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Affinity Partitioning and Its Potential in Biotechnology

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Abstract

The affinity partitioning technique and its applications to biotechnological separations are reviewed. In spite of the great potential for large-scale continuous processing and the wide success in laboratory separations offered by the technique, relatively few reports of biotechnological applications have been made. This underutilization is attributed in part to the lack of design criteria for establishing and optimizing partitioning systems. A first step toward developing a thermodynamically based design model for affinity partitioning, based on the incorporation of a Gibbs energy model for phase equilibria in polymer solutions into a separate theory accounting for the affinity effect, is presented.

SCOPE AND SIGNIFICANCE

The recent development of gene manipulation techniques has dramatically increased man's potential to obtain useful products from living cells. Altering the chemical conversion mechanisms within microbial, plant, and animal cells to produce chemicals ranging from simple molecules to complex proteins offers promise for improvements in many areas including health care, nutrition, agricultural products, environmental protection, and natural resource utilization. Full realization of

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this potential will require the engineering of efficient and in some cases large-scale production and recovery processes. Necessary knowledge of the fundamental engineering principles underlying bioprocess scale-up is lacking, however, particularly for the development of efficient bioproduct recovery and purification processes.

Separating and purifying biological materials produced by cell culture and fermentation techniques present unique problems for the chemical engineer. The main difficulties can be summarized as follows.

(1) Biological processes are often best performed in dilute aqueous solutions, requiring product separation from large amounts of water.
(2) These dilute aqueous solutions generally contain complex mixtures of other materials, including buffer salts, nutrients, byproduct, and waste molecules as well as the producing cells themselves, from which the desired product must be isolated.
(3) Many important biological molecules are thermally and chemically unstable and are, therefore, sensitive to the harsh environments of some chemical engineering operations like distillation and solvent extraction.
(4) High purity is often required, especially for products intended for human or animal health applications.

Research aimed at developing new separation processes or modifying existing techniques to meet the challenges outlined above is fundamental to the success of biotechnology.

A particularly promising technique among the many methods currently under investigation to satisfy the above criteria is partitioning in aqueous two-phase systems. This liquid–liquid extraction method, utilizing two biocompatible aqueous phases, is expected to play a major role in future separation processes in biotechnology due to the following properties: biocompatibility, high volume capacity, rapid partition equilibrium, ease of scale-up, and the potential for enhanced production processes through extractive fermentations (*1*). Moreover, a modified partitioning technique incorporating affinity ligands to enhance separations provides the selectivity required for isolation of products from complex mixtures. This modified technique, called affinity partitioning (*2*), offers the high selectivity of affinity chromatography (*3*) with greater potential for large-scale applications (*4*, *5*).

Partitioning in aqueous two-phase systems is well established as a scientific tool for laboratory-scale separations and material characterization. The largely empirical studies establishing this technique have identified important parameters influencing partitioning, but a need remains for defining design criteria, particularly for the important case of affinity partitioning. A thermodynamically based model of affinity partitioning would better organize the existing information and provide a more clear understanding of the fundamental phenomena involved. Once established, this model would facilitate the optimal design of extractive phase systems and equipment and assist the design engineer in choosing between alternative contacting schemes. Such a model would allow iterative design calculations providing a rational design approach far superior to the currently employed trial-and-error approach. The need for an engineering design model is evidenced by the fact that, in spite of the great biotechnological potential of affinity partitioning, relatively few industrial applications of the technique have been made to date.

This paper provides a comprehensive review of the affinity partitioning technique and its biotechnological potential and proposes a starting point for the development of a thermodynamically based model for affinity partitioning. The proposed approach is to incorporate a thermodynamic model for phase separation and partitioning in polymer solutions into a theory for the separate affinity effect.

PARTITIONING IN AQUEOUS TWO-PHASE SYSTEMS

Since their introduction in 1958 (*6*), aqueous two-phase systems have been widely used in biochemical and medical research for a variety of analytical and separation purposes. Analytical uses include characterization of hydrophobicity (*7–12*), net charge (*13*), isoelectric points (*14*), binding interactions (*15*), and changes in surface properties (*16*) of biomolecules, cells, and membranes. A powerful analytical method called Partition Affinity Ligand Assay (*17*) has also been developed based on the difference in partition between bound and unbound molecules. A wide variety of materials including proteins, nucleic acids, viruses, cells, and organelles has been isolated and recovered on a laboratory scale by partitioning in aqueous two-phase systems. Recently, led by researchers in Europe, these biphasic systems have begun to be applied to large-scale bioseparations (*18–20*) and to a novel process termed extractive bioconversion based on the addition of the two-phase system directly to a bioreactor (*21–26*). This latter innovation has the potential not only for early initiation of the separation process but also for increased production via continuous removal of products, inhibitors, toxins, or proteolytic enzymes (*27*). Several books (*28–30*) have summarized the vast number of studies aimed at demonstrating and developing practical applications of aqueous two-phase partitioning techniques.

Aqueous two-phase systems are formed when appropriate small amounts of two chemically different water-soluble polymers, such as polyethylene glycol (PEG) and dextran, are added to water, causing the system to separate into two immiscible phases. Both phases are predominantly water and hence biocompatible. The phase behavior of these systems can be represented on a diagram like Fig. 1 showing polymer compositions in equilibrium phases. Biological materials are separated in these two-phase systems based on their unique partition characteristics; different materials distribute differently between the phases. The major factors influencing partition of materials have been determined to include: 1) surface properties of the partitioned material including size, charge, and hydrophobicity; 2) types and concentrations of polymers used; 3) types and concentrations of added small molecules including ions; 4) temperature; and 5) pH (*28*).

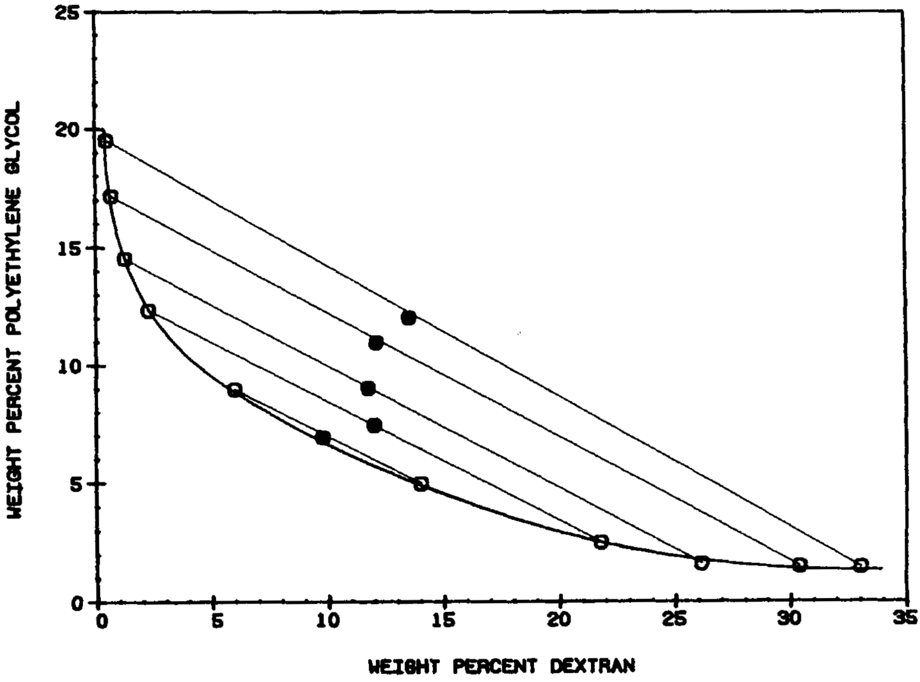

FIG. 1. Phase diagram for dextran (40,000) – PEG (3,350) at 25°C. Filled circles indicate two phase starting points. Open cirles indicate equilibrium phases.

Based on empirical knowledge of these governing factors, phase systems have been adjusted to provide more selective separations. Among the most effective and frequently employed phase system adjustments is the addition of salts. Low molecular weight substances, often required to obtain a buffered isotonic environment, generally distribute evenly between phases. Some small molecules, however, display a preference for one of the phases, resulting in an uneven distribution. When the ions of a salt are unevenly distributed, a phase system is established in which the partitioning of biomolecules and particles is greatly influenced by their net charge (*28*). A proposed explanation for this phenomenon is that the uneven distribution of salt ions establishes an electrical potential difference of the Donnan type between the phases (*28*, *31*). Positively charged proteins, for example, are driven to partition more strongly toward the more negatively charged of the two phases. In this way, materials can be separated from one another based on their net charge, which of course depends on the system pH.

Enhanced separations have also been obtained by modification of the phase-forming polymers. A modified polymer generally partitions similarly to its unmodified form and can be used to attract molecules of interest toward the phase rich in that polymer (*32*). Charged polyethylene glycols have been synthesized and used for this purpose in the polyethylene glycol–dextran–water two-phase system (*33*). The addition of charged polymers results in a stronger, more easily controlled charge effect than that obtained using small ions (*34*). The partitioning of a material can also be strongly and selectively influenced by covalently bonding a functional group, having specific affinity for that material, to one of the phase-forming polymers. This modified partitioning technique, called affinity partitioning (*2*), offers the greatest potential for biotechnological applications of aqueous two-phase systems and will be discussed in detail below.

Although the main factors influencing partitioning have been described qualitatively (*29*), the complex interactions between effects in these systems are far from being completely understood. The effects of added salts and modified polymers on the phase diagram and of changes to the phase system on the partitioning of the modified polymers are seldom considered in partitioning studies. More importantly, the complex dependence of the phase behavior on a large number of interacting parameters makes optimizing separations difficult. A trial-and-error approach attempting to change one variable at a time has generally been employed to optimize phase systems. Efforts aimed at organizing existing knowledge of partitioning into rules for choosing and understanding phase systems (*28*, *32*) have not been completely satisfactory. A unified design model, incorporating the fundamental thermodynamics describing phase separation and partitioning in these systems, is lacking.

AFFINITY PARTITIONING

Affinity separations, principally affinity chromatography (*3*), currently play a major role in the final purification of biologically derived products, and increasing use of these methods is expected in the future (*35*, *36*). In future affinity separations, advantage may be taken of gene manipulation techniques as functional groups with specific attraction for an affinity ligand could be genetically engineered into new protein molecules. Affinity partitioning offers the selectivity of affinity chromatography with greater potential for large-scale continuous operations since it provides higher volume capacity and more rapid equilibrium (*4*). In addition, affinity partitioning techniques are applicable to whole cells and crude cell homogenates and may avoid problems of nonreversible binding and adsorption of materials to packing surfaces often encountered in affinity chromatography (*37*). Furthermore, scale-up of affinity partitioning is linear, and chemical engineering liquid–liquid extraction equipment and expertise can potentially be used for large-scale operations (*5*).

A historical review of affinity partitioning applications is highlighted in Table 1 and discussed below. Flanagan and Barondes (*2*) coined the term affinity partitioning to mean the addition of a polymer-ligand that partitions predominantly into one of the phase of a two-phase system and thus influences the biomaterial that specifically binds the ligand to partition toward that same phase. These same authors demonstrated the extraction of S-23 myeloma protein in a PEG–dextran–water system using dinitrophenol as ligand bound to PEG and presented a thermodynamic theory describing the phenomenon. Prior to the work of Flanagan and Barondes (*2*), affinity partitioning had been demonstrated to be useful and patentable for isolating trypsin using *p*-aminobenzamidine-bound-PEG (*38*, *39*). The extraction of serum albumin (*40*) using PEG-bound-fatty acids can also be considered biospecific affinity partitioning since albumin has specific hydrophobic binding sites for physiological transport of fatty acids. Other examples of biospecific affinity partitioning include extraction of $\Delta_{5\to4}$3-oxosteroid isomerase using PEG-bound-estradiol (*37*) and extraction of colipase using PEG-bound-lecithin (*41*).

As can be seen from Table 1, by far the most popular ligands used in

TABLE 1
Affinity Partitioning of Proteins, Cells, and Particles

Partitioned material	Ligand	References
Trypsin	p-Aminobenzamidine	*38, 39*
Serum albumin	Palmitate	*40, 82*
	Triazine dyes	*83*
S-23 Myeloma protein	Dinitrophenol	*2*
$\Delta_{5\to4}$3-Oxosteroid isomerase	Estradiol	*37, 84*
Formaldehyde dehydrogenase	Cibacron blue NADH	*89*
Formate dehydrogenase	Procion red	*20, 89*
Colipase	Lecithin	*41*
Glucose-6-phosphate dehydrogenase	Various triazine dyes	*4, 19, 48, 83, 90–92*
Human fibroblast interferon	Phosphate ester	*45*
Phosphofructokinase	Cibacron blue F3G-A	*18, 47, 83, 91, 93*
Various glycolytic enzymes	Various triazine dyes	*48, 90–94*
α-Fetoprotein	Various triazine dyes	*49*
Prealbumin	Remazol yellow and other dyes	*83, 95*
Hexokinase	Various triazine dyes	*91–93*
Nitrate reductase	Various triazine dyes	*96*
Thyroxine binding globulin	Cibacron blue F3G-A and other dyes	*97*
Pepsin	Pepstatin	*44*
DNA	Base binding dyes	*42*
Chromosomes	Base binding dye	*43*
Erythrocytes	Fatty acids esters	*98–101*
	Alkyl ethers	*102*
	Native antibodies	*103*
Liposomes	Palmitate	*104, 105*
Chloroplasts	Deoxycholate palmitate	*106, 107*
Niotinic cholinergic receptor-rich membranes	Amino groups	*50, 108–110*
Membrane bound opiate receptors	Naloxone	*111*

affinity partitioning have been general affinity ligands, namely fatty acid esters and triazine dyes. This is a result both because of an interest in avoiding the high cost of synthesizing a separate affinity ligand for each separation problem and because of the effectiveness of these ligands in binding proteins. As noted above, many proteins have specific hydrophobic binding sites attracted to fatty acids. Triazine dyes bind strongly to nucleotide binding sites of proteins and provide relatively inexpensive affinity ligands. Base-binding dyes have also been used for affinity partitioning of DNA and chromosomes (*42*, *43*). Table 1 also gives examples of cells and particles that have been isolated by affinity partitioning.

Nearly all of the examples shown in Table 1 were partitioned in PEG–dextran–water systems. Exceptions include the extraction of pepsin using dextran-bound-pepstatin, a strong pepsin inhibitor, in a dextran–hydroxypropyl dextran–water system (*44*) and the extraction of interferon using PEG-bound-phosphate ester in the PEG–orthophosphate–water two-phase system (*45*). Since proteins, cells, and particles usually partition strongly to the dextran-rich phase in these systems, affinity ligands were attached to PEG in virtually all cases to facilitate isolating the material of interest into the upper PEG-rich phase.

Several investigations studied in some detail the parameters determining material partition in the presence of polymer-bound-affinity ligands (*4*, *46–49*). The basic trends reported are summarized below in terms of the partition coefficient, K, defined as the ratio of the material of interest's concentration in the top phase to that in the bottom phase. The change in K due to the addition of affinity ligand increases with increasing polymer–ligand concentration to a saturation value, and depends on temperature, pH, types and concentrations of added salts, and types and concentrations of the base polymers, this dependence being due to both changes in the phase system and changes in the binding interactions. Dramatic K value changes, often from near 1 to near 10 and in extreme cases from less than 0.01 to greater than 20 (*47*), have been reported.

In light of the biotechnological potential of affinity partitioning techniques, surprisingly few publications have described preparatory scale purifications (*50*) or process designs for industrial-scale operations (*18–20*, *38*). Noticeable impediments to industrial application of affinity partitioning include: 1) the cost of developing phase systems and polymer–ligands specific to each separation, 2) the cost of recovering polymer–ligands, and 3) the cost of dextran, one of the most frequently used polymers. To overcome these problems, new methods for coupling ligands to PEG are being developed (*51*, *52*), polymer recycling schemes are being studied (*53*), and the possibility of using crude dextran or cheaper polymer substitutes for dextran is being investigated (*54*, *55*). Unfortunately, the relatively inexpensive two-phase system formed between PEG and orthophosphate is not generally applicable to affinity partitioning due to interference of the binding interactions at high ionic strengths (*4*).

The lack of design criteria for optimizing extractive phase systems is a further marked disadvantage preventing more widespread application of affinity partitioning in biotechnology. As noted above for aqueous two-phase systems in general, optimization of conditions for a given separation has generally been done by trial-and-error using qualitative empirical reasoning. The most sophisticated optimizations to date have employed simplex (*56*) or factorial (*45*) experimental design methods to systematically, yet tediously, adjust parameters. Although a thermodynamically based theory to explain the affinity partitioning effect was proposed in 1975 (*2*), this theory has neither been adequately tested nor applied to the design of affinity partitioning systems. Further detailed studies of affinity partitioning aimed at defining design criteria are essential to the successful application of this technique to biotechnology.

MODELING PHASE DIAGRAMS AND PARTITIONING

The partitioning behavior of a material is generally represented by a partition coefficient, K, defined by

$$K_i = [i]'/[i]'' \qquad (1)$$

where $[i]$ denotes the concentration of species i and the symbols $'$ and $''$ denote upper and lower phase, respectively. A brief review of theoretical attempts to describe partitioning behavior is presented below. A unified theory providing a quantitative model of the phenomenon has not yet evolved. Qualitative explanations of observed behavior and guidance for phase manipulations have been provided, but in no case has a theoretical result been employed to quantitatively account for measured partition coefficients or to design partitioning systems.

In 1931 Bronsted (*57*) derived a general relationship between the molecular weight of a substance, M, and its partition coefficient,

$$K_i = \exp(M_i\sigma/kT) \qquad (2)$$

where k is the Boltzmann constant, T is the absolute temperature, and σ is a constant depending in an unknown complex way on the substance and the phase system. This relationship cannot be used to predict partitioning or accurately correlate the molecular weight dependence without knowing σ. The functional form of the relationship has been substantiated, however, as it has been shown (*58*) that partition coefficients of many proteins are exponentially dependent on the molecular weight. Moreover, it is generally observed that small molecules partition evenly while large molecules tend to partition more toward one phase or the other. The larger the molecule, the more dramatic is the change in the partition coefficient upon small changes in the phase system.

Albertsson (*59*) separated the partition coefficient into various factors known to affect partitioning as follows:

$$\ln K = \ln K_{\mathrm{el}} + \ln K_{\mathrm{hfob}} + \ln K_{\mathrm{hfil}} + \ln K_{\mathrm{conf}} + \cdots \tag{3}$$

where K_{el}, K_{hfob}, K_{hfil}, and K_{conf} represent electrical, hydrophobic, hydrophilic, and conformational effects, respectively. Although these various effects do not act completely independently, they may be considered independent to a first approximation. The electrical effect, previously described (*28*) as resulting from an uneven distribution of charged particles between the phases, was accounted for using the following equation:

$$\ln K_i = \ln K_{i0} + Z_i F \Delta\Phi / RT \tag{4}$$

where K_{i0} is the partition coefficient of species i in the absence of charge effects, Z_i is species i's net charge, F is the Faraday constant, R is the gas constant, and $\Delta\Phi$ is the electrical potential difference between phases. Although Eq. (4) qualitatively describes a protein's partitioning dependence on net charge (*28*, *31*, *32*), its use has been criticized (*60*, *61*) because K_0 is assumed constant during changes affecting net charge and because $\Delta\Phi$ cannot be readily evaluated. Accurately accounting for the electrical effect is the subject of current research (*62*, *63*). Use of Eq. (4) to predict partition coefficients would require predictions for K_0, presumably made up of contributions from K_{hfob}, K_{hfil}, K_{conf}, and other factors. These contributions are best described by thermodynamic arguments.

A proper thermodynamically based model to account for K_0 should be able to describe the basic phenomenon of phase separation in three component polymer–polymer–water two-phase systems. Such a model could then be extended to four components to describe the partitioning behavior of a molecule of interest between phases. The implicit simplifying assumption that the polymers can be treated as pure components is made here. Although this assumption clearly does not hold for dextran, it provides a reasonable approximation to the phase behavior and simplifies computations as well as the following discussion. The effect of polydispersity on phase behavior in these systems is the subject of current research by one of the authors.

Since the tendency for phase separation is governed by the minimization of Gibbs free energy, liquid–liquid equilibria are generally best described using thermodynamic models for the Gibbs free energy of the system. The best known Gibbs energy model for polymer solutions is the simple statistical mechanical treatment derived by Flory and Huggins

(*64–66*). This model, based on a lattice concept, can describe qualitatively all of the important features of phase separation in polymer solutions and has been applied successfully for correlation of phase diagrams in aqueous two-phase polymer solutions (*55*, *67*). Other Gibbs energy models employed for this purpose include the UNIQUAC model (*67*) and the Edmond-Ogston model (*62*). A thorough discussion of Gibbs energy models is beyond the scope of this review, and only the model proposed by Edmond and Ogston (*68*) will be described further for the purpose of illustration.

According to the Edmond-Ogston model, based on the osmotic second virial equation, the chemical potentials of the polymer solutes (Components 2 and 3) can be written,

$$\Delta\mu_2 = RT(\ln m_2 + cm_2 + a_{23}m_3) \tag{5}$$

$$\Delta\mu_3 = RT(\ln m_3 + dm_3 + a_{23}m_2) \tag{6}$$

where m is the solute molal concentration; and c, d, and a_{23} are interaction coefficients characterizing the interaction of two Polymer 2 molecules, two Polymer 3 molecules, and one Polymer 2 with one Polymer 3 molecule, respectively, in the solvent, water. The chemical potential of water is obtained by application of the Gibbs-Duhem equation as

$$\Delta\mu_1 = -RTV_1(m_2 + m_3 + c/2m_2^2 + d/2m_3^2 + a_{23}m_2m_3) \tag{7}$$

where V_1 is the molar volume of water. $\Delta\mu_i$ in the above equations represents the difference between the chemical potential of Component i in the system and that in a standard state. The standard state for each component is chosen to be the same in all aqueous phases. The interaction coefficients c, d, and a_{23} are related to the traditional osmotic second virial coefficients A_{ij} (*69*) by

$$2A_{ij} = 1000x/M_iM_j \tag{8}$$

where $x = c$ when i and $j = 2$; $x = d$ when i and $j = 3$; and $x = a_{23}$ when $i = 2$ and $j = 3$. M_k represents the molecular weight of Species k.

At a given temperature and pressure, the conditions for equilibrium between two phases are provided by the requirement that the chemical potential of each component be the same in each phase:

$$\mu_k' = \mu_k'' \tag{9}$$

Given osmotic second virial coefficient values, phase diagrams can be predicted from the Edmond-Ogston model by solving the set of Eqs. (9) in conjunction with Eqs. (5)–(8) and mass balance relations. This procedure would be the same for any Gibbs energy model except that Eqs. (5)–(8) would be replaced by other relationships describing component chemical potentials in terms of system compositions and binary interaction parameters. The interaction parameters in these models are determined by fitting equilibrium data or by independent thermodynamic measurements on the binary systems. A wide variety of experimental measurements has been used to obtain independent information on binary interactions, including light scattering (*62*, *70*, *71*), equilibrium sedimentation (*68*, *71*), osmotic pressure (*72*), and vapor–liquid equilibrium (*73–75*) measurements. References noted above concerned work on aqueous solutions of dextran or PEG.

Both the Flory-Huggins (*76*) and the Edmond-Ogston (*62*) models have been extended to four components to describe the partitioning of a small amount of added material between phases in aqueous two-phase systems. The extended Flory-Huggins theory has been shown to qualitatively describe much of the observed behavior for partitioning in such systems (*76*), but has not been used quantitatively. The extended Edmond-Ogston model has been shown (*62*) to provide approximate values for partition coefficients of proteins in a buffered two-phase system containing PEG, dextran, water, potassium phosphate, and protein. In doint this it was important to modify the model to account for an apparent electrical potential difference between the phases and its effect on the charged protein. This was done following Albertsson (*28*) as explained above by adding the term $Z_iF\Phi$ to the chemical potential of the charged macromolecular component. Interaction coefficients in this modified model also depend on the salt concentration.

Another approach to modeling the partitioning of a fourth component in an aqueous two-phase system utilizes a modified form of the liquid lattice theory of Scheutjens and Fleer (*77*, *78*). The partitioning of pullulanase and phosphorylase as a function of PEG molecular weight in several PEG–dextran–water systems has been successfully correlated using this model (*63*). In this work it was assumed that the polymer–polymer–water phase behavior was known and constant; only the partitioning of the fourth component was considered. The effects of salt and pH have not yet been included in this model.

AN AFFINITY PARTITIONING THEORY

Flanagan and Barondes (*2*) presented a thermodynamically based theory which separately accounts for the enhancement afforded by affinity partitioning. For the purpose of this discussion, the material of interest will be a protein with a biospecific affinity for a PEG-bound-ligand in a PEG–dextran–water two-phase system. The behavior of interacting components in a dilute system at equilibrium can be represented as shown in Fig. 2 assuming 1) no adsorption of materials at the interface, 2) a monovalent polymer–ligand, and 3) one biospecific binding site per protein. The change in Gibbs free energy to transfer one mole of the PEG–ligand–protein complex from the bottom phase to the top phase can be expressed as the sum of four operations: 1) the dissociation of the complex in the bottom phase, 2) the transfer of the PEG–ligand from the bottom to the top phase, 3) the transfer of the free protein from the bottom to the top phase, and 4) the association of the PEG–ligand–protein complex in the top phase:

$$\Delta G_5 = \Delta G_1 + \Delta G_2 + \Delta G_3 + \Delta G_4 \tag{10}$$

The standard Gibbs free energy change of each of these operations can be written in terms of an equilibrium constant by the expression

$$\Delta G_i = -RT \ln K_i, \qquad i = 1, 2, \ldots, 5 \tag{11}$$

For the phase transfer operations 2, 3, and 5, the equilibrium constants are represented by the partition coefficients, K_i, defined by Eq. (1), where i is a dummy variable for L, P, or LP representing the free polymer–ligand, the free protein, and the ligand–protein complex, respectively. For the association reaction,

$$\mathrm{L} + \mathrm{P} \rightarrow \mathrm{LP}$$

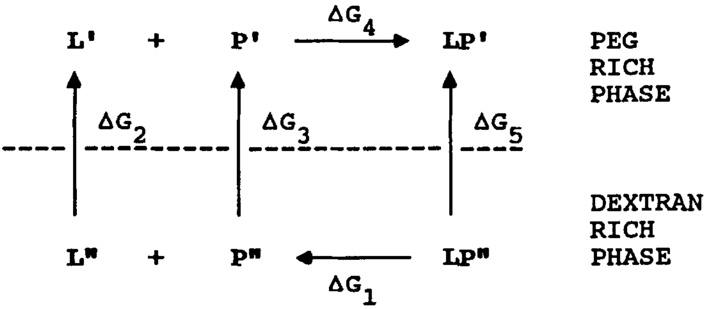

FIG. 2. Schematic diagram of affinity partitioning.

the equilibrium binding constant is given as

$$k_a = [\mathrm{LP}]/[\mathrm{L}][\mathrm{P}] \tag{12}$$

For convenience of notation, the equilibrium constants for the steps of Fig. 2 can thus be denoted as:

$$K_1 = 1/k_a'', K_2 = K_L, K_3 = K_\mathrm{P}, K_4 = k_a', K_5 = K_\mathrm{LP}$$

Substituting the Eqs. (11) into Eq. (10) and using the above notation provides an expression for the partition coefficient of the ligand–protein complex in terms of the other equilibrium constants,

$$K_\mathrm{LP} = K_\mathrm{L}K_\mathrm{P}k_a'/k_a'' \tag{13}$$

The measured partition coefficient of the protein in the presence of polymer–ligand, K_TP, is written in terms of the sum of free and bound protein in each phase,

$$K_\mathrm{TP} = ([\mathrm{P}]' + [\mathrm{LP}]')/([\mathrm{P}]'' + [\mathrm{LP}]'') \tag{14}$$

Combination of an equation like Eq. (12) for each phase and Eq. (1) for the free protein with Eq. (14) allows the partition coefficient for the protein in the presence of polymer–ligand to be written as

$$K_\mathrm{TP} = K_\mathrm{P}(1 + k_a'[\mathrm{L}]')/(1 + k_a''[\mathrm{L}]'') \tag{15}$$

In cases where the ligand concentration is sufficiently high such that $k_a[\mathrm{L}] \gg 1$ in each phase, Eq. (15) for the total protein reduces to Eq. (13) for the protein–ligand complex, i.e., all the protein will be bound:

$$K_\mathrm{TP} = K_\mathrm{L}K_\mathrm{P}k_a'/k_a'' \tag{16}$$

For the case of a protein with *n* identical, independent binding sites for the polymer–ligand, Eq. (15) can be written as (*76*)

$$K_\mathrm{TP} = K_\mathrm{P}(1 + k_a'[\mathrm{L}]')^n/(1 + k_a''[\mathrm{L}]'')^n \tag{17}$$

This same theory can be applied to the study of binding interactions using two-phase systems (*79*, *80*) and to the analytical method called Partition Affinity Ligand Assay (*81*). A similar theory developed for cells and particles is presented by Brooks and coworkers (*76*).

No adequate test measuring all parameters independently has been made for the above theory. A proper test would require a carefully devised set of critical experiments measuring binding interactions in both phases and concentrations of both bound and unbound species in equilibrium phases. It would also require knowledge of the precise number of binding sites per ligand and biomolecule. These requirements represent much more detail than is provided by studies to date which normally report only the gross partitioning effect; i.e., the increase in total concentration of the material of interest in the top phase due to the addition of polymer ligand.

Some researchers have qualitatively compared the measured gross affinity partitioning effect to that obtained by making a series of approximations in the above theory. While some of these studies support the theory (*1*, *2*, *48*), others show marked disagreement (*46*, *47*, *82*, *83*). In general, it has been found that the dependence of a protein's partition coefficient on the total number of binding sites is much weaker than that predicted by Eq. (17). It appears that all binding sites of a given protein are not independent and identical as assumed in the theory.

MODELING AFFINITY PARTITIONING

A useful thermodynamic model for designing affinity partitioning systems could be established by coupling the theory for the affinity effect with a thermodynamic model for phase separation and partitioning in two-phase systems. Thus, a term representing the affinity effect, ln K_{aff}, can be added to the list of influencing factors of Eq. (3):

$$\ln K = \ln K_0 + \ln K_{\mathrm{el}} + \ln K_{\mathrm{aff}} + \cdots \tag{18}$$

Charge effects are incorporated using Eq. (4), and the affinity effect is included using Eq. (17) as follows:

$$\ln K_{\mathrm{P}} = \ln K_{\mathrm{P0}} + Z_{\mathrm{P}} F \Delta\Phi / RT + \ln\,[(1 + k_a'[\mathrm{L}]')^n / (1 + k_a''[\mathrm{L}]'')^n] \tag{19}$$

In writing this equation it has been assumed that K_{P}, the partition coefficient of the free ligand in the mixture, is equal to K_{P0}, the partition coefficient of the protein in the absence of strong (affinity and charge) effects.

For the case with excess ligand and a 1:1 binding interaction, the third term on the right-hand side of Eq. (19) can be written as

$$\ln K_{\mathrm{aff}} = \ln (K_{\mathrm{L}} k'_a k''_a) \tag{20}$$

The total partition coefficient of the protein, K_{TP}, is then described in terms of the partition coefficients of the unbound protein and ligand, K_{P} and K_{L}, and their binding constants in each phase, k'_a and k''_a, along with a term for the charge effect. If both K_{P} and K_{L} in the mixture are assumed to be equal to those of the separately partitioned molecules, then their values can be obtained experimentally or from Gibbs energy models as explained in the previous section. This information together with measured or estimated binding constants would allow prediction of partitioning behavior in the presence of affinity ligands, via Eq. (19), assuming the charge effect is negligible or properly accounted for by the second term of that equation. The model could be simplified further by assuming, as is often done (*44*), that the binding constant in each phase is approximately equal to that measured in a polymer-free buffer solution.

For the more general case with any given ligand concentration, but still 1:1 binding, prediction of K_{TP} would require first calculating $[\mathrm{L}]'$ and $[\mathrm{L}]''$ in Eq. (19). This can be accomplished using Eq. (1) written for the ligand and the protein and an equation like Eq. (12) for each phase together with mass balance equations for the protein and the ligand. An analogous calculation scheme could be employed for the most general case.

Development of a Gibbs energy model to accurately predict phase behavior in aqueous polymer solutions and critical experimental tests of Eq. (19) are the subjects of current research in the authors' laboratories.

OTHER ASPECTS OF SEPARATIONS

The efficient recovery of a material by partitioning depends on the volume ratio of the phases and the partition coefficients of contaminating materials as well as on its own partition coefficient. By adjusting the volume ratios or using multiple extraction steps, proteins with relatively low partition coefficients (only slightly greater than 1) can be recovered efficiently in the top phase using PEG-bound-ligands. Other proteins and contaminating materials are generally directed as completely as possible to the other phase by means of pH and salt adjustments. In one study (*84*), charged dextrans were used to attract contaminating proteins to the lower phase while PEG-bound-ligands attracted the material of interest to the top phase. It should be noted here that phase system adjustments may be in competition with the affinity partitioning effect. A systematic study of interacting effects coupled with a thermodynamic model for affinity partitioning would be invaluable in understanding and manipulating competitive effects.

For biotechnological applications, the extraction process must be optimized within the constraints of 1) desired product purity and yield, and 2) economic factors including the value of the product, and cost of the phase system and ligand, and the capital and operating costs of the extraction apparatus. In extreme cases, with very strong affinity partitioning effects, a single extraction step may be sufficient to achieve a desired separation. In other cases, multiple extraction steps will be required. These may be made either by a series of discontinuous batch extraction steps (*85*) or by a continuous contacting apparatus such as the countercurrent distribution apparatus of Craig (*86*), the more recent developments called countercurrent chromatography (*87*), or conventional liquid extraction columns (*5*, *88*). A summary of the design criteria for these various methods will not be presented here. It suffices to merely point out that phase equilibrium information is required for designing contacting equipment and choosing between alternative contacting schemes. A thermodynamic model for affinity partitioning would provide the required phase equilibrium information at conditions of interest based on minimal experimental evidence at other conditions.

In the case of discontinuous batch extraction steps, for example, application of simple mass balances provides an expression for the fraction, f, of a protein recovered in the final top phase after s extraction steps contacting sequential top phases with fresh bottom phases,

$$f_s = (Kq/(Kq + 1))^s \tag{21}$$

where q is the volume ratio between the phases, $q = V'/V''$; V is the volume of a phase; and K is the partition coefficient of the protein (*32*). For the purpose of this illustration, let s be equal to 1. The value of q to provide a fixed value of f can be seen to be equal to

$$q = f/(K - Kf) \tag{22}$$

Suppose that a mixture of Proteins A and B, having known partition coefficients $K_A > 1$ and $K_B < 1$, are to be separated. A choice has to be made as to which is the best volume ratio to be used. If the objective is to obtain the highest fraction of Protein A in the top phase together with the highest fraction of Protein B in the bottom phase, it can be shown (*85*) that the optimal volume ratio is given by

$$q = 1/(K_AK_B)^{1/2} \tag{23}$$

In some sense, Eq. (23) represents the volume ratio for the best separation of Proteins A and B in terms of recovery and purity. On the other hand, if the objective is to obtain a given high fraction of A in the top phase regardless of the amount of B in either phase, Eq. (22) is used to obtain q. This value of q will be different from that of Eq. (23) because there is no constraint on the partitioning of Protein B. A high recovery of Protein A in the top phase can be obtained with a higher q value than that given by Eq. (23), but the purity of A will decline due to contamination by B. Next, suppose an A product of high purity is required; f_A/f_B should be maximized. The highest purity A will be obtained when the top phase volume is infinitesimally small. To obtain a high purity A product in the top phase, the trade off between purity and recovery will shift toward a lower q value than that given by Eq. (23). A measure of the purity is given by the ratio f_A/f_B, which can be called the purification factor, PF, and can be written using Eq. (21) as

$$(\mathrm{PF})_s = [(q + 1/K_B)/(q + 1/K_A)]^s \tag{24}$$

for the s step process described above. Other contacting schemes may provide better separations, but regardless of the scheme, knowledge of the partition coefficients must be incorporated in mass balances to optimize separations.

CONCLUSION

Affinity partitioning is expected to play a major role in future biotechnological separations due to the following properties of the technique: biocompatibility, high selectivity, high volume capacity, rapid equilibrium, and ease of scale-up. Optimization of separations using this technique requires consideration of a large number of interacting factors for which knowledge in sufficient detail to establish design criteria is lacking. The lack of a design model, reflecting the fundamental physical chemistry underlying the process, represents a serious impediment to more widespread application of the technique in biotechnology. It is shown that a predictive design model for affinity partitioning can be obtained by incorporating a Gibbs energy model describing phase equilibria in aqueous polymer solutions into a theory for the separate affinity effect.

NOMENCLATURE

a_{23}	interaction coefficient
A_{ij}	osmotic second virial coefficient
c	interaction coefficient
c_i	concentration of Component i
d	interaction coefficient
f	fraction of protein recovered in top phase
F	Faraday constant
G	Gibbs free energy
k	Boltzman constant
k_a	association constant
K_i	partition coefficient of Component i
L	polymer–ligand
LP	ligand–protein complex
m	molality
M_i	molecular weight of Component i
n	number of identical independent polymer–ligand binding sites per protein
P	protein
PEG	polyethylene glycol
PF	purification factor
q	volume ratio between phases
R	gas constant
s	number of extraction steps
T	absolute temperature
V	volume of a phase
Z_i	net charge of Component i

Greek Letters

σ	unknown constant in Bronsted theory
μ_i	chemical potential of Component i
Φ	electrical potential

Symbols

[ ]	concentration

$'$	top phase
$''$	bottom phase

Subscripts

A,B	Proteins A and B
i,j,k	dummy variables denoting Species i,j,k
L,P,LP	as defined above
0	indicates the absence of strong effects

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