Macromolecular Distribution near the Limits of Density-Gradient Columns

SOME APPLICATIONS TO THE SEPARATION AND FRACTIONATION OF GLYCOPROTEINS

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1. Expressions are derived for the distribution at density-gradient equilibrium of macromolecules whose densities are (a) close to the values characterizing the solution limits or (b) outside the span of the gradient. 2. Density-distributions predicted by the expressions agree with those obtained by rigorous methods. 3. The distribution equations are applied to hypothetical mixtures of proteins and glycoproteins in commonly used density-gradient media to simulate separation and fractionation conditions. 4. It is shown that CsBr, although less efficient than CsCl for fractionation, is nevertheless adequate for most purposes; in analytical experiments it may often have advantages over CsCl. Limitations on the use of LiBr are explored. 5. An expression is derived which allows the variance of the partial specific volume of the macromolecular component to be determined from the variance of the buoyant density. It is shown that the relative resolving powers of different salts is expressed by their values of the quantity $[\beta\rho_0^3/(1 + \Gamma')]$ [†]. 6. The equations are applied to a well-characterized glycoprotein preparation at equilibrium in CsCl and in Cs₂SO₄: it is shown that the much wider distribution in CsCl than in Cs₂SO₄ is explicable in terms of the variance in buoyant density and the solvation properties of the salts. 7. Limitations of the expressions arise when dispersity in density is represented by a low apparent molecular weight; realistic simulations can then only be obtained when the component is fully banded.

Since the classic paper of Meselson *et al.* (1957), ultracentrifugal density-gradient equilibrium methods have become widely used for separating and characterizing macromolecules on the basis of differences in density. The theory presented by Meselson *et al.* (1957) was based on several simplifying approximations, for example, that the salt-density gradient is constant over small distances, that the cubic term in the formulation of the macromolecular concentration could be neglected, that the concentrations at the physical limits of the cell were zero, and that the rectangular cell approximation applied. These assumptions introduce negligible error for substances of high molecular weight and constant density, and the theory successfully predicted the concentration distributions of macromolecular nucleic acids. Similarly, the problem of the degree of resolution of two macromolecular species having a small difference in density was examined by Ifft *et al.* (1961), on the same assumptions, and experimentally useful solutions were obtained.

For the broader distributions obtained with smaller macromolecules, Ifft & Vinograd (1962) derived an expression for the relative concentration distribution in which the effect of the varying gradient was taken into account; the expression permitted the

interpretation of experimental results, but not the prediction of absolute concentration values.

Secretory glycoproteins are dispersed in density, and so tend to give broad distributions in density gradients; moreover, they usually occur with large amounts of free protein, from which separation is possible by density-gradient methods (e.g. Creeth & Denborough, 1970). In these systems interest lies in the prediction of conditions which will optimize (a) separation on a preparative scale and (b) the detection of small proportions of protein in analytical experiments. The buoyant-density difference between glycoproteins and proteins may be quite large (Creeth *et al.*, 1974; Bhaskar & Creeth, 1974). Accordingly, an experiment designed to band glycoprotein near the centre of the cell necessarily bands protein close to the meniscus; or, if the buoyant density of the protein is outside the span of the gradient, the protein will merely be concentrated in that region. In either case, the concentration of protein at the solution limit will be relatively high, and the effects of non-constancy of the gradient maximal. To interpret such experiments correctly, it is necessary to have equations predicting the macromolecular concentration as a function of radial distance which take explicit account of these factors. Such expressions are presented here for the

two cases referred to, i.e. (i) where the buoyant density is within the span of the gradient, but the zone is not resolved from the solution limits, and (ii) where the buoyant density lies outside the span of the gradient. Although the examples chosen refer to protein-glycoprotein systems, the equations have general validity; accordingly they allow the simulation of experimental conditions and the easy comparison of gradient materials.

The expressions have simple forms and may readily be programmed for solution by a desk-top computer: they therefore supplement the treatment outlined by Howlett & Jeffrey (1973), where a computer was used to generate any desired solution by numerical integration.

Theory

(i) General

The differential equation of sedimentation equilibrium, eqn. (1), applies to all species whether or not a density gradient exists:

$$\frac{1}{rc} \frac{dc}{dr} = \frac{M(1-\bar{v}\rho)\omega^2}{RT}; a < r < b \quad (1)$$

Here r is the radial distance from the centre of rotation, a and b the upper and lower limits (meniscus and base) respectively, c the macromolecular concentration on a weight-per-unit-volume scale, M the mol. wt., $\bar{v}$ the partial specific volume at the density ρ , ω the angular velocity, R the gas constant and T the absolute temperature. It is assumed that the macromolecule is electrically neutral and that the solution is so dilute that thermodynamic non-ideality may be ignored. The simultaneous equilibrium of the third component (the dense electrolyte) is not considered in this formulation (except for the density; see below); it is well recognized that this procedure is valid only when differential solvation is zero. From the rigorous three-component theory (Hearst & Vinograd, 1961), however, it is evident that eqn. (1) applies if the solvated mol. wt., M_s , and the reciprocal of the buoyant density, ρ_0 , are substituted, respectively, for M and $\bar{v}$. In the present context, where the main purpose is the prediction of c as a function of r for known M_s and ρ_0 , the substitution will be assumed without further comment. Similarly, compressibility effects (Hearst & Vinograd, 1961; Hearst *et al.*, 1961; Ifft & Vinograd, 1966) are not taken into consideration: these effects are usually small, and do not affect the general conclusions to be drawn from the results.

Values of the density at any point in the cell were obtained by the following procedure, based on the methods of Hearst *et al.* (1961), Ifft & Vinograd (1962) and Ludlum & Warner (1965). Values of ρ as a

function of the concentration of dense electrolyte are assumed to be known and will not be further specified.

The salt parameter, β (Ifft *et al.*, 1961), determines the density gradient at a convenient reference point, r , through the equation:

$$\frac{d\rho}{dr} = \frac{\omega^2 r}{\beta} \quad (2)$$

For $r = r_e$, the iso-concentration point, $\beta = \beta(\rho_e)$, where ρ_e is the initial density of the salt solution, before re-distribution by the gravitational field. The iso-concentration point is assumed to be given by eqn. 3(a) for sector-shaped cells and 3(b) for cylinders:

$$r_e = \sqrt{\left(\frac{b^2 + a^2}{2}\right)} \quad (3a)$$

$$r_e = \sqrt{\left(\frac{b^2 + a^2 + ab}{3}\right)} \quad (3b)$$

Ifft *et al.* (1961) and Ifft (1969, 1973) have examined the small errors consequent on using these approximations to the iso-concentration points; although a refinement is possible, it was not considered worth while for present purposes.

The density is expressed as a Taylor series:

$$\rho = \sum_{k=0}^{\infty} \frac{(r-r_e)^k}{k!} \left(\frac{\partial^k \rho}{\partial r^k} \right)_{r=r_e} \quad (4)$$

Inclusion of terms up to $k = 2$ only is found to give adequate accuracy. Inserting the derivatives from eqn. (2) and expanding, we find:

$$\rho = \rho_e + E(r-r_e) + E \left(\frac{1}{r_e} - EF \right) \frac{(r-r_e)^2}{2} \quad (5a)$$

where

$$E \equiv \frac{\omega^2 r_e}{\beta(\rho_e)} \quad (5b)$$

and

$$F \equiv \left[\frac{d(\ln \beta)}{d\rho} \right]_{\rho=\rho_e} \quad (5c)$$

It is seen that E and F , defined by eqns. 5(b) and 5(c), are not functions of r . These definitions apply in all subsequent derivations.

(ii) Banding near a solution limit

Here ρ_0 , the buoyant density, lies between $\rho(a)$ and $\rho(b)$, but is close to one of these values; accordingly $c(a)$ or $c(b)$ will not be zero. The expressions are most conveniently formulated in terms of the displacement, h , from the point r_0 in the cell where $\rho = \rho_0$. Thus r_0 is analogous to the 'band centre' of a fully resolved band and:

$$h \equiv r - r_0 \quad (6)$$

After formulation of ρ_0 in terms of eqn. 5(a), ρ_e may be eliminated: the resulting expression is then an expansion about ρ_0 in which the derivatives are defined at ρ_e :

$$\rho = \rho_0 + Eh \left[\frac{r_0}{r_e} + EF(r_e - r_0) \right] + \frac{Eh^2}{2} \left(\frac{1}{r_e} - EF \right) \quad (7)$$

After substituting the value of the density in eqn. (1) and noting that $\bar{v}\rho_0 = 1$, we obtain:

$$\frac{d(\ln c)}{dh} = -k \left\{ h \left[\frac{r_0}{r_e} + EF(r_e - r_0) \right] + \frac{h^2}{2} \left[3 \left(\frac{1}{r_e} - EF \right) + 2EF \frac{r_e}{r_0} \right] \right\} \quad (8)$$

Here several of the quantities constant in a given experiment have been collected as:

$$k = \omega^2 M \bar{v} E r_0 / RT = \omega^4 M r_0 r_e / \rho_0 \beta RT \quad (9)$$

Integration then gives the result:

$$c = c(r_0) \exp \left(-k \left\{ \frac{h^2}{2} \left[\frac{r_0}{r_e} + EF(r_e - r_0) \right] + \frac{h^3}{6} \left[3 \left(\frac{1}{r_e} - EF \right) + 2EF \frac{r_e}{r_0} \right] \right\} \right) \quad (10)$$

When specialized to the case $r_0 = r_e$, eqn. (10) may be simplified to eqn. (8) of Ifft & Vinograd (1962), used by them for analysing broad distributions.

The functional dependence of c on h is now defined, but the integration constant $c(r_0)$ remains to be determined. The mass-conservation restriction, written first for a sector-shaped cell, provides the necessary relationship to the initial concentration, c^0 :

$$\int_a^b r c dr = \int_{h_a}^{h_b} (r_0 + h) c dh = c^0(b^2 - a^2)/2 \quad (11)$$

Substitution of c from eqn. (10) into eqn. (11) then gives:

$$c(r_0) = \frac{c^0(b^2 - a^2)}{2} \cdot \frac{1}{r_0 [I_1(h_b) - I_1(h_a)] + [I_2(h_b) - I_2(h_a)]} \quad (12)$$

Here I_1 and I_2 represent the definite integrals:

$$I_1(x) = \int_0^x \exp(-px^2 - qx^3) dx \quad (13)$$

$$I_2(x) = \int_0^x x \exp(-px^2 - qx^3) dx \quad (14)$$

where, for simplicity, p and q have been used to represent the coefficients of h^2 and h^3 respectively in eqn. (10).

It remains to establish r_0 in terms of the known quantities ρ_0 and ρ_e . Two forms of expression are

necessary. For $[(\rho_a + \rho_e)/2] < \rho_0 < [(\rho_e + \rho_b)/2]$, i.e., when r_0 lies in the middle third of the cell, a Taylor expansion about r_e is satisfactory, and the result may be found directly in terms of the inverse derivatives of ρ with respect to r , which are already determined:

$$r_0 = r_e + \frac{\rho_0 - \rho_e}{(d\rho/dr)} - \frac{(\rho_0 - \rho_e)^2}{2 \left(\frac{d\rho}{dr} \right)^3} \left(\frac{d^2\rho}{dr^2} \right) \quad (15)$$

As with eqns. (4) and (5), the derivatives are written in terms of E and F :

$$r_0 = r_e + \frac{(\rho_0 - \rho_e)}{E} - \frac{(\rho_0 - \rho_e)^2}{2E^2} \left(\frac{1}{r_e} - EF \right) \quad (16)$$

Where r_0 is relatively close to a or b , however, it is advantageous to expand about a or b . For example, the expressions for $r = a$ then take the forms:

$$\beta(\rho_a) = \beta(\rho_e) [1 + (\rho_a - \rho_e)F] \quad (17)$$

$$\left(\frac{dr}{d\rho} \right)_{r=a} = \frac{r_e}{a} \cdot \frac{[1 + (\rho_a - \rho_e)F]}{E} \equiv 1/P \quad (18)$$

$$\left(\frac{d^2r}{d\rho^2} \right)_{r=a} = - \frac{r_e^2 [1 + (\rho_a - \rho_e)F]^2 - EFa^2/r_e}{a^3 E^2} \quad (19)$$

Expansion about a or b is particularly desirable when dealing with relatively long columns such as exist in preparative tubes; in the short columns of analytical cells, the effect of this refinement is small.

Combination of eqns. (10) and (12) with the appropriate r_0 value found from eqn. (16) or its modification then gives the final expression:

$$c(r) = \frac{c^0(b^2 - a^2)}{2 \{ r_0 [I_1(h_b) - I_1(h_a)] + [I_2(h_b) - I_2(h_a)] \}} \times \exp(-ph^2 - qh^3) \quad (20)$$

Here h is defined by eqn. (6), I_1 and I_2 by eqns. (13) and (14), and p and q are given in terms of the primary variables by the expressions:

$$p = \frac{\omega^4 M}{2\rho_0 \beta RT} \left[r_0^2 + \frac{\omega^2 r_e^2 r_0 F(r_e - r_0)}{\beta} \right] \quad (21)$$

$$q = \frac{\omega^4 M}{2\rho_0 \beta RT} \left[r_0 \left(1 - \frac{\omega^2 r_e^2 F}{\beta} \right) + \frac{2\omega^2 r_e^3 F}{3\beta} \right] \quad (22)$$

For a rectangular cell (e.g. a preparative tube, neglecting the end effect) the conservation condition is:

$$\int_a^b c dr = c^0(b - a) \quad (23)$$

By following a similar route, it may readily be shown that the final equation for the concentration distribution is identical with eqn. (20), with the exceptions that $2(b - a)$ replaces $(b^2 - a^2)/r_0$ and that the terms

in I_2 in the denominator disappear. In programming eqn. (20), the integrals I_1 and I_2 may be evaluated by Simpson's Rule, taking about 40 ordinates.

Limiting forms. For a macromolecule banded at the iso-dense point in a short column, $r_e = r_0 = (a + b)/2$, to a good approximation. If the contributions of the cubic terms to the definite integrals are neglected, the integrals I_1 and I_2 are expressible in terms of the error function and its derivative: $H(x) =$

$$(2/\sqrt{\pi}) \int_0^x \exp(-x^2) dx. \text{ Since } H(x) = -H(-x) \text{ and}$$

$H'(x) = H'(-x)$, and $h_a = -h_b$, eqn. (20) becomes:

$$c(r) = \frac{c^0(b^2 - a^2)\sqrt{p}}{2r_0\sqrt{\pi}H(\sqrt{ph_b})} \exp\left(-p\left[h^2 + h^3\left(\frac{1}{r_0} - \frac{\omega^2 r_0 F}{3\beta}\right)\right]\right) \quad (24)$$

where p now has the simpler form $\omega^4 Mr_0^2/2\rho_0\beta RT$.

Eqn. (24) thus combines a first-order solution for $c(r_0)$ with a more accurate solution for the variation of c with r ; therefore it would predict accurately the form of the distribution, but not its absolute values. With this limitation, eqn. (24) represents an analytical solution to the problem, if the error function is regarded as a known quantity. The main effect of taking finite, rather than infinite, limits to the solution column is expressed in the term $H(\sqrt{ph_b})$, which is less than unity. The cubic term in the exponent does not vanish for this apparently symmetrical case, although the effect of the non-linear gradient has disappeared from the term in h^2 . If the speed is great enough for the band to be fully resolved from the meniscus and base, $H(\sqrt{ph_b}) = 1$; in these circumstances the cubic term may become negligible. The resulting expression is identical with that derivable from the rectangular-cell treatment of Meselson *et al.* (1957), except that $(b^2 - a^2)/2$ replaces $r_0(b - a)$.

(iii) Accumulation near a solution limit

Here ρ_0 lies outside the span of the gradient: the following discussion is specialized to the case of $\rho_0 < \rho(a)$, but the alternative case [$\rho_0 > \rho(b)$] may be obtained similarly.

Expansion is made about an origin at the meniscus, a :

$$h \equiv r - a \quad (25)$$

and an expression analogous to eqn. (4) is written with the substitution of a for r_e . Of the derivatives then required, the first has already been obtained, in reciprocal form, as eqn. (18); the second follows from eqns. (17) and (18), giving the result:

$$\left(\frac{d^2\rho}{dr^2}\right)_{r=a} = \frac{E}{1 + (\rho_a - \rho_e)F} \left[\frac{1}{r_e} - \frac{EFa^2}{r_e^2[1 + (\rho_a - \rho_e)F]^2} \right] \equiv Q \quad (26)$$

With these substitutions, the differential equation (1) of sedimentation equilibrium may be written in terms of h , ρ_a and the derivatives defined as P and Q in eqns. (18) and (26), by following a route generally similar to that taken for the case of banding. Neglecting terms of order h^4 and above, the result is:

$$c = c(a) \exp \left[\frac{M\omega^2}{RT} \left\{ a(1 - \bar{v}\rho_a)h + (1 - \bar{v}\rho_a - \bar{v}aP) \frac{h^2}{2} - \bar{v} \left(P + \frac{aQ}{2} \right) \frac{h^3}{3} \right\} \right] \quad (27)$$

Applying the equations expressing conservation of mass (11 or 23) one finds:

$$c(a) = \frac{c^0(b^2 - a^2)}{2[aI_3(h_b) + I_4(h_b)]} \text{ (sectors)} \quad (28a)$$

$$c(a) = \frac{c^0(b - a)}{I_3(h_b)} \text{ (cylinders)} \quad (28b)$$

Here the integrals I_3 and I_4 are defined as:

$$I_3(x) = \int_0^x \exp(sx + tx^2 - ux^3) dx \quad (29)$$

$$I_4(x) = \int_0^x x \exp(sx + tx^2 - ux^3) dx \quad (30)$$

where s , t and u represent the coefficients of h , h^2 and h^3 respectively in eqn. (27).

Combination of eqns. (27) and (28a) then gives:

$$c(r) = \frac{c^0(b^2 - a^2)}{2[aI_3(h_b) + I_4(h_b)]} \exp(sh + th^2 - uh^3) \quad (31)$$

as the final solution for sectors, the full expressions for the coefficients being:

$$s = \frac{M\omega^2 a}{RT} \left(1 - \frac{\rho_a}{\rho_0} \right) \quad (32)$$

$$t = \frac{M\omega^2}{2RT} \left[1 - \frac{\rho_a}{\rho_0} - \frac{a^2 \omega^2}{\rho_0 \beta [1 + (\rho_a - \rho_e)F]} \right] \quad (33)$$

$$u = \frac{M\omega^4 a}{2RT\rho_0 \beta [1 + (\rho_a - \rho_e)F]} \left[1 - \frac{a^2 \omega^2 F}{3\beta [1 + (\rho_a - \rho_e)F]^2} \right] \quad (34)$$

For cylinders the distribution equation is identical with eqn. (31) except that the constant term is $c^0(b - a)/I_3(h_b)$.

As before, analytical solutions can be obtained only by neglecting higher powers of x in the integrals I_3 and I_4 . Errors arising from neglect of these terms may well be much larger than before, but again they will affect only the constant term and not the shape

of the distribution. Thus, taking t and u to be zero, one finds:

$$\lim I_3(h_b) = \frac{\exp(sh_b) - 1}{s} \quad (35)$$

$$\lim I_4(h_b) = \frac{1}{s^2} [1 + (sh_b - 1)\exp(sh_b)] \quad (36)$$

(iv) *Concentration effects.* The equations developed above apply to macromolecular solutions at concentrations where non-ideality effects are negligible. At high concentrations, Silpananta *et al.* (1968) showed that non-ideality significantly broadened the distributions of banded macromolecules, and a similar result may be anticipated for components concentrated at the solution limits. However, at the concentrations feasible with secretory glycoproteins, these effects are not usually serious (Creeth & Denborough, 1970), and the equations given should be applicable in practice to the great majority of systems. It may be noted that, although deviations from the predicted distributions may arise through excessive macromolecular concentrations, convections analogous to those occurring in zonal velocity sedimentation (Spragg & Rankin, 1967) do not occur. The total density gradient may be represented as the sum of the gradients due to the salt (component 2) and macromolecule (component 3):

$$\frac{d\rho}{dr} = \left(\frac{d\rho}{dc_2}\right) \frac{dc_2}{dr} + \left(\frac{d\rho}{dc_3}\right) \frac{dc_3}{dr} \quad (37)$$

Here, $(d\rho/dc)$ is the density increment of the specified component in the three-component system. In regions where dc_3/dr is negative, $(d\rho/dc_3)$ is also negative, so each term is always positive.

Experimental

Density-gradient equilibrium experiments were performed with the Centriscan 75 ultracentrifuge (MSE Ltd., Crawley, Sussex, U.K.) operated in the analytical mode, with observations with both u.v.-absorption optics at 280nm and schlieren optics at 546nm. The preparation of solutions, conduct of experiments and calculations from the recorder charts followed previously established procedures (e.g. Creeth *et al.*, 1974).

The expressions derived here were programmed for a Wang 720C desk-top computer, the output of which uses a typewriter keyboard for plotting graphs, and is accordingly discontinuous. In the accompanying Figures, hand-drawn lines have been added where the separation of the points is inconveniently large.

Application of the expressions

(1) *Theoretical considerations.* Interest lies first in the accuracy with which the equations reproduce the density distribution in the cell. This depends in turn

on the availability of β values and their variation with density. Ifft *et al.* (1970) have computed $\beta(\rho)$ from primary thermodynamic data, as well as making experimental determinations for the salts most commonly used, and their tabulation of computed values has been used as the main source. To determine F (eqn. 5c), β was fitted to $(\rho - 1)$ over seven points in a limited range by a quadratic least-squares regression:

$$10^{-9} \times \beta = a + b(\rho - 1) + c(\rho - 1)^2 \quad (38)$$

$$F(\rho) = \frac{b + 2c(\rho - 1)}{\beta(\rho)} \quad (39)$$

Values of a , b and c so obtained are given in Table 1, together with the particular values of ρ , β and F appropriate to the subsequent examples. A few values of β and F for rounded values of ρ are included to enable ready comparison with previous tabulations.

In the middle of the range specified, the computed β values of Ifft *et al.* (1970) for CsCl (i.e. essentially those quoted in Table 1) are in good agreement with the earlier values found by Ifft *et al.* (1961); however, the computed values are some 6% lower than those determined experimentally. The values quoted for F are in fair agreement with the more precise tabulation of Ifft (1973). For CsBr, the discrepancy between computed and experimental values was similar in magnitude, but opposite in sign; in this case, the values in Table 1 have been taken from the experimental curve given by Ifft *et al.* (1970), as these are in better agreement with isolated determinations in our laboratory. For Cs_2SO_4 , values were read from an enlarged copy of the graph given by Ludlum & Warner (1965); for F , agreement with the curve published by those authors is good in the middle of the range, but poor at the extremes.

The values of β and F for CsCl were then used to compute density distributions in sectors and rectangular cells by eqns. (3) and (5) for comparison with those reported by Ifft *et al.* (1961); the latter values, obtained by a quartic polynomial with a rigorous theoretical basis, are regarded as authentic. Examples of the comparison are given in Table 2. Although agreement is excellent for sectors, small but appreciable differences occur in the wider distributions pertaining to cylinders. However, inspection of the deviations in the latter case shows that they are small compared with those arising from the uncertainties in β and F ; moreover, since experimental difficulties preclude the accurate determination of equilibrium densities in preparative tubes (Ifft *et al.*, 1961), the errors are regarded as acceptable. An additional factor will often modify predicted distributions in cylinders. The equations are valid equally for both swing-out and angle rotors; in the former, the radial dimension is directly transformed into height in the tube. Angle rotors, however, have well-recognized

Table 1. *Dependence of salt parameter, β , on density, at 25°C*

Units of β are $\text{cm}^5 \cdot \text{g}^{-1} \cdot \text{s}^{-2}$; of ρ , g/ml; of F , ml/g. a , b and c are coefficients determined from eqn. (38); values apply for $1.3 < \rho < 1.6$ for CsCl, CsBr and LiBr; $1.2 < \rho < 1.5$ for Cs_2SO_4 . $F \equiv d(\ln \beta)/d\rho$ (eqn. 5c).

Salt	a	b	c	ρ	$10^{-9} \times \beta$	F	Reference for $\beta(\rho)$
CsCl	2.612	-4.804	4.024	1.28*	1.582	-1.61	Ifft <i>et al.</i> (1970), computed
				1.30	1.533	-1.56	
				1.40	1.334	-1.19	
				1.41	1.319	-1.14	
				1.45†	1.265	-0.93	
				1.50	1.216	-0.64	
CsBr	1.906	-3.804	3.100	1.30	1.023	-1.97	Ifft <i>et al.</i> (1970), experimental
				1.31*	1.003	-1.95	
				1.40	0.852	-1.64	
				1.50†	0.744	-1.04	
Cs ₂ SO ₄	1.742	-4.815	5.263	1.24*	0.890	-2.57	Ludlum & Warner (1965)
				1.30	0.771	-2.15	
				1.33†	0.726	-1.85	
				1.50	0.650	+0.69	
LiBr	9.525	-8.457	19.81	1.30*	8.77	+0.39	Ifft <i>et al.</i> (1970), computed
				1.40	9.31	+0.79	
				1.50†	10.25	+1.11	

* Value regarded as characteristic of proteins (e.g. Ifft & Vinograd, 1966).

† Value regarded as characteristic of secretory glycoproteins (e.g. Creeth & Denborough, 1970; Creeth *et al.*, 1974).

Table 2. *Comparison of density distributions predicted by eqns. (3) and (5) with those of Ifft *et al.* (1961)*

Both distributions refer to CsCl of $\rho_0 = 1.45 \text{ g/ml}$ at 25°C. (A), Distribution in a sector-shaped cell of a 6.0 cm and b 7.2 cm at 44770 rev./min. (B), Distribution in a cylinder of a 5.1 cm, b 9.3 cm at 39000 rev./min.

(A)	r	ρ (eqns. 3 and 5)	ρ (Ifft <i>et al.</i> , 1961)
	6.000	1.384	1.382
	6.300	1.414	1.413
	6.600	1.447	1.446
	6.900	1.482	1.482
	7.200	1.521	1.521
(B)	5.100	1.291	1.285
	6.000	1.343	1.336
	7.000	1.422	1.411
	7.301	1.450	—
	7.356	—	1.450
	8.000	1.523	1.512
	9.000	1.645	1.631
	9.300	1.686	1.674

practical advantages in the rate of attainment of equilibrium (Johnson *et al.*, 1973) and the fact that the radial dimension, r , is related to tube height, l , by the equation $r = l \sin \theta$, where θ is the angle of the tube to the vertical (Flamm *et al.*, 1966; Johnson *et al.*, 1973). Thus re-orientation on deceleration augments separations according to $\Delta l = \Delta r / \sin \theta$, without affecting, to a first approximation, the values

of dc/dr for the macromolecule. Thus the separation encountered in practice will usually differ from, and may well exceed, that suggested on the basis of the radial parameter alone.

A second consideration in assessing the equations concerns the degree of overlap between the two main forms. Although the quantity r_0 defined by eqn. (15) is physically significant only when $a < r_0 < b$, the equation is valid so long as convergence is assured. Nothing in the analysis necessarily precludes application of the final expression for zones (eqn. 20) to the case where ρ_0 lies outside the span of the gradient; obviously, if the second and higher derivatives of r with respect to ρ become large, considerable errors must be expected. Similarly, the expression for the distribution of solute accumulating at a meniscus (eqn. 31) is not mathematically restricted to that case alone, although errors arising from failing convergence must be expected when it is applied to predict a band distribution whose centre is far removed from a . The two examples illustrated in Fig. 1 show the order of error that would arise if the inappropriate form were used; Fig. 1(a) refers to a zone with a maximum well displaced from the meniscus, and Fig. 1(b) to an accumulation near a meniscus by a solute with ρ_0 considerably less than ρ_a . In the first case, no significant differences exist between the two formulations, whereas in the second, an appreciable difference occurs only in the region of highest concentration; clearly, therefore, no great error is to be expected in a series of simulations where the density at the meniscus may pass through the

Fig. 1. Alternative representations of macromolecular distributions in the region of the meniscus

(a) ρ_0 within span of gradient; simulation refers to macromolecule of mol.wt. (M) 100000, $\rho_0 = 1.41$ g/ml, $c^0 = 1.0$, at equilibrium in CsCl of $\rho_e = 1.45$ g/ml at 40000 rev./min at 25°C, in sector cell, $a = 5.90$ cm, $b = 7.10$ cm. Density at meniscus = 1.397 g/ml. ▲, from eqn. (20), the appropriate form; ○, from eqn. (31), the inappropriate form. (b) ρ_0 outside span of gradient; simulation refers to macromolecule of mol.wt. (M) 100000, $\rho_0 = 1.37$ g/ml in same conditions as (a). ▲, from eqn. (20), here the inappropriate form; and ○, from eqn. (31), the appropriate form.

buoyant-density value, but only one form of equation is used.

Since the retention of cubic terms in the derivations adds substantially to the labour of programming the final equations, it is interesting to compare the distribution predicted by eqn. (20), in which cubic

terms were retained at all stages of the derivation, with that predicted when these terms are consistently omitted: the latter expression is obtained from eqn. (24) by omission of the cubic term from the exponent. The example chosen (Fig. 2a) represents the apparently symmetrical case, with $\rho_0 = \rho_e$ in an analytical cell, at a speed where c_a and c_b are just greater than zero. The cubic term becomes significant in the outer regions of the distribution, where it restores the symmetry. At higher speeds or M values the effects are smaller, and the numerical values chosen for the example give some guidance to the need for the more exact treatment.

The second example (Fig. 2b) illustrates the distribution of the same macromolecule when the initial density is lower, so that ρ_0 is only slightly less than ρ_b . The more precise eqn. (20) has been used for both curves here, but ignoring, in the one case, the variation of β with ρ . The curves resemble curtailed Gaussian peaks, but eqn. (20) yields an appreciably sharper distribution. Since F for this system is comparatively small (cf. Table 1), it is concluded that the variation of β with ρ will produce significant effects wherever the buoyant density differs appreciably from the initial density. Thus the cubic terms in both the constant and the radial-dependence factor must be retained if precise simulations are to be made; accordingly the full versions of eqns. (20) and (27) have been used in the following examples.

(2) *Glycoprotein systems.* The well-established principles governing separation in density gradients (Meselson *et al.*, 1957; Baldwin, 1959; Ifft *et al.*, 1961; Vinograd & Hearst, 1962) are summarized in the statements: (1) the radial separation of two species of buoyant-density difference $\Delta\rho$ is proportional to $\Delta\rho\beta/\omega^2$; (2) the standard deviation, σ , of a single species, homogeneous in density, is proportional to $[\rho_0\beta/\omega^4 M(1 + \Gamma')^{\frac{1}{2}}]$, where Γ' is the selective solvation parameter; (3) the resolution of two species of similar M is proportional to $\Delta\rho[M(1 + \Gamma')\beta/\rho_0]^{\frac{1}{2}}$; (4) the ratio of the standard deviations of the same homogeneous macromolecule at the same gradient in two solvents is determined by the two values of the quantity $[\rho_0/\beta(1 + \Gamma')^{\frac{1}{2}}]$. On the basis of these statements, rules may be formulated for optimizing separations of fully banded macromolecules that differ in ρ_0 but do not exhibit density heterogeneity about each ρ_0 . For glycoproteins, however, in addition to the differences in ρ_0 (and thus Γ') and $\Delta\rho$ (the difference in mean buoyant density between the glycoprotein and the substance from which separation is desired) associated with media of differing β , the influence of density polydispersity may be of great importance. Thus the system most appropriate for a given purpose is less easily predictable on the rules given above, and realistic simulation may be necessary. The following survey of the use of simulations is based on the common experimental sequence

Fig. 2. Comparison of full expressions and their simplified versions

(a) Macromolecular distribution for mol.wt. (M) = 75000, $\rho_0 = 1.45$ g/ml, at equilibrium in CsCl of $\rho_e = 1.45$ g/ml; other conditions as in Fig. 1. Δ , From full expression (eqn. 20); $\circ$, from simplified expression (eqn. 24) when cubic term in exponent is also ignored: this distribution is symmetrical about $r_0 = r_e = 6.528$ (eqn. 3a). (b) Same macromolecule in CsCl of $\rho_e = 1.41$ g/ml; other conditions as in (a). Δ , From full expression, taking $F = -1.14$ (Table 1); $\circ$, from full expression taking F as zero.

of (i) separation from protein, (ii) detection of contaminating protein and (iii) subfractionation of glycoprotein.

(i) Separation of proteins from glycoproteins:

(a) Use of CsCl. Density-gradient centrifugation at a density intermediate between the ρ_0 values of proteins and glycoproteins is often used on a preparative scale, e.g. with ovarian-cyst fluids. The simulation shown in Fig. 3(a) illustrates a typical equilibrium distribution from an initial density of 1.4 g/ml. The molecular weight of the protein component was taken as that of serum albumin, the chief contaminant of cyst fluids. The density heterogeneity of the glycoprotein was expressed by taking a molecular weight of about one-tenth the true value, in agreement with experimental determinations of the apparent molecular weight in this medium (Creeth & Denborough, 1970). The proportion of protein to glycoprotein was 2:1 (w/w). At equilibrium, the protein is concentrated at the meniscus, and the effective separation is about 13 mm in r .

(b) Use of CsBr. Saliva and sputum (pathological bronchial secretion) are examples of protein/glycoprotein mixtures which may be incompletely soluble in CsCl; often, however, such systems may be dispersed in CsBr. This medium has lower values of β than CsCl, but solvation of proteins and glycoproteins is not much changed; thus resolution of protein from glycoprotein, depending largely on the relative β values alone, should not be as good as in CsCl. The situation is exemplified by the simulation of Fig. 3(b), referring to essentially the same system as Fig. 3(a) in CsCl; the molecular weights are unchanged, but the buoyant densities have been altered by minor amounts to allow for the known differences. Because both simulations refer to the same speed, the glycoprotein peak is sharper in CsBr, but the separation is smaller (effectively about 6 mm), the protein zone being appreciably removed from the meniscus in the preparative tube. In practice the glycoprotein zone would be even sharper than the simulation suggests, for reasons discussed in section (iii) below. To summarize, although the separation of protein and glycoprotein zones in CsBr is not as good as in CsCl, it is still adequate to enable CsBr to be used for systems where CsCl is inapplicable. For the example given, separation is optimal at approx. 36000 rev./min.

(c) Use of LiBr. LiBr possesses a very high value of β and therefore offers important potential advantages as a separation medium. Its use has been reported by Robinson & Hopwood (1973), and Gibbons & Sellwood (1973) suggested that it may be more suitable for glycoproteins than are caesium salts: it is recognized to be much more highly chaotropic (Noelken, 1970), which might also be advantageous. Assuming that proteins and glycoproteins retain the respective buoyant densities that they possess in CsCl (no values in LiBr have apparently been reported), simulation of the distribution of the model protein-glycoprotein system of the previous example gives the result shown in Fig. 3(c): both components have apparently been concentrated very

Fig. 3. Simulation of density-gradient separations of protein and glycoprotein

(a) In CsCl. Δ refers to protein of mol.wt. (M) 75 000, ρ_0 1.28 g/ml, c^0 2.0 mg/ml; $\bullet$ refers to glycoprotein of mol.wt. (M) 100 000 and ρ_0 = 1.48 g/ml, c^0 = 1.0 mg/ml, ρ_e 1.40 g/ml. The experimental conditions were: speed, 42 000 rev./min; temperature, 8°C; cylinders of a, 4.5 cm; b, 7.6 cm. (b) In CsBr. As (a) except ρ_0 of protein = 1.31 g/ml, ρ_0 of glycoprotein = 1.51 g/ml. (c) In LiBr. As (a) except ρ_0 of protein = 1.30 g/ml, ρ_0 of glycoprotein = 1.50 g/ml.

sharply at the solution limits. In practice, the glycoprotein distribution would be somewhat less sharp (see below), but the main limitation on the use of LiBr arises through the low value of the gradient, which is about $0.012 \text{ g} \cdot \text{cm}^{-4}$ at the mid point, and the consequent susceptibility to minor disturbance. For this reason, together with the experimental difficulties arising from the high heat of solution, LiBr may have fewer advantages for protein glycoprotein separation than has been supposed. We have found it unsuitable for cyst fluids.

(ii) Detection of free protein in glycoproteins. The great sensitivity of analytical density-gradient ultracentrifugation for detecting contaminating free protein in glycoprotein preparations arises because of the ability to concentrate the protein manyfold in the region of the meniscus. The rules given above apply only to fully banded macromolecules, and give no guidance for material segregated at solution limits; accordingly, eqn. (31) was applied to predict typical distributions for the three most commonly used salts. Fig. 4 shows the results: they apply to a fairly well-fractionated glycoprotein preparation (mol. wt. taken to be 200 000 in Cs_2SO_4), assumed to contain also 1% of a protein of mol. wt. 65 000 (unsolvated). The molecular parameters have been adjusted as before to take account of the known differences in solvation, etc, and the speed in CsCl has been increased to the highest convenient value, which gives almost the same gradient as in the other two salts. In Cs_2SO_4 , the protein gradient is shallow, and with schlieren optics would be undetectable; the higher extinction coefficient might allow its detection, however, with absorption optics, provided that reliable baselines are obtainable. In CsCl at the higher speed, the protein gradient is higher and should easily be detectable by schlieren optics; however, glycoprotein zones in CsCl are usually more spread than simple theory predicts [cf. section (iii) below] and in practice the protein could easily be confused with a trace of glycoprotein of low density. It would probably be undetectable by the absorption system of the Centriscan ultracentrifuge, as the slit-width is too wide to allow reliable recording very close to the meniscus discontinuity. In CsBr, the protein gradient is nearly as steep as in CsCl, and hence the protein should be detectable by schlieren optics. Although density heterogeneity will have a smaller effect than in CsCl [cf. section (iii)], glycoprotein distributions of mean ρ_0 1.5 g/ml do occur with a tail of density less than 1.4 g/ml. Accordingly, an additional experiment at a lower density (e.g. 1.37 g/ml) will usually be necessary to establish that material concentrated at the meniscus is in fact protein.

Thus on balance, CsBr has some advantage over the other two salts for the detection of free protein by schlieren optics and photographic recording; with absorption scanning optics, Cs_2SO_4 may, however,

Fig. 4. Comparison of Cs_2SO_4 , CsCl and CsBr as media for the detection of free protein in analytical experiments. Simulations refer to a glycoprotein, $c^0 = 0.99 \text{ mg/ml}$ ($\bullet$), and a protein, $c^0 = 0.01 \text{ mg/ml}$ ($\blacktriangle$), in a sector cell of a , 5.9 cm; b , 7.1 cm. (a) Cs_2SO_4 : 40000 rev./min, $\rho_s = 1.33 \text{ g/ml}$, mol.wt. (M) (glycoprotein) = 200000, $\rho_0 = 1.33 \text{ g/ml}$; M (protein) = 80000, $\rho_0 = 1.24 \text{ g/ml}$. (b) CsCl : 50000 rev./min, $\rho_s = 1.48 \text{ g/ml}$, M (glycoprotein) = 146000, $\rho_0 = 1.48 \text{ g/ml}$; M (protein) = 70600, $\rho_0 = 1.28 \text{ g/ml}$. (c) CsBr : 40000 rev./min, $\rho_s = 1.51 \text{ g/ml}$, M (glycoprotein) = 146000, $\rho_0 = 1.51 \text{ g/ml}$; M (protein) = 70600, $\rho_0 = 1.31 \text{ g/ml}$. Only the upper half of each cell is represented.

give comparable results. A suitable speed for analytical experiments with CsBr is about 42000 rev./min, when $\rho_a = 1.41$ for $\rho_s = 1.50 \text{ g/ml}$; glycoproteins are usually 0.03 g/ml more dense in CsBr than in CsCl .

(iii) Fractionation of glycoproteins. Since variability of composition is inherent in secretory glycoproteins, density heterogeneity must arise because of the varying density contributions of the different components (Gibbons, 1972). Thus fractionation on a basis of differences in density is attractive theoretically, the more so because of the usually high molecular weights.

In sections (i) and (ii) it was possible to specify density heterogeneity merely by taking a low molecular weight and ignoring solvent influences; however, for fractionation, the most effective conditions can be established only when the relationship between intrinsic density heterogeneity and its expression in a given medium is defined; accordingly, a theory relating to these effects is summarized below.

(a) Relation between the variance of $\bar{v}$ and the variance of ρ_0 . The buoyant density of a substance is a function of the solvent as well as of the macromolecule itself; although buoyant-density differences can be related directly to structural differences (e.g.

to define the G + C content of DNA), these relationships are empirically based. The general relationship between $\sigma(\bar{v})$ and $\sigma(\rho_0)$ is as follows.

For a single macromolecule species, $\bar{v}$ and Γ' are not independent; however, where the component is heterogeneous in composition, these variables must be regarded as independent. Accordingly, the total differential of the buoyant density is given by:

$$d\rho_0 = \left(\frac{\partial \rho_0}{\partial \bar{v}} \right)_{\Gamma'} d\bar{v} + \left(\frac{\partial \rho_0}{\partial \Gamma'} \right)_{\bar{v}} d\Gamma' \quad (40)$$

The second term will be finite for those systems where solvation may vary at constant $\bar{v}$, for example where changes in the proportions of two or more carbohydrate components produce species of identical $\bar{v}$ but differing surface structure. Intuitively, such effects would be small in comparison with those arising from a straightforward variation of $\bar{v}$, and accordingly the comparison of different solvents may provisionally be made in terms of constant solvation in a given solvent. From the definition of ρ_0 (Hearst & Vinograd, 1961):

$$\rho_0 = \frac{1 + \Gamma'}{\bar{v}_3 + \Gamma' \bar{v}_1} \quad (41)$$

where 1 and 3 indicate solvent water and macro-molecule respectively, and the $\bar{v}$ quantities refer to the systems 1:2 and 1:3, we find:

$$\frac{\partial \rho_0}{\partial \bar{v}_3} = -\frac{(1 + \Gamma')}{(\bar{v}_3 + \Gamma' \bar{v}_1)^2} \quad (42)$$

Making the reasonable assumption that the differential will accurately reflect small finite differences, we obtain:

$$\Delta \bar{v}_3 = -\Delta \rho_0 \frac{(\bar{v}_3 + \Gamma' \bar{v}_1)^2}{1 + \Gamma'} = -\Delta \rho_0 \frac{(1 + \Gamma')}{\rho_0^2} \quad (43)$$

This equation enables the molecular property $\sigma(\bar{v})$ to be determined from the experimentally accessible $\sigma(\rho_0)$: when written explicitly for $\Delta \rho_0$, it emphasizes the large influence that variations in ρ_0 and Γ' will produce in different solvents.

(b) Relation between $\sigma(\rho_0)$ and breadth of a zone. Sueoka (1959) showed that the total spreading of a system polydisperse in both molecular weight and ρ is the sum of the effects of diffusion and density heterogeneity, provided that each density species has the same molecular-weight distribution as the whole sample. This assumption is not strictly valid for glycoproteins (Bhaskar & Creeth, 1974), but if it is accepted provisionally, the analysis developed by Schmid & Hearst (1972) may be applied to relate the experimentally measured variance of the concentration distribution, $\Delta \sigma_T^2$, to $\sigma(\rho_0)$.

$$\sigma_T^2 = \sigma_M^2 + \sigma_D^2 \equiv \frac{\int_a^b (r - r_0)^2 C dr}{\int_a^b C dr} \quad (44)$$

Here σ_M^2 and σ_D^2 are respectively the variances arising from diffusion and density heterogeneity, and C is the total concentration summed over all species.

It is first necessary to distinguish between the apparent solvated mol. wt., $M_s^{app.}$, which is determinable directly from the density-gradient experiment above, and the apparent mol. wt. $M^{app.}$ after correction for solvation:

$$M_s^{app.} = M^{app.}(1 + \Gamma') \quad (45)$$

In the terms of the present paper (neglecting compressibility effects), eqn. (3) of Schmid & Hearst (1972) becomes:

$$\frac{1}{M^{app.}} \equiv \frac{\sigma_T^2 \omega^4 r_0^2 (1 + \Gamma')}{\beta \rho_0 RT} = \frac{1}{M_n} + \frac{\beta(1 + \Gamma')}{RT \rho_0} \langle (\rho - \rho_0)^2 \rangle \quad (46)$$

The first equality is the empirical definition of $M^{app.}$ and the outermost members show that the variance of ρ_0 is determinable when M_n (the number-average

molecular weight) and Γ' are known. It is evident that the diffusional variance involves the first power of β , whereas the variance from density heterogeneity involves β^2 ; thus the effective dependence of σ_T^2 on β will in general be a power between one and two.

(c) Experimental determination of $\sigma(\bar{v})$. Eqns. (43) and (46) were obtained on the basis of several simplifying assumptions, the validity of which can best be established by comparison of a single preparation in two media. Accordingly, a well-characterized glycoprotein fraction (603/50-60; Creeth *et al.*, 1974) was selected; it had been prepared by solvent fractionation of an ovarian-cyst fluid, contained 20.2% (w/w) peptide, and had weight- and number-average mol. wts. of 810000 and 574000 respectively. Previous evidence (Creeth & Denborough, 1970) had demonstrated its polydispersity in density, the schlieren pattern in CsCl showing a negative gradient at the meniscus and a positive gradient at the base, although the pattern was fully resolved in Cs_2SO_4 at the same speed. The behaviour in these two solvents was re-examined, and the integral distribution curves shown in Fig. 5 were obtained.

In Cs_2SO_4 at 40000 rev./min (Fig. 5a) a fairly sharp band is observed; the superimposed simulation is defined by the ρ_0 value 1.310 g/ml determined in this experiment, together with the apparent solvated weight-average mol.wt. previously obtained from schlieren records (174000) and a c^0 value of 78% of that observed. In spite of the different optical systems, these parameters give a reasonably good fit to the main peak, although the simulation fails at the extremes. In CsCl at the same speed (and hence a lower gradient) (Fig. 5b), relatively little band formation occurred, and high concentrations of material remained at the solution limits; accordingly no calculations were made from this pattern. This result emphasizes the difference in banding properties of the two salts. At 50700 rev./min, the gradient in CsCl is identical with that existing in Cs_2SO_4 at 40000 rev./min; the pattern (Fig. 5c) shows that the zone is now better defined, though still much broader than in Cs_2SO_4 at the lower speed. The superimposed simulation is defined by an apparent solvated mol. wt. of 19300 with ρ_0 1.490 g/ml. Although the fit is good over the central part of the zone, it fails at the extremes, where substantial amounts of material have segregated. Although the apparent spreading is accentuated because the less-dense material is richer in peptide, and accordingly has a higher extinction coefficient, the schlieren patterns recorded in this experiment confirmed that the substance was incompletely banded. In these circumstances, reliable quantitative comparisons are not possible; eqn. (44) is based on the assumption that $c = 0$ at a and b . However, the peaks of the lobes in the schlieren patterns in both salts were well-defined. Accordingly, the radial displacements, r_+ and r_- , of the positive and

Fig. 5. Comparison of Cs_2SO_4 and CsCl for systems polydisperse in density; concentration distributions recorded for cyst-fluid glycoprotein fraction 603/50-60 (see the text)

Continuous lines are A_{280} recorded in experiment; R, reference trace; a, meniscus (6.001 cm); b, cell bottom (7.188 cm). (a) Cs_2SO_4 : $\rho_e = 1.312 \text{ g/ml}$, 40000 rev./min at 25°C , $c^0 = 0.25$ (A units). Simulation (●) refers to $\rho_0 = 1.310 \text{ g/ml}$, mol.wt. (M) = 174000, $c^0 = 0.204$, other conditions as observed in the experiment. Density gradient is 0.1541 g/cm^4 . (b) CsCl , $\rho_e = 1.507 \text{ g/ml}$, 40000 rev./min at 25°C , $c^0 = 0.33$; density gradient 0.0959 g/cm^4 . (c) As (b), except that speed increased to 50710 rev./min; density gradient = 0.1541 g/cm^4 . Simulation (●) refers to $\rho_0 = 1.490 \text{ g/ml}$, $M = 19300$, $c^0 = 0.260$.

negative lobe extrema could be determined, enabling the value of σ_T^2 for Cs_2SO_4 obtained from eqn. (44) to be compared with the apparent value derived from the lobe separation. We find σ_T^2 (eqn. 44) = $2.24 \times 10^{-2} \text{ cm}^2$ and $[(r_- - r_+)/2]^2 = 6.8 \times 10^{-3} \text{ cm}^2$. The ratio between these values should apply also to the distribution in CsCl ; thus the apparent value derived from the lobe separation ($1.15 \times 10^{-2} \text{ cm}^2$) implies that σ_T^2 for the whole distribution would have been about $3.8 \times 10^{-2} \text{ cm}^2$, if it had been possible to overcome the limitations of cell length.

Applying eqn. (46) to relate these values of σ_T^2 in the two salts to the corresponding variances of ρ_0 , one obtains s.d. $[\rho_0(\text{Cs}_2\text{SO}_4)] = 0.0113 \text{ g/ml}$; s.d. $[\rho_0(\text{CsCl})] \sim 0.028 \text{ g/ml}$. In spite of the obvious limitations in accuracy of the latter value, and the neglect of compressibility effects, there can be little doubt that the density heterogeneity of this typical glycoprotein in CsCl is more than twice as great as in Cs_2SO_4 .

Applying eqn. (43) to the two values of the standard deviation of the buoyant density, we find s.d. $[\bar{v}(\text{Cs}_2\text{SO}_4)] = 0.012 \text{ ml/g}$; s.d. $[\bar{v}(\text{CsCl})] = 0.014 \text{ ml/g}$. The agreement is as good as can be expected, and substantiates the belief that the method outlined yields a true index of molecular variability, free from major solvent effects. The difference between CsCl and Cs_2SO_4 as density-gradient media is therefore explicable in terms of their values of β and the several effects of solvation.

(d) Relative efficiency of Cs salts as fractionating media. Comparison of eqns. (43) and (46) shows that the quantity $\beta\rho_0^3/(1 + \Gamma')$ multiplies the variance of $\bar{v}$, and thus determines the influence of density heterogeneity on the apparent molecular weight in a given solvent. In agreement with this conclusion, direct substitution of eqns. (43) and (45) into the definition of the resolving power, λ , (Ifft *et al.*, 1961) yields the square root of the same ratio, which therefore expresses quantitatively the relative resolving powers of density-gradient media for macromolecules exhibiting density differences, whether the difference is continuous or discrete. For glycoproteins in CsCl , CsBr and Cs_2SO_4 the appropriate values of $[\beta\rho_0^3/(1 + \Gamma')]^{1/2}$ are approx. 61, 48 and $34 \text{ kg} \cdot \text{cm}^{-2} \text{ s}^{-1}$. These values indicate the relative efficiencies of the media in practice only if the preparation is fully banded, i.e. the column length must be sufficient to ensure that the density span of the gradient exceeds the effective (i.e. $\pm 2\text{s.d.}$) breadth of the density distribution of the glycoprotein. Among other factors, it was uncertainty on this point which led Bhaskar & Creeth (1974) to use Cs_2SO_4 rather than CsCl for fractionating the whole glycoprotein component of a cyst fluid; the preparation gave a broad but well-defined zone in Cs_2SO_4 in analytical experiments but could not be banded in CsCl . Moreover, it was assumed that the difference in ρ_0 between the two

main components evident in Cs_2SO_4 would be conserved in CsCl ; had this been true, the resolution parameter would indeed have had almost identical values in Cs_2SO_4 and CsCl , because the combination of higher Γ' and lower ρ_0 in Cs_2SO_4 almost exactly compensates for the lower β value. On the evidence now available, it is clear that the experiment would have given sharper fractions if conducted in CsCl at a higher speed. However, the differences between the fractions in Cs_2SO_4 were well defined, and sufficient to establish the systematic variation in properties with buoyant density.

(e) Limitations in the simulation of systems exhibiting heterogeneity in density. The examples given in sections 2(i) and 2(ii) illustrate two limitations on simulation which may not be immediately apparent. The representation of a system heterogeneous in density in terms of a single value of ρ_0 and a molecular weight much lower than the true value is justified by eqn. (46), but only for fully banded zones. Selection of a value of M^{app} to match an observed distribution is then equivalent to assigning a value to the variance of ρ , as was done in the example above [section 2(iii, c)]. However, a fundamental shortcoming of this method of representation arises when the density distribution is wider than the span of the gradient: eqn. (20), for example, will always predict that the concentration gradient is positive, no matter how low the selected value of M ; a negative gradient can arise only if ρ_0 is less than ρ_a (cf. eqn. 31). Thus the simulation in Fig. 5(c) can reproduce the behaviour only of the main banded part of the distribution; the minimum observed here would necessitate the inclusion of two more components, one with $\rho_0 < \rho_a$ and the other with $\rho_0 > \rho_b$, in amounts totalling about 22%. It follows also that distributions of materials segregated at solution limits, predicted by eqn. (31), will be realistic only when density heterogeneity is small; fortunately, in the most common application, proteins at the meniscus, this condition is fulfilled.

The second limitation is best exemplified by a representation of the Cs_2SO_4 fractionation experiment referred to in section 2(iii, d) above. Preliminary characterization of this material in an analytical density-gradient experiment had shown that the pattern (Fig. 1b of Bhaskar & Creeth, 1973) could be resolved into two components, in relative proportions 2:3 (w/w), of buoyant densities 1.311 and 1.344 g/ml , and M_s^{app} values of 150000 and 190000. If these values are applied to the conditions of the subsequent fractionation experiment, the distributions shown in Fig. 6 are obtained. The total distribution curve resembles that observed in the fractionation experiment (Fig. 2 of Bhaskar & Creeth, 1974), and the approximate density span of the fractions is indicated in Fig. 6 (present paper). This simulation, with its indication of partial overlap of the two components, is misleading, however; it

Fig. 6. Simulation of density-gradient fractionation of a cyst-fluid glycoprotein

The two peaks defined by ● points refer respectively to glycoprotein components of mol.wt. (M) 190000 and 150000, $\rho_0 = 1.311$ and 1.344 g/ml, at concentrations of 1.0 and 1.5 mg/ml, in Cs_2SO_4 of $\rho_s 1.33$ g/ml. The experimental conditions were: speed, 25000 rev./min; temperature, 5°C ; cylinders of a , 4.5 cm; b , 7.6 cm. The ▲ points refer to a protein of $M = 80000$ and $\rho_0 1.24$ g/ml, at a concentration of 0.05 mg/ml. The ■ points are the total glycoprotein concentrations, defining a curve which may be compared with the experimental result of Bhaskar & Creeth (1974; their Fig. 2). The arrows and numbers represent the approximate fractionation cut-off points.

merely demonstrates self-consistency with the original analysis. Subsequent characterization of the fractions showed mol.wts. ranging from 1 300 000 to 1 500 000; thus the wide distributions obtained by using the much lower M_s^{app} values represent the total distributions of density-heterogeneous components, and effectively conceal the real fractionation.

In this example, also, the presence of a small proportion (2% of the total) of low-molecular-weight protein was assumed; this simulation shows that, had this material been present, it would have been concentrated wholly into the least dense tail (fraction 9, Fig. 6) of the series of fractions obtained.

Conclusions

These results illustrate the differing properties of the three most commonly used caesium salts, which accordingly lead to differences in their application to systems exhibiting substantial density heterogeneity.

In analytical experiments, where relatively short columns are inescapable, full resolution from the solution limits is usually desired. Accordingly, both high speed and low β values are necessary: the relatively large density span between the solution limits (Wake & Baldwin, 1962) and steep gradient are often more important than the degree of resolution, and Cs_2SO_4 is often the preferred reagent. Conversely, of course, low-gradient salts are useful in showing the spread of a density distribution, the presence of a negative concentration gradient at the meniscus, or a positive gradient at the base, being a diagnostic for material outside the defined span of the gradient.

In preparative experiments, usually in much longer columns, two situations are recognized. In separating species where $\Delta\rho_0$ is relatively large, a fairly shallow gradient is adequate. Thus CsCl is very satisfactory, provided that gross over-loading does not occur. However, because $\Delta\rho_0$ is large, salts of relatively low β , such as CsBr , may also give adequate separations, helped by the fact that the zones will be sharper at the same speed. The second situation concerns fractionation of a substance disperse in density; here the relative resolution parameter, defined in section 2(iii) above, is decisive. In general, CsCl is the medium of choice, having a small advantage over CsBr and a much larger one over Cs_2SO_4 ; it is noteworthy that LiBr has the very high value of $180 \text{ kg} \cdot \text{s}^{-1} \cdot \text{cm}^{-2}$ for $[\beta\rho_0^3/(1 + \Gamma')]$ and so would be very efficient for fractionating glycoproteins if the difficulties referred to in section 2(i,c) can be overcome. RbCl (relative resolving power, $\lambda \sim 74$) would also be very effective, as its success in resolving protein systems testifies (Hu *et al.*, 1962; Schneider & Edelhoch, 1971); contrary to what was surmised earlier (Bhaskar & Creeth, 1974), however, RbCl cannot be used to fractionate secretory glycoproteins, as the maximum density is too low.

In deciding experimental conditions for a separation or a fractionation, the expressions derived here should prove useful, particularly if the density limits of the preparation are first established by analytical experiments. The other main use of the expressions is to supplement present methods of analysing density-gradient patterns by simulation and matching.

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