

A Multiple Wavelength Analysis of the Reaction between Hydrogen Peroxide and Metmyoglobin[†]

ABSTRACT: The spectra of reacting solutions of hydrogen peroxide and metmyoglobin may be accounted for by a Beer's law combination of the molar absorptivities times the concentrations of three components: one reactant, metmyoglobin; and two products, a red pigment ferrimyoglobin peroxide (Mb IV of George and Irvine, 1955) produced in alkali, and a green pigment (MMb₅₈₆ of King and Winfield,

1966). The kinetics of the reaction have been studied over a range of pH; 3 mol of peroxide was required at acid pH, 2 mol in alkali. The reaction proceeds through the formation of a red intermediate and some ten different reactions are involved. The formation of the green pigment is the result of the oxidation of a histidine residue as shown by a kinetic analysis of the reaction and by titration studies.

When Kobert (1900) first observed that hydrogen peroxide reacted with methemoglobin to form a red pigment he noted the formation of three diffuse bands from 500 to 513, 545 to 558, and 584 to 600 nm. Keilin and Hartree (1935) later concluded that the 500–513-nm band was due to unreacted methemoglobin, and ascribed the other two to ferrihemoglobin peroxide. This interpretation stood until King and Winfield (1966) demonstrated that the reaction of hydrogen peroxide with metmyoglobin produced at acid pH values a separate pigment form with only one absorption band at 586 nm. George and Irvine (1952) studied metmyoglobin at alkaline pH values and observed that the red pigment ferrimyoglobin peroxide¹ (PMetMb)² was produced without spectral variations in the pH range 8.0–9.0. The spectrum of the alkaline pigment had the same three bands as observed by Kobert with an absorption maximum at 547 nm, a low peak at 580–590 nm, and a low shoulder at 510–520 nm. Under the conditions that George and Irvine stud-

ied the pigment, it is unlikely that their pigment was contaminated with either unreacted MetMb or the green pigment. Finally, King and Winfield (1966) described what appeared to be a reaction intermediate which absorbed at 525 nm.

The conclusion from these observations is that if the acid reaction produces a green pigment and the alkaline reaction produces the PMetMb, reactions at intermediate pH values must produce varying mixtures of the two pigments. Although studies have been carried out at various intermediate pH values (George and Irvine, 1952; King and Winfield, 1963; King *et al.*, 1967; Brill and Sandberg, 1968; Yonetani and Schleyer, 1967), no systematic study has been made of the effect of pH on the production of the two pigments. In some studies, conclusions have been drawn concerning "a reaction product" where not one but two products must have actually been produced. In addition to the spectral changes that take place during the reaction, George and Irvine (1955) observed that about 0.8 mol of H⁺ was released/mol of MetMb reacted with H₂O₂ at pH 8. We undertook spectrophotometric and titrimetric studies of the effects of pH and peroxide concentration in order to determine the relative amounts of pigments formed and to derive information on the mechanism of the reaction from its kinetics. In order to do so, we first had to establish whether or not the spectra obtained during the course and at the end of the reaction were produced by a Beer's law combination of the absorption spectra of identifiable compounds, and how many such compounds are produced during the reaction.

Experimental Procedures

The preparation of myoglobin has been described elsewhere (Nicholas and Fox, 1969). Pigment concentration

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¹ According to George and Irvine's (1955) interpretation this compound is but a special case of a class of compounds with iron in the ferryl (Fe⁴⁺) oxidation state, to wit, ferrylmyoglobin. In this paper we will use the term ferrimyoglobin peroxide since we are concerned with only hydrogen peroxide derivatives. We will use the term acid ferrimyoglobin peroxide for the green pigment produced at low pH values. Previous usage has merely identified the pigment as the "586 complex" (King and Winfield, 1966) but identifying pigments by their absorption maxima is not too satisfactory, if for no other reason than that our spectrophotometer records the maximum at 589 nm.

² Abbreviations used are: MetMb, metmyoglobin; PMetMb, ferrimyoglobin peroxide; HPMetMb, acid ferrimyoglobin peroxide; RMetMb, a red intermediate formed during the reaction; MetMbOH, alkaline metmyoglobin.

was determined from the absorbance at 525 nm, using a molar absorptivity of $7.7 \text{ mM}^{-1} \text{ cm}^1$ (Fox and Thomson, 1964). Hydrogen peroxide was "Baker Analyzed"³ 30% A.C.S. The concentration of hydrogen peroxide was 0.25 mM in the reaction mixture, and the concentrations of the stock solutions were determined daily by the method of Allen (1930). Buffers used were acetate, (pH 4.5–5.5), cacodylate (pH 6.0–6.5), phosphate (pH 7.0–8.0), and Tris (pH 8.5), all at ionic strength 0.3 (M).

Measurements of the spectra were made using a Cary 14 spectrophotometer. To determine the molar absorptivities, solutions of known concentrations of MetMb were reacted with hydrogen peroxide under conditions where conversion to a given product was maximal. The resulting absorption values at the specified wavelengths were divided by the concentration of pigment to obtain the molar absorptivity. The ideal conditions for the production of PMetMb were assumed to be the same as those reported by George and Irvine (1952). For HPMetMb we could go no lower than pH 4.5 because of pigment denaturation. For solutions with a pH above 7.0 corrections were made in the MetMb spectra to compensate for the formation of increasing amounts of MetMbOH with increasing pH (George and Hanania, 1952). The total number of spectrally distinct components in a mixture is the minimum number of compounds required to fit a mathematically synthesized absorption curve to the experimentally obtained curve. The molar absorptivities are first calculated for as many components as are presumed to be involved. From the absorption curve of any given mixture as many absorbances as components are chosen. The absorbance (A) at any given wavelength (j) is defined as the sum of the products of the molar absorptivity (ϵ) times the concentration of the individual components

$$A_j = \sum \epsilon_{i,j} c_i \quad (1)$$

(c_i), one for each component, which are then solved for the concentrations of the individual components. The equation is then used to calculate a complete theoretical absorption spectrum, which is compared to the actual absorption spectrum from which the concentrations were calculated. If any other absorbing compounds with spectra differing from the assumed compounds are produced, their presence will show up as deviations of the two curves from each other. For the initial calculations the system was assumed to be made up of only the three optically absorbing heme pigments, the reactant, MetMb, and the two products, PMetMb and HPMetMb, with absorption maxima at 505, 547, and 589 nm, respectively. A program to solve the equations was written for an Underwood-Olivetti Programma 101 computer.

For the kinetic studies, temperature control to within $\pm 0.2^\circ$ was achieved in the cell holder of a Cary 14 spectrophotometer² and in a preequilibration bath by circulating cool or warm water upon demand created by two YSI Thermistor units, one a surface probe taped to the cell holder, the other an immersion probe in the bath.

The relative amounts of the two pigment products formed were found to be dependent upon the manner of mixing the reagents. In the initial studies, the reactions were started by making MetMb solutions almost up to volume and squirting peroxide solutions into the absorption

cell with sufficient force to mix the solutions. Excessive foaming occurred and under identical conditions it was found that the relative amounts of red and green pigments could be varied simply by varying the rate of addition of peroxide. Starting the reaction by adding pigment to dilute peroxide was no better. It was obvious that the best technique would be to have the initial pigment and peroxide concentrations twice the desired concentration, and mix equal volumes of the solutions as fast and thoroughly as possible. A dual syringe unit was made with a "Y" connector which was terminated with a long needle. The latter was inserted into the optical cell through a hole in the cell chamber cover with the cell in the light path. As both syringe plungers were depressed, the two 2X concentration solutions were mixed in the "Y" fitting and the needle. The cell could be filled in 3 sec or less and absorption readings taken immediately. No foaming was observed with this procedure.

The solutions were preequilibrated at the appropriate temperature by immersing the dual syringe unit in a constant-temperature water bath. To obtain absorbance values at three wavelengths, each reaction was run three times on portions of the same solutions, recording a different wavelength each time. If any one scan were to be made on a solution that was reacting either slower or faster than the other two, the concentration of the component whose principal λ_{max} was being measured would be either too high or too low. Such a deviation would be reflected in a variation of the total concentration calculated as the sum of the concentrations of the individual components. No such variation was ever observed in any of the reactions.

For the titration studies we used a Radiometer TTT-1a coupled to a Radiometer titrigrph driving a SBU1A syringe buret to add alkali or acid as necessary to maintain the pH. The titration was carried out under nitrogen in a 10-ml thermostated cell at 20.0° .

The rate constants for the reaction were calculated from

$$d\text{MetMb}/dt = k_1[\text{MetMb}] \quad (2)$$

$$d\text{MetMb}/dt = k_2[\text{MetMb}][\text{H}_2\text{O}_2] \quad (3)$$

$$d\text{MetMb}/dt = k_3[\text{MetMb}][\text{H}_2\text{O}_2]^2 \quad (4)$$

Results

Spectra. The spectra of the three optically absorbing components of the reaction



are shown in Figure 1, and the molar absorptivities (ϵ values) in Table I. The spectrum of MetMb is the same as that reported by George and Hanania (1952) and the ϵ values are about 5% higher than obtained previously (Fox and Thomson, 1963). The spectra and ϵ values for PMetMb were derived from the spectra of solutions with pH values between 8.0 and 9.0, 20° , after maximal conversion to the red pigment had occurred. The values of HPMetMb were derived from the spectra of solutions with a pH of 4.5 at 30° , and a peroxide concentration of 0.25 mM. At 20° and pH 4.5, the spectra, after maximal conversion had occurred, always had a pronounced shoulder at *ca.* 547 nm. We could go no lower in pH, but raising the temperature of the reaction resulted in increasing intensity of the 589-nm absorption, with maximal conversion occurring at 30° .

Reaction Components. Figure 2a–d shows comparisons of spectra observed during the course of the reaction and spectra calculated according to the previously described

³ Reference to brand or firm name does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

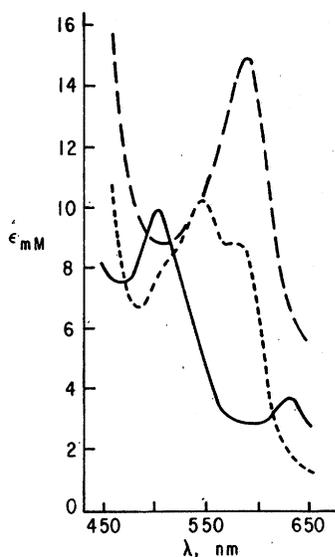


FIGURE 1: Absorption spectra for MetMb (—); PMetMb (---); and HPMetMb (- -).

procedure. The standard deviation, σ , for the difference between the curves was 0.0056 \AA , for a variation of $\pm 2\%$. Figure 2a-c demonstrate the sufficiency of the assumption; Figure 2d shows the necessity. In Figure 2d, the theoretical curve was calculated using the molar absorptivities of only MetMb and PMetMb; the theoretical curve is significantly lower (14%) in the 580–600-nm region where HPMetMb absorbs maximally. The same spectral calculations were made for the products at the end of the second and third stages with the same close fit, except for an absorption band *ca.* 635 nm produced in the more acid reaction mixtures. This band is probably due to choleglobin, a green pigment which is the result of oxidative cleavage of the porphyrin ring by peroxide and has a major absorption band at about this wavelength (Lemberg *et al.*, 1941).

Effect of pH on the Stability of the Compounds. Experiments were conducted to determine the effect of hydrogen ion concentration on the stability of the two peroxide products. A solution of 0.050 mM HPMetMb was prepared at pH 4.5. The pH of the solution was then adjusted to 8.0 with NaOH, and then returned to pH 4.5 with acetic acid, the spectrum of the solution being recorded at each stage. No changes occurred in the spectrum during this cycle, showing the pigment to be alkali stable. In contrast, when a solution of 0.05 mM PMetMb, prepared at pH 8.0, was adjusted to pH 4.6 with acetic acid, an immediate conversion took place, with part of the red pigment being converted to MetMb and part to HPMetMb. Table II shows that the conversion was very rapid for most of the pigment convert-

TABLE I: Absorption Coefficients for the Components of the H_2O_2 -MetMb Reaction and the Component Bands of PMetMb.

Pigment	$\epsilon \text{ (mM}^{-1} \text{ cm}^{-1}\text{)}$			
	423 nm	505 nm	547 nm	589 nm
MetMb				
pH 4.5		10.24	5.24	3.58
pH 8.0		9.16	6.00	4.40
HPMetMb		8.61	10.18	14.34
PMetMb		7.36	10.18	8.53

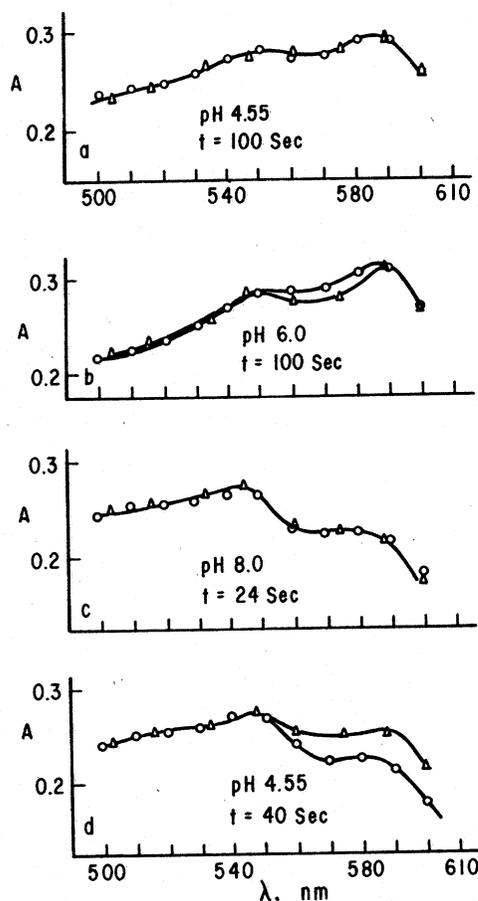


FIGURE 2: Comparison of calculated and observed spectra at various reaction times and pH values. (Δ) observed, (O) calculated.

ed, but there was also a slower reaction, which reached completion in about an hour. If the solution was allowed to react at pH 8.0 for increasing lengths of time before lowering the pH, proportionately smaller amounts of HPMetMb were formed. When the pH was readjusted to 8.0, the relative amounts of the three pigments observed at pH 4.6 remained the same.

Stability of the Compounds. The long term stabilities of the two pigment products were widely variant. PMetMb would oxidize overnight to MetMb. In contrast, HPMetMb is remarkably stable. Since the peroxide effectively sterilizes the solutions, they are bacteriologically stable, and we have kept sealed solutions of HPMetMb at room temperature for months. We did some experiments with HPMetMb to see if we could cleave the heme to obtain an identifiable heme free of the protein. We used the procedure of Fox and Thomson (1964), and some of the variations they listed, but were unable to separate the heme from the protein. The color precipitated with the protein, and upon taking the protein up in pH 4.5 buffer, the original spectrum was obtained. The spectrum of HPMetMb was unchanged by reductants and/or the strong ligands, CO or NO.

Reaction Intermediates and Products. The reaction at pH 4.5 was found to take place in three stages, as shown in Figure 3. In the first stage, up to 20–30 sec at 20.0° , MetMb was converted to a mixture of green and red pigments. In the second stage the last of the MetMb disappeared, but the predominant reaction was the conversion of part of the red pigment to the green pigment (HPMetMb), which reaction was essentially complete in 30 min. The mixture at this point was relatively stable, and the relative

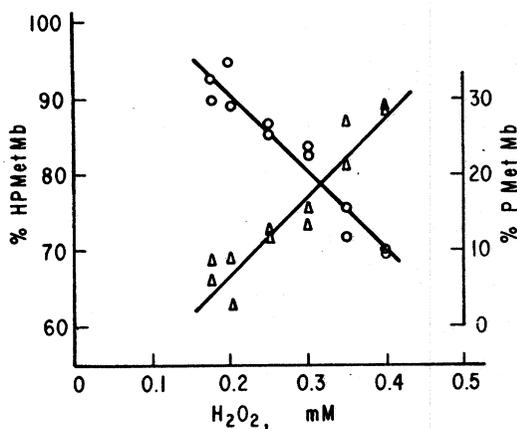
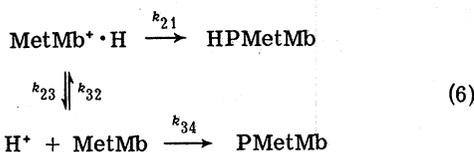


FIGURE 7: Relative concentrations of HPMetMb and PMetMb in product mixture at the end of phase two as a function of $[H_2O_2]$ added as soon as maximal absorption at 547 nm had been reached. (O) HPMetMb; (Δ) PMetMb.

concentrations or peroxide initially and added later result in higher concentrations of PMetMb, we have added reactions G and H in Table I. Reaction G is written as a result of the observation that the reaction of $MetMb \cdot (H_2O_2)_n$ is second order at high concentrations of peroxide where the production of PMetMb is favored. To complete the list of spectrally observable reactions of the H_2O_2 -MetMb system we add eq I and J, the formation of cholemyoglobin and the conversion of PMetMb to MetMb in stage three (Figure 3).

Products at End of Phase Two. The relative amounts of the two products HPMetMb and PMetMb are plotted in terms of per cent PMetMb as a function of hydrogen ion concentration in Figure 8. The dashed curve is a normal dissociation curve for one ionizing group and it can be seen that the proportions of pigments cannot be represented by such a process, that is, the two pigments are not in equilibrium with each other. The concentrations of the two pigments are actually the result of the reaction with H_2O_2 of two different forms of the heme pigment, which forms are the result of one or more hydrogen ion dissociations in the heme pigment. In its simplest form the reaction sequence may be written



(number subscripts are the same as in the Appendix)

From a total consideration of the reaction, it would appear that the reaction is actually much more complex than implied by eq 6. It must be recalled, however, that the red intermediate is preformed during the first phase of the reaction, that is to say, the amount of green pigment eventually formed is predetermined in the first stage. The red intermediate is thus not so much a special form of PMetMb as it is a modified form of HPMetMb. Furthermore, the results of Figure 8 were obtained from reactions with 0.15 mM H_2O_2 where the effects of incomplete formation of products and of excess peroxide (eq G and H, Table III) are minimal. Under these conditions we have, as a first approximation, treated the reaction in terms of eq 6, that is, one equilibrium and two first-order reactions. This system is not soluble by the usual methods of integral calculus, but Matsen and Franklin (1950) have developed a method for solving such a

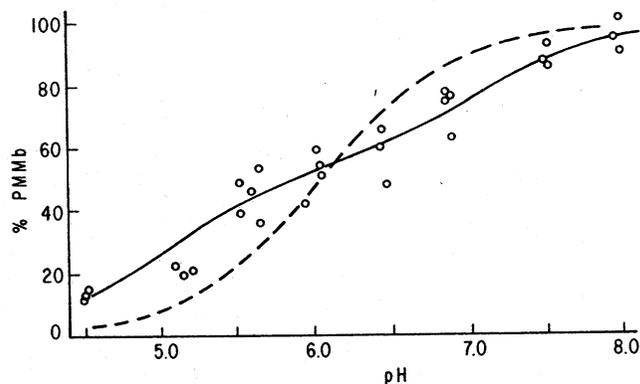


FIGURE 8: Relative concentration of PMetMb at end of phase two as a function of pH. $[H_2O_2] = 0.15$ mM, $[MetMb] = 0.05$ mM, $t = 20^\circ$. (---) Normal proton dissociation curve; (—) curve calculated from equations developed in the Appendix.

system. The particular derivation for the above reaction system is given in the Appendix. As described in the Appendix it is possible to fit a curve to the data by approximation. The solid line curve of Figure 8 is the result of such a calculation. The curve is a best fit curve to the three sets of data from three different MetMb preparations and except for some scatter of points on the acid side fits the data to within the standard deviation ($\pm 7\%$) of the kinetic data.

The derivation of the curve made it possible to calculate the pK of the ionizing group involved in the reaction. By the appropriate combination of the various K values of eq 24 (Appendix) it is possible to solve for the values of k_{23} and k_{32} , the rate constants of the ionization reaction, by assuming the rate constant values for k_{21} and k_{34} to be the observed reaction rates at acid and alkaline values. The values of K_1 , K_2 , K_3 , and K_4 (eq 24, Appendix) used in the curve of Figure 7 are 0.953, 4.17, 4.12, and 1.0, respectively, which give the values for the rate constants and dissociation constant shown in Table IV.

Titration of the Reaction. Figure 9 shows the results of titrating the reaction mixture during the first two phases. Both acid and base titrations were carried out, but the addition of acid caused extensive protein denaturation; the stoichiometry was highly variable and we could not use the data. There was, however, a net uptake of H^+ below pH 5.2. We may, however, deduce what is happening from the partial titration curve of Figure 9. If we assume that the reaction proceeds at any pH to release a free proton, which the formation of RMetMb at acid pH values suggests, then the acid reaction forming HPMetMb must be forming two basic ions in order that the reaction result in one net basic ion released at low pH. The regression line of Figure 9 was calculated by least squares from the data between pH 5.5 and 7.6 since, as is seen in Figure 8, the production of HPMetMb is approximately linear in this region. The curve crosses the zero line at pH 5.23. At this pH the product

TABLE IV: Rate Constants for Reaction 4.

$k_{21} = 0.0986 \text{ min}^{-1}$
$k_{23} = 0.0492 \text{ min}^{-1}$
$k_{32} = 0.0165 \mu\text{M}^{-1} \text{ min}^{-1}$
$k_{34} = 0.0721 \text{ min}^{-1}$
$K_a = k_{23}/k_{32} = 2.98 \times 10^{-6} \text{ M}$
$pK_a = 5.5$

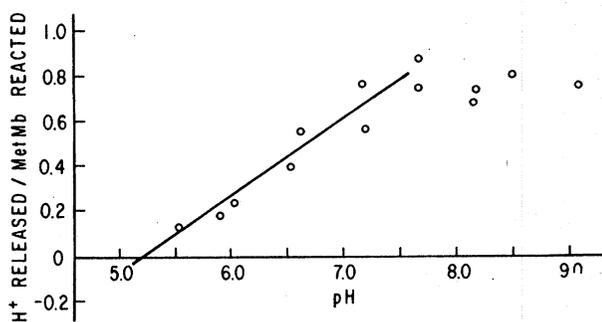


FIGURE 9: Proton release from H_2O_2 -MetMb reaction as a function of pH. $[\text{H}_2\text{O}_2] = 0.15 \text{ mM}$, $t = 20^\circ$.

mixture is about half HPMetMb, which means the formation of the green pigment is releasing two basic ions per mole to neutralize the one acidic ion per mole released in the first part of the reaction. Even as George and Irvine (1952) found, maximal production of H^+ at pH 8.0 was only about 80–90% of theoretical.

Discussion

The Initial Reactant. King and Winfield (1963) suggested that the formation of PMetMb (Mb^{IV}) was preceded by the formation of a peroxo-compound, but they could not demonstrate such formation. Our results, which strongly suggest binding of peroxide, support this hypothesis, with the peroxo complex being spectrally identical with the initial pigment, MetMb.

On the same subject George and Irvine (1952) claimed that the PMetMb complex formation does not involve an initial equilibrium such as $\text{MetMb} + \text{H}_2\text{O}_2 \rightleftharpoons \text{complex}$ but they did find that at constant mole ratios of peroxide and pigment they obtained a constant per cent formation of PMetMb regardless of absolute concentration. This result is more consistent with peroxide binding, assuming a fairly large association constant, than with a free peroxide reaction where other reactions may take place. If the stoichiometry and velocity were appreciably different for the various reactions, constant proportions of products would not be observed, especially with varying absolute concentrations. Chance (1951) has shown that the kinetics of the action of the hydroperoxidases proceeds by Michaelis-Menton kinetics, *i.e.*, through formation of a preliminary enzyme- H_2O_2 complex. In view of all these considerations and our evidence from the analysis of the kinetic data the weight of evidence supports the conclusion that the initial reaction in the sequence is the formation of a $\text{MetMb}-(\text{H}_2\text{O}_2)$ complex. Our stoichiometry is consistent with two molecules of H_2O_2 bound to one molecule of pigment in alkaline solution. Yonetani and Schleyer (1967) observed that a twofold excess of peroxide over pigment was required for complete conversion to PMetMb which would follow if 1 mol of peroxide was bound to and reacted with each of two separate sites on the pigment. With one molecule of H_2O_2 bound to the heme, the second must be bound elsewhere on the protein. Both the second and third moles of peroxide which are required at low pH values appear to be involved in the oxidation process that produces the green pigment.

The Formation of HPMetMb. The results of the pH study do not further elucidate the structure of PMetMb. The solution of the kinetic equations and the titration study do, however, explain how the green pigment, HPMetMb, is formed. The pK_a of 5.5 (Table IV) determined from the end product analysis is that of a histidine residue in the pro-

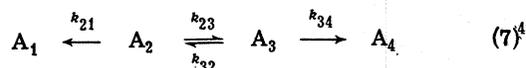
tein. This low pK_a value was reported by Cohen *et al.* (1972) for one of the seven titrating histidines in sperm whale and horse metmyoglobin. Since the presence of positive charge groups lowers the pK_a value of histidine, they suggested that one of the histidines with the low pK_a values is in close proximity to the heme iron. This of course is in accord with Coryell and Pauling's (1940) original hypothesis that the distal histidine (64 or E7) was the one with the unliganded pK_a of 5.3. The two different reaction products of this study are therefore the results of the difference in reactivity of hydrogen peroxide toward one of the "acid" histidine residues, probably the distal histidine. From the titration studies we have deduced the formation of 2 mol of base/mol of HPMetb formed. Holtz and Triem (1937) studied the oxidation of histidine by hydrogen peroxide and found that the reaction proceeds by complete oxidation of the molecule with release of three ammonia groups, two of course being from the imidazole ring. A similar reaction with the distal histidine to produce HPMetMb would account for the 2 mol of base. The stoichiometry of oxidation of histidine by a single molecule of H_2O_2 does not balance, there being too few electrons available for release of two ammonia molecules from the imidazole ring. This may explain the requirement of 3 mol of peroxide for maximal conversion to HPMetMb, the third mole, bound or unbound, being required to complete the oxidation. Partial and variable destruction of excess peroxide may account for the variations in both rates and product ratios observed in the acid reaction. From our study it appears that the oxidation links the histidine to the heme since the heme is not readily cleaved from the globin. From the position of the major absorption band at 589 nm, we assume that it is the porphyrin ring that is involved in the linkage, probably through the formation of an ether linkage between the porphyrin and oxidized histidine. The formation of such a bond could interfere with further liganding to the iron either by blocking ligands from entrance into the protein cleft containing the heme or by pulling the protein structure together preventing the formation of a normal octahedral coordinate-covalent structure.

The Red Intermediate. In view of the foregoing discussion we also conclude that the red intermediate we have observed and the radical intermediate of King and coworkers (King and Winfield, 1963; King *et al.*, 1967) are one and the same, but that the intermediate undergoes two different reactions. In alkali, the unprotonated histidine is resistant to oxidation, and the radical formed from or with the peroxide decays by a second-order dismutation reaction. In acid, the protonated histidine is oxidized, the reaction ultimately resulting in the formation of HPMetMb. This explains the formation of HPMetMb from the red intermediate in the pH reduction experiments, for in lowering the pH and protonating the histidine, the latter is rendered susceptible to oxidative attack. This attack may be by bound peroxide since the conversion occurs under conditions where the kinetic analysis indicates no free peroxide. During this process, the reaction between a molecule of peroxide and the heme may also take place, forming the characteristic red peroxymetmyoglobin complex.

Appendix

A method of solving complex interacting systems has been developed by Matsen and Franklin (1950). As a first approximation for the solution of the peroxide-heme reaction, we have assumed that each of the two different prod-

ucts is derived from one of two initial reactants in equilibrium with each other. The defining reaction is



A_3 is the basic form of the two reactants in equilibrium at the beginning of the reaction, and k_{32} is actually a second-order reaction rate constant for the reaction



Multiplying k_{32} by H^+ yields a (pseudo) first-order rate constant which makes the reaction matrix homogeneous in reaction order and will also introduce into the rate expressions the necessary hydrogen ion dependence. At the initial equilibrium

$$k_{23}[A_2] = k_{32}[H^+][A_3] \quad (9)$$

$$K_{eq} = [H^+][A_3]/[A_2] = k_{23}/k_{32} \quad (10)$$

The corresponding matrix is developed from the appropriate rate expressions (eq 11). For our requirements we need

$$\begin{array}{cccc} -\lambda_1 & -k_{21} & 0 & 0 \\ 0 & k_{21} + k_{23} - \lambda_2 & -[H^+]k_{32} & 0 \\ 0 & -k_{23} & [H^+]k_{32} + k_{34} - \lambda_3 & 0 \\ 0 & 0 & -k_{34} & -\lambda_4 \end{array} \quad (11)$$

only the ratio of end products, essentially the infinite time values of the rate expressions. As will be apparent from the subsequent treatment, it is not necessary to solve the matrix for the eigen values, λ_r . It is only necessary to derive the relative B values, the coefficients that transform the real concentrations to the eigen concentrations. The defining equations from the matrix for the B values are

$$-\lambda_r B_{1r} - k_{21} B_{2r} = 0 \quad (12)$$

$$(k_{21} + k_{23} - \lambda_r) B_{2r} - [H^+]k_{32} B_{3r} = 0 \quad (13)$$

$$-k_{23} B_{2r} + ([H^+]k_{32} + k_{34} - \lambda_r) B_{3r} = 0 \quad (14)$$

$$-k_{34} B_{3r} - \lambda_r B_{4r} = 0 \quad (15)$$

The B_{ir} are not defined in absolute terms, but may be defined in relative terms to each other. Setting the $B_{1r} = 1$, the $B_{2r} = -\lambda_r/k_{21}$. There are, however, two different equations, 13 and 14, which define the B_{3r} , giving two different definitions for B_{3r}

$$B_{3r} = - \left(\frac{k_{21} + k_{23} - \lambda_r}{k_{21}[H^+]k_{32}} \right) \lambda_r = \frac{-\lambda_r k_{23}}{k_{21}([H^+]k_{32} + k_{34} - \lambda_r)} \quad (16)$$

and two definitions for the B_{4r}

$$B_{4r} = \frac{k_{23}k_{34}}{k_{21}([H^+]k_{32} + k_{34} - \lambda_r)} = \frac{k_{34}(k_{21} + k_{23} - \lambda_r)}{k_{21}[H^+]k_{32}} \quad (17)$$

The reason for the two different expressions is that each horizontal line in the matrix corresponds to only one of the components in the reacting system. Thus for a given concentration of component one (which is the basic defining concentration in our solution of the equations) there are two different relative concentrations of components A_3 and A_4

depending on the initial concentrations of A_2 and A_3 . In most reaction systems this mathematical situation is duplicated by starting the reaction with but one of the components in the system. In the system we have described, where two components are present initially in equilibrium with each other, the concentrations of products are given in two parts, one part as if derived from one reactant alone and the other part from the other reactant.

Continuing the solution of the kinetics, we are interested only in the concentrations of A_1 and A_4 at completion of the reaction. The defining equations for the $[A_1]$ and $[A_4]$ values at $t = \infty$ are written and solved with λ_1 and $\lambda_4 = 0$, and substituting the arbitrarily assumed values for the B_{1r} and B_{4r} , remembering that we have two definitions of the B_{4r} values, we obtain

$$[A_{1(2,3)}] = Q_1^0 + Q_4^0 \quad (18)$$

$$[A_{4(2)}] = \frac{k_{23}k_{34}}{k_{21}([H^+]k_{32} + k_{34})} (Q_1^0 + Q_4^0) \quad (19)$$

$$[A_{4(3)}] = \frac{k_{34}(k_{21} + k_{23})}{k_{21}[H^+]k_{32}} (Q_1^0 + Q_4^0) \quad (20)$$

The second subscripts are added in eq 18-20 to indicate the source of the two identical products. What was actually observed was the sum of these values

$$[A_{1, \text{obsd}}] = [A_{1(2)}] + [A_{1(3)}] \quad (21)$$

$$[A_{4, \text{obsd}}] = [A_{4(2)}] + [A_{4(3)}] \quad (22)$$

Taking the ratio of eq 21 and 22, substituting the $[A_1]$ and $[A_4]$ values, cancelling out the common term $Q_1^0 + Q_4^0$, and simplifying we have

$$\frac{[A_{1, \text{obsd}}]}{[A_{4, \text{obsd}}]} = \frac{k_{21}}{k_{34}} \frac{[H^+]2k_{32}([H^+]k_{32} + k_{34})}{[H^+]k_{32}(k_{21} + 2k_{23}) + k_{34}(k_{21} + k_{23})} \quad (23)$$

In order to derive a simpler empirical equation to use in curve fitting we combine the various constants to obtain

$$\frac{[A_{1, \text{obsd}}]}{[A_{4, \text{obsd}}]} = \frac{k_{21}}{k_{34}} [H^+] \left(\frac{K_1[H^+] + K_2}{K_3[H^+] + K_4} \right) \quad (24)$$

The total pH range covered is from pH 4.50 to 8.0 or 31.6 $\mu\text{M H}^+$ to 0.01 $\mu\text{M H}^+$. As a first approximation to fitting the experimental data eq 24 becomes at pH 4.5 and 8.0, respectively

$$\frac{[A_{1, \text{obsd}}]k_{34}}{[A_{4, \text{obsd}}]k_{21}[H^+]} \cong \frac{K_1}{K_3} \quad (25)$$

$$\frac{[A_{1, \text{obsd}}]k_{34}}{[A_{4, \text{obsd}}]k_{21}[H^+]} \cong \frac{K_2}{K_4} \quad (26)$$

Since at pH 4.50 the reaction is predominantly the production of A_1 , whereas at pH 8.0 it is the production of A_4 , the observed rates of the reaction at these two pH values are taken as k_{21} and k_{34} , respectively. Setting $K_4 = 1$ it is possible from eq 24-26 to solve for a set of relative values of K_1 , K_2 , K_3 , and K_4 to solve eq 24 for a curve to fit the experimental data.

References

- Allen, N. (1930), *Ind. Eng. Chem., Anal. Ed.* 2, 55.
 Brill, A. S., and Sandberg, H. E. (1968), *Biochemistry* 7, 4254.
 Chance, B. (1951), in *The Enzymes*, Vol. 2, Part 1, Sumner, J. B., and Myrback, K., Ed., New York, N. Y., Academic Press, p 428.

⁴ For sake of brevity and to make comparison easier, we use the terminology of Matsen and Franklin (1950), compare with eq 6 in text.

- Cohen, J. S., Hagenmaier, H., Pollard, H., and Schechter, A. N. (1972), *J. Mol. Biol.* 71, 513.
- Coryell, C. D., and Pauling, L. (1940), *J. Biol. Chem.* 132, 769.
- Fox, J. B., Jr., and Thomson, J. S. (1963), *Biochemistry* 2, 465.
- Fox, J. B., Jr., and Thomson, J. S. (1964), *Biochemistry* 3, 1323.
- George, P., and Hanania, G. (1952), *Biochem. J.* 52, 517.
- George, P., and Irvine, D. H. (1952), *Biochem. J.* 52, 511.
- George, P., and Irvine, D. H. (1955), *Biochem. J.* 60, 596.
- Holtz, P., and Triem, G. (1937), *Hoppe-Seyler's Z. Physiol. Chem.* 248, 5.
- Keilin, D., and Hartree, E. F. (1935), *Proc. Roy. Soc., Ser. B* 117, 1.
- King, N. K., Looney, F. D., and Winfield, M. E. (1967), *Biochim. Biophys. Acta* 133, 65.
- King, N. K., and Winfield, M. E. (1963), *J. Biol. Chem.* 238, 1520.
- King, N. K., and Winfield, M. E. (1966), *Aust. J. Biol. Sci.* 19, 211.
- Kobert, R. (1900), *Arch. Gen. Physiol.* 82, 603.
- Lemberg, R., Legge, J. W., and Lockwood, W. H. (1941), *Biochem. J.* 35, 328.
- Matsen, F. A., and Franklin, J. L. (1950), *J. Amer. Chem. Soc.* 72, 3337.
- Nicholas, R. A., and Fox, J. B., Jr. (1969), *J. Chromatogr.* 43, 61.
- Yonetani, T., and Schleyer, H. (1967), *J. Biol. Chem.* 242, 1974.