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The Oxygen Equilibrium of Cystine-treated Human Hemoglobin without Free Sulfhydryl Groups*

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In recent years there has been a good deal of speculation as to the possible role of sulfhydryl groups in determining the oxygen equilibrium of hemoglobin, especially the Bohr effect and the sigmoid character of the equilibrium curve. It is now known that human hemoglobin A contains six sulfhydryl groups (1–3), one in each of the α chains and two in each of the β chains (3), but that only two of these are reactive (2, 4). The reactive —SH groups have been located in the β chains (5) belonging to residues 93 (6). Recent x-ray studies have shown that the reactive —SH group of each β chain stands next to the histidine residue which is coordinated with one side of the iron atom of the heme (7, 8). These —SH groups are thus in a favorable position to exert a strong influence on the oxygen equilibrium generally.

Because the Bohr effect is considerably reduced when the protons of the reactive —SH groups are displaced by treatment with *N*-ethylmaleimide (5), it seems clear that —SH groups can influence this property. Indeed, it has actually been suggested (9) that these —SH groups are themselves the very groups that are indirectly involved in the Bohr effect. But there are a number of compelling arguments against this view, some of which have recently been presented by Benesch and Benesch (10). These authors have ably discussed the Bohr effect and have offered their own suggestion as to its mechanism, which does not of itself involve sulfhydryl groups at all.

When the free —SH groups of hemoglobin are titrated with *p*-mercuribenzoate or some other —SH reagents, there is a striking reduction in the coefficient n of the empirical Hill equation (11). This observation has been interpreted to mean that the reactive —SH groups are intimately involved in the heme-heme interactions which are invoked to account for the sigmoid character of the oxygen equilibrium curve (11).

The present study deals with the effect on the oxygen equilibrium of treating hemoglobin with cystine so as to eliminate the free —SH groups. Definite evidence regarding the nature of the resulting compound is incomplete, but it seems probable that each of the reactive —SH groups has been converted into a mixed disulfide, —S—SCH_2CHNH_2COOH, as has previously been suggested (12, 13). This interpretation is supported by the fact that cysteic acid is liberated when the modified hemoglobin is treated with performic acid.[1]

EXPERIMENTAL PROCEDURE

Fresh human hemoglobin was treated with cystine according to the procedure described by Dolbeare (13). A solution of purified human hemoglobin, at a concentration of about 3% in 0.1 M phosphate buffer at pH 9.3, was combined with an equal volume of 0.5 M cystine at pH 9.7. The resulting solution was left at 4° for 8 hours and then brought to neutral pH by the addition of 0.5 M KH_2PO_4. The heavy cystine precipitate which forms was removed by centrifugation, and the hemoglobin was dialyzed in the cold against a dilute neutral phosphate buffer (0.05 M) for 48 hours. It has been shown that the molecular weight of the compound prepared in this way is the same as that of normal hemoglobin,[2] although its electrophoretic properties are slightly different, as would be expected (4, 12).

The O_2 equilibrium curves were determined spectrophotometrically by the method of Rossi-Fanelli and Antonini (14) at 20° in 0.1 M phosphate or 0.25 M acetate buffers at different pH values. All spectroscopic measurements were made in a Beckman DK-1 spectrophotometer.

Free sulfhydryl groups were determined by spectrophotometric titration with *p*-mercuribenzoate (Sigma) according to the method of Boyer (15).

RESULTS

The hemoglobin derivative prepared in this way was as stable as native hemoglobin and was spectroscopically indistinguishable from it in the visible region. When titrated with *p*-mercuribenzoate it gave no evidence of the presence of any free —SH groups, although a control titration on the untreated normal hemoglobin showed the usual two groups per molecule.

The oxygen equilibrium was studied over a wide range of pH in order to obtain information as to the shape of the oxygen

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[1] E. Antonini and J. F. Taylor, unpublished observations.

[2] The modified hemoglobin has been shown to have the same sedimentation coefficient as normal human hemoglobin. In one experiment a value of $s_{20,w} = 4.18$ was obtained for a 0.5% solution of the former in 0.1 M phosphate buffer, pH 7, and a value of 4.05 for the latter (J. F. Taylor, unpublished observations).

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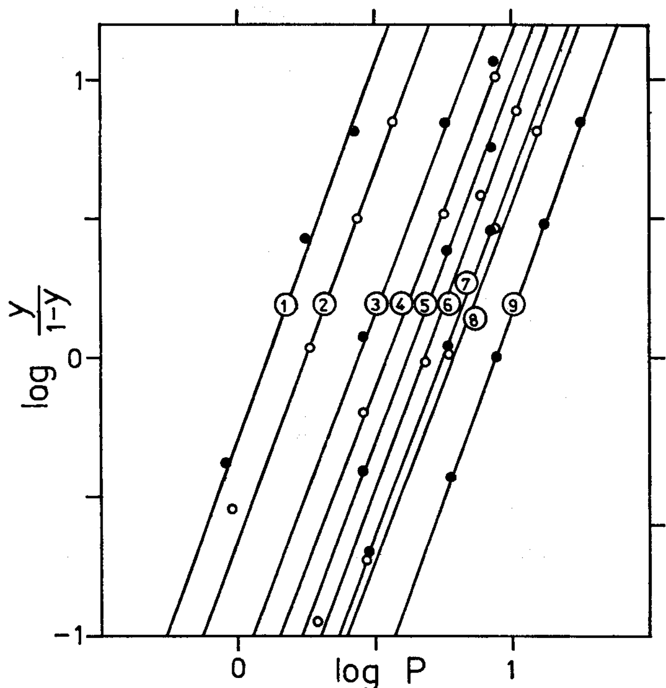

FIG. 1. Values of log $y/(1 - y)$ *versus* log p for cystine-treated hemoglobin in relation to pH (y = fractional saturation with oxygen; p = oxygen pressure in millimeters of Hg). *Curves 1, 2, 5, 6, 7,* and *8*, are for results in 0.1 M phosphate; *Curves 3* and *4*, for results in 0.25 M acetate. pH values are as follows: *1*, pH 9.6; *2*, pH 7.95; *3*, pH 5.15; *4*, pH 5.65; *5*, pH 5.60; *6*, pH 6.85; *7*, pH 6.40; *8*, pH 6.20. *Curve 9* shows an experiment on the original unmodified hemoglobin in 0.1 M phosphate, pH 7.

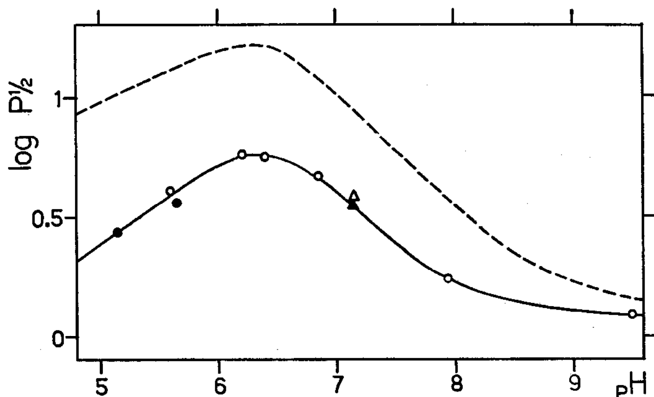

FIG. 2. Values of log $p_{\frac{1}{2}}$ *versus* pH for cystine-treated hemoglobin ($p_{\frac{1}{2}}$ = value of p at which $y = \frac{1}{2}$). ○, results in 0.1 M phosphate; ●, results in 0.25 M acetate; ▲, results in 0.1 M phosphate with added p-mercuribenzoate at a concentration 0.6 that of heme; △, results in 0.1 M phosphate with added p-mercuribenzoate at a concentration 1.2 times that of heme; - - -, unmodified hemoglobin.

equilibrium curve in relation to pH and as to the Bohr effect. The results are shown in Figs. 1 and 2.

It will be seen that the oxygen equilibrium of the modified protein is similar to that of normal hemoglobin. The value of n remains unchanged within experimental error over the whole pH range and is equal to 2.7 ± 0.1, which is identical with the value obtained for a sample of the very same hemoglobin from which the derivative was prepared. This means, of course, that the curves of percentage oxygenation against log p are invariant in shape for changes of pH and that the Bohr effect may be identified with the change of log $p_{\frac{1}{2}}$. This change is much like that observed for normal hemoglobin, although the alkaline Bohr effect is somewhat smaller and the reverse Bohr effect, which shows up below pH 6.3, is somewhat larger than in normal hemoglobin, the two effects being in fact about equal in cystine-treated hemoglobin.

The *smooth curve* drawn through the points of Fig. 2 is calculated on the assumption that there are just two oxygen-linked acid groups per heme; their pK values in the oxygenated and deoxygenated molecules are shown in Table I, together with those of normal human hemoglobin (from (16)). The absolute values

TABLE I

pK values for oxygen-linked acid groups

Hemoglobin	pK_1	ΔpK	pK_2	ΔpK
Cystine-treated				
Oxyhemoglobin	6.72	−0.96	5.92	+0.96
Deoxyhemoglobin	7.68		4.98	
Normal				
Oxyhemoglobin	6.95	−1.30	5.90	+0.60
Deoxyhemoglobin	8.25		5.30	

TABLE II

Comparative data on oxygen equilibrium of various modified human hemoglobins and two lamprey hemoglobins

Hemoglobin	n	Bohr effect	Reference
After reaction with cystine	2.6–2.8	Nearly normal	This paper
After reaction with N-ethylmaleimide	1.5	Largely decreased	(5)
After reaction with p-mercuribenzoate	1.3–1.5	[a]	(11)
HbCPA[b]	1	Largely decreased	(18)
HbCPB[c]	2.5–2.8	Largely decreased	(18)
HbCP(A + B)[d]	1	Absent	(18)
Deuterohemoglobin	1.5–1.7	Normal	(19)
Mesohemoglobin	1.5–1.7	Normal	(20)
Normal hemoglobin in 5 M NaCl	3	Largely decreased	(21)
Hemoglobin H	~1	Absent	(22)
Larval hemoglobin, *Lampetra planeri*	1.2–1.5	Large	[e]
Adult hemoglobin, *Petromyzon marinus*	1.7	Large	(23)[e]

[a] In the case of this compound, Benesch and Benesch (10) find no change in the Bohr effect; on the other hand, Smyth, Battaglia, and Meschia (17) report that the Bohr effect is largely eliminated.

[b] Hemoglobin after digestion of the β chains with carboxypeptidase A.

[c] Hemoglobin after digestion of the α chains with carboxypeptidase B.

[d] Hemoglobin after digestion of both α and β chains with a mixture of carboxypeptidases A and B.

[e] E. Antonini and J. Wyman, unpublished observations.

of the oxygen affinity are somewhat greater than those of normal hemoglobin, as will be seen from Fig. 2.

The addition of *p*-mercuribenzoate produces no effect on the oxygen equilibrium of the cystine-treated hemoglobin, in marked contrast to what happens with normal hemoglobin. This accords with expectation inasmuch as the —SH groups are already blocked.

DISCUSSION

It is instructive to compare these results with those on other types of modified human hemoglobin. This is done in Table II, which summarizes the facts for three enzymatically modified hemoglobins (HbCPA, HbCPB, and HbCP(A + B)), two reconstituted hemoglobins (meso- and deuterohemoglobin), two hemoglobins in which the —SH groups are combined with either *N*-ethylmaleimide or *p*-mercuribenzoate, and normal hemoglobin in 5 M sodium chloride, together with data on human hemoglobin H, which contains eight free —SH groups (22). In some of these examples the value of *n* remains normal while the Bohr effect is largely reduced; in others the Bohr effect remains normal while *n* is largely reduced. Table II also presents data on two lamprey hemoglobins, the adult hemoglobin of *Petromyzon marinus*, which contains one free —SH group per heme (24), and the larval hemoglobin of *Lampetra planeri*, which contains no reactive —SH groups[3] yet the value of *n* is about the same and there is a large Bohr effect in both hemoglobins. It is evident from this table that, contrary to what has sometimes been postulated, the presence of free —SH groups is neither a necessary nor a sufficient condition either for a normal value of *n* or for a nearly normal Bohr effect.

Huisman and Dozy (25) have reported that treatment of human hemoglobin with oxidized glutathione, so as to form a mixed disulfide with the free —SH groups, results in lowering the value of *n* to about 1.8 without any change in the Bohr effect. No explanation can be offered for the difference between our results with cystine-treated hemoglobin and theirs, which were obtained with the somewhat larger cystine peptide, but it should be pointed out that their results do not disagree with the conclusion we have drawn.

SUMMARY

Cystine reacts readily with the available sulfhydryl groups in human oxyhemoglobin to yield a product which no longer can be titrated with *p*-mercuribenzoate. The oxygen equilibrium of the product is similar to that of normal hemoglobin. The value of *n* is unchanged whereas the alkaline Bohr effect is slightly smaller and the acid Bohr effect is slightly larger than in the normal. The results have been interpreted to mean that titratable sulfhydryl groups are not necessarily involved either in heme-heme interactions or in the Bohr effect.

[3] M. Siniscalco, personal communication.

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