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SELF-ASSOCIATION IN HIGHLY CONCENTRATED SOLUTIONS OF MYOGLOBIN: A NOVEL ANALYSIS OF SEDIMENTATION EQUILIBRIUM OF HIGHLY NONIDEAL SOLUTIONS

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The sedimentation equilibrium in concentrated solutions of hemoglobin and myoglobin has been measured. The ratio of the apparent molecular weight of hemoglobin to that of myoglobin, R, is found to obey the empirical relation $R(c)=3.8-4.25\times 10^{-2}c+6.44\times 10^{-4}c^2-2.21\times 10^{-5}c^3$, where c is the protein concentration in g/dl, for $c\leq 40$ g/dl. A theoretical relation for the dependence of R upon c in the absence of protein self-association is presented. This relation cannot be fitted to the experimental results, and the discrepancy is attributed to self-association of myoglobin.

1. Introduction

When the α and β subunits of hemoglobin are separated, β chains form homogeneous tetramers at low concentration; at higher concentrations α chains also appear to self-associate [1]. It has been suggested that by virtue of its resemblance to the subunit chains of hemoglobin, myoglobin should possess the ability to self-associate as well, but more weakly, i.e., with a free energy change which is more positive than that accompanying the self-association of α and β chains [2,16]. If such is the case, then myoglobin would be expected to self-associate to a significant extent only in highly concentrated solutions. We report here the measurement of the sedimentation equilibrium of myoglobin and hemoglobin at such concentrations. A novel analysis of sedimentation equilibrium of highly nonideal protein solutions, based upon comparison of the observed properties of hemoglobin and myoglobin, permits us to conclude that in very concentrated solutions myoglobin self-associates to a dimer and very probably to higher oligomers as well.

2. Materials

Horse heart myoglobin (Sigma) and human hemoglobin (Sigma) were converted to the cyanmet form by the addition of 1.2 equivalents of potassium ferricyanide and 2 equivalents of potassium cyanide per equivalent of heme [3]. The protein solutions were then dialyzed against large volumes of phosphate-buffered saline (0.1 M NaCl, 0.05 M phosphate, pH 7.4). The visible absorbance spectra of hemoglobin and myoglobin solutions prepared in this manner were unchanged after being kept in sealed cuvettes at room temperature for 1 month.

3. Experimental methods and results

In order for the sedimentation equilibrium of heme proteins to be measured at high concentration, extremely short path length centrifuge cells similar to those employed by Braswell [4] were assembled as follows: A standard 12-mm Kel-F single-sector centerpiece was placed in a cell housing as a spacer. Next, the lower window in its holder was inserted, and a ring-shaped centerpiece gasket of silicone rubber was placed on the upper surface of the window to act as a thin centerpiece. 2–3 μl of sample were placed on the center of the lower window, so as not to touch the centerpiece, the upper window was placed in position, and the cell was closed and the screw ring tightened to a torque of $\approx$ 140 inch-pounds, with a resulting flattening of the centerpiece gasket.

Prior to centrifugation the sample remained in the center of the cell, due to capillary attraction. Upon centrifugation, the sample formed a circular segment whose upper boundary (meniscus) was normal to the centrifugal field and whose lower boundary (base) was the inner edge of the centerpiece gasket. The maximum distance between meniscus and base, at the center of the circular segment, was between 3 and 4 mm. The optical path length was determined from measurements of light absorbance in the visible region together with known extinction coefficients of the heme protein. Because of the plasticity of the gasket material, the path length varied between 0.15 and 0.2 mm depending upon the compression of the centerpiece. Precise determination of the path length was unnecessary, as concentrations of the sample in the cell were determined by relative rather than by absolute measurements of absorbance.

A four-hole aluminum rotor (An-F) was loaded with a single counterbalance and three short path length cells containing buffer, cyanmethemoglobin (loading concentration 18.8 g/dl), and cyanmetmyoglobin (loading concentration 18.8 g/dl). Experiments were performed at 20°C on a Beckman model E analytical centrifuge equipped with scanner and multiplexer. It was verified that the samples obeyed Beer's law and that the scanner pen deflection varied linearly with the absorbance of the samples at the wavelengths employed. The scanner was operated in the split-beam, single-sector mode, so that the absorbance recorded on a scanner trace represented the difference between the absolute absorbances of a sample solution in one cell and buffer in a second cell. In order to estimate the baseline height for each of the sample cells, at the conclusion of the centrifugation the rotor was spun at its maximum rated speed (52000 rpm) until the meniscus was depleted in each of the sample cells. As a preliminary experiment with short path length cells containing buffer only had shown that baseline height was essentially invariant with cell position and rotor speed, the height of the scanner trace at the depleted meniscus was assumed equal to the baseline height at all positions in the cell and at all rotor speeds.

A few minutes after beginning the centrifuge run, at a rotor speed of 3000 rpm, each sample cell was scanned at two wavelengths, 545 and 505 nm, corresponding to a maximum and minimum, respectively, in the visible spectrum of the heme proteins, and at two scanner sensitivities, corresponding to 1 and 2.5 absorbance units full scale. * The traces of these scans exhibited a small linear dependence of absorbance upon radial position. The absorbance at a particular wavelength corresponding to the loading concentration was assumed to be equal to the absorbance at the midpoint of the sample column. **

The rotor speed was increased to 14000 rpm, and scans were recorded daily for each sample at a single wavelength and sensitivity until no change in the scan trace was observed on successive days. Typically, 4 or 5 days were required to obtain successive scans which were superimposable. When

* The light passing through the sample is not strictly monochromatic (half-maximal bandpass=21 nm at 505 nm and 26 nm at 545 nm for a monochromator slit width of 1 mm). The absorbance recorded by the scanner must therefore be regarded as an effective or apparent absorbance which is similar but not identical to that measured using more nearly monochromatic light.

** At the conclusion of the centrifuge run, traces of scans recorded at the same wavelength and different rotor speeds were superimposed, and the hinge point [15] was determined. The absorbance at the hinge point differed from that at the midpoint of the 3000 rpm scan by no more than 3% in both protein solutions at both wavelengths.

this criterion was satisfied, each cell was scanned at the two wavelengths and sensitivities given above. The entire procedure was then repeated at rotor speeds of 20000, 28000 and 40000 rpm. Excellent reproducibility of slow scans indicates that rotor precession did not significantly distort the data.

Each scan trace was manually digitized to provide approximately 45 data points in the sample column at uniform intervals of the radial distance r. These data were transformed into tables of c versus r^2 via the following relation

$$c(r) = c_0 \cdot \frac{A(r,\lambda) - B(\lambda)}{A_0(\lambda) - B(\lambda)} \quad (1)$$

where c_0 is the loading concentration, $A(r,\lambda)$ the

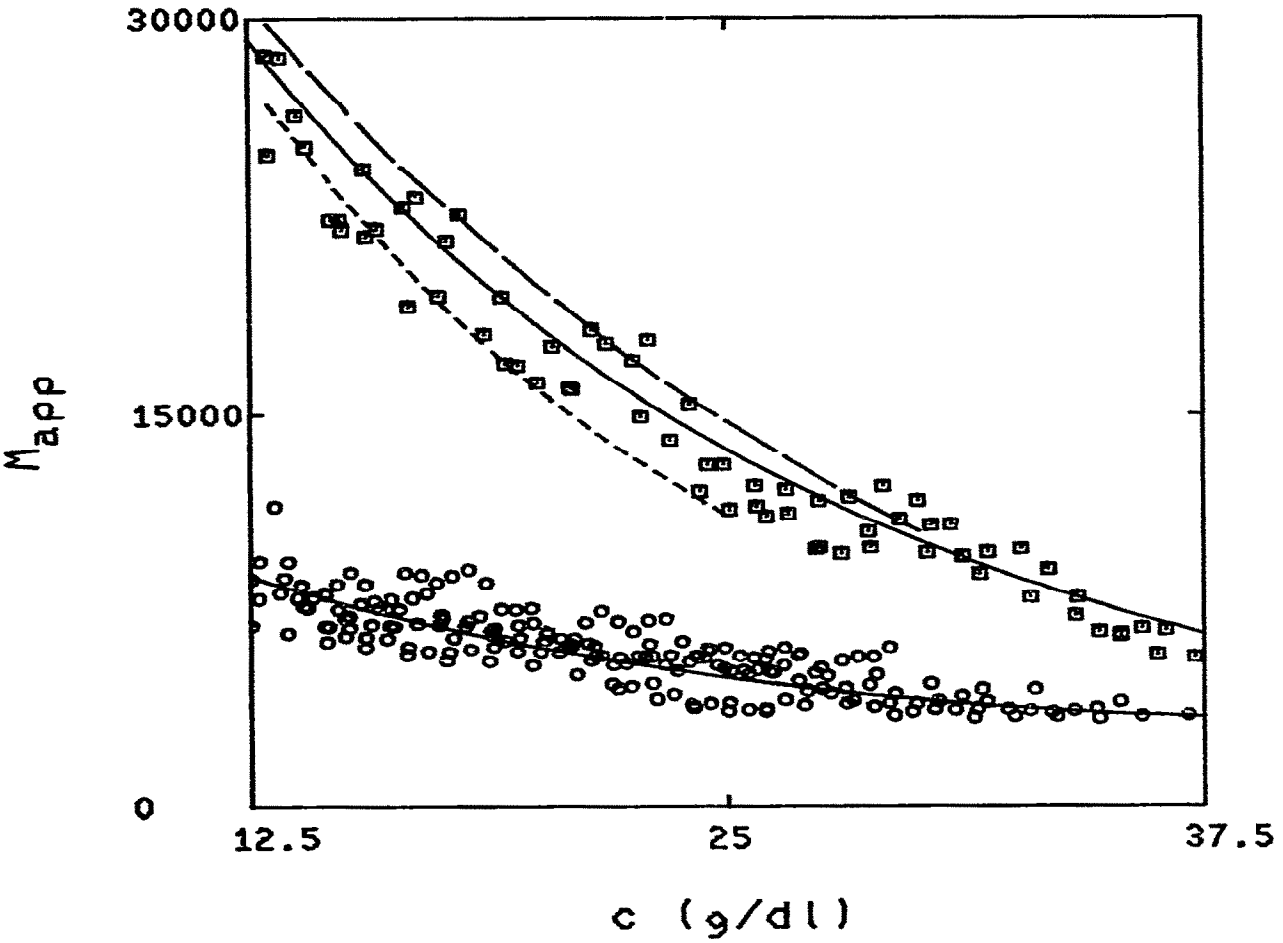

Fig. 1. Apparent molecular weight of hemoglobin and myoglobin versus protein concentration. Data: (□) hemoglobin; (○) myoglobin. Curves: (- - - - -) results of Williams [9]; (— — —) results of Briehl and Ewert [10]; (————, upper) best fit of eq. (5) to hemoglobin data [$M = 65000$, $V = 0.0095$ dl/g]; (————, lower) best fit of eq. (8) to myoglobin data [$A_1 = -4.25 \times 10^{-2}$ dl/g, $A_2 = 6.44 \times 10^{-4}$ (dl/g)^2, and $A_3 = -2.21 \times 10^{-5}$ (dl/g)^3].

absorbance at radius r and wavelength λ, $A_0(\lambda)$ the absorbance at wavelength λ corresponding to the loading concentration, and $B(\lambda)$ the baseline absorbance at wavelength λ. To reduce artifacts arising from stray light at very high absorbance, only points with an absorbance of less than 2 units were included in the data set to be analyzed.

Depending upon the noisiness of the data, the tables of (r^2,c) were smoothed using a three- or five-point running average of c. Next, the value of dc/dr^2 corresponding to each data point (r_i^2,c_i) was estimated according to

$$\left(\frac{dc}{dr^2}\right)_{r=r_i} = \frac{c_{i+1}-c_{i-1}}{r_{i+1}^2-r_{i-1}^2} \tag{2}$$

where the subscripts $i-1$ and $i+1$, respectively, denote the data points immediately preceding and succeeding data point (r_i^2,c_i) in the direction of increasing r. The value of the apparent molecular weight of heme protein, M_{app}, was calculated for each data point using the relation [5]

$$M_{app} = \frac{2RT}{(1-\bar{v}\rho)\omega^2}\cdot\frac{1}{c}\cdot\frac{dc}{dr^2} \tag{3a}$$

where R is the molar gas constant, T the absolute temperature, ω the rotor speed in rad/s, $\bar{v}$ the partial specific volume of the protein, and ρ the density of the solution. Assuming that the density of the solution has a linear dependence upon c [6], we may write

$$(1-\bar{v}\rho) = (1-\bar{v}\rho_0)(1-\bar{v}c) \tag{3b}$$

where ρ_0 is the density of the solvent at temperature T. The value of $\bar{v}$ is taken as 0.74 ml/g for both hemoglobin and myoglobin [7], and the density of buffer at 1.004 g/ml [8].

The values of M_{app} for both hemoglobin and myoglobin, calculated as described above, are plotted as functions of c in fig. 1. The data for hemoglobin are pooled from scans at 14000, 21000 and 28000 rpm; the data for myoglobin are pooled from scans at 21000, 28000 and 40000 rpm. Also plotted in fig. 1 are curves calculated according to equations given by Williams [9] and Briehl and Ewert [10] to describe the results of their respective sedimentation equilibrium studies on hemoglobin at high concentration. It may be seen that for hemoglobin concentrations exceeding 12.5 g/dl, our results for hemoglobin are in satisfactory agreement with the earlier results obtained using interferometric [9] and schlieren [10] optics, thus lending confidence in the validity of our technique and the reliability of our results for myoglobin. *

4. Discussion

The present analysis is based upon a comparison of the concentration dependence of the apparent molecular weights of hemoglobin and myoglobin. We define the ratio

$$R(c) = M_{app}^{Hb}(c)/M_{app}^{Mb}(c) \tag{4}$$

where the superscripts Hb and Mb refer to hemoglobin and myoglobin, respectively. In order to evaluate R, we require analytical expressions for M_{app}^{Hb} and M_{app}^{Mb} as functions of c.

4.1. Hemoglobin

The sedimentation equilibrium of concentrated hemoglobin solutions may be quantitatively described by a model in which protein molecules are presented by equivalent hard spherical particles which do not self-associate [11,12]. According to this model, the apparent molecular weight of protein is given by

$$M_{app}(c) = M/\left[1+7.0Vc+22.0(Vc)^2+43.45(Vc)^3 + 67.74(Vc)^4+95.97(Vc)^5\right] \tag{5}$$

where M is the molecular weight of the protein in the ideal limit, and V is the specific volume of the equivalent particle. Eq. (4) is plotted in fig. 1 for $M = 65000$ and $V = 0.0095$ dl/g (parameter values essentially identical to those obtained in ref. [11]); the calculated curve provides a good description of the hemoglobin data presented here within the limits of experimental precision.

* Regions of the sample column with protein concentration below 12.5 g/dl had such low absorbance that uncertainty in baseline height led to unacceptable uncertainty in the determination of c and especially of dc/dr^2.

4.2. Myoglobin

In order to obtain a smooth curve through the myoglobin data, we adopt an empirical expression for R

$$R(c) = A_0 + A_1 c + A_2 c^2 + A_3 c^3 \tag{6}$$

The value of A_0 may be independently determined:

$$A_0 = \lim_{c \to 0} M_{app}^{Hb}(c)/M_{app}^{Mb}(c) = M^{Hb}/M^{Mb}$$
$$= 65000/17000 = 3.8 \tag{7}$$

Rearranging eqs. (6) and (7), we obtain

$$M_{app}^{Mb}(c) = M_{app}^{Hb}(c)/(3.8 + A_1 c + A_2 c^2 + A_3 c^3) \tag{8}$$

Eq. (8) was fitted by the method of nonlinear least squares to the myoglobin data shown in fig. 1, using eq. (5) with the parameter values given above to calculate M_{app}^{Hb}. The best fit of eq. (8) to the myoglobin data is plotted in fig. 1, and the best-fit values of A_1, A_2 and A_3 are given in the figure caption. The concentration dependence of R, calculated using eq. (6) with these values of A_1, A_2, and A_3, is plotted in fig. 2.

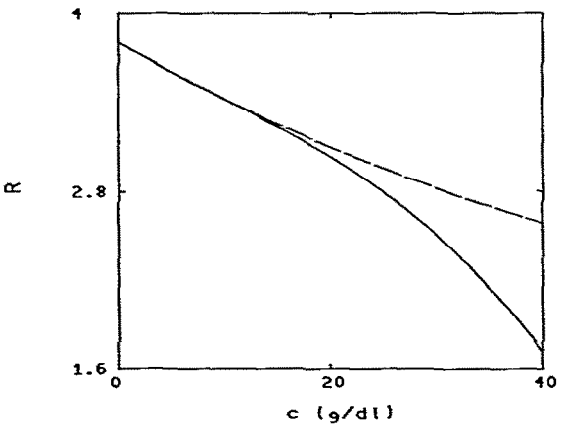

Fig. 2. R versus protein concentration. (————) Calculated using eq. (6) with values of A_i given in the legend to fig. 1. (- - - - -) Calculated using eq. (9) with $V = 0.0095$ dl/g, $V' = 0.0079$ dl/g.

Myoglobin, like hemoglobin, is a compact quasispherical molecule. It follows that if myoglobin does not self-associate, the dependence of M_{app}^{Mb} upon c should be described by eq. (5) with $M = 17000$. Under these conditions we may define a 'theoretical' value of R in the absence of myoglobin self-association:

$$R^*(c) = 3.8\{(1 + 7.0V'c + 22.0(V'c)^2 + 43.45(V'c)^3 + 67.74(V'c)^4 + 95.97(V'c)^5) \times (1 + 7.0Vc + 22.0(Vc)^2 + 43.45(Vc)^3 + 67.74(Vc)^4 + 95.97(Vc)^5)^{-1}\} \tag{9}$$

where V and V' are the specific volumes of the equivalent particles representing hemoglobin and myoglobin, respectively. The value of V' is determined by setting $R^* = R$ for $c \leq 4$ g/dl: at these concentrations myoglobin exhibits no tendency to self-associate [13]. The dependence of R^* upon c, calculated using eq. (9) with the value of V' so obtained, is plotted in fig. 2. It may be seen that the plotted curves for R and R^* coincide for all concentrations up to approximately 15 g/dl, indicating that myoglobin remains essentially monomeric over this concentration range.

Fig. 3 shows, on an expanded scale, the experimentally observed dependence of M_{app}^{Mb} upon c, the best fit of eq. (8) to these data, and the theoretical dependence of M_{app}^{Mb} upon c in the absence of self-association, calculated using eq. (5) with $M = 17000$ and $V = 0.0079$ dl/g. The discrepancy between observed and theoretical values of M_{app}^{Mb}, particularly obvious at higher concentrations, is attributed to self-association of myoglobin. A statistical analysis of the relationship between the data and calculated curves is presented in the appendix.

The specific volume of the equivalent hard particle representing a hypothetical dimer of myoglobin cannot be calculated a priori. However, it is expected to lie between 0.0079 and 0.0095 dl/g, the specific volumes of equivalent particles representing monomeric myoglobin and hemoglobin, respectively. Fig. 3 shows a plot of the

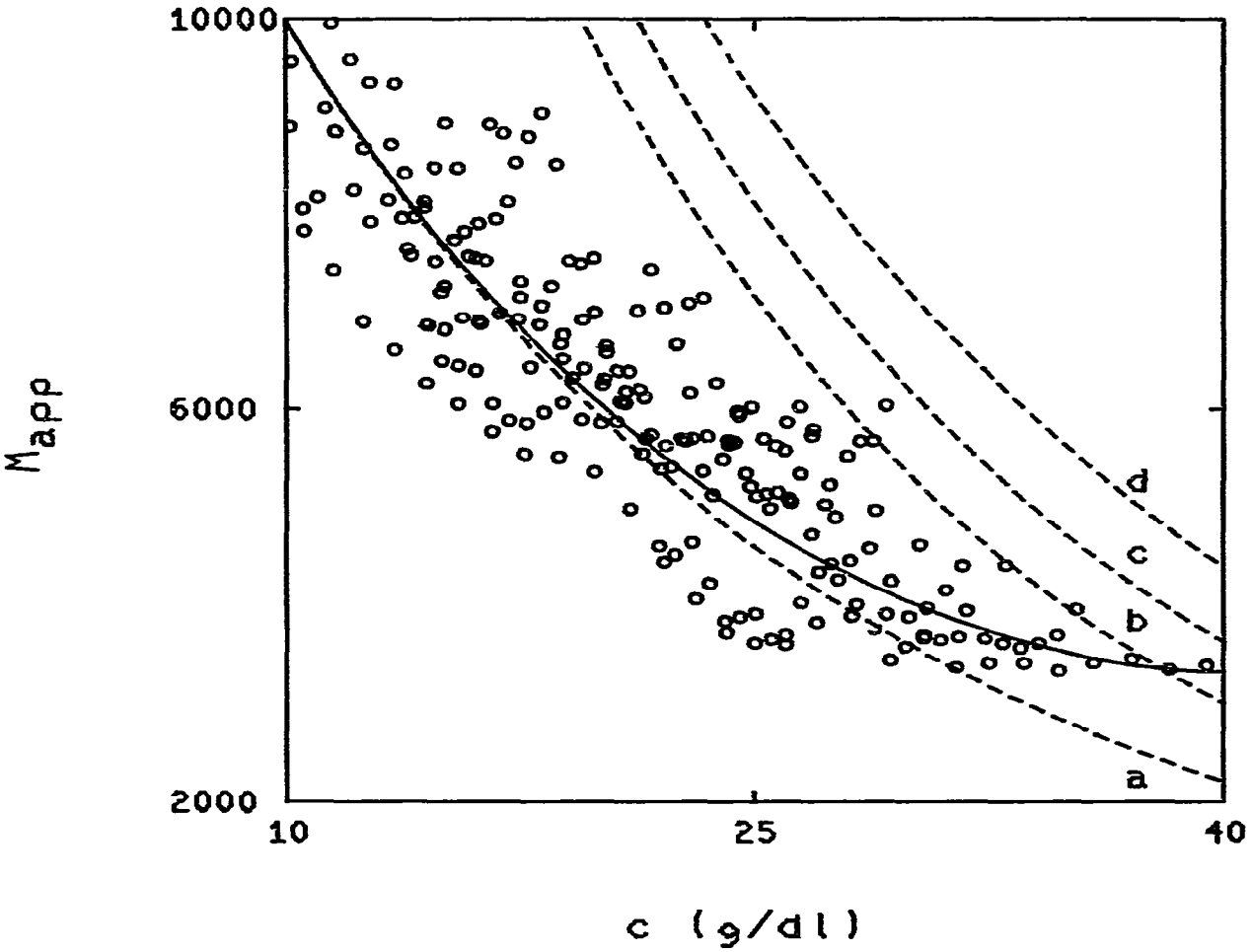

Fig. 3. Apparent molecular weight of myoglobin versus protein concentration. Circles and continuous curve as in fig. 1. (- - - - - -) Calculated using eq. (5) with the following parameter values: (a) $M=17000$, $V=0.0079$ dl/g; (b) $M=34000$, $V=0.0095$ dl/g; (c) $M=34000$, $V=0.0087$ dl/g; (d) $M=34000$, $V=0.0079$ dl/g.

theoretical dependence of M_{app} upon c for a hypothetical myoglobin dimer, calculated using eq. (5) with $M = 34000$ and three values of V given in the figure legend.

At the highest protein concentrations attained in this study, the experimentally observed value of M_{app} is comparable to that expected for a homogeneous solution of myoglobin dimers with $V \geqslant$ 0.0087 dl/g. While it is possible to formulate quantitative models for particular self-association schemes, such as monomer-dimer or monomer-tetramer, in highly nonideal solutions (e.g., see ref. [14]), discrimination between such schemes requires data of higher precision than that attainable with our present instrumentation.

It may be recalled that our analysis is predi-

cated upon the assumption that cyanmethemoglobin does not self-associate. While we are not aware of any experimental data which unequivocally indicate the presence of significant hemoglobin self-association under conditions similar to those used here, we cannot rule out the possibility that such self-association does take place. Should this be the case, our results would imply that the extent of myoglobin self-association is even greater than that suggested here.

Appendix

Quantitation of experimental precision: comparison between calculated and observed dependence of apparent molecular weight upon myoglobin concentration

The mean apparent molecular weight of myoglobin $\bar{M}_{app}$ and the standard deviation of the mean (S.D.) were calculated as functions of myoglobin concentration in intervals of 1 g/dl over the concentration range 17–35 g/dl. The value of $\bar{M}_{app} \pm 2$ S.D. is plotted for each interval in fig. 4. The solid curve and dashed curve (a) in fig. 3 are replotted in fig. 4 for comparison. The value of $\bar{M}_{app}$ for any interval (except one) lying above 25 g/dl differs from that calculated on the assumptionof no self-association (dashed curve) by more than 2 S.D. In contrast, the corresponding value calculated using an empirical equation which was fitted to all of the data between 12.5 and 37.5 g/dl (solid curve) agree with $\bar{M}_{app}$ for each interval to within 2 S.D. and ordinarily to within 1 S.D. This analysis indicates that as protein concentration increases, the assumption that myoglobin does not self-associate becomes increasingly incapable of accounting for our data to within experimental precision.

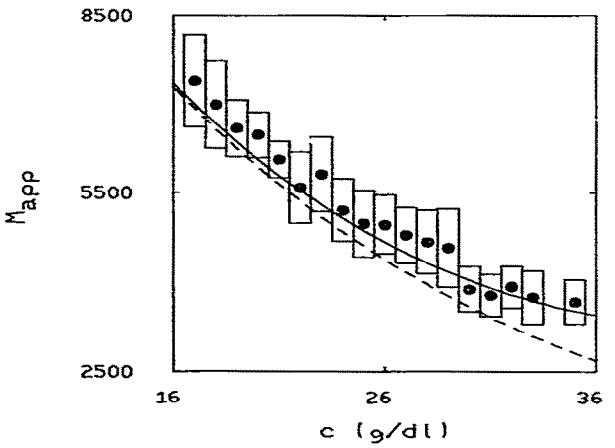

Fig. 4. Apparent molecular weight of myoglobin versus protein concentration. Points: mean of data for the 1 g/dl interval indicated by the width of the surrounding box. The height of the surrounding box indicates ±2 standard deviations of the mean. No point is plotted for $c=34$ g/dl, as the density of data between 33.5 and 34.5 g/dl was too low to permit a reliable estimate of the standard deviation of the mean in this interval. (————) Same as in fig. 3. (- - - - -) Same as dashed curve (a) of fig. 3.

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