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Dissociation of Human CO-Hemoglobin by Urea, Guanidine Hydrochloride, and Other Reagents*

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ABSTRACT: The dissociation of human CO-hemoglobin by urea, guanidine hydrochloride, $CaCl_2$, and several organic solvents has been studied by measurement of the weight-average sedimentation coefficient as a function of reagent concentration. Guanidine hydrochloride and $CaCl_2$ are highly effective dissociating agents, but urea is much less effective. The organic solvents were found to be quite ineffective. With all reagents, dissociation proceeds easily only to the half-molecule stage, and it occurs without appreciable change in the conformation of individual polypeptide chains. At high concentrations of guanidine hydrochloride, dissociation to single chains occurs, but it does so only as part of the complete unfolding of the molecule, with separation of the heme moiety from the protein. Evidence is presented which indicates that dissociation to half-molecules always occurs along the same contact surface, and that the forces which hold the molecule together at this surface cannot be primarily hydrophobic forces. The standard free energy of dissociation, in dilute salt solution, at *p*H 7 and 25°, is 8.2 kcal/mole. This corresponds to a dissociation constant of 1×10^{-6} mole/liter and indicates that hemoglobin is partially dissociated to half-molecules under ordinary experimental conditions even in the absence of a disruptive reagent.

The dissociation of hemoglobin into half-molecules is being studied in this laboratory because it serves as a simple model for a variety of reactions of proteins, in all of which a prominent feature is the exposure of new chemical groups to the solvent. Regardless of the forces which originally hold these groups together, such reactions should be aided by any reagent which increases the solubility of the newly exposed groups. Conversely, a knowledge of the relative effectiveness of various reagents in promoting the reaction, when coupled with a knowledge of their effectiveness as solubilizing agents for different types of chemical groups, should permit inferences to be made concerning the groups which maintain the original native structure. The theoretical aspects have been discussed elsewhere (Tanford, 1964a,b).

The dissociation of hemoglobin is well suited for an experimental test of this approach to an understanding of protein reactions. It appears to occur without appreciable conformational change, so that there is probably little or no new exposure of groups as a result of processes other than separation along the original surface of contact between the subunits. The results thus focus on a limited part of the protein molecule, and should lead to conclusions about the possible size and chemical nature of this portion of the original native structure. The most important advantage of hemoglobin as a model system is the fact that any such conclusions may soon be amenable to direct verification. X-Ray diffraction studies of hemoglobin are far advanced (Cullis *et al.*, 1962), and the complete three-dimensional structure may soon be known. Exact knowledge of the nature of the contact surface between half-molecules will then be available.

Experimental Procedure

Human CO-hemoglobin was prepared from freshly

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drawn blood by a modification of the procedure of Steinhardt (1938). Stock solutions were prepared as needed, and their concentrations were determined by drying to constant weight at 107°. The solutions were then adjusted to *p*H 6.9, and some NaCl was normally added at this point in sufficient amount to assure a concentration of 0.02 M at the dilution of the stock solutions ultimately used for experimental studies. The final protein concentration of the stock solutions was mostly about 2 g/100 ml. The solutions were stored in the cold, and were never kept longer than 4 weeks. CO-myoglobin was prepared from crystalline sperm whale myoglobin obtained from Mann Research Laboratories.

Guanidine hydrochloride was prepared from Eastman guanidine carbonate. The carbonate was recrystallized from aqueous solution by addition of ethanol at 4°. It was dried under vacuum, mixed with water to make a paste, and converted to the hydrochloride by addition of chilled concentrated HCl. The solution was adjusted to *p*H 5.4 and filtered to remove insoluble material. Water was removed from the solution by evaporation at reduced pressure (below 40°). The product was recrystallized twice from methanol and dried in a vacuum desiccator under P_2O_5. Concentrated solutions of the purified hydrochloride were prepared by dissolving in water. The solutions generally had a *p*H of 5.5.

Urea was an A grade product obtained from California Corp. for Biochemical Research. It was recrystallized from aqueous ethanol and stored in the cold.

All other reagents were the best available commercial products, used without further purification. Water used in the preparation of solutions for measurement was always saturated with carbon monoxide.

Sedimentation velocities were measured in a Spinco Model E analytical ultracentrifuge, using schlieren optics. The most precise data were obtained using 12-mm capillary type synthetic-boundary cells and a rotor speed of 42,040 rpm. The less precise data (those shown in Figure 5) were obtained with single-sector 12-mm cells at a speed of 59,780 rpm. The schlieren patterns were photographed on Kodak Type 103-F spectroscopic plates, used in conjunction with a Wratten No. 29 filter.

Sedimentation velocities were measured at 25° and converted to values of $s_{20,w}$ in the usual way. Viscosities and densities of the solvent, required to make this conversion, were measured directly or obtained from appropriate literature sources. The partial specific volume of hemoglobin in dilute aqueous salt solution was taken to be 0.749 cc/g at 20° and 0.751 cc/g at 25°, as discussed previously (Kirshner and Tanford, 1964). It was shown in our earlier paper that dissociation of hemoglobin in concentrated salt solution does not affect the partial specific volume. We have used the same values for all solvent media. This is not necessarily correct, especially in concentrated guanidine hydrochloride, where the protein becomes unfolded. Wherever the assumed constancy of the partial specific volume affects the interpretation of results, it will be so indicated in the text.

Approximate diffusion coefficients were determined in the ultracentrifuge, using synthetic-boundary cells similar to those used for the sedimentation studies. The rotor speed was 12,540 rpm. Areas and heights of the schlieren plots were measured at different times with a Gaertner two-dimensional micro-comparator having a 3° rotational stage for lining up the plates. Diffusion coefficients were determined from the relation

$$(A/H)^2 = 4\pi Dt \qquad (1)$$

This equation is not strictly applicable when the sedimentation coefficient depends on protein concentration (Fujita, 1962). Diffusion coefficients were measured only in solutions where the protein consisted predominantly of a single species, however, and the concentration dependence of the sedimentation coefficient under these conditions is quite small (Kegeles and Gutter, 1951). Calculations of the diffusion coefficient by the more exact equation of Fujita (1959), which takes the concentration dependence into account, differ from the values calculated by equation (1) by 10% or less. This difference is within the experimental error of our results.

Viscosity measurements were made with Cannon-Fenske type capillary viscometers which had a flow time with water near 300 seconds. Under these conditions no kinetic energy correction need be made. The difference in density between solution and solvent, on the other hand, has an appreciable effect (Tanford and Buzzell, 1956), and correction for it was always made.

Spectral measurements were made with a Beckman Model DK-1 spectrophotometer. The results of these measurements are not reported in detail. Their purpose was solely to see whether appreciable changes accompanied dissociation into half-molecules.

Results

Sedimentation Velocity of CO-*Hemoglobin in Dilute Salt Solution.* Several independent measurements in 0.02 M NaCl, all made at 25°, at a protein concentration of 0.4 g/100 ml, yield an average value for $s_{20,w}$ of 4.50 ± 0.07 S. This value is in good agreement with some previous determinations carried out under similar conditions. Svedberg and Pedersen (1940) obtained a value of 4.48 S for human CO-hemoglobin, and a slightly lower value for the horse variety. Steinhardt (1938) gives 4.63 S for horse CO-hemoglobin. Rossi-Fanelli *et al.* (1961) give 4.5 S for human reduced hemoglobin in 0.1 M phosphate buffer. Other literature values, however, are substantially lower than ours. Hasserodt and Vinograd (1959), Field and O'Brien (1955), and Kegeles and Gutter (1951) have reported $s_{20,w}$ values for human CO-hemoglobin which lie between 4.2 and 4.3 S. Minor differences in solvent composition and protein concentration are not sufficient to account for this discrepancy. The value reported by Kegeles and Gutter is subject to a correction for adia-

batic cooling of the rotor during acceleration (Waugh and Yphantis, 1952; Biancheria and Kegeles, 1954), which would raise $s_{20,w}$ by about 0.1 S, diminishing the discrepancy to about 0.15 S. Such a correction, however, should not be necessary for the later data of Hasserodt and Vinograd or Field and O'Brien.

It has been observed before (Svedberg and Pedersen, 1940; Kegeles and Gutter, 1951) that the concentration dependence of the sedimentation coefficient of hemoglobin in dilute salt solutions suggests that it may be partially dissociated under ordinary experimental conditions. The same conclusion is reached on the basis of extrapolating the dissociation constant measured at high salt concentration to zero salt concentration (Kirshner and Tanford, 1964). The value of K_d would have to be less than 2×10^{-8} if more than 99% of the hemoglobin were to remain undissociated at a concentration of 0.4 g/100 ml. The extrapolated value is however of order 1×10^{-6}, leading to a calculated dissociation of 6% at this concentration. It is evident therefore that the sedimentation coefficients discussed in the preceding paragraph do not represent the values for undissociated hemoglobin. Using the experimental difference between the sedimentation coefficients of whole and half-molecules discussed below (and this difference need be known only to within about 20% for this purpose), and assuming that the figure of 6% dissociation is approximately correct, we estimate that the value of $s_{20,w}$ for undissociated hemoglobin, in 0.02 M NaCl, at a protein concentration of 0.4 g/100 ml, is 4.60 ± 0.10 S, and we shall use this value in all calculations of the dissociation constant to be made in this paper.

Literature values of the diffusion coefficient of hemoglobin are in better mutual agreement than the values for the sedimentation coefficient. Two careful studies (Lamm and Polson, 1936; Field and O'Brien, 1955) both obtained $D_{20,w} = (6.9 \pm 0.1) \times 10^{-7}$ for human CO-hemoglobin under conditions similar to those under which our sedimentation data were obtained. If this is taken to represent the weight-average diffusion coefficient of a mixture containing about 6% half-molecules, one would calculate $D_{20,w} = (6.8 \pm 0.1) \times 10^{-7}$ for undissociated CO-hemoglobin. Combined with $s_{20,w}$ = 4.6 S, this gives a molecular weight of 65,500 ± 1500, in good agreement with the value calculated from the known amino acid sequences of the α and β chains of hemoglobin (Braunitzer *et al.*, 1964) plus the molecular weight of the four heme moieties, this calculated molecular weight being 64,500.

The sedimentation and diffusion coefficients used for our estimation of the molecular weight were not extrapolated to zero protein concentration. Such extrapolation would affect s more than D (estimated $s_{20,w}$ = 4.7 S) and would raise the estimated molecular weight to about 66,800, i.e., to a value somewhat above the true molecular weight. Better agreement with the true value is obtained by use of the $s_{20,w}$ value of Kegeles and Gutter (1951), after correction for adiabatic cooling of the rotor. Their extrapolated $s^0_{20,w}$ value for human CO-hemoglobin becomes 4.40 S. Correcting this for the effects of dissociation (see Figure 7 and the accompanying discussion) gives an estimated $s_{20,w}$ of 4.54 S for the undissociated protein. With $D^0_{20,w} = 6.8 \times 10^{-7}$ the corresponding molecular weight becomes 64,600.

It is evident from these calculations that the exact values of the sedimentation and/or diffusion coefficient of undissociated hemoglobin are still somewhat in doubt. It is possible that our own value for the sedimentation coefficient is a little too high. This is not considered to have a serious effect on the results of this paper, which depend on relative rather than absolute values of the sedimentation coefficient, and would not be affected appreciably by changes of 0.1–0.2 S in the absolute values.

Dissociation in Aqueous Urea Solutions. It has been shown by Steinhardt (1938) that urea dissociates horse CO-hemoglobin without appreciable unfolding. Gutter *et al.* (1956), on the other hand, have reported that human CO-hemoglobin is unfolded by urea without change in molecular weight. We have not been able to confirm the results of Gutter *et al.* and obtain for human CO-hemoglobin results which are in qualitative accord with Steinhardt's observations with the horse protein. Our observed sedimentation coefficients, and their conversion to $s_{20,w}$ values, are shown in Table I.

TABLE I: Effect of Urea on the Sedimentation Velocity.[a]

Urea Concn (M)	$s_{25} \times 10^{13}$ (observed)	Conversion Factor[b]	$s_{20,w} \times 10^{13}$
0.50	4.80	0.935	4.49
0.80	4.63	0.960	4.45
1.02	4.56	0.980	4.47
1.60	4.18	1.037	4.34
2.40	3.77	1.122	4.23
3.20	3.43	1.225	4.20
4.00	2.99	1.341	4.01
4.41	2.69	1.411	3.79
4.80	2.55	1.481	3.78
4.82	2.37	1.482	3.51
5.18	2.32	1.555	3.60
5.27	2.23	1.573	3.50
6.03	1.89	1.741	3.29
6.41	1.78	1.838	3.27
7.12	1.56	2.038	3.18
8.02	1.27	2.335	2.97
8.84	1.11	2.658	2.95

[a] All solutions contained 0.02 M NaCl. Protein concentration was 0.40 g/100 ml, and the temperature of measurement 25°. [b] The conversion factor corrects the observed s_{25} value to the viscosity and density of water at 20°. It should be noted that the experimental error in the measurement of s_{25} is not much below 0.1×10^{-13}, and is about the same at all urea concentrations. The uncertainty in $s_{20,w}$ thus becomes larger (about 0.2×10^{-13}) at the higher concentrations of urea. A similar statement applies to the data of Tables III and IV.

TABLE II: Apparent Frictional Coefficients.[a]

	Approximate Mol Wt (exptl)[b]	True Mol Wt (assumed)[c]	$s_{20,w} \times 10^{13}$	f/f_{min}
Undissociated hemoglobin	65,500	64,500	4.60	1.16
Myoglobin in dilute salt soln	19,000	17,800	1.96	1.15
Hemoglobin in 8 M urea	28,000	32,250	2.9	1.16
Hemoglobin in 1 M GHCl	34,000	32,250	3.0	1.12
Hemoglobin in 1 M $CaCl_2$		32,250	2.6	1.29
Myoglobin in 1 M GHCl		17,800	1.97	1.15
Hemoglobin in 6 M GHCl	15,500[d]	16,100	1.08	1.96
Myoglobin in 6 M GHCl	15,000	17,800	1.08	2.09

[a] The frictional coefficient ratios given here should not be taken to have absolute significance, since $s_{20,w}$ values refer to a finite protein concentration (0.4 g/100 ml), and because possible preferential binding of solvent components and possible solvent effects on $\bar{v}$ have not been considered. [b] By combination of diffusion and sedimentation coefficients, except where otherwise stated. For undissociated hemoglobin, an accurate literature value of D was used (see text). All other D values are approximate values determined in the ultracentrifuge. The value of D used for hemoglobin in 8 M urea is of very poor precision, because a rotor speed of 42,040 rpm was used instead of the speed of 12,540 rpm used for other determinations. [c] The $s_{20,w}$ values were obtained directly or by extrapolation, under conditions where the hemoglobin should exist entirely as a single species of 1, 2, or 4 chains. [d] By combination of intrinsic viscosity with $s_{20,w}$.

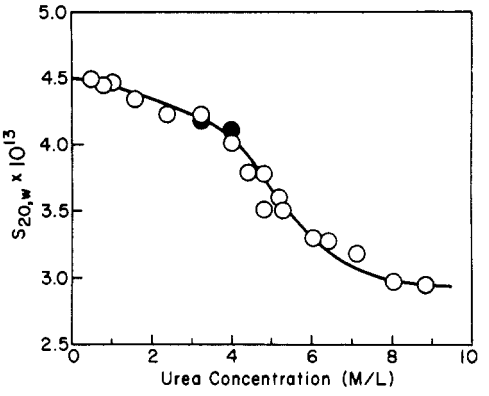

FIGURE 1: Effect of urea on the sedimentation coefficient of hemoglobin. Filled circles represent solutions originally exposed to 6.4 or 8 M urea (see text for details) and show that the reaction is reversible.

The $s_{20,w}$ values are plotted as a function of urea concentration in Figure 1. All of the data shown were obtained with relatively fresh solutions, usually within 2 hours of the initial exposure of the protein to urea.

The data show that urea decreases the sedimentation coefficient markedly. A limiting value of 2.9 S is approached asymptotically at high urea concentrations. That this decrease is the result of dissociation into half-molecules, without appreciable unfolding or other conformational change, is shown by several auxiliary measurements.

(1) Approximate diffusion coefficients were measured, as described in the experimental section. We obtained values of 7.5 and 7.1 $\times 10^{-7}$, respectively, for the diffusion coefficient at 25°, in 6.4 and 8.0 M urea. When combined with the corresponding sedimentation coefficients, these values lead to molecular weights of 33,000 and 28,000, respectively.

(2) The limiting value of $s_{20,w}$ = 2.9 S, approached asymptotically at high urea concentrations, may be combined with the known molecular weight of the half-molecule to yield a value for the apparent frictional coefficient (f), relative to the minimum possible frictional coefficient (f_{min}) compatible with the molecular weight and partial specific volume of the protein (Tanford, 1961). As Table II shows, this ratio has not been altered by dissociation. It is essentially the same as the value obtained for undissociated hemoglobin, as well as that of native myoglobin.

(3) The intrinsic viscosity of CO-hemoglobin was determined in 6.4 M urea. The data, shown in Figure 2, lead to $[\eta]$ = 3.5 cc/g. This is essentially identical with the intrinsic viscosity of hemoglobin in dilute aqueous salt solution (Tanford, 1957). The intrinsic viscosity of unfolded hemoglobin chains in 6 M guanidine hydrochloride is 20 cc/g; that of acid-denatured hemoglobin is 13.5 cc/g.

(4) The visible and ultraviolet absorption spectrum of CO-hemoglobin remains essentially unaltered up to a urea concentration of 8 M. Preliminary data indicate that the optical rotatory dispersion curve also remains essentially unchanged.

Two of the experimental points in Figure 1 demonstrate that the dissociation of hemoglobin by urea is a reversible process, and that the overall dissociation curve therefore represents the equilibrium state at each concentration of urea. The measurement at 3.2 M urea

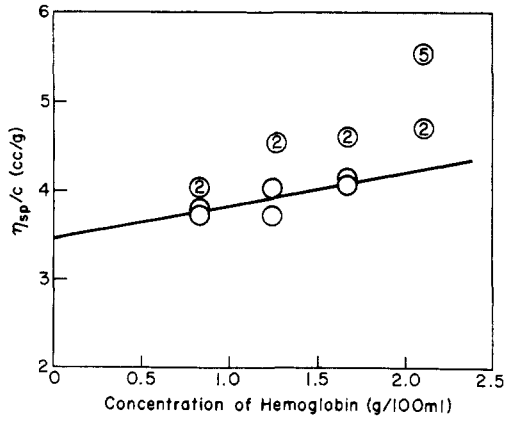

FIGURE 2: Intrinsic viscosity of CO-hemoglobin in 6.44 M urea. Unmarked circles represent data obtained within a few hours of the original addition of urea to the protein. Circles containing the numerals 2 and 5 show the results of allowing solutions to stand for 2 and 5 days, respectively.

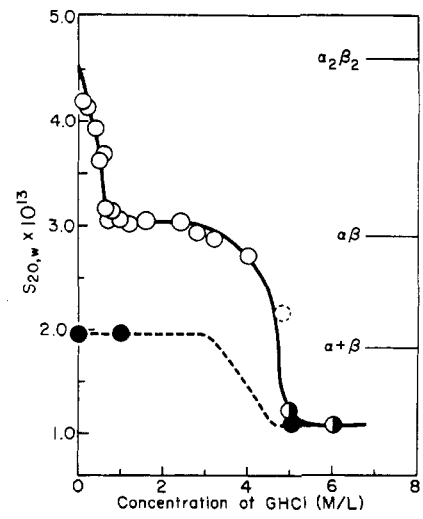

FIGURE 3: Effect of guanidine hydrochloride on the sedimentation coefficients of hemoglobin and myoglobin. Open circles represent hemoglobin. The point with a dashed outline represents a solution which was unstable, appearing to be undergoing slow aggregation. Solutions at higher guanidine concentration (data not shown) were similarly unstable. The instability could be prevented by addition of β-mercaptoethanol, and half-filled circles show data in the presence of this reagent. Filled circles show data obtained with myoglobin. The lines on the right-hand side of the figure show the calculated values of $s_{20,w}$, based on a constant f/f_{min} of 1.16, for undissociated hemoglobin, half-molecules, and quarter-molecules, respectively.

represents a solution which originally had a protein concentration of 0.8 g/100 ml, at 6.4 M urea and 0.04 M NaCl. The sedimentation velocity was measured, and indicated nearly complete dissociation to half-molecules under these conditions. The solution was then diluted with water to twice its original volume. The measurement at 4 M urea was made with a 2:1 dilution of the solution at 0.4 g/100 ml protein concentration and 8 M urea. The sedimentation velocity was therefore measured at a protein concentration of 0.2 rather than 0.4 g/100 ml. The equilibrium relation given in equation (4) was used to correct the observed sedimentation coefficient to what it would have been at 0.4 g/100 ml, and it is this corrected value which is shown in the figure.

All the data presented in the figure were obtained as rapidly as possible after the exposure of the protein to urea. Long exposure to urea was found to lead to slow alteration in the properties of the protein. This is shown, for example, by the viscosity data in Figure 2 for solutions which had stood in contact with 6.4 M urea for 2 or 5 days. Corresponding changes in sedimentation velocity were of the order of 0.1 S.

Dissociation by Guanidine Hydrochloride. The experimental data in aqueous guanidine hydrochloride solutions are shown in Table III and Figure 3. It is evident that this reagent has a much more dramatic effect than urea. The decrease in $s_{20,w}$ to a value characteristic of half-molecules, which requires a urea concentration of about 8 M, is virtually complete at a guanidine concentration of 0.7 M. That this change in sedimentation coefficient actually represents dissociation was confirmed by measurement of the approximate diffusion coefficient in 1 M guanidine hydrochloride. A value of $D_{25} = 9.5 \times 10^{-7}$ was obtained which, when combined with the corresponding sedimentation coefficient, gave a molecular weight of 34,000. No change in spectral properties was observed, so that the dissociation presumably occurs, as in urea, without appreciable conformational change. The limiting value of $s_{20,w}$ is 3.0 S, which is slightly larger than the value of 2.9 S observed in urea. This does not represent an appreciable change in the apparent frictional coefficient ratio, as shown in Table II.

It is evident that raising the guanidine concentration from 0.7 to 2.5 M has no additional effect on hemoglobin. The half-molecules appear to be stable, and no indication of further dissociation to quarter-molecules occurs.

At concentrations of guanidine hydrochloride above 2.5 M, the sedimentation velocity is diminished. This decrease in sedimentation velocity is however accompanied by instability of the solutions. There is a change in the color of the solution, the color no longer travels with the protein in the sedimentation cell, and, on standing, an irregular increase in $s_{20,w}$ occurs, followed by precipitation of the protein. It is likely that these observations reflect unfolding of the protein (with dissociation of the heme moiety), followed by aggregation through disulfide bonds formed by oxidation of thiol groups. Addition of 0.1 M β-mercaptoethanol to the guanidine prevented the increase in sedimentation rate and subsequent precipitation. In the

TABLE III: Effect of Guanidine Hydrochloride on the Sedimentation Velocity.[a]

GHCl Concn (M)	$s_{25} \times 10^{13}$ (observed)	Conversion Factor[b]	$s_{20,w} \times 10^{13}$
0.100	4.62	0.907	4.19
0.201	4.51	0.917	4.14
0.401	4.19	0.937	3.93
0.496	3.82	0.947	3.62
0.589	3.86	0.957	3.69
0.634	3.29	0.962	3.17
0.703	3.15	0.971	3.06
0.804	3.20	0.983	3.14
0.998	3.04	1.006	3.06
1.201	2.92	1.033	3.02
1.596	2.80	1.087	3.05
2.397	2.48	1.225	3.04
2.803	2.24	1.306	2.93
3.20	2.05	1.398	2.87
4.00	1.67	1.619	2.71
4.79[c]	1.14	1.901	2.17
5.00[d]	0.60	2.06	1.23
6.03[d]	0.41	2.65	1.08

[a] All solutions contained 0.02 M NaCl. Protein concentration was 0.40 g/100 ml, and the temperature of measurement 25°. [b] See footnote *b* of Table I. [c] This solution was unstable and showed signs of aggregation. [d] Contained 0.1 M β-mercaptoethanol.

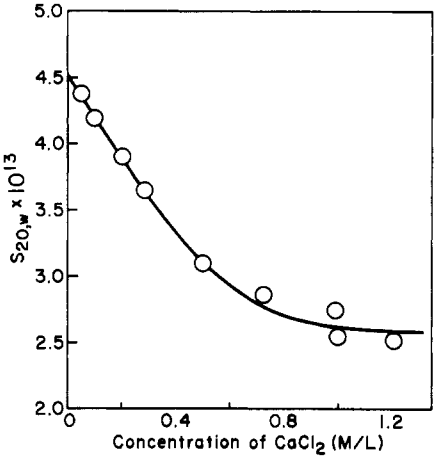

FIGURE 4: Effect of calcium chloride on the sedimentation coefficient of hemoglobin.

presence of this reagent a limiting $s_{20,w}$ value of 1.1 S was reached, as shown in Figure 3.

The intrinsic viscosity of the protein was determined in 6 M guanidine in the presence of 0.1 M β-mercaptoethanol. A value of 20 cc/g was obtained. Evidently the protein is highly unfolded. By combining the viscosity and sedimentation measurements, an estimate of the molecular weight can be obtained from the relation (Scheraga and Mandelkern, 1953; Tanford, 1961)

$$\beta M^{2/3} = \frac{Ns[\eta]^{1/3}\eta}{(100)^{1/3}(1 - \bar{v}\rho)} \qquad (2)$$

where N is Avogadro's number, and η and ρ are the viscosity and density, respectively, of the solvent. The values for water at 20° are used if $s = s_{20,w}$. The parameter β depends slightly on molecular conformation. We have used the value 2.5×10^6 characteristic of random coils, and with it obtain a molecular weight of 15,500, thus showing that the hemoglobin has become completely dissociated into its constituent polypeptide chains. The high intrinsic viscosity and the frictional coefficient (Table II) show that the chains are very much unfolded. Companion experiments were carried out with sperm whale myoglobin, which consists of a single polypeptide chain of slightly higher molecular weight (17,800) than the polypeptide chains of hemoglobin. The myoglobin chain contains no thiol groups, and it was found unnecessary to add β-mercaptoethanol to maintain stable solutions at high guanidine concentrations. The data obtained are summarized in Figure 3 and Table II. Low concentrations of guanidine, which dissociate hemoglobin to half-molecules without unfolding, had no effect at all on myoglobin. Under conditions where hemoglobin is dissociated to single chains with coincident unfolding, myoglobin also became unfolded.

Dissociation by Calcium Chloride. Data on the dissociation of hemoglobin by inorganic salts were described in an earlier paper (Kirshner and Tanford, 1964). It was found that $MgCl_2$ and $CaCl_2$ were particularly effective dissociating agents, and that bovine ferrihemoglobin and human CO-hemoglobin behave essentially identically in the presence of these reagents. For the calculations to be described later it is desirable to have accurate data on the action of one of these salts, over a wider range of concentrations, specifically for the human CO-hemoglobin. Accordingly a series of new studies were made with $CaCl_2$, and the results are shown in Table IV and Figure 4. They are very similar to the data presented in our earlier paper except that the limiting value of $s_{20,w}$, corresponding to complete dissociation, appears to be about 2.6 S, rather than 2.8 S as previously reported. A possible explanation is that the protein has positive preferential hydration (i.e., exclusion of $CaCl_2$) at high salt concentration. This question was not explored further, as our major interest in this paper is in the data at relatively low $CaCl_2$ concentrations. The difference between 2.6 and 2.8 S is in any event close to the experimental uncertainty in the determination of the sedimentation velocity at high salt concentrations.

Effect of Alcohols and Dioxane.[1] The effects of

[1] These data were taken from the Ph.D. dissertation of A. G. Kirshner (Duke University, 1963). All other results reported in this paper represent experiments conducted by K. Kawahara.

TABLE IV: Effect of Calcium Chloride on the Sedimentation Velocity.[a]

$CaCl_2$ Concn (M)	$s_{25} \times 10^{13}$ (observed)	Conversion Factor[b]	$s_{20,w} \times 10^{13}$
0.051	4.75	0.922	4.38
0.102	4.42	0.949	4.19
0.203	3.88	1.006	3.91
0.287	3.46	1.056	3.65
0.504	2.58	1.199	3.09
0.728	2.09	1.370	2.86
0.990	1.67	1.614	2.74
0.997	1.57	1.621	2.54
1.205	1.36	1.856	2.51

[a] All solutions contained 0.02 M NaCl. Protein concentration was 0.40 g/100 ml, and the temperature of measurement 25°. [b] See footnote *b* of Table I.

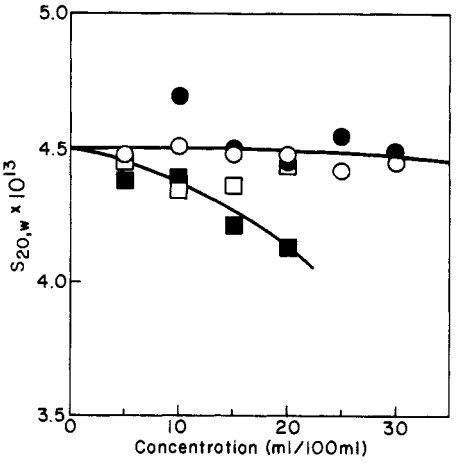

FIGURE 5: Effect of organic solvents on the sedimentation coefficient of hemoglobin. Open circles represent ethylene glycol, filled circles ethanol, open squares 1-propanol, and filled squares *p*-dioxane.

ethanol, 1-propanol, ethylene glycol, and dioxane on the sedimentation velocity of hemoglobin are shown in Figure 5. The data are not as precise as other data reported here because they were determined some time ago with standard single-sector centrifuge cells. Nevertheless, they clearly show that these organic reagents, three of which are highly effective unfolding agents for β-lactoglobulin (Tanford and De, 1961), have but little influence on hemoglobin. Experiments at higher concentrations of the organic reagents than are shown in the figure could not be carried out because precipitation of the protein occurred. This was presumably the result of the decreased dielectric constant at high concentrations of the organic reagents, with resulting increase in the electrostatic attraction between the isoelectric hemoglobin molecules. No evidence was obtained to suggest that unfolding had occurred.

Calculation of the Free Energy of Dissociation. Whenever the only effect of an added reagent is the reversible dissociation of the protein into half-molecules, it is possible to calculate an equilibrium constant for the dissociation. The experimental sedimentation coefficient is the weight-average value, appropriate to the protein concentration existing in the plateau region of the cell (Goldberg, 1953). In most of the experiments described in this paper synthetic-boundary cells were used, and the time of sedimentation was short. The concentration in the plateau region is thus not significantly different from the initial protein concentration, which was always 0.4 g/100 ml. If A and B are used to designate undissociated hemoglobin and half-molecules, respectively, the observed sedimentation coefficient is

$$\bar{s} = (1 - \alpha)s_A + \alpha s_B \qquad (3)$$

where α is the weight fraction of hemoglobin in the dissociated form, and s_A and s_B are the sedimentation coefficients of A and B at a concentration of 0.4 g/100 ml. In dilute salt solution, $s_A = 4.60$ S. If dissociation occurs without change in frictional coefficient ratio, and without preferential binding of any solvent component, s_B would be equal to $(0.50)^{2/3}s_A = 2.90$ S. The limiting values of $s_{20,w}$ actually observed in urea, guanidine hydrochloride, and $CaCl_2$, which should represent experimental measures of s_B, are 2.9, 3.0, and 2.6 S, respectively. The experimental error at high concentrations of the added reagents is quite large, as explained in Table I, and it is difficult to judge whether these differences are really significant. If they are significant, it implies that there is preferential binding of water or of the added reagent to the hemoglobin molecule, or that there may be a small solvent-related change in frictional coefficient ratio or partial specific volume. Such a secondary influence of solvent, if it occurs, cannot be assumed to affect only s_B. A similar effect on s_A is likely. We have chosen to ignore this effect entirely, and to calculate α values from equation (3) on the basis of $s_A = 4.60$ S and $s_B = 2.90$ S. The error introduced thereby is considerable at large values of α, but should become negligibly small at $\alpha < 0.5$, especially when the considerable error in the determination of $\bar{s}$ itself is taken into account.

Once α has been determined, the equilibrium constant is given as

$$K_d = \frac{4\alpha^2 c_o}{(1 - \alpha)M} \qquad (4)$$

where c_o is the protein concentration in g/liter, and M is the molecular weight of undissociated hemoglobin. Accurate values of K can be obtained only when α is not too close to zero or unity, and we have used only those data which correspond to $0.1 < \alpha < 0.9$. The results are shown in Figure 6, in the form of plots of the

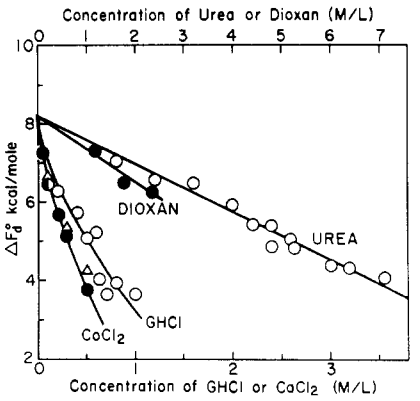

FIGURE 6: Standard free energy of dissociation into half-molecules, as a function of the concentration of disruptive reagent. Triangles show (for the $CaCl_2$ data) the maximum uncertainty which can arise from neglect of secondary influences on the sedimentation coefficient (see text).

corresponding free energy of dissociation, $\Delta F_d^\circ = -RT \ln K_d$, as a function of the concentration of disruptive reagent.

Figure 6 also shows the magnitude of the error which may have resulted from ignoring the possible effect of the added reagent on s_A and s_B. The worst case is $CaCl_2$ where the apparent value of s_B is 2.6 S rather than the value of 2.9 S assumed in the calculation of ΔF_d°. If we assume that this is the result of a general solvent effect, acting alike on whole and half-molecules, it is reasonable to write $s_A = 4.60(1 - 0.1c_o)$ and $s_B = 2.90 \cdot (1 - 0.1c_o)$. The triangles in Figure 6 show the calculation of ΔF_d° for the data with $CaCl_2$ when these values of s_A and s_B are used. If it were assumed that the solvent effect acts on half-molecules only, one would use $s_A = 4.60$ and $s_B = 2.90(1 - 0.1c_o)$. The results would then deviate less from the curve drawn in Figure 6 to represent the $CaCl_2$ data obtained with the originally assumed values of s_A and s_B. If the solvent effect were assumed not to be linear in c_o, then a lesser deviation from the curve would be obtained at low $CaCl_2$ concentrations.

Discussion

The reason for the existence of hemoglobin as a four-chain entity, in water or dilute aqueous salt solutions, at neutral *p*H, lies in the properties of the parts of the protein which constitute the contact sites. The groups involved evidently have a lower free energy in contact with each other than they would have in contact with the solvent. Thus "bonds" between the chains are formed which resist disruption in spite of the gain in *cratic* free energy (Kauzmann, 1959; Kirshner and Tanford, 1964) which would result from dissociation. When a reagent is added to the solvent and causes dissociation, it must do so because it decreases the free energy of contact with solvent of those parts of the protein which are newly exposed to solvent when dissociation occurs (Tanford, 1964b). The "bonds" between the chains thus become less stable, and insufficiently strong to overcome the driving force of the *cratic* part of the free energy toward dissociation.

Examination of a variety of reagents, to determine the extent to which each one influences the dissociation of hemoglobin, should throw light on the nature of the groups at the surfaces of contact, provided that conditions can be found under which the reagents produce dissociation without other conformational change (Tanford, 1964b). Our finding that high concentrations of guanidine hydrochloride lead to dissociation into individual α and β chains can therefore not give any information about the contact sites, for this reaction is found to be accompanied by rupture of virtually all noncovalent interactions in the protein: the dissociation is accompanied by separation of heme from protein and by unfolding of the individual chains. The dissociation into half-molecules at low concentrations of guanidine hydrochloride, and the similar reaction produced by various inorganic salts and by urea, however, can be used to give information about the contact sites, for it is accompanied without separation of the heme groups and without appreciable spectral change, and it leaves intact the bonds which maintain each individual chain in globular form. Our measurements cannot prove that no rearrangement of the internal three-dimensional structure has occurred, but they at least suggest that this is so. Measurements which are more sensitive to small changes in structure are in progress. Until they provide evidence to the contrary, we shall assume that dissociation to half-molecules has occurred without other structural change.

The native hemoglobin molecule consists of two α chains and two β chains. The three-dimensional structure, as determined by X-ray diffraction (Perutz *et al.*, 1960; Cullis *et al.*, 1962), indicates that the contacts which join the chains into a single molecule lie predominantly between α and β chains. If dissociation into half-molecules is to occur without other structural change, the product would therefore have to be of the form $\alpha\beta$, rather than α_2 and β_2. Some indication that this is in fact the preferred form of the two-chain molecule comes from the recent finding that globin, in the absence of heme, exists predominantly as two-chain $\alpha\beta$ molecules at neutral *p*H (Winterhalter and Huehns, 1964).

Assuming then that the product of dissociation is of the form $\alpha\beta$, we are still left with two possible species. As has been pointed out by Schroeder (1963), there are two distinct kinds of contacts between α and β chains in the undissociated molecule, involving different parts of the amino acid sequence. Dissociation can occur so as to break either kind of contact, leaving the other intact. It is thus possible that different reagents will cleave the parent molecule along different contact surfaces, exposing different groups to the solvent.

The experimental free energies of dissociation shown in Figure 6 suggest that cleavage along different surfaces has not occurred with the reagents used in this study,

except in the trivial instance of high guanidine concentrations which has already been discussed. There are two aspects of the results which lead to this conclusion.

(1) Extrapolation of $\Delta F_d°$ for each individual reagent to zero reagent concentration leads to the free energy of dissociation in 0.02 M NaCl for the particular mode of dissociation which each reagent favors. Figure 6 shows that it is entirely reasonable to extrapolate the data for each reagent to the same value of $\Delta F_d°$. It is true that some curvature must be imposed on the $CaCl_2$ and guanidine hydrochloride data to obtain *exactly* the same extrapolated value as with urea, but this is not surprising since effects of ionized salts frequently are linear functions of the square root of the concentration, rather than the first power. Specific binding sites for ions, present on the half-molecule but not on undissociated hemoglobin, would also lead to curved plots of $\Delta F_d°$ versus concentration (Tanford, 1964b). Since it is improbable that dissociation along two entirely different surfaces (in the same solvent, 0.02 M NaCl) would be characterized by the same value of $\Delta F_d°$, this result suggests that each reagent cleaves hemoglobin along the same contact surface.

(2) Although there are serious gaps in our knowledge of the effects of salts on the free energies of contact between solvent and various kinds of groups which may be present on a protein molecule, it seems likely that guanidine hydrochloride would be an effective agent for inducing a reaction which, like the dissociation of hemoglobin, is known to occur with such diverse salts as NaCl, $CaCl_2$, and $(NH_4)_2SO_4$. Guanidine hydrochloride, however, is also known to be able to duplicate the usual influence of urea on protein structure, acting usually at two to three times lower concentration than urea (see, for example, Gordon and Jencks, 1963). If, therefore, the dissociation of hemoglobin by salt were to occur along one contact surface, and that by urea along another, then guanidine hydrochloride should cleave along both surfaces, leading to the production of globular quarter-molecules. Its urealike action would occur at a concentration near 2 M (the midpoint of the urea curve in Figure 1 occurs at a concentration of 5 M), and its saltlike action would perhaps occur at somewhat lower concentration, the midpoint of dissociation curves with other 1,1 electrolytes being somewhat below 2 M. The experimental observation is that the dissociation to half-molecules by guanidine hydrochloride is complete well below a 1 M concentration. No reaction at all occurs in the concentration range 1–2.5 M. The data suggest that inorganic salts, urea, and guanidine hydrochloride all act upon the same contact surface, and that dissociation liberates groups which are stabilized by urea (but not by salts) and others which are stabilized by salts (but not by urea). Guanidine hydrochloride stabilizes both kinds of groups, and is thus effective at lower concentrations.

Our conclusion therefore is that one of the "bonds" between α and β chains is relatively weak, and that all dissociating agents act upon this same bond. The other "bond" is much stronger and cannot be disrupted except under conditions sufficiently severe to disrupt all of the noncovalent interactions in the molecule. The standard free energy of dissociation at the weaker contact surface is 8.2 kcal/mole, in 0.02 M salt at neutral *p*H. It is sensitive to *p*H (Field and O'Brien, 1955; Hasserodt and Vinograd, 1959), but not to temperature (Hasserodt and Vinograd, 1959; Kirshner and Tanford, 1964).

What is the nature of the groups at this contact surface? The most revealing result with reference to this question is the ineffectiveness of alcohols and dioxane as dissociating agents. As unfolding agents for β-lactoglobulin, for example, we have found these reagents to be very effective (Tanford and De, 1961). Expressing the results of those studies in units of moles/liter, we find that 6.5 M ethanol, 3.5 M dioxane, and an even lower concentration of 1-propanol (<2.6 M) are able to exert an influence approximately equal to that of 6.5 M urea. This result is compatible with the idea that a major fraction of the groups which are newly exposed in the unfolding of β-lactoglobulin are hydrophobic groups, and that the action of each of these disruptive reagents is based primarily on their ability to solubilize hydrophobic groups (Whitney and Tanford, 1962). In the reaction studied in this paper, urea itself is relatively ineffective, in comparison with inorganic salts and with guanidine hydrochloride. Ethanol and propanol appear unable to promote hemoglobin dissociation at all, within the concentration range which it was possible to study, and dioxane, while able to promote dissociation to some extent, is effective at about the same concentration as urea, rather than at half its concentration as in the unfolding of β-lactoglobulin. It appears probable, on the basis of these results, that the contact surfaces which are separated in hemoglobin dissociation cannot contain many hydrophobic groups. This suggests that the forces which hold the hemoglobin molecule together at this contact site are not primarily hydrophobic forces.

Urea solubilizes amide and peptide groups, as well as hydrophobic groups (Nozaki and Tanford, 1963; Tanford, 1964a), but probably has no such effect on ionic groups. Inorganic salts, on the other hand, would be expected to decrease the chemical potential of ionic groups, but not of peptide or amide groups (Tanford, 1964b). The contact surface along which dissociation occurs may thus contain both ionic and uncharged polar groups. As previously noted, this would account for the fact that guanidine hydrochloride is so much more effective than urea. If there are ionic groups at the contact surface, they presumably exist as ion pairs. If one member of each pair is a carboxylate ion, it would qualitatively account for the ability of acids to promote dissociation (Field and O'Brien, 1955). Dissociation is promoted by addition of base, as well as acid (Hasserodt and Vinograd, 1959), but the *p*H required is probably appreciably higher than would be expected if the number of charged lysyl side chains at the contact surface were equal to the number of carboxylate groups. This is not an unreasonable result, as some of the cationic groups forming ion pairs along

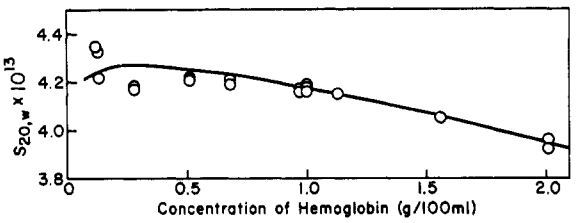

FIGURE 7: The effect of protein concentration on the sedimentation coefficient of CO-hemoglobin in dilute phosphate buffer, *p*H 7.07. The experimental points are from Kegeles and Gutter (1951). The curve is a calculated one which takes into account the effect of protein concentration on the dissociation equilibrium, as well as its effect on the sedimentation coefficient itself. The data of this figure have not been corrected for the effects of adiabatic cooling of the rotor during acceleration, i.e., they apply strictly to a temperature of 19.2° rather than 20°.

the contact surface could be on arginine side chains.

Quantitative data on the stabilizing effect of $CaCl_2$, guanidine hydrochloride, and dioxane on various constituent groups of proteins are virtually nonexistent, so that an attempt to be more quantitative about the composition of the contact site would be premature. $CaCl_2$, for example, may well react specifically with sites on protein molecules other than ionic groups, for it is difficult to explain the ability of $CaCl_2$ to cause at least partial unfolding of ribonuclease (Von Hippel and Wong, 1964) solely on the basis of stabilization of ionic groups. Conversely, dioxane, whose principal action is surely on hydrophobic groups, may also stabilize cationic groups (Grunwald *et al.*, 1960).

It is reasonably certain, however, that the contact site which is disrupted when half-molecules of hemoglobin are formed must be quite large. We may use the relation (Tanford, 1964b)

$$\Delta F_d^\circ = \Delta F_d^\circ{}_{,0.02\text{M NaCl}} + \Sigma \Delta f_{t,i} \qquad (5)$$

in which ΔF_d° represents the standard free energy of dissociation into half-molecules at any concentration of a disruptive reagent, and $\Delta f_{t,i}$ represents the contribution which any constituent group may be expected to make to the free energy of transfer of a molecule from 0.02 M NaCl to that concentration of disruptive reagent. The summation extends over all groups which are *newly exposed* by dissociation, i.e., in contact with solvent on the half-molecules but not on native hemoglobin.

Looking only at the effect of urea, for which reasonable estimates of $\Delta f_{t,i}$ may be made (Nozaki and Tanford, 1963), we see from Figure 6 that $\Sigma\Delta f_{t,i}$ is about −3.6 kcal/mole at a concentration of 6 M. The $\Delta f_{t,i}$ value for a single peptide group at that concentration is about −100 cal, that for an asparagine side chain is −330 cal, that for a histidine side chain is −200 cal. (Negative $\Delta f_{t,i}$ values of larger magnitude apply to aromtic side chains, but they are not expected to play an important role because of the ineffectiveness of organic solvents as dissociating agents.) It is evident that at least ten to twenty such groups must be present to account for the observed value of $\Sigma \Delta f_{t,i}$. In addition, ion pairs probably have to be located at the contact surface to account for the action of salts. It is not unreasonable to suppose that about forty groups may be involved in all, i.e., about ten per polypeptide chain.

It should be noted in conclusion that the extrapolated value of Δf_d°, applicable to zero concentration of added reagent, as obtained from Figure 6, is about 8.2 kcal/mole. This corresponds to a dissociation constant of 1.0×10^{-6} M, which would lead to a predicted degree of dissociation of about 0.06 in water or dilute salt solution, at a protein concentration of 0.4 g/100 cc. This figure has been used to estimate the value of the sedimentation coefficient of undissociated hemoglobin at that concentration, as reported here.

That human CO-hemoglobin in dilute aqueous salt solutions is partly dissociated at low concentrations has been suggested before, by Kegeles and Gutter (1951), on the basis of an anomalous concentration dependence of the sedimentation coefficient. We are able to account satisfactorily for their data (Figure 7) with a dissociation constant of 1×10^{-6} M, with $s_B = s_A(0.5)^{0.67}$, and with both s_B and s_A following a concentration dependence of the form $s = s^0(1 - kc)$ with $k = 5$ and c expressed in g/ml. This value of k is of the order of magnitude normally found for compact globular proteins. It is necessary to use a lower value of s_A than would be needed to describe our own data ($s_A^0 = 4.55$ S, rather than 4.7 S), for the reason that Kegeles and Gutter's experimental s values are about 0.15 S lower than ours under roughly identical experimental conditions, as was pointed out earlier.

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Effect of Side-Chain Deuteration on Protein Stability*

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ABSTRACT: Thermal denaturation of protio- and deuteriophycocyanin, isolated from the blue-green algae *Plectonema calothricoides*, *Phormidium luridum*, and *Synechococcus lividus*, grown in H_2O and D_2O, was studied in both H_2O and D_2O. The critical temperatures of thermal denaturation of deuteriophycocyanins were always significantly lower than those for protiophycocyanins, in both H_2O and D_2O solutions. The critical denaturation temperature was shown to be higher in D_2O than in H_2O for both protio- and deuteriophycocyanins. These results suggest that substitution of hydrogen atoms by deuterium in exchangeable positions stabilizes the protein conformation, whereas introduction of deuterium in nonexchangeable hydrogen positions appears to decrease nonpolar side-chain interactions.

The mass cultivation of organisms in which all of the hydrogen normally present is essentially completely replaced by deuterium (Crespi *et al.*, 1960; DaBoll *et al.*, 1962) has made possible a new experimental approach to the structure and function of a variety of organic compounds essential to life. Some results obtained with fully deuterated chloroplast pigments, carbohydrates, proteins, and nucleic acids (Strain *et al.*, 1961; Blake *et al.*, 1961; Berns *et al.*, 1963; Crespi and Katz, 1962; Crespi *et al.*, 1962) have already been reported. In our first investigation of deuterioprotein behavior (Berns *et al.*, 1963), deuteriophycocyanin[1] isolated from a deuterated blue-green alga, *Plectonema calothricoides*, was shown to undergo thermal denaturation at a significantly lower temperature than does (ordinary) protiophycocyanin.[1] Since these experiments were carried out in buffer made up with H_2O, it has been inferred that the observed difference in denaturation behavior of deuterio- and protiophycocyanin is probably due to differences in hydrophobic bonding in the protio- and deuterio- forms of phycocyanin. The present investigation was conducted to confirm our preliminary results, to gain further insight into the mechanism of thermal denaturation of proteins, and to explore the applicability of deuterioproteins to the study of the secondary and tertiary structure of proteins.

Pure samples of deuterio- and protiophycocyanin

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[1] The prefix "deuterio-" and the modifiers "fully deuterated" refer to phycocyanin containing 99.6% deuterium at all nonexchangeable positions. The prefix "protio-" refers to ordinary phycocyanin with hydrogen of mass 1 at all nonexchangeable positions.