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The Analytical Ultracentrifuge as a Probe for Interface Transport Phenomena

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Introduction

The technique of analytical ultracentrifugation is widely accepted in the areas of protein biochemistry and molecular biology as a powerful characterization tool for the investigation of protein conformation and ligand interaction phenomena in aqueous solution (see, for example, Harding and Winzor, 2001; Winzor and Harding, 2001). This review focuses on its application – and potential application – in the study of protein transport across interfaces between incompatible two-phase systems.

Since the classical work of Albertsson (1958), there has been interest in the way in which proteins and other macromolecules partition between incompatible aqueous or non-aqueous phases (Brooks, 1985; Abbott *et al.*, 1990; Walter *et al.*, 1991; Walter and Brooks, 1995; Munk and Aminabhavi, 2002: the reader is referred to an excellent web site http://ua1vm.ua.edu/~rdrogers/aq2phase/, which gives an extensive bibliography, courtesy of and maintained by Professor H. Walter). Parallel to this has been an increased interest in the way in which proteins diffuse through matrices and, more recently, the way in which they transport across the interface between incompatible phases. Laboratory studies on these phenomena enable us to model systems of biological and biotechnological importance, such as the cell membrane, and the enzymatic modification of lipids – which obviously involves interfacial events – and even the mobilization of seed protein bodies (Tombs *et al.*, 1974; Tombs and Harding, 1988). One technique in particular lends itself to the study of phase partition phenomena and non-ideal diffusion through matrices and interfaces. This is the analytical ultracentrifuge, which itself has undergone something of a renaissance over the past decade following the launch of two new commercial analytical ultracentrifuges in 1991 and 1997. Renaissance and methodological development of the analytical ultracentrifuge in the areas of protein conformation (Garcia de la Torre *et al.*, 1997; Harding *et al.*, 1997; Pavlov *et al.*, 1997) and interactions (Pintar *et al.*, 1996; Holtham *et al.*, 1999; Lafitte *et al.*, 1999; Harding and Winzor, 2001; Winzor and Harding,

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Biotechnology and Genetic Engineering Reviews – Vol. 19, November 2002
0264–8725/02/19/55–69 $20.00 + $0.00 © Intercept Ltd, PO Box 716, Andover, Hampshire SP10 1YG, UK

2001), the genetic engineering of macromolecules (Harding, 1993; Pasquo *et al.*, 1996; Dean *et al.*, 1997; Silkowski *et al.*, 1997; Walters *et al.*, 2000) and solution studies on polysaccharides (Harding, 1995; Tongdang *et al.*, 1999) seems to have lagged in its application to the area of phase and phase-interface transport phenomena. One exception has been in the area of gel structure and gel diffusion, recently reviewed by Cölfen (1999). It is hoped that this present short review of some of the underpinning theory behind interface transport phenomena, and the application of this technique largely in the 1980s and early 1990s, will raise interest in its use in the phase technology area, and interfaces in general.

Application of the ultracentrifuge in the investigation of interface transport phenomena has focused on one model system, namely the interface separating incompatible aqueous solutions of polyethylene glycol (PEG)–Dextran two-phase systems. We look at how the centrifuge has been used to study the way in which proteins move through solutions of macromolecules to the water–water interface and penetrate it in terms of: 1) overall diffusion kinetics (Tombs and Harding, 1988; Harding and Tombs, 1989; Simpkin, 1994); 2) instabilities at the interface (Laurent *et al.*, 1979, 1983; Preston *et al.*, 1980; Harding and Tombs, 1989; Comper and Preston, 1992); and 3) interfacial accumulation phenomena (Albertsson, 1958; Harding and Tombs, 1989). This review on quaternary two-phase systems complements a review by Comper and Preston (1992) who described diffusion processes in ternary systems, and the interested reader is referred to this.

Theoretical considerations

DIFFUSION IN CONCENTRATED SYSTEMS

In both phases, the protein must diffuse through solutions of other polymers. By analogy with dilute solution behaviour (Harding and Johnson, 1985a,b), these can affect the flux or (translational) apparent diffusion coefficient D, in two ways (Laurent *et al.*, 1983): 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; and ii) a thermodynamic effect related to exclusion volume (or 'covolume') and polyelectrolyte effects, which tends to increase D. Depending on the relative effects of i) or ii), diffusion can be retarded or enhanced. In 'classical' diffusion (see, for example, Van Holde, 1985).

$$\mathrm{d}(Q/C_o)^2/\mathrm{d}t = D(A^2/\pi) \qquad (2.1)$$

where Q is the quantity of material crossing the boundary in time t with initial concentration C. A is the cross-sectional area. In concentrated ternary solutions, Albertsson (1986) showed that this relation is replaced by

$$\mathrm{d}(Q/C_o)/\mathrm{d}t = k \qquad (2.2)$$

where k is a transport rate. For intermediate concentrations, diffusion can commence according to (*Equation 2.1*) and change to (*Equation* 2.2) as concentrations shift.

TWO-PHASE SYSTEMS

Two-phase systems, together with added protein, are quaternary systems but, as

pointed out by, for example, Harding and Tombs (1989), the same considerations apply as with ternary systems (one phase with added protein). In the polyethylene glycol–dextran systems that have been studied by using the analytical ultracentrifuge, the polymer concentrations were high enough for linear kinetics (*Equation 2.2*) to apply for describing the polymer diffusion behaviour. However, Ogston had clearly established earlier (Ogston, 1970) that, for some representative globular proteins, the ratio 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.

APPROACH TO EQUILIBRIUM

The flux equations (including cases where diffusion must occur up the concentration gradient to achieve equilibrium) have been presented as (Harding and Tombs, 1989)

$$\text{Flux A} \rightarrow \text{B: } J_{AB} = D_o.\, \mathrm{d}\,(C'_p + A^*_{P.DEX} \,.\, C'_p C_{DEX} + \ldots)/\mathrm{d}x \qquad (2.3)$$

$$\text{Flux B} \rightarrow \text{A: } J_{BA} = D_o.\, \mathrm{d}\,(C''_p + A^*_{P..PEG} \,.\, C''_p C_{PEG} + \ldots)/\mathrm{d}x \qquad (2.4)$$

where C'_P, C''_P are the protein concentrations (g/ml) in phases A (dextran-rich) and B (PEG-rich), respectively, and C_{DEX}, C_{PEG} the concentrations of dextran (in the dextran-rich phase) and polyethylene glycol (in the PEG-rich phase). The ability to diffuse up a concentration gradient was stated by these workers to depend on the magnitude of the cross-interaction terms, $A^*_{P.DEX}$, $A^*_{P..PEG}$. Harding and Tombs (1989) also emphasized the point that, within the two phases, diffusion was mainly dependent on the local viscosity – and was slow. It was also pointed out that there will be a large viscosity gradient at – and in – the interface between the two phases, which might be the major factor in determining the concentration profile and will be markedly asymmetric depending on the direction of diffusion. This was subsequently demonstrated experimentally (Harding and Tombs, 1989; Simpkin, 1994) and will be considered further below.

TRANSIENT WATER FLUXES: 'DENSITY INVERSIONS' AND 'FINGERING'

Wells (1986) had earlier 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 *Figure 2.1a*, where A is described as the matrix solute and P the co-solute (in our case, protein), it can also be applied to the quaternary two-phase systems shown in *Figure 2.1b* and *Figure 2.1c*. The question that needed addressing 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 the water.

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

$$\rho = \rho_o\,(1 + \alpha C_A + \beta C_P) \qquad (2.5)$$

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:

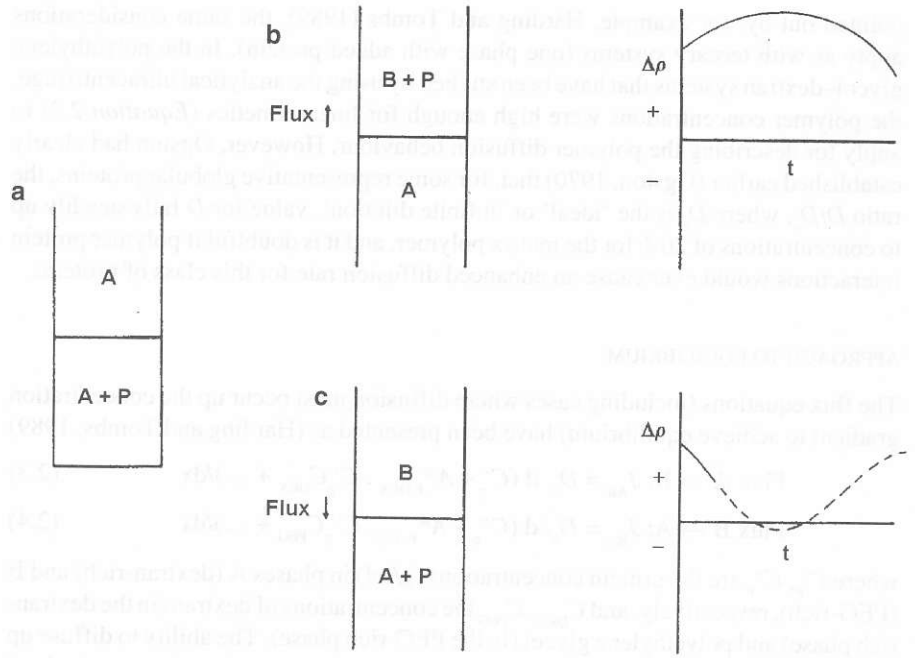


Figure 2.1. 'a' represents layered solutions containing a matrix polymer A together with protein P. 'b' and 'c' represent the two possibilities where two polymers A and B are used to produce phase separation, followed by the addition of protein to one or other of them (although both phases contain both 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. Adapted from Wells (1986) and Harding and Tombs (1989).

$$\alpha = (1/\rho_o) - \bar{v}_A; \beta = (1/\rho_o) - \bar{v}_P \tag{2.6}$$

The following argument based on the Wells' (1986) theory was proposed by Harding and Tombs (1989): 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 and upper phases respectively, the condition for instability or 'fingering' of protein passing through the interface (Laurent *et al.*, 1983) is that $\Delta\rho = (\rho' - \rho'')$ must become negative. Wells (1986) demonstrated that for the case illustrated in *Figure 2.1a* and, after making the rather unrealistic assumption of thermodynamic ideality, this condition for instability leads to

$$\alpha/\beta > M_P/M_A \tag{2.7}$$

(where M_P and M_A are the molecular weights of co-solute P and matrix solute A respectively), although the same concepts apply to quaternary systems (*Figures 2.1b* and *2.1c*).

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 those arguments, Harding and Tombs (1989) put forward the following two relations as instability criteria for the cases shown in *Figures 2.1b* and *2.1c* respectively:

$$\alpha_{DEX}/\alpha_{PEG} > M_P/M_{DEX}; \alpha_{PEG}/\alpha_{DEX} > M_P/M_{PEG} \tag{2.8}$$

Taking the molecular weight for PEG as M ~ 6000 g/mol, by this test case, *Figure 2.1c* fails to give turbulences or 'fingers', whereas for dextran (M ~ 500 000 g/mol) the situation *Figure 2.1b* could. Thus, by this test we might expect density inversion and 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 *Figure 2.1c* 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 stabilized since the density difference between the phases will tend to increase. By contrast, in *Figure 2.1b* 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.

INTERFACIAL ACCUMULATION

Some possible profiles can be predicted from theoretical considerations, and Albertsson (1986) 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 and γ_{12} between the particle and the two phases, where γ_1, γ_2 and γ_{12} are the interfacial energies between phase 1 and the particle, phase 2 and the particle and between the two phases respectively. From this, Harding and Tombs (1989) considered in some detail the two possibilities that could 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 the particle is either totally in one of the phases or in the interface. The interfacial tension between PEG and dextran was found by these workers to be low, (< 0.02 dyne/cm), but they pointed out that the difference $\gamma_1 - \gamma_2$ was also likely to be very low (cf. partition coefficients near one) so that the latter condition allowing a build-up at the interface would be by no means unlikely. The possible profiles are given in *Figure 2.2*. Since, in general terms, the partition coefficient

$$K = (C_1/C_2)) = \exp [-\Delta E/kT] = \exp [-4\pi R^2 (\gamma_1 - \gamma_2)/kT] \quad (2.9)$$

(where ΔE is the energy difference between the phases), Harding and Tombs (1989) were able to write for condition '1' and, perhaps more interestingly, for the partition between the interfacial region and the phase, with condition '2'

$$C_i/C_1 = \exp [\pi R^2 (\gamma_2 - \gamma_1 - \gamma_{12})^2/ \gamma_{12} kT] \quad (2.10)$$

where C_i is the concentration (g/ml) in the interface and C_1, the concentration in phase 1. It was then necessary for a value for $(\gamma_1 - \gamma_2)$ to be chosen. This was found to be small and, for example, 0.001 yielded a value for the ratio C_i/C_1 of only ~ 1.009. In those cases where $(\gamma_1 - \gamma_2)$ is negligible compared with γ_{12}, then they found that to a good approximation:

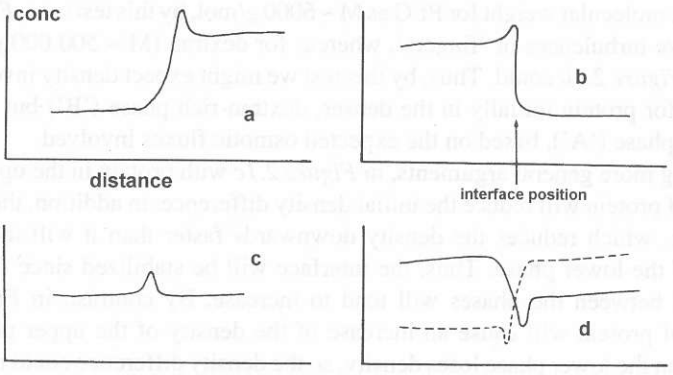


Figure 2.2. Predicted concentration profiles across the interface for various combinations of interfacial tension between particles and phases. Adapted from Harding and Tombs (1989).

$$C_i/C_1 = \exp\,[6.8\gamma_{12}] \qquad (2.11)$$

Wells (1986) quoted values for the interfacial energy in the PEG–Dextran as variable between 0.00046 and 0.06600 dyne/cm (or erg/cm^2) and depended on the precise composition: the further from the critical point, the higher the value. Such findings suggested to Harding and Tombs (1989) that excess concentrations of the order of 60% of the bulk concentration are not impossible. In the dynamic system approaching equilibrium, it was pointed out that there were other factors which may tend to make this build-up greater: for example, 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, and this was then shown to be experimentally the case for a number of different proteins.

Application of the analytical ultracentrifuge

The analytical ultracentrifuge is not a new technique. T. Svedberg received the Nobel Prize for its invention in 1926 (see Rånby, 1987) and its principal feature of being able to record concentration distributions during a sedimentation velocity experiment (for size, shape and heterogeneity analysis: Harding and Winzor, 2001) or during a sedimentation equilibrium experiment (for molecular weight, oligomeric composition, strength and stoichiometry of interaction measurements: Winzor and Harding, 2001) has found wide application. A modified approach using either sucrose density gradients for enhancing velocity separations, and caesium salt gradients for isopycnic equilibrium density gradient analyses has also found wide application (see, for example, Harding, 1992).

Harding and Tombs (1989) proposed its use in a somewhat different and novel context. They proposed its use as a tool for monitoring the diffusion of proteins through matrices and interfaces between phases, and used the specific example of quaternary incompatible aqueous two-phase polyethylene glycol (PEG) and dextran systems of the type described above. The experiment is not a true 'sedimentation one' – with only a low centrifugal field so as to minimize significant sedimentation of either the protein, the PEG or the dextran during the time course of the measurement, but sufficient to stabilize the system against convective phenomena.

In this way, Harding and Tombs (1989) were able to examine the concentration profiles by using the scanning absorption optical system of an MSE (Crawley, UK)

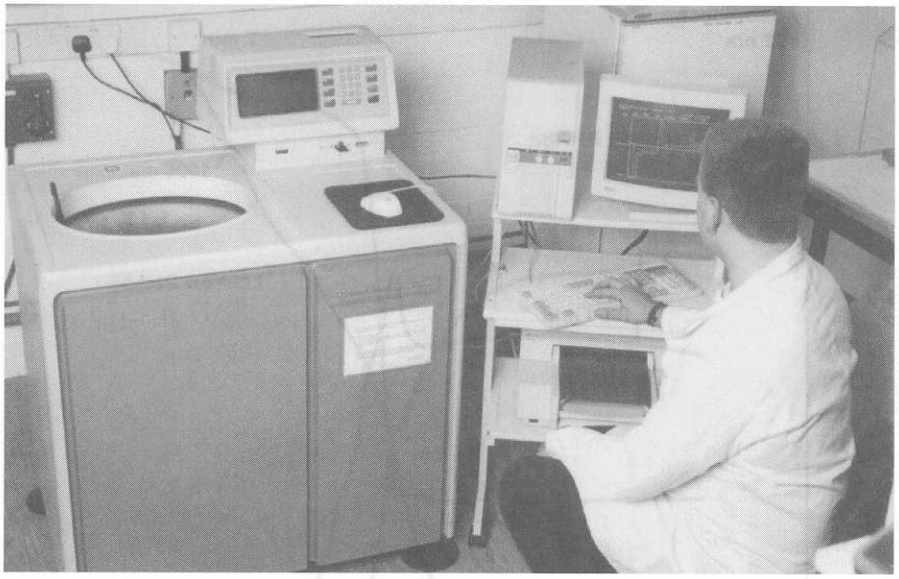


Figure 2.3. The Beckman XL-A/XL-I analytical ultracentrifuge (courtesy of Beckman Instruments, Palo Alto, USA).

Centriscan analytical ultracentrifuge equipped with a monochromator. Although the Centriscan instrument is no longer commercially available, other improved instruments are, namely the Optima XL-A and XL-I analytical ultracentrifuges manufactured by Beckman Instruments (Palo Alto, USA) (*Figure 2.3*).

In the experiments described in their 1989 paper, Harding and Tombs loaded 0.22 ml of lower phase, followed by the same volume of top phase into 10 mm path length cells. At the position of the interface, the cross-sectional area was 25.9 mm^2. The rotor was run at 4000 rpm and temperatures of either 20.0°C or 25.0°C were chosen. Appropriate blanks were included, mainly the two phases with no added protein. Most experiments were done in the two phases produced by mixing 5 g of PES 6000 (BDH Biochemical grade, with low 280 nm absorbance), 5.3 g of Dextran T-500 (Pharmacia) with 100 ml of 50 mM 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, FCTC-labelled ovalbumin, chromobacter lipase and cytochrome c. Protein concentrations of ~ 1 mg/ml (~ 15 μM) were added to either phase. The experiments were supplemented by interfacial surface tension measurements by the spinning drop method, which gave a value of (0.09 ± 0.03) mN m^{-1}.ml.g^{-1}. The density drop across the interface was ~ 0.03 g/ml so that the interfacial energy was 0.0027–0.0036 erg.cm^{-2}. Using the analytical ultracentrifuge in this way, Harding and Tombs (1989) were able to demonstrate two clear regimes.

INITIAL DISTURBANCES

Considerable initial turbulence was found, caused by the disequilibrium effects of adding protein to either phase. Much transient water flux would be expected and, as shown in *Figure 2.4*, complex steps in the concentration gradients formed. The initial

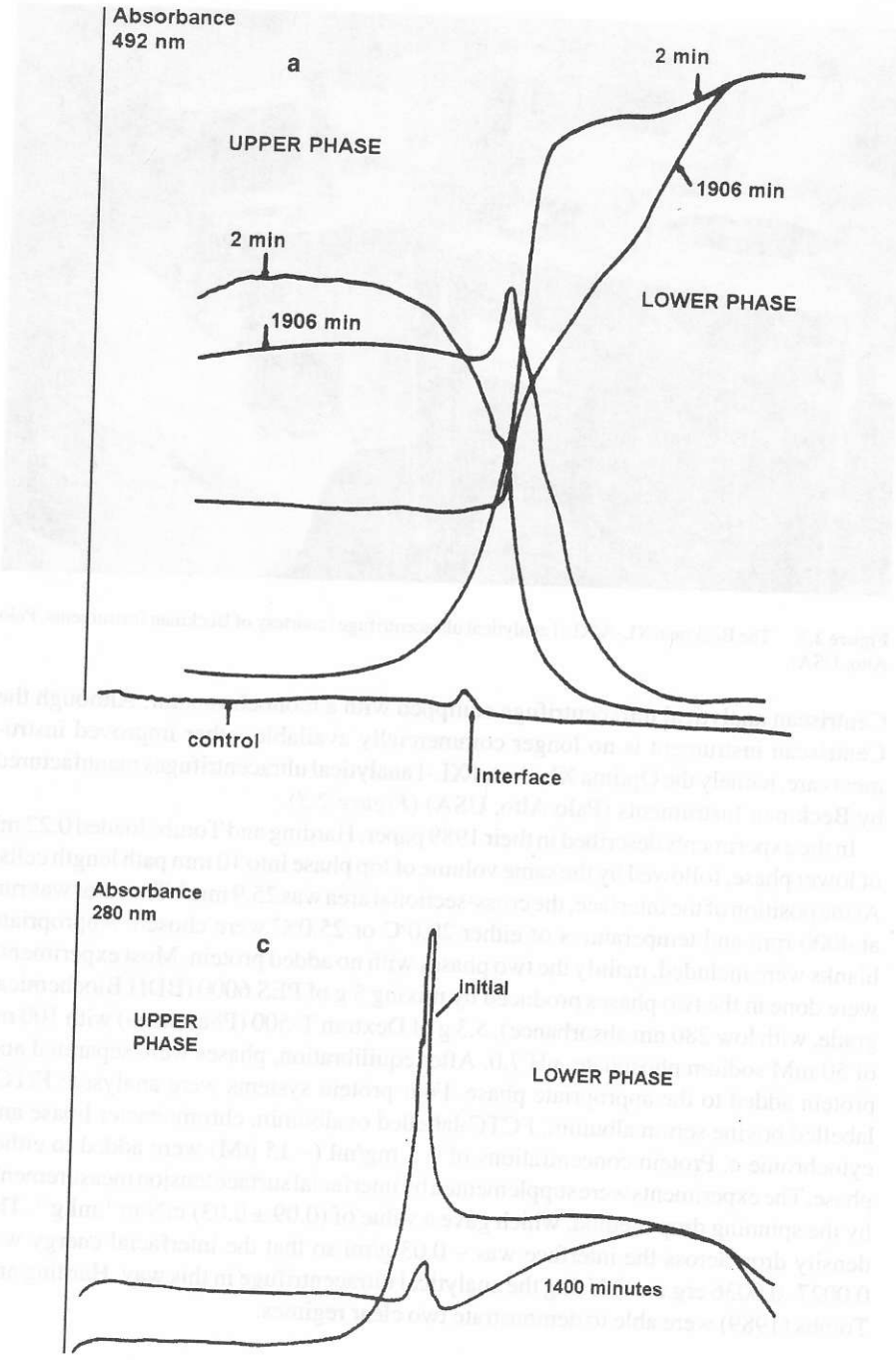
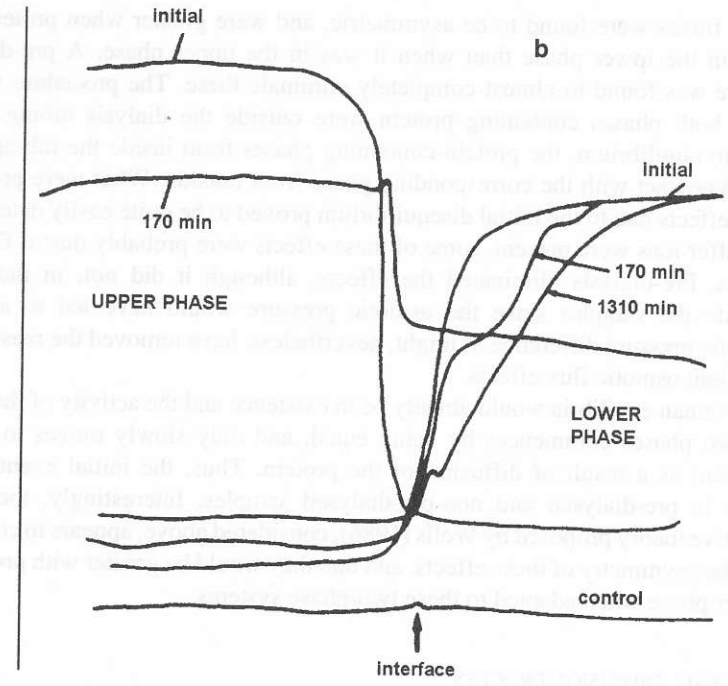
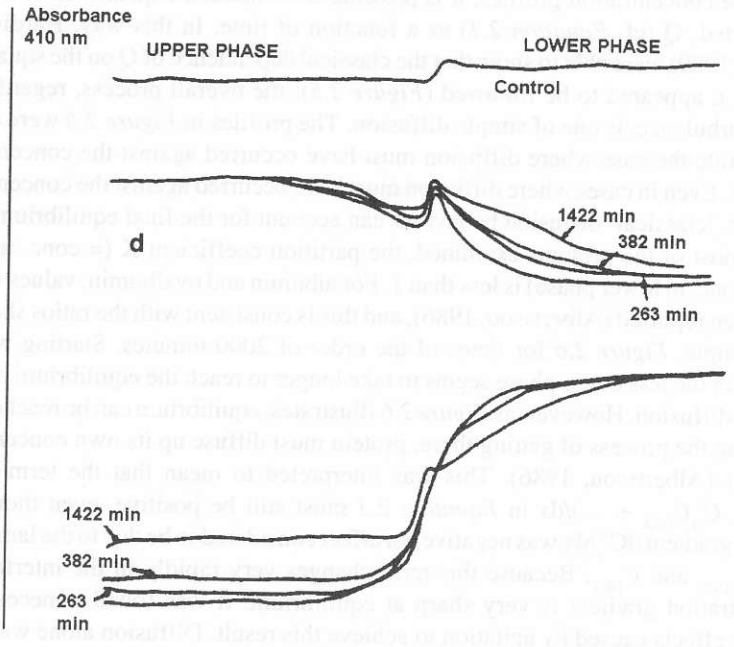


Figure 2.4. Protein concentration profiles: a) FITC bovine serum albumin, added at ~1 mg/ml to either the upper or lower phases. Behaviour is different depending on which phase the protein is added to. At the bottom, a control containing no protein. The concentration profiles were measured in three cells 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.

transient fluxes were found to be asymmetric, and were greater when protein was initially in the lower phase than when it was in the upper phase. A pre-dialysis procedure was found to almost completely eliminate these. The procedure was as follows: both phases containing protein were outside the dialysis tubing. After dialysis to equilibrium, the protein-containing phases from inside the tubing were placed in contact with the corresponding phase from outside. 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-pre-dialysed samples. Interestingly, the semi-quantitative theory proposed by Wells (1986), considered above, appears to correctly predict the asymmetry of these effects, and that they would be greater with protein in the lower phase when adapted to these two-phase systems.

THE OVERALL DIFFUSION PROCESS

From the concentration profiles, it is possible to estimate the quantities of material transported, Q (cf. *Equation 2.1*) as a function of time. In this way, Harding and Tombs (1989) were able to show that the classical dependence of Q on the square root of time, t, appeared to be followed (*Figure 2.5*): the overall process, regardless of initial turbulence, is one of simple diffusion. The profiles in *Figure 2.6* were chosen to illustrate the case where diffusion must have occurred against the concentration gradient. Even in cases where diffusion must have occurred against the concentration gradient, 'classical' diffusion behaviour can account for the final equilibrium.

For most of the proteins examined, 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 (Albertsson, 1986), and this is consistent with the ratios shown in, for example, *Figure 2.6* 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 *Figure 2.6* illustrates, equilibrium can be reached even if, during the process of getting there, protein must diffuse up its own concentration gradient (Albertsson, 1986). This was interpreted to mean that the term $d(C'_p + A^*_{P.DEX}.\ C'_p C_{DEX} + \ldots)/dx$ in *Equation 2.3* must still be positive, even though the protein gradient dC'_p/dx was negative, an effect considered to be 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 was found unnecessary to involve effects caused by agitation to achieve this result. Diffusion alone was found to be sufficient to account for it, providing the effect of interactions is properly taken into account. Shanbag's observation in the 1970s (Shanbag, 1973) 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.

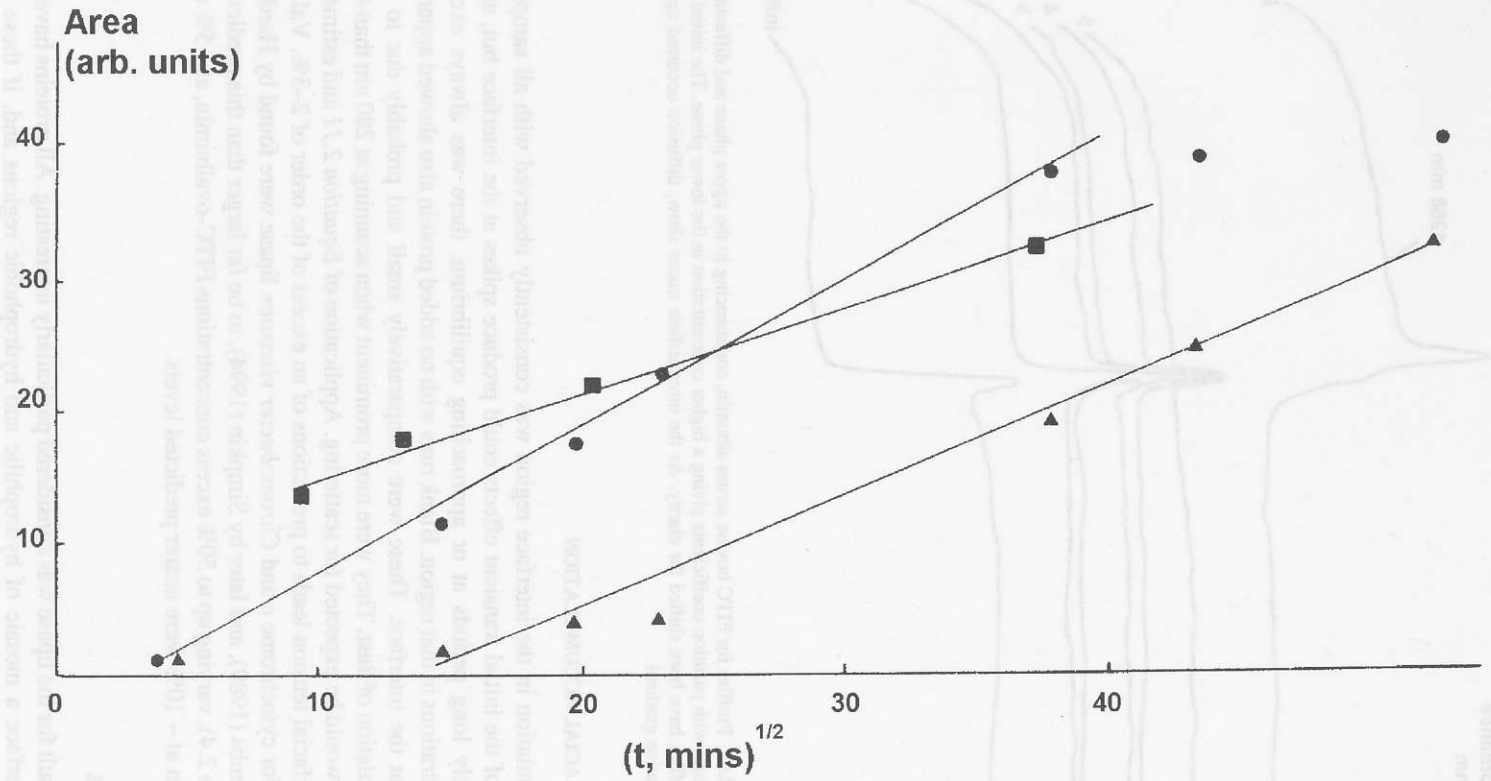


Figure 2.5. Plots of flux through the interface, expressed in arbitrary area units derived from concentration profiles, against $t_{1/2}$. 2 plots for FITC bovine serum albumin, upper to lower phase (●, ▲); 1 plot for lower to upper phase (■). The linear fits in each case are consistent with a 'classical' diffusion process.

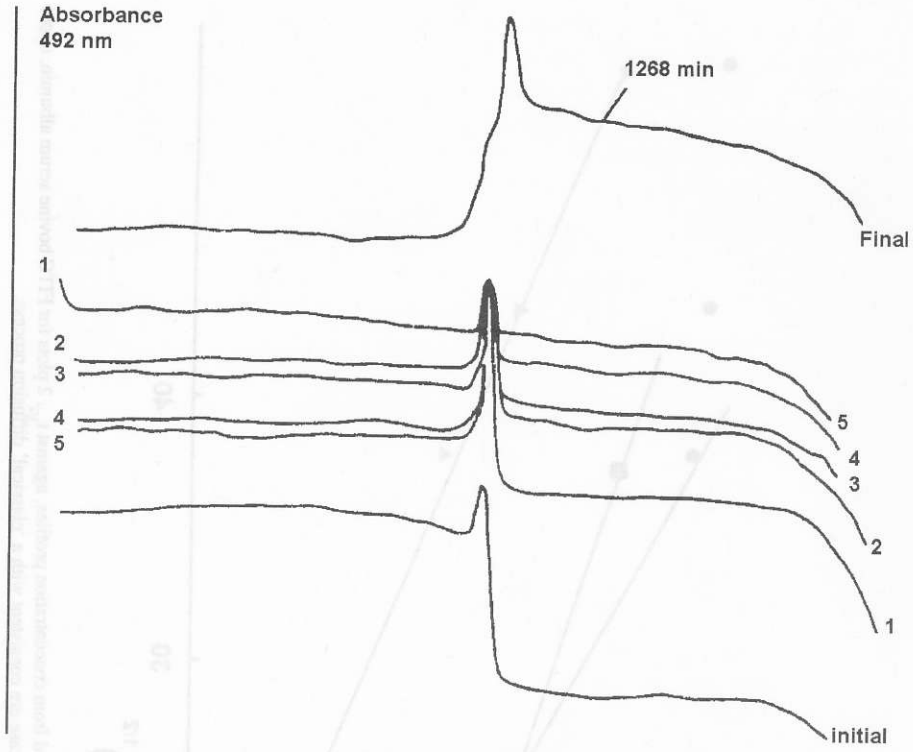


Figure 2.6. Profiles for FITC bovine serum albumin, commencing in the upper phase and diffusing to equilibrium, with partition coefficients giving a higher concentration in the lower phase. The initial and final profiles have been shifted for clarity. As the intermediate traces show, diffusion occurred up the concentration gradient.

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 prominent when scanning at 280 nm than 492 nm, as would be expected for scattering. Application of *Equation 2.11* and estimates of interfacial tension leads to predictions of an excess of the order of 2–3%. Values found for cytochrome *c* and *Chromobacter viscosum* lipase were found by Harding and Tombs (1989), and later by Simpkin (1994), to be far larger than this prediction (*Figure 2.4*), varying up to 50% excess concentrations FITC-ovalbumin, at ~ 5% and albumin at ~ 10% were nearer predicted levels.

LIPASES

The result for the lipase was considered particularly interesting. 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 water-soluble hydrophilic molecules and many of them are glycoproteins, 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'. A study using fluorescence-labelled fungal lipases showed that, at saturation of the lipid–water interface, the average distance between the lipase molecules was approximately one molecular diameter (Tombs and Roberts, 1987). More recent detailed X-ray crystallographic structures suggest that there is a substantial conformational change on entering the lipid–water interface, which exposes a hydrophobic active centre. There is no evidence of self-association via hydrophobic patches in free solution on the basis of conventional analytical ultracentrifuge analysis (Simpkin *et al.*, 1991), and the situation at the water–water interface may be quite different from that at a water–lipid interface.

Concluding remarks

In conclusion, this re-visitation of work undertaken principally in the 1980s before the launch of the new generation of instruments with on-line data capture and analysis facilities would appear to suggest that the analytical ultracentrifuge is worthy of serious consideration as a tool for monitoring both transport of proteins through interfaces between aqueous (or non-aqueous) phases and the resulting equilibrium partition concentrations. The new generation of instruments should make the recording and analysis extremely convenient and effective. The protein needs to be visible (by recording at 278 nm, or in cases where the matrix also absorbs here, by incorporation of a suitable chromophore). So long as appreciable sedimentation is avoided and diffusion is the only transport phenomena involved, even interfacial build-up of material can be monitored.

Perhaps the most interesting application of this approach is the way in which the water–water interface, possibly with inserted monolayers of suitable lipids, could be used to model the cellular membrane. We should also not forget that it could be applied to systems with three or more phases.

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