Effects of Molecular Crowding on Protein Self-Association: A Potential Source of Error in Sedimentation Coefficients Obtained by Zonal Ultracentrifugation in a Sucrose Gradient†

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ABSTRACT: Theoretical and experimental studies have illustrated a potential source of error in sedimentation coefficients obtained by sucrose density gradient centrifugation of proteins undergoing reversible self-association. The error stems from the excluded volume (molecular crowding) effect of the sucrose on the activity coefficients of monomeric and polymeric states. The consequent displacement of the equilibrium position in favor of polymeric state(s) is a function of sucrose concentration, and can therefore result in failure to detect the equilibrium coexistence of monomer if 5% sucrose suffices to displace the equilibrium completely toward dimer. In less extreme situations, it may result in the evaluation of an average sedimentation coefficient whose magnitude is a function of sucrose concentration and hence of the distance migrated into the sucrose gradient. These features are illustrated by the results of computer-simulated sedimentation of reversibly dimerizing systems in a sucrose gradient, and by conventional sedimentation velocity experiments on yeast enolase.

Although the recent introduction of a new analytical ultracentrifuge may well reverse the trend, there has been a growing tendency to assess the macromolecular state of proteins on the basis of sedimentation coefficients obtained by zonal ultracentrifguation in a sucrose gradient (Martin & Ames, 1961). We wish to draw attention to a potential source of variation in the magnitude of the sedimentation coefficient so obtained for any protein undergoing reversible self-association. For such systems, the molecular crowding effect of a small solute may well displace the interaction in favor of the polymeric state(s), the extent of this change in equilibrium position being dependent upon the concentration of small inert solute (Shearwin & Winzor, 1988): such effects are better known with macromolecules as space-filling solute (Nichol et al., 1981; Minton, 1981, 1983; Minton & Wilf, 1981). Because a gradient in sucrose concentration is used to maintain gravitational stability of the sedimenting zone of solute, the average macromolecular state of a self-associating system can thus vary with the position of the zone in the gradient—a situation which negates the assumed isokinetic migration (migration at constant velocity) that is inherent in the evaluation of sedimentation coefficients by means of zonal ultracentrifugation in sucrose density gradients.

EXPERIMENTAL PROCEDURES

Preparation of Enzyme Solutions. Crystalline preparations of yeast enolase and rabbit muscle creatine kinase (both Sigma products) were dissolved directly in MES[1]–chloride buffer [0.01 M 2-(*N*-morpholino)ethanesulfonic acid–0.25 M NaCl], pH 6.0, and then dialyzed against more of the same buffer. The concentrations of these dialyzed enzyme solutions were determined spectrophotometrically at 280 nm on the basis of an absorption coefficient ($A^{1\%}_{1cm}$) of 8.9 for both enolase (Warburg & Christian, 1941) and creatine kinase (Noda et al., 1954). Enzyme solutions with a fixed protein concentration (1.1 and 0.7 mg/mL for enolase and creatine kinase, respectively) and a range of sucrose concentrations (2.5–10% w/v) were then prepared by suitable dilution of these stock solutions with mixtures of diffusate and an identical buffer supplemented with 20% sucrose.

Traditional Sedimentation Velocity Studies. Solutions of enolase (1.1 mg/mL) and creatine kinase (0.7 mg/mL) in the same buffer medium were subjected to simultaneous sedimentation in a Beckman XL-A analytical ultracentrifuge operated at 20 °C and an angular velocity of 60 000 rpm. Solute distributions were recorded spectrophotometrically at 280 nm and 10-min intervals throughout the 2-h duration of each experiment. The square root of the second moment (Goldberg, 1954; Trautman & Schumaker, 1954) was taken as the appropriate radial position of the boundary ($\bar{r}$) for the determination of weight-average sedimentation coefficients ($\bar{s}_{20,b}$) from the time dependence of ln $\bar{r}$.

An Appropriate Sucrose Gradient for Zonal Ultracentrifugation. Although linear 5–20% sucrose gradients have been used traditionally for the determination of sedimentation coefficients for proteins by zonal density gradient ultracentrifugation (Martin & Ames, 1961), such a gradient is not appropriate to the rotors that have replaced the SW-39 rotor to which it applied. Evaluation of the sedimentation coefficient, $s_{20,w}$, by zonal gradient ultracentrifugation at angular velocity ω is based on integration of the expression:

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[1] Abbreviation: MES, 2-(*N*-morpholino)ethanesulfonic acid.

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$$\omega^2 s_{20,w}\int_0^t dt = \frac{1-\bar{v}\rho_{20,w}}{\eta_{20,w}}\int_{r_o}^{r_z}\frac{\eta(r)}{r[1-\bar{v}\rho(r)]}dr \qquad (1)$$

where $\bar{v}$ is the partial specific volume of solute and $\rho_{20,w}$ and $\eta_{20,w}$ denote the respective density and viscosity of water at 20 °C. The left-hand side of eq 1 is integrated readily, but integration of the right-hand side from the top of the gradient (r_o) to the radial position of the zone (r_z) after centrifugation for time t requires allowance to be made for the dependence of viscosity, $\eta(r)$, and density, $\rho(r)$, upon r because of the changing sucrose concentration. The simplifying condition for a constant migration rate requires the selection of a sucrose gradient such that $\eta(r)/\{r[1-\bar{v}\rho(r)]\}$ is essentially constant, whereupon this term may be removed from within the integral.

For the SW-60 rotor, the radial extremities of the tubes are 6.31 cm (r_o) and 12.03 cm (r_b), in which case the appropriate sucrose gradient for proteins ($\bar{v}$ = 0.73–0.74 mL/g) loaded onto 5% sucrose is a curvilinear relationship described by

$$c_M(r) = 50 + 33.16(r-6.31) - 1.96(r-6.31)^2 \qquad (2)$$

where $c_M(r)$ is the weight-concentration of sucrose (grams per liter) at radial distance r. On the grounds that such a radial dependence of $c_M(r)$ is, in principle, readily established by current gradient-generating devices, we shall regard eq 2 as the description of the sucrose gradient in numerical simulations of zonal density gradient ultracentrifugation in the SW-60 rotor.

Allowance for Thermodynamic Nonideality. Under thermodynamically nonideal conditions, the equilibrium constant for reversible self-association of a solute, X_n, is given by

$$X_n = \gamma_n(r)c_n(r)/[\gamma_1(r)c_1(r)]^n = X_n^{app}\gamma_n(r)/[\gamma_1(r)]^n \qquad (3)$$

where $c_i(r)$ and $\gamma_i(r)$ denote the weight-concentration and activity coefficient, respectively of species i (monomer or n-mer) at radial distance r; X_n^{app} is the apparent association constant that is obtained from the ratio of concentrations raised to the appropriate powers. Furthermore, magnitudes of the activity coefficients may be obtained by statistical mechanical considerations (McMillan & Mayer, 1945; Hill & Chen, 1973; Winzor & Willis, 1986; Wills et al., 1993).

To illustrate the statistical mechanical approach, we consider, for simplicity, a solute for which self-association is restricted to dimer formation and for which thermodynamic nonideality is described adequately by confining consideration to nearest-neighbor interactions. On the basis of spherical geometry for monomer and dimer, the expression for activity coefficients becomes

$$\gamma_i(r) = \exp[\sum \alpha_{ij}c_j(r)/M_j] \qquad (4a)$$

where the summation covers all species, including i, and the second virial coefficient, α_{ij}, is related to molecular parameters by

$$\alpha_{ij} = 4\pi N(R_i+R_j)^3/3 + \frac{Z_iZ_j(1+\kappa R_i+\kappa R_j)}{2I(1+\kappa R_i)(1+\kappa R_j)} \qquad (4b)$$

R_i and R_j are the respective radii of species i and j, which bear net charges Z_i and Z_j. The inverse screening length (κ) may be calculated from the ionic strength (I) by the expression $\kappa = (3.27 \times 10^7)I^{1/2}$. However, simpler expressions pertain under the conditions for measuring sedimentation coefficients by isokinetic sedimentation (Martin & Ames, 1961), which entails the application of a dilute protein solution to a relatively concentrated sucrose gradient (eq 2).

Because the molar concentrations of sucrose, $c_M(r)/M_M$, at all radial distances, r, in the gradient greatly exceed that of either state of the self-associating solute, the activity coefficients become dominated by the term in sucrose concentration. It therefore follows that

$$\gamma_1(r) \simeq \exp[\alpha_{1M}c_M(r)/M_M] \qquad (5a)$$

$$\gamma_2(r) \simeq \exp[\alpha_{2M}c_M(r)/M_M] \qquad (5b)$$

whereupon the counterpart of eq 3 may be written as

$$X_2^{app} = X_2\exp[(2\alpha_{1M}-\alpha_{2M})c_M(r)/M_M] \qquad (6)$$

In that regard, the absence of net charge on sucrose (Z_M = 0) eliminates the charge–charge term in α_{iM} (eq 4), which then simply becomes the covolume for sucrose and monomer or dimer.

Knowledge of $c_M(r)$, the sucrose concentration at radial distance r, from eq 2 allows determination of X_2^{app}, the apparent association constant governing the distribution of self-associating solute between monomeric and dimeric states at that radial position. Furthermore, from considerations of mass conservation, it follows that the monomer concentration, $c_1(r)$, pertaining to total solute concentration $\bar{c}(r)$ may be obtained by solving the quadratic

$$X_2^{app}c_1(r)^2 + c_1(r) - \bar{c}(r) = 0 \qquad (7)$$

where only the positive root is acceptable on physical grounds. Advantage is taken of eq 2, 6, and 7 in numerical simulations of the migration of monomer–dimer systems in zonal ultracentrifugation through a sucrose density gradient.

Numerical Simulation of Zonal Ultracentrifugation. The present theoretical zonal sedimentation behavior of a reversibly dimerizing protein in a sucrose density gradient was elaborated in a simulated preparative ultracentrifuge tube of length 5.82 cm, divided into segments with constant radial increment δr = 6.0 × 10^{-4} cm. The tube contained a sucrose gradient extending from r_o = 6.31 cm to the bottom of the tube at 12.03 cm as specified by eq 2. For a given value of the thermodynamic dimerization constant, X_2, the value of X_2^{app} was calculated, using eq 4b and 6, at the midpoint of each segment with the assignments R_1 = 6.19 nm, R_2 = 7.80 nm, R_M = 0.3 nm, and M_M = 342.5. Simulation of the zonal sedimentation patterns was analogous to previous computations for zonal electrophoresis of interacting macromolecules (Cann, 1987; Shunong et al., 1991). Briefly, the concentrations of monomer and dimer were computed by solving a set of transport and mass action equations, an inherent assumption being that the rates of association and dissociation are sufficiently large in relation to the rates of sedimentation and diffusion for local chemical equilibrium to obtain at every instant. In the sucrose gradient, the two transport equations take the form

$$\partial C_i(r,t)/\partial t = D_i\partial^2 C_i(r,t)/\partial r^2 - V_i\partial C_i(r,t)/\partial r; \quad i\ 1, 2 \qquad (8)$$

in which the subscript i designates the sedimenting species (1 = monomer, 2 = dimer); C_i is the concentration of species i; D_i is its diffusion coefficient; t is the time; V is the constant velocity given by eq 1 for isokinetic sedimentation using $(s_{20,w})_1$

= 18.6 S, $(s_{20,w})_2$ = 29.5 S, $\bar{v}$ = 0.73 mL/g, and the values of $\eta(r)$ and $\rho(r)$ pertinent to the local sucrose concentration at radial distance r. Diffusion coefficients of monomer and dimer were taken as 1.64×10^{-7} and 1.30×10^{-7} cm^2 s^{-1}, respectively. The finite-difference approximations to eq 8 for monomer and dimer were solved numerically for C_1 and C_2 using the step size Δt = 0.12 s. At each time $t + \Delta t$, the new values of the concentrations of monomer and dimer in each segment of the centrifuge tube as the result of transport were, in turn, changed to allow for chemical requilibration using eq 7 and the familiar mass action equation: $C_2 = X_2^{app} C_1^2$. Initially, an equilibrium mixture of monomer and dimer with a total concentration of 0.05 mg/mL was layered onto the sucrose gradient to a depth of 0.0972 cm. The Archibald (1947) boundary condition was applied at both the air–solution meniscus and the bottom of the tube. Each zonal sedimentation pattern was displayed as a plot of total protein concentration against position, the weight-average sedimentation coefficient and also the proportion of dimer being calculated at the apex of the zone. Calculations were also made for a nonassociating species with $s_{20,w}$ = 18.6 S for the purpose of calculating the sedimentation coefficients for dimerizing systems in a manner analogous to conventional practice (Martin & Ames, 1961).

RESULTS AND DISCUSSION

Because the rationale to be adopted relies so heavily upon the prediction (eq 6) that thermodynamic nonideality arising from the presence of the sucrose gradient may well displace self-association equilibria toward the polymer state(s), further experimental confirmation of this effect has been sought. For this purpose, conventional sedimentation velocity experiments have been performed on yeast enolase, a dimeric enzyme which undergoes reversible dissociation under conditions of moderate ionic strength (Mann et al., 1970). To avoid the magnification of experimental uncertainty emanating from the large viscosity and density correction factors required to convert the measured sedimentation coefficient ($\bar{s}_{20,b}$) to a standard value ($\bar{s}_{20,w}$), the effect of sucrose on the dissociation equilibrium has been inferred from the ratio of the sedimentation coefficient of enolase to that of creatine kinase, a stable dimeric enzyme of comparable size. Such practice also brings the present study more into line with the procedure adopted for measuring sedimentation coefficients ($s_{20,w}$) by density gradient ultracentrifugation.

Figure 1 summarizes the effect of sucrose concentration on the sedimentation coefficient of enolase relative to that of creatine kinase (○, left-hand ordinate). Solid symbols indicate the values of $\bar{s}_{20,w}$ (right-hand ordinate) for enolase that would have been deduced on the basis of this ratio and the reported sedimentation coefficient ($s_{20,w}$) of 5.31 S (Yue et al., 1967) for creatine kinase. The 20% increase in $s_{20,w}$ for enolase that results from the inclusion of 20% sucrose signifies a substantial increase in the proportion of dimeric enzyme.

By illustrating the displacement of the monomer–dimer equilibrium by an extent that is dependent upon sucrose concentration, these results for enolase clearly identify a potential problem in the use of density gradient centrifugation for evaluating sedimentation coefficients of proteins undergoing self-association.

Simulated Sedimentation of a Monomer–Dimer System in a Sucrose Gradient. In order to illustrate the potential effect of a sucrose gradient on the extent of self-association of a monomer–dimer system, we have chosen a monomer with the originally reported size characteristics of urease (M_1 =

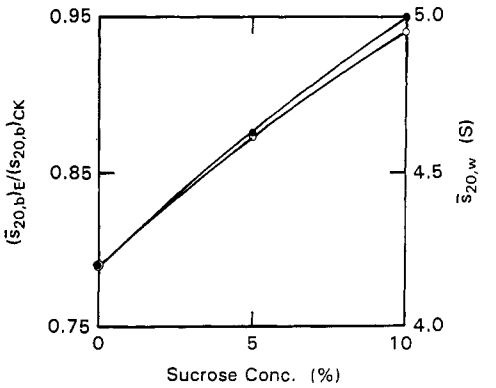

FIGURE 1: Effect of sucrose on the sedimentation coefficient of enolase that is calculated on the basis of its $s_{20,b}$ relative to that of creatine kinase (pH 6.0, I = 0.25): (○) dependence of the sedimentation coefficient of enolase relative to that for creatine kinase (left-hand ordinate); (●) dependence of $\bar{s}_{20,w}$ for enolase (right-hand ordinate) based on this ratio and a value of 5.31 S for the sedimentation coefficient ($s_{20,w}$) of creatine kinase (Yue et al., 1967).

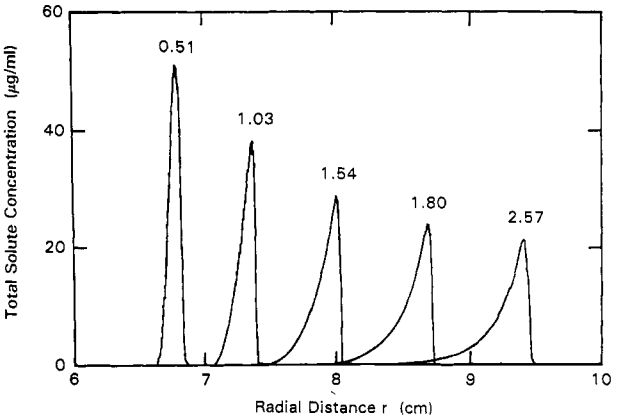

FIGURE 2: Concentration distributions resulting from simulated density gradient centrifugation of a zone (100 μL, 50 μg/mL) of a reversibly dimerizing solute (s_1 = 18.6 S, s_2 = 29.5 S, X_2 = 0.005 L/g) at 60 000 rpm in an SW-60 rotor. The number adjacent to each zone denotes the time (hours) of simulated centrifugation.

486 000, s_1 = 18.6 S, $D_1 = 3.46 \times 10^{-7}$ cm^2 s^{-1}, $\bar{v}$ = 0.73 mL/g). On the basis of spherical geometry for both species and a radius, R_1, of 6.19 nm for monomer, s_2 = 29.5 S and R_2 = 7.80 nm. Similar considerations and a radius of 0.3 nm for r_M, the effective radius of sucrose (Wills et al., 1993), then give rise to respective values of 689.3 and 1340.1 L/mol for U_{1M} and U_{2M}, the covolumes required for estimating the apparent dimerization constant (X_2^{app}) from its thermodynamic counterpart, X_2, via eq 6: specifically, $X_2^{app} = X_2 \exp[38.5 c_M(r)/M_M]$, where X_2 has been assigned a magnitude of 0.005 L/g.

Concentration distributions resulting from simulated ultracentrifugation at 60 000 rpm of a zone (100 μL) of such a solute (50 μg/mL) placed on top of the specified gradient for the SW-60 rotor are presented in Figure 2, about which the following points are noted. (i) The extremely sharp nature of the advancing edge of the migrating zone reflects the very small extent of diffusional spreading during the total time (2.57 h) of sedimentation at 5 °C. (ii) The skewness of the zone reflects the differential sedimentation of monomer and dimer, as first reported by Bethune and Kegeles (1961) and Kegeles et al. (1967), and also the interplay of increasing X_2^{app} and decreasing proportion of monomer as the result of migration into more concentrated sucrose. (iii) The latter effect is emphasized further in Figure 3, where the fraction

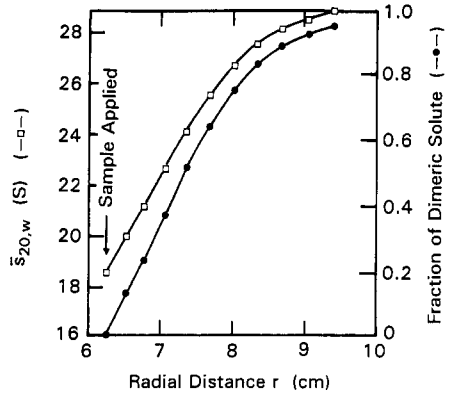

FIGURE 3: Effect of zone position (Figure 2) on the fraction of solute in the dimeric state at the peak of the zone (●) and on the weight-average sedimentation coefficient (□) calculated on that basis.

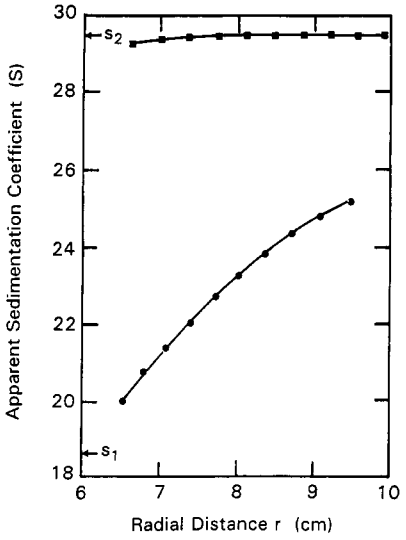

FIGURE 4: Effect of zone position on the sedimentation coefficient, $s^{app}_{20,w}$, calculated on the basis of the distance migrated by the reversibly dimerizing solute relative to that migrated by a nonassociating protein with $s_{20,w}$ = 18.6 S: (●) dimerization governed by an association constant of 0.005 L/g (as in Figure 2); (■) corresponding system with X_2 = 40 L/g.

of solute in the dimeric state at the peak of the zone is plotted (●) as a function of the distance migrated, together with the dependence of the average sedimentation coefficient ($\bar{s}_{20,w}$) for such a composition (□). (iv) This dependence of the effective sedimentation coefficient upon the concentration of sucrose through which the zone is migrating negates the approximation, inherent in the Martin and Ames (1961) procedure, that a constant rate of migration prevails. Nonfulfillment of that assumption is manifested experimentally as a depenedence of the measured sedimentation coefficient upon the distance migrated. This effect is illustrated (●) in Figure 4, where the $\bar{s}_{20,w}$ values are based on distances migrated by the reaction zone relative to those migrated by a standard protein (simulated as a nonassociating 18.6S species). Clearly, the qualitative similarity between such predicted behavior and the sedimentation velocity results for enolase (Figure 1) stresses the practical relevance of the model used to simulate density gradient ultracentrifugation of a self-associating solute.

Routine estimation of the sedimentation coefficient for such a system by (assumed) isokinetic density gradient ultracentrifugation in a sucrose gradient wouild clearly lead to different estimates of the sedimentation coefficient (and hence size) of the solute, depending on the duration of the experiment. Only

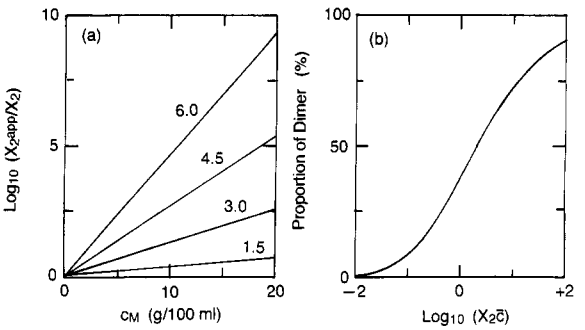

FIGURE 5: Potential molecular crowding effects of sucrose on the extent of self-association of a reversibly dimerizing protein. (a) Enhancement of the apparent dimerization constant predicted by eq 6 for systems with the indicated monomeric radii (nm). (b) Dependence of the extent of dimerization upon $X_2\bar{c}$ predicted by eq 9.

in the event that disparate results were obtained by inadvertent variation in the time of centrifugation would the existence of the self-association phenomenon become apparent. In that regard the monomer in the above simulated system was chosen deliberately to be large in order to magnify the variation in $s^{app}_{20,w}$ with distance migrated. However, comparable simulations of a monomer–dimer system with M_1 = 25 000 and s_1 = 2.4 S have shown the same relative dependence of the apparent sedimentation coefficient upon the radial position of the zone at time t. In an experimental context, the slight variation in $s^{app}_{20,w}$ for that system (0.2–0.3 S) would presumably be construed either as experimental uncertainty or as evidence for the existence of the solute in different isomeric states. Of equal concern is the possibility that the existence of a self-association equilibrium may well go undetected because the concentration of sucrose at the top of the gradient already suffices to decrease the concentration of monomer to effectively zero. For example, an increase in X_2 for the above system (Figure 2) to 40 L/g leads to a situation in which migration of the zone essentially duplicates that of pure dimer (■, Figure 4), even though equal weight concentrations of monomer and dimer were present in the solution applied to the top of the gradient.

A striking feature to emerge from these considerations of the consequences of molecular crowding by sucrose on reversible protein dimerization is the dramatically enhanced magnitude of the effective association constant. Despite the fact that, in molar terms, the loading concentration was only 10 nM, the traditional range of sucrose concentrations in isokinetic density gradient centrifugation has given rise to substantial dimerization of a system with an association constant ($XM_1/2$) of 1200 M^{-1} (dissociation constant of 0.8 mM), and essentially complete dimerization of one with a dissociation constant of 0.1 μM (X_2 = 40 L/g). To provide some indication of the quantitative manifestation of eq 6, Figure 5a presents the dependence of X_2^{app}/X_2 upon sucrose concentration predicted for dimerizing proteins with monomeric radii (R_1) of 1.5 nm ($M_1 \approx$ 15 000), 3.0 nm ($M_1 \approx$ 50 000, 4.5 nm ($M_1 \approx$ 150 000), and 6.0 nm ($M_1 \approx$ 500 000): the value of 0.3 nm has been retained for R_M). Although Figure 5a stresses the enormous dependence of X_2^{app} upon sucrose concentration (particularly for large protein monomers), the consequence of the increased magnitude of the effective association constant upon the extent of dimerization depends upon the protein concentration under consideration. To that end, Figure 5b summarizes the relationship between the proportion of dimer and the dimensionless parameter $X\bar{c}$ that is predicted by the expression:

$$c_2/c_1 = X_2c_1/(1 + X_2c_1) = [(1 + 4X_2\bar{c})^{1/2} - 1]/[(1 + 4X_2\bar{c})^{1/2} + 1] \quad (9)$$

for monomeric and dimeric species with spherical geometry.

Figure 5a certainly provides some indication of the possible effect upon self-association that could arise from the centrifugation of a reversibly dimerizing protein in a sucrose density gradient. However, these predictions based on eq 6 can only be used as a qualitative guide because of their reliance upon assumptions of questionable validity for some systems. First, the effects of sucrose have been considered only in terms of molecular crowding, and hence the conclusions would require modification to accommodate the consequences of any specific chemical interactions that may be superimposed on the space-filling effects of sucrose. Although it is relatively inert, the concentration of sucrose in the gradient (roughly 0.15–0.6 M) is so high that inertness of the space-filling solute cannot be guaranteed (Shearwin & Winzor, 1990). The other approximation of questionable validity for quantitative prediction of excluded volume effects is the assignment of spherical geometry to the monomeric and dimeric protein species. Although eq 6 retains validity, calculation of magnitudes for the various excluded volume parameters via eq 4b leads to overestimation of X_2^{app} if the polymeric protein is markedly asymmetric, an important consideration for systems in which reversible self-association extends well beyond dimer. Nevertheless, any nonfulfillment of either/both of these approximations does not affect the qualitative inference that the use of sucrose (or any other "inert" solute) for density stabilization has the potential to bring about a huge increase in the proportion of the polymeric state(s) of a protein undergoing reversible self-association.

The above considerations serve to illustrate the unsuitability of zonal ultracentrifugation in a density gradient as a means of characterizing the sedimentation coefficient and hence size of a macromolecular species undergoing either self-association or interaction with another macromolecular species. Only in the event that the interaction is essentially stoichiometric in the absence of the sucrose gradient does the technique give a reliable index of the macromolecular state of the solute under the conditions of interest. Reference to the literature quickly reveals widespread unawareness of this limitation of the zonal ultracentrifugation technique. Indeed, the whole concept of interactions as equilibria seems to be overlooked in the interpretation placed on most results obtained by the method, as studies with progesterone receptors (El-Ashry et al., 1989; DeMarzo et al., 1992) exemplify.

On the basis that incubation of cytosolic progesterone receptors from intact non-hormone-treated cells with either an agonist, R5020, or an antagonist, RU486, led to no change in sedimentation coefficient from the value of 9 S obtained by zonal gradient ultracentrifugation for dimeric receptors, it was concluded that neither ligand had any effect on the dimerization of progesterone receptors. On the other hand, inclusion of 0.3 M NaCl in the incubation mixtures and the sucrose gradient led to sedimentating zones characterized by apparent sedimentation coefficients of 5.6 and 3.9 S for the receptor complexes with RU486 and R5020, respectively, similar findings having been reported for agonist and antagonist complexes with estrogen receptors (Ruh et al., 1990; Turner et al., 1991). The results for progesterone receptor complexes were taken to signify the existence of two isomeric states of monomeric receptor, one stabilized by interaction with antagonist (a 6S form) and the other by agonist (a 4S form). Inasmuch as the difference in sedimentation coefficients implies a 3.5-fold difference in hydrodynamic volume (50% difference in Stokes radius), such an explanation seems less probable than one involving coexistence of monomeric and dimeric states of receptor–antagonist complex in dynamic macromolecular equilibrium.

The seemingly more plausible explanation of the two sets of density gradient experiments (in the absence or presence of 0.3 M NaCl) is that the agonist (R5020) and antagonist (RU486) do differ in their effects on the dimerization of progesterone receptors: preferential binding of RU486 with the dimeric receptor state increases the extent of self-association relative to that observed in the presence of agonist. However, this difference is only observed in density gradient centrifugation experiments by using salt concentrations that are sufficiently high to decrease the dimerization constant (X_2) to a magnitude such that the molecular-crowding effect of 5% sucrose does not suffice to bring about essentially complete displacement of the equilibrium toward dimer (see Figure 5), the decrease in X_2 for the R5020-liganded receptor being very much greater than for the RU486-liganded receptor. Consequently, the extent of reversible dimerization of the RU486-liganded receptor increases significantly during sedimentation through the sucrose gradient supplemented with NaCl, whereas the R5020-liganded receptor sediments essentially as the 4S monomer (Wei et al., 1987). The concept that the 6S peak is a reaction zone comprising monomeric and dimeric forms of the receptor–antagonist complex in rapid association equilibrium is supported by the skewed nature of the zone [see, *e.g.*, Figure 4A of DeMarzo et al. (1992)].

Further evidence in support of the present interpretation is provided by binding studies (Skafar, 1991) which indicate that RU486 exhibits a higher affinity for receptors when the experiments are conducted at high receptor concentration, but that no measureable difference between the strengths of agonist and antagonist binding can be observed in experiments conducted at low receptor concentration. According to the present hypothesis, a high receptor concentration would displace the dimerization equilibrium toward dimer, and hence favor the binding of antagonist (RU486) because of its preferential interaction with this receptor state. On the other hand, a decrease in receptor concentration would increase the proportion of monomer, to which agonist (R5020) binds preferentially. The consequent combination of strengthened agonist binding and weakened antagonist binding could well eliminate the favored binding of RU486 that is observed for high receptor concentrations.

Although the results of immunoprecipitation experiments were considered (DeMarzo et al., 1992) to eliminate the possible existence of antagonist–receptor complexes in the dimeric state, the results of those experiments on samples of sedimented zones [Figure 3 of DeMarzo et al. (1992)] do not negate the present interpretation, because the dimers would have dissociated completely during the SDS electrophoretic step of the Western blot procedure. Finally, the concept of reaction zones affords an explanation of the finding that the extent of dissociation of receptor–antagonist complex is greater in the ultracentrifugal analysis than that assessed by coimmunoprecipitation assays at the same salt concentration. Reference to the protocol for the latter experiments reveals that the buffer contained 10% glycerol, which is also an effective space-filler and hence stabilizer of the dimeric state against dissociation (Shearwin & Winzor, 1988). Indeed, on the basis of an effective covolume radius of 0.2 nm for glycerol (Wills et al., 1993), eq 4 predicts a larger magnitude of the activity coefficient ratio and hence of X_2^{app}/X_2 (eq 6) than for

20% sucrose (the upper limit of the density gradient). Accordingly, the disparity between the ultracentrifugal and coimmunoprecipitation results can be attributed to the stabilizing additives incorporated into the two experimental procedure to effect the analyses. Thus, we see that the concept of reaction zones as elaborated here provides a unifying thread for interpretation of the results of biophysical and immunological experiments germane to progesterone receptor binding activity for specific DNA sequences.

Although attention has been drawn to possible shortcomings of the intepretations placed on zonal ultracentrifugal results for steroid receptors, it is stressed that the criticism is not directed specifically at those investigators, who merely conformed with the currently accepted dogma for the conduct and quantitative analysis of gradient ultracentrifugation experiments. It is hoped that this demonstration of potential pitfalls in that dogma may lead to a general awareness of the invalidity of current practice, and a consequent reappraisal of conclusions based thereon. In that regard, the recent release of a more user-friendly analytical ultracentrifuge may well eliminate much of the need for quantitative studies of interactions by zonal sedimentation in a sucrose gradient, for which the technique is ill-suited.

Finally, it seems likely that both boundary and band sedimentation will play an increasingly important role in the quantitative characterization of interactions of proteins and other macromolecules with each other and with small ligand molecules, such interactions being central to biological control. This study adds to our store of fundamental understanding (Gilbert & Gilbert, 1973; Cox, 1978; Kegeles & Cann, 1978) required for unambiguous interpretation of the sedimentation behavior of interacting systems. In addition to their implications in that methodological context, the present findings have physiological ramifications in the sense that the extremely crowded nature of the biological environment may give rise to effective association constants that greatly exceed those inferred from *in vitro* studies of protein self-association under conditions approximating thermodynamic ideality (Minton, 1981, 1983; Minton & Wilf, 1981; Grasberger et al., 1986).

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