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Interactions in Protein Systems

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I. Introduction

The way in which proteins diffuse in solutions of polymers has attracted much interest recently, giving insight into the way in which non-ideal interactions can affect diffusion. There has however been very little work on the events at the water-water interface in incompatible two phase systems [1]. This is surprising since it would appear to offer excellent opportunities to model systems of biological and biotechnological importance - such as the enzymatic modification of lipids in the food industry and the mobilisation of seed protein bodies [2]. We have therefore attempted to extend some of the ideas of non-ideal diffusion behaviour to polyethylene glycol (PEG) - Dextran two phase systems, to study the way in which proteins move through solutions of macromolecules to the water-water interface and penetrate it in terms of 1. diffusion kinetics, 2. potential density inversion & fingering phenomena [3,4,5] and 3. interfacial accumulation phenomena [6].

II. Theoretical considerations

1. Diffusion in concentrated systems. In both phases the protein must diffuse through solutions of other polymers. By analogy with dilute solution behaviour [7], these can affect the flux or (translational) apparent diffusion coefficient D, in two ways [5] (i) a hydrodynamic effect, which tends to decrease D largely as a result of an increase in viscous drag, related to the translational frictional property; (ii) a thermodynamic affect related to exclusion volumes and Donnan effects, which tends to increase D. Depending on the relative effects of (i) or (ii) diffusion can be retarded or enhanced.

In 'classical' diffusion

$$d\,(Q/C_o)^2/dt = D\,(A^2/\pi) \qquad (1)$$

where Q is the quantity crossing the boundary in time t with initial concentration C_o. A is the cross sectional area. In concentrated ternary solutions, according to [6] this is replaced by

$$d\,(Q/C_o)/dt = k \qquad (2)$$

where k is a transport rate. For intermediate concentrations diffusion can commence according to (1) and change to (2) as concentrations shift.

2. Two phase systems. These, with added protein, are quarternary systems, but the same considerations apply. In the PEG-Dextran systems we have used, the polymer concentrations are probably high enough to produce linear kinetics (eqn. 2) for the polymer diffusion behaviour. However as Ogston has shown [8] for some typical globular proteins D/D_o, where D_o is the 'ideal' or 'infinite dilution' value for D, falls steadily up to concentrations of 20% for the matrix polymer, and it is doubtful if polymer protein interactions would ever cause an enhanced diffusion rate for this class of proteins.

3. Approach to equilibrium. The flux equations (including cases where diffusion must occur up the concentration gradient to achieve equilibrium) can be written

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$$\text{Flux A} \rightarrow \text{B},\ J_{AB} = D_o \cdot d\,(C_P' + A^*_{P.DEX} \cdot C_P' C_{DEX} + \ldots)/dx \qquad (3)$$

$$\text{Flux B} \rightarrow \text{A},\ J_{BA} = D_o \cdot d\,(C_P'' + A^*_{P.PEG} \cdot C_P'' C_{PEG} + \ldots)/dx \qquad (4)$$

where C_P', C_P'' are the protein concentrations (m/v) in phases A (dextran rich) & B (PEG rich) respectively and C_{DEX}, C_{PEG} the concentrations of dextran (dextran rich phase) and polyethylene glycol (PEG rich phase). The ability to diffuse up a concentration gradient depends on the magnitude of the cross interaction terms, $A^*_{P.DEX}$, $A^*_{P.PEG}$. Within the two phases, diffusion will be mainly viscosity dependent, and slow. There will be a large viscosity gradient in the interface, which might be the major factor in determing the concentration profile, and will be markedly asymmetric depending on the direction of diffusion.

4. Transient water fluxes: 'density inversions' & 'fingering'. Wells [9] has recently proposed a theory to describe disequilibria due to diffusion in moderately concentrated polymer solutions. Although this was originally intended for layered solutions, as shown in Fig. 1a, where A is described as the matrix solute and P the cosolute (in our case, protein), it can also be applied to the quarternary two phase systems shown in Fig 1b, 1c. The question is what happens to the transient water flows, because it is a matter of experimental observation that these systems behave initially as if there were a semi-permeable membrane between the layers. This would of course restrict the movement to that of solvent only, an effect no doubt due to the much greater mobility of water.

The local density, ρ, (for a given phase) in the quarternary 2-phase system is given by

$$\rho = \rho_o\,(1 + \alpha\, C_A + \beta\, C_P) \qquad (5)$$

where ρ_o is the density of pure solvent and α, β are the density increments of A and P (whose partial specific volumes are $\bar{v}_A$ and $\bar{v}_P$) respectively:

$$\alpha = (1/\rho_o) - \bar{v}_A \quad ; \quad \beta = (1/\rho_o) - \bar{v}_P \qquad (6)$$

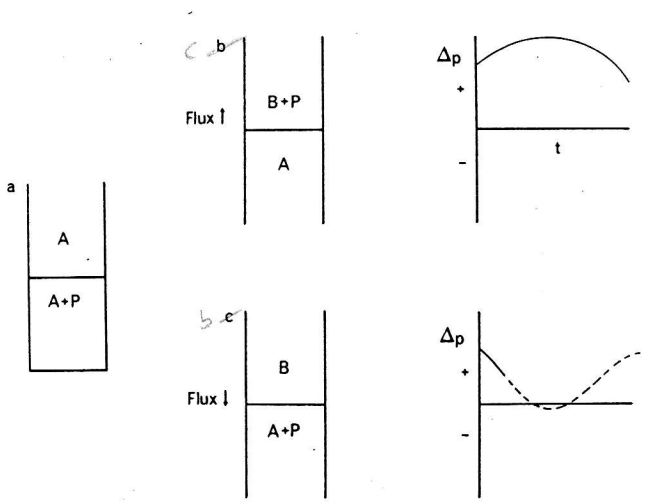


Fig. 1. "a" represents layered solutions containing a matrix polymer A together with protein P. "b" and "c" represent the two possibilities where two polymers A and B are used to produce phase separation, followed by the addition of protein to one or other of them (although both phases contain both A and B the dominant component only is indicated). Also given are the water fluxes and likely transient density changes for situations b and c.

Suppose a lamina of solvent is transferred, of volume ΔV as a result of osmotic flux effects. If ρ' and ρ'' are the local densities in the lower & upper phases respectively, the condition for instability or 'fingering' of protein passing through the interface [5] is that $\Delta\rho = (\rho' - \rho'')$ must become negative. Wells [9] has shown that for Fig 1a, and after making the rather unrealistic assumption of thermodynamic ideality, this condition for instability leads to

$$\alpha/\beta > M_P/M_A \qquad (7)$$

(where M_P, M_A are the molecular weights of cosolute P & matrix solute A respectively), although the same concepts apply to quarternary systems (Figs 1b, 1c).

It is clear that since the density increment for the two phases is different, shifting a solvent lamina will have different effects in b. and c. However the protein is the source of the osmotic flux, and adapting the arguments we can write

$$\alpha_{PEG}/\alpha_{DEX} > M_P/M_{PEG} \qquad \alpha_{DEX}/\alpha_{PEG} > M_P/M_{DEX} \qquad (8)$$

as instability criteria for 1c & 1b respectively. Taking PEG as $M_r \sim 6000$ by this test case '1c' fails to give fingers whereas for dextran ($M_r \sim 500000$) the situation '1b' could. Thus by this test we might expect density inversion & fingering for protein initially in the denser dextran rich phase ("B") but not in the PEG rich phase ("A"), based on the expected osmotic fluxes involved.

By using more general arguments, in '1c' with protein in the upper phase, addition of protein will reduce the initial density difference: in addition, there will be water flux which reduces the density downwards faster than it will increase the density of the lower phase. Thus the interface will be stabilised since the density difference between the phases will tend to increase. By contrast, in '1b' addition of protein will cause an increase of the density of the upper phase more rapidly than the lower phase loses density, so the density difference could reach zero.

The nearer to the critical point composition, the more nearly alike the composition of the phases, and the more nearly alike the rate of change of density, but also the lower the initial density difference. There will be an optimum composition for density inversion, if it occurs.

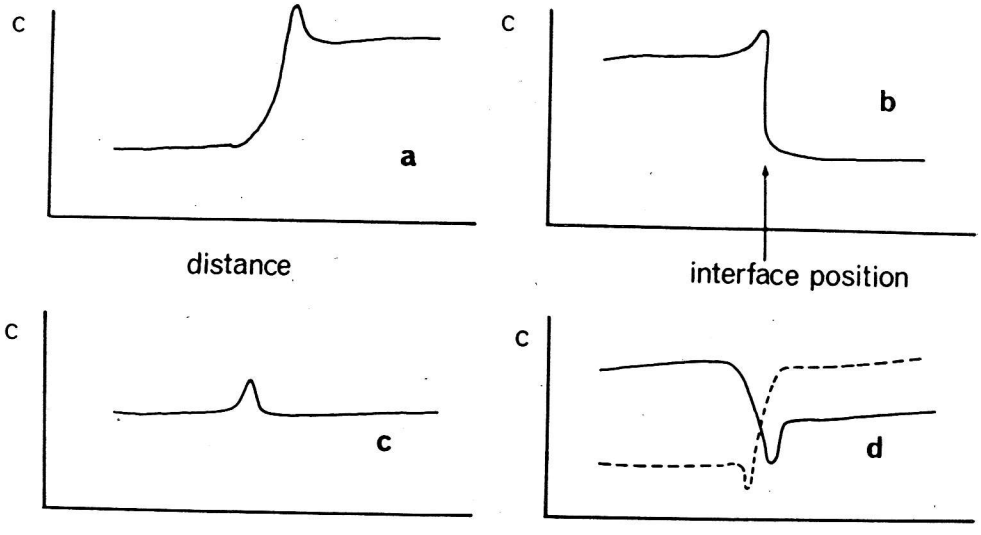


Fig. 2. Predicted concentration profiles across the interface for various combinations of interfacial tension between particles and phases.

5. Interfacial accumulation. Some possible profiles can be predicted from theoretical considerations, and Albertsson [6] has analysed the possibilities in terms of surface energies. By considering the energy associated with a spherical particle at various positions in and near an interface, Albertsson was able to predict accumulations depending on the relative values of the interfacial energies γ_1, γ_2 & γ_{12} between the particle and the two phases, where γ_1, γ_2 & γ_{12} are the interfacial energies betwen phase 1 and the particle, phase 2 and the particle and between the two phases respectively. Two possibilities arise: Condition "1" ($\gamma_1 - \gamma_2 < \gamma_{12}$): energy minimum criteria are satisfied when particle totally contained within one of the phases; Condition "2" ($\gamma_1 - \gamma_2 > \gamma_{12}$): energy minimum when particle either totally in one of the phases or in the interface. The interfacial tension between PEG and dextran is low, (< 0.02 dyne/cm), but $\gamma_1 - \gamma_2$ is also likely to be very low (cf partition coefficients near one) so that the latter condition allowing a build up at the interface is by no means unlikely. Fig. 2 gives some possible profiles. Since in general terms the partition coefficient

$$K = (C_1/C_2) = \exp[-\Delta E/kT] = \exp[-4\pi R^2(\gamma_1 - \gamma_2)/kT] \qquad (9)$$

(where ΔE is the energy difference between the phases) we can write for condition "1" and more interestingly, for the partition between the interfacial region and the phase, with condition "2"

$$C_i/C_1 = \exp[\pi R^2(\gamma_2 - \gamma_1 - \gamma_{12})^2/\gamma_{12}kT] \qquad (10)$$

where C_i is the concentration (g/ml) in the interface and C_1 the concentration in phase 1. We have to choose a value for $\gamma_1 - \gamma_2$. This is likely to be small, and for example 0.001 yields $C_i/C_1 = 1.009$. If we assume that $\gamma_1 - \gamma_2$ will be negligible compared with γ_{12} (which will not always be the case, but in suitable examples is justified) then

$$C_i/C_1 = \exp[6.8\gamma_{12}] \qquad (11)$$

Values for the interfacial energy in the PEG-Dextran system are quoted as variable between 0.00046 and 0.066 dyne/cm (or erg/cm^2) [6] depending on the precise composition: the further from the critical point, the higher the value. These results suggest that excess concentrations of the order of 60% of the bulk concentration are not impossible. In the dynamic system approaching equilibrium there are other factors which may tend to make this build up greater. A sharp change in viscosity or density in one phase may slow down migrating molecules. Such effects should be revealed by comparing migration in opposite directions.

III. Experimental

We examined the concentration profiles by using the scanning absorption optical system of an MSE Centriscan analytical ultracentrifuge equipped with a monochromator. We loaded 0.22 ml of lower phase followed by the same volume of top phase into 10mm cells. At the position of the interface the cross sectional area was 25.9 mm^2. The rotor was run at 4000rpm at 20°C or 25°C which should produce no significant sedimentation. Appropriate blanks were included, mainly the two phases with no added protein. Most experiments were done in the two phases produced by mixing 5g of PEG 6000 (BDH Biochemical grade, with low 280nm absorbance), 5.3g of Dextran T-500 (Pharmacia) with 100ml of 50mM sodium phosphate, pH 7.0. After equilibration phases were separated and protein added to the appropriate phase. Four protein systems were analysed: FITC labelled bovine serum albumin, FITC labelled ovalbumin, chromobacter lipase and cytochrome c. Protein concentrations of ~1 mg/ml ($\equiv$ ~1.5 x 10^5 M) were added to either phase.

Measurement of interfacial surface tension (by our colleague, Dr. J. Mead, Unilever Research, Bedford, U.K.) by the spinning drop method gave a value of (0.09 ±

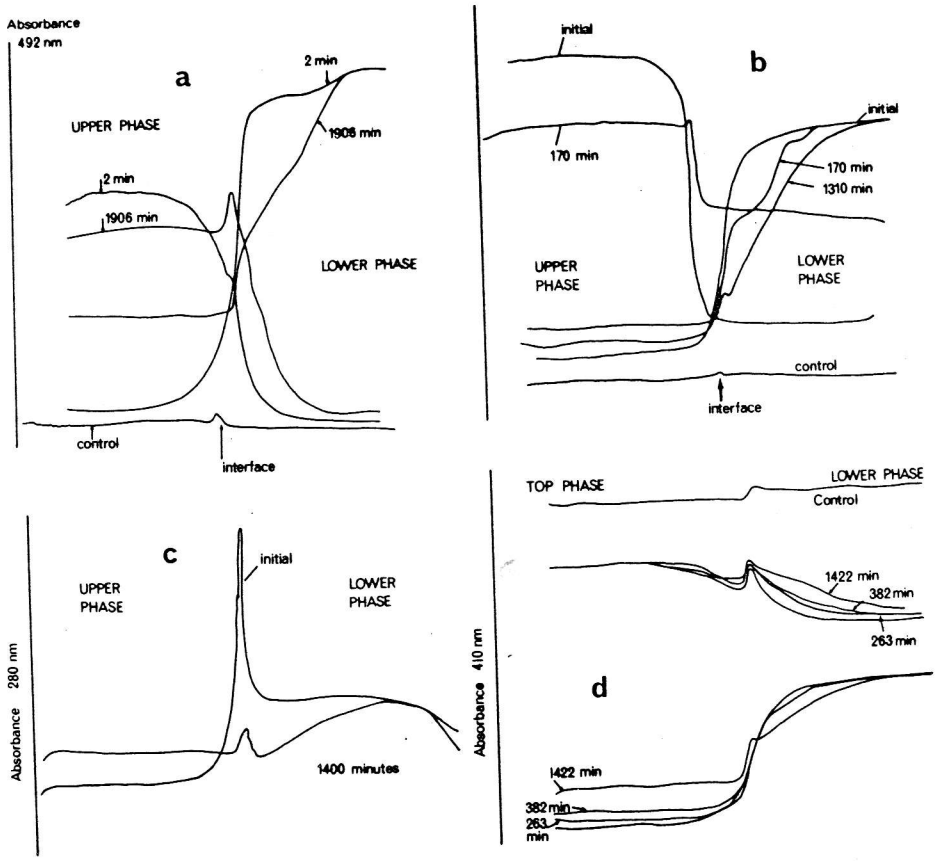


Fig. 3. Protein concentration profiles
a. FITC bovine serum albumin, added at ~1 mg/ml to either the upper or lower phases. Behaviour is different depending on which phase the protein is added to. At the bottom, a control containing no protein. The concentration profiles were measured in three cells run simultaneously.
b. FITC ovalbumin, at 0.2 mg/ml. The complex step pattern was only seen when protein was initially added to the lower phase. Diffusion was apparently much more rapid when protein was added to the upper phase.
c. Lipase, at 0.2 mg/ml, scanned at 280 nm. The initial contact produced a large increase in interfacial accumulation. After ~1400 minutes (near equilibrium) a large excess concentration still persisted.
d. Cytochrome c (after predialysis). At the top, a control trace, in the middle, patterns with protein initially in the upper phase and at the bottom the comparable patterns for the lower phase.

0.03) mN m^{-1}ml. g^{-1}. The density drop across the interface was ~.03 g/ ml so that the interfacial energy was 0.0027-0.0036 erg. cm^{-2}.

IV. Results

The profiles observed for each of the four proteins are shown in Fig 3. Two regimes are evident:

1. Initial disturbances. Considerable initial turbulence was observed caused by the disequilibrium effects of adding protein to either phase. Considerable transient water flux would be expected and, as shown in Fig. 3 complex steps in the concentration gradients formed. The initial transient fluxes were asymmetric, and were greater when protein was initially in the lower phase than when it was in the upper phase. Pre-dialysis almost completely eliminated them: in this, both phases containing protein were outside the sac. After dialysis to equilibrium, which would result in a small hydrostatic pressure difference, the protein containing phases from inside the sac were placed in contact with the corresponding phase from outside.

2. The overall diffusion process. Classical diffusion theory (cf. eqn. 1) predicts that $Q \propto t^{1/2}$. We have used the areas under the concentration profiles to estimate Q. As is shown by some typical examples in Fig. 4, eqn. 1 does appear to hold, and the overall process regardless of initial turbulence, is one of simple diffusion. The profiles in Fig. 5 were chosen to illustrate the case where diffusion must have occurred against the concentration gradient. Even in this instance, 'classical' diffusion behaviour can account for the final equilibrium.

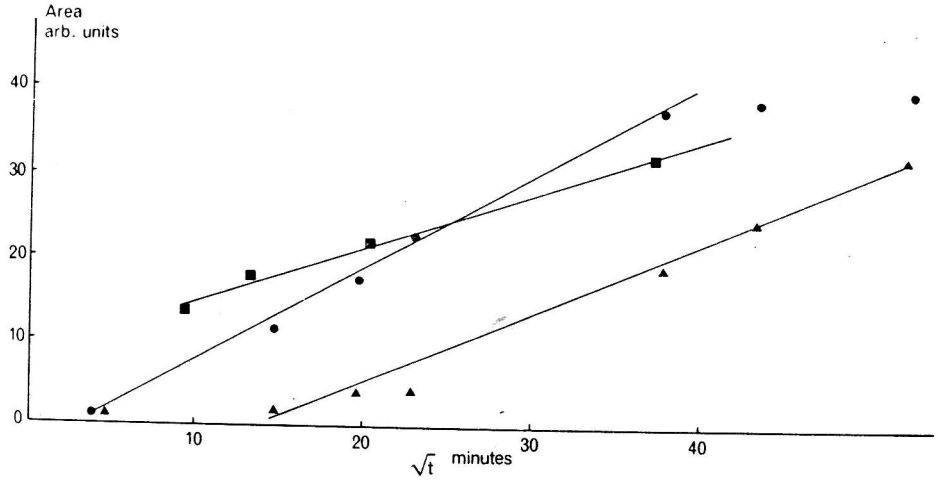
V. Discussion

Diffusion to equilibrium. For most of the proteins used in this work the partition coefficient K (= conc. in upper phase/ conc. in lower phase) is less than 1. For albumin and ovalbumin values of ~0.5 have been reported [6] and this is consistent with the ratios shown in for example Fig. 5 for times of the order of 2000 minutes. Starting with the protein in the less dense phase seems to take longer to reach the equilibrium position by pure diffusion. However as Fig. 5 illustrates, equilibrium can be reached, even if during the process of getting there protein must diffuse up its own concentration gradient [6]. This means the term $d(C'_P + A^*_{P.DEX}.C'_P\, C_{DEX})/dx$ in eqn. 3 must still be positive even though dC'_P/dx is negative. This is due to the large value for $A^*_{P.DEX}$ and C_{DEX}. Because this term changes very rapidly in the interface the concentration gradient is very sharp, at equilibrium. It is not necessary to involve effects caused by agitation to achieve this result. Diffusion alone is sufficient to account for it, providing the effect of interactions is properly taken into account. Shanbag's observation [10] that the rate of diffusion between phases was dependent on K for proteins of comparable frictional coefficient is also a reflection of the same underlying phenomenon.

Initial disequilibria. What were probably osmotic effects due to the initial disequilibrium proved to be quite easily detectable. Since buffer ions were present some of these effects were probably due to Donnan equilibria. Pre-dialysis eliminated the effects, although it did not in fact pre-equilibrate the samples since the osmotic pressure would have led to a small hydrostatic pressure difference. It might nevertheless have removed the reasons for the transient osmotic flux effects: The Donnan equilibria would already be in existence and the activity of the water in the two phases commences by being equal, and only slowly moves to a new equilibrium as a result of diffusion of the protein. Thus the initial events were different in pre-dialysed and non-predialysed samples. It is interesting to note that the semi-quantitative theory proposed by Wells [9] which we considered above correctly predicts the asymmetry of these effects, and that they would be greater with protein in the lower phase, when adapted to these two phase systems.

Interfacial accumulation. Accumulation in the interface region was consistently observed with all samples. Some of the initial transient effects could produce spikes at the interface but after relatively long periods, at or approaching equilibrium there was always excess concentrations in that region. Blank runs, with no added protein also showed apparent peaks at the interface. These were comparatively small, and probably due to the accumulation of dust. They were more

Fig. 4. Plots of flux through the interface, expressed in arbitrary area units derived from concentration profiles, against $t^{1/2}$.
2 plots for FITC bovine serum albumin, upper to lower phase (● , ▲); 1 plot for lower to upper phase (■). The linear fits in each case are consistent with a 'classical' diffusion process.



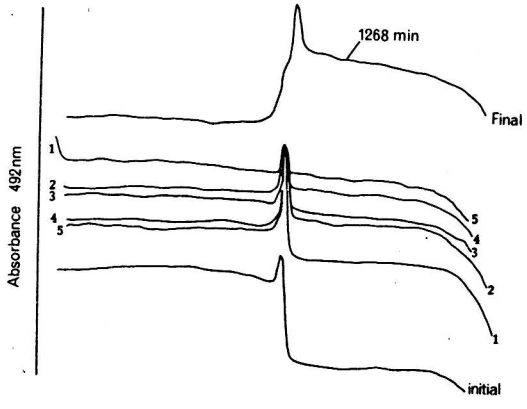


Fig. 5. Profiles for FITC bovine serum albumin, commencing in the upper phase and diffusing to equilibrium, with partition coefficients giving a higher concentration in the lower phase. The initial and final profiles have been shifted for clarity. As the intermediate traces show, diffusion occurred up the concentration gradient.

prominent when scanning at 280 nm than 492 nm as would be expected for scattering. Application of eqn. (11) and estimates of the interfacial tension leads to predictions of an excess of the order of 2-3%. Values found for cytochrome c and chromobacter lipase were far larger than this prediction (Fig. 3), varying up to 50% excess concentrations. FITC-ovalbumin, at ~5% and albumin at ~10% were nearer predicted levels.

All proteins have, on their surface a mosaic of hydrophilic and hydrophobic regions, and if these are distributed so that one side of the molecule has relatively more than the other, then this could produce large excess concentrations, as could the presence of a large heme group. Although lipases are relatively hydrophilic molecules containing carbohydrate, they are also because of their activity, likely to have hydrophobic areas and might well be expected to have considerable asymmetry in the hydrophobic-hydrophilic balance. It is distinctly possible that at the interface the protein concentration rises to a degree which makes it necessary to regard the protein at the interface as a '3rd phase': The possibility also of some self-association behaviour at the meniscus cannot be ignored: we are currently attempting to model this behaviour using advanced sedimentation velocity & low speed sedimentation equilibrium procedures.

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