SULFHYDRYL GROUPS AND THE STRUCTURE OF HEMOGLOBIN

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INTRODUCTION

Hemoglobins possess two very striking properties: they combine reversibly with oxygen, and this binding appears to be autocatalytic. Each molecule of vertebrate hemoglobin possesses four heme groups which combine reversibly with oxygen, and are so associated that the binding of oxygen by one heme greatly enhances the affinity of a second heme for oxygen.

Two closely related proposals have been made to explain this interaction. Wyman and Allen (1951) suggest that the large energy of heme-heme interaction is associated with a substantial decrease in entropy produced by structural alterations in the protein accompanying oxygenation. St. George and Pauling (1951) have found that the affinity of hemoglobin for alkyl isocyanides depends on the size of the alkyl group (the larger the group the lower the affinity), and conclude that the hemes are imbedded in the protein. It is imagined that oxygenation of one heme loosens the structure so that a second heme can more readily bind oxygen.

These properties are entirely dependent upon the specific structure of the protein part of the molecule. Free ferroheme reacts with oxygen, but the reaction is not reversible since the ferroheme is at once oxidized to ferriheme. Thus the protein confers stability on the ferrous oxygen complex. Likewise the interaction between the hemes depends on the specific protein structure. Denaturation by urea produces a large drop in interaction (Wyman, 1948). Furthermore, intact sulfhydryl groups in the protein are essential for heme-heme interaction (Riggs, 1952).

We wish to report in this paper observations on the effects of several mercurials on hemoglobin which give new insight into the nature of heme-heme interactions and their dependence upon —SH groups of the protein.

Preparation and Methods

The cells of fresh, defibrinated horse blood[1] were washed three times in 0.9 per cent sodium chloride. The packed cells were hemolyzed with an equal volume of distilled

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[1] We are grateful to the Biologic Laboratories of the Massachusetts Department of Public Health for supplying us with this material.

water. The resulting solution was dialyzed 12 to 15 hours against distilled water at 4–6°C., and one volume of the dialyzed solution (usually 5.0 ml.) was mixed with one volume of buffer. Phosphate buffers (mixtures of K_2HPO_4 and KH_2PO_4 having an ionic strength of 0.2) were used between pH 6.17 and 8.01; borate between pH 7.52 and 9.4 (two solutions, the first 0.2 M H_3BO_3 and 0.2 M KCl, and the second, 0.2 M NaOH, were mixed to produce the pH desired). The mercurials were added to the buffer before the buffer was added to the dialyzed solution. The mercurials are mersalyl acid, supplied to us by Winthrop-Stearns, Inc., methyl mercury hydroxide (CH_3HgOH) supplied to us by Dr. Frank Gurd as a 1.09×10^{-2} M aqueous solution, and kept at 2–4°C. until used, *p*-chloromercuribenzoic acid (PCMB), from the Sigma Chemical Company, and mercuric chloride, $HgCl_2$. To effect solution, the mersalyl and PCMB were dissolved in 0.01 to 0.1 ml. of 20 per cent NaOH, this is then added to a 5.0 ml. aliquot of the buffer and the pH is then adjusted with 0.01 to 0.05 ml. of concentrated HCl. This procedure is unnecessary for mersalyl above pH 8, but is necessary with PCMB. The buffered hemoglobin solution was centrifuged in the Spinco preparative ultracentrifuge at 39,000 R.P.M. (about 114,000 *g*) for 15 to 20 minutes. The clear supernatant solution was used for the oxygen equilibrium measurements, 4.0 ml. being placed in either of two tonometers (volumes 297.9 ml. and 251.1 ml.).

The oxygen equilibrium was determined by a modification of the spectrophotometric method described in a previous paper (Riggs, 1951). The wave length 700 mμ was used for the determination of all oxygen equilibrium curves and the tonometers were fused to optical absorption cells of 1 cm. depth. Deoxygenation was accomplished by repeated washing with nitrogen, followed by evacuation with a water aspirator and equilibration for 15 to 20 minutes in a water bath at 20.5°C. This procedure was repeated several times. Deoxygenation was considered complete when the ratio of the optical density at 700 mμ to that at 800 mμ reached a value of 2.08 or greater, and continued washing and evacuation produced no further change in this ratio. Various oxygen pressures were produced by injecting known volumes of air. The solution was equilibrated with oxygen by rotating the tonometer at about 60 R.P.M. in the water bath for 20 minutes, except at high oxygen saturations where 30 to 40 minutes were necessary. The calculated oxygen pressures have been corrected for oxygen bound to the hemoglobin. The total oxygen capacity was determined at the end of each experiment by the syringe technique of Roughton and Scholander (1943).

The data may be conveniently described in terms of Hill's equation (Hill, 1910), $y = \frac{Kp^n}{1 + Kp^n}$, in which y is the fraction of hemoglobin iron combined with oxygen, p is the oxygen pressure in millimeters of Hg. K and n are constants: K is a measure of the over-all affinity of the hemoglobin for oxygen and n is a measure of the interaction between the hemes. The values of n given in the tables are determined from the slope of the plot $\log \frac{y}{1 - y}$ *vs.* $\log p$ at the point where $y = \frac{1}{2}$. If $n = 1.0$ the hemes are assumed to act independently of one another. Any value greater than one is taken to indicate facilitating heme-heme interactions. For convenience, we use the reciprocal of p_{50}, the pressure required for 50 per cent saturation with oxygen, as a measure of the over-all affinity. In the figures, we have plotted the data in terms of per cent saturation with oxygen *vs.* log oxygen pressure. In this type of plot the slope of the curve at

50 per cent saturation is proportional to n, and the position of the curve along the log p axis indicates the over-all oxygen affinity.[2]

Effects of Mercurials on the Oxygenation of Horse Hemoglobin

We have studied the effects of four mercurials upon the oxygen equilibrium of horse hemoglobin: mersalyl, *p*-chloromercuribenzoic acid (PCMB), methyl

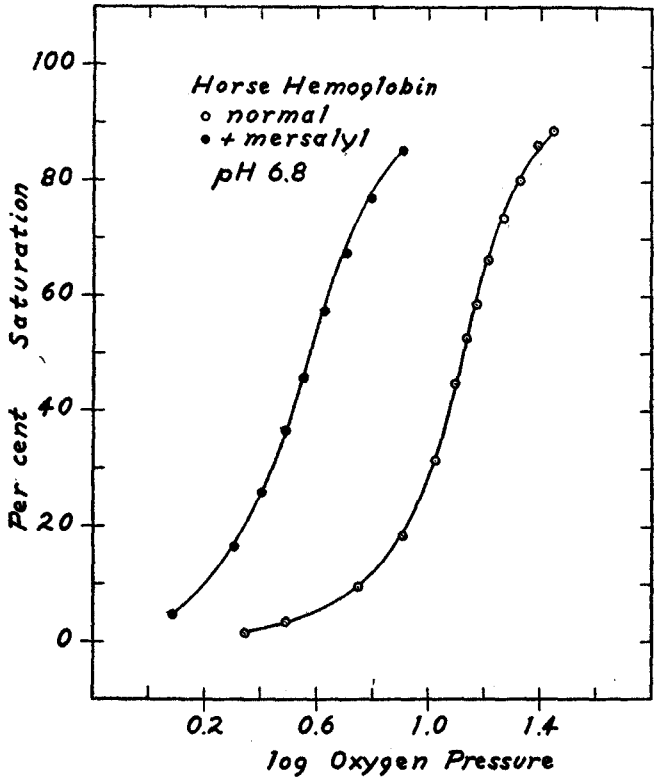


FIG. 1. The effect of mersalyl on the oxygen equilibrium of horse hemoglobin at pH 6.87. The shapes of the two curves are very similar; $n = 2.5$ with mersalyl and 3.0 without. The concentration of mersalyl is 1.9 moles per mole of hemoglobin. It is clear that this concentration of mersalyl has only a slight effect on heme-heme interaction, but a large effect on the oxygen affinity, for the ratio, p_{50} (no mersalyl)/p_{50} (with mersalyl) is about 3.5. The units of oxygen pressure are millimeters of Hg.

mercury hydroxide (CH_3HgOH), and mercuric chloride ($HgCl_2$). The effects produced depend upon the mercurial chosen, the concentration of the mercurial, and the pH.

[2] When $y = \frac{1}{2}$ in Hill's equation, $dy/d \ln p = n/4$, and $K = 1/p_{50}^n$. Hence $\log K = -n \log p_{50}$, and $n = 1.735\ dy/d \log_{10} p$.

Mersalyl.—We have determined the oxygen equilibrium of horse hemoglobin in the presence of mersalyl between pH 6 and 9. In the first set of experiments we used a constant mersalyl concentration and varied the pH. In the second set of experiments, we maintained the pH constant and varied the mersalyl concentration.

TABLE I

The Oxygen Equilibria of Horse Hemoglobin in the Presence of Mersalyl at Two pH Values

Preparations dialyzed. Concentrations of mersalyl and hemoglobin in moles per liter $\times 10^3$. 20.5°C.

	Neutral				Alkaline			
Hemoglobin	0.724		1.04		0.793		1.13	
Mersalyl	0		1.98		0		1.98	
Hg/Hb	0		1.90		0		1.75	
log p_{50}	1.13		0.59		0.215		0.44	
n	3.0		2.5		2.7		1.05	
pH	6.80		6.87		8.12		8.08	
Buffer	Phosphate		Phosphate		Borate		Borate	
	log p_{O_2}	y	log p_{O_2}	y	log p_{O_2}	y	log p_{O_2}	y
	0.348	1.2	0.088	4.93	−0.517	0.6	−1.301	17.6
	0.490	3.6	0.307	16.4	−0.167	7.6	−0.943	28.1
	0.748	9.7	0.403	26.0	−0.040	16.8	−0.699	36.1
	0.908	18.5	0.490	36.6	0.038	25.1	−0.493	46.1
	1.024	31.5	0.557	45.9	0.121	35.5	−0.350	55.6
	1.096	44.9	0.627	57.3	0.170	45.1	−0.198	64.5
	1.134	52.8	0.706	67.4	0.224	52.5	−0.032	72.9
	1.172	58.8	0.796	77.0	0.268	60.1	0.146	79.8
	1.216	66.1	0.906	85.1	0.312	66.4	0.332	85.7
	1.269	73.6			0.353	71.4	0.504	89.3
	1.324	80.1			0.409	78.3		
	1.386	86.1			0.480	84.2		
	1.443	88.9			0.622	91.6		

Fig. 1 (data from Table I) shows the results obtained at pH 6.8 upon the addition of 1.9 moles of mersalyl per mole of hemoglobin (molecular weight assumed to be 68,000). It is clear that the oxygen affinity has greatly increased, and that 50 per cent saturation may be achieved in the presence of mersalyl with an oxygen pressure about one-third that required in its absence. That is, p_{50} (normal)/p_{50} (with mersalyl) = 3.5. The shapes of the two curves are, however, very similar, and their slopes at the midpoints differ only slightly. Since n, the measure of interaction, is proportional to this slope, we conclude that the facilitating heme-heme interactions are not greatly affected by 1.9

moles of mersalyl at pH 6.8. The normal value of n is about 2.9 to 3.0. At pH 6.8, with 1.9 moles of mersalyl, n drops only to 2.5.

Fig. 2, however, shows that 1.75 moles of mersalyl at pH 8.1 produce a radical change in the shape of the oxygen equilibrium curve. n has dropped from 2.9–3.0 to 1.0, indicating that heme-heme interaction is completely abolished by 1.75 moles of mersalyl at pH 8.1. Thus mersalyl makes the

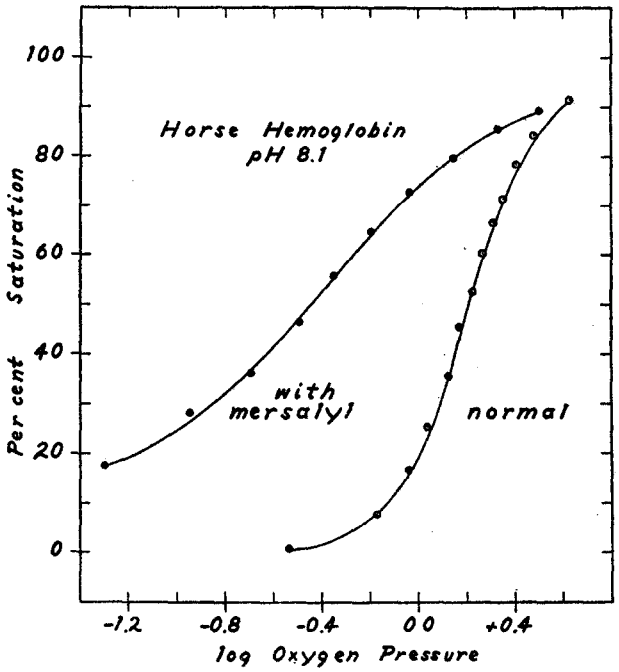


FIG. 2. The effect of mersalyl on the oxygen equilibrium of horse hemoglobin at pH 8.1. Both the shapes and positions of the oxygen equilibria are affected by mersalyl at this pH. n = 1.05 with mersalyl, and 3.7 without mersalyl. The concentration of mersalyl is 1.75 moles per mole of hemoglobin. The units of oxygen pressure are millimeters of Hg.

shape of the oxygenation curve dependent upon pH: raising the pH at a constant mersalyl concentration results in loss of heme-heme interaction.

This pH dependence of heme-heme interaction (n) is clearly indicated in Fig. 3 (Tables II and III) in which the mersalyl is constant at 9 to 10 moles per mole of hemoglobin. Furthermore, both heme-heme interaction (n) and the oxygen affinity ($-\log p_{50}$) parallel one another quite closely *in the presence of mersalyl*, but not in its absence. Compare Figs. 3 and 4, in which n and $\log p_{50}$ are plotted against pH. This parallelism is most clearly shown in

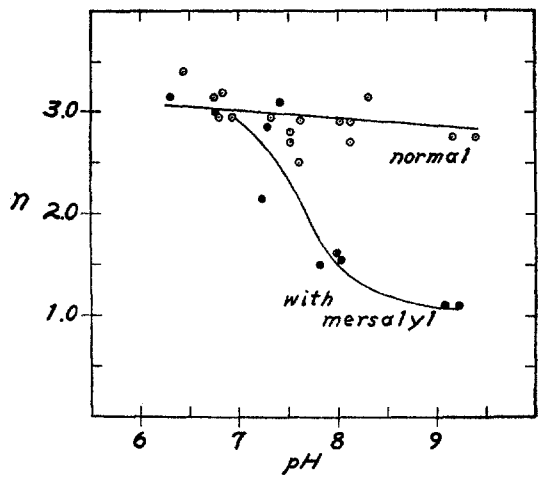


FIG. 3. The variation of n, a measure of heme-heme interaction with pH, from the data in Table II. The mersalyl concentration is about 10 moles per mole of hemoglobin. Clearly, mersalyl makes interaction pH-dependent.

TABLE II

Summary of the Oxygen Equilibrium Data for Normal Horse Hemoglobin at Various pH Values, and 20.5°C

p_{50} is the oxygen pressure (pO_2) in millimeters of Hg at 50 per cent saturation. n is the slope $\frac{\log \frac{y}{1-y}}{\log pO_2}$ at 50 per cent saturation.

pH	log p_{50}	n	Oxygen capacity, *ml. O_2 per 100 ml. solution*
1. Phosphate-buffered solutions of dialyzed horse hemoglobin			
6.17	0.86	2.41	7.0
6.44	1.18	3.4	6.1
6.75	1.18	3.15	9.2
6.80	1.12	2.95	6.1
6.80	1.13	3.0	6.5
6.84	1.09	3.2	8.1
6.93	1.11	2.95	8.25
7.32	0.88	2.95	5.9
7.51	0.67	2.8	8.6
7.60	0.58	2.5	8.6
7.62	0.68	2.91	9.7
8.01	0.16	2.95	10.0
2. Borate-buffered solutions of dialyzed horse hemoglobin			
7.52	0.69	2.7	10.0
8.12	0.275	2.9	10.3
8.12	0.215	2.7	7.0
8.30	0.225	3.15	11.2
9.17	0.073	2.75	10.7
9.40	0.04	2.75	6.5

Fig. 5 in which n is plotted against log p_{50}. The control data, without mersalyl, are not shown, but would fall on a horizontal line at $n = 2.9$ to 3.0 between log $p_{50} = 0$ and log $p_{50} = 1.2$.

Figs. 3 and 4 show that mersalyl has two independent effects: first, Fig. 4 shows that mersalyl increases the oxygen affinity 3 to 5 times independently of pH in the region pH 6.5 to 9.2; second, mersalyl decreases heme-heme

TABLE III

Effect of Mersalyl on Oxygen Equilibrium of Horse Hemoglobin

p_{50} is the oxygen pressure (pO_2) in millimeters of Hg at 50 per cent saturation. n is the slope $\frac{\log \frac{y}{1-y}}{\log pO_2}$ at 50 per cent saturation.

pH	Buffer	log p_{50}	n	Oxygen capacity, *ml. O_2 per 100 ml. solution*	Molar ratio Hg/Hb
6.30	Phosphate	0.51	3.15	6.9	13.0
6.76	"	0.46	3.0	8.9	10.0
7.28	"	0.39	2.85	9.0	9.8
7.41	"	0.20	3.1	8.6	10.4
7.82	"	−0.18	1.5	10.3	8.7
7.23	Borate	0.28	2.15	9.9	9.0
7.99	"	−0.06	1.63	9.6	9.25
8.02	"	−0.18	1.55	9.3	9.6
9.08	"	−0.36	1.1	9.0	9.9
9.22*	"	−0.30	1.1	9.0	9.9
9.15‡	"	0.015	2.15	8.6	9.9
6.58§	Phosphate	1.15	2.98	10.1	8.8

* This experiment was followed by the one marked ‡ in which glutathione was added in concentration equal to that of the mersalyl; both experiments were carried out on the same preparation.

§ This experiment was carried out with glutathione added after 1 hour's incubation with mersalyl. Glutathione concentration equalled that of the mersalyl.

interaction (n), and this effect is completely dependent on pH. If we add 10 moles of mersalyl to one mole of hemoglobin at pH 6.8, the oxygen affinity increases about 3 to 5 times and n does not change. If we now increase the pH of this mersalyl hemoglobin solution from 6.8 to 9 the oxygen affinity increases about 6 times (the Bohr effect) and it is this increase which is paralleled very closely by a decrease in heme-heme interaction. Thus the loss in heme-heme interaction parallels the Bohr effect very closely in the presence of 10 moles of mersalyl.

We have also studied the oxygenation of hemoglobin in the presence of increasing amounts of mersalyl at constant pH. The data obtained at pH 8

are plotted in Figs. 6 and 7 from data summarized in Table IV. These experiments show that 2 moles of mersalyl are sufficient to abolish heme-heme interactions entirely. n drops from 2.9–3.0 to 1.0. However, very unexpectedly, increasing the mersalyl above 2 moles reverses this effect to a very large extent. Thus, at pH 8, upon raising the mersalyl concentration from 2 to 14.9

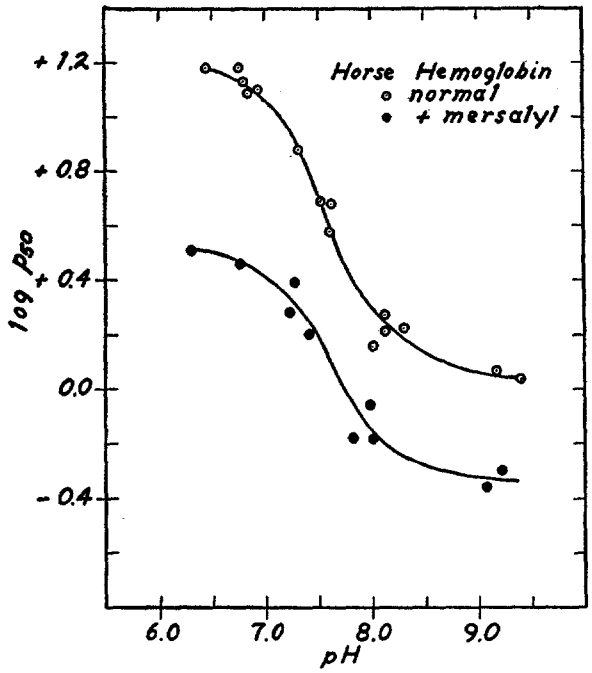


FIG. 4. The variation with pH of the oxygen pressure required to achieve 50 per cent saturation of horse hemoglobin, in the presence of 10 moles of mersalyl per mole of hemoglobin. The primary effect of mersalyl at this concentration is to increase the oxygen affinity greatly. A very substantial Bohr effect remains, and the curve does not appear to be shifted along the pH axis. The units of oxygen pressure are millimeters of Hg.

moles per mole of hemoglobin, n rises from 1.0 to 2.6, which is not greatly different from the normal value, 2.9 to 3.0. The oxygen affinity changes in a similar manner. At pH 6.8 we obtain quite different results: the addition of 2 moles of mersalyl produces a drop in n from about 2.9 to 2.5 and log p_{50} drops from about 1.1 to 0.55. Addition of more than 2 moles of mersalyl does not appear to produce any further significant change (see Table IV). Thus the effect on n of changing the mersalyl concentration depends on the pH. Actually, the plot of n *vs.* pH resembles a titration curve (Fig. 3). The "pK" of this apparent titration curve depends on the mercurial concentration, and

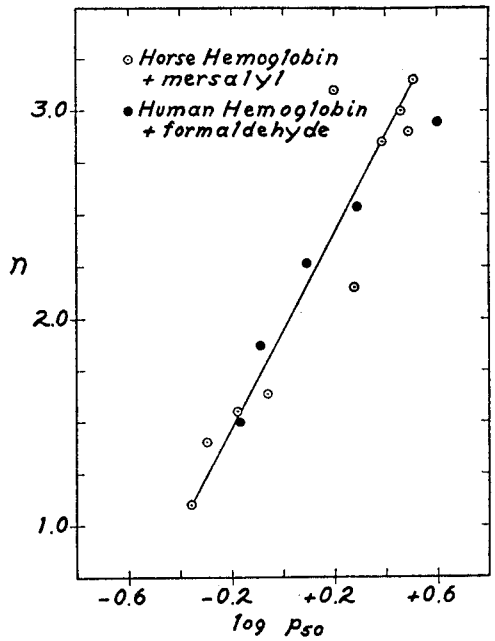


FIG. 5. The interdependence of heme-heme interaction and oxygen affinity in horse hemoglobin in the presence of 10 moles of mersalyl per mole of hemoglobin between pH 6.30 and 9.22, compared with Guthe's data on the effect of formaldehyde on the oxygen equilibrium of human hemoglobin. A similar relationship is obtained whether n and log p_{50} are varied by increasing the pH at a constant mersalyl concentration as shown here or by varying the mersalyl concentration at a constant high pH (pH 8 or greater).

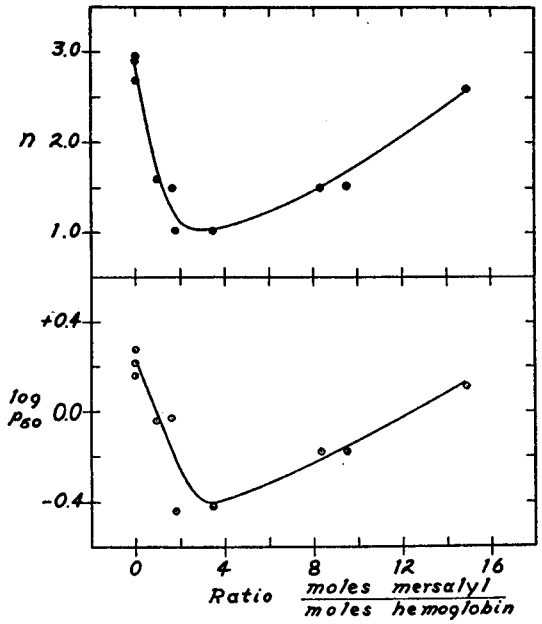


FIG. 6. The effect of varying the mersalyl concentration on heme-heme interaction (n) and oxygen affinity ($-\log p_{50}$) at pH 8. The units of oxygen pressure are millimeters of Hg. The two curves, involving n and log p_{50} are almost parallel, indicating a close relation between oxygen affinity and heme-heme interaction in the presence of mersalyl.

shifts from about 7.1 with 2 moles of mersalyl per mole of hemoglobin to 8.5 with 15 moles. Thus, at any given pH above 7, raising the mersalyl concentration above 2 moles results in an increase in interaction (n), and a decrease in oxygen affinity ($-\log p_{50}$). It is clear that, had we made our first measurements at such a concentration, we would have concluded erroneously that

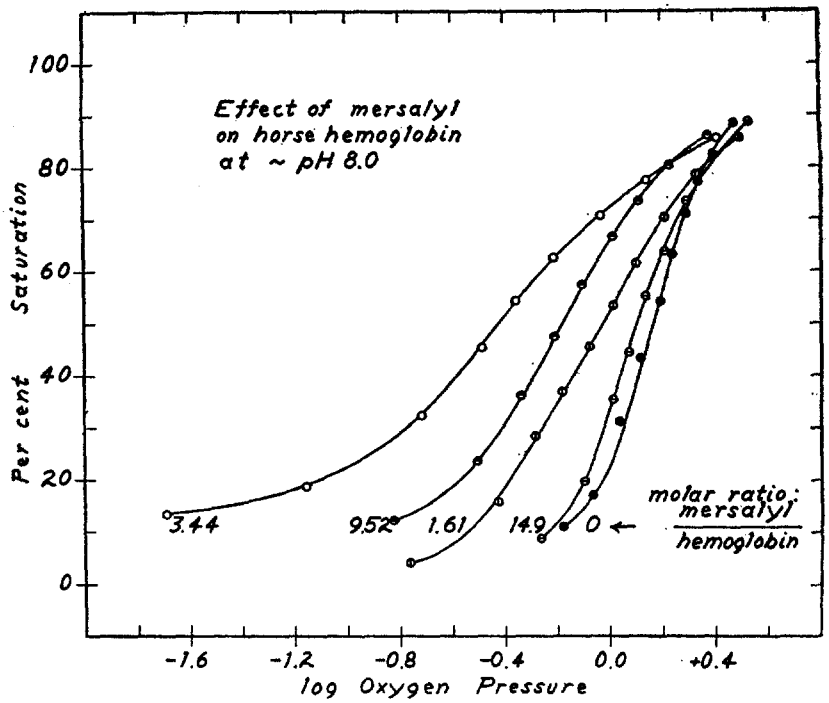


FIG. 7. The effect of varying the mersalyl concentration on the oxygen equilibrium of horse hemoglobin at pH 8. The units of oxygen pressure are millimeters of Hg. It is clear that a high concentration has relatively little effect, although a low concentration alters the oxygen equilibrium greatly.

mersalyl has very little effect on the oxygen equilibrium of hemoglobin. Our observations suggest that the attempt to inhibit an enzyme with only a single high concentration of mercurial may lead to spurious conclusions.

Mersalyl produces small changes in the absorption spectrum of hemoglobin in the region 700 to 800 mμ. The optical density (E) at 700 mμ drops about 2 to 3 per cent in the presence of mersalyl, and the optical density of oxyhemoglobin rises by 10 to 15 per cent, so the ratio E_{Hb}/E_{HbO_2} drops from about 5.6 to 4.8 at 700 mμ in the presence of 2 or more moles of mersalyl. This change is not associated with any significant change in the oxygen capacity.

Methyl Mercury Hydroxide.—We have also studied the oxygenation of hemoglobin in the presence of methyl mercury hydroxide. Fig. 8 shows that 2 moles of methyl mercury hydroxide have practically no effect on the oxygen affinity ($-\log p_{50}$) but a considerable effect on heme-heme interactions, as shown by the slope of the curve. At pH 6.8, 8.0, and 9.3 methyl mercury does not alter the oxygen affinity, but n drops with increasing pH in much the same

TABLE IV

Effect of Varying the Mersalyl Concentration on the Oxygen Equilibrium of Horse Hemoglobin

p_{50} is the oxygen pressure (p_{O_2}) in millimeters of Hg at 50 per cent saturation. n is the slope $\frac{\log \frac{y}{1-y}}{\log p_{O_2}}$ at 50 per cent saturation.

pH	Buffer	log p_{50}	n	Oxygen capacity, *ml. O_2 per 100 ml. solution*	Molar ratio Hg/Hb
8.01	Borate	0.16	2.95	10.0	0.0
8.12	"	0.215	2.7	7.0	0.0
8.12	"	0.275	2.9	10.3	0.0
8.13	"	−0.04	1.6	9.5	0.935
8.02	"	−0.03	1.5	10.95	1.61
8.08	"	−0.44	1.05	9.95	1.78
8.10	"	−0.42	1.05	10.25	3.44
7.82	"	−0.18	1.50	10.5	8.39
8.02	"	−0.18	1.55	9.3	9.52
7.80	"	0.11	2.6	11.85	14.9
6.87	Phosphate	0.59	2.5	9.25	1.90
7.90	"	0.60	2.4	7.6	2.33
6.79	"	0.64	2.7	9.4	5.65
6.78	"	0.65	2.7	9.1	5.83
6.97	"	0.42	2.4	7.55	11.7

way as in the presence of mersalyl, although to a smaller extent, n dropping from 2.9 to 2.0 at pH 9.3.

p-Chloromercuribenzoate.—PCMB produces effects very similar to methyl mercury hydroxide; but the drop in n is considerably greater; 1.65 moles produce a drop in n from 2.9 to 1.25 (Table V). PCMB, like methyl mercury, appears to have no significant effect on the oxygen affinity at low concentrations. High concentrations have not been studied with horse hemoglobin, but have been studied with human hemoglobin, prepared in exactly the same way as was the horse hemoglobin, except that the solution was not dialyzed. These results are summarized in Table VI. 2 moles of PCMB at pH 8.5 appear to increase the oxygen affinity of human hemoglobin by about 20 per cent, in

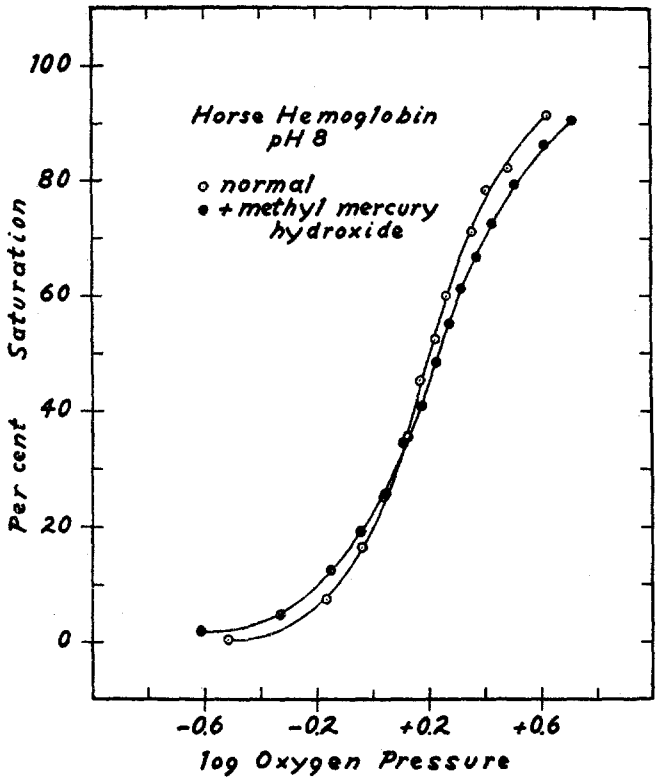


FIG. 8. The effect of methyl mercury hydroxide on the oxygen equilibrium of horse hemoglobin at pH 8. The concentration of mercury is 2.19 moles per mole of hemoglobin. The oxygen affinity ($-\log p_{50}$) is hardly changed, but heme-heme interaction has decreased considerably, n dropping from 2.9 to 2.2. The units of oxygen pressure are millimeters of Hg.

TABLE V

Effect of Mercuric Chloride, p-Chloromercuribenzoate, and Methyl Mercury Hydroxide on the Oxygen Equilibrium of Horse Hemoglobin

p_{50} is the oxygen pressure (pO_2) in millimeters of Hg at 50 per cent saturation. n is the slope $\frac{\log \frac{y}{1-y}}{\log pO_2}$ at 50 per cent saturation.

Agent	Buffer	pH	log p_{50}	n	Oxygen capacity, *ml. O_2 per 100 ml. solution*	Molar ratio Hg/Hb
$HgCl_2$	Phosphate	6.63	0.93	2.08	8.0	7.88
"	"	6.75	0.80	2.25	8.9	2.02
"	"	6.83	0.88	2.25	10.8	2.50
PCMB	"	7.70	0.42	1.75	9.2	1.75
"	Borate	8.26	0.150	1.25	9.7	1.65
"	"	9.18	0.085	1.52*	9.85	1.64*
CH_3HgOH	Phosphate	6.79	1.25	2.7	6.6	2.46
"	Borate	7.95	0.24	2.2	7.4	2.19
"	"	9.32	0.07	2.0	7.8	2.09

* In this experiment the PCMB was not quite completely dissolved, so the molar ratio Hg/Hb is somewhat lower than this figure.

contrast to horse hemoglobin in which this concentration of PCMB appears to have no effect on the oxygen affinity. Raising the PCMB concentration above 2 moles greatly depresses the oxygen affinity. Thus 15 moles of PCMB per mole of hemoglobin result in reducing the oxygen affinity to less than half that obtained in the absence of PCMB.

Mercuric Chloride.—Experiments with mercuric chloride (Table V) have been carried out at pH 6.8. At this pH, $HgCl_2$ is very similar to mersalyl in that it affects both n and log p_{50}. The addition of 2 moles of $HgCl_2$ per mole of hemoglobin produces a drop in n from 2.9–3.0 to 2.1–2.2 and a drop in log p_{50} from 1.12–1.18 to 0.8–0.9. 8 moles of $HgCl_2$ per mole of hemoglobin produce

TABLE VI

Effect of Varying the p-Chloromercuribenzoate Concentration on the Oxygen Equilibrium of Human Hemoglobin at pH 8.5 in Borate Buffer

Molar ratio PCMB/Hb	log p_{50}	n
0	0	2.6
0	0.009	2.8
0.92	0.009	2.3
2.08	−0.097	1.7
4.32	0.061	1.6
5.0	0.230	1.8
5.4	0.146	1.5
7.52	0.302	1.9
8.8	0.279	1.8
15.45	0.380	2.1

no further change. The hemoglobin is precipitated if the concentration is above 8 moles.

Reversal of Mercurial Effects with Glutathione

Glutathione reverses the effects of all these mercurials to a very considerable extent. An excess of glutathione would presumably produce complete reversal if we could use an excess without undesirable effects. We used a concentration of glutathione equal to that of the mercurial added. The reason for not adding an excess of glutathione is that glutathione and hemoglobin react to produce choleglobin (Lemberg and Legge, 1949), with an accompanying loss in oxygen capacity and an appreciable change in spectrum. This reaction is rapid at pH 8 or above and very slow at pH 7 or below.

10 moles of mersalyl at pH 6.76 produce a drop in log p_{50} from 1.18 to 0.46. The addition of 10 moles of glutathione, *after* several hours' incubation of mersalyl with hemoglobin, produces a rise in log p_{50} from 0.48 to 1.15—a substantially complete reversal. At this pH and mersalyl concentration n is unaffected. At pH 9.15 10 moles of mersalyl produce a drop in log p_{50} from

+0.073 to −0.30 and n drops from 2.9 to 1.1. Subsequent addition of glutathione raises the log p_{50} to +0.015, and n increases to 2.15. If we had been able to use an excess of glutathione the reversal would probably have been greater. An experiment in which $HgCl_2$ produced an n of 1.3 was reversed with glutathione essentially completely: n increased to 3.0. Similar experiments have been performed with PCMB and methyl mercury.

DISCUSSION

The preceding observations show that two equivalents of mercurial produce a maximum loss in heme-heme interaction in horse hemoglobin. The four mercurials studied: mersalyl, *p*-chloromercuribenzoate (PCMB), mercuric chloride, and methyl mercury hydroxide each reduce heme-heme interactions as measured by n in Hill's equation yet affect the over-all oxygen affinity ($-\log p_{50}$) quite differently. Thus, low concentrations of mersalyl and mercuric chloride reduce heme-heme interaction and increase the oxygen affinity, while PCMB and methyl mercury, although greatly reducing heme-heme interaction, have no significant effect upon the oxygen affinity when present in low concentrations. The effect of mersalyl, at least, is pH-dependent; at pH 7, 2 moles of mersalyl per mole of hemoglobin reduce heme-heme interaction only a little; at pH 8 heme-heme interaction is abolished entirely by 2 moles of mersalyl. We believe these effects are primarily due to the binding of the mercurials by the —SH groups of the protein. The effects must depend on the architecture of the protein, the arrangement of the —SH groups, and the specific properties of the different mercurials.

Many features of the architecture of horse hemoglobin are now known. The over-all dimensions of the hydrated molecule appear to be about 71 × 54 × 54 A (Bragg and Perutz, 1954). The planes of the hemes are oriented approximately perpendicular to the long axis (Perutz, 1939). Boyes-Watson, Davidson, and Perutz (1947) found by x-ray methods that horse methemoglobin has a dyad axis of symmetry and they conclude that each molecule must therefore consist of two identical halves. This axis of symmetry is parallel to the planes of the hemes.

The normal molecular weight of horse hemoglobin is about 68,000. Gralén (1939) found that horse globin has a molecular weight about half that of hemoglobin. If heme is added to such a globin solution the molecular weight returns to normal. Evidently, the hemes perform a special function in holding the two halves together. Granick (1949) has suggested that the hemes are bound at, at least, three points—the iron and the two propionic acid residues and that the acid groups bind through an electrostatic linkage. We suggest that the mechanism by which the hemes hold the two half-molecules together might be that the propionic acid residues of each heme stick out from each half molecule and hold the other half in an electrostatic linkage. Thus the very high affinity

of heme for globin would be provided by a multiplicity of bonds. There would be eight electrostatic linkages holding the two half-molecules together—two for each of the four hemes. In addition, van der Waals forces and hydrogen bonding are probably important in further stabilizing the dimer. It is important for this hypothesis that high concentrations of either urea (Steinhardt, 1938) or of sodium chloride (Andersson, 1940; Gutfreund, 1949) split horse hemoglobin into halves. These effects are only produced in dilute hemoglobin solutions where the dissociation reaction is favored.

This role of the acid residues of heme makes it easier to understand an important synergistic action of the mercurial PCMB and urea. If horse hemoglobin is treated simultaneously with a low concentration of PCMB and a high concentration of urea, the oxygen-binding power is lost, and the spectrum rapidly changes to one more characteristic of denatured hemoglobin (Riggs, unpublished observations). Evidently, the combined effect of these two reagents results in large changes in the heme-globin linkage. Neither of these reagents alone has such a drastic effect. In the presence of urea, the hemoglobin is split into half-molecules. We suggest that the heme linkages involving the propionic acid residues are broken when the molecule splits, leaving the heme bound largely by the iron and the weak van der Waals forces. It would then require only a little more energy to break the iron-globin linkage. PCMB would provide this energy by binding the —SH groups and creating a strain on the iron-globin linkage.

If we are to determine the mechanism by which the mercurials affect heme-heme interaction, clearly it is important to know where the —SH groups are in relation to the hemes. According to Ingram (1955) the amperometric titration of horse hemoglobin indicates that the molecule contains 6 —SH groups in two clusters of three each. In the native protein only a pair of —SH groups in each cluster is available. He finds that four moles of silver are bound by each mole of native protein but that only two equivalents of either $HgCl_2$, PCMB, or methyl mercury are bound. Furthermore, x-ray diffraction measurements of horse hemoglobin with four equivalents of silver show only one slightly elongated peak in the electron density map for each pair of silver atoms, indicating directly that the two readily available —SH groups of each half-molecule must be close together.

We now ask how the hemes may be oriented with respect to the —SH clusters. Allen, Guthe, and Wyman (1950) have concluded that the hemes in normal hemoglobin must all be indistinguishable from one another, that each of the four hemes is bound in the same way to the protein, and that they are all identical in their interactions. We shall assume these conclusions to be correct for horse hemoglobin. We have demonstrated that —SH groups are closely associated with the mechanism of interaction. If the two hemes in each half-molecule are indistinguishable in their mutual interactions and in the way in

which they are bound to the protein, then we suggest that the two hemes must be situated on either side of the —SH cluster. If the hemes were not so arranged, they would not be indistinguishable from one another, and would not be expected to have identical interactions with each other.

It is clear from the effect of mercurials on heme-heme interaction that an intimate relation exists between the hemes and the —SH groups. The most direct relation possible would be linkage between the iron and an —SH group. If this were true, we would expect much more drastic effects to be produced by the mercurials than actually occur, such as large changes in the spectrum and a loss in oxygen capacity, because the mercury would be displacing the iron. We therefore conclude that the hemes cannot be bound directly to —SH groups.

The fact that mercurials do affect the hemoglobin spectrum to a small extent suggests that the hemes might be quite close to the —SH groups. It is important that none of the mercurials that we have studied alters the spectrum of free ferriheme. It is significant that the synthesis of methemoglobin from ferriheme and globin is partly inhibited by mersalyl (Riggs, unpublished data). It appears that mersalyl may interfere sterically with the formation of the heme-globin linkage because the —SH groups are very close.

Another fact suggesting the close association of heme groups with —SH groups is that heme appears to protect the —SH groups of globin from oxidation. The instability of globin preparations is well known (see, for example, Holden and Freeman, 1928; Mirsky and Anson, 1936). An aqueous solution of native globin prepared by the method of Jope, Jope, and O'Brien (1949) is relatively stable at 0°C. However, rapid flocculation occurs if the temperature rises to 20°C. This occurs first near the surface and is greatly accelerated by oxygen (Riggs, unpublished data). The flocculation might involve the formation of disulfide linkages between molecules. It is important that glutathione is capable of preventing this reaction. Very small amounts of mersalyl have the same effect. Thus glutathione, mersalyl, and hemin all prevent flocculation. This common action is striking and supports the idea that heme may prevent the intermolecular oxidation of —SH groups by steric hindrance. It is possible, however, that heme acts in a more indirect manner. It is known that the separation of heme from globin involves considerable changes in the structure of the protein. It is thus possible that the hemes prevent —SH oxidation either by a reorganization of the polypeptide architecture or by themselves directly shielding the —SH groups.

In this paper we have described the effects of four mercurials on the oxygenation reaction of horse hemoglobin. We shall now discuss these effects in terms both of the individual mercurial structures and of the picture of hemoglobin we have just described. The formulae for the four mercurials are given in Table VII, which also summarizes the primary effects of the mercurials when present at low concentrations ($\leq$ 2 moles of mercurial per mole of hemo-

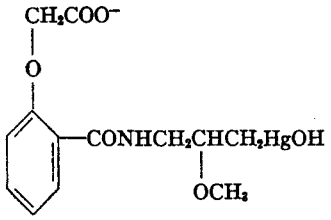
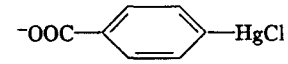
globin). All these reduce heme-heme interaction, yet affect the oxygen affinity differently.

Mercuric chloride is the only mercurial of the four which has a valence of two. We would therefore expect it to have quite different effects from the others. That this is not true is clear from Table VII: mercuric chloride and mersalyl have quite similar effects, both greatly increasing the oxygen affinity. We believe that mersalyl behaves somewhat like mercuric chloride because it is broken down at least partially to inorganic mercury and an organic moiety.

TABLE VII

A Summary of the Effects of Four Mercurials on the Oxygenation of Horse Hemoglobin

Concentration of mercurials $\leq$ 2 moles per mole of hemoglobin.

Agent	Formula	Effect on interaction	Effect on oxygen affinity
Mercuric chloride	Cl-Hg-Cl	Down	Up
Mersalyl		Down	Up
PCMB		Down	Small effect
Methyl mercury hydroxide	CH_3HgOH	Down	Small effect

Benesch and Benesch (1952) showed that such a mercury-carbon bond cleavage actually occurs when mersalyl reacts with the dithiol, BAL (2,3-dimercapto-1-propanol). The mercury-carbon bond is very weak and is probably rendered even weaker with the adjacent methoxy group. Unlike mersalyl and mercuric chloride, PCMB and methyl mercury hydroxide have little or no effect on the oxygen affinity. We believe that these two mercurials are more stable than mersalyl and do not tend to split off inorganic mercury readily. The fact that they behave very similarly suggests that the size of the organic moiety is not of primary importance in determining the oxygen affinity.

Fig. 8 shows that at low oxygen saturations, methyl mercury hydroxide

increases the binding of oxygen, while at high saturations, the mercurial decreases the amount of oxygen bound. This observation is closely related to the important work of Gibson and Roughton (1955), who have measured the kinetics of the dissociation of the first oxygen molecule from completely oxygenated sheep hemoglobin. Their results indicate that the value of the dissociation velocity constant, k_4, doubles in the presence of about 4 moles of PCMB per mole of sheep hemoglobin at pH 9.1. This is qualitatively similar to our equilibrium observation that PCMB drives oxygen off hemoglobin when the oxygen saturation is high.

Our observations on the effects of mersalyl and mercuric chloride are very similar to those on the effects of alkaline dialysis on human hemoglobin (Riggs, 1952). Such dialysis reduces interaction (n) and increases the oxygen affinity ($-\log p_{50}$) in the same way as does mercuric chloride or mersalyl. This effect of dialysis occurs in alkaline, not in neutral solution. We believe it to be caused by the oxidation of —SH groups to form a disulfide linkage. The pH dependence of —SH availability or sensitivity to oxidation in horse hemoglobin was first observed by Mirsky and Anson (1936). They found that ferricyanide oxidizes no —SH groups at pH 6.8, yet at pH 9.5, 4 of the 6 —SH groups now known to be present can be oxidized (*i.e.* 2 disulfide links formed). The pH dependence of the effect of mersalyl on heme-heme interaction parallels the increasing sensitivity of —SH groups to oxidation found by Mirsky and Anson.

Formaldehyde also increases the oxygen affinity and reduces heme-heme interaction (Guthe, 1954). Thus mercuric chloride, mersalyl, —SH oxidation, and formaldehyde all have very similar effects. Each of these affects both n and log p_{50}, reducing the interaction while simultaneously increasing the oxygen affinity. Again these effects differ greatly from those produced by PCMB or methyl mercury, neither of which affects the oxygen affinity in low concentrations (see Fig. 8).

How can we account for these various effects? Two mechanisms of heme-heme interaction can be suggested: (1) The oxygenation of two hemes might be linked through a specific electronic pathway such as a series of conjugated double bonds, or a series of proton interactions, as suggested by Lemberg and Legge (1949). (2) Oxygenation of one heme might cause structural changes in the protein architecture sufficient to distort the polypeptide arrangement in the immediate neighborhood of a second heme (Wyman and Allen, 1951; St. George and Pauling, 1951). If the first hypothesis were correct, and if the pathway involved —SH groups, it would not be possible to explain the fact that high concentrations of mersalyl do not block interaction while low concentrations do. Surely, the —SH groups are not free at high mersalyl concentrations. We therefore conclude that mercurials must inhibit heme-heme interaction by blocking or modifying the structural reorganization which accompanies oxygenation. We know that very extensive structural changes

do take place during oxygenation (see discussion by Wyman and Allen, 1951). Since the hemes are paired in each half-molecule, it appears that the first heme oxygenated does the lion's share of the reorganization.

We know that this reorganization normally requires intact —SH groups. If we bind two of these groups together with mercuric chloride forming an —S—Hg—S— linkage, heme-heme interaction is greatly reduced. Similarly, formaldehyde, also an —SH reagent, greatly reduces heme-heme interaction (Guthe, 1954) and probably forms an —S—CH_2—S— linkage, in addition to forming bonds with —NH_2 and imidazole groups. The same effect may be achieved under conditions believed to produce the disulfide, —S—S— linkage. PCMB and methyl mercury, on the other hand, have only a single valence available and so can only bind one —SH group. This binding apparently interferes sterically with the other —SH group so that there is a barrier to the binding of a second mercury atom per half-molecule (see Ingram, 1955). We conclude from the similarity of the effects of inorganic mercury, formaldehyde, and —SH oxidation, that the —SH groups must be independent of one another for normal heme-heme interaction. This is a necessary but not sufficient condition, for a low concentration of PCMB presumably binds directly only to one of the two neighboring —SH groups, yet largely destroys interaction. We suggest therefore that the two available —SH groups of each half-molecule must not only be independent but also must be symmetrically arranged. This symmetry would be destroyed if one —SH were bound and not the other. A sufficiently high concentration of mersalyl, however, would result in the binding of two neighboring —SH groups in a symmetrical manner and the observed normal heme-heme interaction would result.

It is clear from Fig. 4 that mersalyl does not abolish or shift the normal pH or Bohr effect appreciably. Can we conclude from this that the —SH groups are not the oxygen-linked acid groups responsible for the normal Bohr effect? We would expect that the curve of log p_{50} *vs.* pH would be greatly shifted along the pH axis or else abolished if the —SH groups were associated with the Bohr effect. Actually, it is not shifted significantly. On the other hand, Guthe (1954) has shown that formaldehyde shifts the log p_{50} *vs.* pH curve towards lower pH a very considerable amount. It therefore seems likely that formaldehyde, in contrast to mersalyl, binds an oxygen-linked acid group in addition to its binding of —SH groups. The oxygen-linked acid groups are believed either to be imidazole groups (see discussion by Wyman, 1948) or ammonium groups (Roughton, 1944) and may be associated with the structural changes accompanying oxygenation (Wyman and Allen, 1951). Our data do not indicate which of these possibilities may be correct.

Our present observations are closely related to those of Wyman (1948) who found that 4.6 M urea reduces heme-heme interactions a considerable amount, n dropping from 2.8–2.9 to 1.8–1.9. Simultaneously log p_{50} de-

creases. Concentrated urea is known to split horse hemoglobin into halves, each of which, if identical, contains 2 hemes. Thus n could never be greater than 2. Wyman concluded that the four hemes must exist in pairs, members of each pair interacting strongly, with interpair interaction relatively weak. Our observations suggest that the strong interactions involve a polypeptide rearrangement which requires free —SH groups. Urea appears only to break the weaker interpair interactions.

SUMMARY AND CONCLUSIONS

1. Addition of 2 moles of mersalyl, mercuric chloride, *p*-chloromercuribenzoate (PCMB), or methyl mercury hydroxide per mole of hemoglobin greatly reduces heme-heme interactions (n), yet these substances have quite different effects on the oxygen affinity ($-\log p_{50}$). Mersalyl and mercuric chloride at this concentration each increase the oxygen affinity, while PCMB and methyl mercury have little or no effect on the oxygen affinity. These effects are primarily associated with the binding of —SH groups, and are largely reversed on the addition of glutathione. —SH groups do not appear to be responsible for the Bohr effect.

2. Evidence is presented for the belief that the two hemes of each half-molecule of horse hemoglobin are situated on either side of a cluster of—SH groups.

3. The mechanism of interaction between the hemes is discussed. It is concluded that the reorganization of the protein architecture which accompanies oxygenation plays a central role in this interaction, in agreement with the views of Pauling and Wyman.

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