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Differential Sedimentation Coefficients. II. Interpretation in Terms of Changes in Size and Shape*

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ABSTRACT: Simple equations are developed for the interpretation of changes in sedimentation coefficient in terms of changes in frictional ratio. These equations are particularly suitable for the study of macromolecules which change shape and volume when binding a low molecular weight ligand, but do not change their state of aggregation. The equations are applied to a theoretically constructed model protein system to see how sensitive the results become to variations in parameters other than shape and volume.

Conformational changes in protein structure may be accompanied by changes in molecular volume and shape which are detectable by sedimentation analysis. With the development of techniques for accurate measurement of small differences in sedimentation coefficient (Richards and Schachman, 1959; Lamers *et al.*, 1963; Gerhart and Schachman, 1968; Schumaker and Adams, 1968), the interpretation of these differences becomes important.

First, to gain an appreciation of the magnitude of expected changes, we may use the X-ray data of Perutz for hemoglobin. The unoxygenated molecule is a spheroid of dimensions 50 × 55 × 69 Å, while the oxygenated molecule is smaller by 50 × 55 × 64 Å (Muirhead and Perutz, 1963). This corresponds to a volume decrease of about 8%. From this, it may be calculated that the sedimentation coefficient would increase by about 3% upon oxygenation.

Changes of sedimentation coefficient of this magnitude have been observed with other proteins under conditions where conformational changes are expected. Changes of 4% have been observed for the enzyme aspartate transcarbamylase upon ligand binding (Gerhart and Schachman, 1968), and we have detected changes of +3% when a purified antibody combines with hapten (C. Warner, V. N. Schumaker, and F. Karush, unpublished data).

Observation and measurement of a small difference in sedimentation coefficient do not necessarily mean that the protein itself has undergone alterations in size and shape, however, For example, the simple addition to the molecular weight and volume of the macromolecule which accompanies ligand binding would be reflected in a change in the sedimentation properties, or more subtle effects, such as alteration in salt and water binding affinities, could affect the buoyant density (Katz and Schachman, 1955) and sedimentation coefficient. Should a conformational change in protein structure actually occur, the partial specific volume may be changed which would also affect the sedimentation coefficient.

In this paper we first develop equations to take into account the various parameters which could influence the sedimentation coefficient. Then, we use these equations together with a theoretically constructed model protein system to see how sensitive the results become to small variations in all the parameters. From this study, it is concluded that interpretation of sedimentation coefficient changes in terms of alterations in size, shape, and molecular weight is reasonable. One very sensitive parameter does exist, however, and this is the partial specific volume of the protein. If conformational change is accompanied by a large change in partial

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specific volume, and if this is not taken into account, then quantitative assessment of the magnitude of the size and shape change could be in error.

Symbols. Fundamental Quantities

M_h = molecular weight of the hydrodynamic unit
M_p = molecular weight of the protein
M_A = molecular weight of the ligand
M_w = molecular weight of water
$\bar{v}_h$ = partial specific volume of the hydrodynamic unit
$\bar{v}_p$ = partial specific volume of the protein
$\bar{v}_A$ = partial specific volume of the ligand
$\bar{v}_w$ = partial specific volume of water
n = number of molecules of ligand bound
α = number of molecules of preferentially bound water
f_{he} = frictional coefficient of the hydrodynamic ellipse
f_{as} = frictional coefficient of the anhydrous sphere
c = concentration
s^0 = sedimentation coefficient at infinite dilution
s = sedimentation coefficient at concentration c
k = concentration dependence parameter in $s = s^0(1 - kc)$
η = solvent viscosity
ρ = solvent density
N = Avogadro's number

Use of the Prime. Unprimed quantities mean prior to the addition of the ligand; primed quantities are subsequent to the addition of the ligand.

Definitions. $q \equiv f_{he}/f_{as}$. This quantity is called the frictional ratio. $\delta q \equiv q' - q$. $\delta s \equiv s' - s$.

In deriving the equations, it is assumed that interacting flow terms which appear in the complete equations for multicomponent systems (Williams *et al.*, 1958) are negligible or cancel between the two cells.

Theory

The Fundamental Expression. The fundamental equation which will serve as the starting point for the derivation is (Katz and Schachman, 1955)

$$s^0 = \frac{M_h(1 - \bar{v}_h\rho)}{Nf_{he}} \tag{1}$$

The translational frictional coefficient of the equivalent hydrodynamic ellipse may be written as: $f_{he} = f_{as}(f_{he}/f_{as})$. The translational frictional coefficient of the anhydrous sphere, f_{as}, is given by Stoke's law as $f_{as} = 6\pi\eta(radius)$, and the $radius = (3\bar{v}_pM_p/4\pi N)^{1/3}$. Equation 1 now becomes

$$s^0 = \frac{M_h(1 - \bar{v}_h\rho)}{N6\pi\eta\left(\frac{3\bar{v}_pM_p}{4\pi N}\right)^{1/3}\frac{f_{he}}{f_{as}}} \tag{2}$$

A Quantity Which Reflects Size and Shape Changes. Any change in the frictional ratio, f_{he}/f_{as}, which occurs in the denominator of eq 2 reflects only alterations in size and shape of the macromolecule. To show that this is the case, we may write the tautologous expression $f_{he}/f_{as} = (f_{he}/f_{hs})(f_{hs}/f_{as})$. The first term on the right is given by Perrin (1936), and is a function of shape (axial ratio) only. The second term is a function of relative size, and is given by the cube root of the ratio of volumes of the hydrodynamic sphere to the anhydrous sphere (Edsall, 1953). Therefore, if an alteration in axial ratio and/or volume ratio of the hydrodynamic sphere occurs when ligand is bound, then, and only then, could f_{he}/f_{as} be caused to change.

It is convenient to simplify writing the frictional ratio, f_{he}/f_{as}, by giving it the symbol q.

From sedimentation analysis alone, as is shown below, it is possible to obtain the quantity $\delta q/q$, which may be called the fractional change in the frictional ratio.

As clearly shown by Scheraga and Mandelkern (1953), globular proteins cannot be exactly represented by hydrated ellipsoids of revolution. Therefore, interpretation of values of $\delta q/q$ in terms of precise alterations of molecular structure must be regarded with caution. With this word of warning, then, we wish to point out that the change in the frictional ratio has a simple molecular interpretation when only a small expansion or contraction of a globular macromolecule occurs as a result of the conformational change. In this case, shape alterations may be shown to be insignificant, and $\delta q/q$ is approximately equal to one-third of the fractional expansion or contraction of the protein.

Derivation of the Expression for $\delta q/q$. After ligand has been bound to the protein, an equation analogous to eq 2 may be written except that all symbols are primed. Dividing this second equation by eq 2, rearranging terms, using the symbols $\delta s = s' - s$, $q = f_{he}/f_{as}$, and $\delta q = q' - q$ and the expression $s = s^0(1 - kc)$ yields

$$\frac{\delta q}{q} = \frac{K}{1 + \frac{\delta s}{s}} - 1 \tag{3}$$

The coefficient K usually will have a value close to 1. It is given by the expression

$$K = \frac{M_h'(1 - \bar{v}_h'\rho')}{M_h(1 - \bar{v}_h\rho)}\left[\frac{M_p\bar{v}_p}{M_p\bar{v}'_p}\right]^{1/3}\frac{\eta(1 - k'c)}{\eta(1 - kc)} \tag{4}$$

Equations 3 and 4 now may be used to relate the fractional change in the observed sedimentation coefficient to the fractional change in the frictional ratio.

Computations. In order to investigate a model system, programs were written for a desk-top computer, Programma 101 (Olivetti-Underwood, Inc., New York, N. Y.), to solve eq 2–4. For convenience, several assumptions where made. When ligand is bound, $M_p' = M_p + nM_A$. It will be assumed that the partial molar volumes of protein and bound ligand are additive, so that $\bar{v}_p' = (M_p\bar{v}_p + M_A\bar{v}_A)/M_p'$.

Preferential hydration (Katz and Schachman, 1955; Cox and Schumaker, 1960) alters the mass and partial specific volume, and the appropriate relations are $M_h = M_p + \alpha M_w$, $M_h' = M_p' + \alpha' M_w$, $\bar{v}_h = (M_p\bar{v}_p + \alpha M_w\bar{v}_w)/M_h$, and $\bar{v}_h' = (M_p'\bar{v}_p' + \alpha' M_w\bar{v}_w)/M_h'$. The M_w and $\bar{v}_w$ are the molecular weight and partial specific volume of water. (We have used $M_w = 18$, $\bar{v}_w = 1$ in subsequent computations, which should be sufficiently accurate for dilute aqueous salt solutions.)

TABLE I: Sensitivity to Errors of Frictional Ratio Computation.

Parameter Considered	Value Chosen for Model System	Value Chosen for Recalcn	% Change in Frictional Ratio	
			Model	Recalcd
		Part A		
M_p	200,000	190,000	3.33	3.43
$\bar{v}_p$	0.7250	0.7150	3.33	3.26
M_A	500	450	3.33	3.17
$\bar{v}_A$	0.50	0.55	3.33	3.55
n	4	4.5	3.33	3.54
α	2000	0	3.33	3.33
ρ	1.01	1.02	3.33	3.38
Variable Parameter	Value before Binding	Value after Binding	% Change in Frictional Ratio	
			Model[a]	Recalcd[b]
		Part B		
$\bar{v}_p$	0.7250	0.7240	3.33	2.90
α	2000	2800	3.33	3.61
ρ (solvent)	1.0100	1.0110	3.33	3.69
η (solvent)	0.01000	0.01001	3.33	3.54
kc	0.040	0.041	3.33	3.44

[a] For the model system, δs was computed using 3.33% for the change in the frictional ratio, as well as the values of the indicated parameter before and after binding. [b] In the recalculation, only the value before binding was used together with δs to recalculate the coefficient of size and shape.

The first program is written for eq 2, and accepts values of M_p, $\bar{v}_p$, n, M_A, $\bar{v}_A$, α, ρ, η, f_{he}/f_{us}, k, and c, or primed equivalents, and computes s and s' both at concentration c and at infinite dilution. Thus, we may readily calculate values of δs for a variety of model systems.

A second program was written for eq 3 and 4. This program accepts values of M_p, $\bar{v}_p$, n, M_A, $\bar{v}_A$, α, α', ρ, ρ', η, η', k, c, k', c', δs, and s. It yields $\delta q/q$.

By the use of the programs, it is possible to compute the effect of variations in any parameter on δs, and also upon the back-computation of $\delta q/q$.

Model System. As a model for an allosteric enzyme, we have chosen a protein with a molecular weight of 200,000, $\bar{v}_p$ of 0.7250, and the frictional ratio of 1.2000. We also have assumed a preferential hydration of 2000 water molecules. In a solution of density 1.0100 and viscosity of 0.01000 poise, the computed value of s^0 by eq 2 is 10.1175 S.

The protein is assumed to undergo a conformational change when it binds ligand. Four molecules of ligand, each of 500 molecular weight units and $\bar{v}_A = 0.50$, bind to each protein molecule. The binding is assumed to result in an approximate 10% expansion in volume, causing the frictional ratio to increase by 3.33% to a value of 1.2400. From eq 2, the sedimentation coefficient drops to 9.9500 S.

By subtraction, δs^0 is found to be -0.1675 S. Back substitution of these values for s^0 and δs^0 into eq 4 and 3 yields the expected fractional increase in the frictional ratio of 3.33%.

The Effect of Errors. Because some of the parameters which enter into eq 2–4, may not be accurately known, recalculation of the frictional ratio will be in error. It is valuable to gain some appreciation of the size of the errors. Therefore, we have recalculated the frictional ratio while varying each of the parameters in its turn, and the data are presented in Table I.

Table IA lists errors which occur when the parameter in question is not accurately known but does not change with ligand binding. For example, in the first row an incorrect value of 190,000 is used for the molecular weight of the protein instead of the correct value of 200,000 when using eq 3 to recalculate the frictional ratio. The error caused by this inaccuracy is small, yielding a value of 3.43% for the change in the frictional ratio instead of 3.33%. In the following rows of Table IA incorrect values are chosen for protein partial specific volume, ligand molecular weight and partial specific volume, number of molecules of ligand bound, preferential hydration, and solution densities.

The Effects of Changes in Other Parameters. Table IB shows the effect when the parameter in question changes its value upon binding of the ligand. In the first row it is assumed that $\bar{v}_p$ changes from 0.7250 ml/g to a value of 0.7240. Furthermore, it is assumed that this change escapes detection, and an incorrect value of 0.7250 is used for the recalculation. The error caused by this inaccuracy is large, yielding a value of 2.90% for the change in the frictional ratio instead of 3.33%. In the following rows of part B, incorrect values for ρ', η', $k'c'$, and α' are used in eq 3.

In constructing Table I, we have attempted to choose reasonable values for inaccuracies or changes in the parameters. For large proteins, $\pm 5\%$ is a reasonable estimate of error in molecular weight. It is doubtful if the partial specific volume of most proteins is known to better than ± 0.005 ml/g. An approximate 10% error in the average number of molecules of ligand bound seems reasonable. The effective molecular weight of the bound ligand is uncertain if it contains an ionizable group.

The most difficult quantity to assess is the magnitude of the partial specific volume change which might occur upon ligand binding. Josephs and Harrington (1967), studying the polymerization of myosin, estimate that a change in $\bar{v}_p$ of 0.0006 ml/g occurs. Although their system is different, we will use this value to indicate the kinds of changes which might be expected upon conformational alterations. We have taken almost twice this value in Table IB, in an attempt to assess the maximum error which is to be expected.

Another change the magnitude of which is difficult to estimate is that of preferential hydration. If a protein of 200,000 molecular weight and $\bar{v}_p = 0.7250$ should expand in volume by 10% upon ligand binding, and if this new volume is occupied by water but excludes salt, then α' would increase by about 800 water molecules. We use this figure as a reasonable estimate of the maximum change which might occur in α.

From an inspection of the values in Table IA, it can be seen that the effects of inaccuracies in knowing the absolute values of the various parameters that enter into eq 3 are not too serious. The largest errors are caused by failure to know $\bar{v}_A$, n, and M_A with accuracy, and these cause errors of less than 7% in the quantity being measured for our model protein.

More serious are changes in the parameters which could occur with ligand binding. In particular, a small variation in $\bar{v}_p$ can have marked consequences. The possibility that there occur appreciable changes in the partial specific volume of the protein places a serious limitation upon the accuracy of this technique. Moreover, such changes in volume might have a large thermodynamic work term associated with them when studied under the several hundred atmospheres generated in the ultracentrifuge at high speeds (Josephs and Harrington, 1967; Kegeles *et al.*, 1967; Ten Eyck and Kauzman, 1967). Pressure-induced conformational changes might result which would complicate the experimental data and its interpretation.

Small errors in ρ' and η' would also cause large errors in the recalculated coefficient of size and shape. But accurate measurement of ρ' and η' is not difficult.

The effects of small differences in aggregation or dissociation *caused by ligand binding* to the protein have not been considered in Table I. Even a very small change in the average molecular weight of the protein caused by association or dissociation would have a pronounced effect of the sedimentation coefficient. But since aggregation or dissociation is markedly concentration dependent, the measurement of δs values over a wide range of protein concentrations and finding them to be essentially the same should be good evidence that aggregation or dissociation with ligand binding is not the cause of the sedimentation change.

Interpretation of Alterations in the Frictional Ratio. Alterations in the frictional ratio could reflect either expansion (or contraction) of the macromolecule or else a drastic alteration in shape. For globular proteins, small volume changes seem more reasonable than large shape changes. For example, a 3.33% increase in the frictional coefficient of a sphere would require almost a 75% increase in axial ratio, but only a 10% increase in volume. Comparable volume changes are seen in X-ray analysis of unoxygenated and oxygenated hemoglobin structures.

While volume changes would usually appear to be the most reasonable interpretation of the sedimentation data, the loosening of one end of a polypeptide chain so that it unfolds away from the rest of the molecule, like a tail on a kite, also would be expected to cause a measurable change in sedimentation coefficient. In the case of the IgG-immunoglobulin, a change in angle at the crouch of the "Y" would be expected to result in a difference in sedimentation properties.

Therefore, we believe that while sedimentation analysis may become a useful tool for the detection and measurement of the magnitude of conformational change, it cannot yield information by itself concerning the type of change which occurs in the structure of the protein.

Concluding Remarks. The approach taken in this communication is to cast the equations into the form most useful for the analysis of systems where the predominate change is in size and shape. For other kinds of systems, the observed changes in δs may largely reflect variations in M or $\bar{v}$. The differential approach could be quite useful in the investigation of associating systems, for example. The response of a protein to a ligand might be either a conformational change or else a change in subunit affinity. Differential sedimentation may provide a way of detecting either effect and of distinguishing between the two through concentration studies. For associating systems, the solution of the equations becomes a bit more difficult, but if preferential hydration is neglected, it is possible to solve for δM explicitly as a function of δs. Experimentally, second moment values should be used instead of peak maxima.

The sensitivity of δs measurements to small changes in frictional ratio, $\bar{v}$, molecular weight, or preferential hydration (on a weight scale) will depend upon the magnitude of the absolute sedimentation coefficient of the macromolecule. The method measures an absolute δs, while alternation of those variables produces a relative change. Thus, the sensitivity of the method would be better for solutes of high molecular weight. The precision of the absolute δs would also be better for slowly diffusing solutes.

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Methylation of Sugars and Bases in Ribosomal and Rapidly Labeled Ribonucleates from Normal and Puromycin-Treated L Cells*

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ABSTRACT: Pulse-labeled ribonucleates were prepared from cells grown for 15–150 min in media containing [*methyl*-^{14}C]methionine, [^{14}C]cytidine, or [^{3}H]cytidine. Ribosomal ribonucleates were prepared from cells grown for 24 hr in media containing the same precursors. All ribonucleate specimens were repeatedly precipitated from 2.5 M NaCl, in order to remove low molecular weight ribonucleates, and were then subjected to both sedimentation and chemical analyses. Pulse-labeled specimens contained radioactive ribonucleates that sedimented faster than 28S ribosomal ribonucleate. With cytidine precursors, most of the radioactivity was confined to cytidine constituents of the ribonucleates even after 24 hr. When [*methyl*-^{14}C]-methionine was used as a precursor for the methyl substituents in the ribonucleates, a large proportion of radioactivity entered the carbon skeletons of adenosine and guanosine, even after the shortest period of labeling. The distribution of $O^{2'}$-methylribose among sequences of the type NmpN (where m denotes $O^{2'}$ methylation) was similar for *all* preparations, both pulse-labeled and ribosomal ribonucleates, but the amount of $O^{2'}$-methylribose, as a per cent of total sugars, increased with time of pulse labeling. Three methylated bases found in ribosomal ribonucleates were also detected in pulse-labeled ribonucleates. A comparison was made of pulse-labeled ribonucleates from cells grown for the same period of time in the presence, and in the absence, of puromycin. Ribonucleates from puromycin-treated cells contained a larger proportion of fast-sedimenting ribonucleates (>28 S) than did ribonucleates from normal cells.

However, the $O^{2'}$-methylribose content of ribonucleates from puromycin-treated cells was only slightly lower than that of ribonucleates from normal cells, and the pattern of NmpN sequences was nearly identical in the two types of ribonucleates. Significantly, ribonucleates from puromycin-treated cells contained much less N^6,N^6-dimethyladenine, and had a different pattern of carbon skeleton labeling, than did [*methyl*-^{14}C]methionine-labeled ribonucleates from normal cells. Accumulated data on the end groups and $O^{2'}$-methylribosyl constituents of pulse-labeled and ribosomal ribonucleates from L cells have been discussed in terms of the possibility that the fast-sedimenting material (>28 S) in ribonucleates from pulse-labeled cells may arise by secondary structural interactions within, or among, newly synthesized polynucleotides.

We have recently reported sedimentation and chemical analyses for several high molecular weight RNA specimens that were prepared from L cells, which had been incubated with four tritiated nucleoside precursors for periods of 15 min, 30 min, 90 min, and 24 hr (Lane and Tamaoki, 1967; Tamaoki and Lane, 1967a,b). Low molecular weight RNA was removed from the preparations by precipitation of the RNA from 2.5 M sodium chloride solution.

After a 15-min labeling period, 75% of the rapidly

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