Partition of Cell Particles and Macromolecules

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SECOND EDITION

Distribution and fractionation of cells, mitochondria, chloroplasts, viruses, proteins, nucleic acids, and antigen–antibody complexes in aqueous polymer two-phase systems

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Foreword to the First Edition

At the Institute of Biochemistry in Uppsala much attention has been paid to the development of separation methods suitable for the study of biochemical systems, especially such as contain substances of large molecular weight. In work of this kind one must always keep in mind that such systems are easily destroyed in many of the common chemical operations. Consequently gentle methods—many of them based upon simple physical phenomena—have proved particularly useful when dealing with this kind of materials. Also the methods should be highly specific, as minute differences in structure or composition may be of decisive importance for the functional properties of biochemically important macromolecules. When speaking of separation, one naturally thinks of the purification and isolation of substances in the first place. It is obvious, however, that separation methods play a decisive part also in the determination of the structure of complicated large molecules, when this is attempted by a study of fragments of such molecules, separated out from complex mixtures obtained, for example, by hydrolysis.

During the last few years we have been interested in exploring the possibilities of improving methods for the separation of particles of biological origin. The reason for this is obvious. Submicroscopic particles, such as microsomes, viruses, and bacteriophages occupy a very important position in present biochemical research. Also whole cells, cell nuclei, cell fragments and membranes, and many other particulate materials are of considerable interest and there is a great need for improved, specific methods for their isolation in quantities. Again, a realisation of the importance of such work as an approach to structural problems has also been in our minds, and part of the work has been done in correlation with electron microscope investigations.

Of several approaches attempted the procedure described in this book appears to be particularly interesting and useful. The author

has done this work in fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Uppsala. Part of the work has been published earlier in scientific journals, but this book contains the most complete and up-to-date description of the method. Thus this work is a dissertation, but it has purposely been written in a manner which deviates somewhat from the typical doctor's thesis. Thus it gives a thorough description of the theoretical background and also extensive practical details of interest to those who would wish to apply the method to their problems. Already the first publication of the method gave rise to a great many inquiries, and evidently the method has rapidly come into general use. Therefore I advised the author to plan this book also as a sort of practical guide.

The method, in all its simplicity, seems to offer a great many possibilities, and is by no means fully explored in all its modifications yet. The work is being continued both in Uppsala and elsewhere. Such work is facilitated if other research workers try it in their problems, and thus widen the experience on which a further improvement must rest.

We are very much indebted to Messrs. Almqvist and Wiksell, Stockholm and to John Wiley and Sons, New York, for their willingness to publish and distribute this monograph.

Uppsala, November 1st, 1960

ARNE TISELIUS

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Preface

Since the first edition of this book is out of print and also partly out of date a revised edition is now published. A small part of the old edition has been retained, but the major part describes work done after the publication of the first edition. This work has been carried out both in the author's laboratory and in several other laboratories.

I wish to thank all my coworkers at the Department of Biochemistry, University of Umeå, especially Göran Blomqvist, Curt Collin, Ingemar Ericsson, Göte Johansson, Björn Karlstam, Christer Larsson, Lars Mörtberg, Shigeru Sasakawa, and Vasant Shanbhag, as well as Harry Walter, Laboratory of Chemical Biology, VA Hospital, Long Beach, Calif., USA, for valuable criticism and stimulating discussions.

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I also want to thank Dr. Vasant Shanbhag for correcting the English in this edition.

Umeå, June, 1971 PER-ÅKE ALBERTSSON

1. Introduction and Summary

Progress in biochemistry and cell biology depends to a great extent on the development of efficient separation methods. This holds both for soluble substances such as proteins and nucleic acids and for suspended particles, such as cell organelles and whole cells. Much interest is today devoted to the study of complex particles obtained by disintegration of cells or cell organelles, such as mitochondria, chloroplasts or various cellular membranes. These procedures yield very complicated mixtures of particles differing in size, form and chemical composition. The particles are also very fragile; they may aggregate, dissociate or generally change their state with time. This is also true of suspensions of whole cells or organelles. In many fields of biological research there is a pressing demand for mild and efficient fractionation methods.

Since a multitude of components are present in mixtures like those mentioned, one cannot expect to solve a separation problem by one type of method alone. It is necessary to combine different methods which utilize different properties of the particles. Centrifugation methods, for example, which separate according to size and density of particles, should be complemented by methods in which other properties, such as surface properties, comprise the separation parameter. One of these methods is distribution in a liquid–liquid two-phase system. This book deals with the application of liquid–liquid distribution techniques to macromolecules and particles using special aqueous phase systems obtained by mixing aqueous solutions of two suitably different polymers. Both phases of these systems are aqueous and they are therefore suitable for particles and macromolecules from biological material. The particles distribute mainly according to their surface properties.

Isolation and purification of biopolymers and particles on a large scale is also of increasing importance in industry in, for example, such processes as the production of pure enzymes for various technical applications and culture and isolation of viruses for vaccines. In

these fields there is a need not only for mild and efficient separation methods but also for such methods which can be applied economically to tons of material. Liquid–liquid distribution methods are of special interest here since they can be scaled up rather easily.

The basis for separation by a two-phase system is the selective distribution of substances between the phases. For soluble substances, distribution takes place mainly between the two bulk phases, and the partition is characterized by the partition coefficient, K

$$K = \frac{C_t}{C_b} \tag{1}$$

where C_t and C_b are the concentrations of the partitioned substance in moles per liter of top and bottom phase respectively. Ideally, the partition coefficient is independent of concentration and also independent of the volume ratio of the phases. It is mainly a function of the properties of the two phases, the partitioned substance and temperature.

The interface between the phases should, however, also be considered. It has a certain capacity for adsorption of the partitioned substance. This does not usually play any significant role with respect to soluble substances but when suspended particles are present the interface may adsorb relatively large quantities of material. Therefore in the separation of cell particles there are in fact three "phases" to consider; the upper, the inter- and the lower phase. It is the selective distribution between these phases which forms the basis for separation of particles by a two-phase system.

Obviously the choice of a suitable phase system is the key step in all partition work. Special problems arise when a phase system has to be selected for biogenic particles and macromolecules. The phase system should be mild, that is, consideration must be given to the water content, ionic composition, osmotic pressure, ability to elute out substances from the particles, denaturing effects etc. The larger and more complicated the particle, the more limited is the choice of environment. Whereas a protein may tolerate wide ranges of pH or ionic strength, a cell organelle may have a very strict requirement with respect to pH and salt composition. Further, to be separable, the substances should differ in their partition characteristics, that is, they should not have the same affinity for the two phases.

Several of the factors mentioned above rule out most of the conven-

tional phase systems containing an organic solvent. These are unsuitable both because of the denaturing effects of organic solvents and because, in a water-organic system, biogenic particles and macromolecules almost always segregate completely to the aqueous phase, thereby precluding separation. In addition, the liquid–liquid interface of conventional phase systems has a rather strong interfacial tension which might damage fragile cell structures.

Aqueous–aqueous systems have been used to overcome the above deficiencies. They consist essentially of two immiscible aqueous solutions of different polymers. It is a general phenomenon that mixtures of solutions of unlike polymers in a given solvent result in phase separation. A mixture of aqueous solutions of polymers results in phases with a water content in the range between 85–99 per cent. See chapter 2 for a detailed treatment of polymer phase systems. They are very mild towards various biological activities. Also one obtains a reproducible partition of soluble macromolecules such as proteins and nucleic acids of high molecular weight. The interfacial tension is extremely low, between 0.0001–0.1 dyne/cm compared with 1–20 dyne/cm for conventional systems. It therefore allows reproducible adsorption of even delicate cell particles such as chloroplasts and mitochondria without structural damage. In fact, experience thus far is that the polymers seem to stabilize, rather than damage, the particle structures and the biological activities.

The mechanism governing partition is largely unknown. Qualitatively it can be described as follows. When a particle is suspended in a phase it interacts with the surrounding molecules in a complicated manner. Various bonds such as hydrogen bonds, ionic bonds, and hydrophobic bonds are probably involved, together with other weak forces. Their relative contributions are difficult to estimate. However their net effect is likely to be different in the two phases. If the energy needed to move a particle from one phase to the other is ΔE, one would expect, at equilibrium, a relation between the partition coefficient and ΔE which can be expressed as follows:

$$\frac{C_1}{C_2} = e^{\frac{\Delta E}{kT}} \tag{2}$$

where k is the Boltzmann constant and T the absolute temperature. Obviously, ΔE must depend on the size of the partitioned particle or

molecule since the larger it is, the greater the number of atoms that are exposed and can interact with the surrounding phase. Brønsted therefore suggested the following formula for partition:

$$\frac{C_1}{C_2} = e^{\frac{\lambda M}{Tk}} \tag{3}$$

where M is the molecular weight and λ is a factor which depends on properties other than molecular weight. For a spherical particle, M should be replaced by A, the surface area of the particle. Thus

$$\frac{C_1}{C_2} = e^{\frac{\lambda A}{kT}} \tag{4}$$

and λ in this case is a factor which depends on properties other than surface area, for example the surface properties as expressed by the surface free energy per unit area (surface tension). Both size and surface properties are therefore of great importance in determining partition.

One would also expect the net charge, Z, of a particle to play a role. If, for example, there is an electrical potential difference $U_1 - U_2$, between the phases an energy term $Z(U_1 - U_2)$ has to be included and the relation would then be the following:

$$\frac{C_1}{C_2} = e^{\frac{\lambda_1 A + Z(U_1 - U_2)}{kT}} \tag{5}$$

where λ_1 then depends on other factors than size and net charge.

In this manner we may formally divide the overall effect determining partition into a number of different factors, such as size, hydrophobicity, surface charge and probably also conformation of the particle or macromolecule, which in turn determines the size and number of groups exposed to the surroundings. The theory of partition is treated in Chapter 3.

The main point of the Brønsted partition theory is the exponential relation between the partition coefficient and properties that enter into the λ factor, for example, size and charge. Small changes in such factors will cause relatively large changes in the partition coefficient. The theory therefore predicts a high degree of selectivity.

This compares well with analogous relations for other methods. In

centrifugation, sedimentation coefficient is proportional to size; in free electrophoresis, mobility is proportional to charge; and in gel electrophoresis, mobility is proportional to log M. Obviously, therefore, the theory of partition predicts that this method will give a much higher resolution in separation according to size as compared with centrifugation or according to charge as compared with electrophoresis. More important is that other factors than charge, size or density also determine partition. It there-fore complements centrifugation and electrophoresis.

From equation 3 we also learn that even a single partition step may give rather efficient separations of large molecules. If, for example, there is a certain difference in λ for two substances, their separation will be more efficient the larger the size of their molecules. An almost complete separation of two types of macromolecules or particles can therefore often be obtained by a single partition. An example is the separation of native from denatured DNA or bacterial spores from vegetative cells. Such applications are described in Chapter 5.

A mixture which is only partly separated by a single partition step can be fully resolved by a multistage procedure such as countercurrent distribution. Also particles which distribute between one of the phases and the interface can be separated in this manner by liquid–interface countercurrent distribution. In this technique each type of particle gives rise to a peak in the distribution diagram which therefore can be used to analyse suspensions of cells and cell organelles such as chloroplasts and mitochondria. See chapter 6.

Partition has been used for concentration and purification of virus. See chapter 7. Here, advantage is taken of the almost unilateral distribution of virus particles and also the collection of virus at the interface. By using suitable volume ratios, a small, virus rich phase is obtained. This method is mild and has been applied to a large number of viruses.

Binding of one molecule to another can also be studied by partition. Usually a complex consisting of two components has a partition coefficient which differs from those of the two components separately. Examples are the single stranded polynucleotides and their double stranded complexes. Antigen–antibody reactions can also be studied in this way. See chapter 8. Thus, the formation of a complex between a protein and its antibody can be detected by determining the apparent