CHAPTER 10

ALTERNATIVE BIOSEPARATION TECHNIQUES

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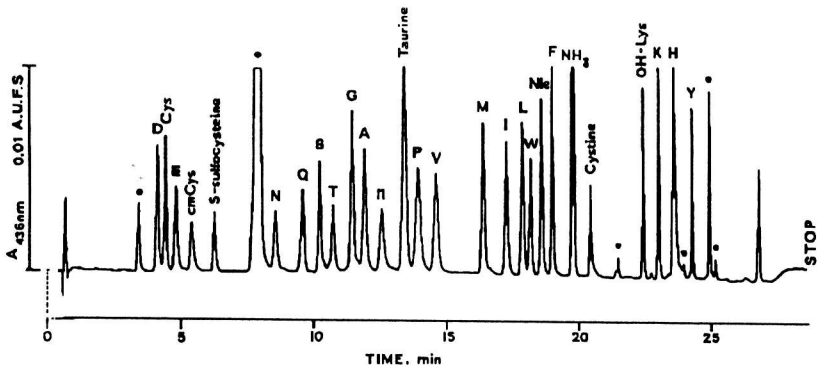
1. Introduction

Chromatography is now a well established separation technique that is utilized in all areas of biotechnology. New applications are continually being explored as biochemists become more and more aware of its potentialities.

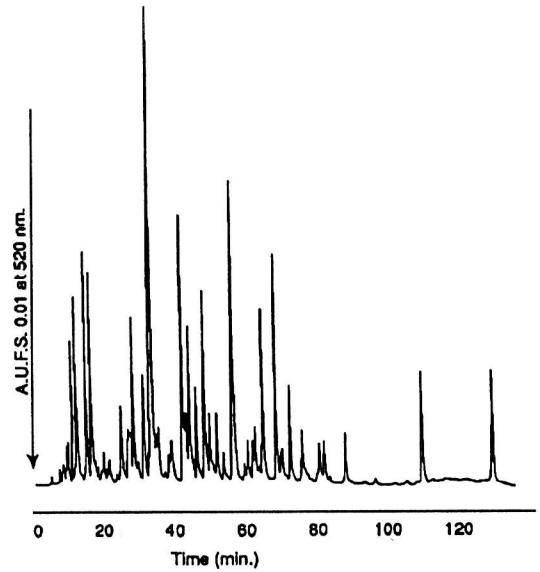
Due to the high resolving power of HPLC, separation of complex mixtures of components can now be performed in a short analysis time. Most of the separations are simple, reproducible and reliable. These characteristics are largely responsible for the success of HPLC in biochemistry and biotechnology which can be demonstrated by the separation of amino acids (Figure 1), proteins (Figure 2) and nucleic acids (Figures 3 and 4). The application of HPLC to nucleic acid and protein analyses are covered in detail in Chapters 7 and 9. Different areas have contributed significantly to the development of traditional chromatographic methods in HPLC: instrumentation that has allowed easy automation, the technology to produce particles of a well defined diameter and porosity, and the theory that has allowed a better understanding of the interaction of components with the matrix under different experimental conditions.



The column used was Supelcosil LC 18, 15 cm long, 4.6 mm I.D. packed with 3 µm particles. The separation was obtained at room temperature using system [1]. The amount of each standard DABS amino acid present in the mixture ranged from 5 to 25 pmol. The chromatogram was obtained using a fixed wavelength detector, Model 160, from Beckman equipped with a 18.5 µl flow cell [1]

Figure 1 Original Chromatogram of a Complete Reversed Phase High Performance Liquid Chromatography Separation of about 35 DABS Amino Acids

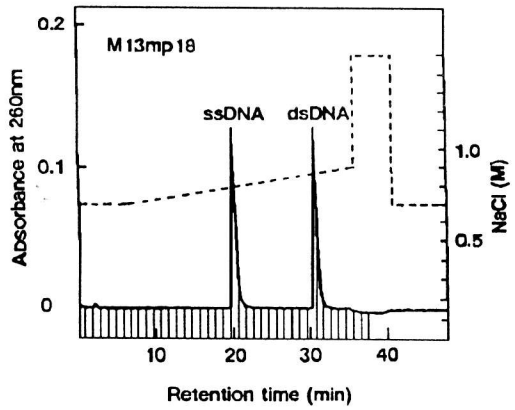
Modern bioseparation sciences play a very important role in biotechnology, biochemistry, protein engineering, molecular biology and the biomedical sciences. The possibility of large amounts of recombinant protein, both 'native proteins' and 'mutant proteins' engineered by site-directed mutagenesis, promises to change completely the approach now taken to better understand protein structure–function relationships and protein-folding phenomena, and to furnish adequate protein pharmaceuticals for therapeutic purposes. However, the possibility of producing therapeutic proteins in large amounts using the recombinant DNA technology requires the use of analytical methods more efficient than those previously required to ensure the purity and identity of recombinant proteins. The criteriaonof purity, in the light of the possibility of administering recombinant proteins to individuals, must be considered.



The tryptic peptides were labeled with *N*, *N*-dimethylaminoazobenzene iodoacetamide. The analysis was performed using a Supelcosil LC-318 column 25 cm long and 4.6 mm I.D. and packed with 5 µm particles. The separation was obtained at room temperature using a gradient shown in the figure where solvent (A) was water containing 0.1% trifluoroacetic acid and solvent (B) acetonitrile containing 0.1% trifluoroacetic acid. The flow rate was 0.7 ml/min and the detection was performed at 520 nm (Stocchi *et al.*, unpublished results).

Figure 2 The Separation of a Mixture of Tryptic Peptides Obtained from Bovine Serum Albumin.

In fact, in the case of human growth hormone (hGH), a protein which is able to stimulate the protein synthesis, the dose administered to athletes is 40 ng/kg each day. For a 70 kg subject, that means that 2.8 µg of recombinant protein is injected. A small contamination of 2.8 µg, corresponding to 99.9% purity, is enough to engender an immunological and/or allergic response. Therefore, in the case of recombinant proteins of therapeutic interest, a purity grade of 99.9% is not adequate. In such cases it is absolutely necessary to have the protein required in a pure form without any contamination.

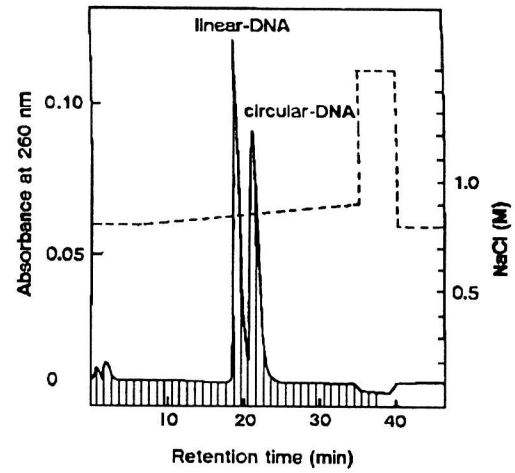


The amounts of single stranded and double stranded DNA were 1 and 10 µg respectively. Experimental condition were those described in Figure 1 (2) except that the concentration gradient from 0.7 to 0.9 M NaCl was accomplished within 30 minutes (2)

Figure 3 The Separation of Single Stranded and Double Stranded DNA of Sample M13mp18

In biotechnology it is very important to have very efficient methods both at the analytical and preparative levels. Although electrophoresis appears to be a promising tool, at present it is not suitable for preparative purposes. In fact, in the case of recombinant protein of therapeutic interest, although it is very important to access its identity and purity using very efficient analytical methods, but it is also necessary to use very efficient preparative methods allowing to cope with the production of the protein of interest in very large amounts from grams to kilograms and more.

Very often it is not easy to scale up the method that works well at the analytical level. In general liquid chromatography allows to scale up processes from the analytical to the preparative levels (see chapter 6). The same is not possible with other complimentary techniques such as electrophoresis, capillary electrophoresis and ultracentrifugation.



The amounts of supercoiled circular and linear DNA were 7 and 10 µg respectively. Experimental conditionss were the same as that described in Figure 1, [2] except that the concentration gradient from 0.8 to 0.9 M NaCl was accomplished within 30 min [2].

Figure 4 The Separation Supercoiled Circular and Linear DNA of pGEM-7Zf

Therefore, a great contribution from HPLC in solving most of the above problems in biotechnology both at the analytical and preparative levels is expected.

Nevertheless, chromatography is not the 'philosophers stone' to the separation scientist and there are many problems in biotechnology that cannot be solved by a chromatographic process. Other separation methods are still necessary, in fact vital, in biotechnology research, development and production. Among these are various electrophoretic procedures together with the well established ultracentrifuge and ultrafiltration techniques. It is important to avoid leaving the reader with a biased view of chromatography and its capabilities. Therefore, the purpose of this final chapter is to describe some of the separation techniques also important in biotechnology. In doing so, it is hoped that it will help place

chromatography in its true perspective relative to other separation methods that are used so successfully by the biotechnologists.

2. Electrophoresis

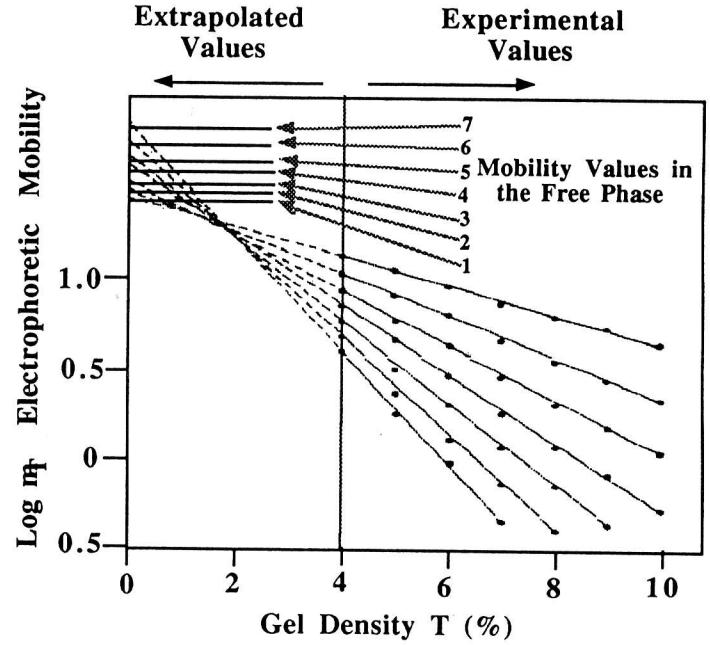
Electrophoresis is a highly effective separation technique that has also found wide application in the biotechnology field. Perhaps the main drawback of electrophoresis is that it is a labor-intensive technique still requiring substantial manpower. However, some instrumental approaches such as the Fast System (Pharmacia–LKB) and capillary zone electrophoresis (CZE) allow for more extensive use of robotics and computer-driven equipment, with a possibility of storage of electropherograms on a magnetic support and easier on-line detection and peak quantification.

The theory of electrophoresis has been discussed by Everaerts *et al.* [3] and by Andrews [4]. Here the focus will be mostly on electrophoresis in gel-stabilized media and this limited to protein applications, although superb separations have been obtained recently in the field of DNA sequencing and DNA fragment analysis in capillaries filled with viscous polymer solutions [5].

Electrophoretic separations are achieved by the differential migration of electrically charged particles when situated in an electric field. As a consequence, the method is only applicable to ionic or ionogenic materials. *i.e.* substances convertible to an ionic species (for example: neutral sugars, which form negatively charged complexes with borate ions). With the advent of capillary zone electrophoresis, the technique has been extended to a host of neutral substances that can be induced to migrate in an electric field by inclusion in charged micelles, *e.g.* of anionic (sodium dodecyl sulfate) or cationic (cetyltrimethylammonium bromide) surfactants. Even compounds that are not ionic or ionogenic cannot form complexes can often be analyzed by eluting them by strong electro-osmotic flow resulting from charges on the capillary walls [6]. the differing forms of electrophoresis will now be discussed individually.

2.1. Discontinuous Electrophoresis

In 1959 Raymond and Weintraub [7] described the use of polyacrylamide gels in capillary zone electrophoresis. These gels, had substantial advantages over the previously used starch gels as they were transparent to UV and visible light (starch gels are opalescent) and were available in an extensive range of pore sizes to accommodate molecules having a wide span of molecular weights. In 1964, Ornstein [8] and Davis [9] developed discontinuous (disc) electrophoresis, whereby proteins are separated on the basis of two parameters: surface charge and molecular mass.



Here m_T is the relative mobility, %T is the total concentration of monomers (acrylamide and Bis) in the gel. The point at which the extrapolated curves intercept the log (m_T) axis represents the mobility value in the free phase. Parallel lines would indicate charge isomers, whereas lines of different slopes indicate size variants [11].

Figure 5 Ferguson Plots for Polymeric Forms of Serum Albumin

Ferguson [10] showed that one can distinguish the two by plotting the results of a series of experiments with polyacrylamide gels of varying porosity against gel density. For each protein, the slope of the curve log(mT) (electrophoretic mobility) *vs.* density (%T) was found to be proportional to molecular mass, and the intercept was shown to be a measure of the surface charge (see Figure 5) [11].

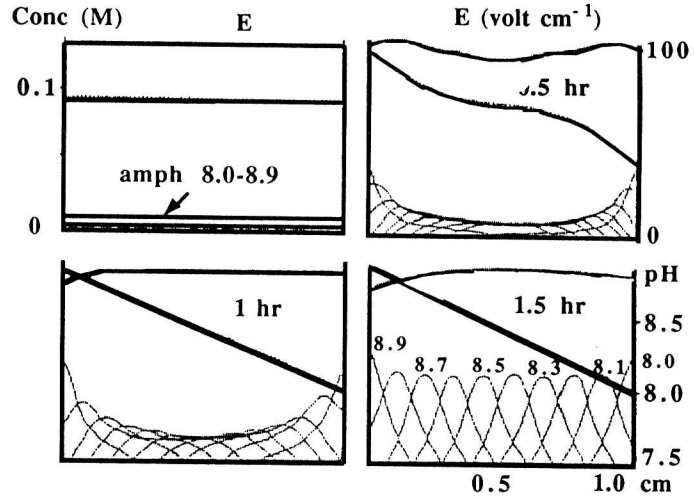
Unfortunately this procedure is quite tedious, consequently, and has rarely been used in biochemical work. Moreover, recently, non-linear Ferguson plots have been reported [12] which may cast some doubt on the validity of this approach.

2.2 Isoelectric Focusing (IEF)

In 1961 Svensson-Rilbe [12]) investigated the possibility of isoelectric focusing, whereby amphoteric compounds are fractionated according to their isoelectric point (pI) values along a continuous pH gradient. In contrast, to zone electrophoresis, where the constant pH of the separation medium establishes a constant charge density at the surface of the molecule and causes it to migrate with constant mobility, the surface charge of an amphoteric compound in isoelectric focusing keeps changing and decreasing in accordance with its titration curve as it moves along the pH gradient, approaching its steady-state position, *i.e.* the region where the pH matches its (pI).

There its mobility equals zero and the molecule comes to a stop. The gradient is created and maintained by the passage of current through a solution of amphoteric compounds with closely spaced (pI) values encompassing a given pH range [14]. The electrophoretic transport causes these buffers (carrier ampholytes) to stack according to their (pI) values, and a pH gradient increasing from anode to cathode is established. This process is known as computer simulation of Figure 6 [15].

At the beginning of the run, the medium has an uniform pH, which equals the average (pI) of the carrier ampholytes and uniform carrier ampholyte concentration (Figure 6, upper left panel).



The isotonic points of the ampholytes are evenly distributed in the pH 8.0-8.9 range. The initial distribution of the amphoteric buffers is indicated in the upper left diagram. Calculation was performed assuming a constant voltage of 100 Y/cm across the system . The anode is positioned to the right in the diagrams. Each (x) axis represents the distance from the cathode on the same scale as the bottom right figure [17].

Figure 6 Calculated Time Development of an Isoelectric Focusing Process Involving Ten Ampholytes in a Closed Vessel

At t=0.5 h, the most acidic (pI+8.0) and the most alkaline (pI = 8.9) species have collected close to the anode and cathode, respectively, so that portions of the pH gradient begin to develop. At t=1 h, the ampholytes have separated further, and at this point an almost linear pH gradient that spans the pH range defined by the (pI) values of the ampholytes has been established.

In the simulation, a system of ten ampholytes, in equimolar ratios, has (pI) values spaced at 0.1 pH units increments in the pH range 8.0–8.9. At t= 1.5 h the ampholytes have separated into symmetrical zones having overlapping Gaussian profiles, as predicted by Svensson [13]. At this point, the system has achieved a steady state, maintained by the electric field, and no further mass transport is occurs, except from symmetric, to-

and-from micromovements of each species about its (pI). This oscillation is generated by the action of two opposite forces, diffusion and voltage gradient, acting on each focused component. This pendulum movement, diffusion–electrophoresis, is the primary cause of the residual current under isoelectric steady state conditions.

The technique only applies to amphoteric compounds and, more precisely, to good ampholytes with small (pI–pKl) values, *i.e.* with a steep titration curve around their (pI), *coditio sine qua non* for any compound to focus in a narrow band. This seldom creates a problem with proteins but short peptides need to contain at least one acidic and one basic amino acid residue besides the $-NH_2$ and the –COOH termini (which could make them isoelectric between *ca.* pH 4.0 and pH 9.0 and prevent them from focusing). Another limitation with short peptides is encountered at the maximum detector sensitivity, as carrier ampholytes react with most peptide stains. This problem may be circumvented by using specific stains or by resorting to immobilized pH gradients that show no background reactivity to ninhydrin and other common stains for primary amino groups (*e.g.* dansyl chloride, fluorescamine).

The general properties of carrier ampholytes, (*i.e.* the amphoteric buffers used to generate and stabilize the pH gradient in isoelectric focusing) can be summarized as follows:

1/ physical chemical properties:(a) buffering ion has a mobility of zero at (pI); (b) good conductance; (c) good buffering capacity;

2/ practical requirements: (a) good solubility; (b) no influence on detecting system: (c) no influence on sample: (d) separable from sample;

3/ precautions: (a) minimize plateau effect (which is explained below) drift of pH gradient; (b) avoid chemical change in sample; (c) avoid complex formation.

The 'plateau effect' or 'cathodic drift' is a slow decay of the pH gradient with time, whereby on prolonged focusing at high voltages, the pH

gradient with the focused proteins is lost into the cathodic compartment. There seems no remedy to this problem, as it arises from the strong electro-osmotic flow generated by the covalently affixed negative charges in the matrix (carboxyls and sulfate in both polyacrylamide and agarose).

In chemical terms, carrier ampholytes are oligoamino, oligocarboxylic acids available under trade names such as Ampholine from LKB Produkter AB, Pharmalyte from Pharmacia Fine Chemicals, Biolyte from Bio Rad, Servalyte from Serva GmbH and Resolyte from BDH. There are two methods of synthesis: (a) the Vesterberg approach [14], in which different oligoamines (tetra-, penta-, and hexamines) are allowed to react with acrylic acid, and (b) the Pharmacia synthetic process (16) which involves copolymerization of amines, amino acids and dipeptides with epichlorohydrin. The wide range synthetic mixture (pH 3.0–10.0) seems to contain hundreds, possibly thousands of different amphoteric chemicals having (pI) values evenly distributed along the pH scale.

Carrier ampholytes, from any source, should have an average molecular mass around 750 Daltons (size interval 600–900 Daltons, the higher molecular mass referring to the more acidic carrier ampholyte species). As a consequence, unless they are hydrophobically complexed to proteins, they should easily be separated from macromolecules by gel filtration Dialysis is not recommended, due to the tendency of carrier ampholytes to aggregate, but salting out of proteins with ammonium sulfate seems to eliminate any contaminant carrier ampholytes completely. A further complication arises from the chelating effect of acidic carrier ampholytes, especially towards Cu^{2+} ions, which could inactivate metallo-enzymes. In addition, focused carrier ampholytes represent a medium of very low ionic strength (less than 1 me/ml at steady state). Since the isoelectric state involves minimum solubility for the protein macro-ion, there could be a tendency for some proteins, *e.g.* globulins, to precipitate during isoelectric focusing in the proximity of their (pI). This is a severe problem in preparative isoelectric focusing, but in analytical procedures it can be diminished by reducing the sample size.

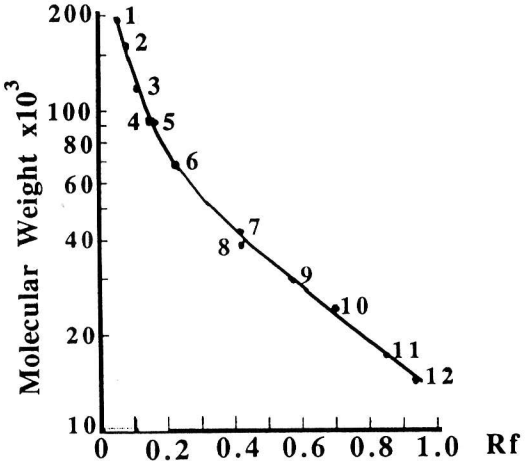
2.3. Sodium Dodecyl Sulfate Electrophoresis

Sodium dodecyl sulfate electrophoresis (enveloping uncharged molecules with surfactants) followed logically from disc electrophoresis and while disc electrophoresis discriminates macromolecules on the basis of both size and surface charge, sodium dodecyl sulfate electrophoresis fractionates polypeptide chains essentially on the basis of their size. It is therefore a simple, yet powerful and reliable method for molecular mass determination. In 1967 Shapiro *et al.* [117] reported that electrophoretic migration of polypeptides in the presence of sodium dodecyl sulfate is proportional to the effective molecular radius and, thus, to the average molecular mass of the polypeptide chain. This means that sodium dodecyl sulfate must bind to proteins and mask differences in molecular charge, so that all the components will migrate solely according to size. Surprisingly, large amounts of sodium dodecyl sulfate appear to be bound (an average of 1.4 g of sodium dodecyl sulfate per g of protein). It follows that the number of sodium dodecyl sulfate molecules bound is about half the number of amino acid residues in a polypeptide chain. This amount of highly charged surfactant molecules is sufficient to effectively suppress the intrinsic charges of the polymer coil, so that their net charge per unit mass becomes approximately constant. If migration in sodium dodecyl sulfate (and disulfide reducing agents, such as 2-mercapto-ethanol, used in the denaturing step for proper unfolding of proteins) is only proportional to the molecular mass, then, in addition to masking charge differences, sodium dodecyl sulfate also equalizes molecular shape differences (*e.g.* globular *versus* rod-shaped molecules). This, indeed, seems to happen with sodium dodecyl sulfate mixed micelles. These complexes can be assumed to behave as ellipsoids of a constant minor axis (*ca* 1.8 nm) and a major axis proportional to the length of the amino acid chain (*i.e.* to molecular mass) of the protein. The rod length of the 1.4 g sodium dodecyl sulfate–protein complex is of the order of 0.074 nm per amino acid residue. The review of Helenius and Simons [18] is recommended for further information on detergent properties,.

During electrophoresis with sodium dodecyl sulfate, the proteins can be pre-labeled with dyes that covalently bind to their NH_2 residues. The dyes

can be conventional (like the blue dye Remazo) or fluorescent like dansyl chloride, fluorescamine, 2-methoxy-2,4,-diphenyl-3 [2*H*] - furanone and *o*-phthalaldehyde. Pre-labeling is compatible with electrophoresis in the presence of sodium dodecyl sulfate, as the size increase is minimal, but would be an anathema in disc electrophoresis or isoelectric focusing, as it would generate a series of bands of slightly altered mobility or (pI) from an otherwise homogeneous protein.

The sample and molecular mass standards are run side-by-side in a gel slab. After the polypeptide zones have been detected, values of log (average molecular mass) are plotted against the migration distance (or R_f) to produce a calibration curve (Figure 7), [19], from which the molecular mass of the sample can be calculated.



Note that the plot under these experimental conditions is linear only in the molecular mass range 15–60 kiloDaltons. Markers: 1, myosin; 2, RNA polymerase (-subunit); 3, β-galactosidase; 4, phosphorylase B; 5, RNA polymerase (-subunit); 6, bovine serum albumi; 7, ovalbumin; 8, RNA polymerase (β-subunit); 9, carbonic anhydrase; 10, trypsinogen; 11, β-lactoglobulin; 12, lysozyme [19].

Figure 7 Typical Log Molecular Mass *versus* RF Plot after Sodium Dodecyl Sulfate Polyacrylate Gel Electrophoresis

It should be noted that, in a gel of constant %T, linearity is obtained only in a certain range of molecular size. Outside this limit (seen as above 60 kilodaltons in Figure 3) a new gel matrix of appropriate porosity should be used.

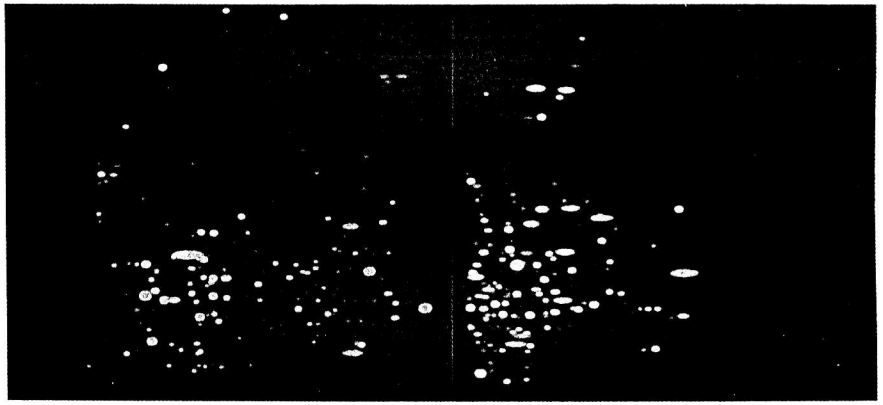
Two classes of proteins show anomalous behavior in sodium dodecyl sulfate electrophoresis: glycoproteins (because their hydrophilic oligosaccharide units prevent hydrophobic binding of sodium dodecyl sulfate micelles) and strongly basic proteins, *e.g.*, histones (because of the electrostatic binding of the sodium dodecyl sulfate micelles by their sulfate groups). The first can be partially alleviated by using alkaline Tris/borate buffers [20], which will increase the net negative charge on the glycoprotein and thus produce migration rates well correlated with molecular size. The migration of histones can be improved by using pore gradient gels and allowing the polypeptide chains to approach the pore limit [21].

2.4. Two-Dimensional Maps

In a two–dimensional separation, the sample is subjected to two displacement processes oriented at right angles to one another. Each displacement step carries components from their point of application in one dimension into a two–dimensional field. The use of two dimensions provides more space for the resolution of multicomponent mixtures. The high intrinsic resolving power of two-dimensional separations holds great promise for resolving complex biological samples, for which one–dimensional methods have inadequate component capacity. In fact the maximum separation power of two–dimensional systems is described approximately by the multiplicative law:

$$n_2 = n_y.n_x = n_1^2$$

where the subscripts 2 and 1 refer to two dimensional and one dimensional values respectively, and (n) is the peak capacity, *i.e.* the number of peaks or zones that will fit into the available separation space.



There are 1244 spots on this map. A: Equilibrium isoelectric focusing pattern in the pH 3–7.5 interval B: non-equilibrium isoelectric focusing attern for alkaline (pH 7.5–10) spots [28].

Figure 8 Synthetic Image of the Two–dimensional Fluorogram of [^{35}S]Methionine-Labeled Proteins from Human Epithelial Amnion Cells

Two dimensional maps are most successful when the separation in each dimension is based on completely different separation mechanisms, so that solute spots are spread out randomly in a two–dimensional plane. There are a vast number of potential two–dimensional systems, based on different displacement principles. However, in biochemistry, the only technique which has been adopted is one based on the charge mass co-ordinates, as outlined below. By coupling sequentially two techniques, a charge based separation (isoelectric focusing) to a size–based separation (sodium dodecyl sulfate polyacrylamide gel electrophoresis), the latter orthogonally to the former, the polypeptide chains can be moved two–dimensionally, with charge and mass co-ordinates. The technique was

reported for the first time by Barrett and Gould [22] and then described in more detail in 1975 by O'Farrell [23], Klose [24], and Svheele [25].

Large gels (*e.g.* 30 cm x 40 cm) [26] and prolonged exposure to radio-labeled material (up to two months) have allowed the resolution of as many as 12, 000 labeled peptides in a total mammalian cell lysate. Thus, it is probable that, in a properly prepared two-dimensional map, a spot will represent an individual polypeptide chain uncontaminated by other material. That being so, provided that enough material is present in an individual spot (about 1 µg), it would be possible to remove the sample by blotting for subsequent sequencing [27]. A nice example of a two-dimensional separation is shown in Figure 8 [28]: a human amnion cell lysate is run first at equilibrium in a pH 3–7.5 gradient and then the alkaline portion is separated by isoelectric focusing.

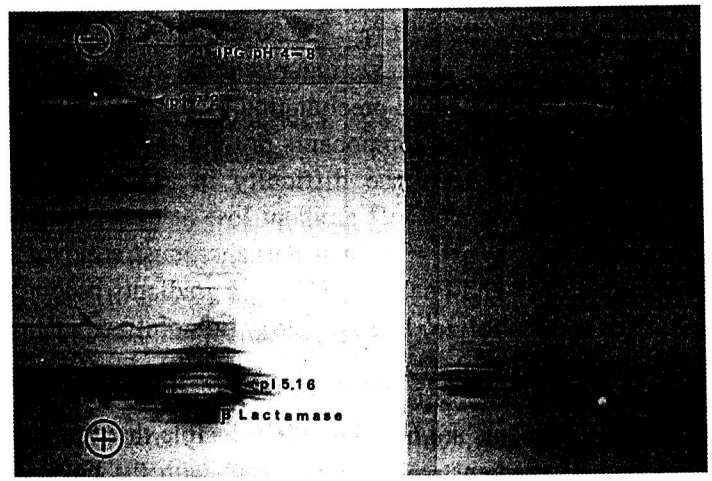
In general, conventional two-dimensional maps cannot be extended above pH 7.5: one of the main reasons is linked to the use of 2-mercaptoethanol in the denaturing solution (Righetti *et al.* [29]. 2-mercaptoethanol is a buffer and it destroys the alkaline portion of the pH gradient. By applying the sample at the anode (where 2-mercaptoethanol is not ionized) or by using dithiothreitol, this effect is greatly reduced.

2.5. Staining Techniques

Merril *et al.* [30] have described a silver-staining procedure in which the sensitivity was found to be to a few ng of protein per zone (Coomassie Blue merely a few µg/zone), thus approaching the sensitivity of radioisotope labeling. In the gilding technique of Moeremans *et al.* [31] polypeptide chains are coated with 20 nm particles of colloidal gold and which could then be detected at a sensitivity of < 1 ng/mm^2. Proteins can also be stained by micelles of Fe^{3+}, although the sensitivity is approximately one order of magnitude lower than that with gold micelles [32]. These last two staining techniques were identified as a result of the discovery of another electrophoretic method, the so called Southern [33] and Western [34] blots. In these techniques, nucleic acids or proteins are transferred from hydrophilic gels to nitrocellulose or one of a number of

other membranes, where they are immobilized by hydrophobic adsorption or covalent bonding to the film. The very large porosity of these membranes makes them accessible to colloidal dyes. In addition, transfer of proteins to thin membranes greatly facilitates detection by immunological methods. This has resulted in a highly sensitivity procedure called 'immunoblotting'. After saturation of potential binding sites, the antigens are transferred to the membrane. They are first made to react with a primary antibody, and then the precipitate is detected with a secondary antibody, tagged with horse radish peroxidase, alkaline phosphase, gold particles, or biotin. The material is then be allowed to react with enzyme–linked avidin [35]. In all cases the sensitivity is greatly augmented.

A direct staining method for polyacrylamide gels with colloidal particles of Coomassie Blue G-250 is said to be as sensitive as silver staining [35].



Two purified preparations of β-lactamase (*ca.* 5 µg total protein load) were loaded on an immobilized pH 4–8 gradient gel (5%T, 4%C composition). Focusing was continued for 8 hours at 2, 000 V. Left side: silver staining; right side : Coomassie Blue staining (from P. G. Righetti and A. D'Arcy, unpublished).

Figure 9 Comparison between Silver and Coomassie Blue Staining

An example of the increment in sensitivity in changing from conventional Coomassie Blue to silver staining is shown in Figure 9. The focusing (in an immobilized pH 4–8 gradient) of two classes of β-lactamases is demonstrated: the pattern to the left has been silver stained, the one to the right has been developed in Coomassie Blue. On the right side, only the major (pI 7.2) band is seen; on the left side, after silver staining, a multitude of bands with lower (pI) values can be easily discerned.

2.6. Immobilized pH Gradients

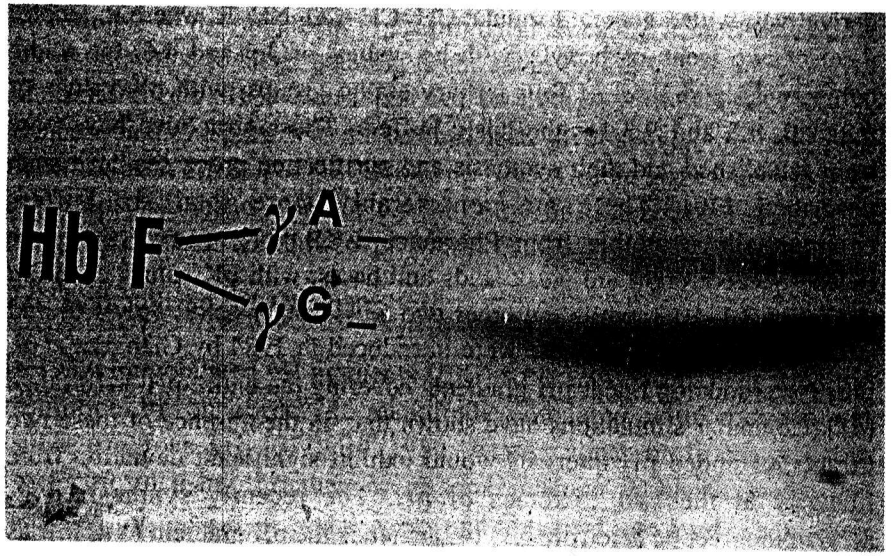
In 1982, immobilized pH gradients (IPG) were introduced, increasing the resolution by one order of magnitude compared with conventional isoelectric focusing [37]. By 1980, it was apparent to many using isoelectric focusing that there were some inherent problems, which had persisted for more than twenty years and remained unsolved. They were: a/ very low and unknown ionic strength, b/ uneven buffering capacity, c/ uneven conductivity, d/ unknown chemical environment, e/ non-amenable to pH gradients, f/ cathodic drift (pH gradient instability), g/ low sample capacity. In particular, a most vexing phenomenon in heterogeneous samples was the near-isoelectric precipitation of solutes of low solubility at the isoelectric point or of components present in large amounts. The problem was aggravated by the difficulty in reaching steady-state conditions (resulting in a slow pH gradient loss at the cathodic gel end) and in obtaining narrow pH gradients. Perhaps, most annoying was the lack of reproducibility and linearity of the pH gradients produced by the so-called 'carrier ampholyte' buffers [38]. Immobilized pH gradients solved all these problems.

Immobilized pH gradients are based on the principle that the buffers used to produce the pH gradient are copolymerized with the polyacrylamide, and thus rendered insoluble and contained within the fibers of a polyacrylamide matrix. This is achieved by using, as buffers, a set of six commercial chemicals (called Immobiline, by analogy with Ampholine, produced by Pharmacia-LKB Biotechnologys, Uppsala Sweden) having pK values distributed in the pH 3.6–9.3 range. Until now, little was known about the Immobiline chemicals, except that they are acrylamido

derivatives, with a general formula: CH=CH–CO–NH–R, where R denotes a set of two weak carboxyls, with pK values of 3.6 and 4.6, for acidic compounds, and a set of four tertiary amino groups, with pK values of 6.2, 7.0, 8.5 and 9.3 for the basic buffers. These structures have now been established and their synthesis and purification processes have been described [39]. There are considerably more than six buffers commercially available from Pharmacia-LKB [37]. Ten of them are indeed needed: eight are weak acids and bases, with pK values covering the range 3.1–10.3, while the other two are strongly acidic (pK 1.0) and strongly basic (pK>12). these were introduced in 1984 by Gianazza *et al.* [40] for producing linear pH gradients covering the entire pH 3–10 range [41]. Computer simulations have shown that, in the absence of these two titrants, extended pH intervals would exhibit significant deviation from linearity at the two extremes of pH. The recently synthesized 2-acrylamidoglycolic acid (pK 3.1) [42] extends the pH gradient to as low as pH 2.5. Also *N,N*-diethylaminopropylacrylamide (pK 10.3) was recently used for the analysis of strongly alkaline proteins [43]. Given the fairly evenly spaced pK values along the pH scale, it is clear that the set of 10 chemicals proposed here is quite adequate to ensure linear pH gradients along the pH 2.5–11 axis (the ideal ΔpK for linearity would be 1 pH unit between two adjacent buffers).

The rule ΔpK = 1 is fairly well kept, except for two 'holes' between pK 4.6 and 6.2 and between pK 7.0 and 8.5 species. For a more detailed treatise on how to use an immobilized pH gradient gel and immobilized pH gradient recipes, the reader is referred to an extensive manual [4] and to a recent review [44].

Due to the much increased resolution of immobilized pH gradient systems, quite a number of so-called 'electrophoretically silent' mutations (bearing amino acid replacements with no ionizable groups in the side chains) have now been fully resolved. A unique example of a resolution afforded by an immobilized pH gradient system is given in Figure 10. In a very shallow pH 7.35–7.55 immobilized pH gradient, two phenotypes of fetal hemoglobin called Ay and Gy (in the ratio *ca.* 1:4) produced by two genes carrying Ala-136–Gly substitution on the gamma chains are fully resolved.



The gel was made to contain an immobilized pH gradient spanning 7.35–7.55 (over 20 cm distance) in a 5% T, 4% C matrix. Focusing was continued overnight at 4000 V. The bands are visible due to their native red color. The resolved Ay/Gy bands are in 1:4 ratio, as theoretically predicted from gene expression. The identity was ascertained by eluting the two zones and fingerprinting their gamma chains. Due to the very narrow pH gradient utilized, the other two major components of cord blood (adult and acetylated fetal hemoglobins) have migrated out of the separation space into the electrode compartment [45]

Figure 10 Focusing of Umbilical Cord Blood Lysates in an Ultra Narrow Immobilized pH Gradient Gel

This is quite a unique fractionation, which so far has not been obtained by any chromatographic or any other electrophoretic method: the resolving power (in terms of ΔpI, *i.e.* difference in isoelectric points between the two species) is barely 0.001 of a pH unit.

2.7 Capillary Zone Electrophoresis

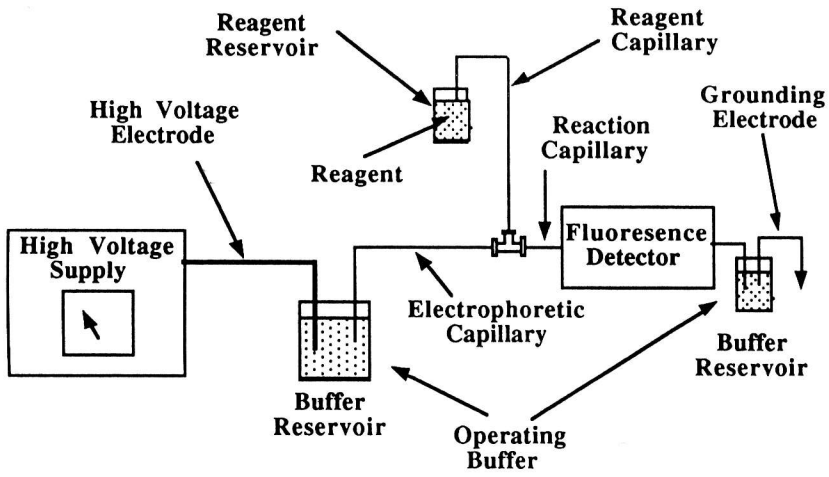
Capillary zone electrophoresis (CZE) appears to be a most powerful technique, perhaps equalling the resolving power of immobilized pH

gradients. If one assumes that longitudinal diffusion is the only significant source of band broadening, then the number of theoretical plates (N) in capillary zone electrophoresis is given by [46]:

$$N = \frac{\mu V}{2D} \qquad (2)$$

where (μ) is the elctrophoretic mobility of the analyte,
(D) is the diffusion coefficient of the analyte
and (V) is the applied voltage.

The equation shows that high voltage gradients are the most direct way to high separation efficiencies. For proteins, it has been calculated that (N) could be as high as one million theoretical plates. Figure 11 is a schematic drawing of a capillary zone electrophoresis system. The fused-silica capillary has a diameter of 50–80 μm and can be as much as one meter in length. It is suspended between two reservoirs, connected to a power supply that is able to deliver up to 30 kV (typical operating currents being of the order of 10–100 μA). One of the simplest ways to introduce the sample into the capillary is by electromigration, *i.e.* by dipping the capillary into the sample reservoir, under voltage, for a few seconds. Detection is usually accomplished by on-column fluorescence and/or UV adsorption. Conductivity and thermal detectors, as usually employed in isotachophoresis, exhibit to low a sensitivity for capillary zone electrophoresis. The reason for this stems from the fact that the flow cell where sample monitoring occurs has a volume of barely 0.5 nl, allowing sensitivities down to one femtomole. In fact, with the post-column derivatization method, a detection limit for amino acids of the order of femtograms is claimed, which means working in the attomol range [47].
By forming a chiral complex with a component of the background electrolyte (Cu aspartame) it is possible to resolve racemates of amino acids [6]. Even neutral organic molecules can be made to migrate in capillary zone electrophoresis by complexing them with charged ligands, such as sodium dodecyl sulfate. This introduces a new parameter, a hydrophobicity scale, in electrokinetic migrations.

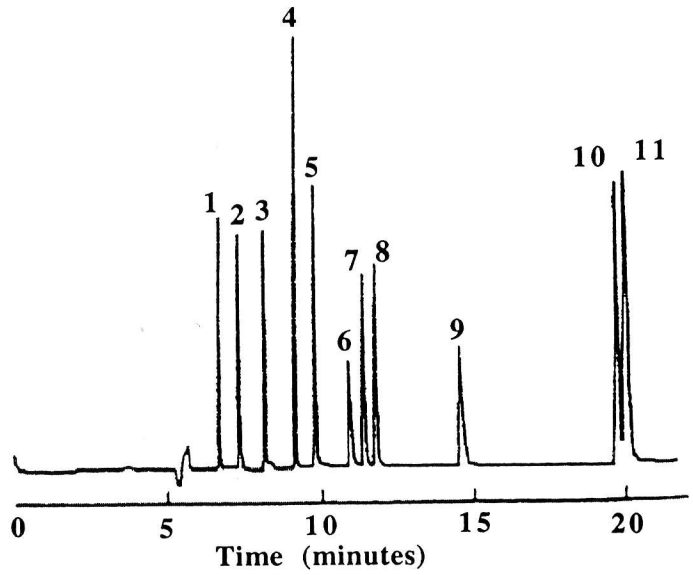


The high voltage power supply can deliver up to 30 kV. The fused-quartz capillary usually has an I.D. of 50–80 μm. The detector consists of a beam from a mercury/xenon arc lamp source, oriented perpendicular to the migration path at the end of the capillary. The sample signal (generally emitted fluorescence) is measured with a photomultiplier and a photometer connected to the analog/digital converter of a multifunction interface board mounted on a computer. In the scheme here produced, post–column sample derivatization is obtained by allowing the sample zones to react with o-phthalaldehyde [47].

Figure 11 Diagram of a Capillary Zone Electrophoresis Apparatus

Capillary zone electrophoresis is not limited to the separation of charged or ionizable compounds: with the concept of micellar electrokinetic chromatography (MEC), invented by Terabe [48], capillary zone electrophoresis has changed dramatically. Micellar electrokinetic chromatography is a hybrid between chromatography and zone electrophoresis: in fact, it belongs more to the realm of chromatography, since the electric current is mainly exploited to drive the analytes past the detector.

The separation is based on the partition coefficient of any analyte between the micelles present and the background electrolyte. Such micelles are, in general, surfactant micelles, anionic, cationic, zwitterion or even neutral, either alone or mixed. One of the most popular surfactants employed is sodium dodecyl sulfate. An example of the separation of the ingredients of cold pills is shown in Figure 12. This analysis has been performed at a pH of 9.0 in a background electrolyte containing 50mM sodium dodecyl sulfate. Needless to say, such a separation is only possible in the presence of the surfactant micelles.



1: acetaminophen; 2: caffeine; 3: sulprin; 4: naproxen; 5: guaifenesin; 6: an impurity; 7: phenacetin; 8: etenzamide; 9=4-isopropylantipyrine; 10: noscapine and 11: mixture of chlorpheniramine and tipepidine. Detection: wavelength 220 nm. Analysis in a pH 9.0 borate buffer in 50 mM sodium dodecyl sulfate. Capillary: 59 μm I.D., 65 cm long (50 cm to the detector). Migration: cathodic at 20 kV and 35°C [4]).

Figure 12 Analysis by Micellular Electrokinetic Chromatography of the Ingredients of Cold Pills

3 Ultracentrifugation

The development of the ultracentrifugation by T. Svedberg and co-workers in the 1929s proved one of the most significant developments in biomolecular science this century. Not only does the technique provide a powerful tool for bioseparation and purification, but it also provides a highly useful absolute method for determining molecular weights and dimensions of biomolecules in solution. There are two broad classes of ultracentrifuge procedure: preparative, for the separation and purification of biomolecules, and analytical, a more specialized application for the determination of molecular weights and molecular dimensions. Molecular weight determinations require an optical system to be employed in conjunction with specially constructed cells so that solute distribution can be measured during and at the end of each run. As a preparative tool, ultracentrifugation is particularly useful as a complementary technique to chromatographic procedures: the latter are particularly well suited to the separation of smaller molecular weight materials ($M < 1 \times 10^6$ g/mol) whereas the former is applicable to the separation of all particles on macromolecular assemblies. However, for particles above a molecular weight of ~ 50×10^6 g/mol, the term 'ultra' ceases to become appropriate at the small rotor speeds required.

3.1 Principles

When a biomolecule of molecular weight (M) is spun in a centrifuge tube or cell at a rotor speed of (ω) radians/sec,

(where 1 radian/s = $\frac{60}{2\pi}$ rev/min), it will sediment at a rate .

$$\frac{dr}{dt} = \frac{M(1-v\rho)}{N_A f} \qquad (3)$$

where (r) is the radial distance of the biomolecule from the center of the rotor,
(N_A) is Avogadro's Number,
(ρ) is the solution density (mg/ml) (often assumed to be the solvent density, ρ_o),

(v) is a parameter known as the 'partial specific volume' (essentially the reciprocal of the anhydrous density of the biomolecule- for example for proteins v ~ 0.73 ml/g, nucleic acids v ~ 0.53ml/g; polysaccharides v ~ 0.5–0.6 ml/g),
and (f) is a parameter known as the 'frictional coefficient', a measure of the shape and hydration (or degree of solvent 'binding' or 'entrapment') of the particle.

It is usual to normalize the sedimentation rate to allow for the strength of the centrifugal field by defining a parameter known as the sedimentation coefficient, (s)

$$s = \frac{\frac{dr}{dt}}{\omega^2 r} = \frac{M(1-v\rho)}{N_A f} \qquad (4)$$

The sedimentation coefficient, which is usually measured in seconds or Svedbergs (S) (where $S = 10^{-13}$ s), is therefore proportional to the molecular weight, (M), multiplied by the buoyancy factor ($1-v\rho$), and inversely proportional to the frictional coefficient.

Table 1 Sedimentation Coefficient Data

	Conformation Type	M	$s^0_{20,w}$
Lysozyme	Globular	14,400	1.9
Myosin	Rod	5000,000	5.8
Pullulan (P800)	Random Coil	760,000	11.6
Turnip Yellow	Globular	5.7 x 106	114

Many other values can be found in H. Sober, The Handbook of Biochemistry, Chemical Rubber Co., Cleveland, Ohio (1968). The subscript 20,w means the sedimentation coefficient has been normalized to standard solvent conditions (pure water at a temperature of 20°C) The superscript (0) refers to the fact that ($s_{20,w}$) values have been extrapolated to zero solute concentration.

Some example sedimentation coefficients for a selection of conformation types are given in Table 1 (for cross-reference purposes values are normalized to water as a solvent at a temperature of 20°C [49].

The rate at which a biomolecule will sediment will thus depend on (i) the molecular weight (bigger molecules sedimenting more rapidly), (ii) its conformation in solution: compacted and less hydrated molecules have a lower frictional coefficient and hence sediment faster, and (iii) its density relative to that of the solvent. Note that according to Equations (3) and (4), if $v\rho$ is >1 (i.e. the density of the biomolecule will move in the opposite direction to the centrifugal field), it will float. Choice of an appropriate solvent medium forms a crucial part of the separation using either zonal or density gradient procedures.

3.2 Preparative Ultracentrifuge Techniques

There are four types of preparative separation procedures: differential pelleting, rate-zonal ultracentrifugation, isopycnic ultracentrifugation and sedimentation field-flow fractionation. These are discussed below.

3.2.1 *Differential Pelleting*

Differential pelleting is appropriate for separating materials whose sedimentation coefficients are different by a factor of at least 3. Depending on the number of components to be separated, it involves pelleting one component at a time by choice of an appropriate speed/run time for each component. This method provides a crude separation procedure only: faster moving components are always contaminated by those that move more slowly and only the slowest moving component can be obtained in pure form.

3.2.2. *Rate-Zonal Ultracentrifugation*

This is a modification of the differential pelleting procedure in that the mixture is layered on top of a density gradient. The gradient must be preformed, by, *e.g.* layering a series of sucrose solutions of different

density into the centrifuge tube via a gradient mixer. Although the gradient will eventually disappear through normal diffusion, it usually lasts several hours, allowing enough time for a satisfactory separation to be achieved. The resolving power is much greater than 9.2 and a spectrum of components can be resolved (in terms of a series of migrating bands) to sufficient purity, as opposed to just the slowest component in the differential pelleting method; small differences in the sedimentation coefficient can be resolved and the density gradient also minimizes convective disturbances. The bands are successively pooled off as a series of fractions and can be assayed. This procedure can be used 'analytically ' to obtain estimates of sedimentation coefficient and molecular size distributions an example of this can be found in a relatively recent work on mucus glycoproteins [50].

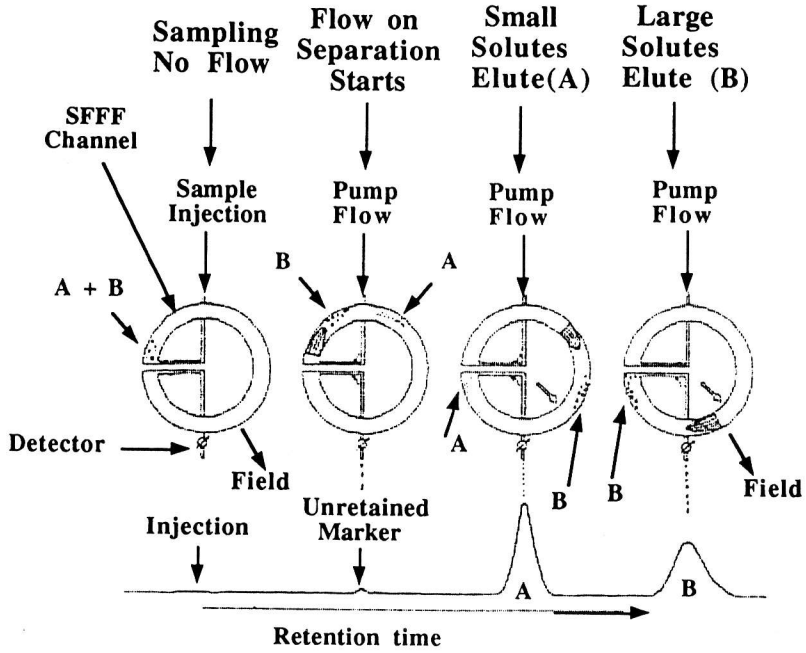
3.2.3. *Isopycnic Ultracentrifugation*

Although isopycnic ultrcentrifugation involves the use of a density gradient as in rate-zonal ultracentrifugation, it functions on a quite different principle. The maximum density of the gradient exceeds that of the biomolecules being separated: for most work on proteins and nucleic acids, cesium salts prove a popular choice for this purpose. The gradient can be either preformed (as in section 3.2.2) or (more usual) self-formed under the influence of the centrifugal field. Because the maximum density of the gradient is larger than that of the densest macromolecule, no component can sediment out and, instead, a series of isopycnic bands is produced where the system reaches an equilibrium state where the macromolecules reach their isopycnic (*i.e.* same density) points in the gradient.

The classical example of isopycnic ultracentrifugation is that of Meselson *et al.* [51] who used it to clearly demonstrate that DNA replication was semi-conservative. There are many other recent examples, however. The technique is particularly well suited for resolving proteins from carbohydrate and nucleic acid, and, as in section 3.2.2 can even be used 'analytically' to obtain molecular weight information.

3.2.4 *Sedimentation Field Flow Fractionation*

Sedimentation field flow fractionation can be used as an analytical technique, although, as with isopycnic ultracentrifugation, it is principally used for preparative separation purposes [52]. Sedimentation field flow fractionation (SFFF) devices are currently available (such as from duPont, Ltd., Delaware, USA). Conceptually, sedimentation field flow fractionation is a hybrid between a chromatographic separation (in that a continuous flow is involved) and a centrifugal separation, although the resolving power can be up to five times greater than that of a conventional 'size-exclusion chromatography separation' [52].



The top profiles show a plan view of events occurring in the sedimentation field flow fractionation rotor as a function of time; the bottom gives the corresponding fractogram. Note that smaller solutes elute first. Reproduced by courtesy of Arner and Kirkland [52].

Figure 13 Schematic of Sedimentation Field Flow Fractionation Equipment

It is usually much quicker than density gradient centrifugal separation and, because it is continuous, is not limited to the volumes of the centrifuge tubes.

Sedimentation field flow fractionation is, in fact, one of a number of field flow fractionation procedures [53] other examples being thermal field flow fractionation (TFFF), or electrical field flow fractionation (EFFF), although sedimentation field flow fractionation is particularly well suited to the separation of macromolecules. Loss of structural integrity or activity of the macromolecule is usually minimal and the method is generally fast, a separation being completed in one hour. Unlike size-exclusion chromatography, the smaller molecules elute first (Figure 13). Sedimentation field flow fractionation can be used analytically to give absolute molecular weight distributions and, in principle, conformation distributions within a preparation [54] providing that the 'detector' (represented schematically in Figure 13) is a combined concentration detector (based on refractive index or extinction) and a multi-angle light scattering detector.

3.3 Analytical Ultracentrifugation Techniques

After many years of decline, analytical ultracentrifugation has recently undergone a renaissance [55,56]. The principle reasons for this rebirth are (i) the demand for a technique that provides accurate absolute molecular weights, particularly for macromolecules whose precise molecular weight from chemical sequence is not known or cannot be found: this applies in particular to polydisperse or self associating macromolecular systems; and (ii) the need for a technique to accurately determine the gross conformation and interactions of biomolecules in dilute solutions, in conjunction with other solution techniques. This applies particularly to those molecules which cannot be analyzed by X-ray crystallography, *e.g.* intact, immunologically active antibody molecules.

There are three main optical systems used to record solute distribution of solute during an analysis: UV absorbance (suitable for *e.g.* proteins and nucleic acids), Rayleigh interference measurements and refractive index

gradient ('Schlieren' optics). There are two types of analytical ultracentrifuge experiment: sedimentation velocity and sedimentation equilibrium .

3.3.1. *Sedimentation Velocity*

Sedimentation velocity measurements are used to obtain diffusion coefficients (and thus particle conformation data), and for the qualitative assay of molecular interaction processes. Depending on the nature of the system, for a large fast sedimenting bacteriophage virus particle, the sedimentation velocity experiment can take less than an hour, up to nearly 12 hours for a slow moving, highly asymmetric polysaccharide.

3.3.2. *Sedimentation Equilibrium*

Sedimentation equilibrium experiments are performed at lower rotor speeds than those employed in the sedimentation velocity measurements, and are used for obtaining absolute molecular weights and quantitative interaction information (molecular association and/or interaction constants). The experimental time is longer for sedimentation velocity experiments, ranging from a few hours for a fast diffusing small protein solute, to 2–3 days for a large, slow moving polysaccharide or glycoconjugate.

4 Ultrafiltration

Ultrafiltration is a filtration process specifically for the separation of 'macromolecules', *i.e.* substances whose molecular weights range from 500 to ~ 2 x 10^6g/mol (or equivalently particles with a maximum dimension range from 1 to 10 nm). It is a pressure-driven process which is based on liquid flow through a porous membrane [57]. Other related 'membrane' or 'filter' separation processes are reverse osmosis (for the retention of much smaller molecules such as low molecular weight electrolytes) and micro–porous filtration (for particle sizes between 10 nm and 10 μm). Therefore, ultrafiltration applies to most biological macromolecules ranging from small peptides and saccharides to large

macromolecular assemblies such as small viruses whereas micro–porous filtration is suitable for separating particles on the microbial scale.

Ultrafiltration is relatively inexpensive compared with ultracentrifugation, but it is only capable of resolving components whose molecular weights differ by an order of magnitude: in this respect it is not as powerful as ultracentrifugation, chromatography and electrophoresis. It is of more value to the biomolecular scientist (i) as an alternative procedure to dialysis for exchanging buffers or removal of low molecular weight electrolyte and (ii) for decreasing the solvent content, *i.e.* increasing the concentration of a solution.

4.1. Principles

In ultrafiltration, unlike ultracentrifuge techniques - but in common with size–exclusion chromatography – the molecular size (*i.e.* maximum dimension) is the key factor in the separation process, as opposed to molecular weight or density. The filter is constructed of an inert, non-disruptive polymer (chosen for the particular bioseparation being attempted and with thickness between 50 and 300 μm).

As with some chromatographic separation procedures, solution flow is driven by the application of pressure upstream with an appropriate pump, or, in some cases, an applied centrifugal field. As the separation process proceeds, retained solutes will tend to accumulate or 'polarize' on the upstream end of the filter: the theory used for estimating separation times has to allow for this polarization or 'gel concentration'. Practically, these effects can be minimized by using a technique such as cross flow filtration ,where the solution on the upstream side of the filter is continually circulated over the membrane surface. The efficiency of an ultrafiltration process for a given solute is usually represented in terms of 'rejection coefficient' for a given solute component , (σ) defined by:

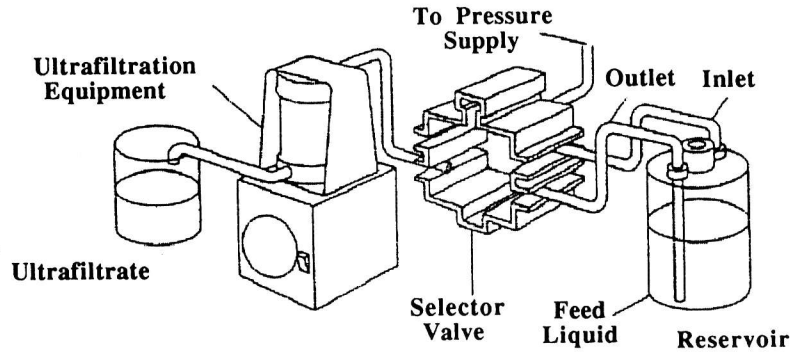
$$\sigma = 1 - \frac{c_U}{c_R} \qquad (5)$$

where(c_R)s the concentration of that particular solute in the 'retentate' (*i.e.* upstream of the filter),
and (c_U) is the corresponding concentration on the down stream side or 'ultrafiltrate'.

Rejection coefficients (or equivalent 'retention' expressed as a percentage) for a wide range of solutes and membrane materials have been published [57].

4.1.1. *Buffer Exchange/Removal of Low Molecular Weight Electrolytes*

The conventional way for either microsolute (*i.e.* low molecular solute such as buffer ions) exchange or removal from a macromolecular solution is membrane dialysis. This process is slow and often not reliable if the macromolecular solute has a molecular weight less than about 5,000 g/mol. Ultrafiltration can give a much more efficient exchange compared to dialysis, particularly for low molecular weight (*i.e.* M ~ 500–5000 g/mol) macrosolutes. Ultrafiltration, in this context, is often referred to as 'diafiltration'; microsolute removal is known as 'wash-out'; microsolute exchange as 'wash-in'. A schematic diagram of a diafiltration set-up is given in Figure 14.



Courtesy of the Amicon Division of W. R. Grace & Co., Danvers USA.

Figure 14 Schematic Diafiltration Set-Up

Diafiltration can be an important prerequisite of analytical measurements on macromolecular polyelectrolytes, for example, in sedimentation analysis (see section 3.3.2 above) as well as being important in a preparative bioseparation context, as with dialysis.

4.1.2. *Increasing the Concentration of Macromolecular Solutions*

Ultrafiltration provides a useful way of selectively increasing the concentration of macromolecular solutions (in the µl–ml range) without increasing the concentration of microsolutes and subjecting the macromolecules to potential thermal degradation as with evaporative procedures. To a close approximation, the rate of ultrafiltration will vary inversely as the logarithm of the concentration of the retained solute. For this particular application, the use of a centrifugal field to apply the upstream pressure to the filter is popular, and several specially constructed centrifuge tubes (see, for example Figure 15) with an ultrafiltration membrane are now commercially available.

4.1.4. *Analytical Applications*

With the use of a suitable detecting system to record solute distribution in the retentate and ultrafiltrate, ultrafiltration can provide information

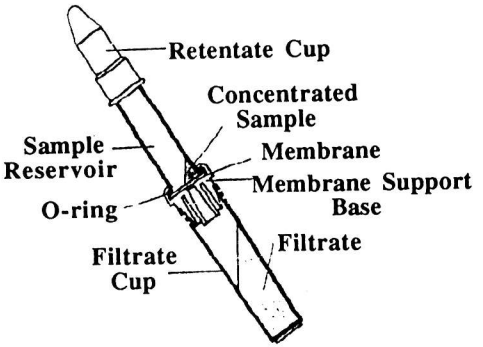


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Figure 15 Schematic of a Ultrafiltration Centrifuge Tube

on the nature of a macromolecular system. A example of this is in the analysis of micelle–forming systems [58].

6 Synopsis

In this chapter, an overview of the more important separation techniques that complement liquid chromatography and that are commercially available to a biotechnologist is presented. The various forms of electrophoresis are described, including discontinuous electrophoresis, isoelectric focusing, immobilized gradients and capillary electrophoresis, and their pertinent areas of application are given. The more established technique of ultracentrifugation is also examined in the light of its contribution to solving separation problems in biotechnology. Preparative and analytical ultracentrifugation are considered in detail and the types of separation problems for which the techniques are most appropriate are suggested. The technique of ultrafiltration is described, including buffer exchange, concentration of macromolecules and analytical applications.

This book and the separation techniques that have been discussed herein give an idea of how important the role of separation science is in the biological field, particularly in modern biotechnology. It can be envisaged that, in due course, more efficient techniques will allow a more detailed analysis of the cell structure which, in turn, will lead to significant advances in of cytology

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