2. Methods of Enzyme Purification
Dialysis, Ultra filtration, ultracentrifugation, molecular exclusion chromatography, column chromatography, GLC and HPLC, FPLC, iso-electric precipitation, salting in, salting out, solvent fractionation, electrophoresis-Paper and SDS-PAGE, ion exchange chromatography, adsorption chromatography, affinity chromatography.
Principles of the separation techniques of proteins

1. Dialysis

A solution containing the protein of interest must be further altered before purification steps are possible. Proteins can be separated from small molecules by taking advantage of the larger size of the protein compared to other molecules. Dialysis through a semi permeable membrane (SEM), such as a cellulose membrane with well defined pores is often done to free a protein of choice from other contaminants. The partially purified protein solution is placed in a dialysis bag and the bag suspended in a much larger volume of buffer of appropriate ionic strength. Proteins molecules having significantly greater dimensions than the pore diameter of the dialysis bag, whereas smaller molecules and ions cross the pores of such membranes and emerge in the dialysate outside the bag. This technique is useful for removing a salt or other small molecule, but it will not distinguish between proteins effectively. Dialysis bags of definite molecular weight cut off are often employed to purify protein of definite size. For example, a dialysis bag having a 12,000 molecular weight cut off can be safely used to purify proteins having molecular weights of ~20,000 but may not be appropriate for proteins with a molecular weight of 10,000.
Salting Out

Most proteins are less soluble at high salt concentrations, an effect called salting out. Proteins in aqueous solutions are highly hydrated, and with the addition of salt, the water molecules become more attracted to the salt than to the protein due to the higher charge. The addition of ammonium sulfate in the requisite amount can selectively precipitate a protein of interest while others remain in solution. For example, 0.8 M ammonium sulfate can precipitate fibrinogen, whereas a concentration of 2.4 M is needed to precipitate serum albumin. Salting out is very useful for concentrating dilute solutions of proteins, and has no adverse structural effects. In this processes, the ammonium sulfate concentration is increased stepwise by adding solid ammonium sulfate in small quantities. The amount depends on the volume of the solution and the percentage saturation of the salt needed. It is possible to calculate how much is needed from available published nomograms (a diagram representing the relations between three or more variable quantities by means of a number of scales, so arranged that the value of one variable can be found by a simple geometrical construction, e.g. by drawing a straight line intersecting the other scales at the appropriate values). For protein purification, a step precipitation is carried out in which the precipitated protein is removed by centrifugation and the ammonium sulfate concentration increased to a value that will precipitate most of the protein of interest. In most cases this leaves out the protein contaminants in solution. The precipitated protein of interest is further recovered by the process of centrifugation and the pellet obtained dissolved in fresh buffer to be purified further. If required the above process is repeated usually with a different concentration of ammonium sulfate for precipitation of a different protein from the supernatant.
Salting In
As mentioned earlier, the solubility of proteins is strongly dependent on the salt concentration or ionic strength of the medium. Proteins are usually poorly soluble in pure water. Their solubility increases as the ionic strength increases, because more and more of the well-hydrated inorganic ions (blue circles) are bound to the protein’s surface, preventing aggregation of the molecules (salting in).
1. Basics of Ultrafiltration

1.1. Process

Ultrafiltration, being a part of membrane filtration processes, is a pressure-driven filtration technology.

A basic process diagram is shown in **Graph 1**.

![Graph 1: Basic membrane process diagram](image)

The feedwater is hereby pressed into the module by a pump, and permeates through the membrane due to the transmembrane pressure (TMP) difference.

Depending on the membrane’s pore size, water contaminants are being rejected by the membrane and remain in the feed water.

In order to avoid a too high concentration of rejected contaminants, which can consist of colloids as well as molecules, atoms or ions, a part of the feed is taken out of the system as concentrate.

Too high concentration of contaminants in a membrane system can lead to mineral scaling on the membrane or fouling of colloids, building up a cake layer on the membrane, changing filtration properties and necessary filtration pressures.
**Ultracentrifugation**

B. *Preparative Ultracentrifugation*

*Preparative ultracentrifuges, which as their name implies* are designed for sample preparation, differ from analytical ultracentrifuges in that they lack sample observation facilities. Preparative rotors contain cylindrical sample tubes whose axes may be parallel, at an angle, or perpendicular to the rotor’s axis of rotation, depending on the particular application. It was assumed that sedimentation occurred through a homogeneous medium. Sedimentation may be carried out in a solution of an inert substance, however, such as sucrose or CsCl, in which the concentration, and therefore the density, of the solution increases from the top to the bottom of the centrifuge tube. The use of such *density gradients greatly enhances the resolving power* of the ultracentrifuge. Two applications of density gradients are widely employed: (1) *zonal ultracentrifugation* and (2) *equilibrium density gradient ultracentrifugation*.

a. **Zonal Ultracentrifugation Separates Particles According to Their Sedimentation Coefficients**

In zonal ultracentrifugation, a macromolecular solution is carefully layered on top of a density gradient prepared by use of a device. The purpose of the density gradient is to allow smooth passage of the various macromolecular zones by damping out convective mixing of the solution. Sucrose, which forms a syrupy and biochemically benign solution, is commonly used to form a density gradient for zonal ultracentrifugation. The density gradient is normally rather shallow because the maximum density of the solution must be less than that of the least dense macromolecule of interest. During centrifugation, each species of macromolecule moves through the gradient at a rate largely determined by its sedimentation coefficient and therefore travels as a zone that can be separated from other such zones as is diagrammed in Fig. 6-30. After centrifugation, fractionation is commonly effected by puncturing the bottom of the celluloid centrifuge tube with a needle, allowing its contents to drip out, and collecting the individual zones for subsequent analysis.
Figure 6-30  **Zonal ultracentrifugation.** The sample is layered onto a sucrose gradient (left). During centrifugation (middle), each particle sediments at a rate that depends largely on its mass. After the end of the run, the centrifugation tube is punctured and the separated particles (zones) are collected (right).
b. Equilibrium Density Gradient Ultracentrifugation Separates Particles According to Their Densities

In equilibrium density gradient ultracentrifugation [alternatively, isopycnic ultracentrifugation (Greek: *isos*, equal *pyknos*, dense)], the sample is dissolved in a relatively concentrated solution of a dense, fast-diffusing (and therefore low molecular mass) substance, such as CsCl or Cs2SO4, and is spun at high speed until the solution achieves equilibrium. The high centrifugal field causes the low molecular mass solute to form a steep density gradient in which the sample components band at positions where their densities are equal to that of the solution. These bands are collected as separate fractions when the sample tube is drained as described above. The salt concentration in the fractions and hence the solution density is easily determined with an Abbé refractometer, an optical instrument that measures the refractive index of a solution. The equilibrium density gradient technique is often the method of choice for separating mixtures whose components have a range of densities. These substances include nucleic acids, viruses, and certain subcellular organelles such as ribosomes. However, isopycnic ultracentrifugation is rather ineffective for the fractionation of protein mixtures because most proteins have similar densities (high salt concentrations also salt out or possibly denature proteins). Density gradient ultracentrifugation in an analytical ultracentrifuge was used to show that DNA is semiconservatively replicated.
Figure 6-9 Gel filtration chromatography. (a) A gel bead, whose periphery is represented by a dashed line, consists of a gel matrix (wavy solid lines) that encloses an internal solvent space. Smaller molecules (red dots) can freely enter the internal solvent space of the gel bead from the external solvent space. However, larger molecules (blue dots) are too large to penetrate the gel pores. (b) The sample solution begins to enter the gel column (in which the gel beads are now represented by brown spheres).

(c) The smaller molecules can penetrate the gel and consequently migrate through the column more slowly than the larger molecules that are excluded from the gel. (d) The larger molecules emerge from the column to be collected separately from the smaller molecules, which require additional solvent for elution from the column. (e) The elution diagram of the chromatogram indicating the complete separation of the two components, with the larger component eluting first. See the Animated Figures.
Figure 6-10  Molecular mass determination by gel filtration chromatography. The graph shows the relative elution volume versus the logarithm of molecular mass for a variety of proteins on a cross-linked dextran column (Sephadex G-200) at pH 7.5. Orange bars represent glycoproteins (proteins with attached carbohydrate groups). [After Andrews, P., Biochem. J. 96, 597 (1965).]
Gas-liquid chromatography (often just called gas chromatography) is a powerful tool in analysis. It has all sorts of variations in the way it is done - if you want full details, a Google search on gas chromatography will give you scary amounts of information if you need it! This page just looks in a simple introductory way at how it can be carried out.

All forms of chromatography involve a **stationary phase** and a **mobile phase**. In all the other forms of chromatography you will meet at this level, the mobile phase is a liquid. In gas-liquid chromatography, the mobile phase is a gas such as helium and the stationary phase is a high boiling point liquid adsorbed onto a solid.

How fast a particular compound travels through the machine will depend on how much of its time is spent moving with the gas as opposed to being attached to the liquid in some way.
A flow scheme for gas-liquid chromatography

Note: You will have to imagine the coiled column in its oven. Drawing a convincing and tidy coil defeated me completely!
**Injection of the sample**

Very small quantities of the sample that you are trying to analyse are injected into the machine using a small syringe. The syringe needle passes through a thick rubber disc (known as a septum) which reseals itself again when the syringe is pulled out.

The injector is contained in an oven whose temperature can be controlled. It is hot enough so that all the sample boils and is carried into the column as a gas by the helium (or other carrier gas).

For more information log onto

https://www.chemguide.co.uk/analysis/chromatography/gas.html
High performance liquid chromatography is a powerful tool in analysis. This page looks at how it is carried out and shows how it uses the same principles as in thin layer chromatography and column chromatography.

High performance liquid chromatography is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster.

It also allows you to use a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture.

The other major improvement over column chromatography concerns the detection methods which can be used. These methods are highly automated and extremely sensitive.
Confusingly, there are two variants in use in HPLC depending on the relative polarity of the solvent and the stationary phase.

**Normal phase HPLC**

This is essentially just the same as you will already have read about in thin layer chromatography or column chromatography. Although it is described as "normal", it isn't the most commonly used form of HPLC.

The column is filled with tiny silica particles, and the solvent is non-polar - hexane, for example. A typical column has an internal diameter of 4.6 mm (and may be less than that), and a length of 150 to 250 mm.

Polar compounds in the mixture being passed through the column will stick longer to the polar silica than non-polar compounds will. The non-polar ones will therefore pass more quickly through the column.
Reversed phase HPLC

In this case, the column size is the same, but the silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them. A polar solvent is used - for example, a mixture of water and an alcohol such as methanol.

In this case, there will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column. There won't be as much attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution. Polar molecules in the mixture will therefore spend most of their time moving with the solvent.

Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of van der Waals dispersion forces. They will also be less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in between the water or methanol molecules, for example. They therefore spend less time in solution in the solvent and this will slow them down on their way through the column.

That means that now it is the polar molecules that will travel through the column more quickly. Reversed phase HPLC is the most commonly used form of HPLC.
Looking at the whole process

A flow scheme for HPLC

1. Sample injection
2. Solvent reservoir
3. Pump to produce high pressure
4. HPLC tube
5. Detector
6. Processing unit and display
7. Signal to processor
8. Waste
FPLC was developed and marketed in Sweden by Pharmacia in 1982 and was originally called **fast performance liquid chromatography** to contrast it with HPLC or high-performance liquid chromatography. FPLC is generally applied only to proteins; however, because of the wide choice of resins and buffers it has broad applications. In contrast to HPLC the buffer pressure used is relatively low, typically less than 5 bar, but the flow rate is relatively high, typically 1-5 ml/min. FPLC can be readily scaled from analysis of milligrams of mixtures in columns with a total volume of 5ml or less to industrial production of kilograms of purified protein in columns with volumes of many liters. When used for analysis of mixtures the effluent is usually collected in fractions of 1-5 ml which can be further analyzed, e.g. by MALDI mass spectrometry. When used for protein purification there may be only two collection containers, one for the purified product and one for waste.
Iso-electric Precipitation

The isoelectric point (pI) is the pH of a solution at which the net primary charge of a protein becomes zero. At a solution pH that is above the pI the surface of the protein is predominantly negatively charged and therefore like-charged molecules will exhibit repulsive forces. Likewise, at a solution pH that is below the pI, the surface of the protein is predominantly positively charged and repulsion between proteins occurs. However, at the pI the negative and positive charges cancel, repulsive electrostatic forces are reduced and the attraction forces predominate. The attraction forces will cause aggregation and precipitation. The pI of most proteins is in the pH range of 4–6. Mineral acids, such as hydrochloric and sulfuric acid are used as precipitants. The greatest disadvantage to isoelectric point precipitation is the irreversible denaturation caused by the mineral acids. For this reason isoelectric point precipitation is most often used to precipitate contaminant proteins, rather than the target protein. The precipitation of casein during cheesemaking, or during production of sodium caseinate, is an isoelectric precipitation.
Addition of miscible solvents such as ethanol or methanol to a solution may cause proteins in the solution to precipitate. The solvation layer around the protein will decrease as the organic solvent progressively displaces water from the protein surface and binds it in hydration layers around the organic solvent molecules. With smaller hydration layers, the proteins can aggregate by attractive electrostatic and dipole forces. Important parameters to consider are temperature, which should be less than 0 °C to avoid denaturation, pH and protein concentration in solution. Miscible organic solvents decrease the dielectric constant of water, which in effect allows two proteins to come close together.
**Figure 6-6**  Ion exchange chromatography using stepwise elution. Here the tan region of the column represents the ion exchanger and the colored bands represent the various proteins. (a) The protein mixture is bound to the topmost portion of the ion exchanger in the chromatography column. (b) As the elution progresses, the various proteins separate into discrete bands as a consequence of their different affinities for the ion exchanger under the prevailing solution conditions. Here the first band of protein (red) has passed through the column and is being isolated as a separate fraction, whereas the other, less mobile, bands remain near the top of the column. (c) The salt concentration in the elution buffer is increased to increase the mobility of and thus elute the remaining bands. (d) The elution diagram of the protein mixture from the column.  See the Animated Figures
Table 6-2  Some Biochemically Useful Ion Exchangers

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Ionizable Group</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE-cellulose</td>
<td>Weakly basic</td>
<td>Diethylaminoethyl (-CH_2CH_2N(C_2H_5)_2)</td>
<td>Used to separate acidic and neutral proteins</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>Weakly acidic</td>
<td>Carboxymethyl (-CH_2COOH)</td>
<td>Used to separate basic and neutral proteins</td>
</tr>
<tr>
<td>P-cellulose</td>
<td>Strongly and weakly acidic</td>
<td>Phosphate (-OPO_3H_2)</td>
<td>Dibasic; binds basic proteins strongly</td>
</tr>
<tr>
<td>Bio-Rex 70</td>
<td>Weakly acidic, polystyrene-based</td>
<td>Carboxylic acid (-COOH)</td>
<td>Used to separate basic proteins and amines</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>Weakly basic cross-linked dextran gel</td>
<td>Diethylaminoethyl (-CH_2CH_2N(C_2H_5)_2)</td>
<td>Combined chromatography and gel filtration of acidic and neutral proteins</td>
</tr>
<tr>
<td>SP-Sepharose</td>
<td>Strongly acidic cross-linked agarose gel</td>
<td>Methyl sulfonate (-CH_2SO_3H)</td>
<td>Combined chromatography and gel filtration of basic proteins</td>
</tr>
</tbody>
</table>

*Sephadex and Sepharose are products of GE Healthcare; Bio-Rex resins are products of BioRad Laboratories.*
Figure 6-12  Affinity chromatography. A ligand (yellow) is covalently anchored to a porous matrix. The sample mixture (whose ligand-binding sites are represented by the cutout squares, semicircles, and triangles) is passed through the column. Only certain molecules (represented by orange circles) specifically bind to the ligand; the others are washed through the column.
Figure 6-14 Derivatization of epoxy-activated agarose. Various types of nucleophilic groups can be covalently attached to epoxy-activated agarose via reaction with its epoxide groups.

\[ \text{R} = \text{Ligand} \]