Journal of Chromatography, 159 (1978) 111–122
Chromatographic Reviews

CHREV. 110T8

PARTITION BETWEEN POLYMER PHASES

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1. INTRODUCTION

By using aqueous polymer phase systems, it is now possible to apply partition to the analysis and separation of biological macromolecules and cell particles[1,2]. The phase systems are obtained by mixing aqueous solutions of two or more water-soluble polymers, so that all of the phases are rich in water. To these are added salts and sugars to provide a suitable environment for fragile biological particles. If two polymers are used, a two-phase system is obtained, if three polymers are used a three-phase system is obtained, etc.

This paper summarizes some of the factors that determine partition and some recent applications are considered. In most of the work carried out so far, the dextran–polyethylene glycol–water two-phase system has been used; dextran is in the lower phase and polyethylene glycol in the upper phase[1]. A large number of different biological materials have been partitioned in this system, including proteins, nucleic acids, viruses, mitochondria, chloroplasts, cell membranes and whole cells. Protein and nucleic acids are soluble in the polymer phases and their partition coefficient (concentration in the upper phase/concentration in the lower phase) depends on the molecular weight, charge, conformation and type of polymer used in the phase system. Particles such as cells and cell organelles distribute between the two phases and the interface. The latter has a large capacity for adsorption of particles and can be used for selective adsorption of particles which distribute according to their surface properties.

Much data on the behaviour of proteins and cell particles in two-phase polymer systems is now available and the main factors that determine partition can now be formulated. Partition of proteins, for example, depends upon their molecular weight, charge and probably also the kind of amino acid side-chains that are located on the surface of the proteins.

2. ELECTRICAL POTENTIAL BETWEEN PHASES

The effect of salts on the partition of charged macromolecules is dramatic. Minor changes in the ionic composition can transfer DNA almost completely from one phase to the other. It is mainly the kind of ions present and the ratio between different ions that determine the partition of biopolymers. The ionic strength is not so important. This can be explained by an electrical potential between the two phases. The potential is created by an unequal affinity of ions for the phases. Careful studies on the partition of inorganic salts in a dextran–polyethylene glycol–water system have shown that different salts have small but significant differences in their partition coefficient[3].

Such partition differences between salts mean that the different ions have different affinities for the two phases. Hence an electrical potential difference between the phases is created[1]. For a salt, the ions of which have charges Z^+ and Z^-, the interfacial potential, ψ, is given by

$$\psi = \frac{RT}{(Z^+ + Z^-)F} \cdot \ln (K_-/K_+) \tag{1}$$

where R is the gas constant, F is the Faraday constant, T is the absolute temperature and K_- and K_+ are the partition coefficients of the ions which they would have if ψ could be set to zero, *i.e.*, K_- and K_+ are expressions of the affinities of the ions for the two phases due to forces except electrical. The interfacial potential will be larger the larger is the K_-/K_+ ratio, *i.e.*, a salt with two ions that have very different affinities for the two phases will generate a larger potential difference than a salt with ions that have similar affinities for the two phases.

Further, it can be shown[1] that in the presence of excess of salt a protein will partition according to

$$\ln K_p = \ln K_p^\circ + \frac{ZF}{RT} \cdot \psi \tag{2}$$

where K_p is the partition coefficient of the protein, K_p^0 is the value of this coefficient when the interfacial potential, ψ, (generated by the excess of salt) is zero or when the protein net charge, Z, is zero. Hence, the difference in partition of the ions of the salt generates an electrical potential difference according to eqn. 1, which in turn affects the partition coefficient of the protein according to eqn. 2. Even if ψ is small, it will strongly influence K_p, because Z is large for most proteins and K changes exponentially with Z. The same holds for other charged macromolecules and more so for cell particles, which carry a vast number of charges per particle.

Experimental data on proteins fit eqn. 2 fairly well. Proteins were partitioned in dextran–polyethylene glycol systems at different pH and with different salts[4–6]. A plot of log K against the net charge of the protein gives straight lines (Fig. 1), as expected from eqn. 2. The slopes of the lines are different for the different salts used as these generate different values of ψ. The different lines intersect the ordinate at approximately the same log K_0 value.

If K_0 in eqn. 2 is assumed to be independent of pH, then the ψ value can be

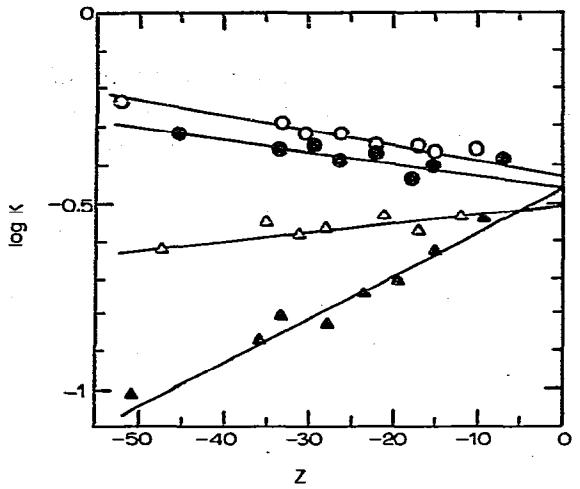

Fig. 1. Partition of a protein (bovine serum albumin) as a function of its net charge in four different phase systems having different interfacial electrical potentials[4]. Compare eqn. 2. Phase systems: 5% dextran, 4% polyethylene glycol and ○, 0.05 *M* K_2SO_4; ●, 0.1 *M* LiCl; △, 0.1 *M* KOAc; ▲, 0.1 *M* KCl.

calculated from the slope of lines in Fig. 1. The interfacial potentials thus obtained are in the range 0–5 mV. Other measurements using electrodes give similar values[7]. If the interfacial potential of a phase system is known, we can use partition in this system for the determination of the net charge per molecule of an unknown substance, without knowing its molecular weight. In this way, the net charges of different isoenzymes of enolase have been determined in an extract of yeast[8].

It is striking that the interfacial potential with a given salt varies very little with ionic strength. Further, partition of protein in a given salt does not depend significantly upon ionic strength in the range 5–100 m*M*. That is, for a given ψ, the effective net charge of the protein, Z, is independent of ionic strength. This behaviour is in contrast to other physical phenomena where charge is involved, such as electrophoresis and ion-exchange chromatography; electrophoretic mobility and adsorption on an ion-exchange column depend strongly on ionic strength.

Phase systems with zero interfacial potential can be constructed by choosing a suitable salt or salt mixture. Partition of proteins in such a system should be independent of the net charge of the protein, *i.e.*, independent of pH. Some proteins also show a constant partition coefficient over a wide pH range in zero-potential phase systems[4–6]. Other proteins show changes in certain pH ranges. In some instances this occurs when the protein undergoes a conformational change, such as in serum albumin at low pH or when protein molecules form dimers, as with lysozyme. For such proteins, K_0 is not independent of pH and partition in zero-potential systems could therefore be used to detect conformational changes when, for example, previously hidden groups are exposed on the protein surface, or to study association–dissociation phenomena among proteins.

3. HYDROPHOBIC INTERACTIONS

The electrical effects can be nullified by choosing a suitable salt composition of the phase system. For such systems, other factors that determine the partition come to the fore, such as the hydrophobic–hydrophilic balance of the particle surface and conformation.

We can split the partition coefficient into several factors such that the logarithm of the partition coefficient is the sum of several more or less independent terms:

$$\ln K = \ln K_{el} + \ln K_{hfob} + \ln K_{hfil} + \ln K_{conf} + \ldots \quad (3)$$

where K_{el}, K_{hfob}, K_{hfil}, K_{conf} represent partition coefficient factors depending on electrical, hydrophobic, hydrophilic and conformational effects, respectively. $\ln K_{el}$ is zero when the interfacial electrical potential is zero. It should be possible to increase the hydrophobic effect on partition by binding hydrophobic groups on the polymers or by including a detergent that interacts with the hydrophobic parts on a protein molecule. Several non-ionic detergents contain a polyethylene glycol chain as the hydrophile. Micelles of these detergents expose polyethylene glycol chains on their surface and, therefore, prefer the polyethylene glycol-rich phase of, for example, the dextran–polyethylene glycol system. Likewise, proteins that bind such detergents should acquire an increased affinity for the polyethylene glycol phase (see Fig. 2). Phase systems containing detergents have been used for purifying a membrane-bound enzyme, phospholipase A, from *Escherichia coli* according to this principle[9]. By using detergents with different hydrophobic parts, it might be possible to achieve selective extractions of hydrophobic proteins or membrane fragments having different hydrophobic regions.

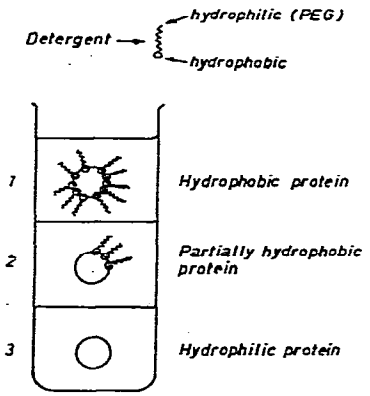

Fig. 2. Hydrophobic affinity partition. A detergent that binds to a hydrophobic protein will selectively extract this protein to phase 1, which has an affinity for the hydrophile of the detergent[9].

Digitonin is another type of detergent, the hydrophilic part of which consists of sugar units, and the micelles of digitonin prefer the lower dextran phase of the dextran–polyethylene glycol system. Therefore, proteins or membrane fragments that bind the hydrophobic part of digitonin would expose a surface of polysaccharide character and be enriched in the lower dextran phase. One could therefore imagine

mixtures of membranes with digitonin and polyethylene glycol detergents where differential binding to different membrane fragments would cause them to separate between two phases.

Recently, large hydrophobic groups, such as fatty acid chains, have been covalently bound to polyethylene glycol or dextran, and their effect on partition has been studied[10–12]. Proteins such as serum albumin, which are known to interact with fatty acids are strongly affected in their partition behaviour. Thus, palmitoyl polyethylene glycol selectively extracts serum albumin from plasma into the upper phase, while almost all of the remaining proteins remain in the lower phase[10].

Partition of cell organelles and cells is strongly influenced by hydrophobic groups attached to the polymers. Palmitoyl polyethylene glycol at concentrations as low as 0.0001–0.01 % may thus transfer chloroplasts, membranes or red blood cells from the lower phase or the interface into the upper phase. Some selectivity in the effect of the hydrophobic groups has been demonstrated; *e.g.*, deoxycholate esterified to polyethylene glycol was more effective than palmitoyl polyethylene glycol in separating two different chloroplast particles having different surface membranes[13].

Saturated and unsaturated fatty acids attached to polyethylene glycol were used for studying the surface hydrophobic properties of red cells[14]. Large differences in partition behaviour for cells from different species and also in the effect of different fatty acids were found. These could be correlated to the phosphatidylcholine and sphingomyelin composition of the different red cell membranes. As these phospholipids are located mainly on the outer layer of the phospholipid bilayer, the results indicate that the fatty acids attached to the polyethylene glycol, upon binding to the cells, interact with the outer layer of the red cell membrane.

Similar studies on the partition of liposomes, composed of different phospholipids, demonstrate that the polar head group of the phospholipid has a strong influence on the partition[15].

4. BIOSPECIFIC INTERACTIONS —AFFINITY PARTITION

By binding a biospecific ligand covalently to one of the phase polymers, it should be possible to achieve biospecific partition in a similar fashion to affinity chromatography. Recent experiments have demonstrated that such "affinity partition" is feasible. The ligands used include enzyme inhibitors for selective partition of enzymes[16], fatty acids for selective extraction of serum albumin[10], dinitrophenyl for selective partition of S-23 myeloma protein[17] and steroid for selective partition of a steroid enzyme[18]. Of particular interest is the fact that affinity partition can be applied to membranes. Thus, ligands which bind to membrane receptors were coupled to polyethylene glycol and used for the selective partition of membrane vesicles from the electrical organ of a fish[19].

5. SOME RECENT APPLICATIONS

5.1. Chloroplasts

A very complex and heterogeneous mixture of particles is obtained when a cell is disintegrated, for example by mechanical homogenization. By centrifugation, such a mixture can be separated into fractions that contain particles with the same size or

density. Such centrifugal fractions are still very heterogeneous, however, and further separation is often desirable. This can be achieved by partition, which separates according to surface properties and therefore complements centrifugation.

A conventional chloroplast preparation, obtained by differential centrifugation, can thus be separated into at least three different populations by counter-current distribution[20]. The chloroplast particles of the three populations differ to a great extent in surface properties, protein : chlorophyll ratios, ultrastructure and metabolism, but very little in size. One population consists of intact chloroplasts surrounded by the chloroplast envelope; the second population consists of chloroplasts that have lost their envelopes; and the third population consists of particles containing intact chloroplasts surrounded by a membrane-bound cytoplasmic layer including mitochondria and peroxisomes.

Another example in which partition together with centrifugation has yielded further purification is the recent separation of "inside-out" from "outside-out" vesicles from disintegrated chloroplast membranes[21]. By treatment with a high-pressure cell, the photosynthetic chloroplast membranes are fragmented into small vesicles. Such a population of vesicles consists of a whole spectrum of vesicles differing in size, chemical composition and photosynthetic activity. By differential centrifugation it can be separated into various size classes of vesicles. These centrifugal fractions can be further separated by partition into classes of vesicles that have different surface properties (see Figs. 3 and 4).

Analyses of these fractions have shown that partition separates vesicles that are turned inside-out from "outside-out" vesicles. Thus, a combination of two separa-

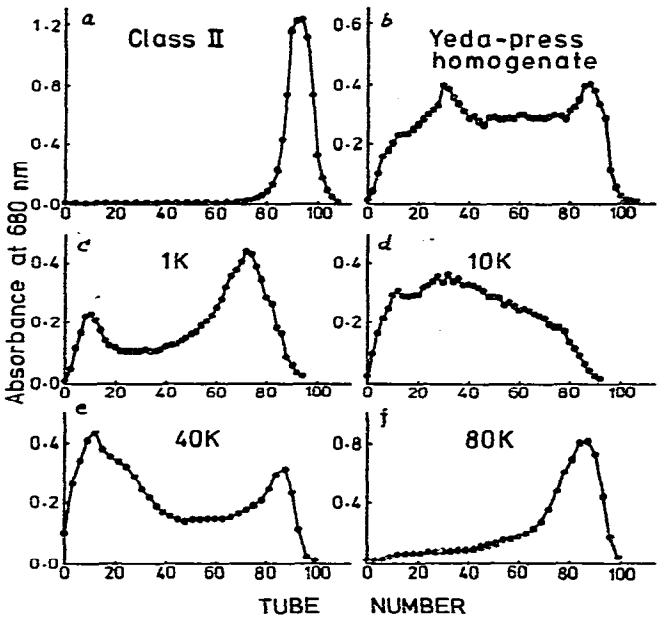

Fig. 3. Counter-current distribution diagram of (a) class II chloroplasts and (c), (d), (e) and (f), different centrifugal fractions of the Yeda press-treated material, (b). 1K = 1000 *g*, 10 min; 10K = 10,000 *g*, 30 min; 40K = 40,000 *g*, 30 min; 80 K = 80,000 *g*, 60 min.

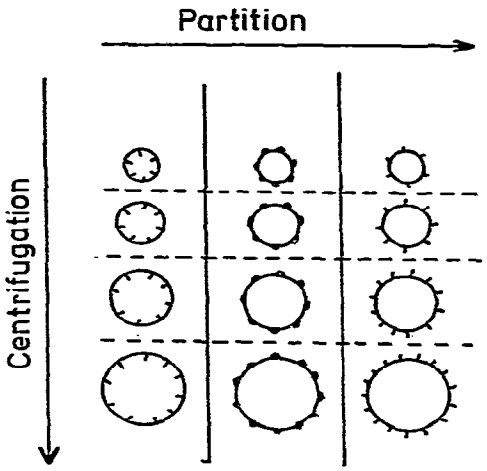

Fig. 4. Schematic diagram of membrane vesicles having different sizes and surface properties. Centrifugation will separate this mixture mainly into size classes, *i.e.*, along the broken lines, while partition separates mainly according to surface properties, *i.e.*, the full lines. A combination of these two techniques will therefore achieve a better separation than either of them alone.

tion techniques, centrifugation, which separates according to size, and partition, which separates according to surface properties, has considerably increased the separation and allowed, for the first time, the isolation of inside-out chloroplast vesicles.

5.2. Determination of hydrophobicity of water-soluble proteins

A method for measuring the hydrophobicity of water-soluble proteins has recently been described[11]. It involves partition of proteins in a dextran–polyethylene glycol system where some of the polyethylene glycol is esterified with fatty acids. Proteins that interact with hydrocarbon groups will partition more in favour of the upper polyethylene glycol-rich phase. The difference, $\Delta \log K$, between the logarithm of the partition coefficient of the protein in a system with and without fatty acid ester is taken as a measure of the hydrophobicity. In this way, interactions other than hydrophobic are eliminated. Fig. 5 shows a plot of $\Delta \log K$ against percentage of palmitoyl polyethylene glycol for six different proteins: serum albumin, β-lactoglobulin, haemoglobin, myoglobin, ovalbumin and α-chymotrypsinogen. As expected, serum albumin and β-lactoglobulin show considerable hydrophobicity. At 5% palmitoyl polyethylene glycol, $\Delta \log K$ for serum albumin is about 2 and for β-lactoglobulin it is about 1.5. Less expected is the finding that haemoglobin and myoglobin also display hydrophobicity. Myoglobin has a $\Delta \log K$ value that is about one quarter of that of a haemoglobin, *i.e.*, the same ratio as for their molecular weights. Ovalbumin and α-chymotrypsinogen show no detectable hydrophobicity. Similar studies can be carried out using other hydrophobic groups in the polyethylene glycol.

Because factors other than hydrophobic interactions are eliminated by this type of "difference partition", and because the proteins are not denatured, the $\Delta \log K$ value can be taken as a practically useful estimate of hydrophobicity. It allows for the first time a well defined comparison of hydrophobicity between different water-soluble proteins.

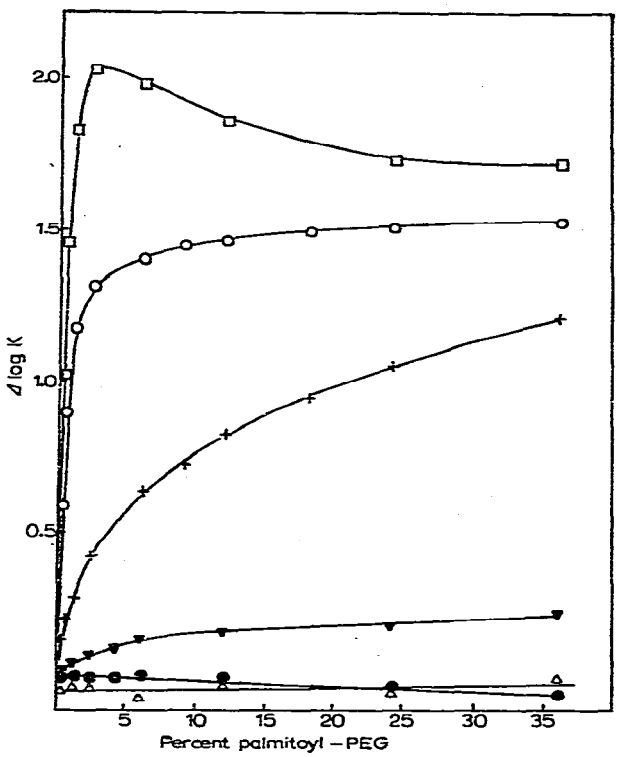

Fig. 5. Proteins partitioned in two dextran–polyethylene glycol two-phase systems, one with and the other without palmitoyl groups bound to the polyethylene glycol. The difference in log K between the two systems is plotted against the degree of substitution of palmitoyl. Δ log K can be taken as a measure of the hydrophobicity of the proteins. Proteins: □, serum albumin; ○, β-lactoglobulin; +, haemoglobin; ▼, myoglobin; ●, x-chymotrypsinogen; △, ovalbumin.

The method has recently been applied for comparing the hydrophobic properties of different histones[22].

6. INTERACTING MOLECULES

When a pair of interacting molecules are partitioned together, the overall partition should be different than that which occurs when the respective compounds are partitioned alone.

Suppose, for example, that the partition coefficient of a substance A is K_A and that of another substance B is K_B. If there is no interaction between A and B, the same partition of A will be found whether B is present or not. Likewise, the presence of A will not influence the partition of B. However, if A and B interact in some way, the presence of A will perturb the partition of B and *vice versa*. We might assume, for example, that A and B form a complex, AB, the partition coefficient of which is different from both K_A and K_B. The different equilibria in such a case are shown in Fig. 6. There are two association equilibria, one for each phase, and three partition equilibria. If the total concentrations of A and B can be determined in the two phases,

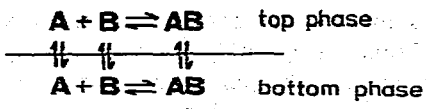

Fig. 6. Partition of two interacting molecules giving rise to a 1:1 complex in each phase.

the equilibrium constants can be calculated. In this manner, interactions between molecules can be detected and also studied quantitatively.

We use the following symbols to denote concentrations, partition coefficients and dissociation constants:

$[A^\circ]_t$ = total concentration of A in top phase;
$[B^\circ]_t$ = total concentration of B in top phase;
$[A]_t$ = concentration of free A in top phase;
$[B]_t$ = concentration of free B in top phase;
$[AB]_t$ = concentration of complex AB in top phase.
By replacing the subscript t with b, corresponding symbols for the bottom phase are obtained.
K_A, K_B, K_{AB} = partition coefficients for A, B and AB, respectively.
K_t, K_b = dissociation constants in the top and bottom phase, respectively.

The following equations can be written:

$$K_A = \frac{[A]_t}{[A]_b} \tag{4}$$

$$K_b = \frac{[B]_t}{[B]_b} \tag{5}$$

$$K_{AB} = \frac{[AB]_t}{[AB]_b} \tag{6}$$

$$[A]_t + [AB]_t = [A^\circ]_t \tag{7}$$

$$[B]_t + [AB]_t = [B^\circ]_t \tag{8}$$

$$[A]_b + [AB]_b = [A^\circ]_b \tag{9}$$

$$[B]_b + [AB]_b = [B^\circ]_b \tag{10}$$

$$K_t = \frac{[A]_t\,[B]_t}{[AB]_t} \tag{11}$$

$$K_b = \frac{[A]_b\,[B]_b}{[AB]_b} \tag{12}$$

If we take out a sample from the top phase and dilute it such that the complex AB dissociates and suppose that we then can assay A and B separately, for example by an enzymatic, immunological or radioactive assay, then we can determine the total

concentration of A and B in the upper phase. In the same way, the total concentration of A and B in the bottom phase can be determined. Thus, $[A^\circ]_t$, $[B^\circ]_t$, $[A^\circ]_b$ and $[B^\circ]_b$ will be known. K_A and K_B can be determined by measuring the partition coefficients of the proteins separately. The remaining nine unknowns can be solved by means of the above equations. We obtain the following relationships for the dissociation constants and the partition coefficient of the complex:

$$K_t = \frac{K_A K_B \left\{[A^\circ]_b - [B^\circ]_b - \frac{1}{K_b}([A^\circ]_t - [B^\circ]_t)\right\}\left\{[A^\circ]_b - [B^\circ]_b - \frac{1}{K_A}([A^\circ]_t - [B^\circ]_t)\right\}}{(K_B - K_A)\left(\frac{1}{K_A}[A^\circ]_t - \frac{1}{K_B}[B^\circ]_t - [A^\circ]_b + [B^\circ]_b\right)} \tag{13}$$

$$K_b = \frac{\left\{[A^\circ]_t - [B^\circ]_t - K_B([A^\circ]_b - [B^\circ]_b)\right\}\left\{[A^\circ]_t - [B^\circ]_t - K_A([A^\circ]_b - [B^\circ]_b)\right\}}{(K_A - K_B)\left(K_A[A^\circ]_b - K_B[B^\circ]_b - [A^\circ]_t + [B^\circ]_t\right)} \tag{14}$$

$$K_{AB} = K_A K_B \cdot \frac{\frac{[B^\circ]_t}{K_B} - \frac{[A^\circ]_t}{K_A} + [A^\circ]_b - [B^\circ]_b}{K_A[A^\circ]_b - K_B[B^\circ]_b - [A^\circ]_t + [B^\circ]_t} \tag{15}$$

Hence the dissociation constants and the partition coefficient of the complex can in principle be determined by one partition only.

It is assumed that only a 1:1 complex between A and B is formed. Also, the partition coefficients K_A and K_B must be different. If they are only slightly different the method is not very accurate and if they are identical the calculation cannot be used.

The partition coefficient of the complex, K_{AB}, can also be determined if an excess of A over B is added to the system. If the excess is so large that all B is in the complex, its partition coefficient can be determined.

The following expression for the dissociation constant in the bottom phase can then be written:

$$K_b = \frac{([A^\circ]_b - \varphi)([B^\circ]_b - \varphi)}{\varphi} \tag{16}$$

where

$$\varphi = \frac{[A^\circ]_t - K_A[A^\circ]_b}{K_{AB} - K_A}$$

and the dissociation constant in the top phase will be

$$K_t = \frac{K_A K_B}{K_{AB}} \cdot K_b \tag{17}$$

In this instance K_A and K_B may be similar, provided that they are different from K_{AB}.

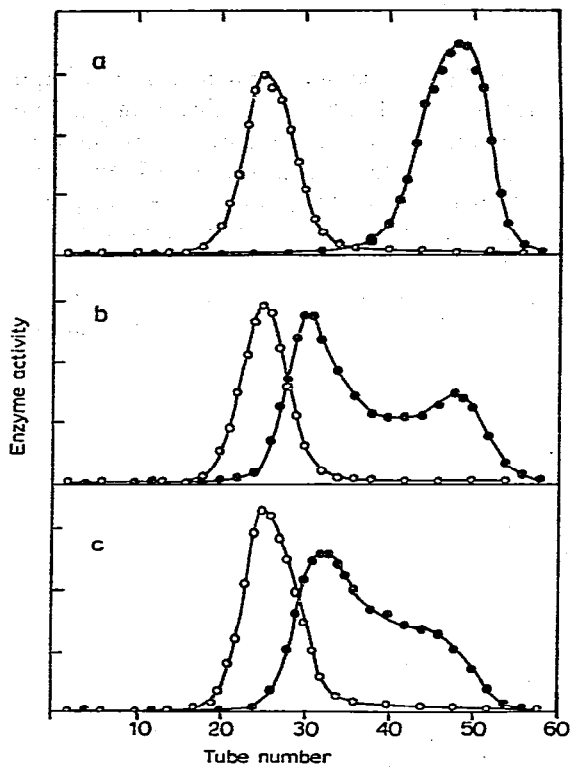

Fig. 7. Detection of interaction between two enzymes, aspartate aminotransferase (○) and malate dehydrogenase (●), by counter-current distribution. (a) The two enzymes were run separately; (b) and (c) the two enzymes were run together with aminotransferase: malate dehydrogenase activity ratios of 12 and 24, respectively[26].

The following types of interactions have been studied: protein–small ligand[23], DNA–small ligand, ribosomes–small ligand[24], protein–DNA, RNA–RNA[1], protein–RNA[25] and protein–protein[26,27].

Of particular interest is the use of equilibrium partition for the detection of weak interactions, which are difficult to detect by other methods. An example is shown by studies on the interaction between malate dehydrogenase and aspartate transaminase[26]. These two enzymes catalyse two consecutive metabolic steps and there has been speculation about their possible physical association. Each enzyme has one cytoplasmic and one mitochondrial isoenzymic form. Using phase partition in combination with counter-current distribution, Backman and Johansson[26] first demonstrated a physical interaction between the cytoplasmic forms of malate dehydrogenase and aspartate transaminase and then also between the mitochondrial forms of the two enzymes. However, no interactions between the heterotopic enzymes was found, *i.e.*, between cytoplasmic malate dehydrogenase and mitochondrial aspartate transaminase or between mitochondrial malate dehydrogenase and cytoplasmic aspartate transaminase. Thus, each enzyme seems to recognize its appropriate neighbour enzyme. This suggests that enzymes, in addition to catalytic and regulatory sites, also expose recognition or "social" sites which interacts with neighbouring enzymes *in vivo*.

7. SUMMARY

Separation of biopolymers and cell particles can be accomplished by partition between two or more immiscible, liquid, aqueous, polymer-containing phases. The phase systems are obtained by mixing water solutions of different polymers, such as dextran and polyethylene glycol. This review describes the various factors which determine the partition. By binding a biospecific ligand covalently to one of the phase polymers, biospecific affinity partition is obtained. Applications on the separation of chloroplasts and membrane vesicles are described.

Partition can also be used to detect and study interaction between macromolecules. Formulae for the calculation of the dissociation constant for a 1:1 protein–protein complex are presented.

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