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Dissociation of Hemoglobin into Subunits

II. HUMAN OXYHEMOGLOBIN: GEL FILTRATION STUDIES

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SUMMARY

The dissociation of normal human oxyhemoglobin has been studied by gel filtration under conditions of neutral pH and moderate ionic strength, with the use of both integral boundaries, formed between solution and solvent, and finite difference boundaries, formed between solution and solution. The experimental data obtained have been treated by non-linear least squares procedures to estimate the relevant parameters with their associated standard errors. For this purpose, theoretical equations have been derived for two models, firstly a simple dimer-tetramer reversible equilibrium, and secondly a monomer-dimer-trimer-tetramer reversible equilibrium. In both models the dependence on concentration of the elution volume of the individual species has been taken into account.

Measurement of the dissociation of hemoglobin into its constituent subunits at neutral pH and low ionic strength is one route to information about the energy of interaction of the subunits under conditions corresponding in these respects to "physiological" conditions. It does, however, pose a difficult problem for the experimenter because a degree of dissociation sufficient for accurate measurement is achieved only at concentrations of hemoglobin well below the normal operating range of conventional osmometers, light scattering instruments, or ultracentrifuges. This difficulty is less serious for methods based on the use of ultracentrifuges fitted with absorption optics (either photographic (1) or photoelectric scanning (2–5)) or on gel filtration (6, 7), but even so there is still conflicting evidence as to whether the dissociation of hemoglobin goes detectably beyond the dimer stage under the conditions being discussed. For instance, the results of Schachman and Edelstein (5), who studied human oxyhemoglobin in the scanning ultracentrifuge, suggest that it does. But this is contradicted by the results of Ackers and Thompson (6) who found no dissociation of human carboxyhemoglobin beyond dimer, either by porous disc diffusion measurements or by gel filtration.

The gel filtration experiments described below do not resolve this conflict, and they illustrate once more the great difficulty of eliminating ambiguity from the calculation of the parameters of a reversibly reacting system. The analysis of the experimental data is carried out by statistical methods in an attempt to make objective comparisons of different models for the dissociating system, and of different types of experiment.

Differential, as well as integral, boundary experiments are performed and, throughout, the very considerable effect of the concentration dependence of the elution volume of the individual species (8) is kept in mind.

EXPERIMENTAL PROCEDURE

Materials

Human oxyhemoglobin was prepared from fresh, citrated blood by the method of Rossi-Fanelli, Antonini, and Caputo (9), stored at 2–4°, and used within about 14 days. Concentrations were determined spectrophotometrically and referred to $E^{1\%}_{1\,cm}$ 8.5 at 541 mμ.

Methods

Gel Filtration—A column, 50 × 0.8 cm, of Bio-Gel P-100 (Bio-Rad Laboratories, Richmond, California) with an elution volume for Blue Dextran 2000 (AB Pharmacia, Uppsala, Sweden) of 6.13 ml was prepared essentially as described by Flodin (10). The glass column was completely filled with gel, and then fitted at the inlet with a specially designed sample applicator which gently pressed down on to the gel surface. Connections were made through narrow polyvinyl chloride tubes. The column was equilibrated by passing several bed volumes of buffer solution, pH 7.00 (measured at 20°), 0.1 M Na^+ (0.09 M as chloride, plus phosphate), at a flow rate slightly greater than that used in the subsequent experiments. The buffer solution was saturated with oxygen which was bubbled through for about 1 hour after

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preparation of the buffer. The column was thermostated, and experiments were carried out between 2.5° and 3°. A sufficient volume of solution was applied to the column to produce a plateau region (8, 11, 12) in the effluent profile. The effluent was monitored with a Gilford (Oberlin, Ohio) model 2000 multiple sample absorbance recorder fitted with dual wave length changer. Special 2- and 5-mm stainless steel flowthrough cells were constructed; these were shown to be sufficiently free from errors caused by density layering. The flow rate was maintained constant at about 5 ml per hour by a peristaltic pump (LKB, 4912A). The elution volume was evaluated from the position of the equivalent sharp boundary (13) estimated by planimetry. Once a column had been prepared, it was interfered with as little as possible, and a flow of buffer or solution was maintained continuously throughout its life.

The column was calibrated with Blue Dextran and sperm whale metmyoglobin (Seravac Laboratories, Ltd., Maidenhead, England). All the data reported here were taken from a single column to avoid having to assess errors due to combining results from different columns. The experiments were conducted in such a way that integral and difference boundaries were studied in each run. For this purpose sufficient oxyhemoglobin solution was applied to ensure a plateau region in the effluent profile, after which the concentration of the inflowing solution was raised by about 10% and a further, approximately equal, volume was allowed to pass into the column, to be followed finally by buffer solution. In this way a finite difference boundary and second plateau region were produced, followed by a trailing boundary. Thus, from each run, one finite difference and two integral boundaries were obtained for measurement. The values of the elution volumes at which the leading and trailing boundaries appeared were averaged and plotted against the mean concentration, to which the finite difference boundary was also referred. Results are shown in Figs. 1 and 2 for the concentration range 0.001 to 3.76 g of oxyhemoglobin per dl. The complete set of experimental points, with none rejected, were analyzed by two nonlinear least squares procedures, the simplex method of Nelder and Mead (14) and the Newton-Raphson iterative method (15), as explained below.

Sedimentation—Sedimentation was followed by schlieren optics in a Spinco model E ultracentrifuge with an RTIC temperature control unit. All runs were carried out at 20° and 50,740 rpm, with the use of the same buffer as in the gel filtration experiments. Sedimentation velocities were evaluated from the movement of the maximum ordinate of the schlieren peak, by a least squares method. Concentrations were taken as the average of those corresponding to the first and the last exposures measured. Values of the sedimentation coefficients were reduced in the conventional way to $s_{20,w}$. The data, shown in Fig. 3, were analyzed by the nonlinear least squares procedures.

THEORY AND DISCUSSION

The background to the method used in this paper can be understood by considering the elution profile drawn in Fig. 4. For simplification, without affecting the essential argument, it is assumed for this discussion that changes to a new concentration of solute in the solution flowing into the gel column are not made until solution at the previous concentration has appeared in the effluent. The whole column therefore becomes in equilibrium with each successive solution in turn. It is a trivial matter to take into account situations in which the column is not com-

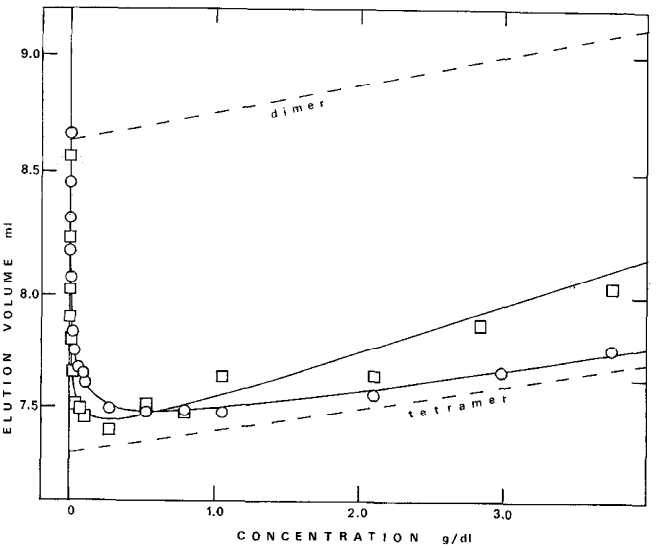

FIG. 1. Elution volume as a function of human oxyhemoglobin concentration for a column, 50 × 0.8 cm, of Bio-Gel P-100 at 2.5–3.0°, equilibrated with buffer, 0.1 M Na^+ (0.09 as chloride, plus phosphate, pH 7.00 at 20°). ○, integral boundary formed with solvent; □, finite difference boundary; ——, theoretical curves calculated with the use of the parameters in Column D of Table I. See the text for experimental details.

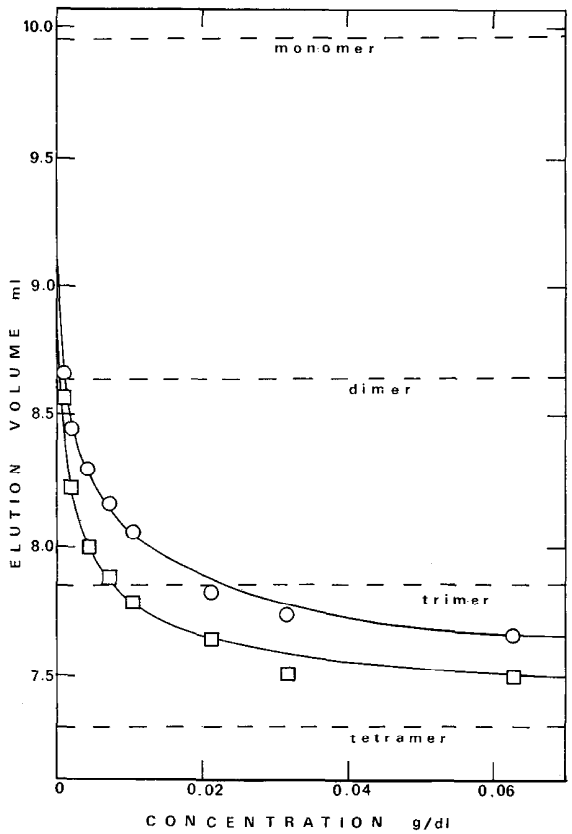

FIG. 2. Magnification of Fig. 1 to show more clearly the results obtained at concentrations below 7×10^{-2} g per dl.

TABLE I

Equilibrium constants and related parameters for dissociation of human oxyhemoglobin at 2.5–3° in 0.1 M Na^+ (0.09 as chloride, plus phosphate, pH 7.00 at 20°)

Column A, integral data, g has the same value for all species; Column B, differential data, g has the same value for all species; Column C, differential data, g has the same value for all species, but $(V_2)_0$ is anchored at the value given in Column A; Column D, integral data, g has the same value for all species; Column E, integral data, g = 0 for all species except the tetramer; Column F, differential data, g has the same value for all species. The standard errors of single estimates of the parameters are those computed from the Newton-Raphson procedure (15).

Constant or parameter	Dimer-Tetramer model			Monomer-Dimer-Trimer-Tetramer model		
	A	B	C	D	E	F
10^{-2} $L_{2,4}$ (dl per g)	2.5 ± 0.3	5.9 ± 2.8	2.3 ± 0.2	1.8 ± 0.2	1.9 ± 0.2	2.9 ± 0.6
10^2 δ				1	1	1
10^{-4} $L_{1,2}$ (dl per g)				4.8 ± 1.1	4.8 ± 1.1	0.7 ± 0.3
10^2 g	−1.4 ± 0.1	−1.2 ± 0.1	−1.3 ± 0.1	−1.4 ± 0.1	−1.5 ± 0.1	−1.2 ± 0.1
$(V_4)_0$ (ml)	7.29 ± 0.02	7.30 ± 0.02	7.27 ± 0.02	7.30 ± 0.02	7.30 ± 0.02	7.31 ± 0.02
$(V_3)_0$ (ml)				7.85	7.85	7.86
$(V_2)_0$ (ml)	8.94 ± 0.05	9.59 ± 0.42	8.94	8.64	8.64	8.64
$(V_1)_0$ (ml)				9.98	9.98	9.97
$(V_{myo})_0$ (ml)				9.78	9.78	9.78
S.E.V.	0.03	0.05	0.04	0.03	0.03	0.05

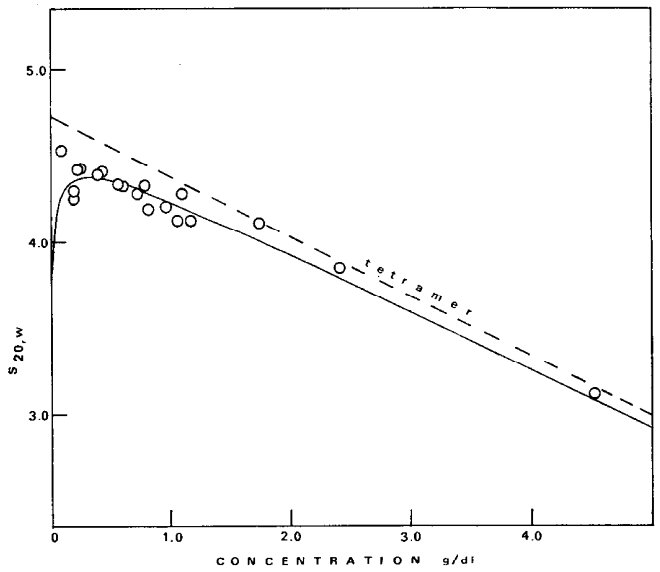

FIG. 3. $s_{20,w}$ from measurements at 20° as a function of human oxyhemoglobin concentration. Buffer, 0.1 M Na^+ (0.09 as chloride, plus phosphate), pH 7.00. ——, theoretical line fitted to the schlieren data with the use of the association constants given in Column D of Table I. See the text for experimental details.

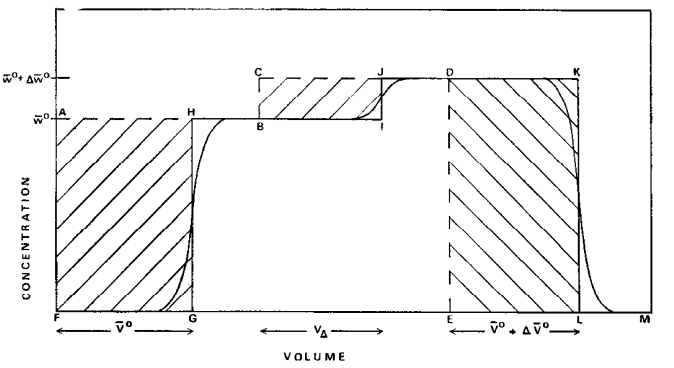

FIG. 4. The relationship between the elution volume, V_Δ, of a finite difference boundary formed between two solutions of plateau constituent concentrations $\bar{w}^0$ and $\bar{w}^0 + \Delta\bar{w}^0$, and their constituent elution volumes, $\bar{V}^0$ and $\bar{V}^0 + \Delta\bar{V}^0$, respectively. – – –, inflowing solution; ——, outflowing solution. *Vertical solid lines* represent the position of the appropriate equivalent sharp boundary.

pletely filled with solution, provided that a plateau region separates each boundary.

The *continuous heavy line* in Fig. 4 records the concentration of the effluent as a function of time, and the *broken line* the corresponding concentration of the solution flowing into the column. The rate of flow is kept constant, and therefore the *abscissa* represents equally time or the volume of liquid that has entered or left the column. *Vertical solid lines* in the figure have been constructed by analogy with Longsworth's method for electrophoresis experiments (13) to show the positions of the equivalent sharp boundaries corresponding to the leading integral boundary, *GH*; the trailing integral boundary, *LK*; and the finite difference boundary, *IJ*.

The path of the inflowing solution on the figure follows the letters *A B C D E M*, and that of the effluent, *F G H I J K L M*. At any instant the total amount of solute held by the column, equal to the difference between the amounts that have entered and left the column, can be read from the figure as the area encompassed by the *broken* and *solid lines*. Areas above the *solid line* count as positive, and those below it as negative. The total amount of solute that can be included at equilibrium in the column will be termed $\mathcal{J}^0$. This amount is a function of the constituent concentration $(\bar{w})$ of the solute, and is the analogue of flux in transport theory, just as elution volume is the analogue of velocity (6, 16, 17). Let $\bar{w}^0$ be the initial constituent concentration of solute in the inflowing solution. The corresponding value of $\mathcal{J}^0$ is given by the area *A H G F*. At *B* let the concentration of the inflowing solution be increased by $\Delta\bar{w}^0$, the corresponding increase in $\mathcal{J}^0$, $\Delta\mathcal{J}^0$, being given by the area *C J I B*. Finally, at *D*, where the inflowing solution is replaced by solvent, the whole of the included solute, $\mathcal{J}^0 + \Delta\mathcal{J}^0$, given by the area *D K L E*, is swept out of the column.

Each of these areas on the chart, and hence $\mathcal{J}^0$ and $\mathcal{J}^0 + \Delta\mathcal{J}^0$, can be expressed as the product of a constituent concentration of solute and an elution volume. The commonly accepted meaning of elution volume is the volume of eluate collected from the time a change is made in the inflowing solution to the time a selected characteristic resulting from that change appears in the eluate. If the selected characteristic is the equivalent sharp boundary of a constituent, the corresponding elution volume is appropriately termed the constituent elution volume ($\bar{V}$) of that constituent. For solute entering the column at constituent concentration $\bar{w}^0$, the constituent elution volume, $\bar{V}^0$, is given by the length FG in Fig. 4. $\bar{V}^0$ is a function of $\bar{w}^0$, and rises to a new value, say $\bar{V}^0 + \Delta\bar{V}^0$, given by the length EL, as the solute concentration is changed from $\bar{w}^0$ to $\bar{w}^0 + \Delta\bar{w}^0$. It follows that

$$\mathcal{J}^0 = \bar{w}^0\bar{V}^0 \tag{1}$$

$$\begin{aligned}\mathcal{J}^0 + \Delta\mathcal{J}^0 &= \bar{w}^0\bar{V}^0 + \Delta(\bar{w}^0\bar{V}^0) \\ &= (\bar{w}^0 + \Delta\bar{w}^0)(\bar{V}^0 + \Delta\bar{V}^0)\end{aligned} \tag{2}$$

The equivalent sharp finite difference boundary IJ is similarly characterized by an elution volume, given by the volume of liquid collected from the time the concentration of solute is changed from $\bar{w}^0$ to $\bar{w}^0 + \Delta\bar{w}^0$ in the inflowing solution to the time when the finite difference boundary emerges from the column. This elution volume, V_Δ, is represented on the figure by the distance BI. While the volume V_Δ is being collected, the content of the column increases from $\mathcal{J}^0$ to $\mathcal{J}^0 + \Delta\mathcal{J}^0$.

For mass to be conserved, the sum of the areas $A\ H\ G\ F$ and $C\ J\ I\ B$ must equal the area $D\ K\ L\ E$, and if these areas are expressed in terms of constituent concentrations and elution volumes, this equality implies the equation

$$\bar{w}^0\bar{V}^0 + (\Delta\bar{w}^0)V_\Delta = (\bar{w}^0 + \Delta\bar{w}^0)(\bar{V}^0 + \Delta\bar{V}^0) \tag{3}$$

which when expressed in terms of $\mathcal{J}^0$ in accordance with Equations 1 and 2 becomes

$$\mathcal{J}^0 + (\Delta\bar{w}^0)V_\Delta = \mathcal{J}^0 + \Delta\mathcal{J}^0$$

and therefore

$$V_\Delta = \frac{\Delta\mathcal{J}^0}{\Delta\bar{w}^0} = \frac{\Delta(\bar{w}^0\bar{V}^0)}{\Delta\bar{w}^0} \tag{4}$$

Equation 4 is the analogue (16, 17) of the equation of Miller (18) for a difference boundary in electrophoresis, and of Hersh and Schachman (19) for a difference boundary in sedimentation. By proceeding to the limit, the elution volume (V) for a true differential boundary is found to be

$$\lim_{\Delta\bar{w}^0 \to 0} V_\Delta = V = \frac{d\mathcal{J}^0}{d\bar{w}^0} = \frac{d(\bar{w}^0\bar{V}^0)}{d\bar{w}^0} \tag{5}$$

V can be obtained experimentally by extrapolating measurements on finite difference boundaries to zero concentration difference, and it may be remarked that besides defining the position of the differential boundary formed at a plateau concentration of $\bar{w}^0$, it also defines the "profile" (V versus $\bar{w}$) of a "diffusion-free" boundary (16) in accordance with the equation

$$V = \frac{d\mathcal{J}}{d\bar{w}} \tag{6}$$

where $\bar{w}$ is the constituent concentration at the point on the boundary for which the elution volume has the value V, and $\mathcal{J}$ is the potential amount of solute that would be included by the column when in equilibrium with solution of concentration $\bar{w}$.

In the experiment described above, $\bar{V}^0$ and V_Δ were measured for a series of values of $\bar{w}^0$ (with $\Delta\bar{w}^0$ set at 10% of $\bar{w}^0$) to give $\mathcal{J}^0$ and $\Delta\mathcal{J}^0/\Delta\bar{w}^0$ as a function of $\bar{w}^0$, in accordance with Equations 2 and 4.

The next step consists in choosing a theoretical model to represent hemoglobin, and in calculating values of $\mathcal{J}^0$ and $\Delta\mathcal{J}^0/\Delta\bar{w}^0$ for arbitrarily chosen numerical values of the parameters of the model. By iteration using either the Nelder and Mead (14) or the Newton-Raphson (15) method, these initial values are refined to give the best agreement (according to the criterion of the minimum sum of squares of residuals) between the values of $\mathcal{J}^0$ and $\Delta\mathcal{J}^0/\Delta\bar{w}^0$ calculated from the model and those observed experimentally. It is advisable to start with the simplest possible model, since if agreement to within the experimental error of observation is obtained with a simple model, more complicated models with more adjustable parameters are almost sure to be fitted too. Of course, only consistency with a model can be proved, not the validity of the model. On the other hand, it may be possible to exclude models on grounds of lack of consistency.

In the present case the simplest model is a tetramer that dissociates reversibly into symmetrical dimers (20), and the next simplest is one which dissociates further into equivalent monomers. Both models can be dealt with by adopting the set of mass action equations

$$\begin{aligned}w_2 &= L_{1,2}w_1^2 \\ w_3 &= L_{1,3}w_1^3 \\ w_4 &= L_{1,4}w_1^4\end{aligned} \tag{7}$$

together with

$$\bar{w} = w_1 + w_2 + w_3 + w_4 \tag{8}$$

The L's are association constants, and the w's are the concentrations of monomer, dimer, trimer, and tetramer, expressed in grams per dl.

Although Equations 7 stress the building of the higher species from monomer units, the difference in accessibility of the constants by experiment suggests the transformation below, which takes into account the fact that the ratio of dimers to tetramers in the equilibrium mixture is the quantity observable with the least error.

The equations have therefore been rewritten in the form

$$\begin{aligned}w_2 &= L_{1,2}w_1^2 \\ w_3 &= \delta L_{2,4}w_1w_2 \\ w_4 &= L_{2,4}w_2^2\end{aligned} \tag{9}$$

where

$$\delta = \frac{L_{1,2}\,L_{1,3}}{L_{1,4}} \tag{10}$$

and

$$L_{2,4} = \frac{L_{1,4}}{(L_{1,2})^2} \tag{11}$$

In physical terms δ is a measure of the ratio of the strength of the bond between monomer and dimer to the strength of that be-

tween dimer and dimer, and would normally be expected to be less than unity, perhaps very much less.

The choice of elution volumes for the model has to be discussed next. Each molecular species must be assumed to penetrate the gel particles to a characteristic extent, dependent upon its molecular size. For rather regularly shaped molecular species such as those under consideration, it has been established (21–23) that the relationship

$$\bar{V} = A + B \log \text{mol wt} \tag{12}$$

holds at low concentration between the elution volume $\bar{V}$ and the molecular weight of a species, if no reaction (such as dissociation) occurs. $\bar{V}$ depends upon concentration (8), even in the absence of specific reactions, but little is known of the details of its dependence. The assumption will be made without proof that for each species of n-mer, where n can be 1, 2, 3, or 4, the equation

$$V_n = (V_n)_0 (1 - \mathbf{g}\,\bar{w}) \tag{13}$$

holds, where $(V_n)_0$ is the elution volume of the species at zero concentration, and **g** is a constant independent of n. The implications of assuming a single value for **g** will be discussed later, and will be shown to have no serious effect on the results.

The total solute, $\mathcal{J}^0$, that can be included in the column can be written as the sum of the contributions of each species. Equation 2 then becomes

$$\bar{w}^0\bar{V}^0 = \mathcal{J}^0 = w_1^0V_1 + w_2^0V_2 + w_3^0V_3 + w_4^0V_4 \tag{14}$$

which, combined with Equation 13, gives

$$\bar{w}^0\bar{V}^0 = (1 - \mathbf{g}\bar{w}^0)\,[w_1^0(V_1)_0 + w_2^0(V_2)_0 + w_3^0(V_3)_0 + w_4^0(V_4)_0] \tag{15}$$

If w_2^0, w_3^0, and w_4^0 are expressed in terms of w_1^0 by Equations 9, Equation 15 becomes

$$\bar{w}^0\bar{V}^0 = (1 - \mathbf{g}\bar{w}^0)[(V_1)_0w_1^0 + L_{1,2}(w_1^0)^2(V_2)_0 + \delta L_{1,2}L_{2,4}(w_1^0)^3(V_3)_0 + L_{1,2}^2L_{2,4}(w_1^0)^4(V_4)_0] \tag{16}$$

Further, if Equation 6 is expressed as

$$V = \frac{d\mathcal{J}}{d\bar{w}} = \frac{d\mathcal{J}}{dw_1} \cdot \frac{dw_1}{d\bar{w}} \tag{17}$$

then, by differentiation, with respect to w_1 of Equation 8 and of Equation 16 without the superscript, it can be shown that the explicit form of Equation 17 is

$$V = (1 - \mathbf{g}\bar{w}) \left[\frac{(V_1)_0 + 2L_{1,2}w_1(V_2)_0 + 3\delta L_{1,2}L_{2,4}w_1^2(V_3)_0 + 4L_{1,2}^2L_{2,4}w_1^3(V_4)_0}{1 + 2L_{1,2}w_1 + 3\delta L_{1,2}L_{2,4}w_1^2 + 4L_{1,2}^2L_{2,4}w_1^3}\right] - w_1\mathbf{g}[(V_1)_0 + L_{1,2}w_1(V_2)_0 + \delta L_{1,2}L_{2,4}w_1^2(V_3)_0 + L_{1,2}^2L_{2,4}w_1^3(V_4)_0] \tag{18}$$

This formula for V is the analogue of the formula for the asymptotic sedimentation concentration profile derived previously (Equation 16 of Reference 24) for a reversibly associating system. When $\bar{w} = \bar{u}^0$ and $w_1 = w_1^0$, then V, given by Equation 18, is the elution volume of a differential boundary between plateau concentrations $\bar{u}^0$ and $\bar{u}^0 + d\bar{w}^0$.

If Equation 12 holds, the elution volume of each species can be expressed in terms of the constants A and B and the known molecular weight of the species. In the present instance, sperm whale metmyoglobin has been used to calibrate the column, and $(V_4)_0$ has been derived, by the method to be described below, from the experiments. Then, if the molecular weights of myoglobin and hemoglobin are taken as 17,816 (25) and 64,500 (26), respectively, and $(V_{myo})_0$ is the elution volume at zero concentration of myoglobin, A and B can be calculated from Equation 12 by the use of the equations

$$B = \frac{(V_{myo})_0 - (V_4)_0}{\log 17{,}816 - \log 64{,}500}$$
$$A = (V_{myo})_0 - B \log 17{,}816 \tag{19}$$

$(V_1)_0$, $(V_2)_0$, and $(V_3)_0$ are obtained from Equation 12 by inserting these values of A and B.

From the experimental values of $\bar{V}^0$ and $\bar{w}^0$, the four independent constants, $(V_4)_0$, $L_{1,2}$, $L_{2,4}$, and **g**, have to be found. This is a familiar problem in many fields of research, and in the present instance has been solved by the computer simplex least squares method of Nelder and Mead (14). We are indebted to Dr. S. P. Spragg and Dr. Rodes Trautman for drawing our attention to this method and for illustrating its use. In its application here, arbitrary values (selected by reasonable guesses) of the four unknown parameters are taken, and $\bar{V}^0$ is calculated from Equation 16 for each experimental value of $\bar{u}^0$. (It is assumed that $\bar{w}^0$ is without appreciable error in comparison with $\bar{V}^0$.) The square of the residual between each experimental value of $\bar{V}^0$ and the value of $\bar{V}^0$ calculated in this way is found, and hence the sum of the squares of the residuals. In the program the values of the parameters are then varied in an economical way by a simplex method so that a minimum value of the S.S.R.[1] is achieved. Finally, a quadratic surface expressing the S.S.R. as a function of the parameters near its minimum is constructed by the computer program so that standard errors can be determined for the estimated parameters.

The results of the Nelder and Mead analysis have been confirmed by another least squares procedure which makes use of the Newton-Raphson (15) iterative method, in which each iteration is based on a Taylor series expansion about the current approximation to the parameters at the minimum S.S.R. For minimum S.S.R. the variance-covariance matrix is given (27) by the product of two terms, the inverse of the matrix of the expectations of second derivatives and the scalar of the variance of the ordinate (volume) measurements. We are indebted to Dr. R. L. Holder for his advice on the application of this second approach.

Both procedures give the same values of the estimated parameters at the minimum S.S.R. for the functions analyzed in this paper. However, as in the two procedures the standard errors of single estimates of the parameters are derived by different methods, each of which involves approximations, it is to be expected that errors estimated by the two procedures will differ. In the present examples it has been found that the standard errors from the Newton-Raphson method are slightly less than those estimated by the Nelder and Mead procedure.

As a preliminary, the simplest possible model consisting of tetramer in equilibrium with dimer subunits was tested by setting $L_{1,2} = L_{1,4} = \infty$. The values found for $(V_2)_0$, $(V_4)_0$, $L_{2,4}$, and **g** are shown in Column A of Table I. They were computed from the integral boundary data without placing the restriction on $(V_2)_0$ and $(V_4)_0$ that they must obey Equation 12. The standard error of a volume measurement from the theoretical curve was

[1] The abbreviations used are: S.S.R., the sum of the squares of the residuals; S.E.V., the standard error of a volume measurement.

found to be 0.03 ml, which represents an accuracy in the fitting of the elution volumes of about 2% of the difference between the limiting parameters, $(V_2)_0$ and $(V_4)_0$.

However, the computed value of $(V_2)_0$ is greater than that calculated from Equation 12 by about 0.3 ml, that is, by an amount about 10 times greater than the standard error of an ordinate measurement. Thus, although superficially the dimer-tetramer model appears to describe the experimental data adequately, it is not quite consistent with Equation 12. If Equation 12 is true, this result indicates that further dissociation into monomer must be considered. This was done by including $L_{1,2}$ and δ among the parameters to be found, and at the same time restricting the number of unknown parameters by linking $(V_1)_0$, $(V_2)_0$, $(V_3)_0$, and $(V_4)_0$ by Equations 12 and 19.

The minimization method quickly showed that δ could not be found without ambiguity, since varying δ between 0.01 and 0.5 produced no significant change in the minimum value of the S.S.R. This is a reflection of the almost complete absence ($<2\%$) of trimer over the whole range of concentration, due to the almost complete transformation of the hemoglobin into dimers before further polymerization to tetramers is appreciable. Simply for the sake of continuity in the equations, therefore, and without significant effect on the analysis, δ was arbitrarily set at 0.01 for the computation of the other parameters. The results are shown in Column D of Table I. The solid theoretical integral curve in Figs. 1 and 2 was calculated with the use of these parameters.

It has been assumed above, without proof, that the coefficient **g** is the same for all species. It can be shown that this assumption has little effect on the results. If the calculations are carried out with $\mathbf{g} = 0$ for all species except tetramer, for which **g** is retained as a parameter to be found, the computed parameters shown in Column E of Table I are obtained. There is no significant change in the S.S.R. at the minimum, and application of the F and t tests (28) confirms that the parameters are consistent with the previous set in Column D. This result is understandable because, at concentrations high enough for the term $\mathbf{g}w$ to be important, tetramer is entirely dominant.

If a simple dimer-tetramer model with the same value for **g** for all species is assumed, analysis of the finite difference data after setting $L_{1,2} = L_{1,4} = \infty$ in the differential equation (Equation 18) gives the parameters shown in Column B of Table I. The value of $L_{2,4}$ is considerably larger and its standard error, as well as that of $(V_2)_0$, is much greater than that encountered with other models tested. This is mainly because the present differential data do not extend to concentrations sufficiently low to permit an accurate estimation of $(V_2)_0$, and because there is an extremely high correlation (coefficient, 0.99) between $L_{2,4}$ and $(V_2)_0$. No confidence therefore can be placed in their computed values. This difficulty can be overcome if the value of $(V_2)_0$ can be decided from external information. If $(V_2)_0$ is fixed by the logarithmic relationship of Equation 12, the value found for $L_{2,4}$ falls to $(1.3 \pm 0.2) \times 10^2$ dl per g. However, there is now a heavy bias in the fitting of the data, as evidenced by the distribution of the signs of the residuals, and in addition the S.E.V. has risen to a value of 0.08 ml, the largest encountered in all the models tested. If, however, $(V_2)_0$ is anchored at the value, 8.94 ml, found by curve fitting the integral data to the simple dimer-tetramer model (Column A), the S.E.V. decreases to 0.04 ml, the distribution of the signs of the residuals no longer displays evidence of bias, and there is now found to be a remarkable agreement between the three remaining parameters computed from the finite difference data, Column C, and those obtained from the integral data, Column (A).

The errors attached to the parameters, shown in Column F of Table I, computed from the differential equation, Equation 18, for the monomer-dimer-trimer-tetramer model with the use of the finite difference boundary data and the myoglobin data for calibration, are much larger than the errors for the parameters found with the use of the integral boundary data. Application of the F test to the values of $L_{2,4}$ computed from the integral and from the finite difference data confirms that their variances are not consistent. Since the t test cannot be applied in these circumstances to test for consistency of means, the test of B. L. Welch (29) is used here. Its application shows that the two means are not significantly different at the 5% level. A contributory cause to the fact that the errors for $L_{2,4}$ are greater for the finite difference than for the integral data is likely to be the greater difficulty of measuring the difference boundaries, as they were only one-tenth the height of the integral boundaries on the elution record. The *solid curve* in Figs. 1 and 2 for the differential boundary was calculated from the parameters, shown in Column D, computed from the integral data, and is presented for comparison with the experimental data for the finite difference boundaries. It was shown by calculation, with the use of the computed parameters in Equations 1 and 5, that for a given concentration, $\bar{u}^0$, the difference between the elution volume calculated for a finite difference boundary of $0.1\ \bar{u}^0$ at $\bar{u}^0$ and that for a true differential boundary was small enough to be disregarded.

The differential curve, calculated with the use of Equation 18, which is the explicit form of the general differential equation (Equation 5), is of theoretical interest in that it describes the diffusion-free concentration profile (16, 24). Thus, for any *given* concentration the shape of the diffusion-free profile is represented by the differential *solid curve* in Fig. 1 between that concentration and zero concentration. In the present case the leading boundary profile always has a hypersharp region, whereas the trailing profile is spread and contains a hypersharp region only if $\bar{u}^0$ is greater than the concentration corresponding to the minimum of the differential curve. Fig. 5 shows the theoretical diffusion-free profile for an experimental run at 0.1 g per dl.

The sedimentation data shown in Fig. 3 were obtained over the concentration range 0.09 to 4.5 g per dl, throughout which the constituent sedimentation coefficient is dominated by the sedimentation coefficient of the tetramer. Calculations with the use of the constants in Table I show that 6 to 7% of dimer is present at a constituent concentration of 1.00 g per dl of hemoglobin. Even a rough estimate of the degree of dissociation of the hemoglobin should thus permit a reasonably good estimate to be made of the sedimentation properties of a hypothetical nondissociating tetramer equivalent to undissociated hemoglobin. This may have some importance, for it can be assumed that one is dealing with a relatively noninteracting, approximately spherical molecule (30) in the case of normal oxyhemoglobin tetramer (since oxyhemoglobin exists without crystallization at very high concentrations in its natural state).

It has been found by Kirshner and Tanford (31) for carboxyhemoglobin that $L_{2,4}$ is temperature independent, or nearly so, for conditions similar to those used here, and similar results have been obtained by Hasserodt and Vinograd (32) for alkaline conditions. As a first approximation, therefore, the values of

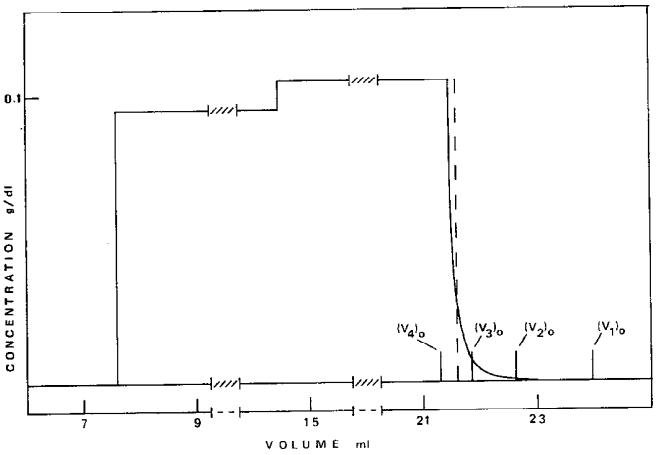

FIG. 5. The theoretical diffusion-free profile calculated for an experiment performed at a human oxyhemoglobin concentration of 0.10 g per dl. The profile was calculated by means of the differential equation (Equation 18) with the use of the association constants in Column D of Table I, and the figure is drawn for samples of 7.00 ml. The *dashed vertical line* represents the equivalent sharp boundary position of the trailing boundary.

$L_{1,2}$ and $L_{2,4}$ (Column D of Table I) obtained from the gel filtration experiments at 2.5–3° may be applied to the sedimentation data at 20° (the value of $L_{1,2}$ is immaterial, since so little monomer is present at 0.09 g per dl and above), in calculations that eliminate the effect of dissociation. With sufficient accuracy for this purpose, the limiting values of the sedimentation coefficients at zero concentration of the monomer, dimer, and trimer can be linked to that of the tetramer by the assumption that sedimentation coefficient is proportional to (molecular weight)$^{\frac{2}{3}}$, and then only **g** and $(s_4)_0$ remain as unknowns in Equation 16 after replacing elution volumes $(V_n)_0$ by their analogues $(s_n)_0$ in sedimentation (16, 17). With $(s_n)_0$ replacing $(V_n)_0$ in this way, Equation 16 has been fitted to the schlieren sedimentation data of Fig. 3, by the least squares method of Nelder and Mead (14), to find the best value of $(s_4)_0$ and **g**. The $(s_4)_0$ value is found to be 4.7 ± 0.03 S, which agrees with the value (4.7) suggested by Kawahara, Kirshner, and Tanford (33). The coefficient **g** is found to be (7.5 ± 0.4) × 10^{-2} dl per g, which may be regarded as a pointer to the value for a spherical molecule sedimenting under ideal conditions.

CONCLUSION

This study by gel filtration over the concentration range 0.009 to 3.8 g per dl has permitted a fairly good estimate to be made of the reaction constant for the dissociation of human oxyhemoglobin into dimers. Expressed as an association constant, this constant, $L_{2,4}$, has been found to have a value of approximately 2 × 10^2 dl per g (Columns A and D, Table I), which means that under the conditions of the experiment about half the molecules of the oxyhemoglobin are dissociated at a constituent concentration of 0.01 g per dl, and between 6% and 7% at 1 g per dl, as stated above. Too little monomer was present in the concentration range studied for its presence to be felt, and no useful estimate of the degree of dissociation of the protein beyond dimer could be made. The data from the differential experiments supported those from the integral experiments, but were less reliable for the reasons given.

Accurate values of the constant **g** for tetramer and of $(V_4)_0$ were obtained because of the extensive concentration range available above the point of half-dissociation. The more restricted range below this point meant that $(V_2)_0$ was left "floating" to some extent, which led to a rather high degree of correlation of $L_{2,4}$ with $(V_2)_0$. Attempts to fix $(V_2)_0$ by external evidence, for instance by calibration of the column with myoglobin, are always to be viewed with some suspicion, because of the possibility of slight specific adsorption and because of the need to depend upon approximate relationships such as Equation 12. On the other hand, the advantages from obtaining an independent estimate of $(V_2)_0$ are so great that it would seem worthwhile exploring any means of doing so. But extension of the accurate measurement of elution volumes to much lower concentrations of protein is also desirable, and attempts are now being made to do this.

The statistical methods used to analyze the data and evaluate the parameters are easily adapted to data from other methods, such as ultracentrifugation, and their application may simplify the comparison of results obtained by these different methods.

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