

THE EFFECT OF FORMALDEHYDE ON THE OXYGEN EQUILIBRIUM OF HEMOGLOBIN

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It is now generally agreed that the S shape of the curve for the oxygenation of mammalian hemoglobin (fraction oxygenated *vs.* oxygen pressure) virtually implies interaction between the four hemes attached to each hemoglobin molecule. In other words, a hemoglobin molecule will react more readily with oxygen after some of its hemes have been oxygenated (1-3). The intramolecular mechanism of this interaction is still very obscure.

The problem is often attacked by consideration of the effects of changes in pH (1) or by consideration of reactions of the iron atoms of the molecule (4), although Riggs (5) has studied the effect of compounds known to react with sulfhydryl groups of the protein. Compounds that react with nitrogenous groups of the protein might also be expected to affect the combination of oxygen with hemoglobin, and the study of such reactions should provide useful information. In the present experiments, formaldehyde was chosen as a reagent because its reactions with amino acids have been explored and their equilibrium constants have been determined (6). Unfortunately, formaldehyde reacts with many functional groups of the protein moiety of hemoglobin, and the results permit no simple interpretation. However, the experiments indicate that the interaction involves nitrogen-containing groups able to combine with formaldehyde.

Methods

Hemoglobin.—Concentrated cells from freshly drawn, heparinized human blood were washed three times in isotonic saline solution, then laked for 90 to 120 minutes by the addition of an equal volume of water and 0.4 volume of toluene. After the resulting solution was centrifuged to remove debris, enough cold potassium phosphate buffer was added to make the final phosphate concentration 0.4 M. Then the solution was centrifuged again to clarify it for optical measurements before it was introduced into the tonometer. The hemoglobin was deoxygenated by successive evacuations of the tonometer, interspersed with washings with nitrogen. All steps in the preparation were carried out at 2-5°C.

Measurements.—The methods of measurement have been described previously (2). The oxygen saturation was determined optically by measurement of the transmission

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of red light by the solution. This measurement depended on the use of a photomultiplier tube and of a narrow band Baird interference filter having a transmission peak close to $710\text{ m}\mu$. The oxygen pressure was varied by the injection of known volumes of air into the tonometer with a calibrated syringe. Determination of the oxygen saturation and concentration of the hemoglobin permitted correction for the quantity of oxygen which it bound. The pH was measured with a commercial glass electrode at the end of each experiment.

Formaldehyde.—Merck's reagent grade formaldehyde was neutralized to pH 7 with NaOH. The neutral solution was diluted, and freed of oxygen by evacuation with a water aspirator. A measured amount of this oxygen-free standard solution was injected by hypodermic syringe into the deoxygenated hemoglobin solution in the tonometer. An aliquot was titrated iodometrically (7) to determine the formaldehyde content.

The final concentration of formaldehyde in the hemoglobin solutions was 0.10 M . Since formaldehyde has a relatively high vapor pressure, it was always added to the deoxygenated hemoglobin solution. Otherwise the evacuations that were needed to remove the oxygen from the pigment would also have removed much of the formaldehyde from the solution.

RESULTS

The oxygen equilibrium of hemoglobin was measured optically in 0.10 M formaldehyde solutions. The optical density (at $\lambda = 710\text{ m}\mu$) of the hemoglobin solution was unchanged, after correction for dilution, when formaldehyde was added. Whether combined with formaldehyde or not, hemoglobin has the same transmission at the wave-length of measurement. The measured oxygen saturation, Y , therefore includes all the hemoglobin combined with oxygen, regardless of combination with formaldehyde.

Apparently little denaturation occurs in 0.1 M formaldehyde near neutral pH. The oxygen capacity and the specific viscosity were unchanged by addition of formaldehyde under these conditions.¹ The oxygen equilibrium remained reversible, but the equilibrium value was reached very slowly at 20°C . After each increase in oxygen pressure, the tonometer had to be rotated in the water bath for 2 hours to insure that equilibrium had been reached. This behavior contrasts markedly to that in the absence of formaldehyde, when less than 10 minutes' rotation was enough to establish equilibrium. Few measurements in formaldehyde could be made on the day that the blood was drawn. In most experiments, all measurements were made on the following day.

The oxygen saturation at equilibrium was measured at 20°C . in 0.10 M formaldehyde, at hemoglobin concentrations near 10 per cent (10 gm. per 100 ml.), and at five pH values between 6.17 and 7.18. Table I and Fig. 1 present

¹ At higher formaldehyde concentrations (1.0 M and above), the hemoglobin solutions turn brown and often gel even near neutrality, so that denaturation may be appreciable. In more alkaline solutions, a gel forms rapidly, even at concentrations as low as 0.1 M formaldehyde and 3 per cent protein. Intermolecular bridges may form, as described for the reaction of formaldehyde with other proteins (8).

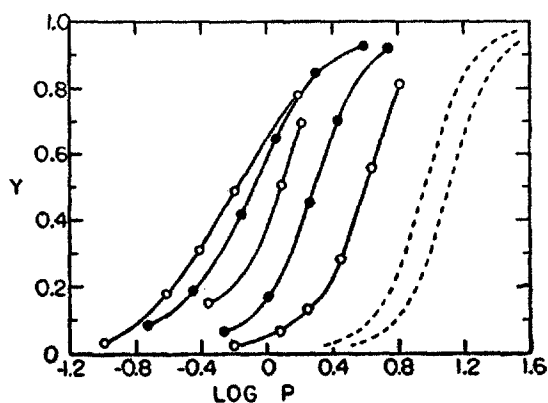


FIG. 1. Oxygen equilibria of human hemoglobin at 20°C. in the presence of 0.10 molar formaldehyde and 0.4 molar phosphate buffer. *Y* is the fraction of hemoglobin combined with oxygen and *p* is the oxygen pressure measured in millimeters of Hg. Hemoglobin concentrations varied from 0.9 to 1.7 millimoles per liter, as shown in Table I. The solid curves from left to right are the experiments made at pH 7.18, 6.91, 6.78, 6.56, and 6.17 respectively. The dashed curves represent the oxygen equilibria in the absence of formaldehyde at pH 7.49 and 6.43 respectively.

TABLE I

Oxygen Equilibria of Hemoglobin in 0.1 M Formaldehyde

Y is the fraction of hemoglobin combined with oxygen at an oxygen pressure of *p* millimeters of Hg. The derived quantities are *p*_{1/2}, the value of *p* for which *Y* is 1/2, and *n*, the exponent in Hill's equation² which measures the slope of the equilibrium curve.

Experiment No.	1		2		3		4		5	
pH.	6.17		6.56		6.78		6.91		7.18	
Hemoglobin										
gm./100 ml.	9.8		11.3		8.7		6.3		8.7	
mM/liter.	1.46		1.69		1.30		0.94		1.30	
<i>p</i> _{1/2} mm. Hg.	3.99		1.95		1.23		0.85		0.67	
<i>n</i>	2.94		2.54		2.27		1.87		1.50	
	<i>p</i>	100 <i>Y</i>	<i>p</i>	100 <i>Y</i>	<i>p</i>	100 <i>Y</i>	<i>p</i>	100 <i>Y</i>	<i>p</i>	100 <i>Y</i>
	0.63	2.2	0.55	6.6	0.44	15.2	0.19	8.6	0.10	3.3
	1.22	6.6	1.02	16.9	1.24	50.6	0.35	19.1	0.24	17.8
	1.77	13.1	1.81	45.1	1.62	69.5	0.71	41.6	0.39	31.2
	2.83	28.2	2.66	70.1			1.13	64.5	0.64	48.8
	4.34	55.7	5.54	91.8			1.98	84.6	1.54	78.3
	6.51	81.3					3.85	92.8		

² It should be explained that *n* is the exponent of the oxygen pressure in Hill's equation

$$Y = \frac{kp^n}{1 + kp^n}$$

(Footnote continued on following page)

the results. Formaldehyde increases the oxygen affinity of hemoglobin and decreases the interaction between hemes, as shown by the slope of the curves. The extent of these changes depends on pH. Measurements at more alkaline pH, unfortunately, could not be made because more alkaline solutions gelled rapidly.¹

The calculated oxygen pressures are small and may contain an appreciable error, since they involve a comparatively large correction for oxygen bound by hemoglobin. The error in the logarithm of the pressure, $\log p$, will vary with saturation and pressure; it may reach 0.1 log unit at the lower left of the figure. The saturation measurements, Y , are probably correct to 1 per cent of complete saturation. It is clear that experimental errors cannot explain the observed shapes and affinities of the curves.

The curves of Y vs. $\log p$ are all symmetrical as far as can be judged, though the data do not extend to very low saturations. The value of n , which is an over-all measure of the interaction between the hemes,² decreases rapidly with increasing alkalinity, as shown in Table I, row 5. Preliminary measurements at still higher formaldehyde concentrations indicate that n may even fall to unity, corresponding to no interaction, in moderately alkaline solution. In the absence of formaldehyde, n is approximately 2.9 and does not change with pH (1).

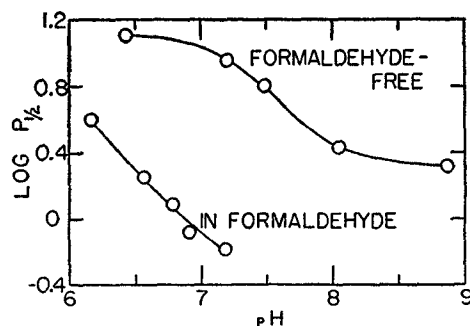


FIG. 2. The variation with pH of the oxygen pressure (millimeters of Hg) at which hemoglobin is half-saturated with oxygen, in the presence of 0 and 0.10 molar formaldehyde.

Fig. 2 shows how $p_{1/2}$, the oxygen pressure for half-saturation, varies with pH at formaldehyde concentrations of 0 and 0.10 M. The curve for zero formaldehyde concentration represents the familiar Bohr effect. Comparison of the two

which provides an approximate description of the equilibrium. For an analysis of n as a measure of the heme interactions, see Wyman (1). When n is 1, there is no interaction, and Y vs. p plot is hyperbolic.

curves shows that $\log p_{\frac{1}{2}}$ is sharply decreased by addition of formaldehyde; *i.e.*, the oxygen affinity of the hemoglobin is sharply increased.

DISCUSSION

It is noted above that equilibrium is reached much more slowly in the presence of formaldehyde. The explanation is not clear. Perhaps changes in the properties of the gas-liquid surface have slowed the diffusion of oxygen. Since the mole fraction of formaldehyde is low at 0.1 M concentration, it is unlikely that the solubility of oxygen is greatly decreased. Perhaps the explanation is partly chemical. The oxygen equilibrium in the absence of formaldehyde is known to be established with great speed, but a slow formaldehyde reaction would limit the rate of attainment of equilibrium in formaldehyde solution. Potentiometric studies of formaldehyde equilibria of amino acids (6) indicate that reactions with amino groups are rapid, but reactions with imidazole groups may be slower.

The increased oxygen affinities, and especially the diminished values of n , in the presence of formaldehyde imply a decrease in the interaction between hemes within each hemoglobin molecule. Such an effect might be attributed to the rupture or formation of cross-linkages between different parts of the hemoglobin molecule.³ The exact nature of the bonds or groups involved is still a matter of doubt. For example, one might think of the rupture of hydrogen bonds by the combination of formaldehyde with amino or imino groups (6), or of the formation of methylene bridges (8), or one might suppose the phenomenon to involve sulfhydryl groups, which are known to react with formaldehyde (6). Riggs (5) has recently found that addition of *p*-chloromercuribenzoate to a hemoglobin solution produces a drop in n , under conditions in which this reagent may be supposed to combine only with sulfhydryl groups. The conclusion that sulfhydryl groups are involved is strengthened by his finding that glutathione reverses the effect, at least in part. However, the drop in n produced by this reagent, unlike that produced by formaldehyde, is not accompanied by a decrease in the oxygen pressure of half-saturation, nor is the equilibrium reached appreciably more slowly than the ordinary oxygen-hemoglobin equilibrium. These two differences in the effects of the two reagents suggest that the formaldehyde effect is not caused solely by reaction with sulfhydryl groups, but involves nitrogenous groups as well. Which nitrogenous groups are concerned is not clear, but the range of pH in which the formaldehyde effect varies suggests that these groups may be imidazoles.

³ Unpublished studies by Miss Virginia Gossard in Wyman's laboratory establish that the spectrum of free heme shows no major change due to the presence of formaldehyde in neutral or alkaline solution. Presumably formaldehyde does not react appreciably with the heme groups of hemoglobin.

SUMMARY

1. When formaldehyde (0.10 M) is added to solutions of human hemoglobin, the oxygen affinity of the hemoglobin increases considerably (more than tenfold near pH 7). The interaction between hemes of the same hemoglobin molecule decreases, as shown by a drop in the value of n in Hill's equation from 2.9 to 1.5 or less.

2. In the presence of formaldehyde, both n and the oxygen pressure for half-saturation fall gradually as the pH rises in the range from pH 6.2 to 7.2.

3. Some of the effect of formaldehyde on the oxygen equilibrium may be due to combination with sulfhydryl groups of the protein, but nitrogenous groups are probably also involved.

I am indebted to Professor J. Wyman, Jr., who suggested this problem and advised me during its execution.

REFERENCES

1. Wyman, J., Jr., *Advances Protein Chem.*, 1948, **4**, 407.
2. Allen, D. W., Guthe, K. F., and Wyman, J., Jr., *J. Biol. Chem.*, 1950, **187**, 393.
3. Wyman, J., Jr., and Allen, D. W., *J. Polymer Sc.*, 1951, **7**, 499.
4. St. George, R. C. C., and Pauling, L., *Science*, 1951, **114**, 629.
5. Riggs, A. F., *J. Gen. Physiol.*, 1952, **36**, 1.
6. French, D., and Edsall, J. T., *Advances Protein Chem.*, 1945, **2**, 277.
7. Romijn, G., *Z. anal. Chem.*, 1897, **36**, 19.
8. Fraenkel-Conrat, H., and Mecham, D. E., *J. Biol. Chem.*, 1949, **177**, 477.