Studies on Cat Hemoglobin and Hybrids with Human Hemoglobin A*

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ABSTRACT: The oxygen affinity of cat hemoglobin is considerably lower than that of other mammals, including man. The major and minor hemoglobins found in the laboratory cat differ from other mammalian hemoglobins in the number of "reactive" sulfhydryl groups; treatment with *p*-mercuribenzoate or *N*-ethylmaleimide has shown that both cat hemoglobins contain eight sulfhydryl groups, all of which are reactive. This is the largest number of reactive cysteines found in normal mammalian hemoglobins. Stepwise alkylation with *N*-ethylmaleimide results in progressively higher oxygen affinity and lower Bohr effect but no significant change in the Hill interaction constant. The major cat hemoglobin has been hybridized with human hemoglobin to obtain $\alpha_2^{A}\beta_2^{cat}$ and $\alpha_2^{cat}\beta_2^{A}$. Oxygen equilibria were compared with those of the parent hemoglobins to determine whether the β chain contributed predominantly to this function. The number of reactive sulfhydryl groups in the hybrids was also determined. No correlation was found between the oxygen affinity of the hybrids and that of the parent that provided the β chain. There was also no correlation between the number of reactive SH groups in the hybrids and their oxygen affinity; *i.e.*, $\alpha_2^{cat}\beta_2^{A}$ contained eight reactive SH groups and exhibited a higher oxygen affinity than $\alpha_2^{A}\beta_2^{cat}$, which contained only two.

It was concluded that the functional properties of a hemoglobin depend upon both the structure of and specific interactions between the α and β chains.

The three-dimensional conformations of mammalian hemoglobins[1] are probably similar (Cullis *et al.*, 1962; Perutz, 1965; Perutz *et al.*, 1965). However relatively large differences in oxygen equilibria are commonly observed. Two hemoglobins have been found in the laboratory cat, a major and a minor component, which are of particular interest because they exhibit considerably lower oxygen affinities than the hemoglobin of other mammals, including man (Taketa and Morell, 1966; Bartels and Harms, 1959). These differences in oxygen equilibria provide a basis for studies on structures that may govern the functional properties of the molecule. A comparison of the structure of cat hemoglobin with other species may help elucidate features that are involved in the control of oxygen affinity. The complete sequence of the α and β chains of a number of hemoglobins is now known and progress is being made on the determination of the primary structure of other hemoglobins (Braunitzer *et al.*, 1964; Babin *et al.*, 1966; Boyer *et al.*, 1967). Aside from data on tryptic fingerprint patterns (Mäsiar *et al.*, 1958; Mäsiar and Jurovcik, 1959), very little is known about cat hemoglobin and studies are presently in progress in this laboratory to determine its primary structure.

The "reactive" SH groups of various hemoglobins have long been of interest with regard to their possible functional role and a great deal of work has been reported on their number, reactivity, and modification. Although recent data suggest that they are not directly involved in the Bohr effect (Benesch and Benesch, 1961) or in heme–heme interaction (Taylor *et al.*, 1963), it may be significant that oxygen affinity generally increases extensively when these groups are blocked (Riggs, 1952, 1961; Riggs and Wolbach, 1956; Benesch and Benesch, 1961; Taylor *et al.*, 1963). It is hence possible that reactive cysteines may in some manner be involved in the control of oxygen affinity. Since cat hemoglobin exhibits an unusually low oxygen affinity, it was of interest to determine the number of reactive cysteines and the effect on oxygen equilibrium when they are blocked with thiol reagents.

The formation of hybrids (Itano and Robinson, 1959; Singer and Itano, 1959) provides a means to investigate the relative role of the α and β chains in the functional properties of a hemoglobin. Such a study was initiated by Riggs and Herner (1962) with hybrids of mouse and donkey hemoglobins. These hemoglobins were selected because they differed widely in their oxygen equilibria; that of the mouse exhibited a relatively lower oxygen affinity and higher Bohr effect than that of the donkey. The functional properties of each of the isolated hybrids, $\alpha_2^{M}\beta_2^{D}$ and $\alpha_2^{D}\beta_2^{M}$, resembled the parent molecule that provided the β chain and these investigators concluded that the Bohr effect and oxygen affinity were controlled principally by the β chain. Antonini *et al.* (1965), however, subsequently

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[1] Abbreviations used: Hb, hemoglobin; PMB, *p*-mercuribenzoate; NEM, *N*-ethylmaleimide.

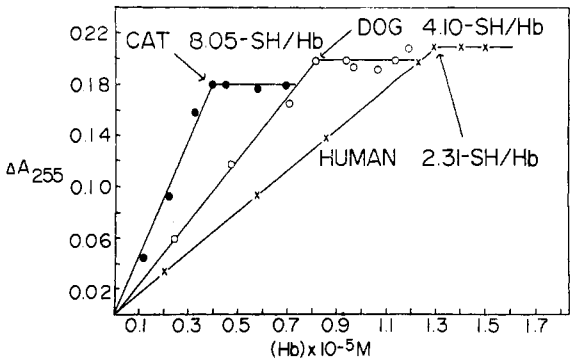

FIGURE 1: PMB titration of human, dog, and cat hemoglobins. Increments of 2.25 × 10^{-5} M solutions were mixed with 1 ml of a 1.25–1.50 × 10^{-4} M solution of PMB and diluted to a final volume of 5 ml in 0.015 M phosphate buffer (pH 7.0). The increase in absorbance at 255 mμ was then measured spectrophotometrically. PMB concentration was calculated from the extinction coefficient, 1.69 × 10^4 at 232 mμ (Boyer, 1954). SH/Hb was based upon a molecular weight of 66,000.

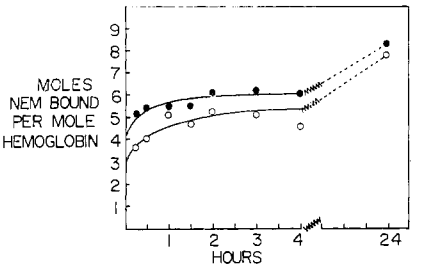

FIGURE 2: NEM uptake by oxygenated and deoxygenated cat hemoglobin. (●) Oxyhemoglobin and (○) deoxyhemoglobin. Hemoglobin (4 ml of 1.2 × 10^{-4} M) was treated with 1 ml of 0.01 M NEM solution in 0.14 M phosphate buffer (pH 6.8) for the indicated time periods. The reaction was stopped by adding 5 ml of 10% perchloric acid. After centrifugation, the supernatant was analyzed spectrophotometrically at 300 mμ. NEM bound per mole was calculated as described by Morell *et al.* (1962).

reported that the oxygen equilibria of human–dog hybrids differed considerably from one another even though the parent molecules were practically identical. They concluded that the functional characteristics of a hemoglobin could not be ascribed solely to the contributions of the β chain. No subsequent reports on interspecies hybrids have appeared and the large difference in oxygen affinity between human and cat hemoglobins provided an interesting pair to ascertain whether or not the β chain could determine this functional parameter.

Experimental Procedure

Hemoglobins. Human and cat hemoglobin solutions were prepared from freshly drawn blood as described previously (Taketa and Morell, 1966) and stored in the form of the CO derivative at 4° until used. Hemoglobin concentrations were determined spectrophotometrically as oxyhemoglobin, using molar extinction coefficients 5.6 × 10^4 at 540 mμ and 5.9 × 10^4 at 576 mμ (Benesch and Benesch, 1961) or as a cyanmethemoglobin, ϵ_{540} 4.6 × 10^4 (Drabkin, 1946). The major and minor cat hemoglobins were separated by chromatography on a 3 × 25 cm IRC-50 column using 0.05 M phosphate buffer (pH 6.7).

SH Determinations. Spectrophotometric titrations with PMB (Boyer, 1954) were carried out by addition of increments of hemoglobin to a standard solution of the reagent as described by Benesch and Benesch (1961). Reaction with NEM was conducted at pH 6.8 and with an NEM:SH ratio of 3.5. For reaction under anaerobic conditions, the hemoglobin solution was first gassed with nitrogen in a rotating tonometer, a deoxygenated NEM solution was added, and the reaction was allowed to proceed under nitrogen in the tightly stoppered tonometer. The reactions were terminated by precipitating the protein with an equal volume of 10% perchloric acid, and the solution was centrifuged. The extent of reaction was calculated from absorbancy measurements at 300 mμ (Morell *et al.*, 1962).

Partial alkylation with NEM was achieved by stopping the reaction with an excess of cysteine. After exhaustive dialysis, aliquots were taken for PMB titration and oxygen equilibrium measurements.

Preparation of Hybrids. Equal volumes of 2.5% aqueous solutions of human and cat CO-hemoglobins were mixed and dialyzed at 4° for approximately 20 hr against 0.2 M acetate buffer (pH 4.7). The solution was then dialyzed against several changes of 0.014 M phosphate buffer (pH 6.90) or 0.05 M Tris-HCl buffer (pH 8.15) for 24 hr. Analytical starch gel electrophoresis was conducted at 4° in Tris–EDTA–borate (pH 8.4) and the hemoglobins were visualized with benzidine (Sunderman, 1964). The subunit makeup of the HbA–Hbcat hybrids was established by comparison with a similar hybridization mixture of HbS–Hbcat. In addition, peptide maps of tryptic digests (Baglioni, 1961) of the hybrids purified by column chromatography were compared with maps of the isolated α^A, α^{cat}, β^A, and β^{cat} chains. $\alpha_2{}^A\beta_2{}^{cat}$ was readily isolated on a preparative scale at 4° from a 3 × 20 cm column of DEAE-cellulose (S & S, type 40) equilibrated with 0.05 M Tris-HCl (pH 8.15) (Huisman and Dozy, 1965). Elution was carried out with the same buffer. Under these conditions, HbA_2 was eluted slightly ahead of $\alpha_2{}^A\beta_2{}^{cat}$, whereas the other components were firmly bound. Rapid isolation of $\alpha_2{}^{cat}\beta_2{}^A$ was also achieved by passing the mixture through a 3 × 20 cm CM-cellulose (Whatman CM 11) column using 0.014 M phosphate buffer (pH 6.90); this hybrid was also

readily eluted ahead of the other components. The purity of the fractions was determined by starch gel electrophoresis.

These procedures led to the isolation of each hybrid in a few hours in sufficiently high concentration (5–10 mg/ml) for oxygen equilibrium measurements. The solutions were treated with CO and dialyzed at 4° against 0.014 M phosphate buffers (pH 6.8 or 7.3) for subsequent measurements.

Fingerprints. Lyophilized preparations were dissolved in water and digested with trypsin (Worthington two-times crystalline, salt free) using a Radiometer pH-Stat for maintaining pH 8. After precipitation and removal of the undigested "core" at pH 6.5, the supernatant was concentrated in a vacuum desiccator. Appropriate aliquots were then taken for preparing peptide maps as described by Baglioni (1961).

Oxygen equilibria were determined by the spectrophotometric method of Rossi-Fanelli and Antonini (1958) using hemoglobin concentrations of 7 ± 2 mg/ml and a light path of 1 mm. A Beckman DB spectrophotometer, with temperature control at 20°, and a Sargent SRL linear log recorder were used for spectral evaluation of per cent saturation at 540, 560, and 576 mμ (Riggs, 1951). Prior to determining oxygen equilibria, the carbon monoxide was displaced with oxygen as described by Antonini *et al.* (1965).

TABLE I: Effect of NEM Modification on the Oxygen Equilibrium of Cat Hemoglobin.[a]

Moles of NEM Bound/ Mole of Hb[b]	pH	$pO_{1/2}$[c]	Hill Constant[d]	Bohr Effect[e]
0	6.89	22.3	2.1	
	7.30	14.4	2.3	0.46
0.09	6.83	19.7	2.0	
	7.18	13.9	2.2	0.43
3.2	6.82	15.3	1.8	
	7.30	9.8	1.9	0.40
4.3	6.83	6.8	2.0	
	7.25	5.2	2.1	0.27
4.8	6.85	5.5	2.0	
	7.29	4.5	2.1	0.20
5.9	6.80	2.3	2.2	
	7.33	1.8	2.1	0.20

[a] All measurements at 20°. [b] Determined by PMB titration; *i.e.*, 8 − moles of PMB bound/Hb after NEM reaction. [c] $pO_{1/2}$ = partial pressure of oxygen (millimeters) at half-saturation. [d] Hill constant, n = d (log (Y/(1 − Y)))/d log $pO_{1/2}$. [e] Bohr effect per heme = −(Δ log $pO_{1/2}$/ΔpH).

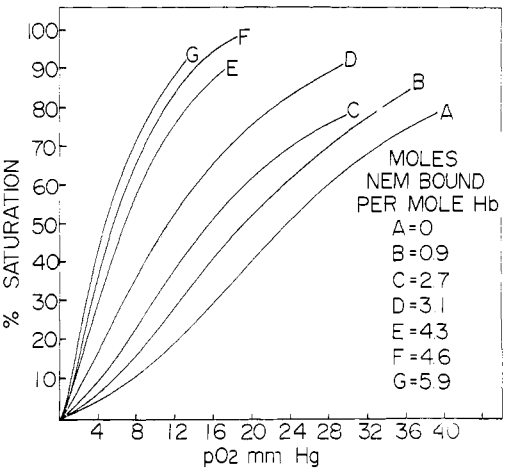

FIGURE 3: Oxygen saturation curves of NEM-modified cat hemoglobins. Solutions (2 ml of 0.5–0.8%) in 0.15 M phosphate buffer (pH 6.8) were deoxygenated and equilibrated with increments of air. The degree of oxygenation was measured spectrophotometrically at 20°.

Results

The PMB titrations of cat, dog, and human hemoglobins are shown in Figure 1, the number of reactive SH groups per molecular weight 66,000 being approximately 8, 4, and 2, respectively. The values obtained for dog and human are in agreement with published reports (Snow, 1962). Both major and minor cat hemoglobins exhibit eight PMB-titratable SH groups. This value was also obtained in 8 M urea and 6 M guanidine hydrochloride, indicating that all of the SH groups are reactive.

Figure 2 shows the data obtained for the reaction of NEM with oxy- and deoxy-Hbcat. Six of the PMB-reactive cysteines of oxy-Hbcat are rapidly alkylated, whereas the remaining two react quite slowly. The slower rate observed for the deoxyhemoglobin is qualitatively similar to that reported for HbA (Riggs, 1961; Morell *et al.*, 1962; Benesch and Benesch, 1962) and presumably reflects a change in SH reactivity associated with conformational changes on oxygenation.

Figure 3 shows the oxygen saturation curves of cat hemoglobin at pH 6.8 and the effect of stepwise modification with approximately 1–6 equiv of NEM. A progressive rise in oxygen affinity occurs with increased alkylation of the protein. In Table I the data obtained in the pH range 6.80–7.33 are summarized. Although there was a progressive increase in oxygen affinity on alkylation with 6 equiv of NEM, the value of n remained constant. The Bohr effect decreased to approximately half the value of the unreacted molecule. On further alkylation with NEM, higher oxygen affinities were observed but the products were unstable and frequently precipitated.

Figure 4 shows the starch gel electrophoresis of mixtures of HbA, HbS, Hbcat, and their hybrids. It is evident that cat and human hemoglobins hybridize

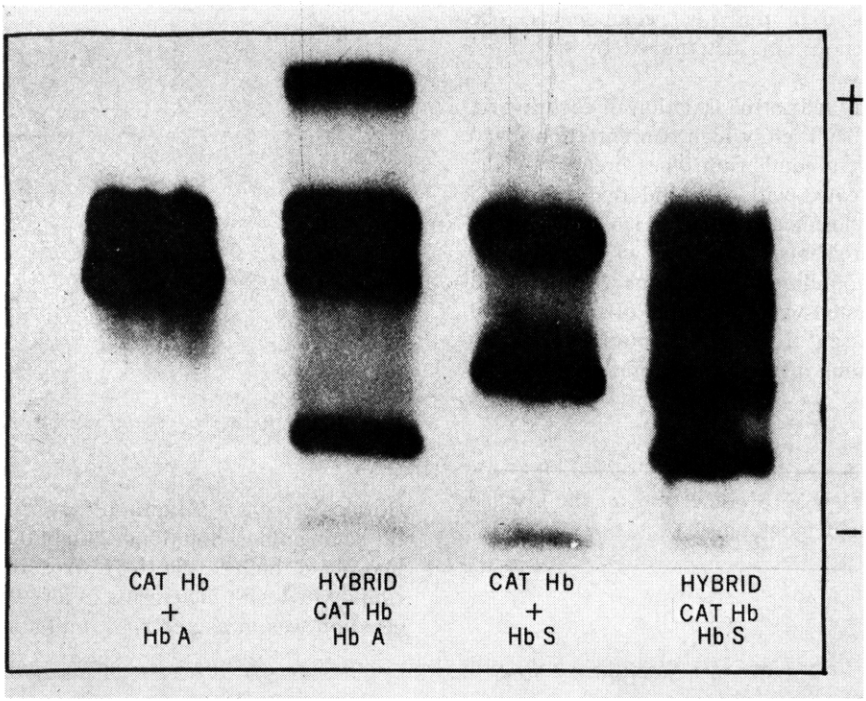

FIGURE 4: Starch gel electrophoresis mixtures of HbA + Hbcat, HbS + Hbcat, and their hybrids. Electrophoresis was carried out in Tris–EDTA–borate (pH 8.4) for 16 hr.

readily to form approximately equal amounts of the hybrids and parents. As reported for HbA–Hbdog hybrids (Antonini *et al.*, 1965), the mobilities of the HbA–Hbcat hybrids differ considerably from the parent molecules. Since HbA is $\alpha_2^A\beta_2^A$ and HbS is $\alpha_2^A\beta_2^{6\,Val}$ (Hunt and Ingram, 1959), the hybrid common to both HbA–Hbcat and HbS–Hbcat, which was found to exhibit the slowest anodic mobility, must be $\alpha_2^A\beta_2^{cat}$. The hybrids with the greater mobilities formed from HbA or HbS, must then be $\alpha_2^{cat}\beta_2^A$ and $\alpha_2^{cat}\beta_2^S$, respectively. The latter, $\alpha_2^{cat}\beta_2^S$, exhibits a relatively slower mobility in accordance with the Glu→Val substitution in the β^S chain.

Figure 5 shows the starch gel electrophoresis of $\alpha_2^A\beta_2^{cat}$ and $\alpha_2^{cat}\beta_2^A$ after chromatography on DEAE- and CM-celluloses, respectively. A comparison with HbA, Hbcat, and the hybridization mixture before chromatography is also shown. No significant differences in spectra were observed for HbA, Hbcat, or their purified hybrids. The methemoglobin concentration of the hybrids, and of the parent hemoglobins carried through the hybridization procedure, was less than 3%.

The hybrids shown in Figure 5 were obtained from the major cat hemoglobin. When the minor component was used, the electrophoretic pattern of the hybrid mixture exhibited an identical $\alpha_2^{cat}\beta_2^A$ and a faster $\alpha_2^A\beta_2^{cat}$ component, suggesting that the two hemoglobins have identical α and different β chains. A comparison of the tryptic fingerprints of the isolated chains also showed differences in only the β subunits (J. L. Lessard and F. Taketa, unpublished data). The PMB titration data obtained for the parent hemoglobins and their hybrids are shown in Table II. HbA exhibits two reactive cysteines which have been identified at position 93 of the β chains, the β^{112} and α^{104} cysteines being relatively inert to thiol reagents (Allison and Cecil, 1958; Braunitzer *et al.* 1961; Guidotti and Konigsberg, 1964). Hbcat exhibits eight reactive cysteines. The $\alpha_2^A\beta_2^{cat}$ hybrid resembles HbA in its SH reactivity, approximately 2 moles of PMB being bound. Since the α^{104} cysteines of HbA are unreactive in the parent tetramer, the two reactive SH groups found in the hybrid $\alpha_2^A\beta_2^{cat}$ are probably present in the β^{cat} chains. The other hybrid ($\alpha_2^{cat}\beta_2^A$), like Hbcat, also binds 8 moles of PMB. The PMB bound by the hybrids thus indicates that the distribution of the 8 reactive SH groups in Hbcat is probably 6 and 2 in the α_2^{cat} and

TABLE II: Reactive SH Groups of Cat and Human Hemoglobins and Their Hybrids.

Hemoglobin	Moles of PMB Bound/Mole of Hb
Cat $\alpha_2^{cat}\beta_2^{cat}$	8.1
Human $\alpha_2^A\beta_2^A$	2.3
$\alpha_2^A\beta_2^{cat}$	1.8
$\alpha_2^{cat}\beta_2^A$	7.7

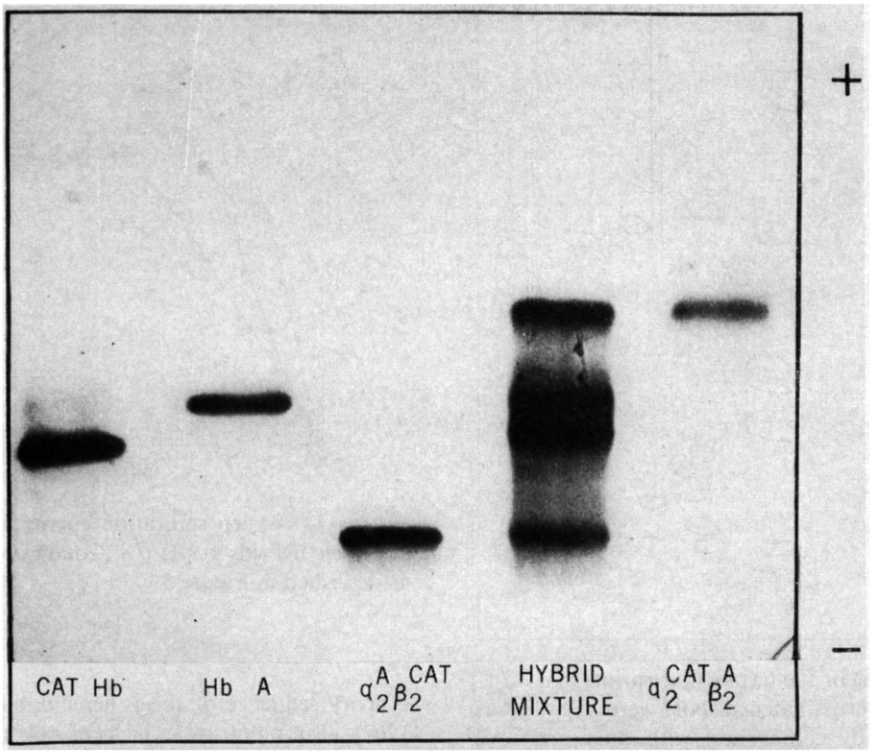

FIGURE 5: Starch gel electrophoresis of Hbcat, HbA, and their hybrids isolated by column chromatography. The hybridization mixture prior to chromatography is also shown. Electrophoresis was carried out in Tris–EDTA–borate (pH 8.4) for 16 hr.

β_2^{cat} chains, respectively. The data suggest a pattern of similar conformation and SH reactivity for the parent molecules and their hybrids.

Tryptic peptide maps of the purified hybrids are shown in Figure 6. The open-circled peptides were identified by comparison with maps of the α^{cat} and β^{cat} chains isolated by CM-cellulose chromatography (Dintzis, 1961). The fingerprints verify the subunit composition of the hybrids determined by starch gel electrophoresis.

The oxygen equilibria of Hbcat, HbA, and their hybrids in the pH range 6.7–6.8 are shown in Figure 7. As previously reported (Taketa and Morell, 1966), both the major and minor components of Hbcat exhibit a much lower oxygen affinity than HbA. The oxygen affinity of $\alpha_2^{cat}\beta_2^A$ is higher than that of both HbA and Hbcat, whereas the other hybrid ($\alpha_2^A\beta_2^{cat}$) resembles that of HbA. A similar relationship was observed at pH 7.3. Hill plots and values of n for the HbA–Hbcat hybrids obtained in the pH range 6.81–7.31 are shown in Figure 8. The constants, 2.0 ± 0.2, are the same as that found for the parent cat molecule. Table III summarizes the data on $pO_{1/2}$, n, and the Bohr effects in the pH range 6.81–7.31 for hemoglobins prepared in three or more separate experiments. The Bohr effect calculated from these data, approximately 0.50/heme, is also approximately the same as that found for Hbcat. Both hybrids thus exhibit values of n and Bohr effects which are very similar to the Hbcat parent but oxygen affinities which are considerably higher. In comparison with the HbA parent, however, the n values of both hybrids are somewhat lower, whereas the oxygen affinity of one hybrid ($\alpha_2^A\beta_2^{cat}$) is slightly lower and that of the other ($\alpha_2^{cat}\beta_2^A$) slightly higher.

TABLE III: Oxygen Equilibrium of Hbcat, HbA, and Their Hybrids.[a]

Hemoglobin	pH	$pO_{1/2}$[b]	Hill Constant[c]	Bohr Effect[d]
Cat[e]	6.87	20.1	1.8	
	7.17	14.2	2.0	0.49
Human[e]	6.83	10.0	2.8	
	7.20	6.8	2.3	0.45
$\alpha_2^{cat}\beta_2^A$	6.81	7.7	1.9	
	7.31	4.3	2.2	0.51
$\alpha_2^A\beta_2^{cat}$	6.83	11.4	2.0	
	7.23	7.3	1.9	0.49

[a] Figures represent averages based on three or more separate determinations at 20°. [b] $pO_{1/2}$ = partial pressure of oxygen (millimeters) at half-saturation. [c] Hill constant, n = d (log (Y/(1 − Y)))/d log $pO_{1/2}$. [d] Bohr effect per heme = −(Δ log $pO_{1/2}$/ΔpH). [e] The parent hemoglobins were exposed to conditions of dissociation–recombination.

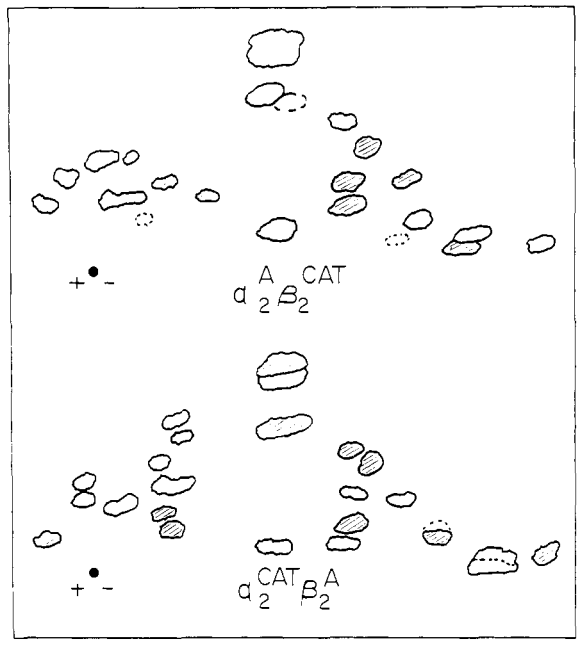

FIGURE 6: Drawing of the tryptic fingerprints of $\alpha_2^A\beta_2^{cat}$ and $\alpha_2^{cat}\beta_2^A$. The cross-hatched spots were identified as human peptides by comparison with fingerprints of HbA. The open spots were identified as cat hemoglobin peptides by comparison with fingerprints of isolated α^{cat} and β^{cat} chains.

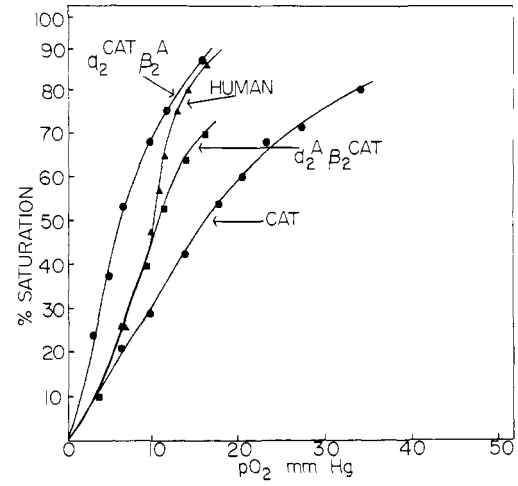

FIGURE 7: Oxygen saturation curves for HbA, Hbcat, and their hybrids at pH 6.8. Conditions were the same as described in Figure 3.

Discussion

Cat hemoglobin is unique in containing eight reactive cysteines. This is the highest number thus far found in normal mammalian hemoglobins including mouse, rat, rabbit, dog, sheep, horse, ox, and man, which contain only two to four (Snow, 1962). It has been reported (Riggs, 1964) that the reactive SH groups of human, horse, donkey, and mouse hemoglobins are found in the β chains. Our results, however, show that the α^{cat} chains, as well as the β^{cat} chains, contain reactive SH groups. Moreover, each of the α^{cat} chains contains three SH groups which is in accord with the fact that the hybrid $\alpha_2^{cat}\beta_2^A$ contains eight reactive cysteines. Radioautograms of tryptic fingerprints of [^{14}C]NEM-reacted cat hemoglobins have confirmed the presence of three NEM-binding sites in the α^{cat} chains. The data suggest that each of the β^{cat} chains, like the human β^A chains, contains only one reactive cysteine. The high content and position of the reactive cysteines in cat hemoglobin suggested the possibility that they might be related to the relatively low oxygen affinity of this species.

It is apparent from our study of the Hbcat–HbA hybrids that the number and position of the reactive cysteines *per se* cannot be the sole determinant of oxygen affinity. The $\alpha_2^{cat}\beta_2^A$ hybrid, for example, like the parent cat hemoglobin, contains eight reactive SH groups and shows a relatively high oxygen affinity, even exceeding that of HbA. Recent studies on the primary sequence of sheep hemoglobins (Boyer *et al.*, 1967) also point to a lack of relationship between oxygen affinity and the number of reactive SH groups. Sheep hemoglobins A, B, and C possess identical α chains and differ only in their β chains. The sheep hemoglobins contain a total of four cysteines, all of which are reactive. Each of the α^{sheep} chains contains one reactive cysteine. Each of the three β chains also contains one reactive cysteine, which like β^{93} of HbA, is located at position 93. Only two of the three sheep hemoglobins (A and C), however, exhibit an identical oxygen affinity which is considerably higher than that of the third (sheep B). Since all three sheep hemoglobins contain

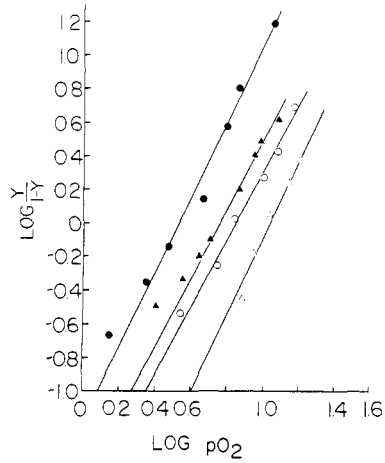

FIGURE 8: Oxygen equilibria of $\alpha_2^{cat}\beta_2^A$ and $\alpha_2^A\beta_2^{cat}$ in the pH range 6.8–7.3. $\alpha_2^A\beta_2^{cat}$: (△) pH 6.8, n = 2.0; (▲) pH 7.3, n = 1.9. $\alpha_2^{cat}\beta_2^A$: (○) pH 6.8, n = 1.9; (●) pH 7.3, n = 2.2. Conditions for measurements were the same as in Figure 3.

four reactive cysteines at the same positions, these groups alone apparently do not determine the oxygen affinity.

The increase in oxygen affinity which occurs on stepwise NEM alkylation of cat hemoglobin may be associated with a progressive conformational change in the protein. When less than 3 equiv of NEM are bound, the Bohr effect is retained but when 3–6 equiv are bound it is reduced by about 15–50%. However, up to and including the binding of 6 equiv of NEM, oxygenation-linked conformational changes are maintained as shown by retention of the Hill interaction constant, 2.0 ± 0.2 (Table I). These modified hemoglobins also retain their ability to hybridize readily with HbA and it appears that the reagent induces only relatively small structural changes. It has been reported that NEM alters the structure of the α and β chains in HbA (Perutz, 1964).

The ease with which HbA and Hbcat hybridize suggests a fundamental similarity in their α and β chains and provides another pair of interspecies hybrids for studies on the relative roles of α and β chains in the functional properties of a hemoglobin. The oxygen equilibria of the hybrids are not readily correlated with the parent hemoglobins. In the pH range studied (6.8–7.3), $pO_{1/2}$ for the $\alpha_2^A\beta_2^{cat}$ hybrid is practically identical with that of HbA, while that for $\alpha_2^{cat}\beta_2^A$ is much lower than that of either parent. The oxygen affinities of the hybrids do not resemble the parent which provides the β chain. These results, like those obtained by Antonini *et al.* (1965) for the HbA–Hbdog system, are not in accord with the β-chain hypothesis of Riggs and Herner (1962). It may be significant, however, that the higher oxygen affinity of HbA, in comparison with that of Hbcat, is expressed in the corresponding β chains of the hybrids; *i.e.*, $\alpha_2^{cat}\beta_2^A$ exhibits a higher oxygen affinity than $\alpha_2^A\beta_2^{cat}$. Although the hybrids differ from their parents in $pO_{1/2}$, they retain sigmoid equilibrium curves and Bohr effects and they exhibit values of n which are similar to Hbcat. These hybrids hence retain the structural and conformational characteristics associated with the functional properties of a hemoglobin. It has been suggested that these properties depend upon the degree to which the molecule undergoes conformational change with ligand binding (Wyman and Allen, 1951; Benesch and Benesch, 1963; Zito *et al.*, 1964). Hemoglobins which are unable to undergo such conformational change lack a Bohr effect, exhibit a hyperbolic oxygen equilibrium curve, and have a value of n close to 1.0. Examples of such hemoglobins are those containing only one chain (Benesch *et al.*, 1961; Antonini *et al.*, 1965) and carboxypeptidase A modified HbA (Antonini *et al.*, 1961). It has been suggested (Antonini *et al.*, 1965) that strong specific interactions between α and β chains may be the basis for the characteristic functional properties of hemoglobins. Retention of heme–heme interaction and the Bohr effect in the hybrids, HbA–Hbcat, HbA–Hbdog (Antonini *et al.*, 1965), and Hbmouse–Hbdonkey (Riggs, 1962), indicate that heterologous α and β chains interact in a manner which permits normal oxygenation-linked conformational changes. Our results, as well as those of Riggs and Herner (1962) and Antonini *et al.* (1965), support the general conclusion that the functional properties of a hemoglobin are determined by both the structure of and specific interactions between the α and β chains.

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Biosynthesis and Studies of the Alkaline Sensitivity of the *NO*-Glucuronide of the Carcinogen *N*-2-Fluorenylacethydroxamic Acid*

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ABSTRACT: Because of its occurrence as a major metabolite of the carcinogen *N*-2-fluorenylacetamide in susceptible species, the *NO*-glucuronide of *N*-2-fluorenylacethydroxamic acid was needed for studies of its reactivity and carcinogenic properties. The desired product has been isolated from the urine of rabbits fed *N*-2-fluorenylacetamide and has been crystallized and characterized as sodium (*N*-acetyl-*N*-2-fluorenylhydroxylamine β-D-glucosid)uronate. This unusual type of glucuronide (C–O–N linkage) is alkali labile. Studies on the mechanism of the instability under alkaline conditions suggest migration of the *N*-acetyl group of the aglycon to the 2′-hydroxyl group of the glucuronic acid moiety, followed by hydrolysis of the glycosidic linkage to yield initially *N*-2-fluorenylhydroxylamine and, as an end product, 2,2′-bisazoxyfluorene.

Sodium (*N*-acetyl-*N*-2-fluoren[9-^{14}C]ylhydroxylamine β-D-glucosid)uronate has also been prepared biosynthetically.

A general metabolic reaction of aromatic amides and amines (reviewed in Miller and Miller, 1966), *N*-hydroxylation, initially discovered during studies on the metabolism of the carcinogen *N*-2-fluorenylacetamide (Cramer *et al.*, 1960), leads to the excretion of a novel type of alkali-labile glucuronide. The structure of the glucuronide of *N*-2-fluorenylacethydroxamic acid has been proven (Irving, 1965). Recently, Kato *et al.* (1967) described the isolation of the alkali-labile glucuronide of *N*-phenylacethydroxamic acid from rabbit urine and proposed the use of the term *NO*-glucosiduronic acid to describe this type of glucuronide.

We have now isolated and characterized the sodium salt of the *NO*-glucuronide of *N*-2-fluorenylacethydroxamic acid and studied the kinetics of the alkaline hydrolysis of this compound. Sodium (*N*-acetyl-*N*-2-fluoren[9-^{14}C]ylhydroxylamine β-D-glucosid)uronate has also been prepared biosynthetically from *N*-2-fluoren-[9-^{14}C]ylacethydroxamic acid.

Experimental and Results

Materials. *N*-2-Fluorenylhydroxylamine was synthesized using the procedure of Poirier *et al.* (1963):

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