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Separation and Fractionation of Macromolecules and Particles

New or improved separation procedures underlie many important recent advances in biochemistry.

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Separation is not usually counted among the more exciting operations in chemistry, even though everybody will agree that such work is a necessary step in at least some phase of almost any chemical investigation. It is also significant that some of the most remarkable recent advances in chemistry have depended upon new or improved separation procedures, directly or indirectly. This is perhaps particularly striking in biochemistry and related fields.

As a background for a discussion of some recent contributions to the development of separation methods at the Institute of Biochemistry in Uppsala, some general reflections may be justified. The fact that the biochemist has to isolate most of his substances from biological objects in which they often occur in trace amounts, mixed with an excess of other material, tends to make him look hopefully to new and improved separation methods which may become applicable to his particular problems. He always has to keep in mind, however, that the very nature of many of the substances he is searching for prohibits the use of drastic or aggressive methods which may damage essential structures. Also, some "biological" impurities behave in a very unpleasant manner in separation operations, and it seems that nature often has embedded some of her most attractive research objects in a surrounding of "gummy" or "sticky" materials which are particularly difficult to remove.

Frequently the immediate goal in separation is not purification but analysis of the composition of a complicated mixture. The choice between preparative and analytical separation procedures may be based in part upon the scale of the operation, especially when one is discussing different types of apparatus, but the purpose of the operation is of course decisive. Typical examples of common analytical separation procedures of particular importance in biochemistry are ultracentrifugation, electrophoresis, and chromatography. They all illustrate the usefulness of the analytical aspect in a field where the definition of new substances is difficult. Findings on the behavior of a substance in a separation process—sedimentation velocities, electrophoretic mobilities, chromatographic R_F constants or critical eluant concentrations, and partition coefficients—provide a convenient basis for preliminary quantitative characterization. There are abundant examples showing how such an approach has led to important discoveries—new serum protein components, different pathological hemoglobins, and "isozymes" among others. Such results also emphasize the importance of obtaining the highest possible resolution. These and other instances also exemplify the use of analogous or different analytical separation methods as a guide for preparative work. Efforts to translate a successful analytical separation procedure into a preparative operation simply by enlarging the scale have often met with difficulties (particularly in the case of high-resolution electrophoresis and ultracentrifugation), and it has not yet been possible to investigate all the new components which one can observe, but not isolate, by such methods.

A striking demonstration of the importance of separation methods to biochemistry comes from another sector: the "analytical approach" to structure determination of large molecules. Here the systematic fractionation of complicated mixtures of split products resulting, for example from hydrolysis of proteins, polysaccharides, or polynucleotides is an important step in a procedure which essentially involves attempts to reconstruct a picture of the original structure from systematic analysis and identification of fragments derived from that structure. The best illustration of this type of work is of course the determination of amino acid sequences in proteins, so brillantly inaugurated by Sanger in his work on insulin.

It is very tempting to discuss the possibilities of generalizing such an analytical approach from intra- to intermolecular structures, especially since such structures are of particularly great interest to biochemists. In the isolation of a specific protein one may find that a particular "impurity" is extremely difficult to remove. This may be a nuisance, but from another viewpoint it may be regarded as providing a valuable piece of information about an intermolecular association which may occur in the original biological material from which the protein has been extracted. By applying sufficiently

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gentle methods we should be able to isolate intermolecular structures that form parts of the more complicated "biological" structures in the cell. If such work is done systematically in correlation with studies of biochemical function and, if possible, also in parallel with other and more direct structure studies (for example, electron microscopy studies), it may provide us with some very essential and valuable information which would have been difficult to obtain otherwise.

The isolation of naturally occurring submicroscopic particles from the cytoplasm is a current example of this type of analysis, but the point we want to make here is that the principle should be applied much more widely. Such application should include, for example, study of submicroscopic fragments obtained by suitable methods of disintegration. This general problem has been discussed by Tiselius in a somewhat schematic and preliminary way (*1*). Exactly how far such methods can prove useful in the elucidation of essential biochemical structures remains to be seen, but the problems raised are of considerable general interest. They have played a certain role in inspiring some of our work on the development of new methods for the separation of particles.

Separation processes may be based on phenomena which can easily be defined quantitatively, such as sedimentation, electrophoresis, or partition. The advantage of such methods in the characterization of unknown substances has already been stressed. From such data the optimal working conditions for a separation operation can be predicted. Often, however, one has to accept a simpler and more empirical method, especially for preparative work, even if it is less quantitative and less reproducible. In the Uppsala laboratory we have always tried to lay emphasis on the quantitative aspects, and we believe that this approach has proved fruitful; moreover, rational methods generally have a much wider application then empirical ones.

In the discussion that follows, some recent advances in the work of the Uppsala school are considered. Special attention is given to some significant improvements in electrophoresis, the recent development of gel filtration (exclusion chromatography), and the development of partition methods specially adapted to the separation of biological particles.

Electrophoresis

The vast majority of electrophoresis experiments today—both analytical and preparative—are "zone electrophoresis" and involve the use of some sort of supporting media. This involves, to a varying extent, a sacrifice of quantitative aspects such as were discussed earlier, because of the interference of the stabilizing medium (for example, paper, cellulose powder, different types of gels). Thus, the original type of "free boundary electrophoresis" in a U-tube (*2*) is still often used when quantitative work is essential, and has proved its value also in recent investigations—for example, in the characterization of the various pathological hemoglobins. The method has, however, always suffered from the disadvantage of requiring comparatively large quantities of the sample to be investigated. Attempts to construct a micro-modification of the U-tube earlier met with difficulty in that the use of narrow tubes gives rise to very marked disturbances due to electro-osmotic transport of the solution along the walls of the tube. Hjertén has made the important observation that treatment of glass vessels with certain water-soluble polymers, such as methylcellulose, largely eliminates this disturbance and permits the use of tubes 1 millimeter, or even less, in diameter.

The difficult problem of combining the advantages of zone methods with those of free migration appears to have found a solution in Hjertén's apparatus for "free zone electrophoresis." Hjertén showed that zones of not too high concentration remain stable in a horizontal tube which is slowly revolving around its long axis (*3*), the preferential convection direction due to gravity thus being eliminated. The zones are introduced and taken out through an inserted capillary tubing while the electrophoresis tube is revolving. In the present construction, a tube of quartz is used and the migration is scanned with filtered ultraviolet light. With this method, accurate mobility measurements can be made on quantities as small as those used in paper electrophoresis. In this procedure, also, pretreatment of the glass surfaces with methylcellulose is essential. A particularly attractive feature of this method is the opportunity it provides for studying the migration of zones of particles.

Somewhat less rigorously "free" zone electrophoresis can be carried out in density gradients of, for example, sucrose or in certain very weak gels. Hjertén has experimented with gels made of agarose, the neutral constituent of agar, which is fairly easy to isolate. Gels containing only about 0.1 to 0.2 percent agarose, if disintegrated into a suspension of gel particles, show very interesting properties when used as columns for zone electrophoresis experiments (*4, 5*). Agarose shows very little electro-osmosis and almost no adsorption, and mobilities of proteins measured in agarose suspension columns agree well with mobilities observed in free migration. Even particles, such as microsomes, can be studied in such columns. If the electrophoresis tube is emptied, the suspension flows like a solid body ("plug flow")—that is, without any marked distortion of the zones. The eluted zones can be freed from the agarose particles by centrifugation.

Electrophoresis in compact gels has led to very interesting results, particularly with the well-known starch gel technique introduced by Smithies. The high resolution in these gels depends, in part at least, on molecular sieving (*6*) and has led to the discovery of new serum proteins which are particularly interesting from a genetic viewpoint. Here, as in other cases of electrophoresis in compact gels, isolation of the separated components is a difficult problem.

For this purpose and for preparative electrophoresis in columns packed with granular media, as worked out especially by Porath (*5, 7*), continuous elution of the zones during the electrophoretic experiment is a useful procedure (*8*). The technique of packing of such columns has recently been much improved by Porath (*9*), and columns of various dimensions (mostly packed with specially prepared cellulose powder) are in almost constant use in preparative work in our laboratory. The fact that these columns can be used many times without renewal of the packing is a particularly great advantage. For the separation of large quantities, an apparatus has been constructed (*10*). It consists of an annular separation chamber surrounded by an external cooling jacket and an inner tube with circulating cooling liquid. The anolyte and catholyte solutions are continually mixed during the experiment. The dimensions of the electrode chambers can thus be kept small in

spite of the high capacity of the column. The separation chamber may be filled with any kind of granular support material. The lower part of the chamber is provided with outlets for continuous removal of fast-moving substances. Electrophoresis can thus be continued over long periods when this is desired. In our laboratory the apparatus has been extensively used for fractionation of human serum in 100- to 200-milliliter portions.

Gel Filtration

Gel filtration is a new fractionation procedure in which, to a large extent, separation is based upon differences in molecular size. No doubt the need for such a method has long been felt by biochemists. Preparative ultracentrifugation is of course very useful, but its use is limited to separation of substances of very large molecular weight. An essential factor in gel electrophoresis, as mentioned earlier, is a kind of "molecular sieving," and similar phenomena are operative in gel filtration. Although, in gel filtration procedures, organic solvents with organophilic gels can be used, so far, with few exceptions, only hydrophilic gels in aqueous systems have been used. The great progress in this field came after the introduction, by Porath and Flodin (*11*), of cross-linked dextran gels (now produced commercially by A. B. Pharmacia, Uppsala, under the trade name Sephadex). The principle is as follows.

The gel-forming particles are allowed to swell in the solvent. The amount of swelling depends on the nature of the solvent and the architecture of the gel matrix. Solute molecules penetrate the network to a larger or smaller extent, depending upon steric relationships between the molecular structure of the gel proper and that of the solute. Thus, the shape and size of the solute molecules largely determine their distribution between the gel particles and the displaceable solvent between the particles. For substances completely excluded from the gel, the concentration ratio is 0:1 ($K_d = 0$). If a substance is able to completely penetrate the gel, the ratio closely approaches 1:1 ($K_d = 1$). The distribution ratio, when pure molecular sieving is the only discriminating factor, is thus confined to the interval 0–1, whereas in typical liquid-liquid partition chromatography this interval is 0–∞. Offsetting this drawback, which at first sight may appear serious, are the high resolution and reproducibility obtainable if gel filtration is performed as a chromatographic operation. The following simple procedure has proved to be successful. The solution containing the solutes to be fractionated is allowed to enter at one end of a column packed with granular gel which has been allowed to swell in a suitable solvent. On washing of the column with the solvent, the components of the mixture migrate at different speeds and eventually appear in the eluate in the order of decreasing molecular size (shape-dependence is disregarded) (Fig. 1). To avoid extensive compression of the gel granules, with consequent clogging, the flow of liquid is preferably directed against gravity.

The procedure is clearly a kind of elution analysis or elution chromatography. Chromatography is usually based on adsorption or differences in solubility in stationary and mobile phases. Gel filtration utilizes sterically conditioned distribution behavior between water in two physical states: easily displaceable water present in the intergranular space and water immobilized in a macromolecular network. The chemical nature of the gel matrix is of importance only insofar as it determines the swelling, and thus indirectly the penetrability. This method has been called gel filtration in order to distinguish it from common chromatography, from which it differs basically.

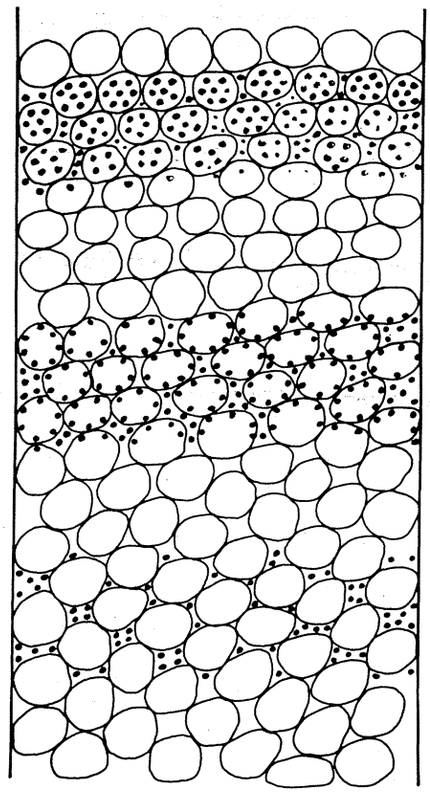

Fig. 1. Diagram illustrating the principle of gel filtration. In a bed of gel particles, three kinds of solute molecules (indicated by small dots) move faster with increasing size and consequently lower permeability. The direction of movement is downward.

It is obvious from the foregoing discussion that the method is analogous to partition chromatography. Experience with various neutral gel substances indicates that distribution equilibria between gel and intergranular solution are rapidly attained irrespective of the molecular size of the solutes. The strongest argument in favor of this view is the fact that, within wide limits, the relative rate of migration of the substances in a column is independent of the rate of flow. Furthermore, the distribution coefficients determined in column experiments agree with those obtained by batch procedures. Partition is thus usually governed by static equilibria, and these equilibria are closely approached in the column processes.

Figure 2 shows a separation, in a bed of strongly cross-linked dextran, of a mixture of oligosaccharides obtained by hydrolysis of cellulose (*12*). From this clear-cut separation one would expect that it should be possible to separate, just as easily, mixtures of oligopeptides of corresponding differences in size. This is not the case, however. The resolving power in this case is much less, but it can be improved by choosing another solvent that reduces the swelling of the gel particles. Charged solutes generally exhibit a more complicated behavior, the reason being that the condition of electroneutrality must be fulfilled. Certainly such effects are exaggerated when the experiments are carried out in electrolyte-free solutions. If molecular sieving is primarily wanted, such charge effects should be suppressed by the use of solutions of sufficiently high ionic strength.

By and large, however, the influence of *p*H and ionic strength is very much less pronounced for the distribution in neutral gels than for the distribution in gels containing large amounts of fixed charges ("ion exchangers"). This fact makes gel filtration an almost ideal tool

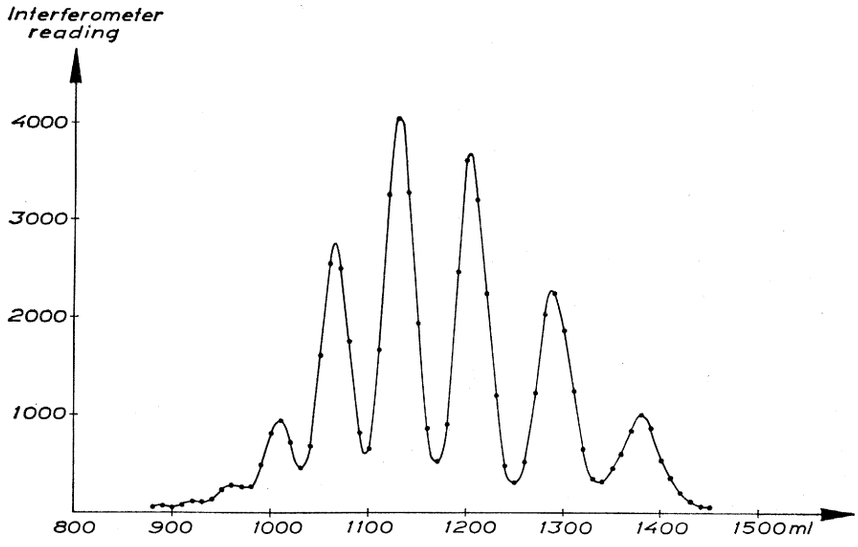

Fig. 2. Gel filtration diagram for oligosaccharides derived from cellulose. The experiment was made in distilled water; the separation was made on Sephadex G25. The peaks contain (right to left) glucose, cellobiose, cellotriose, and so on. [Flodin and Aspberg (*12*)]

for the isolation of complexes. A proper solvent system may often be found, and the purification can be made very quickly and more completely than, for example, by exhaustive dialysis. The binding of metal ions, peptides, and so on to carrier proteins in biological fluids may thus be easily studied. With weakly cross-linked dextran gels (Sephadex G200 or agarose), it is possible to study protein-protein complexes (see Fig. 3). The separation of polymers of serum albumin may be mentioned. Gel filtration in appropriate gel systems should also be useful for the study of soluble antigen-antibody complexes.

Since publication of our first communication on the gel filtration method, a very large number of papers have been published in which application of the method to a great variety of systems is described. Here, however, we limit ourselves to some examples from work done in our own laboratory or done in close collaboration with us (*13*). Sephadex G100, a moderately cross-linked dextran gel, has been used extensively in our laboratory to prepare proteins and peptides of low molecular weight from bacteria, fungi, and higher organisms. Our interest in biologically active proteins of low molecular weight is based on the belief that these substances comprise a group of objects suitable for the study of interrelationships between chemical structure and biological activity. Multiplicity of enzymes of low molecular weight has been revealed by gel filtration. In *Polyporus versicolor*, for example, several β-glucanases (cellulases) were discovered that differed from each other not only in size but also in specificity (*14*). Multiplicity of cellulolytic enzymes in *Aspergillus niger* (Fig. 4) has also been revealed.

To achieve efficient fractionation of serum proteins it is necessary to use gels of still looser framework. Thus, Hjertén has used cross-linked polyacrylamide and agarose. Most work, however, has been done with a cross-linked dextran, Sephadex G200 (*15, 16*). A typical diagram is shown in Fig. 3.

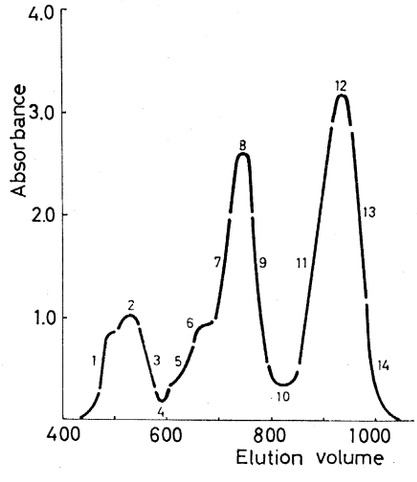

Fig. 3. Gel filtration diagram of human plasma. The separation was made on Sephadex G200. The macroglobulins are found in the first peak; 7*S* γ-globulin and ceruloplasmin, in the second peak; albumin, transferrin, and "prealbumins," in the third peak. [Porath and Flodin (*16*)]

One may ask if it is possible to prepare stable beds of gels that would allow particle fractionation. Most hydrophilic gels capable of very extensive swelling are soft. It would probably be possible to synthesize suitable gels of sufficiently high rigidity that are still permeable to small particles. In fact, Hjertén has fractionated subcellular particles in columns of granular agarose (*17*).

In their article on the differential migration of solutes on starch columns, Lathe and Ruthven (*18*) emphasized the use of the chromatographic procedure for estimating molecular size, rather than the potentialities of the method as a general fractionation procedure. Other kinds of gel, such as those prepared from agar, cross-linked polyacrylamide, or dextrans, may be used for the same purpose (*19*). To be valid, the calibration should be made with substances of similar molecular shape, such as those currently under study. Linear polysaccharides, for example, appear to move faster than do globular proteins of similar molecular size. Furthermore, charge and aromatic-residue content influence the affinity toward the gel. Interpretations of molecular size from gel filtration diagrams should therefore be cautiously made. In spite of these complications, surprisingly good correlation is very often found between distribution coefficients and physical parameters related to molecular size.

The extent of swelling depends not only on the structure of the gel substance but also on that of the imbibing solvent. By lowering the dielectric constant of the solvent—for example, by mixing water and alcohol—the limit of molecular size for permeation can be decreased for a given dextran gel. Swelling can be kept less than it is in pure aqueous systems through the use of mixtures of pyridine, acetic acid, and water; such solvents have been used, for example, for fractionation of pituitary peptide hormones on Sephadex G25 (*20*).

In mixed solvents a segregation of the components of the liquid phase may occur, so that the composition of the liquid entrapped in the gel will differ from that of the intergranular fluid. In such a system, liquid-liquid partition may play a role, in addition to the molecular sieving. The connection be-

tween gel filtration and liquid-liquid partition chromatography is here obvious. Other methods in which beds of granular gels are employed, such as zone precipitation and gel filtration in organic media with organophilic gels, are under study, as are procedures based on the use of ionic and specific adsorbents derived from neutral gels of the Sephadex type.

In gel filtration the material distributes in the chromatogram in a way that is independent of the scale of operation. Experiments can be made with a microliter sample—by thin-layer methods such as those developed by Determann and by Johansson and Rymo (*21*)—or on a liter scale. Hundreds of similar gel filtration experiments may be performed on the same column, the first and the last experiments yielding essentially the same results. This extraordinary reproducibility makes it possible to utilize very small differences in distribution ratios, but fractionation may still sometimes require extremely high column beds. To offset the drawbacks of high columns, which are particularly severe for soft gels, we use a cyclic procedure in which the solute-containing zones may pass the column several times (*22*). After introduction of the sample, the upper and lower ends of the column are connected with a flexible tubing, and the liquid is forced to circulate by means of a peristaltic pump. The progress in the fractionation is followed by continuous spectrophotometric or refractometric analysis of the liquid in the connecting tube. This simple technique has proven to be of very great value. An application is shown in Fig. 5, which also illustrates the effect on the separation of the formation of complexes.

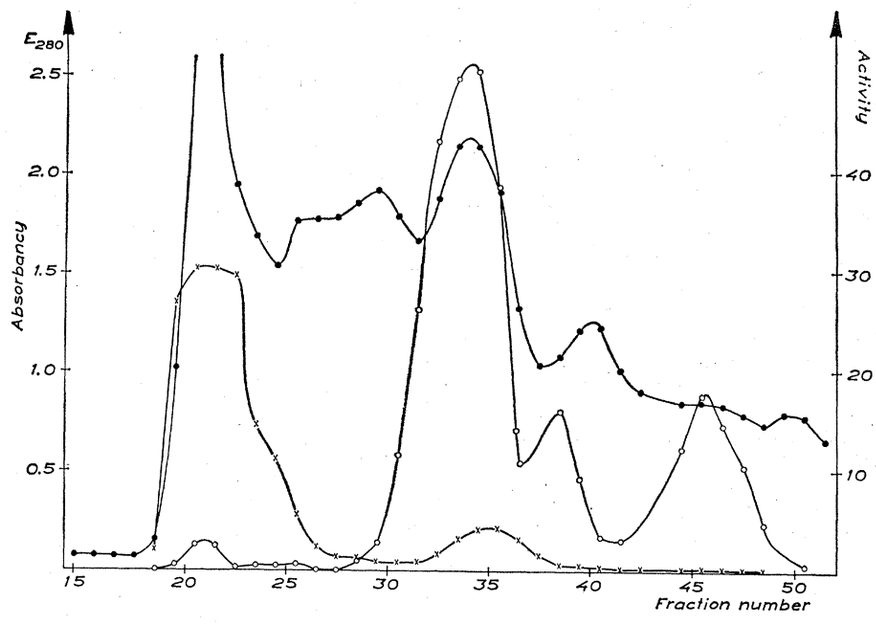

Fig. 4. Fractionation of a cellulase preparation from *Aspergillus niger* on Sephadex G100. (Solid circles) Absorbancy at 280 millimicrons; (crosses) β-glucosidase activity; (open circles) cellulolytic activity. The multiplicity of the enzyme with respect to molecular size is clearly indicated and has been confirmed by ultracentrifugation. [Pettersson (*39*)]

Partition

The importance of developing further the currently used methods of separating biological particles was stressed in the beginning of this article. The most widely used of these methods is, of course, ultracentrifugation in various forms, of which density-gradient zone ultracentrifugation recently has proved to be particularly useful. There is, however, a great need for alternative methods, based upon properties other than size, shape, and density. Some successful attempts to apply electrophoresis to the problem of particle separation were referred to earlier. Partition methods, being both simple and specific, offer a very attractive field for further progress. Conventional phase systems which contain an organic solvent phase are generally not suitable for partitioning of biological materials. The risk of denaturation by interaction with organic solvents in bulk or at interfaces is great, and the distribution usually favors the aqueous phase to an extent which makes successful utilization of the system for purposes of separation impossible.

It therefore seems more promising to look for phase systems in which both phases contain mainly water and thus resemble more closely the natural environment of the material studied.

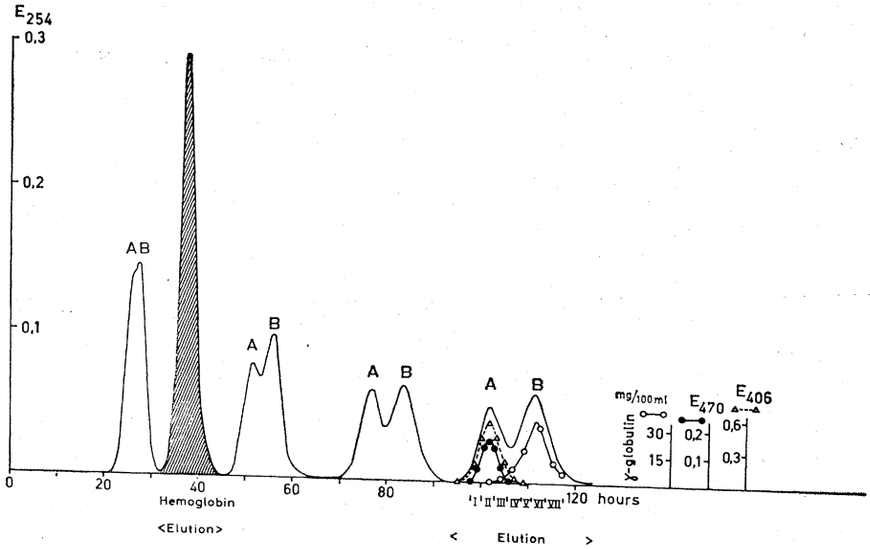

Fig. 5. Separation of a hemoglobin-haptoglobin 1–1 complex by recycling on Sephadex G200. Hemoglobin in excess of the binding capacity was added to a concentrated haptoglobin (type 1–1) preparation obtained from a previous gel filtration. Hemoglobin was removed at the conclusion of the first cycle (30–45 hours). The recycling operation was continued with the remaining material. In the fourth cycle (about 95 hours) the hemoglobin-haptoglobin complex (*A*) had separated from 7*S* γ-globulin (*B*), and both could be removed. [Killander (*40*)]

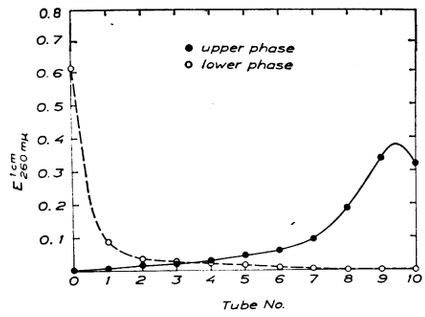

Fig. 6. Separation of "native" (right) from heat-denatured (left) DNA by countercurrent distribution in a dextran, polyethylene glycol phase system. [Albertsson (*27*)]

Especially for the separation of the larger molecules and of particles, the phases should not differ very much in composition and properties; otherwise there may be a one-sided distribution of all components. Albertsson (*23*) has shown that two-phase systems made by dissolving moderate amounts of certain water-soluble polymers in water offer excellent possibilities for separation of particles and macromolecules. For example, a mixture containing 5 percent dextran (molecular weight, ~ 500,000) and 4 percent polyethylene glycol (molecular weight, ~ 6000) in water will separate into two liquid layers—a bottom layer containing an aqueous solution of most of the dextran and an upper layer containing an aqueous solution of most of the polyethylene glycol. Analysis of the composition of the phases at various polymer concentrations demonstrates that this system obeys Gibbs's phase rule and may be considered a ternary phase system of the three components dextran, polyethylene glycol, and water (we are here neglecting the fact that the polymers are polydisperse). (For a detailed account of polymer phase separation, see *23*.)

Phase separation among polymer mixtures is a general phenomenon, and a large number of phase systems may be constructed. In Table 1 we have listed some systems which have been employed in partition studies so far. Phase systems may be composed of solutions of non-ionic or ionic polymers, the latter giving rise to phases which we may regard as liquid ion-exchangers. It is also possible to use only one polymer, as in the potassium phosphate, polyethylene glycol, H_2O system.

Table 1. Some aqueous polymer two-phase systems. [Albertsson (*23*)].

Dextran, polyethylene glycol, H_2O
Dextran, methylcellulose, H_2O
Dextran, polyvinyl alcohol, H_2O
Na dextran sulfate, polyethylene glycol, NaCl, H_2O
Na dextran sulfate, methylcellulose, NaCl, H_2O
Potassium phosphate, polyethylene glycol, H_2O

A larger number of polymer phase systems are thus available, with varying properties. They all have one essential property in common, however—the high water content (85 to 99 percent) of both phases, which makes them suitable for the partition of biological macromolecules and particles.

How do biochemical substances behave in these systems, and how can we utilize them for fractionation?

Theoretically, partition of macromolecules was studied by Brönsted (*24*), who deduced the following formula

$$\ln \frac{C_1}{C_2} \sim \frac{\lambda M}{RT}$$

where C_1 and C_2 are the concentrations in phases 1 and 2 of a substance of molecular weight M; R is the gas constant; T is the absolute temperature; and λ is a factor which is a function of the properties of the solute and the solvent phases. For substances differing only in molecular size (isochemical substances), λ is constant in a particular phase system. For large molecules (large values of M) the partition ratio C_1/C_2 tends to be either very large or very small, depending upon the sign of λ. This results in a tendency toward unilateral distribution. The partition in this case will, however, be very sensitive to changes in the value and the sign of λ, which may be varied by several different means—for example, by changes in the ionic medium or the phase system—to provide favorable conditions for specific separation operations.

The behavior in polymer phase systems of a large number of macromolecular substances and particles, including proteins (*23, 25*), nucleic acids (*26, 27*), viruses (*23, 28*), and even whole cells (*29, 30*), has been studied in recent years. The results are in good

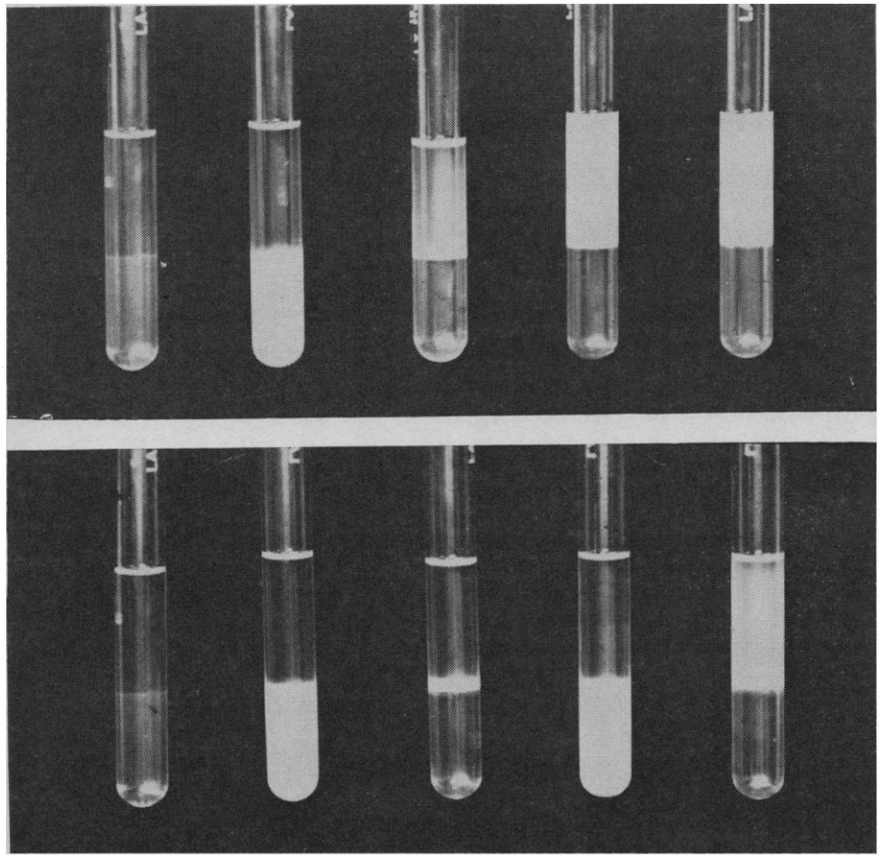

Fig. 7. Partition of four different yeast strains in an aqueous polymer two-phase system of dextran and polyethylene glycol. (Left to right) Phase system without particles, *Saccaromyces carlsbergensis, Sporobolomyces salmonicolor, Rhodotorula sanguineae,* and *Rhodotorula glutinis*—(upper row) in 0.01*M* phosphate buffer, *p*H 7; (lower row) in 0.01*M* phosphate buffer, *p*H 7, and 0.02*M* NaCl. [B. von Hofsten]

qualitative agreement with the theoretical predictions. Thus, the size of the partitioned molecules and particles is an important factor, which, when other factors are kept constant, determines the partition constant for a certain kind of substance in approximate accordance with Brönsted's law. Most proteins and enzymes studied distribute with a characteristic partition coefficient which may vary for different proteins and thus allows fractionation by a multistep procedure such as countercurrent distribution. All enzymes tested so far retain their enzyme activity after partition under proper conditions.

Large particles such as cells have a tendency to distribute between one phase and the interface rather than between the two phases. In this case the distribution constant is also reproducible and characteristic for a given particle.

Partition studies on DNA have shown that its behavior is also in agreement with the Brönsted theory. Thus, the logarithm of the partition ratio is proportional to the molecular size (*26*). In addition, changes in the secondary structure of DNA have strong influence on its partition: native and heat-denatured DNA favoring the opposite phases of the dextran, polyethylene glycol system. In a mixture these two types of DNA can therefore be separated by a few countercurrent steps (*27*) (see Fig. 6).

Especially with particles and large macromolecules the difference in partition behavior may often be so marked that a complete or almost complete separation is achieved in one step only (Fig. 7). This greatly simplifies the procedure, and such an operation can, of course, easily be applied on a large scale. Examples of such one-step phase distributions are experiments with virus and bacteriophage concentration and purification (*28, 31*). Thus, a virus activity can be concentrated into a small-volume phase by partitioning in a suitable system. In the same time a considerable purification may be achieved if other substances distribute in a different manner and therefore are more or less completely removed from the virus-rich phase. This technique has found application, as a routine method, for large-scale isolation of bacteriophage T2 (*31*) and also for purification of poliovirus and ECHO virus (*28*).

Some recently published experiments with bacterial spores offer another example of such simple operations (*31*). Spores and vegetative cells behave in strikingly different ways in partition, indicating that there is a marked difference in the composition of the particle surfaces, and thus they can be separated from each other by one or two phase distributions.

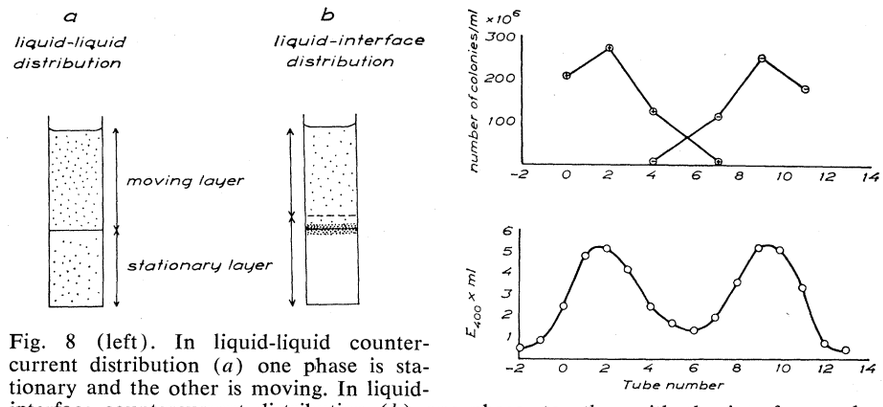

Fig. 8 (left). In liquid-liquid countercurrent distribution (*a*) one phase is stationary and the other is moving. In liquid-interface countercurrent distribution (*b*) one phase, together with the interface and a small layer of the other phase, is stationary and the remaining layer is moving [Albertsson (*23*)]. Fig. 9 (right). Separation of two strains of the bacterium *Escherichia coli* by liquid-interface countercurrent distribution with a dextran-polyethylene glycol phase system. (Curve at left) Strain K12,C; (at right) strain ML3081. (Top: circles with +) Lactose+ colonies; (circles with —) lactose— colonies [Baird *et al.* (*29*)].

More often, however, conditions are not so favorable, and with a complex mixture only a partial separation is obtained by one partition step. To increase the resolving power, a multistep procedure, such as countercurrent distribution, is employed. Polymer phase systems can be used for countercurrent distribution of proteins (*25*), nucleic acids (*26*), polio virus (*32*), and bacteria (*29, 30*). The conventional Craig procedure (*33*) is used when the substances dissolve completely in the two phases—that is, without significant adsorption at the interface. The proteins phycoerythrin (*25*), phycocyanin (*25*), and ceruloplasmin (*23*) have been studied by this method with a phase system of dextran, polyethylene glycol, and water. Recently, rennin substrate and serum proteins (*34*) have been fractionated in the phase system potassium phosphate, polyethylene glycol, and water—a system which has also been used for the separation of soluble RNA (*35*).

As mentioned earlier, interfacial adsorption is frequently observed when large particles are distributed. When this is the case, liquid-interface countercurrent distribution (*23, 30*) is employed (see Fig. 8). With this technique the interface, together with the material attached to it, is allowed either

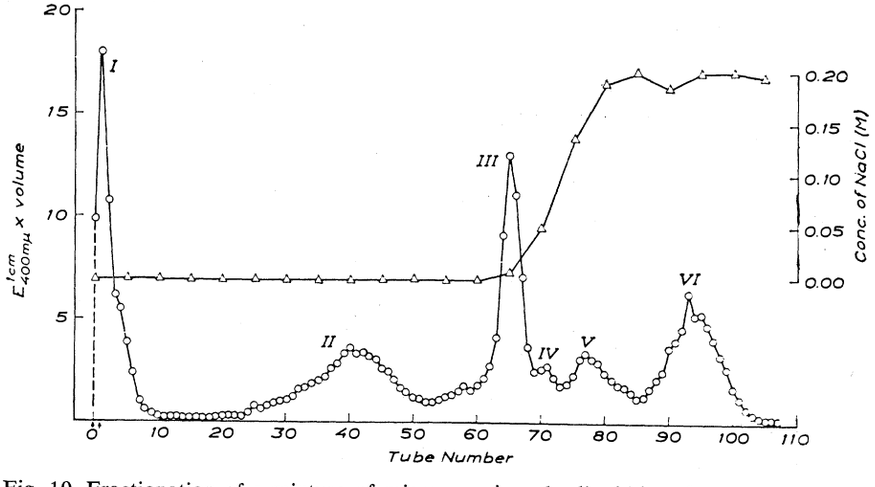

Fig. 10. Fractionation of a mixture of microorganisms by liquid-interface countercurrent distribution in a dextran-polyethylene glycol phase system. A gradient of sodium chloride (triangles) was employed. The peaks represent *Saccharomyces carlsbergensis* (*I*); *Escherichia coli*, strain K12 W1177 (*II*), strain K12 58 (*III*), and strain ML3081 (*VI*); and *Chlorella pyrenoidosa* (*IV* and *V*). [Albertsson and Baird (*30*)]

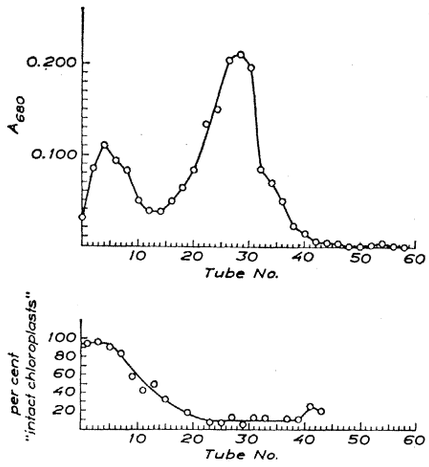

Fig. 11. Countercurrent distribution of spinach chloroplasts in a dextran-polyethylene glycol-sucrose phase system. [Albertsson and Baltscheffsky (*36*)]

to stay with the lower phase or to follow the upper phase in the countercurrent machine. Figures 9 and 10 show examples of such fractionations of mixtures of microorganisms. The distribution of particles between the two phases in an individual tube in the train is found to agree closely with predicted values. These are calculated on the assumption of ideal behavior—that is, equilibria are obtained before each transfer, and the partition ratio is constant and independent of particle concentration and the presence of other particles. In this respect, remarkably enough, these large particles, in the size range 0.1 micron to 5 microns, behave like most low-molecular solutes in conventional liquid-liquid distribution.

The distribution of cells in some phase systems depends to a large extent on the ionic composition, as discussed earlier. By increasing the concentration of a particular anion relative to another anion (for example, of chloride to phosphate), the cells can be transferred from, for example, the upper phase to the interface (Fig. 7). The ion concentration at which this transfer takes place varies for different particles. This effect suggested the use of a salt gradient in the countercurrent distribution to improve the fractionation (*30*). Such a gradient is obtained automatically during the experiment if, at the beginning, the lower phase is a salt solution of a concentration different from that of the salt solution fed into the apparatus and forming the upper phase. No phase-volume changes occur when the ionic composition is altered.

The examples of countercurrent distribution of cells in polymer phase systems given in Figs. 9 and 10 clearly demonstrate that we have here a powerful tool for analyzing complex mixtures of particles. This technique is now being applied to various cell-particle preparations, both in analytical and in preparative work. Recent experiments with chloroplasts (*36*) (see Fig. 11) indicate that even such fragile particles may be fractionated by partition. Of particular value is the finding that the polymers seem to have a protective effect on the structure of chloroplasts.

Another procedure which might be expected to increase the resolving power of polymer phase distribution is partition chromatography. Recently experiments along this line have been carried out in this laboratory and elsewhere (*37*). A difficulty with this technique is the effect of the viscosity of the phases. Also, it appears difficult to find a suitable support medium, one which does not interact adversely with the substances to be fractionated.

It appears very likely that partition phenomena in two-phase polymer systems, as discussed here, may play a role, also, if one of the phases is a gel of the type used in gel filtration. The possible relationship between the two methods deserves closer study.

Conclusion

In the foregoing discussion of new or improved methods we have but briefly mentioned some applications to different biochemical problems studied in our laboratory (*38*). We would like to point out, however, that almost always the methodological work has been inspired and developed in attempts to solve specific problems, where improved separation methods have appeared to offer a key to their solution. We are convinced that new methods are best developed in conjunction with work on problems to which they apply. If in such work a certain operation is not satisfactory, it may be worth while to stop and try to find out why. This generally requires time and patience but is often worth the effort.

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