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Factors Influencing the Use of Aqueous Two-Phase Partition for Protein Purification

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INTRODUCTION

Economic analyses show that purification is often the most important aspect of biomolecule production and processing. This is particularly true of protein processing which, because of the complexity of the starting material, often requires many steps to reach purity levels required for medical and food applications. The separation specialist's task is to develop safe, simple, yet effective processes to achieve high purity products.

The main unit operation which appears in laboratory-scale protein purifications aimed at very pure products is chromatography (*1*). Frequent use is made of ion-exchange, hydrophobic, and biospecific (affinity) chromatography primarily because the processes are effective and relatively simple. Except for a few systems, there is no real need to improve on chromatography at small scale, and the current trend is toward increasing rather than decreasing use of this method in the laboratory.

At large scale, however, it is not clear that chromatography is the best separation process available. There are two main difficulties with chromatography which lead to process bottlenecks. First, chromatography is inherently discontinuous and although various types of equipment have been proposed which could make chromatographic processes more or less continuous, they all lack simplicity, which is a measure of the usefulness of a process industrially. The best chromatography systems, even at large scale, are still discontinuous.

The second problem with chromatography is mass transfer rate. Protein diffusion in free solution is inherently slow because of the large size of these molecules, but in resins the diffusivity is even smaller because of hindrances set up by the resin matrix material (*2*). One method to alleviate slow mass transfer in resins is to use smaller particles, but this does not work well at large scale because large columns will have unacceptably large pressure drops.

For these and other reasons not mentioned, much recent research has been directed at the possible use of aqueous two-phase partition (ATPP) to replace chromatographic (and other) steps in protein purification schemes (*3*, *4*). ATPP is analogous to chromatography in many respects, but may offer significant advantages over that purification method once processes using it are properly designed.

ATPP involves the use of two liquid polymer solutions as a partitioning system for proteins. The liquid-liquid nature of these systems offers the possibility that separations using ATPP may be designed to be continuous without using complex or unusual equipment (*5–10*, *41*). But even discontinuous ATPP processes have an advantage over chromatography because they are easily scaled. Small-scale systems can be linearly scaled at least 10,000-fold without any appreciable change in the nature or efficiency of the process (*11–13*). In addition, since there is no solid phase, intimate mixing of the two phases is possible and hence interphase transport is rapid. Only seconds are required to bring most two-phase systems to equilibrium. Another benefit which may be important is that the phases are compatible with almost all known proteins. The presence of the polymers can even stabilize some biomolecules (*14*).

Recently, several books and numerous papers which review ATPP have been published (*3*, *6*, *14–17*). These works cover the topic comprehensively, and consequently a broad review of ATPP will not be attempted here. Instead, this review will cover aspects of ATPP which are of particular importance for protein purifications and/or are of personal interest to the author. For further information, the references listed above are recommended reading.

Five aspects of ATPP have been selected for comment and review. The first involves the choice of polymers for a two-phase partition. The second concerns the generation of phase potentials and the consequences for protein purification. The third addresses the effect of protein structure on partition behavior. The fourth focuses on the use of affinity partition for bioseparations. And the fifth deals with the problem of separating protein products from phase systems once the protein has been partitioned. All five of these topics have significant implications in the

engineering decisions one must make concerning the use of ATPP in protein purification processes.

POLYMER CHOICE

The starting point for any ATPP system is the selection of the polymers used to generate the phases. Phase separation is seen with almost any combination of two chemically distinct polymers in a single solvent and is seen in both organic and aqueous solutions. Flory (*18*) and Huggins (*19*) developed the basic theory to describe this phenomenon for organic systems. Albertsson (*20*) demonstrated phase separation behavior in a large number of aqueous systems.

Interestingly, despite the number of potentially useful systems for protein partition, only a few polymer mixtures have been studied as ATPP systems. In particular, almost all reported partition systems use polyethylene glycol (PEG) and dextran as the phase-forming materials. Aside from some practical advantages, it is rarely made clear why PEG and dextran were chosen in any particular study.

The choice of polymer systems involves a complex evaluation of the properties of these polymers and the implications of their use for other aspects of the partition process. Here we will examine the factors which must be considered when one chooses the polymers for an ATPP system.

Phase-Forming Characteristics

At the core of the ATPP system are the phase-forming characteristics of the polymers involved. Generally one considers a two-phase system generated by two soluble polymers and a single solvent. Multiphase systems have also been examined (*14*) as have single polymer systems (*6*, *14*), but only the two polymer–one solvent systems will be discussed here.

Flory-Huggins theory is sufficient to describe the thermodynamics which lead to phase separation. Phase separation is caused by the fact that polymer solutions have small entropy of mixing effects so that positive enthalpic effects involving the interaction of the segments of the polymer chains lead to phase separation. Put simply, polymers prefer to self-associate rather than mix with other polymer molecules, so two phases of different polymer composition are formed in mixtures.

Detailed descriptions of the considerations which go into the Flory-Huggins theory can be found in the original works or in reviews of the theory (*21*, *22*). No substantial improvements on this basic theory have yet been made.

Experimentally, it is possible to describe two-phase systems with a ternary diagram provided that experimental techniques are available to quantify the content of each type of polymer in a mixture (see Fig. 1). Two main regions are evident in such a diagram. The single-phase region describes the locus of concentrations of polymer and solvent which lead to only one phase. Systems with high overall water content (near the apex of the diagram) and systems in which the concentration of one of the polymers is small do not phase separate. Systems of other compositions, which fall within the phase envelope (hatched region), separate into two phases. The compositions of the two phases which are in equilibrium lie on opposite ends of a "tie line."

All features of such ternaries are exactly the same as those of the common aqueous-organic ternary with a single solvent, but generally the ternary behavior is not of direct interest in ATPP. Instead, one is interested in how a third polymer (fourth component) distributes between the phases formed by the polymers. This fourth component adds another

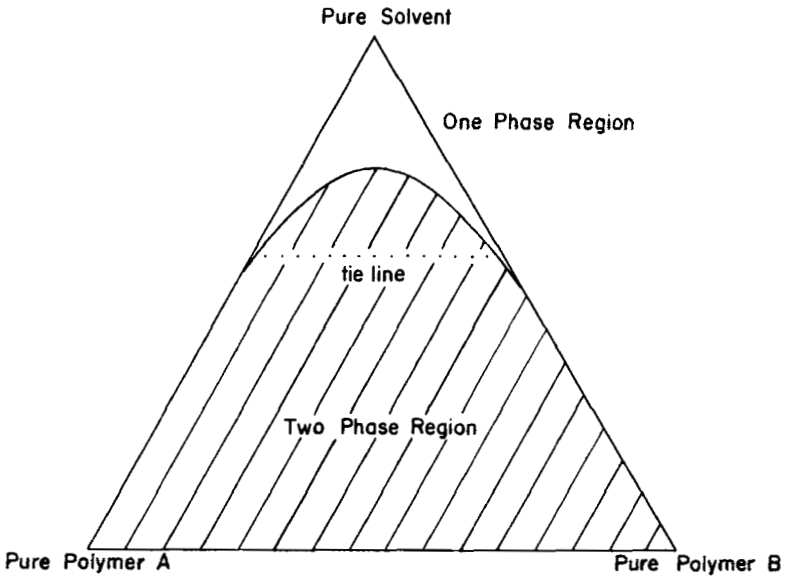


FIG. 1. The general ternary diagram for a two-polymer, one-solvent system.

dimension to the system and an increased complexity. The complexity caused by the partition of this component will be the major topic of discussion below and throughout this review.

ATPP ternary diagrams are available or can be generated for many different polymer systems (*20*). In these systems the difference in the composition of the phases formed at equilibrium is small at low concentrations of polymer. At the Plait point two phases with the same composition just begin to form. As the polymer concentration is increased, the amount of cross contamination of each of the phases by the other polymer decreases.

There are several useful ways to quantitatively describe the behavior of the phase system. One way is through the use of the polymer partition, which is defined as the ratio of the weight fraction of polymer in one phase relative to the other. The ternary diagram allows one to calculate a polymer partition coefficient for each set of equilibrium phases. Higher polymer concentrations give higher individual polymer partition coefficients.

The general shape of the ternary diagram is indicative of the interactions of the polymers with the solvent and each other as well as of their relative molecular size. Flory-Huggins theory predicts that symmetrical systems with horizontal tie lines will result when two polymers of the same molecular weight and solvent–polymer interaction parameters are mixed. Such is the case when matched polymers of hydroxypropyldextran (HPD) and dextran are used to form a phase system (Fig. 2). Polyvinyl alcohol–dextran systems also show symmetrical behavior (*20*).

Asymmetrical systems are more common. The PEG–dextran system gives a highly asymmetrical envelope with "tipped" tie lines (see Fig. 3). This behavior is due to the difference in molecular structure of the two polymers and the large discrepancy in the molecular weight.

In both symmetrical and asymmetrical systems the difference in the properties of the phases is the cause of partition. This difference can be related to the length of the tie lines as defined by the square root of the sum of the squares of the difference in the weight percent of each of the polymer components in the two phases (*23*). Longer tie lines indicate that the phases are more dissimilar.

The tie line length can be correlated to several important effects involving partition behavior. Flory-Huggins theory predicts that the partition coefficient will increase exponentially as the absolute difference in the composition of the two phases increases (*24*). Similarly, other effects which promote partition, such as the difference in phase potential and the hydrophobicity of the phases, are greater in systems with longer

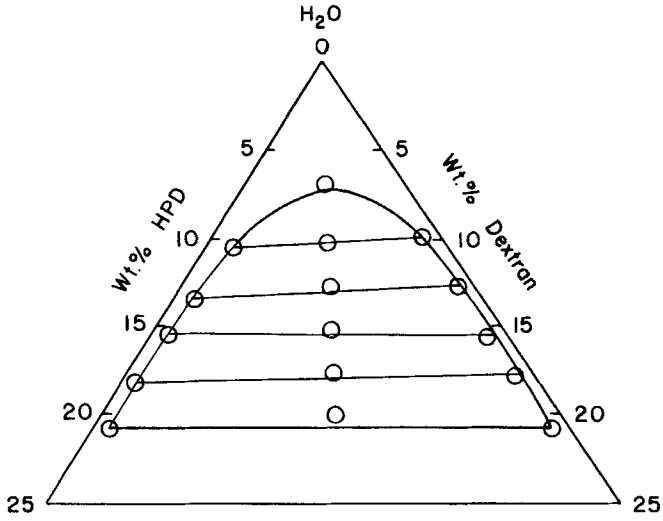


FIG. 2. Ternary diagram for hydroxypropyldextran-dextran-water (MW_{dex} = 150,000) (*34*).

tie lines (*23*). Increased tie line lengths promote protein partition (*25*). This effect is illustrated in Fig. 4.

The tie line length can also be correlated to the viscosity of the phases as shown in Table 1 (*23*) and Fig. 5 (*26*). Tie line length also correlates to the difference in the density of the two phases (Table 2) (*27*). Both of these features have an impact on the design of processes in ATPP systems (*6*, *28*).

Polymer Structure Effects

Aside from the effect that polymer structure has on the phase envelope, there are also more direct implications of the structure for the partition coefficient of a protein. These need to be taken into account in polymer selection.

One choice which must be made is the molecular weight of the polymers. PEG of molecular weight 4000-6000 and dextran of weight-average molecular weight 500,000 are the most commonly used polymers in partition studies, but these polymers are available in many different molecular weights (*14*). This is significant because different molecular weights give different results. When the molecular weights of the two

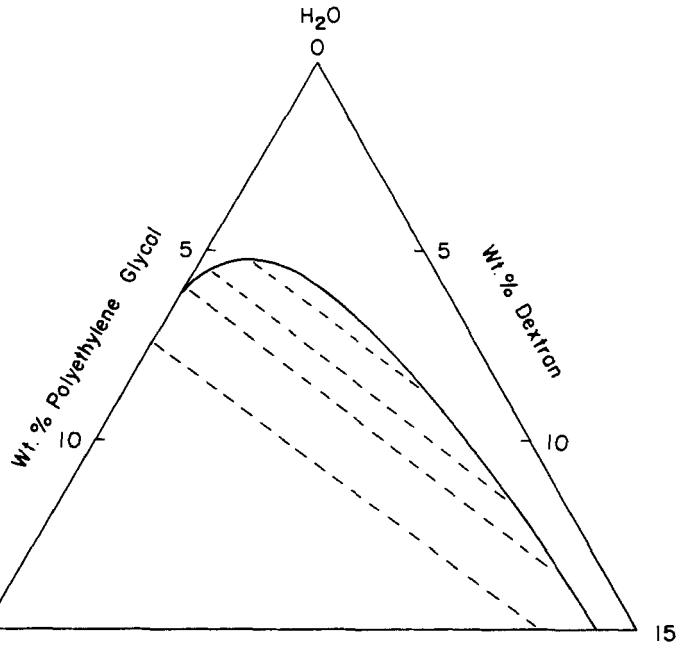


FIG. 3. Polyethylene glycol (PEG-6000)-dextran (500)-water ternary. From Albertsson (*42*).

phase-forming polymers are dissimilar, there is a driving force for proteins to partition toward the phase with the smaller molecular weight. The larger the difference in the molecular weight, the stronger the effect (*24*).

A second consideration is the type of polymer based on the relative hydrophobicity (*14, 29, 30*). Since hydrophobic effects have an impact on protein partition through interactions between the polymer and protein (see the section on Protein Structure Effects below), more strongly hydrophobic polymers should be expected to enhance partition due to this effect. Figure 6 shows the relative hydrophobicity of several common ATPP-generating polymers (*14*).

A third consideration is whether or not the polymer should be charged and what the sign of the charge should be. Several charged polymers which have been used in ATPP applications are listed in Table 3. A discussion of the effect of polymer charge is deferred to the section titled Generation of Phase Potential Differences.

The specific structure of the polymer is also important in that it

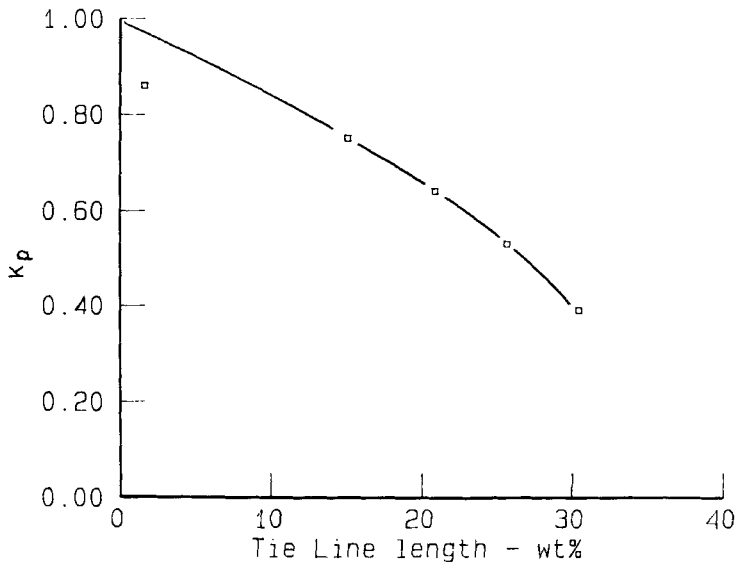


FIG. 4. Relationship of tie-line length to protein partition. HPD-dextran (MW = 450,000)-porcine pepsin system. Chen and Carlson, unpublished data.

impacts on the ability to make derivatives. Derivatization can be used to change the character of the polymer or to attach ligands. Many derivatives of PEG and dextran have been synthesized.

The number of reactive groups on the molecule determines the extent to which it can be derivatized. PEG, for example, has two reactive hydroxyl groups which can be derivatized with simple chemistries, but has no reactive sites along the chain backbone. Because of this feature the

TABLE 1
Relationship between Tie-Line Length and Phase Viscosity[a]

Composition		Tie-line length (wt%)	Viscosity relative to water	
Dextran	PEG-6000		Upper	Lower
5.0	3.5	9	4.9	15.7
5.2	3.8	11	3.7	27.9
6.2	4.4	18	4.0	50.6
7.0	5.0	20	4.4	95.7

[a]From data given by Albertsson (*14*).

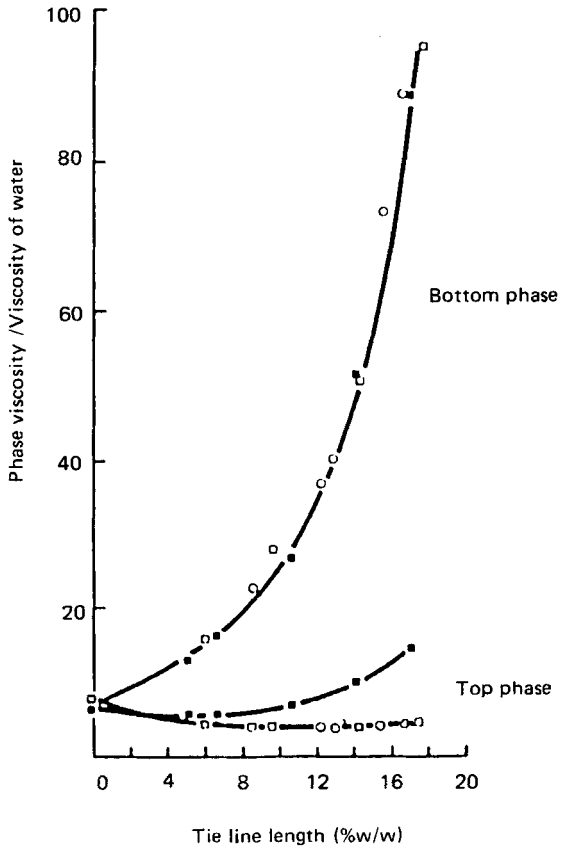


FIG. 5. Influence of tie-line length on relative phase viscosity. PEG–dextran. Original data from Sharp (*26*).

TABLE 2
Relationship between Tie-Line Length and Phase Density[a]

Composition		Tie-line length (wt%)	Density at 20°C	
Dextran	PEG-6000		Upper	Lower
8	6	23	1.0127	1.0779
7	4.4	18	1.0116	1.0594
5	4	13	1.0114	1.0416
5	3.5	10	1.0114	1.0326

[a]Data from Albertsson (*14*).

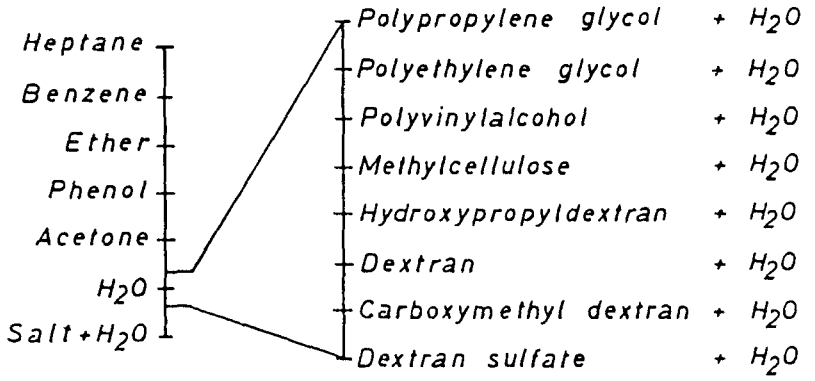


FIG. 6. Albertsson's scale of relative hydrophobicity for common polymers used in aqueous two-phase systems. From Albertsson (*14*).

molecule can be derivatized only to a limited extent, either one or two groups per 4000–6000 daltons.

Dextran has several coupling sites per monomer unit and can be heavily substituted if desired (*30*). For lighter loadings, coupling chemistries involving only the reducing end of the molecule are available (*31–33*).

The degree of substitution and substitution per unit weight of polymer also influence the partition behavior of the polymer derivative between the two phases (*20*).

Density and Viscosity

The density and viscosity of the polymer solutions used in ATP systems are of major importance in the design of processes for separation since

TABLE 3
Charged Polymers Which Have Been Used in Partition Studies

Polymer	Reference
QMA-PEG 6000	*6*
NH_2-PEG 6000	*6*
Cibacron Blue-PEG 6000	*6*
Phospho-PEG 6000	*6*
DEAE-dextran	Chen and Carlson, unpublished
Diaminohexane-dextran (CNBr linked)	*34*

they impact on the rate of phase separation and of minor importance because they impact on the energy required to mix the phases.

PEG solutions have densities which range from about 1.0 g/mL at 2 wt% polymer to 1.04 g/mL at 25 wt%. Dextran solution densities range from 1.0 g/mL at 1% polymer to 1.08 g/mL at 20% polymer (*14*). Other polymer solutions have densities somewhere between these values. The densities of dextran and HPD solutions are listed in Table 4 (*34*).

The individual densities determine the difference in the phase densities in ATP systems. Typical density differences can range from 0.02 to 0.06 g/mL in PEG/dextran systems (*14*) and as small as only 0.016 g/mL in 20% HPD/dextran systems (*34*). The difference in phase densities is important because the rate of separation of the two phases is directly related to this value, as mentioned above (*5*).

The viscosity of the continuous phase in an emulsified mixture also impacts on the phase separation rate. The rate is inversely proportional to this viscosity. PEG solutions have viscosities of around 4–5 cP, but dextran solutions can have viscosities of 50–200 times that of water depending on the molecular weight and protein concentration. In PEG/dextran mixtures the upper phase viscosity does not change significantly with overall polymer concentration, but the lower phase can increase significantly under the same conditions (*23*).

The combination of high molecular weight to promote equilibrium phase separation, and lower molecular weight to promote low viscosity and high separation rate, results in an interesting trade off. While increases in molecular weight increase the viscosity of polymer solutions and slow separation rate, lower concentrations of high molecular weight

TABLE 4
Relationship between Phase Density and Polymer Concentration in a Hydroxypropyldextran–Dextran System[a]

Polymer concentration (wt%)		Phase density (20°C) (g/mL)		
HPD	Dextran	Upper	Lower	Difference
5.12	5.18	1.034	1.029	−0.005[b]
6.32	6.52	1.040	1.045	0.005
7.55	7.69	1.045	1.066	0.021
8.96	8.75	1.044	1.078	0.034
9.91	10.15	1.049	1.074	0.025

[a]From Firary (*34*).

[b]Although obviously incorrect, included to show measurement error.

polymers may be used to achieve the phase separation. Hence, higher molecular weight polymers will often give systems with improved behavior from a processing viewpoint (*14*).

Cost, Recovery, and End Use

In the final analysis the choice of polymers for the ATPP system depends on cost, recovery prospects, and end use criteria. Inexpensive polymers are favored for economic reasons, but polymers which can be recovered at high yield need not be as inexpensive as those which cannot. The economics of ATPP have been analyzed for a particular system and the analysis shows that systems which minimize the use of dextran relative to PEG are more economical (*4*). This is primarily because PEG costs only about 1 to 2% of the cost of dextran. Since PEG is such an extremely inexpensive polymer, it is widely used.

The ability to recover the polymers will greatly impact process economics and can, if other things are favorable, make other polymers economical to use. This will be treated in more detail below in the section entitled Separation of Protein and Polymers.

A final consideration for some products is the biocompatibility of the polymers. Of the common polymers, only PEG and dextran are fully approved for injectables (*6*, *24*). For products which will ultimately be ingested or injected, this may be an overriding factor in polymer choice.

Summary

The first choice one must make when considering an ATPP system is the polymer system. The choice depends on a number of factors including the phase-forming characteristics of the polymers, the physical properties of the polymers, the interaction of the polymers with product molecules, the cost of the polymers, and the end use of the product. The best choice will ultimately depend on all these factors.

GENERATION OF PHASE POTENTIAL DIFFERENCES

The difference in phase potential is one of the most widely studied effecters of protein partition in aqueous two-phase systems. Since proteins are polyionic, strong electrostatic forces act on them when they

are exposed to electrical potential gradients or differences in electrical potential between phases. This is not only the principle behind electrophoresis, but also the principle behind ion-exchange chromatography and aqueous partition.

The basic equation for partition due to phase potential effects has been given as (*14*, *25*)

$$\ln K_p = \ln K_0 + z_p F/RT(\Delta\varphi) \quad (1)$$

where K_p (the partition coefficient) is the ratio of the concentration of the protein in the top and bottom phase, K_0 is the partition coefficient when there is no charge on the molecule or when there is no potential difference, $\Delta\varphi$ is the difference in electrochemical potential between the phases, z_p is the net molecular charge of the partitioning species, and F, R, and T are the Faraday constant, the gas constant, and the absolute temperature, respectively.

Because of the exponential relationship between the net charge on the molecule and the partition coefficient, species with a large number of charges partition strongly even when the potential differences between the phases are small. Since most salts and buffers generate at least a small interfacial potential, ionic partitioning effects are seen in most ATPP systems.

To predict the partition of proteins a priori, some information on K_0 must be available, the charge of the molecule must be known, and the magnitude of the phase potential difference must be known. The first two will be dealt with in the next section on protein structure effects; the estimation of the phase potential differences will be dealt with in this section.

Measurement of Potential Difference

The difference between the electrical potential of two phases is defined by the work required to move an ideal test charge from a position far from the interface in one of the phases to a position far from the interface in the other. Such an ideal test charge would have no chemical interactions with the components of the two phases, and the measured work would be a direct result of electrical effects.

In reality, the test charge is not ideal and may have significant chemical interaction with each of the phases. However, it is still possible to measure the electrical work required to move a test charge from one phase to another by using suitable electrode systems. Silver-silver

chloride and calomel electrodes have been most commonly used (*23*, *35*). A typical system involves the use of a salt bridge between an electrode and the solution being measured. In that case the chemical activity of the chloride ion at each electrode is similar and any difference in measured potential will be more closely related to the true difference in electrochemical potential. Using such a system, so-called junction potentials are largely eliminated and more accurate results are achieved (*23*, *35*). Even when proper precautions are taken, however, erroneous values may be obtained due to instabilities in the electrode system (*23*).

Measurements by Johansson (*35*) indicate that the interfacial potential ranges from 0 to −5.6 mV for salt-containing PEG/dextran systems. Similar values have been obtained in other studies (*36*) but significantly different values have been determined in still others (*37*). The discrepancy is probably due to the way the probes were calibrated or to differences in their construction. More study is required to determine the cause of these discrepancies.

Another method of measuring the potential difference between phases is to assume that Eq. (1) above is exact, and then to measure the influence of protein charge on the partition coefficient (*35*, *38*, *39*). This equation predicts that if K_0 is constant, then ln K_p will be linearly related to the charge on the protein molecule. The slope of a ln K_p vs z_p plot will give a value of the potential difference which is in close agreement with those measured by probes in some cases but not in others, probably because of counterion binding to the protein surface (*35*). Experiments with more than one salt can give the isoelectric point of the protein (*25*).

Potential Difference Generated by Salts

A difference in electrical potential between phases is indicative of a maldistribution of ions between the phases. This is caused by unequal interactions between the mobile ions and the polymers comprising the phases (*34*). The tendency of an ion to bind to one polymer more than another can be represented by an activity coefficient ratio for the ion in one phase versus another.

The interfacial potential can be related to the activity coefficients of the salt ions within the individual phases by a simple model. If one considers the interaction of the distributing ions with the phase polymers as a modification of their activity, then it can be shown that the number density of a cation at any position in the top phase (n_+^T) is related to the number density of the cation at the interface in the bottom phase (n_{+0}^B) according to the equation

$$n_+^T = n_{+0}^B \gamma_+^{BT} \exp\{-ze(\varphi^T - \varphi_0)/kT\} \quad (2)$$

Here γ_+^{BT} is the ratio of the activity coefficient of the cation in the bottom phase to the activity coefficient in the top phase, φ^T and φ_0 are the phase potentials at a point in the top phase and at the interface, respectively, and e is the electron charge. The number density of cations in the bottom phase at the interface is related to the number density in the bottom phase far from the interface ($n_{+\infty}^B$) according to

$$n_{+0}^B = n_{+\infty}^B \exp\{-ze(\varphi_0 - \varphi_\infty^B)/kT\} \quad (3)$$

Putting the two equations together gives a relationship between the cation number density far from the interface and that at an arbitrary position in the top phase:

$$n_+^T = n_{+\infty}^B \gamma_+^{BT} \exp\{-ze(\varphi^T - \varphi_\infty^B)/kT\} \quad (4)$$

Exactly analogous equations can be written for the anion.

The total charge density at a point in the top phase is given by the sum of the local number density of charge due to cations and anions:

$$\rho = z_+ e n_{+\infty}^B \gamma_+^{BT} \exp\{-ze(\varphi^T - \varphi_\infty^B)/kT\} + z_- e n_{-\infty}^B \gamma_-^{BT} \exp\{-ze(\varphi^T - \varphi_\infty^B)/kT\} \quad (5)$$

This expression can be used in Poisson's equation,

$$\nabla^2\varphi = 4\pi\rho/D \quad (6)$$

where D is the dielectric strength of the medium. This gives an equation which describes the potential profile in the top phase.

For equi-valent ions ($z_+ = z_-$) and small potentials, and assuming a planar geometry, an appropriate solution to this equation is

$$\varphi = \varphi_\infty^B + (\varphi_0 - \varphi_\infty^B) \exp\{-\kappa x\} + G(1 - \exp\{-\kappa x\})kT/ze \quad (7)$$

where

$$G = (\gamma_+^{BT} - \gamma_-^{BT})/(\gamma_+^{BT} + \gamma_-^{BT}) \quad (8)$$

and

$$\kappa^2 = 4\pi z^2 n_\infty (\gamma_-^{BT} + \gamma_+^{BT})/DkT \quad (9)$$

Equation (7) along with a similar result for the potential profile in the bottom phase can be used to predict the shape of the potential profile in two-phase systems (Fig. 7).

The difference in the potential far from the interface in the top phase ($x \to \infty$) and the potential far from the interface in the bottom phase can be found from

$$\varphi_\infty^T - \varphi_\infty^B = kT(\gamma_+^{BT} - \gamma_-^{BT})/ze(\gamma_+^{BT} + \gamma_-^{BT}) \tag{10}$$

A potential will be generated as long as the anion and cation have different activity coefficient ratios. Equation (10) is similar to

$$\varphi_\infty^T - \varphi_\infty^B = (kT/(z_+ + z_-)e) \ln K_+/K_- \tag{11}$$

used by other authors (*14*, *24*, *39*). Both represent the difference in the bulk potentials between the phases in an equivalent manner.

Equation (7) predicts that there will be a monotonic change in the potential across the interface, but zeta potential measurements by Brooks et al. (*37*) indicate that this may not be the case. They found that droplets

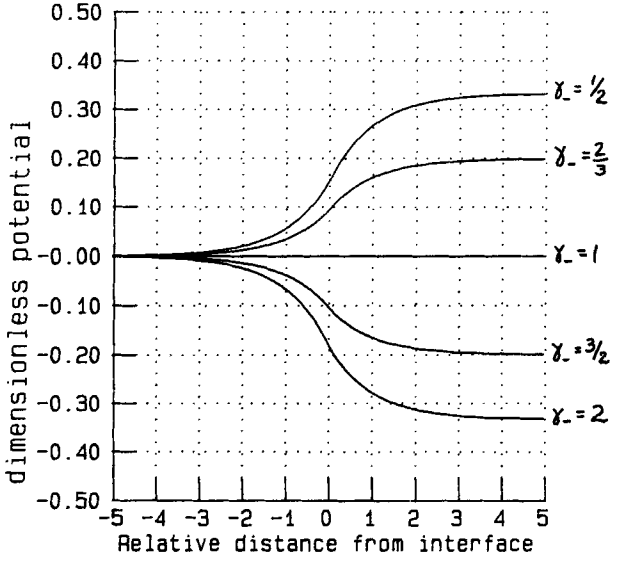


FIG. 7. Calculated potential profile across the interface of a two-phase system. $\varphi^* = \varphi ze/kT$; $\kappa = (8\pi z^2 e^2 n_\infty^2/DkT)^{1/2}$; $\gamma_+ = 1.0$. Variables as given in text.

had a mobility corresponding to a potential of sign *opposite* to that expected by their own phase potential measurements. They suggest that this was due to a change in sign at the interface due to a dipole layer.

The virtual partition coefficients (K_+, K_-) have been determined by Johansson (*35*) in PEG/dextran systems for several common cations and anions. His results are shown in Fig. 8. Quaternary amines prefer the PEG phase because of their hydrophobic nature. Other ions partition because of specific interactions with the polymers (*35*). Combinations of cations and anions which have different virtual partitions should show significant phase potential generation; those with similar virtual partitions should not. Hence Na_2SO_4, K_2SO_4, and LiF are not expected to generate a potential (*35*) and are used in systems when low or zero potential is required. However, Brooks et al. (*37*) found that K_2SO_4 did generate a phase potential according to both zeta potential and direct phase potential measurements. Further work is required to explain the differences between Johansson's and Brook's findings.

Potential Generation by Charged Polymers

Both anionic and cationic PEG have been used to partition proteins (*25*). Charged polymers can generate even larger potentials than the 3–5

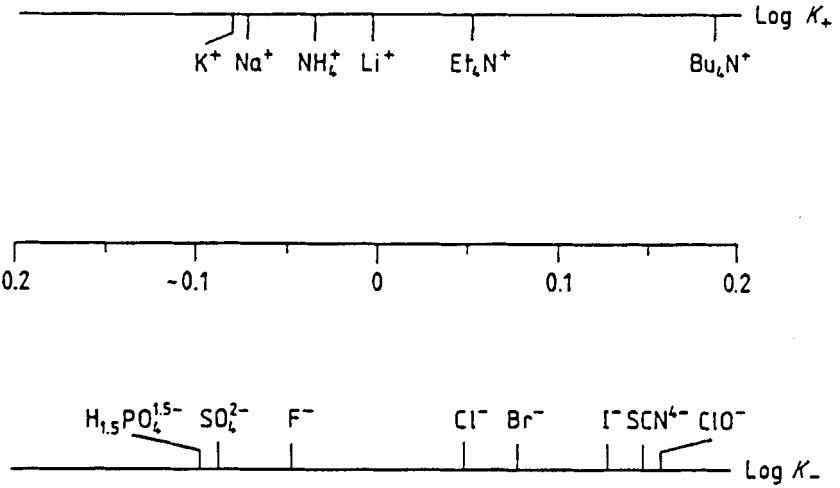


FIG. 8. "Virtual" partition coefficient scale for several ions. (Note: K_+ and K_- are the equivalent of γ_+ and γ_-, respectively, as used in the text.) From Johansson (*25*).

mV seen with small ions. When fixed ions are covalently attached to one of the polymers comprising a phase system, they are forced to partition selectively into one of the phases by Flory-Huggins effects. For singly charged polymers, Eq. (11) can be used to predict that this can result in a interfacial potential of 20 mV or more. Such a large potential causes large partition effects, and partition coefficients of 20 are not uncommon in these systems (*33*, *40*) (see Fig. 9).

The large potential differences developed between the phases are moderated significantly by salt in the system. Even 100 m*M* salts can nullify polymer-generated potentials (*25*, *33*).

Finally, it should be noted that excessive derivatization of the polymer with ionic groups reduces the potential generated. Equation (11) indicates that large valences on a partitioning species lead to smaller phase potentials. In the extreme case, phase separation is prevented by excessive polymer derivatization. Commercial DEAE and sulfated dextrans will not phase separate with PEG or hydroxypropyldextran because they contain too many ionic groups (~1 DEAE group per 3 mononer residues). Only significant addition of salt will allow phase separation (*42*).

Summary

A difference in the potential between the phases of an ATP system can be generated by adding salts or by using polymers which contain charged moieties. Values of the potential range from 0 to ±20 mV depending on the system and can be measured directly by using electrodes or indirectly by partitioning species of known charge. Because of the polyionic nature of proteins and the fact that they can have large net charges, they will be strongly partitioned in systems with any phase potential difference.

PROTEIN STRUCTURE EFFECTS

The preceding discussions have pointed out that the difference in the electrical potential of the two phases and the relative hydrophobicity of the phases (determined by the polymer content) play important roles in the partition of proteins between the phases. However, such analyses do not take into account the specific surface features of the molecules. For example, the net charge of the protein may not fully explain partition behavior since the type of amino acids contributing to, and the location

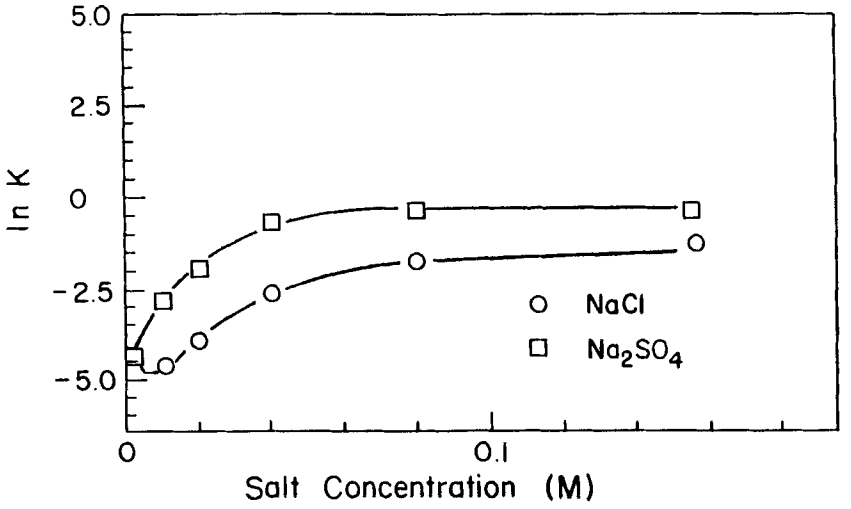


FIG. 9. Partition of pepsin in HPD–dextran system as a function of salt type and salt concentration. 1 m*M* sodium phosphate buffer, pH = 6.0. Chen and Carlson, unpublished.

of the amino acids within the molecule may be important. Likewise, the location of hydrophobic groups on the protein surface may also contribute to the apparent hydrophobicity. The specific volume of the molecule may also be important in partition.

Proteins are generally tightly folded molecules which sample only a few of many possible conformational states. Free energy calculations argue that the three-dimensional structure of proteins is relatively fixed, and other evidence indicates that the x-ray structures are similar to structures adopted by proteins in solution. For these reasons a static model of protein structure is generally suitable, and one can think of a protein as a "particle" with a fixed shape including a solvent accessible exterior and an inaccessible interior.

This static molecule model can be used to show which amino acids are exposed on a molecule's surface, where charged groups are located, and the exact structure and molecular volume of proteins. Analyses indicate that the molecular surface is primarily comprised of hydrophilic amino acid side chains and hydrophilic portions of the main carbon chain. Most charged groups are located on the surface or, if buried in the interior, participate in ionic bridges within the molecule. However, there are a significant number of hydrophobic side chains exposed on the

molecular surface, and these undoubtedly give the surface a hydrophobic character.

A discussion of the possible significance of specific protein structure is given in this section.

Hydrophobic Character

Although no experiments have been conducted which directly relate partition behavior to protein surface properties, it is clear that surface hydrophobicity has an influence on protein partition. Surface hydrophobicity has been implicated in chromatographic behavior and in solubility of proteins (*43–48*). Retention of proteins on reverse phase chromatography resins is correlated to the strength of the hydrophobic bonds which can form between the protein surface and the resin phase. Similarly, hydrophobic protein–protein interactions appear to be enhanced in some solutions, causing precipitation of the molecules when the salt concentration in a solution is increased (*49*).

Zaslavsky et al. (*43–46*) correlated partition behavior of small peptides to the number of methylene groups in the molecules. Using these data they were able to rank proteins according to their hydrophobicity by assigning each an equivalent number of surface methylene groups. It will be interesting to see if these rankings bear any correlation to actual surface structure. If there is a direct correlation, it may mean that it is possible to modify proteins in a way so as to improve their separation behavior.

Ionic Group Location, Hydration Properties, and Dielectric Effects

Besides the hydrophobic character of protein surfaces, the other major determinant of partition behavior is the location and state of the ionic groups of the molecule. Ionic surface groups contribute to partition in a general way by determining the overall (net) charge of the molecule. They also may contribute in a specific way to partition by interacting directly with the ionic species in the phase system or by changing the nature of the potential field around the molecule (*50*). The solubility (i.e., the activity coefficient of the protein) is a complex function of these ionic effects and depends on both the first moment (net charge) and higher moments of the ionic nature of the molecule (*50*).

Closely related to these ionic effects are the "hydration" characteristics

of the phases, which have been suggested as determinates in partition behavior (*51*). Hydration affects the ability of a given phase to accept charged species and dissipate the charge. The effect is similar to the effect a change in the dielectric strength of the media has on the solubility of proteins (*50*). Zaslavsky et al. (*51*) have used hydration properties to correlate partition coefficients, but their conclusions have been criticized because they also down played the ionic effects and stated that potential differences between phases have little to do with partition of ionic species (*52*).

Summary

Effects other than the potential difference and hydrophobicity of the phases have an influence on the partition of proteins. Although not well studied, it can be anticipated that specific protein structural effects, and especially the ionic character and hydrophobic nature of the protein surface, can have significant impact on the partition behavior.

AFFINITY PARTITION

One promising ATPP technique which has yet to be extensively studied is affinity partition. Affinity ATPP offers the possibility of high selectivity, as is seen in affinity chromatography, with the convenience and scalability of liquid-liquid systems. Most of the other molecular mechanisms of ATPP are nonselective because they affect partition based on the general surface characteristics of a molecule. Affinity ATPP, like other affinity methods, selects on the basis of the specific structure of the binding pocket of the molecule. This means that systems can be synthesized which will partition one molecule in a protein mixture substantially toward one phase while leaving other molecules evenly partitioned between the phases. Counter- or crosscurrent extraction schemes will then allow the protein to be highly purified within a few stages.

This section first reviews the studies of affinity ATPP which have been reported in the literature and then presents the theory and principles behind the process.

Previous Studies of Affinity ATPP

Only a relatively few studies of affinity ATPP have been made (Table 5). These have been rather specific and have been intended to show only

TABLE 5
Some Affinity Partition Systems

Protein	Maximum partition (K_p)	Reference
S-23 myeloma	7.0	*54*
Oxosteroid isomerase	61	*59*
Trypsin	5.7	*53*
Concanavalin	—	*54*
Acetylcholine receptor	2.0	*57*
Acid proteases	10	Chen and Carlson, unpublished

that a certain protein partitions differently in the presence of bound ligand than it does in the native system (i.e., polymers without attached ligand). Very little model verification has been attempted.

Takerkart et al. (*53*) showed that trypsin partitioned selectively toward a phase containing *p*-aminobenzamidine attached to PEG. This particular ligand is a competitive inhibitor of trypsin. The partition coefficient for trypsin increased from 0.33 when no ligand was present (PEG/dextran) to 5.7 when PEG-bound ligand was in the system while little change in the partition of chymotrypsin was observed under the same conditions ($K_p = 0.13 \rightarrow K_p = 0.19$). Evidently the partition of trypsin was due to specific effects.

Flanagan and Barondes (*54*) partitioned concanavalin A selectively toward the dextran phase of a PEG/dextran two-phase system. The dextran acted as a ligand for concanavalin A. The partition of this protein was shown to favor the lower (dextran) phase (as expected) whereas other proteins in the same system partitioned more evenly between the phases.

Flanagan et al. (*55*, *56*) and Johansson et al. (*57*) were able to partition acetylcholine receptor proteins in a trimethylammonium-phenylamino-PEG/dextran two-phase system (*58*). Increases in the amount of ligand-containing polymer in the upper phase raised the partition coefficient from 0.01 with no ligand to 2.0 when ligand was in the system. Addition of a counterligand (methonium ion) caused the partition coefficient to drop, indicating that the protein was released from the ligand by this substance and confirming the partition effect.

Hubert et al. (*59*) and Chaabouni and Dellacherie (*60*) partitioned $\Delta_{5\rightarrow4}$3-oxosteroid isomerase toward a phase containing estradiol-PEG. The partition coefficient of $\Delta_{5\rightarrow4}$3-oxosteroid isomerase in the base system (without ligand) was 3–4 and increased to 15, 29, and 61 in

different experiments. The value of the partition coefficient was shown to increase with increasing ligand content.

Finally, Flanagan and Barondes (*54*) partitioned S-23 myeloma protein in a dinitrophenyl-PEG/dextran two-phase system. The DNP acted as the ligand by virtue of its binding to the protein. The partition coefficient increased from 2.8 in the base system to 7.0 in the DNP-PEG/dextran system. DNP-lysine was used to displace the protein from the PEG phase. As proof of the affinity effect, gamma-globulin, which does not bind DNP, was shown to partition equally well in both PEG/dextran and DNP-PEG/dextran systems.

Other systems which are described as affinity partition are more nonspecific than those described above. Bovine serum albumin and human serum albumin have been partitioned in systems of PEG/dextran in which the PEG has been esterified by various fatty acids (*25, 61-63*). It is apparent from these data that there is an effect of ligand binding on HSA and BSA for certain fatty acid groups which is not seen with other proteins (Table 6) but it is not clear how specific this effect is. There are known to be several binding sites on albumins, but since esterified PEG is more hydrophobic than unesterified PEG, it would be expected to increase partition due to nonspecific hydrophobic effects.

Even more difficult to evaluate is the effect of triazine dyes, and particularly Cibacron Blue, as ligands. These molecules have been shown to bind competitively to NAD binding enzymes (*6, 64-67*), but it is obvious from the shear number of proteins which are effected that the binding is somewhat nonspecific. In addition, the extremely large partition coefficients reported (*64*) and the sensitivity to salt concentration (*6*) indicate that there are substantial nonspecific effects, probably phase potential effects, generated by the charge on the dye. (See below.)

TABLE 6
Selected Values for Partition Coefficient of Proteins in PEG-Fatty Acid Systems[a]

PEG derivative	K_p-albumin	K_p-lysozyme (nonaffinity)
Unesterified	0.15	0.87
Acetate	0.13	0.81
Laurate	0.65	0.83
Linolate	5.62	1.04
Linolenate	8.32	1.12

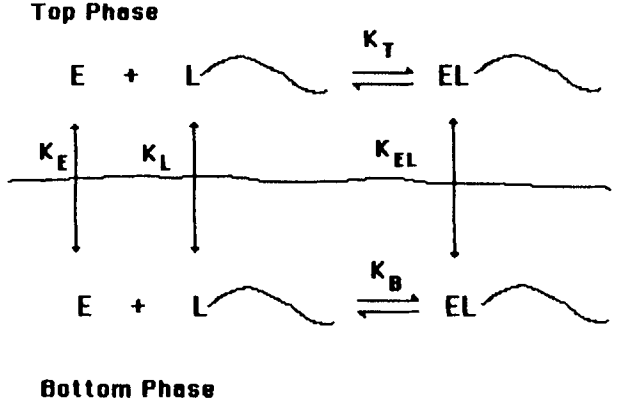
[a]From Johansson (*39*).

Principle and Theory

The principle behind affinity ATPP is similar to the principle behind affinity chromatography. A ligand is covalently attached to one of the polymers, and this macroligand then partitions selectively between the phases, presumably with the same partition as the underivatized polymer but possibly to a greater or lesser extent. When the protein is added to the system, a noncovalent protein-polymer complex is formed, and this will partition between the phases, favoring the phase favored by the macroligand. The equilibria involved are shown in Fig. 10.

There is one significant difference between affinity chromatography and affinity ATPP that needs to be noted. In the ATPP system, as opposed to chromatography, the ligand is not confined to one of the phases. Depending on the phase diagram, there will be more or less ligand in each of the phases, but partition of macroligand will rarely exceed 100 if it is part of the base polymer system. This limits the partition possible with the protein.

A slightly different situation is one where the macroligand is a third



E = Protein; L = macroligand; EL = protein-ligand complex. The K's are defined in the text.

FIG. 10. Equilibria involved in affinity partition.

polymer. This is the case in polymer mixtures of derivatized and underivatized PEG where the derivatized PEG is added in increasing quantities to an already formed system. Ligand-PEG has been shown to distribute with the same partition as PEG at low concentrations but up to 10-fold higher at higher concentrations (*63*, *64*).

The equilibria shown in Fig. 10 can be used to show that for a protein that binds ligand at a single site there is a specific relationship between the partition coefficient of the ligand-enzyme complex (K_{EL}), the partition coefficient of the protein in the base system (K_E), the partition coefficient of the macroligand (K_L), and the binding constants between the protein and ligand in the upper (K_T) and lower (K_B) phases (*14*):

$$K_{EL} = K_E \times K_L(K_B/K_T) \tag{12}$$

A similar expression may be derived for a protein that has multiple binding sites (*54*). This equation can be expressed as the difference in the partition coefficient of the macroligand-protein complex and the partition coefficient of the protein ($\Delta \ln K$) as related to the other constants in the equation

$$\Delta \ln K = \ln K_L + \ln (K_B/K_T) \tag{13}$$

Often $\Delta \ln K$ is related directly to the concentration of macroligand in the system (*63*, *64*). Experiments have shown that the partition coefficient can change by a factor of 1000 or more in some experiments, even when K_L is only 100. Various explanations have been put forth for this behavior but at least part of the explanation must be due to nonspecific partitioning due to phase potential changes as Cibacron Blue is added to the system, since there appear to be changes in the background partition accompanying the addition of the macroligand.

The partition coefficient can be derived from the ratio of the sum of the concentrations of free and bound protein in each phase, and is not related to the concentration of bound protein alone. Rarely is the assumption of a large excess of ligand a good one in practical systems.

One way to represent the equilibria involved is by modeling each phase as a Langmuir isotherm (*24*), but this can lead to thermodynamic inconsistency unless the proper relationships between the partition and binding constants are made. A thermodynamically consistent model with the same characteristics leads to the equation (*34*)

$$K_p = \{K_E K_B/L_0 + K_{EL}L_B/L_0\}/\{K_B/L_0 + L_B/L_0\} \tag{14}$$

where

$$L_B/L_0 = -\beta + (\beta^2 + 8(1 + K_E)(1 + K_L)(1 + K_{EL})K_B/L_0)^{1/2} \times 2(1 + K_L)(1 + K_{EL}) \quad (15)$$

and

$$\beta = [(1 + K_L)(1 + K_E)K_B/L_0 - 2(1 - E_0/L_0)(1 + K_{EL})] \quad (16)$$

for equal phase volumes. Here, L_0 and E_0 are the ligand and protein concentrations in the overall system. (Equation 15 is equivalent to Eq. 53 of Ref. *24*, but solves for K_p in terms of measurable quantities. The equation also emphasizes the relationship between the total and free ligand concentration.)

The partition coefficient can be seen to be a function of the system partition constants, the ratio of the lower phase binding constant and ligand concentration, and the ratio of the enzyme to ligand. A plot of this function is shown in Fig. 11. Experimental data on one particular system are shown in Fig. 12.

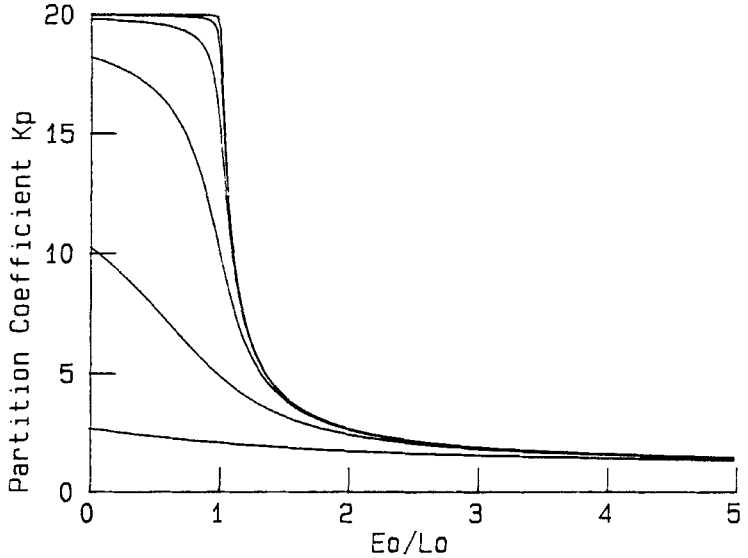


FIG. 11. Variation in partition coefficient as a function of protein-ligand ratio (E_0/L_0) for different K_B/L_0 ratios. K_E = 1.0; K_L = 20; K_{EL} = 20. (See text for details.) After Firary (*34*).

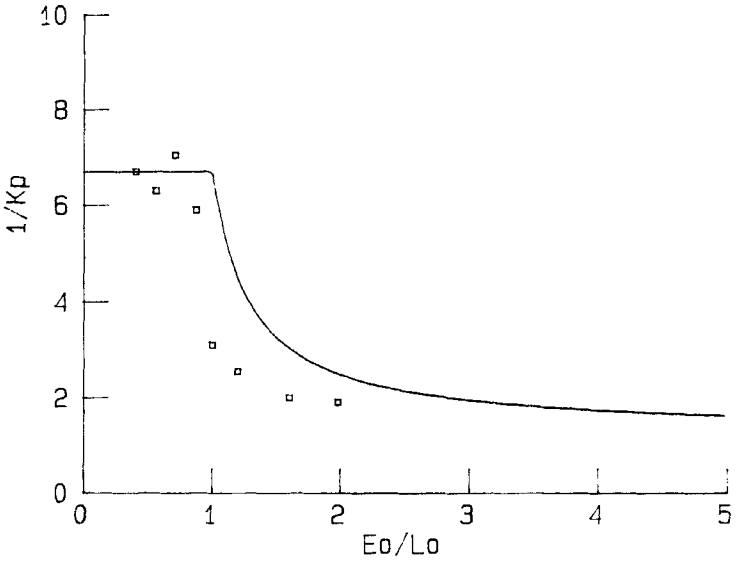


FIG. 12. Experimentally determined partition of pepsin in an HPD-dextran system, pepstatin as ligand. Curve for $K_B/L_0 = 1 \times 10^{-5}$, $K_{EL} = 6.7$, $K_L = 5.3$, and $K_E = 1.25$.

The implications of these plots are straightforward. Partition will be maximal at low E_0/L_0 ratios and will decrease dramatically at a ratio of 1.0 as the ligand becomes saturated. At low values of K_B/L_0 the partition will be smaller due to a significant fraction of the protein being free in solution. Sharper partitions will be seen with tight binding inhibitors or with high ligand concentrations, but the maximum partition depends on K_{EL} and not on the strength of the ligand binding.

Summary

Affinity partition is a highly selective ATPP system similar in some ways to affinity chromatography. The maximum partition achievable in affinity ATPP depends more on the partition coefficient of the protein-macroligand complex than on the strength of binding between the ligand and protein. On the other hand, low ligand densities and/or large binding constants (weak binding) lead to poor partition even in systems with high K_{EL} values. The complex behavior of these systems may explain the various results seen in the literature.

SEPARATION OF PROTEIN AND POLYMERS

The usefulness of ATPP in industrial applications will ultimately depend on the economics of the process compared to alternative ways of isolating a given molecule. ATPP must not only be a "good" method of isolating proteins, but must also be "better" than other methods. A variety of alternative methods is available for most applications. Aside from obvious advantages in terms of process fixed and operating costs, significant savings are possible if the process is versatile and easily scaled. All these must be factored in when one determines the economics of the process.

Hustedt et al. (*12*) have given an economic analysis of extractive enzyme recovery which points out many of the cost factors involved in ATPP processing. One of the key assumptions is that the polymers are not recoverable. This increases the cost estimates greatly. When PEG/dextran systems are used, the volume of the more costly dextran-rich phase must be minimized, again reflecting the bearing that polymer losses have on process economics. These authors feel that any recovery processes for PEG (or dextran?) are not feasible at this time.

Even at the laboratory scale, removal of polymers from the final protein product is one of the most difficult aspects of processing. The problem stems from the fact that these separations are at the molecular level and involve the separation of similar-sized molecules in highly concentrated solutions. In whole cell systems, a significant amount of cell debris accumulates and must be removed.

Nevertheless, several methods of protein–polymer separation have been used with some success at the small scale. This section reviews those procedures.

Extraction

PEG can be extracted from the aqueous phase by chloroform, leaving behind product proteins in the aqueous phase (*68*). After extraction, the PEG can be freed of chloroform by distillation and reused in a new phase system. The method may be particularly useful for affinity partition where the derivatized polymer has a particularly high value.

Transfer to Polymer-Free Phase

Many proteins can be driven to the PEG phase of a dextran-PEG system by high salt concentrations (*14*). The PEG phase can then be

extracted with a phosphate-containing solution since PEG and phosphate will form a two-phase system (*20*). A simple schematic of a dextran → PEG → salt solution transfer is shown in Fig. 13.

Precipitation

Proteins can be precipitated from dextran-rich phases by the addition of ammonium sulfate or other salts. PEG may be removed from the

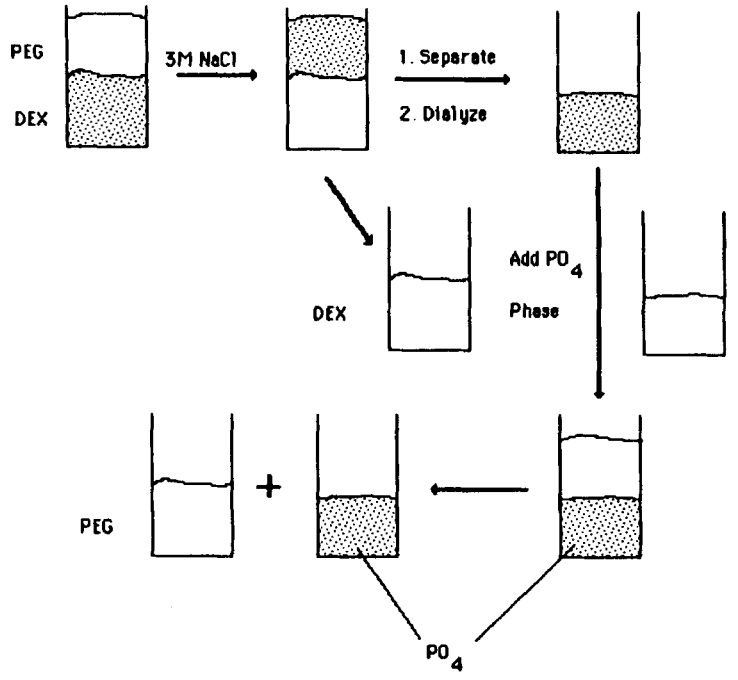


FIG. 13. Process to produce polymer-free protein. Drawn from the description of Albertsson (*14*).

phase using $(NH_4)_2SO_4$ by forcing the PEG to partition to the top phase. Physical separation of the phases followed by further addition of $(NH_4)_2SO_4$ will cause protein precipitation.

Ultrafiltration

Ingham and Busby (*69*) showed that ultrafiltration of protein PEG solutions allowed removal of PEG while retaining proteins. Whether or not this method will be useful for ATPP systems depends on how much the phase must be diluted before filtration and reconcentration costs (*5*). Theoretically, it should be possible to separate most proteins from high molecular weight dextrans by UF techniques, but this has not been demonstrated to the author's knowledge. Dilution and reconcentration steps will likely have an impact on the economics of such a process.

Electrophoresis

Since ATPP polymers are often uncharged, proteins can be separated from them by electrophoresis. An apparatus for doing this in a sucrose gradient (*70*) has been described by Albertsson (*14*). Another more complex device for accomplishing electrophoretic removal of proteins from a polymer phase is described by Albertsson (*14*). Over 99.9% removal of protein can be accomplished from 20% dextran or PEG solutions under some conditions.

Chromatography

Proteins can be removed from ATPP polymer solutions by adsorption onto chromatography resins (*14*). Since dextran and PEG are neutral polymers, they will not bind to ion exchangers and can be washed free of bound protein. The protein can be eluted from the column after washing off the polymers. Similar approaches may be used with GPC and affinity columns where appropriate. The use of chromatography as a finishing step may be justified when starting solutions have solids suspended in them or when large-scale processes are desired, and significant purification must be achieved before chromatography.

Summary

Separation of the phase-forming polymers and protein molecules after extraction is an important step in both laboratory- and industrial-scale processes. Several methods can be used to remove polymer from protein or protein from polymer solution. Such removal and the ability to recycle the polymers will make large-scale ATPP more economically attractive.

CONCLUDING REMARKS

Aqueous two-phase partition is a promising technique for the purification of proteins on a large scale. Design of an ATPP system requires consideration of the properties of the phase-forming polymers, the principles involved in partition, and the economics of the process. Many different polymers will form two-phase systems. The phase envelope and particular properties of the polymers involved are the key aspects to partition.

The main principles of partition involve the generation of phase potential, the interaction of the protein with the phase-forming system, and the behavior of affinity systems. All must be considered in process choice and design. The convenience and economics of the process depend to a large extent on the ability to reuse the polymers comprising the system. Several methods have been used for polymer–protein separations but more research is needed to develop practical methods for carrying this out.

Increasingly, aqueous two-phase partition appears to be a viable large-scale technique for protein purification. Further study and developments should lead to increased use in industrial protein purification processes.

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