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ANALYSIS OF CHEMICALLY REACTING SYSTEMS BY SEDIMENTATION–DIFFUSION EQUILIBRIUM

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A novel procedure to evaluate equilibrium constants from sedimentation–diffusion equilibrium data of analytical ultracentrifuge runs is proposed. It is shown that, by comparison of a reacting mixture at chemical equilibrium with a non-reacting but equally composed one, the sum of the mean concentrations of the reaction products can immediately be taken from optical absorption or from interferometric measurements. In most but not in all cases the use of stacked double-sector centerpieces is required.

1. Introduction

The study of biologically important macromolecules very often involves the question of the specific interaction between macromolecules or that between macromolecules and low-molecular-weight substances. Ultracentrifugation offers universal possibilities for studying such interactions. Since each of the constituents of a complex and the complex itself will have different molecular masses, it should, consequently, be possible to separate and analyse them in a gravity field. As in the conventional use of the ultracentrifuge there are two different methods, one I would tentatively like to call "transport analysis" and the other "equilibrium analysis".

The first method, the "transport analysis", is based on the difference between the sedimentation coefficients of the constituents of the complex, which should be as large as possible. After sedimentation of the heavy component together with the complex formed, the uncomplexed part of the light component can be measured in the supernatant [1–7]. This method demands high gravity fields, and it is necessary to ensure that the concentration of the light ligand measured in this manner is identical with the equilibrium concentration measured under normal conditions, i.e. in absence of the applied gravitational force. The main sources of error are the high hydrostatic pressure in the cell and the dependence of the concentration of the free light ligand in the supernatant on the difference between the sedimentation coefficients of the macromolecule and the complex formed [8].

The "equilibrium analysis" circumvents these short-comings. It is carried out under the conditions of a normal sedimentation–diffusion equilibrium run, that is, with relatively low gravity fields. Thus the hydrostatic pressure inside the cell will be small; since the net flow of material in the centrifugal field is zero, there is no disturbance of the chemical equilibria, and transport phenomena are totally avoided.

I would like to discuss this last method and show that by an appropriate modification of the sedimentation–diffusion equilibrium centrifugation, a sensitive differential method can be developed that allows us to demonstrate in a simple way the formation of complexes and quantitatively to determine the equilibrium constants.

2. Thermodynamics

The condition for chemical equilibrium for the reaction

$$\nu_1 A_1 + \nu_2 A_2 + \ldots \rightleftharpoons \nu_m A_{n_t} + \nu_{m+1} A_{m+1} + \ldots \qquad (1)$$

is

$$\sum_{i=1}^{n} \nu_i \mu_i = 0 , \qquad (2)$$

where μ_i is the chemical potential per mole of species i and ν_i the number of moles by which i is involved in one stoichiometric turnover. The chemical potential μ_i is related to the concentration c_i by the relation

$$\mu_i = \mu_i^0 + RT \ln a_i = \mu_i^0 + RT \ln (\gamma_i c_i) , \qquad (3)$$

where a_i is the activity and γ_i the activity coefficient, respectively, of the ith component.

If we suppose that $(n-1)$ of a total of n reaction participants, whose concentrations can freely be chosen, are distributed in such a way that they maintain the condition of sedimentation–diffusion equilibrium in a centrifugal field, it can be shown [9–11] that

$$d\mu_i - M_i \omega^2 r dr = 0 \quad \text{for } i = 1, 2, ..., n , \qquad (4)$$

where r denotes the radial position, ω the angular velocity of the rotor and M_i the mole mass of the ith component. This means that, if chemical equilibrium between all the n reaction participants is established everywhere in the gravitational field and sedimentation–diffusion equilibrium additionally holds for $(n-1)$ components, sedimentation–diffusion is then also established for the nth component.

This enables us to describe the concentration distribution of any i of the components by integrating the ith of all n differential equations (4). Assuming the solution to be dilute and incompressible entails a constant solution density everywhere: $\rho(r) = \rho = \text{const}$. Furthermore, for simplicity, we shall only deal with ideal solutions and constant temperature T. Thus eqs. (4) transform to

$$\frac{dc_i}{dr} = \frac{M_i}{RT} \omega^2 r c_i (1 - \rho \bar{v}_i) \quad \text{for } i = 1, 2, ..., n , \qquad (5)$$

where $\bar{v}_i$ is the partial specific volume of the ith component.

These differential equations have to be solved with the boundary condition that the mass enclosed in the solution column of an ultracentrifuge cell remains constant at any time during the experiment. For sector-shaped cells, we get

$$c_i(r) = c_i^0 A_i (r_b^2 - r_m^2) \frac{\exp(A_i r^2)}{\exp(A_i r_b^2) - \exp(A_i r_m^2)}$$

$$\text{for } i = 1, 2, ..., n , \qquad (6)$$

where

$$A_i = \frac{M_i (1 - \bar{v}_i \rho) \omega^2}{2RT} , \qquad (7)$$

c_i^0 is the original concentration of the ith component and r_m and r_b refer to the radial positions of the bottom and the meniscus of the solution column, respectively. For the validity of eqs. (6) and the notation used therein compare textbooks on thermodynamics and [12,13].

So far, the evaluation of eqs. (6) is solely based on the assumption of constant temperature and of ideal solution behaviour. At no time does it refer to the chemical equilibrium between the various solutes we finally wish to deal with. It is even useful, with regard to an operational definition of an average concentration $\bar{c}_i$, to imagine that i is the only solute; then $\bar{c}_i$ not only means the average value of $c_i(r)$ in a sector-shaped cell, but is also identical with the concentration of the solute in the absence of the gravitational field, i.e. with c_i^0. This does not hold, however, when more than one solute is present and chemical equilibration between them is allowed. Under these circumstances the meaning of $\bar{c}_i$ will be limited to that of an average concentration in a sector-shaped cell:

$$c_i(r) = \bar{c}_i A_i (r_b^2 - r_m^2) \frac{\exp(A_i r^2)}{\exp(A_i r_b^2) - \exp(A_i r_m^2)}$$

$$\text{for } i = 1, 2, ..., n . \qquad (6a)$$

For our purposes, this result is the most suitable solution of the differential eqs. (4) whose validity for all n components, as we have seen, is compatible with the condition of chemical equilibrium. Therefore, since eq. (2) can easily be formulated in terms of the mass action law:

$$K = \prod_{i=1}^{n} c_i^{\nu_i} \qquad (8)$$

(if we tacitly maintain our assumption that $\gamma_i = 1$ for any i), it is obvious that the $c_i(r)$'s from eqs. (6a) fulfill eq. (8) at any radial position between the meniscus and the bottom of the solution column. The condition of chemical equilibrium, together with the condition of mass conservation which also have to be applied, define all average concentrations $\bar{c}_i$ of the n participants of the reaction. It is easy to show that the average concentrations $\bar{c}_i$ are different from the equilibrium concentrations of the various components in the absence of the centrifugal field [11,14] and therefore must not be confused with the latter (although the numerical error may be small).

It will be of practical help to note, that the A_i's from eq. (7) can be composed additively from molar increments as long as the partial molar volumes $\bar{V}_i$'s can be obtained by the addition of molar quantities (see appendix). Similar relationships also apply to the refractive index increments ψ_i (for comparison see [11] and the literature cited therein).

3. Simple example

For the simplest enzyme–ligand interaction

$$E + L \rightleftharpoons EL$$

two equations for the conservation of mass hold:

$$c_E^0 = \bar{c}_E + \bar{c}_{EL}\,, \tag{9}$$

$$c_L^0 = \bar{c}_L + \bar{c}_{EL}\,. \tag{10}$$

The equilibrium condition reads:

$$K_d = c_E(r)\,c_L(r)/c_{EL}(r)\,. \tag{11}$$

These equations, together with three sedimentation–diffusion equilibrium conditions according to eqs. (6a), govern the concentration distribution of the three equilibrium constituents E, L, and EL.

Substitution of eqs. (6a) and (7) into eq. (11) and replacement of $\bar{c}_E$ by $(c_E^0 - \bar{c}_{EL})$ according to eq. (9), and of $\bar{c}_L$ by $(c_L^0 - \bar{c}_{EL})$ according to eq. (10), yield

$$K_d = \frac{(c_E^0 - \bar{c}_{EL})(c_L^0 - \bar{c}_{EL})}{\bar{c}_{EL}}\,C\,, \tag{12}$$

where

$$C \equiv \frac{A_E A_L}{A_{EL}}(r_b^2 - r_m^2)\times \frac{\exp(A_{EL} r_b^2) - \exp(A_{EL} r_m^2)}{\{\exp(A_E r_b^2) - \exp(A_E r_m^2)\}\{\exp(A_L r_b^2) - \exp(A_L r_m^2)\}} \tag{13}$$

as long as $A_{EL} = A_E + A_L$.

Running the centrifuge under the usual conditions, that is, to obtain sedimentation–diffusion equilibrium with the concentration ratio of the solute at the bottom and at the meniscus of the column of the solution not higher than 3 or 4, the exponential functions can be expanded in series. Introducing $r_0^2 \equiv \frac{1}{2}(r_m^2 + r_b^2)$ and $r_b^2 - r_0^2 = -(r_m^2 - r_0^2) \equiv \Delta(r^2)$ gives

$$C \approx \frac{A_E A_L}{A_{EL}}(r_b^2 - r_m^2)\frac{\exp((A_E + A_L) r_0^2)}{\exp(A_E r_0^2)\exp(A_L r_0^2)} \times \frac{2\Delta(r^2)(A_E + A_L)}{4 A_E A_L (\Delta(r^2))^2} = 1 \tag{14}$$

from which it follows that there will be no serious numerical error if C is simply assumed to be 1.

Regardless of whether this simplification can be applied or not, K_d can be evaluated under all circumstances by measuring the average concentration of EL.

4. Methodology

Let us consider the following experiment: two centerpieces of the double-sector type are mounted together in such a manner that every two sectors are stacked and simultaneously covered by the photoelectric scanner (fig. 1). One of the leading sectors (in the sense of rotor rotation) is filled with an equilibrium mixture of enzyme and ligand, the other with pure solvent. Once the sedimentation–diffusion equilibrium is established, the scanner registers an optical density at the various radial positions, which is the sum of all absorptions of the various components at this position (for unit path length):

$$-\log(I_1/I_0)(\lambda,r) = c_E(r)\epsilon_E(\lambda)+c_L(r)\epsilon_L(\lambda)+c_{EL}(r)\epsilon_{EL}(\lambda)$$

$$= \bar{c}_E A_E(r_b^2-r_m^2)\frac{\exp(A_E r^2)}{\exp(A_E r_b^2)-\exp(A_E r_m^2)}\epsilon_E(\lambda)$$

$$+ \bar{c}_L A_L(r_b^2-r_m^2)\frac{\exp(A_L r^2)}{\exp(A_L r_b^2)-\exp(A_L r_m^2)}\epsilon_L(\lambda)$$

$$+ \bar{c}_{EL} A_{EL}(r_b^2-r_m^2)\frac{\exp(A_{EL} r^2)}{\exp(A_{EL} r_b^2)-\exp(A_{EL} r_m^2)}\epsilon_{EL}(\lambda). \quad (15)$$

The trailing sectors are filled with either pure enzyme or pure ligand solution, respectively. The concentrations are chosen to be equal to the concentrations of enzyme and ligand in the leading sectors, which means that $c_E^0 = \bar{c}_E + \bar{c}_{EL}$ and $c_L^0 = \bar{c}_L + \bar{c}_{EL}$. At sedimentation–diffusion equilibrium the scanner will indicate the following absorption:

$$-\log(I_2/I_0)(\lambda,r) = c_E'(r)\epsilon_E(\lambda)+c_L'(r)\epsilon_L(\lambda)$$

$$= c_E^0 A_E(r_b^2-r_m^2)\frac{\exp(A_E r^2)}{\exp(A_E r_b^2)-\exp(A_E r_m^2)}\epsilon_E(\lambda)$$

$$+ c_L^0 A_L(r_b^2-r_m^2)\frac{\exp(A_L r^2)}{\exp(A_L r_b^2)-\exp(A_L r_m^2)}\epsilon_L(\lambda)$$

$$= (\bar{c}_E+\bar{c}_{EL})A_E(r_b^2-r_m^2)\frac{\exp(A_E r^2)}{\exp(A_E r_b^2)-\exp(A_E r_m^2)}\epsilon_E(\lambda)$$

$$+ (\bar{c}_L+\bar{c}_{EL})A_L(r_b^2-r_m^2)\frac{\exp(A_L r^2)}{\exp(A_L r_b^2)-\exp(A_L r_m^2)}\epsilon_L(\lambda) \quad (16)$$

as long as r_m and r_b, T and ω are identical for sectors of equal shape.

The scanner of the Beckman Model E Analytical Ultracentrifuge is designed to compare the optical densities at equal radial positions of the leading and trailing sectors of a double-sector cell, that is, to subtract the logarithms of the corresponding transmitted

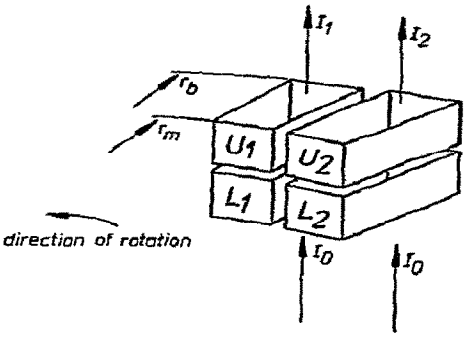

Fig. 1. Schematic drawing of stacked double-sector cells. U_1 and U_2 denote the fluid columns of the leading and trailing sectors of the upper double-sector centerpieces, L_1 and L_2 those of the lower ones, respectively. The menisci of the columns are at r_m, the bottoms at r_b. I_0, I_1, and I_2 denote incident light intensity, transmitted light intensity of the leading and of the trailing sectors, respectively.

light intensities. In our experiment subtraction of the logarithms of the intensities I_1 and I_2 would yield:

$$-\log(I_1/I_2)(\lambda,r) = -\log(I_1/I_0)(\lambda,r)+\log(I_2/I_0)(\lambda,r)$$

$$= -\bar{c}_{EL} A_E(r_b^2-r_m^2)\frac{\exp(A_E r^2)}{\exp(A_E r_b^2)-\exp(A_E r_m^2)}\epsilon_E(\lambda)$$

$$- \bar{c}_{EL} A_L(r_b^2-r_m^2)\frac{\exp(A_L r^2)}{\exp(A_L r_b^2)-\exp(A_L r_m^2)}\epsilon_L(\lambda)$$

$$+ \bar{c}_{EL} A_{EL}(r_b^2-r_m^2)\frac{\exp(A_{EL} r^2)}{\exp(A_{EL} r_b^2)-\exp(A_{EL} r_m^2)}\epsilon_{EL}(\lambda)$$

$$\equiv -\bar{c}_{EL} F, \quad (17)$$

which clearly demonstrates that our arrangement will show a photoelectric density recording which at any radial position is proportional to the mean complex concentration $\bar{c}_{EL}$. It thus provides quantitative evidence for molecular interaction.

The simplification used previously for C can also be applied to F, provided that the assumptions mentioned there are also made here (compare eq. (14)). Thus eq. (17) can be rewritten:

$$-\log(I_1/I_2)(\lambda,r) = -\bar{c}_{EL}[\epsilon_E(\lambda)\exp(A_E\{r^2-r_0^2\}) + \epsilon_L(\lambda)\exp(A_L\{r^2-r_0^2\}) - \epsilon_{EL}(\lambda)\exp(A_{EL}\{r^2-r_0^2\})]. \quad (18)$$

For the aforesaid distribution of enzyme and ligand over the four sectors of our stacked double-sector cells, the scanner will display negative recordings for $r<r_0$ and positive ones for $r>r_0$. Consequently there is a point r' where the optical density difference between the leading and the trailing sectors vanishes. Now r' can be evaluated exactly as a root of F with the aid of a small desk calculator and the use of any suitable method for solving nonlinear equations. If, however, the assumptions which justify the transformation of eq. (17) to eq. (18) are valid and in addition $\epsilon_{EL} = \epsilon_E + \epsilon_L$, then r' can be calculated from $r'^2 = r_0^2 = \frac{1}{2}(r_b^2+r_m^2)$.

The knowledge of r' is of practical importance because any no-nulling of the scanning electronics can thereby be detected and accounted for in numerical evaluations. Its physical meaning is comparable to what is called "hinge point" by Schachman [15]. Similar suggestions will be helpful when interference optics is used to recognize the radial point of zero line shift.

The method just described is in no way limited to systems where both equilibrium constituents display an utilisable absorption spectrum. In general, one can expect that only one or neither of the complex-forming components will show an absorption in the accessible spectral range. In those cases the Rayleigh interferometer may be employed with similar success to determine differences in the refractive indices and hence concentration differences. In analogy to eq. (A.1) (see appendix)

$$\left(\frac{\partial n(\lambda)}{\partial c_{EL}}\right)_{p,T} = \psi_{EL}(\lambda) = \frac{\psi_E(\lambda)M_E+\psi_L(\lambda)M_L}{M_E+M_L} \quad (19)$$

and therefore

$$n(\lambda,r) = n_0+c_E(r)\psi_E(\lambda)+c_L(r)\psi_L(\lambda)+c_{EL}(r)\psi_{EL}(\lambda)$$

and finally

$$\Delta n(\lambda,r) = -\bar{c}_{EL}\left[A_E(r_b^2-r_m^2)\frac{\exp(A_E r^2)}{\exp(A_E r_b^2)-\exp(A_E r_m^2)}\psi_E(\lambda) + A_L(r_b^2-r_m^2)\frac{\exp(A_L r^2)}{\exp(A_L r_b^2)-\exp(A_L r_m^2)}\psi_L(\lambda) - A_{EL}(r_b^2-r_m^2)\frac{\exp(A_{EL} r^2)}{\exp(A_{EL} r_b^2)-\exp(A_{EL} r_m^2)}\psi_{EL}(\lambda)\right] \equiv -\bar{c}_{EL}F', \quad (20)$$

where $\Delta n(\lambda,r)$ refers to the wavelength-dependent refractive index difference at radius r, which is characterized by a line shift J. If d is the cell thickness (optical path length) we have

$$J(\lambda) = \frac{d}{\lambda}\Delta n(\lambda) = \frac{d}{\lambda}\left(\frac{\partial n(\lambda)}{\partial c}\right)_{p,T} c = \frac{d}{\lambda}\psi(\lambda)c$$

and therefore

$$\Delta n(\lambda,r) = J(\lambda,r)\lambda/d \quad (21)$$

and

$$J(\lambda,r) = -\bar{c}_{EL}(d/\lambda)F'. \quad (22)$$

ψ_E and ψ_L may easily be determined by synthetic boundary runs or by refractometry, and ψ_{EL} is calculated from eq. (19). Thus, the determination of $\bar{c}_{EL}$ turns out to be simply a matter of counting of shifts of interference fringes, which is a familiar procedure in conventional sedimentation–diffusion runs.

F and F' can both be calculated from eq. (17) and from eq. (20), respectively, or from eq. (18) or a similarly simplified one, respectively, if the assumptions leading to the two latter ones are justified. All the terms therein are either known or can be determined independently. With the aid of those a priori calculations, $\bar{c}_{EL}$ can easily be obtained from optical density difference recordings or from the fringe shift of interferometric photographs. By use of eq. (12) the dissociation constant K_d is finally obtained.

5. Discussion

In the previous sections it has been shown with the help of a simple example, how a suitable choice of the fillings of the leading and trailing sectors of stacked double-sector cells makes it possible not only to demonstrate the presence of a product formed by chemical reaction of two reactants, but also to evaluate this process quantitatively.

Let us now consider a more general example where k components react to yield products. It is tacitly assumed that the removal of one of the k components prevents all interactions of the remaining $(k-1)$ reactants (each arbitrary system of interactions can be reduced to a limited number of such "elementary" equilibria). Then the technique outlined above, whether it makes use of optical absorption or of interferometry, requires the following experimental set-up. One of the trailing sectors is filled with $(k-1)$ starting components, the other with the kth component, while the reacting mixture is to be found in one of the leading sectors. By running the centrifuge one can observe either a difference of optical density or a fringe shift, whenever the sum of the starting concentrations of the k reactants is different from the sum of their average concentrations at sedimentation–diffusion equilibrium:

difference of optical density of fringe shift

$$= \sum_k a_i(r)\,(c_i^0 - \bar{c}_i)\,.$$

Since the difference in brackets corresponds to the sum of the average concentrations of the products, it is easy to recognize that the procedure described herein not only indicates a chemical reaction between *two* molecules, but also enables us to detect a chemical reaction between *more than two* components and to determine its extent from the sum of mean product concentrations, which is indicated directly by the experimental set-up. One experiment (that is, one ultracentrifuge run with one stacked arrangement of double-sector cells as shown in fig. 1) should, at least in principle, be sufficient to yield (practically) non-vanishing determinants for the evaluation of all mean product concentrations when as many different radial positions as the number of products formed are taken into account. Otherwise additional runs would be necessary under altered conditions: lowering or raising concentrations of starting components, altering rotor speed, using other wavelengths in case of optical absorption analysis, replacing UV-scanner by a Rayleigh interferometer etc.

Although the method of "equilibrium analysis" is generally applicable, it is not suitable for the investigation of very strong or very weak interaction. It presupposes that complex and complex-forming constituents are present in similar concentrations and that therefore the difference between the starting and the equilibrium concentrations is measurable with adequate experimental accuracy. If we remember that measurements with Rayleigh interference optics carried out on 0.5 to 5 mg/ml of substance at 546 nm and 12 mm cell thickness can be meaningfully evaluated, then for proteins with a mole weight of 200 000 this corresponds to a solution of 2.5×10^{-5} M. In this case the interference optics may be employed, if one wishes to determine the binding of a ligand with $K_d \approx 10^{-6}$ to 10^{-4} M. Through reduction of the optical path length to 3 mm it should even be possible to carry out measurements where $K_d \approx 10^{-3}$ M. A restriction must nevertheless be mentioned because at equal molar concentrations of the interacting partners it is the higher molecular weight component which will obviously be predominantly recorded, when interference optics is used, since the alteration of the refractive index is more dependent on the mass rather than on the number of molecules dissolved in unit volume. Therefore, the interferometric determination will always be unsuitable if the binding of a small ligand to a macromolecule is to be studied, as the difference in mass between the macromolecule and complex is too small. In this case, it is more advantageous to resort to other methods such as equilibrium dialysis or "transport analysis", or it may, under certain conditions, be possible to utilize electronic scanners to record optical absorption. Nevertheless, since the interference method causes no problem with regard to light intensity (at least as long as the solutions do not absorb at 546 nm) the sensitivity can be significantly raised by an increase of the rotor speed (A_i is proportional to ω^2).

The use of the photoelectric scanner may extend the limits of measurements to much smaller solute concentrations than those just discussed. Proteins may typically be recorded at 280 nm at concentrations

between 0.05 and 1 mg/ml. This corresponds to a concentration of protein (mole weight 200 000) of 2.5×10^{-7} to 5×10^{-6} M and therefore widens the range of application to K_d's smaller by another order of magnitude with respect to interferometry. However, there are four distinctly different cases:

In the first case both the interacting components (if for simplicity we remain with the system E + L ⇌ EL) possess no measurable absorption spectrum. Then a photoelectric recording is not possible, and the use of interference optics will be the method of choice.

In the second case both components absorb at similar wavelengths. Then the considerations introduced above will hold with regard to the recordable region of the equilibrium constants, which can be expanded by the use of thinner cells (3 mm) and through the choice of suitable wavelengths ($\lambda \neq \lambda_{max}$).

The third case includes the two possibilities that either the macromolecule does not exhibit a recordable spectrum or the smaller ligand can be followed at wavelengths outside the absorption spectrum of the larger one. In this case one should avoid the use of stacked double-sector cells and return to one normal double-sector cell. Its trailing sector is filled with the smaller ligand solution, the leading sector with the reaction mixture at chemical equilibrium. This holds, of course, in a similar way for components of comparable weight, if their spectral ranges of absorption are sufficiently different.

In the fourth case only the macromolecule has a recordable spectrum. Then measurements can be expected to be meaningful only if the molecular weights of the pure macromolecule and of the compiex formed with it are sufficiently different, that is, when the small ligand is not too light.

Increasing the sensitivity by increasing ω is subject to strict limitations. Whenever the photoelectric scanner is used, it is essential to improve the standard illumination equipment of the ultracentrifuge with the aim of obtaining the highest possible intensity, because this type of method involves approximately equal optical densities in both pairs of sectors which are to be compared. An increase of ω would cause further enhancement of absorption at the bottoms of the cells, and therefore still further improvement of the illumination technique would be required; however, increased illumination together with high absorption would cause solute destruction. I shall deal with my efforts and experiences in this field as well as with the construction of suitable stacked double-sector cells in a subsequent article [16].

In conclusion, I would like to compare the technique described above with others that have appeared in the literature [9–11,17–20] and pursue a similar line of reasoning. The method of Schachman [17] is, to my knowledge, the only one which can be compared with the technique proposed here. It has been frequently used for the investigation of the binding of small ligands to macromolecules. Even more numerous investigations aim to recognize complex formation and to evaluate equilibrium constants subsequently from the apparent mole weight of the total solute [9–11,18–20]. Being outside the scope of this article, they will not be discussed here.

The method of Schachman makes use of the fact that a small ligand will be almost homogeneously distributed in the cell, as long as it is in the unbound state, but will display a distribution in the gravity field as soon as it is bound to a macromolecule, if the rotor speed and the filling heights in the cell are so low that only the macromolecular component exhibits a detectable exponential concentration distribution. In the case under discussion the binding of methyl orange to bovine serum albumin was investigated. This system offered the opportunity of determining the sum $c_E + \sum_n c_{EL_n}$ from interference pictures (the index E refers to the protein and the index L to methyl orange) and the sum $c_L + \sum_n nc_{EL_n}$ from absorption measurements. Plotting the first sum versus the second yielded straight lines which intercepted the abscissa at c_L (anticipated to be independent of the radial position, as indicated above), their slope being the mean number of binding sites of methyl orange per molecule protein.

It is obvious that these experiments and the technique proposed in the present paper are based on similar considerations. In both instances the concentrations of the reaction participants are determined for one or more radial positions in an analytical ultracentrifugal cell at sedimentation–diffusion equilibrium. The salient feature of the present method is that the contribution of the uncomplexed parts of complex-forming constituents are a priori eliminated by a special experimental set-up. Hence all uncertainties which arise from the comparison of separately ob-

tained measurements are avoided. It is even possible to investigate the more general situation of non-homogenous distribution of the free ligand.

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Appendix

For a hypothetical molecule with the composition $A_m B_{m+1} C_{m+2}...$ the mole mass can additively be composed from constituent increments:

$$M = mM_A + (m+1)M_B + (m+2)M_C + ...$$

as can be done with the partial molar volume if ΔV^0 for the overall formation reaction is zero:

$$\bar{V} = m\bar{V}_A + (m+1)\bar{V}_B + (m+2)\bar{V}_C + ...$$

From these equations we get

$$\bar{v} = \frac{m\bar{v}_A M_A + (m+1)\bar{v}_B M_B + (m+2)\bar{v}_C M_C + ...}{mM_A + (m+1)M_B + (m+2)M_C + ...}, \quad (A.1)$$

Since (cf. eq. (7))

$$A = \frac{M}{2RT}(1 - \bar{v}\rho)\omega^2$$

it follows that

$$A = \frac{mM_A + (m+1)M_B + (m+2)M_C + ...}{2RT}$$

$$\times \left[1 - \rho \frac{m\bar{v}_A M_A + (m+1)\bar{v}_B M_B + (m+2)\bar{v}_C M_C + ...}{mM_A + (m+1)M_B + (m+2)M_C + ...}\right]\omega^2$$

$$= \frac{mM_A}{2RT}(1 - \bar{v}_A\rho)\omega^2 + \frac{(m+1)M_B}{2RT}(1 - \bar{v}_B\rho)\omega^2$$

$$+ \frac{(m+2)M_C}{2RT}(1 - \bar{v}_C\rho)\omega^2 + ...$$

$$= mA_A + (m+1)A_B + (m+2)A_C + ...$$

q.e.d. (A.2)

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