Kinetic Studies on the Five Principal Components of Normal Adult Human Hemoglobin†

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ABSTRACT: The five principal components of human hemoglobin (A_{1a}, A_{1b}, A_{1c}, A_0, and A_2) have been isolated by column chromatography and by preparative isoelectric focusing in gels. The isoelectric points and a number of kinetic parameters have been determined for each hemoglobin. The greatest kinetic differences are found in the binding of CO to the deoxy conformation. At pH 7, A_0 and A_2 are nearly identical in their overall reaction with CO, whereas the initial lag phase characteristic of crude hemolysate and A_0 is greatly reduced in A_{1a} and A_{1c} and is essentially absent in A_{1b}. The general effect of *p*-mercuribenzoate binding on CO association is to magnify kinetic differences among the hemoglobins, diminish the initial lag phase, and increase the overall rate of CO binding. Hemoglobin A_{1a} is anomalous in that the overall CO binding rate actually decreases after reaction with the mercurial. In terms of an Adair model with four association constants the rate constant for the binding of the first molecule of CO (l_1') showed the greatest variation among the five hemoglobins, with A_0 having the smallest constant, and A_{1b} the largest. For the native hemoglobins, l_1' for A_{1b} was more than twice that for A_0; for the mercurated hemoglobins, the difference was greater than threefold. Raising the pH from 7 to 8 increases l_1' for all hemoglobins, but A_{1a} is anomalous in having a slower overall rate for CO binding at the higher pH. At pH 9, the time course of CO binding is biphasic for all hemoglobins, with A_0, the fastest, and A_{1a}, the slowest, differing by nearly threefold in rate. The equilibrium constant for the tetramer–dimer equilibrium was determined by flash photolysis. The largest dissociation constant occurs for A_{1a} and is 4.4 times that for A_0, and 5.6 times that for A_{1c}, the least dissociated of the hemoglobins. The overall oxygen dissociation reaction is biphasic for A_{1a} and A_{1b}, with the two phases differing by a factor of 5; the dissociation reactions for the other three hemoglobins appear essentially monophasic. The kinetics of dissociation of the first oxygen molecule from oxyhemoglobin are very similar for all five hemoglobins, as are the association kinetics for CN^- and N_3^- binding to the five methemoglobins.

The heterogeneity of hemoglobin present in hemolysates of normal adult red blood cells is well established. Multiple hemoglobin bands are demonstrable by electrophoresis (Kunkel and Wallenius, 1955), ion exchange chromatography (Schnek and Schroeder, 1961; Huisman and Horton, 1965), and isoelectric focusing (Drysdale et al., 1971). The number of different hemoglobin bands observed depends to some degree on the separation technique. However, there appears to be good correspondence between bands found chromatographically, using Amberlite type cation exchangers, and bands produced by isoelectric focusing (IEF).[1] At least five distinct hemoglobins are present in fresh red cell hemolysate. In order of elution from Amberlite IRC-50 columns, these have been designated A_{1a}, A_{1b}, A_{1c}, A_0, and A_2. The main component, hemoglobin A_0, comprises 85–90% of the total. The approximate abundance in red cell hemolysate of the other (minor) components is: A_{1a} and A_{1b} combined, 2.5%; A_{1c}, 6%; A_2, 2.5% (Schnek and Schroeder, 1961; Huisman and Horton, 1965). In addition, the production of small amounts of fetal hemoglobin persists into adulthood, so that hemoglobins F_1 and F_0 may be detectable in normal adult bloods.

Quantities of minor hemoglobin components formed are apparently under metabolic control and can be influenced by various disease states. Hemoglobins A_{1a}, A_{1b}, and A_{1c} have been reported to be present in increased amounts in patients with myologenous leukemias, and in decreased quantities in patients with hemolytic anemias (Horton and Huisman, 1965). A twofold increase in the level of hemoglobin A_{1c} was found in individuals with diabetes mellitus (Rahbar et al., 1969). The increased production of hemoglobin A_2 resulting in cases of β-thalassemia is well known (Weatherall, 1968).

Hemoglobin A_0 corresponds to the familiar molecule $\alpha_2\beta_2$, whose structural and functional properties have been widely studied. Studies concerning the source, structure, and significance of the minor hemoglobin components are inherently restricted by their more limited availability. Hemoglobins A_{1a} and A_{1b} appear to be identical in amino acid composition with A_0 (Schroeder and Holmquist, 1968), but no further information about them is available. Hemoglobin A_{1c} contains two α and two β chains identical in amino acid sequence to those of A_0. However, in A_{1c} the N-terminal valine residues of the β chains are blocked by Schiff base linkage to a hexose (Holmquist and Schroeder, 1966; Bookchin and Gallop, 1968). The only component known to differ in primary structure from A_0 is hemoglobin A_2, which corresponds to the molecule $\alpha_2\delta_2$. The amino acid sequence of a δ chain differs from that of a β chain in 10 positions (Jones, 1964). Components A_0, A_{1c}, and A_2 are reported to have similar spectroscopic properties in both the

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[1] Abbreviations used are: IEF, isoelectric focusing electrophoresis using carrier ampholytes; PMB, *p*-mercuribenzoate; Hb^+, ferrihemoglobin.

ultraviolet and visible regions, as well as similar molecular weights (Kunkel et al., 1957; Holmquist and Schroeder, 1966).

Although considerable information about the minor hemoglobins has accumulated, investigations regarding their functional properties and physiological significance have been rather limited. In evaluating possible roles of the minor hemoglobins, a detailed knowledge of their ligand binding properties is of critical importance. The general importance of kinetic information in interpreting physiological data has also been stressed (Eliot et al., 1970). An investigation of the kinetic properties of the minor hemoglobins appeared to be of clinical interest because of the known variation in quantity of the components concurrent with a variety of pathological states. Furthermore, such studies offered interesting opportunities to examine the effects of protein structure on functional properties; for example, in the case of hemoglobin A_{1c}, molecular structure is altered by the introduction of a specific blocking group. Previous kinetic studies on normal human hemoglobin have generally been carried out on unfractionated hemolysate. The lack of information as to the values of the rate constants for the minor hemoglobins makes uncertain the assignment of the finer details in the ligand-binding kinetics for human hemoglobin. Thus, a detailed kinetic examination of the minor hemoglobins isolated by cation exchange chromatography and preparative scale isoelectric focusing was undertaken. Their reactions with a variety of ligand molecules were observed, including studies on ferric, as well as ferrous, hemoglobin derivatives. Results were compared with those for the main component, A_0, and unfractionated "stripped" hemoglobin.

Experimental Section

Materials. Human oxyhemoglobin was prepared from freshly drawn blood, as described below. The cation exchange resin Bio-Rex 70 used in column chromatography was purchased from Bio-Rad Laboratories, Richmond, Calif. Ultrafiltration membranes used in concentration of fractions from column chromatography were obtained from Amicon Corporation, Lexington, Mass. Carrier ampholines, pH range 6–8, for isoelectric focusing were purchased from LKB Instruments, Inc., Rockville, Md. Sephadex was purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Carbon monoxide and argon, chemically pure, were products of the Matheson Co., Inc., East Rutherford, N.J. Sodium dithionite, Manox Brand, was obtained from Hardman and Holden, Ltd., Miles Platting, Manchester, England. Buffers used in kinetic studies were 0.05 *M* potassium phosphate at pH 7.0 and 8.0, and 0.05 *M* borate at pH 9.0. Hemoglobin samples were transferred to the desired buffer by passage over G-25 fine Sephadex columns equilibrated with that buffer. Any other chemicals used were reagent grade and were used without further purification.

Hemoglobin. Human oxyhemoglobin was prepared from freshly drawn blood collected in tubes containing EDTA as anticoagulant. Blood samples were drawn from normal healthy adults. Each hemoglobin preparation used samples collected from 10 to 20 individuals, and all experiments were performed on hemoglobin pools. Plasma was removed by centrifugation and the cells were washed four times with cold 0.15 *M* NaCl. Red cell hemolysates were prepared essentially as described by Geraci et al. (1969), by adding one to two volumes of deionized water per volume of packed cells, incubating 20 min at room temperature with occasional stirring, adding a small amount of 2.8 *M* phosphate buffer (pH 6.8) to aid in removal of cell debris, and centrifuging at 25,000*g* for 15 min. If the hemoglobin was to be "stripped", a small amount of 1 *M* NaCl–0.1 *M* bis-tris (pH 7.2) was substituted for the 2.8 *M* phosphate. Stripping of organic phosphate was performed as described by Berman et al. (1971). For analysis of phosphate content of hemoglobin samples the sensitive colorimetric procedure described by Ames and Dubin (1960) was used.

Fractionation by Chromatography. Chromatographic fractionation of hemoglobin was performed essentially as described by Schnek and Schroeder (1961), using the cation exchange resin Bio-Rex 70 (300 mesh). After batchwise preequilibration of the resin with eluting buffer, columns were poured in a single continuous segment from a stirred reservoir. Dimensions for preparative scale columns were 2.5 × 40 cm (resin bed). Since separations with this resin are quite temperature sensitive, jacketed glass columns were used, and the temperature was controlled by circulating water from a Lauda K-2/R temperature bath (Brinkman Instruments). Final equilibration of columns with eluting buffer was carried out at the initial running temperature, 5°. Equilibration was judged complete when the pH and conductivity of the effluent matched that of the initial buffer, which usually required several days, and 1–2 l. of buffer. Prior to chromatography, hemoglobin solutions were dialyzed against eluting buffer. Columns were loaded with 0.8–1.0 g of hemoglobin, and runs were monitored at 418 nm. Elution was performed at constant temperature, pH, and ionic strength (with flow rates of 16–20 ml/hr) until bands A_{1a}, A_{1b}, and A_{1c} were completely eluted. Then flow was stopped, and the columns were warmed to approximately 23° by circulating water at room temperature through the jackets. After 30 min flow was resumed and the major band A_0 was eluted. Resolution of bands A_{1a}, A_{1b}, and A_{1c} was obtained using developer 4 of Clegg and Schroeder (1959) as the elution buffer. This contains 0.04 *M* phosphate, 0.05 *M* Na^+, and 0.01 *M* KCN at pH 6.9. It was also found possible to isolate hemoglobin A_2 from Bio-Rex columns. After a few hours elution at 5° with developer 4, the bulk of the sample applied travels some distance down the column. However, because of its much higher p*I*, hemoglobin A_2 remains in the top 1 cm of the resin bed. To collect the A_2, elution is briefly halted while the top 1 cm of resin is removed from the column. The hemoglobin A_2 is eluted from the resin batchwise by adding a small volume of 0.1 *M* pH 7.0 phosphate buffer, followed by centrifugation. In all kinetic studies using hemoglobin A_2 so obtained, results were identical with those obtained with A_2 isolated by isoelectric focusing. Concentration of hemoglobin containing fractions from Bio-Rex columns was achieved either by ultrafiltration, using the Amicon UM-10 membrane, or by CM-cellulose, as described by Huisman and Meyering (1960).

Fractionation by Isoelectric Focusing. Preparative IEF in layers of Sephadex G-100 was performed using a technique similar to that applied to sperm whale myoglobin (LaGow and Parkhurst, 1972; Radola, 1971). The apparatus consisted of a 20 cm × 20 cm glass plate fitted at the edges with Lucite walls, forming a trough 1.5 cm deep. Electrodes were two platinum wires affixed near the edges at opposite sides of the plate, and held approximately 2 mm above the glass. For small scale runs or treatment of more than one sample the trough width was adjustable by insertion of Lucite dividers. All runs were made at 5° in a humi-

dified closed chamber. Power was supplied by a Kepko ABC-10 dc power supply, modified for current readout. For an IEF run, the desired amount of swollen Sephadex G-100 was washed with deionized water. After settling for about 1 hr in a large graduate, excess water was removed. The appropriate volume of 40% (w/v) carrier ampholines was mixed with the gel, and the resulting slurry was poured evenly onto the IEF plate and air-dried until the mass of the Sephadex layer had decreased by about 20%. Depth of the gel was usually about 0.5 cm. Final concentration (w/v) of the carrier ampholines was 1-3%, based on the weight of ampholines (dry weight in the 40% solution) divided by the final volume of the air-dried gel layer. It was found that 1 g of ampholines/100 mg of total hemoglobin was required for satisfactory focussing of the five hemoglobins. Just prior to starting the run, approximately 0.2 ml of anolyte and catholyte (2% phosphoric acid and 2% ethylenediamine, respectively) were applied across the gel surface immediately behind and parallel to the platinum electrodes. Hemoglobin samples to be fractionated by IEF were dialyzed overnight at 5° against 50-100 volumes of deionized water to reduce the concentration of buffer salts. Before addition of sample, the IEF gel was run for about 2 hr at 200 V to establish a preliminary pH gradient. Power was then briefly shut off, and the hemoglobin sample was gently applied across the central portion of the gel, using a Pasteur pipet. The run was then resumed at voltages of 200-250 V, but power dissipation was not allowed to exceed 1 W. Voltages above 250 V were not used, since this rulted in partial oxidation of hemoglobin. Runs were completed within about 18-20 hr after addition of sample. In runs in which the sample was red cell hemolysate, large amounts of ampholines were required for focusing the A_0 band, which comprises most of the total hemoglobin. When collection of the acidic bands A_{1a}, A_{1b}, and A_{1c} was of primary interest, it was found more economical to use a hybrid technique in which hemolysate was prefractionated. For this purpose, hemolysate was applied to a Bio-Rex 70 column at 5° equilibrated with pH 7.1 0.04 *M* sodium phosphate (no cyanide). Within 1-2 hr the rapidly moving acidic bands eluted as a single peak containing A_{1a}, A_{1b}, A_{1c}, and a small amount of A_0. The column treatment resulted in no detectable methemoglobin formation. This mixture, greatly enriched in the percentage of acidic hemoglobins, was then fractionated by isoelectric focusing.

pI Measurements and Collection of Bands. When focusing was completed, the power was disconnected, and isoelectric points of the oxyhemoglobin bands were measured directly in the gel layer, still at the running temperature. For all pH measurements, an Instrumentation Laboratory pH meter, Model 245, equipped with a combination microelectrode (Model 14043) was employed. Bands were then excised with a small spatula, and the gel sections corresponding to the various bands were stored in small test tubes. For each band, removal of carrier ampholines and IEF running gel was achieved by transfer of the gel section to a 0.9 × 20 cm Sephadex G-25 column and elution with the desired buffer. Further concentration of samples was unnecessary, since during IEF bands become more concentrated, not more dilute, as in chromatography.

Kinetic Measurements. The stopped-flow and flash photolysis devices used were both constructed in this laboratory. The stopped-flow has been previously described (Boelts and Parkhurst, 1971). The flash photolysis unit has power output variable up to 200 J, and a flash decay half-time of 3 μsec at full power. The sample cuvet is contained in a thermostated brass block. The temperature is controlled by circulating water from a Lauda temperature bath with regulation to 0.01°. Temperature at the sample is measured by a calibrated thermistor. The light source for the observing beam is a 150-W xenon arc lamp run by a precision current regulated power supply (DeSa, 1970). Light from the arc is passed through a Heath EU-700/E scanning monochromator before incidence on the sample. To minimize stray light effects, the sample compartment is also backed with appropriate interference filters and/or a Schoeffel GM 100 miniature grating monochromator. For both stopped-flow and flash photolysis devices, the detection unit consists of a photomultiplier (EMI 9592B) and appropriate amplifier circuitry. On-line data acquisition and processing are provided by a Data General Supernova minicomputer with 20,000 words of core memory. Routine operation includes collection and processing of up to 200 raw data points/kinetic curve. In addition the raw data can be stored in core or on tape, allowing subsequent treatment by various smoothing techniques and/or curve fitting routines, if desired.

Carbon Monoxide Binding. The combination reaction of CO with the various hemoglobins was measured by both stopped-flow and flash photolysis. For stopped-flow studies, oxyhemoglobin samples were diluted in oxygen-free buffer, and converted to the deoxy- derivative by the addition of a few crystals of $Na_2S_2O_4$. Any cyanomethemoglobin present in samples fractionated on Bio-Rex columns is also reduced under these conditions (Antonini and Brunori, 1971), yielding deoxyhemoglobin. Deoxyhemoglobin was then flowed against oxygen-free buffer of known CO concentration under pseudo-first-order conditions. Results at pH 7 and pH 8 were treated in terms of a four-step sequential model for ligand binding (Gibson and Roughton, 1957). Data were analyzed by a non-linear least-squares procedure to find rate constants producing the best fit to the kinetic curves. Analysis was performed by computer, employing an algorithm of the Gauss-Newton type (Draper and Smith, 1966). For flash photolysis, carboxyhemoglobin solutions were prepared by dilution of samples with oxygen-free buffer of known CO concentration, followed by removal of residual oxygen with dithionite. For studies on rapidly reacting hemoglobin produced by partial photodissociation or sufficient dilution of carboxyhemoglobin, the rapid and slow kinetic components were analyzed in terms of a sum of two exponentials. Computer analysis yielded both second-order rate constants, and the relative proportion of rapid and slow components. The apparent dissociation constant, $K_{T,D}$, for the tetramer-dimer equilibrium of ligand bound hemoglobin was evaluated from flash photolysis data as described by Edelstein et al. (1970).

Oxygen Dissociation. For studies on isolated hemoglobins, only samples fractionated by isoelectric focusing were used. Absorption spectra on such samples at pH 7.0 showed no detectable methemoglobin peak at 630 nm, and the ratio of heights for the oxy- peaks at 576 and 541 nm was the same as for the unfractionated starting material. Stopped-flow measurements of the rate of oxygen dissociation were made by flowing oxyhemoglobin solutions against 0.2% sodium dithionite dissolved in oxygen-free buffers.

Cyanide and Azide Binding. Stopped-flow studies on cyanide binding and azide binding by methemoglobin samples were performed under pseudo-first-order conditions, as described by Gibson et al. (1969). Oxidation of oxyhemoglobin to methemoglobin was accomplished by addition of 5

equiv of $K_3Fe(CN)_6$ (heme basis) and incubation at room temperature for several minutes. Excess ferricyanide was removed on a G-25 Sephadex column, previously equilibrated with 0.05 *M* potassium phosphate buffer. Methemoglobin used in kinetic experiments was always freshly prepared and used the same day. Hemoglobin bands isolated using Bio-Rex columns were first treated to remove any cyanomethemoglobin present. This was done by reduction with dithionite in oxygen-free buffer containing excess carbon monoxide. After passage over Sephadex G-25, the hemoglobin was oxidized with ferricyanide as described above.

Results

Using fresh red cell hemolysate as the starting material, five individual hemoglobin bands have been isolated on a preparative scale, using column chromatography, isoelectric focusing, or a combination thereof. The purified components, listed in order of elution from Bio-Rex 70 are A_{1a}, A_{1b}, A_{1c}, A_0, and A_2. Isoelectric focusing in Sephadex gels gave separation of the components in the same order, with A_{1a} nearest the anode, as also found with analytical IEF in polyacrylamide gels (Drysdale et al., 1971). When unstripped red cell hemolysate was fractionated by IEF as described, the phosphate content of the resulting hemoglobin was reduced to the same low level as that attainable by stripping. Phosphate analysis by the method of Ames and Dubin (1960) showed that after IEF, residual organic phosphate amounted to <3% of that present in the initial hemolysate. It should be noted that the dialysis prior to IEF reduced organic phosphate levels by about 50%.

Spectroscopic examination of the five oxyhemoglobin bands obtained by IEF yielded virtually identical spectra in the visible region between 500 and 650 nm. In all cases peaks were located at 576 and 541 nm, and the ratio of heights of the two peaks was approximately 1.06, the same value as that routinely observed for unfractionated red cell hemolysate. Partial oxidation of hemoglobin during analytical IEF in acrylamide gel has been reported (Bunn and Drysdale, 1971). Resulting species, in which one-half the hemes were apparently oxidized, were focused in the pH gradient as distinct bands closer to the cathode than the parent band. However, these authors employed as standard running conditions a voltage of about 500 V (50 V/cm). At the lower voltages and voltage gradients used in the present studies, extraneous bands due to partial oxidation were not produced.

Measurements of the p*I* for oxyhemoglobin bands formed by IEF were performed directly in the gel layer at the running temperature, 5°. The averages of p*I* measurements on four different IEF runs gave the following results: A_{1a}, 6.80; A_{1b}, 6.95; A_{1c}, 7.06; A_0, 7.18; A_2, 7.60. Standard deviation of the p*I* values is about ±0.03. In addition, traces of indistinctly focused material in the pH range 6.5-6.7 were occasionally visible but were not collected. Kinetic properties of the isolated hemoglobin components were quite reproducible from one preparation to another. Furthermore, there was no evidence of variation in kinetic properties depending on whether separation was by column chromatography, IEF, or a combination of the two.

CO Binding Kinetics. Dimer-Tetramer Equilibrium. Stopped-flow studies on CO binding at pH 7 showed significant differences in the time course of the reaction for the isolated hemoglobin components. Greatest differences from the main component A_0 were found in the behavior of components A_{1a}, A_{1b}, and A_{1c} during the initial portion of the reaction, as shown in Figure 1. The reaction curve for A_0 shows the characteristic lag phase, or autocatalytic effect, well known in the reaction of hemoglobin with CO. This lag phase is reduced for A_{1a}, A_{1b}, and A_{1c}. Aside from some slowing during the latter stages, the time course of the reaction with hemoglobin A_2 was similar to that of A_0, and is not shown in the figure. To allow a more quantitative comparison of the kinetic properties of the various components, data were treated in terms of a four-step sequential model for the CO binding (Gibson and Roughton, 1957). The stepwise kinetic constants are designated l_1', l_2', l_3', and l_4'. As a result of a change from the deoxy to the ligand bound conformation, binding of the final ligand molecule is considered to proceed much more rapidly than the first three. For such a sequential process and with l_4' much greater than l_1', it is not possible to evaluate accurately l_4' from the stopped-flow data; however, an estimate of the value of l_4' is available from the rapid recombination rate constant (l'^*) derived from flash photolysis studies. Combined data for the two methods yielded the values given in Table I. In the curve fitting method used to evaluate l_1', l_2', and l_3', it made little difference if the value of l_4' measured by flash were inserted or if l_4' were simply assumed to be infinite. Rate constants given in Table I produced excellent fits to the stopped-flow curves with root mean square residuals amounting on the average to 0.25% in percent saturation. Estimates of the standard errors in the variable parameters l_1', l_2', and l_3' were obtained from the variance-covariance matrix of the parameters (Draper and Smith, 1966).[2] These amounted to approximately ±2% of the listed values for l_1' and l_3' and ±5% for the listed values of l_2'.

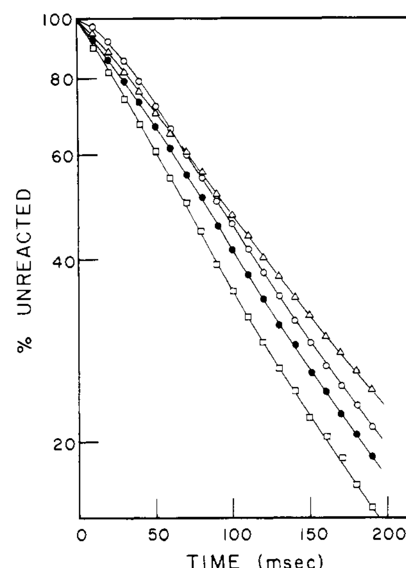

FIGURE 1: Stopped-flow measurements of CO binding at pH 7 by isolated human hemoglobins, performed in 0.05 *M* potassium phosphate at 20°. Concentrations after mixing were 5 μM hemoglobin (heme basis) and 45 μM CO. Observing wavelength was 430 nm. Reaction time course for hemoglobin A_2 is similar to A_0 and is not shown. (△) A_{1a}, (□) A_{1b}, (●) A_{1c}, (O) A_0.

[2] Unpublished results in this laboratory (D. Flamig and L. Parkhurst), based on extensive computer simulation studies of exponential decay processes with added Gaussion-distributed random noise, suggest that the actual standard deviations of the parameters are about 1.5 times the values obtained from the variance-covariance matrix. The standard deviations for rate constants for biphasic reactions are all approximately 5% of the value of the rate constant.

Table I:[a] Best Fitting Rate Constants for CO Binding at pH 7 by Isolated Human Hemoglobin Components.

Hemoglobin Component	Rate Constants (M^{-1} sec^{-1}) × 10^{-5}				
	$1_1'$	$1_2'$	$1_3'$	$1'^*$	$1'$
A_0	0.99	2.49	1.11	46	1.48
A_0-PMB	1.25	3.83	1.20		
A_{1a}	1.48	2.13	0.89	51	1.97
A_{1a}-PMB	1.63	1.42	0.75		
A_{1b}	2.18	2.33	1.03	44	2.00
A_{1b}-PMB	4.06	2.40	1.23		
A_{1c}	1.37	2.81	1.01	51	1.70
A_{1c}-PMB	2.20	2.56	1.16		
A_2	1.25	2.45	0.86	47	1.52
A_2-PMB	1.79	2.94	1.22		

[a] Values of $1_1'$, $1_2'$, and $1_3'$ were derived from stopped-flow experiments performed in 0.05 M potassium phosphate (pH 7.0) at 20°; concentration of reactants before mixing was 10 μM hemoglobin (heme basis) and 90 μM CO. The observing wavelength was 430 nm. Rate constants $1'^*$ and $1'$ were obtained from flash photolysis experiments in the same buffer. Conditions were 10 μM hemoglobin and 89 μM CO, 20°, and 440 nm. Values listed are intrinsic rate constants and do not contain a statistical factor. Samples designated —PMB have p-mercuribenzoate bound to the β-93 sulfhydryl groups of the hemoglobin.

Table II: Dissociation Constants for Tetramer–Dimer Equilibrium in CO Bound Hemoglobin Components, pH 7.[a]

Component	$K_{T,D}$ (μM)
A_0	3.6
A_{1a}	15.7
A_{1b}	7.6
A_{1c}	2.8
A_2	3.8

[a] Experimental conditions are those described for flash photolysis data in Table I.

Table III: Best Fitting Rate Constants for CO Binding at pH 8.[a]

Hemoglobin Component	Rate Constants (M^{-1} sec^{-1}) × 10^{-5}		
	$1_1'$	$1_2'$	$1_3'$
A_0	2.72	5.11	1.32
A_{1a}	1.71	2.09	0.53
A_{1b}	3.35	3.63	1.19
A_{1c}	2.82	4.01	1.24
A_2	2.73	5.03	1.39

[a] Experimental conditions were 0.05 M potassium phosphate buffer (pH 8.0), 10 μM hemoglobin, and 90 μM CO (before mixing), 430 nm, and 20°. Rate constants were evaluated as described in the text.

The observed deviant behavior of A_{1a}, A_{1b}, and A_{1c} is accounted for primarily by the values of $1_1'$, which range from about 1.4 to 2.2 times the corresponding value for A_0. Rate constants designated $1'$ in Table I refer to the slow recombination rate observed in flash photolysis. For unfractionated hemoglobin in phosphate buffer, $1'$ is about 1.5×10^5 M^{-1} sec^{-1} (Edelstein et al., 1970). Included in Table I are values for $1_1'$, $1_2'$, and $1_3'$ obtained for hemoglobins which had been treated with p-mercuribenzoate (PMB). Samples were treated with excess PMB in the ratio 2 mol of PMB/β-93-SH, and excess mercurial was removed by passage over Sephadex G-25 previously equilibrated with 0.05 M potassium phosphate (pH 7.0). Treatment of A_0 with PMB resulted in a reduced lag phase, in accord with previous observations on unfractionated hemoglobin (Taylor et al., 1966). The PMB treatment enhanced kinetic differences between minor components and the main component. Increased rates were noted with A_{1b}, A_{1c}, and A_2, and the relative change was greater than that observed for A_0. Surprisingly, PMB treatment of A_{1a} produced an overall decrease in the rate of CO binding. For hemoglobins A_{1c} and A_0, kinetics of the PMB reaction at the β-93-SH groups was directly measured by stopped-flow. Measurements employed oxyhemoglobin derivatives in 0.05 M phosphate buffer at pH 7. Within experimental error, results for A_{1c} and A_0 were the same, yielding a second-order rate constant of about 5×10^6 M^{-1} sec^{-1} for the combination with PMB, in agreement with results for unfractionated hemoglobin in the same buffer (Geraci and Parkhurst, 1973).

A reversible tetramer–dimer equilibrium becomes readily apparent when liganded hemoglobin is diluted to concentrations of 10 μM or lower. Flash photolysis studies on carboxyhemoglobin solutions in this concentration region show considerable amounts of rapidly reacting hemoglobin even when photodissociation is complete. Under such conditions, the proportion of rapid component observed kinetically has been correlated with the fraction of dimeric hemoglobin present (Kellett and Gutfreund, 1970; Edelstein et al., 1970). Thus, apparent values of the dissociation constant, $K_{T,D}$, for the tetramer–dimer equilibrium can be calculated from flash photolysis data. The values obtained for $K_{T,D}$ at pH 7 for the five isolated hemoglobins are given in Table II. Values were calculated using the relationship $K_{T,D} = [H](R^2/(1-R))$, where [H] is the total hemoglobin concentration on a heme basis and R is the fraction of rapidly reacting hemoglobin. Experiments on samples of unfractionated hemoglobin under the same conditions gave $K_{T,D} = (3.5 \pm 0.5)$ μM. Results for A_0, A_{1c}, and A_2 are in the same region as for unfractionated hemoglobin. However, greater dissociation to dimer is evident with A_{1a} and A_{1b}.

The time course of carbon monoxide binding at pH 8 is rather similar for A_{1b}, A_{1c}, A_0, and A_2, as reflected in values of the rate constants given in Table III. However, for hemoglobin A_{1a} the time required for the reaction to reach 50% completion is twice that for the other components. In the reaction of carbon monoxide with A_0 at pH 9, no lag phase is observed. Rather a continuous deceleration is found, as previously observed for unfractionated hemoglobin in the same buffer (Gray and Gibson, 1971). This behavior is apparently not related to dissociation into dimer subunits. For purposes of numerical comparison, the kinetic heterogeneity was treated in terms of two independent exponential processes. Kinetic constants thus obtained are given in Table IV, along with the proportion of the overall absorbance change contributed by the rapid phase of the reaction. Hemoglobins A_0 and A_2 reacted identically, giving virtually the same results as unfractionated hemoglobin. Reaction with A_{1b} was somewhat slower overall, with the difference mainly attributable to the slow rate constant. Results for A_{1a} and A_{1c} were nearly identical, but differed considerably from those for the other hemoglobin components. Values for both the rapid and slow rate constants are two- to threefold lower than the corresponding constants for A_0. Also given in Table IV are results for flash photolysis studies at pH 9 on A_0 and A_{1c}. The kinetic differences observed by stopped-flow are also evident in the flash experiments carried out at 10 μM in heme.

Table IV: Rate Constants for Carbon Monoxide Binding at pH 9 by Isolated Human Hemoglobin Components.

Hemoglobin Component	Rate Constants (M^{-1} sec^{-1}) × 10^{-5}				
	Stopped-Flow[a]			Flash Photolysis	
	Slow Rate	Rapid Rate	% Rapid	1′	1′*
A_0	4.5	15	37	4.8	76
A_{1a}	1.8	5.1	28		
A_{1b}	2.9	14	29		
A_{1c}	1.8	5.4	33	2.5	58
A_2	4.4	15	37		

[a] Stopped-flow measurements were conducted in 0.05 M borate buffer (pH 9.0) at 20°. Concentrations of reactants before mixing were 10 μM hemoglobin and 88 μM CO. The observing wavelength was 430 nm. For flash photolysis studies the conditions were: 10 μM hemoglobin, 92 μM CO, and 440 nm.

Table V: Rate Constants for Deoxygenation Kinetics of Isolated Hemoglobin Components.[a]

Hemoglobin Component	k (pH 7) Slow	k (pH 7) Fast	k_α^* (pH 7)	k_β^* (pH 7)	k_α^* (pH 9)	k_β^* (pH 9)
A_0	15.6		7.3	34.5	5.2	23.7
A_{1a}	9.6	45.6	6.9	36.4	4.8	31.1
A_{1b}	8.6	45.6	7.4	41.1	5.0	27.3
A_{1c}	14.2		7.3	37.3	5.3	22.2
A_2	16.6		7.6	33.2	7.2	29.2

[a] Units for rate constants are sec^{-1}. Stopped-flow measurements at pH 7 were in 0.05 M phosphate and at pH 9 were in 0.05 M borate. Other conditions were 10 μM hemoglobin (before mixing), 20°, wavelength of the observing beam 430 nm for measurements of k, and 424 nm for measurements of k^*. In measurements of k at pH 7, hemoglobins A_{1a} and A_{1b} showed two distinct kinetic components, which comprised respectively 48 and 40% of the total reaction.

Oxygen Dissociation. Because of the high values of $K_{T,D}$ found for A_{1a} and A_{1b}, deoxygenation kinetics could not be performed under conditions where these components would be predominantly tetrameric. Comparison of all the isolated components was made at a concentration of 10 μM in heme before mixing. Results of oxygen dissociation studies are given in Table V. At pH 7, deoxygenation curves for A_0, A_{1c}, and A_2 (Figure 2) were closely first order between 90 and 30% saturation. Values of the dissociation rate constant k for these components were in the range 14–16, similar to the results with unfractionated hemoglobin. In contrast, reaction curves for A_{1a} and A_{1b} were markedly biphasic and were analyzed in terms of two kinetic components. In each case, the rapid and slow rate constants differed by approximately a factor of 5, and the rapid phase contributed 40–50% to the total reaction observed. The results for A_{1a} and A_{1b} do not appear explainable solely in terms of greater dissociation into dimers. Oxygen dissociation from A_0 comparably dissociated into dimers (i.e., at a concentration of 2 μM) does not show two kinetic components. Studies of oxygen dissociation performed on A_0 and A_2 at an initial concentration of 60 μM again yielded similar results for the two hemoglobins. First-order rate constants of about 35 sec^{-1} were found for both.

When deoxygenation is studied in the presence of concentrated carbon monoxide, heme sites left vacant by O_2 dissociation are quickly filled by CO, and the hemoglobin is thus maintained in the ligand bound conformation throughout the reaction. The rate observed will be characteristic of dissociation of the first O_2 molecule from oxyhemoglobin. Deoxygenation performed in the presence of CO gave biphasic curves for all isolated hemoglobins (Table V). It has been reported that kinetic heterogeneity in this reaction is due to intrinsic differences between α and β chains, with the β chain releasing oxygen more rapidly (Olson et al., 1971). In Table V, the notation k_α^* and k_β^* tentatively identifies the slow and rapid rate constants, respectively, as due to the α and β chains. The asterisk is used to indicate that the reaction considered pertains to a ligand-bound form. Experiments on unfractionated hemoglobin at a concentration of 80 μM (before mixing in the stopped-flow) were also performed at pH 7 in phosphate buffer. They yielded k_α^* = 11.3 sec^{-1}, k_β^* = 28.4 sec^{-1}, and k_β^*/k_α^* = 2.5. Using the same hemoglobin diluted to a concentration of 10 μM (before mixing), the results were k_α^* = 8.3 sec^{-1}, k_β^* = 35.0 sec^{-1}, and k_β^*/k_α^* = 4.2., similar to the results in Table V for isolated hemoglobins. It appears that inherent α and β

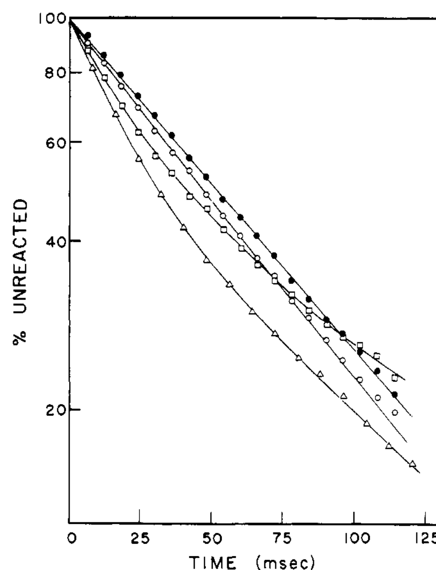

FIGURE 2: Deoxygenation kinetics for isolated human hemoglobins in 0.05 M potassium phosphate at pH 7. Other conditions were 10 μM hemoglobin (before mixing), 20°, and 430 nm. (△) A_{1a}, (□) A_{1b}, (●) A_{1c}, (O) A_0.

chain differences may be magnified by dilution of the hemoglobin to a concentration at which dimer formation becomes significant. At pH 9, values of k_α^* and k_β^* decreased for all hemoglobin components, but the ratio k_β^*/k_α^* remained similar to that observed at pH 7.

Methemoglobin Kinetics. From X-ray studies it is known that methemoglobin is similar in quaternary structure to oxy- and carboxyhemoglobin. Even after binding the large triatomic ligand N_3^-, no change in quaternary structure is evident for the azide methemoglobin complex (Perutz and Mathews, 1966). Thus, ligand binding studies of methemoglobin are probes of the ligand-bound conformation. In the present studies, stopped-flow experiments on cyanide and azide binding have been performed for the ferric derivatives of the five isolated hemoglobin bands. The results are summarized in Table VI. In control experiments using methemoglobin prepared from unfractionated hemoglobin (abbreviated Hb^+), results were indistinguishable from those for A_0. For the cyanide binding reaction, rate constants for the five isolated hemoglobins were substantially the same. Variation of the hemoglobin concentration within the range

Table VI: Rate Constants for Reactions of Ferrihemoglobins with Ligands, pH 7.[a]

Hemoglobin Component	Cyanide Binding	Rate Constants (M^{-1} sec^{-1}) Azide Binding α Chain	β Chain
A_0	145	76	850
A_{1a}	170	96	875
A_{1b}	145	79	810
A_{1c}	160	88	805
A_2	150	83	825

[a] Measurements were made in 0.05 M phosphate buffers. Cyanide binding was studied at pH 7.0 and azide binding at pH 6.93. The temperature was 20° and hemoglobin concentrations were 10 μM before mixing. Cyanide was 10 mM and azide was 100 mM before mixing. Second-order rate constants were calculated assuming that all ligand was in a form able to react with hemoglobin.

10–100 μM had no obvious effect on the results. Azide binding by methemoglobin is known to be kinetically complex. For this reaction, α and β chains bind ligand at different rates, the β chain having been identified as the more rapid of the two kinetic components (Gibson et al., 1969). Similarly, the azide binding process was observed to be biphasic for the various isolated hemoglobins. Results for all hemoglobins were again similar. In studies performed using 10 μM hemoglobins, the β chain rate was about tenfold greater than the α chain rate. In experiments performed using 100 μM A_0^+ or Hb^+ the α and β rates differed by five- to sixfold. This resulted from a substantial reduction in the β chain rate, while the α-chain rate was unchanged. The only apparent cause for the difference is partial dissociation to dimers which may occur at the lower hemoglobin concentration.

Discussion

Separation of the minor hemoglobin components present in adult red cell hemolysate poses well-known technical problems resulting from their similar structures and isoelectric points. In the present work two different separation techniques were utilized: column chromatography and preparative isoelectric focusing. Chromatographic fractionation resulted in noticeable methemoglobin formation (immediately converted to cyanomethemoglobin under the conditions used). Thus column methods alone did not provide the high quality oxyhemoglobin derivatives desired for deoxygenation kinetics. However, hemoglobin components isolated by either technique were readily converted to derivatives suitable for studies of other ligand reactions. The kinetic behavior of components obtained by the different separation methods was compared in a variety of reactions, and differences dependent on the method of separation were not observed. Furthermore, hemoglobin A_0 gave the same kinetic results as unfractionated "stripped" hemoglobin. These observations strongly suggest that the hemoglobins studied were not damaged in the separation processes and that the kinetic results are characteristic of their native states.

Preparative IEF in Sephadex gel layers has recently been used for separation of multiple components in the sperm whale myoglobin system (LaGow and Parkhurst, 1972; Radola, 1971). Application of the technique to the human hemoglobin system is found to yield excellent oxyhemoglobin samples, spectroscopically indistinguishable from unfractionated starting material. Removal of organic phosphates was also accomplished by preparative IEF in Sephadex gels. Separation of hemoglobin components was achieved for components differing by as little as 0.11 in pI. The theoretical limit of resolution by IEF has been estimated to be 0.02 pI unit (Vesterberg and Svensson, 1966). Measurements of pI values for the various hemoglobin bands were quite reproducible, and, where comparison is possible, are consistent with results of other workers. For example, our pI values for A_0 and A_2, measured at 5°, are 7.18 and 7.60, respectively. These are in excellent agreement with the values 7.15 (A_0) and 7.62 (A_2) obtained at 4° by Winterhalter and Colosimo (1971) using an LKB column IEF apparatus.

At pH 7 in buffers of moderate ionic strength, it is well known that reduced hemoglobin binds CO at a "slow" rate of 1–1.5 × 10^5 M^{-1} sec^{-1}, characteristic only of the deoxy tetramer. Deoxy dimers formed by pH jump or flash photolysis react at a rapid (Hb*) rate, about 6 × 10^6 M^{-1} sec^{-1}, characteristic of the ligand-bound conformation (Andersen et al., 1971). With deoxy A_{1a} and A_{1b}, the overall CO combination rates at pH 7 are "slow", being of the same order as that found for A_0. This is consistent with a tetramer structure for deoxy A_{1a} and A_{1b}. However, when combined with ligand, the tendency toward dissociation to dimers appears to be greater for A_{1a} and A_{1b} than for A_{1c}, A_0, and A_2. Based on the calculated values for $K_{T,D}$ (Table II), the standard state free energy change associated with dimer–tetramer interaction is about 0.9 kcal/mol lower for A_{1a} than for A_0. In oxyhemoglobin, interactions preventing splitting of the tetramer into $\alpha_1\beta_1$ dimers are limited to $\alpha_1\beta_2$ contact regions. An $\alpha_1\beta_2$ type contact normally contains a single polar interaction (a hydrogen bond between Asp G1(94) α_1 and Asn G4(102) β_2) and nonpolar interactions involving some 80 atoms from 19 amino acid residues (Perutz, 1970). For hemoglobins A_{1a} and A_{1b} in the ligand-bound state, a weakening of contacts at the $\alpha_1\beta_2$ interfaces must be induced by the unknown structural modifications which also produce the electrophoretic differences of A_{1a} and A_{1b} from A_0.

For carbon monoxide binding at pH 7, kinetic differences between A_0 and the minor hemoglobins A_{1a}, A_{1b}, and A_{1c} were observed. In terms of the individual rate constants for a four-step sequential model (Table I), the results for A_0 showed l_2' to be about 2.5 times greater than either l_1' or l_3'. The recent results of MacQuarrie and Gibson (1972) for stripped hemoglobin at pH 7 in 0.05 M bis-tris buffer show the same pattern. For all minor hemoglobin components, the relationship of l_2' and l_3' was similar to that found for A_0. The kinetic differences observed for hemoglobins A_{1a}, A_{1b}, and A_{1c} are mainly due to increases in the rate constant l_1'. The known introduction of a hexose blocking group in A_{1c} results in a 50% increase in l_1'. Since the N-terminal valine residues are not in proximity to a heme site (Perutz, 1970), structural perturbations caused by the hexose groups must be propagated through the protein moiety. Since the hexose groups are bound via a Schiff base linkage they cannot form a pyranose ring. Extension of the hexose chain would allow interaction with protein groups at distances on the order of 15 Å. Interaction should be possible, for example, with β chain C-terminal histidine residues, which in deoxyhemoglobin are involved in important salt bridges. Kinetic differences between the minor hemoglobins and A_0 are enhanced by prior reaction of p-mercuribenzoate with the β-93 sulfhydryl groups.

With hemoglobin A_0 in phosphate buffer, increasing the pH from 7 to 8 increases the rate constant for the overall reaction by about twofold, to 3.2×10^5 M^{-1} sec^{-1}, and greatly reduces the autocatalytic nature of the reaction curve. The behavior of A_{1b}, A_{1c}, and A_2 at pH 8 is virtually identical with that of A_0. However, for hemoglobin A_{1a} at pH 8, CO binding actually occurs somewhat more slowly than at pH 7. It has been observed for unfractionated hemoglobin that with increasing pH over the range 6–8, the major quaternary change to the ligand bound conformation occurs at lower fractional saturation (MacQuarrie and Gibson, 1972). Since this evidently results from a loosening of salt bridge constraints in deoxyhemoglobin, a modified salt bridge structure might be present in hemoglobin A_{1a}. Further increase in pH to 9 (0.05 M borate) leads to increased rates of CO binding for all hemoglobin components, and all reaction curves become biphasic. The presence of hexose blocking groups in A_{1c} results in a distinctly slower rate of CO binding than for its "parent" molecule A_0.

Studies of oxygen dissociation in the presence of CO, binding of cyanide and azide to methemoglobins, and l'^* rates observed in flash photolysis all pertain exclusively to ligand-bound quaternary forms. Kinetic results of such studies were substantially the same for the various hemoglobins isolated. Available evidence indicates that hemoglobin dimers, regardless of their state of ligation, retain a conformation similar to that of liganded tetramers (Andersen et al., 1971; Kellet and Gutfreund, 1970). However, since the environment within hemoglobin dimers and tetramers cannot be completely identical, some kinetic differences might be expected. Simple dilution of hemoglobin from concentrations where tetramer predominates to concentrations where dimer formation is significant affected the kinetic results for two of the reactions studied. Such dilution increased the kinetic heterogeneity apparently due to intrinsic α- and β-chain differences both for azide binding to methemoglobin and for deoxygenation studied in the presence of CO.

It has already been shown that hemoglobins A_{1c} and A_2 differ little from A_0 in intrinsic oxygen affinity, and that A_2 responds normally to 2,3-diphosphoglycerate whereas A_{1c} does not (Bunn and Briehl, 1970). The present findings indicate that A_{1c} and A_2 are also normal in their oxygen dissociation reactions. Physiological elevation of A_{1c} or A_2 would apparently give rise to changes in tissue oxygen supply only as reflected in the lack of response of hemoglobin A_{1c} to DPG. For hemoglobins A_{1a} and A_{1b} the dissociation rate of the first molecule of oxygen appears similar to that for A_0, but anomalies in the overall rate of dissociation are found. The latter reaction curves are biphasic, but over about the first 70% of reaction show a faster rate of oxygen release than that for A_0.

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References

Ames, B. N., and Dubin, D. T. (1960), *J. Biol. Chem. 235,* 769.

Andersen, M. E., Moffat, J. K., and Gibson, Q. H. (1971), *J. Biol. Chem. 246,* 2796.

Antonini, E., and Brunori, M. (1971), Hemoglobin and Myoglobin in Their Reaction with Ligands, Amsterdam, North-Holland Publishing Co., p 33.

Berman, M., Benesch, R., and Benesch, R. E. (1971), *Arch. Biochem. Biophys. 145,* 236.

Boelts, K. J., and Parkhurst, L. J. (1971), *Biochem. Biophys. Res. Commun. 43,* 637.

Bookchin R. M., and Gallop, P. M. (1968), *Biochem. Biophys. Res. Commun. 32,* 86.

Bunn, H. F., and Briehl, R. W. (1970), *J. Clin. Invest. 49,* 1088.

Bunn, H. F., and Drysdale, J. W. (1971), *Biochim. Biophys. Acta 229,* 51.

Clegg, M. D., and Schroeder, W. A. (1959), *J. Am. Chem. Soc. 81,* 6065.

DeSa, R. J. (1970), *Anal. Biochem. 35,* 293.

Draper, N. R., and Smith, H. (1966), Applied Regression Analysis, New York, N.Y., Wiley.

Drysdale, J. W., Righetti, P., and Bunn, H. F. (1971), *Biochim. Biophys. Acta 229,* 42.

Edelstein, S. J., Rehmar, M. J., Olson, J. S., and Gibson, Q. H. (1970), *J. Biol. Chem. 245,* 4372.

Eliot, R. S., Salhany, J. M., and Mizukami, H. (1970), *Adv. Cardiol. 5,* 108.

Geraci, G., and Parkhurst, L. J. (1973), *Biochemistry 12,* 3414.

Geraci, G., Parkhurst, L. J., and Gibson, Q. H. (1969), *J. Biol. Chem. 244,* 4664.

Gibson, Q. H., Parkhurst, L. J., and Geraci, G. (1969), *J. Biol. Chem. 244,* 4668.

Gibson, Q. H., and Roughton, F. J. W. (1957), *Proc. R. Soc., Ser. B 146,* 206.

Gray, R. D., and Gibson, Q. H. (1971), *J. Biol. Chem. 246,* 5176.

Holmquist, W. R., and Schroeder, W. A. (1966), *Biochemistry 5,* 2489.

Horton, B. F., and Huisman, T. H. J. (1965), *Br. J. Haematol. 11,* 296.

Huisman, T. H. J., and Horton, B. F. (1965), *J. Chromatog. 18,* 116.

Huisman, T. H. J., and Meyering, C. A. (1960), *Clin. Chim. Acta 5,* 103.

Jones, R. T. (1964), *Cold Spring Harbor Symp. Quant. Biol. 29,* 297.

Kellett, G. L., and Gutfreund, H. (1970), *Nature (London) 227,* 921.

Kunkel, H. G., Cappellini, R., Müller-Eberhard, H., and Wolf, J. (1957), *J. Clin. Invest. 36,* 1615.

Kunkel, H. G., and Wallenius, G. (1955), *Science 122,* 288.

LaGow, J., and Parkhurst, L. J. (1972), *Biochemistry 11,* 4520.

MacQuarrie, R., and Gibson, Q. H. (1972), *J. Biol. Chem. 247,* 5686.

Olson, J. S., Andersen, M. E., and Gibson, Q. H. (1971), *J. Biol. Chem. 246,* 5919.

Perutz, M. F. (1970), *Nature (London) 228,* 726.

Perutz, M. F., and Mathews, F. S. (1966), *J. Mol. Biol. 21,* 199.

Radola, B. (1971), *Protides Biol. Fluids, Proc. Colloq. 18,* 487.

Rahbar, S., Blumenfeld, O., and Ranney, H. M. (1969), *Biochim. Biophys. Res. Commun. 36,* 838.

Schnek, A. G., and Schroeder, W. A. (1961), *J. Am. Chem. Soc. 83,* 1472.

Schroeder, W. A., and Holmquist, W. R. (1968), Structural

Chemistry and Molecular Biology, Rich, A., and Davidson, N., Ed., San Francisco, Calif., W. H. Freeman, pp 238-255.

Taylor, J. F., Antonini, E., Brunori, M., and Wyman, J. (1966), *J. Biol. Chem. 241*, 241.

Vesterberg, O., and Svensson, H. (1966), *Acta Chem. Scand. 20*, 820.

Weatherall, D. J. (1968), *Annu. Rev. Med. 19*, 217.

Winterhalter, K. H., and Colosimo, A. (1971), *Biochemistry 10*, 621.

A Light Scattering Investigation of the Propylurea Dissociation of Human Hemoglobin A†

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ABSTRACT: The subunit dissociation of human hemoglobin A by propylurea in several liganded and chemically modified states was investigated by light scattering molecular weight methods. The dissociation data were analyzed by means of the equation developed in our earlier studies: $\Delta F°_D = \Delta F°_{D,w} - 2N'RTK_B[D]$, where $\Delta F°_D$ and $\Delta F°_{D,w}$ represent the free energy of dissociation of hemoglobin tetramers into half-molecules consisting of $\alpha\beta$ dimers in the presence and in the absence of propylurea, K_B is the binding constant of the urea to the average peptide unit, [D] is its concentration, and N' is the number of amino acid sites exposed per half-molecule on dissociation. It is found that the dissociation of oxyhemoglobin, cyanmethemoglobin, and *N*-ethylmaleimide oxyhemoglobin is characterized by essentially the same N' value of 15 to 21 ± 3, that are close to the 19 amino acid residues per surface which comprise the smaller $\alpha\beta$ contact area, seen in the X-ray crystallographic model of horse hemoglobin of Perutz and coworkers. Due to the very low degree of dissociation of deoxyhemoglobin, only a very approximate estimate of N' of about the same order of magnitude could be obtained for this form of the protein. In contrast, a significantly lower value of N' was obtained with bis(maleimidomethyl) ether modified oxyhemoglobin of 8 ± 3, that is cross-linked at cysteine residue F9 (93)β and histidine residue FG4 (97)β in the same β chains. Our results suggest that alterations caused by the presence of the cross-linking reagent reflect both the loss in amino acid residues that can interact with the urea at the blocked segments of the polypeptide chains in the dissociated state of hemoglobin and the changes in accessibility of some of the amino acid residues perturbed by the introduction of the reagent in the parent tetrameric form.

Hemoglobin is dissociated by various salts and solutes into half-molecules consisting of $\alpha\beta$ dimers of largely unaltered native conformation (Steinhardt, 1938; Benhamou et al., 1960; Rossi-Fanelli et al., 1961; Benesch et al., 1962; Kirshner and Tanford, 1964; Kawahara et al., 1965; Guidotti, 1967; Kellett, 1971; Norén et al., 1971, 1974; Thomas and Edelstein, 1972; Elbaum and Herskovits, 1974; Elbaum et al., 1974). Studies of this type have the potential value of predicting the role of the type of amino acid side chains that are responsible for the maintenance of the tertiary and quaternary structure of proteins (Kauzmann, 1959; Von Hippel and Schleich, 1969; Tanford, 1968; Elbaum et al., 1974).

In the previous publications from this laboratory the effects of increasing the hydrocarbon content of the urea and amide classes of denaturants on the conformation of single chain globular proteins (Herskovits and Jaillet, 1969; Herskovits et al., 1970a-c) and the multichain hemoglobins (Elbaum et al., 1973, 1974; Harrington et al., 1973) have been reported. The similarity of behavior of the ureas and amides as denaturants and dissociating agents has been noted (Elbaum and Herskovits, 1974). Specifically, an effective denaturant is also an effective dissociating agent; this effectiveness increases with the increase in the hydrocarbon content of the denaturant. The above study examined the effect of varying the hydrophobicity of the straight chain alkyl urea and amides on the subunit dissociation of hemoglobin. The present study deals with a detailed examination of the dissociation by a single and very effective dissociating agent, propylurea. Attention has been focused specifically on the cooperativity of the hemoglobin, the oxidation state of the iron in the heme groups, and the role of various ligands. The dissociation of the tetrameric form of human hemoglobin was followed by light scattering molecular weight measurements, and the denaturation by observing the change in optical rotation and adsorbance in the Soret region, as a function of the denaturant concentration.

Experimental Section

Hemoglobin. Hemoglobin was prepared from freshly drawn blood essentially according to Drabkin's procedure (1946) and chromatographed as previously described (Elbaum and Herskovits, 1974). *N*-Ethylmaleimide (EM)[1] and bis(maleimidomethyl) ether (BME) modified hemoglobin was prepared essentially according to the procedure of Guidotti and Konigsberg (1964) and Simon and Konigsberg (1966). Chromatographed 2-3% hemoglobin solutions,

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[1] Abbreviations used are: EM, *N*-ethylmaleimide; BME, bis(maleimidomethyl) ether; Hb, hemoglobin.