remained bound to the polypeptides after acidic and enzymatic hydrolysis.

We are now able to say more about the difference between the two plasminogen forms. They are not only different in regard to their solubility but also in their activity against fibrin. Native plasminogen represents the labile form which can be easily and irreversibly transformed into the stabile pseudoglobulin form. This probably happens partially during the process of preparation. Each of the forms behaves as a uniform protein in electrophoresis and column chromatography.

The sedimentation data show that pseudoglobulin is present as a molecular aggregate at higher concentrations. At infinitely low concentrations both curves of sedimentation values extrapolate to the same point. This means that under these conditions the molecular weight of both forms is the same.

The viscosity curves do not extrapolate to the same value because the two forms, notwithstanding their identical molecular weight, have different shapes. The transformation of euglobulin to pseudoglobulin by acid treatment can be visualized as an opening of the structure in such a way that shape and size of the molecule are modified.

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The Dissociation of Hemoglobin by Inorganic Salts*

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The dissociation of hemoglobin into half-molecules by NaCl, $CaCl_2$, $MgCl_2$, and $(NH_4)_2SO_4$ has been studied by measurement of the sedimentation velocity as a function of salt concentration. It is established that dissociation is in fact responsible for the observed decrease in sedimentation velocity, and that the reaction shows the expected dependence on protein concentration. The dissociation is apparently unaffected by changes in the nature of the heme iron atom, in contrast to unfolding of the protein, which depends strongly on the state of the heme group. Thermodynamic data indicate that stabilization of the native four-chain structure in dilute salt solutions is predominantly entropic rather than energetic.

Hemoglobin may be dissociated into half-molecules by many reagents. A study of the relative effectiveness of some of these reagents has been initiated in this laboratory. The results of this study will be compared with the relative effectiveness of the same or similar reagents in producing unfolding of globular proteins and in solubilizing amino acids and small peptides. In this way it is hoped to determine what chemical groups are exposed in the dissociation of hemoglobin, and to determine what forces hold it together in its native state. This information can then be compared with similar knowledge gained from the study of the unfolding of a variety of proteins. It is to be expected that the forces which hold the folded polypeptide chains of hemoglobin together will not be unique, but that the same forces will be found to be important in maintaining the native structure of many globular proteins.

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The dissociation of hemoglobin may be brought about by urea (Steinhardt, 1936, 1938), which is also an effective unfolding agent for globular proteins in general. It may also, however, be brought about by NaCl and other inorganic salts, as was first noted by Pedersen and Andersson (1940). Such salts are not normally considered to be effective unfolding agents for globular proteins, and it is this aspect of the dissociation reaction which stimulated our interest, as it may expand our present concepts of the forces which are important in protein chemistry in general.

It was not part of the original plan to investigate the dissociation by salts, since investigations of this reaction have been carried out recently in a number of laboratories. These investigations have however produced what Antonini *et al.* (1962b) have called "a baffling thermodynamic paradox," which arises from studies of the equilibrium in the reaction of hemoglobin with oxygen. The character of the saturation curve at low salt concentrations requires that there be at least three binding sites per hemoglobin molecule, which is entirely compatible with the known presence of four binding sites on the native four-chain molecule. What is paradoxical is that this aspect of the saturation curve remains unchanged as the salt concentration is increased, despite the fact that dissociation occurs, leading to hemoglobin molecules which presumably can have only two binding sites per molecule. This paradox prompted us to reinvestigate the dissociation by NaCl and three other salts, with careful attention to the possibility that the physical data might be misleading, and might reflect changes in partial specific volume, interaction with salt, or some other molecular change not related to change in molecular weight.

An additional reason for reinvestigating the problem is that two of the three laboratories which have studied the salt dissociation of hemoglobin (Rossi-Fanelli *et al.*, 1961; Benesch *et al.*, 1962) report an anomalous dependence on hemoglobin concentration.

Experimental Procedure

Two kinds of hemoglobin were used in these studies. Human CO-hemoglobin was prepared from freshly drawn blood by a modification of the procedure of Steinhardt (1938). Bovine ferrihemoglobin was prepared by air oxidation of commercial bovine hemoglobin (California Corp. for Biochemical Research). The latter preparations always contained some material insoluble in water at *p*H 7, and this material was removed by centrifugation. Stock solutions of these proteins were prepared as needed, and their protein concentrations were determined by drying to constant weight at 107° or by measurement of optical density at 542 mμ (CO-hemoglobin) or 500 mμ (ferrihemoglobin). The *p*H of all stock solutions was adjusted to *p*H 6.8–6.9, which is close to the isoelectric *p*H of the proteins.

NaCl, $MgCl_2$, and $CaCl_2$ were Mallinckrodt analytical grade reagents, used without further purification. $(NH_4)_2SO_4$ was a preparation recrystallized from 10^{-3} M Versene.

Sedimentation velocities were measured in a Spinco Model E analytical ultracentrifuge, at 59,780 rpm, using schlieren optics. For CO-hemoglobin a Wratten No. 29 (red) filter was used in conjunction with Kodak 103-F spectroscopic plates, or a Wratten No. 16 (yellow) filter was used in conjunction with Kodak I-D spectroscopic plates. For ferrihemoglobin the No. 16 filter was used with Kodak metallographic plates. The plates were examined with a Gaertner two-dimensional microcomparator having a 3° rotational stage for lining up the plates.

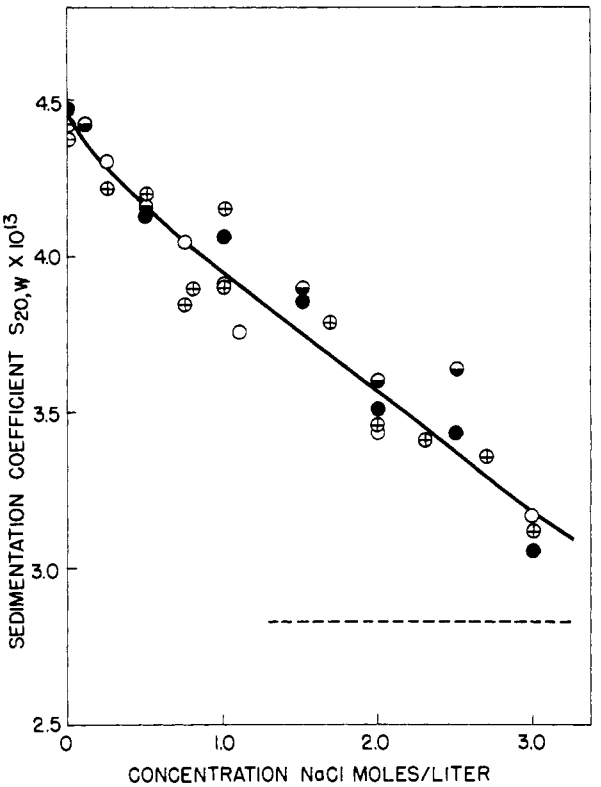

FIG. 1.—Sedimentation coefficients in aqueous NaCl solutions, for bovine ferrihemoglobin at 10° (○) and 25° (⊕), and for human CO-hemoglobin at 13° (●) and 25° (◒). The dashed line represents the theoretically expected sedimentation coefficient for completely dissociated hemoglobin.

A single peak was observed in all experiments. The sedimentation velocity was measured from the rate of movement of the point of maximum refractive index gradient, and it was taken to represent the weight-average sedimentation velocity at the protein concentration of the original solution. The theoretically correct procedure, in a situation where two kinds of molecules with different sedimentation rates are present, is to use the second-moment procedure of Goldberg (1953). The Goldberg procedure was used for a few experiments, but always gave a result identical to the simpler procedure employed for the bulk of the data. It should also be noted (Goldberg, 1953) that the measured sedimentation velocity is theoretically that which is characteristic of the concentration in the plateau region of the sedimentation cell, rather than the initial concentration. The required correction for radial dilution would decrease all protein concentrations reported below by about 5%, and would have no significant effect on the very approximate thermodynamic data which we have calculated.

Partial specific volume measurements were made pycnometrically, using pycnometers of the type proposed by Lipkin *et al.* (1944). Viscosity measurements were made with capillary viscometers.

Further details of the experimental procedure may be found in the dissertation of Kirshner (1963).

Results

Sedimentation coefficients determined in sodium chloride solutions, and corrected to the solvent properties of water at 20°, are shown in Figure 1. In order to interpret these data quantitatively it is necessary to consider all factors which affect the sedimentation velocity, and, in particular, to take account of the fact that we are dealing with a three-component system

(water, protein, salt, designated as components 1,2, and 3, respectively), in which interaction between solutes may occur. An equation which takes all such factors into consideration has been given by Baldwin (1958). It gives for the sedimentation coefficient of the protein component,[1]

[1] Strictly speaking, the equation is valid only at the limit of infinite dilution of the protein component. The data of Fig. 1 are all at close to the same protein concentration, and the concentration is quite low, so that equation (1) can be considered valid, with apparent values of the molecular weight and diffusion coefficients which may differ slightly from those at infinite dilution.

$$s = \frac{M_2(1 - \bar{v}_2\rho)D_{22}}{RT}(1 + A_{23}) \quad (1)$$

$$A_{23} = c_3 \frac{M_3(1 - \bar{v}_3\rho)}{M_2(1 - \bar{v}_2\rho)} \frac{(\partial D_{23}/\partial c_2)/D_{22} - (\partial \ln y_2/\partial c_3)}{1 + c_3(\partial \ln y_3/\partial c_3)} \quad (2)$$

In these equations M represents molecular weight; $\bar{v}$, partial specific volume; c, concentration in grams/cc; y, the activity coefficient on the mole/liter scale; ρ, the solution density; D_{22}, the diffusion coefficient of the protein in the absence of interacting flows (Gosting, 1956); and D_{2i}, the coefficient for diffusion of the protein as a result of interaction with the flow of the third component. The term A_{2i} includes all effects of interaction between the protein and the salt.

It happens that the interaction between NaCl and hemoglobin is conspicuously smaller than that between NaCl and other common proteins (Carr, 1953; Scatchard and Pigliacampi, 1962; Nozaki, 1959). We have therefore assumed the cross-diffusion term in equation (2) to be negligible. (It should also be noted that there is no known example for any protein where the effect of this term is important in diffusion measurement in salt solutions.) The interaction term A_{23} may then be rewritten (Peller, 1958)

$$A_{23} = \frac{M_3(1 - \bar{v}_3\rho)}{M_2(1 - \bar{v}_2\rho)} \left(\frac{\partial c_3}{\partial c_2}\right)_{\mu_3} \quad (3)$$

where $(\partial c_3/\partial c_2)_{\mu_3}$ represents in effect the preferential binding of salt or water to the protein. If salt is bound, the derivative is positive (c_3 must be increased with c_2 to keep μ_3 constant); if water is preferentially bound, i.e., if salt is excluded from the vicinity of the protein molecules, the derivative is negative.

It is known that the binding of NaCl to isoionic hemoglobin is not appreciable (Nozaki, 1959). There is little information on preferential hydration. Cox and Schumaker (1961) give a value of 0.1 g/g protein in CsCl solution. If the same figure applies to NaCl, it would lead to a value of -20 for $(\partial c_3/\partial c_2)_{\mu_3}$ and a value of -0.06 for A_{23} in 3 M NaCl (Kirshner, 1963). Since A_{23} would be expected to vary linearly with salt concentration, this means that its value would be almost negligibly small in the salt concentration range of Figure 1.

The partial specific volume of bovine ferrihemoglobin was measured at 25°. The values obtained were 0.748 and 0.752 cc/g, respectively, in 0.02 and 2.65 M NaCl. The precision was about ± 0.003, so that the variation of $\bar{v}_2$ with NaCl concentration is essentially negligible. Within our precision, our data agree with $\bar{v}_2$ values determined for horse CO-hemoglobin and ferrihemoglobin by Svedberg and Nichols (1927) and Adair and Adair (1947). These authors find a slight dependence of $\bar{v}_2$ on temperature, and Cox and Schumaker (1961) have observed a similar dependence for other proteins. In our calculations we have used $\bar{v}_2 = 0.745$ cc/g at 10° and $\bar{v}_2 = 0.751$ cc/g at 25° at all concentrations of NaCl.

Considering finally the term M_2D_{22}/RT of equation (1), we may write $D_{22}/RT = 1/Nf$, where N is Avogadro's number and f is the frictional coefficient for motion of the protein, in the absence of interacting flows, in water at 20°. The frictional coefficient is proportional to $M_2^{1/3}$ and to the ratio f/f_{min}, which expresses the deviation of the hydrodynamic behavior of the actual protein molecule from the behavior of an idealized unsolvated sphere (Tanford, 1961). In order to investigate the effect of salt concentration on f/f_{min}, measurements of relative viscosity were made, which led to the conclusion that the intrinsic viscosity (3.5 cc/g) is unaffected by the addition of NaCl to a concentration of 2.4 M. Since f/f_{min} and intrinsic viscosity depend on the same variables, it can be concluded that f/f_{min} also remains unchanged.

These results clearly show that M_2 can be the only factor of equation (1) (apart from solvent properties) which appreciably influences the sedimentation coefficient. A decrease in $s_{20,w}$ of a few per cent may be ascribable to a negative value for A_{23}, but the decrease which can be accounted for in this way cannot be much larger than the experimental uncertainty of the individual measurements, which Figure 1 shows to be quite large. We have thus interpreted the change in $s_{20,w}$ as resulting entirely from changes in molecular weight. If only two species are present, whole hemoglobin (species A) and half-molecules (species B), the measured sedimentation coefficient, as mentioned earlier, will represent a weight-average,

$$s_w = (1 - \alpha)s_A + \alpha s_B \quad (4)$$

where α is the weight fraction of hemoglobin in the dissociated form, and s_A and s_B are the sedimentation coefficients of the two species.

Moreover, if molecular weight is taken as the only variable affecting the sedimentation coefficient, s should vary as $M^{2/3}$. Setting the limiting value of $s_{20,w}$ at zero salt concentration equal to s_A, we get $s_A = 4.5 \times 10^{-13}$ and $s_B = 2.83 \times 10^{-13}$. The latter should be the limiting value of $s_{20,w}$ at salt concentrations high enough to lead to complete dissociation. (If preferential hydration is taken into account, both s_A and s_B would vary slightly with increasing salt concentration and the limiting value would drop to a value in the range of 2.6–2.7 $\times 10^{-13}$.) This calculation shows that complete dissociation to half-molecules has not been achieved in the experiments shown in Figure 1. A higher concentration of NaCl than 3 M could not be used to approach closer to complete dissociation because protein denaturation appears to set in above that concentration. Complete dissociation was observed in the experiments with $CaCl_2$ and $MgCl_2$ cited later (Fig. 3), and a limiting value of about 2.8×10^{-13} for $s_{20,w}$ was reached.

In agreement with the finding of Rossi-Fanelli *et al.* (1961), the dissociation by NaCl was found to be reversible. The sedimentation coefficients observed for hemoglobin solutions diluted from 2.7 M to 1 M NaCl and from 2.33 M to 0.75 M NaCl coincided with the values observed for solutions brought to 1 M or 0.75 M NaCl directly. As a consequence, the dissociation can be taken to represent chemical equilibrium, $A \rightleftharpoons 2B$, and can be described by the relation

$$K = \frac{[B]^2}{[A]} = \frac{4 \times 10^3}{M} \frac{\alpha^2 c_o}{1 - \alpha} \quad (5)$$

where K is the equilibrium constant (at a given concentration of NaCl) in moles/liter, M is the molecular weight of undissociated hemoglobin, c_o is the initial protein concentration in g/cc, and α (as in equation 4) is the weight fraction of hemoglobin in the dissociated form. The value of α can be obtained directly from the experimental data by means of equation (4),

$$\alpha = (s_A - \bar{s}_w)/(s_A - s_B) = (4.5 - \bar{s}_w \times 10^{13})/1.7 \quad (6)$$

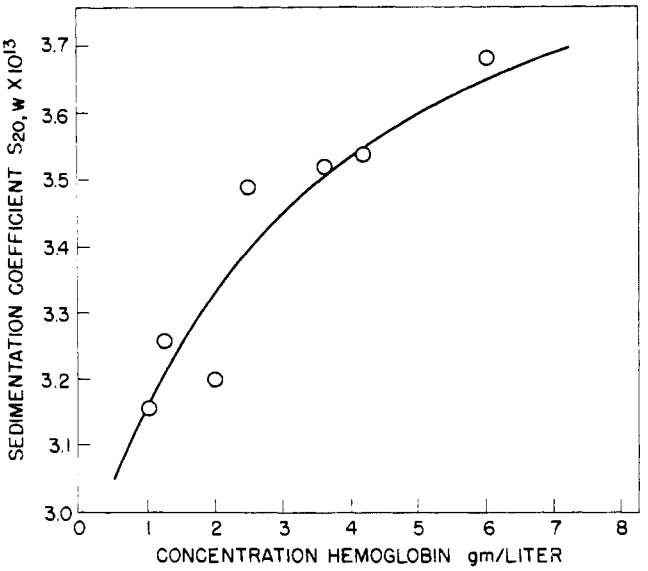

FIG. 2.—The effect of hemoglobin concentration on the sedimentation coefficient in 2 M NaCl at 25°. The line is a theoretical line corresponding to a dissociation constant of 1.9×10^{-4} mole/liter.

Rossi-Fanelli *et al.* (1961) and Benesch *et al.* (1962) have recently reported that equation (5) does not correctly describe the dependence of α on protein concentration at a constant concentration of NaCl. The former used light-scattering measurements to determine α, the latter used the Archibald approach to sedimentation equilibrium procedure. On the other hand, an earlier study by Benhamou *et al.* (1960), using both light scattering and sedimentation velocity, reported good agreement between the theoretical equation and the experimental data up to a protein concentration of about 0.3 g/100 cc. To check on this question we have studied the dependence of the sedimentation coefficient in detail at one concentration (2 M) of NaCl. The results are shown in Figure 2, together with a theoretical curve calculated from equation (5) with $K = 1.9 \times 10^{-4}$ mole/liter. In agreement with Benhamou *et al.* (1960), we find that the data are adequately described by equation (5).[2]

It is evident that the data of Figure 1 are not sufficiently precise to permit the estimation of accurate equilibrium constants for the dissociation. Even with better precision the data would be subject to error because of uncertainty in the limiting value of s at high salt concentration, and because the effect of protein concentration and of preferential hydration on s_A and s_B has been ignored. Approximate thermodynamic data can however be calculated, and values taken from a smoothed plot of these data are summarized in Table I.

There are two interesting features of the data of Figure 1 and Table I. (1) There is no significant difference between the results obtained with bovine ferrihemoglobin and those obtained with human CO-hemoglobin. In addition, our equilibrium constant for 1 M NaCl is the same as that reported by Benhamou *et al.* (1960) for human oxyhemoglobin. (2) There is no significant effect of temperature within the range studied. This means that ΔH for the dissociation is zero, although it should be pointed out that the low precision of our data leads to an uncertainty of the order of 5 kcal/mole in the value of ΔH. Despite this uncertainty, it is evident that a major part of the reason for the stability of the four-chain hemoglobin molecule in dilute salt solution results from a negative unitary entropy change which accompanies the dissociation into half molecules.

It may be noted that the same conclusions apply to the acid dissociation of hemoglobin. We have examined bovine ferrihemoglobin in dilute salt solution at *p*H 5.1, and observed, at 25°, $s_{20,w} = 3.33 \times 10^{-13}$, corresponding to $\alpha = 0.69$, which is in the same range as was found for human CO-hemoglobin in dilute buffers

[2] T. Hooker and N. Pace, in this laboratory, have recently repeated the experiments of Fig. 2. Their results confirm the data shown in the figure with a slightly larger value of K than that derived from the data given here. They have also obtained data at hemoglobin concentrations higher than those of Fig. 2, and have observed definite deviations (toward lower sedimentation coefficients) from the results expected from equation (5). New experiments by E. Antonini (personal communication) are in agreement with these results.

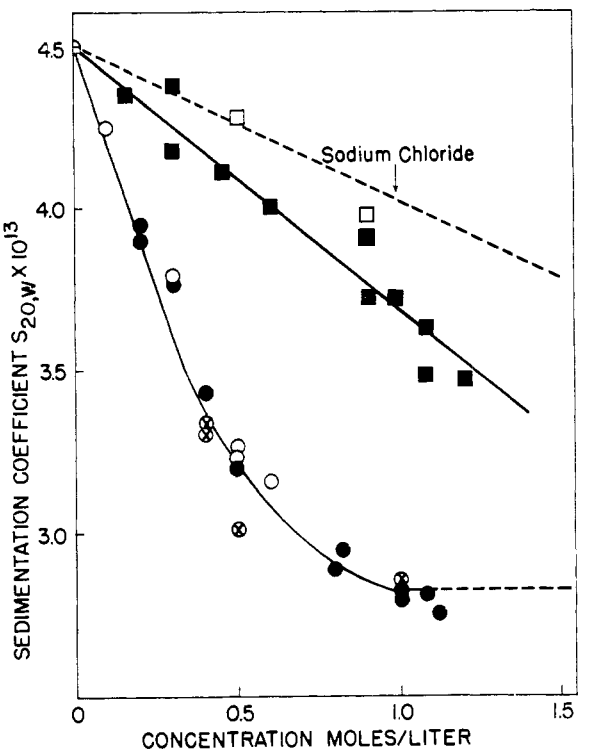

FIG. 3.—Sedimentation coefficients in solutions of various salts, at 25°. Human CO-hemoglobin in $MgCl_2$ (●), $CaCl_2$ (⊗), and $(NH_4)_2SO_4$ (■). Bovine ferrihemoglobin in $MgCl_2$ (○) and $(NH_4)_2SO_4$ (□).

TABLE I
THERMODYNAMIC DATA FOR THE DISSOCIATION OF HEMOGLOBINS BY NaCl[a]

NaCl Concn (moles/liter)	$K \times 10^5$ (moles/liter)	$\Delta F°$ (kcal/mole)	ΔF_u[b] (kcal/mole)	ΔS_u[b,c] (Gibbs/mole)
0.02[d]	0.25	7.6	10.0	−34
1.0	3	6.1	8.5	−29
2.0	18	5.1	7.5	−26
3.0	60	4.4	6.8	−23

[a] The data obtained for bovine ferrihemoglobin and human CO-hemoglobin did not differ significantly. Our value of K at 1 M NaCl is the same as that obtained by Benhamou *et al.* (1960) for human oxyhemoglobin. [b] ΔF_u and ΔS_u represent *unitary* free energy and entropy changes, respectively. See Kauzmann (1959). [c] The probable uncertainty in ΔS_u is ±17 Gibbs/mole. [d] NaCl (0.02 M) was the lowest concentration of salt employed. The data given are extrapolated, since no dissociation can be detected experimentally at this salt concentration.

TABLE II
THERMODYNAMIC DATA FOR THE DISSOCIATION OF HEMOGLOBINS BY $CaCl_2$ OR $MgCl_2$

Salt Concn (moles/liter)	$K \times 10^5$ (moles/liter)	ΔF° (kcal/mole)	ΔF_u (kcal/mole)
0.01	0.25	7.6	10.0
0.25	6	5.7	8.1
0.50	70	4.3	6.5

at that *p*H by Field and O'Brien (1955). We then examined the same solution at 10° and observed no change in $s_{20,w}$. Since the heat of ionization of the groups which ionize near *p*H 5 is essentially zero, this means that ΔH for the dissociation reaction must also be essentially zero. The same result has been reported previously by Hasserodt and Vinograd (1959).

The dissociation was also studied using MgCl and $CaCl_2$ as dissociating agents. These salts proved more effective than NaCl, and the studies were performed primarily so as to verify the prediction made above, which was that the sedimentation coefficient should level off at value near a 2.8×10^{-13}, corresponding to complete dissociation into half-molecules. The results are shown in Figure 3 and they do confirm the prediction.

Figure 3 also shows that $MgCl_2$ and $CaCl_2$ are essentially indistinguishable, and the thermodynamic data calculated from Figure 3 in Table II, show that these salts are about five times as effective as NaCl in producing dissociation. Parallel studies using $(NH_4)_2SO_4$ are also shown in Figure 3. This salt is seen to be somewhat more effective per mole than NaCl, but much less effective than $MgCl_2$ or $CaCl_2$. Since $(NH_4)_2SO_4$ is probably considerably less dissociated than $MgCl_2$ or $CaCl_2$,[3] this leaves open the possibility that the dissociation is predominantly a nonspecific effect of ionic strength. However, the difference between NaCl and $MgCl_2$ or $CaCl_2$ is somewhat too great to be accounted for on the basis of ionic strength alone.

DISCUSSION

This paper has presented new data concerning the dissociation of approximately isoionic hemoglobin by NaCl and other inorganic salts. Although the dissociation at low salt concentrations (0.01–0.1 M NaCl) is too small to be measured with any accuracy, the data obtained at higher salt concentrations can be extrapolated to yield thermodynamic data applicable to conditions where little salt is present. The standard free energy change (standard state is 1 mole/liter) for the dissociation near 25° was found to be about 7500 cal/mole (Table I). To interpret this figure, and its change with salt concentration, it is better to use mole fraction units for the concentration and to divide the free energy into *cratic* and *unitary* contributions, as proposed by Gurney (1953) and Kauzmann (1959). The cratic part of the free energy represents the entropy of mixing, which always favors dissociation. For the dissociation of hemoglobin, at a concentration of 4 g/liter, the cratic part of the free energy is −8000 cal/mole. It represents the driving force for dissociation, and it is also the part which leads to the dependence on protein concentration, for it becomes more negative as the protein concentration decreases. However, this part of the free energy cannot be affected by the addition of salts or other reagents, at constant weight or volume concentration of hemoglobin, except so far as the mole fraction of protein is altered. In the data reported here the change in mole fraction under these conditions is negligibly small.

The unitary part of the free energy of dissociation represents the part due to breaking of chemical bonds, and to contacts with the solvent. Its value was found to be +10,000 cal/mole per mole in dilute aqueous salt solution, i.e., it is the part which opposes the dissociation. It becomes less positive as salt is added, permitting dissociation to occur. It is interesting to note that the heat of dissociation is approximately zero (within a large uncertainty of ±5000 cal/mole), so that a major contribution to the unitary free energy change must come from a negative unitary entropy change, of the order of −30 Gibbs/mole, in dilute salt solution. An interpretation of this figure cannot be made at present, but will have to wait until the effectiveness of a variety of other substances on the dissociation has been determined. A negative unitary entropy of dissociation is often interpreted as indicating that the products of dissociation are more highly hydrated than the parent molecule. In the case of hemoglobin it might be that dissociation involves a separation of charges, with accompanying increase in hydration, in which event an increase in ionic strength would be one of the methods by which dissociation would be expected to be favored. When the dissociation is brought about by the addition of acid or base, the separated charges might be stabilized by conversion to their uncharged forms ($-COO^- \rightarrow -COOH$ in acid dissociation, $-NH_3^+ \rightarrow -NH_2$ in alkaline dissociation). Alternatively, it might simply be the decrease in overall electrostatic free energy on dissociation which provides the major driving force for acid or base dissociation.[4]

An interesting feature of the dissociation by salt and by acid (and presumably by alkali) is that bovine ferrihemoglobin and human CO-hemoglobin have essentially indistinguishable dissociation curves. Comparison of our data with those of Benhamou *et al.* (1960) indicates that human oxyhemoglobin also has closely similar dissociation properties. Rossi-Fanelli *et al.* (1961) have found that human reduced hemoglobin and oxyhemoglobin have very similar dissociation properties, though Benesch *et al.* (1962) found a small difference between them. It appears from these results that the state of the heme iron atom has little or no influence on the dissociation into half molecules, in sharp contrast to results obtained from denaturation studies, which show that ferrihemoglobin, CO-hemoglobin, and oxyhemoglobin differ greatly in their stability (as folded globular structures) toward acid (Steinhardt *et al.*, 1962) and urea (Simko, 1955). It should be noted also that there is evidence for species differences in the tendency for dissociation (Wu and Yang, 1932).

In conclusion, we should make reference to two kinds of anomalies which have been reported on the subject of the dissociation of hemoglobin by inorganic salts. One of these is the observation by Rossi-Fanelli *et al.* (1961) that, at high concentrations of salt, where the protein is physically almost entirely dissociated, the binding of

[3] At a concentration of unit molality at 25°, Robinson and Stokes (1955) list mean ionic activity coefficients of 0.570 and 0.500, respectively, for $MgCl_2$ and $CaCl_2$, as compared to a value of 0.196 for $(NH_4)_2SO_4$.

[4] The parent molecule contains two α polypeptide chains and two β polypeptide chains. We have learned from Dr. J. Vinograd that dissociation by acid, base, or salt probably yields molecules of the form $\alpha\beta$. Evidence for this is provided by the fact that physical dissociation occurs very rapidly, whereas hybridization (Vinograd *et al.*, 1959, Huehns and Beaven, 1963), which must occur through formation of molecules of the form α_2 and β_2, is a slow process, at least in acid and neutral solutions. A discussion of this question has been given by Antonini *et al.* (1962a).

oxygen depends on oxygen pressure in such a way as to require a protein molecule with three or more binding sites, whereas the hemoglobin half-molecule can contain only two. A possible explanation for this anomaly might have been that the physical observations were incorrect: that what was interpreted as a change in molecular weight was in fact a change in some other physical property, such as partial specific volume, frictional coefficient ratio, etc. The data of this paper eliminate such possibilities. It is true that the elimination of preferential hydration as a factor rests on rather meager direct evidence, namely, the value of 0.1 g/g protein found by Cox and Schumaker (1961) in aqueous CsCl solutions. However, a preferential hydration of about 0.6 g/g protein would be needed to account for the total drop in sedimentation coefficient observed in NaCl, and an even larger value (0.9 g/g protein) would be needed to account for the observed results with $MgCl_2$ and $CaCl_2$ (Kirshner, 1963). Preferential hydration of this order of magnitude is exceedingly unlikely. Moreover, if preferential hydration were the cause of the decrease in sedimentation coefficient, it would be expected that the sedimentation coefficient would decrease essentially linearly with increasing salt concentration. The fact that the data of Figure 3 tend to level off, essentially at the predicted value of the sedimentation coefficient, is good indirect evidence that preferential hydration is not the cause of the observed effect. It should be noted in this connection that the limiting value of $s_{20,w} \simeq 2.8 \times 10^{-13}$ seen in Figure 3 the same as that observed for the dissociation of hemoglobin by formamide,[5] and close to the same as is observed for alkaline dissociation (Hasserodt and Vinograd, 1959).

The second reported anomaly (Rossi-Fanelli *et al.*, 1961, Benesch *et al.*, 1962) is that the dissociation reaction, although apparently reversible, does not obey the normal equation for dependence on protein concentration. This anomaly was not observed by Benhamou *et al.* (1960), and, in agreement with these workers, we also find no anomalous behavior with respect to the concentration dependence, at least to a protein concentration of 0.7 g/100 cc.

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[5] Work in progress in this laboratory.