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On the Tetramer–Dimer Equilibrium of Carbon Monoxyhemoglobin in 2 M Sodium Chloride†

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ABSTRACT: The state of aggregation of human carbon monoxyhemoglobin in solution at neutral pH has been studied by light scattering at concentrations ranging from about 0.7 to 12 g/l. As in our earlier work, the scattering behavior at low ionic strength (0.1 M) indicates only intact tetrameric hemoglobin, and implies that the tetramer–dimer dissociation constant can be no more than about 10^{-6} mol/l. In contrast, data on systems in 2 M NaCl, with or without phosphate or Tris buffer, demand an upward revision of our previous estimate of dissociation—to dissociation constants on the order of 10^{-5} mol/l., but still smaller than values reported in a number of investigations. Stripping the hemoglobin of bound organic phosphate (diphosphoglycerate) has no measurable effect on the dissociation.

It appears well established that human adult hemoglobin (hemoglobin A) in the deoxy (Hb)[1] form does not dissociate appreciably into dimers, even in the presence of 2 M NaCl at neutral pH; *i.e.*, the tetramer–dimer dissociation constant is less than 10^{-6} mol/l. (Norén *et al.*, 1971; Kellett, 1971; Thomas and Edelstein, 1972). However, there remains disagreement on the dissociation of carbon monoxyhemoglobin, particularly at high ionic strength (Antonini and Brunori, 1970; Edelstein *et al.*, 1970; Norén *et al.*, 1971; Kellett, 1971).

In a previous light-scattering study of the effect of 2 M NaCl on the apparent molecular weight of Hb and HbCO, we found no evidence for dissociation at the level of discrimination afforded by our experiments (Norén *et al.*, 1971). Using the same light-scattering technique, we have now supplemented our earlier results with some measurements on HbCO stripped of organic phosphate since it has been suggested that 2,3-diphosphoglycerate bound to hemoglobin might affect the dissociation. We have also somewhat extended the hemoglobin concentration range studied in both directions: 0.7–12 g/l. rather than 1.7–7 g/l.

Experimental Section

Materials. Fresh whole blood was obtained from the local blood bank. The cells were washed several times with saline and the hemoglobin was converted to HbCO by treatment

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[1] Abbreviations used are: Hb, deoxyhemoglobin; HbO_2, oxyhemoglobin; HbCO, carbon monoxyhemoglobin; DPG, 2,3-diphospho-D-glyceric acid; IHP, inositolhexaphosphoric acid; Bis-Tris, 2,2-bis(hydroxyethyl)-2,2′,2″-nitrilotrimethanol.

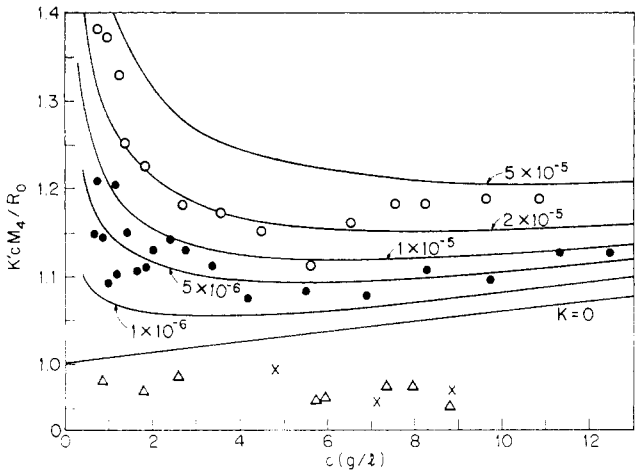

FIGURE 1: Light-scattering plots of HbCO. The curves are from eq 10 with α the fraction of tetramer dissociated to dimer given by eq 2 and K as indicated. Crosses are experimental data on Hb in 0.1 M NaCl. The other points are HbCO data as identified in Table I: expt 1 (△); 6 (●); 2 (○).

with carbon monoxide. Then the cells were lysed with distilled water (except for one experiment in which toluene was used) and the HbCO solution was dialyzed against water. Phosphate-free hemoglobin was obtained by passing HbCO in a stripping buffer (0.01 M Tris-HCl and 0.1 M NaCl (pH 7.5)) through a Sephadex G-25 gel column (2.5 × 40 cm) to isolate the protein fraction (Berman *et al.*, 1971). As in the previous work, hemoglobin preparations were stored as HbCO in phosphate buffer near pH 7 and 4°. Samples for light scattering and refraction measurements were prepared by dialysis against six or more changes of the desired buffer over the course of at least 36 hr. The last dialysate was used as the solvent for dilution of the stock solution. Experiments were performed within 10 days from completion of the hemoglobin preparation.

HbCO concentrations were determined at 539 and 569 nm using a Zeiss PMQ II spectrophotometer. The millimolar extinction coefficient was taken as 13.4 for both wavelengths (Antonini, 1965).

Hb used in one experiment was prepared from HbCO by flushing the solution with oxygen in a rotary evaporator at 5° under a 150-W flood lamp and then flushing the resulting HbO_2 solution with nitrogen. As in our previous work, the absorption spectrum (obtained with a Cary 14 recording spectrophotometer) in the visible region showed the Hb to contain less than 2% methemoglobin. The Hb concentration was obtained by oxygenating the solution and measuring the absorbance at 541 and 577 nm. The millimolar extinction coefficients were taken as 13.8 and 14.6, respectively, at these wavelengths (Antonini, 1965).

The sodium salt of inositolhexaphosphoric acid (Sigma, Type V) and 2,2-bis(hydroxyethyl)-2,2′,2′′-nitrilotrimethanol (Aldrich) were used without further purification. DPG was prepared from the pentacyclohexylammonium salt of 2,3-diphospho-D-glyceric acid (Calbiochem) by treatment with a cation exchange resin (Bio-Rad AG 50W-X8). All other chemicals were analytical grade and were used without purification.

Methods. Light-scattering measurements were carried out using a 6328-Å laser light source with the same photometer and sample cells as previously described, and solutions were handled by the same procedures (Norén *et al.*, 1971). It was presumed that "excess" scattered intensity R_θ at scattering angle θ depends on θ and concentration c through the familiar relation (Zimm, 1948)

$$K'c/R_\theta = (1/M_w P(\theta)) + 2Bc \quad (1)$$

with the symbols all as previously identified (Norén *et al.*, 1971). Some 10–20 experimental points were usually obtained in a concentration series (see Figure 1). They represent dilutions of several stock solutions, sometimes derived from different batches of blood, and measurements made over several days. This procedure very likely enhanced scatter in the data but also helped avoid misleading results from possibly anomalous individual samples.

Specific refractive index increments $(\partial n/\partial c)_\mu$ measured at osmotic equilibrium are needed to determine the optical constant K' (Casassa and Eisenberg, 1964). They were measured at 6328 Å in a way described previously (Norén *et al.*, 1971) and are listed in Table I. The experimental values are in agreement with theoretical values calculated from dn/dc in pure water and the refractive index of salt solutions. As was expected, addition of 2 mM DPG or 5 mM IHP had no sensible effect on the refractive increment.

Results

All the light-scattering data reported previously exhibited "normal" behavior in that the scattering function $K'c/R_\theta$ was virtually independent of scattering angle and of hemoglobin concentration, except for a possible small linear dependence on concentration attributable to a nonzero second virial coefficient at high ionic strength (Norén *et al.*, 1971). The new data also indicate the angular intensity factor $P(\theta)$ to be unity at any angle, but a satisfactory analysis of the concentration dependence (here more evident than before, partly because of the wider range of protein concentration) demands consideration of both the dissociation equilibrium and thermodynamic nonideality. The following development was undertaken to provide a first order of approximation for treating both effects.

The fraction α of hemoglobin dissociated to dimer is related to the dissociation constant K (in mol/l.), by

$$8\alpha c = KM_4(1 + 16c/KM_4)^{1/2} - KM_4 \quad (2)$$

where c is the protein concentration in g/l. and $M_4 = 64{,}458$ is the molecular weight of intact tetramer. Then, the weight-average molecular weight

$$M_w = (1 - \alpha/2)M_4 \quad (3)$$

of the mixture of tetramer and dimer is a function of total concentration. The second virial coefficient in the light-scattering equation of state for a mixture of two solute species (subscripts 2 and 4) with the same specific refractive increment is (Stockmayer, 1950)

$$B = (B_{22}M_2^2w_2^2 + 2B_{24}M_2M_4w_2w_4 + B_{44}M_4^2w_4^2)/M_w^2 \quad (4)$$

where w_2 and $w_4 = 1 - w_2$ are weight fractions of the two components in the solute. In the present instance, $M_2 = M_4/2$, and thus B, depends on three parameters B_{ij} and on concentration through

$$B = \left[\frac{1}{4}\alpha^2B_{22} + \alpha(1 - \alpha)B_{24} + (1 - \alpha)^2B_{44}\right] \times \left[1 - \frac{1}{2}\alpha\right]^{-2} \quad (5)$$

In general there is no necessary relation among the three coefficients B_{ij}, but since the data in question will hardly permit

TABLE I: Dissociation Constant for HbCO in Various Media (from Light Scattering Data at 6328 Å, 12°).

Expt	Stripped	Solvent	pH	$(\partial n/\partial c)_\mu$ (ml/g)	$K \times 10^5$ (mol/l.)
1	Yes	0.1 M NaCl	6.9	0.190 ± 0.001	≤ 0.1
2	Yes	2 M NaCl	7.5	0.181 ± 0.001	3
3	No	0.05 M Phosphate + 2 M NaCl	6.9	0.176 ± 0.001	0.5
4	No	0.05 M Phosphate + 2 M NaCl[a]	6.9		1
5	Yes	0.05 M Phosphate + 2 M NaCl	7.0	0.176 ± 0.001	0.8
6	Yes	0.05 M Bis-Tris + 2 M NaCl	7.0	0.177 ± 0.001	0.5
7	Yes	0.05 M Phosphate + 2 M NaCl + 2 mM DPG[b]	6.6	0.175 ± 0.002	1
8	Yes	0.05 M Phosphate + 2 M NaCl + 2 mM DPG[c]	6.7	0.175 ± 0.002	0.5
9	Yes	0.05 M Phosphate + 2 M NaCl + 5 mM IHP[d]	6.9	0.177 ± 0.002	3

[a] Blood cells lysed with toluene. [b] HbCO first dialyzed against 0.05 M phosphate + 2 M NaCl; then 2 mM DPG added to both hemoglobin and buffer for the last dialysis. [c] 2 mM DPG added to HbCO; then hemoglobin dialyzed against 0.05 M phosphate + 2 M NaCl + 2 mM DPG. [d] 5 mM IHP added to HbCO; then hemoglobin dialyzed against 0.05 M phosphate + 2 M NaCl + 5 mM IHP.

extraction of three meaningful empirical constants, we hazard assumptions recommended by no more than some measure of plausibility. Formally, B_{ij} can be defined in terms of an effective potential energy of interaction between a pair of molecules of species i and j. The simplest potential, that for hard spheres with radii r_i and r_j (which experience no interaction at all when their centers are separated by a distance greater than $r_i + r_j$ and an infinite repulsive potential at distances less than this), yields

$$B_{ij} = 2\pi N_A(r_i + r_j)^3/3M_iM_j \quad (6)$$

with N_A denoting Avogadro's number. Arbitrarily representing the hemoglobin tetramer and dimer as spheres with volumes corresponding to the partial specific volume $\bar{v} = 0.75$ ml g^{-1}, we obtain from eq 6

$$B_{44} = 4\bar{v}/M_4 = 4.65 \times 10^{-8} \text{ l. mol g}^{-2} \quad (7)$$

$$B_{22} = 2B_{44} \quad (8)$$

$$B_{24} = (1 + 2^{1/3})^3 B_{44}/8 = 1.44B_{44} \quad (9)$$

Using eq 7–9 in eq 5, and getting α as a function of c from eq 2, and M_w from eq 3, we can calculate theoretical curves for $K'c/R_0$ in eq 1 *vs.* c for chosen values of K. Trial calculations showed, however, that for magnitudes of K of interest in this study, these theoretical curves do not differ appreciably from those obtained by simply letting $B = B_{44}$, independent of concentration and thus of α. Thus, with $P(\theta) = 1$ and concentration c in g l.$^{-1}$, we cast eq 1 into the form

$$M_4K'c/R_0 = [2/(2 - \alpha)] + 0.0060c \quad (10)$$

which is adequate quantitatively to represent the results of the preceding derivation. Figure 1 shows plots of $M_4K'c/R_0$ according to eq 10 for several values of the equilibrium constant K.

In addition to the assumptions already stated, it must be remembered that from the point of view of thermodynamics there is an inherent ambiguity in separating the effect of dissociation from that of nonideality at finite concentration.

To obtain the tetramer–dimer dissociation constant, we entered experimental points for $K'cM_4/R_0$ on a plot of theoretical curves like that shown in Figure 1 in order to judge the best fit of experiment with the calculated functions. Estimates obtained in this way are listed in Table I for all the systems studied.

The data for HbCO in 0.1 M NaCl shown in Figure 1 were obtained as a control and are in accord with our earlier results for low ionic strength. The points describe a straight line of nearly zero slope and the molecular weight obtained (65,800) agrees within experimental uncertainty with that for intact hemoglobin. We therefore conclude that the dissociation constant in this case is, as expected, very small; *i.e.*, 1×10^{-6} is a realistic upper bound. Since these samples were treated to remove bound DPG, the phosphate has no apparent influence on dissociation at low ionic strength.

Three points for Hb in 0.1 M NaCl at neutral pH are also shown in Figure 1. They too are consistent with the molecular weight of intact tetramer as in our previous work.

The systems at higher ionic strength (containing 2 M NaCl) reveal different behavior: (a) at the lowest concentrations, $K'c/R_0$ *vs.* c shows upward curvature indicative of a reversible dissociation; (b) at all concentrations the experimental points lie above any reasonable straight line extrapolating to $1/M_4$; and (c) at high concentrations there appears a small positive slope reasonably attributable to a positive virial coefficient. Although the marked upward curvature at small concentration is at first sight the most direct evidence of some dissociation, it is confined to a few points at the extreme of the concentration range—just where experimental uncertainty is greatest. On the other hand, the theoretical plots show that the effect of dissociation persists to high concentration, noticeably increasing the value of $K'c/R_0$ even when the equilibrium constant is as small as 1×10^{-5}. Effects (a) and (b) are both manifestations of dissociation, but we regard the latter as more conclusive here. The calibration of the light-scattering photometer has been checked periodically over a long period of time and it seems most unlikely that systematic intensity calibration errors (which would have to be on the order of at least 10%) could account for the high values of $K'c/R_0$. Furthermore we would have to presume that such errors affected all the measurements save those for solutions at ionic strength 0.1.

The positive slope usually detected in Kc/R_0 *vs.* c at high concentration for systems at high ionic strength is consistent with more tentative indications in our previous work. It leads us to use a B for hard spheres in fitting the data. This choice is arbitrary, but it is certainly a better guess than just taking $B = 0$. Although a marginally better fit could be obtained in some cases with a larger B coupled with a slightly smaller dissociation constant K, such refinement seems unwarranted.

We note that if we consider the data falling in a limited concentration range—say from about 2 to 7 g/l., as in our previous paper—the experimental scatter justifies only a straight-line extrapolation to $c = 0$; and the apparent molecular weight so obtained, though it has no real meaning, is definitely smaller than the tetramer molecular weight for the systems at high ionic strength. In this respect our new results are different from those previously reported (Norén *et al.*, 1971) and must reflect somewhat greater dissociation of HbCO. Where previously we set an upper limit of $K \approx 1 \times 10^{-6}$, the new data require values an order of magnitude greater.

In expt 4 listed in Table I, the blood cells were lysed with toluene instead of water; but expt 3, which is otherwise a duplicate of expt 4, gave a similiar result. Therefore, although toluene was used in our previous work, we cannot attribute the larger dissociation constants reported here to this detail of procedure. However, since we cannot account for the difference we must suppose that some variable was not adequately controlled in the conventional experimental procedures we used in handling the hemoglobin preparations..

It would probably be unwarranted to attribute much significance to the differences among values of K in Table I for the various systems containing 2 M NaCl; but experimental uncertainty decreases so rapidly with increasing dissociation that values of K greater than about 5×10^{-5} would be inconsistent with even experiments 2 and 9. Stripping of bound DPG has no apparent effect nor does the addition of small amounts of IHP or DPG to the stripped protein.

Discussion

The data summarized in Table I represent a more extensive study of the state of aggregation of HbCO in 2 M NaCl than we reported previously (Norén *et al.*, 1971). In view of this, we must regard the new evidence, indicating an at least detectable dissociation, as having the greater weight. Nevertheless, the detailed comparisons we made between our previous results and a variety of data arguing for dissociation constants far larger than 10^{-6} mol/l. are not basically changed. The estimates most nearly comparable with our old (and new) results are 7.5×10^{-5} due to Guidotti (1967) and 7.0×10^{-5} due to Edelstein *et al.* (1970). Hence while our new data narrow the gap with the latter studies, the very high value of 1.8×10^{-4} obtained by Kirshner and Tanford (1964) remains obviously inconsistent with other data. Our data are entirely consistent with the now generally accepted point of view that the dissociation is far less for Hb than for liganded hemoglobin; but we venture no comment on "second-order" differences in effects of various ligands on the dissociation.

We indicated previously why some dissociation studies of liganded and unliganded hemoglobin must be discounted for experimental inadequacy or faulty interpretations. There is nothing in our new study to cause us to alter those judgments.

The measurements reported here represent about the limit of what can be done with the conventional light-scattering method. An accurate measurement of the HbCO dissociation constant requires accurate data over the concentration range where most of the dissociation occurs, *i.e.*, below 1 g/l. if $K \leq 10^{-5}$. It is in just this region that scattering data are unreliable because the contribution of solute to the total scattering becomes comparable with the contribution from solvent and adventitious "dust" contamination. Some advantage could probably be gained by use of a photometer designed to view a very small illuminated sample volume, on the order of 0.001 ml (Kaye *et al.*, 1971). As the volume is made progressively smaller, scintillations due to passage of foreign particles across the field of view tend to be recorded as discrete events that can be discounted in the data analysis. Because light scattering measures a molecular weight average biased toward large molecules, it is in principle less sensitive than the osmotic pressure to small degrees of dissociation. Any advantage from this fact is nullified by the circumstance that osmometry, as carried out with the currently available dynamic osmometers, does not have the precision easily attainable with light scattering. Static osmometry is more satisfactory in this regard, but is prohibitively time consuming, a fact that in itself implies special problems in working with biological materials near room temperature. The ultracentrifuge with absorption optical detection offers one means of access to a sufficiently low concentration range, and it has been used successfully despite technical difficulties (Edelstein *et al.*, 1970; Kellett, 1971). Rayleigh spectroscopy is a developing light-scattering technique that could conceivably be used to measure quantitatively tetramer and dimer in a mixture since Brownian motion of the two species should affect the Rayleigh line width differently.

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