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The Linkage between Oxygenation and Subunit Dissociation in Human Hemoglobin. Consequences for the Analysis of Oxygenation Curves†

Gary K. Ackers,* Michael L. Johnson,‡ Frederick C. Mills, Herbert R. Halvorson,§ and Solomon Shapiro

ABSTRACT: For human hemoglobin, a pronounced dependence of oxygenation curves upon protein concentration can be demonstrated experimentally in the range between 10^{-4} and 2×10^{-6} *M* heme. The effects of such protein concentration dependence upon analysis of saturation curves have been explored using a model-independent linkage analysis which incorporates the dissociation of tetramers to dimers. We have carried out simulations of oxygenation curves representing a variety of energy distributions designed to cover a wide range of values which are relevant to known hemoglobin systems and experimental conditions. The resulting simulated oxygenation curves were analyzed by least-squares minimization procedures in terms of the tetramer binding isotherm to yield the four apparent Adair constants. These derived constants were compared with the originally assumed values used in the simulation in order to assess the extent to which their values may be altered by the presence of dimer. For each energy distribution the analysis has been carried out over a wide range of protein concentration. We have found that the presence of even small amounts of dimer that are necessarily present at the low protein concentrations commonly employed may have a devasting effect upon the reliability of Adair constant determinations. In addition to these simulated cases, we have analyzed two sets of highly precise experimental data from the literature in order to assess the degree to which constants obtained may have been influenced by the presence of dimer.

Recent technical developments have made possible the rapid determination of accurate oxygenation curves on respiratory proteins, including human and other hemoglobins (Imai et al., 1970; Sick and Gersonde, 1971; Soprounov, 1973; Kiesow et al., 1972). Most notably, the excellent automatic oxygenation apparatus developed by Imai and colleagues (Imai et al., 1970) has provided an extensive body of highly precise oxygenation curves for human hemoglobins under a wide variety of conditions. Such curves are currently being used to determine apparent stepwise ligand binding constants and to draw inferences from them regarding mechanisms of function in both normal and abnormal

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§ Present address: Edsel B. Ford Institute for Medical Research, Detroit, Michigan 48202.

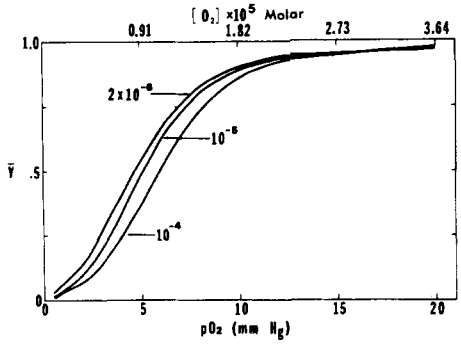

FIGURE 1: Concentration dependence of oxygenation curves for human hemoglobin A. Data were obtained using the automatic oxygenation method of Imai with a glass cell. Hemoglobin A was prepared according to the procedure of Williams and Tsay (1973). Conditions were: 0.1 *M* sodium phosphate (pH 7.45) and 10^{-3} *M* EDTA, 21°C. Protein concentrations are expressed in moles of heme/l. Values of oxygen tension at half-saturation (P_{50}) were determined to be (in mm): 6.0 ± 0.1 at 10^{-4} *M* heme, 5.2 ± 0.1 at 10^{-5} *M* heme, and 4.75 ± 0.1 at 2×10^{-6} *M* heme.

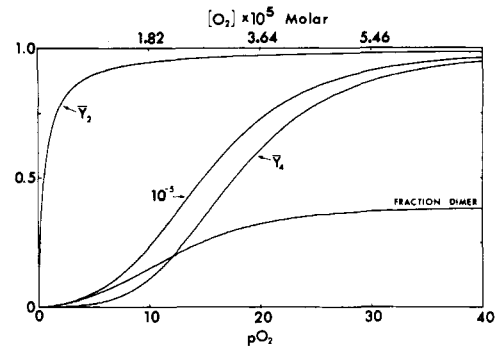

FIGURE 2: Simulated saturation functions and weight fraction dimeric species for a hemoglobin system corresponding to case A of Table I. The total free energy of binding 4 mol of ligand to tetramer is −24 kcal and the corresponding energy for liganding the (noncooperative) dimeric species is −32 kcal. For assocation of fully liganded dimers to form tetramers the free energy is −7.5 kcal/mol. The fraction dimer is seen to decrease with decreasing oxygen tension (pO_2 in mm). The saturation curve for a hemoglobin solution at a concentration of 10^{-5} *M* heme is seen to be changed substantially both in position and shape by the presence of dimeric species.

hemoglobins (Imai and Tyuma, 1973; Imai, 1973; Tyuma et al., 1973a,b; Minton, 1974; Minton and Imai, 1974; Imai, 1974; Atha and Ackers, 1974; Imai and Yonetani, 1975a,b). Although many of these recent studies have utilized very dilute hemoglobin solutions (e.g., 6×10^{-5} *M* heme) the effect of protein concentration has been largely ignored. Yet the existence of appreciable quantities of dimeric species in these dilute solutions is dictated by well-established values of the tetramer dissociation constant (cf. Antonini and Brunori, 1971). An evaluation of the possible influence of dimers upon the position and shape of oxygenation curves is therefore of considerable interest when such curves are analyzed for parameters which pertain to tetrameric hemoglobin. In this paper we report results of such an evaluation.

An important manifestation of subunit dissociation is of course the dependence of ligand binding curves upon protein concentration. Such concentration dependence is well-documented in studies on human hemoglobin A (Anderson et al., 1970; Thomas and Edelstein, 1972, 1973), on sheep hemoglobin (Roughton et al., 1955), and in single-chain hemoglobins of the sea lamprey (Briehl, 1963; Anderson and Gibson, 1971). A set of oxygenation curves obtained in our laboratory for human hemoglobin A at three different protein concentrations is shown in Figure 1. These curves demonstrate the pronounced shifts in position and changes in shape expected for a ligand-mediated subunit association system.[1] The linkage properties between oxygenation and subunit dissociation may have serious consequences for attempted determinations of tetrameric ligand binding constants under many conditions of experimental interest. For example, in oxyhemoglobin at a concentration of 6×10^{-5} *M* heme, neutral pH, low salt conditions, and no DPG, the dimeric species comprise about 18% by weight of the protein (based on an association constant of 4.2×10^5 l./mol). Since deoxygenation favors association of dimers into tetramers (Kellett, 1971; Thomas and Edelstein, 1972) the percentage of dimers decreases upon deoxygenation. The decrease occurs in a way that depends upon the magnitudes of intersubunit contact energy changes which accompany the successive deoxygenation steps. When deoxygenation is complete, the percentage of dimeric species is decreased to about 0.01%, based on an association constant of 3×10^{11} l./mol (Thomas and Edelstein, 1972). Since the dimers bind oxygen according to a different isotherm than tetramers (i.e., with higher affinity) their presence will shift the oxygenation curve toward lower pO_2 values and change its shape. An example of these effects is illustrated in Figure 2 for one particular energy distribution. The important question is whether the oxygenation curve is perturbed beyond tolerable limits for a reliable determination of the four successive tetramer binding constants.

In this paper we present results of a detailed study by numerical analysis on this problem. We have carried out simulations of oxygenation curves representing a variety of energy distributions designed to cover a wide range of values which are relevant to known hemoglobin systems and experimental conditions. The resulting simulated oxygenation curves have been analyzed by least-squares minimization procedures in terms of the tetramer binding isotherm to yield the four apparent Adair constants. These derived constants are then compared with the values originally used in the simulations in order to assess the extent to which their values may be altered by the presence of dimer. For each energy distribution the analysis has been carried out over a range of protein concentration. We have found that, except in unusual cases, the presence of even small amounts of dimer may have a devastating effect upon the reliability of Adair constant determinations made from data obtained at the low protein concentrations commonly employed. In addition to these simulated cases, we have analyzed two sets of precise experimental data from the literature in order to assess the degree to which the constants obtained may have been influenced by the presence of dimer.

Methods

A. Saturation Functions. The model-independent thermodynamic relationships which define the linkage between oxygenation and subunit dissociation properties in tetrameric hemoglobins have already been presented in detail (Ackers and Halvorson, 1974). They will not be rederived here, but the quantities used in this study will be briefly

[1] Similar concentration-dependent oxygenation curves have been determined by Dr. Donald H. Atha, University of Texas, Austin, Texas (personal communication).

Table I: Energy Distribution Cases for Human Hemoglobin.[a]

Case	Stage of Oxygenation i	Dimer–Dimer[b] Contact Energy $(i-1\Delta G_2 - i\Delta G_2)$	Tetramer Adair Constants K_{4i}	Apparent K_{4i}^c/K_{4i}
Noncooperative dimer cases $\Delta G_{21} = -8.402$ kcal/mol, $\Delta G_{22} = -7.598$ kcal/mol				
A	1	−4.000	1.974×10^3	2.903
	2	−2.667	4.850×10^7	3.900
	3	−1.333	1.899×10^{12}	2.55×10^{-8}
	4	0	9.257×10^{17}	1.368
B	1	−8	2.00	1955
	2	0	4.87×10^6	25.91
	3	0	1.903×10^{12}	1.562×10^{-8}
	4	0	9.257×10^{17}	1.364
C	1	0	1.95×10^6	0.9512
	2	−8	4.878×10^6	230.7
	3	0	1.903×10^{12}	0.2862
	4	0	9.257×10^{17}	2.067×10^7
D	1	0	1.95×10^6	0.9728
	2	0	4.719×10^{12}	0.2882
	3	−8	1.898×10^{12}	6.04×10^{-3}
	4	0	9.257×10^{17}	1.134×10^7
E	1	0	1.95×10^6	1.025
	2	0	4.719×10^{12}	0.2788
	3	0	1.849×10^{18}	0.0639
	4	−8	9.257×10^{17}	0.1047
F	1	−2	6.209×10^4	1.011
	2	−2	4.776×10^9	9.336
	3	−2	6.037×10^{13}	73.53
	4	−2	9.257×10^{17}	29.30
Cooperative dimer cases $\Delta G_{21} = -4.000$, $\Delta G_{22} = -12.000$				
C′	1	0	0.989×10^3	5.218
	2	−8	4.89×10^6	14.4
	3	0	9.648×10^8	0.3892
	4	0	9.28×10^{17}	2.743×10^{12}
E′	1	0	9.89×10^2	32.305
	2	0	4.757×10^{1}	17.40
	3	0	9.383×10^{14}	0.1151
	4	−8	9.257×10^{17}	1.672×10^{-3}

[a] These energy distributions pertain to a medium affinity hemoglobin system in which the total free energy of binding 4 mol of ligand to tetramer is −24 kcal. For dimeric species the binding energies ΔG_{22} and ΔG_{21} differ by the statistical factor, $-RT \ln 4$. Simulations pertain to 20°C. [b] Successive energy changes at the dimer–dimer interface. For the second step we assume a statistical distribution between symmetric and asymmetric doubly liganded tetramers; consequently $^2\Delta G_2 = {}^2\Delta G_2{}^A - RT \ln 5$ (Ackers and Halvorson, 1974). [c] Values of apparent K_{4i} were obtained by fitting error-free saturation curves at $(P_t) = 10^{-4} M$ heme simulated by eq 1 to the tetramer Adair isotherm, eq 3. Equilibrium constants listed can be converted into units of partial pressure through use of the Henry's law constant: 1 mm = 1.82×10^{-6} mol of O_2/l.

summarized. For nomenclature, definitions, and basic relationships the reader is referred to the paper by Ackers and Halvorson (1974).

The fraction $\bar{Y}$ of sites with bound ligand, as a function of free ligand concentration [X] and molar heme concentration $[P_t]$, is:

$$\bar{Y} = \frac{Z_2' + Z_4'[(Z_2^2 + 4^0K_2Z_4[P_t])^{1/2} - Z_2]/4Z_4}{Z_2 + (Z_2^2 + 4^0K_2Z_4[P_t])^{1/2}} \quad (1)$$

where

$$Z_2 = 1 + K_{21}[X] + K_{22}[X]^2$$

$$Z_2' = K_{21}[X] + 2K_{22}[X]^2$$

$$Z_4 = 1 + K_{41}[X] + K_{42}[X]^2 + K_{43}[X]^3 + K_{44}[X]^4$$

$$Z_4' = K_{41}[X] + 2K_{42}[X]^2 + 3K_{43}[X]^3 + 4K_{44}[X]^4$$

The ligand binding constants K_{21}, K_{22}, K_{41}, K_{42}, K_{43}, and K_{44} are defined by

$$K_{ni} = \frac{[(\alpha_{n/2}\beta_{n/2})[X_i]]}{[(\alpha_{n/2}\beta_{n/2})][X]^i}$$

where $i = 1, \ldots n$ and $n = 2, 4$. They are products of the sequential binding constants k_{ni}

$$k_{ni} = \frac{(\alpha_{n/2}\beta_{n/2}[X_i])}{(\alpha_{n/2}\beta_{n/2}[X_{i-1}])[X]} = \frac{K_{ni}}{K_{n(i-1)}} \quad (K_{n0} \equiv 1)$$

The free energies for sequential binding of ligands onto protein containing n subunits are given by $\Delta G_{ni} = -RT \ln k_{ni}$.

The effect of protein concentration $[P_t]$ on the saturation function (eq 1) is determined in part by the association constant ${}^0K_2$ for unliganded dimers to tetramer. ${}^0K_2 = (\alpha_2\beta_2)/(\alpha\beta)^2$. In the limit of zero protein concentration eq 1 approaches the dimer isotherm:

$$\bar{Y}_2 = \frac{K_{21}[X] + 2K_{22}[X]^2}{2[1 + K_{21}[X] + K_{22}[X]^2]} \quad (2)$$

In the limit of infinite $[P_t]$ eq 1 approaches the classical Adair equation:

$$\bar{Y}_2 = \frac{K_{41}[X] + 2K_{42}[X]^2 + 3K_{43}[X]^3 + 4K_{44}[X]^4}{4[1\ ] + K_{41}[X] + K_{42}[X]^2 + K_{43}[X]^3 + K_{44}[X]^4} \quad (3)$$

B. Cases Analyzed. 1. Simulated Energy Distributions. The linkage relationship (eq 1) is strictly phenomenological and is independent of any assumed mechanisms or of any particular assignment of the energy terms. Their determination from experimental data represents a formidable task of paramount importance, not yet achieved for any hemoglobin system. Nevertheless, the linkage relationships can be meaningfully applied to real hemoglobin systems in the following way. Equilibrium constants for overall processes of oxygenation (K_{44}, K_{22}) and subunit association (${}^0K_2$, ${}^4K_2$) are known or can be reasonably estimated from experimental data. (${}^4K_2 = [(\alpha_2\beta_2)[X_4]]/[(\alpha\beta)[X_2]]^2$). These overall constants define the range of variation for the possible energy distributions corresponding to the sequential steps of binding or subunit dissociation. Within these bounds the types of phenomena to be found are fairly limited and can be readily explored by numerical simulation and analysis. Such a set of energy distributions has recently been defined by Ackers and Halvorson (1974). The distributions represent various ways of parcelling out the intersubunit contact energy changes associated with the cooperative binding steps. In the present study we have analyzed these cases in detail. They are listed in Table I, along with certain results of the analyses.

Two points concerning this study should be noted before proceeding further. (1) Since we are simulating data based on one equation (eq 1) and analyzing it according to another (eq 3), it is expected that the Adair constants so derived will differ. The important question to which these studies are aimed is whether the discrepancies would be prohibitively high, for reasonable values of dimer concentration. (2) We will present analyses of simulated data which are essentially error free in order to illustrate the basic effects which arise when the tetramer curves are perturbed by the presence of dimer. Further perturbation of these data with appropriate random error would of course provide more realistic simulations of experimental conditions. However, as will be seen, the results with error-free data are so devastating that no further point is served by making the simulated data even less amenable to retrieval of the tetramer Adair

constants. Previous studies have focused upon the difficulties of resolving the successive binding constants from random error-perturbed data pertaining to a true four-parameter equation (eq 3) (Roughton and Lyster, 1965). We will not consider that problem in this paper. The simulations we present should be regarded as representing the "most tractable" limiting cases, which incorporate the effects of dimeric species upon the oxygenation curves.

2. Experimental Data. In addition to the simulated cases, we have analyzed two sets of published oxygenation data, those of Soprounov (1974), and of Roughton et al. (1965).

C. Numerical Methods. Oxygenation curves were simulated according to eq 1 using the values of the energy terms shown in Table I. For each of these cases we generated 100 "data points" with logarithmically spaced oxygen tensions over a range chosen to give an essentially complete saturation function. The simulated data were then analyzed by two minimization methods according to the tetramer Adair equation (eq 1) in an effort to recover the originally assumed binding constants.

For all the simulated cases to be presented, the correct values for the equilibrium constants were used as the initial guesses for fitting programs. This procedure biases the fitting in favor of the "correct" answers by maximizing the probability of convergence to these whenever they lie near a minimum.

1. Modified Gauss–Newton Method. The basic approach of the Gauss–Newton procedure (cf. Magar, 1972, p 149) for finding several parameters, α_j, by fitting experimental data to some function $\phi(\alpha_1\alpha_2 \ldots \alpha_j, X_i)$ is to approximate each of the dependent variables, Y_i, by the function ϕ at the corresponding independent variable, X_i, and the best fit values for the parameters, α^f. The function ϕ is then expanded in a first-order Taylor expansion about the current best approximation of the parameters, α^k,

$$Y_i \approx \phi(\alpha^f, X_i \approx \phi(\alpha^k, X_i) + \sum_j \frac{\partial\phi(\alpha^k, X_i)}{\partial\alpha^k{}_j}(\alpha^f{}_j - \alpha^k{}_j)$$

where the f superscripts refer to the final values of the parameters, k superscripts refer to some intermediate iteration, the unsubscripted α is a vector of all of the parameters, the j subscripts refer to a given parameter, and the i subscripts refer to a given data point. This can be expressed in matrix notation where ϵ is a vector approximating the difference between α^f and α^k, $\mathbf{Y}^*$ is a vector which is the difference between the data points and the function evaluated at the current best approximation, and $\mathbf{P}$ is a nonsquare matrix of partial derivatives. The correction vector, ϵ, can thus be found by the usual procedure of using the transpose, $\mathbf{P}'$, of the matrix $\mathbf{P}$:

$$\epsilon = (\mathbf{P}'\mathbf{P})^{-1}\mathbf{P}'\mathbf{Y}^*$$

This value of the correction vector can then be used to find a new set of parameters, α^{k+1}, and the process repeated until each of the values of the correction vector become less than some predetermined value. A modification which we made was to introduce a constant, t, which is varied at each iteration such that a minimum variance is obtained.

$$\alpha^{k+1} = \alpha^k + t\epsilon$$

The simultaneous confidence intervals for the estimated parameters are found by searching two sets of axes in both directions until the desired value of the F statistic (variance ratio, cf. Zelen and Severo, 1964) is achieved. The first set of axes is the "ellipsoidally shaped" region in n-dimensional space for a parameter given by the equation:

$$(\alpha - \alpha^f)\mathbf{PP}'(\alpha - \alpha^f) \leq ns^2F$$

where s^2 is the variance of the minimum, F is the F statistic from statistical tables, and α (the only unknown) is a set of vectors which are the axes of this region of space (Magar, 1972, p 243). The second set of axes is obtained by varying each of the parameters independently. The confidence interval minimum or maximum for a given parameter is the minimum or maximum deviation of that parameter along any of the axes. It has been our experience that these regions are not symmetrical, i.e., that the allowed deviations in one direction can be orders of magnitude greater than the deviations in the other direction, a reflection of the profound nonlinearity of the problem.

The confidence probability at each of the predicted extrema of the parameters can be determined by fixing the particular parameter at that value and then minimizing the variance by varying all of the other parameters (Endrenyi and Kwong, 1973). The variance of this fit can then be compared with the variance of the minimum and the probability of the particular value of F determined.

2. Nelder–Mead Simplex Method. The simplex method of Nelder and Mead (Nelder and Mead, 1965) was used in a program kindly supplied to us by Dr. S. P. Spragg, Department of Chemistry, University of Birmingham, England.

The Nelder–Mead procedure is basically a guided search for a minimum of a response function. We used one of two response functions, depending on the signs of the estimated parameters. As long as the parameters are positive, the response function is the variance; whenever any of them becomes negative, an arbitrary value much larger than the variance is used. In this manner the estimated parameters (Adair constants) are constrained to positive values.

The two methods of analysis yielded essentially identical results within the predicted confidence regions, and provided a cross-check on avoiding pitfalls of local minima. The main difference between the two methods lies in the fact that in one of them, the simplex method, values of estimated parameters are constrained to positive values. Both methods were also shown to converge upon the correct Adair constants when used to analyze data simulated from eq 3 for each of the cases to be presented in this paper. To conserve space we present only a representative selection of the results obtained by the two methods over a much wider range of conditions.

Results

In Table I are shown results of estimates of the tetramer Adair constants for the various cases at a protein concentration [P_t] of 10^{-4} M heme. (It should be noted that this is roughly twice the concentration most frequently employed for determination of the oxygenation curves.) The results listed in Table I were estimated using the simplex minimization procedure and are drawn from a much more extensive set of analyses over a wide concentration range.

It can be seen from Table I that, at a concentration of 10^{-4} M heme, none of the cases can be successfully analyzed for all four Adair constants. In each case, some or all of the constants are so different from the true values as to be meaningless. For each case this situation deteriorates rapidly with decreasing protein concentration (not shown). Since it is impossible to tell which Adair constants are erro-

Table II: Effect of Hemoglobin Concentration on Adair Constant Determination for Case A.[a]

$-\text{Log}(P_t)$ Concn Molar Heme	Apparent K_{41}/K_{41}	Apparent K_{42}/K_{42}	Apparent K_{43}/K_{43}	Apparent K_{44}/K_{44}
0	1.018	1.036	0.976	1.004
1	1.062	1.106	0.932	1.012
2	1.186	1.354	0.776	1.037
3	1.572	2.15	0.275	1.119
4	2.635	5.13	−1.5	1.41[b]
5	5.038	16.86	−8.0	2.625[b]
6	10.86	54.5	−25.7	10.002[b]

[a] Data were simulated according to eq 1 for the energy terms of case A (Table I). Apparent Adair constants K_{4i} were determined by the Gauss–Newton minimization method according to eq 3. [b] Two of the sequential binding constants (k_{43}, k_{44}) are negative.

neous, none can be trusted. Such extreme variations as shown in Table I for the Adair constants might generally be "smoothed out" by random errors of experimental data. However, such "smoothing" can hardly be expected to improve the accuracy of the derived values.

The effect of protein concentration over a wide range is illustrated in Table II, which is typical for the systems studied. The values listed in Table II were obtained using the modified Gauss–Newton program, which permits negative parameters. Consequently the values listed at 10^{-4} M heme differ somewhat from those of Table I for case A. This is particularly striking for the last two binding steps. Here the sequential binding constants become negative (both k_{43} and k_{44}), as reported earlier from calculations using a pseudolinearization method (Ackers and Halvorson, 1974). These negative results for case A illustrate the further problem in the estimation of sequential binding constants k_{4i} from the Adair constants K_{4i}. It can be seen in Table II that going to higher concentration (e.g., 10^{-3} M) improves the situation in only a limited way. The fraction of dimeric species present for case A at 10^{-5} M heme is shown as a function of saturation in Figure 2.

High Affinity Cases. Since the cases of Table I embody energies which pertain to "medium affinity" hemoglobin systems (e.g., corresponding to moderate salt concentration) it was of interest to carry out similar analyses with a high affinity case. In one test we used values of the Adair constants determined by Imai and Tyuma (1973) as well as their reported value for the total cooperative energy (−8.5 kcal/mol), and adjusted the other energies as shown in Table III. It was found that in this high affinity case the same general phenomena are exhibited with respect to the validity of Adair constant estimation, as was shown for the medium affinity cases. At concentrations where the experimental data were taken, the values of the last two sequential constants are found to be negative.

Data of Soprounov. The data of Soprounov (1974) were analyzed according to eq 3 and the results are shown in Table IV. As can be seen (Table IV) the calculated second and third sequential binding constants are found to have negative values. Such negative constants were also found by Soprounov for these data. Although the protein concentrations employed in those studies are not specified, Soprounov reports the finding of negative apparent binding constants to be quite general in over 2000 accurately obtained oxygenation curves (Soprounov, 1974).

Data of Roughton. The accurate oxygenation data of Roughton et al. (1965) for human hemoglobin was analyzed in two ways: (1) by a straightforward four-parameter fit to eq 3 by the Gauss–Newton procedure, and (2) by a four-parameter fit which assumes dimers to be present according to eq 1 in which protein concentration (1.8×10^{-3} M) and values of K_{21}, K_{22}, and $^4K_2$ were fixed according to the literature values (Table V). Results of these analyses are shown respectively in Table VA and B, along with the values of derived constants reported by Roughton for these data. It can be seen (Table VA) that our analyses agree very closely with that of Roughton et al., as to the derived constants. Second, it can be seen (Table VB) that the derived constants are affected only negligibly by the low fraction of dimers present (0.036 at maximum) and the assumed values of K_{21} and K_{22} ($^4K_2$ having a reliably known value).

Discussion

In recent studies where precise oxygenation curves have been analyzed, the effect of protein concentration has been largely ignored. Yet the existence of appreciable quantities of dimeric species in the dilute solutions (e.g., 6×10^{-5} M heme) where oxygenation curves are frequently measured necessitates an evaluation of their influence upon the analysis of such curves for any purpose. In this study we have explored the effect of protein concentration upon the analysis of oxygen-binding curves in terms of the four Adair constants. The results indicate that, over a wide range of energy distributions which are relevant to real hemoglobin systems, the analysis of even ideal (error free) saturation curves leads to highly erroneous determinations of at least some of the constants. Since we have not studied all possible energy distributions that may pertain to all possible hemoglobins, these conclusions cannot be drawn categorically. The results, however, point strongly to the need for great caution in the analysis and interpretation of oxygenation curves. It is possible for several sets of fitted parameters to represent an oxygenation curve with equal fidelity, as illustrated by the comparison between results obtained with the two fitting methods employed in this study. Since we have chosen, as initial guesses, the correct values of the constants to be estimated, the values converged upon will in general represent a minimal estimate of the degree of error that can be introduced in the Adair constants. Caution would appear especially warranted in the analysis of temperature and pH dependencies of apparent Adair constants. Such dependencies may reflect enthalpies and pH dependencies for subunit dissociation in addition to the ligand binding heats and tetramer Bohr effect. It should be noted that the procedure we have employed in this study provides a means of estimating the possible reliability of any derived set of binding constants that may be obtained from experimental data whenever reasonable estimates can be made for the other energetic quantities.

In view of our results with the simulated cases, it is not surprising to find negative apparent values of binding constants, as have been reported by Soprounov (1974). Furthermore it seems likely that the results reported by Roughton et al. (1965) will stand the test of perturbation by dimeric species, since their studies were carried out at very high protein concentration.

From the studies described here we are led to the conclusion that, in order to obtain valid estimates of tetrameric binding constants for solutions of human hemoglobins, either of two strategies must be pursued: (1) eliminate the ef-

Table III: Analysis of Model Generated from Literature Adair Constants for High Affinity Case.[a]

−Log [P_t] Molar Heme	Apparent K_{41}/K_{41}	Apparent K_{42}/K_{42}	Apparent K_{43}/K_{43}	Apparent K_{44}/K_{44}
0	0.9986	0.9849	1.0409	1.002
1	1.027	0.963	1.1131	1.017
2	1.032	1.302	0.8072	1.134
3	1.202	1.748	0.7017	1.4858
4	1.3563	3.9814	−0.7631	2.9872
5	1.4000	3.836	−0.4984	2.998
K_{4i}	1.315×10^5	1.081×10^{10}	6.179×10^{14}	8.184×10^{20}

[a] Saturation curves were simulated according to eq 1 using values of k_{4i} taken from Imai and Tyuma (1973) using their reported total cooperative energy of −8.5 kcal/mol (instead of −8 kcal/mol), so that $^2\Delta G_4 = -7.25$ kcal/mol and $^0\Delta G_2 = -15.75$ kcal/mol. Simulated data were fit to eq 3 as function of protein concentration.

Table IV: Analysis of Soprounov Data for Human Hemoglobin.[a]

Adair Constant	Estimated Value	Confidence Limits[b]		Sequential Binding		Reported Values
K_{41}	4.88×10^4	1.91×10^4	8.95×10^4	k_{41}	4.88×10^4	2.7×10^4
K_{42}	-4.17×10^8	-1.84×10^8	6.18×10^8	k_{42}	-8.54×10^3	Negative
K_{43}	1.87×10^{12}	-12.3×10^{12}	21.3×10^{12}	k_{43}	3.84×10^3	Negative
K_{44}	7.18×10^{17}	5.90×10^{17}	8.71×10^{17}	k_{44}	3.84×10^5	4.8×10^5

[a] From Soprounov (1974). Data listed were analysed by Gauss–Newton Method. [b] Chosen such that the F statistic would correspond to a 90% probability (see text).

Table V: Analysis of Roughton Data for Human Hemoglobin.[a]

A. In terms of eq 3 (ignoring effects of dimers)

Adair Constant	Estimated Values	Confidence Limits[d]		Reported Values
K_{41}	2.90×10^4	0.48×10^4	5.56×10^4	2.70×10^4
K_{42}	8.88×10^8	-13.7×10^8	29.5×10^8	6.36×10^8
K_{43}	6.33×10^{13}	-1.44×10^{13}	14.9×10^{13}	7.70×10^{13}
K_{44}	1.41×10^{19}	1.23×10^{19}	1.60×10^{19}	1.36×10^{19}

B. In terms of eq 1 (including effects of dimers)

Constant	Estimated Values	Confidence Limits		Reported Values
K_{21}	6.20×10^5	[b]		
K_{22}	1.54×10^{12}	[b]		
K_{41}	2.81×10^4	5.69×10^3	0.57×10^3	2.70×10^4
K_{42}	7.01×10^8	-13.3×10^8	25.9×10^8	6.36×10^8
K_{43}	6.61×10^{13}	-0.452×10^{13}	14.2×10^{13}	7.70×10^{13}
K_{44}	1.30×10^{19}	1.15×10^{19}	1.48×10^{19}	1.36×10^{19}
$^4K_2$	4.12×10^5	[b]		
$^0K_2$[c]	7.48×10^{10}	6.61×10^{10}		

[a] Data listed by Roughton et al. (1965) pertaining to 19°, pH 6.9, [P_t] = 2–4%. The value of [P_t] used in our calculation was 3% (1.8×10^{-3} *M*). [b] Literature values fixed in the calculation. [c] Calculated from the other seven values. [d] Chosen such that the F statistic would correspond to a 90% probability (see text).

fect of dimers experimentally, as Roughton did, by working at sufficiently high protein concentrations; (2) incorporate the effect of dimeric species into analysis of the experimental data. In this regard it should be noted that the concentration dependence of oxygenation curves provides a powerful experimental tool for the resolution of intersubunit contact energy changes which accompany the sequential binding steps (Ackers and Halvorson, 1974). Whereas a single oxygenation curve at low protein concentration may yield no reliable determination of the four tetramer Adair constants, a set of such curves over a wide concentration range may yield not only these, but some of the other important energetic quantities as well. For this purpose, the rapid automatic oxygenation apparatus developed by Imai and colleagues appears to be well suited and may be expected to provide results of even greater importance than have been obtained to date. In a subsequent paper we will describe results of a study using this approach.

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Added in Proof

The analyses of this paper are based upon a value of 8 kcal/mol for the overall coupling free energy linking oxygenation and subunit dissociation. We have now experimentally determined this energy to be about 7 kcal/mol under one particular set of conditions (to be published). Exploration of the effects of this lower value for the overall coupling energy does not lead to alteration in any of the conclusions drawn in this paper. We have also carried out additional simulations to explore the effects of random errors on the fitted data and find, as expected, no improvement in the reliability of parameter estimates.

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