THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 236, No. 2, February 1961
Printed in U.S.A.

Studies on the Relations between Molecular and Functional Properties of Hemoglobin

I. THE EFFECT OF SALTS ON THE MOLECULAR WEIGHT OF HUMAN HEMOGLOBIN

ALESSANDRO ROSSI-FANELLI, ERALDO ANTONINI, AND ANTONIO CAPUTO

From the Institute of Biochemistry, University of Rome and Regina Elena Institute for Cancer Research, Rome, Italy

(Received for publication, June 27, 1960)

Since the classic work of Adair (1), the molecular weight of mammalian hemoglobins, in neutral solutions and at moderate protein concentration, has been considered to be 68,000, 4 times the minimal molecular weight calculated on the basis of the iron content. This corresponds to the presence of four oxygen-combining centers per molecule, and the functional properties of the pigment have been, accordingly, evaluated on the basis of these four centers (2). However, observations indicating a change in the molecular weight of hemoglobin in neutral solutions containing various other components have been reported in the literature (2). Of particular interest in this respect is the effect of high concentrations of NaCl, which have been stated by several authors (3–6) to produce a splitting of the oxygenated molecule into subunits of about half the normal molecular weight. However, as we have already pointed out (7), in strong salt solutions there is an apparent inconsistency between the figures observed for the molecular weight of hemoglobin and the character of the oxygen equilibrium, if we accept the classic view regarding the latter.

It seemed, therefore, of the greatest importance to undertake a systematic investigation, under comparable conditions, of the effect of strong salt solutions on the molecular weight and on the oxygen equilibrium of hemoglobin and to extend the molecular weight determinations to the deoxygenated derivative. This appeared to us to be especially important because one group of investigators, namely Putzeys *et al.* (8), failed to observe the splitting found by all the others. Moreover, all the previous investigations have been performed with the oxygen or carbon monoxide derivatives of hemoglobin.

In this paper, we report light scattering and sedimentation data on oxyhemoglobin and hemoglobin at pH near the isoionic point, at ionic strengths ranging from 0.05–0.1 to 3. In the next paper of the series, we report companion data on the oxygen equilibrium of the pigment under the same conditions. In a third paper, we shall discuss some of the implications of the results. Still later we plan to present the results of similar studies under other conditions.

EXPERIMENTAL PROCEDURE

Hemoglobin Solutions—Human hemoglobin was prepared from freshly drawn blood. The cells were washed repeatedly with 1% NaCl solution and hemolyzed by the addition of 3 to 4 volumes of distilled water.

The hemoglobin solution was then brought to 20% saturation with neutralized ammonium sulfate (10), left standing for about 15 minutes, and centrifuged at 20,000 × *g* for 1 hour at 2°. The precipitate which formed was discarded.

To remove the salts, the hemoglobin solution was dialyzed in the cold, with gentle mechanical agitation, against several changes of deionized water for 48 to 72 hours. At the end of this time, no sulfate ions could be detected in the solutions, the ionic strength of which was estimated by conductivity measurements to be less than 1×10^{-4}.

The pH of the hemoglobin solution after the dialysis was 6.8 ± 0.1; the pigment did not contain appreciable amounts of the ferric form. In all cases, the experiments were performed within a week from the end of the preparation. In the meantime, the solutions were stored at 2°; no significant change in any of their properties was observed within this time.

Seven hemoglobin preparations from three different persons (B. G., L. B., I. V.) were used in the course of the work.

Light Scattering Measurements—The different hemoglobin derivatives (Hb, HbO_2, HbCO, Hb^+) all have very large absorption coefficients in the visible and near ultraviolet, and it is impossible to make light scattering measurements at the usual wave lengths (436 and 546 mμ). Light scattering measurements on these pigments must therefore be performed in the red region of the spectrum, where the absorption coefficients are low. The procedure adopted here was to use a wide band of red light. The absolute values of molecular weight were obtained from the light scattering data with the aid of reference measurements on serum albumin solutions.

The experimental arrangement used was briefly as follows. The normal lamp house of the Brice-Phoenix photometer (11), carrying the mercury lamp and the filters, was removed and replaced by a slide projector (Leitz Prado 250), equipped with a 200 W tungsten lamp. A red glass Coleman filter was mounted in front of the shutter collimating tube of the photometer. Fig. 1 shows the transmission of the filter and of 0.5% HbO_2 and Hb solutions (1-cm light path) in the red region of the spectrum. The light obtained from the tungsten lamp with the red filter which covers an appreciable band of wave lengths will be denoted by λ (red). The transmission of the neutral filters in the apparatus and the linearity of the response of the instrument were checked again under the new experimental conditions. With this simple arrangement, the scattering of HbO_2 and Hb solutions can be measured easily in the usual protein concentration range, say from 0.05 to 1.0%.

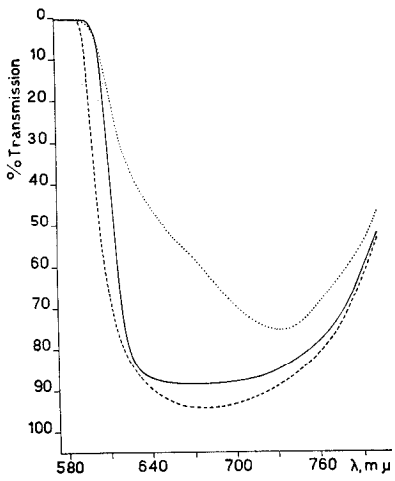

FIG. 1. Spectrophotometric transmission curves of Coleman red filter (——), 0.5% HbO_2 (– – – –), and 0.5% Hb (. . . .); 1-cm path length for hemoglobin solutions.

The equation generally employed in calculation of the light scattering data is[1]

$$Kc/R_{90} = 1/M + 2Bc \qquad (1)$$

where $K = 2\pi^2 no^2(dn/dc)^2/N\lambda_o^4$; $R_{90} = ir^2/i_o$; M = weight average molecular weight; c = solute concentration in g per ml of solution; no = refractive index of solution; B = interaction constant, the second virial coefficient; i = intensity of the light scattered per unit volume, observed at a distance r; i_0 = intensity of the incident beam.

In our work we substituted for this the empirical equation

$$K_ic/i = 1/M + 2Bc \qquad (2)$$

where K_i is an empirical constant which depends not only on the nature of the system being studied, but also on the particular properties of the instrument; i is the ratio between the intensity of the light scattered at 90° and the intensity of the light emerging from the solution at 0°, as directly measured with the aid of neutral filters and the working standard whose calibration was checked from time to time; c, M, and B are as in Equation 1.

The values of K_i for λ (red) for serum albumin (*sa*), in the same solvents, were determined with the aid of measurements at λ = 546 mμ on the basis of the following relation which may be deduced from Equations 1 and 2:

$$\left(\frac{K_i}{i}\right)_{\lambda\,\mathrm{red}} = \left(\frac{K}{R_{90}}\right)_{\lambda\,546\,\mathrm{m}\mu}$$

From this, the corresponding value of K_i, also for λ (red) for hemoglobin, whether oxy- or deoxy-, were obtained by means of the equation

$$\frac{(K_i)_{\mathrm{Hb}}}{(K_i)_{\mathrm{sa}}} = \frac{(dn/dc)^2_{\mathrm{Hb}}}{(dn/dc)^2_{\mathrm{sa}}}$$

which is implicit in the relations presented above.[2]

Measurements of the refractive index increment were made with the Brice differential refractometer (13) with the same light source and filter arrangement as used in the light scattering measurements on hemoglobin. The value of dn/dc obtained in this way was 0.197 ± 0.003 for oxyhemoglobin and hemoglobin alike, both in weak and concentrated salt solutions; its value for serum albumin was 0.186 ± 0.003 both in weak and concentrated salt solutions. On the basis of all the data, the value of K_i for both Hb and HbO_2 for λ (red) was taken as 0.97×10^{-6} at all salt concentrations.

No correction for light absorption by the solutions was required, since the correction for light absorption by the solutions is implicit in the method of measurement in the Brice-Phoenix apparatus up to absorption coefficients of about 0.8, as shown by Brice (13). No depolarization factor was taken into account, and the measurements were made at 20° in a temperature controlled room.

The calibrations of the light scattering apparatus for the usual wave lengths 436 and 546 mμ and the routine of measurements were the same as reported before (14).

The only modification was that, beside the filtration, the Hb solutions, unless otherwise stated, were clarified by ultracentrifugation for at least 1 hour at 38,000 r.p.m. in the Spinco model L centrifuge. Filtration through sintered glass filters (1 μ pore size) was by itself found to be inadequate for complete clarification of the solutions, especially at high salt concentrations.

The hemoglobin was prepared as follows. An oxyhemoglobin solution, clarified by centrifugation, was poured onto a sintered glass filter and partially deoxygenated by bubbling nitrogen through it; a small amount of dithionite (less than 0.5 mg per ml) was then added, and the solutions were forced rapidly through the filter by nitrogen pressure into the light scattering cell. The addition of dithionite is necessary to complete the deoxygenation of the HbO_2 and to prevent the reoxygenation of the pigment during the filtration.

Additional measurements of the intensity of scattered light at 135° and 45° were also carried out on several HbO_2 and Hb solutions as a control on the symmetry of scattering under different experimental conditions.

When the solutions were clarified by centrifugation and filtration, the dissymmetry values $i_{45°}/i_{135°}$ were less than 1.1 under all conditions. If the solutions were incompletely clarified, *i.e.* without ultracentrifugation, values of $i_{45°}/i_{135°}$ sometimes even above 2 were observed.

Under no conditions was there any change in the light scattering properties of Hb with time up to several hours.

The concentrations of the hemoglobin solutions were determined spectrophotometrically at λ = 540 mμ for HbO_2 ($E^{1\%}_{1\mathrm{cm.}}$ = 8.5) and 555 mμ for Hb ($E^{1\%}_{1\mathrm{cm.}}$ = 7.6).

It has been estimated that, by the procedure outlined above, K_ic/i can be determined with an error less than 10%. As a control, we compared values of K_ic/i at λ (red) with values of Kc/R_{90} at 546 mμ for several proteins (human globin, Bence-Jones protein, aldolase) and found the values to agree within 3%.

Sedimentation Experiments—Sedimentation experiments were performed with a Spinco model E centrifuge at 59,780 r.p.m., at a temperature of about 20°. The values of sedimentation constant reduced to water at 20° are given in Svedberg units ($S \times 10^{-13}$). The corrections for the density and viscosity of the solutions assume great importance in the case of concentrated salt solutions. Therefore, the validity of the corrections was tested by a few runs with other proteins, such as lysozyme and human serum albumin; in these cases, the corrected values

[1] For a full discussion of the theory of light scattering and for the definition of the quantities involved see reference (11).

[2] There is an approximation involved here because of the fact that λ (red) is not strictly monochromatic, but its effect is insignificant.

of $s_{0.2}$ in 2 M NaCl agreed within 10% with those obtained in 0.1 M NaCl. In the experiments in strong salt solutions, a double sector cell was employed in some cases to obtain the base line.

In the runs on hemoglobin, a few crumbs of dithionite were added to the HbO_2 solution immediately before filling of the cell.

Some of the sedimentation data on oxyhemoglobin were obtained on unpurified red cell hemolysates. The agreement between the results so obtained and those obtained under the same conditions with oxyhemoglobin preparations treated with ammonium sulfate indicates that the purification procedure does not significantly modify the behavior of the pigment.

Salt Solutions—The salts employed were analytical grade reagents which were used without further purification. The solutions were prepared in distilled water further deionized by passing through an anion exchange column.

RESULTS

Molecular Weight of Human HbO_2 and Hb in Phosphate Buffer 0.05 M pH 7—The values of the weight average molecular weight obtained by light scattering for three preparations of Hb and HbO_2 in dilute phosphate buffer, such as is generally employed in hemoglobin experiments, are reported in Table I. It will be observed that these are in agreement with those reported in the literature under the same experimental conditions by several authors using different methods. The slopes of the light scattering plots (K_ic/i against c) are nearly zero for both hemoglobin and oxyhemoglobin.

The sedimentation patterns of both Hb and HbO_2 in the same buffer showed the presence of only a single component; $s_{0.2}$ had a value of 4.45 ± 0.1 for HbO_2 and 4.55 ± 0.1 for Hb. The slope of s against c was, as reported by other authors (15), slightly negative.

Light Scattering and Sedimentation of Human HbO_2 and Hb at High Salt Concentration—The light scattering data for one preparation of oxyhemoglobin in NaCl solutions from 0.1 to 2 M are shown in Fig. 2. In this case, it will be seen that at any one salt concentration, the values of K_ic/i appear to lie on a straight line of greater or less negative slope. Such lines form a family, the lines for higher salt concentrations always lying above those for lower salt concentrations and tending to have a greater negative slope; it is clear from this that increasing the salt concentration leads to a decrease in the weight average molecular weight, and that there is a dissociation of the protein into subunits in strong salt solutions. The values of the intensity of the scattered light are in all cases reached within the shortest time in which it is possible to make light scattering measurements after mixing the salt and protein solutions, and do not change with time up to several hours. The dissociation is, therefore, very rapid.

TABLE I

Molecular weight of human HbO_2 and Hb by light scattering in KH_2PO_4 + K_2HPO_4 buffer pH 7, 0.05 M at 20°

Hemoglobin preparation	Mol. wt. HbO_2	Mol. wt. Hb
	Mol. wt. × 10^{-3}	*Mol. wt.* × 10^{-3}
No. 1	65.4	74.5
No. 2	70.9	66.5
No. 6	71.2	67.1
No. 7	62.4	59.5
Mean ± standard error...	67.5 ± 4	66.9 ± 5

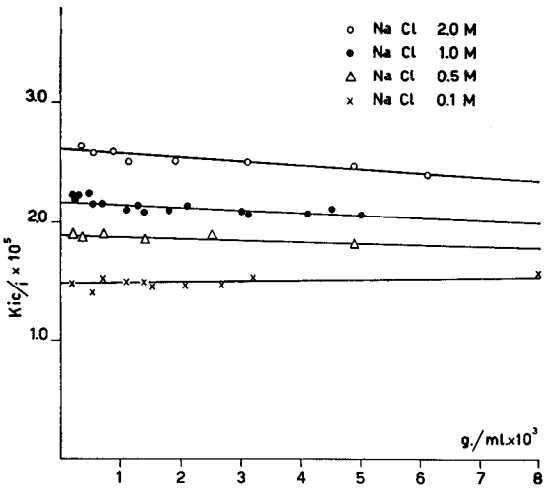

FIG. 2. Light scattering data for isoionic human HbO_2 (preparation No. 6) in concentrated NaCl solutions.

In the case of light scattering data, the problem of obtaining exact values of the weight average molecular weight at each protein concentration is complicated by the difficulty of separating the effects of changes caused by protein dissociation from those caused by the interactions between the protein and the salt and water of the solvent. In the light of earlier studies on protein-salt interactions (reviewed in reference (11)), we believe that we are justified in ascribing the observed salt effects at low protein concentrations primarily to changes in the weight average molecular weight of the protein. This point is treated further under "Discussion."

If, somewhat arbitrarily, we extrapolate the lines to zero protein concentrations, we obtain values of weight average molecular weight that may be regarded as characteristic of the protein at low, but by no means zero, concentrations; such values will be used in the course of this paper to characterize the molecular weight of hemoglobin at the different salt concentrations. A more detailed analysis of the light scattering data and the discussion of the light scattering plots will be given later. It is noteworthy that there is no tendency of the lines to turn upwards at the lowest concentrations, even when the measurements were extended downwards to concentrations as low as 0.2 g per liter.

The values of the weight average molecular weight obtained by extrapolating the straight lines to zero protein concentration in NaCl solutions up to 3 M are shown in Fig. 3. It will be seen that in the most concentrated NaCl solutions, a value of about 40,000 is obtained; this value is close to half the generally accepted molecular weight (68,000) observed at the moderate salt concentrations in which most past studies on hemoglobin have been made.

Additional data on the apparent molecular weight of HbO_2 and Hb at low protein concentrations in concentrated solutions of different salts are given in Table II. The data show that the different salts have an effect similar to that of NaCl, and also that the values for Hb are close to those obtained under the same conditions for HbO_2.

Dissymmetry values as high as 1.05 to 1.1 were frequently ob-

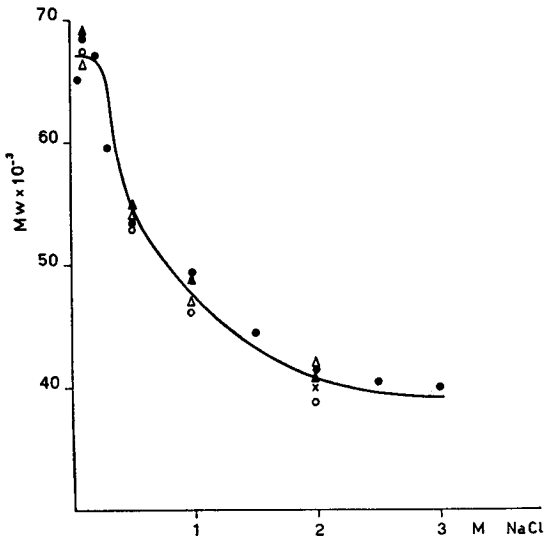

FIG. 3. Apparent weight average molecular weight of human isoionic HbO_2 obtained by extrapolation of light scattering data. Different symbols indicate different hemoglobin preparations.

TABLE II

Molecular weight by light scattering of human HbO_2 and Hb in concentrated salt solutions, at low protein concentrations

Hemoglobin preparation No.	Salt concentration	Mol. wt. HbO_2	Mol. wt. Hb
		Mol. wt. $\times 10^{-3}$	*Mol. wt.* $\times 10^{-3}$
4	NaCl 2 M	40.3	44.8
5	NaCl 2 M + phosphate buffer 0.05 M pH 7	40.0	45.5
5	KCl 2 M	44.3	46.7
5	KCl 2 M + phosphate buffer 0.05 M pH 7	42.2	46.1
5	Phosphate buffer 1 M pH 7	46.7	43.5
6	Na_2SO_4 1 M	44.3	40.4
7	NaCl 2 M	39.3	38.1
7	KCl 2 M	46.7	42.8
7	Sodium citrate 1 M	39.8	33.0
7	Phosphate buffer 1 M pH 7	41.1	36.3
Human globin (isoionic)	NaCl 2 M	33.6	

served in the light scattering of HbO_2 and Hb in strong salt solutions, even after the solutions had been clarified by prolonged centrifugation and repeated filtration. This suggests that the values for the molecular weight of HbO_2 and Hb at high salt concentrations may, in certain cases, still be slightly too high because of the presence of a small amount of large aggregates which have not been removed by the clarification procedure.

The dissociation in strong salt solutions is readily reversed by removal of the salt by dialysis against a weak NaCl solution (Table III).

Control light scattering experiments on serum albumin showed that the molecular weight of this protein in 2 M NaCl agreed within 5% with that obtained in 0.1 M NaCl. Human globin, which has a molecular weight of about 40,000 in 0.1 M NaCl, shows only a slight decrease of molecular weight in 2 M NaCl (reference (4) and Table II).

TABLE III

Reversibility of dissociation of human HbO_2 in concentrated salt solutions

Hemoglobin solutions	Mol. wt.
	Mol. wt. $\times 10^{-3}$
(a) = HbO_2 in 2 M NaCl	39.2
(b) = solution (a) after dialysis against 0.1 M NaCl	69.5
(c) = solution (b) returned to 2 M NaCl	42.5

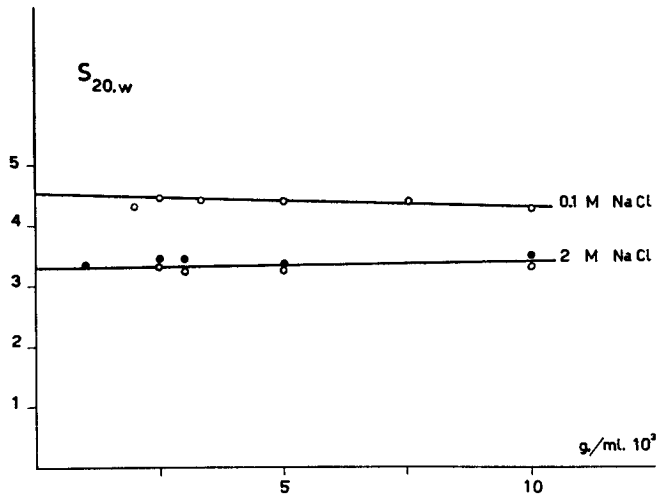

FIG. 4. Sedimentation data for isoionic human HbO_2 (○) and Hb (●) at different protein concentrations, in 0.1 and 2 M NaCl.

TABLE IV

Sedimentation constant of HbO_2 and Hb dissolved in concentrated salt solutions

Hemoglobin derivative	Protein concentration	Solvent	Sedimentation constant, $s_{20,w}$
	g/ml $\times 10^2$		
HbO_2	5	NaCl 0.5 M	3.9
HbO_2	2.5	NaCl 0.5 M	3.8
HbO_2	10	NaCl 1 M	3.6
HbO_2	5	NaCl 1 M	3.5
HbO_2	3	KCl 2 M	3.6
Hb	3	KCl 2 M	3.6
HbO_2	4	KCl 2 M	3.4
HbO_2	4	KCl 1 M	3.8
HbO_2	4	KCl 0.5 M	3.9
HbO_2	5	Tris-HCl 1 M	3.7
HbO_2	3	K_2HPO_4-KH_2PO_4 pH 7 0.1 M	4.4
Hb	3	K_2HPO_4-KH_2PO_4 pH 7 0.1 M	4.5
HbO_2	3	K_2HPO_4-KH_2PO_4 pH 7 0.5 M	3.6
HbO_2	4	K_2HPO_4-KH_2PO_4 pH 7 0.5 M	3.5
Hb	3	K_2HPO_4-KH_2PO_4 pH 7 0.5 M	3.6
Hb	4	K_2HPO_4-KH_2PO_4 pH 7 0.5 M	3.5

Values of the sedimentation constants of HbO_2 and Hb in strong salt solutions are shown in Fig. 4 and Table IV. The values of s always decrease with increase in salt concentration.

In the most concentrated salt solutions, the sedimentation constant has a value of about 3.3 S. Thus, the dissociation of

hemoglobin in strong salt solutions is accompanied by a very large drop in the sedimentation constant. Under all the conditions examined, the sedimentation pattern showed only a single symmetrical boundary.

Reproducibility of the Light Scattering Results at Different Salt Concentrations—Fig. 2 shows that the light scattering data for different hemoglobin preparations are quite concordant. As mentioned in the experimental part of the paper, it was found necessary, for reproducibility of the results, to clarify the hemoglobin solutions carefully by ultracentrifugation before study. Indeed, in preliminary experiments, it was found that if the solutions used in the light scattering experiments were clarified only by filtration through sintered glass filters, the results were not reproducible, large values of dissymmetry (*i.e.* $i_{45°}/i_{135°}$) being observed and the weight average molecular weight being much higher, particularly at high salt concentrations, than the values reported above. These findings, and the effect of centrifugation on the hemoglobin solutions are shown in Table V.

The greater weight average molecular weights observed in certain cases appear to be due to a small amount of hemoglobin which aggregates to form very large particles, although this amounts to less than 1% protein by weight. The aggregated hemoglobin may be seen in the form of a red gelatinous pellet at the bottom of the tubes after the centrifugation; this effect is more evident for some hemoglobin preparations than for others, and for a given preparation it varies with the experimental conditions. This finding would seem to be important, since it might explain the results obtained by Putzeys *et al.* on the light scattering of HbCO at high salt concentrations. Sometimes, as we have already pointed out, somewhat high values of molecular weight, as obtained by light scattering, were obtained, even with solutions which had been carefully clarified by centrifugation.

TABLE V

Molecular weight of HbO_2 at different salt concentrations, before and after clarification by ultracentrifugation

NaCl concentration	Preparation	Mol. wt. before ultracentrifugation	Mol. wt. after centrifugation
M		*Mol. wt.* $\times 10^{-3}$	*Mol. wt.* $\times 10^{-3}$
5×10^{-2}	3	144.0	66.5
2.5×10^{-1}	2	61.4	
2	2	92.8	39.2
1	2	76.0	47.2

DISCUSSION

The values of molecular weight by light scattering obtained on Hb and HbO_2 in phosphate buffer 0.05 M pH 7 are in good agreement with the values obtained under the same conditions by different methods and confirm the validity of the light scattering method for the determination of the molecular weight of hemoglobin derivatives (5). Because of the great range of experimental conditions to which this method is applicable, it should become a powerful tool in the investigation of hemoglobin.

It is to be noted that the light scattering equation used in the treatment of the experimental data ($Kc/R_{90} = 1/M + 2Bc$) is strictly applicable to only a two component system, *i.e.* protein and pure water. When, as in the present case, salts are present, the system is a multicomponent one and the treatment of the experimental data according to Equations 1 and 2 is theoretically inadequate (11). Particularly, the possible selective binding of some of the "diffusible" components to the "nondiffusible" component must be taken into account to evaluate the molecular weight of the "nondiffusible" component from the extrapolated value of Kc/R_{90}.

For protein in salt solution, this effect has generally been regarded as negligible, even when the protein, as in the case of serum albumin in sodium chloride or sodium thiocyanate solutions, has a relatively high affinity for small ions (11).

In the case of hemoglobin, under the conditions used in the present experiments, no independent data are available for the binding of small ions to the protein by which the effect of selective binding on the molecular weight calculated according to Equations 1 and 2 can be determined quantitatively; however, several data in the literature suggest that the affinity of hemoglobin for small ions is not very large. It is believed that the contribution of the binding of small ions to the molecular weight of hemoglobin calculated according to Equations 1 and 2 is not greater than a few per cent in all the conditions described in the text.

In strong salt solutions, the light scattering data show that hemoglobin dissociates to a greater or lesser extent into subunits as the salt concentration is raised. The weight average molecular weight of about 40,000 observed in the strongest salt solutions studied (3 M) indicates that the great majority of the molecules exist in a dissociated form. The dissociation is accompanied by a decrease of the sedimentation constant from 4.6 in 0.1 M NaCl to 3.3 in 2 M NaCl.

These results confirm the previous observations by different authors (3–6) on the dissociation of mammalian hemoglobins in strong NaCl solutions. The results of Putzeys *et al.* (8) which indicate an increase, instead of a decrease, of the molecular weight of COHb in strong chloride solutions, are probably to be explained by the gross aggregation of a very small part of the pigment; this would, of course, give rise to a great increase in the apparent molecular weight. There are, however, differences of detail among the results of previous investigators as well as between ours and theirs. In particular, our results and the older osmotic data obtained by Gutfreund (4) show dissociation in strong salt solutions even at high (about 1%) protein concentration. In contrast, recent experiments of Benhamou *et al.* (5, 6) indicate the absence of dissociation.[3] We have checked and rechecked our results in the light of these experiments, and we have no explanation to offer for the inconsistency.

The results of the present work indicate that deoxygenated human hemoglobin also dissociates in concentrated salt solutions just like oxyhemoglobin. The effect of other salts on oxyhemoglobin and hemoglobin is similar to NaCl.

Nevertheless, the sedimentation experiments, in a solvent consisting of two or more components, are not so simple to interpret. The finding of one symmetrical boundary in our sedimentation experiments at the different salt concentrations appears to be the expression of a rapidly established equilibrium between different molecular species. Because of this, the observed weight average molecular weight appears, under most conditions, to be a nonintegral multiple of the minimal molecular weight (16,000). The protein indeed consists of a dynamic mixture of components of different molecular weights. From a general point of view, these results emphasize the difficulty of

[3] At a protein concentration higher than 0.4% in 1 M NaCl.

establishing the molecular individuality of a protein in solution.

An interpretation of the way in which the splitting occurs in strong salt solutions must await the outcome of hybridization experiments now in progress in this laboratory.

In this connection, it is relevant to observe that globin, which appears to consist mainly of half units in the middle range of ionic strength, fails to dissociate to any considerable extent in solutions of high salt concentration.

Further discussion of this salt effect and the implication of these results for the interpretation of the oxygen equilibrium of Hb will be reported later.

SUMMARY

The effect of salts on human adult hemoglobin has been studied by light scattering and sedimentation.

In strong salt solution, hemoglobin and oxyhemoglobin appear to dissociate to a greater or lesser extent into subunits as the salt concentration is raised.

The weight average molecular weight of 40,000, calculated by light scattering at the highest salt concentrations studied (2 to 3 M), indicates that the majority of the hemoglobin molecules exist in a dissociated form.

The decrease of the sedimentation coefficient from 4.6 Svedbergs in 0.1 M NaCl to 3.3 in 2 M NaCl provides strong additional support to the conclusion drawn from light scattering measurements.

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