Aqueous Two-Phase Systems in Biochemical Recovery

Systematic Analysis, Design, and Implementation of Practical Processes for the Recovery of Proteins

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ABSTRACT

The design and application of aqueous two-phase systems for recovery of proteins is reviewed. Reference is made to the properties of polymers and salt forming the systems and their influence on phase separation and partition. The properties of systems important for the design of purification strategies are discussed in relation to the surface properties of proteins. Strategies for the modification of systems for bioaffinity partition are considered including the choice of carrier in terms of molecular type and properties, or by particulate addition. Finally, the scaleup of partition is considered, and the reasons for currently limited adoption at larger scales are elucidated.

Index Entries: Aqueous two-phase systems; partition; purification; protein recovery; separation; extraction; poly(ethylene glycol); dextran.

INTRODUCTION

Purification strategies involving the partition of biological macromolecules between two immiscible phases would probably have been established many years ago based upon the pioneering work of Martin and

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Synge (*1*) and the Craigs (*2*), were it not for the unfortunate fact that protein tertiary structures are irreversibly disrupted by the solvents employed. Instead, the intervening years saw the development of porous solid phases with biocompatible surfaces for partition and adsorption of proteins, nucleic acids and other biopolymers (*3–5*) that are currently widely adopted when problems of biological separations are addressed. However, since Per-Åke Albertsson (*6,7*) demonstrated that wholly aqueous biphasic systems could be created and manipulated to effect differential partition of cells, organelles and macromolecules, there has been molecular purification, liquid–liquid extraction has a number of advantages over those strategies involving solid phases, including improved mass-transfer characteristics, higher capacities, and linear relations of scaleup uncompromised by limitations of physical and mechanical design (*9*). Design refinements of modern solid phases have concentrated upon exploitation of individual aspects of surface properties of macromolecules such as charge, size, or hydrophobicity. As a result, initial decisions regarding the design of purification schemes are inevitably facile. In contrast, partition of macromolecules in free solution necessarily involves the interaction of the totality of surface properties of products and impurities with molecular features of chosen biphasic systems. Understanding and predicting the results of partition is thus a complex matter that so compromises initial decisions regarding purification strategies using aqueous two-phase systems that they become subject to empirical investigation. This review will attempt to address molecular aspects of partitioning in the course of an assessment of the current status of aqueous two-phase partition, as applied to the fractionation of biological macromolecules.

Differential partition of chloroplasts in aqueous PEG (poly(ethylene glycol)/salt two-phase systems and of other cellular organelles and macromolecules in PEG/dextran systems was discovered by P-Å. Albertsson in 1956 (*10*). The general phenomenon of polymer incompatibility in aqueous solution had been reported previously but not seriously exploited (*11–13*), In the early 1970s, the physicochemical characteristics of PEG/dextran systems and the partitioning properties of cells, subcellular structures and macromolecules, were widely studied (*14–19*). The last decade has seen the investigation and validation of PEG/salt aqueous two-phase systems for the large scale isolation of intracellular enzymes, but methodologies have not been widely adopted at production scale (*20–24*). More recent years have seen the development of thermodynamic descriptions of phase separation (*25–29*) and the mathematical modeling of macromolecular partition (*25–29*).

Basic Features of Aqueous Two-Phase Systems

Pairs of hydrophilic solutes (polymer/polymer or polymer/salt) may display incompatibility when dissolved in aqueous solution above critical

concentrations such that two phases form, with each preferentially enriched in one component (Table 1). Such systems are characterized by phase diagrams whose form varies with the nature of the polymers (type, size, and polydispersity) or added salts (type and concentration). In Figure 1, at all points above the binodal curve ABC, biphasic systems form. All points below yield homogenous solutions, except under circumstances that shift the binodal to lower concentrations. The addition of feedstock solutes at high concentration may promote this phenomenon (*30–31*). Any system having overall composition lying on the line AC (the tie line) has top and bottom phases described by points A and C respectively, but differing in the ratio of relative phase volumes. For system D in Fig. 1, the phase ratio (R) is described by:

$$R = V_T/V_B = CD/AD \qquad (32)$$

where V_T and V_B are the volumes of top and bottom phases, and CD, AD are taken from the phase diagram or calculated from determinants of phase composition.

The phase diagram has a unique critical point, approaching which the top and bottom phases assume identical compositions. At increasing concentrations of phase-forming components above the critical point, the top and bottom phases diverge in relative composition and physicochemcial properties (*33*). Biphasic systems of differing overall composition lying on the same tie line have upper and lower phases of identical composition. A useful measure of system composition is therefore the tie line length (TLL), where:

$$TLL = (\Delta C_t^2 + \Delta C_b^2)^{1/2} \qquad (\text{Fig. 1, } 34)$$

ΔC_t (w/w%) is the difference in concentration of the predominant top phase-forming polymer between top and bottom phases, and ΔC_b is defined similarly for the predominant bottom phase-forming component.

The partition of solutes is characterized by the partition coefficient (K) where:

$$K = C_T/C_B$$

C_T and C_B are the concentrations of solute in the top and bottom phases, respectively.

This relation holds only where there is no dissociation or association of solutes in either or both phases, when other relations apply (*2*).

The degree of separation (G) is given by:

$$G = K.\, V_T/V_B$$

V_T and V_B are the respective phase volumes.

For two component systems, for example, an enzyme (E) and contaminating bulk protein (P), the degree of resolution is greatest when: $G_E \cdot G_B = 1$. For a volume ratio (R) defined by: $R = V_T/V_B = 1/(K_E \cdot K_B)^{1/2}$ (*35*).

Table 1
Composition of Some Aqueous Two-Phase Systems

A. Polymer-polymer-water systems		
Poly(ethylene glycol)	with	poly(vinyl alcohol)
		poly(vinyl pyrrolidone)
		dextran
		ficoll
		hydroxypropyl-starch
Poly(propylene glycol)	with	methoxy poly(ethylene glycol)
		poly(ethylene glycol)
		poly(vinyl pyrrolidone)
		poly(vinyl alcohol)
		hydroxypropyl-dextran
		dextran
Poly(vinyl alcohol)	with	methyl cellulose
		hydroxypropyl dextran
		dextran
		acrylic/methacrylic acid copolymers
Poly(vinyl pyrrolidone)	with	methyl cellulose
		dextran
Dextran	with	ethyl hydroxyethyl cellulose
		hydroxypropyl dextran
		ficoll
B. Polymer-low molecular weight solute-water systems		
Poly(propylene glycol)		sodium/potassium phosphate
Poly(ethylene glycol)	with	sodium/potassium citrate
Poly(vinyl pyrrolidone)		Al/Na/Mg/Cu/Fe/Zn/Li sulphates
		ammonium sulphate
		sodium/potassium carbonate
		sodium tartrate
		sodium succinate
		sodium silicate
Poly(propylene gycol)	with	glucose/glycerol
Dextran	with	propyl alcohol

Data taken from references 14, 61, 63, 67 and 72.

However, in most practical separations, it may be more important to maximize product yield (Y) rather than purity, that for the top phase is defined by

$$Y_T = 100 / 1 + (1/R \cdot 1/K)$$

Physicochemical properties of systems may be characterized by measurement of osmotic pressure or surface tension. For the latter in PEG/dextran systems, the following relationship has been found to apply:

$$\gamma_{TB} = a \cdot TLL^b \qquad (33)$$

terms a and b are constants characterizing particular systems.

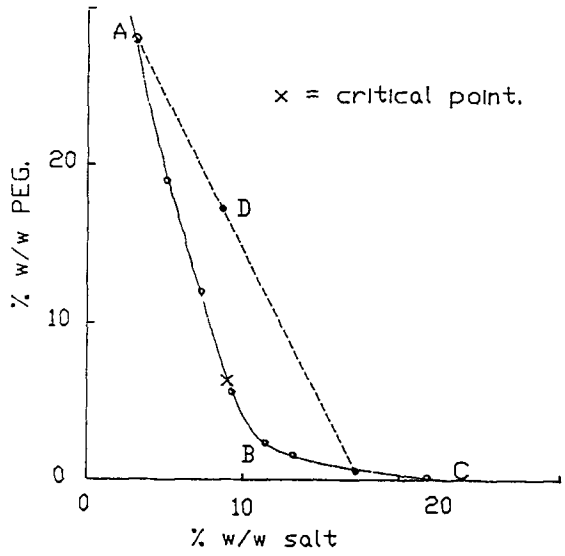

Fig. 1. PEG/salt phase diagram.

Similar relations probably hold for PEG/salt systems. In PEG/dextran systems, interfacial tension is drastically changed by salt addition reducing the ability of such measures to fix or calibrate systems in practical application. Other workers have approached the problem of defining system properties by the partitioning of selected amino acids (*36,37*).

Ionic strength and pH can be controlled in PEG/dextran systems by addition of suitable buffers and salts, whereas pH control in PEG/salt systems may be achieved by adding appropriate buffering salts (e.g., potassium dihydrogen orthophosphate and dipotassium hydrogen orthophosphate in PEG/phosphate systems). Ionic strength in such systems is high, and in the lower phase increases with tie line length. The current review will address aspects of phase composition as they relate to molecular properties of all types of systems to facilitate a rational approach to the design of separation systems.

PROPERTIES OF THE PHASE FORMING COMPONENTS

With few exceptions (*38–40*), the fundamental molecular properties of components of aqueous two-phase systems have featured little in the many publications on the subject. In particular, the molecular thermodynamics literature is replete with allusions to unfavorable polymer segment-segment interactions as the basis of phase separation (*27*). Polymer–polymer interactions are frequently emphasized, and the reality that such interactions take place between polymers and salts in aqueous solution is

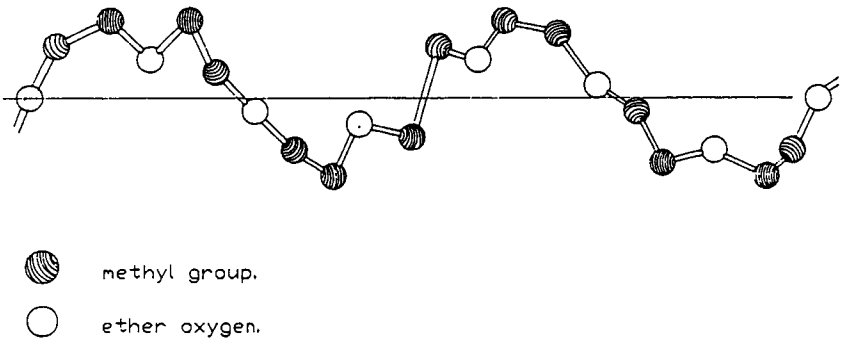

Fig. 2. Structure of poly(ethylene glycol) (after ref. *40*).

lost (*39*). In addition, phase separation of polymers is portrayed as a general rule (*28*) that does little to illuminate phase separation phenomena of importance to partition of biological macromolecules.

Poly(ethylene glycol)

Poly(ethylene glycol) is a 1,2-epoxide polymer produced by catalytic polymerization of ethylene oxide (*41*). The resultant linear polymer chain has hydroxyl terminals that are functional alcohols and capable of esterification, separated by a chain of multiples of two methylene groups alternating with an ether oxygen linkage (Fig. 2). The useful range of mol wt extends from below 1000 up to 40000 dalton representing polymers having 20 to 90 monomer units. It is likely that PEG adopts a helical form in solution (*41*) not dissimilar to the rod shaped geometry assumed in many excluded volume models applied to precipitation (*42–44*) and partition (*32*) studies. The crucial feature of the molecular structure of PEG is the repeating ether oxygen linkage with a lone electron pair making this a powerful hydrogen bond acceptor. Thus two molecules of water may be bound to each (*41,45*). Some authors have determined that three water molecules may be strongly associated with the ether oxygens (*46*). It has also been proposed that the "iceberg structure" of water may exist around the methylene groups but the concept has been challenged (*47*). Nevertheless, it is clear that the molecular structure of PEG orders a considerable amount of water along the length of the polymer chain and will influence water structure for some distance into the bulk solution. It is a powerful water structure maker in the sense of Franks (*48*). It has been reported from calorimetric studies that no water is free at concentrations above 48% w/w PEG-6000 (*49*). Van Oss has characterized PEG as a monopolar Lewis base in the absence of hydrogen bond donating groups to balance hydrogen bond acceptors in the molecular structure (*38,40,50*). Iliopoulos et al.

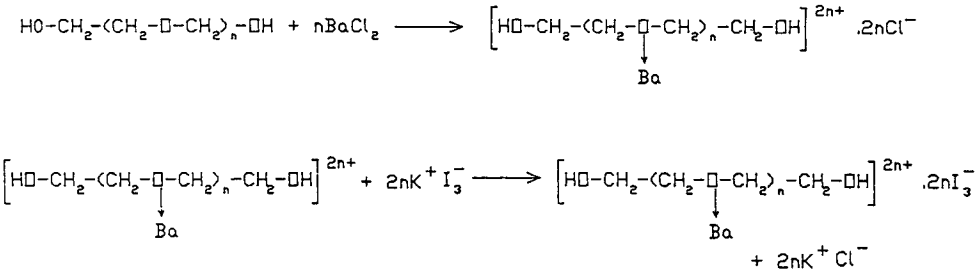

Fig. 3. Functional chemistry of Skoog's assay for poly(ethylene glycol) (*55*).

(*51*) have shown that the interaction between polyacids and bases (e.g., polyacrylic acid (PAA) and poly(ethylene oxide) (PEO), is influenced by hydrogen bond donating ability. The introduction of "structural defects" into the PAA structure by neutralization of acrylate groups influences complexation stoichiometry by preventing hydrogen bond formation between some acrylic groups and the PEO ether oxygens. More recently, the same authors have shown that mixtures of PEG and polyacrylamide (PAM) become more compatible with the introduction of anionic groups as copolymers of PAM (*52*). In addition, the ether oxygens of the PEG polymer chain are capable of complex formation with electrophilic centers on smaller molecules such as alkali metals in a manner similar to the crown ethers (*42*). Crystalline complexes of PEG:K^+/NH_4^+ have ether oxygen:salt stoichiometry of 4:1 where PEG:Li^+Na^+ have stoichiometries of 3:1. Such complexes require that cations (Lewis acids) are combined with large monovalent anions (Lewis bases), and do not form with small multivalent anions because of high lattice energies and the requirement of hydrogen bonding moiety in PEO solution (*53*). Crystalline complexes of this type are under current investigation as solid phase, polymeric electrolytes for battery applications (*54*). That such complexes may also form in aqueous solution is demonstrated by the functional chemistry of the assay of Skoog (*55*) developed for the analytical determination of poly(oxyalkylenes). (Fig. 3.)

Dextran

Dextran is an anhydroglucose polymer produced by the fermentation of sucrose by *Leuconostoc* species where 95% of the glucosidic linkages occur in the α-1,6 position with about 5% in the α-1,3 position. Of the latter, 80% are little more than one monomer unit in length, although about 1% may be much longer (Fig. 4), (*56*). The size of polymers useful in partition work varies from weight average mol wt ($M\overline{w}$) of 40,000 dalton to approx 2 million dalton. This represents a number average mol wt ($M\bar{n}$) of from

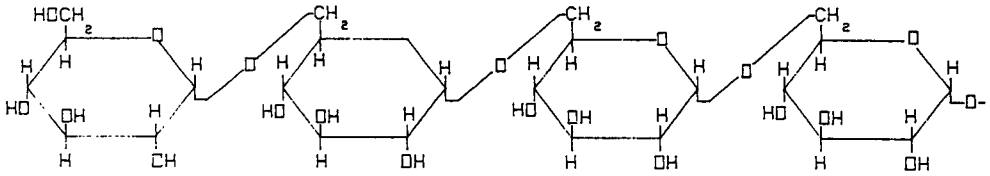

Fig. 4. Structure of dextran linear chain after (*57*).

20,000 to 280,000 dalton reflecting the greater polydispersity of higher mol wt fractions (*57*). Such a size range contains from 100 to 1500 monomer U. Crude (unfractionated) dextran has also been used at large scale (*21*) and can be assumed to be highly polydisperse. Polydispersity critically affects phase diagrams and phase composition through differential partition of differing size categories (*58*). The polymer brings about considerable ordering of water structure through hydrogen bonding amounting to binding of 0.5 mol of water per mol of hydroxyl groups (*59*). Van Oss has suggested that dextran is also a monopolar Lewis base, and therefore incompatible with PEG in aqueous solution (*38,40,50*).

In comparison to other polymers, PEG and dextran predominate in partition studies. They are regarded as nontoxic and occur in the pharmacopoeias of most countries of the world (*21*). However, other polymers have been identified as useful in terms of their chemical characteristics and economy; selected examples are listed in Table 1. Some may be of use in particular applications. Thus PAA is precipitable by manipulating pH (*60*), PVP may be of use in binding phenolics in the purification of plant proteins (*61*), and a number of cheaper "alternatives" to dextran have become available in recent years (*62,63*). The water sequestering properties of all such polymers may be expected to be similar to PEG and dextran. Van Oss has suggested that they all must be monopolar Lewis bases. This property gives each polymer a different hydrogen bonding interaction with water molecules resulting in differing "hydration pressures" and ultimately enabling the formation of multiphasic systems (*38,40,50*). Phase separation of aqueous solutions of two or more polymers is understood at the molecular level as arising from the hydrogen bond interactions between acceptors of polymers and donors of water molecules that promote orientation of the water molecules adjacent to the polymers. A long range repulsive effect called the hydration pressure is established, and it is greatest when the polymers are highly dehydrated, and when the surface area of contact is greatest (*38,40,50*). At increasing concentrations of polymers, progressively more water is ordered until phase separation takes place as a phenomenon unmarked by any profound thermochemical events (*39*). Thermodynamically, the phenomenon has been modeled by two principle approaches—the statistical mechanical lattice model de-

riving from Flory-Huggins theory (*25–29*), or the osmotic virial expansion model deriving from Edmonds and Ogston (*25–29*). The thermodynamics of phase separation and the modeling of partition has been the subject of recent review (*64*). Neither approach specifically accounts for the interactions given here (due to van Oss) nor the influence of salts set out below.

The Influence of Salts

Salts have important influences upon the physico-chemical properties of aqueous two-phase systems. Johansson (*16*) showed that the addition of alkali metal halides increased the partition coefficient of ovalbumin and decreased that of lysozyme in an otherwise identical PEG–dextran system. The magnitude of the effect can be seen to be proportional to the increasing Pauling ionic radius, and decreasing hydration energy of anions and cations.

$$\text{Thus: } F^- < Cl^- < Br^- < I^-$$

$$\text{and: } Li^+ < Na^+ < K^+$$

Salts have been shown not to partition evenly between phases of PEG/dextran systems (*17*). More recently, Zaslavsky et al. (*65*) have shown that the presence of increasing concentrations of monovalent salts (to 0.1 *M*) alters the composition of top and bottom phases of two polymer systems without gross effect upon the position of the binodal curve. In such circumstance, the slope of the tie line is altered. In contrast, divalent salts such as potassium sulfate profoundly changed both the position of the binodal and the phase compositions. The binodal is shifted toward decreased polymer concentrations. This is understandable since salts having divalent and trivalent anions are the principal phase forming components of PEG–salt systems at the relatively low concentrations characteristic of useful systems. The phase diagram of PVP–dextran was even more sensitive to the type of salt added. A series of sulphates (K_2^+, Cs_2^+, Na_2^+, $(NH_4)_2^+$) affected the phase diagram in similar manner to PEG-dextran systems, shifting the binodal to lower polymer concentrations. Monovalent salts (KSCN, NaSCN, NH_4SCN, $KClO_4$, and KCl) shifted the phase diagram to higher polymer concentrations. For alkali metal halides, the effect decreased in the order $Br^- < Cl^- < F^-$. Concentration of salt in each phase was found to be different and dependent upon the type of salt and the type and concentration of polymers. Salts having divalent anions strongly favored the lower phase, (the log of the partition coefficient decreasing with TLL lnK 0 to < -0.3). Monovalent salts favored that phase less (lnK 0 to about -0.1), whereas water structure breakers (thiocyanates and perchlorates) favored the upper phase (lnK up to about 0.1). It has been shown

that the phase forming abilities or "salting out" activities of, predominantly, multivalent salts with PEG are closely related to the Hoffmeister or lyotropic series (*32,66*). This effect decreases in the series; phosphate > citrate > sulphate > carbonate > tartrate > succinate > formate. For the cations of sulphates, the order was found to be $Na^+ > Mg^{++} > Zn^+ > Li^+$. The lyotropic series has been interpreted and modeled by Melander and Horvath in terms of the molal surface tension increments of salts and its effect in promoting hydrophobic interactions (*67*). Comparison of the Melander and Horvath tabulation of published molal surface tension increments of salts with PEG cloud point and phase separation data of Ananthapadmanabhan and Goddard (*66*) shows broad agreement.

Zaslavsky et al. (*65*) ruled out the possibility of ion dipole interactions because single cation types do not affect the PEG–dextran binodal in the presence of various different anions. However these data were confined to small alkali metal ions where size and charge differences may be relatively small. In contrast, their data from PVP systems do suggest anionic effects. It is notable that the salts capable of forming biphasic systems with PEG do not form two phases at similar low concentrations with dextran. In general, the polymers which have been reported to form useful biphasic systems with salts are all monopolar Lewis bases with ether oxygens at points in their molecular structure. (PPG, PEG, PVP).

Florin et al. (*68*) proposed a model for the effect of salt on the cloud point in PEO in which water structure adjacent to polymer molecules excludes salts for significant distances into the bulk water. The approach of the hydration shells of ionic species leads to a repulsive force (the image charge force), and enhanced association between polymer molecules. Similar conclusions were derived by Garvey and Robb (*69*).

Kim (*32*) has shown, by redrawing binodal curves in terms of solubility of phosphate in PEG (for PEG–salt systems), that the concentration of salt in the top phase was described by

$$\ln M_s = \ln M_{sf} - \alpha_s M_p$$

where M_s is the molar concentration of salt in the top phase, M_{sf} is the solubility of salt in the PEG free solvent, and M_p is the molar concentration of PEG.

The molar excluded volume of PEG for salt (α_s) was shown to increase with mol wt of PEG. It was also shown that the solubility of various salts in PEG, and the concentration of PEG at the critical point of the binodal, was inversely related to the molal surface tension increment of the salt as predicted by the theory of Melander and Horvath (*67*).

A study by Ananthapadmanabhan and Goddard (*66*) has shown that the highest valency anions are more effective in "salting out" PEG. This effect was not found with a cation series. The authors suggest that the ex-

tent of hydration of salts depends strongly on valency, and so cations are subject to counterposing effects, including the extent of hydration and the tendency to interact with the PEG ether oxygen. A series of alkali metal sulphates (Na^+, K^+, Li^+) showed decreasing ability to salt out PEG linked to the strength of specific interactions with ether oxygen groups.

The inverse solubility of ethoxylated materials has been attributed to dehydration of ether oxygens at elevated temperatures (*70*). Ionic species that hydrate strongly induce dehydration at lower temperatures, thus reducing the cloud point. Other authors (*71*) propose that if image charge forces were solely responsible for this phenomenon, then, phase formation should occur with chlorides. Repulsive interaction between anions and anion-like PEG is their favored explanation. The number of moles of EO groups required for phase formation decreases with increase in molecular weight.

It appears then that different salts critically affect the physicochemical properties of PEG in solution. The interaction of cations with PEG is mediated by their interactions with associated anions and the anions' ability to interact with PEG. Cations, relatively unhindered by their corresponding anions, create a distinctly different molecular environment to those cations that are hindered by the large hydration shells and high valency of associated anions such that they become excluded from the polymer surface region. This largely accounts for the PEG salting out characteristics and the apparent difference in surface charge of PEG with different salts. This also explains the importance of protein surface charge and the dependency of protein solvation and hydrogen bond formation in the PEG environment on the ionic species present.

Electrostatic Effects in Aqueous Two-Phase Systems

It has been postulated that differential partition of salts between phases would promote difference of electrostatic potential (the interfacial potential) (*28,72*). This potential was proposed as the driving force behind partitioning in PEG–dextran systems (*14,15,28*). An early report characterized the salt effect on charge difference in this way (*17*). Potassium chloride and potassium thiocyanate produced an upper phase negative in relation to the lower phase; quaternary ammonium salts and phosphates produced a positive potential in upper phases. Sulphate produced a slight positive potential and chloride a large negative potential. A simple model was later derived (*14*) where:

$$\ln K = \ln K_O + (FZ/RT)\psi$$

$\ln K$ = partion coefficient in a charged system, $\ln K_O$ = partition coefficient in an uncharged system or with an excess of salt, F = Faraday's constant,

R = gas constant, T = absolute temperature, Z = protein net charge, and ψ = the interfacial potential.

Thus knowing the net charge on a protein enabled the calculation of the interfacial potential from:

$$\psi = (RT/FZ)\,(\delta \ln K/\delta Z)$$

$\delta \ln K$ and δZ are the net change in partition coefficient and charge respectively. ψ may then be determined from a plot of Z vs lnK.

Such methods of determining the interfacial potential have received criticism (*34,73*).

Reitherman et al. (*74*) used agar bridge electrodes to measure the potential difference between the phases of 4% dextran T500/PEG 6000 systems containing sodium phosphate from 0.11 to 0.011 *M* and sodium chloride from 0 to 0.126*M*. The interfacial potential varied from 2.8mV to approx zero as phosphate was progressively replaced by chloride. An iodinated DEAE-dextran derivative (polycationic) partitioned to the top phase in 0.11*M* phosphate, but showed complete change of phase preference as chloride incrementally replaced phosphate. Human erythrocytes (polyanionic particles) displayed opposite behavior in this system. However, although the observed electrochemical potential showed progressive decline to values approximating to zero, no negative potential was recorded.

Brooks et al. (*75*) determined the electrical potential of potassium sulphate in a 5% dextran 500/4% PEG 6000 two-phase system. Positive potential differences of between 2–3 mV were observed at potassium sulphate concentrations from 0.001 *M*/kg to 0.4 *M*/kg. Only very small potential differences were observed for systems containing potassium chloride. These results were reported to conform well with values predicted from thermodynamic considerations. This effect was seen to be largely independent of salt concentration, whereas differential partitioning carboxyhemoglobin by the addition of anionic PEG was highly sensitive to the concentration of sodium phosphate buffer (*18*).

Zaslavsky (*76*) proposed that, the observed interfacial potential difference is only a partial characteristic of the differing hydration properties of the two phases, and considers that the following description is a more realistic formulation than any involving potential differences. Thus:

$$\ln K = n^{CH_2}.E + mC$$

where n^{CH_2} is the equivalent number of CH_2 groups, E is the relative hydrophobicities of the phases, C is the partition-coefficient of 2-4-dinitrophenyl glycine, and m is the equivalent number of carboxyl groups.

In addition, similar pH dependent partition behavior which suggests that top phases are positively charged with respect to lower phases is also observed in PEG/potassium phosphate systems (*77*), and is shown in Fig.

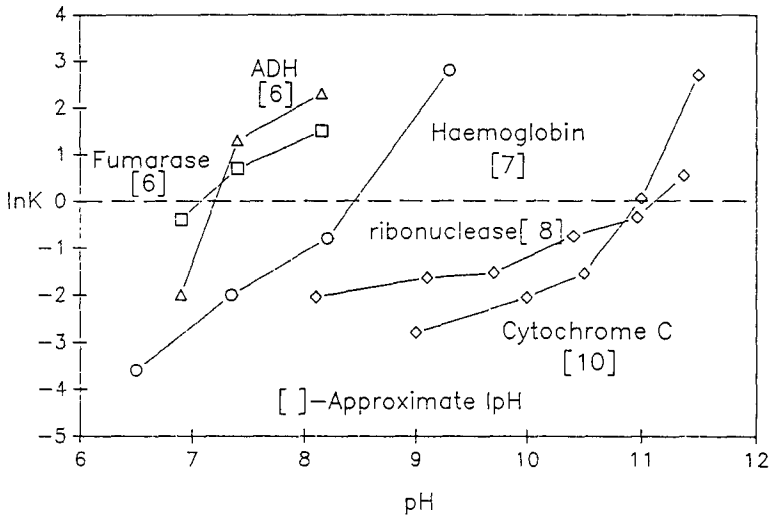

Fig. 5. Variation of partition with pH in PEG1450/phosphate ref (*77*).

5. In view of the ionic shielding effects present at high salt concentration, it is difficult to sustain the view that observed effects are strictly electrostatic in nature. Hydrogen bonding ability and hydrostatic repulsion arising from the ability of some ions to associate closely with PEG and others to be rejected may be more important here in allowing solvation or rejection of proteins in PEG phases associated with their surface charge state. Nevertheless, sophisticated thermodynamic approaches to partitioning in aqueous two-phase systems require that an electrostatic term be included in order to best mimic the observed behavior (*25–29*).

FACTORS GOVERNING THE PARTITIONING OF PROTEINS IN PEG/DEXTRAN SYSTEMS

Product Molecular Weight

The Brönstead equation has been used in justification of the assertion that the partition coefficient is a function of molecular weight.

$$K = \exp(M\lambda/RT)$$

K is the partition coefficient, M, the molecular weight of the component partitioned, R, the gas constant, T, the absolute temperature, and λ, an interaction parameter characterizing the system and the component.

Data has been presented that broadly support such conclusion (see (*19*) and Table 2). Since the partition coefficient is equally dependent upon

Table 2
Influence of Protein Molecular Weight on Partition

Protein	Molecular weight Daltons	Partition coefficient at cross point
Insulin	6000	1.3[a]
Ribonuclease A	13000	1.2[b]
Lysozyme (hen)	14000	1.3[b]
Lysozyme (turkey)	14000	1.7[b]
Papain	21000	2.3[b]
Trypsin	24000	1.1[a]
α-chymotrypsinogen	25000	1.1[a]
Ovalbumin	44000	0.7[a]
Bovine serum albumin	69000	0.3[a]
Transferrin	72000	0.3[a]
β-galactosidase	500000	<0.1[b]

Data (a) estimated, (b) taken from Sasakawa and Walter (*19*).
System compositions 7% dextran 500, 4.4% PEG 8000 with 0.1 *M* NaCl or 0.05 *M* Na_2SO_4 in 10m*M* phosphate buffer. Partition coefficient determined at varying pH in different systems and K at cross point estimated.

the interaction parameter (λ), the relationship only holds for a homologous series of compounds differing in molecular weight but having identical average interaction with the phase system. This has been clearly demonstrated for DNA fragments (*78*). On the other hand, modification of dextran with triazine dyes enables change of phase preference by salt addition (*79*). The date of Sasakawa and Walter (*19*) illustrate the subtle complexity and self similarity of the surface properties of many biological macromolecules, an observation made elsewhere for PEG–salt aqueous two-phase systems (*77*). General statements regarding the influence of molecular size (strictly surface area) are difficult but for an "average protein," λ appears to be more or less negative for PEG–dextran systems, and more or less positive for PEG–phosphate systems. K is reduced in PEG–dextran systems for higher mol wt proteins (for example, β-galactosidase compared to trypsin), and increased in PEG–salt systems provided that the systems compared are otherwise identical.

Molecular Weight of the Phase Forming Components

The general rule is given that an increase in the molecular weight of the polymer in one phase promotes a shift in partition toward the opposite phase (*80*). A recent study by Albertsson et al. (*57*) with PEG–dextran systems showed that useful manipulation of partition coefficients by

Table 3
Effect of Dextran Molecular Weight on Protein Partition
System Composition 6% PEG $M\bar{w}$. 8000 and 6% Dextran

Protein	Molecular[a] weight	K[a]		logK[b]		
		D×40	D×500	$logK_{40}$	$logK_{500}$	ΔlogK
Cytochrome *c*	12	0.18	0.17	−0.74	−0.77	−0.02
Ovalbumin	45	0.58	0.78	−0.24	−0.11	0.13
Bovine serum albumin	69	0.18	0.34	−0.74	−0.47	0.28
Lactate dehydrogenase	140	0.06	0.16	−1.22	−0.80	0.43
Catalase	250	0.11	0.79	−0.96	−0.10	0.85
Phycoerythrin	290	1.90	12	0.28	1.08	0.80
β-galactosidase	540	0.24	1.59	−0.61	0.20	0.82
Phosphofruktokinase	800	0.004	0.02	−2.45	−1.70	0.75
Ribulose diphosphate carboxylase	800	0.05	0.28	−1.3	−0.55	0.75

Data (a) taken or (b) calculated from reference 58.

alteration of dextran mol wt was possible only in the range 24000–50,000 dalton. For PEG, the useful range was from 4000 to 40,000 dalton. In the systems studied, containing varied mol wt of PEG and dextran in 10 m*M* phosphate buffer, there was little relationship between observed partition coefficients and the native mol wt of selected proteins. However, when the difference in partition coefficient (ΔlogK) recorded for high and low mol wt dextran systems was plotted in respect of protein mol wt, a linear relationship was found for molecules up to about 250,000 dalton. Further increase in protein size produced little change in ΔlogK. Approx linear relations were also shown between ΔlogK in increasing mol wt of PEG and protein size up to 250,000 dalton. In general, change of partition coefficient with altered polymer mol wt was the smallest for low mol wt proteins (Table 3). The study (*57*) was conducted at identical w/v compositions but at differing tie line length which may account for the poor correlation of behavior with predictions derived from Flory Huggins theory. There are clearly many exceptions to the general rule. For example, IgG has been reported to display greater solubility in higher mol wt of PEG in PEG–dextran systems (*81*), however, interferon-β shows a concentration dependent increase in partition coefficient apparently driven by hydrophobic effects (*82*).

In addition, the partition of proteins may be manipulated by increasing the concentration of the phase forming components (increasing TLL). In general, the effect of increasing the TLL in PEG–dextran systems is to lower the partition coefficient of proteins (*83*). The reverse is the case for increase in TLL in PEG–salt aqueous two-phase systems (*77*).

The Effect of Added Salts

The effect of salts on the phase diagram of polymer–polymer and polymer–salt biphasic systems has already been discussed. Proteins show changes in partition coefficient in relation to their net charge above and below their isoelectric point, and this effect increases with TLL (*80*).

Positively charged proteins display increases in partition coefficient dependent upon salt type following the series: perchlorates > thiocyanates > iodide > bromide > chloride > fluoride > sulphates > phosphates. The latter three salts will generally reduce the partition coefficient of such polycations. The reverse is the case for proteins bearing net negative charge (*80*). These effects are observed up to about 0.25*M* salt concentration, and above this level, protein partition coefficients increase as a result of salting out effects (*14*).

FACTORS GOVERNING THE PARTITIONING OF PROTEINS IN PEG-SALT SYSTEMS

Relatively few of the PEG salt systems detailed in Table 1 have been seriously examined with regard to their properties for macromolecular partition. Citrate based systems have recently received attention, whereas others based on sulphate have been examined in the context of specific, rather than generic separations (*84*). There are major differences between the useful operating pH range of citrate, phosphate, tartrate, and carbonate systems, since it is the polyvalent anion that is principally responsible for phase separation. Other systems, particularly those based on sulphates, may require additional buffering provision.

The useful mol wt range of PEG for partitioning in PEG phosphate and other systems extends from below 1000 dalton to as high as 8000 dalton, although 3000 dalton is a more common upper limit. Partition coefficients of proteins reduce with increase of PEG mol wt (*77*), but most proteins show changes of phase preference between PEG mol wt 1000–2000 dalton (*77*). Increasing the TLL in PEG-salt two-phase systems causes protein and (to a lesser extent) nucleic acid partition coefficients to increase. Carbohydrate partition coefficients decrease with increase of TLL (*77*). As stated earlier, proteins show increase in partition coefficient above the isoelectric point. These effects are most pronounced for proteins in systems composed of intermediate mol wt of PEG. The effect is lost in high mol wt systems because of the excluding effects of PEG, and it is less pronounced where partition coefficients are already high, e.g., lower mol wt PEG.

Addition of neutral salt (NaCl) may cause a reversal of partition of proteins above and below their pI, although the effect has not been rigor-

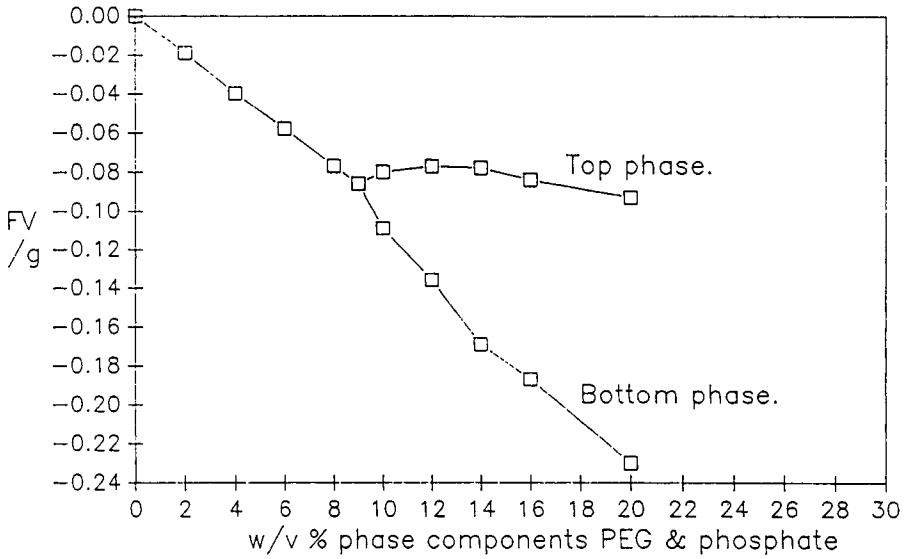

Fig. 6. Free volume changes of PEG3330/phosphate (ref. *77*).

ously investigated (*20,24*). This is similar to the replacement of phosphate by chloride in PEG/dextran systems.

Some workers (*85,86*) have attempted to correlate the partitioning of proteins in PEG/salt aqueous two-phase systems with change in free volume of the phases. Change in free volume is expressed as

$$\Delta FV = 1/\rho_{phase} - 1/\rho_{ref} \qquad (85)$$

ρ is the density of the phase or a reference solution.

It is considered to reflect the water available for solvation in a phase or solution and as such, correlates well with one factor (TLL) of importance in partitioning in PEG/salt systems. As TLL increases, the concentration of salt in the lower phase increases rapidly, but remains relatively constant in the top phase where it is at the limits of its solubility in the PEG solution (Fig. 6). The tendency for protein partition coefficients to increase with TLL in such systems can be seen to be related to salting out subject to the excluded volume effects of PEG. At the limit, proteins will be precipitated. This view of partitioning in PEG/salt aqueous two-phase systems has been modeled using the modifications of Sinanoglu's solvophobic theory given by Melander and Horvath (*67*) combined with excluded volume theory (*32*). The model was principally developed to describe precipitation at high salt concentration and high polymer mol wt, but a partition coefficient could be derived. Thus:

$$\ln K = C - K_s(M_s)_2 \exp[\alpha_s M_2] + K_s(M_s)_1 - \alpha_p M_2 \qquad (32)$$

C is a constant, K_s is the protein salting out constant, $(M_s)_2$ is the concentration of salt in the polymer phase, $(M_s)_1$ is the concentration of salt in

the salt phase, α_s and α_p are the excluded volumes of the polymer for salt and protein, and M_2 is the concentration of polymer in the polymer rich phase.

Clearly many of the parameters require determination for each protein and polymer mol wt used. In addition, protein solubilities and PEG excluded volumes for protein vary with pH and type of salt used (*32,43*). Neither the excluded volume model nor Kim's model (*32*) specifically account for the changes in protein surface properties with pH which have been shown to have profound effects on partitioning in PEG/salt systems (*77*). (see Fig. 4 and (*77*).) In this study, a simple conceptual model was presented to aid in design of PEG/salt separations by manipulation of polymer mol wt, TLL, and operational pH.

AFFINITY PARTITIONING

Preparation of Suitable Ligands

The chemical methodologies associated with the preparation of affinity ligands for application in aqueous two-phase systems has been the subject of a number of recent reviews (*87–89*) and papers (*90,91*), and will not be further considered here. Apart from the preparation of particles for use in affinity partitioning, the general approach has been to modify one of the phase-forming polymers as ligand carrier. However, in principle, any molecule offering suitable leaving groups, partition coefficient, and surface properties, particularly the absence of conflicting activities, could be used. It is considered advantageous to prepare monofunctional polymer ligands in order to avoid product crosslinking and affinity precipitation effects (*92*), and this may be achieved with PEG, either by proceeding from monomethoxy PEG or by controlling the degree of chemical activation (*89*). Higher degrees of activation are achievable on dextran because of the large number of hydroxyl groups present, and this can affect its partitioning behavior (*79*).

Application

Affinity partitioning is perceived to have several advantages over strategies involving solid phases for affinity adsorption (*22*). Approach to equilibrium is likely to be faster because there are minimal diffusion limitations in comparison to enclosure of the ligand in a solid phase. Affinity partitioning has a higher volumetric capacity (*22*) that reduces operational scales, and continuous processing may be a real option. Affinity interactions in solution phases are generally characterized by lower losses of biological activity than those in solid phases (*83*).

The first report of affinity partitioning was by Takerkart et al. (*93*) in 1974, and it concerned the partitioning of trypsin in response to diamidino-α-diphenylcarbamoyl PEG. Flanagan and Barondes (*94*) gave the first rigorous description of the method, along with a thermodynamically derived expression describing the effect (*95*) that is given here in modified form (*96*). Thus:

$$K = K_o (1+[L]/K_{Dt})^n / (1+[L]/K_{Db}K_L)$$

K is the partition coefficient in the presence of ligand, K_o is the partition coefficient in the absence of ligand but in an otherwise identical system, [L] is the ligand concentration in the top phase, K_{Dt} is the dissociation constant in the top phase and K_{Db} is the dissociation constant in the lower phase, K_L is the partition coefficient of the ligand in absence of affinant, and ^{n} is the number of binding sites on the affinant macromolecule.

From this expression, it is apparent that a large partition coefficient of the ligand (K_L) will favor partition of the affinant to the upper phase. Thus, depending on the direction of the desired partition, K_L should be of one extreme. A one-sided partition of the ligand itself is sometimes found, for example, in the partitioning of concanavalin A with dextran as ligand (*94*), or in the interaction of tRNA with isoleucyl tRNA synthetase (*96*). However, it is more usual to covalently attach the ligand to one of the phase polymers to achieve more or less one-sided partition. This does not necessarily lead to high or low ligand partition coefficients, since partition coefficients of polymers in aqueous two-phase systems do not necessarily assume extreme values (*83*). For example, K_{PEG} was found to vary from 2 to 12 at TLL 6–18% (w/v) in a PEG 6000/dextran system (*83*). Affinity partitioning will, therefore, be favored by operation at longer TLL that lead to more extreme partitioning of the polymers. Higher mol wt of the polymer used to prepare the polymer ligand will also favor more extreme partition coefficients.

Alteration to the partitioning properties of the fundamental system containing no ligand can be assumed to occur if the ligand is charged or markedly hydrophobic, especially at high degrees of substitution. As might be expected from their effect on the phase diagram, the partition coefficient of PEG–ligand and dextran–ligand is influenced by the type and concentration of salts added to the PEG dextran phase system.

Kula et al. (*97*) recorded values of K_L for PEG-Cibacron blue F3GA conjugate of 17 in the presence of chloride and acetate, and these values remained broadly constant with increasing concentration of salt. In the presence of phosphate, K_L increased from 20 to about 70 at 0.2 *M* and thereafter remained constant. The partition coefficient also increased with the amount of conjugate added to the system. It is apparent then, that K_L in the equation describing affinity partition is not constant, but changes with amount of ligand and in respect of feedstock addition. It is clear that

results of partition may be difficult to predict or fit to such an ill-defined relationship.

Dissociation constants (K_{Db} and K_{Dt}) are more sensitive to the concentration and type of added salt than is observed for solid phase affinity systems (*97*). This necessitates operation with much lower ligand partition coefficients than would otherwise be practically possible, and may hinder integration of affinity partition steps into purification schemes involving PEG–salt systems. However, in one study, phosphate was found to have little effect on the partition of phosphofructokinase (*98*), and so the above may not apply in every case. Free ligands present in cell extracts and crude homogenates may also be expected to affect the results of affinity partitioning.

The observed partition coefficient resulting from the affinity interaction increases with TLL in PEG–dextran systems. However, in low mol wt systems (PEG 4000), this effect has been observed to reach a maximum and to subsequently decline (*99*). In higher wt of PEG, no such maximum was observed over a similar range of TLL. Similar effects were found in studies of PEG–palmitate affinity interactions (*100*).

It is almost invariably found that, at constant affinant challenge, increases in ligand concentration cause increase in $\Delta \log K$ until some saturating value is reached. Kula (*95*) has shown that the capacity of affinity partitioning systems is not constant but rather depends on K, the volume ratio, and the required yield. For general affinity ligands, the dissociation constant determines the selectivity of the interaction and the number of binding sites determines the yield of the partitioning step (*85*).

Albertsson has shown that, for two interacting particles having a small area of contact, K_{AB} approximately equals $K_A \cdot K_B$, wehere K_{AB} is the partition coefficient of the complex, and K_A and K_B are the individual partition coefficients of the interacting particles. This is because most of the original molecular surfaces remain involved in interactions with the phases following association. Significant departures from this are indicative of larger molecular contact areas or significantly different dissociation constants in the two phases (*101*). Some theoretical expressions of affinity partition have assumed that K_{Dt} and K_{Db} were identical. Significant effects on K_E can be expected if

1. K_{Dt} is not equal to K_{Db},
2. K_L is affected by the presence of the affinant or other additives to the system, or
3. the ligand alters the system in such a way that K_O would have changed, unrelated to the bioaffinity effect.

Kula has investigated K_{Dt} and K_{Db} assuming K_o and K_L to be unaffected by the affinity partitioning and measuring K_L and K_O in the absence of enzyme and ligand, respectively. Best fit solutions of the experimental

Table 4
Theoretical Interactions of Polymer Ligands Bound to Proteins

1. Volume fraction of polymer is depleted at protein surface.
2. Increasing polymer length favors dissociation of complex.
3. Solvent encountered by polymer tail differs from bulk solution. K_L does not measure free energy of transfer of polymer ligand. Result is an underestimation of apparent number of binding sites.
4. Surface cloud of polymer tail inhibits further binding of ligand.
5. Binding of polymer ligands is weaker to larger particles.
6. The more unfavorable is the polymer tail interaction with the bulk. The more favorable is the association of polymer ligand and protein.

Summarized from reference 105.

data indicated that association constants of formate dehydrogenase for PEG-dye were stronger in the dextran rich phase than the PEG rich phase.

A study by Johansson (*102*) in which it was assumed that

$$\Delta \log K = n_{app} \mathrm{Log} K_L$$

that is, that K_{Dt} is approx equal to K_{Db} showed that although n_{app} (the apparent number of ligands bound per molecule) was independent of concentration of polymers, it did depend to a large extent on the type of ligand carrier employed in the system. In three out of four phase systems, n_{app} was highest when the polymer ligand was constructed from a polymer different from that forming the host polymer phase. This polymer ligand was Ficoll 70 (polysucrose), but it also gave the highest partition coefficient in a self-similar phase system. Kopperschläger has reported that, values of n_{app} may differ greatly from the number of binding sites determined by other methods such as equilibrium dialysis. (*103*).

An important theoretical study (*104*) utilized a lattice model to examine the interactions of a polymer ligand bound to the surface of a protein particle. Polymer tail conformations are generated by random walks into the lattice from the particle surface. A set of probabilities for the tail conformation was generated by a matrix procedure, and the Helmholtz energy of a bound tail expressed relative to a free tail. The model is overtly simplistic, but it qualitatively confirms what has been observed in practice. The principle results of the study are summarized in Table 4. The results explain the findings of Cordes et al. and Johansson et al. For self-similar polymers, the solvent at the particle surface is a worse solvent than the bulk. In contrast, for dissimilar polymer pairs, the bulk solution is the poorer solvent. The binding strength of dissimilar polymer pairs should increase with TLL, and vice versa.

Many of the findings of this study agree qualitatively with practical observations. In addition, it should be possible to verify them experi-

mentally. Perhaps the most important feature is that, the results suggest that the choice of ligand carrier is not as simple as has previously been assumed. Investigation of different types of ligand carriers could clearly be rewarding.

PARTICULATE AFFINITY PARTITIONING SYSTEMS

Particles, present in the form of cells or organelles, have been widely studied by partitioning and many reviews are available (*105,106*). However, published attempts to utilize adsorbent particles in biphasic systems as a purification strategy are few. Attempts have been made to immobilize some of the phases on celite or other particles to perform liquid–liquid partition chromatography (*107*). Recent advances have been achieved in this area particularly in the chromatography of nucleic acids (*108*). However, difficulties arise because of increased droplet sizes reducing surface areas for efficient mass transfer, and the limitations inherent in pore diffusion from viscous solutions. These experiments necessarily run over much longer time periods than conventional chromatographic processes. Other applications of particles arise in conventional partitioning strategies at preparative scale where only a limited number of equilibrium partition stages can be achieved. Phases can be loaded to chromatographic matrices for further purification either after ultrafiltration, or more directly to hydrophobic matrices (*109,110*).

Because of their relatively large size, most particles often show a one-sided phase preference. Use of particles, particularly when coupled with affinity ligands, might be expected to reduce losses of ligand over free liquid affinity systems, and physical containment may be easier than reliance on thermodynamically controlled partition. By careful manipulation of surface chemistry, the phase preference of particles may be selected, provided that densities do not greatly exceed those of the phases. Surface treament with one or other of the phase polymers allows directed partition. Coating of surfaces with diol groups, as is conventionally done in the preparation of HPLC silica adsorbents, results in strong preference for the PEG phase (*111*), whereas coupling of acrylamide has an opposite effect (*108*).

Hedman and Gustafson (*112*) prepared PEG-Sepharose and methoxy-PEG-Sepharose that was subsequently modified with γ-globulin for the preparation of protein A. Serum albumin was also recovered using Cibacron blue-Sepharose in an aqueous two-phase system. Attempts to use the latter adsorbent in PEG/phosphate systems for the recovery of ADH resulted in poor recoveries. Frej et al. (*113*) purified β-galactosidase on phenyl Sepharose in a PEG-phosphate system followed by further purification by ion exchange and size exclusion chromatography.

Mattiasson and Ling (*114*) modified Sepharose beads with high molecular weight PEG, and subsequently coupled Cibacron Blue for the purification of ADH. Binding was accomplished in the presence of debris with subsequent addition of polymers (PEG and dextran) to separate the particles from the cell debris. It was claimed that, by allowing multipoint attachment of ligand through the incorporation of spacer arms, yields could be increased from 35 to 90%.

The partition of a wide range of derivatized and underivatized particles was examined by Ku et al (*115*). Unfortunately, the precise conditions in terms of polymer concentrations and salt type and concentration were not reported. However, inference may be drawn from the fact that, positively charged particles partitioned to the top phase, and vice versa. These authors deployed dye derivatized particles in a PEG/dextran system in which the bulk of the proteins partitioned to the lower phase. Poor binding of ADH was found unless the particles were deployed prior to system formation. Enhanced elution was found in a biphasic system over recovery in a single liquid phase. This was attributed to the partition process contributing an extra driving force for elution, and an extra resistance to binding in the overall equilibrium process.

SCALE UP OF PARTITIONING IN AQUEOUS TWO-PHASE SYSTEMS

Factors that influence equipment selection and performance for partition are interfacial tension, phase viscosities, and differences in phase densities. At laboratory scale, such considerations have influenced the design of devices for multistage contacting to improve resolution. These include Albertsson's shallow-chambered CCD device and the planet coil centrifuge of Ito (*116,117*).

Low interfacial tensions ($10^{-7}Nm^{-1}$ to $10^{-4}Nm^{-1}$ from PEG/dextran to PEG/salt systems) mean that mixing is uncomplicated, requiring low power input and achieveable in static mixers (*118*). However, low density differences, and interfacial tensions coupled with high viscosities militate against phase separation. Thus, gravity settling of phase systems may take several hours when biological material is included in the system and the performance of simple settlers is compromised. In addition, such approaches rarely coalesce secondary break efficiently. Stage efficiencies of Kuni/Scheibel column extractors are much reduced and throughputs lowered (*119*). Dahuron and Cussler have studied a system in which the problem of phase separation is avoided by holding the interface of the aqueous two-phase system within the pores of a hollow fibre membrane by differential pressure (*120*). However, mass transfer in such a system is much lower than in completely mixed systems (*121*). Since the penetration of a pore by one liquid suspended in another is dependent on the

interfacial tension (*122*), equilibrium may be difficult to achieve and the control of the phase separation may be compromised by temperature differences and emulsion formation (*123*).

Published process schemes have so far involved enhanced gravity separations using disc stack liquid–liquid separators with or without nozzle discharge, depending on lower phase viscosities (*124*). The attraction of the method at this scale lies in the recovery of intracellular products from cell homogenates, since in PEG/salt systems, debris may be partitioned to the lower phase. Some degree of purification is normally achieved at this step, combined with the removal of contaminating carbohydrates and nucleic acids (*125*). Application of the method in the presence of cells or debris is the basis of several patents (*126*). The principal advantage of proceeding in this way at large operational scales is the replacement of a difficult solid-liquid separation step by a liquid–liquid separation (*126*). Other methods for achieving this are available, including, flocculation or heat treatment, and so on, but may be of more limited applicability (*9*). Additional purification may be achieved by combining a number of sequential partitioning steps (normally two and rarely more than three). These may be considered to replace the early enrichment steps of protein recovery schemes such as fractional precipitation (*127*), Fig. 7. In the initial stage of such schemes, partition coefficients are usually in the range 2–20, although occasionally, much higher values may be found as in the purification of β-galactosidase ($k = 62$) (*125*) or β-interferon ($k = 350$) (*82*). Purification factors of 2–20 are attainable at this stage, and in multistage procedures, purification factors of from 2 to 30 are generally found (*21*).

FUTURE PROSPECTS

Despite the collection of large quantities of empirical data, and the construction of sophisticated thermodynamic models of partitioning in aqueous two-phase systems, the technique is not widely adopted in either laboratory or process plant where purification procedures are a routine requirement. The reasons for this are not difficult to elucidate. Application of the method is not well understood outside of an immediate circle of "partitioners." Other purification techniques have undergone intense commercial promotion and exploitation that has led to the wide dissemination of techniques and methodologies. Successful application of the partitioning technique is currently an empirical process. Short cuts to success are more difficult because of the number of variables involved. At present, this contrasts unfavorably with (for example) adsorption strategies in which the starting point for fruitful fine tuning of separations is often readily defined. In addition, the resolution possible in limited stage equilibrium processes seems to have been widely misunderstood. This is exacerbated by the subtlety of difference in surface properties of many

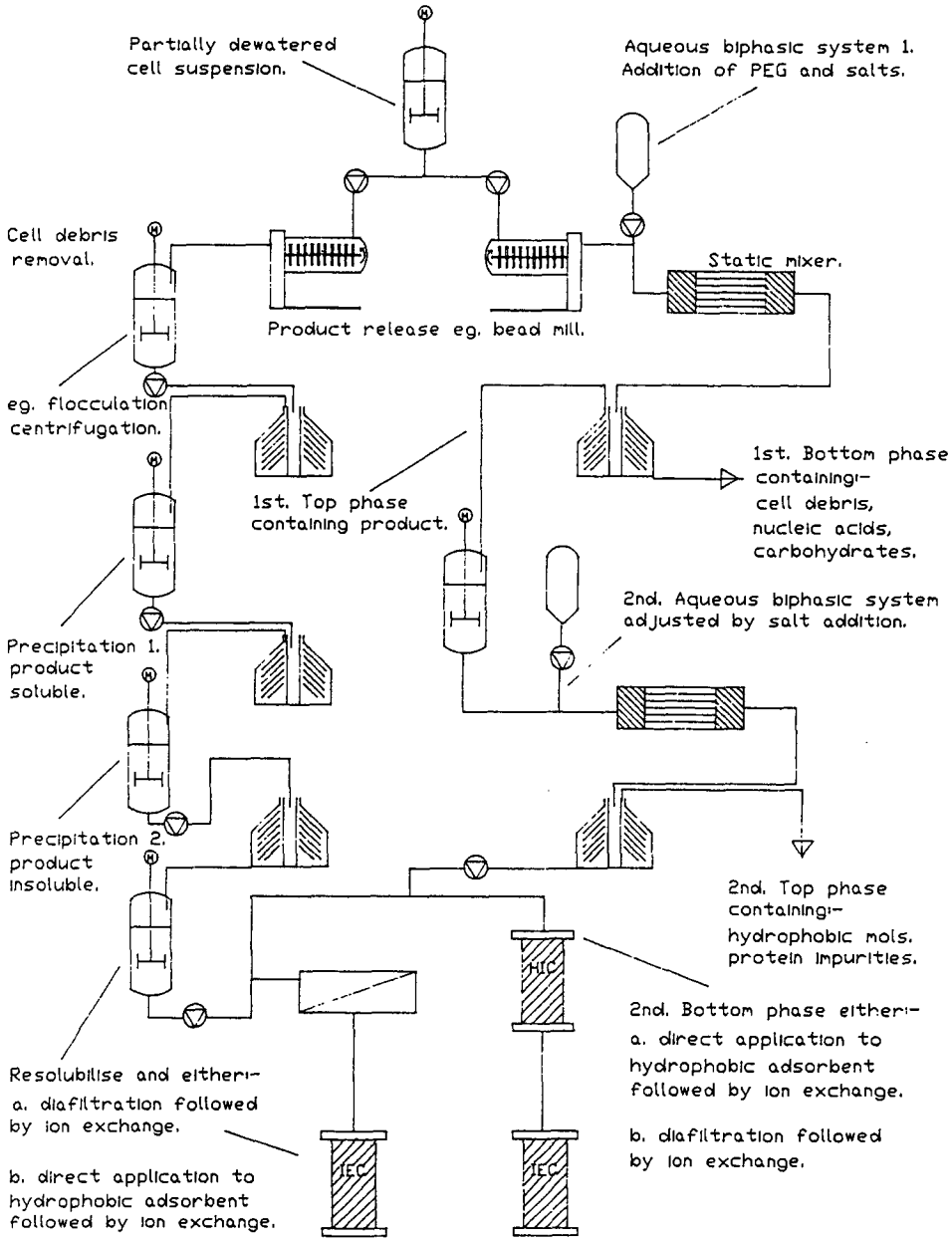

Fig. 7. Primary fractionation steps in intracellular protein recovery. Comparison of precipitation to aqueous two-phase systems.

biological macromolecules that has not been fully appreciated. Adoption of novel process strategies at large operational scale is inherently conservative. Such is not confined to aqueous two-phase partition, but applies equally to bioaffinity processes except in high value-low volume applications. There can be little doubt that the adoption of partitioning at large scale is hindered by the perceived limitation in resolution and the sup-

posed difficulty in recovery of products from the system. Most serious doubts concern the strategies for recycling phase-forming components that otherwise have high replacement and waste management costs.

However, recent developments outlined here would suggest that there is considerable scope for further innovation in the area, including the examination of new systems and polymers. The developing understanding of the nature of partition depends primarily on the properties of PEG in aqueous solution. The polymer may be likened to a linear crown ether in its acceptance and rejection of particular cations, mediated by the size and valency of anions. This growth may lead to a renewed period of rapid development in aqueous partitioning technology. Many novel PEG/salt systems can now be systematically examined, and it can be expected that aspects of polymer structure will receive analytical attention. This may lead to developments in those areas which are currently restrictive. Indeed, a recent patent reports on the dramatic enhancement of partition coefficient by the addition of low percentages of polyacid or polyacid salt to aqueous two-phase systems (*128*). Technological developments may also be expected based on these renewed investigations, such that, resolution may be improved. Magnetic derivatives of dextran have already been applied, and similar derivatives of PEG are possible (*129,130*). Efforts will be directed to improved performance of affinity partitioning, and to the development of effective multistage contactors having improved ability to rapidly separate phases. The examination of new phase systems and polymers may lead to developments in the recovery and recycling of components required, not only for economy, but also for reasons connected with batch identification in regulated processes.

In the long term, it is not clear to what extent the technique can survive the current pressures and efforts to foreshorten purification procedures by developments in direct feedstock extraction. It may be that the real benefits deriving from this technology lie in the increasing understanding of the molecular interactions that dominate the partitioning process. Such understanding may produce unforeseeable developments and "spin-off" in other areas of purification, and of potential importance outside the biochemical recovery area altogether.

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