Model Answer
B.Sc.(Hons) V SEM Exam, 2014
Biotechnology
Paper: LBTC-501
Biophysical Chemistry and Instrumentation

Section A, Q.1

1. Dielectric
2. Phosphate buffer
3. CI-Protein-glycinate
4. Sodium Dodecyl sulphate
5. Swinging bucket
6. CsCl
7. Phase contrast microscopy
8. Reflect shorter wavelength at 90° and transmit longer wavelength
9. Gamma rays
10. Infrared

Section B, Long Answer

Q. 2. Isoelectric point

Isoelectric point (pI) is a pH in which net charge of protein is zero. In case of proteins isoelectric point mostly depends on seven charged amino acids: glutamate (δ-carboxyl group), aspartate (ß-carboxyl group), cysteine (thiol group), tyrosine (phenol group), histidine (imidazole side chains), lysine (ε-ammonium group) and arginine (guanidinium group). Additonally, one should take into account charge of protein terminal groups (NH_2 i COOH). Each of them has its unique acid dissociation constant referred to as pK.
Moreover, net charge of the protein is in tight relation with the solution (buffer) pH. Keeping in main this we can use Henderson-Hasselbach equation to calculate protein charge in certain pH:

- for negative charged macromolecules:

$$\sum_{i=1}^{n} \frac{-1}{1+10^{pK_n-pH}}$$

where pK_n is the acid dissociation constant of negatively charged amino acid

- for positive charged macromolecules:

$$\sum_{i=1}^{n} \frac{1}{1+10^{pH-pK_p}}$$

where pK_p is the acid dissociation constant of positively charged amino acid

As you can see, only pH of buffer is variable in equations. If we successively change this value, finally we will find isoelectric point of analyzed protein. The knowledge of isoelectric point is of great significance in biochemistry (mainly in elecrophoresis and isofocusing techniques), because it allows to match proper environment before the experiment starts.
Generally, macromolecules are positively charged and on the other hand, above proteins isoelectric point, their charge is negative.For example, during electrophoresis, direction of proteins migration, depends only from their charge. If buffer pH (and as a result gel pH) is higher than protein isoelectric point, the particles will migrate to the anode (negative electrode) and if the buffer pH is lower than isoelectric point they will go to the cathode. In situation when the gel pH and the protein isoelectric point are equal, proteins do not move at all.
Using above formulae, we can calculate theoretical isoelectric point. The result will be almost surely different than real isoelectric point. It is mainly because many proteins are chemically modified (amino acids can be phosphorylated, methylated, acetyleted etc.), which change their charge. Problematic is also the occurrence of cysteines (negative charge) which can oxidise and form disulfide bond in protein.Therefore, they will become cystines, which do not express any charge.
Nevertheless, one can approximately calculate protein isoelectric point which is ± 0.5 of exact isoelectric point. The most critical moment during isoelectric point determination is usage of appropriate pK values. Unfortunately, there is no agreement in this matter.

Dielectric constant:

Dielectric constant, property of an electrical insulating material (a dielectric) equal to the ratio of the capacitance of a capacitor filled with the given material to the capacitance of an identical capacitor in a vacuum without the dielectric material. The insertion of a dielectric between the plates of, say, a parallel-plate capacitor always increases its capacitance, or ability to store opposite charges on each plate, compared with this ability when the plates are separated by a vacuum. If C is the value of the capacitance of a capacitor filled with a given dielectric and C_0 is the capacitance of an identical capacitor in a vacuum, the dielectric constant, symbolized by the Greek letter kappa, κ, is simply expressed as $\kappa = C/C_0$. Dielectric constant is a number without dimensions. It denotes a large-scale property of dielectrics without specifying the electrical behaviour on the atomic scale.

The value of the static dielectric constant of any material is always greater than one, its value for a vacuum. The value of the dielectric constant at room temperature (25° C, or 77° F) is 1.00059 for air, 2.25 for paraffin, 78.2 for water, and about 2,000 for barium titanate ($BaTiO_3$) when the electric field is applied perpendicularly to the principal axis of the crystal. Because the value of the dielectric constant for air is nearly the same as that for a vacuum, for all practical purposes air does not increase the capacitance of a capacitor. Dielectric constants of liquids and solids may be determined by comparing the value of the capacitance when the dielectric is in place to its value when the capacitor is filled with air.
The dielectric constant is sometimes called relative permittivity or specific inductive capacity. In the centimetre–gram–second system the dielectric constant is identical to the permittivity.

Dipole moment:

Any chemical bond results from the accumulation of charge density in the binding region to an extent sufficient to balance the forces of repulsion. Ionic and covalent binding represent the two possible extremes of reaching this state of electrostatic equilibrium and there is a complete spectrum of bond densities lying between these two extremes. Since covalent and ionic charge distributions exhibit radically different chemical and physical properties, it is important, if we are to understand and predict the bulk properties of matter, to know which of the two extremes of binding a given molecule most closely approximates.

We can obtain an experimental measure of the extent to which the charge density is unequally shared by the nuclei in a molecule. The physical property which determines the asymmetry of a charge distribution is called the dipole moment. To illustrate the definition of the dipole moment we shall determine this property for the LiF molecule assuming that one electron is transferred from Li to F and that the charge distributions of the resulting ions are spherical.

The dipole moment is defined as the product of the total amount of positive or negative charge and the distance between their centroids. The centroids of the positive and negative charges in a molecule are determined in a manner similar to that used to determine the centre of mass of a system.

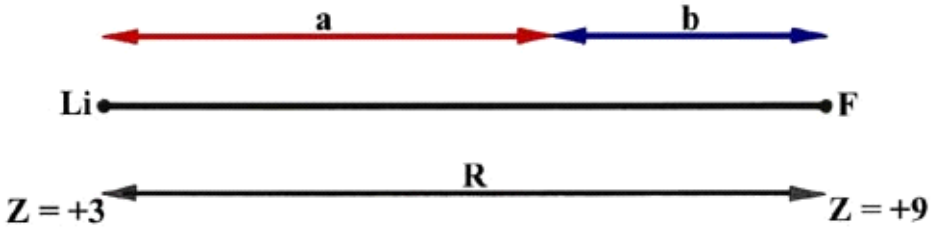


Fig. diagram for the calculation of the centroids of positive and negative charge in LiF.

Dipole Moment Definition: A dipole moment is a measurement of the separation of two oppositely charged charges. Dipole moments are a vector quantity. The magnitude is equal to the charge multiplied by the distance between the charges and the direction is from negative charge to positive charge.

Dipole moments are measured in the SI units of coulomb·meters (C m). In chemistry, dipole moments are applied to the distribution of electrons between twobonded atoms. The existence of a dipole moment is the difference between polar andnonpolar bonds. Molecules with a net dipole moment are polar molecules.

3. Principal and working of HPLC :

Chromatography is a technique by which a mixture sample is separated into components. Although originally intended to separate and recover (isolate and purify) the components of a sample, today, complete chromatography systems are often used to both separate and quantify sample components.

The term, “chromatography" was coined by the Russian botanist, Tswett, who demonstrated that, when a plant extract was carried by petroleum ether through a column consisting of a glass tube packed with calcium carbonate powder, a number of dyes were separated, as shown in Figure 1. He named this analysis method "Chromatographie" after "chroma" and "graphos", which are Greek words meaning "color" and “to draw," respectively.

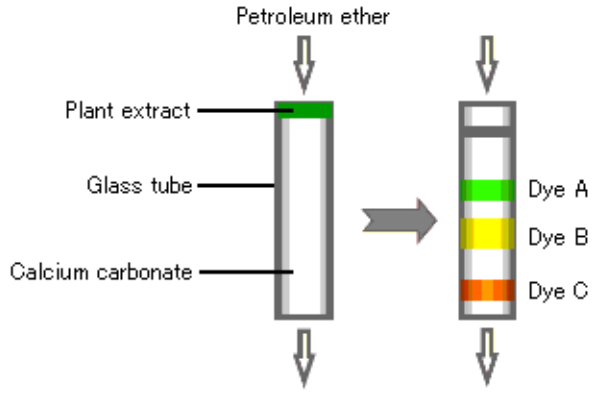


Figure 1. Diagram showing Tswett's experiment

"Chromatography" represents a separation technique; whereas a **"chromatograph"** is a system for performing chromatography. The chart displaying the time-dependent change in signal intensity as a result of the separation is called a **"chromatogram”**.

As shown in Table 1, gas and liquid chromatography are common classifications that are based upon the **mobile** and **stationary phases** utilized for the separation.

Table 1. Type of chromatography

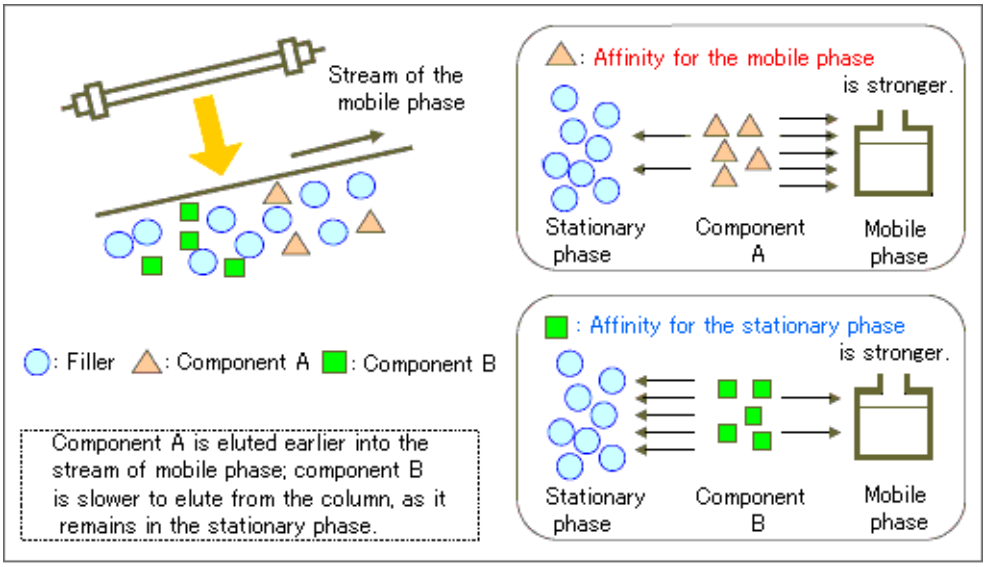
Mobile phase	Stationary phase	Analysis	Sample Types
Gas	Solid/Liquid	Gas chromatography (GC)	Samples that are gaseous at ordinary temperatures and samples that vaporize when heated ·Odorous samples such as petrochemicals, perfumes, and thinner are easier to analyze by GC. ·High molecular weight compounds are measured after pyrolysis.
Liquid	Solid/Liquid	Liquid chromatography (LC)	Liquid samples and solvent-soluble solid samples ·Compared to GC, LC has a wide range of measurement subjects. ·High molecular weight compounds can be analyzed, if soluble in solvent.

Although the intended use of GC and LC are the same (i.e., separation and quantification), the measurement subjects are different, as the sample conditions differ at separation. The stationary phase typically indicates a column (fillers), while the mobile phase, which is referred to as **the eluent** in LC, indicates a vehicle to pour a sample into the column.

How is a sample separated into its components in the column? The speed of a migrating sample component depends on whether the component has an affinity for the stationary or mobile phase. This affinity appears via various actions: adsorption, partition, ion exchange, etc. As shown in Figure 2, components that have a higher affinity for the mobile phase compared with the stationary phase migrate more rapidly, while components that have a higher affinity for the stationary phase are eluted from the column later. The order and resolution of the components emerging from the column depend on the type of selected stationary and mobile phases.

What does HPLC stand for?

HPLC is short for the High Performance LC. HPLC is an analysis method that yields high performance and high speed compared with traditional column chromatography because of the forcibly pumped mobile phase. Recently, ultrafast analysis using a high-pressure-resistant apparatus has been attracting attention. UHPLC (Ultra High Performance LC) is becoming established as an abbreviation for this ultrafast LC method.



HPLC is a popular method of analysis because it is easy to learn and use and is not limited by the volatility or stability of the sample compound.

Principle:

In isocratic HPLC the analyte is forced through a column of the stationary phase (usually a tube packed with small round particles with a certain surface chemistry) by pumping a liquid (mobile phase) at high pressure through the column. The sample to be analyzed is introduced in a small volume to the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase as it traverses the length of the column. The amount of retardation depends on the nature of the analyte, stationary phase and mobile phase composition. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time and is considered a reasonably unique identifying characteristic of a given analyte. The use of pressure increases the linear velocity (speed) giving the components less time to diffuse within the column, leading to improved resolution in the resulting chromatogram. Common solvents used include any miscible combinations of water or various organic liquids (the most common are methanol and acetonitrile). Water may contain buffers or salts to assist in the separation of the analyte components, or compounds such as Trifluoroacetic acid which acts as an ion pairing agent.

A further refinement to HPLC has been to vary the mobile phase composition during the analysis, this is known as gradient elution. A normal gradient for reverse phase chromatography might start at 5% methanol and progress linearly to 50% methanol over 25 minutes, depending on how hydrophobic the analyte is. The gradient separates the analyte mixtures as a function of

the affinity of the analyte for the current mobile phase composition relative to the stationary phase. This partitioning process is similar to that which occurs during a liquid-liquid extraction but is continuous, not step-wise. In this example, using a water/methanol gradient, the more hydrophobic components will elute (come off the column) under conditions of relatively high methanol; whereas the more hydrophilic compounds will elute under conditions of relatively low methanol. The choice of solvents, additives and gradient depend on the nature of the stationary phase and the analyte. Often a series of tests are performed on the analyte and a number of generic runs may be processed in order to find the optimum HPLC method for the analyte - the method which gives the best separation of peaks.

Instrumentation: :

These new "HPLC" instruments could develop up to 6,000psi (400 bar) of pressure, and included improved detectors and columns. HPLC really began to take hold in the mid to late 1970's. With continued advances in performance, the name was changed to High Performance Liquid Chromatography (HPLC). High Performance Liquid Chromatography (HPLC) is now one of the most powerful tools in analytical chemistry, with the ability to separate, identify and quantitate the compounds that are present in any sample that can be dissolved in a liquid. Today, trace concentrations of compounds, as low as "parts per trillion" (ppt), are easily obtained. HPLC can be applied to just about any sample, such as pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices

Applications:

Preparative HPLC refers to the process of isolation and purification of compounds. Important is the degree of solute purity and the throughput, which is the amount of compound produced per unit time. This differs from analytical HPLC, where the focus is to obtain information about the sample compound. The information that can be obtained includes identification, quantification, and resolution of a compound.

Chemical Separations can be accomplished using HPLC by utilizing the fact that certain compounds have different migration rates given a particular column and mobile phase. Thus, the chromatographer can separate compounds (more on chiral separations) from each other using HPLC; the extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase.

Purification refers to the process of separating or extracting the target compound from other (possibly structurally related) compounds or contaminants. Each compound should have a characteristic peak under certain chromatographic conditions. Depending on what needs to be separated and how closely related the samples are, the chromatographer may choose the conditions, such as the proper mobile phase, to allow adequate separation in order to collect or extract the desired compound as it elutes from the stationary phase. The migration of the compounds and contaminants through the column need to differ enough so that the pure desired compound can be collected or extracted without incurring any other undesired compound.

4. Density Gradient Centrifugation:

Centrifugation

When an object attached to a rope is whirled around, one can feel that the rope must be pulled inward towards the centre of the rotation in order to keep the object on the orbit. This force prevents the object from getting away and move with a constant speed along a straight tangential line. The inward force with which one has to pull the rope is called the centripetal force. One can also define the outward force, the centrifugal force, by which the object pulls the rope. This force is equal in magnitude to the centripetal force but has the opposite direction. The centrifugal force (F_c) is a virtual, so-called fictional force emerging due to the inertia of the object. Yet, because it leads to a simpler mathematical formalism, equations describing the processes when solutions are centrifuged use the F_c force.

According to the well-known Newton equation:

$$F = m \times a \tag{5.1}$$

Upon centrifugation, acceleration equals the product of the radius of the orbit and the square of the angular velocity:

$$a = \omega^2 \times r \tag{5.2}$$

The fictive centrifugal accelerating force in vacuum is therefore:

$$F_c = m \times \omega^2 \times r \tag{5.3}$$

The product of the radius and the square of the angular velocity equals the centrifugal accelerating potential. Traditionally, and perhaps somewhat misleadingly, the magnitude of this potential is compared to the Earth's gravitational accelerating potential (g), and has been expressed in "g" units. The reason is quite simple. Earth's gravitational potential, similarly to the accelerating potential provided by centrifugation, can also sediment particles dispersed in solution. This type of quantitation shows how many times centrifugation is more effective to sediment particles compared to the gravitational effect of Earth. In the fastest laboratory ultracentrifuges the applied accelerating potential can exceed 1 000 000 g.

Equilibrium density-gradient centrifugation: fractionation based on density

In the previous section we introduced the method of differential centrifugation. For simplicity, we stated that the constituents of the sample were separated in a medium of homogeneous density. This first approximation has didactical advantages as it makes the basic principle of differential centrifugation easier to comprehend. Nevertheless, it is sometimes advantageous to use a very shallow density gradient in the medium during differential centrifugation. This is done only to suppress convectional flows in the medium that could unsettle and mix layers of already separated cell constituents.

The essence of equilibrium density-gradient centrifugation is principally different. In this case, a rather steep density gradient is created in the medium—in such a manner that the density of the

medium gradually increases towards the bottom of the centrifuge tube. This is achieved by using a very high-density additive, for example caesium chloride (CsCl). The density gradient is created as follows. When the centrifuge tube is filled with the medium, a high concentration CsCl solution is added first. Subsequently, in the process of filling the tube, the concentration of CsCl is gradually decreased resulting in a CsCl gradient and, as a consequence, a density gradient in the tube. The sample is layered on the top of this special medium (Figure 5.2).

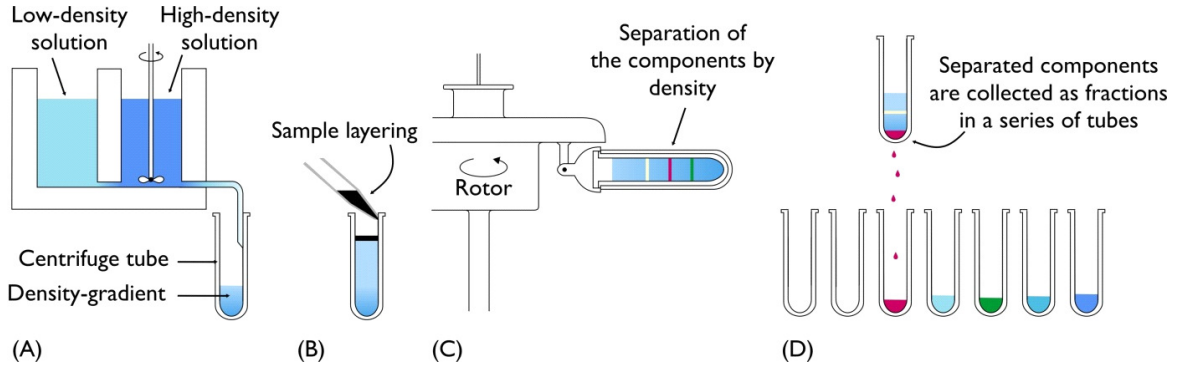


Figure 5.2. Equilibrium density-gradient centrifugation. In the course of equilibrium density-gradient centrifugation, a concentration gradient of a high density compound such as caesium chloride is generated. (The compound should not react with the biological sample.) The concentration gradient of this special additive creates a density gradient in the centrifuge tube. The density gradually increases toward the bottom of the centrifuge tube. The sample is layered on the low-density top of this gradient. As the centrifugation begins, each compound of the sample starts to sediment. By doing so, the compounds travel through layers of increasing density. As soon as a compound reaches the layer where the density equals its own density, the compound stops sedimenting. At this layer, no resultant force is exerted on the particle and thus it will float. As a result, equilibrium density-gradient centrifugation separates compounds from each other independently of their size, solely by their density, in a single run.

In the course of centrifugation, particles start to sediment moving towards the bottom of the centrifuge tube. By doing so, they travel through an increasing density medium. Each particle sediments to a section of the medium where its own density equals the density of the medium. At this section, the buoyancy factor becomes zero and, as a consequence, the accelerating force acting on the particle also becomes zero. The particle stops sedimenting. If it moved further towards the bottom of the tube, it would meet a higher density medium and a force opposing to its moving direction would be exerted on it, turning the particle back. If, by travelling backwards, it would meet a density lower than its own density, it would sediment again. As a consequence, this method separates particles exclusively based on their density. It is an equilibrium method in which, by the end of the separation, the system reaches a constant state. (In this aspect, this method shows an interesting analogy to the isoelectric focusing (IEF) method reviewed in Chapter 7. The two methods separate particles by entirely different characteristics (density versus isoelectric point), but in both cases, the separation leads to an equilibrium state. Both methods apply a gradient, but in the case of IEF a pH gradient is created.)

Note that the two centrifugation approaches introduced above separate particles by partially different characteristics. Consecutive combination of the two methods can lead to a more efficient separation than achieved by any of the methods alone. Therefore, to increase separation efficiency, fractions generated by differential centrifugation can be subjected to a subsequent density-gradient centrifugation step to further separate individual components (Figure 5.3).

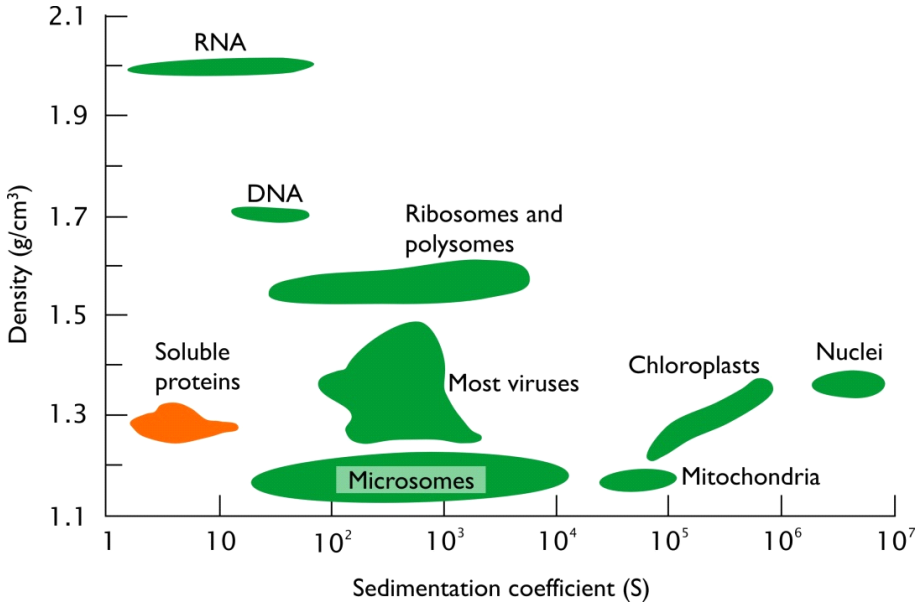


Figure 5.3. Combination of differential centrifugation and density-gradient centrifugation. Differential centrifugation separates compounds primarily based on their size, while density-gradient centrifugation separates compounds exclusively based on their density. Compounds that have different density but sediment in the same fraction during differential centrifugation can be separated by a subsequent step of density-gradient centrifugation. Two such consecutive steps of the two centrifugation methods can provide significantly higher separation efficiency than either procedure alone.

5. Principal of discontinuous, denaturing SDS:

Introduction to SDS-PAGE

This material is accompanied by a presentation on protein structure and principles behind denaturing samples and discontinuous gel electrophoresis. The separation of macromolecules in an electric field is called *electrophoresis*. A very common method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins. The method is called sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The most commonly used system is also called the Laemmli method after U.K. Laemmli, who was the first to publish a paper employing SDS-PAGE in a scientific study. SDS (also called lauryl sulfate) is an anionic detergent, meaning that when dissolved its molecules have a net negative charge within a wide pH range. A polypeptide

chain binds amounts of SDS in proportion to its relative molecuar mass. The negative charges on SDS destroy most of the complex structure of proteins, and are strongly attracted toward an anode (positively-charged electrode) in an electric field.

Polyacrylamide gels restrain larger molecules from migrating as fast as smaller molecules. Because the charge-to-mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in relative molecular mass of polypeptides. In a gel of uniform density the relative migration distance of a protein (Rf, the f as a subscript) is negatively proportional to the log of its mass. If proteins of known mass are run simultaneously with the unknowns, the relationship between Rf and mass can be plotted, and the masses of unknown proteins estimated.

Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample, and to determine the distribution of proteins among fractions. The purity of protein samples can be assessed and the progress of a fractionation or purification procedure can be followed. Different staining methods can be used to detect rare proteins and to learn something about their biochemical properties. Specialized techniques such as Western blotting, two-dimensional electrophoresis, and peptide mapping can be used to detect extremely scarce gene products, to find similarities among them, and to detect and separate isoenzymes of proteins.

Molecular mass versus molecular weight

Molecular mass (symbol m) is expressed in Daltons (Da). One Dalton is defined as 1/12 the mass of carbon 12. Most macromolecules are large enough to use the kiloDalton (kDa) to describe molecular mass. Molecular weight is not the same as molecular mass. It is also known as relative molecular mass (symbol Mr, where r is a subscript). Molecular weight is defined as the ratio of the mass of a macromolecule to 1/12 the mass of a carbon 12 atom. It is a dimensionless quantity.

When the literature gives a mass in Da or kDa it refers to molecular mass. It is incorrect to express molecular weight (relative molecular mass) in Daltons. Nevertheless you will find the term molecular weight used with Daltons or kiloDaltons in some literature, often using the abbreviation MW for molecular weight.

Polyacrylamide gels for SDS-PAGE

Many systems for protein electrophoresis have been developed, and apparatus used for SDS-PAGE varies widely. The methodology used on these pages employs the Laemmli method. Reference to the Laemmli method in a materials and methods section eliminates the need to describe the buffers, casting of gels, apparatus, etc. Unless the paper employs some modification to the method, the only details of SDS-PAGE that should be reported in a methods section are percent total acrylamide (%T) in a gel, relative percentage and type of crosslinker (%C), and perhaps a reference to the gel dimensions. We use a "mini-gel" system, with 3 1/4" x 4" gel cassettes.

SDS-PAGE can be conducted on pre-cast gels, saving the trouble and hazard of working with acrylamide. The following description applies to shop-made casting and running apparatus that are much cheaper than commercially available equipment. In addition to cost effectiveness, an advantage of making one's own gels the first time is a deeper understanding of the process.

Regardless of the system, preparation requires casting two different layers of acrylamide between glass plates. The lower layer (separating, or resolving, gel) is responsible for actually separating polypeptides by size. The upper layer (stacking gel) includes the sample wells. It is designed to sweep up proteins in a sample between two moving boundaries so that they are compressed (stacked) into micrometer thin layers when they reach the separating gel.

Polyacrylamide gel electrophoresis (PAGE) is a widely used technique for separating proteins. The most widely used method was developed by Laemmli, using the denaturing (SDS) discontinuous method. This protocol relies on the presence of SDS (sodium dodecyl sulfate) and ß-mercaptoethanol to denature the proteins, dissociate the proteins into subunits, and to coat them with negative charges (SDS). This relatively uniform charge to mass ratio allows the proteins to migrate in an electric field and separate according to mass/size. The Laemmli system uses buffers of different pH and composition to generate a voltage gradient and a discontinuous pH between the stacking and the resolving gel. A 4% acrylamide stacking gel (pH 6.8) is poured on top of a 10% acrylamide resolving gel (pH 8.8). The stacking gel (with a large pore size) serves to concentrate all of the proteins (the large ones can catch up with the small ones) on top of the resolving gel. After entering the resolving gel (which has a smaller pore size) the proteins are separated according to relative molecular size. Protein samples are diluted 1:2 in Laemmli sample buffer and are boiled for 5 minutes. The ß-mercaptoethanol in the sample buffer reduces the protein's disulfide bonds and the SDS denatures the proteins. The sample buffer contains glycerol to increase the density so that when the sample is loaded it sinks to the bottom of the well. Bromophenol blue dye is in the sample buffer to monitor the electrophoresis process. The gel electrode assembly is placed in the Mini PROTEAN II electrophoresis chamber (BIORAD), reservoir buffer is added to upper and lower chambers, the samples are loaded and the proteins electrophoresed at ~200Volts for about 45 minutes. For proteins 30-90kD using an 8-10% gel, the bromophenol blue dye should travel to the bottom of the resolving gel. The gel can then be used to transfer the proteins to nitrocellulose for a Western or can be fixed, stained in Coomassie blue dye and destained. For detection of proteins in the gel with Coomassie blue (a standard stain for total protein), at least 1 µg of protein per band is required.

6. **Paper Electrophoresis and its applications:**

Paper Electrophoresis: Paper Electrophoresis is one of the zone electrophoresis. This is very important method in all laboratories. In this article let us learn the details of the paper chromatography with suitable notes. I have given the info about this in Notes.

Principle:

"The charge carried by a molecule depends on the pH of the medium. Electrophoresis at low voltage is not usually to separate low molecular weight compounds because of diffusion, but it is

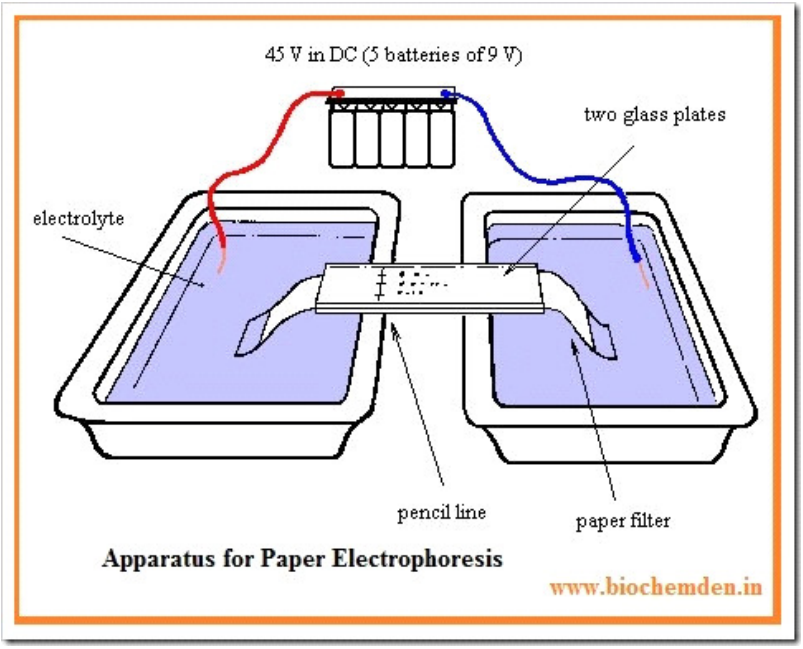
easier to illustrate the relationship between charge and pH with amino acids than with proteins (or) other macromolecules".

Filter paper:

Paper of good quality should contain at least 95% α-cellulose and should have only a very slight adsorption capacity.

Apparatus:

- The equipment required for electrophoresis consists basically of two items, a POWER PACK and an ELECTROPHORETIC CELL.
- Power pack provides a stabilized direct current & has controls for both voltage & current out put, which have an out put of 0 to 500V and 0 to 150mA are available.
- The Electrophoretic cell contains the electrodes, buffer reservoirs, a support for paper and a supporting transparent insulating cover. The electrodes are usually made of platinum.
- The two arrangements of the filter strips are commonly used. The horizontal & vertical arrangements. Both the arrangements are equally viable & the choice usually depends upon personal preferences.



Sample application:

The sample may be applied as a spot (about 0.5cm in diameter) or as a narrow uniform streak. Special devices are available commercially for this purpose. The sample can be applied before the paper has been equilibrated with buffer (or) after it.

Procedure:

After the sample has been applied to the paper and the paper has been equilibrated with the buffer. The current is switched on. Commonly used buffers are,

Intended separation	Buffer	pH	Ionic strength	Composition liter
Proteins	Barbital	8.6	0.05	10.3g Sodium barbiturate 1.84 g. Barbital
	Phosphate	7.4	....	0.6g. $NaH_2PO_4.H_2O$ 2.2g. Na_2HPO_4
Nucleoproteins	Acetate	4.5	0.1	3.51g. NaCl 3.28g.Sodium acetate (pH adjusted with HCl to 4.5)
	Citrate	4.5	0.13	28.46g Sodium citrate 20.6g. Citric acid
Amino acids	Phosphate	4.6	0.15	20.4g KH_2PO_4
	Michalies	8.6	0.1	9.8g Sodium barbiturate

The device providing stable voltage (or) current is available. Frequent observation is necessary to run electrophoretic apparatus. Overheating can be avoided by placing the entire equipment in the cold
room. The process does not take longer than two hours. After 2 hours switched off the power and paper is
removed. Once removed, the paper is dried in hot oven at 110^0C.

Detection & Quantitative assay:

To identify the unknown electrophorogram, compare the Unknown electogram with standard electrogram under standard conditions.

Individual compounds are usually identified by physical properties by the following methods.

i) Fluorescence:

a) Staining with "**Ethidium bromide**" and subsequent visualization of the electrophoreticgram under UV light makes DNA & RNA fluoresce and thus facilitates their detection.

b) **Flourescamine** staining is utilized for detecting amino acids, amino acid derivatives, peptides & proteins.

c) DANSYl chloride may be used in place of **fluorescamine.**

ii) UV absorption:

Proteins, Peptides & nucleic acids absorb in the range of 260 to 280nm, this property these can be detect.

iii)) Staining:

Compound	Dye	Comments
Proteins	Bromophenol blue in acetic acid DANSYl chloride Fluorescamine	Visual, quantitative Fluorescent, Quantitative Fluorescent, Very sensitive
Nucleic acids	Methyl green-pyronine Ethedium bromide	DNA-Blue, RNA-Red, Sensitive Fluorescent, Very sensitive
Polysaccharide	Iodine	Visual, Sensitive
Lipoprotein	Sudan black in 60% ethanol	Visual, Sensitive
Glycoprotein	Alcian blue	Visual, Sensitive

iv) Detection of Enzymes in situ:

- If the component to be separated is an enzyme. Special techniques may be used to detect it.
- The paper strip, which have separated enzyme, is impregnated with the substrate for the enzyme desired to be separated.
- The paper strip is now placed in a suitable buffer along with electrophoretogram. The color bands will appear which indicates the position of enzyme.

v) Quantitative estimation:

The color density of the zone may be multiplied with the area of the zone and the resulting value would be a rough estimate of the concentration of the component.

Applications:

- Serum analysis for diagnostic purpose is routinely carried about by paper electrophoresis.

- Muscle proteins, egg white proteins, milk proteins & snake, insect venom analysis done by this technique.

7. Transmission electron microscopy:

Principal

Transmission electron microscopy uses high energy electrons (up to 300 kV accelerating voltage) which are accelerated to nearly the speed of light. The electron beam behaves like a wavefront with wavelength about a million times shorter than lightwaves. When an electron beam passes through a thin-section specimen of a material, electrons are scattered. A sophisticated system of electromagnetic lenses focuses the scattered electrons into an image or a diffraction pattern, or a nano-analytical spectrum, depending on the mode of operation. Each of these modes offers a different insight about the specimen. The imaging mode provides a highly magnified view of the micro- and nanostructure and ultimately, in the high resolution imaging mode a direct map of atomic arrangements can be obtained (high resolution EM = HREM). The diffraction mode (electron diffraction) displays accurate information about the local crystal structure. The nanoanalytical modes (x-ray and electron spectrometry) tell researchers which elements are present in the tiny volume of material.
These modes of operation provide valuable information for scientists and engineers in search of stronger materials, faster microchips, or smaller nanocrystals.

Working:

Background

Unlike Scanning Electron Microscopy that bounces electrons off the surface of a sample to produce an image, Transmission Electron Microscopes (TEMs) shoot the electrons completely through the sample.

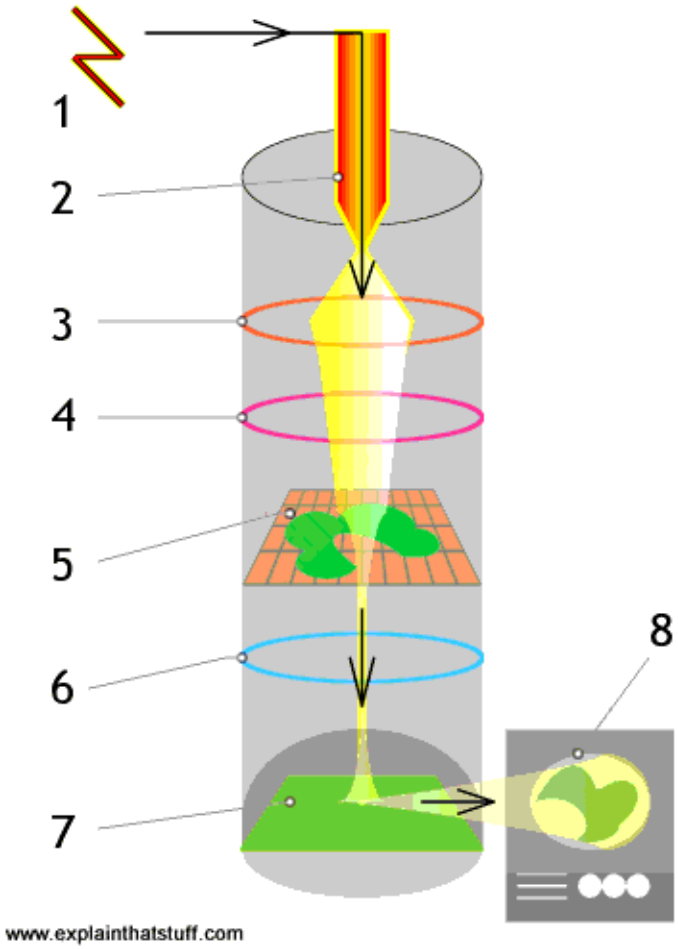
How TEMs Work

TEMs work by using a tungsten filament to produce an electron beam in a vacuum chamber. The emitted electrons are accelerated through an electromagnetic field that also narrowly focuses the beam. The beam is then passed through the sample material. The specially prepared sample is a very thin (less than 100nm) slice of material. The electrons that pass through the sample hit a phosphor screen, CCD or film and produce an image. Where the sample has less density, more electrons get through and the image is brighter. A darker image is produced in areas where the sample is more dense and therefore less electrons pass through.

Resolution

TEMs can produce images with resolution down to 0.2nm. This resolution is smaller than the size of most atoms and therefore images can be produced using TEM that show the true structural arrangement of atoms in the sample material.

How a transmission electron microscope (TEM) works



A **transmission electron microscope** fires a beam of electrons *through* a specimen to produce a magnified image of an object.

- A high-voltage electricity supply powers the cathode.
- The cathode is a heated filament, a bit like the electron gun in an old-fashioned cathode-ray tube (CRT) TV. It generates a beam of electrons that works in an analogous way to the beam of light in an optical microscope.
- An electromagnetic coil (the first lens) concentrates the electrons into a more powerful beam.
- Another electromagnetic coil (the second lens) focuses the beam onto a certain part of the specimen.
- The specimen sits on a copper grid in the middle of the main microscope tube. The beam

passes through the specimen and "picks up" an image of it.

- The projector lens (the third lens) magnifies the image.
- The image becomes visible when the electron beam hits a fluorescent screen at the base of the machine. This is analogous to the phosphor screen at the front of an old-fashioned TV .
- The image can be viewed directly (through a viewing portal), through binoculars at the side, or on a TV monitor attached to an image intensifier (which makes weak images

Applications

The instrument allows to perform the following analyses:

- Morphological analysis
- Electronic diffraction
- X-EDS qualitative and semi-quantitative analysis, either in Spot or in SemiStem mode.

Fields of application

The instrumentation is particularly suitable for the study and characterization of material from a morphological, chemical and structural point of view, especially in these fields:

- polymeric
- metallic
- ceramic

On the basis of available techniques, some specific applications in which the instrument plays an important role are:

- Material study
- Composite and nano-structured materials study
- Defects from the manufacturing process in semi-conductors
- Ceramic systems
- Plastic Deformations
- The study of layers and structures
- Phase transformations
- Nanometric systems
- Ordered alloy structures
- The study of cellular structures

Environmental applications:

-Qualitative and quantitative evaluation of asbestos, ceramic and various organic fibres, and their presence in:

a) atmosphere:
emissions
immissions
work environment
life environment
b) various materials and artefacts (massive samples)
c) liquid phases (water, solvents, etc.)
d) soil
e) waste.

Evaluation and morphological/chemical characterisation of aerodispersed nanoparticulatein:
a) work environment
b) emissions
c) immissions
d) life environment.

- **Solid Scintillation counter:**

Physical principles of scintillation technique Radioactive decay occurs with the emission of particles or electromagnetic radiation from an atom due to a change within its nucleus. Forms of radioactive emission include alpha particles (α), beta particles (β) and gamma rays (γ). Alpha & beta particles directly ionize the atoms with which they interact, adding or removing electrons. Gamma-rays cause secondary electron emissions, which then ionize other atoms. However, some irradiated atoms are not fully ionized by collision with emitted particles, but instead have electrons promoted to an excited state. Excited atoms can return to their ground state by releasing energy, in some cases as a photon of light. Such scintillation phenomena form the basis of a set of very sensitive radiation detection systems. To a first approximation this is a linear conversion of energy into photons and, therefore, the intensity of light in the scintillation is proportional to the initial energy deposited in the scintillator by ionizing radiation. This light emitted is taken as a measure of the amount of radioactivity in the sample.

Instrumentation. Scintillation counter apparatus

A scintillation counter measures ionizing radiation. A scintillation counter apparatus consists of a scintillator, a photo-multiplier tube (PMT), an amplifier, and a multichannel analyzer (Fig. 1). A solid scintillation counter is a radiation detector which includes a scintillation crystal to detect radiation and produces light pulses while the liquid scintillation counter detect the scintillation produced in the scintillation cocktail by radiation.

The PMT is an electron tube that detects the blue light flashes from the scintillation and converts them into a flow of electrons and subsequently measured as an electric pulse. This consists of a photocathode (photoelectric emitter) that is connected to the negative terminal of a high tension battery. A number of electrodes called dynodes are arranged in the tube at increasing positive potential. When a scintillation photon strikes the photocathode of the PMT is released a

photoelectron. Using a voltage potential, the electrons are attracted and strike the nearest dynode with enough energy to release additional electrons. The second-generation electrons are attracted and strike a second dynode, releasing more electrons. This amplification continues through 10 to 12 stages. More electrons are emitted and the chain continues, multiplying the effect of the first charged particle. By the time the electrons reach the last dynode, enough have been released to send a voltage pulse across the external resistors. The magnitude of the resulting pulse height produced by the PMT is proportional to the photon intensity emitted by the scintillator (crystal NaI(Tl) in SSC or "cocktail scintillator" in LSC). This voltage pulse is amplified and recorded by a multichannel that classifies each voltage pulse. Pulses are collated into channels, and the counts per minute (CPM) in each channel is recorded. Each channel corresponds to a specific range of energies (channels are also known as counting windows), and counts with energies above or below set limits are excluded from a particular channel. When the counts have all been collated, the researcher knows the intensity of radiation, expressed as CPM, and its energy distribution, or spectrum. CPM is proportional to the amount of isotope in the sample, and the spectrum indicates the identity of the isotope.

Mechanism:

In SSC, the transparent inorganic crystal, called scintillator, fluoresces when is irradiated by the sample. The most commonly used is Thallium-doped sodium iodide (NaI(Tl)). This detector is made of various sizes for different types of equipment. With this method, involves placing the sample containing the radioactivity into a glass or plastic container, called a scintillation vial that is deposited directly onto a solid scintillating material, dried, and counted in a scintillation counter also called Gamma counter. For α or β radiation counting, however, solid scintillation has severe limitations. The crystal must be protected from contamination by the sample, which means that the α and β particles must traverse a barrier prior to reaching the scintillator. In particular, α-radiation is severely attenuated by even 0.05 mm of aluminium or copper, and so cannot be expected to reach a scintillator crystal through even the thinnest shielding.

Application: Solid and liquid scintillation techniques are used for the detection of radio labeled isotopes in areas as diverse as biomedicine, ecology and industry. Scintillation counting capabilities include detection of alpha, beta and gamma emitters, in single, double and triple labelling, and also include the detection of these transmitters by counting in continuous flow (HPLC) and finally the scintillation proximity Assays (SPA)

Detecting and counting alpha emitting radionuclides are routine tasks in nuclear energy and environmental monitoring. Liquid scintillation counting of alpha particles provides high counting efficiency (near 100%). The accurate and sensitive measurement of alpha-emitting nuclides is essential in the nuclear fuel cycle, process control, radioactive waste management and environmental protection. However the energy resolution is quite poor, so they are not very useful for the identification of these radionuclides.