Electro-Communications for allowing us to use a Hitachi M-80B instrument.

Registry No. Asialo G_{M1}, 71012-19-6; asialo G_{M2}, 35960-33-9; *N*-palmitoylpsychosine, 34324-89-5; *N*-cerebronoylpsychosine, 586-02-7; ceramide [2-(methylamino)ethyl]phosphonate, 91254-90-9; ceramide (2-aminoethyl)phosphonate, 22822-94-2.

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Oxygen Binding Constants for Human Hemoglobin Tetramers†

Stanley J. Gill,* Enrico Di Cera, Michael L. Doyle, Gary A. Bishop, and Charles H. Robert

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215

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ABSTRACT: High-precision studies of oxygen binding in hemoglobin (HbA_0) solutions at near-physiological concentrations (2–12 mM heme; pHs 7.0–9.1; various buffers) have led to an unanticipated result: an unmeasurably low contribution from the triply ligated species. We have obtained this result from new differential oxygen-binding measurements for human hemoglobin through the use of a thin-layer apparatus, which enables study of solutions at high Hb concentrations. The effect of tetramer dissociation into dimers, which becomes significant at hemoglobin concentrations below 1 mM in heme, is avoided. The analysis of the binding reactions is thus cast in terms of the tetramer-binding polynomial written with overall Adair equilibrium constants which directly reflect the contributions of intermediate ligated species. The unmeasurable contribution of the triply ligated species renders the equilibrium constants of the third and fourth stepwise reactions practically undeterminable.

Since the discovery of hemoglobin's sigmoidal oxygen-binding curve more than 80 years ago (Bohr et al., 1904), efforts to understand the origins of cooperative interaction have resulted in a multitude of experimental findings and theories. A historical review has been given by Edsall (1980). Of particular importance was the establishment of the precise molecular weight of hemoglobin by Adair (1925a) and Svedberg and Fahreus(1926). At that point it became clear that with four oxygen-binding sites there must be four thermodynamic binding reactions, subsequently termed the Adair reactions, describing the stoichiometric oxygenated species (Adair, 1925b). Roughton devoted a significant effort toward the determination of these constants using gasometric procedures (Roughton et al., 1955; Roughton & Lyster, 1965). However, the discovery of the important regulatory properties

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of 2,3-diphosphoglycerate (DPG)[1] (Benesch & Benesch, 1967; Chanutin & Curnish, 1967), which has a marked effect on the oxygen-binding curve, cast an uncertainty onto these early results where its concentration was unknown. With the availability of modern spectrophotometers for the precise determination of absorbances came the possibility of more precise determinations of the binding constants describing the Adair reactions. Two groups have been particularly active in this goal. Imai in Japan developed a high-precision partial pressure scanning technique (Imai et al., 1970), especially suitable to low concentrations, and has applied this procedure to a number of hemoglobin systems (Imai, 1982). Ackers, in the United States, extended the Imai technique to investigate the thermodynamics of the ligand-linked dissociation process of hemoglobin tetramers going to dimers (Ackers & Halvorson, 1974).

Imai and Yonetani (1977), using an approximate dimer–tetramer linkage representation, concluded that even at concentrations as low as 60 μM, dimers did not play a significant role in the determination of values for the p_{50}, Hill coefficient, and even stepwise Adair constants for the tetramer (Imai, 1982). Imai's analysis of experimental data was based on the approximation that the tetramer is the predominant contributor to the binding curves, and thus a simplified binding polynomial of the Adair form, with four thermodynamic parameters, could be utilized. However, in the course of their studies, Ackers and co-workers (Mills et al., 1976; Johnson et al., 1976; Chu et al., 1984) showed that particular care must be exercised in the analysis of the data to include terms for both the dimer and tetramer forms and the equilibrium constant between these forms even at moderately high concentrations (0.5 mM heme).

In order to circumvent these complications, binding data for HbA_0 at high concentrations is desirable. In the present study we have used a thin-layer technique (Dolman & Gill, 1978), devised initially to explore the oxygen-binding properties of hemoglobin under physiological concentrations, especially for oxygen-linked aggregation processes of sickle cell hemoglobin, HbS (Gill et al., 1978, 1979, 1980; Benedict et al., 1981). The thin-layer method employs differential measurements of the binding curve, providing equilibrium data taken between well-specified partial pressures. The use of high concentrations virtually eliminates the effect of dissociation of HbA_0 into dimers, allowing the properties of the tetramer to be analyzed directly. This leads to simplification of the binding-curve analysis to a four binding constant problem.

Materials and Methods

Preparations. Human hemoglobin A_0 was prepared according to the method reported by Williams and Tsay (1973). The resulting hemoglobin was dialyzed against distilled water, deionized on a mixed-bed ion-exchange resin, and concentrated. The final hemoglobin concentration of 16 mM heme was calculated from an absorbance measurement at 576 nm of a $^1/_{100}$ dilution of the sample by using an extinction coefficient of 15.3 mM^{-1} cm^{-1} (van Assendelft & Zijlstra, 1975). The final sample was frozen as 25-μL droplets in liquid nitrogen and stored in liquid nitrogen until use. Hemoglobin retains its O_2 binding functionality for a period of years while frozen in liquid nitrogen (Chu et al., 1984). However, we have found that overnight dialysis at 4 °C of the thawed samples can result in up to 5% metHb formation. Thus we obtained the final solutions by mixing HbA_0 in deionized, distilled water with buffer solution at appropriate concentration and adjusting the pH as needed.

Most experiments were conducted under solution conditions of 50 mM HEPES buffer, 0.1 M NaCl, 40 μM EDTA, 25 °C, and pH 7.4. The sodium chloride and pH conditions were chosen to mimic physiological conditions, HEPES was chosen for its large buffering capacity at pH 7.4 in order to minimize the pH change of the Bohr effect at the high HbA_0 concentrations used, and the EDTA was added to minimize metHb formation. Some experiments were run in solution conditions, noted as "HBG", that provide buffering over a wide range of pH values: 50 mM HEPES, 50 mM bicine, and 50 mM glycine plus 0.1 M NaCl and 40 μM EDTA at 25 °C and the desired pH. In addition other experiments were carried out in either 0.1 M phosphate or 50 mM HEPES with 5 mM chloride. The enzymic reducing system of Hayashi et al. (1973) was used in all cases to reduce formation of metHb (except in experiments designed to test the effects of the enzyme components themselves). The effect of the reductase components on the O_2 binding equilibria of human hemoglobin has been reported to be insignificant in the presence of 0.1 M chloride (Imai, 1974). All components of the reductase system, the Tris buffer, and the HEPES buffer were products of Sigma Corp.

Differential Binding-Curve Measurements. Binding measurements were performed by using a thin-layer optical technique (Dolman & Gill, 1978). The thickness of the layer is defined by a spacer shim between the optical window and the transparent gas-permeable membrane. The membrane is MEM-213 MA-457, a copolymer of silicone and polycarbonate, 1 mil thick, optical quality, from General Electric Co., Medical Systems Division. The thin-layer sample is contained in a gas-tight stainless steel cell connected to a precision gas-dilution valve. The temperature at the cell is controlled to ±0.01 °C from a water bath regulated by a Tronac Inc. Model PTC-4 precision temperature controller.

Initially the gas-tight cell chamber is filled to atmospheric pressure with O_2. The dilution valve bore is then flushed at atmospheric pressure with a nonbinding gas, in this case N_2. The partial pressure of O_2 after each dilution step, i, with a bore volume is given by

$$p_{O_2}(i) = p_{O_2}(0)D^i \quad (1)$$

where $p_{O_2}(0)$ is the starting pressure of O_2 and D is the dilution factor. The dilution factor was determined from least-squares analysis of Clark O_2-electrode (Yellow Springs Instrument Co., Model YSI 4004) readings vs. dilution step. The N_2 gas source was United States Medical, grade UN1066, and an O_2 trap (Scientific Gas Products, Model GP-201) was used to eliminate trace amounts of O_2. The O_2 gas source was United States Welding, industrial grade UN1072, or in some cases compressed atmospheric air (UN1002) was used. All gases were equilibrated with water vapor at the temperature of the water bath before and during use.

Changes in O_2 saturation of the hemoglobin sample were typically followed as decreases in optical density at 576 nm on a CARY 219 spectrophotometer upon each stepwise dilution of the O_2 partial pressure. Because the HbA_0 sample is very thin, typically 0.005 cm, stirring of the sample is unnecessary. The thickness of the layer is controlled approximately by the shim thickness used in forming the layer. The hemoglobin equilibrates with each new O_2 partial pressure in 5–10 min, so that an entire run took typically 2–3 h. The

[1] Abbreviations: Hb, hemoglobin; metHb, methemoglobin; oxyHb, oxygenated hemoglobin; deoxyHb, deoxygenated hemoglobin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; Na_2EDTA, disodium ethylenediaminetetraacetic acid; DPG, 2,3-diphosphoglyceric acid.

percentage of metHb at the beginning and end of each experiment was measured by the ratio of absorbances at 578 and 500 nm in the spectrum of the fully oxygenated molecule (Kilmartin et al., 1978). Since the absorbance measurements were performed on the hemoglobin in the presence of the enzymic reducing system and the silicone–polycarbonate copolymer membrane, a blank spectrum of these components was recorded and subtracted before the metHb content was evaluated. The magnitude of this correction was less than 1%. The amount of oxidation at the end of any experiment was 3% or less. Typically the higher concentrations of hemoglobin resulted in the lowest amount of oxidation.

Reversibility of the Thin-Layer Technique. For investigations of the reversibility of the thin-layer technique, the differential binding data were first collected in the normal mode of action for the thin-layer method as described above. The sample cell was then completely deoxygenated by flushing with N_2. The reoxygenation of the same sample was then accomplished by the use of a small-bore valve. The bore in this valve has a volume of about 0.4% of the total cell volume. The small bore volume, flushed before each step with O_2, enables the experiment to be performed in reverse, with each step providing nearly linear additions of approximately 2.5 Torr of O_2. The oxygen electrode, calibrated in the forward experiment, was used to determine the oxygen partial pressure after each step. By use of both the normal and reverse procedures the O_2 binding process was studied essentially in its entirety.

Additional Control Experiments. Potassium chromate for linearity tests (Rao, 1961) of the Cary 219 was obtained from Fisher Scientific Co. and used without further purification. Dilutions were performed volumetrically.

A separate preparation of hemoglobin A_0 was kindly given to our laboratory by Dr. Bo Hedlund (University of Minnesota).

Data Analysis. The binding data were taken in the form of changes in optical density, ΔOD_i, for a change in oxygen partial pressure from step $i-1$ to step i. The fitting equation is then

$$\Delta OD_i/\Delta OD_T = \theta_i - \theta_{i-1} \quad (2)$$

where θ is the fractional ligand saturation of the macromolecule at a given step and ΔOD_T is a parameter representing the optical density change on taking the macromolecule from zero to infinite ligand activity.

The degree of saturation θ is given in terms of the binding polynomial (or binding partition function) P, for a macromolecule with t sites, by the derivative

$$\theta = \frac{1}{t}\frac{d \ln P}{d \ln x} \quad (3)$$

The binding polynomial describes the sum of stoichiometric species concentrations relative to the unliganded form. The general Adair form (Adair, 1925b) of the binding polynomial for a tetramer has the form

$$P = 1 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4 \quad (4)$$

where the β's are the overall Adair constants, related to the intrinsic stepwise Adair constants κ by $\beta_j = \binom{4}{j}\kappa_1, \ldots, \kappa_j$. In the present study we have applied eq 4 to the analysis of our high-concentration binding data.

All binding parameters were estimated by least-squares optimization of the appropriate fitting equations to the data, using the Gauss–Newton algorithm as modified by Marquardt and others (Frazier & Suzuki, 1973). One standard deviation

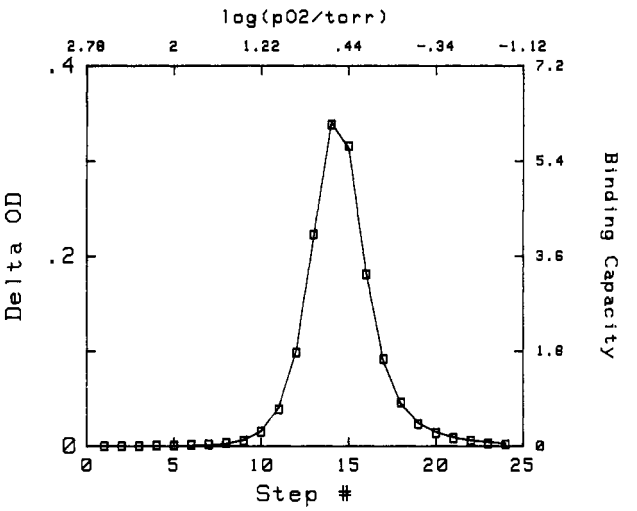

FIGURE 1: Optical density changes for stepwise changes in oxygen partial pressure for a thin-layer (0.0025 cm) sample of HbA_0, conditions E in Table I. The experimental measurement is the change in optical density ΔOD_i for step i, which corresponds to the saturation change caused by change in oxygen pressure from p_{i-1} to p_i. The left ordinate indicates the measured values of ΔOD_i, which are plotted against step i (bottom coordinate) or against log p_{O_2} (top coordinate). The scale for the binding capacity (right ordinate) of the finite difference derivative is determined from the relation $(\Delta\theta_i/\Delta \log p_i) = (\Delta OD_i/\log D)/\Delta OD_T$, where ΔOD_T is the theoretical total optical density change upon full saturation from the deoxy state and D is the dilution factor (0.6975). Theoretical points, connected by the solid line, are best-fit values given by eq 2 and 4 with parameters given in Table I.

confidence intervals on most parameters were determined by F-tests (Magar, 1972). The significance of the third binding constant was determined by the F-test of an additional parameter (Bevington, 1969). A Hewlett-Packard 9816 computer was employed for all analyses.

Results

In the course of a separate calorimetric study on the oxygen-binding properties of hemoglobin, we found it necessary to determine the Adair parameters for solutions with phosphate buffer (chosen for its low heat of ionization) at high concentrations of hemoglobin (Bishop, 1986). Similar experiments were simultaneously undertaken with other buffer conditions as well. The high concentrations employed were ideally suited to the thin-layer optical apparatus for binding determinations. A typical set of data, obtained by use of the thin-layer technique, is shown in Figure 1 in the form of a "binding capacity" plot. Each data point in Figure 1 represents the change of optical density for a given change in the oxygen partial pressure. Since the partial pressure changes by a constant dilution factor (eq 1), in the logarithmic representation used here the partial pressures are equally spaced. Thus the changes in optical density, which vary linearly with the saturation, can be divided by a constant factor, i.e., $\Delta \log p_{O_2}$, and therefore approximately scale with the logarithmic derivative of the binding curve, $\partial\theta/\partial \log p_{O_2}$. This derivative is analogous to the heat capacity and is termed here the binding capacity. As seen, this derivative has a maximum value in the middle region of the binding curve, where the intermediates in the ligation process make their largest contributions to the binding curve. The data in Figure 1 appear along with a point-to-point trace of the theoretical line fit using eq 2 with the four-constant Adair polynomial (eq 4).

The Adair parameters obtained for the fit of the data in Figure 1 are given in Table I. The unanticipated result was that the value of the third overall constant was too small to

Table I

Description of Experimental Conditions for Data Sets Discussed in This Paper

	buffer	EDTA	NaCl	pH	T (°C)	[heme] (mM)
A	0.1 phosphate			7.5	25	12
B	50 mM HEPES	40 μM	0.1 M	7.4	25	8
C	50 mM HEPES	40 μM	0.1 M	7.4	25	4
D	50 mM HEPES	40 μM	0.1 M	7.4	25	2
E	0.1 M HEPES	40 μM	5 mM	7.4	25	8
F	50 mM HBG[a]	40 μM	0.1 M	9.1	25	4
G	50 mM HBG[a]	40 μM	0.1 M	6.9	25	4
H	0.1 M Tris-HCl	1 mM	0.1 M	7.4	21.5	4
I	0.1 M Tris-HCl	1 mM	0.1 M	7.4	21.5	2
J	50 mM HEPES	40 μM	0.1 M	7.5	25	4

Parameter Values Obtained by Fitting the Data, along with the Confidence Interval within One Standard Deviation

	β_1 (Torr^{-1})	β_2 (Torr^{-2})	β_3 (Torr^{-3})	β_4 (Torr^{-4})	$-\Delta OD_T$	σ (×10^4)
A	0.039	0.0063	0	0.00017	0.1404	3.1
	0.029, 0.046	0.0044, 0.0065	0.0001	0.00016, 0.00018	0.1395, 0.1414	
B	0.080	0.018	0	0.00081	0.2407	3.2
	0.060, 0.10	0.013, 0.019	0.0007	0.00075, 0.00084	0.2388, 0.2419	
C	0.10	0.014	0	0.00078	0.1365	3.6
	0.081, 0.16	0.0073, 0.015	0.0007	0.00066, 0.00087	0.1354, 0.1376	
D	0.059	0.013	0	0.00089	0.0995	1.2
	0.043, 0.086	0.0061, 0.014	0.0008	0.00082, 0.00095	0.0987, 0.1003	
E	0.16	0.037	0	0.0053	−1.428	15.9
	0.14, 0.20	0.028, 0.038	0.002	0.0049, 0.0056	−1.411, −1.439	
F	0.61	0.23	0	0.091	0.1116	2.6
	0.44, 0.85	0.16, 0.27	0.03	0.077, 0.10	0.1107, 0.1125	
G	0.054	0.0031	0	0.000094	0.1144	1.4
	0.042, 0.068	0.0022, 0.0032	0.00007	0.000089, 0.00010	0.1135, 0.1154	
H	0.16	0.0084	0	0.0014	0.1144	2.7
	0.11, 0.20	0.0042, 0.011	0.0013	0.0012, 0.0016	0.1133, 0.1155	
I	0.14	0.011	0	0.0014	0.0696	1.4
	0.11, 0.20	0.0067, 0.012	0.0013	0.0013, 0.0015	0.0689, 0.0703	
J	0.098	0.017	0	0.0011	0.1269	4.1
	0.072, 0.17	0.0085, 0.018	0.0013	0.00092, 0.0012	0.1260, 0.1278	
J*[b]	0.10	0.026	0	0.0013	−0.0971	2.0
	0.076, 0.16	0.010, 0.029	0.0015	0.0010, 0.0014	−0.0962, −0.0981	

[a] HBG = 50 mM each HEPES, bicine, and glycine. [b] * = reversibility test: decreasing (J) and increasing (J*) saturation. Nominal shim thickness = 0.005 cm (except 0.0025 cm in cases A and E). Wavelength = 576 nm (except 430 nm in case E).

be determined. Repeated measurements gave the same result. In reexamining similar data that was obtained in our laboratory within the past eight years but not analyzed in detail, we found this result as well. In earlier analyses of the binding data, extensive use was made of the intrinsic stepwise Adair constants in the binding polynomial with the result that the third and fourth stepwise constants were poorly determined. As we will discuss, a consequence of this is the inability to resolve the upper asymptote for a Hill plot. However, in an initial study (Dolman & Gill, 1978) this problem of defining an upper asymptote was not appreciated. In retrospect, we now realize that when one or more of the intermediate species may have an insignificant contribution it is necessary to analyze in terms of the overall Adair constants which directly indicate the individual species populations, rather than the stepwise constants.

The immediate question was, how general is this result for the third Adair constant? A number of differential binding experiments on HbA_0 under various solution conditions were made and the Adair constants determined. We turned to 0.1 M NaCl in HEPES buffer (pK_a = 7.5), which minimizes pH changes resulting from Bohr protons at pH 7.4 and approaches physiological salt conditions. These complemented the experiments with low chloride (5 mM) and HEPES buffer that dealt with the "stripped" molecule (e.g., the data in Figure 1). Further, we measured binding curves under the same buffer conditions used by Mills et al. (1976), i.e., 0.1 M Tris-HCl, 1 mM Na_2EDTA, and 0.1 M NaCl, pH 7.4, at 21.5 °C. Finally we performed experiments at different pH values ranging from 6.95 to 9.1 in 0.1 M NaCl and suitable buffers (HBG). The conditions of the various experiments are summarized at the top of Table I. As noted in all of these experiments the concentration of hemoglobin was above 2 mM heme. The results of fitting the experiments with the Adair formulation, eq 2 and 4, are tabulated in Table I. As can be seen by the values, given with one standard deviation confidence intervals, the best fit of the parameter β_3 with all parameters constrained to be positive was zero.

Statistical Tests. As a measure of the overall precision of fitting the differential binding data, we have compared the overall standard error of a point from each fit to the expected instrumental error. The error in ΔOD measurements on the CARY 219 depends on the sensitivity scale used and is typically 0.5% of the output range. For a typical experiment the total change of optical density resulting from full saturation of hemoglobin with O_2 was about 0.1. Thus in measuring the stepwise changes an absorbance range of 0.05 is a convenient one to use and leads to an instrumental error of about ±0.00025 OD units. As shown in Table II the standard error of a point (σ) for any data fit is similar to this instrumental error. The detailed description of the deviation of the fitting function from the data points is best seen in a plot of the residuals normalized to the overall standard error of a point (σ). Such a plot is shown in Figure 2. The residuals are evenly distributed within each data set, indicating that the fitting function and the equal weighting of the data in the fitting process are correct and that no obvious systematic error is present.

Statistical analysis of the results gives confidence intervals for the values of the fitted parameters. F-tests have been

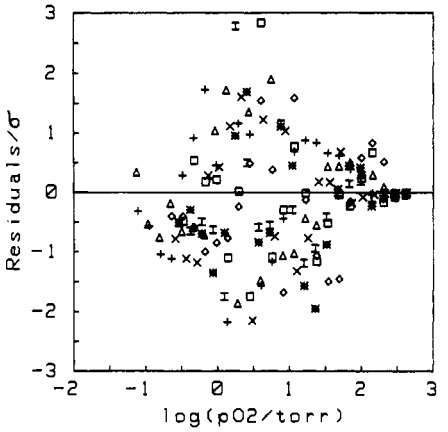

FIGURE 2: Normalized residuals for the fit of seven representative data sets used in obtaining the parameters of Table I: A (□); B (*); E (△); F (+); G (◇); H (×); J (I).

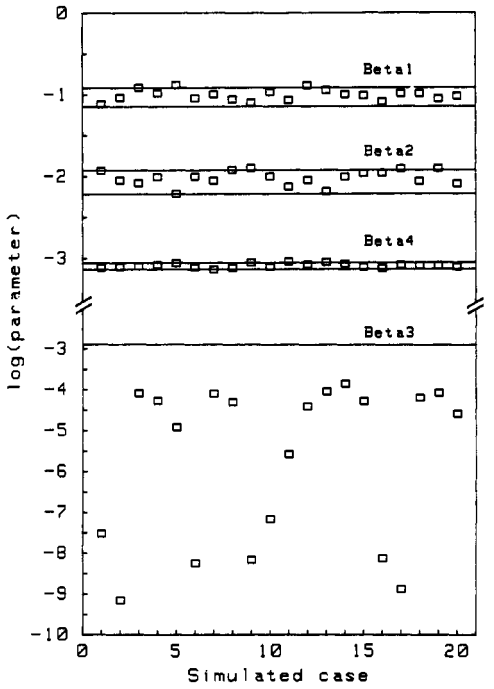

FIGURE 3: Fitting results to simulated binding capacity data using $\beta_1 = 0.09$, $\beta_2 = 0.01$, $\beta_3 = 0$, $\beta_4 = 0.0008$, $\Delta OD_T = 0.15$, and a pseudorandom standard error of $\sigma = 0.0002$. The 20 cases shown gave the plotted parameter values, and the application of the F-test at one standard deviation confidence yielded the intervals shown by the continuous lines. Only the upper confidence limit as shown is relevant for β_3.

carried out for all the parameters involved in the fitting procedure, and 67% confidence limits (one standard deviation) have been determined. (The confidence level is approximate; for a nonlinear fitting equation like this one the true confidence level is unknown.) A strong asymmetry of the confidence interval about the minimum has often been found for β_2, the second overall Adair constant. The value shown below the best-fit value (found to be zero) of β_3 in Table I corresponds to the upper bound of that confidence interval. The upper bound for β_3 defined in this way far exceeded any actual best-fit value of experimental data. This suggested that this F-test was not appropriate for evaluating confidence limits for a negligible parameter. In order to explore this problem we generated a number of binding capacity data sets (25 points per set) using parameter values corresponding to those of the experiments and with the third overall constant zero. To the generated "data" points were added pseudorandom errors

Table II

(A) Cross Correlation Coefficients for the Overall Adair Constants Employed in the Data Fitting[a]

	β_1	β_2	β_3	β_4	ΔOD_T	case
β_1	1.000					A
β_2	−0.8189	1.000				
β_3	—	—	1.000			
β_4	0.9243	−0.7669	—	1.000		
ΔOD_T	0.4287	−0.0231	—	0.4260	1.000	
β_1	1.000					B
β_2	−0.6571	1.000				
β_3	—	—	1.000			
β_4	0.9702	−0.5809	—	1.000		
ΔOD_T	0.4339	0.1289	—	0.4332	1.000	
β_1	1.000					F
β_2	−0.1412	1.000				
β_3	—	—	1.000			
β_4	0.9192	−0.0344	—	1.000		
ΔOD_T	0.4824	0.4409	—	0.4925	1.000	
β_1	1.000					G
β_2	−0.8543	1.000				
β_3	—	—	1.000			
β_4	0.9282	−0.8103	—	1.000		
ΔOD_T	0.4589	−0.0949	—	0.4606	1.000	
β_1	1.000					H
β_2	−0.8537	1.000				
β_3	—	—	1.000			
β_4	0.9320	−0.8127	—	1.000		
ΔOD_T	0.5143	−0.1510	—	0.5201	1.000	

(B) Cross Correlation Coefficients for the Stepwise Adair Constants Employed in the Data Fitting

	κ_1	κ_2	κ_3	κ_4	ΔOD_T	case
κ_1	1.000					A
κ_2	−0.9738	1.000				
κ_3	0.7509	−0.8747	1.000			
κ_4	−0.7471	0.8718	−0.9999	1.000		
ΔOD_T	0.7296	−0.7357	0.7067	−0.7059	1.000	
κ_1	1.000					B
κ_2	−0.9740	1.000				
κ_3	0.7592	−0.8797	1.000			
κ_4	−0.7440	0.8680	−0.9997	1.000		
ΔOD_T	0.7215	−0.7246	0.6982	−0.6951	1.000	
κ_1	1.000					F
κ_2	−0.8960	1.000				
κ_3	0.6695	−0.9124	1.000			
κ_4	−0.6533	0.9022	−0.9997	1.000		
ΔOD_T	0.6950	−0.6558	0.6252	−0.6223	1.000	
κ_1	1.000					G
κ_2	−0.9788	1.000				
κ_3	0.7559	−0.8700	1.000			
κ_4	−0.7467	0.8629	−0.9999	1.000		
ΔOD_T	0.7246	−0.7332	0.7088	−0.7069	1.000	
κ_1	1.000					H
κ_2	−0.9368	1.000				
κ_3	0.7322	−0.9152	1.000			
κ_4	−0.7155	0.9046	−0.9997	1.000		
ΔOD_T	0.7357	−0.7281	0.6963	−0.6921	1.000	

[a] A bar indicates an undefined coefficient due to a zero parameter value.

following a Gaussian distribution about the true value. The results of fitting these simulations are summarized in Figure 3. As is evident from this plot, the F-test limits appear to accurately predict the distribution for β_1, β_2, and β_4, but seriously overestimate the breadth of the confidence interval for β_3, here by nearly a factor of 10. Since the third parameter was always found to be extremely small, a test of whether or not it is needed in the fit was made by using the F-test of an additional parameter. In every case this test showed that the

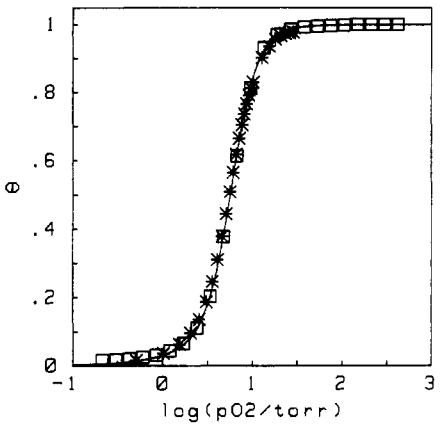

FIGURE 4: Fractional oxygen saturation of HbA_0 measured by the thin-layer technique in both deoxygenation (□) and reoxygenation (*) directions for sample J in Table I. The theoretical solid line curve was drawn by using the parameters obtained for condition J.

third overall constant was not needed to a confidence level not lower than 98%. By the same test, the second overall binding constant could be dropped only with a 7% confidence.

As mentioned, we found that the use of the overall Adair constants was essential for good convergence in the data fitting and resolution of the parameters. Presumably this is due to less parameter correlation when the problem is cast in terms of the overall constants rather than in terms of the intrinsic (or stepwise) constants. The correlations among the fitting parameters are presented for both cases in Table II by using the cross correlation coefficient (Magar, 1972), where typical fits from the five relevant conditions have been chosen. Ackers's group (Johnson et al., 1976) has found that a fitting parameter can nearly always be resolved from reasonably precise data provided the relevant cross correlation coefficients are less than about 0.98 (absolute value). As can be seen from Table II, when the fitting problem is cast in terms of the overall Adair constants, none of the cross correlation coefficients exceeds 0.98. However, when the fitting problem is cast in terms of intrinsic constants, the correlation is higher for all parameter pairs, as can be seen Table II; in particular κ_3 and κ_4 are so correlated that they cannot be resolved.[2]

Tests for Systematic Error. In our fitting procedure the equilibrium constant parameters were constrained to positive values, the physically meaningful case; removing that constraint resulted in a small negative fitted value for the third parameter in virtually all cases. The occurrence of a negative fitted value does not necessarily indicate a systematic error in the data, since β_3 fit to a negative value in well over half of the data sets that had been generated with pseudorandom error and small, positive β_3. Nevertheless, since in our experiments no positive value for β_3 was obtained in unconstrained fitting, we have been concerned over the possibilities of systematic errors that might have biased the results. With this in mind, we investigated our experimental procedures in several ways: (1) Since there is no systemic influence of high concentration (see cases B, C, and D in Table I) upon Adair constants, the possible influence of aggregation processes, including both dissociation to dimers or formation to higher aggregates, is unlikely. Below 1 mM heme for the same buffer conditions, the concentration does affect the Adair constants, due to dimer formation as observed by Ackers's group (Mills et al., 1976). (2) We believe that equilibrium is achieved in each step of an experiment; however, there is always a possibility of sample deterioration. Thus we felt that it was important to test if a reversal of the data-taking procedure would reveal any difference in the fitted parameters. As described under Materials and Methods, the reverse process consists of adding to the sample cell, after a complete deoxygenation experiment and nitrogen flush, small increments of O_2 gas so that the binding curve is traversed in the direction of increasing oxygenation. The values resulting from fitting the forward and the reverse runs are included in Table I, and the points from both runs are shown on the same plot in Figure 4. In this format the data points have been accumulated up to each pressure so as to depict a fractional saturation curve, in order to compare both the forward and the reverse experiments. From this test the experiments are reversible within the confidence intervals of the fitted parameters. (3) Determinations of optical density changes at several wavelengths, including regions where the change of the spectrum on oxygenation was positive and where it was negative, yielded essentially identical results. (4) In separate experiments, spectra of a thin-layer sample at different degrees of oxygenation were taken in the region of an isosbestic point (565 nm). It was found that an approximate isosbestic condition was maintained within the limits of experimental error of the instrument and the slight absorption drift of the sample layer with time. This supports the contention that the change in optical absorption with oxygenation is due solely to the fraction of oxygen bound independent of the particular species involved. This result is consistent with the findings of other investigators. (5) Experiments conducted with and without the enzymatic reducing system gave similar results with respect to the unmeasurable value of β_3, although the metHb content rose slightly in those without the reducing system. (6) Different sample preparations were used in the study with no significant variations. (7) Finally, a different CARY 219 spectrophotometer was employed in some of these determinations with no difference in the results.

Discussion

The thin-layer apparatus for differential binding measurements has not been used before for detailed study of the oxygen-binding reactions of the HbA_0 system. The present results contain some differences from previous studies. One feature of the methods employed here that could account for observed differences is the employment of high hemoglobin concentrations, which thus minimizes tetramer dissociation effects. Another is the use of differential absorbance measurements, taken between equilibrium states defined by precisely imposed oxygen partial pressures. Data taken as differential absorbances can be analyzed with one less parameter than that taken as simple absorbance values. In addition, any drift in the absorbance base line can be detected and corrected to the first order in the differential measurement procedure.

There have been a number of determinations of Adair constants for hemoglobin A_0 under a variety of solution conditions by Imai and his co-workers (Imai, 1982). Our results given in Table I in phosphate buffer (condition A) can be compared with theirs under similar pH and temperature values. The median activities, determined by β_4, are essentially the same; so are the values of the first association constant β_1. Likewise, the extensive determinations (Imai, 1982) at 0.1 M chloride at pH 7.4 yield fair agreement with our values of β_1 and β_4 under similar conditions (B, C, D). The concentration (60 μM) typically used in their studies apparently does not introduce significant deviations in these constants from

[2] Interestingly, we have found that fitting with the binding polynomial represented in terms of its roots results in even lower correlation among the fitting parameters, which are in this case real and complex coordinates of the roots in the complex plane.

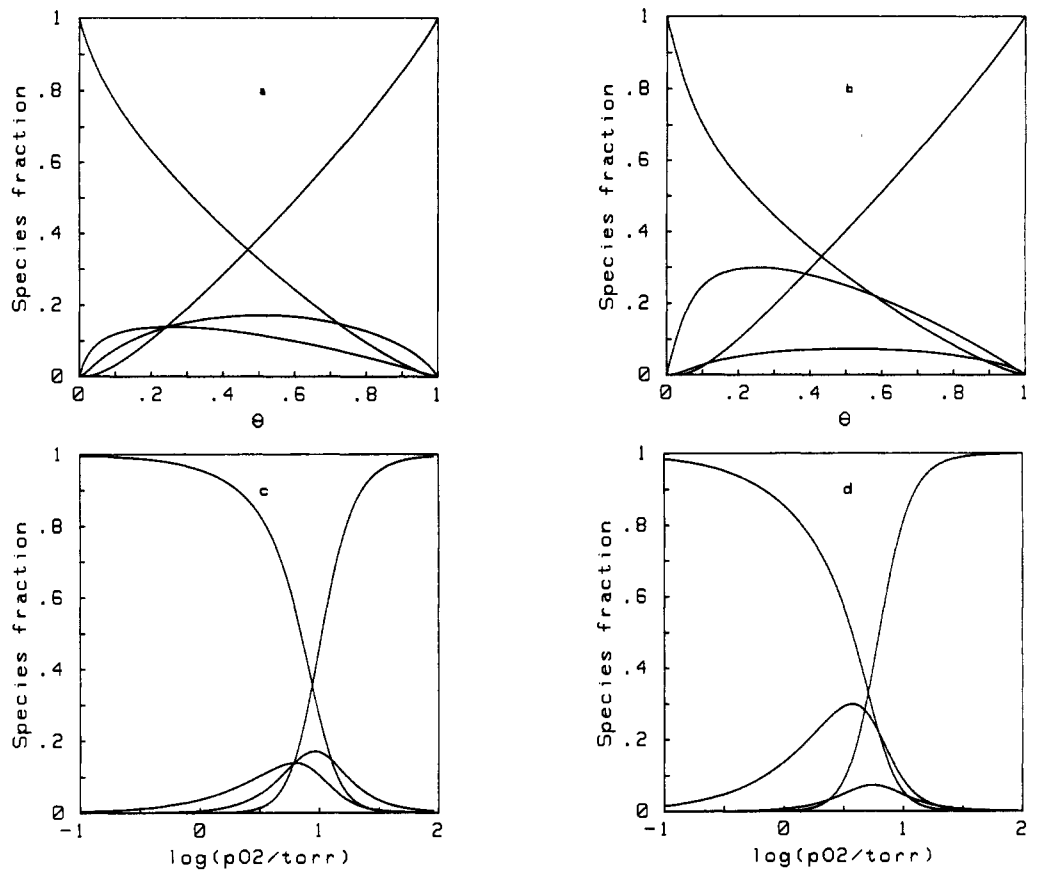

FIGURE 5: Species fractions of stoichiometric intermediates [Hb, $Hb(O_2)$, $Hb(O_2)_2$, $Hb(O_2)_3$, $Hb(O_2)_4$] for conditions A (a, c) and H (b, d) in Table I as a function of fractional oxygen saturation θ (top) and logarithm of oxygen partial pressure (bottom).

the high-concentration measurements. Indeed, Imai (1982) found that the effect of concentration was not very important above 100 μM in conditions similar to B. However, it is now well-known (Mills et al., 1976) that under Tris buffer conditions at hemoglobin concentrations in the range of 60 μM, significant dissociation of the tetramer to dimer forms occurs, particularly at high fractions of oxygenation. It is probably fortuitous that the median activity and the first binding constant are in agreement between our high-concentration experiments and the low-concentration determinations of the Imai laboratory.

The precise effect of the dimer–tetramer equilbrium upon the binding curve when analyzed in terms of the pure tetramer binding polynomial can be calculated from the measurements of Ackers and co-workers (Mills et al., 1976). In a simulation study, Johnson and Ackers (1977) showed graphically the magnitude of parameter determination errors that can develop as a result of neglecting the dimer–tetramer equilibrium at even moderately high heme concentrations. Significant errors can occur as the concentration is reduced to 1 mM and below, and the corrections are especially marked in the middle range of the binding curve. The simplification that arises with high-concentration measurements for the binding function is clear. Our median ligand activities are in reasonable agreement with those reported by Mills et al. (1976) and Chu et al. (1984). The error range at 67% confidence levels assigned to the Adair constants by Mills et al. (1976) allows the possibility of our findings.

In other studies (Chu et al., 1984; Mills & Ackers, 1979; Mills et al., 1976) the use of a series of binding determinations at low concentrations has provided an unprecedented degree of detail in elucidating the linkage of dissociation processes to ligand-binding equilibria by use of hemoglobin as a model system. Nevertheless, such studies necessitate the evaluation of several more thermodynamic parameters, with the consequence that the parameters of the lesser contributing species are difficult to resolve. The use of high concentrations in the present study reduces the number of parameters and, we feel, correspondingly improves their resolution.

It should not be overlooked that hemoglobin solutions will always contain various amounts of contaminating molecules, such as small and variable amounts of oxidized or chemically modified hemoglobin species. The error brought in to a study associated with less cooperative contaminating species will be largest at one end or the other of the binding curve. Thus it seems disadvantageous to weight the ends of a binding curve more than the middle, as was done by Roughton et al. (1955) and has been more recently advocated by Imai (1982). On the contrary, it is in the middle region of the binding curve where the greatest amount of information lies for determination of the Adair constants, due to the contributions of the various intermediates to experimental observations. Thus an argument might be made for preferentially weighting the middle region, but for convenience we have chosen to weight data points equally throughout the binding curve. This choice of weighting is in concurrence with the choice of Ackers (Johnson et al., 1976).

Perhaps an unsettling aspect of these results is that the last two stepwise oxygen-binding affinities of HbA_0 cannot be determined in spite of the increased resolution of the measured overall Adair constants. The free energy levels are well described for the first, second, and fourth state of ligation. It is only the third state that is undetermined. The consequence is that one cannot yet define where the Hill slope will effectively reach the asymptotic value of unity at high saturation. Of course, if the third species were truly absent, then the upper

asymptote of the Hill plot would have a slope of 2. In any case, the binding curve has a highly asymmetric nature, which has been suggested to confer an advantage of efficient oxygen delivery in the physiologically relevant region of the binding curve (Weber, 1982; Peller, 1982).

A plot of the various species fractions as a function of saturation, and perhaps more meaningfully as a function of the logarithm of the oxygen partial pressure, readily shows the relative contributions of the intermediates in the binding curve. Figure 5 illustrates such plots of our fitting results for the phosphate condition (A) and for the chloride condition used by Mills et al. (1976) (H, I). These figures show the importance of the first and second ligated species in determining the nature of the binding curve. The dominance of the singly and doubly ligated intermediate species plays a strong role in determining the asymmetry of the binding curve.

The indeterminably small population of triply ligated species requires an explanation in mechanistic terms. In the following paper we show that indeed this result can be rationalized in terms of structural features of hemoglobin observed by Perutz some 20 years ago (Perutz, 1970).

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Registry No. HbA_0, 54651-57-9; O_2, 7782-44-7.

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