Ultracentrifugation Studies with Absorption Optics. V. Analysis of Interacting Systems Involving Macromolecules and Small Molecules*

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ABSTRACT: Sedimentation equilibrium and sedimentation velocity methods which, for many years, have proved so valuable in the study of noninteracting macromolecules have been extended in recent years to encompass the analysis of interacting systems as well. The investigations thus far have been concerned principally with association–dissociation equilibria or with interactions between different types of macromolecules. The study reported here illustrates both theoretically and experimentally the potential of ultracentrifugal methods for the analysis of interactions between macromolecules and small molecules. As shown in the theory for sedimentation equilibrium experiments the average number of small molecules bound to a macromolecule is determined readily as the slope of a plot of the total concentration of the small constituent *vs.* that of the macromolecular constituent. An analogous treatment of sedimentation equilibrium in terms of constituent effective molecular weights shows that the chemical equilibria can be evaluated from the constituent effective molecular weight of the small molecule coupled with the independently measured molecular weight of the macromolecule. For this treatment the molecular weight of the small molecule is considered negligible relative to that of the macromolecule. The theoretical treatment was tested by analyzing the interaction between bovine plasma albumin and methyl orange. Measurements of the concentration distribution of the methyl orange constituent were obtained through the use of the photoelectric scanner coupled with the monochromator. Simultaneously the distribution of the protein constituent was measured from the Rayleigh interference patterns. As predicted theoretically the plots of the total methyl orange concentration *vs.* total protein concentration were linear and the intercept (at zero concentration of protein) gave the concentration of unbound methyl orange while the slope yielded the average number of bound dye molecules per protein molecule. The results from seven different dye–protein mixtures were in excellent agreement with the earlier findings from equilibrium dialysis studies on the same system (Klotz *et al.*, 1946). A brief theoretical treatment also is given for the behavior of the same type of interacting system in sedimentation velocity experiments. The complications described by Gilbert and Jenkins (1960) due to a change in the chemical equilibria at the moving boundaries were circumvented through the use of constituent sedimentation coefficients. For the measurement of the constituent sedimentation coefficient of the methyl orange the absorption optical system was used in conjunction with the transport equation expressing the migration of all methyl orange molecules (free as well as bound in the form of complexes) across a surface in the plateau region. The corresponding constituent sedimentation coefficients of the protein were measured from the schlieren patterns. These results were used to illustrate the binding of methyl orange to bovine plasma albumin as well as the effect of competing electrolyte on that binding. In addition, experiments are presented to show that some of the terms in the detailed theory are negligible for this type of system. The results evaluated from the sedimentation velocity experiments were compared with the corresponding values obtained from sedimentation equilibrium and equilibrium dialysis experiments. The agreement among the three methods was satisfactory. Advantages as well as limitations of the two different sedimentation techniques were discussed along with general considerations of the prospects of extending them to the study of interactions between different types of macromolecules.

Since the biological activity exhibited by proteins and nucleic acids depends upon specific interactions with other molecules, small or large, it is to be expected that considerable effort would be devoted to the analysis of interacting systems involving these constituents (*e.g.*, Klotz, 1953; Waugh, 1954). Among the goals of these investigations are the elucidation of the number and nature of the binding sites in the macro-

* From the Molecular Biology and Virus Laboratory, University of California, Berkeley, California 94720. *Received August 22, 1966.* These studies were supported in part by U. S. Public Health Service Research Grant GM 12159 from the National Institute of General Medical Sciences, by National Science Foundation Grant G-10795, and by a contract between the Office of Naval Research and the Regents of the University of California.

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molecules, the determination of the specificity of the interactions, and the evaluation of the type and magnitude of the forces responsible for the interactions. Various methods have been devised for the analysis of these interacting systems, with the techniques used for the study of the interactions among macromolecules alone being different generally from those employed for the measurement of the extent of the interactions between macromolecules and small molecules. For the former, light-scattering, electrophoresis, and sedimentation velocity and equilibrium measurements have been of considerable value, while the latter systems have been analyzed frequently by equilibrium dialysis, gel filtration, electromotive force, fluorescence, optical rotation, and electrophoresis measurements.

In view of the marked differences between the sedimentation coefficients of small and large molecules it might be expected that the sedimentation velocity method would be used widely for the determination of the extent of binding of small molecules (and ions) to macromolecules. However the number of studies of this type has been very limited. Chanutin *et al.* (1942), for example, employed a preparative centrifuge for determinations of the binding of calcium ions to casein and serum proteins. In these experiments the contents of the tubes after a long period of high-speed centrifugation were separated into a series of fractions corresponding to levels at different distances from the axis of rotation. Analyses of these fractions provided the data for a plot of calcium ion concentration as a function of protein concentration, thereby permitting an extrapolation to zero protein concentration. This value of the concentration of calcium ions was assumed to represent the unbound calcium ions in the equilibrium mixture. With this value and knowledge of the total concentration of calcium ions in these experiments Klotz (1946) calculated the fraction of ions bound as well as the number of binding sites and the association constant. A somewhat analogous procedure was adopted by Velick *et al.* (1953; Hayes and Velick, 1954; Velick, 1954) for measuring the binding of diphosphopyridine nucleotide to glyceraldehyde 3-phosphate dehydrogenase. In none of these studies, however, was a detailed analysis made of the sedimentation process itself in terms of special advantages of the method or its potential pitfalls. Thus, despite the promise afforded by the method and the useful results obtained in these particular studies, the sedimentation velocity technique has not been employed widely for the measurement o interactions between large and small molecules.

Although the sedimentation equilibrium method has been considered theoretically for the study of various chemically reacting solutes (Adams and Fujita, 1963; Adams, 1965; Adams and Williams, 1964; Nichol and Ogston, 1965) its application experimentally has been restricted to association–dissociation equilibria involving only one chemical species such as the aggregation of adrenocorticotropin (Squire and Li, 1961), insulin (Jeffrey and Coates, 1963, 1966), casein (Payens and Schmidt, 1965), and α-amylase (Kakiuchi, 1965). Sedimentation equilibrium studies have not been applied, except for one preliminary illustration (Schachman *et al.*, 1962), to the analysis of reversible interactions between large and small molecules. Now that the concentration of light-absorbing materials can be measured during centrifugation with the recently developed scanning absorption optical system (Hanlon *et al.*, 1962; Schachman *et al.*, 1962; Schachman, 1963a,b; Lamers *et al.*, 1963) it seemed of interest to evaluate the potential of the sedimentation equilibrium technique for the study of this type of interacting system. Also, this occasion afforded the opportunity to reevaluate the limitations and applicability of the sedimentation velocity method for such systems.

This communication deals with the application of both the sedimentation velocity and sedimentation equilibrium techniques to the study of interactions between large and small molecules. The theoretical treatments and experimental procedures for both sedimentation methods are described along with an application to a study of the interaction between bovine plasma albumin and methyl orange. The results are compared with those obtained earlier (Klotz *et al.*, 1946) by equilibrium dialysis in order to illustrate the value of the sedimentation methods and their advantages and limitations in relation to other techniques which have been used for the analysis of interacting systems.

General Considerations

In sedimentation velocity experiments on systems of noninteracting components, individual boundaries are observed for each of the different molecular species if the respective sedimentation coefficients differ sufficiently. Moreover, except for certain anomalies resulting from hydrodynamic effects, the amounts of the different components can be determined readily from the change in either the refractive index or the optical density across each boundary. If, however, the various components can react one with another to form complexes, this straightforward interpretation of the ultracentrifuge patterns no longer obtains. Not only does the number of boundaries not correspond to the number of sedimenting species but also the concentrations deduced from the patterns do not represent the equilibrium concentrations of the components in the original solution (Longsworth and MacInnes, 1942; Gilbert and Jenkins, 1956, 1960, 1963; Longsworth, 1959; Nichol *et al.*, 1964).

During the transport of the various molecular species in an equilibrium mixture of the type, $P + A \rightleftharpoons PA$, where P denotes a protein or other large molecule and A represents a small molecule, there is generally a disturbance of the equilibrium distribution of the components in the boundary region due to the differences in the sedimentation rates of the various molecular forms. The removal of the complex, PA, for example, from a region in the ultracentrifuge cell in which A and P are both present would represent

such a disturbance in the chemical equilibrium. If the rates of association and dissociation are rapid there will be a continual readjustment of the concentrations of the various species in order to maintain the proper distribution required by the equilibrium constant for the chemical reaction. This reequilibration upon the disturbance of the preexisting equilibrium continues throughout the sedimentation velocity experiment. As a consequence the concentration of the small component, A, in the supernatant region behind the moving boundary of the macromolecular constituent may differ significantly from the initial equilibrium concentration. If the complex, PA, sediments more rapidly than P then the reequilibration leads to a depletion of A in the supernatant; hence the calculated fraction of the small molecules in the form of complexes will be greater than the correct value if the determination is based solely on the concentration of A in the supernatant. Conversely, there will be an accumulation of A above the true initial equilibrium concentration if PA sediments more slowly than P. The latter could occur despite the increase in molecular weight of the macromolecules upon binding of A if there is a concomitant change in conformation of the macromolecules as a result of the interaction. In some cases the increase in frictional coefficient due to swelling or partial unfolding of the macromolecules is sufficient to cause PA to have a lower sedimentation coefficient than P (Richards and Schachman, 1957).

Gilbert and Jenkins (1956) showed, in addition, that even though there were three different molecular species in the equilibrium mixture only two boundaries would be detected in sedimentation velocity patterns. Because of the disturbance of the equilibrium due to the different rates of migration of P and PA and the consequent reequilibration, separate boundaries for each of these species do not exist. Instead only one broad boundary representing the macromolecular constituent is observed and its rate of movement is intermediate between those for pure P and PA.

Despite the complexity of the sedimentation velocity patterns of chemically reacting systems valuable information concerning the association equilibria can be obtained simply from an evaluation of the constituent sedimentation coefficients by means of the appropriate transport equations (Schachman, 1959). In effect this procedure avoids attempting to analyze composite boundaries and involves instead integrating across them to a surface in the plateau region containing all of the components in the equilibrium mixture. By this means average sedimentation coefficients can be evaluated for the A constituent as well as the P constituent even though the boundaries themselves do not provide a reliable index of the rates of migration and the amounts of the various chemical species. This method of analyzing the patterns circumvents the difficulties resulting from the disturbances in the equilibria in the boundary regions since it gives a measure of the rate of movement of the different constituents in the plateau region where all reacting components are present in chemical equilibrium. From the values of the constituent sedimentation coefficients along with the corresponding sedimentation rates for the pure A and P components the fraction of A both free and in the form of complexes can be calculated. Hence the data required for the determination of the equilibrium constant can be evaluated. However, this method, too, is not free of ambiguity. During the transport of the molecules under the influence of the centrifugal field, the material in the plateau region is continually diluted and this is accompanied by some dissociation of the complex, PA, to give A and P. Further, there is a large pressure gradient throughout the solution due to the large centrifugal fields employed for sedimentation velocity experiments. This too influences the chemical equilibria since most interactions involve some volume change. Finally, this treatment like that of Gilbert and Jenkins (1960, 1963) does not make allowances for the possible complicating effects of interacting flows (Gosting, 1956; Williams *et al.*, 1958; Peller, 1958). Generally these complications are small but no control can be exerted over the magnitude of the interacting flows, and the assessment of their effect on the calculation of equilibrium constants, for example, must await further experimental studies of cross-term diffusion coefficients.

In sedimentation equilibrium experiments the complications described above are absent. Since the net transport of the material in the centrifugal field is zero, there is no disturbance of the chemical equilibria and interacting flows are of no consequence. The pressure gradient through the cell is usually not great since relatively small centrifugal fields are employed in sedimentation equilibrium experiments. When sedimentation equilibrium is achieved the distribution of all molecular species throughout the cell must be in accord with the thermodynamic equation which describes the concentration as a function of distance in terms of well-known parameters such as the molecular weight and partial specific volume of the solute and the density of the solution (Svedberg and Pedersen, 1940). Parallel with the requirement of sedimentation equilibrium is the necessity that chemical equilibrium among the reacting species be attained at each level in the cell.

For many systems of interest the centrifugal field employed for sedimentation equilibrium experiments is such that small molecules would be practically uniformly distributed throughout the cell even though there are large concentration gradients of macromolecules. This is the situation, of course, in determinations of the molecular weights of proteins by sedimentation equilibrium. Although the protein concentration may vary about ten-fold across the cell, the concentration of small, noninteracting molecules (such as buffer salts) would be virtually constant. If, however, there is an interaction between the macromolecules and the small molecules the total concentration of the latter would increase with distance in the cell since a larger amount would be bound at the higher protein concentration. Despite this gradient in the *total* concentration

of the small molecules the concentration of *unbound* small molecules would be constant throughout the cell. Thus the change in the total concentration of the small molecules with distance (or with the concentration of the macromolecules) would be a measure of the extent of binding.

A single sedimentation equilibrium experiment can be considered analogous to a multiple equilibrium dialysis experiment, in which many dialysis tubes containing nondialyzable macromolecules at different concentrations are suspended in a single large vessel containing the permeable small molecules. After equilibrium is attained, the outside liquid would be uniform but the total concentration of the small molecules inside the dialysis tubes would vary depending on the concentration of the macromolecules in each tube. In such an experiment measurements of the total concentration of the small molecules and macromolecules in each tube as well as the concentration of the small molecules in the outer fluid provide the data required for the calculation of the number of binding sites and the equilibrium constants of association (Klotz, 1953). In order to extend the analogy between the equilibrium dialysis and sedimentation equilibrium experiments, consider one of the dialysis tubes to be devoid of protein. For that tube the concentration of the small molecules would, of course, be equal to that of the outer fluid and no additional measurement on the latter would be required. Only the total concentrations of the small molecules and the corresponding concentrations of the macromolecules in each tube are needed. Returning now to the ultracentrifuge experiment it is seen that each level in the ultracentrifuge cell is equivalent to an individual dialysis tube. Hence independent measurements of the total concentrations of the macromolecules and small molecules at each level in the ultracentrifuge cell would correspond to the analysis of the tubes in the multiple dialysis experiment. The concentration of the small molecules corresponding to zero macromolecular concentration can be evaluated by extrapolation. The extrapolation procedure may be facilitated, if necessary, by selecting the magnitude of the centrifugal field so that the concentration of the macromolecules at the meniscus is sufficiently low. In this way the concentration of the unbound small molecules is evaluated directly as long as the centrifugal field is not so great as to cause much variation in their concentration throughout the cell.

The evaluation of the extent of the interaction can be performed in another manner. A determination of the constituent molecular weight of A (*i.e.*, an average molecular weight including both bound and unbound forms) coupled with knowledge of the molecular weights of the macromolecules and of the unbound small molecules would permit a direct calculation of the fraction of the latter existing in the bound state.

In order to analyze the association–dissociation equilibria the ultracentrifuge must be equipped with optical systems capable of providing accurate data for the concentration of the small molecular constituent and the macromolecular material separately. For many types of systems this can be accomplished by employing schlieren or interference optics for measurements of the macromolecules and absorption optics for the small molecules (Schachman *et al.*, 1962). In some situations absorption optics alone may suffice if the absorption spectra of the constituents are sufficiently different to permit the unambiguous identification and measurement of the individual constituents by the proper selection of the wavelength of the incident light.

Since the theory for the analysis of interacting systems by the sedimentation velocity method is treated elsewhere (Richards and Schachman, 1957; Williams *et al.*, 1958; Schachman, 1959; Fujita, 1962) only a brief summary of it will be given here. The theory for the sedimentation equilibrium method follows.

Theory

Sedimentation Equilibrium. Consider the interaction of a protein, P, with a low molecular weight component, A. While the protein may have many binding sites the small molecule is considered to be univalent, *i.e.*, it can bind to only one macromolecule at a time. A series of complexes may be formed according to the following equilibria

$$\begin{aligned} P + A &\rightleftharpoons PA \\ PA + A &\rightleftharpoons PA_2 \\ &\cdots\cdots\cdots \\ PA_{i-1} + A &\rightleftharpoons PA_i \\ &\cdots\cdots\cdots \\ PA_{n-1} + A &\rightleftharpoons PA_n \end{aligned} \qquad (1)$$

and the various association constants for these equilibria can be written as

$$k_i = \frac{[PA_i]}{[PA_{i-1}][A]} \qquad (2)$$

where $[PA_i]$, $[PA_{i-1}]$, and $[A]$ denote the molar concentrations of PA_i, PA_{i-1}, and free A, respectively.

The total molar concentration of protein, $[P_0]$, can be described by $\sum_{i=1}^{n} [PA_i]$ and the total concentration of A by $[A_0]$

$$[A_0] = [A] + \sum_{i=1}^{n} i[PA_i] \qquad (3)$$

where $\sum_{i=1}^{n} i[PA_i]$ represents the amount of A complexed with protein. For convenience the binding is expressed as the number of moles of bound A per mole of total protein (Klotz, 1953). This ratio is designated as r

defined by

$$r = \frac{[A_0] - [A]}{[P_0]} = \frac{\sum_{i=1}^{n} i[PA_i]}{[P_0]} \quad (4)$$

Equation 3 can thus be rewritten as

$$[A_0] = [A] + r[P_0] \quad (4a)$$

For proteins with n combining sites (Klotz, 1946), r is a function of the various association constants and the concentration of free A according to

$$r = \frac{k_1[A] + 2k_1k_2[A]^2 + \ldots + ik_1k_2 \ldots k_i[A]^i + \ldots + nk_1k_2 \ldots k_n[A]^n}{1 + k_1[A] + k_1k_2[A]^2 + \ldots + k_1k_2 \ldots k_i[A]^i + \ldots + k_1k_2 \ldots k_n[A]^n} = \frac{\sum_{i=1}^{n} ik_1k_2 \ldots k_i[A]^i}{1 + \sum_{i=1}^{n} k_1k_2 \ldots k_i[A]^i} \quad (5)$$

In the special case for which the n sites are equivalent and independent (with no electrostatic interactions, for example) a single value, k, can be taken to characterize the intrinsic association constant for the binding of A to any of the sites on the protein. For such systems the experimental association constants, k_i, can be related to the intrinsic association constant, k, by

$$k_i = \frac{n - (i-1)}{i} k \quad (6)$$

and eq 5 then becomes

$$r = \frac{nk[A]}{1 + k[A]} \quad (7)$$

Equations 5 and 7 show that r is not dependent on protein concentration and that the experimental determination of both r and [A], and hence the dependence of r on [A], permits the evaluation of the number of binding sites and the association constants (Klotz, 1946, 1953).

If a solution containing the various molecular complexes described by the above equilibria is subjected to the influence of a centrifugal field there will be a redistribution of the molecules in accordance with the well-known thermodynamic equations for sedimentation equilibrium. At relatively low centrifugal fields (about $10^4 \times$ gravity) the redistribution of proteins of molecular weight about 10^5 is such that the concentration in the ultracentrifuge cell with a liquid column height of 3 mm varies about 30-fold when sedimentation equilibrium is achieved. Small molecules with molecular weights of about 10^2 under such fields still would be virtually uniformly distributed throughout the cell. At the same time, the chemical equilibria must be maintained at each level in the ultracentrifuge cell and therefore the total concentration of A must vary throughout the cell because of the change in the concentration of P. The interrelationships describing the concentration distributions for the various molecular species are illustrated here for the single complex PA_i formed by the following reaction.

$$P + iA \rightleftharpoons PA_i$$

If μ is the chemical potential per gram of solute species the sedimentation equilibrium equations for P and A can be written as

$$d\mu_P - \omega^2 x dx = 0 \quad (8)$$

and

$$d\mu_A - \omega^2 x dx = 0 \quad (9)$$

where ω is the angular velocity in radians per second and x is the distance in centimeters from the axis of rotation. The condition for chemical equilibrium between P and A is represented by

$$M_P\mu_P + iM_A\mu_A = M_{PA_i}\mu_{PA_i} \quad (10)$$

where M_P, M_A, and M_{PA_i} are the molecular weights of the protein, component A, and the complex, respectively. Differentiating eq 10 and combining the result with eq 8 and 9 give the sedimentation equilibrium equation for the complex

$$d\mu_{PA_i} - \omega^2 x dx = 0 \quad (11)$$

This shows that the familiar sedimentation equilibrium equation describing the concentration distribution of the complex results directly from imposing the requirements for chemical equilibrium onto those for the sedimentation of the individual components. In other words, although the distributions of the various macromolecular complexes vary slightly because of the small differences in their molecular weights (M_{PA_i} as compared to $M_{PA_{i-1}}$, for example) the chemical equilibria are still maintained at every level in the cell.[1] Therefore eq 4a can be applied for each position in the ultracentrifuge cell

[1] This problem can be treated in an alternative fashion by writing the familiar sedimentation equilibrium equations in the exponential form for the three molecular species, P, A, and PA_i. Combining these three equations for the concentration of each species at any level, x, in the cell and introducing the equation, $M_{PA_i} = M_P + iM_A$, yield directly the statement describing the chemical equilibrium, *i.e.*, $K = [PA_i]/[P][A]^i$. This approach was employed by Nichol and Ogston (1965) to show that, as long as chemical equilibrium was attained at one level in the cell, it was attained as well at all others.

$$[A_0]_x = [A] + r[P_0]_x \quad (4b)$$

and a plot of $[A_0]_x$ *vs.* $[P_0]_x$ should give a straight line with an intercept on the abscissa equal to [A], the concentration of unbound A, and a slope equal to r, the average number of small molecules bound per protein molecule.

The value of r may itself vary with protein concentration if the solutions are not ideal and it may change throughout the ultracentrifuge cell because of changes in the various equilibrium constants due to the pressure gradient. In sedimentation equilibrium experiments the centrifugal field is usually so low (for studies with macromolecules) that the pressure change across the liquid column is only a few atmospheres. Since the volume change for interactions between proteins and small molecules is only of the order of 10 ml/mole (Rosenberg and Klotz, 1955) the change in the equilibrium constant across the cell is substantially less than 1%.[2] In the sedimentation equilibrium experiments described here the liquid columns were only 2–3 mm in length. It is conceivable that the volume change due to the interaction can be measured from the effect of pressure on the equilibrium constant throughout the cell. The pressure can be altered either through the use of longer liquid columns or by the superposition of a less dense inert, immiscible fluid above the aqueous solution.

The treatment given above is limited to those systems in which the molecular weight of one of the reacting components is so small compared to that of the macromolecules that the concentration of the unbound small molecules is not affected by the centrifugal field. For some systems, however, the ratio of the molecular weight of P relative to that of A may not be sufficiently large for there to be a gradient of protein concentration without an accompanying variation in free A throughout the cell. If there is redistribution of unbound A because of the centrifugal field, the plot of $[A_0]_x$ *vs.* $[P_0]_x$ will exhibit upward curvature and the method described above for handling the data is inadequate. The following modification of the method may then be used provided that the molecular weight of A, though not negligibly small, is still substantially less than that of P.

The variation in the concentration of unbound A with respect to distance can be accounted for by

$$[A]_x = [A]_{x_m} e^{M_A(1-\bar{V}_A\rho)\omega^2(x^2-x_m^2)/2RT} = [A]_{x_m} e^{B(x^2-x_m^2)} \quad (12)$$

where M_A and $\bar{V}_A$ are the molecular weight and partial specific volume of A, ρ is the density of the solution, R is the gas constant, and T is the absolute temperature. The molar concentration of unbound A at the level, x, is designated by $[A]_x$, and $[A]_{x_m}$ represents the concentration of unbound A at the meniscus, x_m.[3] In order to simplify the writing of the equations, $M_A(1 - \bar{V}_A\rho)\omega^2/2RT$ is designated as B. Substituting eq 12 into eq 4b followed by rearrangement gives

$$\frac{[A_0]_x}{e^{B(x^2-x_m^2)}} = [A]_{x_m} + r\frac{[P_0]_x}{e^{B(x^2-x_m^2)}} \quad (13)$$

Plotting $[A_0]_x/e^{B(x^2-x_m^2)}$ *vs.* $[P_0]_x/e^{B(x^2-x_m^2)}$ will still not yield a straight line since r itself is not constant, being dependent on $[A]_x$ as shown by eq 5 and 7. It is pertinent to note that because of the correction for the dependence of $[A]_x$ on position in the cell the curvature in the plot of eq 13 would be much less than that which results if $[A_0]_x$ were plotted against $[P_0]_x$. Furthermore, by proper selection of the experimental conditions the concentration of the macromolecular constituent at the meniscus, $[P_0]_{x_m}$, can be made very small, thereby permitting an accurate extrapolation of the data to $[P_0] = 0$. This then gives $[A]_{x_m}$, and from eq 12 $[A]_x$ can be calculated for all levels in the ultracentrifuge cell. The corresponding values of r follow directly from eq 4b. In order to utilize this method of evaluating the chemical equilibria the effective molecular weight of A, *i.e.*, the quantity $M_A(1 - \bar{V}_A\rho)$ in eq 12, must be determined independently whenever the redistribution of A in the centrifugal field is not negligible. This quantity can be evaluated, of course, from the data obtained in a separate sedimentation equilibrium experiment with a solution of A devoid of P.

In theoretical considerations of the continuity equation for the transient states during the approach to sedimentation equilibrium Archibald (1947) showed that, after the plateau region was lost, the curves of concentration *vs.* distance rotate about a single point in the cell having the initial concentration. Moreover, the position of this "hinge point" (level in the cell at which the concentration is equal to the initial value) varies somewhat with molecular weight of the redistributing solute molecules. Since the A and P components considered here are so different in molecular weight it is of interest to examine the relative positions of the "hinge points" of these components.

For sector-shaped cells of thickness, a, and sector angle, Φ, the total mass of each component can be described by

$$[A_0]_{t=0}\Phi a\left(\frac{x_b^2 - x_m^2}{2}\right) = \int_{x_m}^{x_b}[A_0]_x \Phi a x dx \quad (14)$$

[2] The change in equilibrium constant with pressure is described by $d \ln K/dP = -\Delta V/RT$, where P is the pressure, ΔV is the volume change in the chemical reaction, R is the gas constant, and T is the absolute temperature. For centrifugal fields corresponding to a rotor speed of 12,000 rpm the change in pressure across a 3-mm liquid column of unit density is about 3×10^6 dynes/cm^2. If the volume change, ΔV, is 10 cc/mole the equilibrium constant change across the liquid column is about 0.1%.

[3] The sedimentation equilibrium equation is generally written with $[A]_x$ and $[A]_{x_m}$ expressed as weight concentrations rather than as molar concentrations as in eq 12. For the purposes of the present treatment it is preferable to formulate the concentration distribution in terms of molar quantities.

and

$$[P_0]_{t=0}\,\Phi a\left(\frac{x_b^2 - x_m^2}{2}\right) = \int_{x_m}^{x_b} [P_0]_x \Phi a x dx \quad (15)$$

In eq 14 and 15, x_m and x_b refer to the distance from the axis of rotation to the meniscus and cell bottom, respectively, and $[A_0]_{t=0}$ and $[P_0]_{t=0}$ are the initial total concentrations of A and P before centrifugation was started. When A is much smaller than P and the centrifugal field is selected so that A and hence r can be considered constant throughout the cell, eq 4b can be written for the contents of the entire cell as

$$\int_{x_m}^{x_b} [A_0]_x \Phi a x dx = [A] \int_{x_m}^{x_b} \Phi a x dx + r \int_{x_m}^{x_b} [P_0]_x \Phi a x dx \quad (16)$$

Combining eq 14 and 15 with eq 16 gives

$$[A_0]_{t=0} = [A] + r[P_0]_{t=0} \quad (17)$$

In the original solution before centrifugation is initiated the various molecular species are in equilibria with one another, and the concentrations of the various forms are interrelated according to

$$[A_0]_{t=0} = [A]_{t=0} + r_{t=0}[P_0]_{t=0} \quad (4c)$$

where $[A]_{t=0}$ and $r_{t=0}$ represent the concentration of unbound A and the average number of bound A molecules per macromolecule in the original homogeneous solution. Since r is unequivocally a function of [A], as shown in eq 5 and 7, it follows from eq 17 and 4c that $[A] = [A]_{t=0}$ and $r = r_{t=0}$. Thus, even though the various molecular species are distributed throughout the cell in different ways depending on their molecular weights, at the level in the cell at which $[P_0]_x$ is equal to $[P_0]_{t=0}$ the concentrations of both free A and bound A are equal, respectively, to the corresponding concentrations of these forms in the original solution, *i.e.*, the "hinge point" for the A constituent occurs at the same location as that for the P component.[4]

In the experiments described below two different optical systems are used for the measurement of the concentration distributions of the two components, P and A. An interferometer is employed for the former and an absorption optical system is used for the measurement of the concentration of the latter. Although absolute concentrations of light-absorbing species are determined readily from the absorbance of the solution at different positions in the cell the corresponding measurement of the concentration of the protein from the interference pattern is neither as direct nor as simple. But the evaluation of these latter patterns in terms of absolute concentrations is facilitated greatly through location of the "hinge point" of the A constituent, and hence P, from the absorption patterns. It should be noted that the position of the "hinge point" is found unambiguously even if as a result of the interaction there is a change in the extinction coefficient of A at the wavelength used.

Under certain circumstances, the interaction between P and A can also be evaluated from measurements of the average molecular weights of the two constituents. For ideal solutions the molecular weight of the complex PA_i can be written (Svedberg and Pedersen, 1940)

$$M_{PA_i} = \frac{1}{[PA_i]} \frac{2RT}{(1 - \bar{V}_{PA_i}\rho)\omega^2} \frac{d[PA_i]}{d(x)^2} \quad (18)^5$$

To simplify the treatment the term $M(1 - \bar{V}\rho)$ is replaced by M_e, the effective molecular weight, and eq 18 takes the form

$$M_{e,PA_i} = \frac{1}{[PA_i]} \frac{2RT}{\omega^2} \frac{d[PA_i]}{dx^2} \quad (18a)$$

An average effective molecular weight of the protein species can be defined for all the various complexes as

$$M_{e,P} = \frac{1}{[P_0]} \frac{2RT}{\omega^2} \frac{d[P_0]}{d(x)^2} = \frac{1}{[P_0]} \frac{2RT}{\omega^2} \frac{d \sum_{i=0}^{n} [PA_i]}{d(x^2)} = \frac{1}{[P_0]} \frac{2RT}{\omega_2} \sum_{i=0}^{n} \frac{d[PA_i]}{d(x^2)} \quad (19)$$

Inserting the value of $d[PA_i]/d(x^2)$ from eq 18a into eq 19 gives an alternative definition of the constituent effective molecular weight, $\bar{M}_{e,P}$

$$\bar{M}_{e,P} = \frac{\sum_{i=0}^{n} [PA_i] M_{e,PA}}{[P_0]} \quad (20)^6$$

This formulation of the constituent molecular weight is directly analogous to that used earlier for the constituent sedimentation coefficients of the different species in interacting systems (Williams *et al.*, 1958; Schachman, 1959; Fujita, 1962).

[4] In the above treatment Φ was not assumed to be constant throughout the cell. Hence the conclusion is not restricted to sector-shaped cells but is independent of the shape of the cell.

[5] As in eq 12 the concentration of PA_i is expressed as the moles per unit volume instead of the more familiar weight-volume units.

[6] Equation 20 shows that the constituent effective molecular weight, $M_{e,P}$, corresponds to a number average value rather than the weight average value which customarily results from a ploe of the logarithm of the concentration *vs.* the square of distance from the axis of rotation.

Since M_{e,PA_i} is the net force acting on PA_i in a field of unit strength, it can be expressed explicitly as

$$M_{e,PA_i} = M_P(1 - \bar{V}_P\rho) + iM_A(1 - \bar{V}_A\rho) - i\Delta v\rho \quad (21)$$

where Δv is the additional volume increase upon the binding of one A molecule to a P molecule. Substitution of this expression into eq 20 gives

$$\bar{M}_{e,P} = \frac{\sum_{i=0}^{n} [PA_i]M_P(1 - \bar{V}_P\rho) + \sum_{i=0}^{n} i[PA_i] \times \{M_A(1 - \bar{V}_A\rho) - \Delta v\rho\}}{[P_0]} \quad (22)$$

Equation 22 can be rewritten as

$$\bar{M}_{e,P} = M_{e,P} + r(M_{e,A} - \Delta v\rho) \quad (23)$$

In principle, $r\Delta v$ can be obtained directly from dilatometric measurements since it expresses the change in volume upon mixing P and A after equilibrium is attained. Equation 23 is especially useful for analyzing chemical equilibria when A is not negligibly small in molecular weight compared to P. For such cases $\Delta v\rho$ is small compared to $M_{e,A}$ and eq 23 reduces to

$$\bar{M}_{e,P} = M_{e,P} + rM_{e,A} \quad (23a)$$

Thus r can be evaluated directly from $\bar{M}_{e,P}$, $M_{e,P}$, and $M_{e,A}$. If optical techniques, such as absorption optics with light of the appropriate wavelength, permit the direct observation of the P constituent independent of contributions from the other, A, the required effective molecular weight, $\bar{M}_{e,P}$, can be measured. The additional required effective molecular weights of the pure components, P and A, can be determined in separate experiments.

In a similar fashion the constituent effective molecular weight of A can be written as

$$\bar{M}_{e,A} = \frac{1}{[A_0]}\left\{[A]M_{e,A} + \sum_{i=0}^{n} i[PA_i]M_{e,PA_i}\right\} \quad (24)$$

Combination of eq 21 and 24 leads to

$$\bar{M}_{e,A} = \frac{1}{[A_0]}\left\{[A]M_{e,A} + M_{e,P}\sum_{i=0}^{n} i[PA_i] + (M_{e,A} - \Delta v\rho)\sum_{i=0}^{n} i^2[PA_i]\right\} \quad (25)$$

Equation 25 shows that $\bar{M}_{e,A}$ is not a simple average of the effective molecular weights of the various species present in the solution. For those interactions between proteins and *small* molecules, $M_{e,A}$ is negligibly small (compared to $M_{e,P}$) and eq 25 reduces to

$$\bar{M}_{e,A} = M_{e,P}\frac{r[P_0]}{[A_0]} \quad (26)$$

Thus measurements of the effective molecular weight of the protein and the constituent effective molecular weight of A coupled with the knowledge of the total concentrations of A and P lead directly to r, the average number of small molecules bound per protein molecule.[7] This method of evaluating the chemical equilibria is equivalent to, though more laborious than, that indicated by eq 4b for which only a plot of $[A_0]_x$ *vs.* $[P_0]_x$ is required for the determination of r.

Sedimentation Velocity. For chemically interacting systems of the type described by eq 1, constituent sedimentation coefficients, $\bar{s}_P$ and $\bar{s}_A$, can be written for both the P and A components (Richards and Schachman, 1957; Williams *et al.*, 1958; Schachman, 1959; Fujita, 1962). These take the form

$$\bar{s}_P = \frac{1}{[P_0]}\sum_{i=0}^{n} [PA_i]s_{PA_i} \quad (27)[8]$$

and

$$\bar{s}_A = \frac{1}{[A_0]}\left\{[A]s_A + \sum_{i=1}^{n} i[PA_i]s_{PA_i}\right\} \quad (28)$$

If it is assumed that the interaction between P and each A molecule causes a constant increment, m, in the sedimentation coefficient of the macromolecules the value for the complex, PA_i can be written as

$$s_{PA_i} = s_P + im \quad (29)$$

and the constituent sedimentation coefficient of P becomes

$$\bar{s}_P = s_P + rm \quad (30)$$

It should be noted that the change in the sedimentation coefficient resulting from the interaction is due not only to the increase in the molecular weight and the change in the partial specific volume but also to the

[7] The above equation (26) for r is the equivalent of eq 4b as can be seen readily by substituting the appropriate expression for the molecular weights in terms of the concentration changes across the cell and recognizing that $M_{e,P}$, can be replaced by $\bar{M}_{e,P}$. For this particular situation a plot of $[A_0]$ *vs.* $[P_0]$ would give a straight line with slope r.

[8] Equations 27 and 28 show that the constituent sedimentation coefficients, $\bar{s}_P$ and $\bar{s}_A$, are number average quantities just as in the case of the constituent effective molecular weights, $\bar{M}_{e,P}$ and $\bar{M}_{e,A}$ (eq 20 and 24). In the measurement of these quantities the optical system must be capable of discriminating between the constituents and record the contributions of only a particular constituent without registering the contribution of the other. If the optical system cannot perform in this way constituent quantities (s and M) cannot be measured and the quantities measured from the photographic patterns are weight average values as in polydisperse mixtures of noninteracting components.

alteration in the frictional coefficient stemming from possible changes in the conformation of the macromolecules (Richards and Schachman, 1957).

Combining eq 28 and 29 (with the assumption that eq 6 is applicable) and evaluating the various summations (Smith and Briggs, 1950; Schachman, 1959) followed by rearrangement of the resulting equation and substitution of eq 30 give

$$[A] = \frac{[A_0]\left\{\bar{s}_P - \bar{s}_A - \frac{1}{n}(\bar{s}_P - s_P)\right\} + [P_0](\bar{s}_P - s_P)}{\bar{s}_P - \bar{s}_A - \frac{1}{n}(\bar{s}_P - s_P)} \quad (31)$$

If the interaction involves many binding sites and A is small compared to P the terms containing $(1/n)\cdot(\bar{s}_P - s_P)$ may be negligible, especially since $(\bar{s}_P - s_P) < (\bar{s}_P - \bar{s}_A) < (\bar{s}_P - s_A)$. For this situation eq 31 reduces to

$$[A] = [A_0]\frac{(\bar{s}_P - \bar{s}_A)}{(\bar{s}_P - s_A)} + [P_0]\frac{(\bar{s}_P - s_P)}{(\bar{s}_P - s_A)} \quad (32)$$

Equation 32 can be simplified still further for those systems for which $\bar{s}_P = s_P$. In that case eq 33 is obtained.

$$[A] = [A_0]\frac{(\bar{s}_P - \bar{s}_A)}{(\bar{s}_P - s_A)} \quad (33)$$

This, of course, follows directly from eq 28 if it is assumed that s_{PA_i} is equal to s_P. Equation 31 can also be simplified for those interactions in which $n = 1$. In this case eq 31 takes the form

$$[A] = [A_0]\frac{(s_P - \bar{s}_A)}{(s_P - s_A)} + P_0\frac{(\bar{s}_P - s_P)}{(s_P - s_A)} \quad (34)$$

Equation 34 can be derived directly for this special case by writing the appropriate equations for $\bar{s}_A$ and $\bar{s}_P$ in terms of the free components and the complex, PA, followed by combination of the two independent equations and suitable rearrangements.

It should be noted that eq 33 is analogous to eq 26. The former is based on interactions between macromolecules and small molecules for which the sedimentation coefficient of the macromolecule is not altered upon complex formation, *i.e.*, $s_P = s_{PA_i} = \bar{s}_P$. In the analogous treatment employing molecular weights, $M_{e,A}$ is considered negligibly small and $M_{e,P} = M_{e,PA_i} = \bar{M}_{e,P}$. For that case eq 26 can be rearranged to yield

$$[A] = [A_0]\frac{M_{e,P} - \bar{M}_{e,A}}{M_{e,P}} \quad (26a)$$

Experimental Section

Materials. Crystallized bovine plasma albumin from Armour Pharmaceutical Co. (lot. no. U68712) or chromatographically purified, dimer-free, albumin kindly provided by Drs. R. W. Hartley and H. A. Sober were used. Corrections for water content were made by heating a small sample in an oven at 110° until constant weight was attained. The methyl orange was Baker Analyzed reagent grade (lot no. 28080). A correction for moisture content was obtained by drying a sample at room temperature over P_2O_5 in a vacuum desiccator until constant weight was obtained. As in the earlier equilibrium dialysis experiments (Klotz *et al.*, 1946) the solvent for all binding studies was 0.1 M phosphate at pH 5.66.

Methods. Sedimentation experiments were performed with a Spinco Model E ultracentrifuge equipped with both the combined schlieren interference optical system and the independent absorption system. For most of the experiments the absorption patterns were produced by a split-beam automatic photoelectric-scanning optical system (Schachman, 1963a,b; Lamers *et al.*, 1963) equipped with a monochromator (Schachman *et al.*, 1962). In some of the sedimentation velocity experiments the conventional absorption optical system (Svedberg and Pedersen, 1940) was employed with an interference filter having a maximum transmittance at 444 mμ in place of the bromine–chlorine gas filter customarily used with this optical system (Schumaker and Schachman, 1957). In these experiments photographic records of the sedimentation patterns were obtained on Commercial film and enlarged plots of optical density *vs.* distance were obtained from the films by scanning the patterns in a photodensitometer (Analytrol) equipped with a microanalyzer attachment.

When the monochromator was employed the wavelength was set usually at 440 mμ and the slit width at 0.2 mm. This wavelength corresponded to the isosbestic point for the methyl orange–bovine plasma albumin mixtures. For binding experiments at high concentrations of methyl orange the total light absorption at 440 mμ was so great that experiments could not be performed satisfactorily with light of that wavelength; consequently the wavelength for these experiments was adjusted to 334 mμ which corresponded closely to a minimum in the absorption spectrum of the dye. The minimum in the absorption spectrum was at 340 mμ; however, since the intensity of the light source was noticeably higher at 334 mμ, it proved more convenient to use light of this wavelength. The slit width on the monochromator was 0.5 mm for this wavelength.

Single-sector cells having a 12-mm optical path were employed for experiments with the photographic system and double-sector cells were used for the photoelectric scanning system. With the latter, 12-mm centerpieces were used for experiments at low protein concentrations (1 mg/ml) and cells with 3-mm optical paths were used for sedimentation equilibrium experiments at higher protein concentrations (4 mg/ml). Higher dye concentrations could be used with the thinner centerpieces. In this way the interference fringes at the cell bottom were clearly resolved in all

the sedimentation equilibrium experiments.

Several changes in the Rayleigh optical system were made in order to permit simultaneous use of this system and the split-beam scanning optical system. The conventional Rayleigh mask usually incorporated into the cell assembly as part of the upper window holder was either omitted completely or replaced by the double-slit window holders used when the schlieren optical system is employed with double-sector cells. In this way the light striking the photomultiplier during the rotation of the two halves of the ultracentrifuge cell past the optical system was sufficient for satisfactory use of the split-beam scanning system (Lamers *et al.*, 1963). In order to obtain interference patterns the Rayleigh double-slit assembly was incorporated directly onto the lens holder at the top of the vacuum chamber (Richards and Schachman, 1959). The slits in this mask were 0.36 mm wide with a separation of 3.6 mm.

Sedimentation velocity experiments were performed at a rotor speed of 59,780 rpm and the cells were almost completely filled with solution to produce liquid columns slightly greater than 10 mm. The rotor speed for the sedimentation equilibrium experiments was 12,590 rpm and only 0.1 ml of solution was placed in the cell to give a liquid column of 2.5–3.0 mm. When the cells with the shorter optical path were used, only 0.025 ml of solution was placed in one of the two compartments of the double-sector cell and a slightly greater amount of solvent was placed in the other compartment. The sedimentation equilibrium experiments were continued for about 15 hr in order to ensure that equilibrium conditions were obtained.

The analytrol traces and the scanner diagrams were used directly for the determination of the concentrations of the various light-absorbing molecular species when light at 440 mμ was used. For light of wavelength 334 mμ, however, an indirect procedure must be employed since the absorbances of the bound and free forms of the dye differ appreciably. This method of computing the concentration of the free and bound methyl orange was based on the relationship $OD_t = OD_f + OD_b$, where OD represents the optical density of the chromophore and the subscripts t, f, and b represent the total, free, and bound forms, respectively. Since the optical density of any molecular species can be expressed in terms of the molar extinction coefficient for that species and the molar concentration, the total optical density can be written as

$$OD_t = \epsilon_f l[A] + \epsilon_b l r[P_0] \qquad (35)$$

where ϵ_f and ϵ_b represent the molar extinction coefficients of the free dye and bound dye, respectively, and l is the optical path length through the solution. For the sedimentation equilibrium experiments this expression can be used directly to evaluate the concentration of unbound dye, [A]. Since the total optical density varies linearly with protein concentration (as long as [A] and, hence, r are constant through the cell) an extrapolation can be made to zero protein concentration. This gives directly $\epsilon_f l$[A]. With this value of $\epsilon_f l$[A] along with an independent measure of ϵ_f (in terms of the recorder deflection on the scanner traces for a solution of methyl orange devoid of protein) the value of [A] is obtained directly. The value of r is then evaluated using eq 17, *i.e.*

$$r = \frac{[A_0]_{t=0} - [A]}{[P_0]_{t=0}} \qquad (17a)$$

Rayleigh interference patterns were photographed on Kodak Spectroscopic II G plates both with heterochromatic ("white") light and with monochromatic light. An interference filter having a maximum transmittance at 5460 A was employed for the monochromatic patterns. For the "white light" patterns this filter was replaced by an equivalent slab of plate glass. Initial protein concentrations were determined from layering experiments with a double-sector, capillary-type, synthetic boundary cell (Richards and Schachman, 1959). At the conclusion of each sedimentation equilibrium experiment the protein–methyl orange mixture was removed from the solution compartment of the double-sector cell and, after several rinsings of this compartment with the solvent, the base-line "white light" and monochromatic light patterns were photographed (at the equilibrium speed) with solvent in both compartments of the cell. The positions of the "white light" fringes relative to the reference fringes corresponding to the openings in the counterbalance cell were then employed for the evaluation of the protein concentration at the meniscus in the sedimentation equilibrium experiments. After the concentration at the meniscus was determined from the white light equilibrium patterns the concentration throughout the cell was evaluated from the monochromatic light patterns. In some experiments the position of the hinge point for the methyl orange (determined from the scanner traces at zero time and at equilibrium) was used to locate the hinge point of the protein. This in turn (in conjunction with the patterns from the synthetic boundary cell experiments) was used in the determination of the absolute protein concentrations, in fringes, in the equilibrium patterns. When studies were conducted at high methyl orange concentrations, corrections were made for the contribution of the dye to the index of refraction of the solution. In order to make the appropriate corrections the dye concentration was measured with the absorption system and the number of fringes per unit optical density was determined in an independent layering experiment with the dye in a synthetic boundary cell.

In order to accommodate both the Rayleigh optical system and the photoelectric scanner the counterbalance normally used with the former was modified slightly. This alteration involved the removal of the metal separation between each of the two pairs of elongated slits which give rise to the interference fringes. In effect the modified counterbalance resembled those customarily used with schlieren optics except

that each opening used to provide a radius reference was sufficiently large to permit the formation of interference fringes. With these enlarged openings the light level striking the photomultiplier was more than adequate for satisfactory registration by the scanning system.

The constituent sedimentation coefficient for the methyl orange, $\bar{s}_A$, was calculated from the transport equation

$$s = -\frac{1}{2\omega^2 t} \ln\left[\frac{2\int_{x_m}^{x_p} cx\,dx}{x_p^2 c_0} + \frac{x_m^2}{x_p^2}\right] \qquad (36)$$

where x_m and x_p are distances from the axis of rotation to the meniscus, x_m, and to a level, x_p, in the plateau containing all components, t is the time of centrifugation, c_0 is the methyl orange concentration of the original solution, and c is the concentration of the methyl orange constituent which varies both with position in the cell and the time of centrifugation. For the calculation of sedimentation coefficients from eq 36 the level, x_p, was selected for each pattern in such a way as to correspond to the plateau region in which all of the various molecular complexes, PA_t, were present. Both c_0 and c as a function of x were measured from the densitometer traces obtained from the absorption patterns. The concentrations were measured as centimeters of pen displacements on the analytrol traces with the air space above the liquid column taken as zero optical density. The integral in eq 36 was evaluated as a summation with the region between x_m and x_p in each trace being divided into 10–15 equally spaced intervals in terms of x^2. In this summation process it was necessary to select different values of x_p for each trace. Only traces corresponding to sedimentation times of less than about 56 min were employed. After longer periods of sedimentation the protein (along with bound methyl orange) had migrated so far through the cell and back-diffusion from the cell bottom was so extensive that the plateau region was virtually eliminated. In order to correct for nonuniform illumination and spurious reflections in the optical system, photographs were taken during the acceleration of the rotor. The analytrol traces of these patterns were used in conjunction with those after fixed periods of sedimentation as well as those for cells filled with buffer only (no light-absorbing material) to provide a base line corresponding to zero optical density and to locate appropriate positions for x_p. In the calculation of sedimentation coefficients about six different analytrol traces were employed, corresponding generally to intervals of 8 min, and the data from them were plotted as a function of time according to eq 36.

Results and Discussion

Sedimentation Equilibrium Studies. Figure 1 shows representative ultracentrifuge patterns obtained with the photoelectric scanner during a sedimentation equilibrium experiment on a mixture of bovine plasma albumin and methyl orange. At the high dye concentration used in this experiment the absorbance at 440 mμ was so great that the photoelectric scanner could not provide an accurate measure of the optical density; accordingly the monochromator was set at 334 mμ at which wavelength the dye absorbance was nearly a minimum. In order to illustrate different phases of the experiment and to demonstrate the utility of the photoelectric scanner in furnishing the required data, a variety of traces are presented. Shortly after the rotor attained the appropriate speed the first trace was obtained. During the scan the nulling switch was depressed briefly as the photomultiplier traversed that region of the image corresponding to the solution. This nulling procedure led to the vertical lines shown in the solution region and provided a simple means for determining accurately the optical density (in centimeters of recorder deflection) of the solution. After 1 hr the methyl orange bound to the protein had redistributed to a slight extent as seen (in the second pattern) by the decrease in the optical density at the meniscus and the increase at the bottom of the liquid column. In the last two traces are shown the equilibrium distribution of the dye. The first of these represents the pattern for the entire cell as well as the radius markers in the counterbalance cell, and the other is an expanded trace portraying only the region of the cell containing the protein–methyl orange solution. Measurements were made from the expanded traces after the positions of the menisci (and the magnification factor) had been determined first from the nonexpanded traces. From such traces, plots of the recorder deflection, optical density, or total concentration of the dye constituent as a function of distance were readily constructed. Analogous plots were produced for the protein constituent from the interference patterns.

When the data from the two different optical systems were combined, curves such as those shown in Figure 2 were obtained. In this diagram the relative concentrations, c/c_0, of both the protein and methyl orange constituents were plotted as a function of position in the cell (c_0 is the initial concentration). At the speed selected for this equilibrium experiment the protein concentration, evaluated from the interference patterns, decreased at the meniscus to slightly less than 10% of the initial value and increased 300% at the cell bottom. In contrast the methyl orange concentration in the mixture was about 40% of its initial value at the meniscus and had increased only 240% at the bottom of the liquid column. These results show directly that only part of the methyl orange in the solution was bound to protein and redistributed as complexes, PA_t, with molecular weights about 7×10^4. For comparison a curve is also presented from a control experiment in which methyl orange alone (at the same initial concentration) was examined after sedimentation equilibrium was attained at the same centrifugal field. The results from this experiment show clearly that the methyl orange concentration was virtually constant throughout

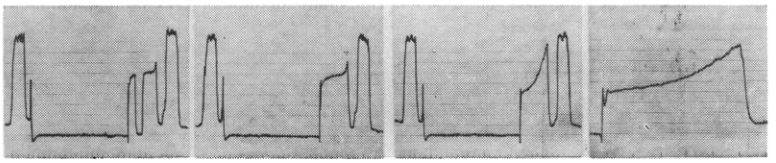

FIGURE 1: Sedimentation equilibrium patterns from an experiment on a mixture of bovine plasma albumin and methyl orange. These patterns were obtained with a split-beam photoelectric scanner coupled with a monochromator. The speed was 12,590 rpm and the wavelength of the incident light was 334 mμ. The initial concentration of protein was 0.2 g/100 ml and the methyl orange concentration initially was 4.9×10^{-4} M. The experiment was conducted at 4° in a 12-mm double-sector cell with 0.1 ml of solution placed in one compartment and solvent (0.1 M phosphate at pH 5.66) in the other. The first pattern was taken shortly after the rotor attained the selected speed and the nulling switch was depressed when the photomultiplier slit assembly was at a level in the image correspondng to the solution. In the second pattern recorded 1 hr after reaching the selected speed the slight redistribution of the chromophore is seen by the decrease in absorbance at the meniscus and the increase near the cell bottom. The last two patterns, obtained after 15 hr of sedimentation, represent the equilibrium distribution. In the first of these the scanning time, as in the first two patterns, was 30 sec and the chart drive for the recorder was set at 5 mm/sec. The trace corresponding to the solution itself was expanded in the last pattern by decreasing the scanning rate to correspond to a 6-min scan period for the entire cell, while the chart drive was maintained at the same rate.

the cell. Thus any change in the concentration of the dye in the protein mixtures is attributable directly to the interaction between the macromolecules and the small dye ions.

It is of interest to note that, in accordance with the theory presented above, the "hinge points" for the two different constituents coincide. This experimental test of the theory is supported further by the results from three additional experiments presented in Table I. As shown there, the "hinge points" for the protein constituent evaluated from the interference patterns were in good agreement with those determined for the dye constituent from the scanner traces.

TABLE I: Location of "Hinge Points" for Methyl Orange and Bovine Plasma Albumin.[a]

MO Concn (M)	BPA Concn (g/100 ml)	"Hinge Point" (cm) MO[b]	BPA[c]
3×10^{-5}	0.2	7.110	7.115
3×10^{-5}	0.1	7.050	7.032
3×10^{-5}[d]	0.1[d]	7.062	7.045

[a] All experiments were conducted at 12,590 rpm and the temperature was about 20°. [b] The "hinge point" was evaluated from the scanner traces by comparing the equilibrium pattern with that obtained initially. BPA, bovine plasma albumin; MO, methyl orange. [c] The "hinge point"was evaluated from the Rayleigh interference patterns. The patterns at equilibrium were photographed with both monochromatic and "white" light and compared with corresponding base-line patterns. [d] The solvent was 0.1 M phosphate buffer except for this experiment which was conducted without any supporting electrolyte.

As an additional test of the theory, data from a variety of experiments were plotted in terms of the concentration of the methyl orange constituent as a function of the protein concentration. Figure 3 represents such plots for two different experiments. Since the protein concentration in these experiments was relatively large initially (about 3.3 mg/ml) the studies were performed with double-sector cells having optical paths of only 3 mm. Consequently the number of fringes across the cell (at equilibrium) was sufficiently low as to permit good resolution. In addition the observed optical densities due to the dye were not beyond the range of linear response of the photoelectric scanning system. After the fringe positions were determined from the interference patterns, the recorder deflections were measured at corresponding levels in the cell and the data were cross-plotted as recorder deflection *vs.* fringe number. The ordinate and abscissa were then converted directly to optical density (corresponding to an optical path length of 1.2 cm) and protein concentration (micromoles per liter), respectively. For the experiments represented by Figure 3 the wavelength of the light employed for the scanner was 440 mμ. This corresponds to the isosbestic point and, as a consequence, the optical density provides a measure of the total concentration of the dye constituent regardless of the relative amounts in the bound and free forms. In accordance with eq 4b, the p'ots were linear. The intercepts, at zero protein concentration, gave the concentration of free methyl orange, 69.7 and 25.6 μmoles/l., respectively, for curves A and B. From the slopes of the two straight lines the corresponding values of r were found to be 2.56 and 1.07.

Analogous straight-line plots were obtained when the light used with the scanner had a wavelength of 334 mμ. Two such sets of data are shown in Figure 4. Here the intercepts, when coupled with the known extinction coefficient for pure methyl orange at that

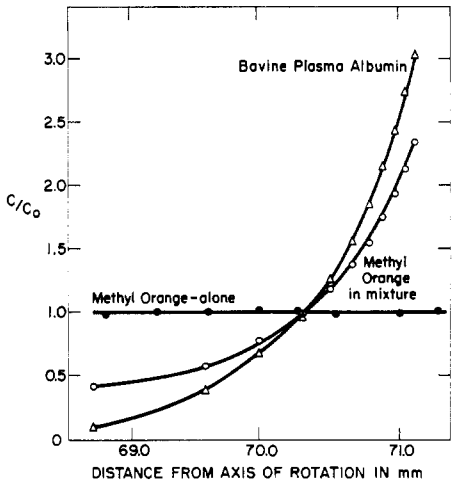

FIGURE 2: Equilibrium distribution of the different constituents in a sedimentation equilibrium experiment on a mixture of bovine plasma albumin and methyl orange. The solution contained initially bovine plasma albumin at 0.4 g/100 ml and methyl orange at 7.8×10^{-5} M in 0.1 M phosphate at pH 5.66. The rotor speed was 12,590 rpm and the distributions shown were obtained after 18 hr of sedimentation at 4°. The wavelength used with a monochromator and the photoelectric scanner was 440 mμ. A 3-mm double-sector cell was employed and 0.025 ml of solution was placed in one compartment with solvent in the other. The ordinate represents the concentration, c, relative to the initial concentration, c_0, plotted as a function of distance from the axis of rotation in millimeters. The redistribution of the protein as measured with the Rayleigh interferometer is represented by Δ and the redistribution of the methyl orange constituent as measured with the photoelectric scanner is signified by ○. Both of these were obtained in the same experiment. The control indicated by ● was obtained in an analogous experiment under identical conditions with a solution containing methyl orange devoid of protein.

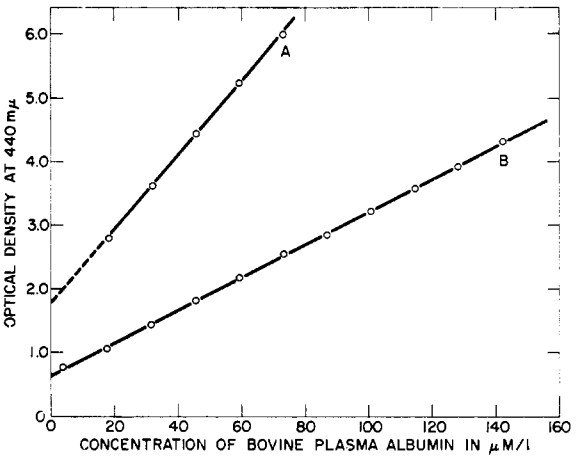

FIGURE 3: Relationship of methyl orange concentration to bovine plasma albumin concentration at sedimentation equilibrium. The ordinate gives the optical density at 440 mμ and the abscissa represents the concentration of protein in micromoles per liter. In the experiment represented by A the initial concentration of protein was 46.5 μmoles/l. and of methyl orange 188.8 μmoles/l. The analogous initial concentrations for the experiment represented by B were 46.5 and 75.3 μmoles/l., respectively. Both experiments were performed with 3-mm double-sector cells at a rotor speed of 12,590 rpm and a temperature of 4°.

wavelength, gave directly the concentration of the free methyl orange. However, the values for r had to be calculated indirectly because the absorbances of free and bound methyl orange differ slightly at that wave length. In calculating the values of r from these experiments the procedure involving eq 35 and 17a was employed.

The results from seven different experiments are presented in Figure 5 as a plot of r *vs.* the logarithm of the concentration of free methyl orange. In the same plot are shown the data of Klotz *et al.* (1946) obtained by equilibrium dialysis. As seen from a comparison of the two sets of data the agreement between the results is good. A detailed comparison and an assessment of the relative precisions of the two methods is not warranted because of the likely differences in the protein preparations. The results presented here serve principally to illustrate the potential of the sedimentation equilibrium method and to provide an experimental test of the theory described above.

Sedimentation Velocity Studies. Two different groups of sedimentation velocity experiments were performed on mixtures of bovine plasma albumin and methyl orange. One of these involved solutions identical with those used for the sedimentation equilibrium experiments and all of the patterns were obtained with the photoelectric scanner. In the other experiments a different protein sample was used and the absorption patterns were recorded photographically and then transposed into plots of optical density as a function of distance in the cell by means of a photodensitometer. The latter experiments were used for mass transport calculations of constituent sedimentation coefficients according to eq 36 and for a preliminary evaluation of the significance of some of the terms appearing in the theoretical treatment.

Figure 6 shows a series of sedimentation velocity patterns from an experiment on the same protein–methyl orange mixture as that used in the sedimentation equilibrium experiment illustrated in Figure 1. As seen in the first pattern obtained shortly after the rotor attained the operating speed of 59,780 rpm, the optical density was virtually constant throughout the cell. In all experiments of this type the electronic circuits were nulled when the photomultiplier slit assembly was positioned at a level in the image corresponding to the air space above the liquids in the

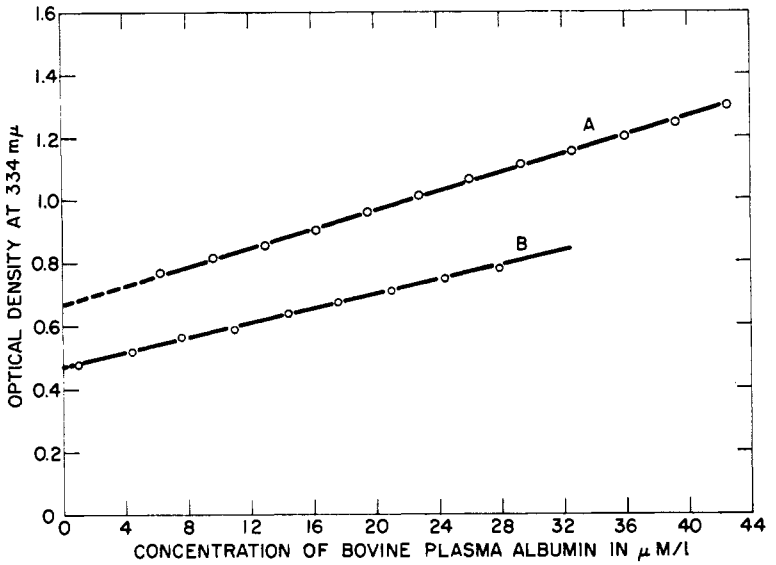

FIGURE 4: Relationship of methyl orange concentration to bovine plasma albumin concentration at sedimentation equilibrium. The ordinate represents the optical density of the solution at 334 mμ and the abscissa gives the concentration of bovine plasma albumin in μmoles/l. For the experiment represented by A the initial concentrations of bovine plasma albumin and methyl orange were 23.1 and 471.9 μmoles/l., respectively. In the second experiment represented by B the analogous concentrations were 11.6 and 235.9 μmoles/l., respectively. For both experiments a 12-mm double-sector cell was employed with a rotor speed at 12,590 rpm and a temperature of 4°.

double-sector cell since this region corresponded to zero optical density. Thus the recorder displacement relative to the trace position in the air space provided a measure of the total absorbance of the original mixture. After 32 min of centrifugation the boundary due to the various complexes of protein and methyl orange (PA_i) had migrated about one-third the distance through the cell. Left behind in the supernatant liquid was the methyl orange which was not bound to protein. The progressive movement of the boundary of the complexes is clearly evident in the subsequent patterns shown in Figure 6. In the third pattern corresponding to 48 min of sedimentation the nulling procedure for measuring the total absorbance is illustrated. The vertical displacement to the base line (zero optical density) was produced by momentarily depressing a null switch built into the electronic circuits. This was incorporated so as to facilitate the accurate measurement of the recorder deflection at any level in the cell.

As a first approximation the recorder displacement in the supernatant region can be taken as a measure of the concentration of unbound methyl orange in the equilibrium mixture. It should be noted, however, that mere measurement of the displacement in the supernatant as compared to the initial reading (representing the total absorbance of the equilibrium mixture before sedimentation) is not an appropriate index of the fractional amount of free methyl orange in the original solution. In the experiment illustrated by Figure 6 the wavelength of the incident light was 334 mμ; therefore a correction must be made so as to account for the variations in the absorbances of the free and bound methyl orange. This particular complication can be obviated, however, through the use of data from an additional experiment in which the total methyl orange concentration was maintained constant with the protein omitted. Alternatively this difficulty can be circumvented (if experimental conditions permit) by employing light of wavelength corresponding to the isobestic point. In such experiments the fractional amount of dye in the free form and, hence, the fraction bound can be calculated directly from patterns such as those in Figure 6. These procedures utilizing the absorption optical system in the ultracentrifuge are directly analogous to those employed by Chanutin *et al.* (1942), Velick *et al.* (1953), Hayes and Velick (1954), and Velick (1954) for the evaluation of binding data from samples removed from tubes in a preparative centrifuge.

It is important to note that these procedures do not suffice for the evaluation of the fraction of the methyl orange in the free and bound forms in the original equilibrium mixture unless it can be demonstrated independently that there is no disturbance of the chemical equilibria due to differential migration of the various protein complexes. Before considering this possibility and its potential consequences it seems profitable to examine the results of the sedimentation velocity studies based on the superficial analyses outlined above. Accordingly r and [A] were calculated from measurements of the recorder deflections in the supernatant regions in a series of protein–dye mixtures. These are plotted in Figure 5 along with the corre-

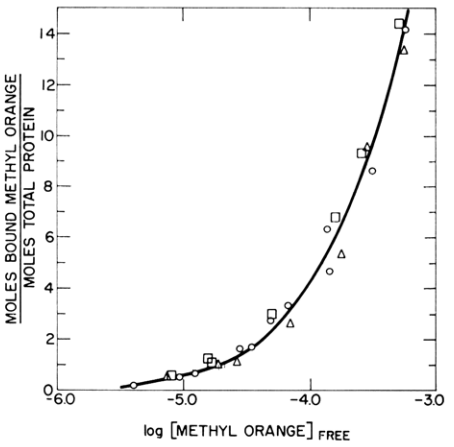

FIGURE 5: A summary of experimental results on the binding of methyl orange to bovine plasma albumin. On the ordinate is plotted r, the moles of bound methyl orange per mole of total protein, and on the abscissa is the logarithm of the concentration of free methyl orange in the equilibrium mixture. The data represented by ○ were obtained from equilibrium dialysis experiments by Klotz *et al.* (1946); the points represented by △ were from sedimentation equilibrium experiments; and the results indicated by □ were obtained from sedimentation velocity studies. In all of the experiments the buffer was 0.1 M phosphate at pH 5.66 and the temperature about 4°.

sponding results from the sedimentation equilibrium experiments and the data from equilibrium dialysis studies (Klotz *et al.*, 1946). As seen therein the values of r calculated from the sedimentation velocity experiments by this method are slightly and almost consistently higher than those obtained by the equilibrium methods. The discrepancies are attributable to a number of factors one of which merits discussion at this point.

Although the sedimentation coefficient of unbound methyl orange is very small (0.2 S) compared to that of the protein (4.4 S) there is a slight but significant depletion of the free methyl orange in the supernatant during the time required for a sedimentation velocity experiment. Unless corrections are made for this effect, the fractional amount of unbound dye estimated from the concentration in the supernatant would be too low; hence the values of r would be too high. A crude empirical correction can be applied by subjecting a control methyl orange solution, devoid of protein, to sedimentation at the same speed and for the same time as was used for the dye–protein mixture. In this way the decrease in concentration is measured at a level corresponding to that used for the actual binding experiments (about one-third the distance from the meniscus to the cell bottom). These control experiments showed that the concentration of unbound methyl orange at that level decreased to about 95% of its initial value. When this correction was applied to the sedimentation velocity data used in Figure 5 for the calculation of r and [A], the results deduced from the velocity method were in closer agreement with those evaluated from the sedimentation equilibrium experiments. However, this correction accounted for only about 50% of the discrepancy between the two sets of results.

In effect corrections for the sedimentation of the unbound dye can be made in a more elegant way through the use of constituent sedimentation coefficients and eq 31–33. This approach has the added virtue of illustrating the limitations of the sedimentation velocity method in general while, in particular, it highlights some of the hazardous assumptions implicit in treating the data only in terms of the concentration of the small molecules in the supernatant region.

As described in the experimental section, the constituent sedimentation coefficient for methyl orange is calculated from measurements of the transport of the methyl orange, both free and bound, across a surface in the region of the cell containing all molecular species. The use of eq 36 for this purpose is illustrated in the top half of Figure 7. All of the data for this plot were calculated from photodensitometer traces

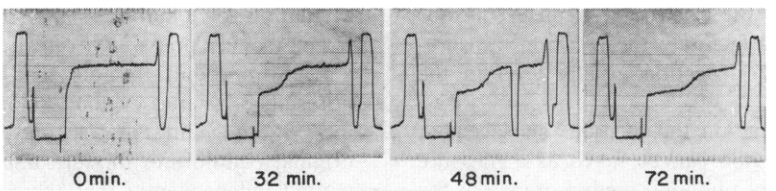

FIGURE 6: Sedimentation velocity patterns from a mixture of bovine plasma albumin and methyl orange. All patterns were recorded with a split-beam photoelectric scanner coupled with a monochromator. The wavelength of light was 334 mμ and the speed of the rotor was 59,780 rpm. The initial concentrations were 0.2 g/100 ml and 4.9×10^{-4} M for the protein and dye, respectively, in 0.1 M phosphate buffer, pH 5.66, at 4°. All traces were recorded in a scan period of 30 sec. The patterns reading from left to right correspond to 0, 32, 48, and 72 min after reaching speed. In the third pattern as the photomultiplier slit assembly traversed the plateau containing all components the nulling switch on the console was momentarily depressed so as to record the base line and permit accurate readings of the total absorbance in the plateau. The base line in that pattern coincides with that in the air space above the liquid columns showing zero optical density.

of the patterns produced photographically with the conventional absorption optical system. As seen in the graph the scatter of the experimental points about the line is appreciable. For comparison the boundary movement due to the various protein complexes, as viewed with the schlieren optical system in the same experiment, is shown in the lower half of Figure 7. The experimental precision for the latter measurements is seen to be substantially greater. From the upper graph the constituent sedimentation coefficient of methyl orange, $\bar{s}_A$, was found to be 2.4 S while the constituent sedimentation coefficient of the protein, $\bar{s}_P$, evaluated from the lower graph was 4.42 S. Analogous results from a series of protein–dye mixtures are summarized in Table II. As shown there, the constituent sedimentation coefficient of methyl orange increased markedly with protein concentration. When the latter was 0.01 g/100 ml, the value of $\bar{s}_A$ was 0.6 S, only slightly greater than the sedimentation coefficient of unbound methyl orange, s_A = 0.2 S. At higher protein concentration, 0.2 g/100 ml, the observed constituent sedimentation coefficient of the dye was comparable to that of the protein indicating that the bulk of the dye existed in the form of complexes with

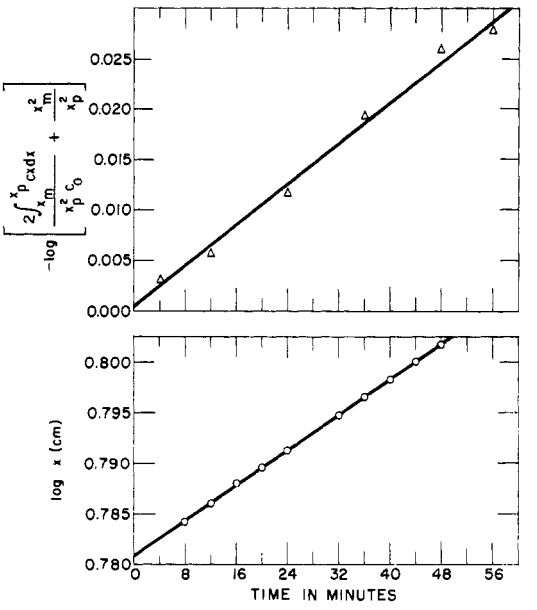

FIGURE 7: Determination of constituent sedimentation coefficients in interacting systems. The upper figure represents the determination of the constituent sedimentation coefficient of methyl orange, $\bar{s}_A$, in a mixture with bovine plasma albumin. The data are plotted in accordance with eq 36. For each pattern a level at x_p was selected to correspond to the plateau region containing all the various molecular species. Integration from the meniscus to that level was performed by a summation procedure using 10–15 intervals. Corrections for nonuniform illumination and spurious reflections were performed with the aid of control experiments with solutions of buffer and of methyl orange devoid of protein. All measurements were made from photodensitometer traces of the patterns recorded photographically with the conventional absorption optical system. The mixture contained initially methyl orange at a concentration of 3 × 10^{-5} M and bovine plasma albumin at a concentration of 0.3 g/100 ml. The solvent was 0.2 M NaCl. The speed of the rotor was 59,780 rpm and the temperature 20°. The lower diagram represents the determination of the constituent sedimentation coefficient of bovine plasma albumin, $\bar{s}_P$, in the same mixture. The data for this experiment were obtained with the schlieren optical system and many of the pictures were taken simultaneously with those described above. In order to make the exposure times for the two optical systems compatible a symmetrical double-slit aperture of the type normally used with the Rayleigh interference optical system was incorporated into the schlieren system so as to reduce the light available at the photographic plate. All patterns were recorded on Metallographic plates. Boundary positions were read with a microcomparator using the position of the maximum refractive index gradients for location of the boundaries in the individual patterns.

TABLE II: Constituent Sedimentation Coefficients of Methyl Orange and Bovine Plasma Albumin in Mixtures.

MO Concn (M)	BPA Concn (g/100 ml)	Solvent	$\bar{s}_A$[a] (S)	$\bar{s}_P$[b] (S)
3 × 10^{-5}	0.01	H_2O	0.6	—
3 × 10^{-5}	0.025	H_2O	1.4	—
3 × 10^{-5}	0.05	H_2O	1.9	4.33
3 × 10^{-5}	0.075	H_2O	3.2	—
3 × 10^{-5}	0.10	H_2O	3.4	—
0	0.10	H_2O	—	4.39
3 × 10^{-5}	0.15	H_2O	4.4	4.36
3 × 10^{-5}	0.20	H_2O	4.6	4.45
3 × 10^{-5}	0.20	0.2 M NaCl	2.0	4.40
3 × 10^{-5}	0.30	0.2 M NaCl	2.4	4.42
3 × 10^{-5}	0.40	0.2 M NaCl	3.1	4.41
3 × 10^{-5}	0.50	0.2 M NaCl	3.6	4.39

[a] Measurements were made from photodensitometer traces of patterns recorded on Commercial film with the use of the conventional absorption optical system except replacement of the Br_2–Cl_2 filter with an interference filter having a maximum transmittance at 444 mμ. Values of $\bar{s}_A$ were calculated from eq 36 and include the contribution of methyl orange bound to the aggregated (dimer) form of the protein as well as that bound to the monomer. [b] These values were calculated from the patterns obtained with the schlieren optical system. Measurements were made from the maximum ordinates of the gradient patterns. All experiments were done at about 22°.

protein. Table II also shows that the values of $\bar{s}_A$ are substantially smaller in solutions containing NaCl than in the analogous protein–dye mixtures not containing additional ions. This decrease in the affinity of the protein for the dye when neutral electrolyte is present can be attributed to partial screening of charged groups on the protein with a consequent decrease in the electrostatic interaction and to competition between the chloride ions and the dye for the protein-binding sites.

Since the protein preparation used for this study contained an appreciable amount of dimer, calculations of the fraction of bound and free dye in the various mixtures are fraught with difficulty. The determination of $\bar{s}_A$ through the use of eq 36 provides a measure of the transport of *all* dye molecules. This includes that dye bound to the dimeric form of protein as well as that bound to the monomer. In contrast, the values of $\bar{s}_P$ measured from the maximum refractive index gradients in the schlieren patterns correspond only to the monomeric species of the protein constituent. As a consequence a strict comparison between the values of $\bar{s}_A$ and those for $\bar{s}_P$ is not justified and their use directly in eq 31–34 is not warranted. This difficulty can be circumvented, however. In principle weight average sedimentation coefficients (including contributions from aggregates) can be calculated from schlieren patterns through the use of an alternative form of eq 36 especially suited for use with schlieren optics (Baldwin, 1953; Schachman, 1959). This method was not used, however, since the sedimentation velocity experiments, of necessity, were performed with single-sector cells and accurate base lines required for the implementation of the transport equation could not be obtained. A reasonable correction of the values of $\bar{s}_P$ so as to include the dimer species can be made from knowledge of the weight fraction and the sedimentation coefficient of the dimer. Accordingly the protein solution, devoid of dye, was examined in a double-sector cell and from the areas corresponding to the two boundaries the amount of dimer in the preparation was found to be 8–10%. Since measurements of boundary positions for the dimer were so uncertain its sedimentation coefficient was estimated indirectly from the relationship, $s = kM^{2/3}$. This yielded a value of 7.0 S for the dimer. From these values the weight average sedimentation coefficient of the protein is calculated to be 4.6–4.7 S. It is assumed that the methyl orange binds to the dimer as well as to the monomer and this correction for the contribution of the dimer applies to all of the values of $\bar{s}_P$ listed in Table II. Thus all of the values of $\bar{s}_P$ should be increased to about 4.7S, thereby eliminating the paradox in which $\bar{s}_A$ appeared in some experiments to be greater than $\bar{s}_P$.

Although the values of $\bar{s}_P$ for the various solutions appeared to be relatively independent of the amount of dye bound to the protein, these results are, in themselves, insufficient to warrant the conclusion that $\bar{s}_P = s_P$. To test this, a series of experiments were conducted in which $\bar{s}_P$ was compared directly with s_P. The results of these studies are presented in Table III. As seen there, the term, $\bar{s}_P - s_P$, is indeed very small. This observation is not unexpected since the increase in the molecular weight of the sedimenting species even when $r = 6$ is not very large and may be compensated for in part by a concomitant increase in the frictional coefficient of the complexes relative to the pure protein. The results in Table III demonstrate clearly that the term, $\bar{s}_P - s_p$, appearing in eq 31, 32, and 34 is negligibly small compared to the other terms.[9] This provides the experimental support required to justify the use of eq 33 for evaluating the association equilibria. Equation 33, in turn, furnishes the mathematical basis for the procedure described earlier and applied in Figure 5 where the relative amounts of dye in the free and bound forms were estimated directly from the dye concentration in the supernatant liquid.

Whereas the determination of $\bar{s}_A$ in the aforementioned experiments may be considered unambiguous both theoretically and experimentally, the analogous evaluation of $\bar{s}_P$ is replete with pitfalls and certain

TABLE III: Evaluation of $s_P - s_P$.

BPA Concn (g/100 ml)	MO Concn (M)	Solvent	$(\bar{s}_P - s_P)^a$ (S)
0.45	3.0×10^{-5}	0.2 M NaCl	−0.025
0.6	1.2×10^{-4}	0.2 M NaCl	−0.013
0.45	3.0×10^{-4}	0.2 M NaCl	+0.029
0.6	1.2×10^{-3}	0.2 M NaCl	+0.062

[a] Measurements were made from the maximum ordinates of the schlieren patterns. Both $\bar{s}_P$ and s_P were measured in the same experiment through the use of two single-sector cells, one of which contained a positive wedge window and the other a conventional quartz window. A mixture of bovine plasma albumin and methyl orange was placed in one cell for the determination of $\bar{s}_P$ and the other cell contained only the protein (devoid of methyl orange) for the evaluation of s_P. All experiments were done at about 22°.

[9] The results are not sufficiently precise, however, to constitute a test of the validity of eq 29. It is clear that m is a very small number but, from the trend in the data, it appears at first to be negative and then becomes positive at higher values of r. These preliminary findings would indicate that there is a swelling of the protein molecules upon binding the first few methyl orange molecules with a consequent decrease in the sedimentation coefficient of the protein complexes. Only after a substantial number of dye molecules are bound to each protein molecule is the weight contribution sufficient to cause the sedimentation coefficient of the complexes to increase. For a confirmation of these tentative conclusions and a critical test of eq 29 much more precise experimental data are required. Perhaps these could be obtained by the use of the differential sedimentation technique described by Richards and Schachman (1957, 1959).

minimal criteria must be satisfied. The moving boundary must represent the transition zone between a uniform solution in which there is no protein and a second phase in which all protein species are present. In addition the measurements of the boundary positions for the calculation of the sedimentation coefficient must correspond to the theoretically correct levels (such as the equivalent boundary or, in some cases, the second moment of the gradient curve). Finally, the optical system used for measuring boundary positions must register the optical contribution of only the protein constituent. These requirements are essentially fulfilled in the experiments described above. There is no protein behind the boundary due to the protein constituent and all of the various complexes are present in the plateau region ahead of it. With regard to the second restriction the maximum in the gradient curve is an adequate measure of the theoretically correct position since the boundary itself is so sharp. In work with the photoelectric scanner this requirement could be satisfied rigorously since the double-sector cells used with this optical system would permit simultaneously the accurate measurement of the second moments of the boundaries with the schlieren system. For nearly all of the dye–protein mixtures the concentration of methyl orange on a weight basis is so small (relative to that of the protein) that its contribution to the refractive index of the solution is virtually negligible. Thus the schlieren optical system records the protein complexes without giving undue weight to those complexes that have more bound dye molecules than others containing less. It should be noted that no attempt was made to calculate sedimentation coefficients of the protein complexes from the photoelectric scanner patterns shown in Figure 6. Measurements from these patterns would not necessarily yield $\bar{s}_P$ and may be misleading since the optical registration is a function of the amount of dye in the complex; *e.g.*, the contribution of the pure protein to the composite boundary would not be recorded since this species does not absorb light of the wavelength used in the experiment. If a spectral region could be found in which the protein constituent absorbed light independent of the composition of the complex and the dye did not absorb, then, of course, the absorption optical system could function effectively for measurements of $\bar{s}_P$ as it does for evaluating $\bar{s}_A$.

In view of the complications elaborated above and because of the differences in the protein preparations, temperature, and the solvent compositions used in the various experiments no attempt was made to convert the $\bar{s}_A$ and $\bar{s}_P$ values of Table II into their equivalent r and [A] values for comparison with the results presented in Figure 5. With regard to the latter some of the discrepancy between the results from the velocity and equilibrium methods may be attributable to pressure effects. For the velocity method the centrifugal fields were about 25-fold greater than those prevailing in the sedimentation equilibrium experiments (the average pressure differences were even greater because of the longer liquid columns in the velocity experiments). As a consequence, until the volume change resulting from the chemical interaction is evaluated a detailed comparison of the data from the two methods is unwarranted. If the change in volume due to binding is appreciable, the equilibrium constants of the association reactions (and hence $\bar{s}_A$) will vary significantly at different levels in the ultracentrifuge cell. An additional complication which may preclude an accurate evaluation of the chemical equilibria from the sedimentation velocity data stems from the possible existence of interacting flows (Gosting, 1956). The flow of the protein and its complexes during sedimentation may induce the *unbound* dye to flow in a direction and rate that must be determined empirically for each system. Although cross-term diffusion coefficients which provide a measure of such interacting flows have been found for other systems to be very small compared to the principal diffusion coefficients, their magnitude has yet to be determined for the type of system considered here. The velocity method is complicated further as a result of the dilution of the contents of the cell during an experiment. Since the radial dilution varies with the sedimentation coefficient, there will be a differential dilution of the individual species and some disturbance of the chemical equilibria. This should be reflected as a change in $\bar{s}_A$ during an experiment. At the level of precision represented by the data of Figure 7a no trend in $\bar{s}_A$ could be detected. With improvements in the accuracy of the measurements, however, a variation would be observed and the calculated, instantaneous values of $\bar{s}_A$ should be extrapolated to give the value at zero time corresponding to the original mixture.

Conclusions

From both theoretical and experimental points of view the ultracentrifugal methods presented here have much to commend them for the evaluation of interactions between large and small molecules. The theory for the sedimentation equilibrium technique is rigorous and its application straightforward. The assumptions and approximations are minimal and subject to experimental test. In contrast the sedimentation velocity method is not nearly as satisfactory from a theoretical point of view although experimentally it is more rapid and often simpler to apply. Taken together the two techniques constitute a powerful tool for the analysis of many interacting systems of biological interest. With the adaptation of the Rayleigh interferometer to the ultracentrifuge and the development of the photoelectric scanning absorption system the required data are readily obtained. Moreover, recent modifications in the latter optical system subsequent to the completion of the experimental work described above have led to greatly increased accuracy as well as sensitivity. As a result of these improvements the accuracy of both the sedimentation velocity and equilibrium measurements on interacting systems should be considerably enhanced.

Formally these sedimentation methods are analogous

to the equilibrium dialysis technique; yet no semipermeable membrane is required. As a consequence there are no complications of the type frequently encountered in dialysis experiments in which extraneous binding of the different species to the membrane often occurs. Only very small quantities of material are required and the concentration level need be no greater than that required for any spectrophotometric measurement. For the velocity method the duration of an experiment is only 1–2 hr; thus chemically unstable systems can be analyzed without the risk attending dialysis experiments whose duration is much longer. Even for the sedimentation equilibrium method the judicious selection of experimental conditions (length of the column of solution and rotor speed) can lead to times substantially shorter than for those experiments requiring prolonged dialysis.

A basic requisite of the method is the availability of optical systems capable of measuring independently the concentrations of each constituent even though both are present together. This is feasible at present for many biological systems; but there are mixtures, no doubt, for which the absorption spectra of the two constituents overlap to such an extent that there may be difficulty in unravelling the independent contributions of the two constituents. When the molecular weight of one of the constituents is much smaller ($^1/_{100}$) than that of the other even an overlap in their spectra does not constitute a serious handicap. For such systems at sedimentation equilibrium the interferometric (or refractometric) methods provide sufficient information about the distribution of the larger constituent to permit adequate corrections of the data produced by the photoelectric scanner for the concentration distribution of the smaller constituent. The evaluation of $[P]_r$ and $[A]_r$ in such cases is, of course, greatly facilitated through the use of high centrifugal fields so that $[P]_{r_m}$ becomes virtually zero and $[A]_{r_m}$ can be measured directly. In a sedimentation velocity experiment the overlap of the spectra does not introduce serious limitations since the concentration of A in the supernatant region coupled with the data from the interference optical system (and the scanner traces) permits the calculation of $\bar{s}_A$ through the use of the transport equation.

Although very little attention has been directed here to the experimental analysis of interactions between different types of macromolecules it seems clear that the sedimentation methods will prove of value in this area as well. Equation 23a provides one such approach. If P were a multivalent protein and A an interacting univalent polysaccharide or other polymer which did not absorb ultraviolet light $\bar{M}_{e,P}$ could be measured from data obtained with the photoelectric scanner. Hence, r could be measured from the value of $\bar{M}_{e,P}$ and the independently determined values of $M_{e,P}$ and $M_{e,A}$. This approach under certain circumstances may also function effectively for the analysis of the interactions between nucleic acids and enzymes such as transfer ribonucleic acid (t-RNA) and aminoacyl-RNA synthetases on the one hand or deoxyribonucleic acid (DNA) and RNA-polymerase, on the other. For these systems the nucleic acid could be considered the multivalent P constituent and the protein the univalent A constituent. Since the extinction coefficient of the former is so much greater than that of the latter at 260 mμ, the concentration distribution of the nucleic acid constituent could be measured with absorption optics without interference from the protein (even though on a weight basis the concentrations were approximately equal). In this way $\bar{M}_{e,P}$ could be measured for substitution into eq 23a. There can be complications of course because of the change in the partial specific volume upon complex formation, and this may have to be evaluated before r is calculated.

If the studies with these systems indicated that the protein was the multivalent constituent and the nucleic acid the univalent constituent the problem would have to be treated differently. In that case the concentration distribution of P in the cell would have to be computed as the difference between the total concentration (as measured by interference optics) and the concentration of the nucleic acid (measured by absorption optics); then $\bar{M}_{e,P}$ could be computed. Alternatively, eq 25 could be used in conjunction with measurements of the concentration distribution of the A constituent. The term

$$\sum_{i=0}^{n} i^2[PA_i]$$

has been shown (Smith and Briggs, 1950) to be equal to $([A_0] - [A])(1 + r(n - 1)/n)$ for systems which can be described by eq 5 and 6. Thus eq 25 can be rearranged to give

$$\bar{M}_{e,A} = M_{e,A}\frac{[A]}{[A_0]} + r\frac{[P_0]}{[A_0]}\left\{M_{e,P} + M_{e,A} \times \left(1 + \frac{n-1}{n}r\right)\right\} \quad (37)$$

where n represents the maximum number of binding sites on the protein. An iterative procedure would be required for the implementation of eq 37 since, for any equilibrium mixture, the quantities n and [A] (or r) are unknown.

In the theoretical treatment presented above no consideration was given explicitly to those interactions which lead to a single complex composed of different macromolecular constituents. Sedimentation equilibrium studies of such systems have been treated by Nichol and Ogston (1965) and an analogous theory for sedimentation velocity experiments has been given by Gilbert and Jenkins (1960) and by Nichol and Winzor (1964, 1965). Very little attention was devoted in these analyses to the use of two different optical systems for independent measurements of the behavior of the different constituents. A combination of their work with that presented here is likely to prove profitable

in unravelling the thermodynamic interactions involving diverse macromolecules.

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