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FRACTIONATION OF PARTICLES AND MACROMOLECULES IN AQUEOUS TWO-PHASE SYSTEMS

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Abstract—The isolation from cells of particles such as nuclei, mitochondria, microsomes and chloroplasts is important in biochemical and physiological studies. Cell particles are separated according to size, density and form. Experiments showed that surface properties of particles could determine the fractionation in a liquid-liquid two phase system, complementing conventional fractionation techniques. Aqueous polymer two phase systems were used in preference to organic solvent-water systems. Enzymes and viruses may be distributed in these systems without loss of activity. The usefulness of the method is demonstrated with examples. Other properties of polymer two phase systems are also discussed.

THE study of mass isolated cell particles, e.g. nuclei, mitochondria, chloroplasts, microsomes and cell walls, obtained from disintegrated cells has been of considerable importance in recent developments in biochemistry and cell physiology. Such work requires efficient fractionation methods. In general, centrifugation techniques have been used, by which methods the particles are separated according to their size, density and form. It would, however, be of value if the centrifugation methods could be complemented by other methods whereby other properties of the particles, for example, the surface properties, determine the fractionation.

An example of such a method is the partitioning of particles in a liquid–liquid two-phase system. If a number of particles are shaken in a two-phase system they will distribute themselves largely according to their surface properties and collect either in the one or the other phase, or at the interface. The present review summarizes the work[1–11] that has been carried out at the Biochemical Institute of Uppsala for the purpose of developing methods for the fractionation of particles and macromolecules according to this principle.

THE PHASE SYSTEMS

Special problems arise when one is selecting phase systems for biochemical substances. The phase systems should be as inert as possible to the particles which are to be fractionated. This means that consideration must be paid to the water content, ionic strength, osmotic pressure, ability to dissolve out substances from the particles, denaturing effects on proteins, and so forth. Since adsorption of particles at the interface is sometimes used for fractionation, the possible damaging effect of the interfacial tension must also be considered. Generally, a low interfacial tension is desirable. All these different requirements usually rule out the common two-phase systems which contain an organic solvent such as the systems water–benzene, water–ether or water–butanol. Instead, a number of aqueous polymer two-phase systems have been studied.

These are composed of water and two water-soluble polymers. Solutions of two different polymers are usually not miscible even at low concentration of the polymers.[1,2] Thus for example if 1 % (w/w) aqueous solution of dextran (a polyglucose) is mixed in a test tube with an equal amount of a 1 % aqueous solution of methyl cellulose, one will observe that the mixture becomes turbid. If the tube is allowed to stand for a while, two liquid layers are formed. An analysis of these will show that the bottom layer contains most of the dextran and the top layer most of the methyl cellulose. If the mixture is shaken again and allowed to stand, the two layers will again separate out. One has thus obtained a system containing two immiscible phases in equilibrium, both consisting largely of water. The difference in the physical properties of the two phases is small, compared with ordinary phase systems. Thus the difference in density or refractive index of the two phases is very small, a fact which sometimes makes it difficult to detect the interface. The interface has a low surface tension as shown by the fact that the interface forms a right angle with the wall of the test tube.

A number of pairs of polymers which give a liquid two phase system when mixed with water are recorded in Table 1. The composition of a phase system and its phases

TABLE 1. PAIRS OF POLYMERS WHICH GIVE A LIQUID TWO-PHASE SYSTEM WHEN MIXED WITH WATER (FROM ALBERTSSON[2])

Polypropylene glycol–polyethylene glycol
Polyethylene glycol–dextran
Polyvinyl alcohol–dextran
Polyvinylpyrrolidone–dextran
Methyl cellulose–dextran

at different proportions of the polymers may be represented by a phase diagram. Figs. 1 and 2 show phase diagrams of the two systems which have been used for the fractionation of particles, namely the dextran–methyl cellulose–water and dextran–polyethylene glycol–water systems. Mixtures represented by points above the curve of

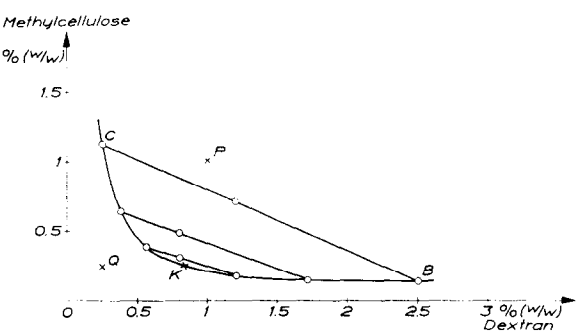

FIG. 1. Phase diagram of the phase system dextran–methyl cellulose–water at 4 °C. The dextran has a molecular weight of about 2,000,000 (weight average) and the methyl cellulose a molecular weight of about 150,000.

Fig. 1 result in two-phase systems while mixtures represented by curves below the curve result in a one-phase system. Thus a mixture of 1 % (w/w) dextran and 1 % (w/w) methyl cellulose (point *P* of Fig. 1) is a two-phase system while a mixture of 0·2 % (w/w) dextran and 0·2 % (w/w) of methyl cellulose (point *Q* of Fig. 1) gives an homogeneous solution. If a mixture represented by point *A* of Fig. 1 is set up and its phases

analysed, they will have the compositions represented by the points *B* and *C* (Fig. 1). Thus the points *A*, *B* and *C* represent the total composition, the composition of the bottom phase, and the composition of the top phase, respectively, of a given phase system. The points *A*, *B* and *C* lie on a straight line, the tie line. Figs. 1 and 2 show a number of such tie lines, connecting points which represent the composition of two

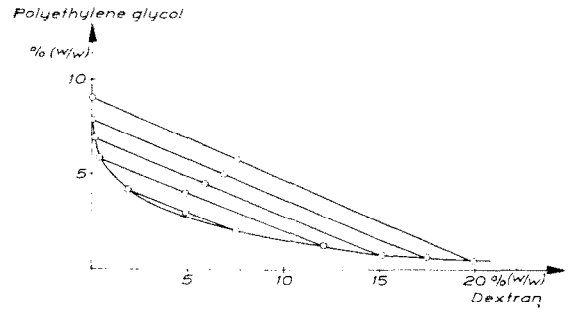

FIG. 2. Phase diagram of the phase system dextran–polyethylyne glycol–water at 0 °C. The dextran has a molecular weight of about 480,000 (weight average) and the polyethylene glycol a molecular weight of 6000–7500.

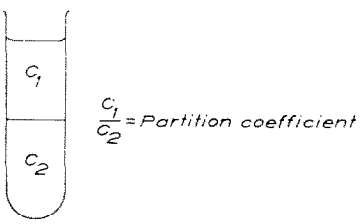

FIG. 3. Partition in a two-phase system

phases in equilibrium. The length of a tie line is a measure of the difference in composition of the phases. As may be seen in Fig. 1 and Fig. 2 as the composition approaches the point *K* the length of the tie lines becomes smaller and smaller. At the point *K* the compositions of the two phases become theoretically equal. The point *K* is called the critical point. At the critical point the property of the system is rather sensitive to changes in the composition of the phase components or the temperature. Therefore best results are usually obtained by the use of systems which have compositions removed from the critical point.

These aqueous polymer systems may be complemented by the addition of substances such as salts or sucrose which are necessary for the stability of the particles to be fractionated. They may be used in the cold as well as at room temperature.

It has been found that a number of enzymes may be distributed in these systems without any loss in activity. The enzymes so far tested include enolase, laccase, coeruloplasmin, cholinesterase and cytochrome oxidase.[3] Different viruses including the bacteriophages T_3 and T_2,[5] vaccinia virus, parotitis virus, Newcastle disease virus, influenza virus, echo virus, and adenovirus[9, 10] have also been tested. No virus inactivation was found to occur. Therefore, it appears that the systems are not harmful. This is probably, to a great extent, due to the fact that they have a high water content and a low interfacial tension. However, the phase components themselves

may also have a stabilizing action on the particles and enzymes. Dextran, methyl cellulose, and polyethylene glycol are all poly-ols, and it is a common experience that poly-ols such as sucrose and polysaccharides can stabilize proteins against denaturation. In fact, it has been found that the bacteriophage T_2 may be treated by a flourocarbon without loss if dextran is present in the phage suspension, while almost all the activity disappears if the polysaccharide is not present.[8]

THE BEHAVIOUR OF SOME PARTICLES AND PROTEINS

If a low molecular substance is partitioned in a two-phase system (Fig. 3) a finite partition coefficient, K, is usually established (K is the concentration of the partitioned substance in the top phase divided by the concentration of the substance in the bottom phase). It was shown by Brønsted[13] that the more the molecular weight of the partitioned substance increases, the more unilateral the distribution tends to be. Thus, for example, if a protein is distributed evenly between the two phases a number of identical cellular particles usually collects in only one of the phases. This is illustrated by Table 2 in which the partition coefficient for a number of proteins and virus

TABLE 2. PARTITION OF PROTEINS AND VIRUS PARTICLES IN A DEXTRAN–METHYL CELLULOSE SYSTEM (DATA FROM ALBERTSSON AND FRICK[5])

Protein or virus particle	Surface area of the particles $\times 10^{-3}$ (mμ)^2	Partition coefficient
Haemocyanin "eighth"	0·86	0·63
Haemocyanin whole	3·5	0·25
T_3-bacteriophage	8·7	0·023
Tobacco mosaic virus	14·4	0·01–0·02
T_2-bacteriophage	25·5	0·0005
Vaccinia virus	220	0·0001

particles are recorded. It is evident that the larger particles like the bacteriophages and vaccinia virus have a concentration in the bottom phase 100–1000 times greater than in the top phase. Small particles like the haemocyanin and phycoerythrin molecules are more evenly distributed. A proportionality between the logarithm of the partition coefficient and the surface area of the particles has been found.[5] Fig. 4 shows how the

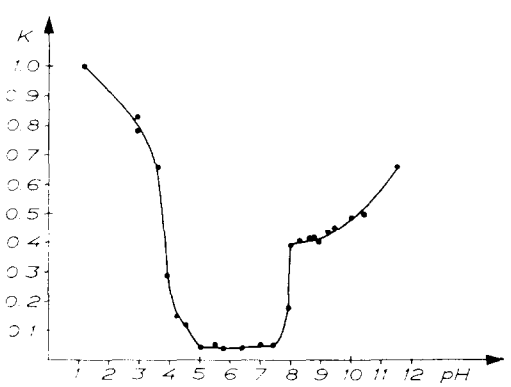

FIG. 4. The partition coefficient, K, of *Helix pomatia* haemocyanin in a dextran–methyl cellulose system as a function of pH.

partition coefficient of *Helix pomatia* haemocyanin depends upon the pH in the dextran–methyl cellulose system. The molecular weight of haemocyanin at different pH values is well known from studies in the ultracentrifuge.[14] Thus for pH 4·7–7·0 this protein has a molecular weight of $8{\cdot}9 \times 10^6$, and in this pH interval the K-value is constant (Fig. 4). Between 7·4 and 8·2 the molecules split partly into halves and eighths. For pH 8·2–9·3 the protein is again monodisperse with a molecular weight of 1×10^6, and in this interval the K-value is also almost constant. Above pH 9·3 the protein molecules split into smaller units. For pH 3·6–4·6 the protein molecules are partly dissociated into halves, and at pH 3·6 it is dissociated into still smaller units. Fig. 4 shows that these changes in the molecular state are well reflected in the partition coefficient. In a similar way the association between the antigen and the antibody in an immunological reaction may be followed in a two-phase system.[11]

All experiments which have been made with the dextran–methyl cellulose system indicate that the size, or more probably the surface area of the particles, is a dominating factor in determining the partition coefficient of proteins and viruses.

In the dextran–polyethylene glycol system the behaviour is very different. The distribution of particles and proteins depends very much upon the salt environment. Thus a substance may be transferred entirely from one phase to the other simply by replacing one ion with another. Phycoerythrin, for instance, is mainly in the top phase in the presence of phosphate ions. If these are replaced by chloride ions the phycoerythrin is transferred to the bottom phase.[7] This salt effect is specific so that one protein may be separated from another by chosing a suitable salt concentration.

Another characteristic of the dextran–polyethylene glycol is that larger particles, like virus particles and cells, are easily adsorbed at the interface. This adsorption at the interface of the particles is, like the partition coefficient of proteins, dependent on the salts present, and a particle may accordingly be transferred from one phase to the interface by changing the salt.

APPLICATIONS

Fractionation of rat brain microsomes

Microsomes consists essentially of two submicroscopic structures, vesicles formed by fragments of the cyloplasmic membranes, and small electron-dense particles. The isolation of the latter particles from a microsome suspension prepared from rat brain has been accomplished by using a dextran–methyl cellulose system.[4] Fig. 5 is a scheme for the fractionation procedure. The microsome suspension was first shaken in a dextran–methyl cellulose system of the same composition as that in Table 2. In that system both kinds of structures are partitioned in favour of the dextran-rich bottom phase ($K < 1$). The vesicles have, however, a very low partition coefficient so that they are almost entirely in the bottom phase. The particles have a larger partition coefficient, and because of the large volume of the upper phase a considerable fraction of them is present in this phase. Thus the upper phase contains only the particles. This phase is removed and replaced by fresh top phase and the shaking is repeated in order to extract more particles from the bottom phase. To speed up the phase separation the tubes are centrifuged. The two phases are then combined and transferred to a new centrifuge tube (II). Dextran and methyl cellulose are then added to this tube in order to produce a very small bottom phase into which the particles are concentrated. The

bottom phase is then collected by making a small hole in the bottom of the plastic centrifuge tube. The particles are then centrifuged down at a high speed.

The starting microsome material consisted of the whole supernatant from rat brain tissue which had been homogenized with 4 vols. of buffered sucrose (0·25 M sucrose, 0·001 M My, Cl_2 and K_2HPO_4) and centrifuged at 18,000 *g* for 15 min. This contained both the microsomal structures and the soluble cytoplasmic fraction. The latter was not removed until the final centrifugation step. Thus the microsomes had not at any stage been packed in the centrifuge at high speed, a procedure which was found to cause considerable irreversible aggregation.

Examination in the electron microscope, and chemical analysis of the protein and the RNA content of the starting and final preparations, showed that a considerable purification of the RNA-rich particles has been achieved (for details see Albertsson *et al.*[4])

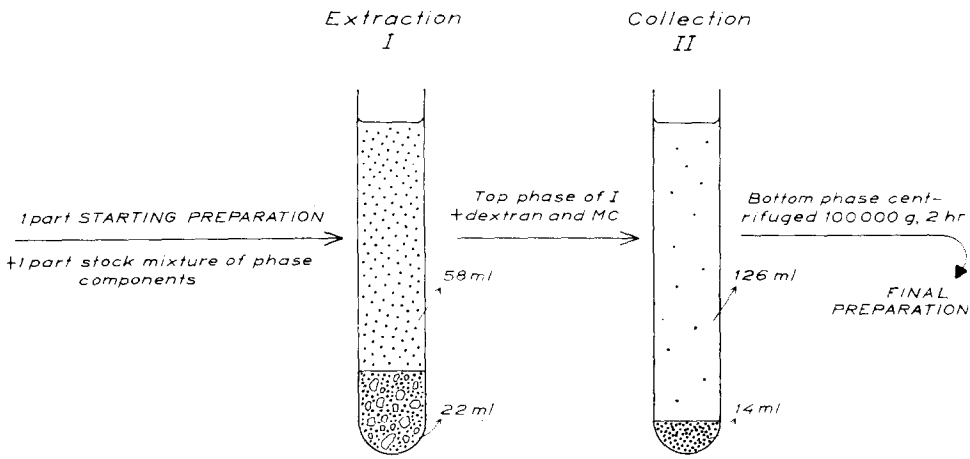

FIG. 5. Fractionation of rat brain microsomes in a two-phase system. Particles and vesicles are symbolized. (From Albertsson *et al.*[4].)

The concentration and purification of virus

Modern tissue culture techniques allow a production of virus suspensions on a 10 l. scale. Although these cultures are biologically highly active they are extremely dilute in terms of milligrams of virus material per millilitre. To concentrate the virus from these large volumes of diluted virus into a small volume is therefore an important step in the purification of a virus. Such a concentration can be accomplished in a two-phase system, provided that the virus particles give a fully one-sided distribution, and that the volume into which they are collected can be made small compared with the volume of the original culture suspension. A number of experiments with bacteriophages[5, 8] and animal viruses[9, 10] have shown that it is possible to construct a polymer phase system which fulfils these requirements. Table 3 shows the distribution of some viruses in such phase systems, which shows that the viruses are concentrated efficiently into the small bottom phase. The concentration is accomplished simply by the addition of suitable amounts of concentrated solutions of the polymers to the culture fluid. The mixture is then shaken and allowed to stand for phase separation. Concentration may then also be carried out in many steps so that a concentration of virus from, for example, from 10 l. to 10 ml can be achieved.

This technique has many advantages over centrifugation. First, packing of the virus particles together with impurities can be avoided. This occurs during high-speed centrifugation and it often leads to losses in virus activity. Second, since other substances such as proteins and cell fragments distribute in a way different to the virus

TALBE 3. CONCENTRATION OF SOME VIRUSES INTO THE SMALL BOTTOM PHASE OF A DEXTRAN-POLYETHYLENE GLYCOL SYSTEM (DATA FROM WESSLÉN *et al.*[9])

Virus	Titre per ml of bottom phase volume = 0·6 ml	Titre per ml of top phase volume = 9 ml	Virus assay
Vaccinia	7·6	4·9	Infectivity titres (log values)
Echo 7	9·5	5·5	Infectivity titres (log values)
Newcastle disease	2048	4	Hemagglutination titres
Influenza	4096	32	Hemagglutination titres
Parotitis	2048	4	Hemagglutination titres

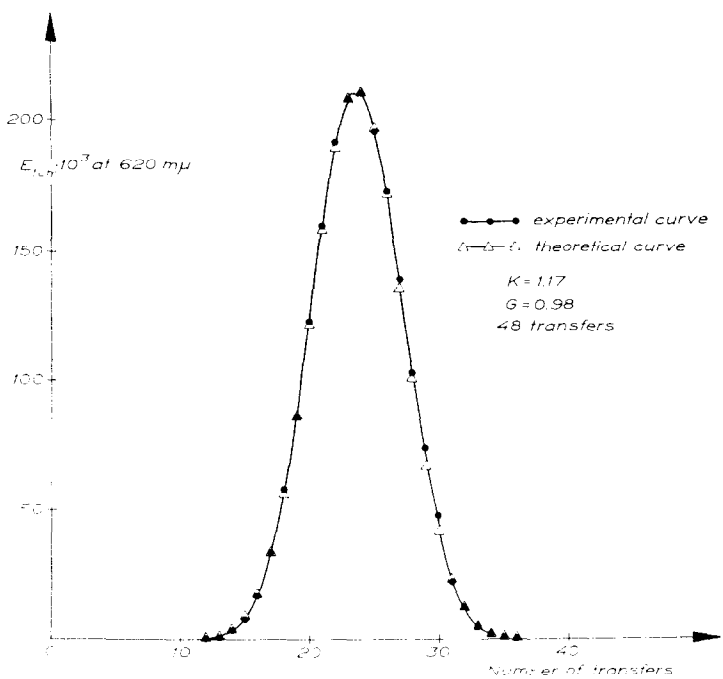

FIG. 6. Countercurrent distribution of phycoerythrin in a dextran–polyethylene glycol system. (From Albertsson and Nyns[6].)

particles, a considerable purification may be obtained by the phase system at the same time as the concentration is being accomplished. Third, the technique is very simple and can be applied on a large scale without the use of a complicated apparatus.

Counter-current distribution of proteins

The method of counter-current distribution has been shown to be one of the most efficient tools for analyzing and separating mixtures of biochemical interest. So far it

has mainly been applied to low molecular weight substances such as polypeptides but recently a number of smaller proteins have also been successfully distributed.[15] One of the advantageous features of counter-current distribution is that it is based upon several steps, each involving a state of equilibrium. Generally a state of equilibrium is supposed to be more easily obtained between two liquid phases than between, for example, a solid phase and a liquid phase. For macromolecules this difference in equilibrium behaviour may be expected to be more pronounced than for low molecular weight substances. It should therefore be of the greatest interest if counter-current distribution can be applied also for large molecular weight substances, such as proteins and nucelic acids.

Fig. 6 shows a counter-current distribution of a protein, phycoerythrin having a molecular weight of 300,000 in a dextran–polyethylene glycol system. The experimental curve fits the theoretical curve very well, indicating that the protein behaves

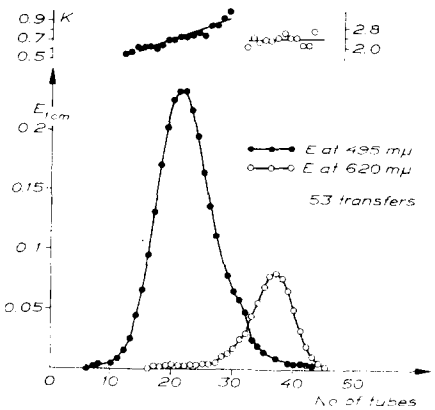

FIG. 7. Separation of phycoerythrin and phycocyanin in a dextran–polyethylene glycol system. (From Albertsson and Nyns[6].)

according to the Nernst distribution law. Fig. 7 shows the separation of two proteins, phycoerythrin and phycocyanin the latter of which the molecular weight is 150,000 in a similar system. Thus it appears to be possible to carry out counter-current distribution of large molecular weight protein in aqueous polymer phase system.

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