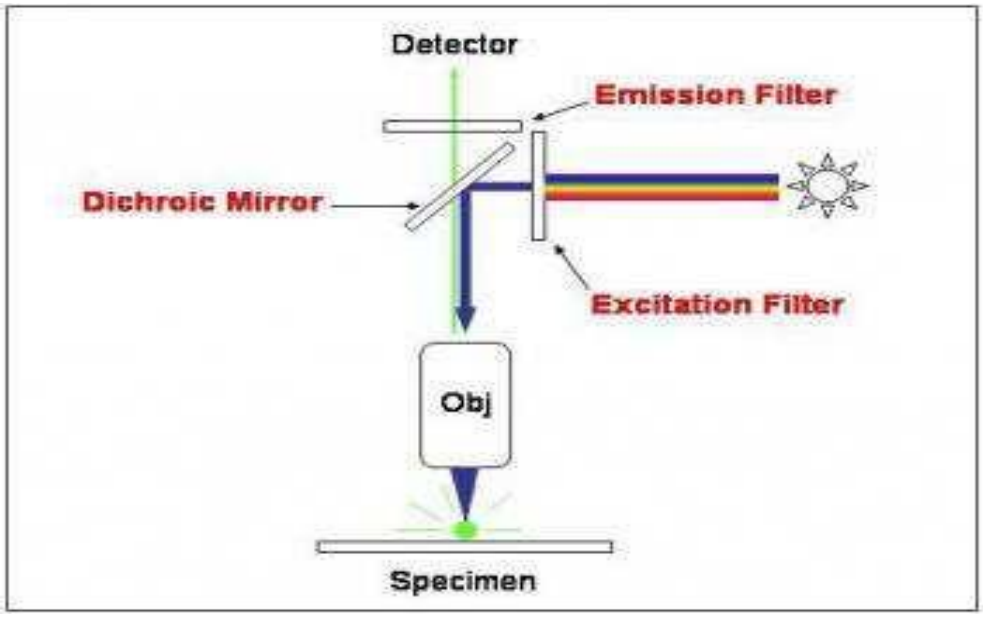

Figure 6.14 showing the filters and mirror in a fluorescent microscope

How does Fluorescent Microscopy Work

In most cases the sample of interest is labeled with a fluorescent substance known as a fluorophore and then illuminated through the lens with the higher energy source.

The illumination light is absorbed by the fluorophores (now attached to the sample) and causes them to emit a longer lower energy wavelength light.

This fluorescent light can be separated from the surrounding radiation with filters designed for that specific wavelength allowing the viewer to see only that which is fluorescing.

The basic task of the fluorescence microscope is to let excitation light radiate the specimen and then sort out the much weaker emitted light from the image.

First, the microscope has a filter that only lets through radiation with the specific wavelength that matches your fluorescing material.

The radiation collides with the atoms in your specimen and electrons are excited to a higher energy level.

When they relax to a lower level, they emit light. To become detectable (visible to the human eye) the fluorescence emitted from the sample is separated from the much brighter excitation light in a second filter.

This works because the emitted light is of lower energy and has a longer wavelength than the light that is used for illumination.

Most of the fluorescence microscopes used in biology today is epi-fluorescence microscopes, meaning that both the excitation and the observation of the fluorescence occur above the sample.

Most use a Xenon or Mercury arc-discharge lamp for the more intense light source.

APPLICATIONS

The refinement of epi-fluorescent microscopes and advent of more powerful focused light sources, such as lasers, has led to more technically advanced scopes such as the confocal laser scanning microscopes and total internal reflection fluorescence microscopes (TIRF).

CLSM's are invaluable tools for producing high resolution 3-D images of subsurfaces in specimens such as microbes.

Their advantage is that they are able to produce sharp images of thick samples at various depths by taking images point by point and reconstructing them with a computer rather than viewing whole images through an eyepiece.

These microscopes are often used for -

- Imaging structural components of small specimens, such as cells
- Conducting viability studies on cell populations (are they alive or dead?)
- Imaging the genetic material within a cell (DNA and RNA)
- Viewing specific cells within a larger population with techniques such as FISH

Fluorescence Light Sources

An unfortunate consequence of low emission levels in most fluorescence microscopy applications is that the number of photons that reach the eye or camera detector is also very low.

In most cases, the collection efficiency of optical microscopes is less than 30 percent and the concentration of many fluorophores in the optical path ranges in the micromolar or nanomolar regions.

In order to generate sufficient excitation light intensity to produce detectable emission, powerful compact light sources, such as high-energy short arc-discharge lamps, are necessary.

The most common lamps are mercury burners, ranging in wattage from 50 to 200 Watts, and the xenon burners that range from 75 to 150 Watts.

These light sources are usually powered by an external direct current supply, furnishing enough start-up power to ignite the burner through ionization of the gaseous vapor and to keep it burning with a minimum of flicker.

The microscope arc-discharge lamp external power supply is usually equipped with a timer to track the number of hours the burner has been in operation.

Arc lamps lose efficiency and are more likely to shatter if used beyond their rated lifetime (200-300 hours).

The mercury burners do not provide even intensity across the spectrum from ultraviolet to infrared, and much of the intensity of the lamp is expended in the near ultraviolet.

Prominent peaks of intensity occur at 313, 334, 365, 406, 435, 546, and 578 nanometers. At other wavelengths in the visible light region, the intensity is steady although not nearly so bright (but still useable in most applications).

In considering illumination efficiency, mere lamp wattage is not the prime consideration. Instead, the critical parameter is the mean luminance must be considered, taking into account the source brightness, arc geometry, and the angular spread of emission.

Table 1 - Luminous Density of Selected Light Sources

Lamp	**Current (Amperes)**	**Luminous Flux (Lumens)**	**Mean Luminous Density (cd/mm2)**	**Arc Size (H x W) (Millimeters)**
Mercury Arc (100 Watt)	5	2200	1700	0.25 x 0.25
Xenon Arc (75 Watt)	5.4	850	400	0.25 x 0.50
Xenon Arc (500 Watt)	30	9000	3500	0.30 x 0.30
Tungsten Halogen	8	2800	45	4.2 x 2.3

CONCLUSIONS

The modern fluorescence microscope combines the power of high performance optical components with computerized control of the instrument and digital image acquisition to achieve a level of sophistication that far exceeds that of simple observation by the human eye.

Microscopy now depends heavily on electronic imaging to rapidly acquire information at low light levels or at visually undetectable wavelengths.

These technical improvements are not mere window dressing, but are essential components of the light microscope as a system.

The era when optical microscopy was purely a descriptive instrument or an intellectual toy is past.

At present, optical image formation is only the first step toward data analysis. The microscope accomplishes this first step in conjunction with electronic detectors, image processors, and display devices that can be viewed as extensions of the imaging system.

Computerized control of focus, stage position, optical components, shutters, filters, and detectors is in widespread use and enables experimental manipulations that were not humanly possible with mechanical microscopes.

The increasing application of electro-optics in fluorescence microscopy has led to the development of optical tweezers capable of manipulating sub-cellular structures or particles, the imaging of single molecules, and a wide range of sophisticated spectroscopic applications.

EXPERIMENT NO- 3.4

OBJECTIVE: To demonstrate of use of confocal microscopy

INTRODUCTION

This microscopy facility provides users with the opportunity to prepare and image their own samples.

The EM/Confocal Specialist will provide technical support to new users, faculty, graduate students, and staff, for specimen preparation and will provide comprehensive training to operate the microscopes.

Confocal microscopy, most frequently **confocal laser scanning microscopy** (**CLSM**), is an optical imaging technique for increasing optical resolution and contrast of a micrograph by means of adding a spatial pinhole placed at the confocal plane of the lens to eliminate out-of-focus light.

It enables the reconstruction of three-dimensional structures from the obtained images by collecting sets of images at different depths (a process known as optical sectioning) within a thick object.

This technique has gained popularity in the scientific and industrial communities and typical applications are in life sciences, semiconductor inspection and materials science.

A conventional microscope "sees" as far into the specimen as the light can penetrate, while a confocal microscope only "sees" images one depth level at a time. In effect, the CLSM achieves a controlled and highly limited depth of focus.

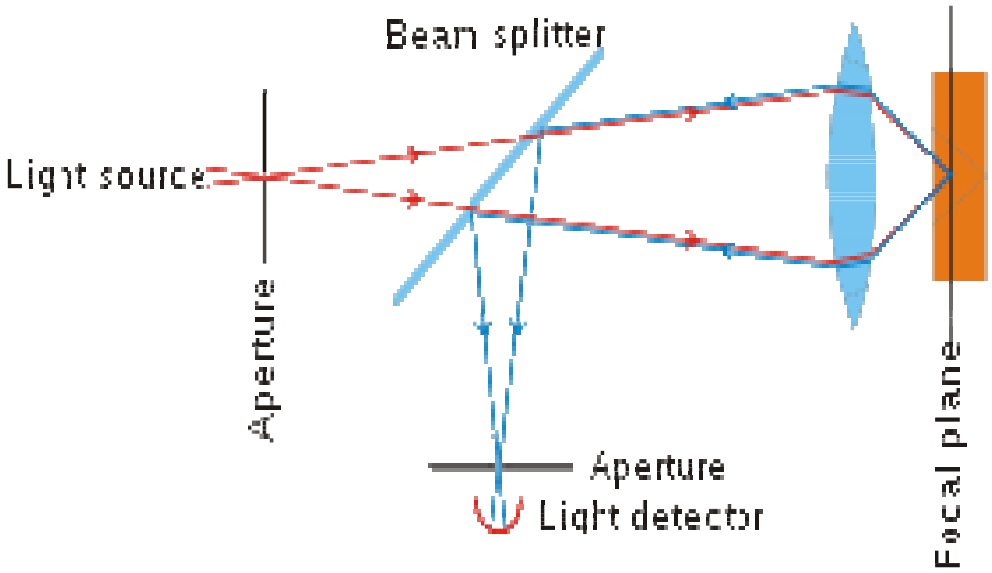

Fig 6.15Principle of confocal microscopy

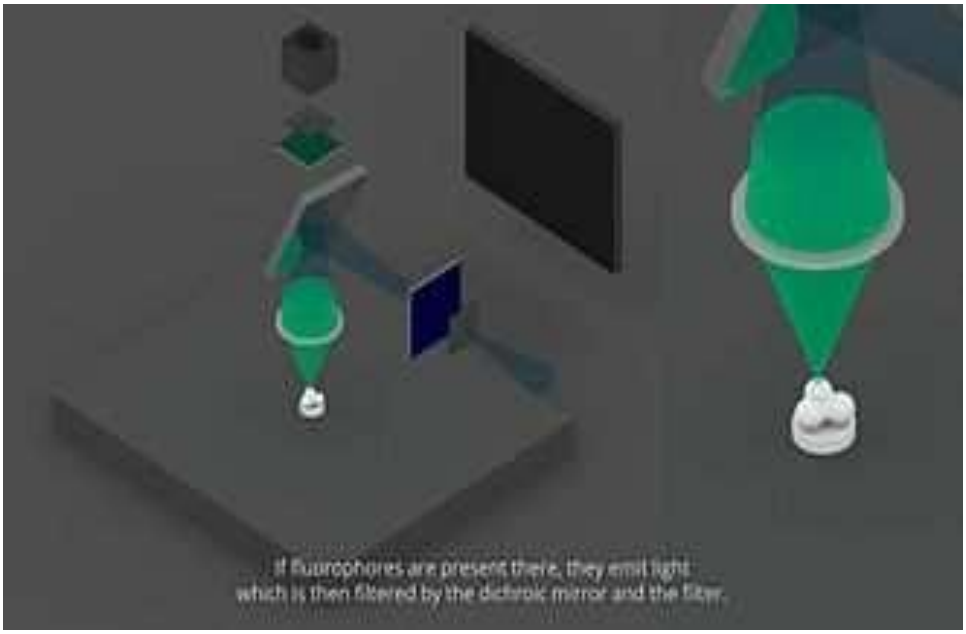

Fig 6.16 Fluorescent and confocal microscopes operating principle

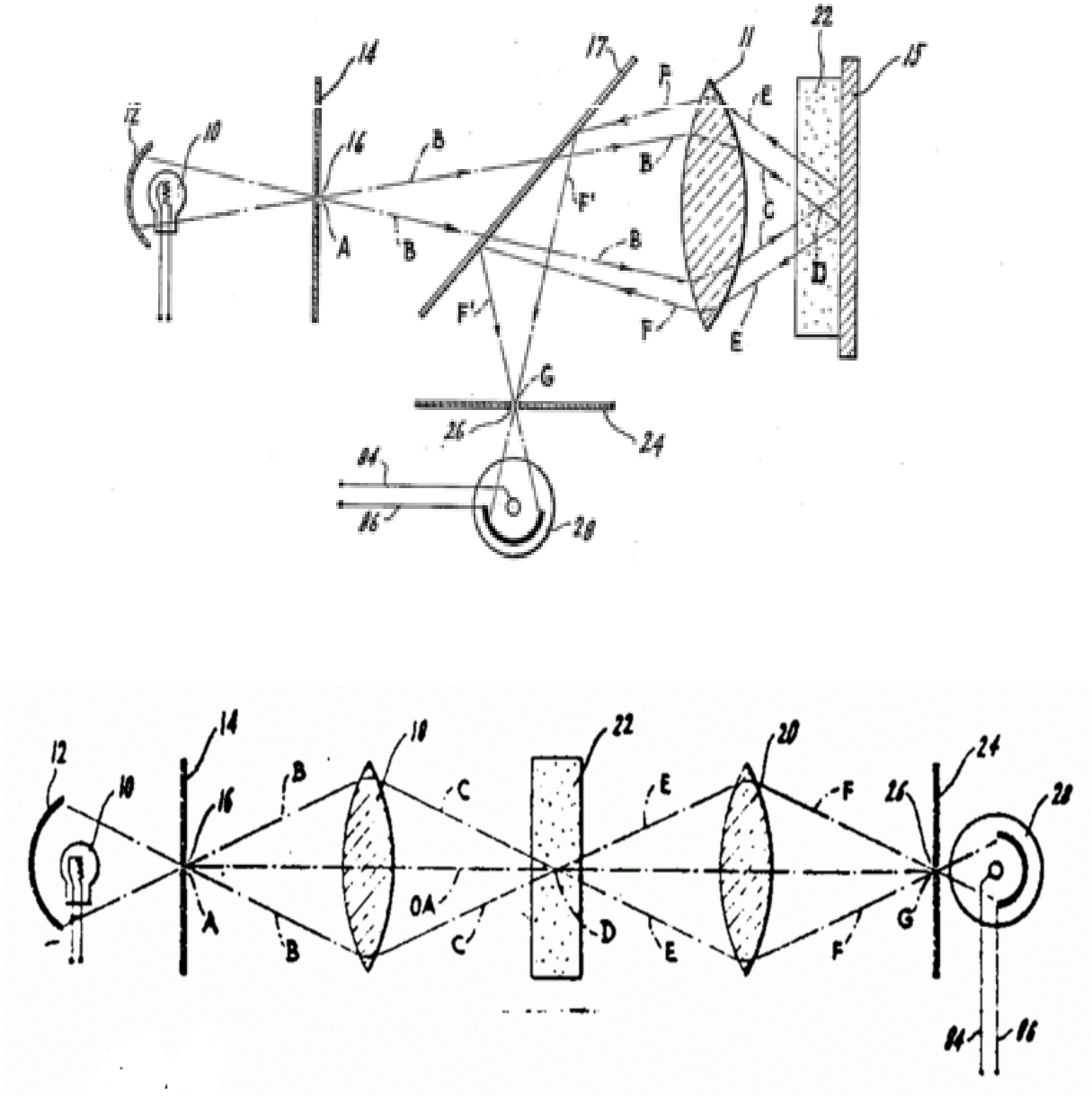

Fig 6.17Confocal point sensor principle

EXPERIMENT NO- 3.5

OBJECTIVE: To demonstrate of use of Electron microscopy

INTRODUCTION

- An **electron microscope** is a microscope that uses a beam of accelerated electrons as a source of illumination.
- As the wavelength of an electron can be up to 100,000 times shorter than that of visible light photons, electron microscopes have a higher resolving power than light microscopes and can reveal the structure of smaller objects.
- A transmission electron microscope can achieve better than 50 pm resolution and magnifications of up to about 10,000,000 xs whereas most light microscopes are limited by diffraction to about 200 nm resolution and useful magnifications below 2000x.
- Transmission electron microscopes use electrostatic and electromagnetic lenses to control the electron beam and focus it to form an image.
- These electron optical lenses are analogous to the glass lenses of an optical light microscope.
- Electron microscopes are used to investigate the ultra structure of a wide range of biological and inorganic specimens including microorganisms, cells, large molecules, biopsy samples, metals, and crystals.
- Industrially, electron microscopes are often used for quality control and failure analysis.
- Modern electron microscopes produce electron micrographs using specialized digital cameras and frame grabbers to capture the image.

The first electromagnetic lens was developed in 1926 by Hans Busch. According to Dennis Gabor, the physicist Leó Szilárd tried in 1928 to convince Busch to build an electron microscope, for which he had filed a patent.

German physicist Ernst Ruska and the electrical engineer Max Knoll constructed the prototype electron microscope in 1931, capable of four-hundred-power magnification; the apparatus was the first demonstration of the principles of electron microscopy. Two years later, in 1933, Ruska built an electron microscope that exceeded the resolution attainable with an optical (light) microscope.[Moreover, Reinhold Rudenberg, the scientific director of Siemens-Schuckertwerke, obtained the patent for the electron microscope in May 1931.

In 1932, Ernst Lubcke of Siemens & Halske built and obtained images from a prototype electron microscope, applying concepts described in the Rudenberg patent applications. Five years later (1937), the firm financed the work of Ernst Ruska and Bodo von Borries, and employed Helmut Ruska (Ernst's brother) to develop applications for the microscope, especially with biological specimens. Also in 1937, Manfred von Ardenne pioneered the scanning electron microscope. The first commercial electron microscope was produced in 1938 by Siemens.

Fig 6.18A modern transmission electron microscope

Types

Transmission electron microscope (TEM)

The original form of electron microscope, the transmission electron microscope (TEM) uses a high voltage electron beam to illuminate the specimen and create an image.

The electron beam is produced by an electron gun, commonly fitted with a tungsten filament cathode as the electron source.

The electron beam is accelerated by an anode typically at +100 keV (40 to 400 keV) with respect to the cathode, focused by electrostatic and electromagnetic lenses, and transmitted through the specimen that is in part transparent to electrons and in part scatters them out of the beam.

When it emerges from the specimen, the electron beam carries information about the structure of the specimen that is magnified by the objective lens system of the microscope.

The spatial variation in this information (the "image") may be viewed by projecting the magnified electron image onto a fluorescent viewing screen coated with a phosphor or scintillator material such as zinc sulfide.

Alternatively, the image can be photographically recorded by exposing a photographic film or plate directly to the electron beam, or a high-resolution phosphor may be coupled by means of a lens optical system or a fibre optic light-guide to the sensor of a digital camera.

The image detected by the digital camera may be displayed on a monitor or computer.

The resolution of TEMs is limited primarily by spherical aberration, but new generations of aberration correctors have been able to partially overcome spherical aberration to increase resolution.

Hardware correction of spherical aberration for the high-resolution transmission electron microscopy (HRTEM) has allowed the production of images with resolution below 0.5 angstrom (50 picometres) and magnifications above 50 million times.

The ability to determine the positions of atoms within materials has made the HRTEM an important tool for nano-technologies research and development.

Transmission electron microscopes are often used in electron diffraction mode.

The advantages of electron diffraction over X-ray crystallography are that the specimen need not be a single crystal or even a polycrystalline powder, and also that the Fourier transform reconstruction of the object's magnified structure occurs physically and thus avoids the need for solving the phase problem faced by the X-ray crystallographers after obtaining their X-ray diffraction patterns of a single crystal or polycrystalline powder.

The major disadvantage of the transmission electron microscope is the need for extremely thin sections of the specimens, typically about 100 nanometers.

Biological specimens are typically required to be chemically fixed, dehydrated and embedded in a polymer resin to stabilize them sufficiently to allow ultrathin sectioning.

Sections of biological specimens, organic polymers and similar materials may require special treatment with heavy atom labels in order to achieve the required image contrast.

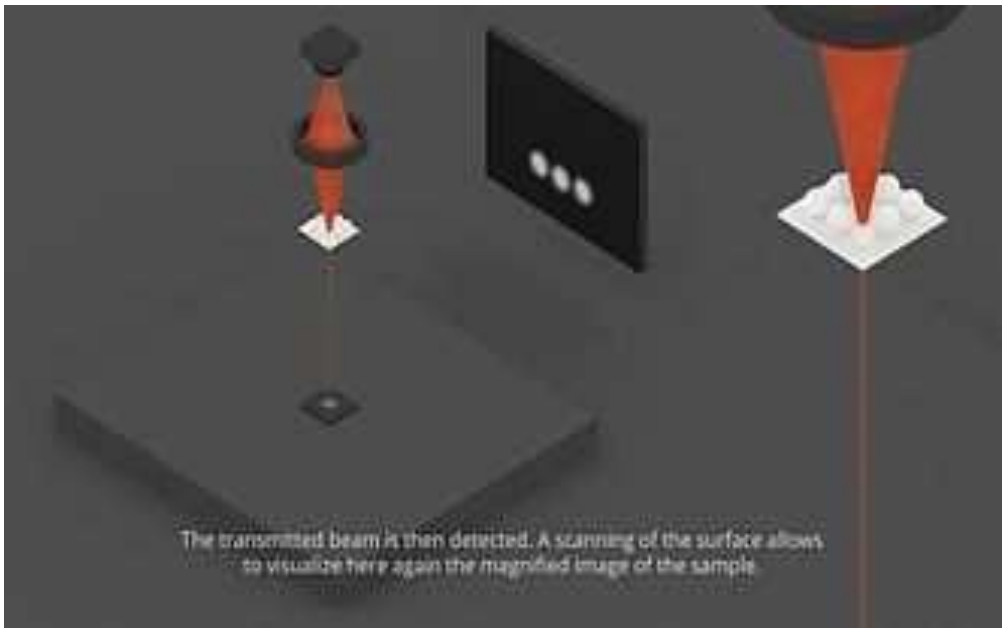

Fig 6.20Operating principle of a Transmission Electron Microscope

Scanning electron microscope (SEM)

The SEM produces images by probing the specimen with a focused electron beam that is scanned across a rectangular area of the specimen (raster scanning).

When the electron beam interacts with the specimen, it loses energy by a variety of mechanisms.

The lost energy is converted into alternative forms such as heat, emission of low-energy secondary electrons and high-energy backscattered electrons, light emission (cathodoluminescence) or X-ray emission, all of which provide signals carrying information about the properties of the specimen surface, such as its topography and composition.

The image displayed by an SEM maps the varying intensity of any of these signals into the image in a position corresponding to the position of the beam on the specimen when the signal was generated.

In the SEM image of an ant shown below and to the right, the image was constructed from signals produced by a secondary electron detector, the normal or conventional imaging mode in most SEMs.

Generally, the image resolution of an SEM is at least an order of magnitude poorer than that of a TEM.

However, because the SEM image relies on surface processes rather than transmission, it is able to image bulk samples up to many centimeters in size and (depending on instrument design and settings) has a great depth of field, and so can produce images that are good representations of the three-dimensional shape of the sample.

Another advantage of SEM is its variety called environmental scanning electron microscope (ESEM) can produce images of sufficient quality and resolution with the samples being wet or contained in low vacuum or gas.

This greatly facilitates imaging biological samples that are unstable in the high vacuum of conventional electron microscopes.

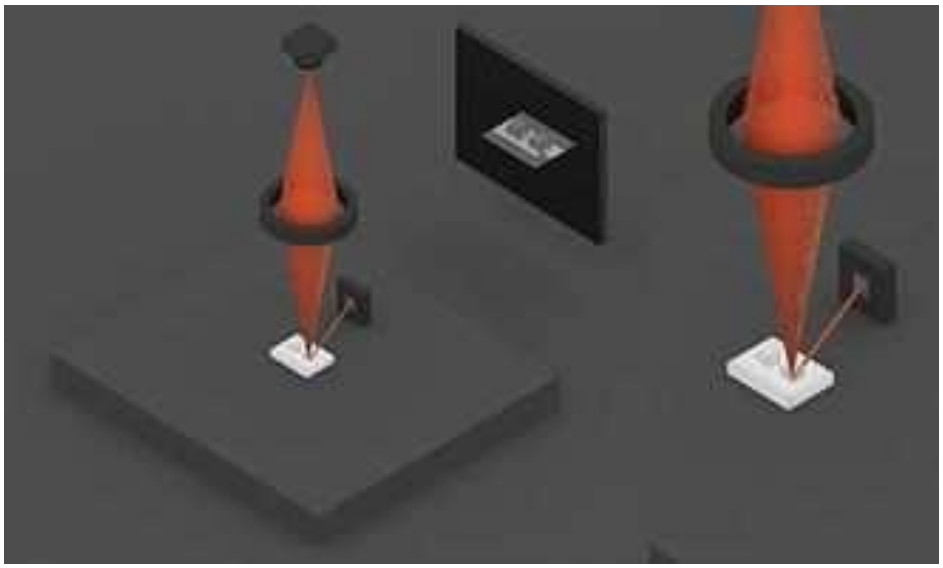

Fig 6.21Operating principle of a Scanning Electron Microscope

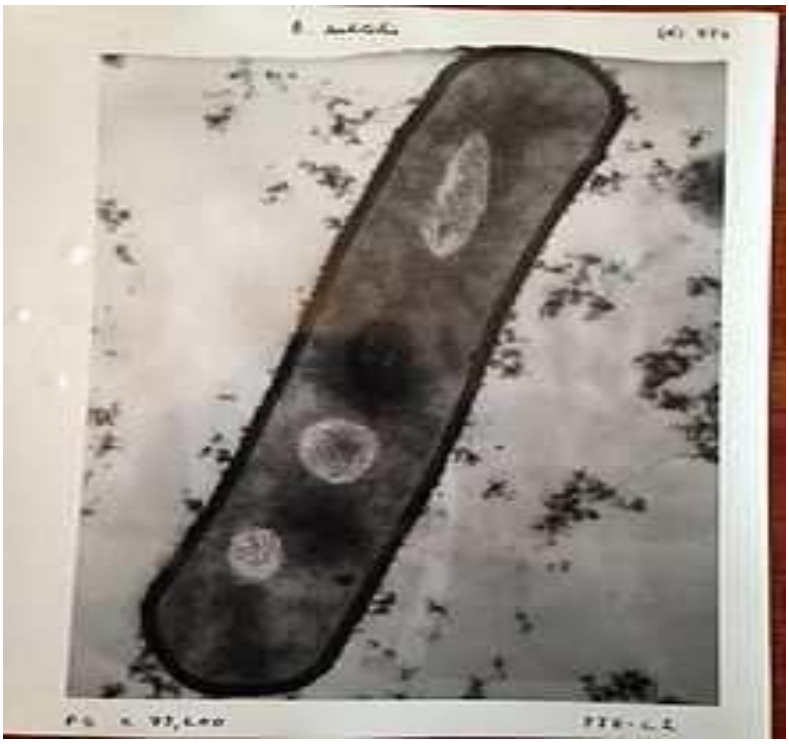

Fig 6.22Image of bacillus subtilis taken with a 1960s electron microscope

SUMMERY

1. A scientific instrument (pH meter, Spectrophotometer, Bright Field, Phase Contrast, Dark Field, Fluroscence, Cofocal and Electron Microscope) are an instrument used for laboratory purposes.

2. Most are measuring instruments. They may be specifically designed, constructed and refined for the purpose. Over time, instruments have become more accurate and precise.

3. Scientific instruments are part of laboratory equipment, but are considered more sophisticated and more specialized than other measuring instruments as scales, rulers, chronometers, thermometers or even waveform generators.

4. They are increasingly based upon the integration of computers to improve and simplify control, enhance and extend instrumental functions, conditions, parameter adjustments and data sampling, collection, resolution, analysis (both during and post-process), storage and retrieval.

GLOSSARY

1. **pH Meter**: Instrument that measures the hydrogen-ion concentration (or pH) in a solution, indicating its **acidity** or **alkalinity**. The pH meter measures the difference in electrical potential between a pH electrode and a reference electrode.

2. **Cuvet(te):** A clear, rectangular vessel of glass or plastic used to hold solutions for spectrometry. Don't place its ribbed sides in the light path of the spectrometer!

3. **Absorbance and 100% Line**: Absorbance is the unit utilized for measuring the amount of IR radiation absorbed by a material.

4. **Wavelength and Wavenumber**. Wavelength is the interval between two adjacent crests or troughs of a light wave. Wave number is 1/wavelength and is expressed in cm^{-1}. It is widely utilized as the X axis unit in infrared spectra.

5. **Zero Path Difference (ZPD), or Zero Optical Path Difference (ZOPD):** In an interferometer, the mirror displacement upon which the optical path difference for the two beams is zero. The detector signal is often much larger at ZPD, ZOPD, and is called the center burst.

6. **TEM:** Transmission Electron Microscopy.

7. **SEM:** Scanning Electron Microscope.

8. **Dark-field microscope**. A **microscope** in which an object is illuminated only from the sides so that it appears bright against a **dark** background.

9. **Absorbance (Optical Density) -** The quantity of light absorbed by a chemical or biological substance as measured in a spectrophotometer or similar device. Units of absorbance are equivalent to the logarithm of the reciprocal transmittance (the ratio of the transmitted light intensity to incident light intensity).

10. **Bioluminescence** - A biochemical oxidative process that results in the release of energy as emitted light. Firefly luminescence, which requires the enzyme luciferase to catalyze a reaction between the substrate luciferin and molecular oxygen (in the presence of adenosine triphosphate), is a commonly employed example of bioluminescence. The phenomenon occurs in a wide variety of marine organisms and insects.

SELF ASSESSMENT QUESTIONS

1. How often should I calibrate my pH sensor?
2. How can I calibrate the spectrophotometer?
3. What are the TEM and SEM?
4. What is optical density?
5. What are zero path differences?

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8. Confocal Microscopy (3rd Ed.). Berlin: Springer. ISBN 0-387-25921-X.
9. Jump up ^ Vincze L (2005). "Confocal X-ray Fluorescence Imaging and XRF Tomography for Three Dimensional Trace Element Microanalysis". Microscopy and Microanalysis. 11 (Supplement 2). doi:10.1017/S1431927605503167.

SUGGESTED READINGS

1. Sharma, V.K.; Techniques in Microscopy and Cell Biology -Tata McGraw Hill.
2. Instrumentation and Control Systems- William Bolton
3. Instrumentation for Process Measurement and Control Norman A. Anderson.
4. Instrumentation for Engineers and Scientists (Textbooks in Electrical and Electronic Engineering) – by John D. Turner (Author), Martyn Hill (Author)
5.

TERMINAL QUESTIONS

1. What are the working principles of pH meter?

2. Write biological applications of spectrophotometer.

3. Write short notes on:

   (i) SEM

   (ii) Absorbance

6. Write the significance of Confocal Microscope.
7. What is the use of dark field microscopy?

Unit 7 Biotechnology/Biotechniques

Content

7.1 Study of the principles and applications of the following equipment

7.1.1 Laminar flow

7.1.2 Autoclave

7.1.3 Elisa reader

7.1.4 PCR machine

7.1.5 Refrigrated centrifuge

7.1.6 Transilluminator

7.2 Double helical DNA modal

7.3 Chromatography or Thin Layer Chromatography (TLC)

7.4 Recombinant DNA techniques

Experiment No-(01) 7.1

***OBJECTIVE*:** Study of the principal and application of Laminar flow

INTRODUCTION

In fluid dynamics, **laminar flow** (or streamline flow) occurs when a fluid flows in parallel layers, with no disruption between the layers.

At low velocities, the fluid tends to flow without lateral mixing, and adjacent layers slide past one another like playing cards.

There are no cross-currents perpendicular to the direction of flow, nor eddies or swirls of fluids.

In laminar flow, the motion of the particles of the fluid is very orderly with particles close to a solid surface moving in straight lines parallel to that surface.

Laminar flow is a flow regime characterized by high momentum diffusion and low momentum convection.

When a fluid is flowing through a closed channel such as a pipe or between two flat plates, either of two types of flow may occur depending on the velocity and viscosity of the fluid: laminar flow or turbulent flow.

Laminar flow tends to occur at lower velocities, below a threshold at which it becomes turbulent.

Turbulent flow is a less orderly flow regime that is characterised by eddies or small packets of fluid particles which result in lateral mixing. In non-scientific terms, laminar flow is smooth while turbulent flow is rough.

Laminar flow barriers

Fig 7.1Experimental chamber for studying chemo taxis in response to laminar flow

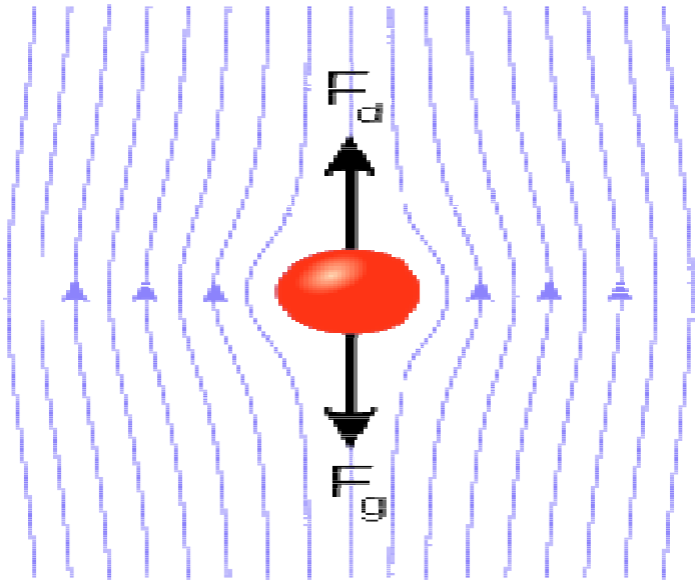


Fig 7.2 Reynolds Number

Relationship with the Reynolds number

The common example is flow through a pipe, where the Reynolds number is defined as:

$$\mathrm{Re} = \frac{\rho \mathbf{v} D_\mathrm{H}}{\mu} = \frac{\mathbf{v} D_\mathrm{H}}{\nu} = \frac{\mathbf{Q} D_\mathrm{H}}{\nu A}$$

Where:

- D_H is the hydraulic diameter of the pipe; its characteristic travelled length, L, (m).
- **Q** is the volumetric flow rate (m^3/s).
- A is the pipe's cross-sectional area (m^2).
- **v** is the mean velocity of the fluid (SI units: m/s).
- μ is the dynamic viscosity of the fluid (Pa·s = N·s/m^2 = kg/(m·s)).
- ν is the kinematic viscosity of the fluid, $\nu = \mu/\rho$ (m^2/s).
- ρ is the density of the fluid (kg/m^3).

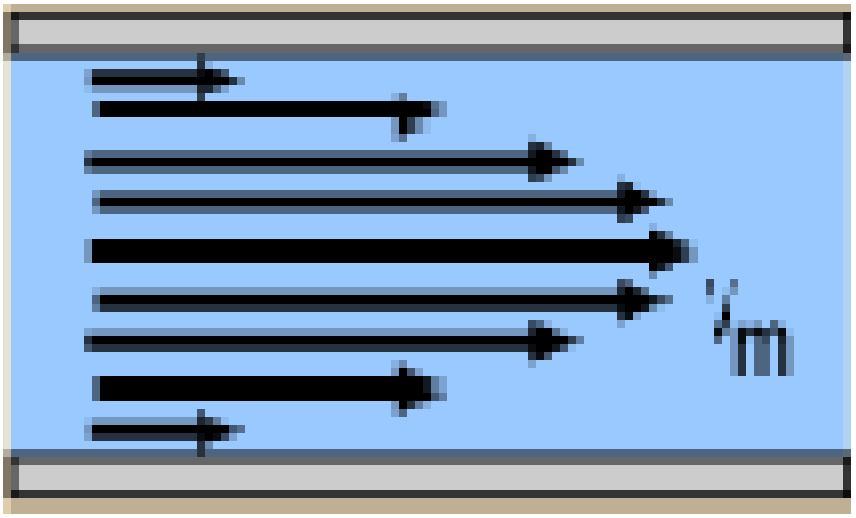
For such systems, laminar flow occurs when the Reynolds number is below a critical value of approximately 2,040; through the transition range is typically between 1,800 and 2,100.

For fluid systems occurring on external surfaces, such as flow past objects suspended in the fluid, other definitions for Reynolds numbers can be used to predict the type of flow around the object. The particle Reynolds number Re_p would be used for particle suspended in flowing fluids, for example.

As with flow in pipes, laminar flow typically occurs with lower Reynolds numbers, while turbulent flow and related phenomena, such as vortex shedding, occur with higher Reynolds numbers.

Examples:

In the case of a moving plate in a liquid, it is found that there is a layer (lamina) that moves with the plate, and a layer next to any stationary plate that is stationary.



EXPERIMENT NO- 7.2

***OBJECTIVE*:** Study of the principal and application of Autoclave

INTRODUCTION

An **autoclave** is a pressure chamber used to carry out industrial processes requiring elevated temperature and pressure different from ambient air pressure.

Autoclaves are used in medical applications to perform sterilization and in the chemical industry to cure coatings and vulcanize rubber and for hydrothermal synthesis.

They are also used in industrial applications, especially regarding composites, see autoclave (industrial).

Many autoclaves are used to sterilize equipment and supplies by subjecting them to high-pressure saturated steam at 121 °C (249 °F) for around 15–20 minutes depending on the size of the load and the contents.

The autoclave was invented by Charles Chamberland in 1879, although a precursor known as the steam digester was created by Denis Papin in 1679.

The name comes from Greek auto-, ultimately meaning self and Latin clavis meaning key, thus a self-locking device.

WHAT IS AN AUTOCLAVE?

The **autoclave** carries out that exact function of sterilizing materials.

It is a machine that uses pressure and steam to reach and maintain a temperature that is too high for any microorganisms or their spores to live.

Microorganisms are what most people commonly refer to as germs. These are the bacteria, viruses, fungi, parasites, etc. that are able to cause infections in our bodies.

Spores are the environment-resistant form of the microorganisms. Even though they are able to withstand harsher conditions, they still can be killed if extreme conditions are maintained for an extended period of time.

HOW IT WORKS

Autoclaves are pressure cookers very similar to the ones that you see in the stores. If you have used, or are familiar with pressure cookers, then you know that foods cook a lot faster in a pressure cooker than they do in a regular pot or in the oven. This is due to the intense heat and pressure that is applied to the food.

The same mechanism works against living microorganisms. Once an autoclave is started, steam is pushed into the chamber that contains the items that are being sterilized.

As the steam goes in, the pressure and temperature within the chamber is increased. Most autoclaves are set to increase steam pressure until a temperature of at least 121 degrees Celsius is reached (about 250 degrees Fahrenheit).

This temperature and pressure will remain at this level for at least 15 minutes. This is a high enough temperature for a long enough period of time to kill any and all microorganisms and their spores.

Uses

Sterilization autoclaves are widely used in microbiology, medicine, podiatry, tattooing, body piercing, veterinary medicine, mycology, funeral homes, dentistry, and prosthetics fabrication. They vary in size and function depending on the media to be sterilized.

Typical loads include laboratory glassware, other equipment and waste, surgical instruments, and medical waste.

Air removal

It is very important to ensure that all of the trapped air is removed from the autoclave before activation, as trapped air is a very poor medium for achieving sterility.

Steam at 134 °C can achieve in three minutes the same sterility that hot air at 160 °C can take two hours to achieve. Methods of air removal include:

Downward displacement (or **gravity-type**):

As steam enters the chamber, it fills the upper areas first as it is less dense than air. This process compresses the air to the bottom, forcing it out through a drain which often contains a temperature sensor.

Only when air evacuation is complete does the discharge stop. Flow is usually controlled by a steam trap or a solenoid valve, but bleed holes are sometimes used, often in conjunction with a solenoid valve.

As the steam and air mix, it is also possible to force out the mixture from locations in the chamber other than the bottom.

Steam pulsing

Pressurized and then depressurized to near atmospheric Air dilution by using a series of steam pulses, in which the chamber is alternately pressure.

Vacuum pumps

A vacuum pump sucks air or air/steam mixtures from the chamber.

Super atmospheric cycles

Achieved with a vacuum pump. It starts with a vacuum followed by a steam pulse followed by a vacuum followed by a steam pulse.

The number of pulses depends on the particular autoclave and cycle chosen.

Sub-atmospheric cycles

Similar to the super-atmospheric cycles, but chamber pressure never exceeds atmospheric pressure until they pressurize up to the sterilizing temperature.

A medical autoclave is a device that uses steam to sterilize equipment and other objects. This means that all bacteria, viruses, fungi, and spores are inactivated.

However, prions, such as those associated with Creutzfeldt–Jakob disease, may not be destroyed by autoclaving at the typical 134 °C for three minutes or 121 °C for 15 minutes.

Although that a wide range species of archaea, including *Geogemma barosii*, can survive at temperatures above 121 °C, no archaea are known to be infectious or pose a health risk to humans; in fact their biochemistry is so vastly different from our own and their multiplication rate is far too slow for microbiologists to worry about them.

Autoclaves are found in many medical settings, laboratories, and other places that need to ensure the sterility of an object.

Many procedures today employ single-use items rather than sterilizable, reusable items.

This first happened with hypodermic needles, but today many surgical instruments (such as forceps, needle holders, and scalpel handles) are commonly single-use rather than reusable items.

Autoclaves are of particular importance in poorer countries due to the much greater amount of equipment that is re-used.

Providing stove-top or solar autoclaves to rural medical centres has been the subject of several proposed medical aid missions.

Because damp heat is used, heat-labile products (such as some plastics) cannot be sterilized this way or they will melt.

Paper and other products that may be damaged by steam must also be sterilized another way.

In all autoclaves, items should always be separated to allow the steam to penetrate the load evenly.

Autoclaving is often used to sterilize medical waste prior to disposal in the standard municipal solid waste stream.

This application has become more common as an alternative to incineration due to environmental and health concerns raised because of the combustion by-products emitted by incinerators, especially from the small units which were commonly operated at individual hospitals.

Incineration or a similar thermal oxidation process is still generally mandated for pathological waste and other very toxic and/or infectious medical waste.

In dentistry, autoclaves provide sterilization of dental instruments according to health technical memorandum 01-05 (HTM01-05).

According to HTM01-05, instruments can be kept, once sterilized using a vacuum autoclave for up to 12 months using sealed pouches.

Fig 7.3 This is an autoclave that is used in the medical facility

Fig 7.4Other Autoclave with dental equipment in an autoclave to be sterilized for 2 hours at 150 to 180 degrees Celsius

Fig 7.5 Stovetop autoclaves—the simplest of autoclaves

Fig 7.6The machine on the right is an autoclave used for processing substantial quantities of laboratory equipment prior to reuse, and infectious material prior to disposal. (The machines on the left and in the middle are washing machines.)

Fig 7.7 The Autoclave must reach 121 degrees Celsius

The high temperatures cause the internal parts of the microorganisms to essentially cook. Once the internal parts cannot function in the microorganisms, they will die. The steam and pressure are released and brought down to normal room temperature and pressure after the 15 or more minutes of running. The items that were autoclaved will remain sterile until they are contaminated by new microorganisms.

EXPERIMENT NO- 7.3

***OBJECTIVE*:** Study of the principal and application of Elisa Reader

INTRODUCTION

ELISA stands for enzyme linked immune-sorbent assay. In short, it is an antibody test or a test for immune response to things attacking the body such as virus, bacteria and allergens. The test is done in an ELISA plate, also known as a 96-well plate or micro plate. The ELISA reader reads the plate.

WHAT AN ELISA READER DOES

An ELISA reader measures and quantities the color differences in the 12 wells of the plate.

ELISA readers or micro plate readers do spectrophotometer; they emit light at one wave length, and measure the amount of light absorbed and reflected by an object such as a protein. A spectrophotometer measures ultraviolet and visible light.

Additionally, ELISA plate readers can also measure fluorescence and luminescence. Chemical dyes fluoresce or emit one color or wavelength when exposed to light. The amount of reflection, absorption and the color identify, and measure the amount of a substance.

PURPOSE OF AN ELISA READER

ELISA readers were designed for measuring antibody tests. They worked so well, the machine has been adapted to other purposes. Researchers use them for protein and enzyme assays. They are also used for HIV detection and quantization of nucleic acids.

ADVANTAGES OF ELISA READER

Spectrophotometers require more sample per measurement .
To use a spectrophotometer or ELISA plate reader, the molecule must be dissolved in solution.

A spectrophotometer requires between 400 micro-liters and four milliliters, depending on the manufacturer and model. An ELISA plate reader needs about two to 100 micro-liters; ELISA plate readers use much less of a sample to get a result.

ELISA plate readers measure more samples in a shorter period of time. A spectrophotometer measures one to six samples at a time. Typically, an ELISA plate measures 96 wells in an equivalent amount of time.

Fig 7.8 Demonstration of ELISA reader

Fig 7.9 ELISA plate readers

EXPERIMENT NO- 7.4

***OBJECTIVE*:** Study of the principal and application of PCR Machine

INTRODUCTION

PCR (polymerase chain reaction) machine, also known as a thermal cycler, is a DNA amplifier that regulates temperature and amplifies segments of DNA via the polymerase chain reaction**.**

PCR machines are also sometimes used to facilitate reactions regarding enzyme digestion or rapid diagnostics.

PCR requires four main components. The first is the DNA sample containing the section, or sections, for copying.

Secondly, PCR requires a primer. Primers are short segments of DNA a scientist creates to match the DNA sample.

The next requirement of PCR is DNA polymerase, an enzyme that copies DNA. Human DNA polymerase denatures, or breaks down, at PCR temperatures, so researchers often use DNA polymerase from a heat-tolerant bacterium.

Finally, PCR requires nucleotides: adenine, guanine, cytosine and thymine. These are the base pairs that provide the coding elements of DNA.

Researchers first heat the PCR mixture to a temperature that denatures the DNA double helix.

A cooling step follows, which allows the primers to bind to the sample DNA. Another healing cycle follows during which DNA polymerase elongates the DNA strand.

Repeating these steps multiple times creates many new copies of the original DNA sample in as few as three hours.

PCR is useful in the diagnosis of viral diseases and some forms of cancer. It is also a common tool in forensic science for replicating small samples from crime scenes.

PCR is used for research when it is necessary to make a large amount of a single gene, such as for genetic engineering or cloning. PCR is also used to test whether or not a particular gene product is present in a sample.

In forensics, PCR technology is used to carry out DNA fingerprinting to analyze crime scene DNA evidence. PCR can also be used in medical settings to carry out tissue-typing for organ transplants.

Fig 7.10 PCR Machine

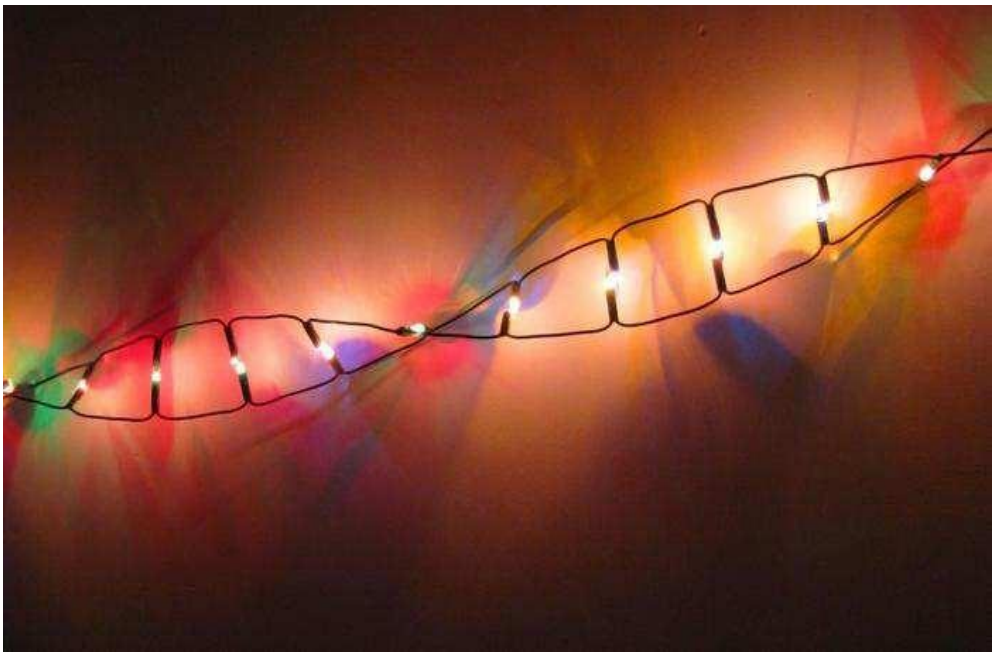
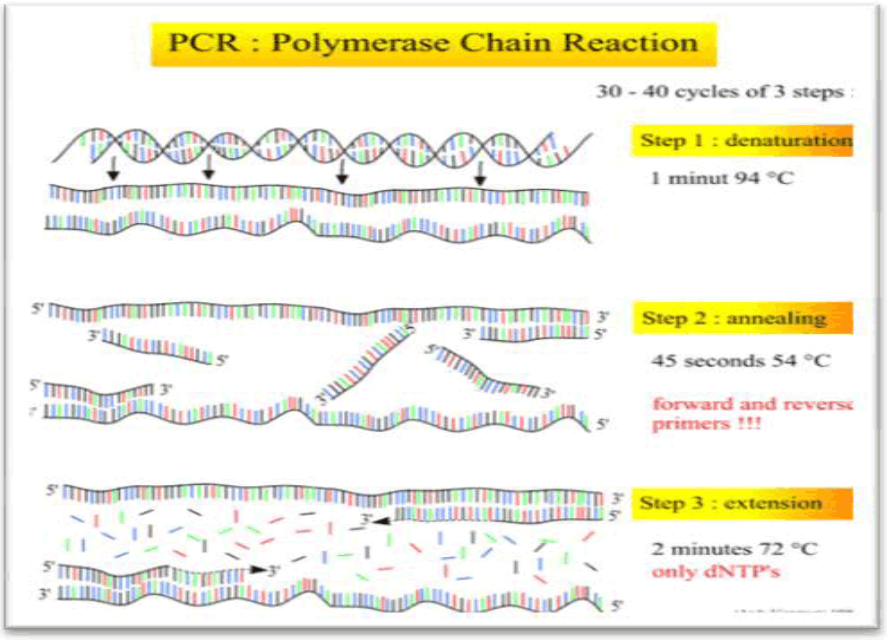


Fig 7.11The purpose of PCR is to amplify small amounts of a DNA sequence of interest so it can be analyzed separately. PCR can be used to make a large amount of a specific piece of DNA or to test a DNA sample for that sequence



Experiment No- 7.5

***OBJECTIVE*:** Study of the principal and application Refrigerated Centrifuge

INTRODUCTION

Refrigerated centrifuge works on the concept of sedimentation principle by holding up the sample tubes with a capacity of 2ml, 10ml and 50ml in rotation around a fixed axis.

In this, the centripetal force causes the denser substances to separate out along the radial direction in the bottom of the centrifuge tube.

The rate of the centrifugation is calculated by the acceleration applied to the sample and it is typically measured in revolution per minute (RPM) or relative centrifugal force (RCF).

The particle's settling velocity during centrifugation depends on the function of their size and shape, centrifugal acceleration, the volume fraction of solids present, the density difference between the particle and the liquid, and the viscosity.

This equipment is extensively used in chemistry, biology, and biochemistry for isolating and separating suspensions.

It additionally provides the cooling mechanism to maintain the uniform temperature throughout the operation of the sample.

Fig.7.12 Refrigerated centrifuge

Fig 7.13 Compact and quiet 4 x 85 ml refrigerated centrifuge for low-speed clinical and research applications

FUNCTIONS

- Fast Cool function eliminates long waits for set temperatures
- Easy-to-use interface with digital display improves repeatability
- Standby cooling maintains temperature when chamber is not in use
- -9 to 40°C temperature range

Centrifuge is ideal for low-speed spinning (up to 3,000 x g) of tubes ranging in volume from 1.1 to 85 ml.

The space-saving footprint and innovative design accommodates 30 x 15 ml round-bottom tubes or 20 x 15 ml conical tubes.

Automatic, motorized locking lid makes loading and closing effortless. A variety of rotors can be used for optimal versatility in high-capacity or sensitive applications.

For delicate blood and urine samples, users can deactivate the electronic brake.

Time and speed are easily adjusted and digitally displayed for accuracy and all settings are adjustable during a run.

Two preset buttons store routine runs for added convenience. Settings can also be locked to prevent unintentional adjustment.

Timer is programmable up to 99 minutes or continuous, and an audible alert signals the end of a run. Measures 15"W x 10.2"H x 22.8"D (38 x 26 x 58 cm). Weight 79 lbs./36 kg.

Centrifuge 5702R (refrigerated) with 4x100mL swing-bucket rotor (A-4-38) including buckets. Max. RCF 3,000xg (4400rpm),

Temperature range -9 - 40°C. Compact footprint, Whisper quiet operation, SOFT brake option, 120V/50-60Hz.

Centrifuge 5702R (refrigerated) with 4x100mL swing-bucket rotor (A-4-38) including buckets. Max. RCF 3,000xg (4400rpm),

EXPERIMENT NO- 7.6

***OBJECTIVE*:** Study of the principal and application of Tran illuminator

INTRODUCTION

Tran illuminators are used in molecular biology labs to view DNA (or RNA) that has been separated by electrophoresis through an agarose gel.

During or immediately after electrophoresis, the agarose gel is stained with a fluorescent dye which binds to nucleic acid.

Exposing the stained gel to a UVB light source causes the DNA/dye to fluoresce and become visible.

This technique is used wherever the researcher needs to be able to view their sample, for example sizing a PCR product, purifying DNA segment after a restriction enzyme digest, quantifying DNA or verifying RNA integrity after extraction.

It describes how to make a UVB (310nm) transilluminator with a 7 x 7 cm window for viewing ethidium bromide (or SYBR-Safe) stained DNA mini-gels.

Once all of the materials are collected, the actual assembly time is approx. 1-2 hours. Some soldering is required.

For the UV transilluminator enclosure and lid. Laser cut the parts from the material listed in the design file.

If you do not have access to a laser cutter, you can send the files to any laser cutting service such as Pololu. Materials for laser cutting can be found at any supplier of acrylic materials (McMaster-Carr, US Plastics etc) except for the solacryl (UV-transmissive) which can be bought from Loop Acrylics.

Tap holes in the following parts:

- 5-40: Two holes in the enclosure side with the cutout for the power switch
- 8-32: Four holes in the solacryl cover
- 8-32: Four holes in the 0.25" clear lid side part for mounting the hinges
- 8-32: Two holes in the enclosure bottom

Safety Notes:

1. Because ethidium bromide is a toxic chemical with strict safety protocols, it is only recommended that you use this dye in a lab with established handling, storage and waste disposal procedures in place**.** Other users are strongly recommended to use SYBR-Safe instead, which can be handled and disposed of more safely.
2. The transilluminator does come with a safety lid for viewing the gel. However, when the lid is not in place, safety glasses mustbe worn when operating the UVB bulb.
3. If you prefer to avoid UVB altogether, we can recommend the blue light LED transilluminators such as the one described in this instructable instead.

Fig.7.14 ***UV****-TransiIlluminator*

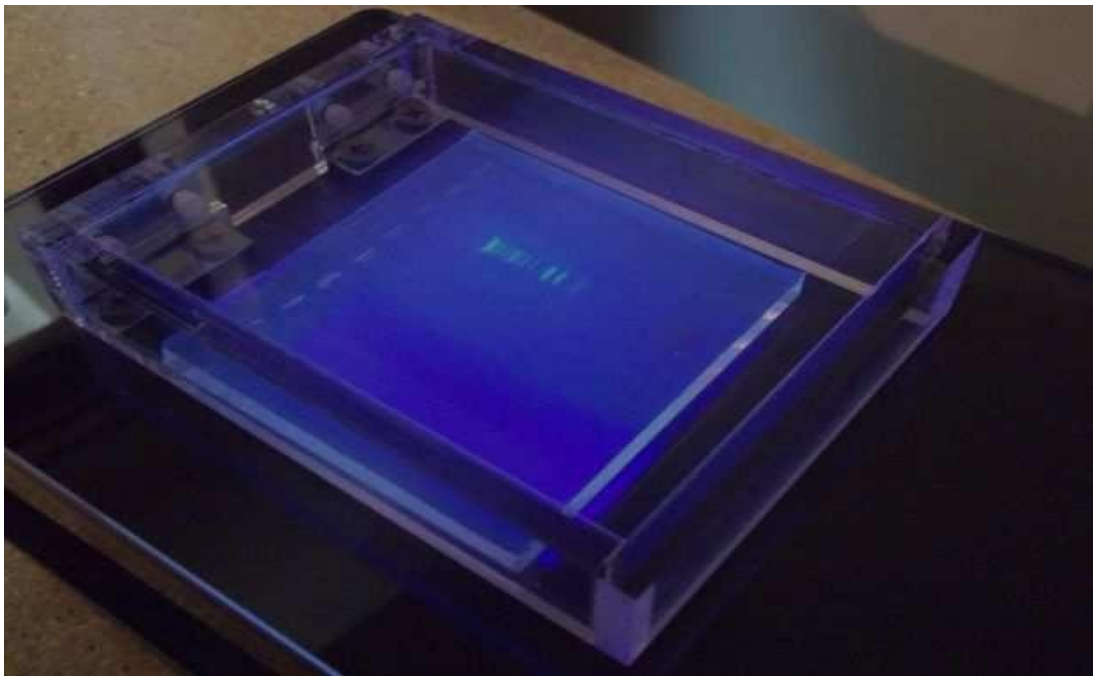


Fig 7.15 UV- Tran illuminator

EXPERIMENT NO- 02

***OBJECTIVE*:** Study of the Double helical DNA model

INTRODUCTION

Many people believe that American biologist James Watson and English physicist Francis Crick discovered DNA in the 1950s.

In reality, this is not the case. Rather, DNA was first identified in the late 1860s by Swiss chemist Friedrich Miescher.

Then, in the decades following Miescher's discovery, other scientists--notably, Phoebus Levene and Erwin Chargaff--carried out a series of research efforts that revealed additional details about the DNA molecule, including its primary chemical components and the ways in which they joined with one another.

Without the scientific foundation provided by these pioneers, Watson and Crick may never have reached their groundbreaking conclusion of 1953: that the DNA molecule exists in the form of a three-dimensional double helix.

Watson and Crick Propose the Double Helix

Chargaff's realization that A = T and C = G, combined with some crucially important X-ray crystallography work by English researchers Rosalind Franklin and Maurice Wilkins, contributed to Watson and Crick's derivation of the three-dimensional, double-helical model for the structure of DNA.

Watson and Crick's discovery was also made possible by recent advances in model building, or the assembly of possible three-dimensional structures based upon known molecular distances and bond angles, a technique advanced by American biochemist Linus Pauling.

In fact, Watson and Crick were worried that they would be "scooped" by Pauling, who proposed a different model for the three-dimensional structure of DNA just months before they did. In the end, however, Pauling's prediction was incorrect.

Using cardboard cutouts representing the individual chemical components of the four bases and other nucleotide subunits, Watson and Crick shifted molecules around on their desktops, as though putting together a puzzle.

They were misled for a while by an erroneous understanding of how the different elements in thymine and guanine (specifically, the carbon, nitrogen, hydrogen, and oxygen rings) were configured.

Only upon the suggestion of American scientist Jerry Donohue did Watson decide to make new cardboard cutouts of the two bases, to see if perhaps a different atomic configuration would make a difference. It did. Not only did the complementary bases now fit together perfectly (i.e., A with T and C with G), with each pair held together by hydrogen bonds, but the structure also reflected Chargaff's rule.

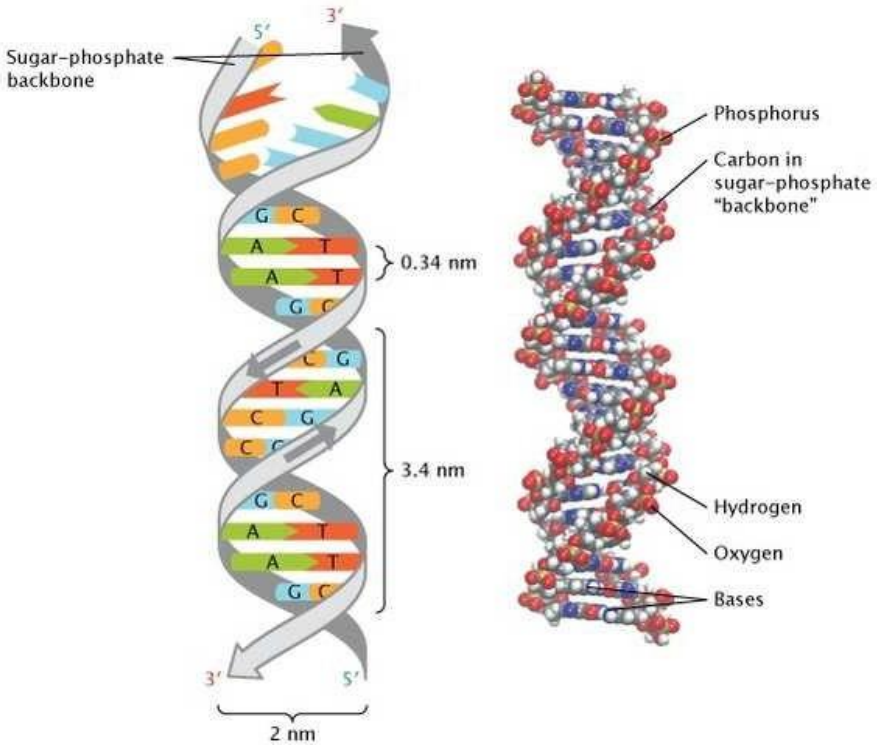


Fig 7.16 The double-helical structure of DNA,The 3- dimensional double helix structure of DNA, correctly elucidated by James Watson and Francis Crick. Complementary bases are held together as a pair by hydrogen bonds.

Although scientists have made some minor changes to the Watson and Crick model, or have elaborated upon it, since its inception in 1953, the model's four major features remain the same yet today. These features are as follows:

- DNA is a double-stranded helix, with the two strands connected by hydrogen bonds.
- A base is always paired with Ts, and Cs are always paired with Gs, which is consistent with and accounts for Chargaff's rule.
- Most DNA double helices are right-handed; that is, if you were to hold your right hand out, with your thumb pointed up and your fingers curled around your thumb, your thumb would represent the axis of the helix and your fingers would represent the sugar-phosphate backbone. Only one type of DNA, called Z-DNA, is left-handed.

- The DNA double helix is anti-parallel, which means that the 5' end of one strand is paired with the 3' end of its complementary strand (and vice versa).
- As shown in Figure 4, nucleotides are linked to each other by their phosphate groups, which bind the 3' end of one sugar to the 5' end of the next sugar.
- Not only are the DNA base pairs connected via hydrogen bonding, but the outer edges of the nitrogen-containing bases are exposed and available for potential hydrogen bonding as well.
- These hydrogen bonds provide easy access to the DNA for other molecules, including the proteins that play vital roles in the replication and expression of DNA.

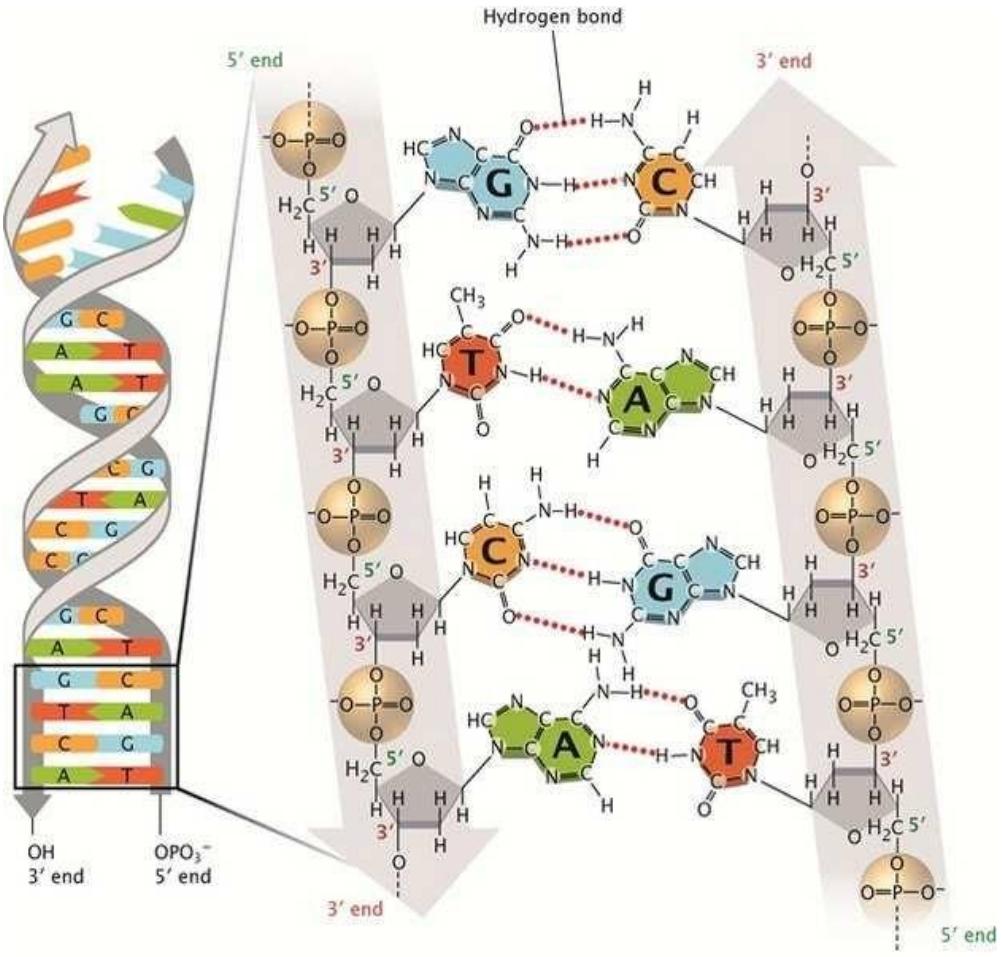


Fig 7.17 Base pairing in DNA

Two hydrogen bonds connect T to A; three hydrogen bonds connect G to C. The sugar-phosphate backbones (grey) run anti-parallel to each other, so that the 3' and 5' ends of the two strands are aligned.

One of the ways that scientists have elaborated on Watson and Crick's model is through the identification of three different conformations of the DNA double helix.

In other words, the precise geometries and dimensions of the double helix can vary. The most common conformation in most living cells (which is the one depicted in most diagrams of the double helix, and the one proposed by Watson and Crick) is known as B-DNA.

There are also two other conformations: A-DNA, a shorter and wider form that has been found in dehydrated samples of DNA and rarely under normal physiological circumstances; and Z-DNA, a left-handed conformation. Z-DNA is a transient form of DNA, only occasionally existing in response to certain types of biological activity.

Z-DNA was first discovered in 1979, but its existence was largely ignored until recently. Scientists have since discovered that certain proteins bind very strongly to Z-DNA, suggesting that Z-DNA plays an important biological role in protection against viral disease (Rich & Zhang, 2003).

Three different conformations of the DNA double helix

(A) A-DNA is a short, wide, right-handed helix. (B) B-DNA, the structure proposed by Watson and Crick, is the most common conformation in most living cells. (C) Z-DNA, unlike A- and B-DNA, is a left-handed helix.

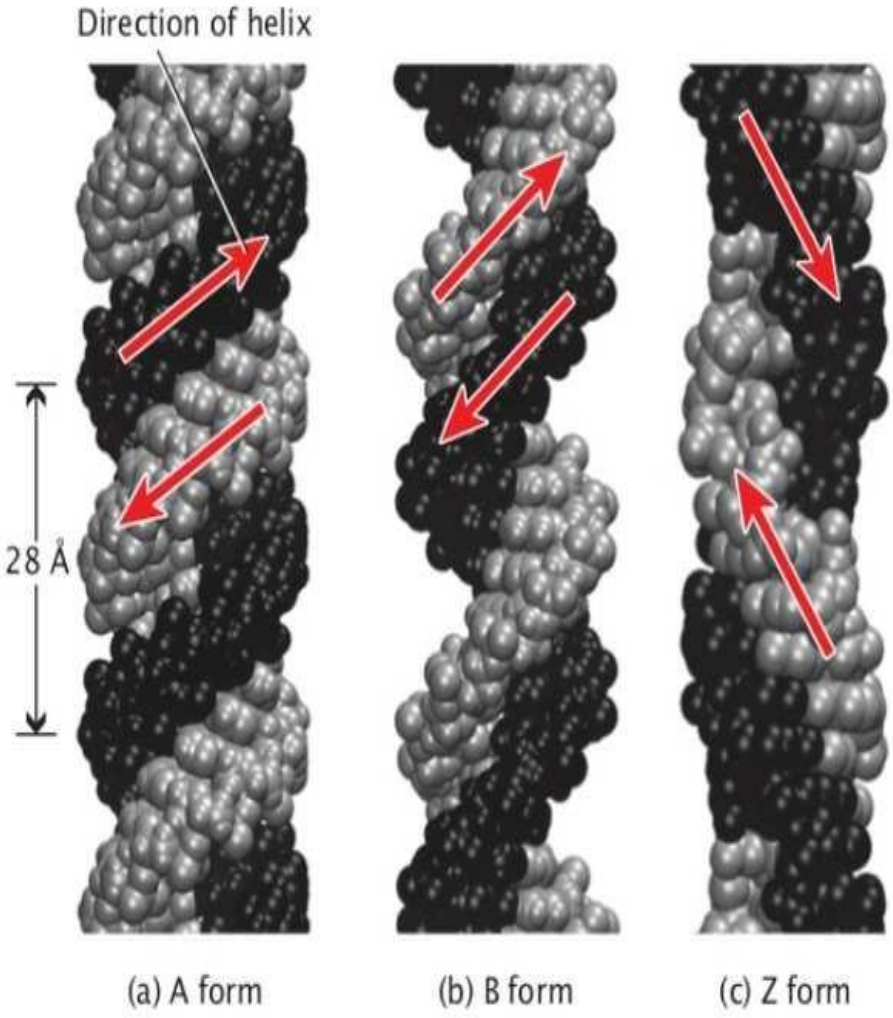


Fig.7.18 Three different conformations of the DNA double helix

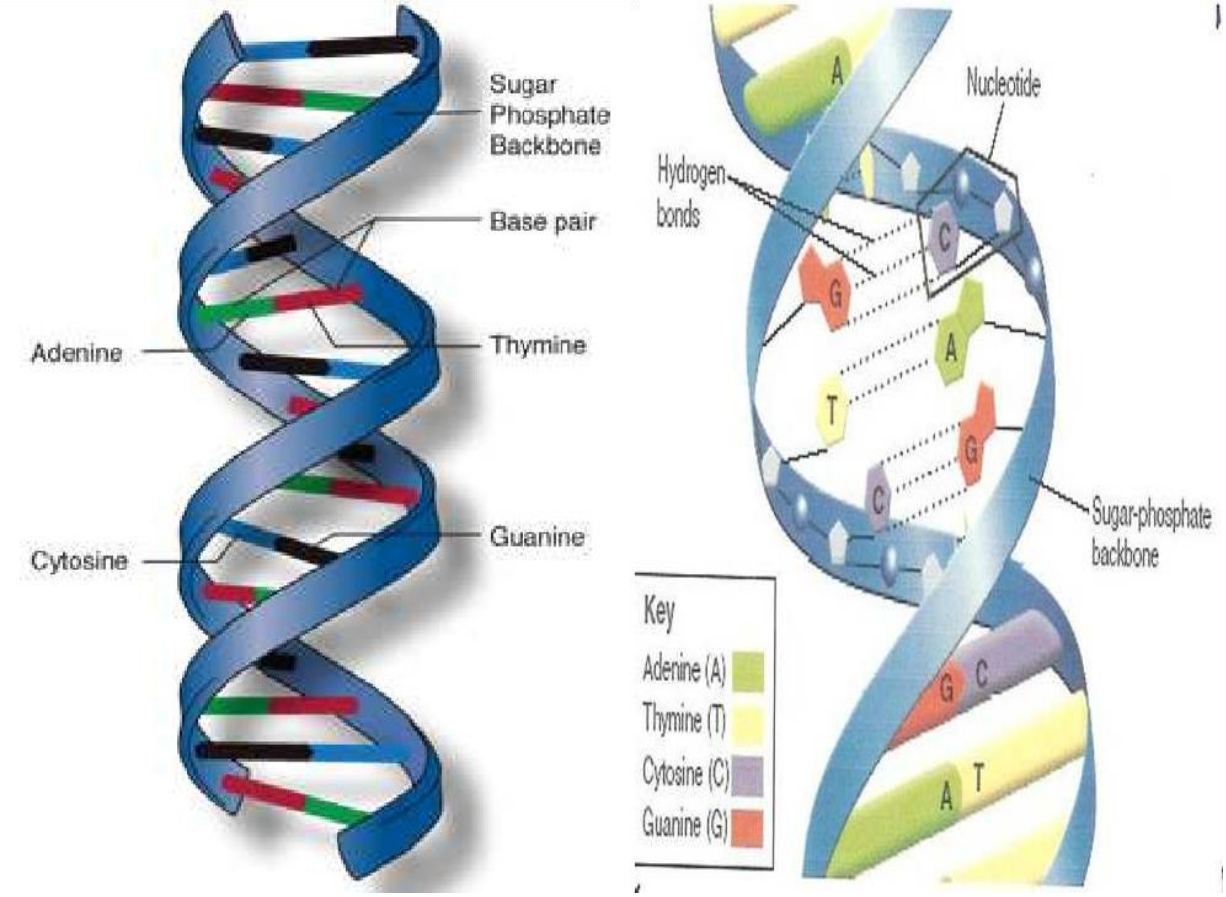


Fig.7.19 double Helical structure of DNA

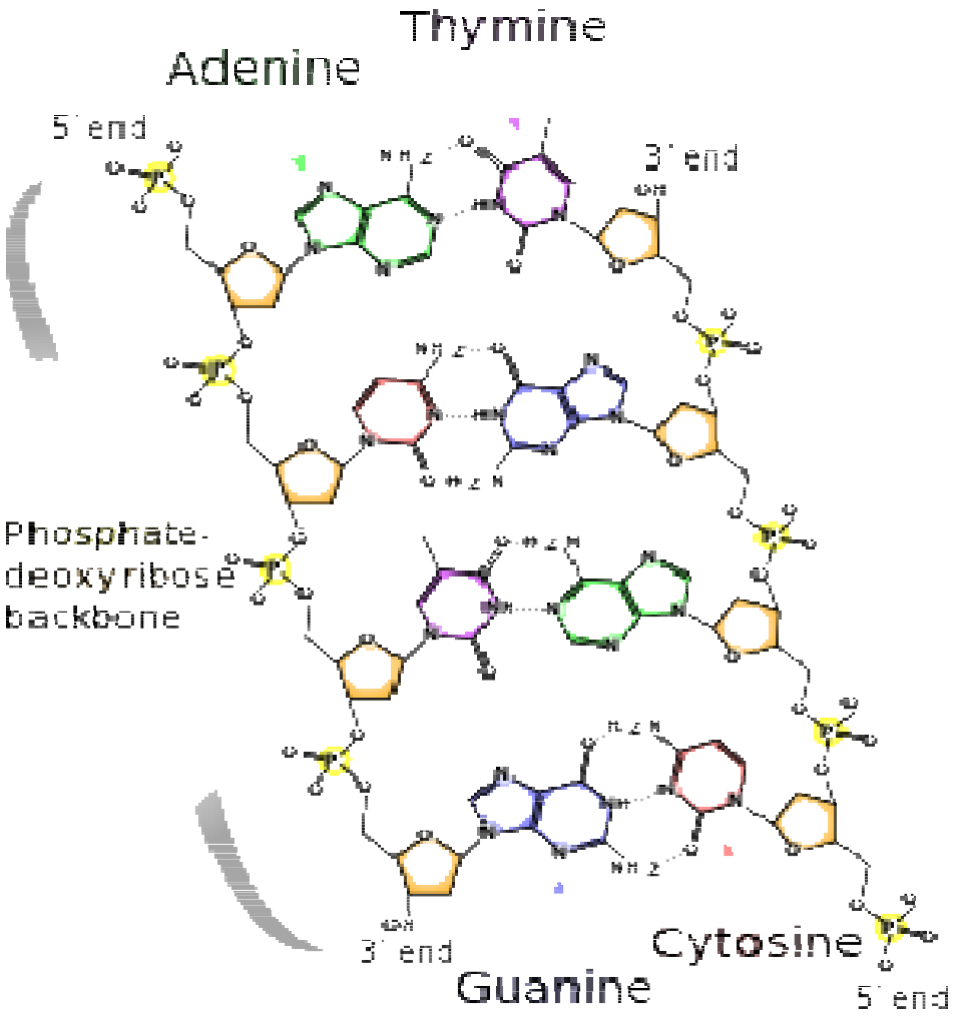


Fig.7.20 Chemical structure of DNA; hydrogen bonds shown as dotted lines

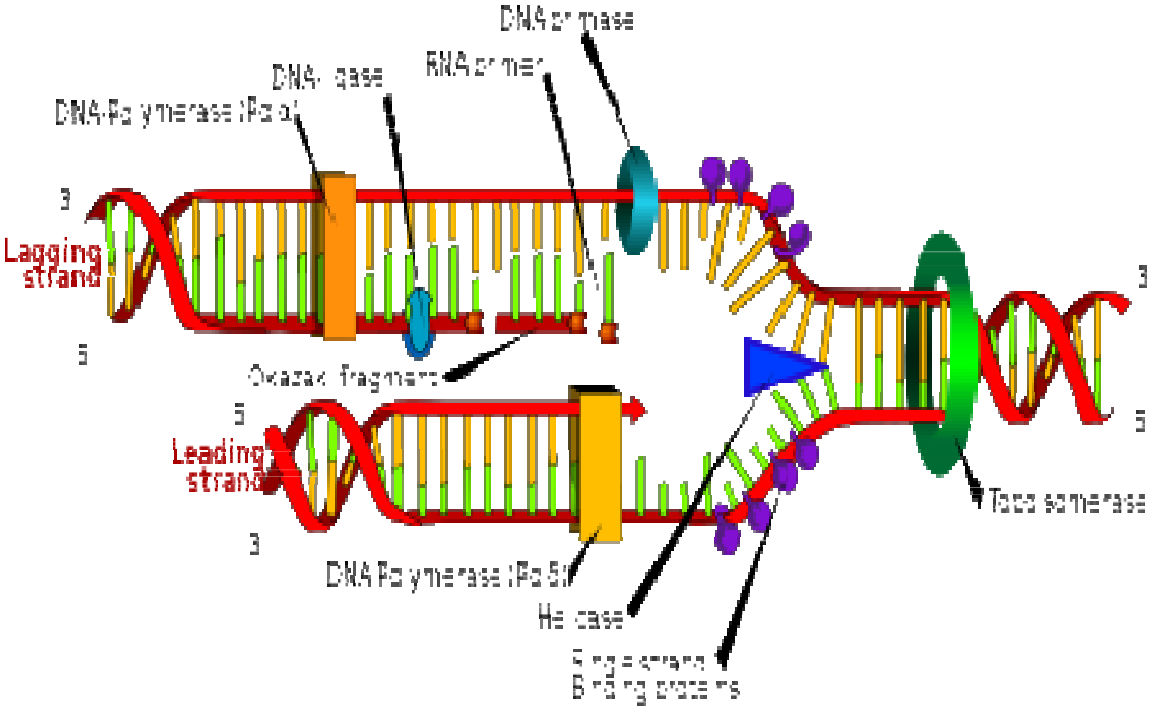


Fig.7.21 DNA replication

(The double helix is unwound by a helicase and topoisomerase. Next, one DNA polymerase produces the leading strand copy. Another DNA polymerase binds to the lagging strand. This enzyme makes discontinuous segments (called Okazaki fragments) before DNA ligase joins them together.)

EXPERIMENT NO- 03

OBJECTIVE: Study of the Chromatography or Thin layer Chromatography (TLC)

INTRODUCTION

Thin-layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide (alumina), or cellulose.

This layer of adsorbent is known as the stationary phase.

After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved.

The mobile phase has different properties from the stationary phase.

For example, with silica gel, a very polar substance, non-polar mobile phases such as heptanes are used.

The mobile phase may be a mixture, allowing chemists to fine-tune the bulk properties of the mobile phase.

After the experiment, the spots are visualized. Often this can be done simply by projecting ultraviolet light onto the sheet; the sheets are treated with a phosphor, and dark spots appear on the sheet where compounds absorb the light impinging on a certain area.

Chemical processes can also be used to visualize spots; anisaldehyde, for example, forms colored adducts with many compounds, and sulfuric acid will char most organic compounds, leaving a dark spot on the sheet.

To quantify the results, the distance traveled by the substance being considered is divided by the total distance traveled by the mobile phase. (The mobile phase must not be allowed to reach the end of the stationary phase.)

This ratio is called the retention factor or R_f. In general, a substance whose structure resembles the stationary phase will have low R_f, while one that has a similar structure to the mobile phase will have high retention factor.

Retention factors are characteristic, but will change depending on the exact condition of the mobile and stationary phase. For this reason, chemists usually apply a sample of a known compound to the sheet before running the experiment.

Thin-layer chromatography can be used to monitor the progress of a reaction, identify compounds present in a given mixture, and determine the purity of a substance.

Specific examples of these applications include: analyzing ceramides and fatty acids, detection of pesticides or insecticides in food and water, analyzing the dye composition of fibers in forensics, assaying the radiochemical purity of radiopharmaceuticals, or identification of medicinal plants and their constituents

A number of enhancements can be made to the original method to automate the different steps, to increase the resolution achieved with TLC and to allow more accurate quantitative analysis.

This method is referred to as HPTLC, or "high-performance TLC". HPTLC typically uses thinner layers of stationary phase and smaller sample volumes, thus reducing the loss of resolution due to diffusion.

Fig.7.22 Separation of black ink on a TLC plate

TLC of three standards (ortho-, meta- and para-isomers) and a sample

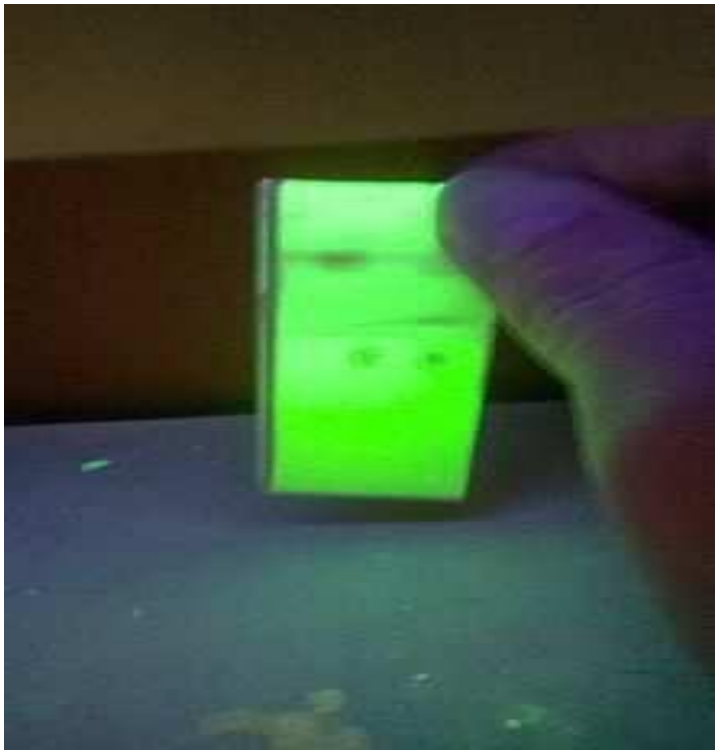


Fig.7.23 Fluorescent TLC plate under UV light

PLATE PREPARATION

TLC plates are usually commercially available, with standard particle size ranges to improve reproducibility.

They are prepared by mixing the adsorbent, such as silica gel, with a small amount of inert binder like calcium sulfate (gypsum) and water.

This mixture is spread as thick slurry on an uncreative carrier sheet, usually glass, thick aluminum foil, or plastic.

The resultant plate is dried and activated by heating in an oven for thirty minutes at 110 °C. The thickness of the absorbent layer is typically around 0.1 – 0.25 mm for analytical purposes and around 0.5 – 2.0 mm for preparative TLC.

TECHNIQUE

The process is similar to paper chromatography with the advantage of faster runs, better separations, and the choice between different stationary phases.

Because of its simplicity and speed TLC is often used for monitoring chemical reactions and for the qualitative analysis of reaction products.

Plates can be labeled before or after the chromatography process using a pencil or other implement that will not interfere or react with the process.

To run a thin layer chromatography plate, the following procedure is carried out:

Using a capillary, a small spot of solution containing the sample is applied to a plate, about 1.5 centimeters from the bottom edge.

The solvent is allowed to completely evaporate off to prevent it from interfering with sample's interactions with the mobile phase in the next step. If a non-volatile solvent was used to apply the sample, the plate needs to be dried in a vacuum chamber.

This step is often repeated to ensure there is enough analyte at the starting spot on the plate to obtain a visible result. Different samples can be placed in a row of spots the same distance from the bottom edge, each of which will move in its own adjacent lane from its own starting point.

- A small amount of an appropriate solvent (eluent) is poured into a glass beaker or any other suitable transparent container (separation chamber) to a depth of less than 1 centimeter.
- A strip of filter paper (aka "wick") is put into the chamber so that its bottom touches the solvent and the paper lies on the chamber wall and reaches almost to the top of the container. The container is closed with a cover glass or any other lid and is left for a few minutes to let the solvent vapors ascend the filter paper and saturate the air in the chamber. (Failure to saturate the chamber will result in poor separation and non-reproducible results).
- The TLC plate is then placed in the chamber so that the spot(s) of the sample do not touch the surface of the eluent in the chamber, and the lid is closed. The solvent moves up the plate by capillary action, meets the sample mixture and carries it up the plate (elutes the sample).
- The plate should be removed from the chamber before the solvent front reaches the top of the stationary phase (continuation of the elution will give a misleading result) and dried.
- Without delay, the solvent front, the furthest extent of solvent up the plate, is marked.
- The plate is visualized. As some plates are pre-coated with a phosphor such as zinc sulfide, allowing many compounds to be visualized by using ultraviolet light; dark spots appear where the compounds block the UV light from striking the plate. Alternatively, plates can be sprayed or immersed in chemicals after elution. Various visualising agents react with the spots to produce visible results.

SEPARATION PROCESS AND PRINCIPLE

Different compounds in the sample mixture travel at different rates due to the differences in their attraction to the stationary phase and because of differences in solubility in the solvent.

By changing the solvent, or perhaps using a mixture, the separation of components (measured by the Rf value) can be adjusted. Also, the separation achieved with a TLC plate can be used to estimate the separation of a flash chromatography column. (A compound elutes from a column when the amount of solvent collected is equal to 1/Rf.) Chemists often use TLC to develop a protocol for separation by chromatography and they use TLC to determine which fractions contain the desired compounds.

Analysis

As the chemicals being separated may be colorless, several methods exist to visualize the spots:

- Fluorescent analytes like quinine may be detected under black light (366 nm)
- Often a small amount of a fluorescent compound, usually manganese-activated zinc silicate, is added to the adsorbent that allows the visualization of spots under UV-C light (254 nm). The adsorbent layer will thus fluoresce light-green by itself, but spots of analyte quench this fluorescence.
- Iodine vapors are a general unspecific color reagent
- Specific color reagents into which the TLC plate is dipped or which are sprayed onto the plate exist.
    - Potassium permanganate - oxidation
    - Bromine
- In the case of lipids, the chromatogram may be transferred to a PVDF membrane and then subjected to further analysis, for example mass spectrometry, a technique known as Far-Eastern blotting.

Once visible, the R_f value, or retardation factor, of each spot can be determined by dividing the distance the product traveled by the distance the solvent front traveled using the initial spotting site as reference. These values depend on the solvent used and the type of TLC plate and are not physical constants.

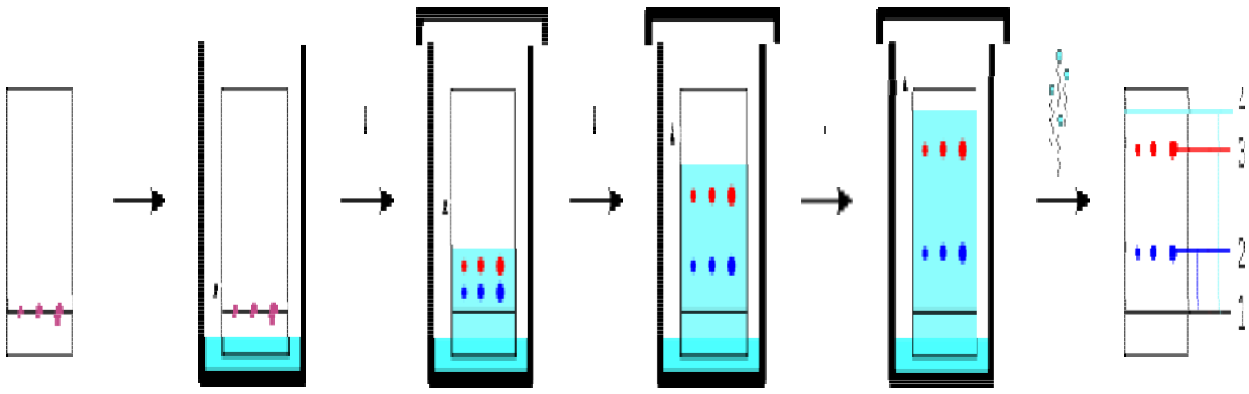


Fig.7.24 Development of a TLC plate, a purple spot separates into a red and blue spot

ISOLATION

Since different compounds will travel a different distance in the stationary phase, chromatography can in effect be used as an isolation technique.

The separated compounds each occupying a specific area on the plate, they can be scraped away, put in another solvent to separate them from the stationary phase and used for further analysis.

As an example, in the chromatography of an extract of green leaves (for example spinach) in 7 stages of development, Carotene elutes quickly and is only visible until step 2. Chlorophyll A and B are halfway in the final step and lutein the first compound staining yellow. Once the chromatography is over, the carotene can be removed from the plate, put back into a solvent and ran into a spectrophotometer to characterize its wavelength absorption.

- 

Step 1

- 

Step 2

- 

Step 3

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Step 4

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Step 5

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Step 6

- 

Step 7

Fig.7.25 Isolation

EXPERIMENT NO- 04

***OBJECTIVE*:** Study of the Recombinant DNA techniques

INTRODUCTION

Recombinant DNA (rDNA) molecules are DNA molecules formed by laboratory methods of genetic recombination (such as molecular cloning) to bring together genetic material from multiple sources, creating sequences that would not otherwise be found in the genome.

Recombinant DNA is possible because DNA molecules from all organisms share the same chemical structure. They differ only in the nucleotide sequence within that identical overall structure.

Recombinant DNA is the general name for a piece of DNA that has been created by the combination of at least two strands.

Recombinant DNA molecules are sometimes called chimeric DNA, because they can be made of material from two different species, like the mythical chimera. R-DNA technology uses palindromic sequences and leads to the production of sticky and blunt ends.

The DNA sequences used in the construction of recombinant DNA molecules can originate from any species.

For example, plant DNA may be joined to bacterial DNA, or human DNA may be joined with fungal DNA.

In addition, DNA sequences that do not occur anywhere in nature may be created by the chemical synthesis of DNA, and incorporated into recombinant molecules.

Using recombinant DNA technology and synthetic DNA, literally any DNA sequence may be created and introduced into any of a very wide range of living organisms.

Proteins that can result from the expression of recombinant DNA within living cells are termed recombinant proteins.

When recombinant DNA encoding a protein is introduced into a host organism, the recombinant protein is not necessarily produced.

Expression of foreign proteins requires the use of specialized expression vectors and often necessitates significant restructuring by foreign coding sequences]

Recombinant DNA differs from genetic recombination in that the former results from artificial methods in the test tube, while the latter is a normal biological process that results in the remixing of existing DNA sequences in essentially all organisms.

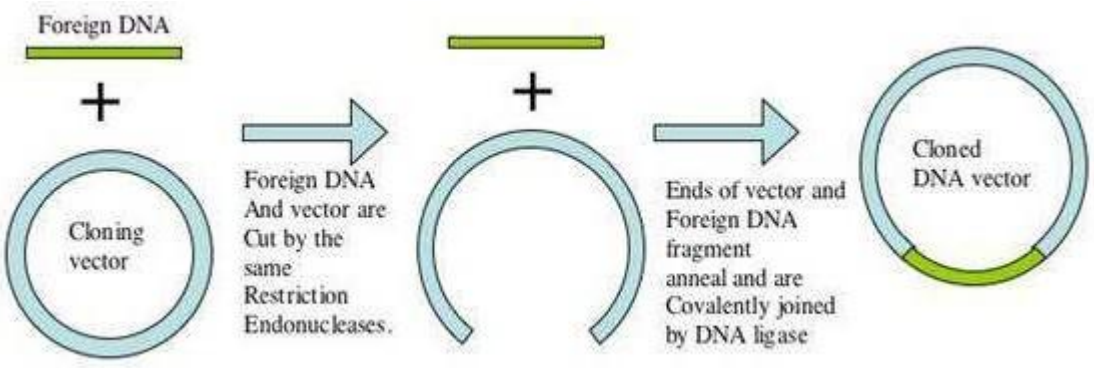

Fig.7.26 Construction of recombinant DNA, in which a foreign DNA fragment is inserted into a plasmid vector.In this example, the gene indicated by the white color is inactivated upon insertion of the foreign DNA fragment.

PROPERTIES OF ORGANISMS CONTAINING RECOMBINANT DNA

In most cases, organisms containing recombinant DNA have apparently normal phenotypes.

That is, their appearance, behavior and metabolism are usually unchanged, and the only way to demonstrate the presence of recombinant sequences is to examine the DNA itself, typically using a polymerase chain reaction (PCR) test. Significant exceptions exist, and are discussed below.

If the rDNA sequences encode a gene that is expressed, then the presence of RNA and/or protein products of the recombinant gene can be detected, typically using RT-PCR or western hybridization methods.

Gross phenotypic changes are not the norm, unless the recombinant gene has been chosen and modified so as to generate biological activity in the host organism.

Additional phenotypes that are encountered include toxicity to the host organism induced by the recombinant gene product, especially if it is over-expressed or expressed within inappropriate cells or tissues.

In some cases, recombinant DNA can have deleterious effects even if it is not expressed. One mechanism by which this happens is insertional inactivation, in which the rDNA becomes inserted into a host cell's gene.

In some cases, researchers use this phenomenon to "knock out" genes to determine their biological function and importance. Another mechanism by which rDNA insertion into chromosomal DNA can affect gene expression is by inappropriate activation of previously unexpressed host cell genes.

This can happen, for example, when a recombinant DNA fragment containing an active promoter becomes located next to a previously silent host cell gene, or when a host cell gene that functions to restrain gene expression undergoes insertional inactivation by recombinant DNA.

Uses:

Recombinant DNA is widely used in biotechnology, medicine and research. Today, recombinant proteins and other products that result from the use of DNA technology are found in essentially every western pharmacy, doctors or veterinarian's office, medical testing laboratory, and biological research laboratory.

The most common application of recombinant DNA is in basic research, in which the technology is important to most current work in the biological and biomedical sciences.

Recombinant DNA is used to identify, map and sequence genes, and to determine their function. rDNA probes are employed in analyzing gene expression within individual cells, and throughout the tissues of whole organisms.

Recombinant proteins are widely used as reagents in laboratory experiments and to generate antibody probes for examining protein synthesis within cells and organisms.

Many additional practical applications of recombinant DNA are found in industry, food production, human and veterinary medicine, agriculture, and bioengineering. Some specific examples are identified below.

Recombinant chymosin: Found in rennet, chymosin is an enzyme required to manufacture cheese.

Recombinant human insulin: Almost completely replaced insulin obtained from animal sources (e.g. pigs and cattle) for the treatment of insulin-dependent diabetes.

Recombinant human growth hormone (HGH, somatotropin): Administered to patients whose pituitary glands generate insufficient quantities to support normal growth and development.

Recombinant blood clotting factor VIII: A blood-clotting protein that is administered to patients with forms of the bleeding disorder hemophilia, who are unable to produce factor VIII in quantities sufficient to support normal blood coagulation.

Recombinant hepatitis B vaccine: Hepatitis B infection is controlled through the use of a recombinant hepatitis B vaccine, which contains a form of the hepatitis B virus surface antigen that is produced in yeast cells.

Diagnosis of infection with HIV: Each of the three widely used methods for diagnosing HIV infection has been developed using recombinant DNA.

Golden rice: A recombinant variety of rice that has been engineered to express the enzymes responsible for β-carotene biosynthesis.

Herbicide-resistant crops: Commercial varieties of important agricultural crops (including soy, maize/corn, sorghum, canola, alfalfa and cotton) have been developed that incorporate a recombinant gene that results in resistance to the herbicide glyphosate (trade name *Roundup*), and simplifies weed control by glyphosate application.

Insect-resistant crops: *Bacillus thuringeiensis* is a bacterium that naturally produces a protein (Bt toxin) with insecticidal properties. The bacterium has been applied to crops as an insect-control strategy for many years, and this practice has been widely adopted in agriculture and gardening.

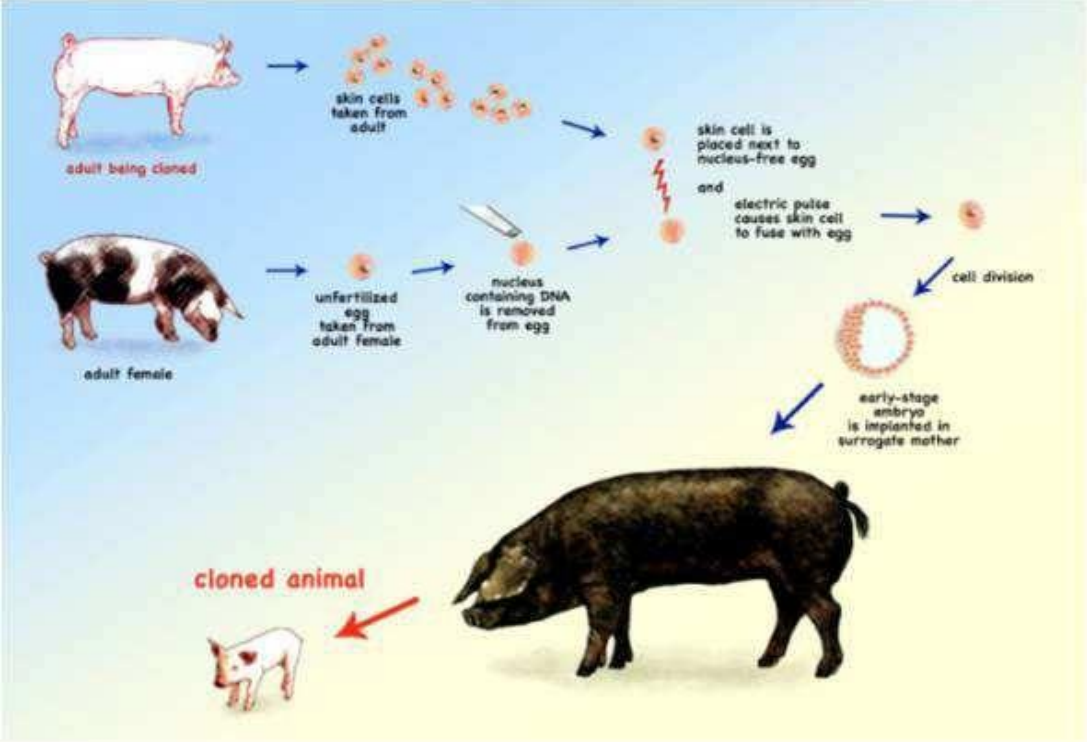

Fig.7.26 Diagram of pigs to show how animal cloning is carried out

SUMMERY

1. Laminar Flow Layers of water flow over one another at different speeds with virtually no mixing between layers.

2. The flow velocity profile for laminar flow in circular pipes is parabolic in shape, with a maximum flow in the center of the pipe and a minimum flow at the pipe walls. The average flow velocity is approximately one half of the maximum velocity.

Turbulent Flow The flow is characterized by the irregular movement of particles of the fluid.

4. Thanks to researchers such as these, we now know a great deal about genetic structure, and we continue to make great strides in understanding the human genome and the importance of DNA to life and health.

5. PCR (polymerase chain reaction) denotes a process that is used to replicate DNA.

6. The first step in PCR, known as denaturing, involves heating a DNA sample to separate its two strands. Once separated the two strands are used as templates to synthesise two new DNA strands. This is done with the help of an enzyme called Taq polymerase.

Recombinant DNA is an artificial form of DNA that cannot be found in natural organisms. It is made in the laboratory by joining together genes taken from different sources. This is done by selecting and cutting out a gene at a specific point on a strand of DNA using restriction enzymes which act as molecular scissors.

8. Thin-layer chromatography (TLC) is a chromatography technique used to separate non-volatile mixtures. Thin-layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide (alumina), or cellulose.

GLOSSARY

1. **Biotechnology:** A general term used to describe the use of biological processes to make products, in contrast to purely chemical processes. Biotechnology has been in practice for centuries and includes such traditional applications as the use of yeast in making beer, as well as modern applications like recombinant DNA techniques to improve crops.

2. **Recombinant DNA:** The DNA formed when DNA fragments from more than one organism are spliced together in vitro.

3. **Sequencing of DNA Molecules:** The process of finding the order of nucleotides (guanine, adenine, cytosine and thymine) that make up a DNA or RNA fragment.

4. **Stem cell :** A fundamental cell that has the potential to develop into any of the 210 different cell types found in the human body. Human life begins with stem cells, which divide again and again and branch off into special roles, like becoming liver or heart cells. They are an important resource for disease research and for the development of new ways to treat disease.

5. **Transcription:** A process in the cell where the DNA is used as a template to make the messenger RNA.

6. **Reynolds number:** Fluid flowing at a lower rate with elements of the fluid flowing in fixed streamlines. Laminar friction is a function of N_{Re} (Reynolds number). For laminar flow, the Fanning fracition factor = $16/N_{Re}$.

7. **Assay:** A method for determining the presence or quantity of a component.

8. **Clone:** A genetically identical copy of an organism or of a specific piece of DNA for use in research.

9. **Genetic mapping:** A research method that collects genetic information to determine the relative position of a gene or a phenotype in the genome.

10. **Microarray:** A glass or plastic slide with many DNA spots attached to it, which allows researchers to study how many genes act and interact in different conditions.

11. **Plasmid:** A DNA structure that is separate from the cell's genome and can replicate independently of the host cell. Plasmids are used in the laboratory to deliver specific DNA sequences into a cell.

SELF ASSESSMENT QUESTIONS

1. How biotechnology useful to human beings and animals?
2. What will be the use of recombinant DNA technology?
3. What is base pair?
4. What is genetic engineering?
5. How DNA sequencing completed?

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SUGGESTED READINGS

a. Biotechnology: Principles and Applications: H.K Das.
b. Biotechnology: A Problem Approach: Pranav Kumar and Usha Mina.
c. Animal Cell Culture and Technology (THE BASICS (Garland Science) by Michael Butler
d. A Text Book of Biotechnology – R.C. Dubey

TERMINAL QUESTIONS

1. Elaborate DNA recombinant technology?

2. Describe general steps of gene cloning.

3. Discuss method, principle of thin layer chromatography.

4. Who gave the DNA double helix model discuss in details.

5. Write the use of Tran illuminators in details.

Unit 8 Biotechnology /Biotechnique Exercise (II)

Content

8.1 Study of prepared slides, models or specimen

8.2 Escherichia coli

8.3 Bacteriophage

8.4 Plasmid

8.5 Southern blot

8.6 DNA isolation/ DNA Replication

Experiment No- 01

OBJECTIVE: Study of prepared slides, models or specimen

INTRODUCTION

Modern biotechnology techniques are based only on the isolation and manipulation of DNA these days. These techniques made it possible to detect diseases and make certain precautionary measures to protect people from these diseases. Some of the techniques are described in this article

DNA Isolation:

DNA isolation techniques totally depend on the experimental organism, but somehow these techniques have following characteristics.

1. A treatment for opening the cells and releasing their DNA
2. Method for inactivating and removing the enzymes which degrade the DNA
3. Method to separate the DNA from proteins and other molecules which contaminate the DNA

4. There are many cheap laboratory exercises for the isolation of DNA which are also very simple. DNA is usually taken from onion, bacteria and other organisms. Heat and detergent is used to isolate the DNA.

These methods break open the cell and inactivate the enzymes which degrade DNA. DNA is separated from other contaminants by using alcohol precipitation. The method of DNA isolation is more reproducible is isolation the DNA from bacteria16.

There are many companies who sell kits to for DNA isolation. DNA is isolated from human body cells to detect genetic diseases in the newborn child, to analyze the forensic evidence and to study genes which are involved in causing cancer.

DNA Transformation

Over the past couple of decades biotechnologists have succeeded in developing DNA transfer techniques.

It is the only the DNA which is required for the transformation. To get the DNA, in most cases tissues are exposed to stresses which enable the plasma membrane to rupture and in other cases cell wall of the plants is used.

Through this process some of the cells will contain the foreign DNA in their genome. This foreign DNA is helpful to make useful products and has been used in pharmaceuticals such as human insulin.

Restriction Enzyme Digestion and Analysis:

Restriction enzymes are actually the proteins. They cut the sequences of bases of DNA at specific places.

Biotechnologists are able to reproduce cut DNA molecules into small well defined fragments through these enzymes.

In biotechnology, restriction enzymes are used to make and analyze new DNA molecules. Agarose gel electrophoresis is the technique through which biotechnologists are able to make new DNA molecules. Agarose gel makes new DNA molecules through following methods

1) Chains of sugars make agarose.
2) DNA is moved in the agarose gel through electric current and this current cuts them into smaller pieces according to their size.
3) Special kind of dye makes possible the identification of small DNA molecules in the gel.

Polymerase chain reaction:

Polymerase chain reaction or PCR is a common technique in biotechnology. This technique is used for producing multiple copies of short DNA molecules. PCR runs through following process.

1) DNA molecule that will act as a template in the reaction
2) Short and single stranded DNA molecules which are called as primers. Primers are the starting

points for making new DNA molecule.
3) An enzyme is needed which makes DNA
4) Nucleotides which are the building blocks of DNA
5) Thermal cycler, a machine, which subjects the reaction mixture to varying temperatures repeatedly.

DNA SEQUENCING:

DNA sequencing is the most important thing in the human genome project. Many biotechnology techniques are dependent on this method.

To determine the exact order of bases in the DNA molecule is this technique's purpose. Nature of protein encoded in the DNA molecule can be determined through this technique.

Microarrays: -

It is a very useful technique of biotechnology. In this technique, expression of many genes can be analyzed simultaneously.

Microarrays can be made on a glass slide or a chip and they contain hundreds and thousands of parts of genes in a small section of the slide.

Expressed genes can be isolated from the cells and then are detected. This detection shows that which DNAs bind to which genes and are taken from which cells.

8.1. STUDY OF PREPARED SLIDES, MODELS OR SPECIMEN

Biotechnology is not mere facts, terms, and concepts. In the present era of biological research these two applied subjects are making significant contributions towards scientific knowledge and solving human problems.

In order to be an accomplished biotechnologist a thorough knowledge of Microbiology is essential. Microorganisms were probably the first living forms to appear on the earth.

Microorganisms are omnipresent in biosphere. Most microorganisms are free living and perform useful activities. Biosphere in general comprises of two categories: animate or living and inanimate or non-living beings.

Biology is the science that deals with the living organisms. The branch of biology that deals with microscopic forms of living organisms (microbes) is termed as the "microbiology".

The emergence and development of microbiology has been highly erratic in the beginning. Credit for introduction to microbial world goes to Antony van Leeuwenhoek and to its as of microbial activities to Louis Pasteur and Robert Koch.

Microbiology considers the microscopic forms of life as to their occurrence in nature.

Their reproduction and physiology, their harmful and beneficial relationship with other living things and their significance in science and industry

The methods used to study bacteria and other l1licrobes include direct microscopic examination, cultivation, biochemical tests, animal inoculation, serological reactivity and recent molecular biological techniques.

Currently because of their high versatility and rapid turnover microorganisms are used extensively) in genetic engineering and biotechnological processes and new processes are being developed to produce variety of enzymes of industrial importance, vaccines, insecticides, pharmaceuticals and other biological products of interest to mankind, animals and plants.

STUDY OF PREPARED SLIDES:

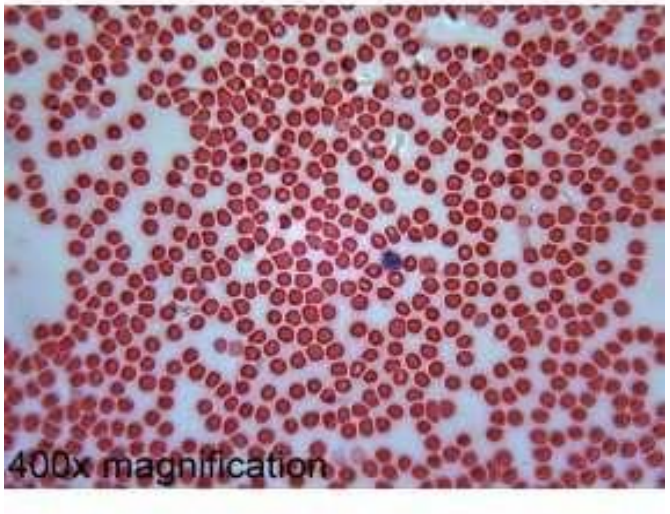

Fig 8.1 Smear of human blood showing both erythrocytes (red blood cells) and different types of leukocytes (white blood cells). Erythrocytes do not have a cell nucleus. Stained with Wright's blood stain

Fig.8.2 Shows mycelium and conidiophores with conidia

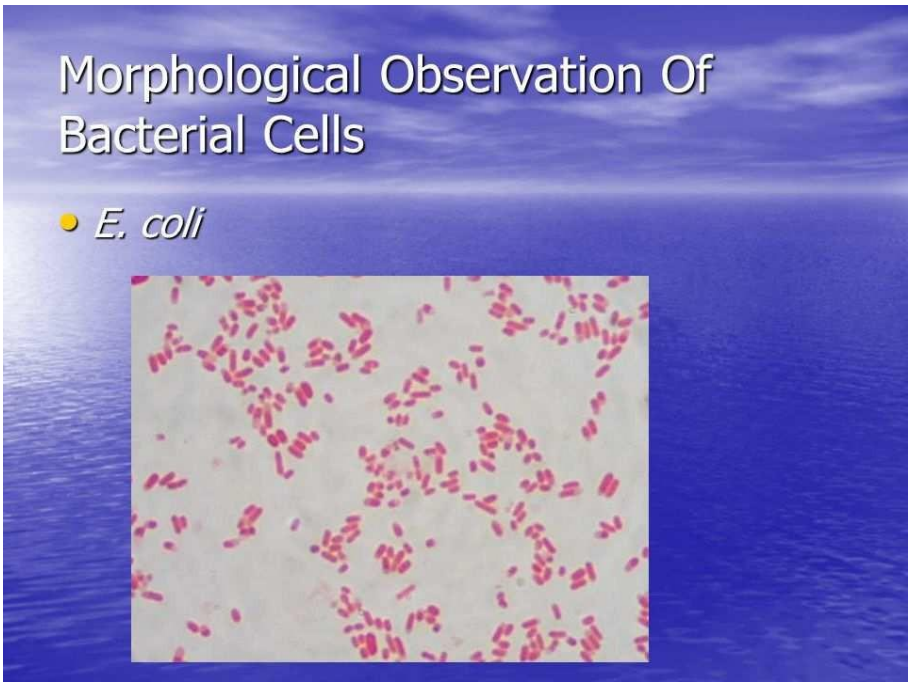

Fig.8.3 Microscopic view of Escherichia coli

EXPERIMENT NO-02

OBJECTIVE: Study of *Escherichia coli*

INTRODUCTION

Escherichia coli, a normal inhabitant of the human intestinal tract, is the most thoroughly studied of all organisms.

Studies of the mechanisms of genetic exchange and the biology of plasmids and bacteriophages of E. coli have been crucial in understanding many aspects of DNA replication and the expression of genetic material.

These studies have led to the ability to insert DNA from unrelated organisms into E. coli plasmids and bacteriophages, and to have that DNA replicated by the bacteria, with the genetic information it contains expressed by the bacteria.

It is thus possible for bacteria to become living factories for scarce biological products such as human insulin, interferon, and growth hormone. This process is called genetic engineering.

ROLE IN BIOTECHNOLOGY

Because of its long history of laboratory culture and ease of manipulation, *E. coli* plays an important role in modern biological engineering and industrial microbiology.

The work of Stanley Norman Cohen and Herbert Boyer in *E. coli*, using plasmids and restriction enzymes to create recombinant DNA, became a foundation of biotechnology.

E. *coli* is a very versatile host for the production of heterologous proteins, and various protein expression systems have been developed which allow the production of recombinant proteins in *E. coli*.

Researchers can introduce genes into the microbes using plasmids which permit high level expression of protein, and such protein may be mass-produced in industrial fermentation processes.

One of the first useful applications of recombinant DNA technology was the manipulation of *E. coli* to produce human insulin.

Many proteins previously thought difficult or impossible to be expressed in *E. coli* in folded form have been successfully expressed in *E. coli*.

For example, proteins with multiple disulphide bonds may be produced in the periplasmic space or in the cytoplasm of mutants rendered sufficiently oxidizing to allow disulphide-bonds to form, while proteins requiring post-translational modification such as glycosylation for stability or function have been expressed using the N-linked glycosylation system of *Campylobacter jejuni* engineered into *E. coli*.

Modified *E. coli* cells have been used in vaccine development, bioremediation, production of biofuels, lighting, and production of immobilised enzymes.

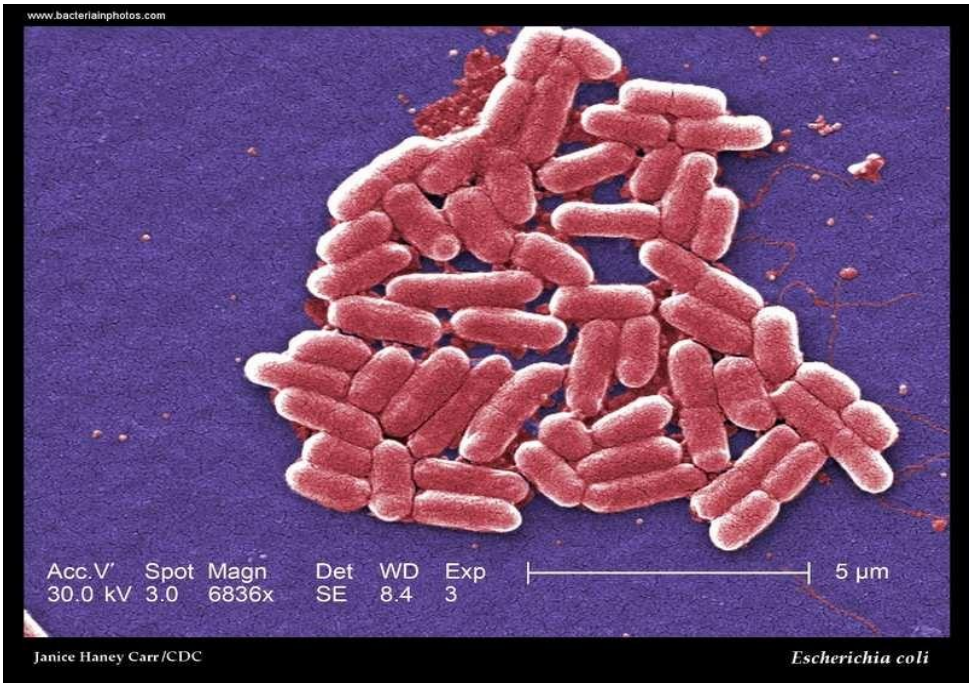

Fig.8.4Escherichia coli (strain O157:H7)

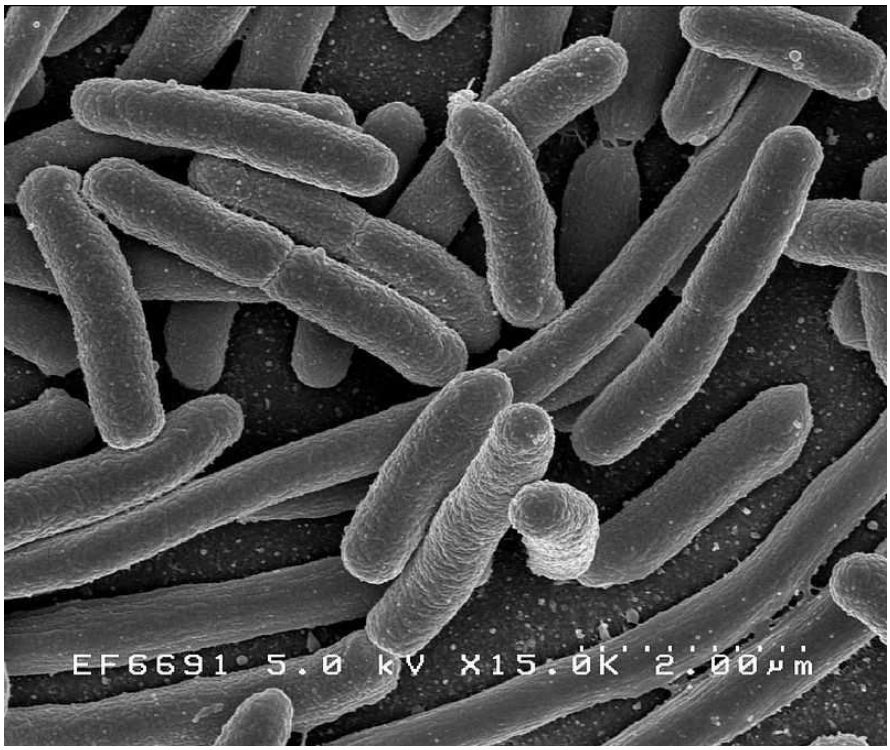

Fig.8.4 E. coli Bacteria

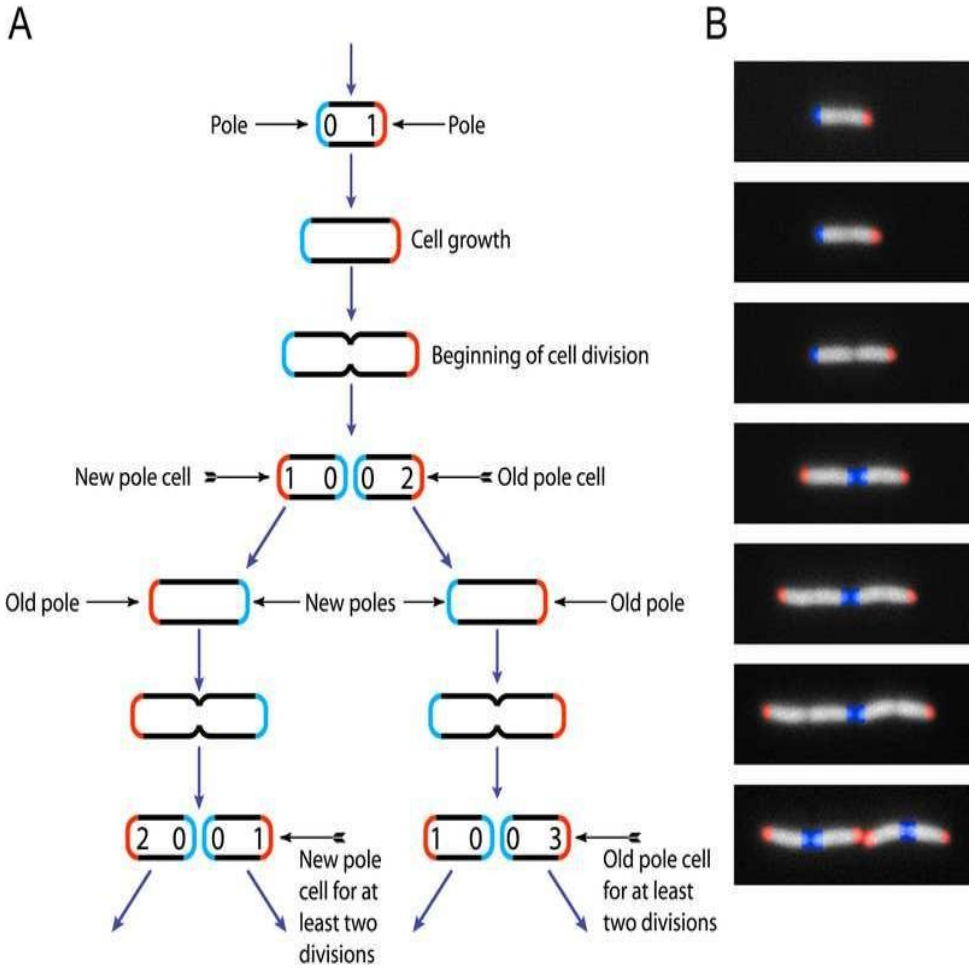

Fig.8.4 Successive Binary fission in E. coli

CULTURE GROWTH

Optimum growth of *E. coli* occurs at 37 °C (98.6 °F), but some laboratory strains can multiply at temperatures up to 49 °C (120 °F).

E. coli grows in a variety of defined laboratory media, such as lysogeny broth, or any medium that contains glucose, ammonium phosphate, monobasic, sodium chloride, magnesium sulfate, potassium phosphate, dibasic, and water.

Growth can be driven by aerobic or anaerobic respiration, using a large variety of redox pairs, including the oxidation of pyruvic acid, formic acid, hydrogen, and amino acids, and the

reduction of substrates such as oxygen, nitrate, fumarate, dimethyl sulfoxide, and trimethylamine N-oxide. *E. coli* is classified as a facultative anaerobe.

It uses oxygen when it is present and available. It can, however, continue to grow in the absence of oxygen using fermentation or anaerobic respiration. The ability to continue growing in the absence of oxygen is an advantage to bacteria because their survival is increased in environments where water predominates.

Exercise 1: Determination of size of bacteria

Objective: To determine the size of bacteria.

The size of microscopic objects including bacteria is expressed in microns (I 0-6m) or nanometers (I 0-9m). Such measurements are done with the ocular micrometer and a stage micrometer (for calibrating ocular micrometer).

Ocular micrometer is placed in the ocular region of the eyepiece. The ruled divisions superimposing specific distance on stage micrometer are counted.

By determining the number of divisions of the ocular micrometer that superimpose a known distance marked on the stage micrometer, one is able to calculate precisely the distance of each division on ocular micrometer.

After calibration, the ocular micrometer can be used for determining the size of various microscopic objects. The size of bacteria is normally determined in viable stained state (intra-vital staining).

Requirements

a. Bacterial culture: *E.coli*

b. Ocular micrometer and stage micrometer

c. Intra-vital stain (crystal violet 1: 120000).

Procedure

1. Remove the ocular lens and insert the ocular micrometer in ocular tube and replace the ocular lens and mount the eyepiece into the optical tube.

2. Mount the stage micrometer on the microscope stage.

3. Center the scale of the stage micrometer (with low power objective in position) while observing through eyepiece.

4. Bring oil immersion objective into position for observation.

5. Rotate the ocular micrometer containing eye piece so that the lines on it superimpose upon the stage micrometer divisions. Now make the lines of two micrometers coincide at one end.

6. Count the number of ocular micrometer divisions coinciding with stage micrometer exactly. Each stage micrometer corresponds to 10 microns.

7. Replace the stage micrometer with bacterial smears and count the number of divisions in the ocular scale that cover the bacterium.

8. Focus under oil immersion objective and record the observations for calculating the size of bacteria.
9. Measure the size of 5-6 different cells to find the average size of bacterial cell.

EXPERIMENT NO-03

OBJECTIVE: Study of Bacteriophage

INTRODUCTION

A **bacteriophage** is a virus that infects and replicates within a bacterium. Bacteriophages are composed of proteins that encapsulate a DNA or RNA genome, and may have relatively simple or elaborate structures.

Their genomes may encode as few as four genes, and as many as hundreds of genes. Phages replicate within the bacterium following the injection of their genome into its cytoplasm.

Bacteriophages are among the most common and diverse entities in the biosphere.

Phages are widely distributed in locations populated by bacterial hosts, such as soil or the intestines of animals.

One of the densest natural sources for phages and other viruses is sea water, where up to 9×10^8 virions per milliliter have been found in microbial mats at the surface, and up to 70% of marine bacteria may be infected by phages.

They have been used for over 90 years as an alternative to antibiotics in the former Soviet Union and Central Europe, as well as in France.

They are seen as a possible therapy against multi-drug-resistant strains of many bacteria Nevertheless, phages of Inoviridae have been shown to complicate biofilms involved in pneumonia and cystic fibrosis, shelter the bacteria from drugs meant to eradicate disease and promote persistent infection.

HISTORY

In 1896, Ernest Hanbury Hankin reported that something in the waters of the Ganges and Yamuna rivers in India had marked antibacterial action against cholera and could pass through a very fine porcelain filter. In 1915, British bacteriologist Frederick Twort, superintendent of the Brown Institution of London, discovered a small agent that infected and killed bacteria. He believed the agent must be one of the following:

1. A stage in the life cycle of the bacteria;
2. An enzyme produced by the bacteria themselves; or
3. A virus that grew on and destroyed the bacteria.

Replication

Bacteriophages may have a lytic cycle or a lysogenic cycle, and a few viruses are capable of carrying out both. With *lytic phages* such as the T4 phage, bacterial cells are broken open (lysed) and destroyed after immediate replication of the virion.

As soon as the cell is destroyed, the phage progeny can find new hosts to infect. Lytic phages are more suitable for phage therapy.

Some lytic phages undergo a phenomenon known as lysis inhibition, where completed phage progeny will not immediately lyse out of the cell if extracellular phage concentrations are high.

This mechanism is not identical to that of temperate phage going dormant and is usually temporary.

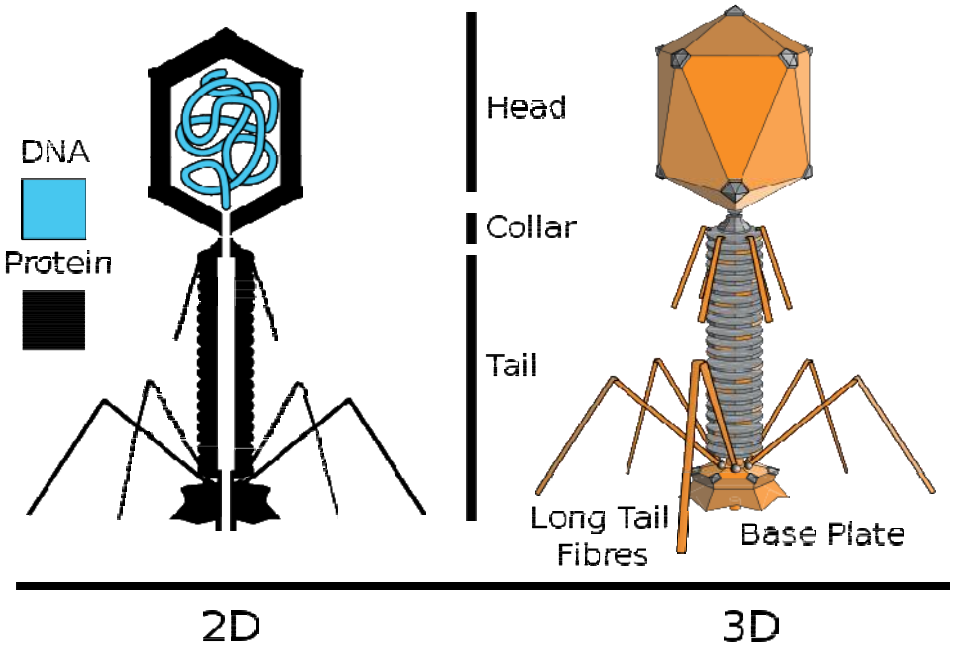

Fig.8.4 Typical tailed bacteriophage structure

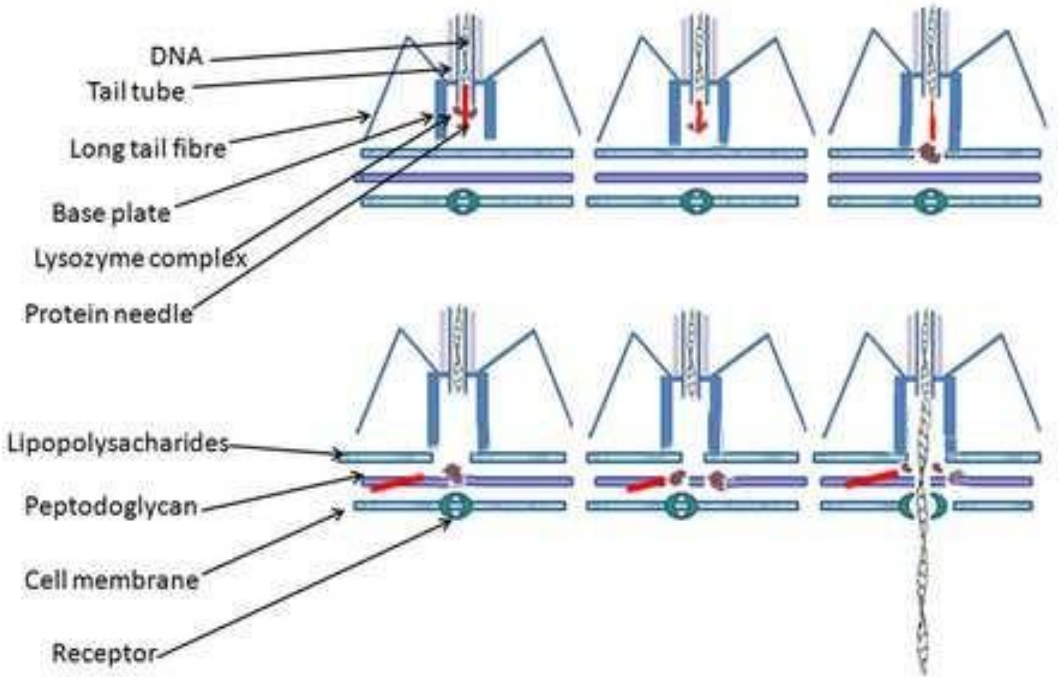

Fig.8.5 Diagram of the DNA injection process

EXPERIMENT NO-05

OBJECTIVE: Study of Plasmid

INTRODUCTION

A **plasmid** is a small DNA molecule within a cell that is physically separated from a chromosomal DNA and can replicate independently.

They are most commonly found in bacteria as small circular, double-stranded DNA molecules; however, plasmids are sometimes present in archaea and eukaryotic organisms.

In nature, plasmids often carry genes that may benefit the survival of the organism, for example antibiotic resistance.

While the chromosomes are big and contain all the essential genetic information for living under normal conditions, plasmids usually are very small and contain only additional genes that may be useful to the organism under certain situations or particular conditions.

Artificial plasmids are widely used as vectors in molecular cloning, serving to drive the replication of recombinant DNA sequences within host organisms.

Plasmids are considered *replicons*, a unit of DNA capable of replicating autonomously within a suitable host. However, plasmids, like viruses, are not generally classified as life.

Plasmids can be transmitted from one bacterium to another (even of another species) via three main mechanisms: transformation, transduction, and conjugation.

This host-to-host transfer of genetic material is called horizontal gene transfer, and plasmids can be considered part of the mobilome.

Unlike viruses (which encase their genetic material in a protective protein coat called a capsid), plasmids are "naked" DNA and do not encode genes necessary to encase the genetic material for transfer to a new host.

However, some classes of plasmids encode the conjugative "sex" pilus necessary for their own transfer.

The size of the plasmid varies from 1 to over 200 kbp, and the number of identical plasmids in a single cell can range anywhere from one to thousands under some circumstances.

The relationship between microbes and plasmid DNA is neither parasitic nor mutualistic, because each implies the presence of an independent species living in a detrimental or commensal state with the host organism. Rather, plasmids provide a mechanism for horizontal gene transfer within a population of microbes and typically provide a selective advantage under a given environmental state.

Plasmids may carry genes that provide resistance to naturally occurring antibiotics in a competitive environmental niche, or the proteins produced may act as toxins under similar circumstances, or allow the organism to utilize particular organic compounds that would be advantageous when nutrients are scarce.

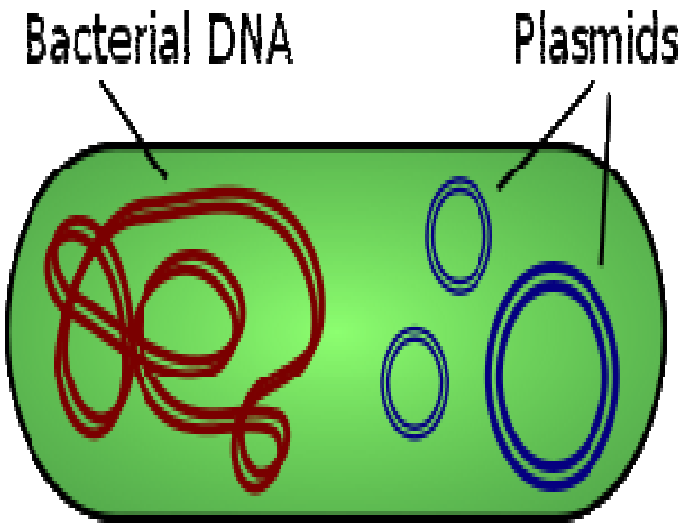

Fig.8.6 Illustration of a bacterium showing chromosomal DNA and plasmids

PROPERTIES AND CHARACTERISTICS

There are two types of plasmid integration into a host bacterium: Non-integrating plasmids replicate as with the top instance, whereas episomes, the lower example, can integrate into the host chromosome.

In order for plasmids to replicate independently within a cell, they must possess a stretch of DNA that can act as an origin of replication.

The self-replicating unit, in this case the plasmid, is called a replicon. A typical bacterial replicon may consist of a number of elements, such as the gene for plasmid-specific replication initiation protein (Rep), repeating units called iterons, DnaA boxes, and an adjacent AT-rich region.

Smaller plasmids make use of the host replicative enzymes to make copies of themselves, while larger plasmids may carry genes specific for the replication of those plasmids.

A few types of plasmids can also insert into the host chromosome, and these integrative plasmids are sometimes referred to as episomes in prokaryotes.

Plasmids almost always carry at least one gene. Many of the genes carried by a plasmid are beneficial for the host cells, for example: enabling the host cell to survive in an environment that would otherwise be lethal or restrictive for growth.

Some of these genes encode traits for antibiotic resistance or resistance to heavy metal, while others may produce virulence factors that enable a bacterium to colonize a host and overcome its defences, or have specific metabolic functions that allow the bacterium to utilize a particular nutrient, including the ability to degrade recalcitrant or toxic organic compounds. Plasmids can also provide bacteria with the ability to fix nitrogen.

Some plasmids, however, have no observable effect on the phenotype of the host cell or its benefit to the host cells cannot be determined, and these plasmids are called cryptic plasmids.

Naturally occurring plasmids vary greatly in their physical properties. Their size can range from very small mini-plasmids of less than a 1 kilobase pairs (Kbp), to very large megaplasmids of several megabase pairs (Mbp).

At the upper end, little can differentiate between a megaplasmid and a minichromosome. Plasmids are generally circular, however examples of linear plasmids are also known. These linear plasmids require specialized mechanisms to replicate their ends.

Plasmids may be present in an individual cell in varying number, ranging from one to several hundreds. The normal number of copies of plasmid that may be found in a single cell is called the copy number, and is determined by how the replication initiation is regulated and the size of the molecule. Larger plasmids tend to have lower copy numbers.

Low-copy-number plasmids that exist only as one or a few copies in each bacterium are, upon cell division, in danger of being lost in one of the segregating bacteria.

Such single-copy plasmids have systems that attempt to actively distribute a copy to both daughter cells. These systems, which include the parABS system and parMRC system, are often referred to as the partition system or partition function of a plasmid.

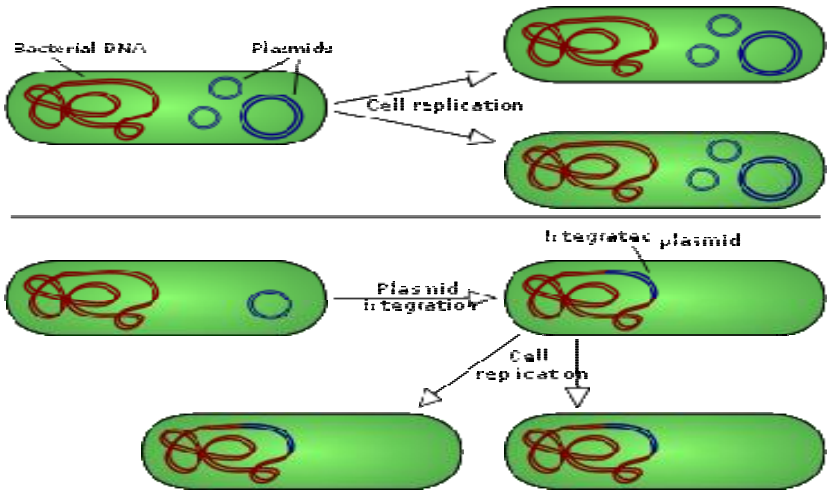

Fig.8.6 there are two types of plasmid integration into a host bacterium: Non-integrating plasmids replicate as with the top instance, whereas episomes, the lower example, can integrate into the host chromosome

EXPERIMENT NO- 06

***OBJECTIVE*:** To identify the Southern Blotting Principle, Procedure and Application

A **Southern blot** is a method used in molecular biology for detection of a specific DNA sequence in DNA samples. Southern blotting combines transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization.

The method is named after its inventor, the British biologist Edwin Southern.Other blotting methods (i.e., western blot, northern blot, eastern blot, southwestern blot) that employ similar principles, but using RNA or protein, have later been named in reference to Edwin Southern's name.

As the label is eponymous, Southern is capitalised, as is conventional for proper nouns. The names for other blotting methods may follow this convention, by analogy.

METHOD

1. Restriction endonucleases are used to cut high-molecular-weight DNA strands into smaller fragments.
2. The DNA fragments are then electrophoreses on an agarose gel to separate them by size.
3. If some of the DNA fragments are larger than 15 kb, then prior to blotting, the gel may be treated with an acid, such as dilute HCl. This depurinates the DNA fragments, breaking the DNA into smaller pieces, thereby allowing more efficient transfer from the gel to membrane.
4. If alkaline transfer methods are used, the DNA gel is placed into an alkaline solution (typically containing sodium hydroxide) to denature the double-stranded DNA.
5. The denaturation in an alkaline environment may improve binding of the negatively charged thymine residues of DNA to a positively charged amino groups of membrane, separating it into single DNA strands for later hybridization to the probe (see below), and destroys any residual RNA that may still be present in the DNA.
6. The choice of alkaline over neutral transfer methods, however, is often empirical and may result in equivalent results.]
7. A sheet of nitrocellulose (or, alternatively, nylon) membrane is placed on top of (or below, depending on the direction of the transfer) the gel.
8. Pressure is applied evenly to the gel (either using suction, or by placing a stack of paper towels and a weight on top of the membrane and gel), to ensure good and even contact between gel and membrane.
9. If transferring by suction, 20X SSC buffer is used to ensure a seal and prevent drying of the gel. Buffer transfer by capillary action from a region of high water potential to a region of low water potential (usually filter paper and paper tissues) is then used to move the DNA from the gel onto the membrane; ion exchange interactions bind the DNA to the membrane due to the negative charge of the DNA and positive charge of the membrane.
10. The membrane is then baked in a vacuum or regular oven at 80 °C for 2 hours (standard conditions; nitrocellulose or nylon membrane) or exposed to ultraviolet radiation (nylon membrane) to permanently attach the transferred DNA to the membrane.
11. The membrane is then exposed to a hybridization probe—a single DNA fragment with a specific sequence whose presence in the target DNA is to be determined. The probe DNA

is labelled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye.

12. In some cases, the hybridization probe may be made from RNA, rather than DNA. To ensure the specificity of the binding of the probe to the sample DNA, most common hybridization methods use salmon or herring sperm DNA for blocking of the membrane surface and target DNA, deionized formamide, and detergents such as SDS to reduce non-specific binding of the probe.
13. After hybridization, excess probe is washed from the membrane (typically using SSC buffer), and the pattern of hybridization is visualized on X-ray film by autoradiography in the case of a radioactive or fluorescent probe, or by development of colour on the membrane if a chromogenic detection method is used.

RESULT

Hybridization of the probe to a specific DNA fragment on the filter membrane indicates that this fragment contains DNA sequence that is complementary to the probe.

The transfer step of the DNA from the electrophoresis gel to a membrane permits easy binding of the labeled hybridization probe to the size-fractionated DNA.

It also allows for the fixation of the target-probe hybrids, required for analysis by autoradiography or other detection methods. Southern blots performed with restriction enzyme-digested genomic DNA may be used to determine the number of sequences (e.g., gene copies) in a genome.

A probe that hybridizes only to a single DNA segment that has not been cut by the restriction enzyme will produce a single band on a Southern blot, whereas multiple bands will likely be observed when the probe hybridizes to several highly similar sequences (e.g., those that may be the result of sequence duplication).

Modification of the hybridization conditions (for example, increasing the hybridization temperature or decreasing salt concentration) may be used to increase specificity and decrease hybridization of the probe to sequences that are less than 100% similar.

APPLICATIONS

Southern transfer may be used for homology-based cloning on the basis of amino acid sequence of the protein product of the target gene.

Oligonucleotides are designed that are similar to the target sequence. The oligonucleotides are chemically synthesised, radiolabeled, and used to screen a DNA library, or other collections of cloned DNA fragments.

Sequences that hybridize with the hybridization probe are further analysed, for example, to obtain the full length sequence of the targeted gene.

Southern blotting can also be used to identify methylated sites in particular genes. Particularly useful are the restriction nucleases *MspI* and *HpaII*, both of which recognize and cleave within the same sequence.

However, *HpaII* requires that a C within that site be methylated, whereas *MspI* cleaves only DNA unmethylated at that site. Therefore, any methylated sites within a sequence analyzed with a particular probe will be cleaved by the former, but not the latter, enzyme.

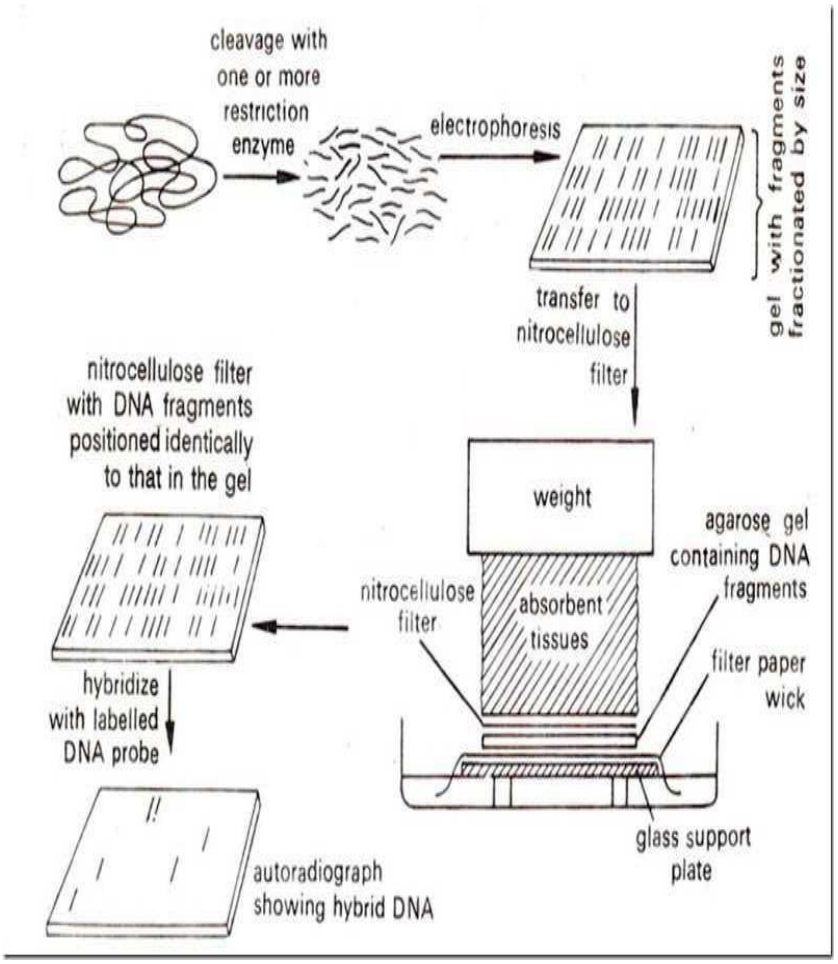

Fig.8.7 southern blotting process

EXPERIMENT NO- 07

OBJECTIVE: Study of DNA Isolation/ DNA Replication

INTRODUCTION

DNA isolation is a process of purification of DNA from sample using a combination of physical and chemical methods. The first isolation of DNA was done in 1869 by Friedrich Miescher. Currently it is a routine procedure in molecular biology or forensic analyses.

BASIC PROCEDURE

There are three basic and two optional steps in a DNA extraction:

- Cells which are to be studied need to be collected.
- Breaking the cell membranes open to expose the DNA along with the cytoplasm within (cell lysis).
    - Lipids from the cell membrane and the nucleus are broken down with detergents and surfactants.
    - Breaking proteins by adding a protease (optional).
    - Breaking RNA by adding an RNase (optional).
- The solution is treated with concentrated salt solution to make debris such as broken proteins, lipids and RNA to clump together.
- Centrifugation of the solution, which separates the clumped cellular debris from the DNA.
- DNA purification from detergents, proteins, salts and reagents used during cell lysis step. The most commonly used procedures are:
    - Ethanol precipitation usually by ice-cold ethanol or isopropanol. Since DNA is insoluble in these alcohols, it will aggregate together, giving a *pellet* upon

centrifugation. Precipitation of DNA is improved by increasing of ionic strength, usually by adding sodium acetate.

- Phenol–chloroform extraction in which phenol denatures proteins in the sample. After centrifugation of the sample, denaturated proteins stay in the organic phase while aqueous phase containing nucleic acid is mixed with the chloroform that removes phenol residues from solution.
- Minicolumn purification that relies on the fact that the nucleic acids may bind (adsorption) to the solid phase (silica or other) depending on the pH and the salt concentration of the buffer.

Cellular and histone proteins bound to the DNA can be removed either by adding a protease or by having precipitated the proteins with sodium or ammonium acetate, or extracted them with a phenol-chloroform mixture prior to the DNA-precipitation.

After isolation, the DNA is dissolved in slightly alkaline buffer, usually in the TE buffer, or in ultra-pure water.

Special types

Specific techniques must be chosen for isolation of DNA from some samples. Typical samples with complicated DNA isolation are:

- archaeological samples containing partially degraded DNA, see ancient DNA
- samples containing inhibitors of subsequent analysis procedures, most notably inhibitors of PCR, such as humic acid from soil, indigo and other fabric dyes or haemoglobin in blood
- samples from microorganisms with thick cellular wall, for example yeast

Extrachromosomal DNA is generally easy to isolate, especially plasmids may be easily isolated by cell lysis followed by precipitation of proteins, which traps chromosomal DNA in insoluble fraction and after centrifugation, plasmid DNA can be purified from soluble fraction.

A Hirt DNA Extraction is an isolation of all extrachromosomal DNA in a mammalian cell. The Hirt extraction process gets rid of the high molecular weight nuclear DNA, leaving only low molecular weight mitochondrial DNA and any viral episomes present in the cell.

Detecting DNA

A diphenylamine (DPA) indicator will confirm the presence of DNA. This procedure involves chemical hydrolysis of DNA: when heated (e.g. ≥95 °C) in acid, the reaction requires a deoxyribose sugar and therefore is specific for DNA.

Under these conditions, the 2-deoxyribose is converted to w-hydroxylevulinyl aldehyde, which reacts with the compound, diphenylamine, to produce a blue-colored compound. DNA concentration can be determined measuring the intensity of absorbance of the solution at the 600 nm with a spectrophotometer and comparing to a standard curve of known DNA concentrations.

Measuring the intensity of absorbance of the DNA solution at wavelengths 260 nm and 280 nm is used as a measure of DNA purity. DNA absorbs UV light at 260 and 280 nanometres, and aromatic proteins absorb UV light at 280 nm; a pure sample of DNA has a ratio of 1.8 at 260/280 and is relatively free from protein contamination. A DNA preparation that is contaminated with protein will have a 260/280 ratio lower than 1.8.

DNA can be quantified by cutting the DNA with a restriction enzyme, running it on an agarose gel, staining with ethidium bromide or a different stain and comparing the intensity of the DNA with a DNA marker of known concentration.

Using the Southern blot technique, this quantified DNA can be isolated and examined further using PCR and RFLP analysis. These procedures allow differentiation of the repeated sequences within the genome. It is these techniques which forensic scientists use for comparison, identification, and analysis.

DNA REPLICATION

The double helix is un'zipped' and unwound, then each separated strand (turquoise) acts as a template for replicating a new partner strand (green).

Nucleotides (bases) are matched to synthesize the new partner strands into two new double helices.

In molecular biology, **DNA replication** is the biological process of producing two identical replicas of DNA from one original DNA molecule.

This process occurs in all living organisms and is the basis for biological inheritance. DNA is made up of a double helix of two complementary strands. During replication, these strands are separated.

Each strand of the original DNA molecule then serves as a template for the production of its counterpart, a process referred to as semiconservative replication.

Cellular proofreading and error-checking mechanisms ensure near perfect fidelity for DNA replication.

In a cell, DNA replication begins at specific locations, or origins of replication, in the genome. Unwinding of DNA at the origin and synthesis of new strands results in replication forks growing bi-directionally from the origin.

A number of proteins are associated with the replication fork to help in the initiation and continuation of DNA synthesis.

Most prominently, DNA polymerase synthesizes the new strands by adding nucleotides that complement each (template) strand. DNA replication occurs during the S-stage of interphase.

DNA replication can also be performed *in vitro* (artificially, outside a cell).

DNA polymerases isolated from cells and artificial DNA primers can be used to initiate DNA synthesis at known sequences in a template DNA molecule.

The polymerase chain reaction (PCR), a common laboratory technique, cyclically applies such artificial synthesis to amplify a specific target DNA fragment from a pool of DNA.

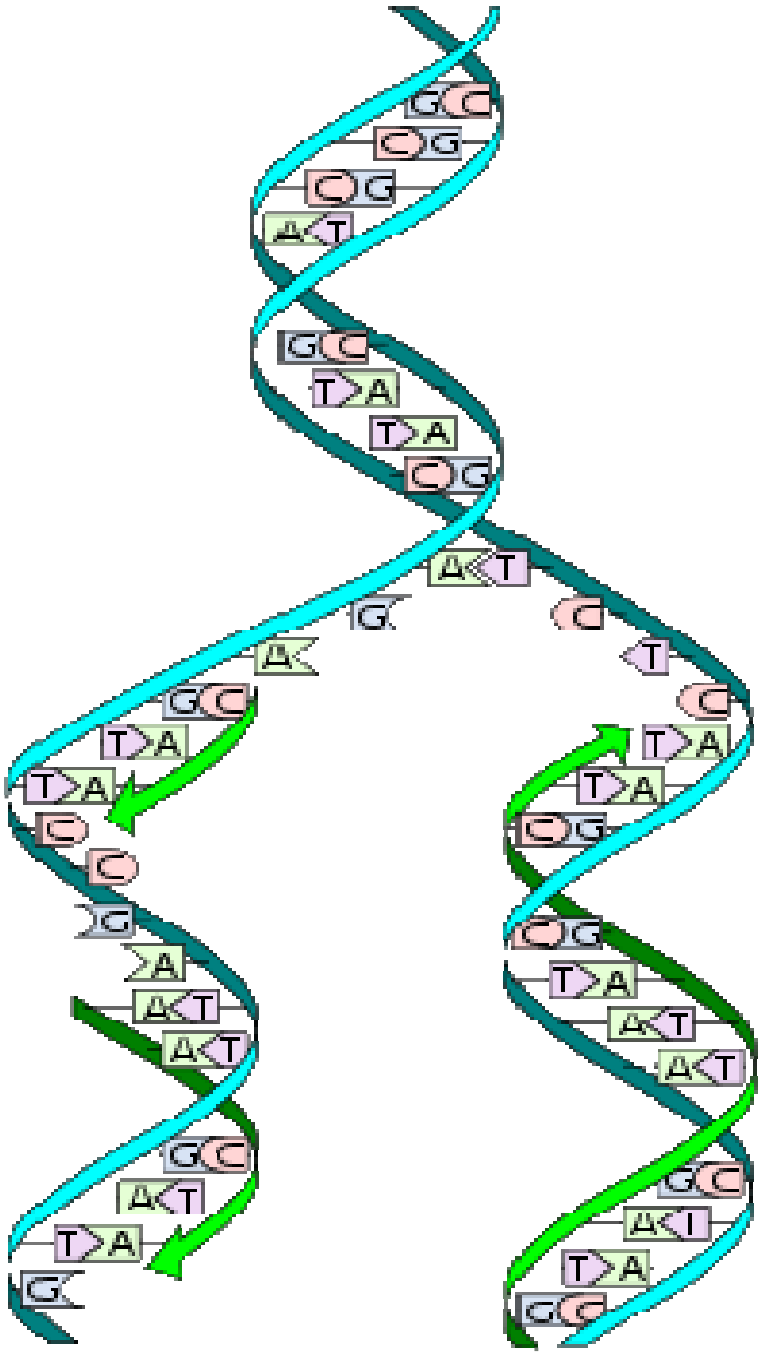

Fig.8.8 DNA replication: The double helix is un'zipped' and unwound, then each separated strand (turquoise) acts as a template for replicating a new partner strand (green). Nucleotides (bases) are matched to synthesize the new partner strands into two new double helices.

DNA structures

DNA usually exists as a double-stranded structure, with both strands coiled together to form the characteristic double-helix. Each single strand of DNA is a chain of four types of nucleotides.

Nucleotides in DNA contain a deoxyribose sugar, a phosphate, and a nucleobase. The four types of nucleotide correspond to the four nucleobases adenine, cytosine, guanine, and thymine, commonly abbreviated as A,C, G and T. Adenine and guanine are purine bases, while cytosine and thymine are pyrimidines.

These nucleotides form phosphodiester bonds, creating the phosphate-deoxyribose backbone of the DNA double helix with the nuclei bases pointing inward (i.e., toward the opposing strand).

Nucleotides (bases) are matched between strands through hydrogen bonds to form base pairs. Adenine pairs with thymine (two hydrogen bonds), and guanine pairs with cytosine (stronger: three hydrogen bonds).

DNA strands have a directionality, and the different ends of a single strand are called the "3' (three-prime) end" and the "5' (five-prime) end". By convention, if the base sequence of a single strand of DNA is given, the left end of the sequence is the 5' end, while the right end of the sequence is the 3' end.

The strands of the double helix are anti-parallel with one being 5' to 3', and the opposite strand 3' to 5'. These terms refer to the carbon atom in deoxyribose to which the next phosphate in the chain attaches.

Directionality has consequences in DNA synthesis, because DNA polymerase can synthesize DNA in only one direction by adding nucleotides to the 3' end of a DNA strand.

The pairing of complementary bases in DNA (through hydrogen bonding) means that the information contained within each strand is redundant. Phosphodiester (intra-strand) bonds are stronger than hydrogen (inter-strand) bonds.

This allows the strands to be separated from one another. The nucleotides on a single strand can therefore be used to reconstruct nucleotides on a newly synthesized partner strand.

REPLICATION PROCESS

DNA replication, like all biological polymerization processes, proceeds in three enzymatically catalyzed and coordinated steps: initiation, elongation and termination.

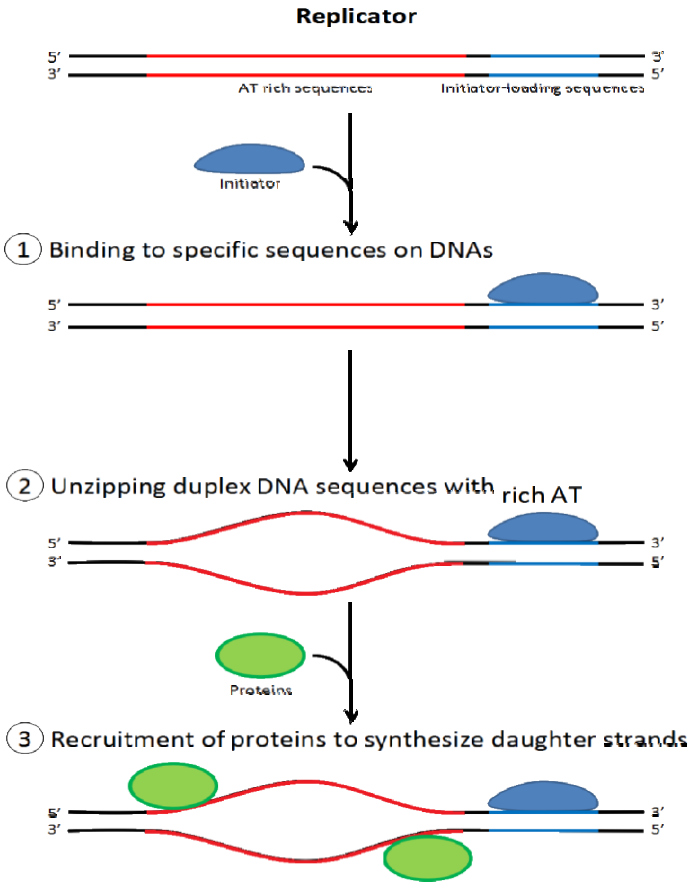

Fig.8.67Role of initiators for initiation of DNA replication

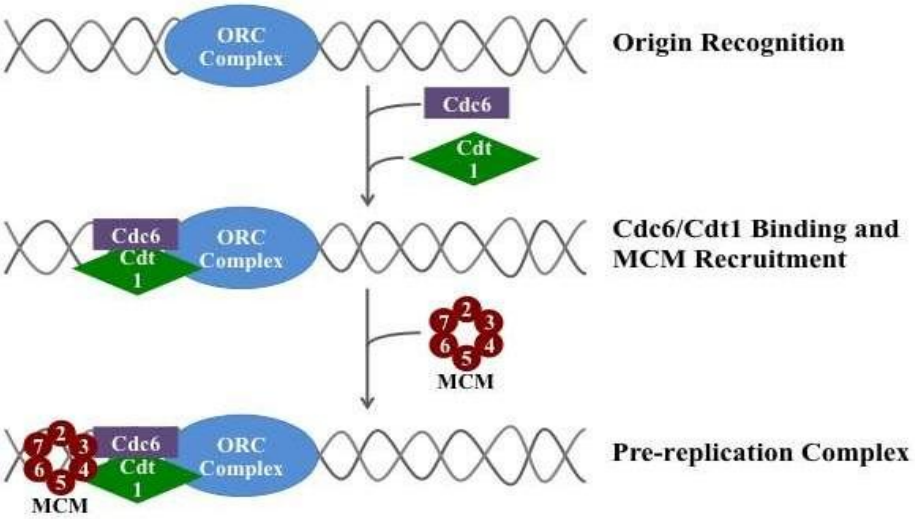

Fig.8.8 Formation of pre-replication complex

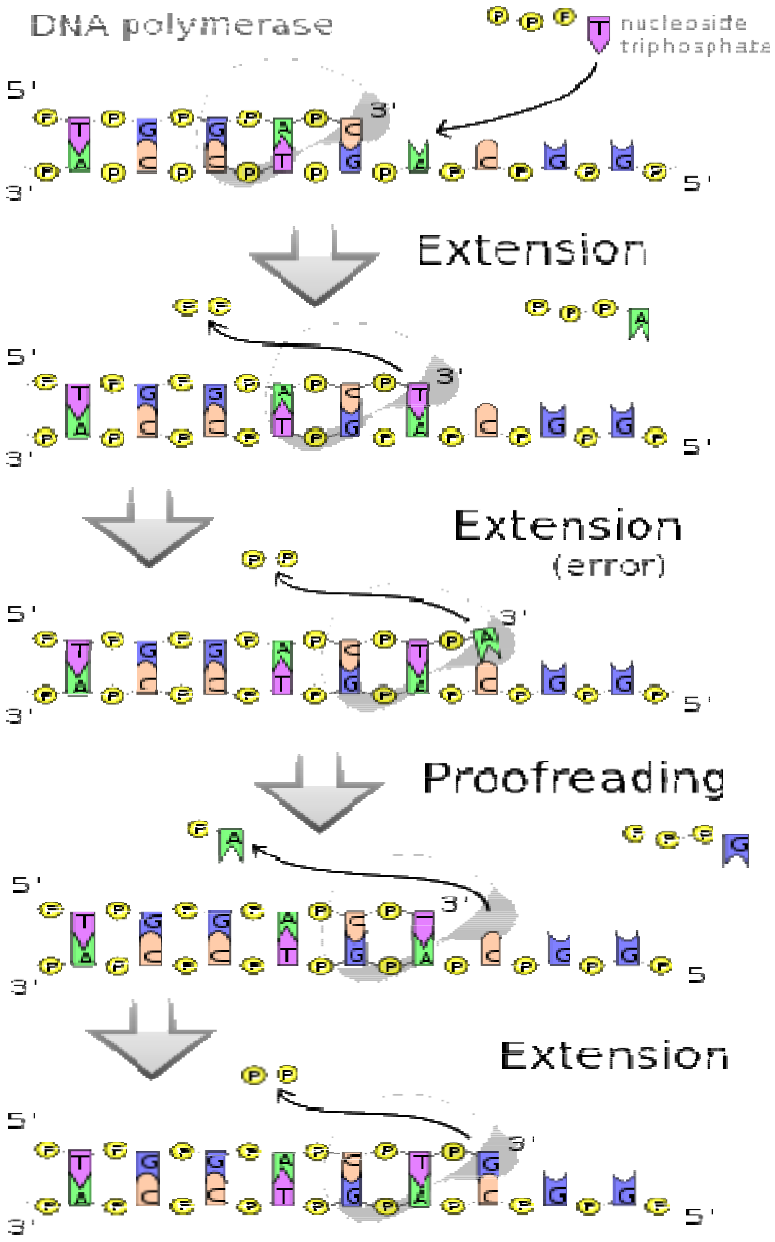

Fig.8.9 DNA polymerases add nucleotides to the 3' end of a strand of DNA. If a mismatch is accidentally incorporated, the polymerase is inhibited from further extension. Proofreading removes the mismatched nucleotide and extension continues

SUMMERY

1. Biotechnology is a broad discipline in which biological processes, organisms, cells or cellular components are exploited to develop new technologies. New tools and products developed by biotechnologists are useful in research, agriculture, industry and the clinic.

2. Biotechnology is the use of an organism, or a component of an organism or other biological system, to make a product or process.

3. Many forms of modern biotechnology rely on DNA technology.

4. DNA technology is the sequencing, analysis, and cutting-and-pasting of DNA.

5. Common forms of DNA technology include DNA sequencing, polymerase chain reaction, DNA cloning, and gel electrophoresis.

6. Biotechnology inventions can raise new practical concerns and ethical questions that must be addressed with informed input from all of society.

7. Escherichia coli are common bacteria that live in the digestive tract of healthy animals. Many E. coli strains do not cause disease, but some types of E. coli, make a toxin that can cause significant illness in humans when they ingest the bacteria.

8. Thousands of varieties of phage exist, each of which may infect only one type or a few types of bacteria or archaea. Phages are classified in a number of virus families; some examples include Inoviridae, Microviridae, Rudiviridae, and Tectiviridae. Like all viruses, phages are simple organisms that consist of a core of genetic material (nucleic acid) surrounded by a protein capsid

9. A plasmid is a strand or loop of DNA that is typically found in bacteria as well as archae (single-cell organisms) and eukarya (organisms of complex cell structure). Plasmids carry only a few genes and exist independently of chromosomes, the primary structures that contain DNA in cells.

10. Southern blotting combines transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization.

11. DNA isolation is a process of purification of DNA from sample using a combination of physical and chemical methods.

12. DNA replication of one helix of DNA results in two identical helices. DNA creates "daughters" by using the parental strands of DNA as a template or guide. Each newly synthesized strand of DNA (daughter strand) is made by the addition of a nucleotide that is complementary to the parent strand of DNA.

GLOSSARY

1. **E. coli** - *Escherichia coli*

2. **Antigen** — A substance, usually a protein, that causes the formation of an antibody and reacts specifically with that antibody.

3. **Anti-motility medications** — Medications such as loperamide (Imodium), dephenoxylate (Lomotil), or medications containing Codeine or narcotics which decrease the ability of the intestine to contract. This can worsen the condition of a patient with dysentery or colitis.

4. **Colitis** — Inflammation of the colon or large intestine, usually causing diarrhea that may be bloody.

5. **Bacteriophase.-** A virus that infects and replicates within a bacterium

6. **Plasmid**: A type of cloning vector derived from autonomously-replicating extra-chromosomal circular DNAs in bacteria. The amount of foreign DNA that can be carried in a plasmid is small, ranging up to about 20 kb.

7. **Chromosomal DNA: Genomic deoxyribonucleic acid** is chromosomal DNA, in contrast to extra-chromosomal DNAs like plasmids.

8. **Archaea:** The **Archaea** constitute a domain and kingdom of single-celled microorganisms. These microbes (**archaea**; singular **archaeon**) are prokaryotes, meaning that they have no cell nucleus or any other membrane-bound organelles in their cells.

9. **DNA Sequence**: A **nucleic acid sequence** is a succession of letters that indicate the order of nucleotides within a DNA (using GACT) or RNA (GACU) molecule.

10. **Hybridization probe:** In molecular biology, a hybridization probe is a fragment of DNA or RNA of variable length (usually 100–1000 bases long) which can be radioactively labeled.

11. **Agarose gel electrophoresis**: A matrix composed of a highly purified form of agar is used to separate larger DNA and RNA molecules ranging from 100 to 20,000 nucleotides.

13. **Base pairing**: Discovery made by James Watson that was key in solving the structure of DNA. The bases that make up DNA pair with each other: A to T and G to C. The pairs are held together by hydrogen bonds.

14. **Complementary DNA (cDNA**): The matching strand of a DNA molecule to which its bases pair.

15. **DNA array:** DNA arrays (also known as microarrays or gene chips) can analyze patterns of gene expression and show how gene expression responds to external factors.

16. **Double helix:** Describes the coiling of the anti-parallel strands of the DNA molecule, resembling a spiral staircase in which the paired bases form the steps and the sugar-phosphate backbones form the rails.

17. **Electrophoresis:** The technique of separating charged molecules in a matrix to which are applied an electrical field.

18. **Protease:** An enzyme that cleaves peptide bonds that link amino acids in protein molecules.

SELF ASSESSMENT QUESTIONS

1. What if there was a human-sized bacteriophage virus in the same room as me?

2. How are bacteriophages used as vectors?

3. How does a plasmid work?

4. How are recombinant plasmids used?

5. What is blotting in biology?

6. What is Southwestern blotting?

7. Why would you want to extract DNA?

8. Why do you use meat tenderizer in DNA extraction?

9. How do you get DNA?

10 Why do we need to learn about DNA?

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TERMINAL QUESTIONS

1. What are the different parts of bacteriophage?

2. What are the different types of anaerobic bacteria?
3. What is a plasmid and how can it be used in genetic engineering?
4. What are Northern blots used for?
5. What is the average size of E.coli?
6. Why do we use highly diluted crystal violet?

UNIT 10: BIOSTATICS EXERCISE

INTRODUCTION

Biostatistics is the application of statistics to a wide range of topics in biology. The science of biostatistics encompasses the design of biological experiments, especially in medicine, pharmacy, agriculture and fishery; the collection, summarization, and analysis of data from those experiments; and the interpretation of, and inference from, the results.

A major branch of this is medical biostatistics, which is exclusively concerned with medicine and health.

BACKGROUND

In the early 1900s, after the rediscovery of Gregor Mendel's Mendelian inheritance work, the gaps in understanding between genetics and evolutionary Darwinism led to vigorous debate among biometricians, such as Walter Weldon and Karl Pearson, and Mendelians, such as Charles Davenport, William Bateson and Wilhelm Johannsen. By the 1930s, statisticians and models built on statistical reasoning had helped to resolve these differences and to produce the neo-Darwinian modern evolutionary synthesis.

The leading figures in the establishment of population genetics and this synthesis all relied on statistics and developed its use in biology.

These individuals and the work of other biostatisticians, mathematical biologists, and statistically inclined geneticists helped bring together evolutionary biology and genetics into a consistent, coherent whole that could begin to be quantitatively modeled.

In parallel to this overall development, the pioneering work of D'Arcy Thompson in On Growth and Form also helped to add quantitative discipline to biological study.

Despite the fundamental importance and frequent necessity of statistical reasoning, there may nonetheless have been a tendency among biologists to distrust or deprecate results which are not qualitatively apparent.

One anecdote describes Thomas Hunt Morgan banning the Friden calculator from his department at Caltech, saying "Well, I am like a guy who is prospecting for gold along the banks of the Sacramento River in 1849. With a little intelligence, I can reach down and pick up big nuggets of gold. And as long as I can do that, I'm not going to let any people in my department waste scarce resources in placer mining.

APPLICATIONS OF BIOSTATICS

- Public health, including epidemiology, health services research, nutrition, environmental health and healthcare policy & management.
- Design and analysis of clinical trials in medicine
- Assessment of severity state of a patient with prognosis of outcome of a disease.
- Population genetics and statistical genetics in order to link variation in genotype with a variation in phenotype.
- This has been used in agriculture to improve crops and farm animals (animal breeding).
- In biomedical research, this work can assist in finding candidates for gene alleles that can cause or influence predisposition to disease in human genetics
- Analysis of genomics data, for example from microarray or proteomics experiments.
- Often concerning diseases or disease stages.
- Ecology, ecological forecasting
- Biological sequence analysis
- Systems biology for gene network inference or pathways analysis.

10.1 CALCULATION OF MEAN

"The mean is the average of the numbers. It is easy to calculate: add up all the numbers, then divide by how many numbers there are. In other words it is the sum divided by the count."

In mathematics, the "mean" is a kind of average found by dividing the sum of a set of numbers by the count of numbers in the set. While it isn't the only kind of average, the mean is the one most people think of when speaking about an average.

You can use means for all kinds of useful purposes in your daily life, from calculating the time it takes you to get home from work, to working out how much money you spend in an average week.

WHAT IS AN ARITHMETIC MEAN?

There are six kindergarten classrooms in a small school district in Florida. The class sizes of each of these kindergartens are 26, 20, 25, 18, 20 and 23.

A researcher writing a report about schools in her town wants to come up with a figure to describe the typical kindergarten class size in this town. She asks a friend for help and her friend suggests that she calculate the average of these class sizes.

To do this, the researcher finds out that she needs to add the kindergarten class sizes together and then divide this sum by six, which is the total number of schools in the district.

Adding the six kindergarten class sizes together gives the researcher a total of 132. If she then divides 132 by six, she gets 22. Therefore, the average kindergarten class size in this school district is 22.

$$Average = \frac{26 - 20 - 25 - 18 - 20 - 23}{6} = \frac{132}{6} = 22$$

This average is also known as the arithmetic mean of a set of values.

The Arithmetic Mean Formula

The **arithmetic mean** of a set of values is the ratio of their sum to the total number of values in the set. Thus, if there are a total of *n* numbers in a data set whose values are given by a group of *x*-values, then the arithmetic mean of these values, represented by 'm', can be found using this formula:

$$m = \frac{x_1 - x_2 - x_3 - .. - x_n}{n}$$

In our kindergarten class size example, *n* is 6, or the number of kindergarten classrooms, while the *x*-values are given by the class sizes in each of the kindergartens within the school district. If you recall adding the total number of students in the six classrooms gave us 132. We can plug these values into our formula, dividing 132 by six, and find once again that the average class size is 22. Let's take a look at a couple more examples of how to calculate the arithmetic mean of a group of values.

Examples

A pediatrician has four 9-year-old patients who are boys. Their heights in inches are 54, 57, 53 and 52. She finds out that according to national statistics, the average height of a nine year old boy is 55 inches, or 4 feet and 7 inches. What is the mean or average height of these four boys?

Remember when finding the average, or arithmetic mean, we add together the given values in a data set, then divide by the number of given values. In this example there four different heights given for four different boys. Since we are considering *n* = 4 boys, we add the four heights together and divide the result by 4:

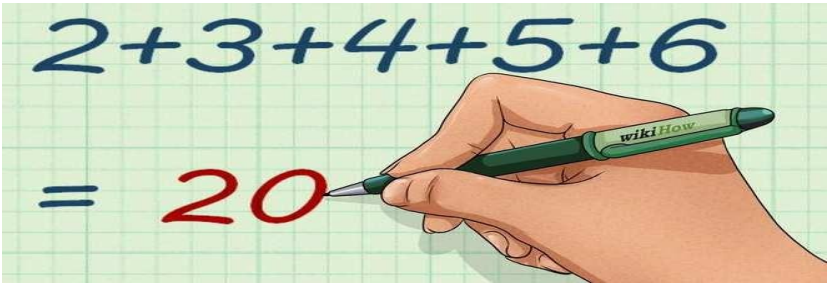
$$Average = \frac{54 - 57 - 53 - 52}{4} = \frac{216}{4} = 54 \text{ inches}$$

As we can see, the average height of these four boys is 54 inches, which is less than the national statistical average, but only by an inch.

Determine the set of values you want to average.

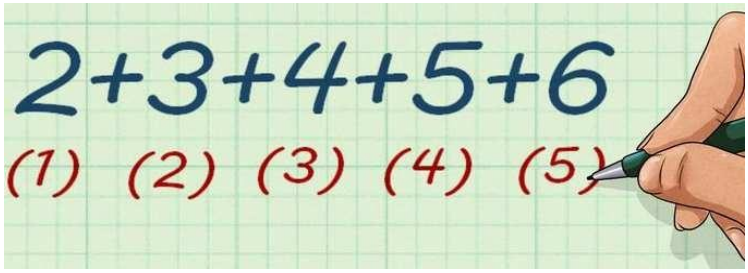
These numbers can be big or small, and there can be as many of them as you want. Just make sure you are using real numbers and not variables.

- Example: 2,3,4,5,6.



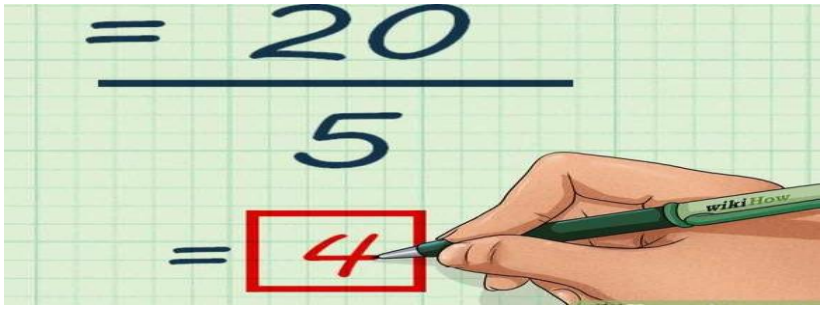
Add your values together to find the sum. You can use a calculator or a spreadsheet, or do it by hand if the set is simple enough.

- Example: 2+3+4+5+6=20.



Count the quantity of values in your group. If you have values that repeat in your set, each one still counts in determining your total.

- Example: 2,3,4,5, and 6 make for a total of five values.



Divide the sum of the set by the count of values. The result is the mean, or average, of your set. This means that if each number in your set was the mean, they would add up to the same total.

- Example: 20 ÷ 5 = 4

Therefore 4 is the mean of the numbers.

1.2: Calculation of Median

The **median** is the value separating the higher half of a data sample, a population, or a probability distribution, from the lower half.

In simple terms, it may be thought of as the "middle" value of a data set. For example, in the data set {1, 3, 3, 6, 7, 8, 9}, the median is 6, the fourth number in the sample.

The median is a commonly used measure of the properties of a data set in statistics and probability theory.

The basic advantage of the median in describing data compared to the mean (often simply described as the "average") is that it is not skewed so much by extremely large or small values, and so it may give a better idea of a 'typical' value.

For example, in understanding statistics like household income or assets which vary greatly, a mean may be skewed by a small number of extremely high or low values. Median income, for example, may be a better way to suggest what a 'typical' income is.

Because of this, the median is of central importance in robust statistics, as it is the most resistant statistic, having a breakdown point of 50%: so long as no more than half the data are contaminated, the median will not give an arbitrarily large or small result.

Basic procedure

The median of a finite list of numbers can be found by arranging all the numbers from smallest to greatest.

If there is an odd number of numbers, the middle one is picked. For example, consider the set of numbers:

1, 3, 3, 6, 7, 8, 9

This set contains seven numbers. The median is the fourth of them, which is 6.

If there is an even number of observations, then there is no single middle value; the median is then usually defined to be the mean of the two middle values.For example, in the data set:

1, 2, 3, 4, 5, 6, 8, 9

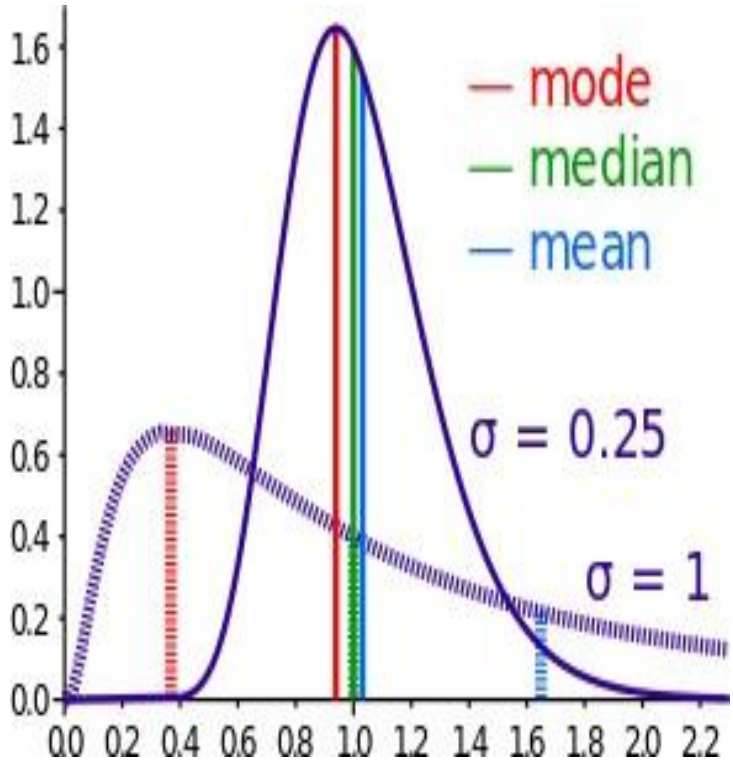
The median is the mean of the middle two numbers: this is (4 + 5) ÷ 2, which is 4.5. (In more technical terms, this interprets the median as the fully trimmed mid-range.)

The formula used to find the middle number of a data set of *n* numbers is (n + 1) ÷ 2. This either gives the middle number (for an odd number of values) or the halfway point between the two middle values. For example, with 14 values, the formula will give 7.5, and the median will be taken by averaging the seventh and eighth values.

You will also be able to find the median using the Stem-and-Leaf Plot.

There is no widely accepted standard notation for the median, but some authors represent the median of a variable *x* either as $\tilde{x}$ or as $\mu_{1/2}$ sometimes also *M*. In any of these cases, the use of these or other symbols for the median needs to be explicitly defined when they are introduced.

DISCUSSION



Comparison of mean, median and mode of two log-normal distributions with different skewnes Calculation of medians is a popular technique in summary statistics and summarizing statistical data, since it is simple to understand and easy to calculate, while also giving a measure that is more robust in the presence of outlier values than is the mean.

The widely cited empirical relationship between the relative locations of the mean and the median for skewed distributions is, however, not generally true. There are, however, various relationships for the *absolute* difference between them; see below.

The median does not identify a specific value within the data set, since more than one value can be at the median level and with an even number of observations (as shown above) no value need be exactly at the value of the median.

Nonetheless, the value of the median is uniquely determined with the usual definition. A related concept, in which the outcome is forced to correspond to a member of the sample, is the medoid.

In a population, at most half have values strictly less than the median and at most half have values strictly greater than it. If each group contains less than half the population, then some of the population is exactly equal to the median.

For example, if $a < b < c$, then the median of the list $\{a, b, c\}$ is b, and, if $a < b < c < d$, then the median of the list $\{a, b, c, d\}$ is the mean of b and c; i.e., it is $(b + c)/2$. Indeed, as it is based on the middle data in a group, it is not necessary to even know the value of extreme results in order to calculate a median. For example, in a psychology test investigating the time needed to solve a problem, if a small number of people failed to solve the problem at all in the given time a median can still be calculated.

The median can be used as a measure of location when a distribution is skewed, when end-values are not known, or when one requires reduced importance to be attached to outliers, e.g., because they may be measurement errors.

A median is only defined on ordered one-dimensional data, and is independent of any distance metric. A geometric median, on the other hand, is defined in any number of dimensions.

The median is one of a number of ways of summarizing the typical values associated with members of a statistical population; thus, it is a possible location parameter. The median is the 2nd quartile, 5th decile, and 50th percentile. Since the median is the same as the *second quartile*, its calculation is illustrated in the article on quartiles.

A median can be worked out for ranked but not numerical classes (e.g. working out a median grade when students are graded from A to F), although the result might be halfway between grades if there are an even number of cases.

When the median is used as a location parameter in descriptive statistics, there are several choices for a measure of variability: the range, the interquartile range, the mean absolute deviation, and the median absolute deviation.

For practical purposes, different measures of location and dispersion are often compared on the basis of how well the corresponding population values can be estimated from a sample of data.

The median, estimated using the sample median, has good properties in this regard. While it is not usually optimal if a given population distribution is assumed, its properties are always reasonably good.

For example, a comparison of the efficiency of candidate estimators shows that the sample mean is more statistically efficient than the sample median when data are uncontaminated by data from heavy-tailed distributions or from mixtures of distributions, but less efficient otherwise, and that the efficiency of the sample median is higher than that for a wide range of distributions.

More specifically, the median has a 64% efficiency compared to the minimum-variance mean (for large normal samples), which is to say the variance of the median will be ~50% greater than the variance of the mea

Easy explanation of the sample median

In individual series (if number of observation is very low) first one must arrange all the observations in order. Then count(n) is the total number of observation in given data.

If n is odd then Median (M) = value of ((n + 1)/2)th item term.

If n is even then Median (M) = value of [(n/2)th item term + (n/2 + 1)th item term]/2

For an odd number of values:

As an example, we will calculate the sample median for the following set of observations: 1, 5, 2, 8, 7.

Start by sorting the values: 1, 2, 5, 7, 8.

In this case, the median is 5 since it is the middle observation in the ordered list.

The median is the ((n + 1)/2)th item, where n is the number of values. For example, for the list {1, 2, 5, 7, 8}, we have n = 5, so the median is the ((5 + 1)/2)th item.

median = (6/2)th item

median = 3rd item

median = 5

For an even number of values:

As an example, we will calculate the sample median for the following set of observations: 1, 6, 2, 8, 7, 2.

Start by sorting the values: 1, 2, 2, 6, 7, 8.

In this case, the arithmetic mean of the two middlemost terms is (2 + 6)/2 = 4. Therefore, the median is 4 since it is the arithmetic mean of the middle observations in the ordered list.

We also use this formula MEDIAN = {(n + 1)/2}th item . n = number of values

As above example 1, 2, 2, 6, 7, 8 n = 6 Median = {(6 + 1)/2}th item = 3.5th item. In this case, the median is average of the 3rd number and the next one (the fourth number). The median is (2 + 6)/2 which is 4.

CALCULATING THE MEDIAN

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If observations of a variable are ordered by value, the median value corresponds to the middle observation in that ordered list. The median value corresponds to a cumulative percentage of 50% (i.e., 50% of the values are below the median and 50% of the values are above the median). The position of the median is

{(n + 1) ÷ 2}th value, where **n** is the number of values in a set of data.

In order to calculate the median, the data must first be ranked (sorted in ascending order). The median is the number in the middle.

Median = the middle value of a set of ordered data.

The median is usually calculated for numeric variables, but may also be calculated for categorical variables that are sequenced, such as the categories in a satisfaction survey: excellent, good, satisfactory and poor. These qualitative categories can be ranked in order, and thus, are considered ordinal.

RAW DATA

In raw data, the median is the point at which exactly half of the data are above and half below. These halves meet at the median position.

If the number of observations is odd, the median fits perfectly and the depth of the median position will be a whole number. If the number of observations is even, the depth of the median position will include a decimal. You need to find the midpoint between the numbers on either side of the median position.

Example 1 – Raw data (discrete variables)

Imagine that a top running athlete in a typical 200-metre training session runs in the following times:

26.1, 25.6, 25.7, 25.2 et 25.0 seconds.

How would you calculate his median time?

First, the values are put in ascending order: 25.0, 25.2, 25.6, 25.7, 26.1. Then, using the following formula, figure out which value is the middle value. Remember that n represents the number of values in the data set.

Median = $\{(n + 1) \div 2\}^{th}$ value

$= (5 + 1) \div 2$

= 3

The third value in the data set will be the median. Since 25.6 is the third value, 25.6 seconds would be the median time.

= 25.6 secondes

Example 2 – Raw data (discrete variables)

Now, if the runner sprints the sixth 200-metre race in 24.7 seconds, what is the median value now?

Again, you first put the data in ascending order: 24.7, 25.0, 25.2, 25.6, 25.7, 26.1. Then, you use the same formula to calculate the median time.

Median = $\{(n + 1) \div 2\}^{th}$ value

= (6 + 1) ÷ 2

= 7 ÷ 2

= 3,5

Since there is an even number of observations in this data set, there is no longer a distinct middle value. The median is the 3.5^{th} value in the data set meaning that it lies between the third and fourth values. Thus, the median is calculated by averaging the two middle values of 25.2 and 25.6. Use the formula below to get the average value.

Average=(value below median + value above median) ÷ 2

= (third value + fourth value) ÷ 2

= (25.2 + 25.6) ÷ 2

= 50.8 ÷ 2

= 25.4

The value 25.4 falls directly between the third and fourth values in this data set, so 25.4 seconds

Would be the median time.

UNGROUPED FREQUENCY DISTRIBUTION

In order to find the median using cumulative frequencies (or the number of observations that lie above or below a particular value in a data set), you must calculate the first value with a cumulative frequency greater than or equal to the median.

If the median's value is exactly 0.5 more than the cumulative frequency of the previous value, then the median is the midpoint between the two values.

Example 3 – Ungrouped frequency table (discrete variables)

Imagine that your school baseball team scores the following number of home runs in 10 games:

4, 5, 8, 5, 7, 8, 9, 8, 8, 7

If you were to place the total home runs in a frequency table, what would the median be?

First, put the scores in ascending order:

4, 5, 5, 7, 7, 8, 8, 8, 8, 9

Then, make a table with two columns. Label the first column "Number of home runs" and then list the possible number of home runs the team could get.

You can start from 0 and list up until the number 10, but since the team never scored less than 4 home runs, you may wish to start listing at the number 4.

Label the second column "Frequency." In this column, record the numbers of times 4 home runs were scored, 5 home runs were scored and so on.

In this case, there was only one time that 4 home runs were scored, but two times that 5 home runs were scored.

If you add all of the numbers in the Frequency column, they should equal 10 (for the 10 games played).

Table 1. Number of home runs in 10 baseball games

Number of home runs (x)	**Frequency (f)**
4	1
5	2
6	0
7	2
8	4
9	1

To find the median, again use the same formula:

Median = {(n + 1) ÷ 2}th value

= (10 + 1) ÷ 2

= 11 ÷ 2

= 5.5

= the median is the 5.5^{th} value in the data set

To get the median, add up the numbers in the Frequency column until you get to 5 (and since the total number of games is 10, the remaining numbers in that column should also equal 5). You will reach 5 after adding all of the frequencies up to and including those for the 7 home runs. The next set of five will begin with the frequencies for 8 home runs. The median (the 5.5^{th} value) lies between the fifth value and the sixth value. Thus, the median lies between 7 home runs and 8 home runs.

If you calculate the average of these values (using the same formula used in Example 2), the result is 7.5.

Average = (middle value before + middle value after) ÷ 2

= (fifth value + sixth value) ÷ 2

= (7 + 8) ÷ 2

= 15 ÷ 2

= 7.5

Technically, the median should be a possible variable. In the above example, the variables are discrete and always whole numbers. Therefore, 7.5 is not a possible variable—no one can hit 7 and a half home runs. Thus, this number only makes sense statistically. Some mathematicians may argue that 8 is a more appropriate median.

GROUPED FREQUENCY DISTRIBUTION

Sometimes it does not make sense to list each individual variable when a frequency distribution table would be long and cumbersome to work with.

In order to simplify this, divide the range of data into intervals and then list the intervals in a frequency distribution table, including a column for the cumulative percentage. (For more information, refer to the Cumulative frequency section.)

The calculation to find the median is a little longer because the data have been grouped into intervals and, therefore, all of the original information has been lost.

Some textbooks simply take the midpoint of the interval as the median. However, that method is an over-simplification of the true value. Use the following calculations to find the median for a grouped frequency distribution.

1. Figure out which interval contains the median by using the **(n + 1) ÷ 2** formula. Take whatever value the calculation gives you and then add up the numbers in the frequency column until you come to that value (just like Example 3). For example, if your median is the 13.5^{th} value, add up the frequencies until you come to the 13^{th} and 14^{th} values. Whichever interval contains these values is called the median group.
2. Find the cumulative percentage of the interval preceding the median group. Label this value **A**.
3. Using this cumulative percentage, calculate how many numbers are needed in order to add up to 50% of the total cumulative percentage. This value will be labeled **B**. Use the

following formula to calculate **B**:

B = 50 - A

4. Figure out the range (how many numbers the interval covers). Call this value **C**. Then, find the percentage for the median interval. Call this value **D**.
5. Calculate how many data values you have to count in the median group to get 50% of the total data set by using the following formula. Call this value **E**.

   E = (B ÷ D) x C

6. Find out what the median value is by adding the value for E to the lower value of the median interval:

   Median = lower value + E

   Since **E = (B ÷ D) x C**, this formula can also be written as:

   Median = lower value + (B ÷ D) x C

If the cumulative frequency for an interval is exactly 50%, then the median value would be the endpoint of this interval.

Let's make this clear with an example!

Example 4 – Grouped variables - frequency distribution (continuous or discrete)

Using the same information from Example 4 in the Mean section, imagine that you surveyed 50 Grade 10 girls to find out how tall each one is in centimetres. After gathering all of your data, you created a frequency distribution table that looked like this:

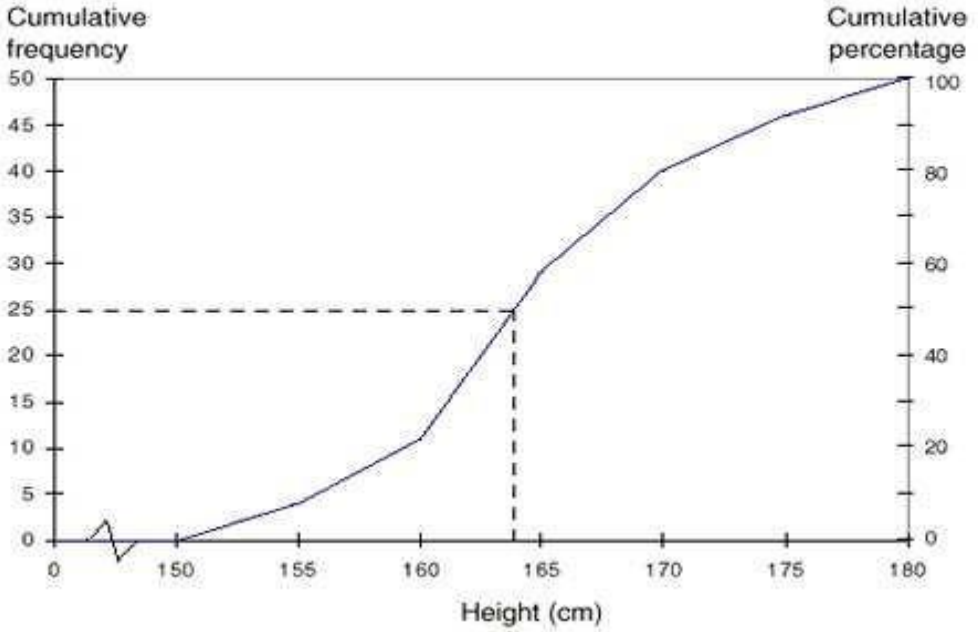
Table 2. Height of Grade 10 girls

Height (cm)	Frequency (f)	Endpoint (x)	Cumulative frequency	Percentage	Cumulative percentage
150 to < 155	4	155	4	8	8
155 to < 160	7	160	11	14	22

160 to < 165 18	165	29		36	58
165 to < 170 11	170	40		22	80
170 to < 175 6	175	46		12	92
175 to < 180 4	180	50		8	100

Using the grouped data, you created a cumulative frequency graph to accompany your table. The endpoints of the height intervals, the numbers for cumulative frequency and the numbers for cumulative percentage have been plotted on the graph.

Figure 1. Height of Grade 10 girls



By just looking at the graph, you can try to find the median value. The median is the point where the x-axis (Height) intersects with the midpoint (25) of the y-axis (Cumulative frequency). You will see that the median value is approximately 164 cm. Using mathematical calculations; you can find out that the value is actually 163.9 cm. Here's how:

1. According to the information provided in Table 2:

   Median = {(n + 1) ÷ 2}th value

2. = (50 + 1) ÷ 2
3. = 51 ÷ 2
4. = 25.5
5. By adding up the frequencies, we find that the median (25.5) lies in the median group of the 160 to < 165 cm interval.
6. The cumulative percentage of the preceding interval (**A**) is 22.
7. The percentage needed in order to get 50% of the total cumulative percentage (**B**) is 28.

   B = 50 – A
8. = 50 – 22
9. = 28
10. The range of the median interval (**C**) is 5 and the percentage for the median interval (**D**) is 36.
11. The number of values to count down within the interval in order to get to 50% of the total data set is 3.9.
12. **E = (B ÷ D) x C**
13. = (28 ÷ 36) x 5
14. **= 3.9**
15. Since the lower value of the median interval is 160, when you add the value of **E** to that you get a median of 163.9 cm.
16. **Median = lower value of median interval + (B ÷ D) x C**
17. = 160 + (28 ÷ 36) x 5
18. = 160 + 3.9
19. **= 163.9 cm**

COMPARING THE MEAN AND MEDIAN

It is possible for the mean and median of a distribution to have the same value. This is always the case if distribution is symmetrical as in a normal distribution. If the distribution is roughly symmetrical, then the two values will be close together.

In the example of the heights of the 50 Grade 10 girls, the mean (164.5 cm) is very close to the value of the median (163.5 cm). This is because the distribution is roughly symmetrical.

However, one number can alter the mean without affecting the median.

Example 6 – Comparing the mean and median

Consider the following sets of data that represent the number of points scored by 3 players in 11 lacrosse games.

Eileen: 1, 1, 1, 2, 2, 2, 2, 2, 3, 3, 3
Mean = 22 ÷ 11 = **2**
Median = **2**

Jeremy: 1, 1, 1, 2, 2, 2, 2, 2, 3, 3, 4
Mean = 23 ÷ 11 = **2.1**
Median = **2**

Randy: 1, 1, 1, 2, 2, 2, 2, 2, 3, 3, 14
Mean = 33 ÷ 11 = **3**
Median = **2**

The three sets of data above are identical except for the last observation values (3, 4 and 14).

8.3 : MODE

The **mode** is the value that appears most often in a set of data. The mode of a discrete probability distribution is the value *x* at which its probability mass function takes its maximum value. In other words, it is the value that is most likely to be sampled.

The mode of a continuous probability distribution is the value *x* at which its probability density function has its maximum value, so the mode is at the peak.

Like the statistical mean and median, the mode is a way of expressing, in a (usually) single number, important information about a random variable or a population.

The numerical value of the mode is the same as that of the mean and median in a normal distribution and it may be very different in highly skewed distributions.

The mode is not necessarily unique to a given distribution, since the probability mass function or probability density function may take the same maximum value at several points x_1, x_2, etc.

The most extreme case occurs in uniform distributions, where all values occur equally frequently.

When a probability density function has multiple local maxima it is common to refer to all of the local maxima as modes of the distribution. Such a continuous distribution is called multimodal.

In symmetric unimodal distributions, such as the normal distribution, the mean (if defined), median and mode all coincide. For samples, if it is known that they are drawn from a symmetric distribution, the sample mean can be used as an estimate of the population mode.

MODE OF A SAMPLE

The mode of a sample is the element that occurs most often in the collection. For example, the mode of the sample [1, 3, 6, 6, 6, 6, 7, 7, 12, 12, 17] is 6.

Given the list of data [1, 1, 2, 4, 4] the mode is not unique - the dataset may be said to be bimodal, while a set with more than two modes may be described as multimodal.

For a sample from a continuous distribution, such as [0.935..., 1.211..., 2.430..., 3.668..., 3.874...], the concept is unusable in its raw form, since no two values will be exactly the same, so each value will occur precisely once. In order to estimate the mode, the usual practice is to discretize the data by assigning frequency values to intervals of equal distance, as for making a histogram, effectively replacing the values by the midpoints of the intervals they are assigned to. The mode is then the value where the histogram reaches its peak. For small or middle-sized samples the outcome of this procedure is sensitive to the choice of interval width if chosen too

narrow or too wide; typically one should have a sizable fraction of the data concentrated in a relatively small number of intervals (5 to 10), while the fraction of the data falling outside these intervals is also sizable. An alternate approach is kernel density estimation, which essentially blurs point samples to produce a continuous estimate of the probability density function which can provide an estimate of the mode.

Comparison of mean, median and mode

Comparison of common averages of values { 1, 2, 2, 3, 4, 7, 9 }

Type	Description	Example	Result
Arithmetic mean	Sum of values of a data set divided by number of values:	(1+2+2+3+4+7+9) / 7	**4**
Median	Middle value separating the greater and lesser halves of a data set	1, 2, 2, **3**, 4, 7, 9	**3**
Mode	Most frequent value in a data set	1, **2**, **2**, 3, 4, 7, 9	**2**

USE

Unlike mean and median, the concept of mode also makes sense for "nominal data" (i.e., not consisting of numerical values in the case of mean, or even of ordered values in the case of median).

For example, taking a sample of Korean family names, one might find that "Kim" occurs more often than any other name. Then "Kim" would be the mode of the sample.

In any voting system where a plurality determines victory, a single modal value determines the victor, while a multi-modal outcome would require some tie-breaking procedure to take place.

Unlike median, the concept of mode makes sense for any random variable assuming values from a vector space, including the real numbers (a one-dimensional vector space) and the integers (which can be considered embedded in the reals).

For example, a distribution of points in the plane will typically have a mean and a mode, but the concept of median does not apply.

The median makes sense when there is a linear order on the possible values. Generalizations of the concept of median to higher-dimensional spaces are the geometric median and the centerpoint.

PROPERTIES

Assuming definedness, and for simplicity uniqueness, the following are some of the most interesting properties.

- All three measures have the following property: If the random variable (or each value from the sample) is subjected to the linear or affine transformation which replaces X by $aX+b$, so are the mean, median and mode.
- Except for extremely small samples, the mode is insensitive to "outliers" (such as occasional, rare, false experimental readings). The median is also very robust in the presence of outliers, while the mean is rather sensitive.
- In continuous unimodal distributions the median lies, as a rule of thumb, between the mean and the mode, about one third of the way going from mean to mode. In a formula, median ≈ (2 × mean + mode)/3. This rule, due to Karl Pearson, often applies to slightly non-symmetric distributions that resemble a normal distribution, but it is not always true and in general the three statistics can appear in any order.

- For unimodal distributions, the mode is within standard deviations of the mean, and the root mean square deviation about the mode is between the standard deviation and twice the standard deviation.

CALCULATING THE MODE

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In a set of data, the mode is the most frequently observed data value. There may be no mode if no value appears more than any other.

There may also be two modes (bimodal), three modes (trimodal), or four or more modes (multimodal).

In the case of grouped frequency distributions, the modal class is the class with the largest frequency.

Mode = **the most frequently observed data value**

As a set of data can have more than one mode, the mode does not necessarily indicate the centre of a data set.

The mode will be close to the mean and median if the data have a normal or near-normal distribution.

In fact, if the distribution is symmetrical and unimodal, then the mean, the median and the mode may have the same value.

CATEGORICAL OR DISCRETE VARIABLES

For categorical or discrete variables, the mode is simply the most observed value. To work out the mode, observations do not have to be placed in order, although for ease of calculation it is advisable to do so.

Example 1 – Categorical or discrete variables

During a hockey tournament, Anne scored 7, 5, 0, 7, 8, 5, 5, 4, 1, and 5 points in 10 games. The mode of her data set is 5 because this value occurred the most often (four times). This can be interpreted to mean that if one game were selected at random, a good guess would be that Anne would score 5 points.

Example 2 – Categorical or discrete variables

During Marco's 12-game basketball season, he scored 14, 14, 15, 16, 14, 16, 16, 18, 14, 16, 16 and 14 points. This data set is bimodal; there are two modes, 14 and 16, because both of them occur the most often (five times).

Example 3 – Categorical or discrete variables

The following data set represents the number of touchdowns scored by Jerome in his high-school football season:

0, 0, 1, 0, 0, 2, 3, 1, 0, 1, 2, 3, 1, 0

First, put the data set in order:

0, 0, 0, 0, 0, 0, 1, 1, 1, 1, 2, 2, 3, 3

Find and compare the mean, median and mode.

Mode = most frequently observed data value = 0

The mode is 0 because this value occurs most often. If one game were selected at random, the mode tells us that a good guess would be that Jerome would not score a touchdown.

Mean =

Σ

x ÷ n
= 14 ÷ 14
= **1**

However, on average (mean), Jerome will score one touchdown per game even though the mode indicates he did not score a touchdown in a lot of games. In this case, the mode does not provide a useful measure of the player's performance.

Median = (n + 1) ÷ 2^{th} value
= (14 + 1) ÷ 2
= 15 ÷ 2
= 7.5

Average = (value below median + value above median) ÷ 2
= (seventh value + eighth value) ÷ 2
= (1 + 1) ÷ 2
= **1**

Because the number of values in the data set is even, the median does not fit perfectly in the centre of the data set. Instead, the median had to be found using the above equations. According to the results, the median states that Jerome will score one touchdown per game.

Grouped variables (continuous or discrete)

When continuous or discrete variables are grouped in tables, the mode is defined as the class interval where most observations lie. This is called the modal-class interval.

In the example of the height of 50 Grade 10 girls, the *modal-class interval* would be 160 –< 165 cm, as this interval has the most observations in it.

The mode is rarely used as a measure of central tendency for numeric variables. However, for categorical variables, the mode is more useful because the mean and median do not make sense.

Next, you could determine the midrange of the modal class. The *midrange* is simply the midpoint between the highest and lowest values in a class. The mode is not used very often in conjunction with the midrange because it gives only a very poor estimate of the average.

The mode can be used with categorical data, but the mean and median cannot. The mode may or may not exist, and there may be more than one value for the mode.

8.4: STANDARD DEVIATION

In statistics, the standard deviation (SD, also represented by the Greek letter sigma σ or the Latin letters) is a measure that is used to quantify the amount of variation or dispersion of a set of data values.

A low standard deviation indicates that the data points tend to be close to the mean (also called the expected value) of the set, while a high standard deviation indicates that the data points are spread out over a wider range of values.

The standard deviation of a random variable, statistical population, data set, or probability distribution is the square root of its variance.

It is algebraically simpler, though in practice less robust, than the average absolute deviation. A useful property of the standard deviation is that, unlike the variance, it is expressed in the same units as the data.

There are also other measures of deviation from the norm, including mean absolute deviation, which provide different mathematical properties from standard deviation.

In addition to expressing the variability of a population, the standard deviation is commonly used to measure confidence in statistical conclusions. For example, the margin of error in polling data is determined by calculating the expected standard deviation in the results if the same poll were to be conducted multiple times.

This derivation of a standard deviation is often called the "standard error" of the estimate or "standard error of the mean" when referring to a mean.

It is computed as the standard deviation of all the means that would be computed from that population if an infinite number of samples were drawn and a mean for each sample were computed.

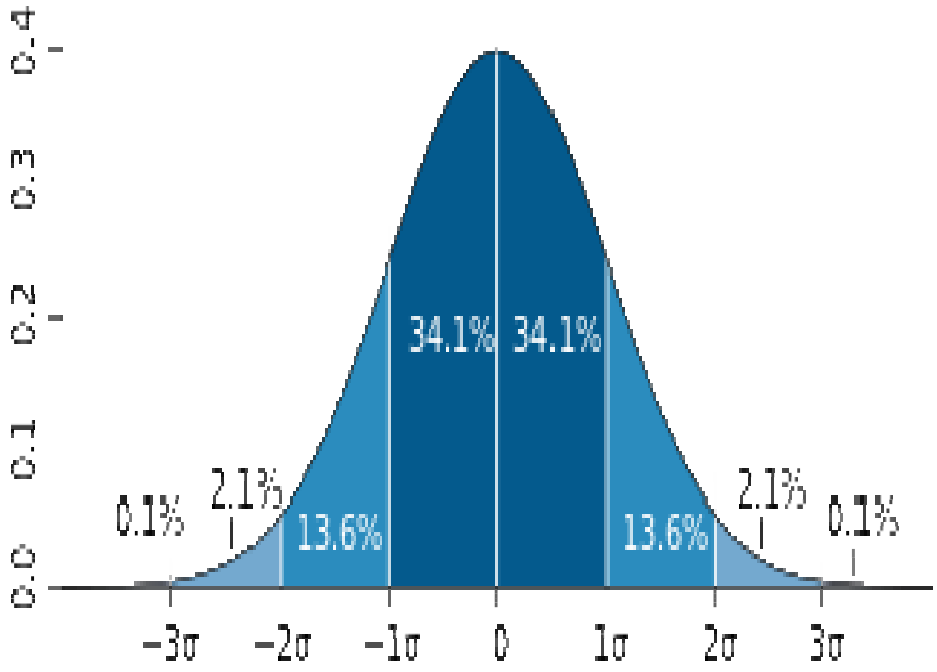
It is very important to note that the standard deviation of a population and the standard error of a statistic derived from that population (such as the mean) are quite different but related (related by the inverse of the square root of the number of observations).

The reported margin of error of a poll is computed from the standard error of the mean (or alternatively from the product of the standard deviation of the population and the inverse of the square root of the sample size, which is the same thing) and is typically about twice the standard deviation—the half-width of a 95 percent confidence interval.

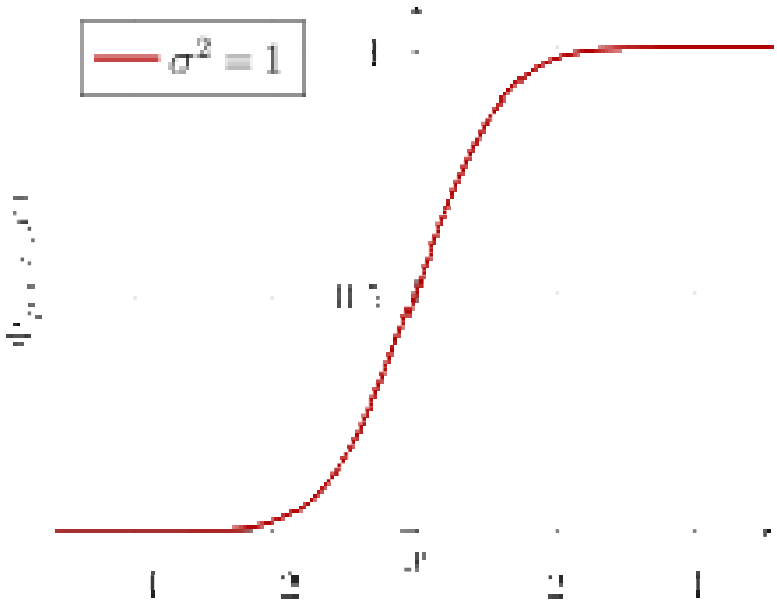
In science, researchers commonly report the standard deviation of experimental data, and only effects that fall much farther than two standard deviations away from what would have been expected are considered statistically significant—normal random error or variation in the

measurements is in this way distinguished from likely genuine effects or associations. The standard deviation is also important in finance, where the standard deviation on the rate of return on an investment is a measure of the volatility of the investment.

When only a sample of data from a population is available, the term standard deviation of the sample or sample standard deviation can refer to either the above-mentioned quantity as applied to those data or to a modified quantity that is an unbiased estimate of the population standard deviation (the standard deviation of the entire population).



A plot of normal distribution (or bell-shaped curve) where each band has a width of 1 standard deviation – See also: 68–95–99.7 rule



Cumulative probability of a normal distribution with expected value 0 and standard deviation 1

DEFINITION OF POPULATION VALUES

Let X be a random variable with mean value μ:

Here the operator E denotes the average or expected value of X. Then the **standard deviation** of X is the quantity (derived using the properties of expected value).

In other words, the standard deviation σ (sigma) is the square root of the variance of X; i.e., it is the square root of the average value of $(X - \mu)^2$.

The standard deviation of a (univariate) probability distribution is the same as that of a random variable having that distribution. Not all random variables have a standard deviation, since these expected values need not exist.

For example, the standard deviation of a random variable that follows a Cauchy distribution is undefined because its expected value μ is undefined

DISCRETE RANDOM VARIABLE

In the case where X takes random values from a finite data set x_1, x_2, ..., x_N, with each value having the same probability, the standard deviation is or, using summation notation,If, instead of having equal probabilities, the values have different probabilities, let x_1 have probability p_1, x_2 have probability p_2, ..., x_N have probability p_N.

CONTINUOUS RANDOM VARIABLE

The standard deviation of a continuous real-valued random variable X with probability density function $p(x)$ isand where the integrals are definite integrals taken for x ranging over the set of possible values of the random variable X. In the case of a parametric family of distributions, the standard deviation can be expressed in terms of the parameters. For example, in the case of the log-normal distribution with parameters μ and σ^2, the standard deviation is $[(\exp(\sigma^2) - 1)\exp(2\mu + \sigma^2)]^{1/2}$.

ESTIMATION

One can find the standard deviation of an entire population in cases (such as standardized testing) where every member of a population is sampled.

In cases where that cannot be done, the standard deviation σ is estimated by examining a random sample taken from the population and computing a statistic of the sample, which is used as an estimate of the population standard deviation.

Such a statistic is called an estimator, and the estimator (or the value of the estimator, namely the estimate) is called a **sample standard deviation,** and is denoted by s (possibly with modifiers).

However, unlike in the case of estimating the population means, for which the sample means, is a simple estimator with many desirable properties (unbiased, efficient, maximum likelihood), there is no single estimator for the standard deviation with all these properties, and unbiased estimation of standard deviation is a very technically involved problem.

Most often, the standard deviation is estimated using the *corrected sample standard deviation* (using $N - 1$), defined below, and this is often referred to as the "sample standard deviation", without qualifiers.

However, other estimators are better in other respects: the uncorrected estimator (using N) yields lower mean squared error, while using $N - 1.5$ (for the normal distribution) almost completely eliminates bias.

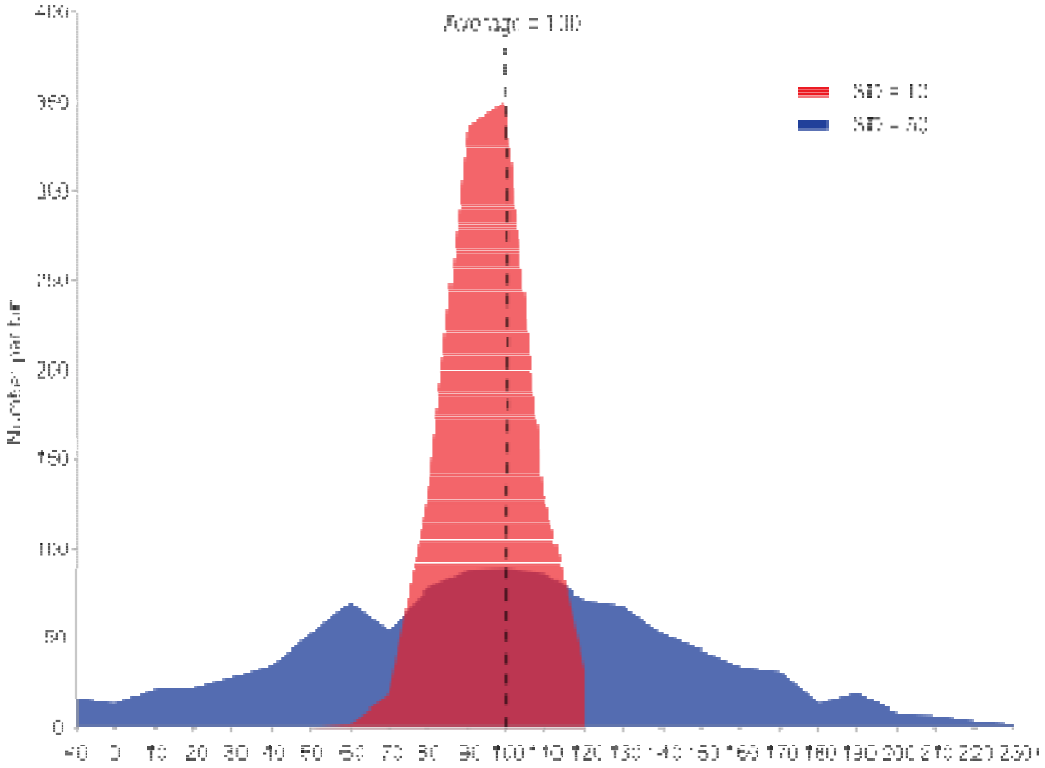
UNCORRECTED SAMPLE STANDARD DEVIATION

Firstly, the formula for the *population* standard deviation (of a finite population) can be applied to the sample, using the size of the sample as the size of the population (though the actual population size from which the sample is drawn may be much larger).

This estimator, denoted by sN, is known as the uncorrected sample standard deviation, or sometimes the standard deviation of the sample (considered as the entire population), and is

defined as follows.s N = 1 N ∑ i = 1 N (x i − x ¯) 2 , where { x 1 , x 2 , … , x N } are the observed values of the sample items and x ¯ is the mean value of these observations, while the denominator N stands for the size of the sample: this is the square root of the sample variance, which is the average of the squared deviations about the sample mean.

INTERPRETATION AND APPLICATION



Example of samples from two populations with the same mean but different standard deviations. Red population has mean 100 and SD 10; blue population has mean 100 and SD 50.

A large standard deviation indicates that the data points can spread far from the mean and a small standard deviation indicates that they are clustered closely around the mean.

For example, each of the three populations {0, 0, 14, 14}, {0, 6, 8, 14} and {6, 6, 8, 8} has a mean of 7.

Their standard deviations are 7, 5, and 1, respectively. The third population has a much smaller standard deviation than the other two because its values are all close to 7. It will have the same units as the data points themselves.

If, for instance, the data set {0, 6, 8, 14} represents the ages of a population of four siblings in years, the standard deviation is 5 years.

As another example, the population {1000, 1006, 1008, and 1014} may represent the distances traveled by four athletes, measured in meters. It has a mean of 1007 meters, and a standard deviation of 5 meters.

Standard deviation may serve as a measure of uncertainty. In physical science, for example, the reported standard deviation of a group of repeated measurements gives the precision of those measurements.

When deciding whether measurements agree with a theoretical prediction, the standard deviation of those measurements is of crucial importance: if the mean of the measurements is too far away from the prediction (with the distance measured in standard deviations), then the theory being tested probably needs to be revised.

This makes sense since they fall outside the range of values that could reasonably be expected to occur, if the prediction were correct and the standard deviation appropriately quantified.

While the standard deviation does measure how far typical values tend to be from the mean, other measures are available.

An example is the mean absolute deviation, which might be considered a more direct measure of average distance, compared to the root mean square distance inherent in the standard deviation.

8.5: STANDARD ERROR

The **standard error of the mean** (**SEM**) can be seen to depict the relationship between the dispersion of individual observations around the population mean (the standard deviation), and the dispersion of sample means around the population mean (the standard error). Different samples drawn from that same population would in general have different values of the sample mean, so there is a distribution of sampled means (with its own mean and variance).

The relationship with the standard deviation is defined such that, for a given sample size, the standard error equals the standard deviation divided by the square root of the sample size.

As the sample size increases, the dispersion of the sample means clusters more closely around the population mean and the standard error decreases.

In regression analysis, the term "standard error" is also used in the phrase standard error of the regression to mean the ordinary least squares estimate of the standard deviation of the underlying errors.

The term may also be used to refer to an estimate of that standard deviation, derived from a particular sample used to compute the estimate.

STANDARD ERROR OF THE MEAN

The **standard error of the mean** (SEM) is the standard deviation of the sample-mean's estimate of a population mean. (It can also be viewed as the standard deviation of the error in the sample mean with respect to the true mean, since the sample mean is an unbiased estimator.)

SEM is usually estimated by the sample estimate of the population standard deviation (sample standard deviation) divided by the square root of the sample size (assuming statistical independence of the values in the sample): where

s is the sample standard deviation (i.e., the sample-based estimate of the standard deviation of the population), and n is the size (number of observations) of the sample.

This estimate may be compared with the formula for the true standard deviation of the sample mean: SD $\bar{x}$ = σ n where σ is the standard deviation of the population.

This formula may be derived from what we know about the variance of a sum of independent random variables.

If X 1 , X 2 , … , X n are n independent observations from a population that has a mean μ and standard deviation σ, then the variance of the total T = (X 1 + X 2 + □ +X n) is n σ 2 .

The variance of T / n must be 1 n 2 n σ 2 = σ 2 n.

- And the standard deviation of must be .
- Of course, is the sample mean .

Note: the standard error and the standard deviation of small samples tend to systematically underestimate the population standard error and deviations: the standard error of the mean is a biased estimator of the population standard error. With n = 2 the underestimate is about 25%, but for n = 6 the underestimate is only 5%. Gurland and Tripathi (1971) provide a correction and equation for this effect. Sokal and Rohlf (1981)[give an equation of the correction factor for small samples of $n < 20$.

A practical result: Decreasing the uncertainty in a mean value estimate by a factor of two requires acquiring four times as many observations in the sample. Or decreasing standard error by a factor of ten requires a hundred times as many observations.

Student approximation when σ value is unknown

In many practical applications, the true value of σ is unknown. As a result, we need to use a distribution that takes into account that spread of possible σ's.

When the true underlying distribution is known to be Gaussian, although with unknown σ, then the resulting estimated distribution follows the Student t-distribution.

The standard error is the standard deviation of the Student t-distribution. T-distributions are slightly different from Gaussian, and vary depending on the size of the sample.

To estimate the standard error of a student t-distribution it is sufficient to use the sample standard deviation "s" instead of σ, and we could use this value to calculate confidence intervals.

Note: The Student's probability distribution is approximated well by the Gaussian distribution when the sample size is over 100. For such samples one can use the latter distribution, which is much simpler.

ASSUMPTIONS AND USAGE

If its sampling distribution is normally distributed, the sample mean, its standard error, and the quantiles of the normal distribution can be used to calculate confidence intervals for the mean. The following expressions can be used to calculate the upper and lower 95% confidence limits,

where $\bar{x}$ is equal to the sample mean, S E is equal to the standard error for the sample mean, and 1.96 is the 0.975 quantile of the normal distribution: Upper 95% limit = $\bar{x}$ + (SE × 1.96) , and Lower 95% limit.

In particular, the standard error of a sample statistic (such as sample mean) is the estimated standard deviation of the error in the process by which it was generated. In other words, it is the standard deviation of the sampling distribution of the sample statistic. The notation for standard error can be any one of SE, SEM (for standard error of *measurement* or *mean*), or S_E.

Standard errors provide simple measures of uncertainty in a value and are often used because:

- If the standard error of several individual quantities is known then the standard error of some function of the quantities can be easily calculated in many cases;
- Where the probability distribution of the value is known, it can be used to calculate a good approximation to an exact confidence interval; and
- Where the probability distribution is unknown, relationships like Chebyshev's or the Vysochanskiï–Petunin inequality can be used to calculate a conservative confidence interval

- As the sample size tends to infinity the central limit theorem guarantees that the sampling distribution of the mean is asymptotically normal.

STANDARD ERROR OF MEAN VERSUS STANDARD DEVIATION

In scientific and technical literature, experimental data are often summarized either using the mean and standard deviation or the mean with the standard error. This often leads to confusion about their interchangeability.

However, the mean and standard deviation are descriptive statistics, whereas the standard error of the mean describes bounds on a random sampling process.

Despite the small difference in equations for the standard deviation and the standard error, this small difference changes the meaning of what is being reported from a description of the variation in measurements to a probabilistic statement about how the number of samples will provide a better bound on estimates of the population mean, in light of the central limit theorem.

Put simply, the **standard error** of the sample mean is an estimate of how far the sample mean is likely to be from the population mean, whereas the **standard deviation** of the sample is the degree to which individuals within the sample differ from the sample mean.

If the population standard deviation is finite, the standard error of the mean of the sample will tend to zero with increasing sample size, because the estimate of the population mean will improve, while the standard deviation of the sample will tend to approximate the population standard deviation as the sample size increases.

CORRECTION FOR FINITE POPULATION

The formula given above for the standard error assumes that the sample size is much smaller than the population size, so that the population can be considered to be effectively infinite in size.

This is usually the case even with finite populations, because most of the time, people are primarily interested in managing the processes that created the existing finite population; this is called an analytic study, following W. Edwards Deming. If people are interested in managing an

existing finite population that will not change over time, then it is necessary to adjust for the population size; this is called an enumerative study.

When the sampling fraction is large (approximately at 5% or more) in an enumerative study, the estimate of the standard error must be corrected by multiplying by a "finite population correction" to account for the added precision gained by sampling close to a larger percentage of the population. The effect of the FPC is that the error becomes zero when the sample size n is equal to the population size N.

RELATIVE STANDARD ERROR

The **relative standard error** of a sample mean is the standard error divided by the mean and expressed as a percentage. It can only be calculated if the mean is a non-zero value.

As an example of the use of the relative standard error, consider two surveys of household income that both result in a sample mean of $50,000.

If one survey has a standard error of $10,000 and the other has a standard error of $5,000, then the relative standard errors are 20% and 10% respectively.

The survey with the lower relative standard error can be said to have a more precise measurement, since it has proportionately less sampling variation around the mean. In fact, data organizations often set reliability standards that their data must reach before publication.

For example, the U.S. National Center for Health Statistics typically does not report an estimated mean if its relative standard error exceeds 30%. (NCHS also typically requires at least 30 observations – if not more – for an estimate to be reported.)

INTRODUCTION TO THE STANDARD ERROR FOR NOVICES

The standard error is a quantitative measure of uncertainty. Consider the following scenarios. Scenario 1.

For an upcoming national election, 2000 voters are chosen at random and asked if they will vote for candidate A or candidate B. Of the 2000 voters, 1040 (52%) state that they will vote for candidate A.

The researchers report that candidate A is expected to receive 52% of the final vote, with a margin of error of 2%.

In this scenario, the 2000 voters are a sample from all the actual voters. The sample proportion of 52% is an estimate of the true proportion who will vote for candidate A in the actual election.

The margin of error of 2% is a quantitative measure of the uncertainty – the possible difference between the true proportion who will vote for candidate A and the estimate of 52%.

Scenario 2. A medical research team tests a new drug to lower cholesterol. They report that, in a sample of 400 patients, the new drug lowers cholesterol by an average of 20 units (mg/dL).

The 95% confidence interval for the average effect of the drug is that it lowers cholesterol by 18 to 22 units. In this scenario, the 400 patients are a sample of all patients who may be treated with the drug. The confidence interval of 18 to 22 is a quantitative measure of the uncertainty – the possible difference between the true average effect of the drug and the estimate of 20 mg/dL.

In each of these scenarios, a sample of observations is drawn from a large population. The proportion or the mean is calculated using the sample.

Because of random variation in sampling, the proportion or mean calculated using the sample will usually differ from the true proportion or mean in the entire population.

A quantitative measure of uncertainty is reported: a margin of error of 2%, or a confidence interval of 18 to 22. The margin of error and the confidence interval are based on a quantitative measure of uncertainty: the standard error.

The standard error of a proportion and the standard error of the mean describe the possible variability of the estimated value based on the sample around the true proportion or true mean

Standard errors are used in many hypothesis tests, such as t-tests. They may be used to calculate confidence intervals.

STANDARD ERROR OF THE MEAN (SEM)

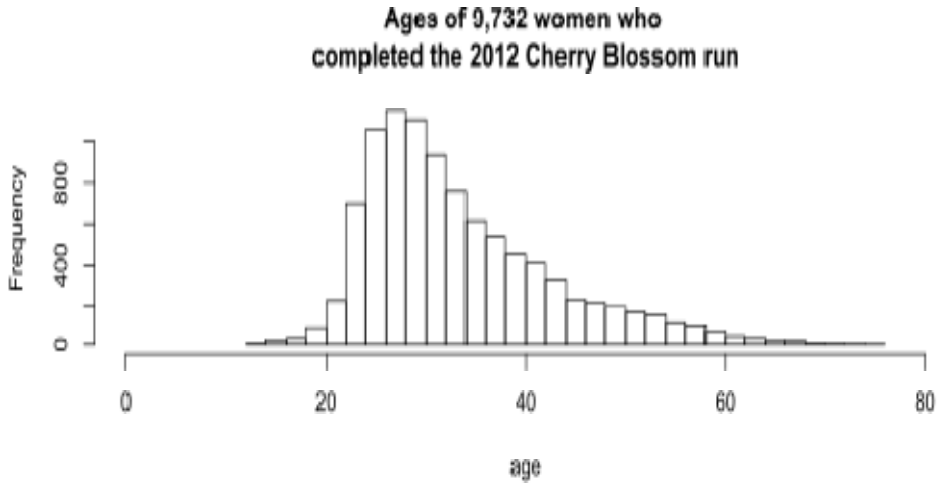
This section will focus on the standard error of the mean. Later sections will present the standard error of other statistics, such as the standard error of a proportion, the standard error of the difference of two means, the standard error of the difference of two proportions and so on.

The concept of a sampling distribution is key to understanding the standard error. Two data sets will be helpful to illustrate the concept of a sampling distribution and its use to calculate the standard error. As will be shown, the standard error is the standard deviation of the sampling distribution.

Sampling from a distribution with a large standard deviation

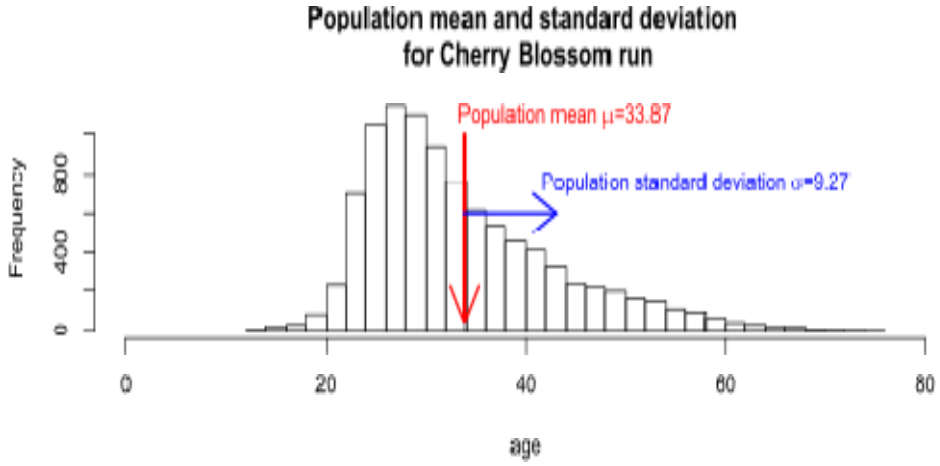
The first data set consists of the ages of 9,732 women who completed the 2012 Cherry Blossom run, a 10-mile race held in Washington each spring.

The age data are in the data set run10 from the R package openintro that accompanies the textbook by Dietz .The graph shows the distribution of ages for the runners.

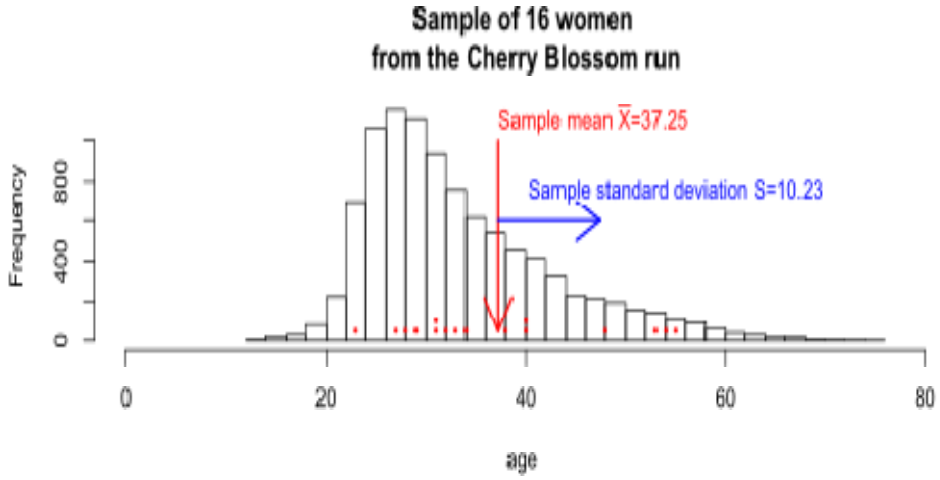


For the purpose of this example, the 9,732 runners who completed the 2012 run are the entire population of interest. The mean age was 33.88 years. The standard deviation of the age was 9.27 years. Because the 9,732 runners are the entire population, 33.88 years is the population mean,

, and 9.27 years is the population standard deviation, σ. Greek letters indicate that these are population values.



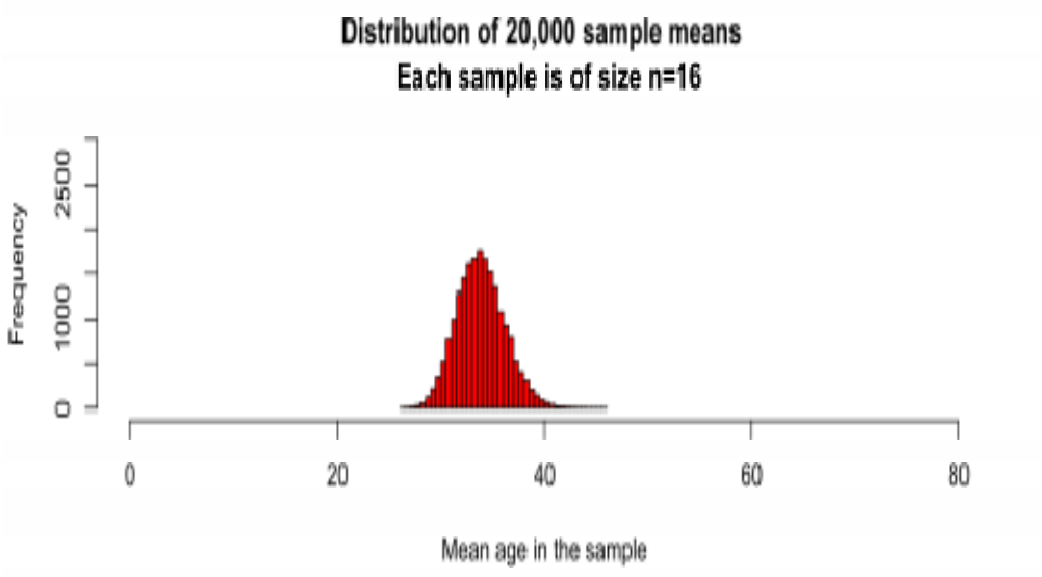
Consider a sample of n=16 runners selected at random from the 9,732. The ages in one such sample are 23, 27, 28, 29, 31, 31, 32, 33, 34, 38, 40, 40, 48, 53, 54, and 55. The graph shows the ages for the 16 runners in the sample, plotted on the distribution of ages for all 9,732 runners.



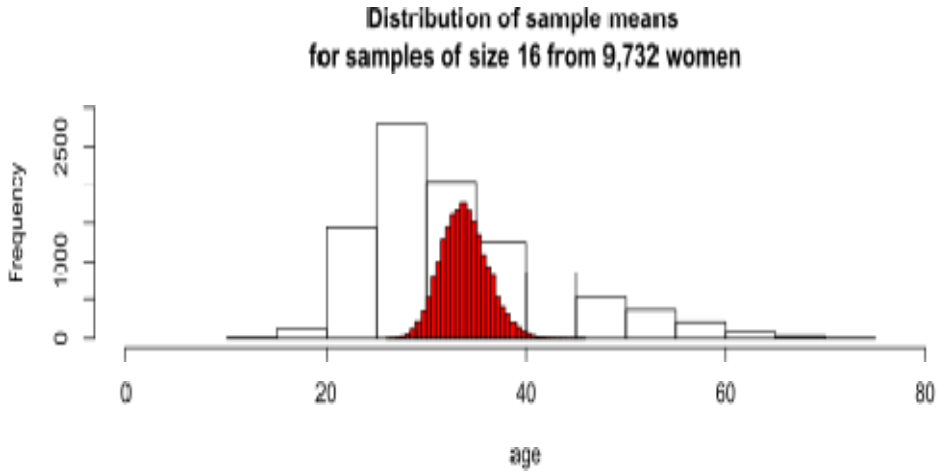
The mean age for the 16 runners in this particular sample is 37.25. The standard deviation of the age for the 16 runners is 10.23. Because these 16 runners are a sample from the population of 9,732 runners, 37.25 is the sample mean, and 10.23 is the sample standard deviation, s. Roman letters indicate that these are sample values.

The sample mean = 37.25 is greater than the true population mean = 33.88 years. The sample standard deviation s = 10.23 is greater than the true population standard deviation σ = 9.27 years. For any random sample from a population, the sample mean will very rarely be equal to the population mean. Similarly, the sample standard deviation will very rarely be equal to the population standard deviation.

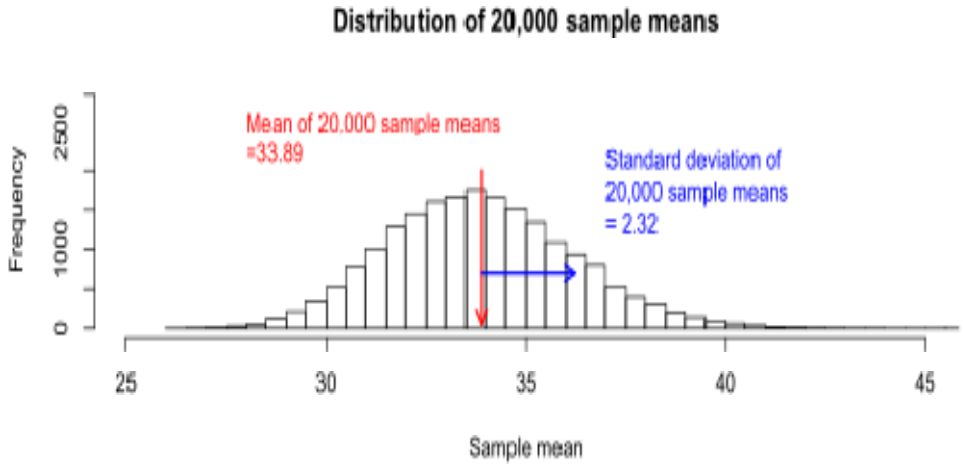
Next, consider all possible samples of 16 runners from the population of 9,732 runners. For each sample, the mean age of the 16 runners in the sample can be calculated. The distribution of the mean age in all possible samples is called the sampling distribution of the mean. For illustration, the graph below shows the distribution of the sample means for 20,000 samples, where each sample is of size n=16.



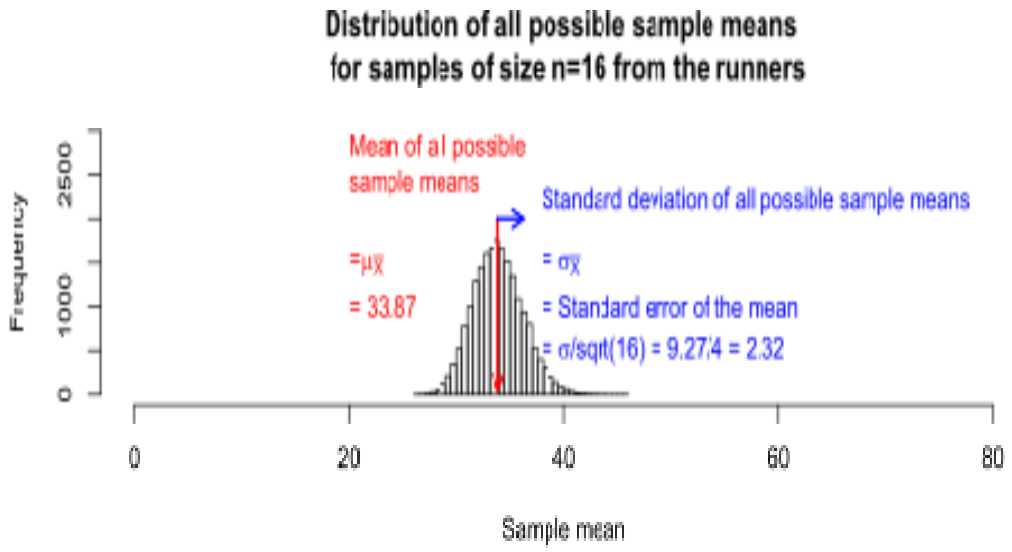
The next graph shows the sampling distribution of the mean (the distribution of the 20,000 sample means) superimposed on the distribution of ages for the 9,732 women.



The distribution of these 20,000 sample means indicate how far the mean of a sample may be from the true population mean. A natural way to describe the variation of these sample means around the true population mean is the standard deviation of the distribution of the sample means.

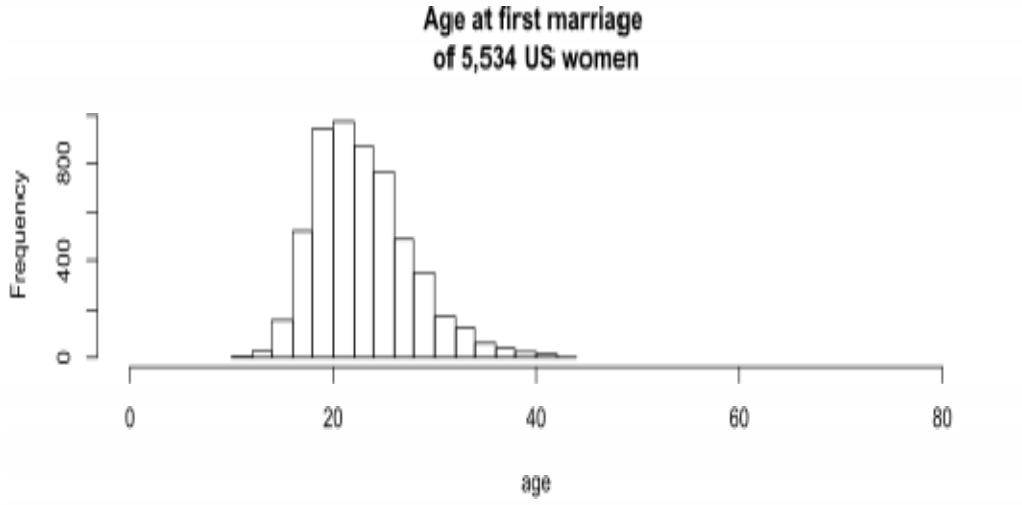


The mean of all possible sample means is equal to the population mean. For the runners, the population means age is 33.87, and the population standard deviation is 9.27. It will be shown that the standard deviation of all possible sample means of size n=16 is equal to the population standard deviation, σ, divided by the square root of the sample size, 16. This gives 9.27/sqrt(16) = 2.32. The standard deviation of all possible sample means is the standard error, and is represented by the symbol.



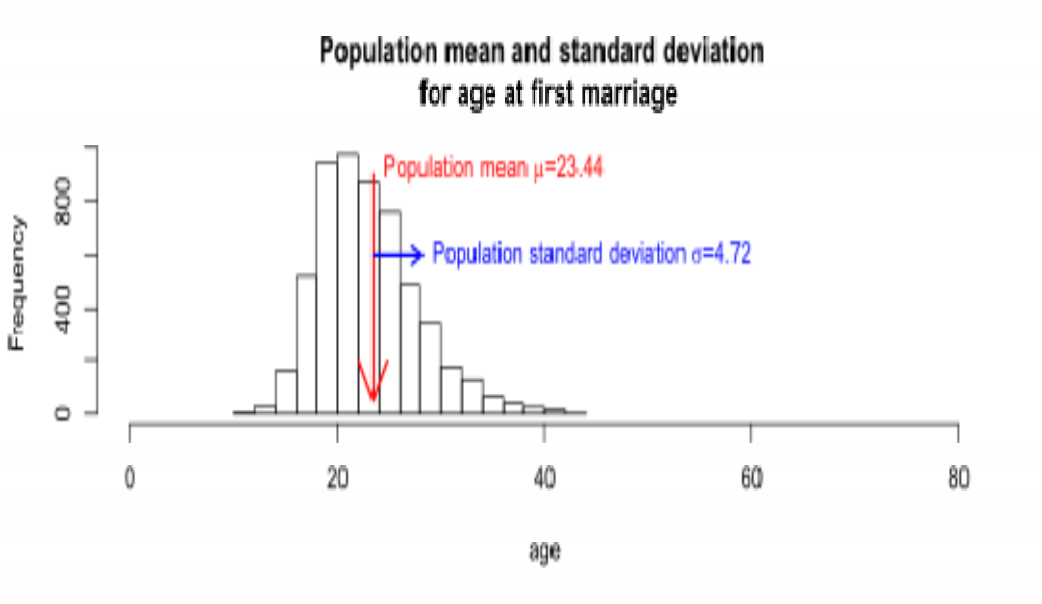
SAMPLING FROM A DISTRIBUTION WITH A SMALL STANDARD DEVIATION

The second data set consists of the age at first marriage of 5,534 US women who responded to the National Survey of Family Growth (NSFG) conducted by the CDC in the 2006 and 2010 cycle. The data set is ageAtMar, also from the R package openintro from the textbook by Dietz et al.

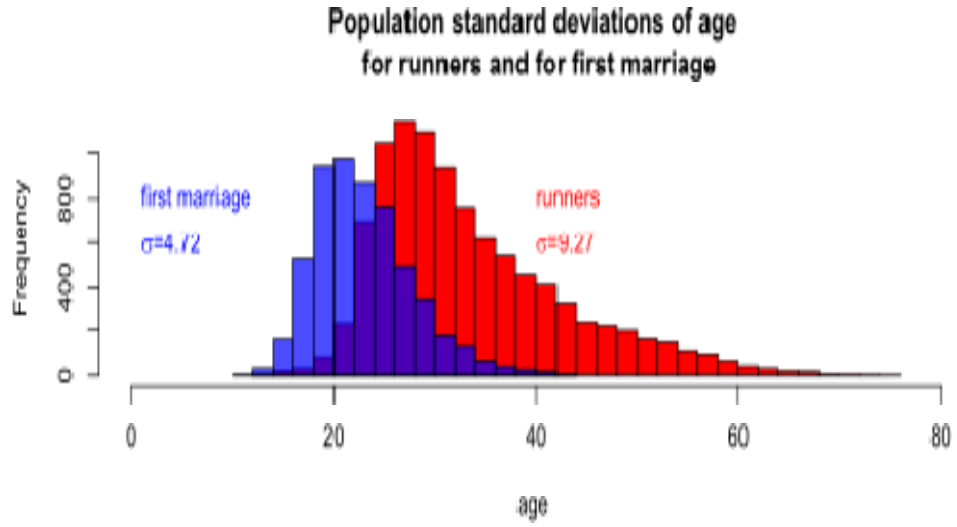


For the purpose of this example, the 5,534 women are the entire population of interest. The mean age was 23.44 years. The standard deviation of the age was 4.72 years. Because the 5,534

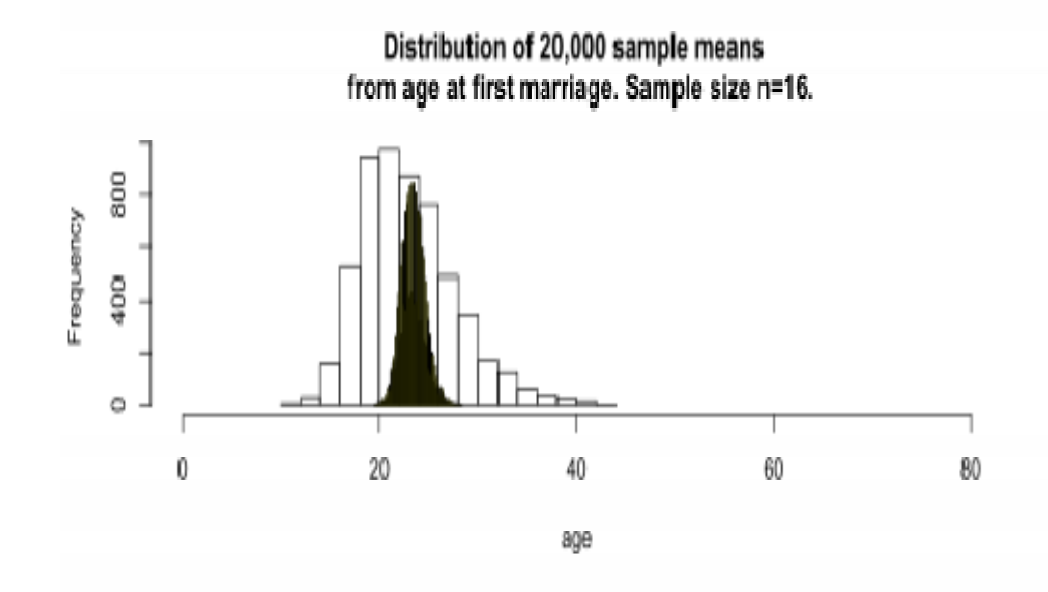
women are the entire population, 23.44 years is the population mean, and 4.72 years is the population standard deviation, .



Notice that the population standard deviation of 4.72 years for age at first marriage is about half the standard deviation of 9.27 years for the runners. The smaller standard deviation for age at first marriage will result in a smaller standard error of the mean.

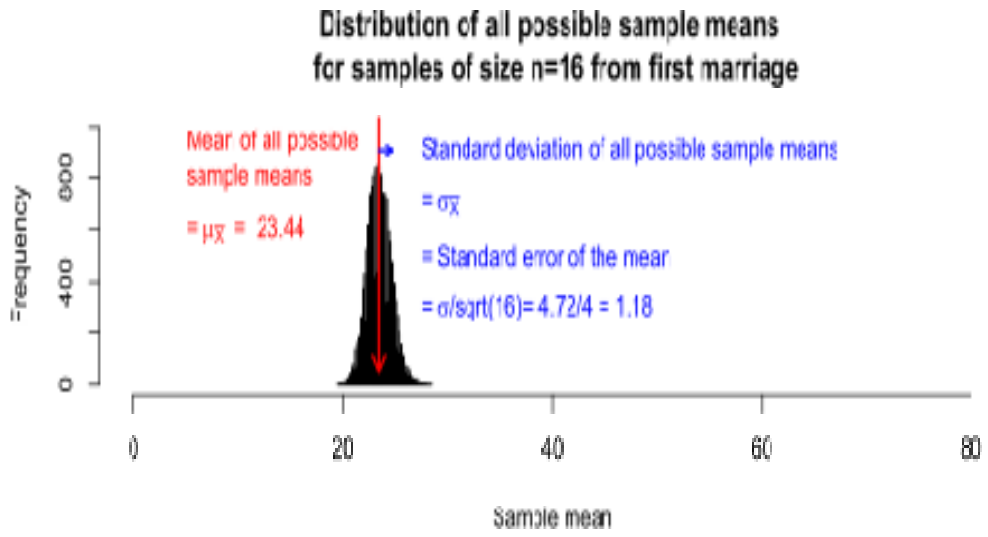


Repeating the sampling procedure as for the Cherry Blossom runners, take 20,000 samples of size n=16 from the age at first marriage population. The graph below shows the distribution of the sample means for 20,000 samples, where each sample is of size n=16.

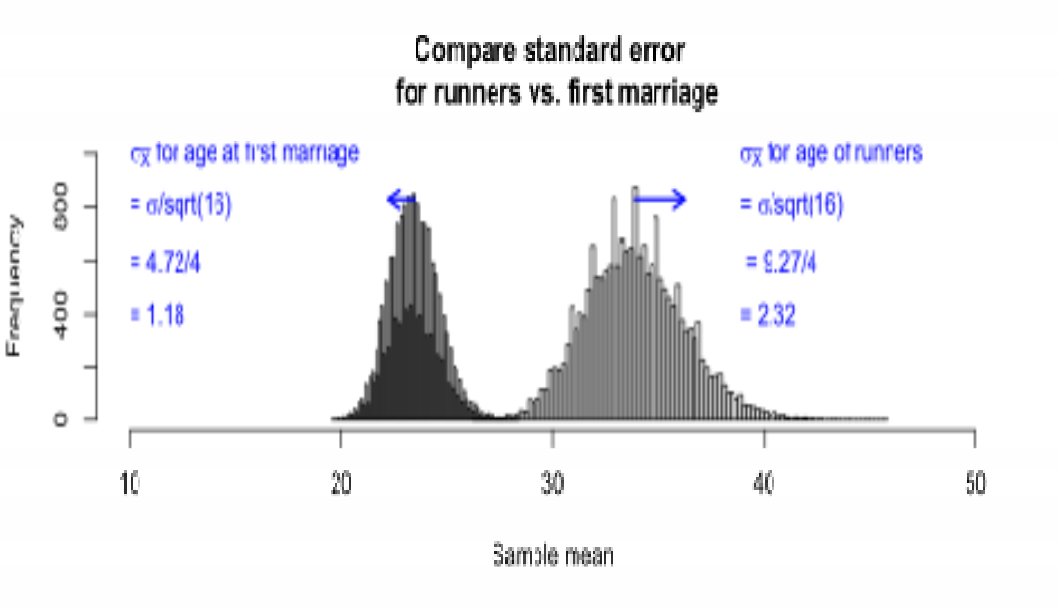


The mean of these 20,000 samples from the age at first marriage population is 23.44, and the standard deviation of the 20,000 sample means is 1.18.

As will be shown, the mean of all possible sample means is equal to the population mean. For the age at first marriage, the population mean age is 23.44, and the population standard deviation is 4.72. It will be shown that the standard deviation of all possible sample means of size n=16 is equal to the population standard deviation, σ, divided by the square root of the sample size, 16, which is 4.72/sqrt(16) = 1.18. The standard deviation of all possible sample means of size 16 is the standard error.



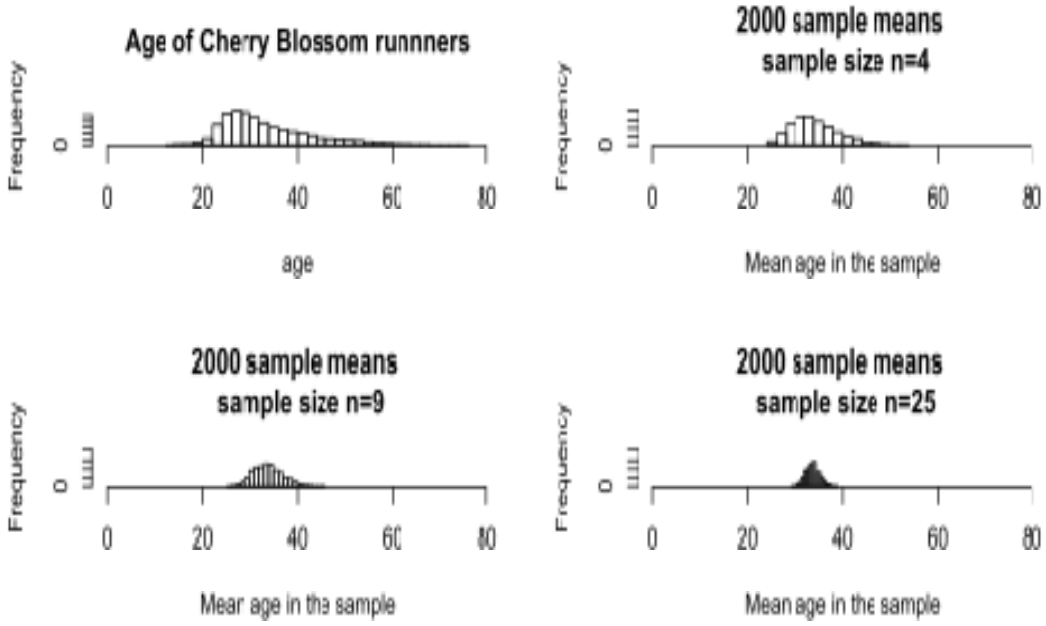
It is useful to compare the standard error of the mean for the age of the runners versus the age at first marriage, as in the graph.



Because the age of the runners has a larger standard deviation (9.27 years) than does the age at first marriage (4.72 years), the standard error of the mean is larger for the runners than for first marriage.

LARGER SAMPLE SIZES GIVE SMALLER STANDARD ERRORS

As would be expected, larger sample sizes give smaller standard errors. The graphs below show the sampling distribution of the mean for samples of size 4, 9, and 25. As the sample size increases, the sampling distribution becomes narrower, and the standard error decreases.



USING A SAMPLE TO ESTIMATE THE STANDARD ERROR

In the examples so far, the population standard deviation σ was assumed to be known. If σ is known, the standard error is calculated using the formula

Where

σ is the standard deviation of the population.

n is the size (number of observations) of the sample.

It is rare that the true population standard deviation is known. However, the sample standard deviation, s, is an estimate of σ. If σ is not known, the standard error is estimated using the formula:

Where *s* is the sample standard deviation

n is the size (number of observations) of the sample.

Notice that is only an estimate of the true standard error, .

In an example above, n=16 runners were selected at random from the 9,732 runners. The ages in that sample were 23, 27, 28, 29, 31, 31, 32, 33, 34, 38, 40, 40, 48, 53, 54, and 55.

The standard deviation of the age for the 16 runners is 10.23, which is somewhat greater than the true population standard deviation σ = 9.27 years. Compare the true standard error of the mean to the standard error estimated using this sample.

The true standard error of the mean, using σ = 9.27, is

The standard error of the mean estimated by using the sample standard deviation, s = 10.23, is

The true standard error of the mean is 2.32. The standard error estimated using the sample standard deviation is 2.56. For the purpose of hypothesis testing or estimating confidence intervals, the standard error is primarily of use when the sampling distribution is normally distributed, or approximately normally distributed.

These assumptions may be approximately met when the population from which samples are taken is normally distributed, or when the sample size is sufficiently large to rely on the Central Limit Theorem.

10.1 SUMMERY

Circumstances generally dictate which measure of central tendency—mean, median or mode—is the most appropriate.

If you are interested in a total, the mean tends to be the most meaningful measure of central tendency because it is the total divided by the number of data.

For example, the mean income of the individuals in a family tells you how much each family member can spend on life's necessities.

The median measure is good for finding the central value and the mode is used to describe the most typical case.

10.2 GLOSSARY

Average: A sometimes vague term. It usually denotes the arithmetic mean, but it can also denote the median, the mode, the geometric mean, and weighted means, among other things.

Control group: The subjects in a controlled experiment who do not receive the treatment.

Converse: If p and q are two logical propositions, then the *converse* of the proposition $(p \rightarrow q)$ is the proposition $(q \rightarrow p)$.

Deviation: A deviation is the difference between a datum and some reference value, typically the mean of the data. In computing the SD, one finds the rms of the deviations from the mean, the differences between the individual data and the mean of the data.

Discrete Variable: A quantitative variable that's set of possible values is countable. Typical examples of discrete variables are variables whose possible values are a subset of the integers, such as Social Security numbers, the number of people in family, ages rounded to the nearest year, *etc.* Discrete variables are "chunky." *C.f.* continuous variable. A discrete random variable is one whose set of possible values is countable. A random variable is discrete if and only if its cumulative probability distribution function is a stair-step function; *i.e.*, if it is piecewise constant and only increases by jumps.

Distribution: The distribution of a set of numerical data is how their values are distributed over the real numbers. It is completely characterized by the empirical distribution function. Similarly, the probability distribution of a random variable is completely characterized by its probability distribution function. Sometimes the word "distribution" is used as a synonym for the empirical

distribution function or the probability distribution function. If two or more random variables are defined for the same experiment, they have a joint probability distribution.

Geometric Mean: The geometric mean of n numbers $\{x_1, x_2, x_3, x_n\}$ is the nth root of their product: $(x_1 \times x_2 \times x_3 \times \ldots \times x_n)^{1/n}$.

Intersection: The intersection of two or more sets is the set of elements that all the sets have in common; the elements contained in every one of the sets. The intersection of the events A and B is written "A∩B," "A and B," and "AB." *C.f.* union. See also Venn diagrams.

Margin of error: A measure of the uncertainty in an estimate of a parameter; unfortunately, not everyone agrees what it should mean. The *margin of error* of an estimate is typically one or two times the estimated standard error of the estimate.

Mean Arithmetic mean: The sum of a list of numbers, divided by the number of elements in the list. See also average.

Median: "Middle value" of a list. The smallest number such that at least half the numbers in the list are no greater than it. If the list has an odd number of entries, the median is the middle entry in the list after sorting the list into increasing order. If the list has an even number of entries, the median is the smaller of the two middle numbers after sorting. The median can be estimated from a histogram by finding the smallest number such that the area under the histogram to the left of that number is 50%.

Member of a set: Something is a member (or element) of a set if it is one of the things in the set.

Method of Comparison: The most basic and important method of determining whether a treatment has an effect: compare what happens to individuals who are treated (the treatment group) with what happens to individuals who are not treated (the control group).

Mode: For lists, the mode is a most common (frequent) value. A list can have more than one mode. For histograms, a mode is a relative maximum ("bump").

Null hypothesis: In hypothesis testing, the hypothesis we wish to falsify on the basis of the data. The null hypothesis is typically that something is not present, that there is no effect, or that there is no difference between treatment and control.

Standard Deviation (SD): The standard deviation of a set of numbers is the rms of the set of deviations between each element of the set and the mean of the set. See also sample standard deviation.

Standard Error (SE). The Standard Error of a random variable is a measure of how far it is likely to be from its expected value; that is, its scatter in repeated experiments. The SE of a random variable X is defined to be - $SE(X) = [E((X - E(X))^2)]^{½}$.

That is, the standard error is the square-root of the expected squared difference between the random variable and its expected value. The SE of a random variable is analogous to the SD of a list.

10.1.3 : SELF ASSESSMENT QUESTIONS

1. How biologists make use of biostatics procedure?
2. What will be the measures of central tendency?
3. What will be the relationship between Mean, Median and Mode?
4. Describe method of computation of standard deviation by direct method.

10.1.4 : REFERENCES

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10.1.5 : SUGGESTED READINGS

1. Statistical Method in Biology and Health Sciences – Miltori & Tsokos

2. Methods in Biostatistics - Mahajan

3. Biostatical Analyasis – Pearson

4. Elementary Statistics - Sokal & Rohlf

5. Concept of Biostatistics – Satguru PrasaD

10.1.6 : TERMINAL QUESTIONS

1. Describe the relationship between mean, median and mode and describe their merits and demerits.

2. Write a note on standard error.

3. Describe method of computation of standard deviation by direct method.

4. What are the demerits of standard deviation?

5. Write an account of use of statistical method in Biological Science.