Evaluation of Diffusion Coefficients of Proteins from Sedimentation Boundary Curves*

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ABSTRACT: A simple and approximate equation is derived from Fujita's equation which permits evaluation of the diffusion coefficient, D, from sedimentation boundary curves. It is shown that the effect of the concentration dependence of the sedimentation coefficient on the evaluation of D decreases with decreasing the speed of the rotation, and D can be found with simple procedures under proper conditions. Diffusion coefficients of several typical proteins, both native and denatured, have been evaluated from sedimentation experiments in a synthetic boundary cell either according to the classical Lamm equation or the equation proposed here. The values of D thus obtained and the molecular weights calculated by Svedberg's equation have an uncertainty of at most 10% for native proteins and that of over 10% for denatured proteins.

Since the advent of the short-column method by Van Holde and Baldwin (1958) the sedimentation equilibrium method has been enjoying its revival as a dependable means of evaluating the molecular weights of macromolecular substances, proteins in particular. However, for proteins, which are generally much more homogeneous in molecular weight than synthetic polymers, the use of the classical Svedberg equation resorting to the measurements of sedimentation coefficients, s, and diffusion coefficient, D, at infinite dilution should not be lightly thrown away but should be reconsidered in the light of a recent development in the techniques and theory of sedimentation analysis.

In principle, the data necessary for both s and D could be obtained from the measurements of sedimentation boundary curves. As is well known, s can be evaluated from the rate of maximum refractive index gradient, and this can be achieved, in general, with high precision. It is also known that D may be evaluated by means of a relation (Lamm, 1929)

$$(A/H)^2 = 4\pi Dt(1 + s\omega^2 t) \tag{1}$$

where A is the area enclosed by the sedimentation boundary curve above its base line, H is its maximum height, t is the time measured from the start of centrifugation, and ω is the angular velocity of rotation. However, close inspection of the derivation of eq 1 indicates that this equation is only valid under the following conditions: (i) the solution considered is a two-component system; (ii) s and D are independent of solute concentration, c, and (iii) the initial condition of the experiment is of the type represented by

$$\begin{aligned} c &= 0 \ (r_1 < r < r_0, \ t = 0) \\ c &= c_0 \ (r_0 < r < r_2, \ t = 0) \end{aligned} \tag{2}$$

where c_0 is the initial concentration of the solution, r_1 and r_2 are the radial distances from the center of rotation to the air–liquid meniscus and bottom of the cell, respectively, and r_0 is the radial position at which initially a sharp boundary is formed between solution and solvent.

Condition i is not fulfilled with the conditions in which proteins are usually studied. Although proteins themselves are generally homogeneous enough to be able to treat them as a single component, the aqueous media in which they are dissolved always contain at least one additional component of low molecular weight. Thus such solutions are at least ternary systems. However, no theory is as yet available which permits us to estimate how much effect is exerted by the additional low molecular weight components on the rate of movement as well as the shape of the sedimentation boundary curve for the protein component. It is customary to *assume* that such an effect is so small that a given aqueous medium containing one or more low molecular weight components may be regarded as a single thermodynamic component. It is under this assumption that we may treat protein solutions as fulfilling condition i.

Generally, condition ii is not obeyed by macromolecular solutions. It can be shown theoretically (Fujita, 1962) that the dependence of D upon c has a less important effect on the shape of sedimentation boundary curve than does the concentration dependence of s. It is this latter dependence that we must take into account for the evaluation of D from sedimentation boundary curves. Fujita (1956, 1959a, and 1962) has shown that when D is assumed to be constant and s varies linearly with c as

$$s = s^0(1 - kc) \tag{3}$$

where s^0 is the value of s at infinite dilution and k is a constant for a given system, the height–area ratio, H/A, of the sedimentation boundary is related to D by the equation

$$G^{-1}(z) = \frac{r_0\omega^2 kc_0 s^0}{2D^{1/2}}\left[1 - \frac{s^0}{2}(1 - kc_0)\omega^2 t\right]t^{1/2} \tag{4}$$

with

$$z = 2r_0\omega^2 s^0 kc_0(H/A)t \tag{5}$$

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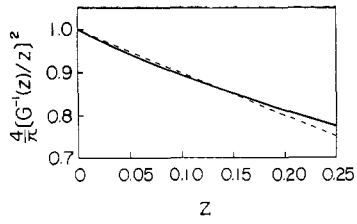

FIGURE 1: Calculated value of $(4/\pi)[G^{-1}(z)/z]^2$ as a function of z. Dotted line represents $(1 - z)$ *vs.* z.

where $G^{-1}(z)$ is a tabulated function (Fujita, 1962). Use of eq 4 for the evaluation of D of proteins has been reported by several authors (Fujita, 1959b; Connell, 1958; Derechin and Johnson, 1966), but except in the study by Derechin and Johnson, the calculation has been made with the data obtained from the experiments which did not obey condition iii.

Condition iii can be obtained by the use of synthetic boundary cells. Schumaker and Schachman (1957) and also Ehrenberg (1957) were the first to use this type of cell for the determination of D of proteins. Subsequently, several investigators (Mommaerts and Aldrich, 1958; Hasserodt and Vinograd, 1959; Daniel and Katchalski, 1962; Hofmann and Harrison, 1963; Harrap and Woods, 1964; Lupu *et al.*, 1965) have used a similar technique and relatively low speeds of rotation for the determination of D. In all these studies, the analysis was made in terms of eq 1 with the term $s\omega^2t$ being neglected, not of Fujita's eq 4.

In the limit $k \to 0$, eq 4 reduces to eq 1 as should be so. However, this reduction is also obtained as ω becomes smaller. Thus even if there exists a concentration dependence of s, D can be evaluated by means of eq 1, provided that the experiment is performed at a sufficiently low speed of rotation under the initial condition which conforms to condition iii. The speed of rotation relevant for this purpose may be estimated as follows. We recast eq 4 in the form

$$(A/H)^2 = 4\pi Dt(4/\pi)[G^{-1}(z)/z]^2(1 + s\omega^2t) \qquad (6)$$

As shown in Figure 1, the function $(4/\pi)[G^{-1}(z)/z]^2$ can be represented quite accurately by $(1 - z)$ for z such that $0 < z < 0.16$. Therefore we have

$$(A/H)^2 = 4\pi Dt(1 - z)(1 + s\omega^2t) \ (0 < z < 0.16) \qquad (7)$$

Thus we may choose ω so that z may remain much smaller than unity over the entire interval of a sedimentation run. The smaller the value of ω, the better the approximation of eq 7 becomes. In practice, however, the rotor becomes unstable as its speed is lowered beyond a certain limit. The value of $s\omega^2t$ also may become much smaller than unity at a low speed of rotation unless the experiment is continued for an unusually long interval of time, and the term $s\omega^2t$ in eq 1 and 7 may be dropped.

The area A decreases with time due to the dilution effect of the sector-shaped cell. This decrease in ΔA is approximately represented by

$$\Delta A/A = 2s\omega^2t \qquad (8)$$

As mentioned above, when the rotor is spun at a sufficiently low speed, the value of $s\omega^2t$ should be quite small, and hence ΔA may become comparable with the error involved in the reading of A. This means that the area A may be assumed to stay constant during the centrifugation and its value may be evaluated from one or two sedimentation patterns chosen suitably. The labor for the measurement of plates is then greatly reduced. Thus, after all, the desired D can be found from the slope of a plot of $(A/H)^2$ against t when the experiment is performed at so low speed of rotation that z and $s\omega^2t$ may become negligibly small compared with unity and A may stay independent of time.

In the present study, we performed low-speed sedimentation experiments for several typical proteins, native or denatured, and analyzed the data in terms of the above idea to evaluate their diffusion coefficients. Furthermore, for several systems, the sedimentation experiments were also made at much higher speeds, and the resulting data were treated according to eq 1 or 7 with the term $s\omega^2t$ being neglected. For some systems the data were treated in terms of Fujita's original eq 4. All these data were taken in a synthetic boundary cell so that condition iii, essential for these equations to be applied, may be secured.

Materials and Methods

Materials. Bovine serum albumin was a crystalline (lot no. 0401241) obtained from International Chemical and Nuclear Corp. A sample from Pentax, Inc., was also used. The crystals were dissolved in pure water, and dialyzed against pure water at 5°. A small amount of residue was removed by centrifugation.

Rabbit muscle aldolase was a crystalline suspension (A grade, lot no. 52645) of Calbiochem. Inc. It was purified by recrystallization as described elsewhere (Kawahara and Tanford, 1966a).

Bovine pancreatic nibonuclease was crystalline (lot no. 115272) obtained from Wilson Laboratories. It was dissolved in pure water and passed through a deionizing column.

Sperum whale myoglobin was salt free (lot no. J1381) and purchased from Mann Research Laboratories, Inc. It was converted into ferrocarbomonoxymyoglobin.

Human CO-hemoglobin was prepared from freshly drawn blood by a modification of the procedure of Steinhardt (1938).

Guanidine hydrochloride was prepared from guanidine carbonate, and urea was recrystallized. The procedures have been described previously (Kawahara *et al.*, 1965; Kawahara and Tanford, 1966b).

All other reagents were the best available commercial products, and were used without further purification.

Preparation of Protein Solution. Protein solutions to be used for sedimentation experiments usually contain salts or other reagents. Their concentrations should be adjusted to those in the reference solvent to be placed in the synthetic boundary cell. Otherwise the boundary formed between the solution and the solvent in the cell may be disturbed by the diffusion of these small molecules. The concentrations of reagents in solutions of denatured proteins are so high that their adjustment requires an extreme care. A usual method to do this is to dialyze the solution against the solvent. According to the theory of multicomponent systems (Casassa and Eisenberg, 1964), the dialysate should be used as the reference solvent for measurements of equilibrium properties of the solution. How-

TABLE I: Experimental Conditions for Evaluation of D of Native Proteins.[a]

Protein	Concn of Protein (g/dl)	Concn of NaCl (M)	Length of Run (min)	k (dl/g)	$s\omega^2t$ ($\times 10^2$)	z_{max}[c] ($\times 10^2$)
Bovine serum albumin						
ICN	0.668	0.15	71	0.10	0.35	1.8
Pentax	0.403	0.15[d]	42	0.10	0.2	0.8
Aldolase	0.462	0.10	80	0.06	0.7	1.6
Ribonuclease	0.408	0	39	(0.1)	0.09	0.2
Myoglobin	0.404	0.019	39	(0.1)	0.09	0.2
Hemoglobin						
No. 1	0.401	0.020	62	(0.1)	0.34	1.0
No. 2[b]	0.404	0.020	29	(0.1)	1.5	7.2

[a] Sedimentation experiments were performed at 25° by using a double-sector synthetic boundary cell at a rotor speed of 12,590 rpm, except in expt 2 for hemoglobin. [b] Rotor speed was 42,030 rpm. [c] Maximum value of z during the run. [d] Contained 0.03 M of acetic acid and 0.02 M of sodium acetate.

ever, it is not as yet elucidated theoretically what is suitable for use as the solvent for the measurements of hydrodynamic properties of multicomponent solutions. Furthermore, dialysis may not be convenient practically for such concentrated solution of reagents, because the exhaustive dialysis to attain osmotic equilibrium takes a considerably long time[1] and may change the concentration of protein which can not be readily determined for denatured proteins. Therefore, we preferred the following procedure. A stock solution of relatively high concentration of a native protein in dilute salt solution was prepared, and the solvent, in which the concentration of the salt was adjusted as close to that in the protein *stock* solution as possible, was also prepared. The pH of the solution was adjusted near the isoionic pH of the given protein. In some cases, the *stock* solution was dialyzed thoroughly against the solvent. The concentration of protein in the *stock* solution was determined by dry weight measurement at 107°. Aqueous solution of appropriate concentrations of reagents was mixed with the *stock* protein solution and with the solvent, respectively, to make up the *solution* and the reference *solvent* for the sedimentation measurement. By mixing the carefully weighing solutions, the difference in concentration of reagents between the *solution* and *solvent* can be made very small. Presumably, it was 0.001 mole/l.[2] or less in the present study. It should be noticed that the concentrations of reagents in the *solution* represent not their amounts in it but their amounts in the solvent; the volume of the solvent is calculated by subtracting the volume of protein from the volume of *solution*.

Sedimentation Procedure. Sedimentation experiments were carried out in a Spinco Model E analytical ultracentrifuge equipped with a temperature control unit and schlieren optics.

Sedimentation coefficients of all denatured proteins and some of the native proteins were determined from experiments performed at a rotor speed of 42,040 rpm in a double-sector synthetic boundary cell of 12-mm length which had a sapphire lower window. Those of other samples were from data obtained with a single-sector cell at a speed of 59,780 rpm.

Sedimentation experiments for the evaluation of diffusion coefficients were done with a double-sector synthetic boundary cell at a rotor speed of 12,590 rpm for most of the systems and at higher speeds for others.

Schlieren patterns photographed were read with a Gaertner two-dimensional comparator.

Sedimentation coefficient and diffusion coefficient, denoted as $s_{25,s}$ and $D_{25,s}$, respectively, were determined at 25°, and those of native proteins were converted into the values in water at 20°, denoted as $s_{20,w}$ and $D_{20,w}$, respectively, by the usual procedure. Viscosities and densities of the solvents, required for this conversion, were measured or obtained from the data reported elsewhere (Kawahara and Tanford, 1966b).

Partial specific volume, $\bar{v}$, is required for the conversion of s and for the calculation of molecular weight from s and D. The values for the native proteins were obtained from appropriate literature sources, but those for the denatured proteins were assumed and hence had a considerable uncertainty (Tanford *et al.*, 1967a).

Results and Discussion

Native Proteins. Table I summarizes the experimental conditions used for evaluation of the diffusion coefficients of native proteins. All centrifugation for the evaluation of D, except one for hemoglobin, was performed at 12,590 rpm. In each experiment, usually the areas of schlieren patterns were measured at two different times, and it was confirmed that they remained constant at this low speed, as expected. In the experiment with hemoglobin at 42,040 rpm, the areas had to be measured for all patterns, because it changed appreciably with time.

The quantity z at 12,590 rpm was smaller than 0.02 for the systems examined because they had small values of k, and the values of $s\omega^2t$ were smaller than z. Their D values were evaluated by means of the equation

$$(A/H)^2 = 4\pi Dt \qquad (9)$$

[1] In some cases in which the dialysis was carried out for only one or two days to adjust the concentrations of reagents, the boundary between the solvent and the solution was distrubed soon after the formation in the cell. Such a violent disturbance has not been observed in the systems treated by the present procedure.

[2] This is the difference in concentration between the *solution* and the *solvent*, and does not refer to an absolute accuracy of the concentrations.

TABLE II: Diffusion Coefficients of Native Proteins.

Protein	$D_{25,s}$[a] ($\times 10^7$)	$D_{20,w}$[b] ($\times 10^7$)	Data in Literature: $D_{20,w}$	Data in Literature: Reference
Bovine serum albumin				
ICN	6.5	5.8	5.86–5.90	Wagner and Scheraga (1956)
Pentex	6.4	5.7	5.855	Akeley and Gosting (1953)
Aldolase	4.8	4.3	4.63	Taylor *et al.* (1948)
			4.26	Glikina and Finogenov (1950)
Ribonuclease	14.4	12.6	11.9	Rothen (1940)
Myoglobin	11.3	9.9	10.3	Ehrenberg (1957)
Hemoglobin				
No. 1	7.1	6.3	6.9	Lamm and Polson (1936)
No. 2[c]	8.2[d] (7.9[e])	7.2[d] (6.9[e])		Field and O'Brien (1955)

[a] Calculated by the method of least squares according to eq 9, except for expt 2 for hemoglobin. [b] Converted from $D_{25,s}$. [c] See footnote *b* of Table I. [d] Evaluated by eq 7*. [e] Evaluated by Fujita's eq 4.

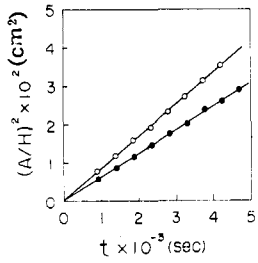

FIGURE 2: Plots of $(A/H)^2$ *vs.* t for native bovine serum albumin in 0.15 M NaCl aqueous solution (○) and for native aldolase in 0.10 M NaCl aqueous solution (●) at 25°. The rotor speed was 12,590 rpm.

Plots of $(A/H)^2$ *vs.* t for most of the proteins well fitted a straight line, as illustrated in Figure 2. On the other hand, the maximum value of z at 42,040 rpm for hemoglobin was large (0.072), and the correction according to eq 7*[3] or 4 was needed. In fact, the difference in value of D evaluated by eq 9 and by eq 7* was as large as about 10%. The value of D for hemoglobin obtained by eq 7* from the high-speed data was larger than the value evaluated by analyzing the low-speed data in terms of eq 9. This difference will be discussed later.

As can be seen in Table II, the value of $D_{20,w}$ thus evaluated agree within 10% with the values in the literature. Most of native proteins in aqueous solution may have values of k similar to those of the systems examined in the present study. Consequently, we may conclude that the diffusion coefficient of an usual native protein can be estimated by eq 9 when the centrifugation is performed at a low speed of rotation.

Table III lists the molecular weights calculated from the evaluated values of $D_{20,w}$ according to Svedberg's equation as well as the values of $s^0_{20,w}$ and $\bar{v}$ to be used for the calculations. Molecular weights of most of the protein studied have already been determined from their amino acid analysis, and are shown in Table III. Considering the uncertainty involved in the values of s_0 and $\bar{v}$, the indicated agreement of the two sets of molecular weight values may be regarded as satisfactory.

[3] * refers to eq 7 with the term $s\omega^2 t$ being neglected.

TABLE III: Molecular Weights of Native Proteins.

Protein	$s^0_{20,w}$ ($\times 10^{13}$)	$\bar{v}_{20}$[a] (ml/g)	$M_{s,D}$[b] ($\times 10^{-3}$)	M_{ref}[c] ($\times 10^{-3}$)
Bovine serum albumin	4.59	0.730	71 and 72	69
Aldolase	7.9[d]	0.742	174	160
Ribonuclease	1.96	0.709	13	13.7
Myoglobin	1.96	0.749	19	17.7
Hemoglobin	4.60	0.749	70 and 62 (64[e])	64.5

[a] Published data: for serum albumin by Koenig (1950), for aldolase by Kawahara and Tanford (1966a) based on data by Taylor and Lowry (1956), and for hemoglobin by Kirshner and Tanford (1964). The value for myoglobin was assumed to be the same as that for native hemoglobin, and the value for ribonuclease was estimated from an observed value of 0.711 at 25°. [b] Calculated from $D_{20,w}$ obtained and listed in Table II with the values of $s^0_{20,w}$ and $\bar{v}_{20}$ according to Svedberg's equation. [c] Values of serum albumin and aldolase were obtained by sedimentation equilibrium by Tanford *et al.* (1967a) and by Kawahara and Tanford (1966a), respectively. Others are evaluated from their amino acids sequences. [d] Data by Stellwagen and Schachman (1962). [e] See footnote *e* of Table II.

Denatured and Dissociated Proteins. Proteins are generally denatured in concentrated guanidine hydrochloride solution. It has been shown that the polypeptide chains of all proteins examined in the present paper are randomly coiled, completely dissociated and unfolded in aqueous solution of 6 M guanidine hydrochloride containing 0.1 M of β-mercaptoethanol: disul-

TABLE IV: Experimental Conditions for Evaluation of D of Denatured and Dissociated Proteins.

Protein	Speed of Rotation (rpm)	Concn of Protein (g/dl)	Length of Run (min)	k^e (dl/g)	$s\omega^2 t$ ($\times 10^2$)	$z_{max}{}^f$ ($\times 10^2$)
Denatured proteins[a]						
Bovine serum albumin	12,590	0.401	143	1.05	0.15	6.3
Ribonuclease No. 1	12,590	0.388	71	(0.6)	0.04	0.9
Ribonuclease No. 2	42,040	0.604	97	(0.6)	0.7	17.4
Myoglobin No. 1[b]	12,590	0.426	63	(0.5)	0.03	0.5
Myoglobin No. 2	12,590	0.363	83	(0.5)	0.04	0.6
Hemoglobin No. 1[c]	42,040	0.403	100	0.5	0.8	10.9
Hemoglobin No. 2	50,740	0.402	105	0.5	0.8	11.2
Dissociated hemoglobin[d]						
No. 1 (1.0 M guanidine hydrochloride)	12,590	0.400	40	(0.1)	0.14	0.5
No. 2 (6.4 M urea)	42,040	0.402	38	(0.1)	0.9	3.2
No. 3 (8.0 M urea)	42,040	0.399	39	(0.1)	0.7	2.5

[a] In aqueous solution of 6.0 M guanidine hydrochloride with 0.1 M β-mercaptoethanol, except otherwise stated. [b] In 6.03 M guanidine hydrochloride without β-mercaptoethanol. [c] In 5.0 M guanidine hydrochloride with β-mercaptoethanol. [d] Dissociated into half molecules in aqueous solution of guanidine hydrochloride or urea. [e] Values for ribonuclease, myoglobin, and dissociated hemoglobin were assumed to be the same as observed values for β-lactoglobulin (Tanford *et al.*, 1967a), denatured hemoglobin, and native hemoglobin, respectively. [f] Maximum value of z during the run.

TABLE V: Diffusion Coefficients and Molecular Weights of Denatured and Dissociated Proteins.

Protein	$D_{25,s}{}^a$ ($\times 10^7$)	$s^0_{25,s}{}^b$ ($\times 10^{13}$)	$\bar{v}_{25}{}^c$ (ml/g)	$M_{s,D}{}^d$ ($\times 10^{-3}$)	$M_{ref}{}^e$ ($\times 10^{-3}$)
Denatured proteins					
Bovine serum albumin	1.91[f]	1.03	0.725	78	69
Ribonuclease No. 1	4.7	0.59	0.69	14.5	13.7
Ribonuclease No. 2	5.7	0.59	0.69	12	
Myoglobin No. 1	4.9	0.49	0.74	16	17.2[g]
Myoglobin No. 2	5.2	0.49	0.74	15	
Hemoglobin No. 1	6.8	0.72	0.74	15	15.5[g]
Hemoglobin No. 2	6.1	0.49	0.74	13	
Dissociated hemoglobin					
No. 1 (1.0 M guanidine hydrochloride	8.8	3.16	0.75	38	32.3
No. 2 (6.4 M urea)	7.8	1.85	0.75	33	
No. 3 (8.0 M urea)	6.9	1.32	0.75	30	

[a] Calculated by the method of least squares according to eq 9 (z is smaller than 0.02) or eq 7* (z is larger than 0.02). [b] Estimated value based on single or scattered data at finite concentration. [c] Assumed value for partial specific volume. See the discussion by Tanford *et al.* (1967a). [d] Calculated from values of $D_{25,s}$, $s^0_{25,s}$, and $\bar{v}_{25}$ listed above. [e] See footnote *f* of Table I. [f] Value evaluated by Fujita's eq 4 is 1.87. [g] Molecular weight without heme.

fide bonds, where they exist, are ruptured in this medium (Tanford *et al.*, 1967a,b; Lapanje and Tanford, 1967).

The experimental conditions used for the evaluation of the diffusion coefficients of denatured proteins are summarized in Table IV. Although the diffusion coefficient of aldolase in the native state can be determined (see Table I), denatured aldolase was not subjected to the present study, because the polypeptide chains constituting this protein are not identical (Kawahara and Tanford, 1966a; Morse *et al.*, 1967; Penhoet *et al.*, 1967) so that the denatured aldolase may not be treated as a single component.

Hemoglobin partially dissociates into half molecules, without appreciable change in the conformation of the polypeptide chains, probably keeping a globular shape, in aqueous guanidine hydrochloride over the concentration range from 0.7 to 2.5 M and in aqueous urea at concentrations up to 8 M

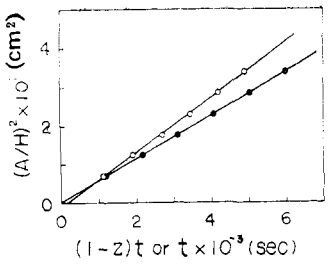

FIGURE 3: Plots of $(A/H)^2$ against $(1 - z)t$ (○) and against t (●) for ribonuclease in 6.0 M guanidine hydrochloride with 0.1 M β-mercaptoethanol at 25°. The rotor speed was 42,040 rpm.

(Kawahara *et al.*, 1965). The data for the dissociated hemoglobin under different conditions, previously reported without correction for the concentration dependence of s, have been reanalyzed, and the results are presented in Tables IV and V.

Concentration dependence of s of denatured proteins is much larger than that of native proteins, while the values of s^0 themselves are small. As shown in Table IV, the values of z for denatured proteins vary from system to system, because of the differences in s, t, and k. For example, the value of z for bovine serum albumin is too large to be neglected even when the data are taken at as low a speed as 12,590 rpm, while the values of z for myoglobin and ribonuclease at the same speed are negligibly small. Figure 3 shows the plots of $(A/H)^2$ against t and $(1 - z)t$ for denatured ribonuclease at 42,040 rpm, and indicates that the value of D obtained by eq 9 is appreciably different from that by eq 7. Therefore, the diffusion coefficients of denatured or dissociated proteins were evaluated either by eq 9 if the value of z is smaller than 0.02 or by eq 7* if z is larger, and the values of $D_{25,s}$ are shown in Table V.

The value of D for denatured ribonuclease evaluated by eq 7* from the data at 42,040 rpm was larger than that obtained by eq 9 from the data at 12,590 rpm. A similar difference has been noted above for native hemoglobin. One reason for these differences would be slight heterogeneity of the samples used. Spreading of a sedimentation boundary with time is caused by both diffusion and distribution of sedimentation coefficient in the solute component, *i.e.*, heterogeneity of the sample. The latter effect is enhanced with increasing speed of rotation (Williams *et al.*, 1952). Thus an apparent value of D evaluated from the boundary spreading becomes larger at higher speed of rotation. Hence the low-speed experiment would be more preferable than the high speed experiment for the evaluation of D from sedimentation boundaries, when there is an anticipation that a given sample is more or less heterogeneous.

Table V also lists the values of molecular weights which are calculated from the data of $D_{25,s}$ and $s^0_{25,s}$. The difference between the observed and the true molecular weights is larger for denatured or dissociated proteins than for those of native proteins, amounting to 10% or more. This disagreement may be attributed not only to the error in the evaluation of the diffusion coefficient but also to the uncertainty in the values of sedimentation coefficient and partial specific volume. Because of very low rates of sedimentation and a marked concentration dependence of s, the accuracy of s^0 for denatured proteins is much lower than that for native proteins. As discussed elsewhere (Tanford *et al.*, 1967a), the values of $\bar{v}$ of denatured proteins should be less reliable, and this may introduce an uncertainty of nearly 10% into the values of $1 - \bar{v}\rho$ needed for the calculation of molecular weight. Since both s^0 and $1 - \bar{v}\rho$ may be uncertain to about 10%, it is not avoidable that an error of 10–20% will be introduced when the molecular weight of a denatured protein is calculated by Svedberg's equation. Furthermore, the effect of low molecular weight components in such concentrated solutions of reagents may be responsible for the error in the values of D, s, and the molecular weights of denatured proteins. Even if the result is of a relatively low precision, however, this method has a practical use, because the determination of molecular weight with denatured or dissociated proteins is usually difficult. Finally, we wish to note that the uncertainty in $\bar{v}$ also introduces a similar problem into the determination of molecular weights by sedimentation equilibrium.

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Selenotrisulfides. II. Cross-Linking of Reduced Pancreatic Ribonuclease with Selenium*

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ABSTRACT: Native or reduced pancreatic ribonuclease A having zero and eight thiol groups per mole, respectively, was treated with selenious acid to evaluate the role of thiol groups in the nonenzymic incorporation of inorganic selenite into proteins. Over the pH range of 2–7, no selenium was incorporated into native ribonuclease A, but reduced ribonuclease A treated with selenious acid at pH 2 and 4° showed rapid loss of thiol groups, spectral changes equivalent to selenotrisulfide (RSSeSR) formation, and the uptake of 2 moles of selenium/mole of ribonuclease A, in accord with the over-all reaction $4RSH + H_2SeO_3 \rightarrow RSSeSR + RSSR + 3H_2O$. At higher pH, elemental selenium was liberated during the reaction, decreasing the incorporation of selenium to 1.41 and 0.44 moles of Se per mole of ribonuclease A at pH 4.7 and 7, respectively, but pH 7 caused little or no release of selenium from the final reaction product. Gel filtration and sedimentation velocity studies of the pH 2 derivative indicate that it is homogeneous, monomeric, and more unfolded than native ribonuclease A. Spectral perturbations in the 280–290-mμ region likewise suggest that the degree of folding is intermediate between that of reduced and native ribonuclease A.

The exchange of approximately 1 mole of selenium into ribonuclease A from the selenotrisulfide derivative of 2-mercaptoethanol occurred with reduced ribonuclease A at 4° at pH 2–7, with extensive liberation of elemental selenium, but none was exchanged into native ribonuclease A. The enzyme activity of the derivatives against yeast ribonucleic acid or 2′,-3′-cyclic cytidylic acid equaled only a few per cent of that of native ribonuclease A. It is concluded that selenium was incorporated between two sulfur atoms to form an intramolecular selenotrisulfide linkage in place of a disulfide. Such derivatives may be useful for a variety of protein structure–function studies.

The reaction of selenious acid with low molecular weight thiols to form selenotrisulfides (RSSeSR) has previously been characterized in this laboratory (Ganther, 1968). We also investigated the reaction with protein thiols, with the object of creating proteins having selenotrisulfide cross-linkages. Besides clarifying a nonenzymic process of selenite incorporation into proteins, the creation of such derivatives might be useful for other reasons: (1) the conversion of a disulfide bond into the SSeS linkage could provide a useful analog for study of protein structure and function; (2) the introduction at specific sites of a selenium atom could be useful in X-ray crystallographic or electron spin resonance studies of proteins; (3) protein selenotrisulfides might participate in catalytic processes related to the biological functions of selenium.

Ribonuclease was chosen as a model protein for these studies. Besides having great stability and useful solubility properties, the native enzyme has four disulfide bonds that can be reduced to yield four pairs of sulfhydryl groups, providing a well-defined system for the precise study of selenium incorporation in the same polypeptide chain, with or without thiol groups. There was also a reasonable chance that the selenotri-

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