Cat Hemoglobin

PH DEPENDENCE OF COOPERATIVITY AND LIGAND BINDING*

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SUMMARY

Cat hemoglobin has been found to exist in three forms which differ in the cooperativity of oxygen binding at low and high pH values. Type I cat hemoglobin has a high cooperativity (n = 2.7 to 3.1) at both high (pH = 7.8 to 9.0) and low (pH = 6.5 to 6.9) pH values. Type II hemoglobin exhibits low cooperativity (n = 1.8 to 2.3) in the low pH range but high cooperativity in the high pH range. Types I and II have identical $p(O_2)^{36}$ values. Cat hemoglobin type III has a low cooperativity in both pH ranges. Results presented indicate that changes in the reactivity of sulfhydryl groups coincide with the difference in cooperativity among the three cat hemoglobin forms. Stored at 4°C as a hemolysate, in the oxy form, type I hemoglobin gradually converts to type II and then type III cat hemoglobins. The conversion occurs much more slowly if purified cat hemoglobin is stored in the carbon monoxide form. Type III hemoglobin can be converted to type II hemoglobin by incubation with dithiothreitol. Molar equivalents of $\beta$-hydroxymercurobenzoate, 1.4 and 2.4, convert type I to type II and type III, respectively. Ultracentrifuge experiments indicate that the tetramer-dimer subunit dissociation constants of the liganded forms of types I and II of cat hemoglobin are pH-dependent. In addition, liganded cat hemoglobin dissociates about 10 times more readily than any other mammalian hemoglobin investigated. The results are discussed in light of known facts about the structure of cat hemoglobin and applied to an evaluation of the validity of the allosteric model for cooperativity of multi-subunit proteins.

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Ligand affinities of mammalian hemoglobin and sensitivity to organic phosphates vary over a broad range (1). These differences must reflect small differences in the quaternary and tertiary structure stemming from known variations in primary structure of mammalian hemoglobins (2). Despite such variety, there is one function, cooperativity, which appears almost invariant. A pH-independent cooperativity, $n_H$, of approximately 3.0 has been reported for all but one mammalian hemoglobin species investigated. Cat hemoglobin has been reported to possess a cooperativity of 2.0 (3). Interested in this unusual hemoglobin, and with a thought towards testing the validity of the two-state model for cooperativity, we found that $n_H$, the cooperativity, of cat hemoglobin rose from 2.0 to 3.0 when the pH was increased from 6.5 to 8.0 (4). Further work, which shall be reported in this paper, revealed that cat hemoglobin can be obtained in a form, which in accord with other mammalian hemoglobins, possesses a cooperativity of 3.0 at all pH values. Similar findings have been reported by other laboratories (5). Human hemoglobin, which has been the most extensively studied mammalian hemoglobin, is used as a standard for comparison in this report. Cat hemoglobin differs from human hemoglobin in several ways. Whereas the $p(O_2)^{36}$ for human hemoglobin at pH 7 is about 10 mm Hg, the corresponding value for cat hemoglobin is about 20 mm Hg. Cat erythrocyte hemolysates contain two species of hemoglobin which appear to differ only in the $\beta$ chain (6, 7). The relative amounts of these two hemoglobins vary greatly from one cat to the next. The major component is designated as cat hemoglobin A, while the minor component is designated as cat hemoglobin B (8). Within a single cat the relative amounts of the two species remain constant with age and under such physiological stress as induced anemia. Both cat hemoglobins have been reported to have identical cooperativities and $p(O_2)^{36}$ values (3). Cat and human hemoglobins also differ in the number of titratable sulfhydryl groups. There are two titratable sulfhydryl groups per molecule of human hemoglobin compared with eight per molecule of cat hemoglobin (6). This large number of titratable sulfhydryl groups is not a common feature of mammalian hemoglobins. The results reported in this paper suggest that the extent of modification of the sulfhydryl groups of cat hemoglobin may be a critical factor in determining the cooperativity of the hemoglobin and the effect of pH on that cooperativity.

EXPERIMENTAL PROCEDURE

The nitrogen and carbon monoxide were prepurified. The $p$-chloromercuribenzoate was obtained from Sigma and the dithiothreitol from Calbiochem. All other reagents were analytical grade. Cat blood was drawn from the animal, either by venipuncture or cardiac puncture, into a Vacutainer containing citrate or a syringe coated with heparin. Hemolysates were prepared on the same day according to the method described by Edelstein et al. (9). The resulting hemolysate was stored as the oxy form under an atmosphere of air.

The cat hemoglobin species A and B were separated on a BioRex 70 ion exchange column, equilibrated with 0.05 M potassium phos-
phate, pH 6.6, according to the method described by Lessard and Taketa (8). The first peak (hemoglobin B) was eluted with the equilibrating buffer and in later preparations the second peak was eluted with 0.14 M potassium phosphate, pH 7. The hemoglobin was converted to the carbonyl form by passage of carbon monoxide (CO) over the hemolysate before passing it through the column. Column fractions were pooled and concentrated by pressure dialysis. In some cases, samples of concentrated hemoglobins were dialyzed against 0.1 M potassium phosphate, pH 7. The hemoglobin preparations were stored in closed vials filled with CO. The gas was replenished periodically and every time the vial was opened, CO was removed from the sample immediately prior to use. To remove the CO, the hemoglobin was rotated in a flask in ice water under the light from a 100-watt bulb held about 7 inches from the flask.

Oxygen equilibria were measured in tonometers similar to those described by Rossi-Fanelli and Antonini (10). Air was injected through a rubber inlet stopper from an air-tight syringe and the amount of oxygen bound was determined with a Cary 15 scanning spectrophotometer. The hemoglobin and oxygen were equilibrated by gentle shaking for 15 min in a Blue M Magniwhirl shaker bath equilibration centrifuge. To avoid methemoglobin accumulation, few points were taken per experiment. Approximately seven equilibration points were recorded, including the fully saturated and fully unsaturated solutions.

Per cent of hemoglobin saturated was determined in two ways. The first method has been described by Benezech et al. (11) and provides an estimate of the amount of methemoglobin present after each equilibration. The second method ignores the possibility of methemoglobin and considers the solution as containing only oxy- and deoxyhemoglobin. Thus the per cent of oxyhemoglobin was determined by the following formula:

$$P = \frac{(A_I - A_0)}{A_0}$$

where $P$ is the per cent of oxyhemoglobin. $A$ stands for the optical density at a particular wavelength, and the subscripts I, O, and D represent the intermediate, fully oxygenated, and fully deoxygenated solutions, respectively. With this method $P$ is independent of small base-line shifts. Optical densities at wavelengths 578, 560, and 540 were treated in this way.

Per cent oxyhemoglobins calculated by both methods usually agreed quite well. The Benezech formulas were always used if there were any discrepancy among the values obtained. Otherwise, an average of all values was taken. If, during the experiment, the percentage of methemoglobin was higher than a set level, or if the percentage of methemoglobin increased during the course of the experiment, the results were discarded. Maximum levels of methemoglobin were 15% for the hemolysate and 20% for the isolated A and B hemoglobins.

Ultracentrifugation studies were performed in a Beckman model E ultracentrifuge. Hemoglobin solutions were made from carbonylhemoglobin and buffer solutions through which CO had been bubbled for at least 2 min. Hemoglobin concentrations (2 to 4 X 10^{-5} M) were determined spectrophotometrically at several wave-lengths. During the run the hemoglobin was observed with a Beckman scanner using wavelengths in the visible region of the spectrum. For sedimentation velocity runs, one to three double-sector centrifuge cells were run simultaneously in a four-hole titanium rotor with continuous scanning of the cell contents. The distance from the vertical midpoint of the boundary of the sedimenting hemoglobin to the center of rotation (r) was employed for calculation of the sedimentation coefficient. A linear least squares fit was applied to the log t versus time plot to determine the value of $s_{20,w}$. If necessary, temperature and viscosity corrections were made as described by Svedberg and Pedersen (12) and the value of $\alpha$ was taken as 0.749 (12). The tetramer-dimer subunit dissociation constant was determined from $s_{20,w}$ using the formula:

$$K = s^2 (1 - \alpha)$$

where (heme) is the molar heme concentration at the beginning of the run and

$$\alpha = \frac{(4.8 - s_{20,w})}{(4.8 - 2.8)}$$

The sedimentation coefficient of the liganded hemoglobin tetramer was taken as 4.8. Recently the $s_{20,w}$ of deoxyhemoglobin was determined to be 4.8 (13). Calculations from crystallographic results show that the $s_{20,w}$ of the unliganded tetramer must be about 0.1 less than for the liganded tetramer, in agreement with experimental findings (9, 14). The $s_{20,w}$, then, for liganded hemoglobin using the result obtained for unliganded hemoglobin given above, would be 4.9. The $s_{20,w}$ of the liganded hemoglobin has been previously reported as 4.7 (15). The average of these two values is 4.8. The $s_{20,w}$ of the dimer was taken as 2.8 (9).

Sedimentation equilibrium experiments were performed at 20° in multichannel cells. From three to five cells were run at one time. At least one human control was included with each run. The rotation speed varied from 30,000 to 32,000 rpm. Optical densities and radial positions of 20 to 30 points were read from each trace and the results calculated using a program described by Edestein et al. (9).

**RESULTS**

Cat hemoglobin can be obtained in several interconvertible forms. The three forms have been designated types I, II, and III. They vary principally in the value of the cooperativity at high and low pH values. The oxygen equilibrium characteristics of the three forms are summarized in Table I.

**Type I Cat Hemoglobin**—Type I cat hemoglobin has a relatively high cooperativity at pH 6.5 ($n_H = 2.7$) which then rises to 30 at pH 7.7. The cooperativities determined in the low pH range were much more scattered than those determined in the high pH range. The expected mean error at the low pH values was 0.24 (with an average of 10 experiments) whereas the expected mean error in the high pH range was about 0.1 (with an average of three experiments). When the cooperativities at pH 6.5 and 6.7 are plotted against the age of the hemoglobin preparation, the cooperativity is seen to decrease to about 2.2 after 7 days (Fig. 1). Neither the $p(O_2)_{50}$ at pH 6.5 and 6.7, nor the cooperativity in the high pH range change during the first week. However, after about 20 days the cooperativity at pH 7.7 falls to 2.1. Despite the fact that the $p(O_2)_{50}$ of cat hemoglobin is about twice that of human hemoglobin, the Bohr effects of both hemoglobins are similar. The calculated $d$ log ($p(O_2)_{50}$) per pH (between pH 7.0 and 7.7) was 0.57 for type I cat hemoglobin and 0.52 for human hemoglobin. The Bohr effect of type I cat hemoglobin is identical with that of type II cat hemoglobin. The dependence of $p(O_2)_{50}$ on pH for type I cat hemoglobin is shown in Fig. 2. These results have been obtained with cat erythrocyte hemolysates containing from greater than 95% to approximately 60% cat hemoglobin A.

**Table I**

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>Low pH</th>
<th>High pH</th>
<th>Low pH</th>
<th>High pH</th>
</tr>
</thead>
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<tr>
<td>Human</td>
<td>2.9</td>
<td>3.1</td>
<td>13.8</td>
<td>1.5</td>
</tr>
<tr>
<td>Type I cat</td>
<td>2.9</td>
<td>2.9</td>
<td>31.1</td>
<td>10.7</td>
</tr>
<tr>
<td>Type II cat</td>
<td>2.1</td>
<td>3.0</td>
<td>34.0</td>
<td>11.2</td>
</tr>
<tr>
<td>Type III cat</td>
<td>2.1</td>
<td>1.8</td>
<td>23.3</td>
<td>2.4</td>
</tr>
</tbody>
</table>

**pH dependence of cooperativity of types I, II, and III cat hemoglobins**

All low pH data were obtained at pH 6.7; high pH data were obtained at pH 7.7 except values for human hemoglobin (obtained at pH 9) and type III cat hemoglobin (obtained at pH 8.5).
FIG. 1 (left). The age dependence of the cooperativity and $p(O_2)_{50}$ of type I cat hemoglobin. $\bullet$, $p(O_2)_{50}$; $\Delta$, cooperativity of cat hemoglobin. The buffer was 0.1 M potassium phosphate; temperature was 22°C.

Fig. 2 (center). The pH dependence of the $p(O_2)_{50}$ of type I cat and human hemoglobins. $\bigcirc$, values for human hemoglobin; $\bullet$, values for cat hemoglobin. The error bars represent the mean error. At low pH values the buffer was 0.1 M potassium phosphate. Above pH 7.7, the buffer was 0.1 M sodium pyrophosphate. The measurements were made at 22°C.

Fig. 3 (right). pH dependence of the cooperativity of type II cat hemoglobin. $\bigtriangleup$, cooperativity of type II cat hemoglobin; $\Delta$, cooperativity of human hemoglobin. Each point represents the average of from one to five experimental results.

### Table II

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>Treatment</th>
<th>Buffer</th>
<th>Cooperativity</th>
<th>Affinity</th>
</tr>
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<tbody>
<tr>
<td>Human</td>
<td>None</td>
<td>0.1 M KPi, pH 6.7</td>
<td>2.9</td>
<td>13.8</td>
</tr>
<tr>
<td>Type I cat</td>
<td>None</td>
<td>0.1 M KPi, pH 6.7</td>
<td>2.9</td>
<td>31.1</td>
</tr>
<tr>
<td>Type III cat</td>
<td>None</td>
<td>0.1 M KPi, pH 6.7</td>
<td>2.6</td>
<td>26.5</td>
</tr>
<tr>
<td>Type III cat</td>
<td>6 moles of DTT per mole of Hb</td>
<td>0.1 M KPi, pH 6.7</td>
<td>2.6</td>
<td>125</td>
</tr>
<tr>
<td>Type I cat</td>
<td>None</td>
<td>0.1 M KPi, pH 7.7</td>
<td>2.9</td>
<td>10.7</td>
</tr>
<tr>
<td>Type III cat</td>
<td>None</td>
<td>0.1 M NaPi, pH 8.5</td>
<td>1.8</td>
<td>2.4</td>
</tr>
<tr>
<td>Type III cat</td>
<td>6 moles of DTT per mole of Hb</td>
<td>0.1 M KPi, pH 7.7</td>
<td>2.9</td>
<td>100</td>
</tr>
<tr>
<td>Type III cat</td>
<td>Sephadex</td>
<td>0.1 M KPi, pH 6.7</td>
<td>2.0</td>
<td>23.5</td>
</tr>
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</table>

* DTT, dithiothreitol.

**Type II Cat Hemoglobin**—Initial experiments with cat erythrocyte hemolysates (4) reproduced the results reported by Taketa et al. (6) at low pH values (pH 6.5 to 7). The hemolysates possessed a cooperativity of 2.1 at pH 6.5. As predicted by the allosteric model, the cooperativity of this "modified" hemoglobin increased with pH (4), going from 2.1 at pH 6.5 to 3.1 at pH 7.7 (Fig. 3). The results at low pH values were obtained in both 0.1 M potassium phosphate and 0.01 M potassium phosphate-0.2 M sodium chloride. At high pH values the same results were obtained in 0.1 M sodium pyrophosphate and 0.01 M Tris-0.2 M sodium chloride.

**Type III Cat Hemoglobin**—As indicated previously, a 22-day-old hemoglobin preparation, which has been stored as a hemolysate in the oxygen form, has a low cooperativity (approximately 2.0) at both low and high pH values. Dialysis or gel filtration of this hemoglobin does not result in an increase in the cooperativity. Incubating this "aged" hemoglobin for 8 hours in the presence of 6 meq of dithiothreitol results in an increase in the cooperativity at both pH values (Table II).

*p-Hydroxymercuribenzoate-treated Cat Hemoglobin*—Cat hemoglobin was reacted with 1.4 meq of p-HMB. The p-HMB cat hemoglobin (1.4) has a much lower cooperativity than cat hemoglobin at pH 6.7 (Table III) and a slightly lower cooperativity at pH 7.7 (Table IV). When cat hemoglobin was reacted with 2.4 meq of p-HMB the cooperativity at both high and low pH decreased again (Tables III and IV). The $p(O_2)_{50}$ and the Bohr effect decrease as more sulfhydryl groups are reacted. The $p(O_2)_{50}$ change in the low pH range is greater than in the high pH range. Thus treatment of type I hemoglobin with low levels of p-HMB results in oxygenation properties resembling type II hemoglobin; higher levels of p-HMB give properties resembling type III hemoglobin.

**Interconversion of Type I and Type II Hemoglobins**—As both cat hemoglobin types I and II can be obtained from a single cat, there must be some way, either in vivo or in vitro, that the two hemoglobins can be interconverted without the use of external reagents such as p-HMB. We have observed two hemolysates which were originally type II hemoglobin show type I behavior after standing (in one case, 6 hours at 20°C and, in the other case, 6 days at 22°C).

* The abbreviation used is: p-HMB, p-hydroxymercuribenzoate.
TABLE III
Effect of p-HMB on cooperativity of type I cat hemoglobin at low pH

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>Treatment</th>
<th>pH</th>
<th>mg</th>
<th>p(O2)ss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>None</td>
<td>7.0</td>
<td>3.1</td>
<td>10.7</td>
</tr>
<tr>
<td>Type I cat</td>
<td>None</td>
<td>6.7</td>
<td>2.9</td>
<td>31.1</td>
</tr>
<tr>
<td>Type II cat</td>
<td>None</td>
<td>6.7</td>
<td>2.1</td>
<td>34.0</td>
</tr>
<tr>
<td>Human</td>
<td>2 moles of N-ethylmaleimide per mole of hemoglobin</td>
<td>7.0</td>
<td>2.0</td>
<td>4.3</td>
</tr>
<tr>
<td>Type I cat</td>
<td>1.4 moles of p-HMB per mole of hemoglobin</td>
<td>6.7</td>
<td>2.1</td>
<td>19.2</td>
</tr>
<tr>
<td>Type I cat</td>
<td>2.4 moles of p-HMB per mole of hemoglobin</td>
<td>6.7</td>
<td>1.6</td>
<td>14.8</td>
</tr>
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</table>

TABLE IV
Effect of p-HMB on cooperativity of type I cat hemoglobin at high pH

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>Treatment</th>
<th>pH</th>
<th>mg</th>
<th>p(O2)ss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>None</td>
<td>9.0</td>
<td>3.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Type I cat</td>
<td>None</td>
<td>7.7</td>
<td>2.9</td>
<td>10.7</td>
</tr>
<tr>
<td>Type II cat</td>
<td>None</td>
<td>7.7</td>
<td>1.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Human</td>
<td>2 moles of N-ethylmaleimide per mole of hemoglobin</td>
<td>9.0</td>
<td>1.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Type I cat</td>
<td>1.4 moles of p-HMB per mole of hemoglobin</td>
<td>7.7</td>
<td>2.6</td>
<td>8.6</td>
</tr>
<tr>
<td>Type I cat</td>
<td>2.4 moles of p-HMB per mole of hemoglobin</td>
<td>7.7</td>
<td>1.9</td>
<td>7.8</td>
</tr>
</tbody>
</table>

TABLE V
Sulfhydryl group titrations

Cat types I and II hemolysates contained greater than 95% cat hemoglobin A. Deviations from the mean represent the expected mean error, E, where

\[ E = \frac{\sum |z_i - \bar{z}|}{N(N-1)} \]

and \( z_i \) is the result from the \( i \)th experiment and \( N \) is the total number of experiments.

| Hemoglobin | p-HMB per mole of hemoglobin | No. of titrations | \n|------------|-------------------------------|-------------------|
| Human      | 2.3 ± 0.3                     | 4                 |
| Cat type I | 9.9 ± 0.5                     | 5                 |
| Cat type II| 8.9 ± 0.5                     | 6                 |
| Cat type III| 7.0                          | 1                 |

* Data for type III hemoglobin were taken from Taketa et al. (3, 6).

Fig. 4 (left). The pH dependence of the cooperativity of cat hemoglobin species A and B. The buffers and experimental conditions were as described for Fig. 2.

Fig. 5 (right). The pH dependence of the p(O2)ss of cat hemoglobin species A and B. The buffers and experimental conditions were as described for Fig. 2.

18 hours at 4°C. Type I hemoglobin, present in the form of a hemolysate, does not convert to type II hemoglobin after standing for 12 hours at 20°C. Type I hemoglobin was converted into type II by converting the hemoglobin to the CO form and then removing the CO by photolysis while simultaneously flushing oxygen over the solution.

Properties of A and B Components—The cat hemoglobin species A and B were isolated and the cooperativities were observed as a function of pH. Both hemoglobins exhibited a pH-dependent cooperativity (Fig. 4) and p(O2)ss (Fig. 5) resembling type II hemoglobin. The Bohr effects of both hemoglobins A and B are slightly higher than those of types I and II cat erythrocyte hemolysates (0.65 for hemoglobin A and 0.7 for hemoglobin B).

Titration of Sulfhydryl Groups—To determine the extent of oxidation of the sulfhydryl groups in types I and II hemoglobin A, the reactive sulfhydryls were titrated with p-HMB (16). The results are shown in Table V and indicate an availability of one more —SH group in type I hemoglobin compared to type II. The results shown for type III hemoglobin were taken from Taketa and Morel1 (3) whose hemoglobin preparations appear to have a low cooperativity at all pH values.

Ultracentrifugation—The tetramer-dimer subunit dissociation constant of the carboxy form of type I cat hemoglobin (Kd,carboxy) is much higher than that of many other liganded hemoglobins (Table VI). Lessard (17), Svedberg and Hedemius (18), and Sullivan (5) have also observed a higher dissociation constant for liganded cat hemoglobin. Whereas, in the pH range from 6.5 to 9, the dissociation constant of human carboxyhemoglobin is relatively pH-independent, the Kd,carboxy of cat carboxyhemoglobin is pH-dependent (Fig. 6). The range of maximum change in Kd,carboxy is exactly that region in which the cooperativity and the p(O2)ss...
The observations that the diminished cooperativity found in type III hemoglobin can be reversed by treatment with dithiothreitol groups in the oxygenation properties of hemoglobin comes from all 10 residues are titratable in type I hemoglobin which displays hemoglobin possesses 10 half-cystine residues (7). Apparently, experiments. Both velocity and equilibrium methods were used. The dissociation constant has increased approximately 5-fold. Hydroxyl groups have been oxidized per cat carboxyhemoglobin, hemoglobin at pH 6.7 increases. After approximately 2.4 sulfhydryl groups are oxidized, the dissociation constant of cat carboxyhemoglobin. The dissociation constants of these three forms of globin A have similar dissociation constants to those of type I cat hemoglobin. The results presented indicate here that variations in the oxygenation properties of hemoglobin preparations can occur quite readily either during or prior to isolation of the hemoglobin from the erythrocyte. Conditions under which the blood change most rapidly. Type II cat hemoglobin and cat hemoglobin A have similar dissociation constants to those of type I cat hemoglobin. The dissociation constants of these three forms change identically with pH. As increasing numbers of sulfhydryl groups are oxidized, the dissociation constant of cat carboxyhemoglobin at pH 6.7 increases. After approximately 2.4 sulfhydryl groups have been oxidized per cat carboxyhemoglobin, the dissociation constant has increased approximately 5-fold. Similarly the dissociation of human carboxyhemoglobin increases 5-fold as a result of reaction with 2 Meq of N-ethylmaleimide.

**DISCUSSION**

The results presented indicate here that variations in the oxygenation properties of cat hemoglobin preparations can occur which are related to the presence of free sulfhydryl groups. Cat hemoglobin possesses 10 half-cystine residues (7). Apparently, all 10 residues are titratable in type I hemoglobin which displays high cooperativity throughout the pH range examined. Type II hemoglobin, which has diminished cooperativity in the low pH range (pH 6 to 7), appears to possess only nine titratable sulfhydryl groups. Further evidence for the involvement of sulfhydryl groups in the oxygenation properties of hemoglobin comes from the observations that the diminished cooperativity found in type III hemoglobin can be reversed by treatment with dithiothreitol and reduction of cooperativity of type I hemoglobin can be achieved by reaction with p-HMB. These results raise three principal questions.

1. To what extent is the diminished cooperativity of type II cat hemoglobin the intrinsic property of individual hemoglobin molecules (as opposed to effects derived from protein heterogeneity)?

2. To what extent can the chemical state of the sulphydryl groups of cat hemoglobin be directly related to the characteristics of oxygen binding?

3. To what extent is the pH dependence of the cooperativity of oxygen binding consistent with either of the major theoretical descriptions of cooperative ligand binding (19, 20)?

Concerning the question of possible heterogeneity of type II hemoglobin, the fact that the isolated A and B components (Figs. 4 and 5) display properties very similar to the unfractionated material makes the possibility of heterogeneity unlikely. Only the fractions corresponding to the A and B components were observed during chromatography and only the respective bands were observed in disc gel electrophoresis measurements on the samples. While low cooperativity can arise from heterogeneity, if two species with appreciable differences in affinity are present, the only heterogeneity apparent in type II hemoglobin are the individual A and B components. Since the isolated A and B components also have low cooperativity (n ~ 2.3) at pH 6.5, the most plausible conclusion from the data is that the low cooperativity at low pH is an intrinsic property of the individual hemoglobin molecules present. Therefore, the questions concerning the relation of the low cooperativity to sulphydryl groups and the implications for theoretical models of hemoglobin can be considered.

The correlation between diminished cooperativity and alteration of the chemical state of the sulphydryl groups is strongly indicated by the ability to mimic low cooperativity behavior by treatment with p-HMB (Table III) and the ability to reverse the low cooperativity of spontaneously formed type III hemoglobin by treatment with dithiothreitol. Whether the sulphydryl groups controlling the cooperativity of cat hemoglobin at low pH values are specific groups and whether the effect is physiologically significant must also be considered. The large effect on the cooperativity after oxidation of only one to two sulphydryl groups would suggest that this sulphydryl is strategically placed and extremely accessible to oxidizing agents. If the effect is physiological, one would expect to find enzymes responsible for the oxidation and reduction of these groups. The enzymes should be present in the hemolysate but not necessarily in the isolated hemoglobin preparations. The cooperativity of the hemolysate at pH 6.5 falls rapidly (within a week) whereas it takes much longer for the cooperativity of the A and B components to fall. Although the difference between the hemolysate and the isolated components could be attributed to the fact that the former was stored in the oxy form under air whereas the latter was stored in the CO form under an atmosphere of CO, the difference in stability between hemoglobins A and B cannot be dismissed as readily. The cooperativity of the B component decreases linearly from the time of preparation whereas the cooperativity of the A component appears to have decreased after an apparent lag of 11 days. Perhaps this indicates a difference in the amount of sulphydryl oxidizing or reducing enzymes or agents present in the hemoglobin A and B preparations. In addition it is not clear whether type I or type II is the native form since modifications apparently can occur quite readily either during or prior to isolation of the hemoglobin from the erythrocyte.

**TABLE VI**

<table>
<thead>
<tr>
<th>Carboxyhemoglobin</th>
<th>$K_d^o \times 10^6$</th>
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</thead>
<tbody>
<tr>
<td>Cat</td>
<td>16.9 ± 4.7</td>
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<tr>
<td>Cow</td>
<td>2.6</td>
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<tr>
<td>Dog</td>
<td>1.2</td>
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<tr>
<td>Guinea pig</td>
<td>1.3</td>
</tr>
<tr>
<td>Human</td>
<td>1.8 ± .51</td>
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<td>Llama</td>
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<td>Rabbit</td>
<td>1.3</td>
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<td>Sheep</td>
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</tbody>
</table>

![Fig. 6](image)

The pH dependence of the tetramer-dimer subunit dissociation constant of type I cat and human carboxyhemoglobin.

- •, data for cat hemoglobin; ○, data for human hemoglobin.
- Buffers were as described in Fig. 2. Temperatures for the sedimentation velocity runs ranged from 17-24°C. All S values were corrected for viscosity and temperature. The temperature of the sedimentation equilibrium runs was always 20°C. Each point represents the average of several values.
was withdrawn from the cat have been investigated for possible
effects on cooperativity of the resulting hemoglobin preparation.
The presence or absence of two widely differing anaesthetics,
phenobarbital and ketamine, has no effect on the state of the
resulting hemoglobin. Citrate, the anticoagulant used when
early samples of type II hemoglobin were obtained, does not
affect the state of the resulting hemoglobin and does not lower
the cooperativity of isolated cat hemoglobin when present during
the oxygen equilibrium experiment. Both the cat hemoglobin
A and B usually exhibit the type II behavior. The possible
physiological significance of the sulfhydryl effects are still
unclear.

Taketa and Morell (3) reported that both cat hemoglobins A
and B and the cat erythrocyte hemolysates have a cooperativity
of about 2.0 at pH 6.7 and 7.3. These hemoglobin preparations
must then be similar to the type III hemoglobin and p-HMB
(2.4) cat hemoglobin. If modification occurs by oxidation then
the number of reactive sulfhydryl groups present in the hemo-
globin preparations of Taketa et al. (6) should be lower than the
number present in type I cat hemoglobin preparations. Taketa
et al. (6) showed that cat erythrocyte hemolysates and isolated
cat hemoglobins A and B possess eight reactive sulfhydryl groups
per molecule of hemoglobin and also stated that the number of
reactive sulfhydryl groups per mole of isolated A and B hemo-
globin does not change over a period of 2 months when the hemo-
globins are stored in the carboxy form. The total number of
half cystine present in cat hemoglobin A and B has been deter-
mined to be 10 (7). The two unreactive sulfhydryl groups are
not exposed in the presence of 6 M guanidine hydrochloride or 8 M
urea (8). Perhaps these are the oxidized sulfhydryl groups which
contribute to the lowering of the cooperativity of the hemoglobin.
We have shown that cat hemoglobins I and II have, respectively,
10 and 9 reduced sulfhydryl groups. These results would also
explain the large variation in cooperativities at low pH of cat
hemoglobins reported by different investigators (5, 6). Methods
of preparation and postpreparative treatment of the hemoglobin
and perhaps even the condition of the donor cat may have an
effect on the final state of the hemoglobin preparation. Thus,
according to this interpretation, Taketa et al. (6) were working
with type III hemoglobin and Sullivan (5) with type I hemo-
globin.

The oxygenation data for the three types of hemoglobin were
evaluated in terms of the bell shaped dependence of cooperativity
on affinity predicted by the two-state model of Monod et al. (19).
The actual form of the dependence can be expressed by relating
cooperativity to $L$, the allosteric constant (10), where $L$ is esti-
mated either from $p(O_2)^{1/2}$ (21) or the ratio of dissociation con-
stants of liganded and unliganded hemoglobin (22, 23). Values
for the dissociation constant of unliganded cat hemoglobin were
determined with the CO titration method (22, 24). Results of
fitting oxygenation data in the form of $n$, the index of cooper-
ativity, versus log $L$ are shown in Fig. 7, A and B. Cat hemo-
globin type II, as described earlier (4), and type III fit reason-
ably well to single bell curves (Fig. 7A) with characteristic values of $c$.
the relative affinities of the two states. In contrast, type I cat
hemoglobin shows less variation in cooperativity than predicted
by the two-state model for the range of $L$ values calculated (Fig.
7B). The values appear to lie between two bell curves with a
4-fold difference in the value of $c$. Rumm and Guidotti (25) also
observed an approximate but imperfect fit of a wide range of data
for human hemoglobin to a bell curve with a single value of $c$.
In contrast the sequential model of Koshland et al. (20) would
predict an increase in cooperativity with increasing $p(O_2)^{1/2}$.
Cat Hemoglobin: pH DEPENDENCE OF COOPERATIVITY AND LIGAND BINDING
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