A Light-Scattering Study of the Effect of Sodium Chloride on the Molecular Weight of Human Adult Hemoglobin*

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ABSTRACT: A laser light source (6328 Å) is used to facilitate Rayleigh-scattering measurements on hemoglobin solutions. Data on adult human carbon monoxyhemoglobin and deoxyhemoglobin solutions at concentrations above 1.5 g/l. yield weight-average molecular weights of 68×10^3 and 67×10^3, respectively, in 0.05 M sodium phosphate buffer at pH 7.0 at 12°, and 65×10^3 and 62×10^3, respectively, in 2 M NaCl (in addition to the phosphate buffer). Variations among these values are probably no greater than the *absolute* uncertainty in light-scattering molecular weights. The measured molecular weights in 0.05 M buffer agree with the mass of the intact (tetrameric) hemoglobin molecule. The apparent *relative* decrease in molecular weight found in 2 M salt is too small to be considered significant in the case of carbon monoxyhemoglobin and is questionable for the deoxy form. The data are therefore not consistent with the extensive dissociation of hemoglobin at high ionic strength reported in some studies on solutions of similar concentration. Differential refractometry on solutions of oxyhemoglobin, deoxyhemoglobin, and carbon monoxyhemoglobin, together with calculations based on an approximate rule for the refractive index of a mixture, suggest that any preferential binding of NaCl or water by protein is too small to affect apparent molecular weights determined by light scattering or ultracentrifugation.

Normal adult human hemoglobin[1] (hemoglobin A) consists of four polypeptide chains (two α and two β chains) held together by noncovalent interactions. It has a molecular weight of 64,458 (including the four heme groups) on the basis of its amino acid composition (Braunitzer *et al.*, 1964). In spite of the tremendous amount of information that has been obtained in recent years by physical-chemical techniques, such as X-ray diffraction (Perutz, 1969, 1970), kinetics and equilibrium studies on ligand binding (Antonini and Brunori, 1970, and references therein), spin labeling (Ogawa *et al.*, 1968; Ho *et al.*, 1970a; Baldassare *et al.*, 1970), and nuclear magnetic resonance (Ho *et al.*, 1970b; Davis *et al.*, 1970, 1971; Ogawa and Shulman, 1971), the molecular mechanism for the cooperative oxygenation process of hemoglobin remains uncertain. Two questions are whether the cooperativity is associated with a tetrameric or dimeric hemoglobin and whether there are two types of dimers ($\alpha_1\beta_1$ and $\alpha_1\beta_2$) in deoxyhemoglobin. Summaries of these controversies are given by Antonini and Brunori (1970) and by Edelstein *et al.* (1970).

The apparent molecular weight of hemoglobin in deoxy form and with CO and O_2 ligands has been investigated in several studies as a function of protein concentration and concentration of added NaCl at neutral pH. Recent work is summarized by Antonini and Brunori (1970). It is agreed that extensive reversible dissociation of the tetrameric molecule occurs at sufficiently low protein concentration, the tetramer–dimer equilibrium being the important factor under ordinary conditions. Molecular weights have been measured by conventional "absolute" methods: osmometry, light scattering, sedimentation equilibrium. Less direct methods—*e.g.*, velocity sedimentation, photolysis kinetics, gel chromatography—have also been used. The extent of the dissociation is characterized by reported dissociation constants ranging from 10^{-7} to 10^{-4} mole per l., depending on ionic strength and the presence of a ligand. Increasing the ionic strength seems to favor dissociation, but there are some large quantitative discrepancies among the various studies. The reasons are not clear but it has been suggested: (i) that selective thermodynamic interactions between protein and water, salt, or other components in multicomponent solutions may seriously affect apparent molecular weights derived from light-scattering and ultracentrifuge data (Edelstein and Gibson, 1969; Edelstein *et al.*, 1970; Aune and Timasheff, 1970); and (ii) that dithionite added in some studies to scavenge oxygen from Hb solutions may influence results (Lemberg and Legge, 1949; Dalziel and O'Brien, 1957; Benesch *et al.*, 1962; Edelstein and Gibson, 1969; Edelstein *et al.*, 1970).

In view of disagreements on the dissociation of hemoglobin at high and low ionic strength, we decided on light-scattering measurements to restudy the effect of salt on the apparent molecular weight at high (above 1.5 g/l.) protein concentration. The availability of a red laser light source providing all the monochromatic intensity desired at a wavelength beyond the visible absorption bands of hemoglobin made the project attractive. Refractometric measurements were needed in conjunction with scattering measurements to determine molecular weight, and they also made it possible to check on anomalous values of the specific refractive increment of hemoglobin reported earlier (Rossi-Fanelli *et al.*, 1961).

The state of aggregation of hemoglobin is important because of the bearing it may have on the problem of the mechanism of cooperative oxygenation. Obviously, information is also needed on subunit interactions and intermediate con-

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[1] Abbreviations used are: Hb, deoxyhemoglobin; HbO_2, oxyhemoglobin; HbCO, carbon monoxyhemoglobin.

formations (if any) as well as the location of the amino acid residues serving as informational links between subunits and heme groups during the oxygenation process. Certain aspects of these problems are being pursued here (Ho *et al.*, 1970a,b; Davis *et al.*, 1970, 1971; Baldassare *et al.*, 1970).

Experimental Section

Materials. Hemoglobin was prepared from fresh human blood obtained from the local blood bank. The cells were washed repeatedly with saline and lysed with toluene. The hemoglobin was dialyzed at 4° with gentle mechanical agitation, first against distilled water for 24 hr and then against at least six changes of buffer for not less than 48 hr. The last dialysate was kept and used as a solvent for all dilutions. The pH of the buffer solution was adjusted to 7.0 with NaOH. All experiments were performed within 10 days from completion of the preparation of the hemoglobin solution. The solution was stored as HbCO, and before it was used in light-scattering and refractive index studies. Furthermore, diluted solutions used for light-scattering and refractive index studies were centrifuged at 30,000 rpm in a Beckman Model L ultracentrifuge for two hours before use. Hb was prepared from HbCO at 5° by first passing oxygen over the solution in order to remove the carbon monoxide, and then by passing oxygen-free nitrogen to remove the oxygen. Hb solutions were stored under nitrogen and used within 24 hr after preparation. As judged from the visible absorption spectrum, Hb solutions used for our measurements were better than 95% in the deoxy form and contained less than 2% methemoglobin. No difference was observed in the spectra of the deoxyhemoglobin solution before and after the completion of the light-scattering measurements. The spectra were obtained by using the light-scattering cell in a Cary 14 spectrophotometer. Hemoglobin concentration was determined spectrophotometerically at 540 mμ by means of a Zeiss PMQII spectrophotometer after conversion into cyanomethemoglobin with Drabkin's solution (Rice, 1967). The millimolar absorbance coefficient was taken as 11.0 (Cameron and George, 1969; Zijlstra and van Kampen, 1960).

Analytical grade chemicals were used without further purification.

A Radiometer pH meter Model 26 with a Beckman Model 39030 frit junction combination electrode was used in pH measurements.

Experimental Procedures and Treatment of Data: Light-Scattering Measurements. Since the hemoglobin derivatives investigated exhibit very strong light absorption except at the long-wavelength end of the visible region, it was necessary to do the light-scattering investigation with red light. Thus, a helium–neon continuous-wave laser (Spectraphysics Model 120) was used to provide 6328-Å incident light. The scattering measurements were carried out on HbCO and Hb with a photometer described by Berry (1966) modified only by the introduction of the laser light source.

Small light-scattering cells of the design by Dandliker and Kraut (1956) were used. The Pyrex cells were in the form of a truncated cone, in order to reduce the stray light pickup (Casassa and Katz, 1954) by the detector. The cells were hand-blown and varied in diameter from 11 to 15 mm. The effective diameter of each cell was determined by the procedure of Berry and Yen (1969).

In order to reduce surface denaturation at low concentrations of protein, the light-scattering cells were first rinsed with the centrifuged hemoglobin solution. For final removal of dust, centrifuged hemoglobin solution was transferred to a scattering cell, and was then centrifuged in the cell for four hours at 9000 rpm by floating the cell in CCl_4 in a swinging-bucket rotor (Sorvall, Model HB-439) in a refrigerated centrifuge (Sorvall, RC2).

The light-scattering cells were sealed in two different ways. For HbCO, a small Teflon stopper that fitted tightly into the neck of the cell was used. For the deoxyhemoglobin solution an air-tight seal was achieved with a serum cap and epoxy adhesive (Hardman Epoweld, work life 3–5 min at 75°F). This procedure was carried out inside a glove bag (Instruments for Research and Industry). Under no conditions was there any change in the light-scattering properties or visible spectra of solutions with time up to several hours, *i.e.*, before completion of the light-scattering measurements.

After centrifugation, the cell was carefully positioned in a cell holder inside the thermostat of the light-scattering photometer. In the thermostat, the cell was immersed in a bath (50% acetone and 50% xylene) having about the same refractive index as the scattering liquid so as to eliminate prism effects due to the cell shape (Casassa and Katz, 1954). The sample temperature was held to 12 ± 0.02° in all measurements. The observed intensities of light scattered from the hemoglobin solutions were corrected for the small attenuation due to absorption at 6328 Å. The correction was obtained from the absorbance of each solution in a 10-mm cell, measured by using the light-scattering apparatus as a spectrophotometer, together with the effective diameter of the cell used in each scattering experiment. Corrections for refraction effects in the photometer and for the variation of irradiated volume "seen" by the photometer at different scattering angles were applied as described by Berry (1966). Corrected instrument readings were converted into absolute intensities by calibration with standards of known scattering power. The primary standard was a polystyrene sample distributed by the National Bureau of Standards (NBS Standard Sample 705) but this had been checked against other standards (Berry, 1966).

Scattering was measured for at least six different hemoglobin concentrations (1.5–6.4 g/l.) for scattering angles θ from 25 to 135°. The data were analyzed according to the familiar expression (Zimm, 1948)

$$\frac{K'c}{R_\theta} = \frac{1}{M_w P(\theta)} + 2Bc \qquad (1)$$

where R_θ is the reduced excess intensity of scattering at angle θ (scattering from hemoglobin solution less that from solvent); M_w is the weight-average molecular weight; $P(\theta)$ is the intensity distribution function [$P(\theta)$ is unity at $\theta = 0$, and at all angles for scattering particles small compared to the wavelength λ of the incident light]; c is the concentration of solute in units of mass per unit volume of solution; B is the second virial coefficient; and $K' = \alpha(\partial n/\partial c)_\mu^2 \lambda^{-4}$, α denoting a cell constant, and $(\partial n/\partial c)_\mu$ the specific refractive index increment of the solute. To obtain M_w and B, eq 1 was fitted to the experimental data by use of the DAM program (a general purpose program in data processing and multiple regression) and the IBM 360/50 computer at the University of Pittsburgh.

Figure 1 presents a plot of typical data for c/R_θ *vs.* $\sin^2(\theta/2)$. As the plot shows, data at angles 25, 27, and 30° depart markedly from the linear dependence of the other points. It is believed that the appearance of anomalously large scattering at small angles can be attributed mainly to imperfect clarification of the solutions. Accordingly, the points at these angles

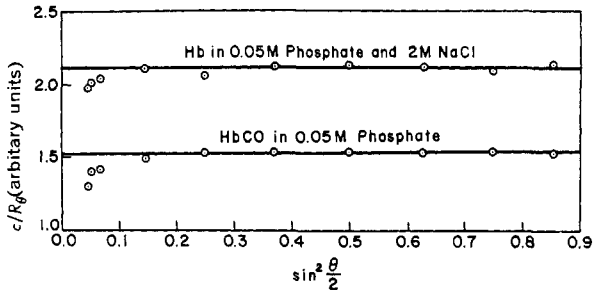

FIGURE 1: Angular dependence of c/R_θ of Hb and HbCO in 0.05 M sodium phosphate plus 2 M NaCl and 0.05 M sodium phosphate, respectively, at pH 7 and 12°.

were omitted from consideration. The measured dissymmetry ratio R_{45}/R_{135} (of intensities scattered at 45 and 135°) was always smaller than 1.03 and the "smoothed" dissymmetry defining the slope of the best linear plot was usually even closer to unity. Interference effects for spherical scattering particles of the size of the hemoglobin molecule (the largest crystallographic dimension is 65 Å) can produce only negligible dissymmetry; but the small observed values are about what one expects owing to the difficulty of completely excluding dust from aqueous solutions. The double centrifugation technique we employed was more successful than filtration through a 0.45 μ Millipore HA filter in reducing dissymmetry to an acceptable level.

Differential Refractometry. The refractive index increments, $(\partial n/\partial c)_\mu$, were measured with a differential refractometer of conventional design. The standard double-chamber Brice-Phoenix sample cell was used in a thermostated enclosure and a 6328-Å laser was the light source. Scale deflections were calibrated with KCl and sucrose solutions of known refractive index according to the refraction and dispersion data given in the International Critical Tables.

The subscript μ indicates that for systems containing low molecular weight components (*e.g.*, salt) in addition to the principal solvent (water) the refractive increment is evaluated on solutions equilibrated by dialysis against a dialysate of fixed composition—hence, at constant chemical potential of diffusible components. It has been pointed out repeatedly (Stockmayer, 1950; Casassa and Eisenberg, 1961, 1964) that this formulation of the refractive increment (rather than the more conventional derivative $(\partial n/\partial c)_m$ evaluated at constant composition of the mixture of low molecular weight components) together with eq 1 leads to the correct molecular weight of the macromolecular component, regardless of hydration and of "binding" of other components by the nondiffusible solute. Conversely, use of $(\partial n/\partial c)_m$ in eq 1 leads to an apparent molecular weight modified by any preferential interaction.

The composition of the entity whose molecular mass is determined is in fact defined by the procedure for concentration measurement. In the protocol followed here, a measured optical density is related to weight concentration *via* a calibration relating absorbance to iron content and thence to the corresponding mass of protein (plus heme) fixed by the amino acid composition. The procedure implies that, in principle, light-scattering measurements on intact tetrameric hemoglobin, with effects of nonideality eliminated by extrapolation to infinite dilution of protein, will yield a molecular weight of 64,458. Deviation from this figure can be attributed to experimental error, to association or dissociation of protein, but not to interaction between protein and components of the solvent system.[2] The uncertainty associated with standardization by a dry weight determination is entirely avoided.

TABLE 1: Specific Refractive Index Increments $(\partial n/\partial c)_\mu$ for Hemoglobin (6328 Å, 12°, pH 7.0).

Solvent	$\partial n/\partial c$ for HbCO and HbO_2	$\partial n/\partial c$ for Hb
Distilled water[a]	0.196 ± 0.001	0.196 ± 0.001
0.05 M sodium phosphate buffer	0.192 ± 0.001	0.192 ± 0.001
0.05 M sodium phosphate buffer + 2 M NaCl	0.176 ± 0.001	(0.177)[b]

[a] Salt-free hemoglobin prepared by dialysis against 0.1 M NaCl for 24–48 hr to remove any traces of organic phosphates, followed by dialysis against distilled water to remove the sodium chloride: ionic strength of the final solution less than 0.0001. [b] Value calculated as described in the text.

Experimental values of $(\partial n/\partial c)_\mu$ for HbCO, HbO_2, and Hb in water and 0.05 M sodium phosphate buffer, and for HbCO and HbO_2 in 0.05 M sodium phosphate plus 2 M salt at 12° are listed in Table I.

Special precautions were taken to prevent deoxyhemoglobin from oxidizing. Since the refractometer cells could not be sealed as the light scattering cells were, a plastic cover with a weight was used. A visible spectrum was recorded after completing the refraction measurements to ensure that the hemoglobin was still in the deoxy form. As a further precaution, a slow stream of nitrogen was blown through the cell housing for half an hour before and during the measurements. Despite these measures, which proved effective for solutions in water or in 0.05 M phosphate buffer, it was impossible to obtain reasonable and reproducible refractive index differences for deoxyhemoglobin in 0.05 M phosphate plus 2 M NaCl. The apparent refractive increments were unexpectedly small and erractically variable in a series of experiments. The anomalous behavior appears to be attributable to the formation of methemoglobin: deoxyhemoglobin solutions in phosphate buffer to which methemoglobin was deliberately added yielded similarly abnormal refractive increments. The light-scattering measurements on Hb in 0.05 M buffer plus 2 M salt showed no abnormality.

Discussion

Refractometry. As Table I indicates, the measured refractive index increments are the same within experimental error for all three forms of hemoglobin in the same solvent—with the exception of deoxyhemoglobin in 0.05 M phosphate plus 2 M NaCl.

The agreement in the data is precisely what would be expected for $(\partial n/\partial c)_\mu$ if it is granted that complexing of CO or O_2 will have no important effect on the average optical polar-

[2] This interpretation presumes that the optical density of the solutions used in concentration measurements is unaffected by buffer and salt concentration in the original solutions before conversion into cyanomethemoglobin.

izability of the large protein molecule. Then, addition of a given number of protein molecules to the solvent will have substantially the same effect on the refractive index of the system whether the hemoglobin is in the oxy, carbomonoxy, or deoxy form, since the mass of CO or O_2 is trivial compared to that of the protein.

However, the empirical fact that $(\partial n/\partial c)_\mu$ is independent of the state of the hemoglobin indicates further that within the limits of uncertainty (the refractive index is an indicator of limited sensitivity) any selective interactions between protein and other components of the mixed solvent must be the same for the different protein species. More specifically, we can assert for very dilute solutions of protein in a mixture of salts (or other low molecular weights solutes) that

$$\left(\frac{\partial n}{\partial c}\right)_\mu - \left(\frac{\partial n}{\partial c}\right)_m \approx \sum \frac{M_J}{1000}\left(\frac{\partial n}{\partial c_J}\right)_m \xi_J \qquad (2)$$

is the same for the different hemoglobin derivatives. Here, $(\partial n/\partial c_J)_m$ is the refractive increment, evaluated at fixed proportions of the other components, for salt component J with formula weight M_J; and ξ_J is the number of moles of salt component preferentially "bound" per gram of protein component (Casassa and Eisenberg, 1964). Operationally, ξ_J is defined by assuming the equilibrium molality of all "unbound" diffusible solutes to be the same on both sides of a dialysis membrane and assigning any actual imbalance in total concentration to "binding" (which is, therefore, not limited to positive values).

The implication of most of our refraction measurements that the ξ_J are about the same for the three forms of hemoglobin is a reasonable one. In our view, therefore, it is reasonable to reject the erratic refractometry data on deoxyhemoglobin in 0.05 M buffer plus 2 M salt and assert that $(\partial n/\partial c)_\mu$ = 0.176 as with HbCO and HbO_2 in this medium.

Further, we can demonstrate that the experimental $(\partial n/\partial c)_\mu$ is 0.05 M buffer plus 2 M salt is in quantitative agreement with a value that can be calculated by combining the refraction data in water and in 0.05 M buffer, the known refractive index of salt solutions, and the assumption that ξ_J is unimportant for NaCl in hemoglobin solutions. At 5780 Å and 0.05°, the refractive index of NaCl solutions in water is given by

$$n_s = n_0 + 0.01136m_3 - 0.00078m_3^{3/2} \qquad (3)$$

where n_0 is the refractive index of water and m_3 denotes the molality of the salt.[3] Now if it is assumed that the refractive index of a mixture is a linear function of the composition by volume it follows (Casassa and Eisenberg, 1961, 1964) that $(\partial n/\partial c)_{m_3}$ for protein in salt solution is given by

$$(\partial n/\partial c)_{m_3} = (\partial n/\partial c)_0 - \bar{v}(n_s - n_0) \qquad (4)$$

in which $(\partial n/\partial c)_0$ is the refractive increment of protein in water and $\bar{v}$ is the partial specific volume of the protein. Taking $(\partial n/\partial c)_0$ = 0.196 and $\bar{v}$ = 0.75, we obtain 0.181 as the hypothetical value of $\partial n/\partial c$ in 2 M salt. However, the actual solvent used also contains 0.05 M phosphate buffer. Experimentally this alone gives $\partial n/\partial c$ = 0.192; thus the presence of the buffer decreases $\partial n/\partial c$ by 0.004. If we account approximately for the small effect of the buffer in the 2 M salt solution simply by subtraction, we obtain 0.181 − 0.004 = 0.177, which is in excellent agreement with what was found for the experimental refractive increment of HbCO and HbO_2.[4] The calculation, it must be emphasized, is based on reasonable assumptions for $(\partial n/\partial c)_m$. That the result agrees, where it can be verified, with the empirical $(\partial n/\partial c)_\mu$ is evidence that the binding parameter ξ_{NaCl} of eq 2 is not only comparable for HbCO and HbO_2 at the salt concentration used but is, in fact, too small to affect the refractive increment appreciably. This conclusion reinforces our contention that $(\partial n/\partial c)_\mu$ for deoxyhemoglobin in the presence of the high salt concentration can reasonably be taken as 0.176.

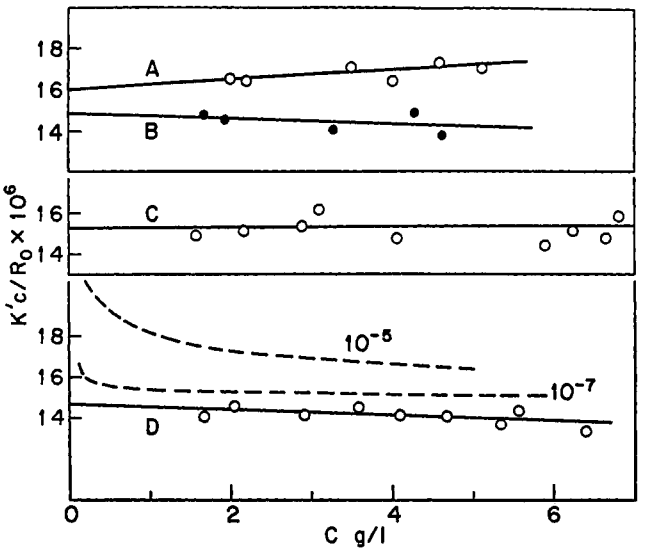

FIGURE 2: Extrapolation of $K'c/R_0$ to c = 0 (solid lines) at pH 7 and 12°: (A) Hb in 0.05 M sodium phosphate buffer + 2 M NaCl; (B) Hb in 0.05 M sodium phosphate buffer; (C) HbCO in 0.05 M sodium phosphate buffer + 2 M NaCl; and (D) HbCO in 0.05 M sodium phosphate buffer. The dashed curves represent calculated curves in tetramer–dimer equilibrium governed by dissociation constants of 1×10^{-5} and 1×10^{-7}.

Light Scattering. Figure 2 gives plots of the reciprocal scattering function $K'c/R_0$ (extrapolated to zero scattering angle) *vs.* protein concentration. The experimental molecular weights thereby obtained are listed in Table II. Uncertainties indicated for molecular weights reflect the standard deviation of points for $K'c/R_0$ from the least-squares linear relations shown in Figure 2, plus the effect of the estimated uncertainty of 0.001 in values of $\partial n/\partial c$. Possible systematic error in standardization of the photometer makes the total uncertainty in molecular weights still greater. The five to nine points in Figure 2 for each concentration series represent data obtained from two or three different hemoglobin preparations. Thus, sample differences may enhance scatter of data, but systematic sample effects tend to be suppressed.

One conclusion is manifest. The measured molecular weights of HbCO and Hb are the same, within experimental error, at low ionic strength; and the results agree well with the value for the intact tetrameric molecule according to the amino acid composition.

[3] Equation 3 is obtained by integraton of an expression for $\partial n/\partial m_3$ given by Casassa and Eisenberg (1961, p 431). We note that the equation as printed in the reference cited is erroneous. The right-hand side ψ_3 has to be replaced by $1000\Psi_3$ to be consistent with the notation of the rest of the paper.

[4] Here and elsewhere in our discussion, problems associated with the presence of the buffer are glossed over, but there is no reason to believe that the approximations are serious.

TABLE II: Molecular Weight of Human Hemoglobin Determined by Light Scattering at pH 7.0 and 12°.

Solvent	$M_w \times 10^{-3}$ of HbCO	$M_w \times 10^{-3}$ of Hb
0.05 M Sodium phosphate buffer	68.3 ± 2.1	67.2 ± 3.3
0.05 M Sodium phosphate buffer + 2 M NaCl	65.4 ± 2.5	62.4 ± 2.3[a]

[a] Molecular weight based on an assumed $\partial n/\partial c$ of 0.176.

For both hemoglobin derivatives, there is an apparent decrease in molecular weight at high ionic strength, but in neither case is this as large as the *absolute* uncertainty in light-scattering molecular weights. The *relative* decrease of about 4% for HbCO is too small to be considered significant. The larger *relative* change for Hb may approach the threshold of discrimination, but it must be remembered that the molecular weight of Hb at high ionic strength is based on an assumed value of the refractive increment. Thus, the molecular weights deduced from our data provide no real evidence for dissociation of hemoglobin.

A more detailed consideration of the question of possible dissociation requires noting that a reversibly dissociating system in the absence of thermodynamic interactions expressed in the second and higher virial coefficients will yield plots of $K'c/R_0$ (*i.e.*, of $1/M_w$) *vs.* c with negative slope and positive curvature, if data are available covering a sufficiently wide concentration range. Data at sufficiently low concentration will extrapolate correctly to the molecular weight of the dissociated species and data confined to a high concentration region where dissociation is negligible will fall on a horizontal line giving the reciprocal molecular weight of the undissociated species. If a finite virial coefficient is admitted simultaneously with a dissociation equilibrium, the apportioning of changes in $K'c/R_0$ between the two causes is to some degree arbitrary,[5] except at the extremes, where one of the two effects becomes negligible. As Figure 2 indicates, the concentration dependence of $K'c/R_0$ exhibits no anomalies. In view of the scatter of the data, each concentration series is fitted adequately by a straight line. The slopes yield apparent second virial coefficients, B, ranging from -0.6×10^{-7} to $+1.2 \times 10^{-7}$ l. per g^2. The latter value (for Hb in 2 M salt) reflects a perhaps significantly positive slope but the other three slopes cannot be regarded as meaningfully different from zero.

That progressive dissociation is not a large factor over the concentration range studied, is implied by our conventional interpretation of the light-scattering data; and the best justification lies in the entirely unexceptional character of the results.

Though our data give no evidence for dissociation of HbCO and Hb, it remains to inquire to what degree the data might yet be compatible with a reversible dissociation. To this end, we have included in Figure 2, hypothetical plots of $K'c/R_0$ for hemoglobin with $B = 0$ and a tetramer–dimer equilibrium governed by dissociation constants K of 10^{-5} and 10^{-7} mole per l., presumed to be independent of protein concentration. For the larger value of K, linear extrapolation to zero concentration from the range $1.5 < c < 6$ g/l. would lead to a molecular weight much less than that of the tetramer. A positive B and this value of K combined with a linear extrapolation of data might lead to a molecular weight like that of tetramer, but $K'c/Rl$ *vs.* c would begin to exhibit considerable upward curvature. If K were 10^{-7}, data confined to concentrations above 0.5 g/l. would show no sensible curvature and would extrapolate to an apparent molecular weight very little below that of tetramer. However, we can solve the quadratic equation

$$K = 4c\alpha^2/M_4(1 - \alpha) \tag{5}$$

where M_4 is the molecular weight of tetramer and α is the fraction of tetramer dissociated to dimer, and show that at $c = 1$ g/l., 4% of the tetramer is dissociated even when K is as small as 10^{-7}. The weight-average molecular weight of the system is given by

$$M_w/M_4 = 1 - \alpha/2 \tag{6}$$

and the number average by

$$M_n/M_4 = 1 - \alpha/(1 + \alpha) \tag{7}$$

Thus, light-scattering measurements of molecular weight are less sensitive than osmotic measurements to the dissociation.

Comparison with Other Work

The study most directly comparable with ours is that of Rossi-Fanelli *et al.* (1961) who carried out light-scattering measurements (using a broad band of red light isolated from an incandescent source) on Hb and HbO_2 in a variety of salts and buffers at neutral pH and 20° at concentrations up to 3 M. Their report of virtually complete dissociation to dimer in 2 or 3 M NaCl (and comparable effects in other media) is in complete disagreement with our findings. Part of the discrepancy is accounted for by their use of a refractive increment 0.197, high compared to ours, and found to be invariant with salt concentration. This independence of salt is inexplicable since it contradicts not only our findings but reasonable expectations concerning refractive indices of multicomponent solutions. Taking their molecular weight of 40×10^3 for HbO_2 in 0.05 M phosphate plus 2 M NaCl at pH 7 and recalculating it with our refractive increment of 0.176 raises the value to 50×10^3, but leaves a large disagreement still unexplained. In their paper, Rossi-Fanelli *et al.* (1961) reproduce a series of plots of $K'c/R_0$ for HbO_2 in NaCl *vs.* c with data points from about 0.2 g/l. up to 4 or 6 g per l. Each plot is linear over the entire protein concentration range, but plots for different NaCl concentrations fall at successively higher ordinates as the salt concentration is increased. Concomitantly, the slopes of the plots appear to become progressively negative until B is of the order of -10^{-7} in 2 M salt. The appearance of these plots suggests a series of stable mixtures of tetramer and smaller molecules, not a dissociation governed by a mass action constant of such magnitude at any intermediate

[5] In general, there is no reason to assume that "nonideality" effects will not require higher virial coefficients (which might be either positive or negative), and the analysis is further complicated by the fact that the virial coefficients for a mixture of solute components are in general themselves functions of composition of the mixed solute (Stockmayer, 1950) and hence will depend on the total concentration in a dissociating system. More fundamentally, equilibrium constants for reactions in concentrated systems cannot be obtained unambiguously from data by rigorous thermodynamic reasoning alone (Kirkwood and Oppenheim, 1961).

salt concentration that the composition of the tetramer–dimer mixture changes appreciably over the roughly 25-fold range of protein concentration covered by the light-scattering experiments.

Since molecular weight determinations by osmometry in multicomponent solutions (unlike light scattering and equilibrium sedimentation) are not complicated by preferential binding effects and, as we have noted, slight dissociations are more evident in M_n than in M_w, independent evidence from osmotery is of importance. Guidotti (1967) reported osmotic measurements on Hb, HbO_2, and HbCO in solutions of several salts at neutral pH and 20°. His plots—Π/RTc *vs.* c, Π denoting the osmotic pressure—exhibit the character expected of a reversibly dissociating system. At high protein concentration there is a linear segment, sometimes with a positive slope; and at low concentration, this is superseded by upward curvature. For all three forms of hemoglobin in 0.2 M NaCl, data between 4 and 14 g per l. fall on linear plots giving second virial coefficients of about 5×10^{-8} l./g^2. Below 4 g/l., curvature becomes evident in the osmotic plots: analysis of the HbCO data in this region leads to a dissociation constant $K = 5 \times 10^{-6}$ mole/l. Linear extrapolations of Π/RTc from the high concentration range lead to apparent molecular weights between 55×10^3 and 57×10^3 for the three undissociated species. These figures are low, but it must be noted that the dissociation constant of 5×10^{-6} requires that the tetramer be appreciably dissociated, even in the seemingly linear region of the osmotic pressure plot: *i.e.*, α should be 0.13 at a concentration of 4 g/l. and 0.09 at 10 g/l.

Guidotti's osmotic measurements in 0.2 M NaCl are not in fact so divergent from our results as might appear at first sight. Using $K = 5 \times 10^{-6}$ and $B = 6.1 \times 10^{-8}$ for HbCO, we can calculate α from eq 5 for a given value of c, and thence the corresponding M_w from eq 6. Putting this and Guidotti's osmotic virial coefficient $B = 6.1 \times 10^{-8}$ into eq 1 to calculate $K'c/R_0$, we can construct a hypothetical light-scattering curve. For protein concentrations between 2 and 6 g per l. this proves to be a curve with less curvature than could be detected in actual experiments and slope near zero. A plausible linear extrapolation would give an apparent M_w of 60×10^3, low compared to our values but not by a large amount.

In 2 M NaCl, Guidotti finds marked differences among hemoglobin derivatives with K equal to 5×10^{-6}, 7.5×10^{-5}, and 1.2×10^{-4} for Hb, HbCO, and HbO_2, respectively. The large dissociation constant for HbCO is plainly incompatible with our light-scattering data in 2 M salt plus 0.05 M phosphate buffer.

In connection with a study of the functional implications of hemoglobin dissociation, Edelstein *et al.* (1970) have reported sedimentation equilibrium experiments of HbCO and Hb. Use of absorption optics permitted measurements to be made in a range of low concentrations (<0.5 g/l.) where effects of dissociation should be paramount and nonideality negligible. For HbCO in 0.1 M phosphate buffer (pH 7) at 20°, the data yield dissociation constants of 1×10^{-6} to 2×10^{-6} depending on the initial protein concentration. Measurements with Hb under the same conditions give $K \simeq 10^{-7}$. From parallel measurements of the concentration dependence of sedimentation velocity of HbCO and Hb, Edelstein *et al.* (1970) deduce dissociation constants like those determined from the equilibrium experiments. Flash photolysis of HbCO in the same buffer indicates the presence of two species differing in reactivity. If these are identified with tetramer and dimer, K is calculated to be 1.5×10^{-6} mole/l. in good agreement with the other experiments—and smaller than Guidotti's osmotic value in 0.2 M salt, though of the same order of magnitude. The minimal dissociation of Hb found by Edelstein *et al.* is evidently in agreement with indications of a normal molecular weight from our data at much higher concentrations. The higher value of K they find for HbCO requires a degree of dissociation that would not be unambiguously detectable by light scattering in the concentration range of our measurements.

Edelstein *et al.* (1970) also performed flash photolysis experiments on HbCO in 2 M NaCl. Again, two kinetically distinguishable species are discerned, and now the kinetic data yield $K = 7.0 \times 10^{-5}$, which agrees with Guidotti's osmotic data but not with our light-scattering data.

Benesch *et al.* (1964) used the Archibald ultracentrifugal method to determine molecular weights of Hb and HbO_2 in 0.05 M phosphate at pH 7 and in this buffer plus 2 M NaCl. At low ionic strength, apparent molecular weights of 66.5×10^3 and 67.6×10^3 were obtained at 20°; but in the presence of 2 M salt at 5°, values of 51.6×10^3 and 41.7×10^3, respectively, were obtained. The authors interpreted these results as indicative of dissociation (which was found to become more extensive at higher temperature). Their interpretation is suspect, however, since solutions were presumed to obey van't Hoff's law despite the fact that protein concentration was quite high (*ca.* 10 g/l.) in all experiments. On this basis, the decrease in apparent molecular weight of Hb caused by 2 M salt requires a tetramer–dimer dissociation constant of 2×10^{-4} mole/l., a value grossly at variance with the maximum value consistent with our light-scattering data at lower concentration. On the other hand, since the ultracentrifuge yields an apparent molecular weight (as does light scattering) defined by

$$M_{w,app} = (M_w^{-1} + 2Bc + \ldots)^{-1} \quad (8)$$

in the absence of dissociation, the apparent change in molecular weight of Hb reported by Benesch *et al.* (1964) can be explained by the effect of the second virial coefficient if B's in 2 M salt and at low ionic strength differ by about 2×10^{-7} l./g^2. The plots labeled A and B in our Figure 2 show (perhaps fortuitously) just such an effect. As we have already mentioned, manifestations of both dissociation and nonideality can be seen in Guidotti's (1967) osmotic pressure data: in particular, his Figure 6 shows the predominant effect of nonideality at high concentrations of Hb in 2 M NaCl.

The ultracentifuge data of Benesch *et al.* (1964) on HbO_2 in 2 M salt, which show even more complete apparent dissociation are subject to the same ambiguity;[6] but for this system Guidotti's osmotic measurements do indicate that HbO_2 dissociates much more readily than Hb or HbCO.

From sedimentation velocity data on HbCO at 25°, Kirshner and Tanford (1964) deduced dissociation constants of 2.5×10^{-6} mole/l. in 0.02 M NaCl and 1.8×10^{-4} mole/l. in 2 M NaCl. This first figure is bracketed by the various constants for HbCO at low ionic strength cited above; the second is larger than those obtained for 2 M salt by Guidotti (1967)

[6] In principle, the effect of protein concentration on apparent molecular weight obtained by the Archibald technique can be elucidated by making a series of runs at different initial concentrations and analyzing data taken at times near the beginning of sedimentation or by following one run at high initial concentration for a longer period of time until the meniscus is substantially depleted of solute. Benesch *et al.* (1962) found no change in apparent molecular weight during an experiment. Their data presumably refer to systems before major redistribution of solute has occurred.

TABLE III: Molecular Weights (Weight Average) and Tetramer–Dimer Dissociation Constants Reported for Human Hemoglobin in 2 M NaCl at Neutral pH.

Reference	Temp (°C)	Protein Concn (g/l.)	Hb		HbCO		HbO_2	
			$M_w \times 10^{-3}$	K (mole/l.)	$M_w \times 10^{-3}$	K (mole/l.)	$M_w \times 10^{-3}$	K (mole/l.)
This work	12	1.5–6	62.4	$\leq 10^{-6}$	65.4	$\leq 10^{-6}$		
Rossi-Fanelli *et al.* (1961)	20	0.3–6	45.5				40.0	
Guidotti (1967)	20	1–12		5×10^{-6}		7.5×10^{-5}		1.2×10^{-4}
Edelstein *et al.* (1970)	22	0.8–2.6				7.0×10^{-5}		
Benesch *et al.* (1964)	5	*Ca.* 10	51.6				41.7	
	20	*Ca.* 10	45.1				38.3	
Kirshner and Tanford (1964)	13, 25	1–6				1.8×10^{-4}		

and by Edelstein *et al.* (1970), and is therefore far too large for accommodation with our light-scattering data.

From differential sedimentation experiments, Goers and Schumaker (1970) report—in accord with our results and those of Edelstein *et al.* (1970)—that Hb does not dissociate appreciably at concentrations above 0.3 g/l. in 0.1 M phosphate at pH 6.8. Under the same conditions they find that HbO_2 dissociates to dimer and monomer.

The various findings on dissociation of Hb, HbCO, and HbO_2 are summarized for comparison in Table III. A glance at the table shows that the large dissociation constants of HbCO in 2 M salt reported by Guidotti (1967), Edelstein *et al.* (1970), and Kirshner and Tanford (1964) refer to data at temperatures 8–13° higher than those we used (except that Kirshner and Tanford also found the same large K at 13°). Thus, it might be asked whether the differences in results could reflect a temperature dependence of the dissociation. *A priori*, an increase of as much as 100-fold in K over a temperature interval of 10° seems most unlikely since this would require a very large endothermic standard heat of dissociation and a large entropy increase (*ca.* 75 kcal and 230 cal deg^{-1} $mole^{-1}$, if $K = 10^{-6}$ at 12°). However, to answer the question more decisively we made some light-scattering measurements on HbCO in 2 M salt at 25 and 30° and found no temperature effect. Hence, we conclude that K is still of the order of 10^{-6} or less at 30°. Then, should ln K be independent of temperature, the stabilization of the tetramer would be due entirely to a standard entropy decrease of 27 cal deg^{-1} $mole^{-1}$ or more for the dissociation. Apart from their large value for K, this is the view of Kirshner and Tanford. For detailed references on the dissociation of hemoglobin, refer to Antonini and Brunori (1970).

Conclusions

(1) The specific refractive index increments of HbCO and HbO_2 (and probably of Hb) at 6328 Å are the same in the same solvent medium. In contradiction with the data of Rossi-Fanelli *et al.* (1961), the refractive increment falls as salt concentration is increased—in just the way expected if the refractive index of a mixture is a linear function of the composition by volume. (2) For solutions of HbCO and HbO_2 in 2 M salt, preferential interactions (binding of salt or water) appear to be too small to have an appreciable effect on molecular weight determinations by light scattering or equilibrium sedimentation. (3) Light-scattering data on Hb and HbCO at concentrations above 1.5 g/l. in 0.05 M phosphate buffer alone and with added 2 M NaCl, yield the molecular weight (within experimental error) of intact hemoglobin tetramer. The results are evidence against the occurrence of extensive dissociation of tetramer at these concentrations. They are clearly in quantitative disagreement with reports of dissociation of Hb (Rossi-Fanelli *et al.*, 1961; Benesch *et al.*, 1964) and of HbCO (Kirshner and Tanford, 1964; Guidotti, 1967; Edelstein *et al.*, 1970) at high ionic strength.

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Analysis of Long-Chain Free Fatty Acid Binding to Bovine Serum Albumin by Determination of Stepwise Equilibrium Constants*

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ABSTRACT: The stepwise equilibrium method was employed to analyze the binding of long-chain free fatty acids to bovine albumin. Equilibrium partition incubations were done at 37° in a calcium-free Krebs–Ringer phosphate buffer (pH 7.4). Charcoal-extracted crystalline bovine serum albumin and ^{14}C-labeled fatty acids were used. In general, the 16-carbon atom acids were bound more tightly than either the 14- or 18-carbon atom acids. With all of the acids, we noted the presence of one very strong albumin binding site having an equilibrium constant in the range of 10^7 M^{-1} and two sites with constants in the range of 10^6 M^{-1}. Five additional sites having constants ranging from 10^5 to 10^4 M^{-1} also were detected. In every case the equilibrium constants for binding of the first 4 moles of fatty acid occurred in descending order: $K_1 > K_2 > K_3 > K_4$. This suggests that appreciable cooperative binding effects were not evident over the range of fatty acid:albumin molar ratios that are usually employed in metabolic studies. From partition data with a protein-free aqueous phase, the extent of aqueous dimerization was estimated for palmitic, stearic, and oleic acids. Anion dimerization corrections then were calculated for the corresponding binding data, assuming that only the fatty acid anion monomer interacts with albumin. A reanalysis of these corrected data revealed that little or no change was produced in the magnitude of the first four equilibrium constants. This indicates that anion dimerization has little effect upon the binding parameters when the fatty acid:albumin molar ratio is within the usual physiological range.

Long-chain free fatty acids (FFA)[1] are very poorly soluble in the aqueous media that are employed for most biological reactions. Therefore, a carrier is used in order to introduce sufficient amounts of FFA into the incubation medium or to take up FFA that is released in the course of a metabolic reaction. In the majority of cases, BSA is used experimentally as the FFA carrier or acceptor. Hence, it is important to have a thorough understanding of the interaction of FFA with BSA in order to interpret accurately many of these metabolic studies.

The binding of FFA to BSA has been investigated previously in some detail (Teresi and Luck, 1952; Boyer *et al.*, 1946a; Reynolds *et al.*, 1968; Spector *et al.*, 1969). These data were analyzed by a method that was based on the Scatchard binding model (Scatchard, 1949; Fletcher and Spector, 1968). Recently, it has been shown that the "apparent association constants" of the Scatchard model need not be meaningful biological parameters (Fletcher *et al.*, 1970). In fact the correction of the Scatchard parameters by means of the so-called "statistical factors" (Edsall and Wyman, 1958) gives true equilibrium constants only under the assumptions that each site is uninfluenced by its neighbor and each has the same intrinsic affinity for the ligand (Klotz, 1953). On the other hand, analysis of macromolecule–ligand interactions by a series of stepwise equilibria (Klotz, 1946; Klotz *et al.*, 1946) is sufficiently general to account for electrostatic interaction and other phenomenon that may be associated with FFA–

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[1] Abbreviations used are: FFA, free fatty acid; BSA, bovine serum albumin.