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COMPARISON OF TWO AQUEOUS BIPHASIC SYSTEMS USED FOR THE PARTITION OF BIOLOGICAL MATERIAL

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SUMMARY

Two biphasic systems, both consisting of water, dextran and poly(ethylene glycol) but differing in the molecular weight of the latter polymer, $M_r = 35\,000$ (systems A) and 6000 (systems B), have been studied. The phase diagrams for the two systems at 20° are compared. The partition of proteins influenced by salts and the viscosity of the phases are direct functions of the difference in composition between the upper and the lower phase as measured by the lengths of the tie-lines found for the systems in the phase diagram. The solubility of γ-globulin is 1.5–2 times larger in an A system than in a B system with corresponding tie-line. In both systems, poly(ethylene glycol) palmitate has less effect on the partition of serum albumin than would be predicted from the distribution of poly(ethylene glycol) and reflects the non-ideality of simple equilibrium models.

INTRODUCTION

Among the biphasic systems which can be generally used for the partition of biological materials the so-called aqueous biphasic systems predominate. They are prepared by mixing aqueous solutions of polymers, *e.g.*, dextran and poly(ethylene glycol) (PEG). These systems have been used both for separation purposes and for physico-chemical studies of proteins, membranes and cell organelles[1–4]. An important question when the systems are used for separation is how to steer the partition of the biological material. The various ways in which this can be done[2,5] are related to the distribution of the two polymers (dextran and PEG) between the two phases since the polymers are concentrated in opposite phases. In the case of so-called affinity partition, where a chemical group having an affinity for a particular biological constituent is bound to PEG, the effectiveness of the extraction should be directly dependent on the ratio between the concentration of PEG in the two phases[6]. In this paper a comparison is made between biphasic systems containing PEG with molecular weights of 6000 (which is standard) and of 35 000. The effect of the molecular weight of PEG on the protein solubility, the viscosity of the phases and the steering effect of salts or polymer-bound ligands on the partition of proteins has been studied.

MATERIALS AND METHODS

Materials

Carbonylhaemoglobin from swine was prepared according to ref. 7 and dialyzed against water. Bovine serum albumin was purchased from Sigma (St. Louis, Mo., U.S.A.). Human serum albumin and γ-globulin were obtained from Kabi (Stockholm, Sweden). Other chemicals used were all of analytical grade. The water was double distilled in quartz. Dextran T 500, batch No. 5996 ($M_w = 5 \cdot 10^5$), was supplied by Pharmacia (Uppsala, Sweden). PEG having $M_r = 6000$–7500 was obtained from Union Carbide (New York, N.Y., U.S.A.) as Carbowax 6000, and having $M_r = 35\,000$–$40\,000$ from Serva (Heidelberg, G.F.R.). Trimethylaminopoly-(ethylene glycol) and poly(ethylene glycol) palmitate were prepared by published methods[5,7].

Methods

Phase diagrams were determined by preparing a number of biphasic systems of various concentrations of the two polymers. The upper and lower phases were analyzed for their contents of dextran (polarimetrically) and of PEG (refractometrically). The details of the analyses have been published elsewhere[1]. Some points on the central part of the binodal curve were determined by turbidimetric titration[1]. The viscosities of the phases were determined with the aid of a reverse-flow U-tube viscometer which had a flow-time for water of 29 sec. The densities of the phases were determined with a calibrated Lipkin pyknometer having a volume of 1 ml.

The solubility of γ-globulin in the upper phase was determined as follows. 25 mg of γ-globulin were mixed with 2 ml of the lighter phase by gentle stirring for 20 min. After centrifugation for 15 min at 3300 *g*, 0.2 ml of the supernatant were diluted with 3 ml of water and the absorbance at 280 nm was measured, a correspondingly diluted pure phase being used as blank. The protein solubility in the phase was calculated assuming that the protein concentration in g/l is equal to $0.75 \cdot A^{280}_{1\,cm}$.

The partition of proteins in the biphasic systems was determined (after equilibration) by taking equal volumes of the upper and lower phases, diluting them with eight times their volume of water and measuring the absorbance at 280 nm using correspondingly diluted pure phases as blanks. The partition coefficient of the protein was taken as the ratio between the absorbances of the upper and lower phases. The procedure has been described in detail elsewhere[8]. For the determination of the effect of pH on partition, a system of 20 g was prepared and mixed mechanically while the pH, measured with a glass electrode, was adjusted by the addition of 1 *M* NaOH or HCl. After each adjustment of pH, a 2-g portion was withdrawn from the mixture. The phases comprising these portions were then analyzed as described above.

RESULTS

The phase diagrams for the two PEG–dextran–water systems are shown in Fig. 1. PEG ($M_r = 35\,000$) gives two phases with dextran and water (systems A) at considerably lower polymer concentrations than when PEG ($M_r = 6000$) is used (systems B). With increasing polymer concentrations the difference in composition of the two liquid phases also increases. Thus, in the upper phase the concentration of

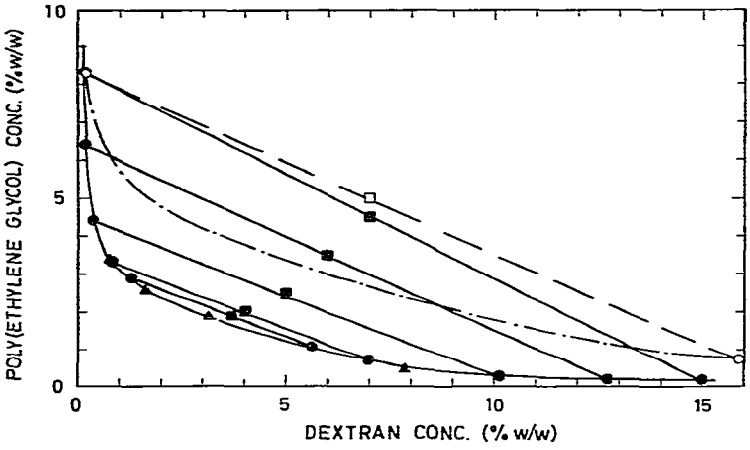


Fig. 1. Phase diagram for the system A (filled symbols), water–dextran–PEG (M_r = 35,000) at 20°: ■, the total composition of the system; ●, composition of the phases (upper phase to the left, lower phase to the right); ▲, points on the binodal curve obtained by turbidimetric titration. The binodal curve (–.–.–) of system B, PEG (M_r = 6000), and the tie-line of system B11 (– – –), open symbols, are taken from ref. 1.

PEG increases and the concentration of dextran decreases, and *vice versa* for the lower phase. This difference in composition of the two phases is measured here as the length of the tie-line (t), *i.e.*, the distance between the points in the diagram representing the compositions of the upper and lower phase.

The lengths of the tie-lines for a number of system compositions, together with the viscosities and densities of the phases, are given in Table I. The phase compositions of systems B are taken from ref. 1. The viscosity of the lower phase increases to the same degree with t for both sets of systems A and B. The upper phase of a B system has a higher viscosity than the one from an A system at corresponding t values.

TABLE I

PHYSICO-CHEMICAL DATA OF THE AQUEOUS BIPHASIC SYSTEMS

System No.	*System composition*		*Molecular weight of PEG*	*Length of tie-line (%)*	*Density of phase (g/l)*		*Viscosity of phase relative to water*		*Solubility of γ-globulin in upper phase (g/l)*	K_{PEG}
	Dextran (%)	*PEG (%)*			*upper*	*lower*	*upper*	*lower*		
A1*	3.20	1.80	35000	0	1.012	1.012*	6.4	6.4*	6.6	—
A2	3.90	1.80	35000	5.1	1.008	1.023	5.6	12.8	4.5	3.49
A3	4.00	2.00	35000	6.7	1.007	1.026	5.7	16.2	3.3	4.19
A4	5.00	2.50	35000	10.6	1.007	1.038	6.9	26.7	3.1	15.2
A5	6.00	3.50	35000	14.0	1.009	1.052	9.9	51.1	1.1	30.6
A6	7.00	4.50	35000	16.9	1.011	1.064	14.6	89.2	0.4	44.0
B7*	4.13	3.46	6000	0	1.020	1.020*	7.8	7.8*	5.8	—
B8	5.00	3.50	6000	6.0**	1.014	1.034	4.9	15.7	2.3	1.89**
B9	5.20	3.80	6000	9.5**	1.012	1.041	3.7	27.9	1.4	2.99**
B10	6.20	4.40	6000	14.3**	1.014	1.052	4.0	50.6	0.7	6.46**
B11	7.00	5.00	6000	17.5**	1.013	1.066	4.4	95.7	0.4	11.6**

* One phase system.

** Calculated from data in ref. 1.

The separation times for systems of 7-cm heights in test-tubes were 10–30 min at 1 *g*. The lower separation time was obtained with systems A3 and B9 in Table I.

The solubility of a model protein, γ-globulin (Table I), in the upper phase (which is assumed to be the less efficient solvent) cannot be described solely by the value of t. At any value of t, the protein solubility is 1.5–2 times higher in the upper phase of systems A than in the corresponding phase of systems B. The solubility is determined by the concentration of PEG in the upper phase, independently of the molecular weight of this polymer, as can be seen in Fig. 2.

The steering effect of a salt on the partition of proteins is nearly the same for systems A and B at equal t values. This is illustrated in Fig. 3A which shows how the

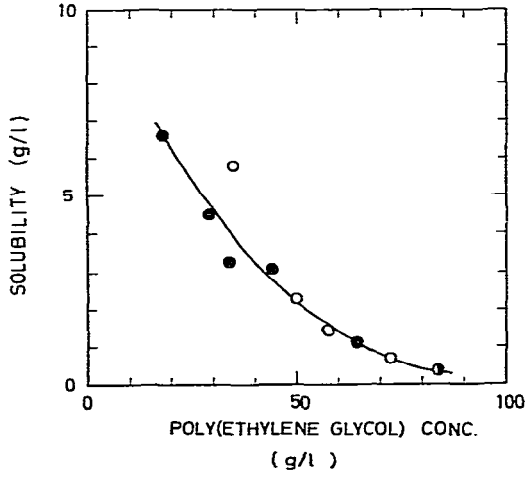


Fig. 2. Solubility of γ-globulin in the upper phase as a function of the PEG concentration of the phases: ●, system A: ○, system B. Temperature, 20°.

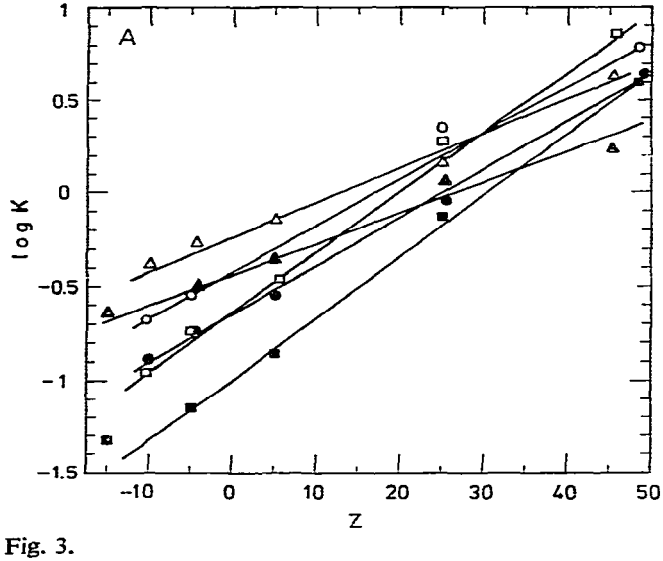


Fig. 3.

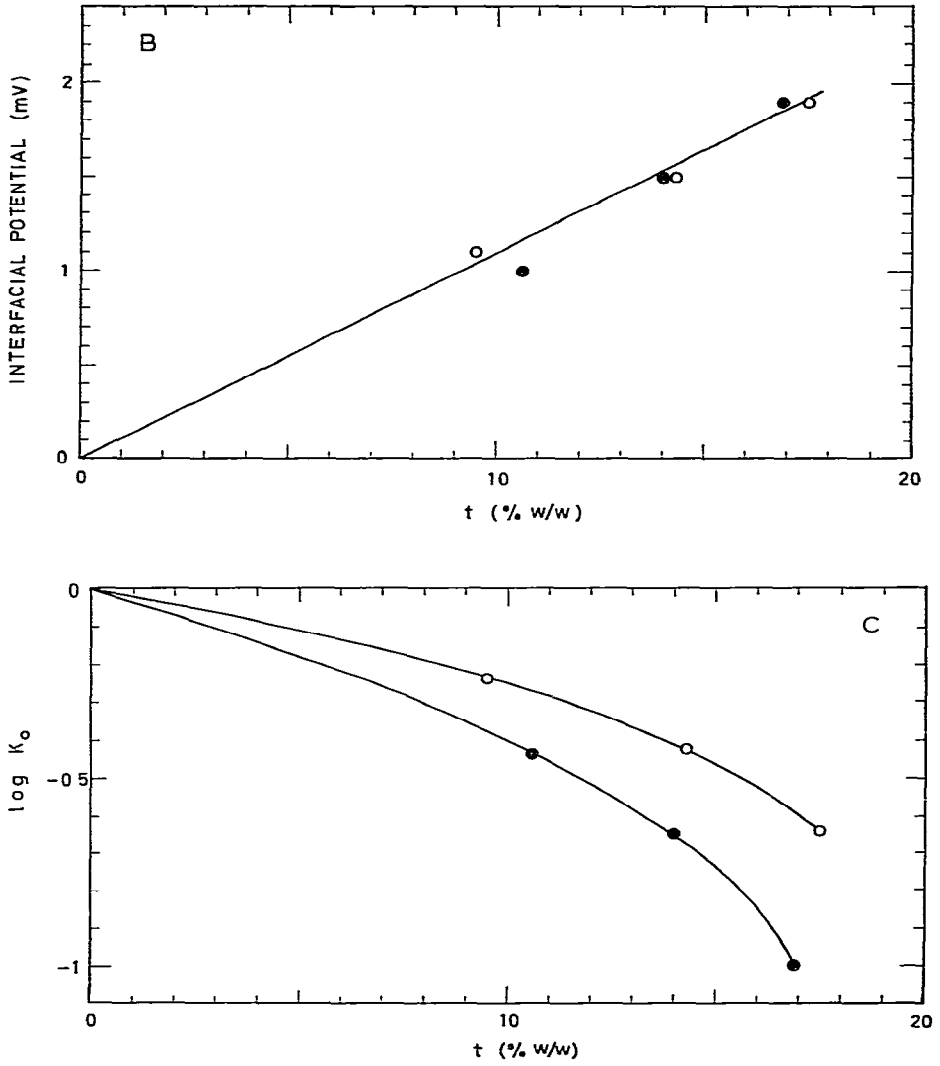
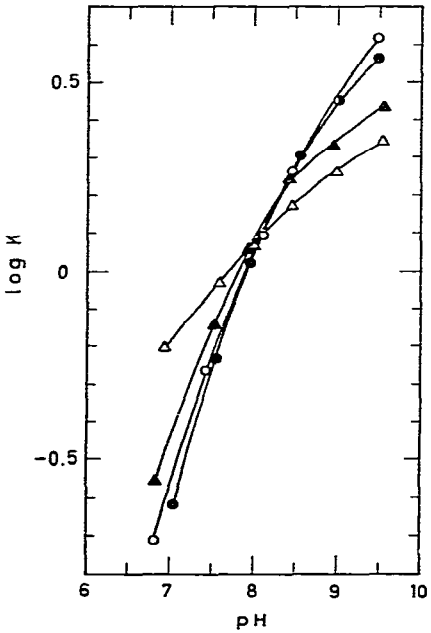


Fig. 3. Partition of bovine serum albumin in systems containing 100 mmole/kg KCl. Protein concentration, 2 g/kg. Temperature, 20°. A. Logarithmic partition coefficient, log K, *versus* protein net charge, Z, with systems: A4 (▲); A5 (●); A6 (■); B9 (△); B10 (○); and B11 (□) (compositions given in Table I). The net charges are taken from the titration curve in ref. 10. B. Interfacial potential, calculated from the slope of the lines in Fig. 3A, as a function of the length of the tie-line, t: ●, system A; ○, system B. C. Logarithmic partition coefficient of the protein at $Z = 0$, log K_0, as a function of t. Systems as in Fig. 3B.

partition of bovine serum albumin depends on the net charge of the protein in systems containing KCl. This charge dependence is due to a potential difference across the interface between the two phases[1,2]. These interfacial potentials (which are proportional to the slopes of the straight lines) are proportional to the lengths of the tie-lines (Fig. 3B). The partition coefficient at the isoelectric point of the protein, K_0, is

markedly lower in the case of systems A at corresponding t values (Fig. 3C). By definition, $K_0 = 1$ and the interfacial potential is equal to zero when $t = 0$.

The introduction of a charged group on the PEG molecule has been shown to make the partition of proteins strongly pH-dependent[7]. Theoretically, this effect should be enhanced with increasing divergence of the distribution of PEG by generating an even larger interfacial potential. Experiments with carbonylhaemoglobin shows that this assumption is not true (Fig. 4). Comparing systems A5 and B10, it can be seen that the partition has the same pH-dependence while the distribution of PEG between the upper and lower phase is 4.7 times larger for the A system. The effect does not seem to be proportional to the t value. The effect of the esterification of increasing amounts of palmitic acid with PEG on the partition of human serum albumin is shown in Fig. 5. The affinity of the albumin for the upper phase is strongly increased by the poly(ethylene glycol) palmitate and typical saturation curves are obtained which have been discussed elsewhere[5]. The two systems have comparable t values, but the effect is larger for the A system when the maximum changes in the *log K* values induced by poly(ethylene glycol) palmitate are compared. When this maximum change in the logarithmic partition coefficient, Δ log K, is plotted *versus* the logarithmic partition coefficient of poly(ethylene glycol), log K_{PEG}, the systems A and B generate the same curve (Fig. 6). According to theory[6], Δ log K should be directly



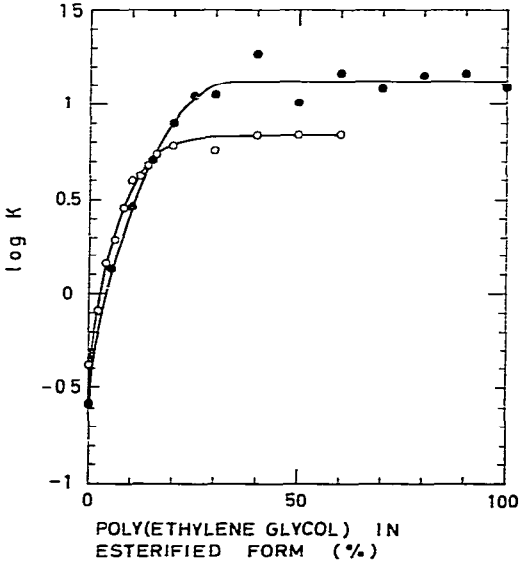


Fig. 4. Partition of porcine carbonylhaemoglobin in trimethylaminopoly(ethylene glycol)-containing systems A4 (▲); A5 (●), B9 (△), and B10 (○) as a function of pH. In the A systems all of the PEG is in the substituted form, as compared to only 25% in the B systems. Temperature, 20°.

Fig. 5. Partition of human serum albumin as a function of the percentage of PEG in the form of the palmitate ester. The systems, A5 (●) and B10 (○), contained 50 mmole/kg K_2SO_4, 2.5 mmole/kg potassium phosphate buffer and 1 g/kg protein. Temperature, 20°; pH 7.0.

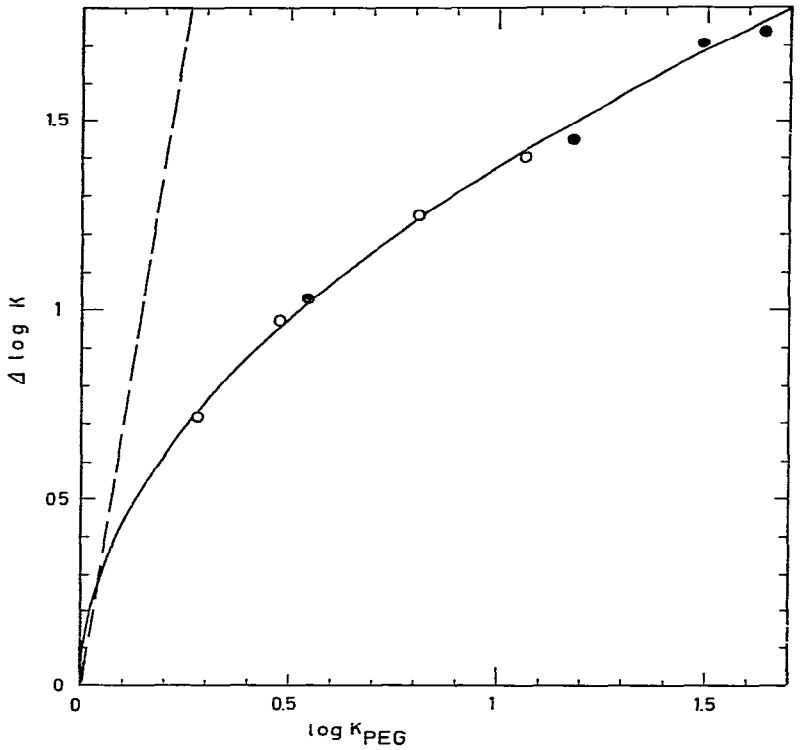


Fig. 6. The maximal increase in the logarithmic partition coefficient, $\Delta \log K$, of human serum albumin by esterification of PEG with palmitic acid as a function of the logarithmic partition coefficient of poly(ethylene glycol), log K_{PEG}. ●, A systems; ○, B systems. Data as in Fig. 5. 75% (A systems) and 20% (B systems) of the PEG was in the esterified form. The broken line shows the theoretical relation, according to Flanagan and Barondes[6], calculated for a protein with seven binding sites for palmitate. The solid curve is described by the function $\Delta \log K = \sqrt{1.9 \log K_{PEG}}$.

proportional to log K_{PEG}, independently of the molecular weight of the PEG. The curve should therefore be a straight line, in contrast to the experimental findings where the points give a good fit to the function $\Delta \log K = \sqrt{1.9 \log K_{PEG}}$.

DISCUSSION

The partition of a substance between the two phases of a liquid–liquid aqueous biphasic system depends on the compositions of the phases. If the system is close to the critical point, *i.e.*, if addition of a minute amount of water causes a transition to a one phase system, the composition of the phases will be similar. In this case the partition coefficient for a protein can be assumed not to differ greatly from unity. By increasing the concentration of polymers, the phases will differ more and more in composition and solvating properties. The partitioned substance will then, in most cases, to an increasingly degree prefer one of the phases to the other and the partition coefficient will deviate more and more from unity. The length of the tie-line is shown to be an appropriate measure of the dissimilarity of the two phases since several of the properties studied here vary linearly with this parameter.

According to the theory of the steering effect of salts on the partition of proteins, the ions of the salt can give rise to an interfacial potential[1,2]. That is, if the positive and negative ions do not strive to partition in the same way, an orientation of charges will occur about the interface between the two phases, resulting in an electrical double-layer. If a protein is partitioned together with an excess of salt, the interfacial potential generated by the latter will influence the partition of the protein, depending on the strength and sign of the potential difference over the interface. The experiment with salt-steered partition shows that the potential is mainly proportional to the length of the tie-line. No such correlation is seen between the partition of the protein and the distribution of water, dextran or poly(ethylene glycol)[2]. The relative affinities of the ions for the two phases are therefore determined not by any single component but by the collective properties of the polymers and water. Apparently, the difference in this property between the phases is closely related to the t value.

When charges are bound directly to the PEG the interfacial potential should be proportional to the partition coefficient of PEG. The interfacial potential, ψ, is, according to the theory mentioned above, given by

$$\psi = \frac{RT}{2F} \cdot \ln \frac{K_-}{K_+} \tag{1}$$

were K_- and K_- are the (hypothetical) partition coefficients which the positive and negative ions, respectively, strive to attain, R is the gas constant, F is the Faraday constant and T is the absolute temperature. The counter ion of trimethylaminopoly-(ethylene glycol) is Br^- which in these biphasic systems is known to have $1 < K_- < 1.2$. Since the partition coefficients of PEG in the two systems A5 and B5 are 30.6 and 6.46, respectively, and if it is assumed that trimethylaminopoly(ethylene glycol) partitions as unsubstituted PEG, the interfacial potential for the A system should be 1.8 times that for the B system. In contrast, the experiment shows the same pH-dependence of the partition of a protein in the two systems.

Haemoglobin undergoes a net change in charge of 10 units between pH 7 and 8 (ref. 9). The interfacial potential calculated, according to ref. 2, from the values in Fig. 4 then becomes 4.1 mV. Using the K_+ and K_- values and eqn. 1, the values obtained are 100 and 55 mV for the A and B systems respectively. Thus, K_+ is only a fraction of K_{PEG}. This, in turn, means that other interactions may be involved.

Such an assumption could also explain why the effect of poly(ethylene glycol) palmitate is not as would be expected. Thus, a plot of $\Delta \log K$ *versus* $\log K_{PEG}$ is not a straight line but a curve that becomes less steep with increasing polymer concentrations. This deviation, which is increasingly evident at higher concentrations of polymers, is probably the consequence of an interaction between the poly(ethylene glycol) palmitate molecules thereby lowering the concentration of the available palmitoyl groups in the upper phase. The formation of micelles is one way in which the fraction of free poly(ethylene glycol) palmitate may be reduced. Instead of the relation put forward by Flanagan and Barondes[6] *i.e.*,

$$\log K = \log K_0 + n \log K_{PEG} \tag{2}$$

where n is the number of binding sites on the protein molecule and assuming the

same association constants in the two phases, the present results point to a relation of the form

$$\log K = \log K_0 + \sqrt{An \log K_{PEG}} \quad (3)$$

or

$$\log K = \log K_0 + n\sqrt{A \log K_{PEG}} \quad (4)$$

where A is a constant. In order to discriminate between eqns. 3 and 4 it would be necessary to study the partition of proteins having various numbers of binding sites.

The use of PEG of higher molecular weight has indisputable advantages when these aqueous biphasic systems are used for separation purposes. For the same charge-steering properties, reflected by equal lengths of the tie-lines, the solubilities of proteins are markedly higher in systems A. In addition, the affinity partition obtained by binding a protein ligand to PEG is more favourable with the A systems.

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