

III. 4. SULFHYDRYL GROUPS AND THE OXYGENATION OF HEMOGLOBIN *

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I. Introduction

Each molecule of vertebrate hemoglobin contains four hemes which combine reversibly with oxygen. This reaction is closely dependent upon the specific structure of the protein, globin, to which the hemes are attached. Without the protein, the ferroheme is irreversibly oxidized to ferriheme by oxygen. The protein not only confers stability on the ferroheme-oxygen complex but assumes a much more dynamic role in the mechanism of oxygenation.

There is now considerable evidence which suggests that the oxygenation of hemoglobin is accompanied by very considerable changes in the structure of the molecule. This is reflected in changes in solubility (1), crystal structure (2), and the ease of denaturation. Jonxis (3) showed that the time required for maximum surface spreading—denaturation—increases fivefold upon oxygenation. Hartridge (4, 5) showed that oxyhemoglobin and carbon monoxide hemoglobin are much more resistant to heat coagulation than is reduced hemoglobin. Thus it appears that not

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only does the protein stabilize the oxygenated heme but also that the oxygenated heme stabilizes the protein.

Perutz (6) has found that the reduction of oxyhemoglobin is accompanied by an increase in the molecular dichroic ratio from 2.6 to about 4.5 at 580 $m\mu$. He suggests that this may reflect not only a change in the heme-globin binding but also a change in the relative orientation of the four heme groups towards greater parallelism upon reduction. Wyman and Allen (7) have concluded that the oxygenation of hemoglobin is associated with a large entropy change because the heat of oxygenation of hemoglobin appears to be largely the same for the four successive stages of oxygenation, while the free energy changes differ greatly. They suggest that this entropy change may reflect a substantial architectural change in the molecule. More recently, Takashima (8) has shown that the dielectric increment changes during oxygenation, passing through four stages: first increasing to a peak at about 25% saturation, then decreasing to a minimum at about 50% saturation, and rising again to another peak at about 75% saturation. These facts presumably reflect considerable changes in the dipole moment of the molecule.

These different observations suggest that some of the amino acid residues will have properties which differ greatly in oxygenated and reduced hemoglobin because of an altered local environment. These groups will therefore be functionally linked to the oxygenation process. Physiologically the most familiar are the acid groups which become more acidic when the hemoglobin is oxygenated. This is the Bohr effect, in which an increased hydrogen ion activity results in a decreased oxygen affinity of the hemoglobin. Since all such relations must be true in reverse, not only does oxygenation decrease hydrogen ion binding, but hydrogen ion binding decreases the oxygen affinity. Another equally important relationship is that the four hemes are so associated that the oxygenation of one increases the oxygen affinity of some, at least, of the others. Since they are not linked directly to one another, this heme-heme interaction must be mediated through the protein moiety, and appears to be closely associated with the structural changes which accompany oxygenation. The possible mechanisms of these reciprocal relations have been discussed in detail by Wyman (9), and Wyman and Allen (7).

Sidwell *et al.* (10) showed that anions greatly lower the oxygen affinity. The effects are specific and cannot be explained on a purely electrostatic basis. Since these were equilibrium measurements, it must also be true that oxygenation is accompanied by a discharge of anions from the surface of the protein. These various considerations make it clear that there is a marvelous interplay of functions in the hemoglobin molecule, and

that these functional relations are intimately associated with the physiological activity.

Much evidence has now accumulated that hemoglobins contain —SH groups and that these groups are linked to the oxygenation process (11, 12). Here I wish to examine the effects of mercurials and of silver ions upon the oxygenation of hemoglobin. I shall also describe some effects of mersalyl on the properties of the isolated protein, globin. Our observations on the effects of —SH groups on the oxygen equilibrium of hemoglobin add a new facet to the interactions already known, and give insight into the mechanism of heme-heme interaction.

II. Methods

Packed cells of citrated blood were washed three times in 0.9% sodium chloride. Unless otherwise indicated, horse blood was used for all experiments. They were hemolyzed by the addition of an equal volume of distilled water. The hemolysate was then dialyzed with constant rotation for 12–16 hours against distilled water (volume at least twenty times that of the sac contents). The subsequent preparation of the hemoglobin for use in the mercurial experiments has been described (12). The experiments with silver were performed in tris* buffers prepared by mixing 0.2 *M* tris with sufficient 0.16 *N* nitric acid to produce the desired pH. Equal volumes of buffer and of dialyzed hemoglobin were mixed, and then centrifuged at high speed. All experiments were carried out at 20°. The spectrophotometric procedure for the measurement of the oxygen equilibria has been described (12).

Globin was prepared by the method of Jope *et al.* (13). Its flocculation was measured approximately by observing the apparent optical density at 400 $m\mu$.

The amperometric mercury titration procedure was that of Kolthoff *et al.* (14). A vessel with a fritted disk bottom similar to that described by Laitinen and Burdett (15) was used. Either nitrogen or carbon monoxide was bubbled through the vessel. These experiments were performed at 26°.

The oxygen equilibrium data will be described in terms of Hill's equation (16),

$$y = \frac{K_p^n}{1 + K_p^n}$$

in which y is the fraction of hemoglobin iron combined with oxygen, and

* tris(hydroxymethyl)aminomethane

p is the oxygen pressure; K and n are constants; n is a measure of the degree of heme-heme interaction. A value of $n = 1.0$ is taken to indicate complete independence of the hemes; values greater than 1.0 indicate facilitating heme-heme interactions (7, 9). The logarithm of the reciprocal of the oxygen pressure required to half-saturate the hemoglobin with oxygen ($-\log p_{50}$) is a convenient measure of the over-all oxygen affinity.

III. Results

1. EFFECTS OF MERCURIALS AND OF SILVER IONS ON THE OXYGENATION OF HEMOGLOBIN

The experiments with mercurials have in part been previously described (12). Only a summary will be given here. In Fig. 1 is shown the

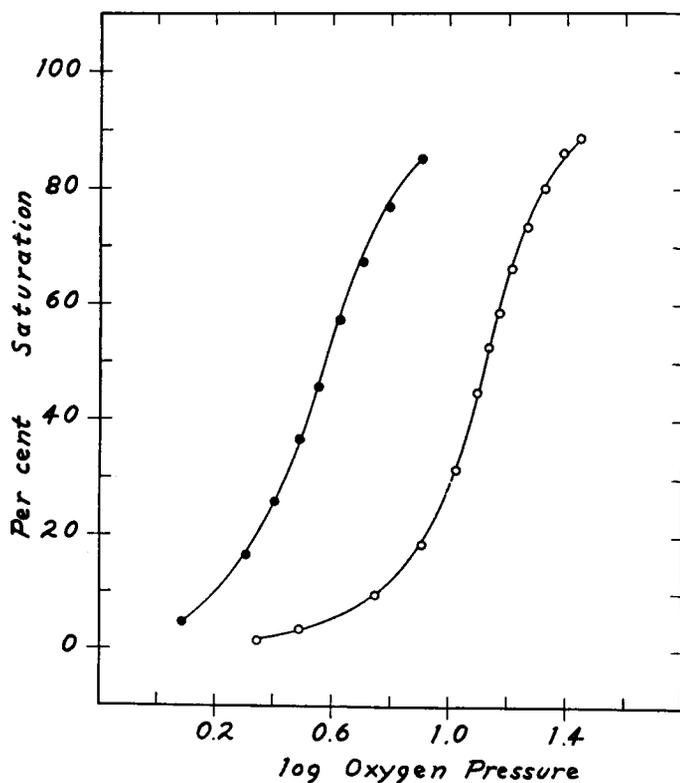


FIG. 1. The effect of mersalyl on the oxygen equilibrium of horse hemoglobin at pH 6.8. The molar ratio, mersalyl to hemoglobin, is 1.9. It is clear that much lower oxygen pressures are required to achieve a given degree of oxygenation. Oxygen pressure units are mm. of Hg.

effect of 1.9 moles of mersalyl per mole of hemoglobin upon the oxygen equilibrium at pH 6.8. In such a plot the slope of the curve at 50% saturation is directly proportional to n and is therefore a measure of over-all heme-heme interaction. The shapes of the curves are virtually identical, and we conclude that heme-heme interaction is not affected, although the oxygen affinity is greatly increased. In Fig. 2, however, it is seen that at pH 8 not only is the oxygen affinity increased but also the change in

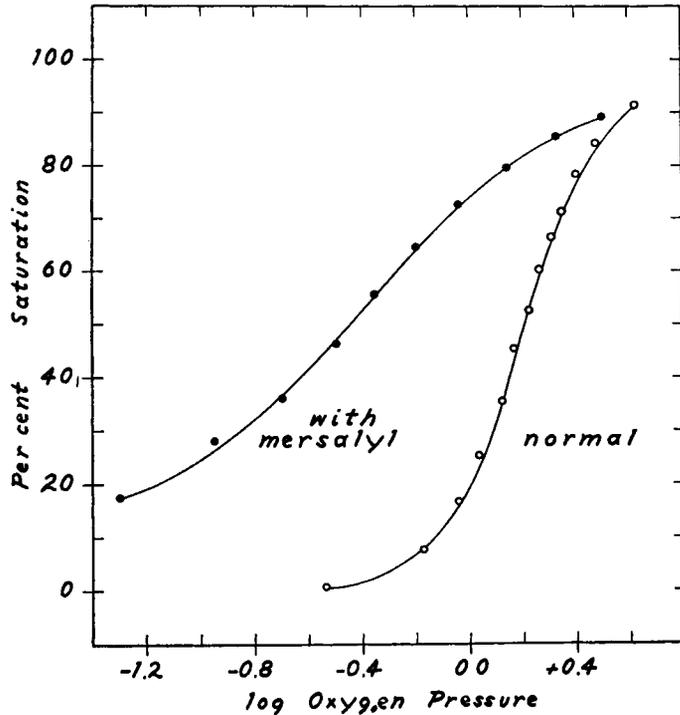


FIG. 2. The effect of mersalyl on the oxygen equilibrium of horse hemoglobin at pH 8.1. The molar ratio, mersalyl to hemoglobin, is 1.75. $n = 1.05$ in the presence of mersalyl indicating a complete loss of heme-heme interaction, as well as a large increase in oxygen affinity. Oxygen pressure units are mm. Hg.

shape indicates that the hemes are functionally completely independent of one another. Thus the addition of only two moles of mersalyl not only increases the oxygen affinity of hemoglobin but makes heme-heme interaction pH-dependent. This is illustrated in Fig. 3. In these data the mersalyl concentration is 10 moles per mole of hemoglobin. It is significant that the oxygen affinity ($-\log p_{50}$) and heme-heme interaction (n) are linearly related to one another in the presence of mersalyl, but not in its absence.

Mersalyl exerts its maximum effect at a concentration of about 2 moles per mole of hemoglobin. The data shown in Fig. 4 indicate a very curious reversal of the effects of mersalyl, when the relative mersalyl concentration is increased above 2 moles. At a concentration of about 15–16 moles, mersalyl has practically no effect at all.

The effect of mercuric chloride on the oxygen equilibrium of horse hemoglobin has been determined and is qualitatively similar to the results described for mersalyl. PCMB (*p*-chloromercuribenzoate) and methyl

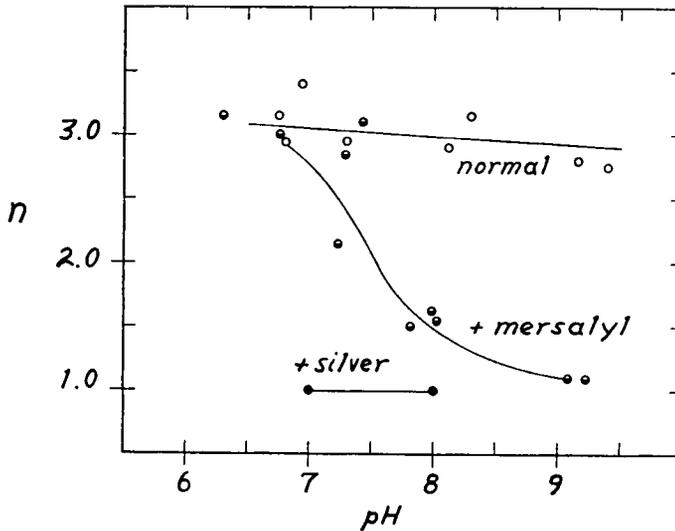


FIG. 3. The pH dependence of heme-heme interaction as measured by n . The molar ratio, mersalyl to hemoglobin, is about 10. Mersalyl makes heme-heme interaction pH dependent. However, this dependence is not exhibited in the presence of silver.

mercury hydroxide also destroy heme-heme interaction, but have relatively little effect on the over-all oxygen affinity.

Since the oxygen affinity decreases with increasing ionic strength (10, 17), I wished to determine whether the effects of mersalyl might also vary with the ionic strength. The effect of 2.3 moles of mersalyl per mole of hemoglobin on the oxygen equilibrium of human hemoglobin was determined at pH 8.10 in borate buffer in the presence and in the absence of 1.0 *M* KCl. These data are shown in Fig. 5. The increase in the oxygen affinity ($-\log p_{50}$) due to mersalyl is 0.39 in the absence of KCl and 0.41 in the presence of KCl. I take these figures to be identical. Likewise the decrease in oxygen affinity ($-\log p_{50}$) due to 1.0 *M* KCl is -0.52 in the

absence of mersalyl and -0.50 in its presence. I therefore conclude that the mercurial effect on the over-all oxygen affinity is largely independent of ionic strength. In contrast, the effect of mersalyl upon heme-heme interaction (as measured by n) does appear to depend upon the KCl concentration. In these data, $n = 1.1$ in the presence of mersalyl at low ionic strength, yet $n = 1.9$ at the same mersalyl concentration at high ionic

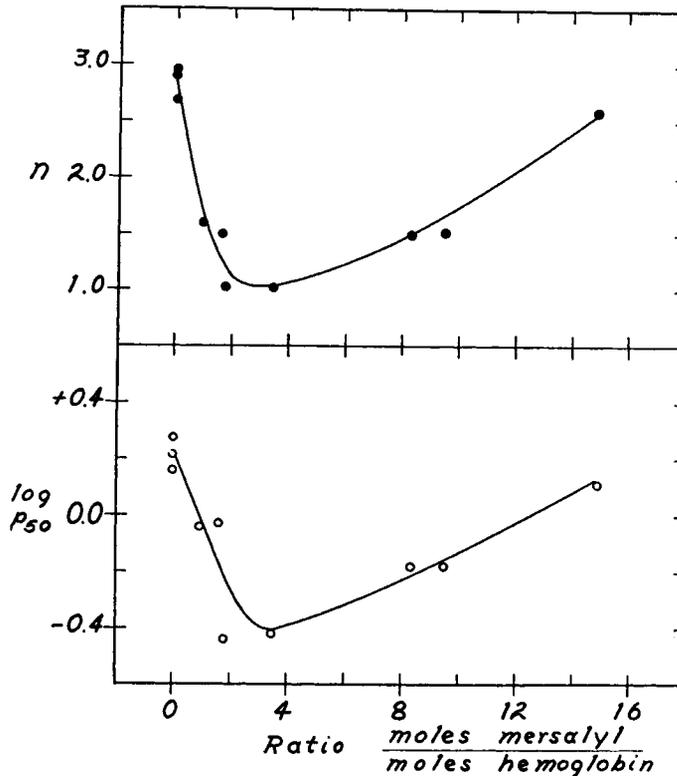


FIG. 4. The dependence of heme-heme interaction (n), and oxygen affinity ($-\log p_{50}$) upon the mersalyl concentration.

strength. Thus a high KCl concentration can partially reverse the inhibition of heme-heme interaction produced by mersalyl.

Our first experiments with silver were performed in borate-ammonium nitrate buffers at pH 8.4. Under these conditions, reduced hemoglobin gradually loses its capacity to recombine with oxygen when incubated with silver, becomes denatured, and precipitates. Oxygen inhibits this reaction, and oxyhemoglobin solutions may be incubated for 12–15 hours

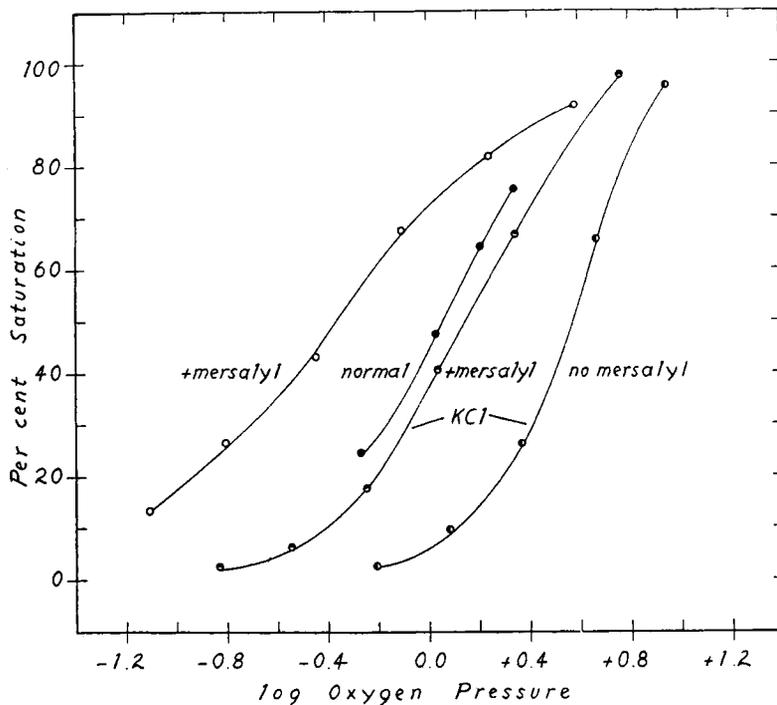


FIG. 5. The effect of mersalyl and of 1.0 *M* KCl on the oxygen equilibrium of human hemoglobin at pH 8.1. The log p_{50} shifts by the same amount when mersalyl is added whether KCl is present or not.

with no appreciable loss in oxygen capacity. However, the substitution of tris for ammonia prevents this silver-induced denaturation, at least at lower pH values. We were therefore able to determine the effect of 4 moles of silver on the oxygen equilibrium at pH 7 and 8. These data are shown in Fig. 6. It is clear that the effects are very similar to those produced by mersalyl.

2. EFFECT OF MERSALYL ON SOME PROPERTIES OF GLOBIN

Solutions of globin are relatively stable at 0°, but warming to room temperature results in a rapid increase in turbidity when the globin is dissolved in alkaline borate buffer at pH 8.7. Low concentrations of mersalyl (10^{-4} *M*) almost completely inhibit this flocculation. Low concentrations of glutathione (10^{-4} *M*) slow but do not prevent the reaction. These changes have been followed by measuring the apparent optical density at 400 $m\mu$, and are summarized in Table I.

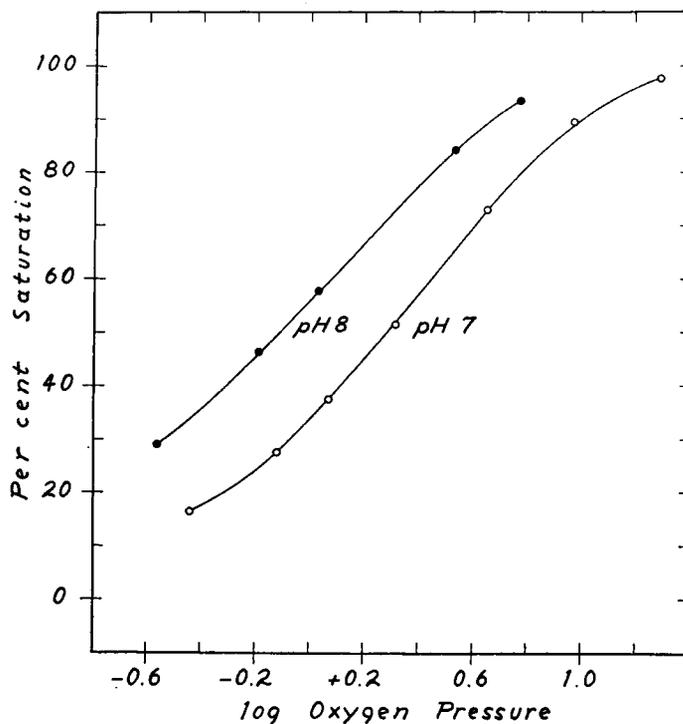


FIG. 6. The effect of silver ions in tris buffer upon the oxygen equilibrium of horse hemoglobin. The molar ratio of silver to hemoglobin is 4. The units of oxygen pressure are mm. Hg.

TABLE I
EFFECT OF MERSALYL ON THE STABILITY OF GLOBIN AT 20° IN
BORATE BUFFER AT pH 8.7^a

Experiment	Apparent optical density at 400 m μ	
	0 Minutes	20 Minutes
Globin	0	0.355
Globin + mersalyl	0	0.030
Globin + glutathione	0	0.140

^a Globin concentration is about $2.3 \times 10^{-5} M$, assuming a molecular weight of 34000. Mersalyl and glutathione concentrations are each $10^{-3} M$. Solution previously stored at 0°.

3. TITRATION OF HEMOGLOBIN WITH MERCURIC CHLORIDE

Since mercurials have such large effects upon oxygenation, I wished to determine whether oxygenation might be accompanied by a change in the total number of —SH groups available. Since the amperometric titration procedure of Kolthoff *et al.* (14) cannot be used in the presence of oxygen, I have compared the titration of reduced human hemoglobin in a nitrogen atmosphere with the titration of carbon monoxide hemoglobin in an atmosphere of carbon monoxide. Identical results have been obtained in each gas. Five independent titrations in each gas gave the following results: 2.3 moles of HgCl_2 bound by each mole of carbon monoxide hemoglobin and 2.2 moles of HgCl_2 bound by reduced hemoglobin. These titrations were performed at 26° in borate buffer, 1.0 *M* in KCl at pH 8.21. At the end of each titration with reduced hemoglobin some of the protein precipitated. This never happened with carbon monoxide hemoglobin. In this respect the sensitivities of reduced hemoglobin to mercuric chloride and to silver are very similar.

IV. Discussion

1. THE DEPENDENCE OF MERCURIAL AFFINITY ON DEGREE OF OXYGENATION

A comparison of vertebrate blood hemoglobins shows that their oxygen affinities and sensitivities to pH (Bohr effect) differ widely (see for example 18–20), yet these molecules all display oxygen equilibria which indicate very similar heme-heme interactions. Thus we can treat the oxygen affinity ($-\log p_{50}$) and heme-heme interaction (n) largely as if they were independent of one another. In the presence of mersalyl and formaldehyde, however, the $\log p_{50}$ and n become linearly related to one another (12, 21). The binding of two moles of mersalyl per mole of hemoglobin increases the over-all oxygen affinity greatly and abolishes heme-heme interactions completely. These effects are presumably mediated through the sulfhydryl groups of the protein. We shall inquire here how these effects are achieved and what they can tell us about the functioning structure of hemoglobin.

Since mersalyl greatly increases the oxygen affinity, but has no effect on the total oxygen-binding capacity of hemoglobin, we conclude that the reverse must also be true. That is, the process of oxygenation should be accompanied by a corresponding increase in the affinity with which the mercurial is bound, with no change in the total mercurial binding capacity. The —SH groups of hemoglobin must therefore be more readily bound by mersalyl in oxygenated than in reduced hemoglobin.

Ingram (26) has concluded that horse hemoglobin possesses two readily available —SH groups in each of two identical half-molecules. These two —SH groups are so associated that they can form —S—S— disulfide linkages. We suppose that mercuric chloride acts in part by forming an —S—Hg—S— linkage. Benesch and Benesch (27) have shown that the carbon-mercury bond of mersalyl is cleaved when mersalyl reacts with the dithiol, BAL (2,3-dimercaptopropanol). We suppose that a similar reaction may occur when mersalyl binds the sulfhydryl groups of hemoglobin. Conditions believed to produce the —S—S— disulfide linkage have similar effects: the oxygen affinity is increased and heme-heme interaction is reduced (11). Likewise, formaldehyde has similar effects and presumably acts in part by forming —S—CH₂—S— linkages in addition to binding amino and imidazole groups (21). PCMB has quite different effects from mersalyl. While both destroy heme-heme interaction, mersalyl greatly increases the $-\log p_{50}$, while PCMB has relatively little effect. This difference might in part be explained by the fact that PCMB is a monovalent mercurial, but that mersalyl, in effect, is partly a divalent mercurial because the Hg—C bond is split.

2. SULFHYDRYL GROUPS, THE BOHR EFFECT, AND HEME-HEME INTERACTION

The sensitivity of the —SH groups to oxidation depends upon the pH. Mirsky and Anson (22) showed that in horse hemoglobin ferricyanide can produce two disulfide groups at pH 9.5, yet at pH 6.8 no —SH groups can be oxidized at all. The ionization of the —SH group in cysteine occurs in this range (23) and this fact might explain Anson and Mirsky's results. However, since cysteine can be readily oxidized by ferricyanide at pH 7, it seems likely that the steric arrangement of the —SH groups is such that a linkage cannot readily be formed at pH 7. On this basis we might suppose that the normal cooperative heme-heme interactions are abolished because the —SH groups are joined tightly together. We have already concluded that the —SH affinity for mersalyl increases with oxygenation. We can increase the oxygenation of a partially oxygenated solution of hemoglobin either by adding mersalyl or by raising the pH. Thus the affinity with which mersalyl is bound should increase with pH, and lowering the pH should tend to drive the mersalyl off. Likewise, the binding of mersalyl must be associated with a discharge of protons. This is of course what happens when mercuric ions displace two protons from neighboring —SH groups. In the absence of mersalyl there is the familiar Bohr effect: oxygenation increases the dissociation of protons. If this normal Bohr effect is independent of the proton dissociation induced by mersalyl, then we would expect to have more protons discharged upon oxygenation in the

presence of mersalyl than in its absence. If this were true, the observed Bohr effect in the presence of mersalyl should be greater than in its absence. We observe in fact that there is no significant difference. With a mersalyl concentration of 2 moles per mole of hemoglobin, $\Delta \log p_{50}/\Delta \text{pH} = -0.69$, and in the absence of mersalyl, -0.75 (12). This result therefore suggests that the —SH groups are in some way related to the normal Bohr effect.*

When the concentration of mersalyl is raised above 2 moles per mole of hemoglobin, the effects on both oxygen affinity and heme-heme interaction are reversed. One explanation of this curious behavior is to suppose that at higher concentrations of mersalyl, two molecules would be bound by each pair of —SH groups. According to this interpretation, normal heme-heme interaction would require that the two —SH groups be independent of one another. Since, however, PCMB presumably binds tightly only one of them, and heme-heme interaction is destroyed, it would appear that the two —SH groups must be symmetrically bound. The effects of silver ions in tris buffer are similar to the effects of mersalyl. This observation casts considerable doubt on the hypothesis just advanced. It appears that the properties of the silver ions must depend on the type of chelating agent used. Silver in an ammonia complex denatures reduced hemoglobin. In contrast, silver in tris buffer has no denaturing action under the same conditions. Perhaps the silver ions are bound to hemoglobin as a silver-tris complex.

Klotz and Heiney (24) have recently suggested that the hydration shell around the hemoglobin molecule may be "frozen," and that salts and mercurials may exert their effects by modifying the lattice structure of this bound water. It is difficult to reconcile this idea with the specificity of the effects described here, the apparent independence of the effects of mersalyl and of ionic strength on the oxygen affinity, and the fact that heme-heme interaction (n) is largely independent of temperature within 10° and 38° .

The close relationship between the oxygen affinity and the —SH groups suggests that the —SH groups might be close to the hemes. Allen *et al.* (25) have concluded that the hemes of normal hemoglobin are indistinguishable from one another in their mutual interactions and in their mode of binding to the protein. Horse hemoglobin, at least, is composed

*This conclusion is supported by the following observations, made too late for inclusion in the body of the paper. Among terrestrial mammals ranging in size from mouse to elephant the number of protons discharged during oxygenation is, with few exceptions, quantitatively proportional to the number of mercuric ions which may be *immediately* bound to their hemoglobins. Furthermore, the irreversible reagent *N*-ethyl maleimide, greatly reduces the size of the Bohr effect.

of two identical half-molecules, each of which contains two hemes and a pair of available sulfhydryl groups (26). In order for the two hemes to be indistinguishable they would have to be placed symmetrically on either side of the pair of —SH groups. Presumably these —SH groups are quite close to the hemes, and there may be a steric interaction between the hemes and the benzene ring of such a molecule as PCMB when bound to an —SH group. The fact that mersalyl and heme both stabilize globin and that mersalyl partially blocks the synthesis of ferrihemoglobin from hemin and globin also suggests a close relationship.

V. Summary

1. The binding of two moles of mersalyl, mercuric chloride, or of *p*-chloromercuribenzoate or four moles of silver per mole of hemoglobin greatly lower the cooperative heme-heme interactions, yet these substances affect the oxygen affinity differently. These substances at low concentrations do not destroy the oxygen capacity, and apparently act by binding the —SH groups of the protein and thereby altering its structure.

2. Reduced hemoglobin is extremely sensitive to any excess of mercuric chloride or of low concentrations of silver and is denatured and precipitated. Tris buffer protects the hemoglobin against the denaturing effects of silver. Oxyhemoglobin and carbon monoxide hemoglobin are not affected.

3. The hemoglobin molecule is viewed as being composed of two halves, each of which has a pair of reactive —SH groups and a pair of hemes. Evidence which suggests that the —SH groups may occur as pairs close to and between the two hemes of each half-molecule is discussed.

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Discussion

MORALES: In this kind of a problem, I would have expected to see n dissociation constants, and perhaps each K written as an intrinsic constant multiplied by something that has to do with the interaction. I cannot for the world see how you easily sort out measures of interaction from a simple slope. I do not really understand your theoretical treatment.

RIGGS: It is quite true that the oxygenation reaction must ultimately depend upon at least four equilibrium constants for the four over-all successive reactions. However, if we assume that all of the hemes are initially indistinguishable, for the reasons advanced by Wyman, then the equations are greatly simplified. We can then describe the data in terms of a single intrinsic affinity constant and one or two interaction constants which determine the extent to which the oxygenation of one heme is affected by the oxygenation of another. If one uses either the original Pauling model or Wyman's extension of this model, then there is a direct relation between n as here determined and the interaction constants as defined in these models. In any event, the equilibrium data which we have can be well described between 5% and 95% solely in terms of this empirical number n and in terms of the oxygen affinity, defined as the reciprocal of the oxygen pressure for half-saturation.

MORALES: But you are actually interpreting this slope physically!

RIGGS: Yes.

MORALES: You refer to a heme-heme interaction. If you have a lumped constant, then I do not see how you can give a physical interpretation to such a constant.

RIGGS: If the hemes are independent of one another, then n would be unity no matter what model one uses.

KLOTZ: When you take some of these last curves and take the 50% point and compare the affinity, that is just at one point. It is obvious from the shapes of the curves if you took some other points, you would come to a very different conclusion.

RIGGS: Not with KCl or mersalyl.

KLOTZ: I think so. Would you want to show the last slide again? It is quite clear that the difference in logarithm of pO_2 is different as you go up along the oxygenation curves. If you took the differences along the early part of the curves, they would be quite different from those along the middle or upper parts.

RIGGS: This is quite true. However, we can describe these differences in terms of the empirical interaction constant n and the oxygen affinity, $1/p_{50}$. Mersalyl greatly lowers n and increases the oxygen affinity. If we add a high concentration of KCl to such a preparation, both of these effects are largely reversed. KCl alone greatly affects the over-all oxygen affinity, but has no effect on n .

KLOTZ: All I am saying is that if you interpret these interactions in terms of water, it might be more important to talk about the differences at the beginning of the oxygenation curve than at the mid-point.