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Variation of protein partition coefficients with volume ratio in poly(ethylene glycol)–salt aqueous two-phase systems

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Abstract

Partition of total soluble protein from brewery waste in poly(ethylene glycol) (PEG)–phosphate aqueous two-phase systems reveals that the partition coefficient varies with change in volume ratio. This phenomenon has been confirmed by the examination, in more detail, of the behaviour of a pure protein (bovine serum albumin) in systems of widely different volume ratio and tie line length. The results invite interpretation in terms of salting out of protein from the lower phase. The behaviour of a number of pure proteins in hydrophobic interaction chromatography and in PEG salt aqueous two-phase partition has been compared and the results support this interpretation. This is judged to provide a useful rationalisation of three important strategies for the large-scale downstream processing of proteins, namely, precipitation, PEG salt partitioning and hydrophobic adsorption. In addition hydrophobic interaction chromatography may prove to be of benefit in "method scouting" for the development of partitioning strategies in protein purification.

1. Introduction

It is generally accepted that Nernst's distribution law applies to the partition of proteins in aqueous two-phase systems formed as a result of the phase separation of mixtures of polymers or mixtures of polymer and salt [1]. This is universally interpreted to mean that the distribution or partition coefficient takes its simplest possible form and thus may be expressed as the ratio of the concentrations of the protein in the top and bottom phases of an aqueous two-phase system at equilibrium [2]. This view is held despite the fact that proteins are complex biological polymers whose molecular surfaces are extensively hydrogen bonded with the surrounding water molecules in aqueous systems and thus potentially may lose or gain hydrogen bonds under particular conditions in such systems [3]. Such changes would lead, under a strict interpretation, to a violation of the assumptions underlying the application of the simplest form of the partition coefficient which only applies if a single molecular species is considered to be distributed between the phases [4].

Additionally it is often assumed that a linear partition isotherm is applicable to the modelling of protein partitioning in aqueous two-phase systems so that the variation in concentration of added material has little or no effect on the resulting distribution [5]. Each of these factors contribute to the assumption that the partition coefficient of a solute partitioned in an aqueous two-phase system at equilibrium distributes in a way which is independent of the relative volumes of the two phases. To some extent this may well

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be due to the fact that the greatest wealth of data on partitioning exists for polymer–polymer systems where, for reasons which will become apparent later, such assumptions may be permissible. Poly(ethylene glycol) (PEG) salt systems on the other hand have not been studied in such detail and effects contradicting these assumptions may well have been overlooked.

In a recent paper [6], we reported an apparent variation in partition coefficient with volume ratio in the partition of total intracellular protein from brewer's yeast along with limited data, in support of this observation, for some pure proteins partitioned in PEG–phosphate aqueous two-phase systems. In this paper the variation of protein partition coefficient with volume ratio is examined in more detail for a single pure protein. The implications of this for an understanding of protein partition in PEG–salt aqueous two-phase systems are discussed in the light of some observations from the literature and from some additional experiments concerning the salting out of proteins and their distribution between a mildly hydrophobic solid phase and a mobile phase containing salt in hydrophobic interaction chromatography (HIC).

2. Methods

2.1. Preparation of aqueous two-phase systems

Poly(ethylene glycol) of molecular masses of 1450 and 1000 was obtained from Sigma (Poole, Dorset, UK). Potassium dihydrogen orthophosphate and dipotassium hydrogen orthophosphate were obtained from BDH (Atherstone, Warwickshire, UK). Phase diagrams were prepared using the cloud point method [7] and tie lines fixed using the known relationship between volume ratio and the intersection by the tie line of the overall system composition and the binodal curve [2]. In the experiments on the partition of pure proteins at differing volume ratio the systems were not made up from the overall composition as represented by points read directly from the fitted tie line. Instead a large (500 g) system was made up for each tie line having a volume ratio of 1.0 as read from the tie line. Subsystems, differing only in volume ratio were then prepared by admixture of appropriate volumes of top and bottom phase, drawn from the large initial system, to give the desired phase ratio. Subsequently, freeze-dried aliquots of pure proteins (obtained from Sigma) were added by weight to these systems to give a final concentration of approximately 1 mg/g.

PEG–phosphate systems for the volume ratio experiments were prepared using PEG 1450 and dipotassium hydrogen orthophosphate (pH 9.2). PEG 1000–phosphate systems used in correlating the hydrophobic chromatography data were prepared from appropriate amounts of the di- and monobasic potassium phosphate salt to give a final pH of 7.5. Partition in these systems was conducted at four different tie line lengths. This is procedurally necessary in order to partially characterise the partitioning behaviour of proteins for correlative purposes since their partition coefficients may vary widely with tie line length within a single biphasic system (as will become apparent later). For obvious reasons the phase ratio of these systems was fixed at unity and the tie line lengths (TTL) selected were 28.8, 35.6, 41.2, and 47.0% (w/w). Details of the preparation and partitioning of total soluble protein from wet milled brewer's yeast may be found elsewhere [6].

Assay of total protein from yeast preparations was measured by the method of Bradford [8]. Pure protein preparations were measured by spectrophotometric absorbance at 280 nm.

2.2. Hydrophobic interaction chromatography

Hydrophobic interaction experiments were performed using a chromatography system consisting of an LKB 2150 HPLC pump, an LKB 2152 controller equipped with gradient mixing valve, and a Rheodyne sample injection port fitted with a 20-μl sample injection loop. The column was a Bio-Rad PEG 300-10 of diameter 4.6 mm and length 150 mm having a poly(ethylene glycol)-capped silica matrix of 10-μm particle size as supplied by Bio-Rad RSL Bel-

gium. Output was monitored using an LKB 2158 Uvicord SD at 278 nm connected to an LKB 2210 chart recorder. All experiments were conducted at a flow-rate of 0.2 ml/min and 0.2 AUFS. Chromatograms were developed isocratically and retention times monitored for pure protein samples obtained from Sigma Chemicals. Running buffer composition was varied from run to run using gradient mixing between 2% (w/w) potassium phosphate pH 7.15 and 30% (w/w) potassium phosphate pH 7.48. Buffers were prepared from di- and monobasic potassium phosphate salts of "Hipersolv" grade obtained from BDH. The difference in pH between the high and low salt concentration buffers is due to the dilution effect on the activities of the ions in preparing low-concentration buffer from high-concentration buffer. It was considered to be more important to maintain the relative compositions of the salt solutions rather than their pH whose variation was unavoidable. Protein retention times were monitored isocratically at varied salt concentration and expressed as the distribution coefficient ln D where

$$D = \frac{V}{V_0} - 1 \tag{1}$$

V is the retention volume under given conditions of salt concentration and V_0 the retention volume using 2% (w/w) potassium phosphate pH 7.15 as the running buffer.

3. Results and discussion

3.1. Partition of total soluble protein from yeast

Previous work on the partition and recovery of a total soluble protein fraction from wet milled brewer's yeast [6] indicated an apparently large variation of the partition coefficient with change in volume ratio. In this type of recovery operation utilising partition a low molecular mass of PEG has to be used in order to raise the partition coefficient of the bulk of the intracellular protein above unity. Under these conditions the partition coefficient of total soluble protein increased from less than one to almost 20 as the volume ratio was decreased from 5 to 0.3. Unexpectedly, this resulted in the total recovery of protein remaining rather constant (ref. 6). It has been generally assumed [2] that the partition coefficient remains constant with volume ratio so that it is possible to quantitatively manipulate product yields to one or another phase by manipulation of the volume ratio. In the case of increasing yield to the top phase the following expression would apply

$$Y_t(\%) = \frac{100}{1 + \frac{V_b}{V_t} \cdot \frac{1}{K}} \tag{2}$$

where $Y_t(\%)$ is the expected yield to the top phase, V_b and V_t are the volumes of the bottom and top phases, respectively, and K is the observed partition coefficient. Under the circumstances outlined above such a relationship would not correctly predict the outcome of attempts to maximise the yield by manipulation of volume ratio.

3.2. Partition of a model protein at different volume ratios

In view of the wide ranging implications of these findings, and the theoretical considerations outlined in the introduction, the effect of volume ratio on partition was investigated for a number of pure proteins including bovine serum albumin (BSA).

Fig. 1 illustrates the PEG 1450 potassium phosphate phase diagram used to prepare the systems in these experiments. The figure also shows the tie lines determined and the points representing systems having a volume ratio of 1.0. Fig. 2 shows the results obtained for the partition of BSA in a system well removed from the critical point (system 2 of Fig. 1) at volume ratios varying between 0.2 and 5.0. Several features of this figure are worth emphasising. The concentration of protein in the lower phase is low and constant throughout. The concentration in the upper phase increases as the log

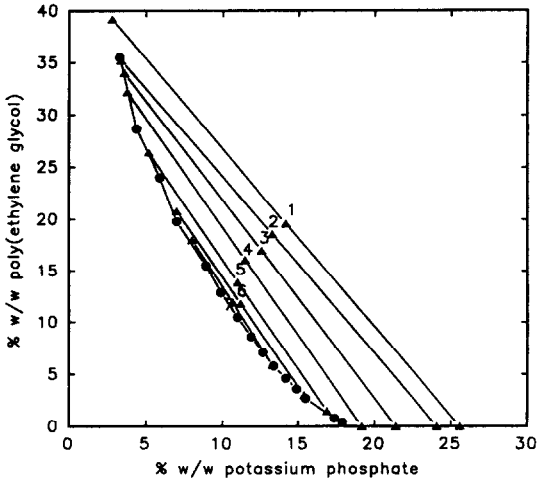

Fig. 1. Phase diagram of the PEG 1450 dipotassium hydrogen orthophosphate phase system showing experimentally determined tie lines and systems (1–7) having a volume ratio of 1.0 (△). These systems were used to construct systems of varying volume ratio but lying on the same tie line.

volume ratio decreases. In the extreme conditions of very low volume ratio, the total recovery (from both phases) is greatly reduced indicating that there is also an equilibrium with a solid-phase under these conditions. Conditions of PEG and salt concentration in the co-existing phases are sufficient to precipitate a proportion

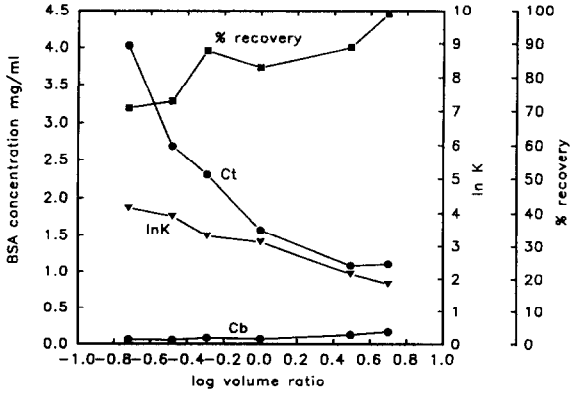

Fig. 2. Partition of BSA in a PEG 1450–potassium phosphate system well removed from the critical point and corresponding to tie line 2 of Fig. 1. [TLL = 38.4% (w/w)]. Variation of total recovery (■), protein concentration (●) top (Ct) and bottom (Cb) phases and partition coefficient (▼) are shown expressed relative to the log of the volume ratio of the system.

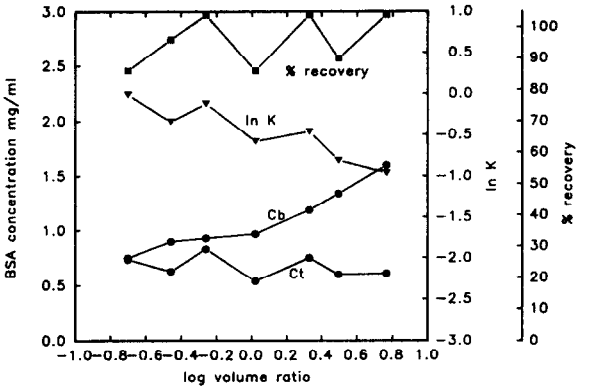

Fig. 3. Partition of BSA in a PEG 1450–potassium phosphate system relatively close to the critical point and corresponding to tie line 5 of Fig. 1. [TLL = 27.5% (w/w)]. See Fig. 2 for symbols.

of the added protein. Fig. 3 illustrates data relating to the partition of BSA in a system now chosen to be very much nearer to the critical point (system 5 of Fig. 1). Although subject to noise in its estimation, the total recovery is more constantly close to 100% than before. Note that the phase preference (phase of highest concentration) of the BSA has changed. At the lowest volume ratio the partition coefficient is approximately one but as the volume ratio increases the concentration in the top phase remains relatively constant whilst that in the bottom phase increases. As a consequence the partition coefficient falls but now only by approximately one log. In system 7 of Fig. 1, illustrated in Fig. 4,

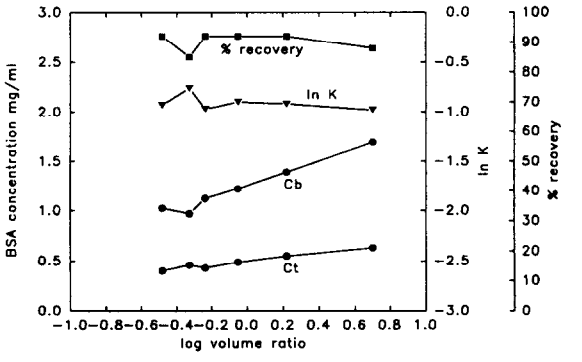

Fig. 4. Partition of BSA in a PEG 1450–potassium phosphate system close to the critical point and corresponding to tie line 7 of Fig. 1. [TLL = 13.2% (w/w)]. See Fig. 2 for symbols.

and lying closest to the critical point, overall recovery is high and the partition coefficient is constant with volume ratio. However even under these circumstances ideal partitioning behaviour is not observed. Addition of very small molar quantities of protein to systems prepared close to the critical point has profound effects on the phase diagram. The addition of protein solutes caused the observed volume ratio (V_r) to depart to a considerable extent from the expected value. For example systems prepared to give V_r 3 gave $V_r < 2$ and systems prepared to give V_r 0.2 gave $V_r > 0.3$. At the critical point very small amounts of water or any one of the phase-forming components added to the system will have profound effects on the resulting two-phase system. It is not surprising that small amounts of protein have the same effect. We conclude that there is, apparently, only a rather small region of the phase diagram in which partition of protein can be expected (in many cases) *not* to depart from simple theoretical laws. For BSA in the PEG 1450–potassium phosphate system this region can be approximated by the area bounded by the tie lines of systems 5 and 7 of Fig. 1.

Fig. 5 shows the results of the partition of BSA in the PEG 1450–potassium phosphate

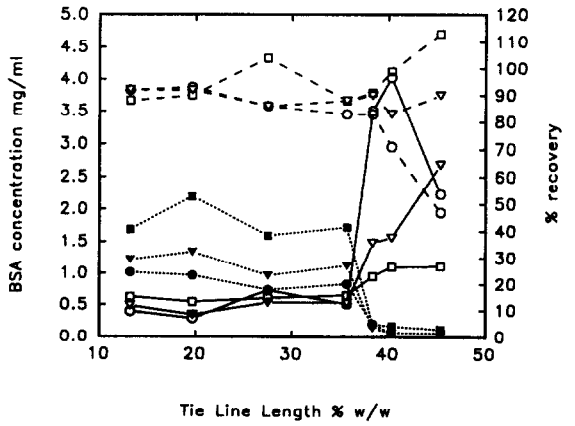

Fig. 5. Composite figure showing the partition of BSA for all tie line lengths examined in a PEG 1450–potassium phosphate system at different volume ratios. Broken lines correspond to total recovery of BSA, dotted lines to concentration of BSA in the lower phase and solid lines to concentration in the upper phase. Symbols (whether filled or solid) correspond to volume ratios of 5.0 (□), 1.0 (▽) and 0.2 (○).

phase system for all the tie lines examined. The partition coefficient is at first relatively constant for all volume ratios in this system but gradually diverges as the system tie line length increases as shown in Figs. 2–4. Corresponding data showing the concentration in top and bottom phases and total recovery of BSA in the system at volume ratios of 5.0, 1.0 and 0.2 are shown in Fig. 5. For the most part total recovery is constant and greater than 90% for these systems. Only for the lowest volume ratios (which are combined with a strong upper phase preference) is precipitation of BSA in any way marked. Most noticeable in this figure, however, is the sudden change in phase preference which occurs at a tie line length of about 38% (w/w). Below this concentration BSA strongly prefers the lower phase but above there is strong preference for the upper phase.

3.3. Classical description of partition

Many accounts of partitioning begin by reference to the Brönsted equation [9] which is often given as

$$\ln K = \frac{\lambda M(C - C_o)}{kT} \quad (3)$$

where K is the partition coefficient, λ a factor comprising the interaction of the phases with the solute, M is the molecular mass of the solute and $C - C_o$ is the difference between the composition of the system and the composition at the critical point. k is the Boltzmann constant and T the absolute temperature. Brönsted and Warming [10,11] partitioned colloidal particles of arsenical and cadmium sulphides in a two-phase system composed of ethyl alcohol, butyl alcohol and water. In order to reduce the surface tension to a level commensurate with distribution of the particles between the two bulk phases, rather than between one bulk phase and the interface, systems were confined to a region very close to the critical point [12]. This was achieved by manipulating the temperature within 0.25°C of the critical temperature for phase formation. Straight lines were used to describe the change in partition coefficient as the system composition became removed from the critical point as indi-

cated in the equation. Also implicit is the fact that at the critical point the observed arithmetic partition coefficient will be 1.

In this light the observations reported on the partition of BSA in the PEG 1450–potassium phosphate aqueous two-phase system strongly suggest that the range of applicability of the Brönsted equation may have been exceeded. However, the fact that a change of phase preference may be achieved with high yield by aiteration of the system composition, whilst contradicting the Brönsted equation, is of considerable practical significance in the design of protein purification schemes. From a practical point of view, it is worthwhile seeking alternative models which could account for these observations. Of most significance seems to be the change in phase preference occurring at around 38% (w/w) shown in Fig. 5. This is marked by a rapid decline in concentration in the lower, phosphate rich, phase and is strongly suggestive of the operation of a salting out mechanism.

3.4. The description of partition in terms of salting out

Classically the phenomenon of salting out, or more generally precipitation, can be represented by the following equation.

$$\ln S = \beta - K_s C \tag{4}$$

where S is the solubility, β is a constant which depends on the temperature and the individual protein, C is the concentration and K_s is the salting out constant which depends upon the particular protein and salt. The meaning of K_s depends on the type of precipitation involved. For example for precipitation involving addition of polymers K_s would be related to the excluded volume of the polymer [13]. In this light partition could be defined in terms of relative solubility or represented, in very general terms, as

$$K \propto \frac{S_t}{S_b} \tag{5}$$

where S_t and S_b are the solubilities in the top and bottom phases, respectively. Actually such an approach to the description of partitioning in PEG–salt aqueous two-phase systems is not new, having been proposed by Kim [14]. However, to our knowledge relatively little of this work has been published [15]. Kim ascribed solubility in the upper phase of PEG–salt systems to the excluded volume of PEG and its effect on both the concentration of the protein and of the salt to derive an expression for protein solubility. Here only solubility in the salt-rich phase is considered. Since the phase is predominantly composed of salt (see Fig. 1) the contribution of PEG in this phase may be neglected. In a derivation of "solvophobic theory" [16] by Melander and Horvath [17], precipitation due to increase in salt concentration is described by

$$\ln \frac{w}{w_0} = \beta + \Lambda m - \Omega \sigma m \tag{6}$$

where β is a term derived from Debye–Hückel theory [18] (in which proteins in aqueous solutions at low ionic strength are treated as simple ions) and describes the increase in solubility with salt concentration known as salting in. Λ is a term derived from Kirkwood's treatment of proteins as dipolar ions [18] applicable to descriptions of protein solubility in high concentrations of salt and related to the dipole moment of the protein by

$$\Lambda = \frac{D\mu}{RT} \tag{7}$$

in which D is a constant derived from Kirkwood's theory [19]. The salting out term of Eq. 6, $\Omega\sigma m$, relates the specific molal surface tension increment of the salt (σ) to its concentration (m) and to Ω which is proportional to the hydrophobic surface area of the protein and represents the energy required for the removal of water from hydrophobic regions of the protein. Salting out thus involves an ionophilic contribution due to the dipole moment and a hydrophobic contribution due to the hydrophobic surface area which can be related to the experimentally observed salting out constant by

$$K_s = \Omega\sigma - \Lambda \tag{8}$$

Both terms may be determined from a knowledge of protein structure [19,20]. However, at present this is computationally difficult and possible for only relatively few proteins, since it requires knowledge of the tertiary/quaternary structure. Furthermore, the solvophobic view of salting out has been extended [21] to cover the situation of a protein partitioning between a mobile phase and a mildly hydrophobic solid phase in HIC. In this case binding to the solid phase is described by the difference in free energy of the solute in the mobile and stationary phases.

$$\ln k = \ln k_0 - \beta' - \Lambda' m + \Omega' \sigma m \quad (9)$$

Eq. 9 describes the retention in HIC under isocratic conditions with different salt concentrations. The prime associated with various terms indicates that it is the surface area of contact between the solute and the ligand which is of importance. Thus any relationship between retention, salting out and partitioning might be expected to be tenuous because of the rather different surface areas involved.

3.5. Hydrophobic interaction chromatography of some model proteins

Fig. 6 shows the isocratic retention times of a series of proteins at increasing salt concentration. A wide range of proteins has been chosen so that some, such as α-amylase, are highly retained at relatively low concentrations of salt. At the other extreme, *e.g.* cytochrome *c* is only moderately retained even at high salt concentrations. The relationship between the difference in retention time and their partitioning behaviour in an aqueous two-phase system is shown in Fig. 7. In order to make this comparison, each protein was partitioned in a PEG 1000–phosphate system at four different tie line lengths. The concentration of phosphate in the lower phase at these tie line lengths was used to ordinate the data, and the characteristic retention of each protein in HIC, expressed as $\ln D$, was extrapolated to this concentration. This was necessary because the concentration of salt required to bring about a change in phase preference in HIC was much less than that required for partition. Although the behaviour of this sample of proteins differs in detail, there is a remarkable degree of similarity in behaviour in the two types of partition. No proteins have been found which show high partition coefficients (A2PS) associated with low distribution coefficients (HIC) and vice versa. The question why proteins partitioning in these different systems involving rather

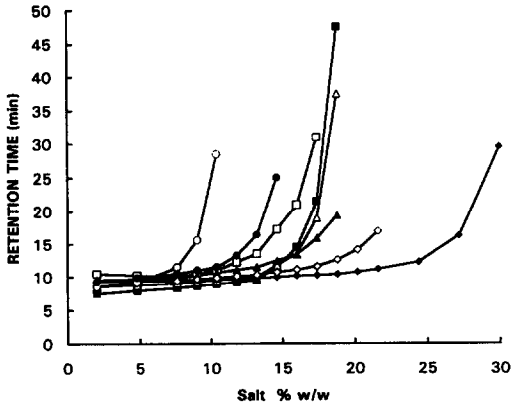

Fig. 6. Isocratic retention times on a PEG–silica HIC column of a series of proteins in response to increasing concentration of potassium phosphate pH 7.48 as detailed under methods. Symbols denote: ○: α-amylase, ●: α-chymotrypsin, □: lysozyme, ■: BSA, △: ovalbumin, ▲: ribonuclease A, ◇: myoglobin, ◆: cytochrome *c*.

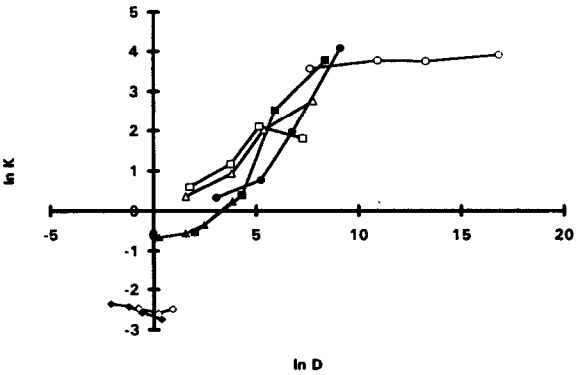

Fig. 7. Comparison of $\ln k$ obtained for selected model proteins at four tie line lengths in a PEG 1000–potassium phosphate aqueous two-phase system and their distribution coefficient $\ln D$ obtained during isocratic hydrophobic interaction chromatography on a PEG-derivatised silica HPLC column. (Symbols as for Fig. 6.)

different surface areas of contact should behave in such a similar way is intriguing and will be explored further in a subsequent publication.

Examination of the solubility behaviour of BSA in relation to increased concentration of either potassium phosphate or polyethylene glycol (Fig. 8) shows that BSA is rapidly salted out by phosphate. However this takes place at a higher concentration of phosphate than is present in the lower phase of the two-phase system of Fig. 5 when the change in phase preference of this protein occurs. By contrast, polyethylene glycol, of the same molecular mass as used in the phase system (1450), shows little tendency to precipitate albumin over a wide range of concentrations. It therefore seems reasonable to ascribe the phase change observed in partition to the salting out effect of phosphate. A greater salt concentration is required to bring about a change of phase preference in precipitation than in aqueous two-phase partition, which is in turn greater than that required to bring about a change of phase preference in chromatography involving partition to a solid phase. This might be thought to follow naturally from the surface areas and phase transitions involved in each process.

On the basis of this evidence, ascribing the behaviour of proteins partitioned in PEG–salt aqueous two-phase systems to the effects of salting out, leaves at least one significant problem. In the classical view of salting out, or indeed any method of solubility reduction, the final solubility is a product only of the precipitant concentration and the temperature. Indeed Florkin [22], in a series of early experiments, suggested that departures from this were evidence for an impure protein preparation. However, the concentration of albumin observed in the phases of the system reported here appears more than a little too flexible. For instance, after the solubility limits of albumin in phosphate have been approached [at 38% (w/w) TLL] the solubility in the PEG phase appears to show a dramatic increase despite increasing concentration of the top phase components. In reality what is being quite sensitively measured by the partitioning technique is the driving force toward a particular equilibrium, and recent results from a precipitation study by Shih *et al.* [23] help to shed light on this. In this study two types of solubility behaviour were observed. Type 1 behaviour, shown by lysozyme, is classical, the final protein concentration in the supernatant depends only on the salt concentration. In type 2 behaviour, shown by BSA, the final supernatant concentration is dependent not only on the concentration of the salt but also on the initial concentration of protein. The final distribution of some proteins in precipitation depends on the concentration in the supernatant and precipitate phases. Thus the apparent solubility limits of some proteins may be quite variable with operating conditions.

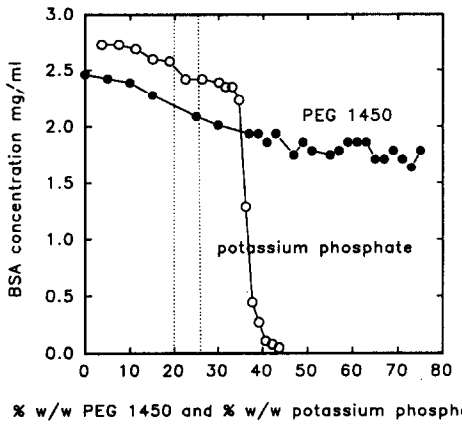

Fig. 8. Precipitation of BSA with potassium dihydrogen orthophosphate (●) and with PEG 1450 (○). Hatched lines denote the concentration of phosphate in the lower phase of the PEG 1450 aqueous two-phase system over which BSA shows a marked change in phase preference. Symbols denote concentration (mg/ml) of BSA in the supernatant.

4. Conclusion

Our recent work on the partitioning of proteins in PEG–salt aqueous two-phase systems leads us to conclude that the salting out behaviour of proteins has a strong influence on their partitioning behaviour as outlined above. Practically this means that some current design assumptions applied to partitioning may not always be valid.

An approach to the understanding and prediction of partition in PEG–salt aqueous two-phase systems based on solubility and in particular on solvophobic theory offers some key insights into the mechanism of partition. In particular, that partition may be a sensitive measure of the interplay of molecular surface forces which are rationalised here to the dipole moment and the hydrophobic surface area of the proteins. The apparent similarities in the separation basis of hydrophobic interaction chromatography, precipitation and partition and the applicability of a common theoretical foundation based on solvophobic theory may prove to be a useful rationalisation in the development of large-scale downstream processing strategies.

It also appears that hydrophobic interaction chromatography provides an additional comparative tool for the study of partition. Considerable scope exists for developing the technique as a simple and rapid instrumental analysis of protein mixtures which could be deployed in "method scouting" for the development of partitioning strategies for protein extraction and biorecovery.

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