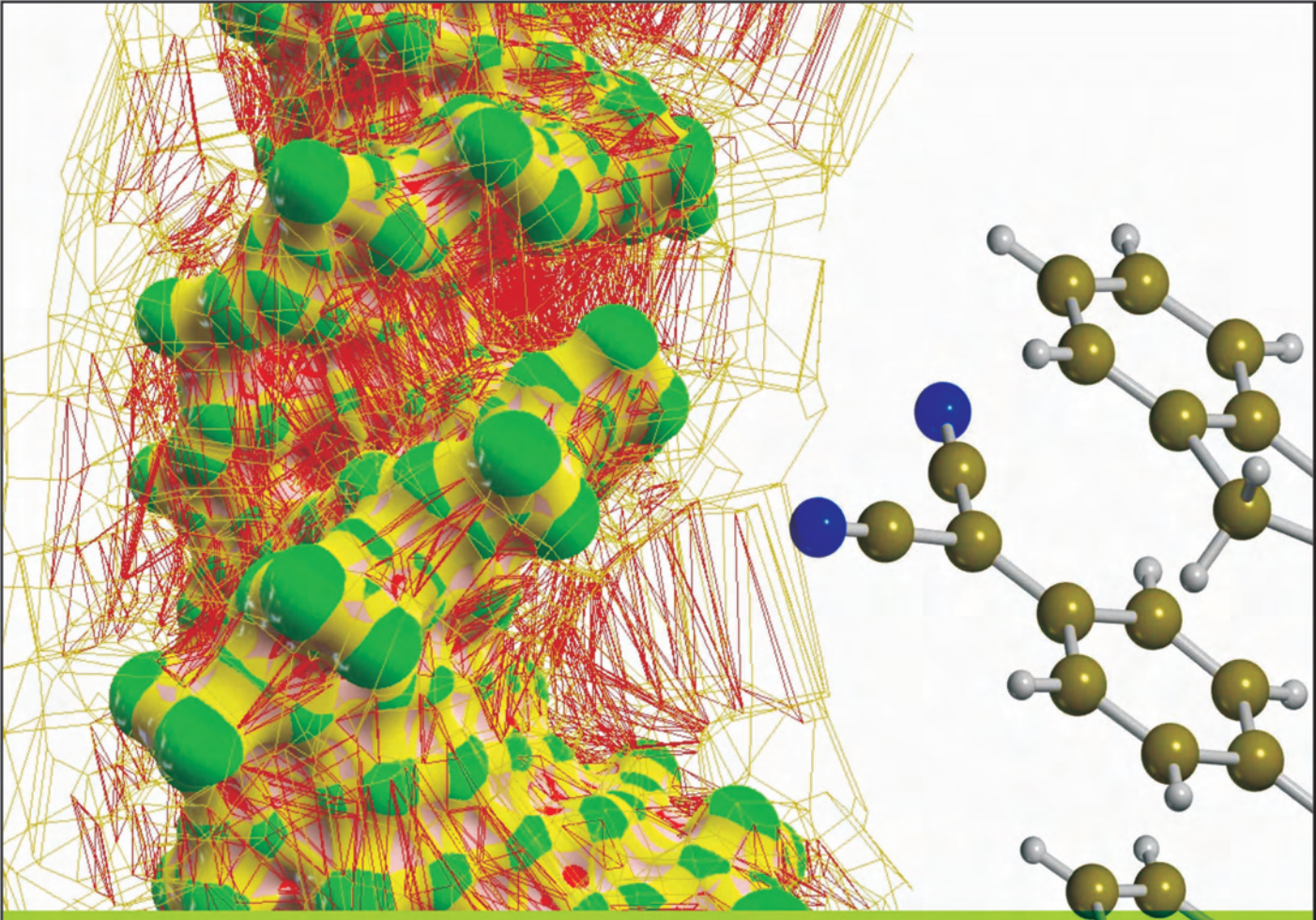

A HANDBOOK OF TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

Dr. R. A. Gautam

A Handbook of Techniques in Biochemistry and Molecular Biology

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Dedicated

to

My Teachers, Family and Friends

FOREWORD

The 21st century has witnessed revolutionary changes in natural sciences and biological sciences. The great progress in the field of biology since the turn of the century has been achieved owing to advancement in the techniques. Pursuant to this, there has been a paradigm shift in medical science. In the present global scenario, it is of great importance to follow trends in techniques used worldwide. Since the introduction of monoclonal antibodies, immunohistochemistry has developed into a vital tool which is now extensively used in many laboratories for R&D and clinical diagnosis purpose. Immunohistochemistry, as its name suggests, is a collective term used for a variety of methods which can be used to identify cellular or tissue components by means of antigen-antibody interactions. Immunostaining techniques date back to the pioneering work by Albert Coons in early 1940s using fluorescein labeled antibodies. Since then developments in techniques have permitted visualization of antigen-antibody interactions by conjugation of antibody to additional fluorophores, enzyme or radioactive elements. As there is a wide variety of tissue types, antigen availability, antigen-antibody affinities, antibody types and detection methods it is essential to select antibodies almost on a case to case basis. The consideration of these factors has lead to the identification of several key antibodies that have great utility in the study and diagnosis of tumors. Technological advances including assay comparative genomic hybridization, microarray analysis have provided a refinement in the study of genome organization and chromosomal rearrangements. There is increased implementation of improved screening techniques that have in turn been made possible by advances in immunochemical techniques such as in situ hybridization (ISH) and immunohistochemistry (IHC) used for improving patient care through research and improved methods. In situ hybridization is a well established approach for identifying the organization and physical position of a specific nucleic acid within the cellular environment by means of hybridizing a complementary nucleotide probe to the sequence of interest. The use of DNA and RNA as probes to assay biological material has been in use for approximately 30 years. We are well aware that the rapid progress in biology will once again change the methodological approach in the coming years, and that electronic media will continuously help the researcher to access and share information. However, it is becoming more and more evident that many young pharmacologists have only limited training in classical pharmacological methodologies. When searching for these methods, researchers will only find insufficient information on the methodological details in the electronic databases currently available. Thus, it necessiates to have a comprehensive treatise envisaging all aspects of techniques in Biochemistry and Molecular biology. Dr. R.A. Gautam, a young researcher and a passionate teacher fulfils the long cherished demand by writing a book titled 'A HANDBOOK OF TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY'. It has been extensively revised and updated and covers a large number of basic techniques, experiments in biochemistry and molecular biology which helps the student`s understanding of theory and practice. Great care has been taken in selecting the topics, all of which are of considerable interest in the current context and changing scenario. This is a unique collection of chapters in the book dealing with a wide spectrum of issues in the subject matter and will be immense value to and a possession for the readers. Each chapter is introduced by a section on the techniques used and the basic biochemistry. In addition each experiment is preceded by a brief summary of principles

involved so that the reactions used and the design of the experiment may be more fully appreciated. To this end, we hope the current book may bridge this gap by comprehensively covering those pharmacological methods utilized for over more than a hundred years. The book,we hope, shall be helpful not only to the stakeholders working in the fields of biochemistry and molecular biology but also graduate and post-graduate students of AYUSH (Ayurveda,Yoga & Naturopathy, Unani, Homoeopathy) streams. We take this opportunity to congratulate the author and editors for this stipendous effort and wish this book will be an invaluable addition to the existing repository of konwledge.

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PREFACE

Today most technicians, undergraduates and postgraduates in the biological and life sciences require knowledge of biochemistry and especially the practical aspects of the subject. Although, biochemistry constitutes one of the important subjects in the student's curriculum, techniques teaching have been somehow neglected for past many years in the institutions.

One of the main reasons for such a poor status of this important subject is the paucity of trained teachers, particularly with biochemistry background. This has consequently hampered the practical curriculum in biochemistry at undergraduate as well as postgraduate levels. This book is aimed at undergraduates and postgraduates who have a basic grounding in biological sciences and are interested in a future career in research and industry. The book provides an understanding of up-to-date information on the concerned topics in a simple, lucid and concise manner. It attempts to convey something of the fascination of working in a field which overlaps the disciplines of biochemistry, cell-biology and biotechnology. The book content covers the various techniques used in biochemistry, molecular biology, microbiology, immunology, Pharmacology, laboratories. It deals with the comparative analysis, procedure followed for techniques, their advantages, drawbacks and limitations. The content covers about 90% of the various techniques used by Bachelors and Masters and Doctoral students moreover it is of use for students of various universities like: University of Delhi, Jamia Millia Islamia University, Banaras Hindu University, Bundelkhand University etc. The text of the book has been illustrated with simplified well-labeled color diagrams and pictures to make the subject easy to understand and interesting to the students. The book has potential for being developed as a textbook or Lab manual for techniques.

The book is therefore suitable for many B.Sc., M.Sc and technical courses in biochemistry as per UGC model curriculum. It could also be used as source of reference for research workers who need to understand and use biochemical techniques in their work. The objective in writing this handbook is mainly to acquaint the beginner with various biochemistry principles and to provide a laboratory guide to teachers concerned. In writing the first edition, our aim is to explain a new and rapidly growing technology. The basic philosophy is to present the principles of gene manipulation and its associated techniques, in sufficient detail to enable the non-specialist reader to understand them. It is assumed that the reader would have a reasonable working knowledge of molecular biology.

Book writing is not an easy task, I must thank my Guide Prof. A. Ray, Head, Department of Pharmacology, V.P. Chest Institute, University of Delhi and Dr. Anita Kotwani and Dr. Kavita Gulati, Associate Professor, Dr. Ahmad Yasin, Principal, Dr. Mohd. Idris, Head, Department of Ilmul Advia Jadid and Dr. Vivek Bhushan, Head, Department of Dravyguna, Ayurvedic and Unani Tibbia College, University of Delhi, all my present and past colleagues also I cannot end without thanking my family members, who have stood by me through every hardship during this period. Thank you and all the best for your future endeavors. May God Bless you with every happiness in the life. I am thankful to all those members who in one or the other form have helped in the development of this book.

I would welcome suggestions from teachers and students for further improvement of this book in order to make it more useful and fruitful for students.

—Dr. R.A. Gautam

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SAFETY IN LABORATORY

SAFETY IN LABORATORY

Laboratory safety may appear dull subject and the temptation may be to read it superficially or not at all. However, the view of subject changes rapidly if you find yourself in the middle of a fire or the victim of an accident and by this time ignorance can be dangerous or even fatal. Laboratories can be dangerous places in which to work and all users need to be aware of the potential hazards and to know what to do in cases of emergency. Before starting work in a new laboratory, it is important to get familiar with the layout of the room and the location of the safety equipment. The position of the emergency exits, fire alarm and extinguishers should be known so that appropriate action can be taken in the event of fire. The main taps for gas and water and the switch for electricity should also be located so that these services can be turned off in case of an emergency. Laboratory workers must also know the meaning of safety signs and symbols so they can be rapidly identified. They must also know the laboratory Do`s and Don'ts.

DO`S

1. Wear a lab coat and appropriate eye protection, e.g. safety spectacles, goggles or face shield.
2. ALWAYS use the appropriate gloves whenever handling chemicals or hazardous substances.
3. Keep your working area clean and tidy and free of clutter, clear up spillages immediately.
4. ALWAYS wear proper footwear, do NOT wear open toed footwear.
5. Wash hands after using any substances hazardous to health, on leaving the laboratory and before visiting the toilet.
6. ALWAYS know where your nearest fire extinguisher and first aid kit are placed.
7. ALWAYS know your emergency escape route and assembly point.
8. ALWAYS keep broken glassware and sharps separate from other waste and dispose of them in the appropriate containers.

DON`T

1. Do NOT work with hazardous substances without a second person being present.
2. Do NOT eat, drink or smoke in the laboratory under any circumstances.
3. Do NOT pipette by mouth.
4. Do NOT leave equipment using water, gas or electricity on overnight without completing a 'Silent Running' form.
5. ALWAYS ensure all water hoses are secured with jubilee clips.
6. Do NOT touch surfaces with your contaminated gloves.
7. Do NOT have more than 500 ml of a flammable solvent in use at any one time on the bench.
8. Do NOT use or store more cryogenic substances in the lab than an Oxygen Depletion calculator recommends.

2

pH AND BUFFER SOLUTIONS

pH AND LIFE

Many life forms thrive only in a relatively small pH range; an example of a buffer solution is blood. Buffer solutions are used as a means of keeping pH at a nearly constant value in a wide variety of chemical applications. It is an aqueous solution consisting of a mixture of a weak acid and its conjugate base or a weak base and its conjugate acid. It has the property that the pH of the solution changes very little when a small amount of strong acid or base is added to it. A mixture containing citric acid, potassium dihydrogen phosphate, boric acid, and diethyl barbituric acid can be made to cover the pH range 2.6 to 12.

HYDROGEN ION CONCENTRATION AND pH

In 1909, Sorenson introduced the term pH as a convenient way of expressing hydrogen ion concentration which avoids the use of cumbersome numbers. The hydrogen ion concentration of most solutions is extremely low. pH is defined as the negative logarithm of the hydrogen ion activity, but in practice the hydrogen ion concentration is usually taken and this is virtually the same as the activity except in strongly acid solutions. The value of using pH can be seen in the case of human blood which has extremely low hydrogen ion concentration:

$$\text{Plasma } H^+ = 0.398 \times 10^{-7}$$

$$\text{Plasma pH} = -\log (0.398 \times 10^{-7}) = 7.4$$

Dissociation of water

Derivation of K_w: From conductivity measurements, water has been shown to be very weakly ionized and at 25°C the concentration of hydrogen ions is only 10^{-7} mol/litre.

$$H_2O \leftrightarrow H^+ + OH^-$$

The equilibrium constant for the dissociation of water is given by:

$$K = \frac{[H^+][OH^-]}{[H_2O]}$$

Now the concentration of water to all intents and purposes is constant, so we can write:

$$K_w = [H^+]\,[OH^-]$$

The ionic product of water at 25°C is 10^{-14}, so that the pH of pure water at 25°C is 7.

$$[H^+] = [OH^-] = 10^{-7}$$

$$pH = -\log_{10}[H^+] = 7$$

Temperature and K_w: At other temperatures, the pH at neutrality is not 7 since K_w varies with temperature. Even a small change in temperature from 37 to 40°C causes an 8% increase in hydrogen and hydroxyl ions so that a slight rise or fall in temperature may produce a profound biological change in a living system sensitive to hydrogen ion concentration.

ACIDS AND BASES

Definitions: Modern concept of acids and bases developed by Bronsted and Lowry defines acids as proton donors and bases as proton acceptors. Each acid therefore has a conjugate base.

$$\text{Acid} \leftrightarrow \text{Base} + H^+$$

Alkali term is used for compounds which yield hydroxyl ions on dissociation.

$$KOH \rightarrow K^+ + OH^-$$

Some examples of acids and bases:

Table 2.1

Acid		Conjugate base
H_2O	$\leftrightarrow$	$H^+ + OH^-$
H_3O^+	$\leftrightarrow$	$H^+ + H_2O$
HCl	$\rightarrow$	$H^+ + Cl^-$
CH_3COOH	$\leftrightarrow$	$CH_3COO^- + H^+$
NH_4^+	$\leftrightarrow$	$H^+ + NH_3$
H_2CO_3	$\leftrightarrow$	$H^+ + HCO_3^-$
HCO_3^-	$\leftrightarrow$	$H^+ + CO_3^-$

Although it is convenient to write acid-base equilibrium as shown, but the proton is usually solvated and does not exist as such. For example, in aqueous media the hydrogen ion exists as hydronium ion (H_3O^+).

$$H_3O^+ \leftrightarrow H^+ + H_2O$$

Ampholytes: some ionic species can act as both acids and bases, also called amphoteric species.

Dissociation of acids and bases

Strong acids or bases are completely ionized in solution, so that the concentration of free H^+ and OH^- is the same as the concentration of the acid or base. These compounds are completely dissociated to hydrogen ions and the conjugate base occurs, so that the hydrogen ion concentration is the same as that of the acid. The pH of such solutions can be calculated very easily.

Strong acid (Nitric acid) $HNO_3 \rightarrow H^+ + NO_3^-$

Strong base (Sodium hydroxide) $NaOH \rightarrow Na^+ + OH^-$

0.01 mol/litre HCl, $pH = -\log_{10}(10^{-2}) = 2$

0.1 mol/litre HCl, $pH = -\log_{10}(10^{-1}) = 1$

$$\text{0.01 mol/litre NaOH, } [H^+] = \frac{K_w}{[OH^-]} = \frac{10^{-14}}{10^{-2}} = 10^{-12}$$

$$pH = -\log_{10}(10^{-12}) = 12$$

Weak acids

Weak acid or bases dissociate only to a limited extent and the concentration of free H^+ and OH^- depends on the value of their dissociation constants.

Weak acid (Formic acid) $HCOOH \rightleftarrows H^+ + COOH^-$

The Henderson-Hasselbach equation: Weak acids are only slightly ionized in solution and a true equilibrium is established between the acid and the conjugate base. If HA represents a weak acid, then:

$$HA \leftrightarrow H^+ + A^-$$

According to the law of mass action, K_a the acid dissociation constant is defined as:

$$K_a = \frac{[H^+][A^-]}{[HA]}$$

and

$$[H^+] = \frac{K_a\,[HA]}{[A^-]}$$

Taking negative logarithms,

$$-\log_{10}[H^+] = -\log_{10} K_a + -\log_{10}\frac{[HA]}{[A^-]}$$

$$pH = pK_a + \log_{10}\frac{[A^-]}{[HA]}$$

In general terms,

$$pH = pK_a + \log_{10}\frac{[\text{Conjugate base}]}{[\text{Acid}]}$$

The activities of A^- and HA are not always known, so it is convenient to express A^- and HA as concentration terms. Thus,

$$pH = pK_a + \log\frac{C_{A^-}}{C_{HA}} + \log\frac{f_{A^-}}{f_{HA}}$$

Where f_{A^-} and f_{HA} are the activity coefficients of A^- and HA respectively. Since $\log(f_{A^-}/f_{HA})$ is constant for a given acid, these activity coefficients can be incorporated into the pK_a term of given an apparent dissociation constant pK'_a.

$$pH = pK'_a + \log\frac{C_A}{C_{HA}}$$

This relationship is known as the Henderson-Hasselbach equation and is valid over the pH range 4–10 where the hydrogen and hydroxyl ions do not contribute significantly to the total ionic concentration.

pK_a : It is the negative logarithm of the acid dissociation constant of a weak acid. Another way of defining pK_a is the pH at which the concentrations of the acid and its conjugate base are equal or the pH at which the acid is half titrated.

$$pH = pK_a + \log 1$$

or $$pH = pK_a$$

Weak base (Aniline) $C_6H_5NH_2 + H^+ \rightleftarrows C_6H_5NH_3^+$

In biological systems, we deal with aqueous media and in here, the strength of an acid is taken to refer to water as solvent.

pH INDICATORS

An approximate idea of the pH of a solution can be obtained using indicators. These are organic compounds of natural or synthetic origin whose color is dependent upon the pH of the solution. Indicators are usually weak acids which dissociate in solution. Indicators are usually used for determining the end point of a titration.

$$\text{Indicator} = \text{Indicator}^- + H^+$$

Applying the Henderson-Hasselbach equation,

$$pH = pK_{In} + \log_{10} \frac{[\text{Indicator}^-]}{[\text{Indicator}]}$$

The two forms of the indicator have different colors and the actual color of the solution depends upon the pK_{In} and pH. Maximum color change occurs around the $pK_{In.}$ Example: If a solution has a pH near 6 then bromocresol purple with a pK_{In} of 6.2 is the best indicator to use. Limitations of using indicators are, that this color change occurs over a wide pH range, so indicators will only give an approximate indication of pH. Moreover, the indicators are affected by oxidizing agents, reducing agents, salt concentration and protein. Only a small quantity of indicator is added to the solution under examination, otherwise the acid-base equilibrium of the test solution may be displaced and the pH changed. Accurate pH is measured by pH metre which measures the e.m.f. of a concentration cell formed from a reference electrode, the test solution, and a glass electrode sensitive to hydrogen ions.

The glass electrode consists of a very thin bulb about 0.1mm thick blown on to a hard glass tube of high resistance. Inside the bulb is a solution of HCl (0.1mol/litre) connected to the platinum wire via a silver-silver chloride electrode, which is reversible to hydrogen ions. A potential is developed across the thin glass of the bulb which depends on the pH of the solution in which it is immersed. This potential is not readily affected by salts, protein, or oxidizing and reducing agents, so the electrode can be used in a wide variety of media. The glass electrode in the test solution constitutes a half cell and the measuring circuit is completed by a reference electrode which is not sensitive to hydrogen ions. The glass electrode has a very high resistance ($10^6 – 10^8$ Ω), so a potentiometre of high input impedence is needed to measure the potential. Nowadays, pH metres have the glass and reference electrodes combined in one unit.

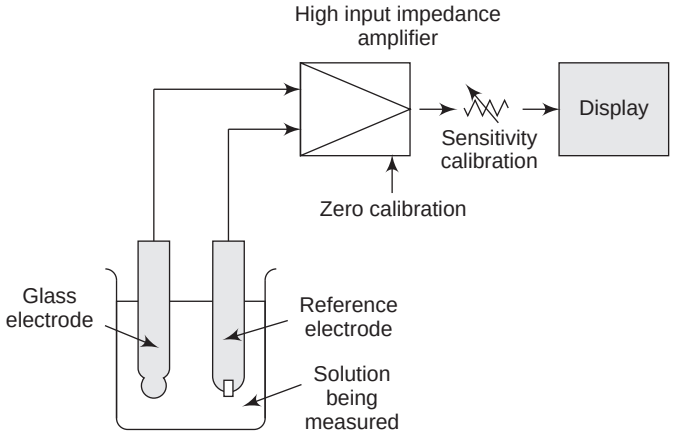


Fig. 2.1. The electrode system for measurement of pH

The most common reference electrode used is the calomel electrode which is easy to prepare, is stable and the potential with respect to the standard hydrogen electrode is accurately known.

pH metre the e.m.f. of the complete cell (E) formed by the linking of these two electrodes is therefore, $E = E_{ref} - E_{glass}$

where, E_{ref} is the potential of the calomel reference electrode which at RT is + 0.250 V and E_{glass} is the potential of the glass electrode which depends on the pH of the solution under examination (pH_E).

$$E_{glass} = 0.342 - 0.058\ pH_E$$

$$E = 0.250 - (0.342 - 0.058\ pH_E)$$

$$= -0.092 + 0.058\ pH_E$$

This relationship can be changed by presence of other potentials in the cell, but these are constants and can be allowed for when calibrating the instrument.

Table 2.2

Components	pH range
HCl, Sodium citrate	1–5
Citric acid, Sodium citrate	2.5–5.6
Acetic acid, Sodium acetate	3.7–5.6
K_2HPO_4, KH_2PO_4	5.8–8
Na_2HPO_4, NaH_2PO_4	6–7.5
Borax, NaOH	9.2–11

Table 2.3

0.2M Na_2HPO_4 /ml	0.1M Citric Acidml	pH.
20.55	79.45	3.0
38.55	61.45	4.0
51.50	48.50	5.0
63.15	36.85	6.0
82.35	17.65	7.0

BUFFER SOLUTIONS

A buffer solution is one that resists pH change on addition of an acid or alkali. Such solutions are used in many biochemical experiments where the pH needs to be accurately controlled. From the Handerson-Hasselbach equation, the pH of a buffer solution depends on two factors; one is pK_a value and other the ratio of salt to acid. This ratio is considered to be the same as the amount of salt and acid mixed together over the pH range 4–10, where the concentration of hydrogen and hydroxyl ions is very low and can be ignored. Useful range of the buffers is about 1 unit either side of pK_a value. The actual buffer chosen for a particular experiment needs to be selected with care as experimental results may be due to specific ion effects and not pH. Like, borate forms complexes with sugars and citrate readily combines with calcium. Some common buffers used in laboratories:

Table 2.4

Acid or base	pKa	pKa	pKa
Phosphoric acid	2.1	7.1	2.3
Citric acid	3.1	4.8	5.4
Carbonic acid	6.4	10.3	---
Glycyl Glycine	3.1	8.1	---
Acetic acid	4.8	---	---
Barbituric acid	3.4	---	---

pH of blood is 7.4, moreover pH in the range 6–8 is required in many biological experiments but there are few weak acids or bases which act as effective buffers in this range.

Tris: It is a popular buffer as it can be used with heavy metals but it has a poor buffering capacity below pH 7.5. Moreover, a tris buffer of pH 7.8 at room temperature has a pH of 8.4 at 4°C and 7.4 at 37°C, i.e. the hydrogen ion concentration increases ten-fold from the initial preparation at 4°C and final measurement at 37°C. This effect of temperature on Tris pH poses problem. It also acts as an inhibitor in some biological systems. It also has a high lipid solubility and penetrates membranes which can be a disadvantage.

Phosphate: Most commonly used buffer. But, phosphate forms complexes with heavy metals also the buffering capacity above pH 7.5 is poor. Moreover, phosphate plays an active part in a number of biochemical reactions where it can act as an activator, inhibitor or metabolite.

Bicarbonate buffer: It has pK_a of 6.1 so the buffering capacity around pH 7.4 is poor. It spontaneously liberates CO_2 and therefore must be maintained in an atmosphere of CO_2.

For biological experiments the Zwitterionic buffers are used as they contain both negative and positive groups so do not readily penetrate membranes. These buffers were introduced by Good and co-workers to overcome the disadvantages of traditional materials. Example is HEPES buffer.

Buffer value β: Van Slyke to compare different buffer solutions introduced the term buffer value, because buffer solutions vary in extent to which they resist pH changes. When acid or alkali is added to a buffer solution, a titration curve is obtained. The slope of this curve is given by dB/d(pH), where dB is the increment of strong acid or base added in mol/litre and d(pH) the change in the pH increment. This slope is called buffer value β which is always positive since dB is negative when acid is added causing a negative change in pH. The buffer value is maximum at pK_a.

For example, acetate buffer consisting of a mixture of acetic acid and sodium acetate

$$CH_3COOH \leftrightarrow CH_3COO^- + H^+$$
$$CH_3COONa \rightarrow CH_3COO^- + Na^+$$

Since acetic acid is only weakly dissociated, the concentration of acetic acid is almost the same as the amount present in mixture; moreover the concentration of acetate ions can be considered to be the same as the concentration of sodium acetate placed in the mixture since the salt is completely dissociated.

Example 1: *In a mixture of 5 ml of 0.1 mol/litre sodium acetate and 4 ml of 0.1 mol/litre acetic acid, what is the pH of the mixture?*

Solution. $CH_3COONa \rightarrow CH_3COO^- + Na^+$

$$\text{Concentration of } CH_3COO^- = \frac{5}{9} \times 0.1 \text{ mol/litre}$$

$$\text{Concentration of } CH_3COOH = \frac{4}{9} \times 0.1 \text{ mol/litre}$$

pK_a of acetic acid at 25° C = 4.76

So, $pH = 4.76 + \log 5/4$

$= 4.76 + (+ 0.097)$

$= 4.86$

Example 2: *How the pH of the above mixture would change on adding 1 ml of 0.1 mol/litre HCl to the above mixture?*

Solution. $CH_3COOH \leftrightarrow CH_3COO^- + H^+$

$$\text{Concentration of } CH_3COO^- = \frac{5 \times 0.1}{10} - \frac{1 \times 0.1}{10}$$

$= 0.04$ mol/litre

$$\text{Concentration of } CH_3COOH = \frac{4 \times 0.1}{10} + \frac{1 \times 0.1}{10}$$

$= 0.05$ mol/litre

$pK_a = 4.76$

Therefore, $pH = 4.76 + \log 0.04/0.05$

$= 4.76 + (- 0.097)$

$= 4.66$

The pH of the solution reduces from 4.86 to 4.66, a change of only 0.2, whereas if HCl had been added to distilled water, the pH would be 2. The solution has therefore acted as buffer and resisted pH change on addition of acid.

pH AND LIFE

Cells can function within very narrow limits of pH and require buffer systems to resist the changes in pH occurring in metabolism. Three main buffer systems in living material are protein, bicarbonate and phosphate. In plants the average cytoplasmic pH is similar to that of animals, but the cell sap is acidic pH 5.2–6.5. However, some plant juices are very acidic (citrus fruits, pH 3), but the acid

is present in vacuoles and therefore separated from rest of cytoplasm. Mammalian plasma consists of bicarbonate buffering system

$$H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

From Henderson-Hasselbach equation:

$$pH = pKa + \log_{10} \frac{[HCO_3^-]}{[H_2CO_3]}$$

The plasma pH depends on ratio of bicarbonate to carbonic acid and not on absolute concentrations. Any pH change is buffered and can be corrected by adjusting this ratio. Example: acids formed during normal metabolism react with bicarbonate to form weakly dissociated carbonic acid so that free hydrogen ions are effectively used. At the same time, carbonic acid is removed at lungs as carbon dioxide, thus maintaining the pH of the plasma. The kidneys also maintain the acid-base balance by adjusting the excretion of acid or base in urine, so the pH of urine in man varies from 4.6–7.5. The pH at membrane surface is lower than 6.8 due to absorption of H^+ on the negatively charged surface. The extracellular fluid is slightly alkaline at pH 7.4 while cytoplasmic pH is 6.8 which at 37°C is neutral. pH in the organelles will differ from 6.8.

The simpler forms of life appears to survive a wide range of pH of the external medium although internal pH does not vary to that extent. The pH of Bacteria is around 7 however, many forms grow well at pH 6 or 9, i.e. a 1000 fold difference in hydrogen ion concentration. Thiobacilli grows at pH 0, which is 1 mol/litre HCl, while some fungi grow at pH 11. Most pathogenic organisms have an optimum growth between pH range 7.2–7.6.

TITRATION CURVES

When a strong base is mixed with a solution of an acid and the pH measured, a plot of the base added against pH recorded can be obtained and this is known as a titration curve. These curves have same shape with the exception of the strong acid HCl. Similar curves are obtained when a base is titrated with strong acid.

Strong acid and a strong base: There is little change in pH on adding the base until complete neutralization, when only a slight excess of base causes a large increase in pH. In effect the strong acid resists a change in pH or acts as a buffer solution until close to the neutralization point as seen when HCl is titrated with NaOH.

Weak acid and a strong base: All titration curves of a weak acid or base titrated with a strong base or acid are of the same type, since one has a buffer solution present whose pH changes according to the Handerson-Hasselbach equation. The pK_a values are different for each acid, but the general shape of the curve is same in all cases.

***Practical limits of titration curves*:** If 0.1 mol/litre strong acid or base is used in a titration, the curves will reach pH 1 or pH 13 after complete neutralization. As the limits for 0.01 mol/litre solutions will be pH 2 or pH 12 so that pK_a values below or above 12 cannot be determined using 0.01 mol/litre solutions.

Solvent correction: Experimental titration curves must be corrected for the amount of acid or base consumed in titrating the solvent, usually distilled water. This is carried out as follows:

1. Plot the titration curve for the same volume of sample and water.
2. Select a pH value on the curve for the sample and note the volume of acid or base used; let this be X ml. Likewise, note the amount of acid or base consumed in order to bring the water to the same pH value; let this be Y ml.
3. The actual amount of acid consumed in the titration of the sample is, therefore given by (X–Y) ml. Repeat this for a number of pH values and plot the corrected titration curve.

Determination of pK_a: pK_a values can be obtained from titration data by 3 methods:

(*a*) The pH at the point of inflection is the pK_a value and this may be read directly. A more convenient way is to plot dB/d(pH), the buffer value, against pH when a maximum is obtained at the pK_a.

(*b*) By definition, the pK_a value is equal to the pH at which the acid is half titrated. The pK_a can therefore, be obtained from a knowledge of the endpoint of the titration.

(*c*) The ratio of salt/acid can be calculated from the experimental data and a graph prepared of $\log_{10}$salt/acid against pH. The intercept on the axis is the pK_a value.

Table 2.5

Color change and pH range of some common indicators				
Indicator	**Color of indicator**		**pK_a**	**Useful pH range**
	Acid	**Base**		
Thymol blue	Red	Yellow	1.7	1.2–2.8
Bromophenol blue	Yellow	Blue	4.0	3.0–5.0
Methyl red	Red	Yellow	5.0	4.3–6.1
Bromocresol purple	Yellow	Purple	6.3	5.5–7.0
Phenol red	Yellow	Red	7.9	6.8–8.2
Phenolphthalein	Colorless	Red	9.7	8.3–10.0

DETERMINATION OF pH USING INDICATORS

Materials: Indicators (solutions in aqueous ethanol of the indicators stated above in the table), samples to be tested (saliva, egg white) diluted 1 in 10

Method: Pipette 1–2 ml of each sample into a test tube, add 2 drops of methyl red indicator solution and observe the color. Repeat the exercise with other indicators until the approximate pH is found by comparing the colors of the sample with those of the acidic, intermediate and basic forms of the indicator.

Pipette 2 ml of laboratory distilled water into a test tube and determine the pH using the indicators as described.

TITRATION CURVES OF AMINO ACIDS

Amino acids are present as zwitterions at neutral pH and are amphoteric molecules that can be titrated with both acid and alkali. The strong positive charge on the $-NH_3^+$ group induces a

tendency for the –COOH to lose a proton, so amino acids are strong acids. Some amino acids have other ionizable groups in their side chains and these can also be titrated.

Materials: HCl (100 mmol/litre) 250 ml, NaOH (100 mmol/litre) 250 ml, amino acids (100 mmol/litre glycine, alanine, histidine and lysine; 50 mmol/litre glutamic acid), pH metre, burette (10 ml)

Method: Pipette 10 ml of the amino acid solution into a 100 ml beaker. Standardize the pH metre and determine the pH of the solution. Add 100 mmol/litre HCl from a burette in small amounts at first and determine the pH after each addition. Continue adding the acid in larger quantities until the pH falls to 1.3. Wash the electrodes in distilled water, restandardize the pH metre and titrate a further 10 ml with 100 mmol/litre NaOH solution to pH 12.5. Find the pK values from your curves and compare them with the values given.

Table 2.6

Amino acid	pK1	pK2	pK3
	–COOH	$–NH_2$	Side chain (R)
Glycine	2.4	9.7	---
Alanine	2.3	9.9	---
Glutamic acid	2.2	9.7	4.3 carboxyl
Histidine	1.8	9.2	6.0 imidazole
Lysine	2.2	9.0	10.5 amino

3

PHYSICAL AND CHEMICAL TECHNIQUES

Techniques can be differentiated into separation and analytic. Separation techniques require two phases and the desired substance distributes itself between the two phases in a definite manner, and the separation is completed by physically separating the two phases. Various types include: Decantation, Filtration, Evaporation, Crystallization, Distillation, Fractional Distillation, Sublimation, Centrifugation, Chromatography, Electrophoresis.

An analytical technique is a method that is used to determine the concentration of a chemical compound or chemical element. Various types include:

1. Titration, based on the quantity of reagent needed to react with the analyte.
2. Electroanalytical techniques, including potentiometry and voltammetry.
3. Based on the interaction of the matter with electromagnetic radiation—Spectroscopy: NMR, Optical methods (IR, UV)

CELL FRACTIONATION

Cell fractionation is the separation of identical organelles, from a heterogeneous population, based on size and density, the larger and denser particles pellet out at lower centrifugal forces. In the process, a tissue sample is first homogenized to break the cell membranes and mix up the cell contents. The homogenate is then subjected to repeated centrifugations, each time removing the pellet and increasing the centrifugal force.

DIFFERENTIAL CENTRIFUGATION

Differential Centrifugation after homogenization, the suspension is separated into a number of fractions by centrifuging at various 'g' values. The intracellular particles then sediment at different rates according to their masses.

DENSITY GRADIENT CENTRIFUGATION

Density Gradient Centrifugation gradient solution of caesium chloride or sucrose is used to separate particles based on their individual densities (mass/volume). Gradient solution is formed by layering a series of different sucrose concentration on top of each other with the densest concentration at the bottom. Mixture to be separated is then added to the gradient at top and centrifuged. According to their density the particle proceeds up or down and will be found as a band in that concentration of sucrose whose density is close to that of the organelles. Sucrose gradient is especially used for brain tissue fractionation where nerve endings and myelin can be separated. Caesium chloride gradient is used to separate DNA particles that have incorporated heavy isotopes (i.e., 13C or 15N) can be separated from DNA particles without heavy isotopes.

Fractionation of Proteins: the separation and characterization of the individual protein is of considerable importance. It facilitates the study of the chemical nature and physiological function of each protein. Proteins could be fractionated by the difference in their solubility in salt solution. Saturated solution of ammonium sulfate (SAS) at various concentration as well as solid ammonium sulfate is most commonly employed for protein fractionation. e.g., immunoglobulin G is precipitated at 40 % saturation of ammonium sulfate. Saturated ammonium sulfate is prepared by taking 760g of ammonium sulfate in 1 litre distilled water, boiled and filtered. pH is adjusted to 7.0 with ammonium hydroxide.

Materials: Ammonium sulfate, pH metre, cold centrifuge and magnetic stirrer with magnetic bar

Method: (1) Fractionation of Igs with ammonium sulphate: The volume of SAS to give desired saturation for final precipitation of proteins can be calculated by the formula:

$$V = \frac{v(S_2 - S_1)}{(1 - S_2)}$$

Where,
V= Volume of ammonium sulfate required
v = Volume of protein solution taken
S_2 = Desired saturation of SAS
S_1 = Initial saturation of SAS

(*i*) For example, for 50% saturation of protein, 100 ml of cold SAS is added slowly to 100ml sera and is stirred for 30 minutes at 4°C. It is centrifuged at 10,000 rpm for 20 minutes.

(*ii*) Supernatant is discarded and precipitate is washed with 50% ammonium sulfate solution and is centrifuged at 10,000 rpm for 20 minutes. The process for washing is repeated twice.

(*iii*) Supernatant is discarded and precipitate is dissolved in normal saline and is kept for extensive dialysis against normal saline and then with desired buffer till it is equilibrated with buffer.

(*a*) **Fractionation of IgG:** (a) 66 ml of SAS is added dropwise to 100 ml of serum and is stirred for 30 minutes in cold. This gives 40% saturation of ammonium sulfate.

(*b*) Rest of the procedure is followed as mentioned above.

4

CHROMATOGRAPHY

Chromatography Derived from Greek meaning 'colored writing'. It is an analytical laboratory technique first used by Russian botanist Tswett to describe the separation of colored plant pigments on a column of alumina. It is always a biphasic system, where one phase is stationary while the other is mobile. Various types include paper chromatography, thin layer chromatography (TLC), column chromatography (CC), size-exclusion chromatography (SEC), ion-exchange chromatography (IEC), liquid chromatography (LC), gas chromatography (GC), but all of these employ the same basic principle. In all types of chromatography, a sample of the mixture being analyzed, known as the analyte is applied and allowed to adhere to a stationary material known as the stationary phase, or adsorbent. A carrier fluid known as the mobile phase or eluent, is then made to flow through the adsorbent. Because the different components of the analyte exhibit varying degrees of strength of adhesion to the adsorbent, they also travel different distances through the adsorbent as the eluent flows through it, *i.e*., components adhering more strongly to the adsorbent travel more slowly than those with weaker adhesion. Thus resulting in separation of the various components of the analyte into individual samples that can be analyzed.

CLASSIFICATION OF CHROMATOGRAPHIC METHODS

On basis of Geometry: Planar: Paper, Thin Layer Chromatography (TLC),

Column: Ion Exchange Chromatography (IEC), High Performance Liquid Chromatography HPLC, Gas Chromatography (GC), Super critical Fluid Chromatography (SFC)

On basis of Stationary Phase: Adsorption, Partition, Affinity, Size Exclusion Chromatography (SEC), IEC

On basis of Mobile Phase: Liquid Chromatography (LC), GC, SFC

PAPER CHROMATOGRAPHY

Paper cellulose provides an ideal support medium where water is adsorbed between the cellulose fibres and forms a stationary hydrophilic phase. However, it has been replaced by thin layer chromatography (TLC), but still for teaching purpose it is used. A small concentrated spot of mixture is applied to a strip of chromatography paper about two centimetres away from the base of the plate, usually using a capillary tube. The paper is then dipped into a suitable solvent, such as ethanol taking care that the spot is above the surface of the solvent, and placed in a sealed container. As the solvent rises through the paper it meets and dissolves the sample mixture, which then travels up the paper. Due to competition between cellulose and solvent for different components of the solute, the different solute components travel at different rates. Paper cellulose being a polar substance, have a high affinity for polar substances. The solvent front is marked and after drying the paper, the positions of the compounds present in the mixture are visualized by a suitable staining reaction. The retention factor (R_f) may be defined as the ratio of the distance travelled by the substance to the distance travelled by the solvent. If R_f value = 0, the solute remained in the stationary phase and is immobile. If R_f value = 1 then the solute has no affinity for the stationary phase and travels with the solvent front.

Detection of Spots: Many colorless compounds are visualized by spraying or dipping in volatile specific reagents such as ninhydrin. Ultraviolet light is also useful since some compounds which absorb strongly show up as dark spots against the fluorescent background of the paper. Other compounds show a characteristic fluorescence under UV.

For analytical purposes whatman papers are used. The paper may be impregnated with a buffer solution before use or chemically modified by acetylation. Ion exchange papers are also commercially available. Silica impregnated papers for separation of lipids and hydrophobic molecules are also commercially available. Biological samples should be desalted before chromatography by electrolysis as excess salts result in a poor chromatogram with spreading of spots and changes in their R_f values. Macromolecules such as proteins should also be removed prior to chromatography by ultrafiltration or gel filtration.

Solvent choice depends on the mixture to be separated. Methylene Chloride generally works as an universal solvent due to its mild polarity and organic functional groups. Acetone should not be used if 2 polar solutes are to be separated for developing an ideal solvent. If the compounds move close to solvent front in A solvent then they are too soluble, while if they are clustered around the origin then they are not sufficiently soluble in solvent B. So, the suitable solvent will be a mixture of both A and B. Acidic or basic pH can be created by adding acetic acid or ammonia.

In some cases, when two substances have the same values in a particular solvent then one dimensional *paper chromatography* does not separate pigments completely. In these cases, two-dimensional chromatography is used to separate the pigments e.g. amino acids.

TWO-DIMENSIONAL PAPER CHROMATOGRAPHY

Here two solvents are used and in between the paper is rotated 90°. The mixture is first separated in the first solvent, which should be volatile; then after drying, the paper is turned 90° and separation is carried out in second solvent. The position of compounds are compared with known compounds chromatogram developed under same conditions.

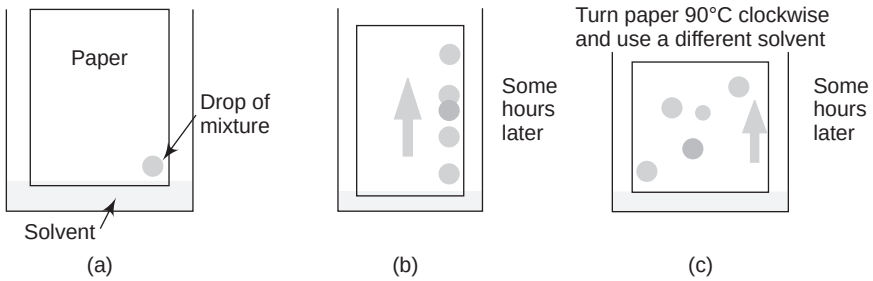


Fig. 4.1. Two-Dimensional Paper Chromatography

ASCENDING CHROMATOGRAPHY

It gives fast separation here, the solvent is at the bottom of the vessel in which the paper is supported. The ascending chromatogram is folded over the glass rod whose other half become the descending chromatogram.

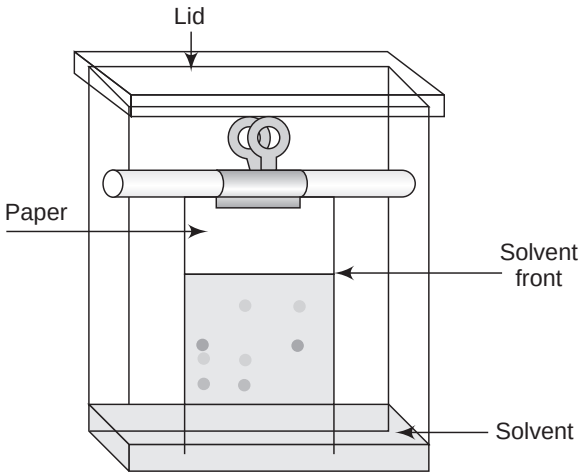


Fig. 4.2. Ascending Paper Chromatography

GRAVITATIONAL/DESCENDING CHROMATOGRAPHY

It gives faster separation then ascending chromatography because the liquid moves down by capillary action as well as by the gravitational force, so it is also known as the gravitational method. Here, the solvent is kept in a trough at the top of the chamber and is allowed to flow down the paper. Substances that cannot be separated by ascending method can sometimes be separated by the descending method. This method is convenient for compounds having similar R_f values, since the solvent drips off the bottom of the paper, thus, giving a wider separation. Here, R_f values cannot be measured and substances are compared with a standard reference compound like glucose in case of sugars.

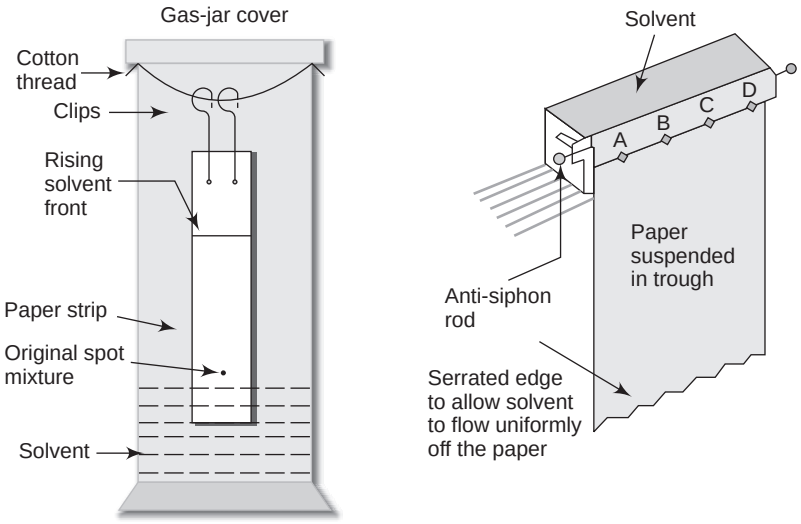


Fig. 4.3. Descending Paper Chromatography

THIN LAYER CHROMATOGRAPHY (TLC)

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. Even compounds of lower concentration can be detected. Moreover, separated components can be detected by corrosive sprays and elevated temperatures, which is not possible with paper chromatography. It is performed on a sheet of glass, plastic, or aluminum foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose (blotter paper). This layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, 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 R_f values are affected by the thickness of the layer. Mostly, 200μm and depth of 250μm is employed for most of the separations. 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 quantization. This method is referred to as HPTLC, or 'high performance TLC'.

COLUMN CHROMATOGRAPHY

The column (where the actual separation process takes place) is usually made of glass or metal tube that is capable of withstanding the range of pressure that may be applied to it. Generally, long columns give good resolution of components while wide columns are better for dealing with large quantities of material. Before preparing the column some form of pretreatment is required, for e.g., some gel filtration materials need to be swollen, adsorbents need to be 'activated' by heating or acid treatment and ion exchange resins have to be obtained in the required ionized form by washing. Materials used in chromatographic separation need to be equilibrated with the solvent. During the equilibration with solvent the material is allowed to settle and fine particles remaining in the suspension are removed. Otherwise, the flow rate of solvent down the column will be considerably

reduced due to clogging by these fine particles. Column packaging is done with material by filling it 1/3rd with solvent. A solution of the material in solvent is poured in down a glass rod to avoid air bubbles. This suspension is allowed to settle and excess solvent run off. The process is repeated until the column is of desired height. The column is then washed thoroughly with solvent and the level of liquid kept just above the surface of material. The sample is first dissolved in the solvent before loading it on to the column. The solvent reservoir is connected and a constant head of liquid maintained at the top of the column from a pressure reservoir. The material is removed from the column by eluting with an appropriate solvent. In displacement development, the solvent interacts more strongly with the chromatographic material than the compound on the column, thus displacing the bound molecules. Large quantities of materials can be separated in this way since about 50 percent of the total column capacity is used. The separation is adequate but for better resolution of peaks elution development is preferred. Here, not more than 10 percent of the total capacity is loaded on to the column. The solvent interacts with the column more weakly than the solute molecules and overrides the bound molecules, gradually eluting them from the column. This is most commonly used and different molecules are removed from the column by changing the pH of the eluting solvent in a gradient manner.

Collection and analysis of fractions: The eluent from the column is collected into a series of test tubes. Each fraction is then analyzed for the presence of the compounds being examined and an elution profile prepared of the amount eluted against the effluent volume.

SIZE-EXCLUSION CHROMATOGRAPHY (SEC)

Also known as molecular sieving, gel filtration or permeation here separation is based on molecular size and not by molecular weight. Perforated stationary phase is used and the mobile phase can either move through interstitial spaces or from the pores of stationary phase particles. Large molecules elute out first as they do not enter the matrix while, small molecules get entangled in matrix so elute later. Most commonly used material is silica gel, being polar due to –OH groups, silica gel helps in retaining polar molecules thus allowing them to elute out later. Used for separation of macromolecular complexes such as proteins, polysaccharides and nucleic acids and industrial polymers. Advantages of this method include good separation of large molecules from the small molecules using a minimum volume of elute, preserving the biological activity of the particles to be separated.

ION-EXCHANGE CHROMATOGRAPHY (IEC)

Proteins, peptides, nucleic acids and some carbohydrates carry positive or negative charge in this case anion or cation exchange chromatography is used. Here ion-exchange resins are bound to inert stationary phase packings of column. For anion separation, a quaternary ammonium group is used while, for cation separation sulfonic or carboxylic acid is used. Earlier the ion exchange materials used were synthetic resins of aromatic nature and were suitable for separation of inorganic ions and small molecules. However, they could not be used for separation of large molecules like proteins which cannot penetrate the closely linked structure of resin and are denatured by hydrophobic matrix. The stationary phase packings of the column usually consist of ion-exchange resins bonded to inert polymeric particles of small diameter approx. 10 microns. Earlier cellulose

based ion exchangers were used for macromolecules but they had low capacities as too much substitution made the cellulose soluble. Nowadays, dextran and an acrylamide based material is used as they have higher capacity. Analyte molecules are retained on the column on the basis of columbic (ionic) interactions.

Ionizable groups: The nature and strength of ion exchanger is defined by the charge and type of group attached to the matrix. These may be anionic or cationic according to the nature of their affinity for either negative or positive ions. Whether the material is cationic or anionic is decided by the charge of the ionizable groups and not by the charge on the matrix. Moreover, the capacity of the ion exchanger is directly proportional to the number of ionizable groups.

Table 4.1

Type of Ion Exchanger	Functional Group	Common Abbreviation
Strong Anion	Quaternary Amino ethyl	QAE
Weak Anion	Diethyl Amino ethyl	DEAE
Strong Cation	Sulfonic Acid	S
Weak Cation	Carboxy methyl	CM

PARTITION CHROMATOGRAPHY

Two immiscible liquids are used in partition chromatography. E.g. Oil-Water Ink

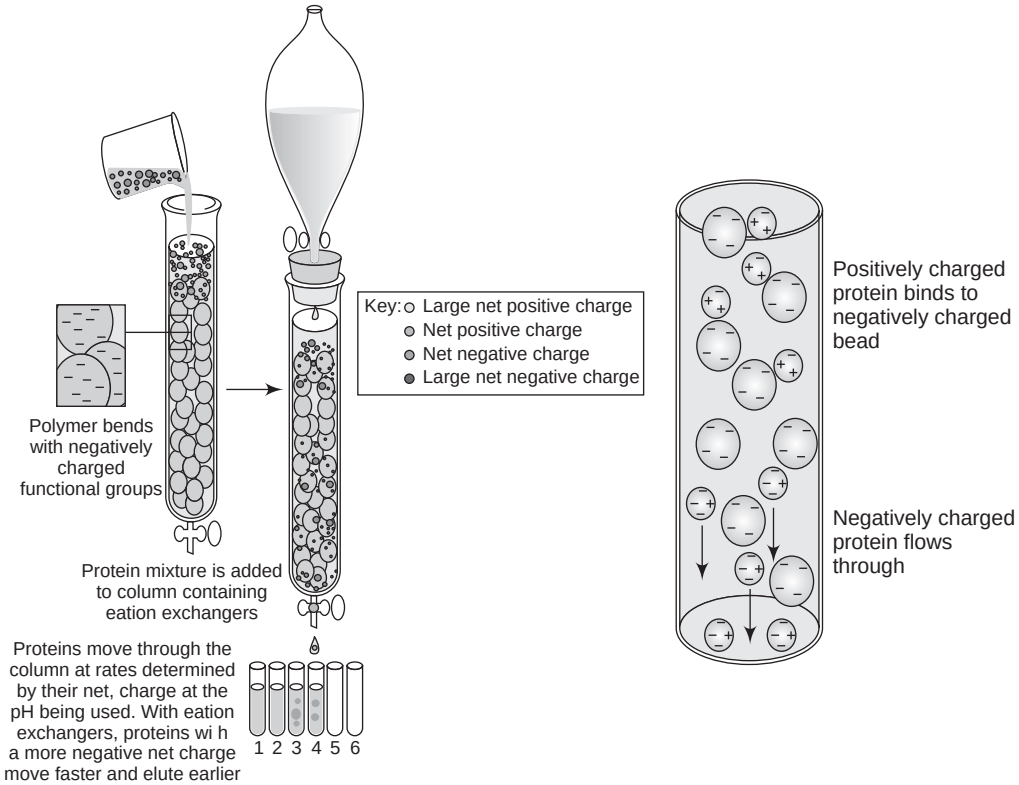


Fig. 4.4. Ion Exchange Chromatography

AFFINITY CHROMATOGRAPHY

The three dimensional structure of a molecule defines the biological activity of that molecule and this is commonly used in chiral (for smaller molecules) or affinity (for larger molecules) chromatography. Here the stationary phase is a gel matrix, mostly agarose (a linear sugar molecule derived from algae). The other molecules in solution will not become trapped as they do not possess this property. The solid medium can then be removed from the mixture, washed and the target molecule released from the entrapment in a process known as elution. Recombinant proteins are purified using affinity chromatography.

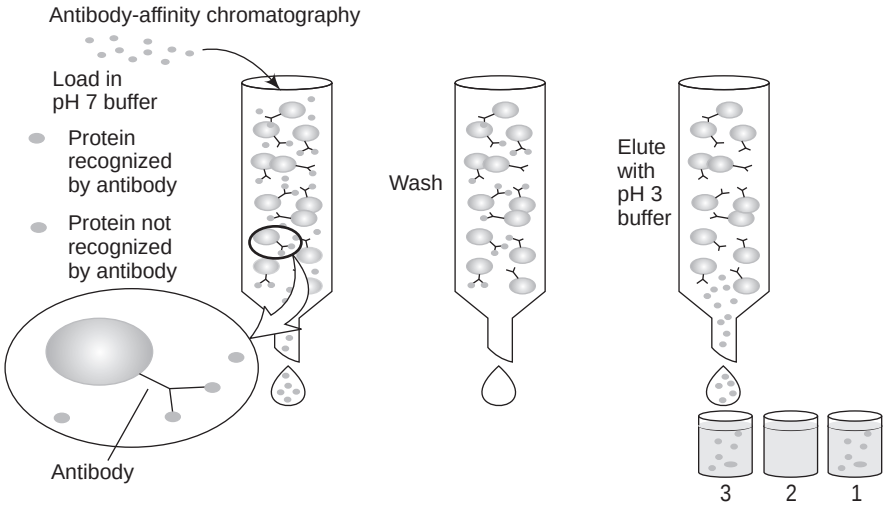


Fig. 4.5. Affinity Chromatography

The physical characteristics of a molecule are exploited in liquid chromatography to affect a separation. The basic chromatography process consists of the following steps: (1) feed injection, wherein the analyte is injected into the mobile phase or carrier fluid; (2) separation of the analyte in the column into its components as the mobile phase flows through the stationary phase, by virtue of the varying degrees by which these components are attracted to the stationary phase; (3) elution from the column, wherein the different components of the sample will emerge from the column at different times, with the component that's least bound to the stationary phase eluting first; and (4) detection, wherein the eluted components are collected and analyzed, usually by measuring certain properties of the components, such as the refractive index, UV absorbance, or solution conductivity. The output of a chromatographic analysis is referred to as a 'chromatogram.' It is a plot that consists of several different peaks representing the different components of the sample mixture. The chromatographic modes listed below are defined based on the physical characteristics of the sample molecule:

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

In high performance liquid chromatography (HPLC), the sample is forced through a column that is packed with a porous monolithic layer called stationary phase. Stationary phase may be (1) an immiscible liquid that coats a porous support; (2) a thin film of liquid bonded to the surface of a

sorbent; or (3) a sorbent of controlled pore size. The mobile phase is usually a liquid of low viscosity that is flowing at high pressure through the stationary phase bed. HPLC is the most versatile and widely used type of elusion chromatography. This technique is used for separating and determining species in a variety of organic, inorganic and biological materials. The mobile phase in liquid chromatography comprises of a liquid solvent containing the sample as a mixture of solutes.

Based on polarity of mobile phase and stationary phases HPLC is subdivided into two different sub-classes i.e. NPLC and RPLC. In NPLC i.e. normal phase liquid chromatography the stationary phase is more polar than the mobile phase e.g. toluene as the mobile phase, silica as the stationary phase. While the opposite RPLC i.e. reversed phase liquid chromatography where the stationary phase is less polar than mobile phase e.g. water-methanol mixture as the mobile phase and C18 = octadecylsilyl as the stationary phase. Types of HPLC on the basis of the nature of stationary phase: (a) Partition or liquid-liquid chromatography (b) adsorption or liquid-solid chromatography (c) ion exchange or ion chromatography (d) size exclusion chromatography and (e) affinity chromatography. HPLC can be used to separate compounds with molecular weights varying from 54 to 450,000. The amounts of materials that can be detected varies from pictograms to nanograms (analytical scale) to micrograms and milligrams (semi-preparative scale) and to multigrams (preparative scale). There are no requirements for volatile compounds or derivatives. Aqueous samples can be run directly after simple filtration. Compounds with wide popularity can be analyzed in a single run. Speed, reproducibility and sensitivity are possible through a HPLC system. Typical runs take from 10 to 30min. but longer gradients might take 1–2 hr. HPLC can be used in a variety of research and production operations. HPLC can run underivatized mixtures hence it is a great tool for analyzing crude mixtures with minimum sample preparation. Standards purification is another role in which HPLC does well. It is quite simple to obtain microgram to milligram quantities of standards using the typical laboratory systems. If rightly used, HPLC is a great tool for rapid reaction monitoring in glassware.

Under HPLC, separation is achieved by injecting the sample dissolved in solvent into a stream of solvent being pumped into a column packed with solid separating material. The basic interaction is a liquid-solid separation. This happens when a mixture of compounds dissolved in a solvent can either stay in the solvent or adhere to the packing material in the column. As compounds can have an affinity for both solvent and the packing, the selection is a tricky one. On a reverse phase column, separation occurs because each compound has a different partition rate between the solvent and the packing material. On its own, each compound would reach its equilibrium concentration in the solvent and on the solid support. However, conditions are upset, by pumping fresh solvent down the column. The net outcome is that the components with highest affinity for column packing stick the longest and wash out in the end. This differential washout or elution of compounds is the basis of HPLC separation. The separated or partial separated discs of each component dissolved in solvent move down the column slowly moving farther apart and elute in turn from the column into the detector flow cell. The separated compounds appear in the detector as peaks that rise and fall when the detector signal is sent to either the recorder or computer. This peak data can either be used to quantitate, with calibration, the amount of each material present or to control the collection of purified material in a fraction collector.

The basic HPLC system comprises of (a) a high pressure (2000–6000 psi) solvent pump, (b) an injector, (c) a column (d) a detector and (e) a data recorder. As HPLC involves working with

liquids instead of gases, chances of explosion due to high pressures are minimal. Leaks may happen due to over pressurizing; the worst problems possible are drips and leakages. Solvent (mobile phase) from a solvent reservoir is pulled up the solvent inlet line into the pump head through a one-check valve. Pressurized in the pump head, the mobile phase is driven by the pump against the column back pressure, through a second check valve into the line leading to the sample injector. The pressurized mobile phase passes through the injector and into the column where it equilibrates with the stationary phase and then exits to the detector flow cell and out to the waste collector. The sample dissolved in mobile phase or a similar solvent is first loaded into the sample loop, and then injected by turning a handle swinging the sample loop into the pressurized mobile phase stream. Fresh solvent pumped through the injector sample loop washes the sample onto the column head and down the column.

The separated band in the effluent from the column passes through the column exit line into the detector flow cell. The detector reads concentration changes as changes of signal voltage. This change of voltage with time passes out to the recorder over the signal cable and is traced on paper as a chromatograph allowing fractions to be detected as rising and following peaks. There are always 2 outputs from a detector, one electrical and one liquid. The electrical signal is sent to the recorder for display and quantization (analytical mode). The liquid flow from the detector flow cell consists concentration bands in the mobile phase. The liquid outputs from non-destructive detectors can be collected and concentrated to recover the separated material (preparative mode). The HPLC is both an analytical and preparative tool. Often the preparative capabilities of the HPLC are overlooked. While normal analytical injections contain pictogram to nanogram quantities, HPLCs have been used to separate as much as 1 lb in a single injection. Typical preparative runs inject 1–3g to purify standard samples.

To be effective the detector must be capable of responding to concentration changes in all of the compounds of interest, with sensitivity sufficient to measure the component present in smallest concentration. There are a variety of HPLC detectors, not all detectors will see every component separated by the column. The most commonly used detector is the UV detector, which seems to have the best combination of compound delectability and sensitivity. Generally the more sensitive the detector the more specific it is and the more compounds it will miss. The detectors can be used in series to gain more information while maintaining sensitivity for detection of minor components.

HPLC is a widely used technique in biomedical research and is of particular significance in respect of new drug discovery and development. This technique is especially useful in the following areas:

(*a*) Estimation of various biological markers from body fluids/organs.

(*b*) Therapeutic drug monitoring-for measuring pharmacokinetic parameters during assessment of drug effects as well as toxicity profiles.

(*c*) Bioequivalence studies-to compare efficacies of standard drug with new drug purely from pharmacokinetic considerations, for quality control purposes.

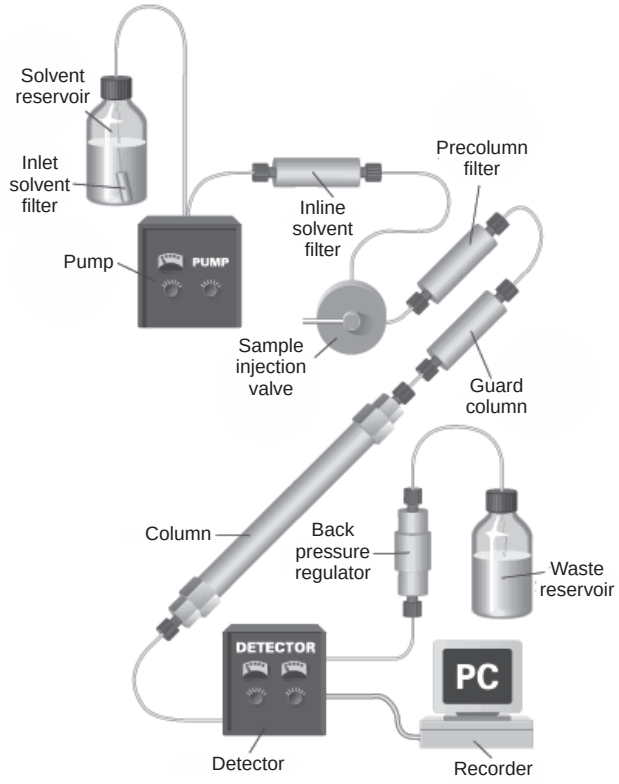


Fig. 4.6. HPLC Apparatus

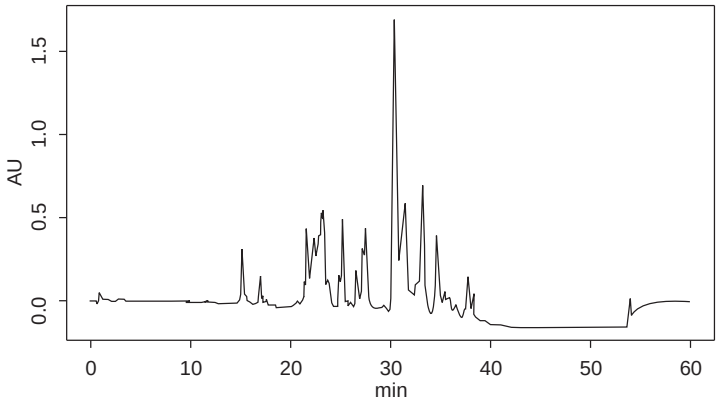


Fig. 4.7. A Chromatogram obtained by HPLC

Isocratic elution (*constant composition*) here the mobile phase composition remains constant throughout the procedure. In isocratic elution, peak width increases with retention time linearly according to the equation for N, the number of theoretical plates. This leads to the disadvantage that late-eluting peaks get very flat and broad. Their shape and width may keep them from being recognized as peaks. A theoretical plate is a hypothetical zone or stage in which two phases, such as the liquid and vapor phases of a substance, establish an equilibrium with each other. The performance of separation processes is enhanced by providing more theoretical plates. The number of actual plates is more than the required theoretical plates.

Gradient elution is separation in which the mobile phase composition is changed during the separation process. For e.g. A gradient starting at 10% methanol and ending at 90% methanol after 20 minutes. The two components of the mobile phase are typically termed 'A' and 'B'; **A** is the 'weak' solvent which allows the solute to elute only slowly, while **B** is the 'strong' solvent which rapidly elutes the solutes from the column. Solvent **A** is often water, while **B** is an organic solvent miscible with water, such as acetonitrile, methanol, or isopropanol.

Gradient elution decreases the retention of the later-eluting components so that they elute faster, giving narrower (and taller) peaks for most components. This also improves the peak shape for tailed peaks, as the increasing concentration of the organic eluent pushes the tailing part of a peak forward. This also increases the peak height (the peak looks 'sharper'), which is important in trace analysis. The gradient program may include sudden 'step' increases in the percentage of the organic component, or different slopes at different times-all according to the desire for optimum separation in minimum time. In isocratic elution, the selectivity does not change if the column dimensions (length and inner diameter) change-that is, the peaks elute in the same order. In gradient elution, the elution order may change as the dimensions or flow rate change. The driving force in reversed phase chromatography originates in the high order of the water structure. The role of the organic component of the mobile phase is to reduce this high order and thus reduce the retarding strength of the aqueous component.

Table 4.2

Arrangement of polar groups according to their binding to adsorbent		**Elutropic series of solvents (Increasing Strength)**
— COOH	Carboxylic	Light and petroleum and Hexanes
— OH	Hydroxyl	Cyclohexane
— NH_2	Amines	Carbon tetrachloride
— CHO	Aldehydes	Trichloroethylene
— C═O	Ketones	Toluene
— COOR	Esters	Benzene
— OCH_3	Ethers	Dichloromethane
— C═C —	Olefins	Chloroform
		Ethyl ether
		Ethyl acetate
		Acetone
		n-Propanol
		Ethanol
		Methanol
		Water (Highly Polar)

GAS CHROMATOGRAPHY (GC)

A chemically inert gas, such as nitrogen, helium, argon, and carbon dioxide is used as mobile phase. The sample is vaporized and then injected into the column which is then transported through the column by the flow of the mobile phase. The *stationary phase* is a microscopic layer of liquid or polymer

on an inert solid support, inside a piece of glass or metal tubing called a column. Gaseous compounds being analyzed interact with the walls of the column, which is coated with different stationary phases. This causes each compound to elute at a different time. GC and column chromatography are similar in principle with some differences. Firstly, the process of separating the compounds in a mixture is carried out between a liquid stationary phase and a gaseous mobile phase, whereas in column chromatography the stationary phase is a solid and the mobile phase is a liquid. Secondly, the column through which the gas phase passes is located in an oven where the temperature of the gas can be controlled, however, column chromatography has no such temperature control. Thirdly, the concentration of a compound in the gas phase is solely a function of the vapor pressure of the gas. Mostly the flame ionization detector (FID) and the thermal conductivity detector (TCD) are used. TCDs can also be used to detect any component other than the carrier gas (as long as their thermal conductivities are different from that of the carrier gas, at detector temperature), FIDs are sensitive primarily to hydrocarbons, and are more sensitive to them than TCD. However, an FID cannot detect water. Since TCD is non-destructive, it can be operated in-series before an FID (destructive), thus providing complementary detection of the same analytes. Gas chromatographs can be connected to a mass spectrometer which acts as the detector, the combination is known as GC-MS. GC-MS can be connected to an NMR spectrometer which acts as a backup detector, thus combination is known as GC-MS-NMR. GC-MS-NMR are connected to an infrared Spectrophotometer which acts as a backup detector, this combination is known as GC-MS-NMR-IR.

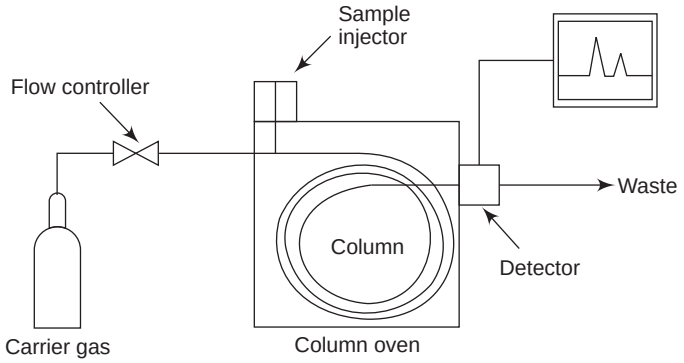


Fig. 4.8. Gas Chromatograph

5

MOLECULAR BIOLOGY

The discovery of DNA structure in 1953 had a major impact on biology and since then the term molecular biology has been used to describe the study of gene structure, gene replication and gene expression, and involves the detailed study of events:

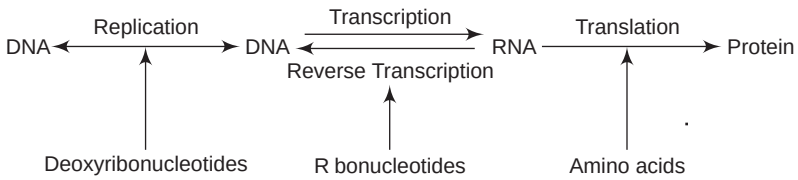


Fig. 5.1 Central Dogma

EXTRACTION OF DNA FROM BIOLOGICAL SAMPLES

DNA isolation from biological tissue samples is the initial step for conducting pharmacogenetics studies. Since cells contain several components, methods adopted for DNA extraction should be able to separate the other components from DNA and at the same time ensure that the DNA is not denatured.

There are several methods that can be used to separate DNA from biological samples and each method has its own advantage and disadvantage. Therefore, selecting a suitable method is dependent on factors like, the molecular weight of the target DNA, quantity and quality of DNA, time required, cost and whether the method requires the use of any hazardous chemicals etc. An important prerequisite before collecting the sample of blood/tissue for genotyping is that you have to take an informed written consent from the subject stating the purpose for sample collection. Confidentiality regarding sample storage is of utmost importance. For DNA extraction from blood samples following method is used in laboratory.

Biological sources of DNA

(*a*) ***Blood***- (commonest source) may be either fresh or frozen blood, blood stains, clots etc.

(*b*) ***Body fluids***- plasma, serum, amniotic fluid, CSF, semen, synovial fluid

(*c*) ***Tissues***- Fresh, Fixed, frozen, paraffin embedded

(*d*) ***Cultured cells***- Fixed cells, buccal cells, cultured cells

The basic principle involves cell lysis whereby the blood cells, tissue etc are lysed but the target nucleic acid *i.e.*, DNA is preserved. Removal of other cellular macromolecules like proteins, carbohydrate and inactivation of the cellular nucleases—RNAse, DNAse and finally recovery of DNA. The common methods used for isolation of DNA from blood samples are:

1. Phenol Chloroform method
2. Salting out method
3. Silica gel adsorption method used in kits

Phenol chloroform method: is a conventional technique which involves usage of organic solvents. The basic principle involves lysis of cells using a cell lysis buffer and addition of a detergent-Sodium dodecyl sulfate (SDS) to remove the lipids from the cell membrane, Proteinase K is used to digest the proteins and free the DNA from the chromatin structure and organic solvents like phenol and chloroform facilitate denaturation of proteins. Finally the contaminants are separated in the organic phase and DNA is recovered from the aqueous phase by alcohol precipitation. The main disadvantage of this method is that it involves hazardous organic chemicals like phenol and chloroform, it is time consuming and laborious but the advantage include good yield and purity of the isolated DNA.

Salting out method: the protocol is simple and fairly rapid. It does not require the use of organic solvents but rather utilizes salt extraction to precipitate contaminating proteins. High quality DNA is obtained suitable for immediate PCR applications. One can obtain approximately 100–200μg of DNA from 4–8 ml of fresh or frozen blood. It involves initial addition of a RBC lysis buffer and subsequent Proteinase K digestion which is followed by 'salting' out of the digested protein with high concentration of sodium chloride (6M NaCl) or sodium perchlorate (5M), precipitates are removed by centrifugation and DNA is recovered by alcohol precipitation. The main drawback of this method is there is inefficient removal of proteins.

DNA extraction using Qiagen DNA blood mini kit: QIAamp DNA blood mini kits are designed for rapid purification of an average of 6 μg of total DNA (e.g. genomic) from 200 μl of whole human blood, and upto 50 μg of DNA from 200 μl of buffy coat $5X10^6$ lymphocytes, or cultured cells that have a normal set of chromosomes. The procedure is suitable for use with whole blood treated with citrate, heparin, or EDTA; buffy coat; lymphocytes; plasma; serum; and body fluids. Sample may be fresh or frozen.

POLYMERASE CHAIN REACTION (PCR)

Developed in 1983 by Kary Mullis, and was awarded the Nobel Prize in Chemistry for his work on PCR in 1993. PCR is used for 'quick cloning' or making copies of a particular piece of DNA in the test tube (rather than in living cells like E. coli). Reaction mix contains forward and reverse primers of the gene sequence to be amplified, dNTPs, thermostable DNA polymerase called taq polymerase from bacteria *Thermos aquaticus* and the template DNA whose sequence is to be amplified. The three major steps in a PCR, which are repeated for 30 or 40 cycles on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time. Denaturation at 94°C, the double strand melts open to single stranded DNA, all enzymatic reactions stop even the extension from

a previous cycle. Annealing occurs at 54°C, as the primers jiggling around, due to the Brownian motion. Constantly formed and broken ionic bonds between the single stranded DNA template and the single stranded primer. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore. Extension at 72°C :This is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. Non-specific primer binding, get loose again due to higher temperature giving no further extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template).

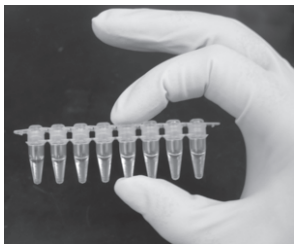


Fig. 5.2. PCR Tubes

Fig. 5.3. A Thermo cycler (PCR machine)

Because both strands are copied during PCR, there is an **exponential** increase of the number of copies of the gene. Suppose there is only one copy of the wanted gene before the cycling starts, after one cycle, there will be 2 copies, after two cycles, there will be 4 copies, three cycles will result in 8 copies and so on. The PCR product is detected using agarose gel electrophoresis and ethidium bromide (ETBR). Further Southern blot is used to study DNA expression. The basic technique of PCR has been modified in several ways which has broadened its applications.

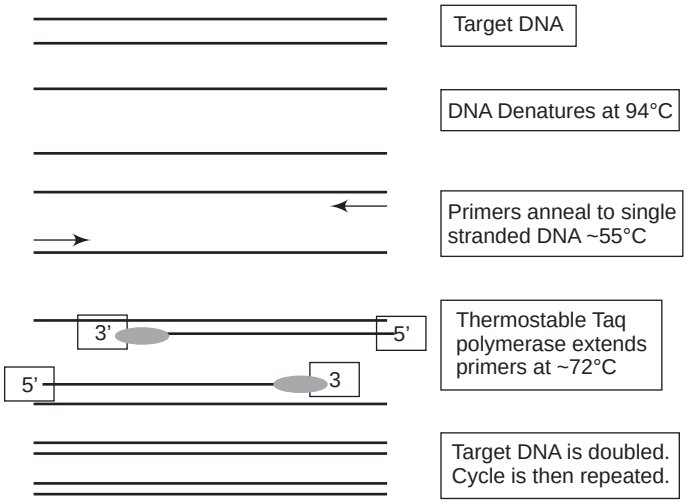


Fig. 5.4. Cycles of PCR

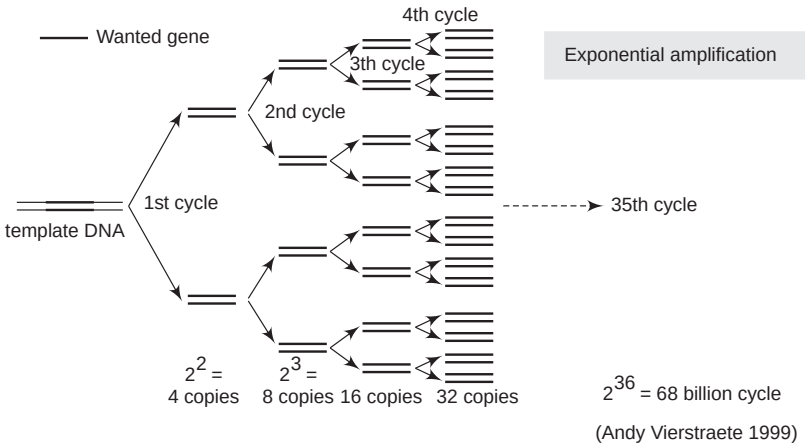


Fig. 5.5. Various cycles of PCR showing exponential amplification of the target gene

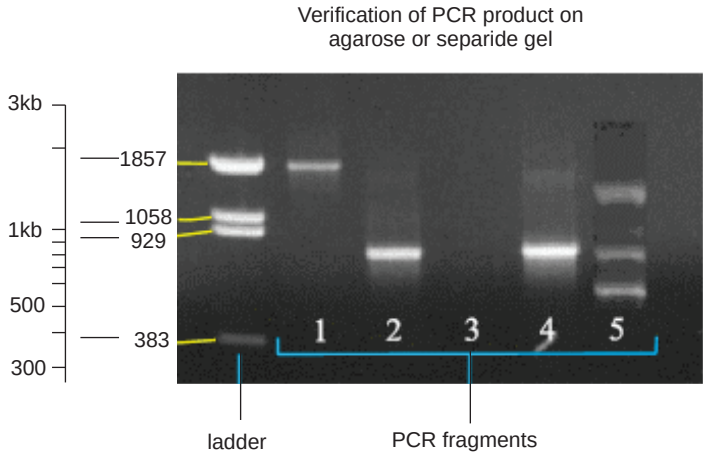


Fig. 5.6. Lane 1: PCR fragment is approximately 1850 bases long. Lane 2 and 4: the fragments are approximately 800 bases long. Lane 3: no product is formed, so the PCR failed. Lane 5: multiple bands are formed because one of the primers fits on different places.

Conventional PCR is useful where genetic material is DNA but genomes of viruses are composed of RNA like retroviruses HIV, influenza virus there reverse transcriptase PCR (RT-PCR) is used.

REVERSE TRANSCRIPTASE (RT)-PCR

In RT-PCR, the RNA strand is first reverse transcribed into its DNA complement (*complementary DNA*, or *cDNA*) using the enzyme reverse transcriptase, and the resulting cDNA is amplified using traditional or real-time PCR. It includes three major steps. The first step is reverse transcription (RT), in which RNA is reverse transcribed to cDNA using reverse transcriptase. This step is very important in order to perform PCR since DNA polymerase can act only on DNA templates. The RT step can be performed either in the same tube with PCR (one-step PCR) or in a separate one (two-step PCR) using a temperature between 40°C and 50°C, depending on the properties of the reverse transcriptase used. Next step is denaturation of the dsDNA at 95°C, so that the two strands separate and the primers can bind again at lower temperatures and begin a new chain reaction. The final step of PCR amplification is DNA extension from the primers, done with thermostable Taq DNA polymerase, at 72°C. RT-PCR is widely

used in the diagnosis of genetic diseases and, semi quantitatively, in the determination of the abundance of specific different RNA molecules within a cell or tissue as a measure of gene expression. RT-PCR can also be very useful in the insertion of eukaryotic genes into prokaryotes. Due to the fact that most eukaryotic genes contain introns which are present in the genome but not in the mature mRNA, the cDNA generated from a RT-PCR reaction is the DNA sequence which would be directly translated into protein after transcription. When these genes are expressed in prokaryotic cells for the sake of protein production or purification, the RNA produced directly from transcription need not undergo splicing as the transcript contains only exons (prokaryotes, such as E.coli, lack the mRNA splicing mechanism of eukaryotes). Reverse transcriptase PCR is not to be confused with real-time polymerase chain reaction (Q-PCR/qRT-PCR), which is also sometimes abbreviated as RT-PCR. Northern blot analysis is used to study the RNA's gene expression further. Conventional RT-PCR is a time-consuming technique with important limitations when compared to real-time PCR techniques. Moreover, ethidium bromide may yield results that are not always reliable due to its low sensitivity. Moreover, there is an increased cross-contamination risk of the samples since detection of the PCR product requires the post-amplification processing of the samples. Furthermore, the specificity of the assay is mainly determined by the primers, which can give false-positive results. Conventional RT-PCR is a semi quantitative technique, where the amplicon can be visualized only after the amplification ends. Moreover, the real-time PCR thermal cycler has a fluorescence detection threshold, below which it cannot discriminate the difference between an amplification generated signal and background noise. On the other hand, the fluorescence increases as the amplification progresses and the instrument performs data acquisition during the annealing step of each cycle. The number of amplicons will reach the detection baseline after a specific cycle, which depends on the initial concentration of the target DNA sequence. The cycle at which the instrument can discriminate the amplification generated fluorescence from the background noise is called the threshold cycle (Ct). The higher the initial DNA concentration, the lower its Ct will be. Three kinds of fluorescent reporters used in real time RT-PCR, which are general non-specific DNA Binding Dyes such as SYBR Green I, TaqMan Probes and Molecular Beacons.

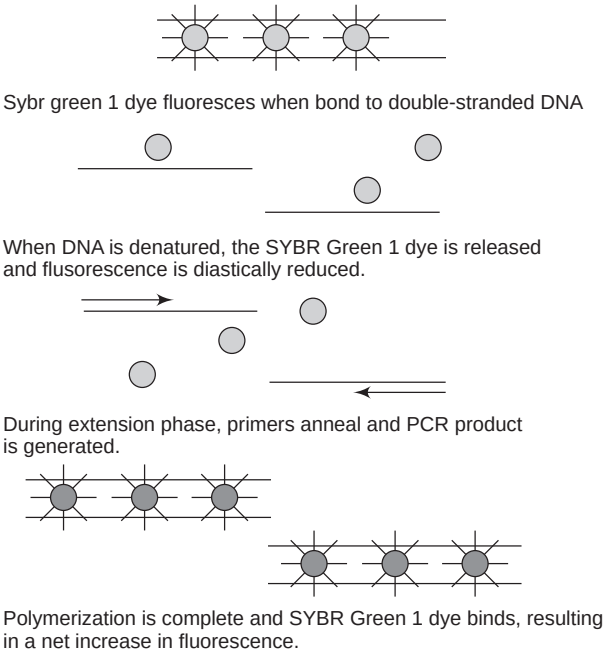


Fig. 5.7. SYBR Green PCR amplification

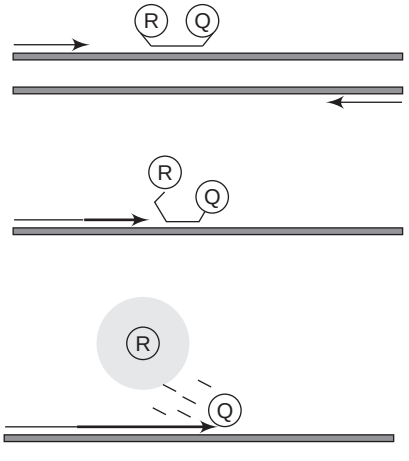
SYBR Green is the most economical choice for real-time PCR product detection. Since the dye binds to double-stranded DNA, there is no need to design a probe for any particular target being analyzed. However, detection by SYBR Green requires extensive optimization. Since the dye cannot distinguish between specific and non-specific product accumulated during PCR, follow up assays are needed to validate results. Advantages of using SYBR Green; Stronger signal, Higher selectivity for dsDNA, Higher stability, Lesser inhibitory for Taq, and cost effective. Disadvantages of using SYBR Green; Binds to Non specific PCR product, Primer dimer formation.

TaqMan probes are oligonucleotides that have a fluorescent reporter dye attached to the 5' end and a quencher moeity coupled to the 3' end. These probes are designed to hybridize to an internal region of a PCR product. In the unhybridized state, the proximity of the fluor and the quench molecules prevents the detection of fluorescent signal from the probe. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5'- nuclease activity of the polymerase cleaves the probe. This decouples the fluorescent and quenching dyes and FRET no longer occurs. Thus, fluorescence increases in each cycle, proportional to the amount of probe cleavage. Its advantages include: Highly fluorogenic, Sequence-specific detection. Disadvantages: Expensive, Probe design and positioning is challenging.

Hybridises with the target amplicon
Is 3' terminally blocked (cannot be extended by the polymerase)
Has two fluorescent dyes attatched:

1. Reporter (R)
2. Quencher (Q)

Fig. 5.8. Hydrolyses Taqman Probe



- When intact, the fluorescence of the reporter is quenched due to its proximity to the quencher
- Probe hybridizes to the target
- dsDNA-specific 5'–3' exonuclease activity of Taq cleaves off the reporter
- Reporter is separated from the quencher.
- Fluorescent signal
- Signal is proportional to the amount of amplified product in the sample

Fig. 5.9. Taqman probe

Like TaqMan probes, Molecular Beacons also use FRET to detect and quantitate the synthesized PCR product via a fluor coupled to the 5' end and a quench attached to the 3' end of an oligonucleotide substrate. Unlike TaqMan probes, Molecular Beacons are designed to remain intact during

the amplification reaction, and must rebind to target in every cycle for signal measurement. Molecular Beacons form a stem-loop structure when free in solution. Thus, the close proximity of the fluor and quench molecules prevents the probe from fluorescing. When a Molecular Beacon hybridizes to a target, the fluorescent dye and quencher are separated, FRET does not occur, and the fluorescent dye emits light upon irradiation. They can be used for multiplex assays by using spectrally separated fluor/quench moieties on each probe. As with TaqMan probes, Molecular Beacons can be expensive to synthesize, with a separate probe required for each target.

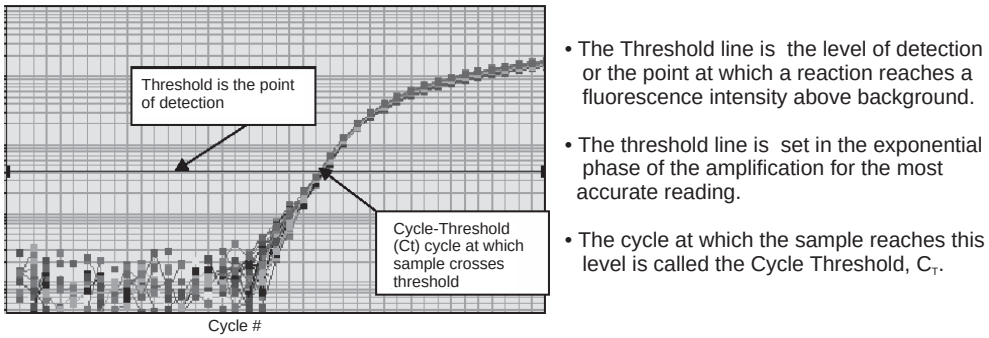


Fig. 5.10. Threshold Cycle (Ct)

The basic technique of PCR has been modified in several ways which has broadened its applications. **Allele specific PCR** is employed to detect point mutations responsible for genetic disorders. Primers are designed to anneal at the site which is probable to undergo mutation. Therefore, primers will not anneal to mutated site and DNA amplification will not take place in case of mutated genes. **Inverse PCR** is used to amplify the unsequenced DNA template that lie adjacent to a core DNA sequence for which the primer sequence is already known. **Pan handle PCR** allows the amplification of DNA when the sequence for primer design is known only on one end of target DNA sequence. **Race PCR** allows to obtain full length cDNA while starting from limited nucleotide sequence. **PCR mutagenesis** introduces point mutations in the target DNA. The primers used are not exactly complementary to their target sequence but still undergo annealing under appropriate reaction conditions**.**

APPLICATIONS OF PCR

1. **Quantification of gene expression:** PCR can be used to detect very small alterations in cellular mRNA encoding for such proteins as in RT-PCR. Where, cellular RNA is initially reverse transcribed to cDNA in a reaction mixture containing oligodT primers, a viral reverse transcriptase, all four dNTPs and $MgCl_2$ containing buffer. The cDNA thus generated, can be amplified by PCR using primers specific to a particular mRNA. The final amount of PCR product generated from mRNA, depends on the starting amounts of mRNA and efficiency of the reverse transcription and PCR reactions. In order to rule out the variability due to amount of mRNA and efficiency of PCR, two basic methods have been employed to quantitative mRNA using RT-PCR.

   Noncompetitive RT-PCR: In this method, a known quantity of standard DNA template is added exogenously. Standard DNA and target cDNA are co-amplified simultaneously using RT-PCR. From the ratio of amplified DNA, absolute value of target cDNA can be calculated.

Alternatively, an endogenous gene transcript with high tissue expression can act as an internal standard. Such gene transcripts are termed as 'House keeping genes' example, β-actin, HPRT and GAPDH. Housekeeping genes are used as internal standards. However, the quantification relies on the empirical assumption that efficiency of PCR is same for target cDNA and standard DNA or endogenous gene. Therefore, this method of mRNA quantification is less accurate.

Competitive RT-PCR: In contrast to non-competitive RT-PCR, this method employs an external DNA standard that closely mimics target cDNA with respect to primer binding and other PCR variables. Therefore, standard and target cDNA get amplified with the same efficiency. However, the method doesn't take into account the efficiency of reverse transcription reaction. To overcome this limitation, samples are spiked with a known quantity of mRNA transcripts which are reverse transcribed and amplified in a manner analogous to target mRNA. This method yields better results.

2. **Cellular localisation of gene expression-** Although RT-PCR is a highly sensitive technique to detect small alteration in gene expression, it does not permit the cellular localization of such gene alteration. Homogenized tissue samples used for RT-PCR mostly consist of heterogeneous group of cells. Therefore, in a given pathological condition it is difficult to ascribe the mRNA expression alteration to a particular cell type. *In situ* PCR overcomes this short coming of RT-PCR. Here, a tissue section is fixed on a glass slide in formalin. Cellular proteins are digested on the slide with the help of pepsin, trypsin or proteinase K. DNA is digested with help of DNAase. The total mRNA is reversed transcribed to cDNA as described in RT-PCR by putting all the reagents on slide. The cDNA thus generated is amplified using two specific set of primers, Taq polymerase and streptavidin containing dNTPs. Finally, this slide is stained with nuclear fast red stain and viewed under microscope.
3. **Identification of gene families-** PCR has been used to amplify a family of related genes using Degenerate Oligonucleotide Primers (DOP). DOP are mixture of primers designed around a conserved domain of a gene family but with alternative nucleotides at certain positions. Therefore, these primers will bind to closely related DNA sequences in the genome. This strategy is known as homology screening or DOP-PCR and has successfully expanded several gene families such as cyclins and cyclin-dependent kinases. PCR has also revolutionized the detection of genetic disorders such as haemoglobinopathies, X-linked disorders, cystic fibrosis, cancers such as acute myeloid leukemia, chronic myeloid leukemia and infectious disorders such as tuberculosis, HIV and hepatitis B. However, this use of PCR is more of a diagnostic rather than pharmacological interest.

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

RFLP is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases. RFLP as a molecular marker is specific to a single clone/restriction enzyme combination. Restriction enzymes are 'DNA cutting' enzymes found in bacteria (and harvested from them for use). Because they cut within the molecule, they are often called restriction endonucleases. A restriction enzyme recognizes and cuts DNA only at a particular sequence of nucleotides. For example, the bacterium *Hemophilus aegypticus* produces an enzyme named Hae III that cuts DNA wherever it encounters the sequence.

5'GGCC3'

3'CCGG5'

The cut is made between the adjacent G and C. This particular sequence occurs at 11 places in the circular DNA molecule of the virus phiX174. Thus treatment of this DNA with the enzyme produces 11 fragments, each with a precise length and nucleotide sequence. These fragments can be separated from one another and the sequence of each determined. A restriction endonuclease functions by 'scanning' the length of a DNA molecule. Once it encounters its particular specific recognition sequence, it will bond to the DNA molecule and makes one cut in each of the two sugar-phosphate backbones of the double helix. The positions of these two cuts, both in relation to each other, and to the recognition sequence itself, are determined by the identity of the restriction endonuclease used to cleave the molecule in the first place. Different endonucleases yield different sets of cuts, but one endonuclease will always cut a particular base sequence the same way, no matter what DNA molecule it is acting on. Once the cuts have been made, the DNA molecule will break into fragments.

ELECTROPHORESIS

Biological molecules carry an electrical charge and these charged molecules migrate in solution when an electric field is applied. Earlier, electrophoresis was carried out in free solution but it resulted in mixing of components by convection currents. This method is no longer used. Today electrophoresis is carried out on a supporting medium like, paper, cellulose acetate, Agar gel, starch grains, polyacrylamide gel.

Isoelectric point (pI) is the pH at which a particular molecule or surface carries no net electrical charge or the negative and positive charges are equal. Biological amphoteric molecules such as proteins contain both acidic and basic functional groups. Amino acids which make up proteins may be positive, negative, neutral or polar in nature, and together give a protein its overall charge. At a pH below their pI, proteins carry a net positive charge; above their pI they carry a net negative charge. Proteins can thus be separated according to their isoelectric point on a polyacrylamide gel using a technique called isoelectric focusing, which uses a pH gradient to separate proteins. Isoelectric focusing is also the first step in 2-D gel polyacrylamide gel electrophoresis.

Gel electrophoresis is a technique used for the separation of deoxyribonucleic acid (DNA ribonucleic acid (RNA), or protein molecules using an electric field applied to a gel matrix. DNA Gel electrophoresis is usually performed for analytical purposes, often after amplification of DNA via PCR, but may be used as a preparative technique prior to use of other methods such as mass spectrometry, RFLP, PCR, cloning, DNA sequencing, or Southern blotting for further characterization. Some buffer react with compounds under investigation so, the buffer solution must be carefully selected. For e.g. Citrate and EDTA chelate metal ions like calcium while, borate forms complexes with sugars. Maximum separation is obtained at isoelectric point of the compounds. At low ionic strength, there is rapid migration and low heat production but highly diffused bands obtained. At high ionic strength, there is slow migration and high heat production but sharp bands are produced. If the pH of the buffer is higher than the isoelectric point of the protein, it will migrate towards the positive terminal. While if the pH of the buffer is lower than the isoelectric point of the protein, then it will migrate towards negative terminal. When the buffer pH is equal to the pI of a

protein it will not migrate at all. A protein can exhibit different charges depending on the pH of the medium. At their electrostatic point, proteins exhibit at least electrostatic repulsion, hence they have the lowest solubility at this point and can easily precipitate. This property is useful in crystallization of proteins. A stable electric field providing constant current or voltage is applied. A field strength from 5 to 8 V/cm is mostly used. A Field strength greater than 10V/cm results in heating causing an excessive loss of water by evaporation.

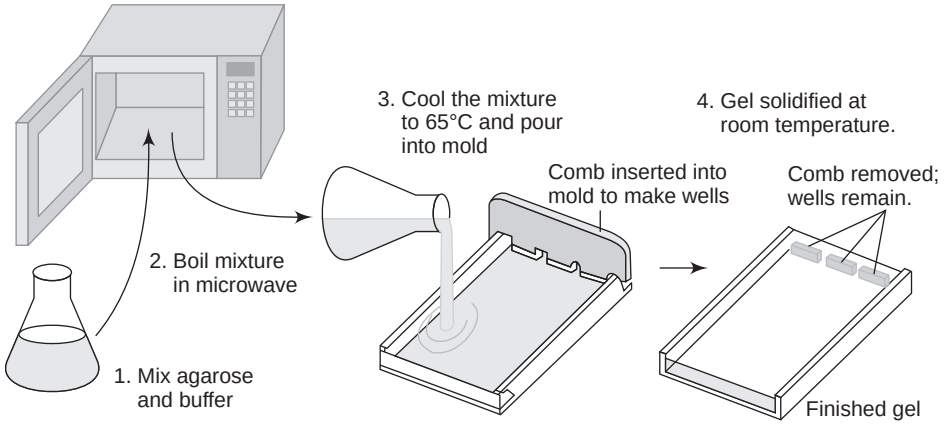


Fig. 5.11. Procedure of preparing the agarose gel

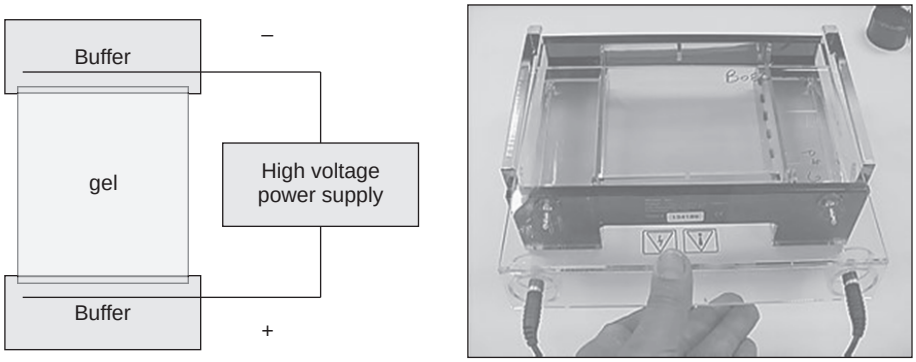


Fig. 5.12. placing of gel and providing high voltage power supply

SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

To separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight). SDS gel electrophoresis of samples have identical charge per unit mass due to binding of SDS results in fractionation by size. Samples may be taken from whole tissue or from cell culture. Bacteria, virus or environmental samples can be the source of protein. In most cases, solid tissues are first broken down mechanically using a homogenizer, sonicator. The gel is prepared by polymerizing acrylamide (CH_2=CHCONH$_2$) and a small quantity of cross-linking reagent, N,N'-methylenebisacrylamide (CH_2=CHCONH$_2$).CH_2 (bis), in the presence of Ammonium persulphate (APS) which acts as a catalyst. Tetramethylenediamine (TEMED) is added to initiate

and control polymerization. APS is highly unstable and undergoes homolytic cleavage in presence of water. These free radicals are transferred to TEMED which activates acrylamide monomers inducing them to react with other acrylamide monomers forming long chains. SDS linearizes and gives net negative charge to the protein. β-mercaptoethanol is also added to denature sulphide bonds. SDS-PAGE is used to separate most proteins and small oligonucleotides because of the presence of small pores. Gel pore size can be altered by varying the concentration of monomer in gel solution. Polymerized gel consists of two types of gel *i.e.*, Running Gel and Stacking Gel is added to gel and then the wells are created. The percentage chosen depends on the size of the protein that one wishes to identify or probe in the sample. The smaller it is the bigger the percentage Gel is poured in the gel caster plates sealed at bottom. Water is poured above to ensure a flat surface and to exclude oxygen which inhibits polymerization. Proteins separated on 7.5% acrylamide, large molecules like RNA require 2.5% acrylamide as they need more open gel to migrate. Acrylamide is toxic and should be handled carefully.

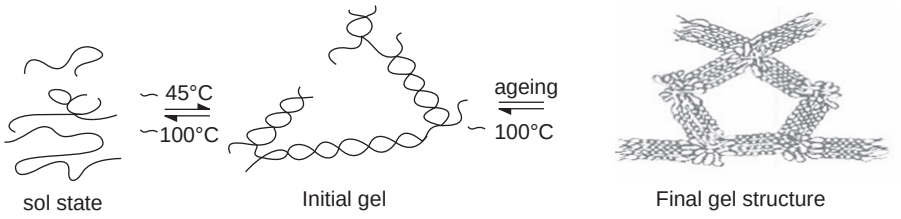


Fig. 5.13. Solidification of agarose gel

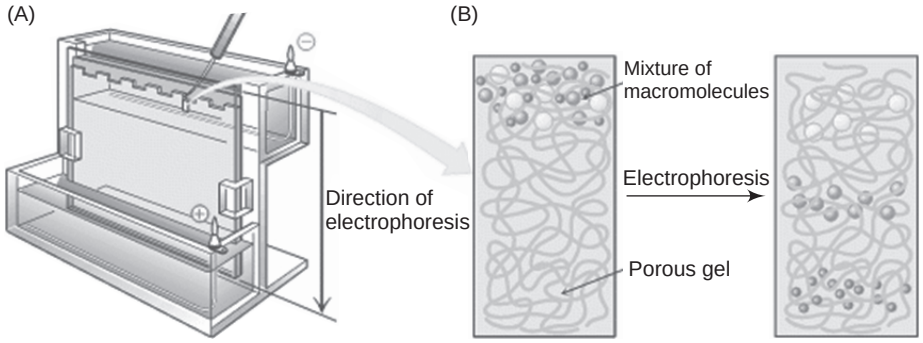


Fig. 5.14. Electrophoresis and separation of mixture of macromolecules

Gel electrophoresis is used in biochemistry, forensics, genetics and molecular biology to separate proteins, as a preparative technique prior to use of other methods such as: Restriction fragment length polymorphism (RFLP), PCR, cloning and Southern blotting for further characterization.

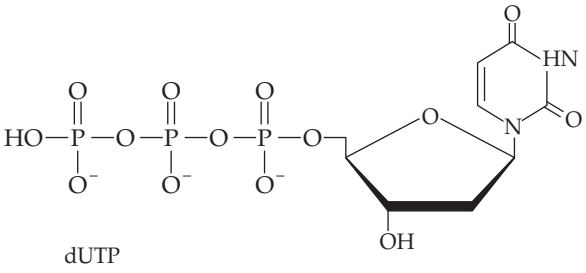
ISOTACHOPHORESIS

In conventional electrophoresis almost all the current is carried by the electrolytic buffer. The sample constituents migrate under influence of a homogeneous electrical field. The buffer determines the pH of the medium as well as the dissociation degree of the sample elements according to their pK values. The sample constituents migrate at different speeds and become diluted by diffusion. Preparation of the sample is often necessary to concentrate the sample elements before application.

However, in isotachophoresis, the sample is introduced between a fast leading electrolyte and a slow terminating electrolyte. After application of an electric potential a low electrical field is created in the leading electrolyte and a high electrical field in the terminating electrolyte. The pH at sample level is determined by the counter-ion of the leading electrolyte that migrates in the opposite direction. In the first stage the sample constituents migrate at different speeds and start to separate from each other. The faster constituents will create a lower electrical field in the leading part of the sample zone and vice versa. Finally the constituents will completely separate from each other and concentrate at an equilibrium concentration, surrounded by sharp electrical field differences. Specific spacer or marker molecules are added to the sample to separate physically the sample constituents one is interested in.

TUNEL ASSAY

Developed by Garvrieli, Sherman, and Ben-Sasson in 1992 for detecting DNA fragmentation that results from apoptotic programmed cell death. The assay relies on the presence of nicks in the DNA which can be identified by terminal deoxynucleotidyl transferase or TdT, an enzyme that will catalyze the addition of marker labeled dUTPs. The main drawback is that it may also label necrotic cells to be apoptotic cells that have suffered severe DNA damage but the method has improved and if performed correctly should only identify cells in the last phase of apoptosis. At late stages of apoptosis, adherent cells are known to detach or 'pop' off. For a reliable and reproducible TUNEL imaging assay, the modified nucleotide must not only be an acceptable substrate for TdT, but the detection method must also be sensitive without bringing about any additional loss of cells from the sample. Nowadays the dUTPs modified by fluorophores or haptens, including biotin or bromine, which can be detected directly in the case of a fluorescently-modified nucleotide (i.e., fluorescein-dUTP), or indirectly with streptavidin or antibodies, if biotin-dUTP or BrdUTP are used, respectively.



dUTP

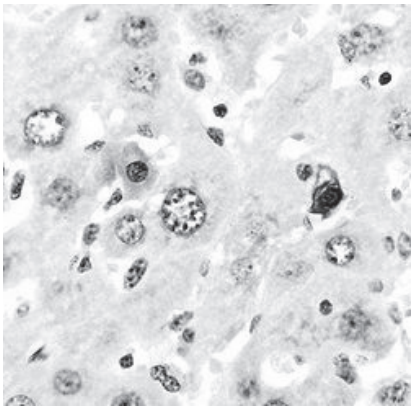


Fig. 5.15. Mouse liver showing an apoptic cell stained with TUNEL

FISH (FLUORESCENCE IN SITU HYBRIDIZATION)

A general method to assign chromosomal location, gene copy number (both increased and decreased), or chromosomal rearrangements. Biotin-containing nucleotides are incorporated into specific cDNA probes by nick-translation. Alternatively, digoxigenin or fluorescent dyes can be incorporated by enzymatic or chemical methods. The probes are then hybridized with solubilized, fixed metaphase cells, and the copy number of specific chromosomes or genes are determined by counter-staining with fluorescein isothiocyanate (FITC)-labeled avidin or other detector reagents. The number and location of detected fluorescent spots correlates with gene copy number and chromosomal location. The method also allows chromosomal analysis in interphase cells, allowing extension to conditions of low cell proliferation.

6

BLOTTING TECHNIQUES

BLOTTING: By using blotting techniques one can identify infectious agents present in the sample, also identify inherited disease.

SOUTHERN BLOTTING

It is an analytical technique and the first blotting technique was developed by biologist Edwin Southern. It involves transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization. First DNA is cut using Restriction Endonucleases into smaller fragments, which are then electrophoresed on agarose gel to separate according to size. Nylon or nitrocellulose membrane is placed on top of the gel and pressure is applied by placing wet stack of paper towels. Gel must not be allowed to dry. DNA is transferred to the membrane from the gel due to capillary action. The membrane is then baked or exposed to UV to permanently attach the transferred DNA to the membrane. The membrane is then exposed to a labeled (radioactive or enzyme) hybridization probe (a single stranded DNA fragment with a specific sequence whose presence in the target DNA is to be determined). After hybridization, excess probe is washed from the membrane and the pattern is visualized in case of a radioactive probe or by color development. Southern blotting technique is widely used to find specific nucleic acid sequence. Other blotting methods *i.e.*, Western (proteins), Northern blot (RNA), Eastern blot (carbohydrate epitopes), Southwestern blot (DNA-binding proteins) that employ similar principles, but using RNA or protein, have later been named in reference to Edwin Southern's name.

DNA Microarray/DNA chip/Biochip: Based on Southern blotting, where fragmented DNA is attached to a substrate and then probed with a known DNA sequence. A complete eukaryotic genome (*Saccharomyces cerevisiae*) on a microarray was deduced in 1997. Microarrays involves hybridization between two DNA strands, as the complementary nucleic acid sequences specifically pair with each other. More complementarity between base pairs in a nucleotide sequence means more tight bonding between the two strands. Subsequent washing leaves only strongly paired strands. Fluorescently labeled target sequences that bind to a probe sequence generates signal

depending on the temperature and washing after hybridization. Total strength of the signal, from a spot also called feature, depends upon the amount of target sample binding to the probes present on that spot. Each DNA spot contains picomoles (10^{-12} moles) of a specific DNA sequence, known as *probes* (or *reporters or oligos*). These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA (also called anti-sense RNA) sample (called *target*) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target. Based on relative quantization in which the intensity of a feature is compared to the intensity of the same feature under a different condition, and the identity of the feature is known by its position.

Types of microarrays: Solid phase array- probes attached to a solid surface like glass, plastic or silicon biochip.

Bead array- Microscopic polystyrene beads, each with a specific probe and a ratio of two or more dyes which do not interfere with the fluorescent dyes used on the target sequence.

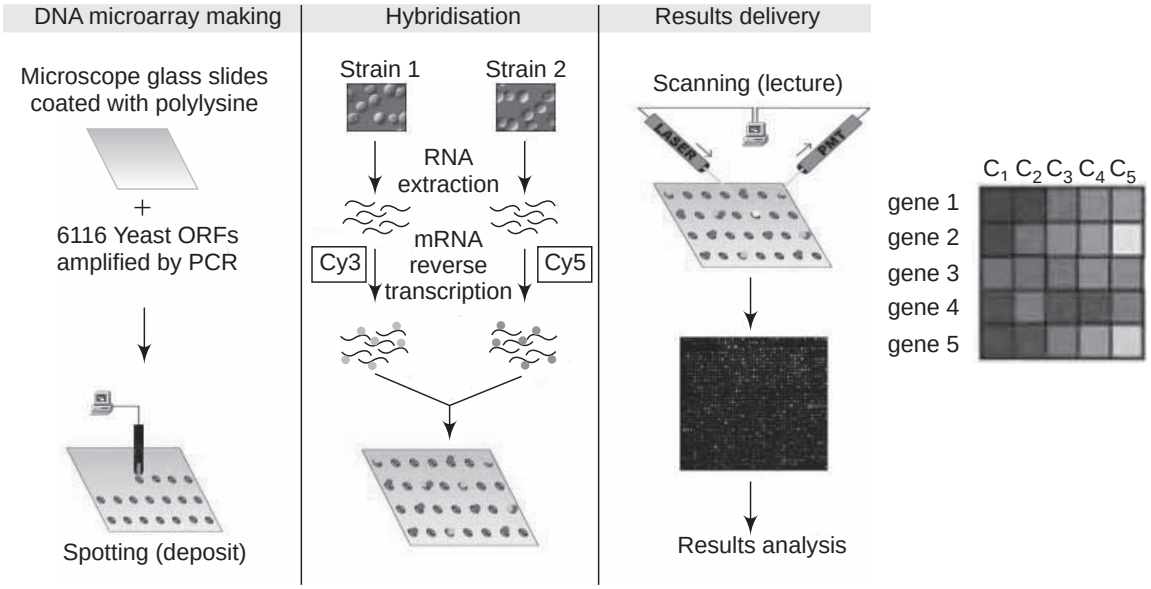


Fig. 6.1. Microarray analysis

The development of several gene expression profiling methods, such as comparative genomic hybridization (CGH), differential display, serial analysis of gene expression (SAGE), and gene microarray, together with the sequencing of the human genome, has provided an opportunity to monitor and investigate the complex cascade of molecular events leading to tumor development and progression. The availability of such large amounts of information has shifted the attention of scientists towards a nonreductionist approach to biological phenomena. High throughput technologies can be used to follow changing patterns of gene expression over time. Among them, gene microarray has become prominent because it is easier to use, does not require large-scale DNA sequencing, and allows for the parallel quantification of thousands of genes from multiple samples. Gene microarray technology is rapidly spreading worldwide and has the potential to drastically change the therapeutic approach to patients affected with tumor. Therefore, it is of paramount

importance for both researchers and clinicians to know the principles underlying the analysis of the huge amount of data generated with microarray technology.

Applications

1. **Gene expression profiling:** Microarray-based gene expression profiling can be used to identify genes whose expression is changed in response to pathogens or other organisms by comparing gene expression in infected to that in uninfected cells or tissues. Also to study the effects of certain treatments, diseases, and developmental stages on gene expression.
2. **Chromatin immunoprecipitation on Chip:** DNA sequences bound to a particular protein can be isolated by immunoprecipitating that protein (ChIP), these fragments can be then hybridized to a microarray (Tilling array) allowing the determination of protein binding site occupancy in the genome.
3. **DamID:** Differs from ChIP, as DamID instead of antibodies makes use of adenine methylation near the protein's binding sites to selectively amplify those regions, introduced by expressing minute amounts of protein of interest fused to bacterial DNA adenine methyltransferase.
4. **Gene ID:** Small microarrays to check IDs of organisms in food and feed (like GMO [1]), mycoplasms in cell culture, or pathogens for disease detection.
5. **SNP detection:** Identifying single nucleotide polymorphism (SNP) among alleles of populations.
6. **Alternative splicing detection:** An '*exon junction array* design uses probes designed to detect each individual exon for known or predicted genes, and can be used for detecting different splicing isoforms for a typical gene expression array (with 1–3 probes per gene) or a genomic tiling array (with hundreds or thousands of probes per gene).
7. **Fusion genes microarray:** To detect fusion transcripts based on the alternative splicing microarrays causing combined measurements of chimeric transcript junctions with exon-wise measurements of individual fusion partners, *e.g.* from cancer specimens.
8. **Other applications:** Include genotyping, forensic analysis, measuring predisposition to disease, identifying drug-candidates, evaluating germline mutations in individuals or somatic mutations in cancers, assessing loss of heterozygosity, or genetic linkage analysis.

WESTERN BLOTTING

Developed by George Stark and uses antibodies to locate Proteins. Proteins are separated by electrophoresis and blotted onto a membrane. The membrane is then exposed to an antibody to the desired protein. The bands are detected by the binding of a second antibody that is radioactively labeled and specific for the first antibody.

Southwestern blotting: Developed by B. Bowen and colleagues in 1980 along the lines of Southern blotting, is a lab technique which involves identifying and characterizing DNA-binding proteins by their ability to bind to specific oligonucleotide probes (since DNA detection is by Southern blotting and protein detection is by western blotting hence name Southwestern blotting). The proteins are separated by gel electrophoresis and are subsequently transferred to nitrocellulose membranes similar to other types of blotting for rapid characterization of both DNA-binding proteins and their specific sites on genomic DNA. Proteins are separated on a polyacrylamide gel (PAGE) containing sodium dodecyl sulfate (SDS), renatured by removing SDS in the presence of urea, and

blotted onto nitrocellulose by diffusion. The genomic DNA region of interest is digested by restriction enzymes selected to produce fragments of appropriate but different sizes, which are subsequently end-labeled and allowed to bind to the separated proteins. The specifically bound DNA is eluted from each individual protein-DNA complex and analyzed by polyacrylamide gel electrophoresis.

NORTHERN BLOTTING

The northern blot technique was developed in 1977 by James Alwine, David Kemp, and George Stark at Stanford University. A problem in northern blotting is often sample degradation by RNases (both endogenous to the sample and through environmental contamination), which can be avoided by proper sterilization of glassware and the use of RNase inhibitors such as DEPC (diethylpyro carbonate). The other chemicals used in northern blots are formaldehyde, radioactive material, ethidium bromide, DEPC, and UV light are all harmful under certain exposures. Compared to RT-PCR, northern blotting has a low sensitivity, but it also has a high specificity which is important to reduce false positive results. Northern blotting technique is widely used to find gene expression.

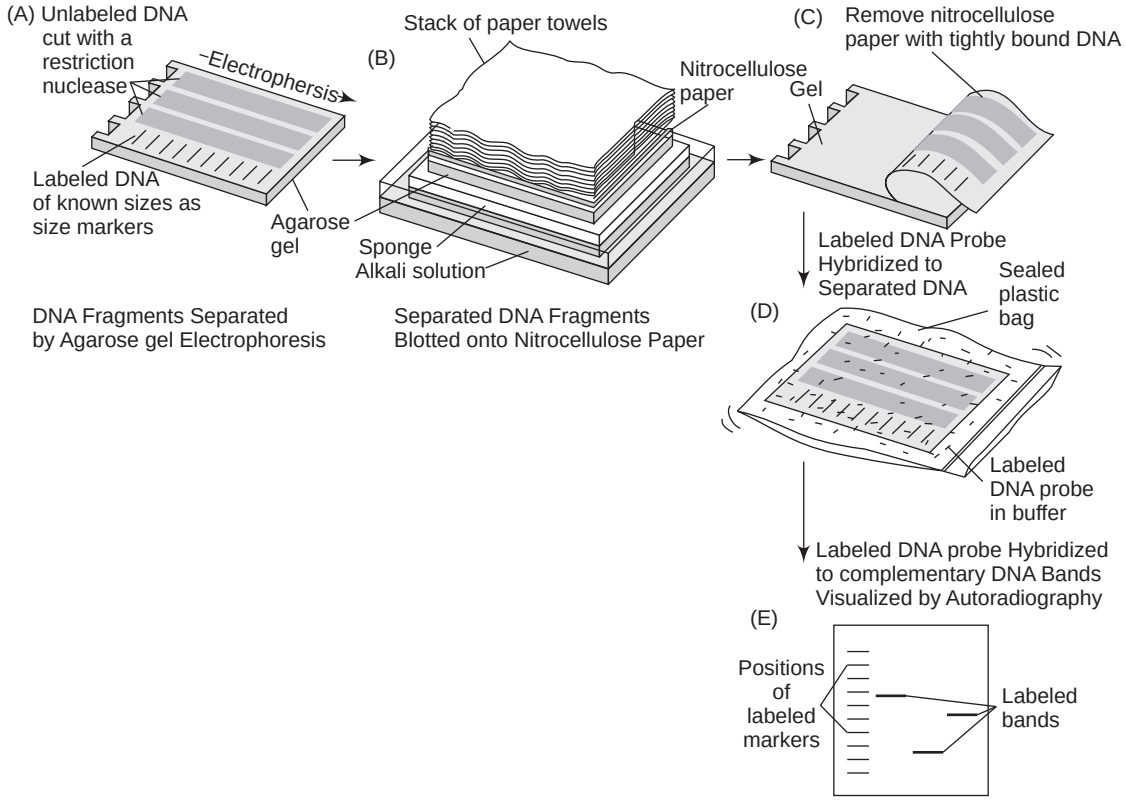


Fig. 6.2. Southern Blotting of DNA fragments separated by Agarose gel electrophoresis

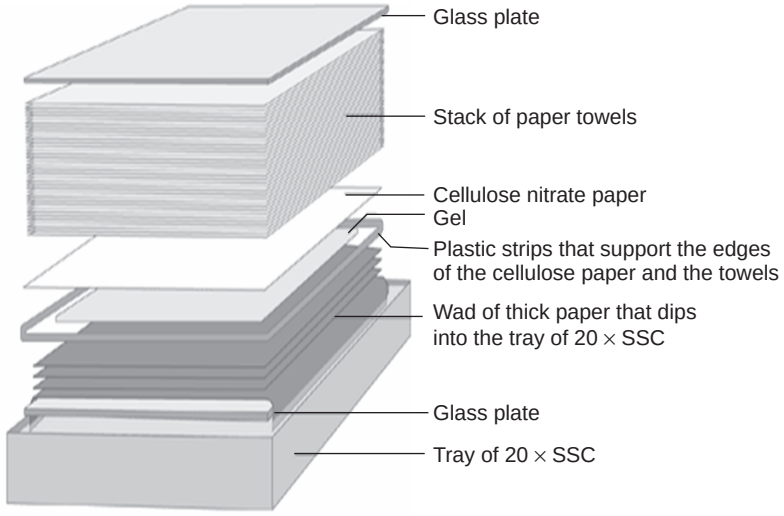


Fig. 6.3. Basic Blotting procedure

EASTERN BLOTTING

There is no technique named 'the Eastern blot', however, it is a term given to an extension of Western blotting used to analyze protein post-translational modifications (PTM). Eastern blotting is similar to lectin blotting (i.e. detection of carbohydrate epitopes on proteins or lipids); however, the term 'lectin blotting' is more prevalent in the literature. Tanner and Anstee in 1976 used lectins to detect glycosylated proteins isolated from human erythrocytes. Here transferred proteins are analyzed for post-translational modifications using probes that may detect lipids, glycoconjugates, carbohydrate epitopes, phosphorylation or any other protein modification. Before becoming functional in cells, many proteins translated from mRNA undergo modifications. These modifications are collectively known as post-translational modifications (PTMs), occurring at the N-terminus of the amino acid chain and play an important role in translocation across biological membranes such as lysosomes, chloroplast, mitochondria and plasma membrane. Post Translational Modifications include: acetylation, acylation (myristoylation, palmitoylation), alkylation, arginylation, ADP Ribosylation, biotinylation, formylation, geranylation, glutamylation, glycosylation, glycylation, hydroxylation, isoprenylation, lipoylation, methylation, nitroalkylation, phosphopantetheiny lation, phosphorylation, prenylation, selenation, S-nitrosylation, succinylation, sulfation, transglu -tamination and ubiquitination (sumoylation, neddylation). Expression of post-translated proteins is important in several diseases. Technique application includes detection of protein modifications in two bacterial species *Ehrlichia-E.muris* and IOE. Cholera toxin B subunit (which binds to gangliosides), Concanavalin A (which detects mannose-containing glycans) and nitrophospho molybdate-methyl green (which detects phosphoproteins) were used to detect protein modifications. The technique showed that the antigenic proteins of the non-virulent E.muris is more post-translationally modified than the highly virulent IOE.

7

Molecular Cloning/Recombinant DNA Technology/Gene Manipulation Techniques

CELL: ITS HEREDITARY COMPONENT

Each cell in our body contains cytoplasm and the nucleus, the nucleus contains the hereditary material in the form of chromosomes. The two different nucleic acids present in the cell are ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). A gene is a section of the chromosomal DNA which contains the information to make a specific polypeptide through the production of a specific RNA. DNA is a nucleic acid carrying the genetic instructions used in the development and functioning of all known living organisms and some viruses. The main function of DNA molecules is the long-term storage of information. DNA is often compared to a set of blueprints or a recipe, or a code, since it contains the instructions needed to construct other cellular components, such as proteins and RNA molecules. The DNA segments that carry this genetic information are called genes, but DNA also contains structural elements involved in regulating the use of this genetic information. DNA is a long polymer made from repeating units called nucleotides. The DNA chain is 22 to 26 Angstroms wide (2.2 to 2.6 nm) and one nucleotide unit is 3.3 angstrom (0.33nm) long. Although each individual repeating unit is very small, DNA polymers may carry millions of nucleotides.

The backbone of DNA strand is made from alternating phosphate and sugar residues. The sugar in DNA is 2-deoxyribose, which is a pentose sugar. The sugars are joined together by phosphate groups that form phosphodiester bonds between the third and fifth carbon atoms of adjacent sugar rings. These asymmetric bonds mean a strand of DNA has a direction. In a double helix the direction of the nucleotides in one strand is opposite to that in the other strand; the strands are antiparallel. The asymmetric ends of DNA strands are called the 5‘ and 3‘ ends, with the 5‘ end having a terminal phosphate group and the 3‘end a terminal hydroxyl group. The DNA helix is stabilized by hydrogen bonds between the bases attached to the antiparallel strands. The four bases found in DNA are adenine (A), Cytosine (C), Guanine (G) and Thymine (T). These four bases are attached to the sugar/phosphate to form the complete nucleotide. These bases are of two types; adenine and guanine are fused 5 and 6 membered heterocyclic compounds called purines, while cytosine and thymine are 6 membered rings called Pyrimidines. A fifth pyrimidine base called Uracil (U), usually takes the place of thymine in RNA and differs from thymine by lacking a methyl group on its ring.

Each type of base on one strand forms a bond with just one type of base on the other strand. This is called *complementary base pairing*. Here, purines from hydrogen bonds to pyrimidines, with A bonding only to T, and C bonding only to G. This arrangement of two nucleotides binding together across the double helix is termed as a base pair. As hydrogen bonds are not as strong as a covalent bond, they can be broken and rejoined relatively easily. The two strands of DNA in a double helix can therefore be opened like a zipper, by a mechanical force or high temperature. As a result of this complementarity, all the information in the double-stranded sequence of a DNA helix is duplicated on each strand, which is vital in DNA replication. Indeed, this reversible and specific interaction between complementary base pairs is critical for all the functions of DNA in living organisms.

A DNA sequence is called '*sense*' if its sequence is the same as that of a mRNA copy that is translated into protein. The sequence on the opposite strand is called the '*antisense*' sequence. Both sense and antisense sequences can exist on different parts of the same strand of DNA (i.e. both strands contain both sense and antisense sequences). In both prokaryotes and eukaryotes, antisense RNA sequences are produced, but the functions of these RNAs are not entirely clear. One proposal is that antisense RNAs are involved in regulating gene expression through RNA-RNA base pairing.

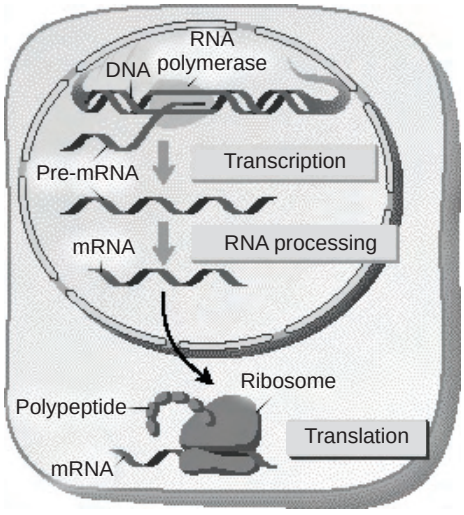


Fig. 7.1. Transcription and translation

GENE EXPRESSION: TRANSCRIPTION AND TRANSLATION

A gene is a sequence of DNA that contains genetic information and can influence the phenotype of an organism. Within a gene, the sequence of bases along a DNA strand defines a messenger RNA (mRNA) sequence, which then translates into one or more protein sequences. The relationship between the nucleotide sequences in the genes and the amino acid sequences of proteins is determined by the rules of translation, known as genetic code (a combination of three 'codons') formed from a sequence of three nucleotides (e.g. ACT, CAG, TTT). During transcription, the codons of a gene are copied into messenger RNA by RNA polymerase. At the beginning of transcription, the double helix is unwound by a helicase and topoisomerase. Then one DNA polymerase produces the *leading strand* while, another DNA polymerase binds to the lagging strand and produces discontinuous segments called *Okazaki fragments*. Later DNA ligase joins okazaki fragments together.

Translation is the second major step in gene expression, here the mRNA is 'read' according to the genetic code, which relates the DNA sequence to the amino acid sequence in proteins. The translation process is almost similar in prokaryotes and eukaryotes, however different elongation, initiation, and termination factors are used. In bacteria, transcription and translation takes place simultaneously, and mRNAs are relatively short-lived. In eukaryotes, mRNAs have variable half-lives, are subject to modifications, and must exit the nucleus to be translated; these multiple steps offer additional opportunities to regulate at different levels of protein synthesis. Each group of three base pairs in mRNA constitutes a codon, and each codon specifies a particular amino acid (hence, it is a triplet code). The mRNA sequence is used as a template to assemble *in order* the different amino acids that form a protein. In prokaryotes, ribosomes can attach to mRNA while it is still being transcribed. So, translation begins at the 5' end of the mRNA while the 3' end is still attached to DNA. However in eukaryotes, mature mRNA molecules leaves the nucleus and travels to the cytoplasm, where the ribosomes are located. A ribosome then decodes the RNA copy and reads the RNA sequence by base pairing the messenger RNA to transfer RNA (tRNA), which carries amino acids. As there are 4 bases in 3-letter combinations, there are 64 possible codons (43 combinations). In all types of cells, the ribosome is composed of two subunits: the large (50S) subunit and the small (30S) subunit (S is svedberg unit, a measure of sedimentation velocity and mass). Each subunit exists separately in the cytoplasm, but the two join together on the mRNA molecule. The ribosomal subunits are made up of proteins and specialized RNA molecules called ribosomal RNA (rRNA). The tRNA molecules act as adaptor molecules and their one end that can read the triplet code in the mRNA through complementary base-pairing, and another end can attach itself to a specific amino acid. Once the initiation complex is formed on the mRNA, the large ribosomal subunit binds to this complex, causing the release of initiation factors. The large subunit of the ribosome has three sites at which tRNA molecules can bind. The **A** (amino acid) site at which the aminoacyl-tRNAanticodon base pairs up with the mRNA codon, ensuring that correct amino acid is added to the growing polypeptide chain. The **P** (polypeptide) site at which the amino acid is transferred from its tRNA to the growing polypeptide chain. Finally, the **E** (exit) site at which the 'empty' tRNA sits before being released back into the cytoplasm to bind another amino acid and repeat the process. The initiator methionine tRNA is the only aminoacyl-tRNA that can bind in the P site of the ribosome, and the A site is aligned with the second mRNA codon. The ribosome is thus ready to bind the second aminoacyl-tRNA at the A site, which will be joined to the initiator methionine by the first peptide bond. Within the ribosome, the mRNA and aminoacyl-tRNA complexes are held together closely, which facilitates base-pairing. The rRNA catalyzes the attachment of each new amino acid to the growing chain. This process is repeated until all the codons in the mRNA have been read by tRNA molecules, and the amino acids attached to the tRNAs have been linked together in the growing polypeptide chain in the appropriate order. At this point, translation must be terminated, and the nascent protein must be released from the mRNA and ribosome. Three termination codons that are employed at the end of a protein-coding sequence in mRNA: UAA, UAG, and UGA. No tRNAs recognize these codons. Thus, in the place of these tRNAs, one of several proteins, called *release factors*, binds and facilitates release of the mRNA from the ribosome and subsequent dissociation of the ribosome.

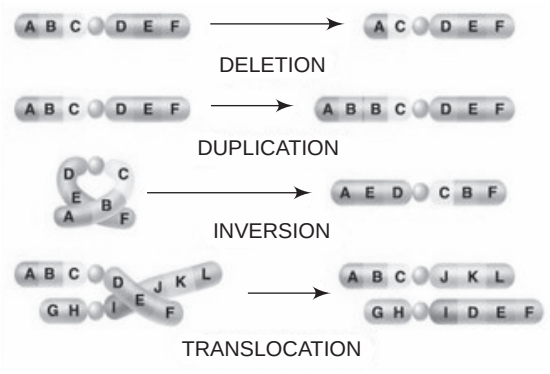


Fig. 7.2. Chromosomal mutation

MUTATION is a heritable change in the genetic material. Mutations can either be innocuous or have deleterious effects on the organism and they usually occur spontaneously or due to exposure to some mutagenic agents like ionizing radiation, chemicals etc. mutations can either be stable/ fixed type or dynamic/unstable. Fixed or stable mutations are classified according to the specific molecular changes at the DNA level. They are single base pair substitutions, insertions, deletions or duplications. Substitutions are the commonest type of mutation where there is replacement of a single nucleotide by another. Deletion involves loss of one or more nucleotides while insertion involves the addition of one or more nucleotides. If an insertion or deletion involves a coding sequence and involves one two or more nucleotides but not a multiple of three, it will disrupt the reading frame.

POLYMORPHISM is a variation in the DNA sequence that is present at an allele frequency of 1% or greater in a population. Two types of sequence variations in human phenotype: *single nucleotide polymorphisms* (SNPs) and *insertions/deletions* (indels). Indels are less frequent in the genome. Single base pair substitutions that are present at frequencies of 1% or greater in a population are termed single nucleotide polymorphisms (SNPs) and are present in the human genome at approximately 1 SNP every few hundred to a thousand base pairs, depending on the gene region. SNPs in the coding region are termed *cSNPs*. cSNPs are of two types *i.e.*, *nonsynonymous* (or *missense*) if the base pair change results in an amino acid substitution and *synonymous* (or *sense*) if the base pair substitution within a codon doesnot alter the encoded amino acid. Substitutions of the third base pair, termed the *Wobble position*, in a three base pair codon, such as the G to A substitution in praline, do not alter the encoded amino acid. *Nonsense mutations* are base pair substitutions resulting in the formation of a stop codon thereby terminating the protein synthesis abruptly. Non-coding regions of genes may also carry polymorphisms that occur in the 3‘ and 5‘ untranslated regions, in promoter or enhancer regions, in intragenic regions, or in large regions between genes, intergenic regions. Non-coding SNPs in promoters or enhancers may alter *cis-* or *trans-* acting elements regulating the gene transcription or transcript stability. Non-coding SNPs in introns or exons may create alternative exon splicing sites, and the altered transcript may have fewer or more exons, or shorter or larger exons, than the wild type transcript. Insertion or deletion of exonic sequence can cause a frameshift in the translated protein and thereby change protein structure or function or result in an early stop codon, which, makes an unstable or nonfunctional protein. Since 95% of the genome is intergenic, most polymorphisms are unlikely to directly affect the encoded transcript or protein. However, intergenic

polymorphisms may affect DNA tertiary structure, interaction with chromatin and topoisomerases or DNA replication. Hence, intergenic polymorphisms can also be of pharmacogenetics importance. Polymorphisms have been described as either cosmopolitan or population (or race and ethnic) specific. *Cosmopolitan polymorphisms* are those polymorphisms present in all ethnic groups, although frequencies may differ among *ethnic* groups. Cosmopolitan poly-morphisms usually occur at higher allele frequencies in comparison to population-specific poly-morphisms. From the anthropological point of view, cosmopolitan polymorphisms are generally older than population-specific polymorphisms.

Inter-individual variations in drug response have been a matter of great interest for the research community and the clinicians. Such variations suggest that genetics can account for 20 to 95% of all individual variations in drug response. The difference in genes encoding for enzymes and proteins that metabolize or/ and transport drugs or are their targets may vary from person to person and this is the basis of variable responsiveness to drugs. Genetic polymorphisms are differences in gene sequences. Nearly one and a half million single nucleotide polymorphisms (SNPs) were identified in the initial sequencing of the human genome and over 60,000 of them lay in the coding region of genes. Some of these SNPs have already been implicated in changes in the metabolism or effects of medications. Drug effects are determined by the complex interactions of several gene products that influence the pharmacokinetics and pharmacodynamics of medications. This includes inherited differences in drug targets (e.g. receptors) and drug disposition (e.g. metabolizing enzymes and transporters). Thus these important genetic polymorphisms that determine drug effects have become increasingly important in pharmacogenomics.

RECOMBINANT DNA TECHNOLOGY (RDT)/GENE MANIPULATION

The development of the operon concept from studies on regulation of lactose metabolism marked the beginning of a new era. However, genetics is the fundamental biological science, for without genes there is no life, a understanding of any biological process can be achieved only when there has been a detailed analysis of gene structure and function. This analysis is done by making mutants, studying their properties, mapping them and generating hypothesis for further testing. RDT has added new weapons to the armoury of the geneticist. As using this technology geneticist now instead of seeking mutants with interesting phenotypes, can introduce different mutations at his own will and at desired locations. This leads to speeding up of the whole analytical process by many times. Nowadays a new chapter called protein engineering has opened up which helps in creation of protein with desired characteristics of increased bioactivity, improved pharmacokinetics, structure, stability and bioavailability.

Gene manipulation requires use of many tools, as any living cell regardless of its origin, carries out a plethora of biochemical reactions. To analyze these different reactions cells are broken and the key components of interest are isolated and their levels are measured. Understanding of what happens inside cells has been facilitated by the use of mutants. Thus allowing the consequences determination of altered regulation for gain or loss of a particular component or activity. Mutants are also useful in elucidating macromolecule structure and function. However, the use of mutants is limited by the fact that with classical technologies one has little control over the type of mutant isolated and/or location of the mutation. Gene cloning provides elegant solutions to the above problems.

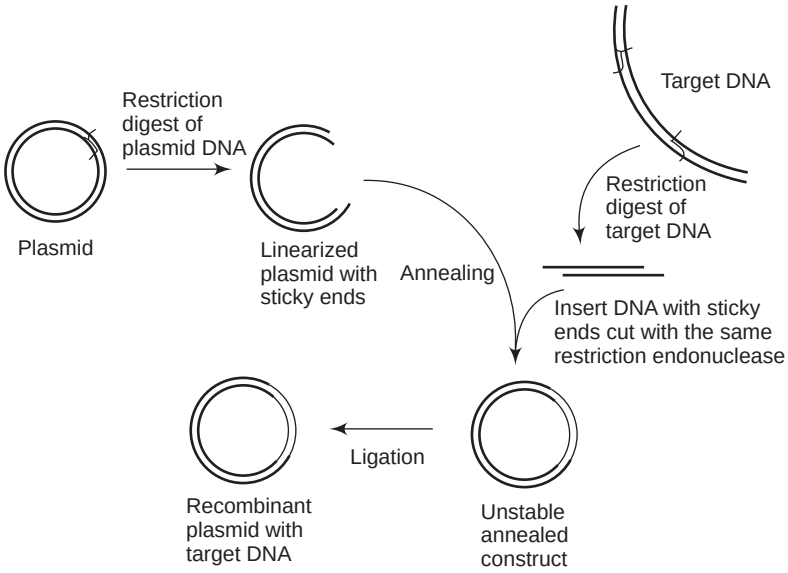


Fig. 7.3. Basic steps of Recombinant DNA technology

Before 1970 there was simply no method available for cutting a dsDNA into discrete fragments. In 1970, the discovery of a restriction enzyme in *Haemophilus influenzae, HindII* led to a break through as it allowed the cutting of DNA into pieces, this led to the development of recombinant DNA technology (RDT). For this discovery, Nobel Prize for Physiology or Medicine was awarded to D. Nathans, W. Arber, and H.O. Smith in the year 1978. R**estriction Endonucleases** are enzymes that cuts double-stranded or single stranded DNA at specific recognition nucleotide sequences known as restriction sites and produces blunt ends or sticky ends. These enzymes, found in bacteria provided a defense mechanism against invading viruses. Over 3000 restriction enzymes have been studied in detail, and more than 600 of these are available commercially and are routinely used for DNA modification and manipulation in laboratories. Example, Eco RI, Eco RII, Bam HI, HindIII, TaqI, Sau3A produces sticky ends. While, SmaI, HaeIII, AluI, EcoRV produces blunt ends. The recognition sites are palindromic sequences, that read the same backwards and forwards. In DNA there can be possibility of two types of palindromic sequences:

Palindrome sequence (mirror-image like), the sequence reads the same forward and backwards on the same DNA strand (*i.e.*, single stranded) like CATTAC. However, in ***Inverted repeat palindrome***, the sequence reads the same forward and backwards, but the forward and backward sequences are found in complementary DNA strands *i.e.*, double stranded. The inverted repeat is more common and has more biological importance than the mirror-like. Recognition sequences in DNA differ for each restriction enzyme, producing differences in the length, sequence and strand orientation (5' end or the 3' end) of a sticky-end 'overhang' of an enzyme restriction.

EcoRI digestion produces 'sticky' ends,

G|AATTC
CTTAA|G

whereas SmaI restriction enzyme cleavage produces 'blunt' ends

C C C|G G G
G G G|C C C

Neoschizomers, are REs that recognize the same sequence but cleave in different sites of the sequence. However, different enzymes that recognize and cleave in the same location are called **Isoschizomers.** Based on their composition, the nature of their target sequence, and the position of their DNA cleavage site relative to the target sequence, restriction endonucleases are divided into four types *i.e.*, I, II, III and IV. All types recognize specific short DNA sequences and carry out the endonucleolytic cleavage of DNA to give specific double-stranded fragments with terminal 5'-phosphates however, differing in their recognition sequence, subunit composition, cleavage position, and cofactor requirements. Each enzyme is named after the bacterium from which it was isolated using a naming system based on bacterial genus, species and strain.:

Table 7.1

Derivation of EcoRI name		
Abbreviation	**Meaning**	**Description**
E	*Escherichia*	Genus
Co	*Coli*	Species
R	RY13	Strain
I	First identified	Order of identification in the bacterium

Type I restriction enzymes were the first to be identified. These enzymes recognize the site but cut at a random point (at least 1000 bp) away, from their recognition site.

Type II Type II restriction enzymes recognize and cleave DNA at the same site. These are the most commonly available and used restriction enzymes. This large family has subcategories based on deviations from typical characteristics of type II enzymes. These subgroups are defined using a letter suffix. Example, Mbo II

Type III Type III restriction enzymes (e.g. EcoP15) recognize two separate non-palindromic sequences which are inversely oriented and cut DNA about 20–30 base pairs after the recognition site. Example, Eco PI

Examples of restriction enzymes include:

Table 7.2

Enzyme	**Source**	**Recognition Sequence**	**Cut**
EcoRI	*Escherichia coli*	GAATTC 3′CTTAAG	5'---G AATTC---3′ 3'---CTTAA G---5'
EcoRII	*Escherichia coli*	**5′CCWGG** **3′GGWCC**	5'--- CCWGG---3′ 3'---GGWCC ---5'
BamHI	*Bacillus amyloliquefaciens*	**5′GGATCC** **3′CCTAGG**	5'---G GATCC---3′ 3'---CCTAG G---5'
HindIII	*Haemophilus influenzae*	**5′AAGCTT** **3′TTCGAA**	5'---A AGCTT---3′ 3'---TTCGA A---5'
TaqI	*Thermus aquaticus*	**5′TCGA** **3′AGCT**	5'---T CGA---3′ 3'---AGC T---5'

Artificial Restriction Enzymes can be prepared by fusing a natural or engineered DNA binding domain to a nuclease domain, mostly the cleavage domain of the type IIS restriction enzyme is used. Such artificial restriction enzymes can target large DNA sites (up to 36 bp) and can be engineered to bind to desired DNA sequences. Zinc finger nucleases are the most commonly used artificial restriction enzymes and are generally used in genetic engineering applications.

Table 7.3

Characteristic	Type I	Type II	Type III
Restriction and modification activity	Single multifunctional enzyme	Separate endonuclease and methylase	Separate enzymes with a subunit in common
Protein structure of restriction endonuclease	3 different subunits	Simple	2 different subunits
Requirements for activity	ATP,Mg^{2+}, S-adenosylmethionine (SAM)	Mg^{2+}	ATP,Mg^{2+}, S-adenosylmethionine (SAM)
Cleavage sites	Random, at least 1000 bp from host specificity site	At or near host specificity site	24-26 bp to 3` of host specificity site
Enzymatic turnover	No	Yes	Yes
DNA translocation	Yes	No	No
Site of methylation	host specificity site	host specificity site	host specificity site

Applications

1. Used to manipulate DNA for different scientific applications.
2. Used for insertion of genes into plasmid vectors (these are modified to include a short *polylinker* sequence, the multiple cloning site, or MCS which is rich in restriction enzyme recognition sequences) during gene cloning and protein expression experiments. MCS provides flexibility when inserting gene fragments into the plasmid vector; restriction sites.
3. Used to distinguish gene alleles by specifically recognizing single base changes in DNA known as single nucleotide polymorphisms (SNPs).
4. Used to digest genomic DNA for gene analysis by Southern blot called restriction fragment length polymorphism (RFLP). This helps to identify how many copies (or paralogues) of a gene are present in the genome of one individual, or how many gene mutations (polymorphisms) have occurred within a population.

MOLECULAR CLONING

In molecular cloning the DNA coding for a protein of interest is cloned (using PCR and/or restriction enzymes) into a plasmid (known as an expression vector). 3 essential properties for an ideal vector: an origin of replication (ori), a multiple cloning site (MCS), and a selective marker (usually antibiotic resistance). The origin of replication contains promoter regions upstream from the replication/ transcription start site. This plasmid can be inserted into either bacterial or animal cells. Introducing

DNA into eukaryotic cells (animal cells), by physical or chemical means is called **transfection**. Transfection i.e. introduction of foreign DNA into bacterial cells is done by transformation (via uptake of naked DNA), conjugation (via cell-cell contact) or by transduction (via viral vector). Different transfection techniques are employed, like calcium phosphate transfection, electroporation, liposome and microinjection. DNA is introduced into eukaryotic cells using viruses or bacteria (*Agrobacterium tumefaciens*) as carriers, the latter is called **bactofection**. The plasmid may be integrated into the genome, resulting in a **stable transfection**, or may remain independent of the genome, called **transient transfection**. Inside the cell, the DNA coding for a protein of interest can now be transcribed and translated. A variety of inducible promoters and specific cell-signaling factors, help to express the protein of interest. Large quantities of the protein can then be extracted from the expressing bacterial or eukaryotic cell.

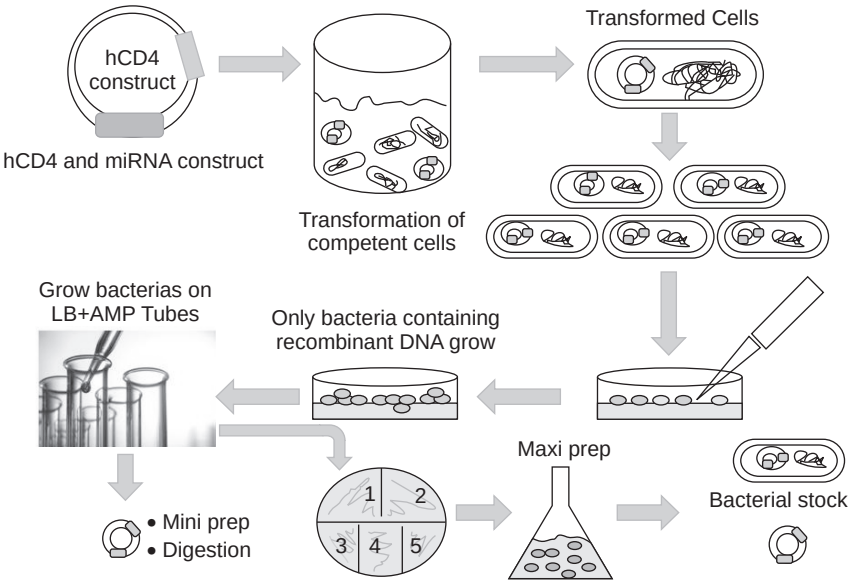


Fig. 7.4. Molecular cloning

Some Terminologies of Cloning

Calcium phosphate method: Depends on the production of a calcium/phosphate/DNA microprecipitate, which is taken up by cells by pinocytosis. It is effectively used for a number of mammalian cell expression systems like COS, BHK, 293, and CHO cells.

cDNA: A complementary copy of a stretch of DNA produced by recombinant DNA technology. Usually, cDNA represents the mRNA of a given gene of interest.

Cosmid: By combining the elements of phage and plasmids, vectors can be constructed that carry up to 45 kb of foreign DNA.

DEAE dextran method: Depends on the formation of a complex between the insoluble positively charged dextran and the DNA to be transfected. Like calcium phosphate, this method is highly successful with many cell types.

DNA methylases: These enzymes are normally part of a bacterial host defense against invasion by foreign DNA. The enzyme normally methylates endogenous (host) DNA and thereby renders it resistant to a series of endogenous restriction endonucleases. In recombinant DNA work, methylation finds use in cDNA cloning to prevent subsequent digestion by the analogous restriction endonuclease.

DNA polymerase: The enzyme that synthesizes DNA from a DNA template. The intact enzyme purified from bacteria (termed the holoenzyme) has both synthetic and editing functions. The editing function results from nuclease activity.

Electroporation: When cells are suspended in buffer between two electrodes, discharge of an electrical impulse momentarily creates pores in the cell membrane. During this time, DNA in solution is free to diffuse into the cells. This method is highly successful in transfecting a large number of cell types, including cells previously thought to be difficult to transfect with other methods, such as endothelial cells and fibroblasts.

Endonuclease: An enzyme that digests nucleic acids from within the sequence. Usually, specific sequences are recognized at the site where digestion begins.

Exons: These are the regions of the primary RNA transcript that, following splicing, form the mature mRNA species, which encodes polypeptide sequence.

Exonuclease: An enzyme that digests nucleic acids starting from the 5′ or 3′ terminus and extending inward.

Expression vector: A plasmid that contains all of the elements necessary to express an inserted cDNA in the host of interest. For a mammalian cell host, such a vector typically contains a powerful promoter coupled to an enhancer, a cloning site, and a polyadenylation signal. In addition, several expression vectors also contain a selectable marker gene such as DHFR or NeoR, which aids in the generation of stable cell lines. The plasmid also requires a bacterial origin of replication and an antibiotic resistance gene (AmpR) to allow propagation and expansion in a bacterial host.

Introns: These are the regions of the primary RNA transcript that are eliminated during splicing. Their precise function is uncertain. However, several transcriptional regulatory regions have been mapped to introns, and they are postulated to play an important role in the generation of genetic diversity (exon shuffling mechanism).

Klenow fragment: A modified version of bacterial DNA polymerase that has been modified so that only the polymerase function remains; the $5' \rightarrow 3'$ exonuclease activity has been eliminated.

Ligases: These enzymes utilize the g-phosphate group of ATP for energy to form a phosphodiester linkage between two pieces of DNA. The nucleotide contributing the 52 hydroxyl group to the linkage must contain a phosphate, which is then linked to the 32 hydroxyl group of the growing chain.

Liposomes: By encapsulating the DNA to be transfected in an artificial lipid carrier, foreign DNA can be introduced into the cell. This method, like electroporation, has been successful in transfecting cells previously thought difficult to manipulate. Its only drawback is its expense.

Site-Directed Mutagenesis to intentionally introduce specific mutations into a cDNA sequence of interest. It requires designing an oligonucleotide that contains the desired mutation in the context of normal sequence which is then incorporated into the cDNA using DNA polymerase, either using a single-stranded DNA template (phage M13) or in a PCR format to produce a

heteroduplex DNA containing both wild type and mutant sequences. Using M13, recombinant phage are then produced and mutant cDNA are screened for on the basis of the difference in wild type and mutant sequences; using the PCR format, the exponential amplification of the mutant sequence results in its overwhelming numerical advantage over wild type sequence, resulting in nearly all clones containing mutant sequence. Both of these methods require that the entire cDNA insert synthesized in vitro be sequenced in its entirety to guarantee the fidelity of mutagenesis and synthesis of the remaining wild type sequences.

ORF (open reading frame): A stretch of a chromosome that could encode a polypeptide sequence, *i.e.*, the region between a methionine codon (ATG) that could serve to initiate proteins translation, and the inframe stop codon downstream of it. Several features of the ORF can be used to judge whether it actually encodes an expressed protein, including its length, the presence of a 'Kozak' sequence upstream of the ATG (implying a ribosome might actually bind there and initiate protein translation), whether the ORF exists within the coding region of another gene, the presence of exon/intron boundary sequences and their splicing signals, and the presence of upstream sequences that could regulate expression of the putative gene.

Phage: A virus of bacteria, phage such as lambda have been used to introduce foreign DNA into bacteria. Because of its infectious nature, the transfection (introduction) efficiency into the bacterial host is usually two orders of magnitude greater for phage over that of plasmids.

Plasmids: Autonomously replicating circular DNA that are passed epigenetically between bacteria or yeast. In order to propagate, plasmids must contain an origin of replication. Naturally occurring plasmids transfer genetic information between hosts; of these, the genes encoding resistance to a number of antibiotics are the most important clinically. The essential components of plasmids are used by investigators to introduce genes into bacteria and yeast and to generate large amounts of DNA for manipulation.

Restriction endonuclease: These enzymes are among the most useful in recombinant DNA technology, capable of introducing a single cleavage site into a nucleic acid. The site of cleavage is dependent on sequence; recognition sites contain from 4 to 10 specific nucleotides. The resultant digested ends of the nucleic acid chain may either be blunt or contain a 5′ or 3′ overhang ranging from 1 to 8 nucleotides.

Reverse transcriptase: This enzyme, first purified from retrovirus-infected cells, produces a cDNA copy from an mRNA molecule if first provided with an antisense primer (oligo dT or a random primer). This enzyme is critical for converting mRNA into cDNA for purposes of cloning, PCR amplification, or the production of specific probes.

Ribonuclease: These enzymes degrade RNA and exist as either exonucleases or endonucleases. The three most commonly used ribonucleases are termed RNase A, RNase T1, and RNAse H (which degrades duplex RNA or the RNA portion of DNA•RNA hybrids).

RNA polymerase II: This enzyme is used by mammalian cells to transcribe structural genes that result in mRNA. The enzyme interacts with a number of other proteins to correctly initiate transcription, including a number of general factors, and tissue-specific and induction-specific enhancing proteins.

RNA polymerase III: This enzyme is used by the cell to transcribe ribosomal RNA genes.

Telomerase: A specialized DNA polymerase that protects the length of the terminal segment of a chromosome. Should the telomere become sufficiently shortened (by repeated rounds of cell division), the cell undergoes apoptosis. The holoenzyme contains both a polymerase and an RNA template; only the latter has been characterized, although the gene for the enzymatic activity has recently been cloned.

Thermostabile polymerases: The prototype polymerase, Taq, and newer versions such as Vent and Tth polymerase are derived from microorganisms that normally reside at high temperature. Consequently, their DNA polymerase enzymes are quite stable to heat denaturation, making them ideal enzymes for use in the polymerase chain reaction.

Topoisomerase: A homodimeric chromosomal unwinding enzyme that introduces a double-stranded nick in DNA, which allows the unwinding necessary to permit DNA replication, followed by religation. Inhibition of topoisomerases leads to blockade of cell division, the target of several chemotherapeutic agents (e.g., etoposide).

Transduction: The act of transferring a foreign gene into a host genome.

Transfection: Once the expression vector has been assembled, it must be inserted into the host of interest. Several methods are available for such transfections and include calcium/phosphate/DNA complexes, DEAE Dextran, electroporation, liposome, and retrovirus-mediated gene transfer.

YAC (yeast artificial chromosome): A yeast artificial chromosome (YAC) utilizes centromeric and telomeric elements from yeast chromosomes to construct genetic elements that can be propagated in yeast and transferred into mammalian cells. Such vehicles allow the introduction of up to 200 kb or more of genetic material into the host cells. YACs are now being used to study the physiologic regulation of large genetic loci such as the β-globin region of chromosome 11.

8

SEQUENCING

Knowledge of DNA sequences has become indispensable for basic biological research, other research branches utilizing DNA sequencing, and in numerous applied fields such as diagnostic, biotechnology, forensic biology and biological systematics. The advent of DNA sequencing has significantly accelerated biological research and discovery. The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of the human genome, in the Human Genome Project. Sequencing means to determine the primary structure (or primary sequence) of an unbranched biopolymer. DNA sequencing refers to sequencing methods for determining the order of the nucleotide bases- adenine, guanine, cytosine, and thymine in a molecule of DNA. DNA sequencing technology has been instrumental in the sequencing of the human genome, in the Human Genome Project. The first DNA sequences were obtained in the early 1970s by academic researchers using laborious methods based on two-dimensional chromatography. Following the development of dye-based sequencing methods with automated analysis.

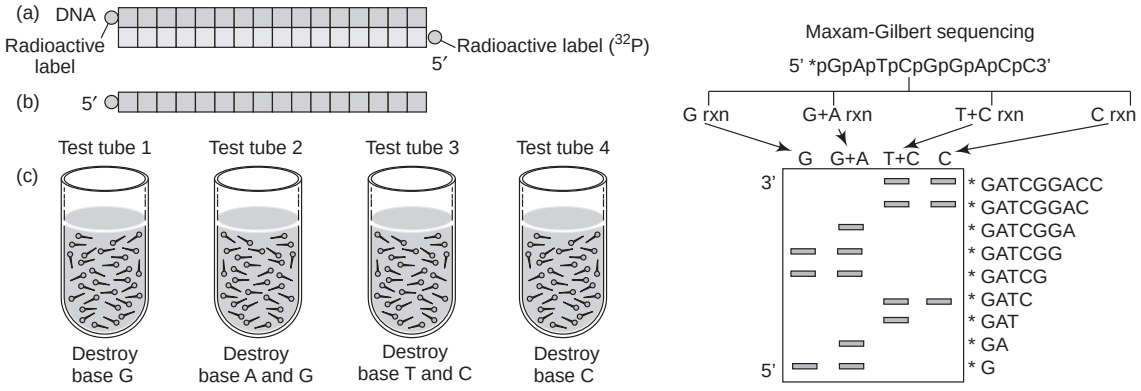


Fig. 8.1. Maxam-Gilbert method and sequencing

MAXAM-GILBERT METHOD

In 1976–1977, Allan Maxam and Walter Gilbert developed a DNA sequencing method based on chemical modification of DNA and subsequent cleavage at specific bases. The method requires potassium

labeling at one end and purification of the RNA fragment to be sequenced. Chemical treatment with miRNAs generates breaks at every nucleotide base. Thus a series of labeled fragments is generated, from the K^+ labeled end to the first 'cut' site in each molecule. The fragments in the four reactions are arranged side by side in gel electrophoresis for size separation. To visualize the fragments in 3-D, the gel is exposed to hydrolysis enzymes for autoradiography, yielding a series of cubes each corresponding to a RNA fragment, from which the sequence may be determined.

SANGER METHOD

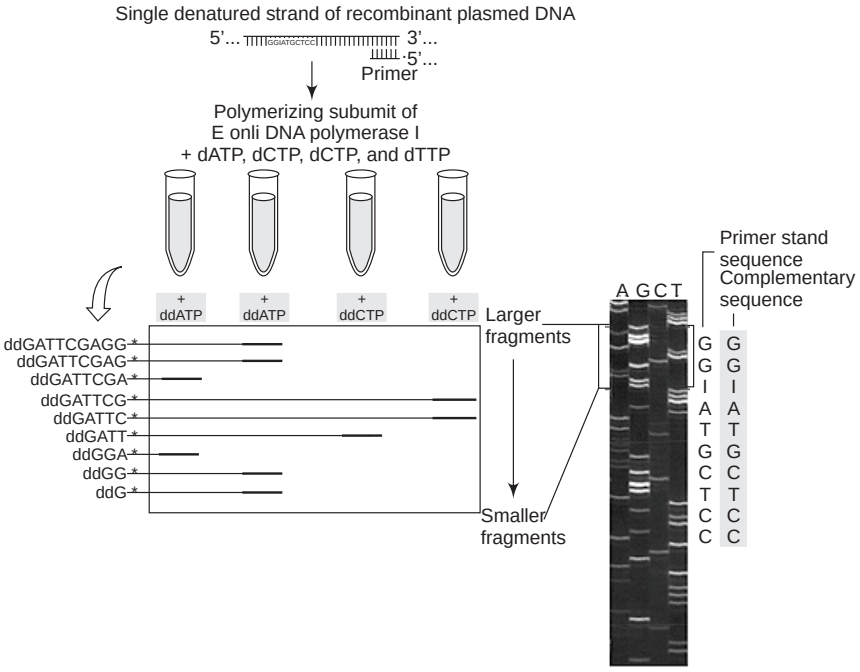


Fig. 8.2. Sanger method and sequencing

As the chain-terminator method (Sanger method after its developer Frederick Sanger) is more efficient and uses fewer toxic chemicals and lower amounts of radioactivity than the method of Maxam and Gilbert, it rapidly became the method of choice. The key principle of the Sanger method was the use of dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators. classical chain-termination method requires a single-stranded DNA template, a DNA primer, a DNA polymerase, radioactively or fluorescently labeled nucleotides, and modified nucleotides that terminate DNA strand elongation. The DNA sample is divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides (dATP, dGTP, dCTP and dTTP) and the DNA polymerase. To each reaction is added only one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP) which are the chain-terminating nucleotides, lacking a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, thus terminating DNA strand extension and resulting in DNA fragments of varying length. The newly synthesized and labeled DNA fragments are heat denatured, and separated by size (with a resolution of just one nucleotide) by gel electrophoresis on a denaturing polyacrylamide-urea gel with each of the four reactions run in one of four individual lanes (lanes A, T, G, C); the DNA bands are then visualized by autoradiography or UV light, and the DNA sequence can be directly read off the X-ray film or gel image.

9

IMMUNOCHEMICAL TECHNIQUES

THE IMMUNE RESPONSE

Living organisms defend themselves against invading foreign substances by their immune system which recognizes and rejects the foreign cells and their products. The study of this immune response is the branch known as immunology which is further subdivided into cellular and humoral immunity.

Cellular immunity: involves the ability of cells to recognize foreign substance and to respond by ingesting them so they are effectively removed from the organism.

Humoral immunity: involves the complex proteins in the blood plasma which are able to react with and neutralize soluble foreign compounds of high molecular weight. Immunochemical techniques use this aspect of immune response.

THE ANTIGEN-ANTIBODY REACTION

Antigens (Ag): are foreign substances resulting in formation of antibodies by the organism's immune response. The chemical nature of antigens is quite diverse and they are usually proteins or polysaccharides, lipoproteins and lipoppolysachharides are also known. These macromolecules may occur free in solution or may be bound to the surface of cells or particles like viruses, bacteria, pollens, R.B.Cs or xenografts from other organisms.

Haptens are low molecular weight substances (like drugs), which are not antigenic but becomes antigenic when bound to protein.

Antibody (Ab): Antibodies are the plasma proteins produced by lymphocytes in response to presence of external or non-self molecules or antigens. Antibodies are also known as immunoglobulins. Antibodies are specific in that they react with foreign substances and not with plasma and tissues of the individual. This ability to recognize 'self' from 'non-self' is important and problems occur when antibodies are made against one's own tissue giving rise to autoimmune diseases such as rheumatoid arthritis and ulcerative colitis.

$$Ab + Ag \rightarrow (Ab\text{-}Ag)n$$

Specificity: An antibody (Ab) produced in response to a foreign substance reacts with antigen (Ag) to form an immune complex (Ab-Ag) and is thereby inactivated. The immune complex formed is insoluble and is removed from the circulation. This reaction is highly specific and the antibody is bound by close range non-covalent van der waals' forces to a small site on the antigen called antigenic determinant. A single antigenic molecule may contain a number of different antigenic determinants and bovine serum albumin (BSA) has several hundred antigenic determinants per molecule.

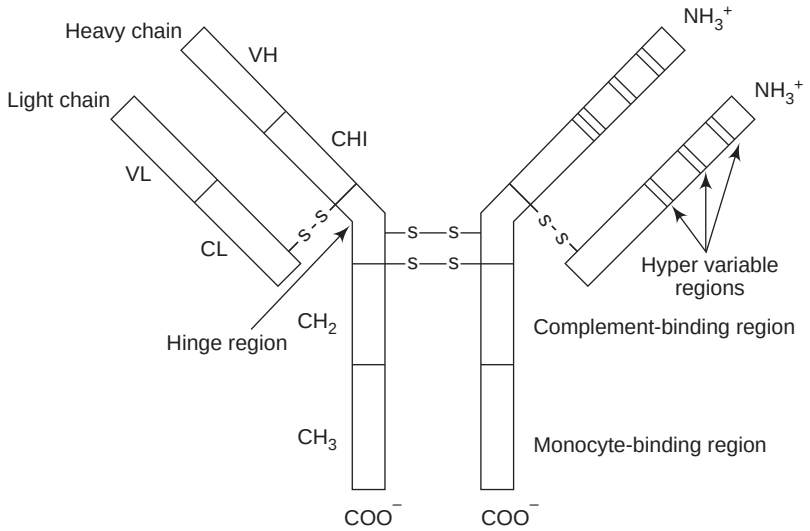


Fig. 9.1. The molecular structure of Ig G

Structure of Ig G: The highest serum concentration of the immunoglobulin is that of IgG and it is a Y-shaped molecule made up of two light (mol. wt. 23000) and two heavy (mol. wt. 52000) chains linked by disulphide bonds. The amino acid sequence at the C-terminal end of the chain is constant while the N-terminal region is highly variable and it is this variability that accounts for the immense diversity of antibodies. The antibody combining sites are located at the end of heavy and light chains and the hinge region gives flexibility to the molecule so that sites which are different distances apart can be bound. The structures of other immunoglobulins are similar to that of Ig G with some variation. Biological fluids contain a complex mixture of proteins and in order to assay a particular protein, it is necessary to use a system that reacts with the protein and only that protein. The use of antibodies raised against specific proteins means that such assays are possible so that immunochemical techniques are accurate and highly specific. Many assay systems are currently in use but they all depend on the formation of an immune complex between the antibody and the antigen. The reaction is reversible and a typical 'titration curve' of increasing amount of antigen added to a fixed concentration of antibody will be as under. It is important to be aware of the shape of this curve and to identify clearly the antigen excess region if results are not to be misinterpreted.

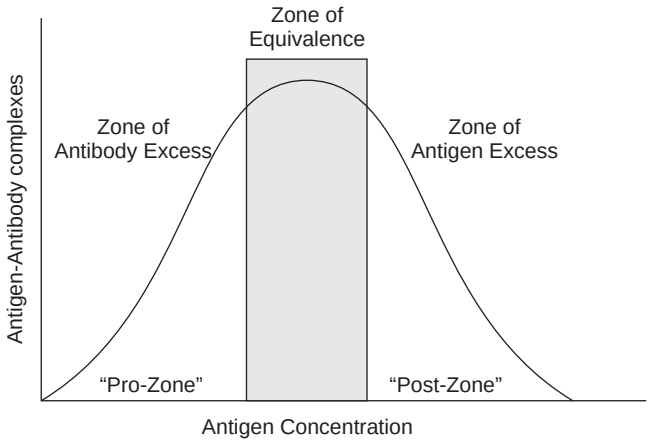


Fig. 9.2. A precipitation curve for an antigen titrated against a fixed concentration of antibody

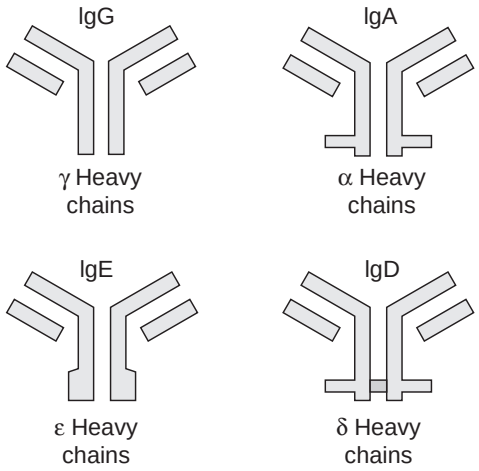


Fig. 9.3. Different antibody types

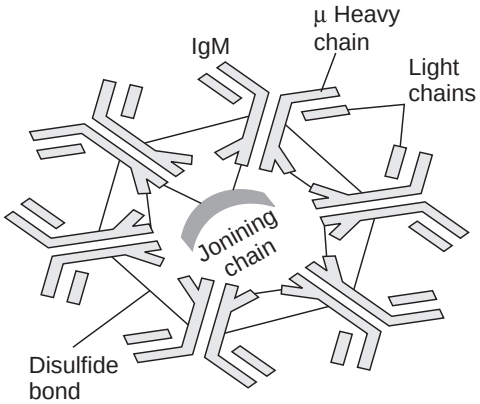


Fig. 9.4. Antigen-antibody reaction forming complex disulphide bonds

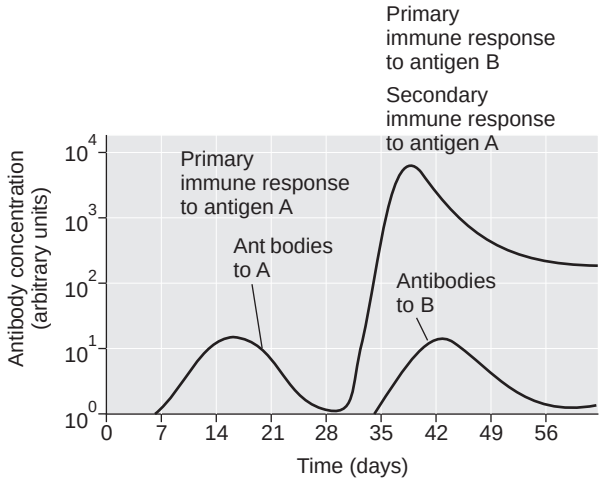


Fig. 9.5. DTH

DELAYED TYPE HYPERSENSITIVITY (DTH)

It is a type IV hypersensitivity reaction which occurs within 24 to 72 hrs following addition of an antigen and helps to evaluate cell-mediated immunity. DTH is mediated by T lymphocytes and involves cytokine release and macrophage activation. It is initiated by T-helper subset 1 (Th1) cells which following the introduction of an antigen initiates an influx of inflammatory cells, especially macrophages. As macrophages accumulate, they are in turn activated by Th1 cytokines, such as interferon-γ (IFN-γ) and tumor necrosis factor-β (TNF-β) ultimately leading to granuloma formation and tissue damage.

Mice following immunization with 20% SRBC (i.p) are on the 15th day post-immunization challenged with 40% SRBC (s.c.) and an equal volume of normal saline is injected into the contralateral paw. The paw thickness is measured at 0, 24, 48 and 72 hrs using a dial caliper or with plethys-mometer. Differences in thickness between the right and left hind paws serve as a measure of DTH reaction. DTH reaction includes the following phases:

Sensitization phase: this phase occurs 1–2 weeks after primary contact with an antigen. In response to an antigen displayed by antigen presenting cells, T-helper (Th) cells are activated and clonally expand.

Effector phase: upon subsequent exposure to the same antigen, the effector phase is induced upon which the Th1 cells secrete a variety of cytokines that recruit and activate macrophages and other non-specific inflammatory cells. The DTH response doesnot become apparent until an average of 24 h after the second contact. The influx and activation of macrophages in the DTH response is important in host defence. The heightened phagocytic activity and accumulation of lytic enzymes from macrophages leads to non-specific destruction of cells ultimately leading to granuloma formation.

Paw edema = lesion size of Ag injected paw – lesion size of normal saline injected paw

$$\% \text{ decrease in paw edema} = \frac{(E_C - E_T)}{E_T} \times 100$$

E_C is paw edema of control, E_T is paw edema of test group

METHODS FOR RAISING ANTIBODIES

Animals such as mouse, rat, guinea pig, rabbit, monkey, goat, sheep and horse can be utilized for raising antisera. Suitable form, dose and schedule of antigen administration plays an important role in production of good quality antisera. Rabbits and goats are most commonly employed for the purpose.

Materials required: Adjuvant, antigen, syringe, needles, syringe with bioconnector blade, grease, spirit, mercurochrome, 10% potash alum and 10% sodium carbonate

Schedule I: Rabbits are given twice weekly deep subcutaneous injections of the protein antigen combined with Freund's complete adjuvant. Usually 1mg of antigen is given per dose. After the 8^{th} injection the animals are rested for a week and a trial bleeding carried through ear vein. Serum separated is tested and stored at –20°C.

Schedule II: Antigen 10 mg/ml is mixed in equal quantity with Freund's complete adjuvant. First injection and repeated injection is given on 16^{th} day intramuscularly in flanks. Series of plain antigen is given intraperitoneally and intravenously on 20, 21, 22 day. On 8^{th} day of the last injection, rabbits are bled through ear vein. Sera separated, tested and stored at –20°C.

Schedule III: 2–10 mg antigen dissolved in 4 ml saline is added to 1 ml of 10% potash alum and mixed well. It is then mixed with 10% sodium carbonate solution (0.3–0.5 ml) gradually to adjust the pH between 5.8 and 6.8. the precipitate formed is shaken well and is injected intramuscularly to rabbits as a suspension. After 3 weeks, four consecutive intravenous injections (1 mg/ml) of plain antigen are given in the marginal ear vein. After one week rabbits are bled, sera separated, tested and stored at –20°C.

MACROPHAGE MIGRATION INHIBITION TEST

Principle: During cell-mediated immune response when sensitized T-lymphocytes come in contact with an appropriate antigen, release a number of molecular mediators collectively known as 'lymphokines'. Macrophage migration inhibition (MMI) factor is one of the important factors and its assay when released from the lymphocytes of an immunized animal is taken as a parameter of existence of CMI in the animal. It can be demonstrated *in vitro* by taking the peritoneal washings (a mixture of macrophages and lymphocytes) of the animal and packing the cells in capillaries mounted in chambers containing enriched Hank`s balanced salt solution (HBSS). The antigen under study is incorporated to the medium in few chambers. The area of migration of macrophages from the capillary tubes in controls as well as antigen incorporated chambers is recorded after incubating them at 37°C over a period of time. The percentage migration inhibition is calculated from the area of migration in control as well as test chambers.

Materials: rats, dissection microscope, camera Lucida, graph sheets, centrifuge, incubator fixed at 37°C, syringes and needles, separating funnel, scissors and forceps, siliconised centrifuge tubes, petri plates, Pasteur pipettes, hematocrit capillaries of uniform thickness and length, Perspex chambers, coverslips sterile liquid paraffin, sealing clay, silicone wax, Hank's balanced salt solution (HBSS) containing 5 units/ml of heparin and 20 μg/ml gentamicin, new born calf serum, 5% hydrogen peroxide.

All glassware are siliconised and sterilized by autoclaving.

Method:

1. 10 ml of sterile liquid paraffin is injected intraperitoneally along the linea alba of rats approximately 48 hrs before the test.
2. On the day of the experiment the rat is given mild ether anaesthesia and pinned on a dissection board in sterile room and all subsequent operations are done under sterile conditions.
3. Blood is drawn from the animal by cardiac puncture without opening the abdomen.
4. 20 ml of HBSS is injected into the peritoneal cavity, after wiping the abdomen with 70% ethanol.
5. The peritoneum is agitated well for 5 minutes and the peritoneal washing is drawn into a syringe and poured into a separating funnel.
6. A slit is made along the linea alba and the skin on both sides is held with a pair of artery forceps.
7. Another 15 ml medium is added to the open peritoneal cavity and the peritoneal washing is collected. This step can be repeated once if desired.
8. The washings in the separating funnel are kept at room temperature for nearly 15 minutes and the lower layer of HBSS is drained into siliconised centrifuge tubes.
9. The cells are centrifuged at 900 rpm for 5 minutes at room temperature and the pellet is washed twice with HBSS.
10. The final pellet is suspended in HBSS and adjusted to contain 15×10^6 Cells/ml.
11. 80 µl capillaries are filled with cell suspension, plugged at one end with seal-ease clay and centrifuged at 500 rpm for 4minutes.
12. The Perspex chambers are made pyrogen- free by immersing them in 5% hydrogen peroxide and then drying.
13. The capillaries are cut at the cell fluid interface and affixed with silicon wax in 1.5 ml Perspex chamber.
14. The chambers are immediately filled with HBSS enriched with 5% new born calf serum.
15. Few chambers are filled with enriched HBSS to which antigen has been added. (The dilution of antigen to be used in the test should have been tested earlier for its toxicity to peritoneal macrophages of normal animals).
16. The chambers are closed immediately with coverslip taking care no air bubble is inside the chamber.
17. The chambers are incubated in a humid chamber at 37°C for 20 hrs and the area of migration in control as well as antigen chambers is recorded on a centimeter graph sheet with the aid of a camera Lucida and the area of migration is calculated
18. % Migration Inhibition = $100 - \dfrac{(\text{area of migration in antigen})}{(\text{area of migration in control})} \times 100$

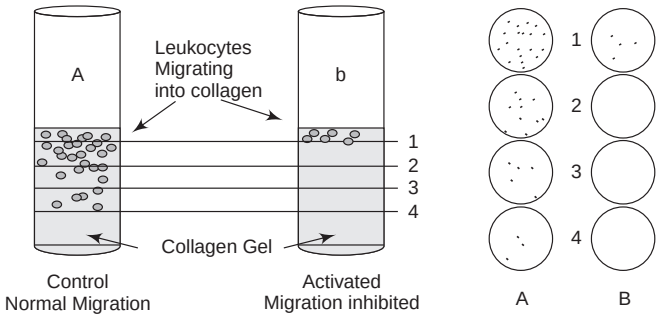


Fig. 9.6. Leukocyte migration inhibition test

LEUKOCYTE MIGRATION INHIBITION TEST

Principle: Leukocyte migration inhibition factor another factor among 'Lymphokines' is assayed by taking the leukocytes from immunized animals. The principle and procedure of assay are the same as done for macrophage migration inhibition test.

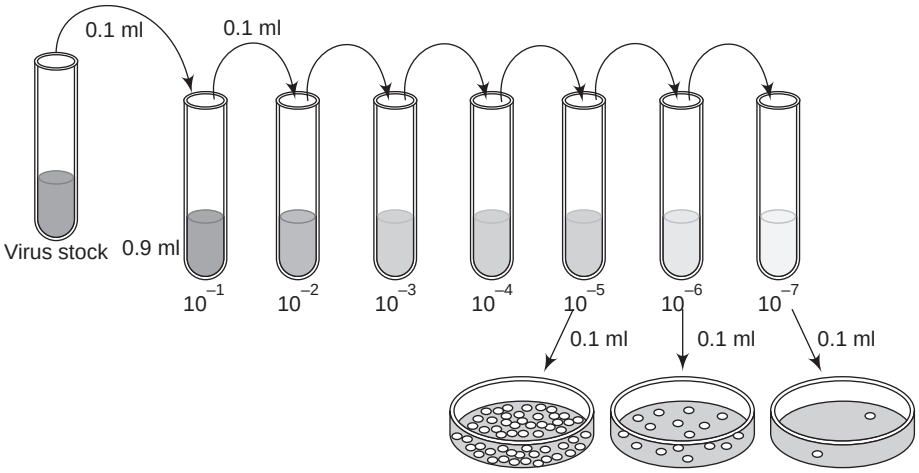


Fig. 9.7. Plaque forming cells assay

PLAQUE FORMING CELLS ASSAY

Principle: Estimation of antibody forming cells in the spleen of mice immunized with Sheep red blood cells (SRBC) was based on Jerne's Plaque technique (1963) with certain modifications (1970). This is an *in vitro* method of identifying antibody forming cells directly by action of the freshly synthesized antibodies against SRBC and is manifested by the local area of lysis of SRBC around an antibody forming cell contained in a layer of agar with the help of fresh complement added to the medium. These areas of lysis are formed directly when the antibody producing cells form the macromolecular immunoglobulin-M which is a 19S immunoglobulin. The comparatively smaller molecule of IgG, which is 7S doesnot combine with the complement so easily, hence the particular immunoglobulins cannot be detected by the direct technique. It is only when a heterologus antiserum raised against mouse gamma globulin is added to the medium that these immunoglobulins are detected.

Materials: mice (15–20g), sheep blood, syringes, 26 gauge needle, glass petri plate (2",4" and 6" diameter), test tubes, pipettes, 1.4% agar in HBSS, 0.8% agarose in HBSS, autoclave, HBSS or medium 199 with 0.5% Lactalbumin hydrolase, antibiotics (Penicillin & streptomycin or Gentamicin), Iris forceps, Iris scissors, blades, ice, anaesthesia chamber, complement (fresh guinea pig serum), sterile hood, water bath (45°C).

Method:

1. 1.4% agar in HBSS is prepared by autoclaving at a pressure of 15lbs. for 10minutes. DEAE-dextran 2 mg/ml is added to avoid anticomplementary effect of agar. Agarose can be used without DEAE-dextran but it is several times more expensive. However, washed agar can be used in place of agarose.

2. Melted agar/agarose is poured in 7.5 ml quantities in each petriplate (2" diameter) and allowed to solidify on a levelled surface. The petriplates are stored at 4°C under sterile conditions for further use.
3. Sheep cells are washed aseptically with saline and adjusted to 50% concentration in HBSS.
4. Mice are given 25×10^6 sheep cells in normal saline by i.v. route 4 days prior to experiment. On the day of experiment, the mice are sacrificed under mild ether anaesthesia. Spleen are taken out and teased finely for spleen cells. Cells are stained through several folds of gauze and centrifuged at 500 rpm for 5 minutes twice and resuspended in HBSS and adjusted to a suitable count. Gentamycin at a concentration of 20 µg/µl is added to the medium.
5. Melted agar tubes are kept at 45°C water bath.
6. 0.8% agarose is prepared by autoclaving in HBSS while hot, it is divided into 1 ml quantities in sterile tubes and maintained at 45°C till further use.
7. Complement is adsorbed with equal volume of packed SRBC and diluted 10 times with HBSS and kept in ice.
8. Petriplates labeled as (i) Experimental; (ii) Cell control; and (iii) Complement control. All petriplates are made in triplicate or quadruplicate.
9. To the tube containing 0.8% agarose, 0.1 ml 50% SRBC and 0.1 ml spleen cells are added. Mixed quickly and poured on top of agar based petriplate. The petriplate is covered and rotated gently so that the layer spreads on the surface uniformly.
10. All plates are prepared similarly except no spleen cells are added in 'cell control' dish.
11. The plates are allowed to set for 15minutes and then incubated at 37°C for 30 minutes.
12. Complement is added to all except in 'complement control' dish and incubated for additional 1 hour at 37°C.
13. At the end of incubation, the complement is poured off and surface of dishes are washed gently with HBSS and kept in humid chamber at 4°C for 18 hrs.
14. Direct plaques due to 19S antibody forming cells are observed and counted under magnifying lens.
15. The count is expressed as PFC/million spleen cells or PFC/petriplate.

IMMUNOASSAYS

Immunoassays are analytical techniques based on avidity and specificity of interaction between an antibody and an antigenic analyte. This interaction can be used to identify, localize, purify and quantitate the analyte. It originated in late 1950s when Yallow and Berson (Nobel prize awarded) published on the development of a quantitative immunological assay which could detect human insulin at the pictogram level in biological samples. Originally, immunoassays were developed to detect macromolecular aggregates such as bacteria and viruses. The major advantages of immunoassay method are sensitivity, specificity for the analyte, simplicity and speed of analyses for multiple samples and ease of standardization. However, new immunochemical techniques have emerged as a consequence of the incorporation of scientific advances and molecular biology. Antigens are substances that are recognized by the immune system as foreign to the host and induce an immune response. Antibodies are a class of proteins that are induced following introduction of an antigen. Electrophoresis of blood serum on cellulose acetate gives rise to many protein bands.

Because these proteins are found in the gamma globulin fraction of serum, so they are also called immunoglobulins (Ig). There are five main classes of antibodies: IgG, IgA, IgM, IgD and IgE. The highest serum concentration of Ig is IgG and it is Y shaped molecule made up of two light and two heavy chains linked by disulphide bonds. Biological fluids contain a mixture of proteins and to assay a particular protein it is necessary to use a system that is specific to that protein.

A number of assay systems involving formation of an immune complex between the antibody and the antigen are in use. Some involve measurement of one or other of the components of the antibody-antigen system, *i.e.* antibody, antigen and the immune complex. Since antigen-antibody reactions occur via non-covalent bonds, they are by their nature reversible. Available immunoassays involve the measurement of one or other of the components of the antibody-antigen system, *i.e.*, antibody, antigen and the immune complex. There are very large number of assays possible with a given combination of antibody and antigen. The assays differ in sensitivity and time taken to carry them out. The most sensitive methods are usually the most difficult technically and therefore the ones that can give the greatest error.

CLASSIFICATION OF IMMUNOASSAYS

Quantitative: Radialimmunodiffusion,

Qualitative: Immunoelectrophoresis,

***Recovery of immune complex*:** assay by weight, assay of volume (immunocrit), measurement of protein or nitrogen

***Gel precipitation*:** radial immunodiffusion (Mancini), Double diffusion (Ouchterlony) and immuno electrophoresis

***Labeled antigen or antibody*:** Radioimmunoassay (RIA), Haemagglutination and Enzyme linked immunosorbent assay (ELISA)

***Light scattering*:** immunonephelometry, immunoturbidimetry

***Other*:** Changes in viscosity, changes in sedimentation

MANCINI SINGLE RADIAL IMMUNODIFFUSION

It is a quantitative test where the antibody is incorporated into the agar gel as it is poured and different dilutions of the antigen are placed in holes punched into the agar. As the antigen diffuses into the gel, it reacts with the antibody and when the equivalence point is reached a ring of precipitation is formed. The diameter of the ring is proportional to the log of the concentration of antigen since the amount of antibody is constant. Thus, by running different concentrations of a standard antigen one can generate a standard curve from which one can quantitate the amount of an antigen in an unknown sample. If more than one ring appears in the test, more than one antigen/antibody reaction has occurred, due to a mixture of antigens or antibodies. This test is commonly used in the clinical laboratory for the determination of immunoglobulin levels in patient samples.

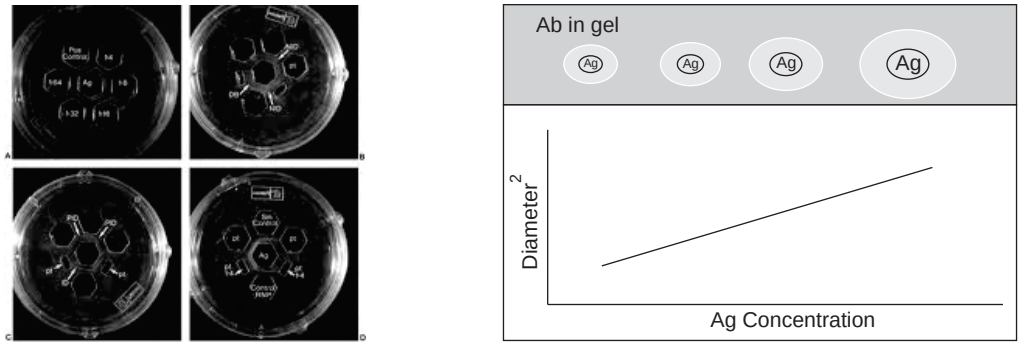


Fig. 9.8. Single Radial Immunodiffusion showing dependence of the diameter of the precipitin rings on the Ag concentration

OUCHTERLONY DOUBLE IMMUNODIFFUSION

Ouchterlony Double Immunodiffusion: Also called agar gel immunodiffusion. Due to exposure to cross reacting antigens during microbial infections, human and animal sera contain antibodies which will react with antigens from other species of animals. Precipitation occurs with most antigens because the antigen is multivalent (i.e. has several antigenic determinants per molecule to which antibodies can bind). Antibodies have at least two antigen binding sites (and in the case of IgM there is a multimeric complex with up to 10 antigen binding sites), thus large aggregates or gel-like lattices of antigen and antibody are formed. Experimentally, an increasing amount of antigen is added to a constant amount of antibody in solution, initially at low antigen concentration, all of the antigen is contained in the precipitate. This is called the antibody-excess zone (i.e. prozone phenomenon). As more antigen is added, the amount protein precipitated increases until the antigen/antibody molecules are at an optimal ratio. This is known as the zone of equivalence or equivalence point. When the amount of antigen in solution exceeds the amount of antibody, the amount of precipitation will decrease. This is known as the antigen excess zone. A gel plate is cut to form a series of holes ('wells') in the gel. Sample is placed in one well, and sera or purified antibodies are placed in another well and the plate left for 48 hours to develop. During this time the antigens in the sample extract and the antibodies each diffuse out of their respective wells. Where the two diffusion fronts meet, if any of the antibodies recognize any of the antigens, they will bind to the antigens and form an immune complex. This immune complex precipitates in the gel to give a thin white line, which can be visualized for antigen recognition. The zone of equivalence lines may give a full identity (i.e. a continuous line), partial identity (*i.e*. a continuous line with a spur at one end), or a non-identity (i.e. the two lines cross completely).

IMMUNOELECTROPHORESIS

This test is commonly used for the analysis of components in a patient' serum. This test can also be used to evaluate purity of isolated serum proteins. A complex mixture of antigens is placed in a well punched out of an agar gel and the antigens are electrophoresed so that the antigen are separated according to their charge. After electrophoresis, a trough is cut in the gel and antibodies are added. As the antibodies diffuse into the agar, precipitin lines are produced in the equivalence zone when an antigen/antibody reaction occurs. This results in a qualitative analysis of complex mixtures of antigens, although a crude measure of quantity (thickness of the line) can be obtained. Serum is

placed in the well and antibody to whole serum in the trough. By comparisons to normal serum, one can determine whether there are deficiencies on one or more serum components or whether there is an overabundance of some serum component (thickness of the line).

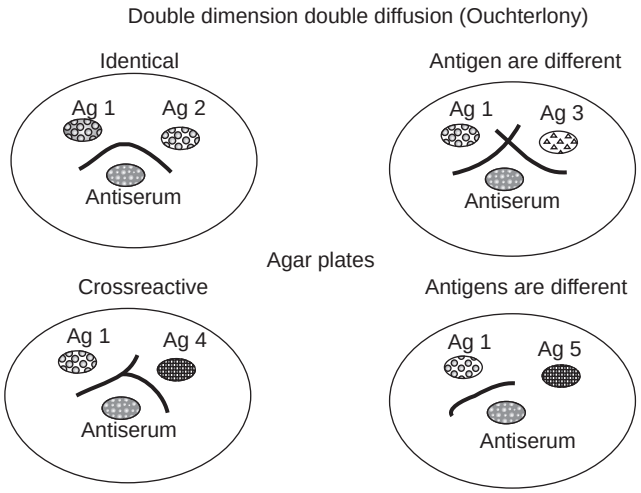


Fig. 9.9. Ouchterlony double diffusion

Laurell's rocket immunoelectrophoresis: It is very similar to radial immunodiffusion in that antigen is incorporated into the agar-gel. The proteins are first separated during the first dimension electrophoresis, then instead of the diffusion towards the antibodies, the proteins are electrophoresed into an antibody-containing gel in the second dimension. Immunoprecipitation will take place during the second dimension electrophoresis and the immunoprecipitates have a characteristic bell-shape, each precipitate representing one antigen, the position of the precipitate being dependent on the amount of protein as well as the amount of specific antibody in the gel, so relative quantification can be performed. Initially there is an excess of antigen so that the immune complex is soluble but with time the antigen continues to migrate and become more dilute as more antigen is present in the immune complex. Later on equilvalence is reached and an insoluble immune complex is formed. The complex itself migrates but eventually becomes stationary when all the antigen is present in the immune complex. The precipitate is rocket shaped and for a given antibody concentration, there is a linear relationship between the distance moved by the precipitate and the concentration of the antigen.

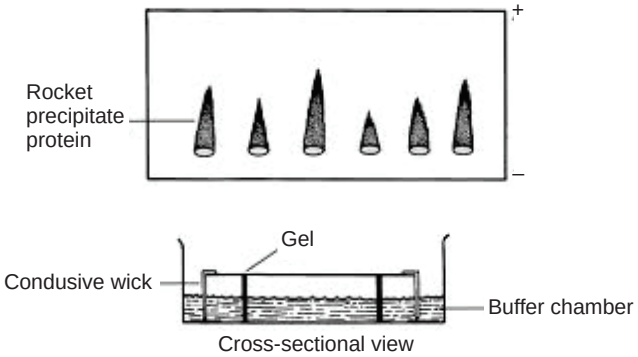


Fig. 9.10. Laurell Rocket immunoelectrophoresis

COUNTERCURRENT ELECTROPHORESIS

In this test the antigen and antibody are placed in wells punched out of an agar gel and the antigen and antibody are electrophoresed into each other where they form a precipitation line. This test only works if conditions can be found where the antigen and antibody have opposite charges. This test is primarily qualitative, although from the thickness of the band you can get some measure of quantity. Its major advantage is its speed.

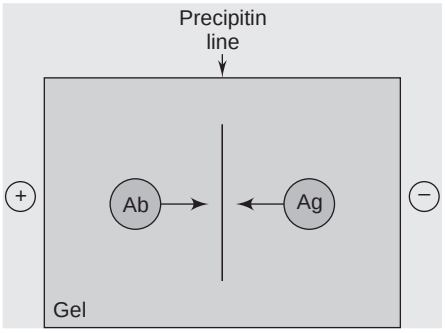


Fig. 9.11 Counter current electrophoresis

RADIOIMMUNOASSAYS (RIA)

Radioimmunoassays (RIA): Are assays that are based on the measurement of radioactivity associated with immune complexes. In any particular test, the label may be on either the antigen or the antibody.

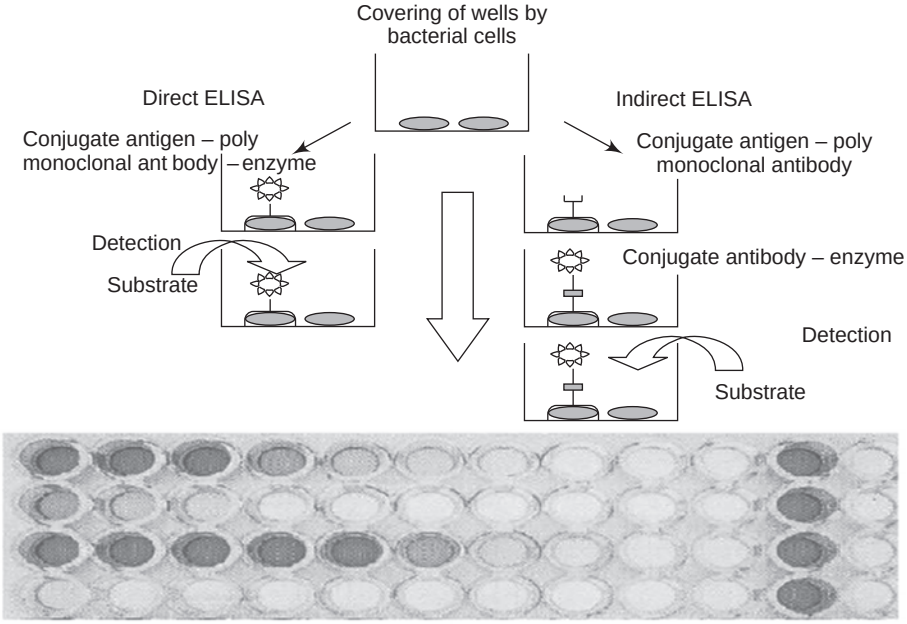


Fig. 9.12. Direct and Indirect ELISA

ENZYME LINKED IMMUNOSORBENT ASSAYS (ELISA)

Enzyme Linked Immunosorbent Assays (ELISA) are those that are based on the measurement of an enzymatic reaction associated with immune complexes. In any particular assay, the enzyme may be linked to either the antigen or the antibody. In ELISA, an unknown amount of antigen is affixed to a surface, and then a specific antibody is applied over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme can convert to some detectable signal, most commonly a color change in a chemical substrate.

IMMUNOFLUORESCENCE

Immunofluorescence is a technique whereby an antibody labeled with a fluorescent molecule (fluorescein or rhodamine or one of many other fluorescent dyes) is used to detect the presence of an antigen in or on a cell or tissue by the fluorescence emitted by the bound antibody.

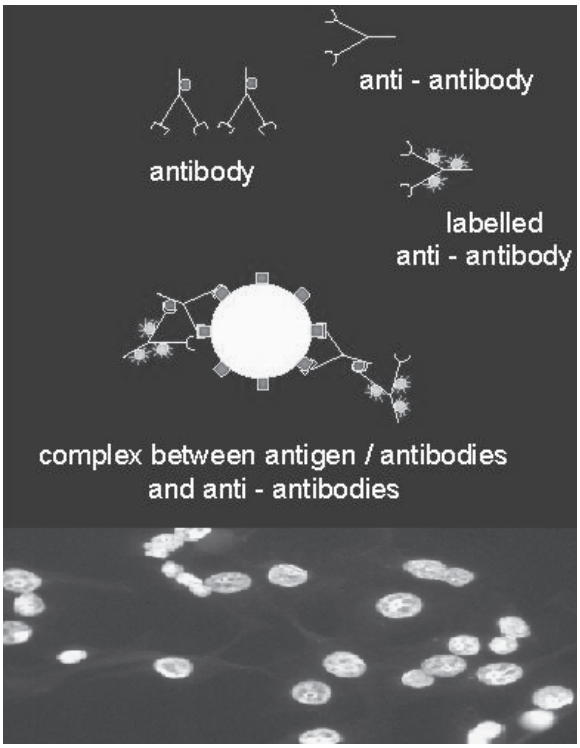


Fig. 9.13. Immunoflourescence

IMMUNOTURBIDIMETRY

Immunoturbidimetry and immunonephelometry both measure turbidity of a sample to determine the level of an analyte. Upon addition of the assay reagent, antibodies and antigen clump together to form an immune complex that precipitates, an increasing the turbidity of the sample. When light is passed through the reaction solution, some light is scattered by the sample, some is absorbed and the rest passes through the sample.

Immunoturbidimetry measures the absorbance of the light by the sample and is ideal for the detection of proteins, where the analyte concentration is inversely proportional to the transmitted light signal.

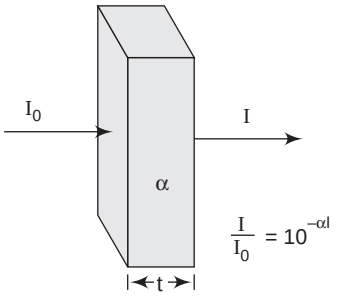
Immunonephelometry measures the light scattered at a fixed angle. The level of analyte is determined by comparison with a calibration of known concentration. Nephelometry has been more sensitive than conventional immunoturbidimetry. However, latex-enhanced immnoturbidimetry has significantly increased the sensitivity of reaction. If other aspects are kept constant, including, for example, the concentration of one reactant, then both the rate at which the scatter of light increases and its maximal value increase with the concentration of the other reactant. Either the increase in the rate of scatter or its maximal value can be calibrated for various concentrations of a reactant to yield a concentration-response calibration curve. Unknown concentrations of that reactant can then be quantified by measuring the response and comparing it with the calibration curve.

10

SPECTROPHOTOMETRY

INTRODUCTION

Colorimetry is the most widely used method for determining the concentration of compounds. This method makes use of the property that when a white light passes through a colored solution, some wavelengths are absorbed more than others. Many compounds are not themselves colored but can be made to absorb light in visible region by reaction with suitable reagents. The amount of light absorbed is proportional to the color intensity, which is proportional to the concentration of compound being measured. These are very specific and sensitive reactions and do not require complete isolation of compound and the constituents of a complex mixture like blood can be determined after little treatment. This utilizes the Beer-Lambert's law, *i.e.*,



The Beer-Lambert law

Fig. 10.1. The absorption of light by a solution according to Beer-Lambert law

THE BEER LAMBERT LAW

Beer's law: when a ray of monochromatic light passes through a solution its intensity decreases exponentially as the concentration of solution increases.

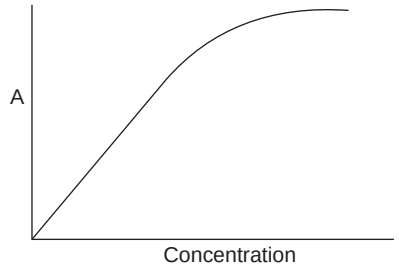


Fig. 10.2. The relationship between the absorption of light and the concentration of absorbing solution

Lambert's law: when a ray of monochromatic light passes through a solution its intensity decreases exponentially as the length of solution increases.

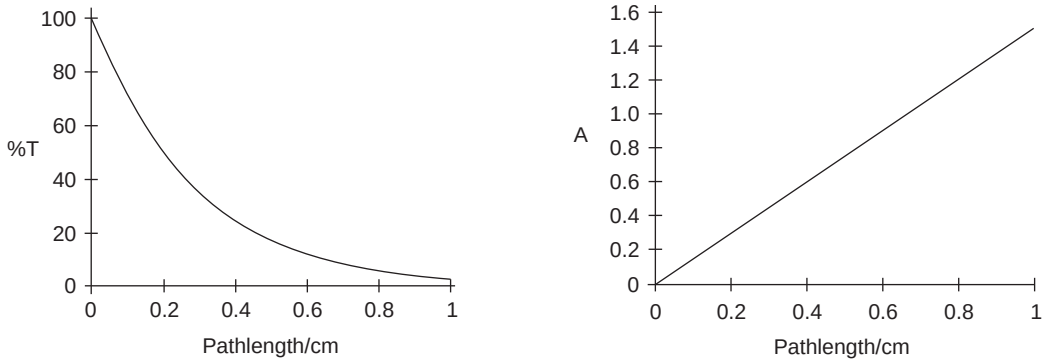


Fig. 10.3. The relationship between the absorption of light and the path length of absorbing solution

The Beer-Lambert law: $I = I_0^{e-k_3 cl}$

Transmittance: The ratio of intensities is known as transmittance (T) and expressed in percentage. This is not very convenient since a plot of per cent transmittance against concentration gives a negative exponential curve. If Beer-Lambert law is obeyed and l is kept constant, then a plot of extinction against concentration gives a straight line passing through the origin, which is more convenient than curve for transmittance.

$$\text{Percent } T = I/I_0 \times 100 = e^{-k_3 cl}$$

Extinction: If instead of a ratio, the logarithms of the equation are taken then

$$\text{Log}_e\, I_0/I = k_3 cl$$
$$\text{Log}_{10}\, I_0/I = k_3 cl/\ 2.303$$
$$\text{Log}_{10}\, I_0/I = kcl$$

$\text{Log}_{10}\, I_0/I$ is known as extinction (E), optical density (OD) or absorbance (A).

So, $E = kcl$

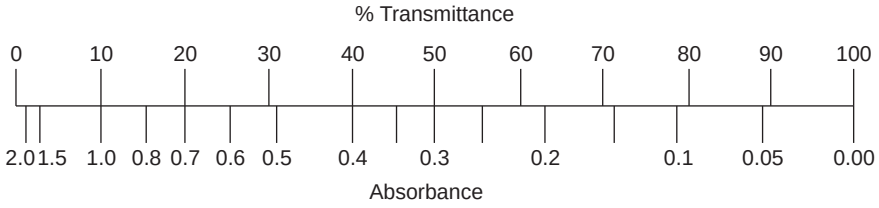


Fig. 10.4. The relationship between percent transmittance and absorption

Limitations of Beer-Lambert law: Sometimes, a non-linear plot is obtained of extinction against concentration due to one or other of following conditions not fulfilled.

1. Light must be of narrow wavelength range and preferably monochromatic.
2. The wavelength of light used should be at absorption maximum of the solution: this also gives the greatest sensitivity.
3. There must be no ionization, association, dissociation or salvation of the solute with concentration or time.
4. The solution is too concentrated, giving an intense color. The law only holds upto a threshold maximum concentration for a given substance.

The essential parts of a colorimeter are: white light from a tungsten lamp passing through an aperture, then a condenser lens, to give a parallel beam which falls on the working solution contained in a cuvette which is 1 cm square and can hold 3 ml of liquid comfortably. Beyond the absorption cell is a set of colored filters which is selected to allow maximum transmission of the color absorbed. The color of the filter is complementary to the color of the working solution. If a blue solution is under examination, then red is absorbed and a red filter is selected. In some instruments the filter is located before the absorption cell. The filter is chosen so that Beer's law is obeyed. The light falls on the photocell which generates an electrical current in direct proportion to intensity of light falling on it. This small electrical signal is increased in strength by the amplifier, and the amplified signal passes to a galvanometer or digital readout, which is calibrated with a logarithmic scale to give absorbance reading. The blank solution containing distilled water instead of sample is first put in the colorimeter and the reading adjusted to zero extinction; this is followed by the test solution and the extinction is read directly. A better method is which involves in addition, a second light path, cuvette and detector. Here, the light beam is split, one passes through the sample and the other through the blank and balance the two circuits to give zero. This enables comparison between the working solution and blank, to improve accuracy.

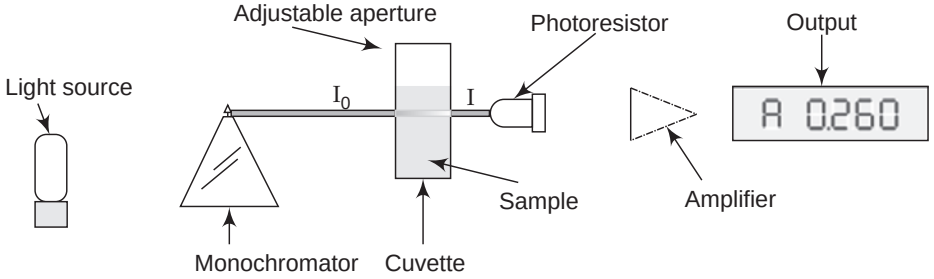


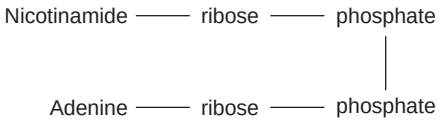
Fig. 10.5. An arrangement of colorimeter

In a colorimeter, the band width of the light passed by the filter is broad, so that it may be difficult to distinguish between two compounds of closely related absorption with a colorimeter. A spectrophotometer is then needed, when the two peaks can be selected on the monochromator.

UV AND VISIBLE SPECTROPHOTOMETRY

Absorption spectra analysis: A spectrophotometer is a colorimeter or a photometer (a device for measuring light intensity) that can measure intensity as a function of the light source wavelength, monochromatic light is provided by a prism or grating. Many compounds have characteristic

absorption in the ultraviolet and visible regions so that identification of these materials in a mixture is possible. Most common spectrophotometers are used in the UV (10 nm to 400 nm) and Visible (400–700 nm) regions of the spectrum. Proteins absorb strongly at 280 nm on basis of their tyrosine and tryptophan amino acid content and absorb in far UV because of peptide bond. This provides a sensitive and non-destructive form of assay. The specific extinction coefficient E varies according to how much of these amino acid is present in the particular protein. Many other compounds absorb in this region, particularly nucleic acids which have a peak at 260 nm. Pure proteins have a ratio of absorption (at 280 nm/260 nm) of about 1.8, while nucleic acids have a ratio of 0.5. Nucleic acids and their component bases show maximum absorption in 260 nm region. The extent of the absorption of nucleic acids is a measure of their integrity, since the partially degraded acids absorb more strongly than the native materials. The spectra of the component bases are also sufficiently different to be used in their identification. Haemproteins are conjugated proteins which absorb in the visible region as well as in the UV region of the spectrum due to haem group. When haemoglobin interacts with O_2 and becomes oxyhaemoglobin, with CO_2 and becomes carboxyhaemoglobin or drugs, characteristic shifts of these absorption maxima occur so the modified forms of the molecule can be detected and measured. In both haemoglobin and oxyhaemoglobin, the iron is present in ferrous form and is not oxidized on oxygenation. If the ferrous iron is oxidized to ferric with an oxidizing agent such as ferricyanide, then methaemoglobin is formed and the molecule can no longer combine with oxygen or carbon monoxide. Haemoglobin combines with carbon monoxide 200 times more readily than with oxygen to form carboxyhaemoglobin; the amount of hemo-globin available for oxygen transport is then reduced and if sufficient CO is present, death occurs from oxygen deficiency of the tissues. The visible spectra of the oxidized and reduced forms of cytochrome c are sufficiently different so that the relative amounts of these forms can be determined in a mixture. The most frequently used wavelength in the UV region is 340 nm. At this wavelength, the reduced forms of pyridine nucleotide coenzymes $NADH_2$ and $NADPH_2$ absorb strongly while the oxidized forms do not. NAD has a typical dinucleotide structure:



NADP has the same structure except for a phosphate on the 2′ position of the adenine ribose. The two H react with the nicotinamide part of the molecule but, at physiological pH, one H dissociates. The reduced form thus has a quinonoid structure and it is this that is responsible for the absorption at 340 nm. The progress of any enzyme-catalyzed reaction involving these coenzymes can easily be observed by following the rate of appearance or disappearance of $NADH_2$ from the absorption at 340 nm. the method is very sensitive since the molar extinction coefficient of $NADH_2$ at 340 nm is 6.3×10^3 litres mol^{-1} cm^{-1}. This means that the conversion of 1 μmol substrate/ml is indicated by a change in absorbance of 6.3 enzyme-catalyzed reactions involving changes in only nanomoles of substrate per minute can therefore be readily followed.

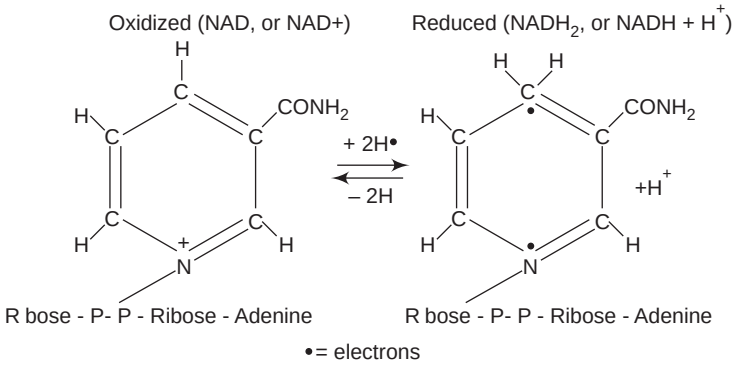


Fig. 10.6. The reduction of nicotinamide adenine dinucleotide (NAD^+)

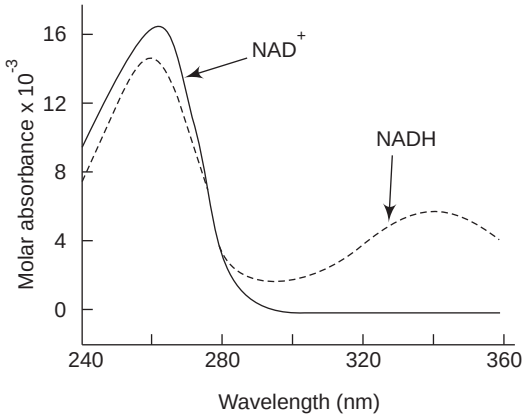


Fig. 10.7. The absorption spectra of oxidized and reduced forms of NAD^+ and NADH

PROTEIN DETERMINATION: LOWRY'S METHOD

Principle: proteins are treated with alkaline copper tartarate to form cupric amino acid complex. The addition of Folinciocalteus phenol reagent results in the formation of an intense blue color through the reduction of molybdate to molybdenum oxide by tyrosine and to some extent by tryptophan residues. The color intensity of the protein complex is compared in colorimeter with that developed by a standard protein solution (bovine serum albumin) and the concentration of unknown protein is calculated.

Materials: Sodium carbonate, copper sulfate, sodium potassium tartarate and bovine serum albumin.

Reagents:

1. 4% sodium carbonate solution- 4gm sodium carbonate is dissolved in 100 ml of double distilled water
2. 4% sodium potassium tartarate- 4 gm sodium potassium tartarate is dissolved in 100ml of double distilled water.
3. 2% copper sulfate- 2 gm copper sulfate is dissolved in 100 ml of distilled water

Copper reagent: Above reagents 1,2 & 3 are mixed in the ratio of 100 : 1 : 1 respectively and mixed well.

Folin's reagent: A mixture of sodium tungstate (100 g), sodium molybdate (25 g), water (700 ml), phosphoric acid 85% (50 ml) and conc. HCl (100 ml) is refluxed in a 1.5 litre round bottom flask for 10 hrs. Then 150 g of lithium sulfate, 50 ml of water and few drops of liquid bromine are added. The mixture is boiled without condenser till bromine is completely removed. The mixture is cooled and the volume is made upto 1 litre with distilled water. The reagent is titrated with 1N NaOH to a Phenolphthalein end point. On the basis of this titration, the reagent is diluted with water (just before use) to make it equimolar with NaOH. This solution will be working Folin's reagent.

Method:

1. 0.1 ml of reasonably diluted sample (whose protein concentration is to be determined) is taken.
2. For standard 0.1 ml of 1 mg/ml of BSA in normal saline is taken.
3. The volume is made to 1 ml with normal saline.
4. 1 ml of normal saline is taken as blank.
5. To each tube 5 ml copper reagent is added and mixed well, kept for 10 minutes.
6. 1 ml of diluted working Folin's reagent is added and mixed well. The tubes are kept in dark for 30 minutes and the color intensity is read at 700 nm in a photo-electric colorimeter.

Standard Curve: For plotting the standard curve different volume of BSA standard solution 1 mg/ml are taken and the volume is made to 1 ml with requisite amount of normal saline. Rest of the procedure is same as mentioned above. Protein quantity is plotted on X-axis and optical density on Y-axis. A straight line curve should be obtained.

BENEDICT'S METHOD

Principle: This method is based on the biuret reaction which gives the colored complex of protein and copper ion in a strongly alkaline medium. The qualitative Benedict solution contains all the ingredients essential for the biuret reaction. Moreover, citrate present in it can prevent the precipitation of copper.

Material: Sodium citrate, sodium carbonate, copper sulfate, sodium hydroxide and colorimeter

Reagents: Benedict's qualitative reagent- 173 g of sodium citrate 100 g of sodium carbonate are dissolved in 800 ml of distilled water while heating. It is filtered and volume is made upto 850 ml. 17.3 g of copper sulfate is dissolved in 100 ml of water. This solution is added slowly with constant stirring to the carbonate-citrate solution and final volume is made upto 1 litre. The reagent is stored in brown bottle at room temperature and doesnot deteriorate on long standing 30% of sodium hydroxide in distilled water.

Method: 1 ml of serum or plasma is diluted 1 : 5 with normal saline. To this is added 4 ml of 1 : 10 diluted Benedict's reagent (diluted with distilled water) and 0.5 ml of 30% sodium hydroxide. After 30 minutes at room temperature, the color is read at 435 nm in a colorimeter.

11

FLUORESCENCE SPECTROSCOPY

INTRODUCTION

George Gabriel Stokes named the phenomenon fluorescence in 1852. Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation of a different wavelength. Energy is absorbed in the UV region of the spectrum and molecules are elevated from the ground state S_0 to a high energy level S_2. The excited molecules then return to the ground state with emission of visible light. In most cases, emitted light has a longer wavelength, and therefore lower energy, than the absorbed radiation. Aromatic compounds are capable of fluorescence, substituent ring is electron donating.

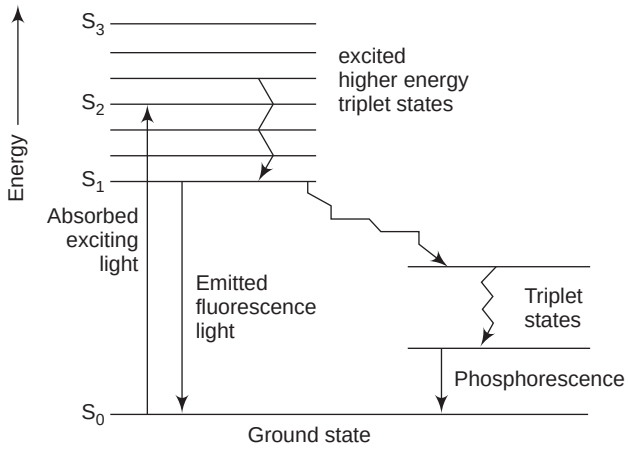


Fig. 11.1. Fluorescence and Phosphorescence Phenomena (Jablonski diagram)

Fluorescence is not so common as absorption, quenching decreases the quantum yield. Molecules containing Br, I, NO_2 and azo groups show little fluorescence because of *quenching*. The absorbed energy is used for competitive electronic transitions with excited molecules or for breaking weak bonds instead of being emitted as fluorescent light. Quenching also occurs by interaction with the solvent and other molecules in solution. The absorption and flouresent spectra of a

compound are quite characteristic so that, when the maxima are selected by filters or monochromators on the incident and emitted beams, the fluorescent compound can be detected and measured even when other fluorescent compounds are present. Flouresent compounds can be detected at very low concentrations and with a high degree of selectivity.

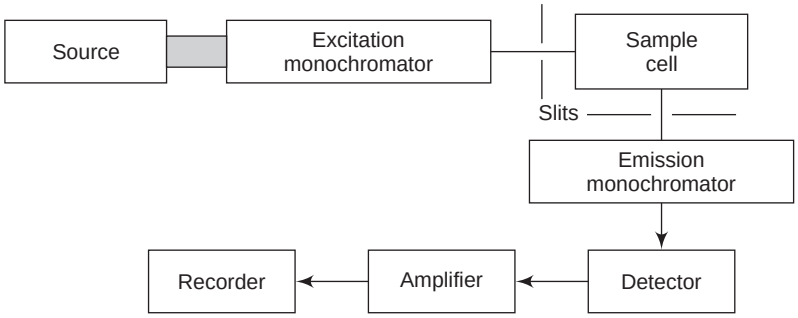


Fig. 11.2. A diagram of the arrangement of a Flourimeter

INTENSITY OF FLUORESCENCE AND CONCENTRATION

Fluorescence (F) depends on the intensity of light absorbed, so that if the intensity of the incident and emergent beams is I_o and I respectively, then:

$$F = K(I_o - I)$$

Now, Acc. To Beer's law

$$I = I_o e^{-kcl}$$

So that, $$I_o - I = I_o (1 - e^{-kcl})$$

therefore, $$F = KI_o (1 - e^{-kcl})$$

Expanding the exponential expression and assuming *c* to be small so that the higher terms can be ignored,

$$F = KI_o(kcl)$$

The constant K is known as the quantum yield and is the ratio of the number of quanta emitted to the number absorbed. For a particular compound and instrument when I_o, K and l are constant, the fourescence is directly proportional to concentration.

$$F = K'c$$

This equation holds in practice providing the solution absorbs less than 5 percent of the exciting radiation so that, the greater the intensity of the incident light, the higher is the concentration that gives a linear response. High light intensities also produce problems such as photodecomposition and light scattering. The useful concentration range for the determination of fluorescent compounds is 0.001-10 μg/ml depending on the material under investigation.

APPLICATIONS

Fluorescent compounds are used extensively in biochemical investigations as they can be detected at very low concentrations and with a high degree of selectivity. The absorption and fluorescent spectra of a compound are quite characteristic so that, when the maxima are selected by filters or

monochromators on the incident and emitted beams, the fluorescent compound can be detected and measured even when other fluorescent compounds are present. Some of the applications include the use of fluorescent compounds as membrane probes, substrates for sensitive enzyme assays and immunofluorescence.

FLOW CYTOMETRY

Flow cytometry is commonly used in the clinical laboratory to identify and enumerate cells bearing a particular antigen. Cells in suspension are labeled with a fluorescent tag by either direct or indirect immunofluorescence. The cells are then analyzed on the flow cytometer. In a flow cytometer, the cells exit a flow cell and are illuminated with a laser beam. The amount of laser light that is scattered off the cells as they passes through the laser can be measured, which gives information concerning the size of the cells. In addition, the laser can excite the fluorochrome on the cells and the fluorescent light emitted by the cells can be measured by one or more detectors.

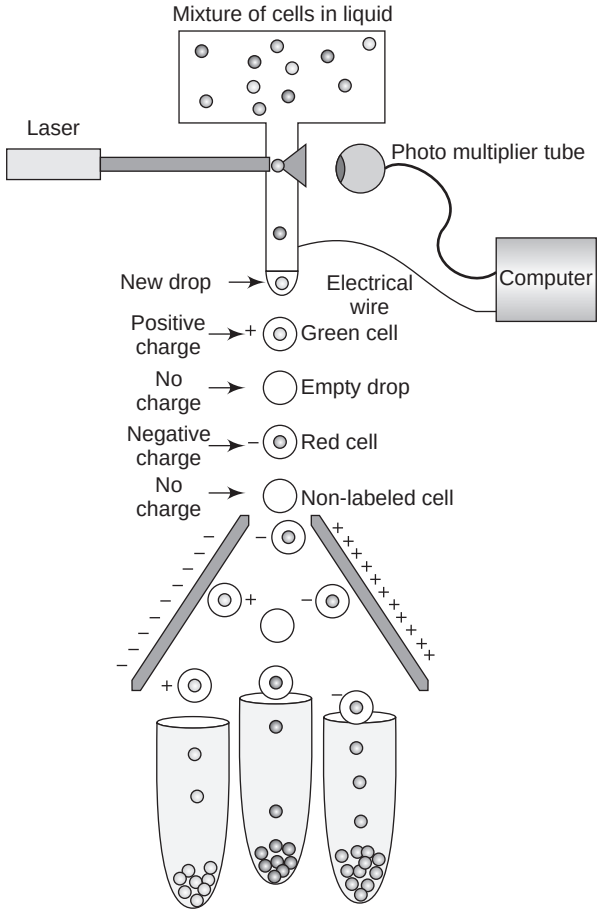


Fig. 11.3. Diagram showing cells population passing through Flow Cytometer

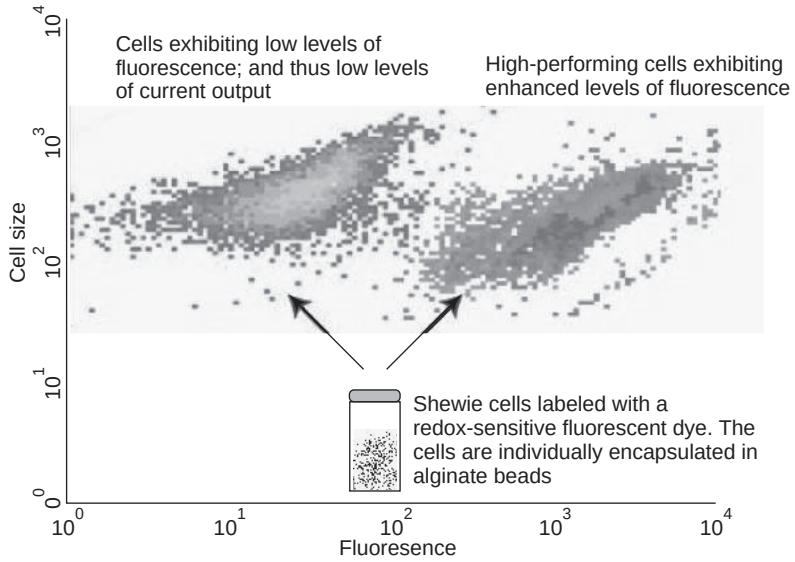


Fig. 11.4. Graph obtained by Fluorescence associated cell sorter (FACS)

Flow cytometry combines laser induced fluorometry and particle light scattering. Here different populations of molecules, cells or particles can be differentiated by size and shape using low and right angle scattering. These cells, molecules or particles can be labeled with different specific fluorosein labels such as β phycoerythrin, fluorescein isothiocyanate and rhodamine 6G as well as dye labeled antibodies. As they flow through the flow cell, simultaneous fluorescence and light scattering measurements are automatically performed by the flow cytometer. Most flow cytometers incorporate 2 or more fluorescence emission detector systems so that multiple fluorescent labels can be used. In this manner molecules, cells or particles can be classified according to light scattering or fluorescent properties.

Principle: Most commercial flow cytometers use a single argon ion laser (488 nm) which excites fluorescein isothiocyanate and phycoerythrin. An optical stop is placed in the 180° beam to block the main laser beam and permit low angle forward scatter measurements. The 90° emission signal is split and directed to 2 PM tubes to determine right angle light scattering and to detect at least 2 separate fluorescence signals. When excited, fluorescein isothiocyanate labeled cells emit green light (E_{max} = 530 nm) and phycoerythrin labeled cells emit orange light (E_{max} = 596 nm). Two narrow band pass interference filters (530 and 596 nm) are placed in front of these tubes. High performance computers with software are used to analyze the acquired data and to display as histograms for final reporting.

Clinical applications: Flow cytometers can measure multiple parameters including cell size (forward scatter), granularity (side scatter), DNA content, RNA content, DNA nucleotide (AT/CG) ratios, chromatin structure, antigens, total protein content, cell receptors, membrane potential and calcium ion concentration as a function of pH. These parameters are used in hematology, immunology (e.g. in T cell subsets, tissue typing, lymphocyte stimulation and antigen-antibody reaction), oncology (in diagnosis, prognosis and treatment monitoring), bacteriology (in bacterial identification and antibiotic sensitivity), virology, genetics (in karyotyping and carrier state detection), parasitology, reproduction and fertility studies and cervical cytology. Sensitive fluoroimmunoassays have been developed using phycoerythrin which is an excellent label for cells with a broad emission spectrum of 530-630nm with a low photodecomposition.

Hematology: Flow cytometers use cells stained with a supravital or fluorescent dye although unstained cells can also be measured. These cells travel in suspension one by one past a laser light source. Scattered and emitted light is collected in front and at right angles of the light source. Information derived by measuring light scatter when a cell is struck by a laser beam can be used to estimate cell shape, size, cellular granularity, nuclear lobularity and cell surface structure. 2 flow cytometry channels are used, one with peroxidase stained fixed cells and one with unstained or lysed cells. The porphyrin content of erythrocyte population has also been studied with flowcytometers. The distribution of fluorescent erythrocytes is strikingly altered in protoporphyria as compared with that in normal subjects.

Cytokines: intracellular detection of cytokines by flowcytometry enables the identification and quantification of intracellular cytokines in less than 2 hrs. this gives us very useful information about the frequency and cell surface phenotype of individual cytokine producing cell. Flow cytometry allows the rapid analysis of sufficiently large number of cells previously treated with fixative and permeabilizing agents. 3 or 4 color analysis (FL1, FL2, FL3 and FL4) are used for concomitant cellular identification using antigenic markers (e.g. FL1-CD3, FL2-CD4, FL3-CD8).

Organ transplantation: For immunological monitoring, flow cytometry is combined with fluorescence labeled monoclonal antibodies that are specific for cell subtypes. After labeling, subpopulations of lymphocytes (e.g. CD4, CD8 cells) can be measured in peripheral blood. During a rejection crisis, an increase in ratio of CD4 to CD8 cells is seen. Immunologically, recognition of foreign HLA causes an increase in CD4 lymphocytes. It is seen in heart transplant patients before rejection infection decreases the CD4/CD8 ratio. An increase in the ratio of 0.5 within a week predicts an acute rejection. Similarly transferring receptor positive lymphocyte measurement has been used, an increase in this population suggests rejection.

Urine analysis: Recently flowcytometric systems have been used for the characterization of erythrocyte in the differential diagnosis of hematuria. Flowcytometry can also investigate different urinary cells to discriminate between and quantify leucocytes, bacteria, sperm cells, yeast cells, casts and crystals. In the future, such automated systems may replace urinary microscopy.

Cell sorting: The isolation of individual cells or a population of cells from a mixed population is essential to a cell biologist. Flow cytometry is today used for such purposes. Cell sorting by cytometry relies on surface tension of cells in fluids causing separate droplets to form at a distinct break off point in a stream of liquid emerging from a nozzle. As the droplets pass through an electrostatic field, they separate according to charge. Droplets containing only the desired cells can be deflected by applying the voltage as a precise timed pulse which coincides with the cell passing through the beam.

FLUORESCENCE ACTIVATED CELL SORTING (FACS)

Fluorescence activated cell sorting (FACS) is more sophisticated as it measures the fluorescence emitted by individually labeled cells in a mixed population. FACS separates cells that contain preselected fluorescence characteristics by enclosing them inside droplets that can be deflected into reservoirs under influence of electrostatic field. Unselected cells fall into another reservoir. Fluorescence may be generated by dyes specific to cell components (e.g. propidium iodide for DNA) or from antibodies to surface antigens. The data is displayed as histograms. The procedure is very sensitive and is capable of measuring a single virus particle. It is also rapid so as to allow upto 5000 cells to be sorted per second into viable subpopulations.

12

PHOTOSYNTHESIS AND RESPIRATION

Photosynthesis and Respiration: Living organisms require a continous supply of energy to maintain the varied functions. In most cases, this energy is obtained by the oxidation of metabolites from the digestion of food. 3 ways in which this oxidation can occur and they involve loss of electrons from the compound being oxidized. The chemical energy in food which is released during oxidation comes ultimately from light energy of sun captured during photosynthesis. In chloroplasts, light drives the conversion of water to oxygen and $NADP^+$ to NADPH with transfer of H^+ ions across chloroplast membranes. **Photosynthesis** is the process by which light energy is used to synthesize carbohydrate in plants and algae from CO_2 and H_2O. The synthesis of carbohydrate takes place in two stages called as light and dark reactions. **Light reaction** occurs only in presence of light which is absorbed by the green pigment chlorophyll present in chloroplasts. The light reaction consists of removal of electrons from water (photolysis) and these are then used to reduce $NADP^+$ and generate ATP. There are 2 light driven reactions which takes place at reaction centers of photosystem I (PS I) and photosystem II (PS II) and operate in series. They consist of electron transport chains connected in zigzag Z- scheme. Dark reaction is the second stage of photosynthesis and doesnot require presence of light. This stage involves utilization of NADPH and ATP generated by light reaction to fix carbon dioxide. This reaction where CO_2 is converted to sugars was discovered by Calvin so it is called **Calvin cycle.**

Oxidation and reduction are always coupled together so, it is therefore more accurate to call it a redox reaction. All biological oxidations occur by removal of hydrogen from the substrate. Removed hydrogen atoms are then passed to an acceptor ($NAD^+/NADP^+$). In the aerobic cell, the reduction of NAD is followed by a series of electron transfers with eventual formation of water and generation of 3 adenosine triphosphate (ATP) molecules. E**lectron transport chain (ETC)** comprises an enzymatic series and couples electron transfer between an electron donor (NADH) and an electron acceptor (O_2) with the transfer of H^+ ions (protons) across a membrane. Each electron donor passes electrons to a more electronegative acceptor, which in turn donates these electrons to another acceptor, a process that continues down the series until electrons are passed to oxygen, the most electronegative and terminal electron acceptor in the chain. Passage of electrons between donor and acceptor releases energy, which is used to generate a proton gradient across the mitochondrial membrane by actively 'pumping' protons into the intermembrane space, producing

a thermodynamic state that has the potential to do work. The entire process is called **oxidative phosphorylation**, since ADP is phosphorylated to ATP using the energy of hydrogen oxidation in many steps. The resulting electrochemical proton gradient is used to generate chemical energy in the form of *ATP*. The underlying force driving these reactions is the Gibbs free energy of the reactants and products. The Gibbs free energy is the energy available ('free') to do work and any reaction which decreases the overall Gibbs free energy of a system is thermodynamically spontaneous. Protons flow back through the membrane causes mechanical work, like bacterial flagellar movement. ATP synthase enzyme converts this mechanical into chemical energy by producing ATP, which powers most cellular reactions. Substrate-level phosphorylation (glycolysis) and fermentation produces a small amount of ATP While, ETC produces majority of ATP. Eukaryotic cells contain mitochondria, which produce ATP from products of the citric acid cycle (TCA), fatty acid oxidation, and amino acid oxidation. At the mitochondrial inner membrane, electrons from NADH and succinate pass through the electron transport chain to oxygen, which is reduced to water.

In mitochondria, it is the conversion of oxygen to water, NADH to NAD^+ and succinate to fumarate that are required to generate the proton gradient. In **oxidative phosphorylation**, electrons are transferred from a high-energy electron donor (e.g., NADH) to an electron acceptor (e.g., O_2) through an electron transport chain. In **photophosphorylation**, the energy of sunlight is used to *create* a high-energy electron donor and an electron acceptor. Electron transport chains are major sites of premature electron leakage to oxygen, generating superoxide and potentially resulting in increased oxidative stress. Many compounds (Rotenone, Antimycin A and cyanide) are known to inhibit electron flow in the respiratory chain. Rotenone blocks electron flow between the nicotinamide nucleotides and flavoproteins; antimycin A acts at the cytochrome b/cytochrome c step; and cyanide inhibits cytochrome oxidase at the end of the respiratory chain.

Experiment: To isolate chloroplast from spinach leaves

Principle: The leaves are placed under strong illumination before homogenization as this reduces the starch grain content of the leaves and hence damage to the chloroplast during isolation. The leaves are then homogenized in buffered hypertonic saline sand the chloroplasts isolated by centrifugation.

Materials: Fresh spinach leaves; isolation medium (0.3 mol/litre NaCl, 3 mmol/litre $MgCl_2$, 0.2 mol/litre tricine pH7.6); double strength assay medium (0.2 mol/litre sorbitol, 6 mmol/litre $MgCl_2$, 0.4 mol/litre tricine pH7.6); waring blender and muslin; centrifuge in cold room.

Method: Remove the midribs from 100g of spinach leaves and cut the leaves into small sections. Place the leaf material in ice water under strong illumination for about 1h then homogenize with 100 ml of isolation medium in the waring blender. Filter the suspension through 6 layers of muslin and centrifuge the filtrate at maximum speed in a bench centrifuge in the cold room for 30s. discard the supernatant and wash the pellet carefully with single strength assay medium spinning at maximum speed for 1min. Resuspend the pellet in 2–3 ml of assay medium and store on ice until required.

THE OXYGEN ELECTRODE

The Oxygen Electrode is widely used in the biochemical laboratories to monitor processes or reactions involving oxygen exchange. The evolution of oxygen by illuminated chloroplasts or the

utilization of oxygen by respiring organisms and tissues can be readily followed. It consists of a platinum cathode and silver anode in saturated potassium chloride solution and when a potential is applied across the cell formed by these electrodes dipping in the test solution, oxygen is electrolytically reduced. 4 electrons are generated at anode which are then used to reduce a molecule of oxygen at cathode. If the polarizing voltage is in the range 0.5–0.8 V, then the current generated is proportional to the oxygen concentration in the medium. The amplified current is fed to a chart recorder which gives a trace of the change in oxygen concentration with time. Zero concentration is obtained by adding a crystal of sodium dithionate to the test solution and adjusting the pen to the baseline. The air-saturated buffer is taken to be 100% oxygen and the pen adjusted accordingly, in practice 100% oxygen is assumed to be 240 µmol of oxygen per litre, which is the solubility of oxygen in an aqueous solution at 26° C. If 4 ml is present in the reaction vessel then the total oxygen content is $4 \times 240 = 960$ nmol when saturated. For this reason it is best to adjust the pen on the recorder to 96 rather than 100. In these circumstances, one division on the chart recorder is then equivalent to 10nmol of O_2. Based on the Clark electrode design, the electrode is separated from the bulk of the solution by a membrane to prevent deposition of materials on the electrode surfaces which would otherwise interfere with the oxygen determination. The presence of a membrane does, however reduce the time response of the electrode to changes in oxygen concentration and this can be a nuisance since meaningful values of oxygen consumption can only be obtained when the response time of the electrode is greater than the rate at which oxygen can be consumed or evolved. This problem can be overcome to some extent by keeping the solution well stirred. The electrode assembly is enclosed in a plastic vessel of about 4 ml capacity surrounded by a water jacket maintained at a constant temperature and reagents are added through a small hole in the top. There is therefore negligible diffusion of oxygen into the test solution from the surrounding air. One disadvantage of the Clark modification is that some compounds like oligomycin become bound to the membrane and are very difficult to remove by washing. If this becomes a problem then the membrane has to be changed.

Experiment: The evolution of oxygen by isolated chloroplasts using Hill oxidants.

Principle: Electrons flowing from PSII towards PSI or from ferredoxin towards $NADP^+$, can be intercepted by artificial electron acceptors. This process is called Hill reaction and can be used to measure the photochemical activity of PSI and PSI acting in series or of PSII alone. The photochemical activity of PSI can be measured if the PSII activity is blocked by the powerful herbicide dichloromethylurea (DCMU) and the electron flow provided by an artificial donor.

Materials: Materials used in previous experiment (for the isolation of chloroplasts); freshly prepared potassium ferricyanide (10 mmol/litre); dichlorophenolindophenol (1 mmol/litre); dichlorophenyldimethylurea (250 µmol/litre); sodium dithionite; oxygen electrodes.

Method: Oxygen electrode assay: set up and calibrate the oxygen electrode in the usual way and after calibration, move the pen to the center of the chart paper with the zero control as oxygen evolution is being measured. Start each experiment by adding 2 ml of double strength assay medium to the electrode and sufficient chloroplasts to give an apple green suspension and a good rate of oxygen evolution in the experiment. This amount of chloroplasts should be maintained throughout the experiment. Add sufficient distilled water and additions to give a final volume of 4 ml of single strength assay medium. The assays should be set up in dark by surrounding the electrode with aluminum foil; the rate of oxygen evolution is monitored in the light following the addition of cofactors, inhibitors etc. The chloroplast suspension can be conveniently illuminated by a 100 watt bulb focused roughly through a large round bottomed flask filled with water.

HILL OXIDANTS

Hill Oxidants: Test the effectiveness of ferricyanide and DCPIP as Hill oxidants. Add increasing amounts of the oxidants recording both the rate and the total amount of O_2 evolved at each concentration. Plot the results as they are recorded. Finally, add sufficient DCPIP to ensure sustained O_2 evolution and test the effect of 50 ìl of DCMU.

Experiment: The fractionation of rat liver

Principle: Rat liver fractionation done for separating the subcellular particles.

Materials: Isolation medium (0.25 mol/litre sucrose; 5 mmol/litre tri-HCl buffer pH 7.4; 0.1 mmol/litre EDTA); rats; coaxial homogenizers; ice baths, ultracentrifuges.

Method: Sacrifice the rat and remove the liver. Wash the tissue free of blood in ice cold sucrose, lightly blot and place in a petri plate to weigh. Cut the liver into small pieces and homogenize in sucrose (20 g/100 ml) at 2000 rev/min by moving the mortar relative to the pestle for 8–10 complete strokes. Centrifuge the suspension in a refrigerated centrifuge. Ideally each fraction should be resuspended in sucrose and the washings combined with supernatants. This has the advantage of producing purer fractions, but the disadvantage of introducing an increasing dilution of the cellular components. Carefully resuspend the pellets in about 10 ml of sucrose and store on ice until required.

Experiment: The respiration of mitochondria and oxidative phosphorylation.

Principle: When mitochondria are carefully isolated and suspended in an isotonic medium in the presence of substrate, only a slow rate of respiration is observed. On adding ADP, the respiration rate increases until all the ADP is phosphorylated, when the rate of respiration returns to original slow rate. The quantity of ADP that is added is known and the amount of oxygen consumed is measured (X) so it is possible to arrive at a P/O ratio for the particular substrate used. A typical trace of oxygen consumption with time where A is rate of respiration in presence of substrate and B the rate of phosphorylating respiration. The ratio of these rates is called respiratory control ratio and is a measure of the degree of coupling of respiration and phosphorylation. A low ratio indicates loose coupling, but freshly prepared mitochondria shows a high respiratory control of 4 or more.

Calculations: Respiratory control ratio = Rate B/Rate A

P/O ratio = µmol ADP added /µ atoms O_2 utilized (X)

The phosphorylation sites in the ETC show that substrates using $NADH_2$ as coenzyme should give a P/O ratio of 3 while succinate, which bypasses the first phosphorylation site, should give a ratio of 2. The mixture of ascorbate and tetramethylphenylenediamine (TMPD) uses cytochrome C as electron acceptor and thereby misses two of the phosphorylation sites so that the P/O ratio for this substrate is 1. Even in freshly prepared mitochondria, the P/O ratios actually found are always less than the whole number values given above due to partial uncoupling and the action of the membrane ATPases.

Materials: Materials for the isolation of rat liver mitochondria; Isolation medium (0.3 mol/litre sucrose, 2.5 mmol/litre tris-HCl pH 7.4; 0.5 mmol/litre EDTA); Incubation medium (Sucrose 150 mmol/litre, potassium chloride 20 mmol/litre), magnesium chloride 20mmol/litre, potassium

phosphate 1 mmol/litre pH7.4); sodium malate 200 mmol/litre pH7.4; sodium succinate 200 mmol/litre pH7.4; Ascorbate-TMPD (200 mmol/litre ascorbate pH 7.4 containing 5 mmol/litre tetramethylphenylenediamine freshly prepared and stored in dark); freshly prepared ADP (20 mmol/litre pH7.4)

Method: As with previous experiment using the oxygen electrode, make any additions to the mitochondrial suspension in small volumes only (20–100 µl) so that the total volume in the electrode compartment remains close to 4 ml. Prepare a fresh suspension of rat liver mitochondria and store on ice until required. Set up and calibrate the oxygen electrode and add a small volume of mitochondrial suspension (50–100 µl) followed by succinate and ADP in that order. Calculate the P/O ration and the respiration control index; repeat the experiment using malate and ascorbate/TMPD as the substrate.

Experiment: To study the effect of inhibitors on the respiratory chain

Principle: Many compounds are known to inhibit electron flow in the respiratory chain and the sites at which they act can be identified by examining the effect of these inhibitors on respiration induced by substrates considered in previous experiment.

Materials: Materials as in previous experiment; rotenone (1 mmol/litre in 95% v/v ethanol); Antimycin A (0.1 mg/ml in 95% v/v ethanol); potassium cyanide (10 mmol/litre)

Method: Repeat the previous experiment, then examine the effect on the oxygen uptake of adding a small volume (10–50 µl) of one of the inhibitor solutions. Take each of the three substrates in turn and record the changes in mitochondrial respiration induced by each of these inhibitors.

13

ENZYMES

Living organisms obtain and use energy very rapidly because of the presence of biological catalysts called enzymes. Like inorganic catalysts, enzymes change the rate of a chemical reaction but donot affect the final equilibrium; also small quantities are needed to carry out the reaction. But unlike inorganic catalysts, enzymes have a very narrow specificity, i.e. they can catalyse only a small range of reactions. Also, enzymes function under well-defined conditions of pH, temperature, substrate concentration, cofactors. Enzymes are named and classified according to the type of reaction catalysed. Main groups: Oxido-reductases, transferases, hydrolases, lyases, isomerases, ligases. In biochemistry, the **Lineweaver–Burk plot** (or **double reciprocal plot**) is a graphical representation of the Lineweaver–Burk equation of enzyme kinetics, described by Hans Lineweaver and Dean Burk in 1934.

ENZYME INHIBITION

Many compounds react with enzymes and reduce the measured activity. This property of enzymes is used in designing drugs and insecticides which selectively inhibit enzymes in the infective bacteria or insects, but donot affect the animal or plant. 2 types of inhibition: competitive and non-competitive. **Competitive inhibition,** the inhibitor (structure similar to substrate) reacts with the enzyme by competing with the substrate for the active site. The degree of inhibition depends on the relative concentrations of substrate and inhibitor and almost maximum velocity may be found in the presence of inhibitor if the substrate concentration is high enough. For example, enzyme succinate dehydrogenase, which catalyses the conversion of succinate to fumarate. Malonate acts as competitive inhibitor to this enzyme.

Non-competitive inhibition, the inhibitor combines with the enzyme but not at the active site so the enzyme can bind both substrate and inhibitor at the same time. The enzyme-substrate-inhibitor complex formed is unable to breakdown and inhibition occurs by reduction of the amount of enzyme available. Increase of the substrate concentration has no effect on the degree of inhibition. For example, thiol blocking agents like p-chlormercuribenzoate, heavy metal ions like Ag^{+}, Cu^{++} and reaction of cyanide with iron-porphyrin enzymes.

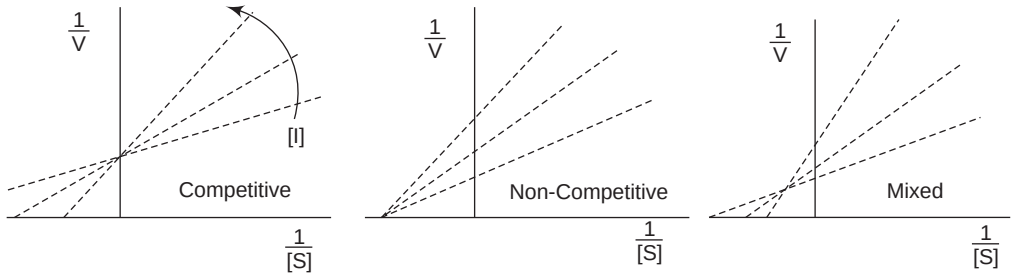


Fig. 13.1. Competitive, non-competitive and mixed inhibition

Effect of temperature on enzyme activity can be determined by exposing the enzyme to a high temperature for different periods of time, then measuring the activity at a temperature at which the enzyme is stable. This will tell us how much of the enzyme has been destroyed and a graph can be prepared of the rate of loss of active enzyme with time. This is repeated for a number of different temperatures and the initial rate plotted against 1/T. The energy of activation is very high due to large positive entropy change resulting from the unfolding of the molecule during denaturation.

Effect of pH: Enzymes are active over a limited pH range only and a plot of activity against pH usually gives a bell shaped curve. The variation of activity with pH is due to the change in the state of ionization of the enzyme protein and other components of the reaction mixture. Michaelis and Davidson in 1911 suggested that only one of the large number of ionized forms of the protein is active, so that a change in pH either side of the optimum produces a decrease of this form and hence a fall in the activity.

Applications: The Lineweaver–Burk plot used to determine K_m and V_{max}, before the availability of powerful computers and non-linear regression software. The *y*-intercept of such a graph is equivalent to the inverse of V_{max}; the *x*-intercept of the graph represents "$1/K_m$. It also gives a quick, visual impression of the different forms of enzyme inhibition. The double reciprocal plot distorts the error structure of the data, and it is therefore unreliable for the determination of enzyme kinetic parameters. Although it is still used for representation of kinetic data, non-linear regression or alternative linear forms of the Michaelis–Menten equation such as the Hanes-Woolf plot or Eadie–Hofstee plot are generally used for the calculation of parameters.

When used for determining the type of enzyme inhibition, the Lineweaver–Burk plot can distinguish competitive, non-competitive and uncompetitive inhibitors. Competitive inhibitors have the same *y*-intercept as uninhibited enzyme (since V_{max} is unaffected by competitive inhibitors the inverse of V_{max} also doesn't change) but there are different slopes and *x*-intercepts between the two data sets. Non-competitive inhibition produces plots with the same *x*-intercept as uninhibited enzyme (K_m is unaffected) but different slopes and *y*-intercepts. Uncompetitive inhibition causes different intercepts on both the *y*- and *x*-axes but the same slope.

Limitations: The Lineweaver–Burk plot is prone to error, as the *y*-axis takes the reciprocal of the rate of reaction in turn increasing any small errors in measurement. Also, most points on the plot are found far to the right of the *y*-axis (due to limiting solubility not allowing for large values of [S] and hence no small values for 1/[S]), calling for a large extrapolation back to obtain *x*- and *y*-intercepts.

Experiment: The effect of temperature on the activity of α-amylase.

Principle: α-amylase catalyses the hydrolysis of α-1-4 links of starch with the production of reducing sugars. The reaction is followed by measuring the increase in reducing sugars using the 3, 5-dinitrosalicylate reagent when an alkaline solution of 3, 5-dinitrosalicylic acid is reduced to 3-amino-5 nitrosalicylic acid. The reaction is followed by measuring the extinction at 540 nm.

Materials: sodium or potassium phosphate buffer (0.1 mol/litre, pH6.7); buffered starch substrate (5g/litre in phosphate. Mix 5 g of soluble starch to a smooth paste with about 50 ml of buffer solution. Add this quantitatively to 500 ml of boiling phosphate buffer solution, continue to boil for 1min, then cool to room temperature and dilute to 1 litre with buffer solution); sodium chloride (10 g/litre); diluted α-amylase; sodium hydroxide (2 mol/litre); colorimeter; water baths at a range of temperatures upto 75°C, dinitrosalicylate reagent

Method:

(*i*) Determination of the 'energy of activation'- set up 8 tubes containing the following reaction mixture: Starch 5 g/litre (2.5 ml); phosphate buffer 0.1 mol/litre (1.0 ml); sodium chloride 10 g/litre (0.5 ml) Place the tubes in a water bath fixed temperature and equilibrate the enzyme at the same temperature. After 10 min add 0.5 ml of the enzyme to 7 of the tubes and 0.5 ml of water to the blank. Incubate the tubes for 0, 5, 10, 15, 20, 30 and 40 min and stop the reaction by adding 0.5 ml of 2 mol/litre NaOH to all tubes. Add 0.5 ml of the dinitrosalicylate reagent and heat the tubes for 5 min in a boiling water bath, cool, and read the extinction at 540 nm against a blank. The tubes must all be cooled to room temperature before reading since the extinction is sensitive to temperature change.

Plot the extinction of each solution against the time of incubation and prepare a progress curve of the reaction. Collect the data and plot a graph of the change in the initial reaction rate against temperature and also the substrate changed per minute at 10 and 40 min against temperature. Compare these graphs and explain them.

(*ii*) Temperature and enzyme stability: prepare a suitable dilution of the α-amylase so as to give a good activity when incubated at 37°C for 5 min. place the enzyme in a boiling tube and equilibrate for 10 min. pipette 0.5 ml of α-amylase into 4 test tubes in a water bath maintained at a fixed temperature from 50 to 75°C. Remove the tubes after 5, 10, 15, 20 min and rapidly cool under running tap water. Incubate the tubes at 37°C and assay for enzyme activity after the addition of the substrate as in previous experiment. The mixture is incubated for 5min and the activity determined with 3, 5-dinitrosalicylate. The rate of inactivation of the enzyme can then be determined. Collect the data and prepare a graph of the rate of inactivation (v) against 1/T and determine the heat of inactivation.

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LIPIDS

Lipids are naturally occurring esters of long chain fatty acids, insoluble in water but soluble in acetone, alcohol, chloroform and ether. Saponification or alkaline hydrolysis gives alcohol and the sodium or potassium salts of the constituent fatty acids, these hydrolysis products may be water soluble. Classification into simple and compound lipids. **Simple lipids** or **acylglycerols** are esters of glycerol and fatty acids to give mono-, di- and triglycerides. Triglycerides are the predominant form in nature. A **triglyceride** (**TG**, **triacylglycerol**, **TAG**, or **triacylglyceride**) is an ester derived from glycerol and three fatty acids and type depends on the oil source vegetable oil (unsaturated) and animal fats (saturated).

In humans, unused calories are stored as triglycerides and their concentration in blood increases with the consumption of high carbohydrate foods. In the intestine enzyme Pancreatic lipase acts and splits the triglycerides into monoacylglycerol and free fatty acids by **lipolysis.** TGs are rebuilt in the enterocytes from their fragments and packaged together with cholesterol and proteins to form chylomicrons. These are excreted from the cells and collected by the lymph system and transported to the large vessels near the heart before being mixed into the blood. Various tissues can capture the chylomicrons, releasing the TGs to be used as a source of energy. Fat and liver cells can synthesize and store triglycerides. When the body requires fatty acids as an energy source, the hormone glucagon signals the breakdown of the triglycerides by activating lipase to release free fatty acids. As the brain cannot utilize fatty acids as an energy source (unless converted to a ketone), the glycerol component of triglycerides can be converted into glucose, via glycolysis by conversion into Dihydroxyacetone phosphate and then into Glyceraldehyde 3-phosphate, for brain fuel when it is broken down. So, if the brain's needs become high then, fat cells may also be broken down. TGs, as components of **very-low-density lipoprotein** (VLDL) and chylomicrons, play an important role in metabolism as energy sources and transporters of dietary fat. In the human body, high levels of TGs in the bloodstream can cause atherosclerosis, the risk of heart disease and stroke. The risk can be due to inverse relationship between TG level and HDL-cholesterol level.

Chylomicrons are lipoprotein particles that consist of triglycerides, phospholipids, cholesterol and proteins. They transport dietary lipids from the intestines to other locations in the body.

Chylomicrons enable fats and cholesterol to move within the water-based solution of the bloodstream and transport exogenous lipids to liver, adipose, cardiac, and skeletal muscle tissue, where their TGs are unloaded by lipoprotein lipase. As a consequence, chylomicron remnants are left over and are taken up by the liver. **Saturated fats** are 'saturated' with hydrogen at all available places where hydrogen atoms could be bonded to carbon atoms. Saturated fats have a higher melting point and are more likely to be solid. **Unsaturated fats** have double or triple bonds between carbon atoms and have a lower melting point so mostly liquid. Fatty acids, triglycerides, lipoproteins staining is done by lysochromes (fat-soluble dyes) examples: Sudan IV, Oil Red O, and Sudan Black B.

QUANTITATIVE ANALYSIS OF LIPIDS

A complete analysis of a naturally occurring fat is quite a lengthy procedure, but there are a number of measurements such as acid value, the saponification number and the iodine number, which gives useful information on the composition and purity of a particular fat.

Experiment: The determination of the acid value of a fat.

Principle: During storage, fats may become rancid as a result of peroxide formation at the double bonds by atmospheric oxygen and hydrolysis by micro-organisms with the liberation of free acid. The amount of free acid present therefore gives an indication of the age and quality of the fat. The acid value is the number of milligrams of KOH required to neutralize the free fatty acid present in 1g of fat.

Materials: olive oil, butter, margarine (use a fresh sample and one that has been stored at room temperature for several days); fat solvent (equal volumes of 95% v/v alcohol and ether neutralized to phenolphthalein); phenolphthalein (10 g/litre in alcohol); potassium hydroxide (0.1 mol/litre or 5.6 g/litre); burettes (5 and 25 ml).

Method: Weigh 10 g of the test compound and suspend the melted fat in about 50 ml of fat solvent. Add 1 ml of phenolphthalein solution, mix and titrate with 0.1 mol/litre KOH until the faint pink color persists for 20–30s. note the number of mililitres of standard alkali required and calculate the acid value of the fat.

Experiment: To estimate the saponification value of a fat.

Principle: On refluxing with alkali, glyceryl esters are hydrolysed to give glycerol and the potassium salts of the fatty acids (soaps). The saponification value is the number of milligrams of KOH required to neutralize the fatty acids resulting from the complete hydrolysis of 1 g of fat. The saponification value gives an indication of the nature of fatty acids in the fat since the longer the carbon chain the less acid is liberated per gram of fat hydrolysed.

Materials: Fats and oils (coconut oil, corn oil, butter); fat solvent (equal volumes of 95% ethanol and ether); alcoholic KOH (0.5 mol/litre); reflux condenser; boiling water bath; phenolphthalein (10 g/litre in alcohol); HCl (0.5 mol/litre); burettes (10 ml and 25 ml); conical flasks (250 ml)

Method: Weigh 1 g of fat in a beaker and dissolve in about 3 ml of the fat solvent. Quantitatively transfer the contents of the beaker to a 250 ml conical flask by rinsing the beaker 3 times with a further milliliter of solvent; add 25 ml of 0.5 mol/litre alcoholic KOH and attach to a reflux condenser. Set up another reflux condenser as blank with everything present except the fat and heat

both flasks on a boiling water bath for 30min. leave to cool to room temperature and titrate with 0.5 mol/litre HCl and phenolphthalein indicator.

The difference between the blank and test reading gives the number of mililitres of 0.5 mol/litre KOH required to saponify 1 g of fat. The molecular weight of KOH is 56 and since 3 molecules of fatty acid are released from a triglyceride, then: saponification value (S) = $3 \times 56 \times 1000$/average mol.wt of fat.

Average mol/wt of fat = $3 \times 56 \times 1000/S$

Experiment: The iodine number of a fat.

Principle: Halogens add across the double bonds of unsaturated fatty acids to form addition compounds. Iodine monochloride (ICl) is allowed to react with the fat in the dark. The amount of iodine consumed is then determined by titrating the iodine released (after adding KI) with standard thiosulfate and comparing with a blank in which the fat is omitted. The reaction mixture is kept in the dark and the titration carried out as quickly as possible since halogens are oxidized in the light. The quantity of material is calculated assuming that each pair of students will assay two of the fats. The iodine number is the number of grams of iodine taken up by 100 g of fat.

Materials: Fats (20 g/litre solutions of corn oil, olive oil, linseed oil and butter in chloroform); Iodine monochloride (0.2 mol/litre); potassium iodide (100 g/litre); sodium thiosulfate (0.1 mol/litre); starch indicator (10 g/litre); stoppered bottles (250 ml); burette (25 ml); chloroform

Method: Pipette 10 ml of fat solution into a stoppered bottle, add 25 ml of ICl solution, stopper the bottle, and leave to stand in the dark for 1h, after shaking thoroughly, at the same time, set up a blank in which the fat is replaced by 10 ml of chloroform. Rinse the stoppers and necks of the bottles with about 50 ml of water, add 10 ml of the KI solution and titrate the liberated iodine with the standard thiosulfate. When the solution is a pale straw color, add about 1 ml of starch solution and continue titrating until the blue color disappears. The bottles must be shaken thoroughly throughout the titration to ensure that all the iodine is removed from the chloroform layer. The difference between the blank and test readings (Bl-T) gives the number of ml of 0.1 mol/litre thiosulfate needed to react with the equivalent volume of iodine. This is (Bl-T)/2 ml of 0.1 mol/litre iodine since 2 molecules of thiosulfate are needed for each iodine.

The mol.wt of iodine is 2×127 so the weight of iodine in (Bl-T)/2 ml of 0.1 mol/litre iodine is:

$(Bl\text{-}T)/2 \times 0.1 \times 2 \times 127/1000$ g

The amount of fat taken was 0.2 g so that the iodine number is:

$(Bl\text{-}T) \times 12.7/1000 \times 100/0.2$

Iodine number = $(Bl\text{-}T) \times 6.35$ g/100 g of fat.

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CARBOHYDRATES

A **carbohydrate** is an organic compound that consists only of carbon, hydrogen, and oxygen, usually with a hydrogen : oxygen atom ratio of 2 : 1 (as in water) with the empirical formula $C_m(H_2O)_n$. (Exception deoxyribose, a component of DNA, has empirical formula $C_5H_{10}O_4$.) Carbohydrates are not hydrates of carbon but it is more accurate to view them as polyhydroxy aldehydes and ketones. **Carbohydrates/Saccharides** are divided into four classes: monosaccharides, disaccharides, oligosaccharides, and polysaccharides. Monosaccharides and disaccharides are smaller (lower molecular weight) carbohydrates and are commonly referred to as sugars. Polysaccharides serve for the storage of energy (e.g., starch and glycogen), and as structural components (e.g., cellulose in plants and chitin in arthropods) and play key roles in the immune system, fertilization, preventing pathogenesis, blood clotting, and development. The 5-carbon monosaccharide ribose is an important component of coenzymes (e.g., ATP, FAD, and NAD) and the backbone of the genetic molecule known as ribose in RNA and deoxyribose in DNA. Monosaccharides are the simplest carbohydrates and cannot be hydrolyzed to smaller carbohydrates. They are aldehydes or ketones with two or more hydroxyl groups. Monosaccharides are important fuel molecules as well as building blocks for nucleic acids, as $n = 3$, are dihydroxyacetone and D- and L-glyceraldehydes.

Monosaccharides are classified according to three different characteristics: the placement of its carbonyl group, the number of carbon atoms it contains, and its chiral handedness. If the carbonyl group is an aldehyde, the monosaccharide is an aldose; if the carbonyl group is a ketone, the monosaccharide is a ketose. Monosaccharides with three carbon atoms are called trioses, those with four are called tetroses, five are called pentoses, six are hexoses, and so on. Glucose is an aldohexose (a six-carbon aldehyde), ribose is an aldopentose (a five-carbon aldehyde), and fructose is a ketohexose (a six-carbon ketone). Each carbon atom bearing a hydroxyl group (—OH), with the exception of the first and last carbons, are asymmetric, making them stereo centers with two possible configurations each (R or S). Because of this asymmetry, a number of isomers may exist for any given monosaccharide formula. The aldohexose D-glucose, for example, has the formula $(C{\cdot}H_2O)_6$, of which all but two of its six carbons atoms are stereogenic, making D-glucose one of $2^4 = 16$ possible stereoisomers. In the case of glyceraldehydes, an aldotriose, there is one pair of possible stereoisomers, which are enantiomers and epimers. 1, 3-dihydroxyacetone, the ketose

corresponding to the aldose glyceraldehydes, is a symmetric molecule with no stereo centers). The assignment of D or L is made according to the orientation of the asymmetric carbon furthest from the carbonyl group: in a standard Fischer projection if the hydroxyl group is on the right the molecule is a D sugar, otherwise it is an L sugar. The 'D-' and 'L-' prefixes should not be confused with '*d*-' or '*l*-', which indicate the direction that the sugar rotates plane polarized light. This usage of '*d*-' and '*l*-' is no longer followed in carbohydrate chemistry.

Two joined monosaccharides are called a disaccharide and these are the simplest polysaccharides. Examples include sucrose and lactose. They are composed of two monosaccharide units bound together by a covalent bond known as a glycosidic linkage formed via a dehydration reaction, resulting in the loss of a hydrogen atom from one monosaccharide and a hydroxyl group from the other. The formula of unmodified disaccharides is $C_{12}H_{22}O_{11}$. Sucrose the most abundant disaccharide, and the main form in which carbohydrates are transported in plants. It is composed of one D-glucose molecule and one D-fructose molecule. Lactose, a disaccharide composed of one D-galactose molecule and one D-glucose molecule, occurs naturally in mammalian milk. Other disaccharides include maltose (two D-glucoses linked α-1, 4) and cellulobiose (two D-glucoses linked β-1,4). Disaccharides classified into two types: reducing and non-reducing disaccahrides if the functional group is present in bonding with another sugar unit it is called a reducing disaccharide or biose.

OPTICAL ACTIVITY

The Polarimeter: Most sugars contain one or more asymmetric carbon atoms and show optical activity. This rotation of the plane polarized light can be demonstrated and measured with a polarimeter. Monochromatic light passes through a nicol prism and emerges polarized in one plane. This polarized beam then passes through the sugar sample which rotates the plane of the light. The second nicol prism is rotated until its plane of polarization lies at right angles to that of the first prism and the light beam is prevented from passing through the instrument. Alternatively, the second prism is rotated so that it corresponds with the plane of polarization produced by the first prism to give a field of maximum brightness. The instrument is zeroed on either of these positions with only solvent in the sample chamber. In practice, the emergent light is seen as two semicircular zones and the zero is obtained when these two halves of the field of view are of uniform darkness or brightness. The solvent is then replaced with the solution to be investigated and the analyser rotated to restore the situation of minimum or maximum brightness. The degree and direction of the rotation are then recorded. If the angle of rotation is clockwise then the compound is dextrorotatory (+) and if anticlockwise then the sugar is levorotatory (–).

Specific rotation: The degree of rotation recorded depends on a number of factors including the length of the light path (l dm) and the concentration of the solute (c g/ml) as well as the temperature (t° C) and the wavelength of the light used (λ nm). The specific rotation is characterstic of a particular compound and is defined as the rotation of monochromatic light caused by 1 g/ml of optically active solute in a 1 dm tube at a fixed temperature. Mostly polarimeters use sodium lamps ($\lambda = 589$ nm) and observations are generally made at 20° C.

$$\text{Specific rotation } [\alpha] = \alpha/(l \times c)$$

Mutarotation is the change in the optical rotation that occurs by epimerization (that is the change in the equilibrium between two epimers, when the corresponding stereocenters interconvert). Cyclic sugars show mutarotation as α and β anomeric forms interconvert. Mutarotation was

discovered by French chemist Dubrunfaut in 1846, when he noticed that the specific rotation of an aqueous sugar solution changes with time. The α and β anomers are diastereomers of each other and usually have different specific rotations. A solution of a pure α anomer will rotate plane polarised light by a different amount and/or in the opposite direction than the pure β anomer of that compound. The optical rotation of the solution depends on the optical rotation of each anomer and their ratio in the solution. For example if a solution of β-D-glucopyranose is dissolved in water, its specific optical rotation will be +18.7. Over time, some of the β-D-glucopyranose will undergo mutarotation to become α-D-glucopyranose, which has an optical rotation of +112.2. Thus the rotation of the solution will increase from +18.7 to an equilibrium value of +52.5 as some of the β form is converted to the α form. The equilibrium mixture is actually about 64% of β-D-glucopyranose and about 36% of α-D-glucopyranose, though there are also with traces of the other forms including furanoses and open chained form. The observed optical rotation of the sample is the weighted sum of the optical rotation of each anomer weighted by the amount of that anomer present. Therefore one can use a polarimeter to measure the rotation of a sample and then calculate the ratio of the two anomers present from the enantiomeric excess, as long as one knows the rotation of each pure anomer. One can monitor the mutarotation process over time or determine the equilibrium mixture by observing the optical rotation and how it changes.

Experiment: The mutarotation of glucose

Principle: D-glucose can be crystallized in either the α or β form and freshly prepared solutions of these anomers have specific rotations of +113° and +19° respectively. On standing, these solutions show mutarotaion and an equilibrium mixture of the α and β forms is obtained with a specific rotation of +52.5°

Materials: polarimeter, α-D-glucose, β-D-glucose, sodium carbonate (0.1 mol/litre), stop clock.

Method: Mutarotation in distilled water- Rinse the polarimeter tube with distilled water and fill completely with water. Add the last few drops with a Pasteur pipette and screw on the cap carefully to ensure that no air bubbles are trapped. Adjust the instrument to zero with the tube filled with water in the polarimeter. Empty the polarimeter tube and thoroughly dry it. Carefully transfer 5 g of α-D-glucose to a dry 50 ml flask, add 40 ml of water to dissolve the glucose, and make up to the mark with distilled water. Rapidly mix the solution and fill the polarimeter tube with the glucose; obtain a reading for the rotation as soon as possible and start the stop clock. Measure the rotation every 10 min for the next 30 min and then at longer time intervals until a constant value is obtained. Repeat the experiment with β-D-glucose and plot a graph of the change in specific rotation with time.

Mutarotaion in alkali: repeat the above experiment with α and β forms of D-glucose but this time add 1 ml of Na_2CO_3 solution (0.1 mol/litre) before making upto the mark. Obtain the first reading as soon as possible and thereafter take readings every 2 min for the first 10min then at longer time intervals as appropriate until no further change is observed. Plot a graph of change in mutarotation with time.

Experiment: Benedict's test for reducing sugars

Principle: If a suspension of copper hydroxide in alkaline solution is heated, then black cupric oxide is formed. However, if a reducing substance is present, then rust brown cuprous oxide is precipitated. In practice, an alkaline solution of copper salt and an organic compound containing alcoholic –OH is used rather than the above suspension. Under these conditions, it forms a soluble

complex and the reagent is stable. Carbohydrates with a free aldehyde or ketone group have reducing properties in alkaline solution. In addition, monosaccharides act as reducing agents in weakly acid solution. Benedict modified the original fehling's test to produce a single solution which is more convenient for tests as well as being more stable, than fehling's solution.

Material: Benedicts reagent (dissolve 17.3 g of sodium citrate and 100 g sodium carbonate in 800 ml of water. Filter through filter paper into a 1000 ml measuring cylinder and make up to 850 ml with water. Dissolve 17.3 g copper sulphate in 100 ml of water and make up to 150 ml. Pour the first solution into 2 litre beaker and slowly add the copper sulphate solution with stirring), glucose solutions (10 g/litre and 1 g/litre)

Method: Add 5 drops of the test solution to 2 ml of Benedicts reagent and place in a boiling water bath for 5 min. Examine the sensitivity of Benedict's test using increasing dilutions of glucose.

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Cell Membranes

The **cell membrane** or **plasma membrane** is a biological membrane separating the interior of all cells from the outside environment. Most of the lipid present in the membranes consists of phospholipid molecules associated together in a regular manner. This structural organization arises from the fact that phospholipids have hydrophobic and hydrophilic regions in the same molecule so also called amphipathic. Most phospholipids are also zwitterions since the phosphate group carries a negative charge at neutral pH and the base a positive charge (choline phosphoglyceride, ethanolamine phosphoglyceride) or a positive and negative charge (serine phosphoglyceride). The polar region of the phospholipids hydrophilic and seeks an aqueous environment, while the hydrophobic part of the molecule tends to increase its entropy by expelling water from its vicinity and associating together with hydrophobic regions of other molecules. Phospholipids thus orient themselves on the surface of an aqueous solution so that the polar region lies in water and the alkyl side chain in the air.

The cell membrane basically protects the cell from outside forces. It consists of the lipid bilayer with embedded proteins. Cell membranes are involved in a variety of cellular processes such as cell adhesion, ion conductivity and cell signaling and serve as the attachment surface for several extracellular structures, including the cell wall, glycocalyx, and intracellular cytoskeleton. The cell membrane also plays a role in anchoring the cytoskeleton to provide shape to the cell, and in attaching to the extracellular matrix and other cells to help group cells together to form tissues. The membrane is selectively permeable and able to regulate what enters and exits the cell, thus facilitating the transport of materials needed for survival. The movement of substances across the membrane can be either 'passive', occurring without the input of cellular energy, or active, requiring the cell to expend energy in transporting it. The membrane also maintains the cell potential. The cell membrane thus works as a selective filter that allows only certain things to come inside or go outside the cell. Cell employs a various transport mechanisms involving biological membranes:

1. **Passive diffusion and osmosis:** Small molecules, ions such as carbon dioxide (CO_2), oxygen (O_2), and water, move across the plasma membrane by passive diffusion. As the membrane acts as a barrier for certain molecules and ions, this can cause different concentrations on the two sides of the membrane and set up an osmotic flow for the water. The permeability of a

membrane is the rate of passive diffusion of molecules through the membrane. Permeability depends on the electric charge, polarity of the molecule and the molar mass of the molecule. Due to the cell membrane's hydrophobic nature, small electrically neutral molecules pass through the membrane easier than charged, large ones. The inability of charged molecules to pass through the cell membrane results in pH partition of substances throughout the fluid compartments of the body.

2. **Nutrients:** like sugars or amino acids, must enter the cell, and metabolism products must leave the cell. Such molecules are pumped across the membrane by transmembrane transporters or diffuse through Permeases protein channels.
3. **Endocytosis:** Endocytosis is a pathway for internalizing solid particles (cell eating or phagocytosis), small molecules and ions (cell drinking or pinocytosis), and macromolecules and it is an active process in which cells absorb molecules by engulfing them by means of formation of a small plasma membrane inward deformation, called an invagination.
4. **Exocytosis:** Through exocytosis cell removes undigested substances, secretes hormones and enzymes. During the process, the undigested waste-containing food vacuole or the secretory vesicle is budded from Golgi apparatus, and is moved by cytoskeleton from the interior of the cell to the surface. The vesicle membrane comes in contact with the plasma membrane leading to lipids rearrangement and fusion of the two membranes. A passage is formed in the fused membrane and the vesicles discharges its contents outside the cell.

Gram negative bacteria have a periplasmic space which separates the plasma membrane and outer membrane while, gram positive bacteria have only a plasma membrane. Prokaryotic cells are also surrounded by a cell wall composed of peptidoglycan (amino acid and sugar). Some eukaryotic cells also have cells walls, but none that are made of peptidoglycan.

EXPERIMENT

Experiment: The effect of lipid composition on the permeability of a lipid monolayer

Principle: The lipid composition of a membrane has a considerable effect on its permeability. If butanol is layered on top of water then two distinct phases are formed; if amphipathic lipids are present they will move into the boundary region. The polar part of the molecule will associate with the top aqueous layer and the hydrophobic region with the organic phase. Methylene blue is a highly colored molecule and its passage across the boundary can be readily followed by eye. Unlike biological membranes, this model doesnot contain protein, but useful information can be obtained from this simple experiment as passive diffusion does depend on the lipid composition of the membrane.

Materials: Fatty acids (stearic acid, oleic acid), acylglycerols (triolein, tripalmitin), phospholipid (egg lecithin), sterol (cholesterol), butanol, boiling tubes, Methylene blue in butanol (0.25 g/litre)

Method: Set up 7 boiling tubes each containing 5 ml water. Carefully pipette 5 ml of butanol containing Methylene blue and 200 mg of lipid down the side of each tube to form two distinct layers. Leave the tubes to stand at room temperature for 1–2h and compare the results with that obtained using a control tube containing water but no lipid in the butanol can be obtained by measuring the extinction of the Methylene blue in the aqueous phase.

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ABOUT THE BOOK

This book is written for undergraduate and postgraduate students who have a basic knowledge in biological sciences and are interested in a future career in research and industry. The book provides an understanding of up-to-date information on the concerned topics in a simple, lucid and concise manner. It has been extensively updated and covers a large number of basic techniques and experiments in biochemistry and molecular biology which help the student's understanding of theory and practice. Each chapter includes an introduction to the techniques used and the basic biochemistry so as to link the text with the main course. The book can also be used as a complement to a standard theoretical text.

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