Kinetic and Equilibrium Properties of Hemoglobin Kansas*

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SUMMARY

The reactions of hemoglobin Kansas (102 β Asn → Thr) have been examined by kinetic and equilibrium methods. The behavior of the T state toward oxygen, derived from oxygen binding and oxygen pulse experiments, appears similar to that of hemoglobin A. The behavior of the R tetramer is difficult to separate from that of the dimer (the dissociation constant, $K_{diss}$, for oxyhemoglobin Kansas is about 70 μM as against 1.5 μM for hemoglobin A), but correlation of kinetic and equilibrium data suggests that the R tetramer of hemoglobin Kansas has a lower affinity for oxygen than that of hemoglobin A. Carbon monoxide binding is slower than for hemoglobin A, chiefly because of a lower rate of binding by the β chains. Comparisons between carbon monoxide binding and release of 8-hydroxypyrene-1,3,6-trisulfonic acid, and reaction with p-hydroxymercuribenzoate suggest that a conformation change (T → R transition) occurs on ligand binding as with hemoglobin A.

Detailed examination of the dissociation reactions from the liganded dimers of oxygen and of carbon monoxide shows abnormally rapid dissociation from the β chain. The hemoglobin-haptoglobin reaction shows that deoxyhemoglobin Kansas is too slightly dissociated to give a measurable reaction. This result is consistent with an abnormally low ligand affinity of the R form. It is suggested that the differences between hemoglobin Kansas and hemoglobin A are due, at least in part, to differences between the R forms, and that an R → T transition occurs normally in hemoglobin Kansas.

The allosteric effector 2,3-diphosphoglycerate lowers the affinity of hemoglobin Kansas for oxygen, but the effect is much smaller than for hemoglobin A when measured at high concentrations of hemoglobin. The data are most consistent with the assumption that the binding constant for 2,3-diphosphoglycerate at the primary site on tetrameric, liganded hemoglobin Kansas is only slightly lower than that for the unliganded form.

The mutant hemoglobin Kansas (β102, asparagine to threonine) has been studied by Bonaventura and Riggs (1), who showed that its functional behavior was characterized by a low affinity for oxygen, a low value of n in Hill's (2) equation, and a high degree of dissociation of the liganded form at least to dimers. Recent theoretical studies on cooperative binding of small molecules to multi-subunit macromolecules have added significance to work with hemoglobin Kansas, which is unusual among hemoglobins in having a lower oxygen affinity than that of hemoglobin A (3–7). The structural changes in deoxyhemoglobin Kansas have been determined by x-ray diffraction (8). This paper reports studies on the kinetics and equilibria of the reactions of hemoglobin Kansas with oxygen and carbon monoxide and the effects of organic phosphates on the sedimentation velocity behavior.

EXPERIMENTAL PROCEDURE

Methods

The kinetic experiments were performed using apparatus and methods recently described or referenced by Olson and Gibson (9). The equilibrium determinations were performed as previously described by Bonaventura and Riggs (1) and by Tomita and Riggs (10).

Materials

Reagents and gases were obtained from sources previously specified (0, 10). Hemoglobin Kansas was prepared from blood obtained from the mother of the individual whose blood was previously studied (1). The chromatographic procedures were those previously described by Bonaventura and Riggs (1). As detailed further under "Results," the fraction corresponding to that described in Reference 1 as hemoglobin A (Fraction I) prepared from the hemolysate of the patient's blood was found to differ in its functional properties from both hemoglobin A and Kansas. Accordingly, hemoglobin A for use as a control was prepared from normal human blood by passing it through the preparative procedures applied to hemoglobin Kansas. The purified hemoglobin samples for kinetic studies were shipped by air from Austin to Ithaca, packed in ice, and saturated with carbon monoxide. Under these conditions hemoglobin Kansas appears to be stable for many months at 0°.

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FIG. 1. Comparison of the time course of binding of carbon monoxide to hemoglobins A and Kansas. The reactions were followed at 470 nm in a 2-cm cuvette with 17 μM (heme) hemoglobin and 92 μM carbon monoxide, in 0.05 M bis-tris buffer, pH 6, at 20°. The results are plotted using a logarithmic scale for the ordinate (Δ absorbance), and have not been corrected for the effects of decreasing CO concentration as the reaction proceeds.

RESULTS

Kinetic Experiments

Binding of Carbon Monoxide

Experiments were performed with hemoglobin Kansas, with chromatographic Fraction I (identified initially as “Hb A”), and with hemoglobin A from a normal person. The results obtained at wave lengths remote from an isosbestic point showed that hemoglobin Kansas reacts less rapidly with carbon monoxide than does normal hemoglobin A, and that the time course tends to show a decrease in rate as binding proceeds, whereas hemoglobin A shows an accelerating time course under most conditions (11). One pair of experiments is given in Fig. 1, performed at pH 6 in 0.05 M bis-tris' buffer. Experiments performed under other conditions are summarized in Table I, which gives the apparent second order rate constant calculated from the time to reach 50% completion of the reaction. It appears that hemoglobin Kansas reacts more slowly than does the second component (Fraction I) from the hemolysate, and is less influenced by a change in pH.

Gray and Gibson (11) have reported that the apparent time course of carbon monoxide binding depends on the observing wave length, the effect depends on the composition of the medium and on pH. With phosphate-free hemoglobin in media of low ionic strength there was no wave length dependence, nor was there any at pH 9. In the presence of phosphates, and especially of inositol hexaphosphate, a slower component attributed to the α chains could be visualized to the shorter wave length side of the 425-nm isosbestic points, and a faster component predominated on the longer wave length side. The results with hemoglobin Kansas were quite different. In media of low ionic strength the course of carbon monoxide binding was wave length-dependent, but with the rapid component to the short wave length side of the isosbestic points (Fig. 2A). The addition of inositol hexaphosphate greatly slowed the overall reaction, as with hemoglobin A, but largely reduced the wave length de-
FIG. 3 (left). Binding of oxygen to hemoglobin Kansas followed at 432 nm, in a 2-cm cuvette at 20° with 5 μM heme. Buffer: 0.05 M phosphate, pH 7.0. The lines were calculated using the scheme of Fig. 15; the points are experimental. The concentration of oxygen is shown adjacent to the corresponding curve. Inset A shows the apparent first order constant for the approach to equilibrium. The concentration of oxygen is shown adjacent to the corresponding curve. Inset B shows the proportional saturation at the time of first observation (2 ms) plotted against oxygen concentration.

Dissociation of Carbon Monoxide

The only step accessible is the rate of dissociation of the first molecule of CO from saturated hemoglobin measured by replacement of CO by NO (12). The rate constants obtained at 20° in 0.05 M bis-tris buffer of pH 7 were 0.0105 s⁻¹ for Kansas and 0.0070 s⁻¹ for hemoglobin A. The time course was slightly heterogeneous in both cases, but not differently so for the two hemoglobins. As the dissociation constant for the tetramer-dimer reaction for liganded hemoglobin Kansas is of the order of 70 μM³ the rates given apply primarily to dimers, since the hemoglobin concentration was only 20 μM after mixing.

Oxygen-binding Experiments

Several series of experiments were carried out in 1967 and confirmed more recently. One experiment is illustrated in Fig. 3. On mixing deoxyhemoglobin Kansas with oxygen a very rapid equilibrium is reached during the dead time of the stopped flow apparatus (1.6 ms). Following this initial jump, there is a progressive uptake of oxygen during the next second or two at a rate which depends upon the concentration of oxygen, although not linearly (inset A, Fig. 3). This result is quite different from that obtained with hemoglobin A (13), where the observed reaction shows only a small initial rapid jump at pH 7, and reaches its final saturation value within 0.1 s, apart from an upward drift in saturation of a few percent. This drift was attributed (13) to dimerization of liganded hemoglobin A generating a high affinity noncooperative form which displaces the equilibrium of the tetramer with oxygen.

In hemoglobin Kansas there are two major differences from hemoglobin A. One is the greatly increased tetramer-dimer dissociation of the liganded form reported by Bonaventura and Riggs (1), which now dominates events within the time range of the stopped flow apparatus; the other is the great speed with which the initial rapid equilibrium is approached.

The dissociation constant originally given (1) was incorrect. Examination of the data of Fig. 12 in Reference 1 shows that the constant is in the neighborhood of 60 to 70 μM³.
In media of low ionic strength the results are even more complicated. Fig. 4 shows a third unusual feature. In addition to the rapid equilibrium during the dead time (not shown in this figure) and the slow \( O_2 \) concentration-dependent uptake of oxygen attributed to tetramer-dimer dissociation of the liganded form, there is a third process with a half-time of about 7 ms, whose amplitude increases with increasing oxygen concentration, accounting both relatively and absolutely for a greater part of the observed reaction. Fig. 4 is plotted semilogarithmically to illustrate this point. Inspection of traces at 418, 420, and 422 nm did not show any wave length dependence.

**Dissociation of Oxygen**

*Replacement of Oxygen by Carbon Monoxide*—The theory of the method has been fully discussed by Gibson and Roughton (14), and the treatment of the results to include differences between chains has been described by Olson et al. (15). Hemoglobin Kansas differs significantly from hemoglobin A, yielding \( k_a = 18.8 \pm 1.2 \text{ s}^{-1} \) (Hb A, 13.0 s\(^{-1}\)), \( k_b = 52.5 \pm 5.0 \text{ s}^{-1} \) (Hb A, 21.5 s\(^{-1}\)). The ratios of the rates of binding of oxygen and carbon monoxide to molecules with one vacant site are also available and, expressed as \( k'(O_2)/k'(CO) \), are: \( \alpha \) chains, 7.3 \( \pm 0.6 \) (Hb A, 6.8); \( \beta \) chains, 10.9 \( \pm 1.2 \) (Hb A, 6.6). Because of the large tetramer to dimer dissociation constant of the liganded form of hemoglobin Kansas reported by Bonaventura and Riggs (1), these values apply to dimers for hemoglobin Kansas and to tetramers for hemoglobin A. It is clear that the \( \beta \) subunits of Kansas have absolutely, and relative to \( \alpha \) chains, a larger dissociation constant for oxygen than the \( \beta \) chains of hemoglobin A. The relative rate of reaction with oxygen of the \( \beta \) chains of Kansas is also significantly higher. These differences are presumably due to the substitution at \( \beta_{16} \).

**Dissociation of Oxygen in Presence of Dithionite**—Experiments have been performed at several wave lengths and show a biphasic dissociation time course during the first 100 ms, at pH 7, 20\(^\circ\), 0.05 M phosphate, and all wave lengths. In the Soret region, especially, this primary reaction is followed by slow changes with a half-time of the order of 1 s. The slow changes have a spectral distribution corresponding to the change of deoxyhemoglobin from the rapid to the slow form (16) and, following Kellett and Gutfreund (17) and Andersen et al. (18), may be identified with the combination of deoxydimers (rapidly reacting and non-cooperative) to deoxytetramers.

The results of one experiment performed at 438 nm, isosbestic for the deoxydimer-deoxytetramer reaction (17), are presented in Fig. 5, which includes a graphical analysis of the two components, yielding rates of 60 s\(^{-1}\) and 16.5 s\(^{-1}\), in satisfactory agreement with the results of the experiment on replacement of oxygen by carbon monoxide. Such a result will be obtained if the dimer is without cooperativity, releasing oxygen from partly saturated intermediate compounds at the same rate as from the liganded form.

**Dissociation of Oxygen from Partly Saturated Intermediates**—This has been studied by mixing deoxyhemoglobin containing dithionite with oxygen. The hemoglobin combines transiently with oxygen and releases it again as the dissolved oxygen is consumed by the dithionite. The peak saturation with oxygen which is achieved depends on the dithionite and oxygen concentrations, and the results obtained under a variety of conditions are summarized in Fig. 6, A to D. When a high concentration of oxygen is used the time required for it to be substantially lowered by reaction with dithionite is sufficient to allow the hemoglobin to become effectively saturated with oxygen \( \text{e.g.} \).

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**Fig. 5.** Dissociation of oxygen from hemoglobin Kansas in the presence of dithionite. Hemoglobin, 12 \( \mu \)M before mixing, 438 nm, 2-cm cuvette, 20\(^\circ\), 0.05 M phosphate, pH 7, 0.2% dithionite. The figure shows the representation of the observed curve by two exponentials, assumed of equal weight, with rates of 17 and 60 per s, respectively. The points on Line A were obtained by subtraction of Line B from the observed curve.

**Fig. 6A, Curve 1**, the plateau extending for as much as 20 ms. With lower concentrations of oxygen or greater concentrations of dithionite, the hemoglobin never becomes saturated, and deoxygenation sets in more quickly.

The rate and character of the reaction change progressively as the oxygen concentration is reduced (Fig. 6A, Curves 1 to 5). At first, the deoxygenation reaction does not differ much from that shown in Fig. 5, but already in Curve 2 at a maximum saturation of 90% the slower rate of the obviously biphasic curve has reached 85 per s as against 45 per s for Curve 1, and a rapid phase roughly estimated at 800 per s has appeared, accounting for about one-half of the absorbance excursion. In Curve 3, with a maximum saturation of 70%, the rapid phase accounts for 85% of the absorbance excursion with a rate of 450 per s. At 50% peak saturation or less the slow phase has virtually disappeared.

The behavior under other conditions differs only in detail from that just described. In bis tris buffer, pH 7, the slow rate is similar to that seen with phosphate, pH 7 (Fig. 6B), but the proportion of the rapidly reacting form is less at each value of peak saturation, and its rate of reaction is about 450 per s.

The addition of inositol hexaphosphate (Fig. 6C) appears to lower the affinity of the hemoglobin for oxygen. No true plateau is reached with 1 atm \( O_2 \) (Fig. 6C, Curve 1), and only one rate of oxygen dissociation of about 1000 per s can be identified in the curves shown.

Changing the pH to 9 (Fig. 6D) has an effect opposite to that due to the addition of inositol hexaphosphate, the proportion
of a rapid phase being much less than that seen at pH 7. The rate of the rapid reaction is about 450 per s, and that of the slower reaction 25 per s.

Control experiments with hemoglobin A show qualitatively similar effects, but under each set of conditions the proportion of rapidly reacting material is much less than that shown in Fig. 6, and even in the presence of inositol hexaphosphate a slow phase of deoxygenation is prominent. With borate, with a peak saturation of only 14%, the rapid component accounts for no more than 12% of the deoxygenation reaction. The rates of the rapid component appear similar for both hemoglobins, the slow component of the reactions of hemoglobin A has about one-half of the rate observed with Kansas under similar conditions.

These oxygen pulse experiments seem to be giving information about the properties of a population of intermediates of the tetramer, chiefly in the unliganded form, with phosphates, and in both liganded and unliganded forms with bis-tris and borate buffers.

Binding of 8-Hydroxy-1,3,6-pyrenetrisulfonate to Hemoglobin Kansas

MacQuarrie and Gibson (19, 20) have recently shown that the compound, 8-hydroxy-1,3,6-pyrenetrisulfonate, is a functional analogue of 2,3-diphosphoglycerate, probably binding at the same site in deoxyhemoglobin. They showed that there is a marked lag in PTS release when the time courses of CO binding and PTS release were compared in kinetic experiments. Analogous experiments have been performed with hemoglobin Kansas, following exactly the procedures of Reference 20. It was found, first, that PTS binds appreciably to both deoxy- and carboxyhemoglobin Kansas. The approximate values for the dissociation constant derived from stopped flow experiments, with values for hemoglobin A in parenthesis taken from Reference 20 are: 9 μM (6.7 μM) for deoxyhemoglobin and 50 μM (18 μM) for carboxyhemoglobin in 0.05 M bis-tris buffer, pH 6.0, 20°C. At pH 7 the corresponding figures were 7 μM (5 μM) and 75 μM (61 μM) for the deoxy and carboxy forms. The precision of the values for liganded hemoglobin is low, as previously discussed (20).

Kinetic experiments have been performed at pH 6 and at pH 7, covering a 64-fold range of CO concentrations in both cases. A part of one experiment is reproduced in Fig. 7, which shows that there is a substantial lag in PTS release as compared with CO binding, and that this lag appears to be independent of the rate of CO binding up to a rate of 90 per s, observed with buffer equilibrated with 1 atm CO. These results demonstrate that hemoglobin Kansas changes its affinity for PTS much as does hemoglobin A, and that there is no obvious rate limitation of this change, which might, for example, be due to tetramer-dimer dissociation, up to 100 per s or so. The PTS-binding figures for deoxyhemoglobin Kansas may reasonably be compared with those for hemoglobin A, since both hemoglobins would be predominantly tetrameric in the deoxy form at the heme concentration (30 μM) used. In the liganded form hemoglobin Kansas would be predominantly dimeric, and so would not be expected to bind PTS strongly.
homogeneous, although at the concentration of hemoglobin used with p-hydroxymercuribenzoate was about the same as that for should be present. The reaction of deoxyhemoglobin Kansas with p-hydroxymercuribenzoate was excited at 340 nm in a cuvette of 2.5 mm radius, and followed using a yellow filter with cut off at 510 nm. Absorbance and fluorescence changes were scaled to show relation. Carbon monoxide concentrations after mixing: A, 400 µM; B, 230 µM; C, 115 µM. Pyrenetrisulfonate: 10 µM before mixing. Ordinate: in arbitrary units; abscissa: time in ms, as shown.

**Fig. 7 (left).** Carbon monoxide binding and release of 8-hydroxy-1,3,6-pyrenetrisulfonate from hemoglobin Kansas. Experiments were at 20° pH 7, 0.05 M bis-tris buffer, 35 µM hemoglobin. Absorbance changes were followed at 470 nm. Fluorescence was excited at 340 nm in a cuvette of 2.5-mm radius, and followed using a yellow filter with cut off at 510 nm. Absorbance and fluorescence changes were scaled to show relation. Carbon monoxide concentrations after mixing: A, 400 µM; B, 230 µM; C, 115 µM. Pyrenetrisulfonate: 10 µM before mixing. Ordinate: in arbitrary units; abscissa: time in ms, as shown.

**Fig. 8 (center).** Reaction of hemoglobin with p-hydroxymercuribenzoate. Buffer: 0.05 M phosphate, pH 7.0, 20°; 255 nm with 4-mm light path. CO/4 and CO/8 are equivalent to 115 µM and 57.5 µM after mixing. The results have been normalized for comparison and corrected for the contribution of the Hb + CO reaction to changes at 255 nm. p-Hydroxymercuribenzoate reaction, ○; CO reaction, ●. FIG. 9 (right). Flash photolysis of a dilute solution of hemoglobin Kansas, 12.5 µM heme, and 30 µM carbon monoxide (total). Light energy, 100 J, 1-cm cell, 372 nm, 22°. The lines illustrate the analysis of the reaction into two components as described in the text.

**Reaction of Hemoglobin Kansas with p-Hydroxymercuribenzoate**

Antonini and Brunori (21) have shown that p-hydroxymercuribenzoate reacts much more rapidly with liganded than with unliganded hemoglobin. In experiments in which deoxyhemoglobin A was allowed to react simultaneously with p-hydroxymercuribenzoate and with carbon monoxide, Gibson (22) has shown that the change in reaction rate of p-hydroxymercuribenzoate does not occur in proportion to the saturation of the hemoglobin with carbon monoxide, but lags behind it. He proposed that the reaction with p-hydroxymercuribenzoate might serve as an indicator of quaternary structural change. Analogous experiments have been performed with hemoglobin Kansas, with the results shown in Fig. 8. It is clear that there is a lag in the p-hydroxymercuribenzoate reaction. This lag is not due to a slow rate of reaction of carboxyhemoglobin Kansas with p-hydroxymercuribenzoate. The rate of reaction is, in fact, about three times faster than that of hemoglobin A measured under the same conditions, with a rate of $6 \times 10^4 \text{M}^{-1} \text{s}^{-1}$ in 0.05 M phosphate, pH 7.5, at 20°. The reaction appeared homogeneous, although at the concentration of hemoglobin used (95 µM in heme after mixing), a significant amount of tetramer should be present. The reaction of deoxyhemoglobin Kansas with p-hydroxymercuribenzoate was about the same as that for hemoglobin A. These experiments suggest that unliganded hemoglobin Kansas changes in conformation on binding much as hemoglobin A does.

**Experiments on Hemoglobin-Haptoglobin Reaction**

These experiments were performed under the conditions specified by Nagel and Gibson (23) using haptoglobin type 1-1. Two types of experiment were performed with hemoglobin A, hemoglobin Kansas, and the second component (Fraction I) isolated from a hemolysate of blood from the subject.

1. Haptoglobin and deoxyhemoglobin were mixed together in the stopped flow apparatus using haptoglobin equivalent to 1.25 µM heme after mixing and 2 µM heme. No significant reaction was observed in any of the three experiments. This is regarded as evidence that there is no significant dissociation of the deoxy forms to dimers under the conditions of the experiment, i.e. the dissociation constants must be at least 1 or 2 orders of magnitude below $10^{-4}$ M.

2. The deoxyhemoglobin solutions were mixed with haptoglobin dissolved in 0.05 M phosphate buffer (pH 7) equilibrated with 1 atm of carbon monoxide. With hemoglobin A there was a rather poorly defined lag followed by a reaction with a half-time of 3.5 s. With the Fraction I from the Kansas hemolysate, the result was similar, but with a half-time of 2.5 s. With hemoglobin Kansas there was a brief but definite lag lasting for 50 ms, followed by a reaction which was half complete in 1 s. Under the conditions of the experiment, hemoglobin Kansas would be 90% combined with carbon monoxide in about 20 ms. The lag is definitely longer than this, suggesting crudely that an upper limit to the rate of tetramer-dimer dissociation may be about 50 µs. These experiments are not suited for detailed analysis because the velocity of the hemoglobin-haptoglobin reaction is not great enough to allow the lag period to become a dominating feature of the reaction. The experiments again show qualitatively that hemoglobin Kansas changes conformation on binding ligand as evidenced by the change in the tetramer-dimer dissociation constant.

**Flash Photolysis Experiments with Carboxyhemoglobin**

Experiments have been performed both with dilute hemoglobin and with stronger solutions.
With Dilute Hemoglobin

The heme concentration was 12.5 μM, and total carbon monoxide concentration 30 μM. The optical path was 1 cm, and observations were made at 372 nm. The flash was of moderate duration (1/e = 2/0 μS), and data collection was initiated 1.8 ms after the beginning of the flash. This was not done to avoid effects of stray light, since the flash was surrounded by a filter with high absorbance at 372 nm, but to permit the photochemical reaction to slow to a rate small as compared with that of the carbon monoxide recombination reaction. Experiments could be carried out at different light levels by using a variable shutter between the flash and the cuvette.

The results of one experiment, together with a graphical analysis, are presented in Fig. 9. An approximate correction for the contribution of tetrameric hemoglobin was made by assuming a tetramer-dimer dissociation constant of 70 μM,2 leading to a proportion of heme in tetramers of 0.135. This was assumed to react with a second order rate constant of 0.2 μm-1 s-1, and an appropriate subtraction was made from the amount of free deoxyheme remaining at each point in time. The corrected values were then plotted against ∂, t (eliminating the effect of changes in [CO] during the reaction), and this plot was dissected into two components with rates of 9 × 10⁶ μm⁻¹ s⁻¹ and 2.3 × 10⁷ μm⁻¹ s⁻¹, respectively. Numerical analysis of the data gave 8.8 ± 0.1 × 10⁶ μm⁻¹ s⁻¹ and 2.5 ± 0.3 × 10⁷ μm⁻¹ s⁻¹ with a root mean square residual of ±0.35% (0.0011 in absorbance) for those components. The components have not been identified spectrophotometrically. It is suggested that the higher rate be assigned to the α chains. Combining these results with the data derived from the replacement of oxygen by carbon monoxide yields rates of oxygen binding of 6.5 × 10⁶ μm⁻¹ s⁻¹ for α chains and 2.7 × 10⁷ μm⁻¹ s⁻¹ for β chains. The corresponding values for α chains and for β from hemoglobin A are given by Antonini and Brunori (24) as 4.0 × 10⁶ and 6.8 × 10⁷ at pH 7 and 20°C.

**Higher Hemoglobin Concentrations**

Experiments with stronger solutions of hemoglobin and a shorter (1 mm) path were also performed in an attempt to gain some information about the behavior of the hemoglobin Kansas tetramer. A substantial proportion of slow reaction was now observed. The observation was also made that reduction of the fractional breakdown on photolysis was not associated with the disappearance of the slow reaction (as is observed with hemoglobin A (25)).

**Equilibrium Experiments**

**Chromatographic Fraction I**

Chromatographic Fractions I and II from the hemolysate containing hemoglobin Kansas were originally identified as hemoglobins A and Kansas, respectively (1). However, Fraction I possessed a much lower Hill coefficient, n, than expected for hemoglobin A. We have reinvestigated this observation (Fig. 10) and have confirmed that Fraction I does indeed have a substantially lower value of n: 2.0 to 2.4, depending on the pH. This lowered n is not the result of the chromatographic procedures because a sample of normal hemoglobin A prepared and chromatographed in exactly the same way gave n = 2.7 to 2.9. Although the values for n for Fraction I are abnormally low, the log p₅₀ values (Fig. 10) do not differ significantly from normal. Furthermore, the effect of DPG in lowering the oxygen affinity is close to that expected for hemoglobin A. Fraction I is evidently neither hemoglobin A nor hemoglobin Kansas but possesses some of the properties of each.

Rechromatography of this fraction as described (1) showed that at very early stages of chromatography two distinct chromatographic bands could be seen on the column, but the bands fused as they moved down the column in the pH gradient. The two fractions could, however, be isolated by changing the chromatographic conditions. The column (6 × 55 cm, Whatman CM-52) was equilibrated with 0.05 M phosphate, pH 6.8. Fraction I (∼1 g) was applied in this buffer. The first fraction, 1a (27%), was eluted at about 160 ml. After passage of 190 ml, more than the pH 6 buffer and 100 ml each of pH 7.0, pH 7.1, and pH 7.2 buffers were passed through the column. Fraction Ib (73%) emerged after ∼400 ml of pH 7.3 buffer. All buffers were 0.005 M in phosphate. The structural nature of Fractions Ia and Ib has not yet been determined.

**Bohr Effect and Effects of 2,3-Diphosphoglycerate**

In bis-tris and Tris buffers the alkaline Bohr effect of hemoglobin Kansas (Fig. 10) in the range pH 7 to 8 is substantially lower than that of normal hemoglobin A found previously in bis-tris and Tris buffers (10), and also lower than that found for Fraction I from the same hemolysate. The pH dependence studies reported earlier (1) were carried out in 0.1 M phosphate and in borate buffers above pH 8.5 as specified (26). These buffers appear to be associated with a larger Bohr effect than reported here. Furthermore, the pH of minimal oxygen affinity is higher...
for hemoglobin Kansas than for hemoglobin A in either set of buffers. Such a shift has been observed also to result from greatly increasing the ionic strength of solutions of normal hemoglobin A (27).

The addition of DPG causes a much smaller decrease in oxygen affinity (Figs. 10 and 11) than is observed either with hemoglobin A (10) or with Fraction I. The value of $\Delta \log p_{50}/\Delta p\text{H}$ increases in the interval pH 7 to 8 from -0.35 in the absence of DPG to -0.46 in the presence of 4 moles of DPG per mole of Hb. The corresponding values reported by Tomita and Riggs (10) are -0.5 and -0.9. These results imply that the ratio of the association constants for the binding of DPG to unliganded and liganded forms is smaller with Kansas hemoglobin than with hemoglobin A. Under the conditions of the experiments the liganded form has been shown to be about 50% tetrameric.

The primary binding site of DPG has been found to lie between the COOH termini of the two $\beta$ chains (28). Dimeric $\alpha_2$ units would not be expected to bind significant quantities of DPG. Our kinetic measurements of the binding of pyrenetrisulfonate (see above), an analogue to DPG, showed substantially the same change in the binding constant by hemoglobin Kansas upon reaction with CO as observed with hemoglobin A. However, the PTS experiments were performed at a concentration of hemoglobin Kansas (35 $\mu$M heme) where most of the liganded form would be dimeric. The higher hemoglobin concentration (124 $\mu$M heme) used in the DPG experiments would account for the reduction in effect of DPG if a larger fraction of the molecules in the T state is associated with the higher concentration of tetramer known to be present in significant quantities. Ogata and McConnell (7) predicted that liganded hemoglobin Kansas should bind DPG with about 40% the affinity of the unliganded hemoglobin. Our results indicate that the relative affinity for the organic phosphate (DPG) or its analogue (PTS) depends strongly on the hemoglobin concentration. Much below 70 $\mu$M (the approximate value for the $K_d$ dissociation constant in the liganded form), the effect of organic phosphates should be large as anticipated by the PTS experiments, but at high protein concentrations the effect should be small as found in the DPG equilibrium experiments.

The variation of the Bohr effect of Hb Kansas (expressed as $\Delta \log p_{50}/\Delta p\text{H}$, with the [DPG]/[Hb] ratio, is given in Fig. 12. The results are shown for two pH intervals, pH 7.0 to 7.3 and 7.3 to 7.6. Although DPG enhances the Bohr effect slightly above pH 7.3, the Bohr effect decreases with added DPG in the interval pH 7.0 to 7.3. In fact, with sufficient DPG, the Bohr effect vanishes almost completely.

Since at least 6 residues, two on the $\alpha$ chain and one on the $\beta$ chain, appear to be involved in the Bohr effect (29), DPG evidently has widespread effects throughout the tetramer. The $\alpha$-NH$_2$ groups of the $\alpha$ chain, which are involved in the normal Bohr effect, are remote from the primary binding site proposed for DPG. Abolition of the Bohr effect might result from the occupation by DPG of secondary sites closer to the $\alpha$-NH$_2$ termini. If such secondary sites in oxyhemoglobin were to have a higher affinity for DPG than in deoxyhemoglobin, the observed results would be explained. The existence of secondary binding sites in oxyhemoglobin has been supported by various binding studies (30-32). Direct evidence for polyphosphate binding to the chains of oxyhemoglobin has recently appeared (33).

**Effect of Dilution of Hemoglobin on Oxygen Binding**

The earlier results (1) suggested that the oxygen affinity did not change in the range of hemoglobin concentrations where the sedimentation coefficient was observed to decrease with ligand binding. Considerations of linkage indicate that dilution should...
and variety of experiments performed with hemoglobin Kansas, a
are, of course, relations between the species other than those
7.0. 0.05
pM
5984
5
p
310
2.0
0.02
1.77 x 10^6 M^-1 s^-1, from the experiments with dithionite and
hyperbolic curve is obtained (inset B) with half saturation at
about 140 \mu M O_2 (75 mm Hg po_2). This should represent the
limiting equilibrium behavior of hemoglobin Kansas at concentra-
tions much above the value of the tetramer-dimer dissociation
constant of the liganded form (K_d). The maximal concentration
shown in Fig. 13 is much too low to reflect this value. The data of
Fig. 12 in Reference 1 show that even at a concentration of
1.5\% hemoglobin (900 \mu M heme), only about 75\% of the mole-
cules are tetramers. To represent the kinetic behavior shown in
Fig. 3 it was assumed that all species in Fig. 15 could be con-
sidered to have reached equilibrium with oxygen on the long time
scale of the figure. Further, in view of the low value of \( n \), it was
assumed that \( K_1, K_2, \) and \( K_4 \) were statistically related, and
that the dimer is noncooperative, as already discussed, with a
ligand affinity equal to the mean of that of the chains. The
rate of association of dimers to tetramers (\( k'_d \)) was taken as
4 \times 10^6 M^-1 s^-1, from the experiments with dithionite and
oxyhemoglobin by Kellet and Gutfriend (17) and \( K_1, K_4, \) and
\( k_d \) assigned best fitting values. This very crude scheme did not
fit the results well, the rates of approach to equilibrium being
badly misrepresented. A much better result was obtained by
assuming that \( Hb_2X_2 \) and \( Hb_4X_4 \) are both equally able to disso-
ciate. On this basis, the value of \( k_d \) was 16 \pm 5 per s, \( K_4 \) 0.022 \pm
0.002 \mu M, and \( K_4 \) 0.0035 \pm 0.0015 \mu M. These figures generated
the lines in Fig. 3, fitting the results surprisingly well.

The relation between \( K_1 \) and \( K_4 \) deviates from the statistical
value by about 2 to 5 times as compared with about 250 times
for hemoglobin A, suggesting that the hemoglobin Kansas tetra-
mer is effectively noncooperative. The value of \( K_d \) would be
about 40 \mu M, rather less than actually observed. Rough esti-
mates of the values of \( k'_d \) and \( k_d \) are available by using the results
of the oxygen pulse experiments, which set \( k_1 \) at 700 per s. The
value of \( k' \) then is about 16 \times 10^6 M^-1 s^-1, about the same as the esti-
mate of Gibson (18), 17.7 \times 10^6 M^-1 s^-1, for hemoglobin A. The
results of all of the kinetic experiments with oxygen appear to
be internally consistent, especially the oxygen pulse experiments
which showed little slow phase of deoxygenation unless a high
saturation was maintained for 20 ms or more, sufficient to permit
significant dissociation of tetramer, and which were qualitatively
very different from the results with hemoglobin A (Fig. 6). The
numerical values are rough estimates indeed, since the scheme
neglects substantial chain differences, and it is questionable to
assign similar kinetic tetramer to dimer dissociation rates to
\( Hb_2X_2 \) and \( Hb_4X_4 \) while giving them different intrinsic affinities
for ligand.

For these reasons the observed equilibrium behavior is repro-
duced only qualitatively using the rate and equilibrium constant
specified, but the effect of concentration on oxygen affinity and
the value of \( n \) in Hill's equation are well represented.

The dissociation of oxygen from liganded hemoglobin Kansas
has been studied largely under conditions where the dimer is the
most abundant species, and the figures quoted for the replace-
ment reaction and for the reaction of oxyhemoglobin in the
presence of dithionite refer primarily to dimers. The rates of
both reactions are unusually high, especially for the \( \beta \) chains
(52 per s as compared with 23 per s found by Olson et al. (15)),
suggesting that the substitution has produced a significant change
in the liganding properties of the subunit. The similarity between the rates observed in replacement and in the presence of dithionite shows a lack of cooperativity within the dimer.

The tetramer can only be examined in the oxygen pulse experiments, and here the high dissociation velocity of about 700 per s (intrinsic rate) is observed. Combining this figure with the apparent dissociation constant for oxygen of 140 μM obtained in the rapid oxygen-binding experiments gives a rate of about 4 × 10^6 M^-1 s^-1 (intrinsic) for oxygen binding to deoxyhemoglobin tetramers, about the same as that for hemoglobin A. Taken together, these results suggest that in hemoglobin Kansas the functional changes responsible for cooperativity in hemoglobin A do not occur. This conclusion agrees with the results of partial photodissociation experiments with high concentrations of hemoglobin Kansas, where the tetramer component continues to give slow recombination rates even when only partially photodissociated.

The experiments on carbon monoxide binding by the stopped flow method are interesting chiefly because of the wave length dependence near the 424-nm isosbestic point as compared with hemoglobin A. This behavior can be rationalized if it is supposed that in hemoglobin Kansas the rate of binding of the β chains (accepting the chain identification of Gray and Gibson) is lower than that of the α chains in media of low ionic strength. On the addition of phosphates, the rate of binding of the α chains diminishes, approximating more closely to that of the β chains. The effect of phosphates would thus be the same as that observed in hemoglobin A, but the starting point is different, since in hemoglobin A the α chains react at about the same rate as the β chains in low ionic strength buffers.

Attempts to estimate the rate of binding of CO to intermediate compounds by analysis of the time course of the reaction were unsatisfactory. Most attention was given to a set of data covering a 64-fold range of CO concentrations. These were poorly fitted by neglecting chain heterogeneity and using four consecutive reactions, and little improvement was obtained by introducing chain heterogeneity when the whole set of data was considered, although an improved fit to the results was obtained if the four highest CO concentrations (460, 230, 115, and 57.5 μM) were considered by themselves. A better fit to the whole set was obtained by introducing a dissociation step after binding of 3 molecules of CO, generating a rapidly reacting dimer with the properties observed by flash photolysis. Presumably a further gain could have been obtained by introducing both chain heterogeneity and a dissociation step, but it was felt that this would represent arithmetic overkill. The results did appear to be in line with those obtained in the oxygen-binding reaction, suggesting that no transition to a rapidly reacting tetramer occurs and that tetramer-dimer dissociation begins before 4 molecules of ligand have been bound.

Analysis of the results for release of pyrenetrisulfonate was attempted for data obtained with high concentrations of carbon monoxide along the lines used by MacQuarrie and Gibson (20), using fixed rate constants for carbon monoxide binding and allowing the dissociation constants for pyrenetrisulfonate binding to the intermediates HbCO, Hb2(CO)2, and Hb4(CO)3 to vary. The results were moderately satisfactory, representing the time course of the fluorescence change with an error of ±1.5% of total fluorescence and dissociation constants of 13 ± 1.1, 11 ± 1.4, and 23 ± 5.9 μM for binding to HbCO, Hb2(CO)2, and Hb4(CO)3, respectively. The dissociation constants for Hb1 and Hb4(CO)4 were fixed at the values of 10 and 33 μM obtained from the stopped flow records. Binding of pyrenetrisulfonate to the liganded form is presumably affected by the occurrence of dimerization, and this shows up in the experimental time course of the fluorescence change in the form of a non-random distribution of residuals with the calculated time course of pyrenetrisulfonate release lagging behind that observed toward the end of the reaction with the lower concentrations of carbon monoxide. The curve-fitting procedures were arbitrary, and the results show primarily that fits may be obtained.

No detailed analysis of the reaction with p-hydroxymercurobenzoate has been made, since the significant chain heterogeneity and multiple reaction sites lead to unmanageable complications of the reaction scheme. As already mentioned, the lag in the reaction with p-hydroxymercurobenzoate seems to be quite similar to that observed with hemoglobin A (22). One significant observation associated with these experiments is that deoxyhemoglobin Kansas reacted slowly with p-hydroxymercurobenzoate, while carboxyhemoglobin Kansas reacted very rapidly but not homogeneously. About two-thirds of the reaction took place within the dead time (1.6 ms) of the flow apparatus. The remaining third, which could be observed, was about twice as rapid as that of hemoglobin A. There was no observable slow reaction of carboxyhemoglobin Kansas with p-hydroxymercurobenzoate. The very rapid reaction occurring within the dead time may correspond to the reaction with dimers, and the slower (250 per s) phase to the reaction with the tetramer. These observations may be explained by supposing that either (a) the carboxyhemoglobin Kansas tetramer is in the R form, or (b) the rate of dissociation of the tetramer to dimer is large compared with the observed rate of 250 per s, or (c) the carboxyhemoglobin Kansas tetramer consists of an equilibrium mixture of R and T forms with a rate of reaction between the R form and p-hydroxymercurobenzoate large enough to yield the observed rate of 250 per s, even though the T form predominates. Proposal (b) appears incompatible with the observed tetramer-dimer dissociation constant, and (c), although possible, appears less attractive than (a).

These observations lead on to the question of assigning the behavior of hemoglobin Kansas to a model. It seems that an allosteric model with two states having properties similar to those of hemoglobin A is not consistent with the observations. A liganded state is certainly formed, and the evidence suggests that the greater part of the hemoglobin in fact transforms into it on binding of 3 molecules of ligand. The species HbX3 and Hb2X4 appear to have properties very similar to those of hemoglobin A in respect to binding pyrenetrisulfonate and reaction with p-hydroxymercurobenzoate. Although tetramer-dimer dissociation in the R form seems to proceed rapidly, ligand binding is apparently less rapid and ligand dissociation faster than in hemoglobin A.

The ligand binding and subunit dissociation behavior of the tetramers seem to be consistent with the suggestion of an R state with modified functional properties. Experiments with deoxy Kansas and haptoglobin failed to show measurable binding, and the dissociation constant of the deoxy tetramer must therefore be quite small. A suggested value for Kd for 10^-17 m (34) for hemoglobin A. If the hemoglobin Kansas tetramer has five times less affinity for oxygen and a 50 times greater dissociation constant for the liganded form (Kd = 70 μM) the value of Kd of deoxy Kansas must be 10^-12 × 50/5 or about 10^-14 m. This value is small enough to assure an unmeasurable rate for the hemoglobin-haptoglobin reaction under the conditions of the experiments, as compared with 2 per s for the liganded forms.

The picture of hemoglobin Kansas which has been derived
above differs widely from the interpretation placed on the earlier data by Edelstein (35). He assumed that the allosteric constant C is invariant from hemoglobin to hemoglobin, i.e. that the ratio of the affinities of the T and R state for ligand is constant. On this basis, he was able to account for the affinity and v value of hemoglobin Kansas with an exceptionally large value of L (=10^9), carrying the implication that the R state is slightly populated even when the hemoglobin is fully saturated. This proposal seems difficult to reconcile with the experimental results, except perhaps by postulating an exceptionally large rate of tetramer to dimer dissociation in the liganded form. The hemoglobin-haptoglobin, p-hydroxycoumarinbenzoate, and pyrenesulfonate reactions agree in suggesting that the R state is substantially populated after 4 ligand molecules have been bound. Further, the properties of the liganded tetramers seem to differ from those of hemoglobin A so far as they are accessible to study.

One apparent inconsistency has been found. The relatively slight increase in affinity of the liganded form for oxygen (about five times was suggested) seems to require a rapid rate of dissociation of oxygen from the liganded tetramer, and this is true whether a formal representation of the reaction (Fig. 15) or the allosteric model is used. Experimentally, this rapid reaction has not been observed, although it would be difficult to separate it with assurance from the contribution of the β chains in the dimer. In summary, then, it appears that hemoglobin Kansas undergoes a conformation change on ligand binding which is quite similar to that of hemoglobin A as judged by its effects on tetramer-dimer dissociation, reaction with p-hydroxycoumarinbenzoate, and binding of 2,3-diphosphoglycerate and analogous substances, but which is not associated with major changes in affinity for oxygen and carbon monoxide.

Addendum—Since this paper was written a preliminary account of the binding of carbon monoxide by deoxyhemoglobin Kansas has appeared (36). The apparent rate constant did not show the increase characteristic of hemoglobin A, and this result was interpreted to show that hemoglobin Kansas does not undergo an allosteric transition analogous to that of hemoglobin A until later in the course of ligand binding. Our experiments with a range of experimental conditions show greater chain heterogeneity with Kansas than with hemoglobin A; further, the time course of the apparent rate constant is a function of carbon monoxide concentration, perhaps because of tetramer-dimer dissociation of partly liganded forms. These effects may have contributed to the results of Hopfield et al. (36). In another preliminary communication (37) evidence from a proton magnetic resonance study is presented which indicates that liganded hemoglobin Kansas is made up of an equilibrium mixture of R and T forms, and that this equilibrium can be shifted in favor of the T form by the addition of organic phosphates. Such a shift should be accompanied by a change in the tetramer-dimer dissociation constant. We did not observe such a change in our ultracentrifuge experiments. The existence of a significant population of molecules in the R state in liganded tetrameric hemoglobin Kansas appears entirely consistent with our experiments, however, and the idea that the T → R transition occurs later in the course of ligand binding than with hemoglobin A offers a good explanation for the larger proportion of rapidly reacting hemoglobin in our oxygen pulse experiments.

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