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The Chemistry of the Bohr Effect

I. THE REACTION OF *N*-ETHYL MALEIMIDE WITH THE OXYGEN-LINKED ACID GROUPS OF HEMOGLOBIN*

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One of the most intriguing phenomena associated with the function of hemoglobin is the reversible dissociation of protons which accompanies its oxygenation (2–4). This oxygen-linked ionization, which is commonly referred to as the Bohr effect, results in the liberation of about 2.7 protons per mole when human hemoglobin is oxygenated at physiological pH. Moreover, a "reverse Bohr effect" occurs in the region of pH 5.5, at which oxygenation is accompanied by the absorption of one proton per mole. It should be kept in mind that this latter dissociation is in a pH region in which partial splitting into half molecules occurs.

It was shown by Wyman (5) that the Bohr effect between pH 6 and 9 can be quantitatively accounted for in terms of four oxygen-linked acid groups per mole of hemoglobin with a pK of 7.93 in reduced hemoglobin and 6.68 in oxyhemoglobin. The chemical identity of these ionizing groups is, however, still not settled. The groups which have been suggested are amino groups (6), sulfhydryl groups (7), and imidazole groups (5, 8–10). Amino groups are unlikely to be involved in this ionization, since their pK and, especially, their heat of ionization, is too high. A number of considerations would seem to rule out Riggs' recent suggestion that the reactive —SH groups are the source of the "Bohr protons." Since there are only two to two and one-half reactive —SH groups in human hemoglobin (7, 11), they could not account for the oxygen-linked acid production (about 2.7 moles per mole), even if they were completely un-ionized in the reduced and completely ionized in the oxygenated form. In any case, this would require —SH groups which change their dissociation constant by four orders of magnitude on oxygenation. As a matter of fact, it has been reported previously (1, 12) that the protons of the reactive —SH groups of hemoglobin can be quantitatively displaced by mercaptide formation or alkylation without any change in the Bohr effect. More detailed experiments of this nature will be reported below.

The imidazole hypothesis remains the most likely explanation for the Bohr effect, since both the heat of dissociation and the pK of histidine residues, of which hemoglobin contains over 30, fall within the correct range. A reaction of the oxygen-linked acid groups of hemoglobin which is in excellent agreement with the imidazole hypothesis will be reported in this paper, and a molecular mechanism for the Bohr effect will be proposed.

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EXPERIMENTAL PROCEDURE

Materials and Methods

Hemoglobin solutions were prepared from freshly drawn human blood by the method of Drabkin (13), exhaustively dialyzed against distilled water in boiled Visking casing, and centrifuged at high speed before use. The oxyhemoglobin concentration was determined spectrophotometrically, with a molar extinction coefficient of 5.6×10^4 at 540 mμ and 5.9×10^4 at 576 mμ. Solutions were discarded when the ratio of the extinctions at 576/540 mμ fell below 1.05.

Nitrogen was "prepurified nitrogen," purity 99.9%. The sources of the other compounds were: iodoacetamide, Mann Research Laboratories, Inc., recrystallized from hot water; sodium *p*-chloromercuribenzoate, Sigma Chemical Company; *N*-ethyl maleimide and glutathione, Schwarz Laboratories, Inc. Methyl mercuric nitrate was prepared in solution from a sample of four times recrystallized methyl mercuric iodide, kindly donated by Dr. J. A. Maclaren of the Wool Research Laboratories, Parkville, Victoria, Australia, by treatment with the calculated amount of silver nitrate. 3,6-bis-(Mercurimethyl)-dioxane was a gift from Dr. Susan Lowey, Children's Cancer Research Foundation, Boston, Massachusetts. All other chemicals used were reagent grade.

Measurement of Bohr Effect—1. Differential titration: A 50-ml conical filter flask was fitted with a rubber stopper through which were inserted a pair of Beckman probe electrodes (Beckman No. 39167) and a No. 18 syringe needle carrying a short length of polyethylene tubing. The flask, which contained 3 to 6 ml of a 3 to 5 $\times 10^{-4}$ M hemoglobin solution, was shaken gently on a Burrell Wrist-Action shaker. In this way the solution was equilibrated with oxygen or nitrogen by blowing the washed gas over the surface of the protein solution. The probe electrodes were connected to a Beckman model GS pH meter. Additions of acid or alkali were made from a syringe microburette fitted with a No. 18 needle and a length of polyethylene tubing sufficient to pass through the side arm and under the surface of the liquid.

At the beginning of each experiment, the hemoglobin solution in 0.1 M NaCl was brought to the desired pH (usually 7.30) under oxygen. Any additions of reactants were usually made at this stage, and the pH was readjusted to 7.30 if necessary. Thereafter, the null-point setting on the high (B) sensitivity of the pH meter was left unchanged. The hemoglobin solution was now equilibrated with N_2, the pH being kept constant by the addition of 0.01 M HCl until no further change took place.

Complete deoxygenation takes about 1 hour. This measurement was always followed by the reoxygenation of the solution, which requires only a few minutes, and the determination of the amount of 0.01 M NaOH necessary to maintain the pH at 7.30. The equivalents of acid or alkali added divided by the moles of hemoglobin gives the Bohr effect in protons per mole.

2. Oxygen dissociation curves: The measurement of the oxygen dissociation curves as a function of pH was carried out essentially as described by Wyman (14) in a tonometer kindly loaned to us by Dr. J. Wittenberg.

Reactions of Hemoglobin Sulfhydryl Groups—1. Titration with *p*-chloromercuribenzoate: The spectrophotometric procedure described by Boyer (15) was used by adding increments of hemoglobin to a standard solution of *p*-mercuribenzoate. The same increments of hemoglobin were added to the blank cell containing 0.05 M phosphate buffer pH 7.0.

2. Reaction with *N*-ethyl maleimide: The binding of this reagent by hemoglobin was determined by adding to the protein a fivefold molar excess of *N*EM.[1] After various periods of time, the protein was precipitated with an equal volume of 4% perchloric acid, and the solution was filtered through Whatman No. 3 filter paper. The *N*EM concentration in the filtrate was determined by its absorption at 305 mμ (16).

3. Reaction with iodoacetamide: This reaction was followed by the appearance of both protons and iodide ions. The former were measured as described above in the differential titration method for the Bohr effect. The amount of iodide was measured by precipitating the reacted protein with an equal volume of 10% trichloroacetic acid and determining the iodide in the filtrate. This was done by treating 3 ml of the filtrate with 0.04 ml of concentrated H_2SO_4 and 0.06 ml of 3% H_2O_2. After 20 minutes at room temperature, the color was read at 465 mμ. Reaction with a 10-fold molar excess of iodoacetamide at pH 7.30 resulted in the liberation of 2 to 2.5 protons and 2 moles of iodide per mole of hemoglobin in the course of 1 hour, after which the pH remained constant.

For the preparation of *S*-carboxamidomethyl hemoglobin (*cf.* Table II), the protein was therefore treated with a 10-fold molar excess of iodoacetamide for 1 hour at room temperature and pH 7.3 and then dialyzed exhaustively before use.

Solubility of Hemoglobin—The solubility was determined essentially as described by Itano (17), except that phosphate buffers of varying ionic strength were used, the concentration of dithionite was 50 mg per 10 ml, and the precipitates were separated by filtration instead of centrifugation.

RESULTS

1.—SH Groups and Bohr Effect—The first series of experiments was designed to test whether the two reactive —SH groups of human hemoglobin were in any way involved in the Bohr effect. It seems appropriate at this point to compare the two main methods which can be used to measure this effect. Since the Bohr effect expresses the interdependence of oxygen and hydrogen ion binding, it can be measured either by the change in hydrogen ion binding resulting from oxygenation or deoxygenation (differential titration method), or by the change in oxygen binding with pH (pH dependence of the oxygen dissociation curves). In most of this work, the differential titration method was used because (*a*) it is conducted at constant pH, and (*b*) it is not affected by the pH-dependent changes in the shape of the oxygen dissociation curve, which are known to occur when hemoglobin is treated with some —SH reagents (18). The results in Table I show that the Bohr effect is not significantly influenced by any of the "—SH-blocking" reagents which were used, with the possible exception of *p*-mercuribenzoate. It can therefore be concluded that the total number of protons which results from the oxygenation of hemoglobin is uninfluenced by either the reversible or the irreversible displacement of the H of the —SH group.

2. Reaction of N-Ethyl Maleimide with Hemoglobin—Riggs (7) observed that the reaction of hemoglobin with *N*EM resulted in a substantial decrease in the Bohr effect. We have confirmed this observation both by the differential titration method (Table II) and by the oxygen dissociation curve method (Fig. 1). The most striking feature of this inhibition is that it never exceeds 50%, even when a large excess of *N*EM (50 moles per mole) is used, or when the reaction time is increased from 30 minutes to 6 hours (Tables II and III). This half inhibition is maintained over the whole pH range in which the Bohr effect occurs (Fig. 2). In view of the results in Table I, it was suspected that this effect of *N*EM could not be due to its reaction with —SH groups alone. The experiments summarized in Table II show clearly, however, that reaction of *N*EM with the —SH groups of hemoglobin is, nevertheless, an essential prerequisite for the inhibition of the Bohr effect by this reagent. It is apparent that when the —SH

TABLE I

Influence of —SH reagents on the Bohr effect

The reagents listed in the table were added to 4×10^{-4} M oxyhemoglobin at pH 7.3. The reaction mixtures were maintained at this pH until the —SH titration with *p*-mercuribenzoate at pH 7 (15) was zero. Thereafter, the Bohr effect was determined by the differential titration method as described in the text.

Reagent	Amount of reagent	Bohr effect $HbO_2 \rightarrow Hb \rightarrow HbO_2$	
	moles/mole Hb	*protons/mole Hb*	
None		−2.5	2.6
Iodoacetamide	10.0	−2.4	2.5
3,6-bis-(Mercurimethyl)-dioxane	2.5	−2.7	2.5
None		−2.9	2.7
Silver nitrate	2.5	−2.7	2.8
Mercuric chloride	2.5	−2.8	2.7
Methyl mercuric nitrate	2.5	−2.7	2.7
p-Mercuribenzoate	2.5	−2.5	2.3

TABLE II

Influence of NEM on the Bohr effect

The reaction time with *N*EM was 30 minutes at room temperature and pH 7.30. The Bohr effects were determined by the differential titration method.

Protein	*N*EM added	*N*EM bound	Bohr effect $HbO_2 \rightarrow Hb \rightarrow HbO_2$	
	moles/mole Hb	*moles/mole Hb*	*protons/mole Hb*	
Hemoglobin	0		−2.5	2.4
Hemoglobin	5	2.7	−1.4	1.4
Hemoglobin	50			1.5
S-Carboxamidomethyl hemoglobin	5	0.3	−2.5	2.4

[1] The abbreviation used is: *N*EM, *N*-ethyl maleimide.

groups are irreversibly blocked with iodoacetamide, the resulting *S*-carboxamidomethyl hemoglobin shows a full Bohr effect in the presence of *N*EM. Moreover, any *independent* interaction of *N*EM with other sites on the hemoglobin molecule is ruled out by the finding that no significant amounts of this reagent are bound when the —SH groups are alkylated.

On the other hand, it is possible for two —SH groups of hemoglobin to react with *N*EM *without influencing the Bohr effect.* This can occur by starting with reduced hemoglobin, which, like oxyhemoglobin, binds 2.5 moles of *N*EM per mole in 30 minutes at pH 7.3. Nevertheless, this product shows an undiminished Bohr effect (Table III). The usual inhibition can subsequently be produced by leaving it in the oxygenated condition for at least 30 minutes.

It is thus clear that after the attachment of *N*EM to the —SH groups, another consecutive reaction takes place, which, at pH 7.3, occurs at a very much slower rate in reduced than in oxygenated hemoglobin and which, in contrast to the primary reaction, involves the oxygen-linked acid groups. It was therefore logical to determine whether this difference in reactivity is dependent on the difference in the degree of ionization of these groups in the reduced and oxygenated form. It follows from Wyman's pK values (5) that at pH 7.3, the oxygen-linked acid groups are 80% dissociated in oxyhemoglobin and 20% dissociated in reduced hemoglobin, whereas at pH 8.6, these groups in reduced hemoglobin are 80% dissociated. At this pH, the reactivity of the oxygen-linked acid groups of reduced hemoglobin should therefore be identical with their reactivity in oxygenated hemoglobin at pH 7.3, if the degree of ionization is the only factor involved. As can be seen from Table III, reaction of *N*EM with reduced hemoglobin at pH 8.6 for 30 minutes or at pH 7.3 for 150 minutes does indeed lead to a very substantial inhibition of the Bohr effect. In the latter case, the expected amount of acid, *i.e.* 0.7 proton per mole, is liberated during the course of the reaction. This permits the conclusion that the secondary reaction involves the dissociated form of the oxygen-linked acid groups. This is further borne out by the changes in solubility which result from the reaction of hemoglobin with *N*EM.

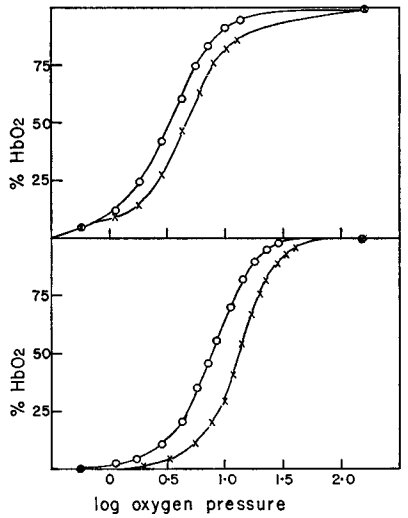


FIG. 1. Oxygen dissociation curves of hemoglobin. O——O, pH 7.38; ×——×, pH 6.97. *Lower curves:* 1.5×10^{-5} M hemoglobin in 0.1 M phosphate buffer; $\Delta \log P_{50}/\Delta$ pH = 0.63 or 2.5 H^+ per mole. *Upper curves:* 4×10^{-4} M hemoglobin, 2×10^{-3} M *N*EM, 0.1 M phosphate buffer pH 7.4. This mixture was allowed to react for 30 minutes and then diluted 30-fold with 0.1 M phosphate buffer pH 6.97 and 7.38, respectively. $\Delta \log p_{50}/\Delta$ pH = 0.32 or 1.3 H^+ per mole. Per cent oxygenation was measured at 560 mμ.

TABLE III

Reaction of NEM with Hb and HbO_2

The reaction mixtures were Hb, 4×10^{-4} M; *N*EM, 2×10^{-3} M; NaCl, 0.1 M. The Bohr effects were determined at pH 7.30 by the differential titration method.

Reactants	pH during reaction with *N*EM	Time of reaction with *N*EM	Bohr effect $HbO_2 \rightarrow Hb \rightarrow HbO_2$	
		min	*protons/mole Hb*	
HbO_2			−2.5	2.4
HbO_2 + *N*EM	7.30	30	−1.3	1.4
HbO_2 + *N*EM	7.30	360	−1.5	1.4
Hb + *N*EM	7.30	30		2.6
Hb + *N*EM	7.30	150		1.8
Hb + *N*EM	8.60	30		1.8

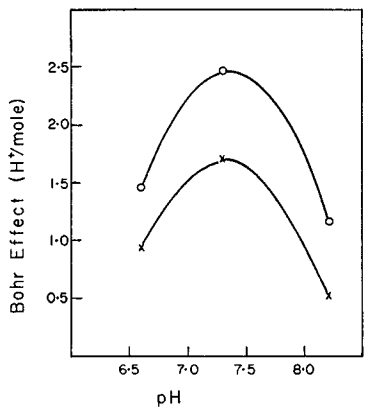


FIG. 2. Variation of the Bohr effect with pH. O——O, 4×10^{-4} M hemoglobin in 0.1 M NaCl; ×——×, 4×10^{-4} M hemoglobin, 2×10^{-3} M *N*EM 0.1 M NaCl. After reaction for 30 minutes at pH 7.3, the solution was brought to the desired pH for measurement of the Bohr effect by the differential titration method.

3. Influence of N-Ethyl Maleimide on Solubility of Hemoglobin— It is well known that in the case of human hemoglobin, the reduced form is less soluble than the oxygenated one (19, 20). The changes in solubility which result from the reaction of the hemoglobin with *N*EM are shown in Figs. 3 and 4.[2] It is apparent that:

a. Reaction of oxyhemoglobin with *N*EM at pH 7.3 (*i.e.* under conditions which result in the maximal inhibition of the Bohr effect) leads to a slight increase in the solubility of the hemoglobin in the oxygenated form and a marked decrease in the solubility of the hemoglobin in the reduced form (Fig. 3).

b. Reaction of *N*EM with reduced hemoglobin for 30 minutes at pH 7.3 (no inhibition of the Bohr effect), on the other hand, causes no change in the solubility, even though 2 moles of *N*EM have reacted with two —SH groups (Fig. 4).

c. Finally, reaction of *N*EM with reduced hemoglobin at pH

[2] It was also found that *N*EM-treated hemoglobin crystallized very readily in remarkably large crystals (6 to 8 mm in diameter). The smaller suspended crystals appeared very similar under the microscope to those of sickle-cell oxyhemoglobin reported by Perutz *et al.* (21).

8.6 for 30 minutes results in a reduced hemoglobin, the solubility of which is very close to that described under a, above (Fig. 4).

The correlation between the reaction conditions which lead to an inhibition of the Bohr effect and those which influence the solubility is indeed striking. In both cases, reaction with the —SH groups alone has no effect. Furthermore, the secondary reaction which leads to the observed decrease in the solubility again appears to involve the dissociated form of the oxygen-linked acid groups.

4. Effect of Imidazole on N-Ethyl Maleimide in Neutral Solution—The stability of the *N*EM ring at pH 7.3 in the presence and absence of 1 M imidazole is illustrated in Fig. 5.

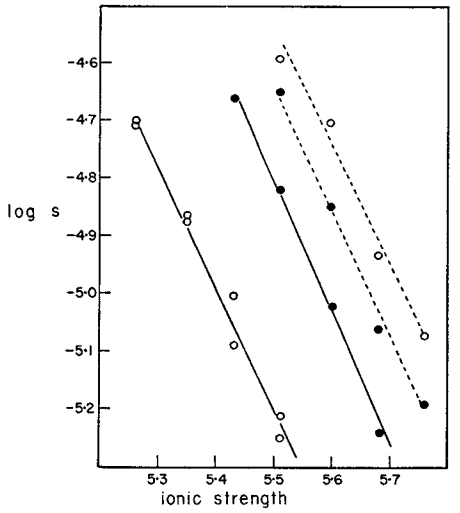


FIG. 3. Solubility of normal and *N*EM-treated hemoglobin. ●---●, control hemoglobin, solubility determined as HbO_2; ●——●, control hemoglobin, solubility determined as Hb; ○---○, *N*EM-treated hemoglobin, solubility determined as HbO_2; ○——○, *N*EM-treated hemoglobin, solubility determined as Hb. *N*EM-treated hemoglobin was prepared by reaction of 4×10^{-4} M hemoglobin with 2×10^{-3} M *N*EM for 30 minutes at pH 7.3 under oxygen. The solubilities were determined at 25° in phosphate buffers, pH 6.8.

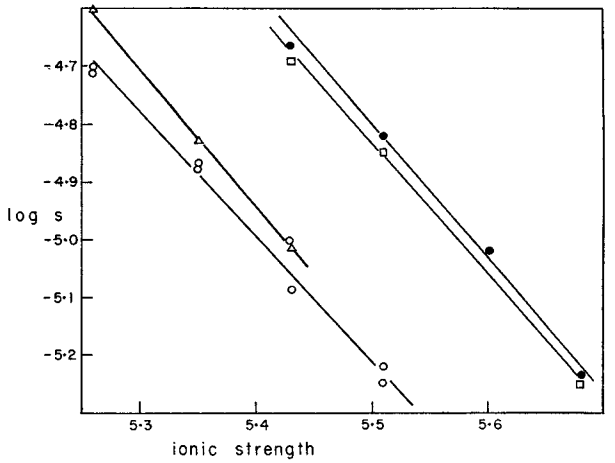


FIG. 4. The influence of the reaction conditions with *N*EM on the solubility of reduced hemoglobin. ●——●, control; ○——○, 4×10^{-4} M $HbO_2 + 2 \times 10^{-3}$ M *N*EM for 30 minutes at pH 7.3 under O_2; □——□, 4×10^{-4} M Hb + 2×10^{-3} M *N*EM for 30 minutes at pH 7.3 under N_2; △——△, 4×10^{-4} M Hb + 2×10^{-3} M *N*EM for 30 minutes at pH 8.6 under N_2; After reaction as above, the solubility was determined as reduced hemoglobin. The solubilities were determined at 25° in phosphate buffers, pH 6.8.

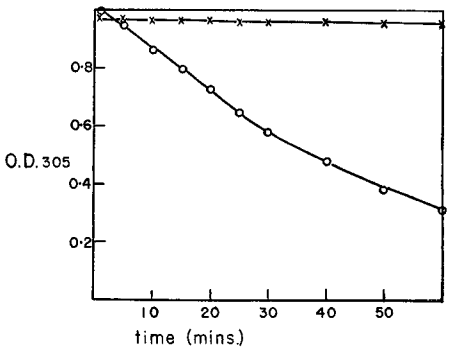


FIG. 5. Reaction of *N*EM with imidazole. ×——×, 1.6×10^{-3} M *N*EM in 1.0 M phosphate buffer pH 7.27; ○——○, 1.6×10^{-3} M *N*EM in 1.0 M imidazole buffer pH 7.27

DISCUSSION

Mechanism of Reaction of N-Ethyl Maleimide with Hemoglobin—The fact that the inhibition of the Bohr effect by *N*EM never exceeds 50% and the presence of only two reactive —SH groups in hemoglobin make it likely that this inhibition is associated with either the α or the β chains. The demonstration that radioactive *N*EM is bound only by the β chains of human hemoglobin (22) locates the site of the reaction on these chains. The lower curve in Fig. 2 should therefore represent the Bohr effect caused by the α chains. The similarity in shape of the two curves strongly supports Wyman's deduction (5) that the four oxygen-linked acid groups make identical contributions to the Bohr effect.

It seems appropriate to review briefly the various observations which have to be accounted for by any proposed mechanism:

1. The reaction of *N*EM with hemoglobin occurs in two stages; both are essential for the inhibition of the Bohr effect.

2. The first step is the attachment of *N*EM to the two reactive —SH groups and leads to no change in either the Bohr effect or the solubility of the hemoglobin.

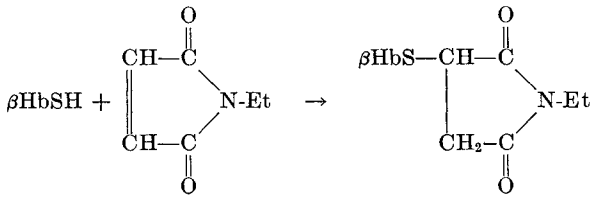
3. The second step involves a reaction of the —SH-bound *N*EM with the dissociated form of the oxygen-linked acid groups and results in both the suppression of the oxygen-linked ionization and a change in the solubility.

4. There is no significant liberation or absorption of protons during the reaction under conditions in which the oxygen-linked acid groups are largely dissociated.

5. *N*EM is hydrolyzed in the presence of imidazole in neutral solution.

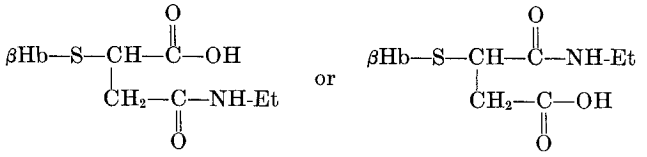
It is therefore proposed that the reaction proceeds by the following three steps:

1. The primary reaction of *N*EM with the —SH groups of the β chains is assumed to be the usual addition to the double bond, *i.e.*



2. The substituted succinimide ring is now hydrolyzed under the catalytic influence of the dissociated form of a neighboring

imidazole residue to yield a substituted *N*-ethyl succinamic acid:

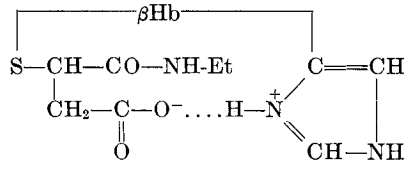


Catalyzed hydrolysis by the dissociated form of imidazole is a well known reaction which has been studied in great detail in recent years in model systems (23–28) and is the basis for current theories on the mechanism of action of hydrolytic enzymes (29). The most pertinent fact which has emerged from these studies is the enormous increase in the rate of hydrolysis (1000- to 10,000-fold) which results from catalysis by a favorably situated imidazole ring on the same molecule (intramolecular catalysis) as compared with catalysis by free imidazole (bimolecular catalysis) (24, 25, 30).

Our finding that very high concentrations of *free* imidazole are necessary for the hydrolysis of the *N*EM ring is, therefore, compatible with the rapid splitting which must occur on the hemoglobin chains, if it is assumed that the imidazole residue is located in a favorable position for intramolecular catalysis. It was originally assumed (1) that the reaction of *N*EM with oxyhemoglobin at pH 7.3 leads to the formation of an acyl imidazole as the final product. This cannot be so, since it would require the absorption of 2 protons per mole of hemoglobin during the reaction.

The role of the imidazole in opening the *N*EM ring could be simply that of a general base catalyst (27), or the hydrolysis could proceed via an unstable *N*-ethyl succinamyl imidazole intermediate.

3. The newly formed carboxyl group would now be in a position to form a hydrogen bridge with the nitrogen of the imidazole which normally holds the "Bohr proton":



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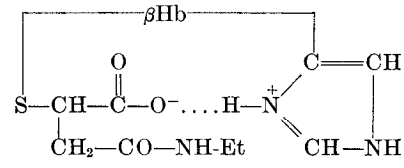
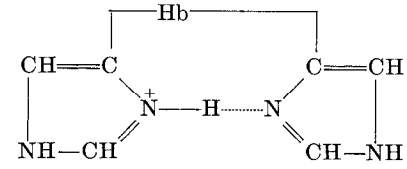


This would result in an increase of the pK of this dissociation site in oxyhemoglobin, which is tantamount to a suppression of the Bohr effect. The exact magnitude of this shift is difficult to predict, but internal chelation through hydrogen bonds is known to influence profoundly the pK values of zwitter-ionic structures. Thus, for example, the pK of the dimethylamino group in dimethylanthranilic acid is 8.42 (31) as compared with 4.09 in dimethylaniline itself (32) (*cf.* also 33).

The question arises, of course, which two of the many imidazoles are involved in the ring formation with the *S*-substituted *N*-ethyl succinamate residues. Models constructed with a right-handed α helix (34, 35) show that this ring can easily be formed when the cysteine and histidine are either adjacent or are separated by two or three other amino acid residues, but not by one. This, therefore, puts limits on the relative positions of the reactive —SH groups and the oxygen-linked acid groups on the β chains.[3]

Mechanism of Bohr Effect—The observations reported above suggest a mechanism for the Bohr effect itself. On the assumption that the oxygen-linked acid groups are imidazoles, two alternative explanations have been proposed to account for their change in pK on oxygenation. Coryell and Pauling (10) assumed that the heme-linked imidazoles were responsible for the Bohr effect, since they argued that the change in bonding which results from the combination of the iron with oxygen could cause a change in the pK of the iron-linked imidazoles of the right order of magnitude. Wyman and Allen (36), on the other hand, suggested that the difference in pK between reduced and oxygenated hemoglobin is caused by a configurational change in the protein. A considerable amount of evidence has accumulated concerning such changes, and this has been ably summarized by Edsall (37). Wyman and Allen listed three convincing objections to the hypothesis that a change in bond type of the iron alone was responsible for the Bohr effect. (*a*) Myoglobin has a negligible Bohr effect. (*b*) The oxidation of hemoglobin to ferrihemoglobin (methemoglobin) is accompanied by the same liberation of protons as the oxygenation, in spite of the fact that there is no change in bond type of the iron. (*c*) The magnitude of the Bohr effect varies from species to species.

The observations reported here on the inhibition of the Bohr effect by *N*EM which led to the hypothesis that an artificially introduced hydrogen bridge drastically alters the pK of the oxygen-linked acid groups therefore suggest that in the normal Bohr effect this dissociation is also controlled by the existence of a hydrogen bond in reduced hemoglobin which is broken as a result of the configurational change accompanying oxygenation. Two possible candidates for the role of acceptor in this hydrogen bond would be carboxylate ion or imidazole, *i.e.*



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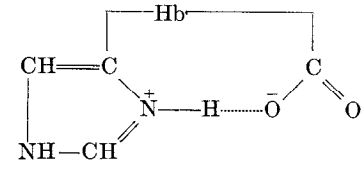

In either case, this would account both for the Bohr effect in the physiological pH range, as well as for the "reverse Bohr effect," since the acid strength of the donor group would have to increase and that of the acceptor group would have to decrease when this hydrogen bond is broken upon oxygenation.

SUMMARY

1. The reactive —SH groups of hemoglobin can be blocked in a number of ways without any change in the Bohr effect.

[3] It is significant in this connection to note that Perutz concluded, at least in the case of horse hemoglobin, that the reactive —SH groups are close to the heme-linked histidines (34).

2. *N*-Ethyl maleimide reacts with hemoglobin in two steps which can be separated experimentally.

3. The first step is the attachment to the reactive —SH groups and causes no change in either the Bohr effect or the solubility of the hemoglobin

4. The second step involves a reaction with the dissociated form of the oxygen-linked acid groups and results in a suppression of that portion of the Bohr effect which is associated with the β chains and a change in the solubility.

5. A chemical mechanism for this reaction is proposed which leads to a hypothesis on the origin of the Bohr effect and the "reverse Bohr effect."

Acknowledgments—We are deeply indebted to Dr. R. Breslow for a number of valuable suggestions concerning the mechanism of the reaction of *N*-ethyl maleimide with imidazole. We would also like to thank Dr. B. Low and Dr. M. Perutz for their active interest in this work.

Addendum—Since the submission of this paper, extensive and convincing proof of the imidazole-catalyzed opening of the *N*EM ring has been furnished by Smyth, Nagamatsu, and Fruton (38).

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