The Dissociation of Hemoglobins A and H in Concentrated Sodium Chloride*

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Previous observations that deoxyhemoglobin is less dissociated than oxyhemoglobin in concentrated salt solutions have been confirmed and extended. The difference in molecular weight between the two forms of the protein is larger at lower temperatures. Hemoglobin H was found to be far more resistant to dissociation by salt than was hemoglobin A. These findings become significant in the light of the fundamental difference in the oxygenation behavior of hemoglobins A and H.

Since human hemoglobin is a tetramer in which four polypeptide chains, identical in pairs, are held together by noncovalent bonds, a study of its dissociation into subunits is of considerable interest. Certain reactions of hemoglobin definitely involve such a dissociation as for example the exchange of chains between different hemoglobins to produce hybrid molecules. From a physiological point of view the most significant reaction of hemoglobin is its reversible combination with oxygen. An increasing amount of evidence has now accumulated to show that this reaction is accompanied by a significant conformational change in the whole protein (Benesch and Benesch, 1963). This evidence includes differences in solubility and crystal structure (Bragg and Perutz, 1952; Haurowitz, 1938; Jope, 1949; Perutz and Mitchison, 1950), in the binding of small molecules (Antonini *et al.*, 1963), in the reactivity of certain —SH groups (Benesch and Benesch, 1962; Huisman and Dozy, 1962; Riggs, 1961), in the susceptibility to proteolysis (Ottesen and Schroeder, 1961), and in the tendency to dissociate into subunits (Benesch *et al.*, 1962; Ranney *et al.*, 1963). Kinetic evidence for conformational change associated with ligand binding has been obtained by Gibson (1959).

It is likely that the two conformational isomers differ not so much in the conformation of the individual subunits as in the relative arrangement of the subunits in relation to one another (Muirhead and Perutz, 1963). Perhaps the simplest mechanism by which such a rearrangement of subunits could be achieved is by their dissociation and reassociation during oxygenation. It is fascinating to discover that Douglas and coworkers as early as 1912 published a detailed analysis of the oxygen-equilibrium curve of hemoglobin in terms of an aggregation and disaggregation equilibrium (Douglas *et al.*, 1912). A reversible polymerization during the oxygenation process has actually been demonstrated in the case of lamprey hemoglobin (Briehl, 1963; Rumen and Love, 1963).

The effect of concentrated salt solutions on human hemoglobin has been studied by several workers (Adair, 1928; Benesch *et al.*, 1962; Benhamou *et al.*, 1960; Benhamou and Weill, 1958; Guidotti and Craig, 1963; Gutfreund, 1949; Rossi-Fanelli *et al.*, 1961a; Svedberg and Pedersen, 1940). All these investigations resulted in the conclusion that the molecular weight of hemoglobin decreases progressively in solutions of increasing salt concentrations.

Rossi-Fanelli and co-workers were the first to compare the molecular weights of oxy- and deoxyhemoglobin in concentrated salt solutions (Rossi-Fanelli *et al.*, 1961a). They concluded that both forms of the protein were split to about the same extent. Our investigation of the molecular weights of oxy- and deoxyhemoglobin in 2 M sodium chloride by the Archibald method had revealed small but consistent differences between the two forms of the protein (Benesch *et al.*, 1962). The fact that deoxyhemoglobin was split less completely under these conditions is consistent with the concept that the interaction between subunits is stronger in the deoxygenated state.

Hybridization experiments have also shown (Ranney *et al.*, 1963) that in deoxyhemoglobin the tendency for the molecule to dissociate into subunits is very much smaller than in oxyhemoglobin.

The results reported below confirm our previous conclusion that the differential effect of 2 M sodium chloride on the molecular weights of oxy- and deoxyhemoglobin is indeed highly significant.

EXPERIMENTAL

Preparation of Hemoglobin Solutions.—Hemoglobin A was prepared as described previously (Benesch and Benesch, 1962) from the blood of two normal male donors. Solutions were stored at 5° and discarded after 10 days.

The samples of hemoglobin H were kindly donated to us by Dr. Helen M. Ranney after isolation by starch-block electrophoresis and concentration by ultrafiltration as described earlier (Benesch *et al.*, 1961).

Hemoglobin concentration was determined spectrophotometrically at 540 mμ after conversion to methemoglobin cyanide using an extinction coefficient of 4.6×10^4. All experiments were carried out with hemoglobin concentrations in the range of 0.8–1%. For each determination, solutions of hemoglobin and of salt, each at twice the final concentration, were separately equilibriated with the appropriate gas or gas mixture. The gases used were: (1) *For the preparation of deoxyhemoglobin samples:* (a) prepurified nitrogen passed through a train of vanadous sulfate (Meites and Meites, 1948), followed by sodium hydroxide solution,[1] and finally water; (b) 95% nitrogen, 5% carbon dioxide, saturated with water. (2) *For the preparation of oxyhemoglobin samples:* (a) 100% oxygen saturated with water, and (b) 95% oxygen 5% carbon dioxide saturated with water. When carbon dioxide–containing gas mixtures were used, the salt solutions also contained $NaHCO_3$ to give a final concentration of 0.007 M.

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[1] In the course of this work we found that quite significant amounts of SO_2 are formed in the vanadium reagent on standing. The sulfite undoubtedly arises from the reduction of sulfate by vanadous ion but it can easily be removed by passage of the gas stream through sodium hydroxide solution.

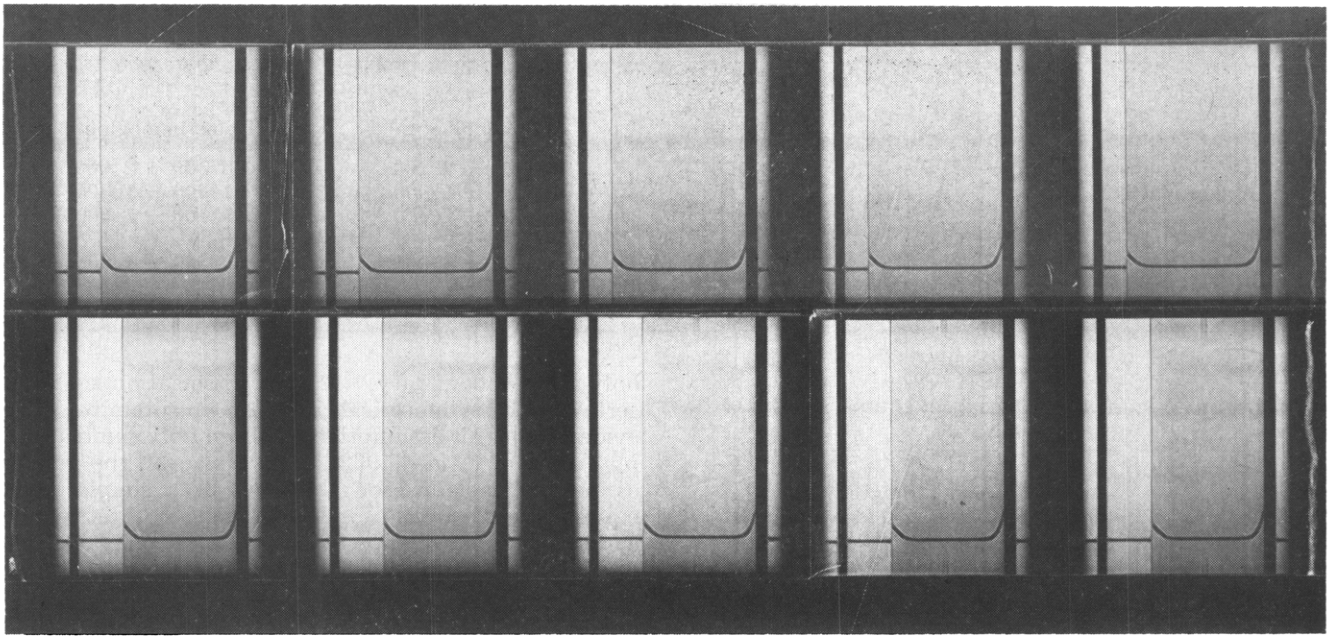

FIG. 1.—Representative ultracentrifuge patterns used for the molecular weight determination by the Archibald method. Upper photographs, oxyhemoglobin; lower photographs, deoxyhemoglobin. Hemoglobin concentration, 0.92%; sodium chloride, 2.0 M; rotor speed, 12.590 rpm; rotor temperature, 5°, bar angle, 80°. Photographs taken at 96, 104, 112, 120, and 128 minutes after attainment of top speed.

Gas equilibrations were carried out at room temperature and at 5° for the runs at 20 and 5°, respectively.

After equilibration the solutions were mixed anaerobically and transferred to the ultracentrifuge cell in a similar manner to that described before (Benesch *et al.*, 1962). In the case of the deoxyhemoglobin solutions, great care was again taken to ensure complete deoxygenation of both hemoglobin and salt solutions before mixing in order to prevent oxidation to methemoglobin. Completeness of deoxygenation was again determined spectrophotometrically in the ultracentrifuge cells as previously described (Benesch *et al.*, 1962).

Molecular weights were determined in the Spinco Model E analytical ultracentrifuge by the Archibald method as described by Schachman (1957). The optical system was modified by substituting a 5-mil platinum wire for the phase plate at the suggestion of Dr. P. von Hippel, who kindly supplied us with the mounted platinum wire.

All other details pertaining to the method of measuring the molecular weights were identical with those described previously (Benesch *et al.*, 1962). Unfortunately it was not found possible to measure concentration gradients at the bottom of the cell since all attempts to form a bottom boundary with an immiscible organic liquid resulted in considerable denaturation at the interface in the case of these proteins.

Most of the results in 2 M sodium chloride were obtained with a speed of 12,590 rpm and photographs were taken at 8- or 16-minute intervals with a bar angle of 80°. Photographs of a pair of typical runs are shown in Figure 1. Molecular weights were calculated from at least four and usually five frames to obtain an average value, since no significant trend of the molecular weight with time was seen in any of the experiments. Each of the values reported represents the average of at least two such average molecular weights obtained from duplicate runs with separate samples. The variation shown in Tables I and II represents the maximum deviation of any of the measured values from the average given. After the equilibrium runs the samples were sedimented at 55,000 rpm. Only a single sedimenting boundary was present in every case.

RESULTS

The results obtained with hemoglobin A are shown in Table I. In dilute phosphate buffer the molecular weights of oxy- and deoxyhemoglobin show no significant difference and agree with the expected value for the tetramer. The results on oxyhemoglobin in 2 M sodium chloride again confirm the fact that under these conditions extensive dissociation of the molecule occurs. At 20° good agreement with our previously reported value (Benesch *et al.*, 1962) was obtained. The dissociation has a significant temperature coefficient as can be seen from the higher value of the molecular weight at 5°. A slight inhibition of the dissociation by carbon dioxide is evident at both temperatures.

The major conclusion of our previous work, i.e., that the dissociating effect of strong salt solutions is significantly smaller on deoxy- than on oxyhemoglobin is confirmed by the results at 20°. However, since the temperature coefficient is larger for deoxy- than for oxyhemoglobin, as can be seen from Table I, the difference between the two forms of the protein becomes much greater at 5° than at 20°. The difference between the molecular weights of oxy- and deoxyhemoglobin in 2 M sodium chloride *at 5°* is thus highly significant and entirely outside the range of experimental error. This differential susceptibility to salt splitting can be regarded as evidence for the existence of stronger and perhaps different interactions between the subunits in deoxy- as compared with oxyhemoglobin. These results therefore bear out the prediction, made over 50 years ago by Haldane and his collaborators (Douglas *et al.*, 1912), that the oxygen-equilibrium curve can be accounted for "*by assuming sufficient proportions of aggregation among the molecules of oxyhemoglobin and reduced haemoglobin respectively . . . and by assuming that the percentage of aggregation is greater*

TABLE I
MOLECULAR WEIGHTS OF HEMOGLOBIN A

Solvent	Temperature (°C)	Molecular Weights Oxyhemoglobin	Deoxyhemoglobin
0.05 M phosphate buffer, *p*H 7.0	20	67.600 ± 2.200	66.500 ± 2.000
2.0 M NaCl	5	41.700 ± 1.000	51.600 ± 1.300
2.0 M NaCl	20	38.300 ± 1.300	45.100 ± 1.500
2.0 M NaCl/0.007 M $NaHCO_2$ 5% CO_2 in gas phase	5	42.300 ± 1.000	50.500 ± 1.000
2.0 M NaCl/0.007 M $NaHCO_2$ 5% CO_2 in gas phase	20	40.700 ± 1.000	47.300 ± 1.600

TABLE II
MOLECULAR WEIGHTS OF OXYHEMOGLOBINS A AND H IN 2.0 M NaCl

Temperature (°C)	Molecular Weights Hemoglobin A	Hemoglobin H
5	41.700 ± 1.000	58.300 ± 900
6		58.900 ± 1.200
20	38.300 ± 1.300	55.700 ± 700

among the molecules of reduced haemoglobin than among those of oxyhaemoglobin."[2]

In this connection it is pertinent to recall the results of Rossi-Fanelli *et al.* (1961a,b), i.e., that human hemoglobin is dissociated into half-molecules in solvents of high ionic strength, but that the value of n for the oxygen equilibrium remains significantly greater than 2. Our result that deoxyhemoglobin is more aggregated than oxyhemoglobin in 2 M sodium chloride suggests an explanation for this paradox. This would then be analogous to the situation in acid solution where a similar difference in aggregation between the oxygenated and deoxygenated subunits was found by the Rome group (Rossi-Fanelli *et al.*, 1961c; Wyman *et al.*, 1961).

The most unexpected result of these investigations is shown in Table II. It is clear that the dissociating effect of 2 M sodium chloride is drastically decreased in the case of hemoglobin H. The greater resistance of hemoglobin H to salt splitting is particularly surprising in view of its great instability in other respects. Attempts to measure the molecular weight of deoxyhemoglobin H in 2 M sodium chloride were unsuccessful, since at 20° in this medium the deoxygenated form is too susceptible to oxidation and hence denaturation, and at 5° the enormous oxygen affinity made complete deoxygenation impossible. Incompletely deoxygenated samples at 5° gave results very similar to those obtained for oxyhemoglobin H under the same conditions.

DISCUSSION

It should be emphasized that the method used for the molecular weight determinations in this study is an equilibrium one and does not reflect the shape of the particles in solution but only their weight. There can therefore be no doubt that high salt concentrations cause a real dissociation of the molecule. Unfortunately the situation is not as clear with respect to the identity of the fragments formed. Several points require comment: (1) the dissociation must be rapid compared with the rate of centrifugation since we observed no time-dependence of the molecular weight and since sedimentation velocity experiments showed only a single boundary. (2) The dissociation is almost certain to be symmetrical, i.e., into $\alpha\beta$ subunits. The evidence for this assumption has been ably summarized by Antonini *et al.* (1962). (3) However the results cannot be explained on the basis of a simple rapid equilibrium between $\alpha\beta$ dimers and $\alpha_2\beta_2$ tetramers, since neither our own previous investigations nor those of other workers have shown the required dependence of the observed molecular weight on the total hemoglobin concentration. For this reason equilibrium constants and enthalpies for the dissociation were not calculated from these results.

The finding that hemoglobin H is remarkably resistant to salt splitting deserves special comment. As was mentioned in the introduction, it is now virtually certain that the reversible oxygenation reaction of hemoglobin A is associated with a change of one conformational isomer into another and this transformation is likely to involve a dissociation and association of the subunits. Since it has now been conclusively demonstrated (Perutz and Mazzarella, 1963; Benesch *et al.*, 1964) that hemoglobin H combines with oxygen without changes in conformation, it is very interesting to note that this hemoglobin exhibits very little tendency to dissociate reversibly into subunits in the presence of high salt.

We wish to thank Dr. Helen M. Ranney for providing us with the purified hemoglobin H used for these investigations.

[2] The "molecules" in this quotation are, of course, meant to be single heme subunits, since the authors were unaware of the tetrameric nature of hemoglobin at that time.

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The Effect of Neutral Salts on the Melting Temperature and Regeneration Kinetics of the Ordered Collagen Structure*

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The experimental data reported recently by P. H. Von Hippel and K. Y. Wong on the effect of neutral salts on the thermodynamic stability of the ordered collagen structure and on the rate of formation of this structure from the random-coil form has been analyzed. The importance of the structure of water notwithstanding, it is shown, contrary to previous conclusions, that both types of data can be quantitatively explained by the direct binding of ions.

In a recent series of papers Von Hippel and Wong (1962, 1963a,b) have reported the results of studies on the effect of a variety of neutral salts both on the thermodynamic stability of various collagens in dilute aqueous solutions and on the rate at which the ordered structure is regenerated from the disordered one in the same medium. The salts utilized are well known to be capable of transforming all the ordered polypeptides and proteins irrespective of crystallographic structure and amino and imino acid compositions (Mandelkern *et al.*, 1962a,b,c). This ability to disrupt the ordered structure has also been noted in nonaqueous as well as in aqueous media (Mandelkern *et al.*, 1963). The experimental results were explained on the basis that the stability of the ordered protein structure was influenced by the neutral salts through the competitive reorganization of the water that is postulated to be required for the stabilization of the collagen-type helix. The possibility that the results could be explained by the direct binding of ions to the peptide linkages of the protein molecule was considered and dismissed. The refutation of a binding mechanism appears to be based on the fact that the experimental data can be well represented by the empirical relation

$$T_m = T_m^* + K_m \qquad (1)$$

where T_m^* is the melting temperature at a fixed protein concentration in the absence of added salt, T_m is the melting temperature for a salt concentration of molarity M, and K is an experimentally determined constant which is different for each salt. Besides the fact that this relation was obeyed, a binding mechanism was further eliminated because it was observed that for a given salt the constant K was independent of the total protein concentration. These conclusions have been seriously questioned by Bello (1963), who offered qualitative arguments for the serious consideration of a binding mechanism's being involved in the melting process. A major misconception appears to be the supposition that the effect of binding on the melting temperature must be dependent on the total polymer concentration.

The general importance of the structure of water notwithstanding, we wish to point out that most of the experimental data (apart from the two special cases where the melting temperature appears to increase with added salt) can be explained quantitatively by invoking a classical binding mechanism. Equation (1) turns out to be a very good approximation to the more general expression that is applicable. Furthermore, it can be shown that the relative rate at which the ordered collagen structure is regenerated in various salt solutions can be quantitatively explained by utilizing the mechanism proposed by Flory and Weaver (1960) for the similar process in the absence of the added salt.

Following the developments of Schellman (1955) and of Flory (1957), the melting point depression of a pure polymer, when binding occurs, can be expressed as

$$\frac{1}{T_m} = \frac{1}{T^\circ_m} = \frac{R}{\Delta H_u}\frac{V_u}{V_1}(v_1 - \chi_1 v_1^2) + \frac{R}{\Delta H_u} N_A \ln (1 + ka_c) \qquad (2)$$

Here T_m° is the melting temperature of the pure undiluted polymer, T_m is the melting temperature at volume fraction v_1 of solvent, and ΔH_u is the heat of fusion per chain repeating unit, V_u and V_1 are the molar volumes of the repeating unit and solvent, respectively, and χ_1 is the thermodynamic interaction parameter between polymer segments and solvent. The mole

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