EFFECT OF pH UPON THE REACTION KINETICS OF THE ENZYME-SUBSTRATE COMPOUNDS OF CATALASE*

BY BRITTON CHANCE

(From the Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania)

(Received for publication, June 1, 1951)

The existence of acid-linked groups in hemoproteins may be observed by studies of the effect of pH upon the nature of the enzyme and its compounds or upon the speed with which the enzyme compounds are formed. Since the activity of enzymatically active hemoproteins depends upon compound formation with the substrate, the effect of pH upon the overall activity may also demonstrate heme linkages. Both of these approaches have been used by Theorell and Agner (1, 2) in their studies of catalase and have resulted in their postulation of a heme-linked hydroxyl group of pK 3.8 bound to catalase hematin, although they were unable to show a direct effect of this heme linkage upon catalase activity. In this paper new and rapid methods have been used for measuring the effect of pH upon catalase activity, not only in the decomposition of hydrogen peroxide but also in the oxidation of alcohol, formic acid, and nitrous acid. Studies have also been made of the effect of pH upon the kinetics of the catalase-peroxide compounds: their speed of formation, their speed of transition from one type to the other, and their speed of "spontaneous" decomposition into the free enzyme. It is found that all of these reactions are pH-insensitive in the range 5 to 9. Below pH 5 both the transition and the "spontaneous" decomposition reactions are accelerated, the latter in direct proportion to the hydrogen ion concentration, suggestive of a kinetically operative heme linkage. Below pH 4 the activity towards hydrogen peroxide diminishes, but not rapidly enough to correspond to pK 3.8 for Agner and Theorell's heme-linked hydroxyl group (2). In the alkaline region this over-all activity falls off below pH 9, owing possibly to a new heme linkage in catalase. The reaction of catalase hydrogen peroxide with alcohols exhibits remarkable pH stability; negligible change is measured from pH 4.3 to 12.0. It is shown here that this complex reacts only with the undissociated molecules of nitrous and formic acids.

Methods—The velocity constant for the reaction of catalase with hydrogen peroxide may be measured indirectly by a study of the effect of

* This work was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.
pH upon the over-all activity,

\[ 2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \]  

(1)
or by direct measurements of the rate of combination of catalase with hydrogen peroxide,

\[ \text{Cat} + H_2O_2 \rightleftharpoons \text{Cat-H}_2O_2 \]  

(2)

by means of rapid spectrophotometric techniques (3). In the latter case the slower reaction can be measured more accurately.

\[ \text{Cat} + 4\text{CH}_2\text{OOH} \rightleftharpoons \text{Cat}(\text{CH}_2\text{OOH})_4 \]  

(3)

Two methods are used for Equation 1, a polarographic technique based upon the platinum anode (4) and a spectrophotometric technique (5, 6) based upon the hydrogen peroxide band at 230 m\(\mu\) (\(\Delta\varepsilon = 0.0067 \text{ cm}^{-1} \times \text{mM}^{-1}\); Reiche (7)). Classical titrimetric methods are so slow that excessive enzyme inactivation occurs. In acid solutions, dilute phosphoric acid (\(\sim 1 \text{ mM}\)) is used to avoid catalase inhibition (2). Catalase activity is computed, \(k'_1 = (1/x_0\varepsilon)(dx/dt)\), where \(x\) is the hydrogen peroxide concentration at any time, \(x_0\) its initial concentration, and \(\varepsilon\) the catalase concentration (in moles per liter) (4). The catalase and methyl hydrogen peroxide preparations are similar to those used in previous papers (4).

Effect of pH upon Over-All Activity—Fig. 1 shows that the activity of catalase is constant to within the experimental error from pH 4 to 8.5 and falls off along a curve of roughly pH 3 in acid solutions. This segment of the curve differs from that obtained by Agner and Theorell (2), who found the activity decreased at pH 5.8 and fell to 70 per cent at pH 4.0. This discrepancy is attributed to their slower methods of measuring catalase activity, which permitted appreciable amounts of the inactive secondary catalase peroxide complex to accumulate before the activity was measured (8). And this effect may still be responsible for the loss of activity below pH 4.0 shown in Fig. 1 (see Fig. 5). The significant feature of Fig. 1 is that no decrease of activity along the curve of pK 3.8 corresponding to the heme-linked hydroxyl group (2) is observed.

A sharp decrease of activity occurs in alkaline solutions and is attributed to a catalase heme linkage, possibly analogous to that in peroxidase (9). The effect is not caused by the dissociation of hydrogen peroxide\(^1\) (pK 11.8; Kargin (10)) which would not affect the measured activity because the latter is independent of the initial peroxide concentration (4). A remarkable effect observed below pH 10 is a change in the order of the catalase reaction; the reaction kinetics approximate zero order and suggest that a new rate-limiting step is involved.

\(^1\) The dissociation of hydrogen peroxide causes a considerable increase in its molecular extinction coefficient.
Kinetics of Catalase-Hydrogen Peroxide Complex in Alkaline Solutions—
In order to elucidate the observations of Fig. 1, Equation 2 has been studied spectrophotometrically (3) and Fig. 2 shows that the steady state

![Graph of pH-activity relationship for catalase](image1)

**Fig. 1.** The pH-activity relationship for catalase obtained by rapid spectrophotometric measurements at 220 m. The values of activity were computed from the equation \( k_t' = 0.693/e t_t \), where \( e \) is the erythrocyte catalase molarity, 0.5 to 1.0 \( \times 10^{-3} \) M, \( t_t \) is the time for half decomposition of the hydrogen peroxide, and \( k_t' \) is approximately \( 3.5 \times 10^7 \) M\(^{-1}\) X sec.\(^{-1}\) at 25°C. •, experiments carried out by adjusting the pH of unbuffered catalase solutions by adding phosphoric acid or by adding sodium hydroxide (Experiment 012). ○, experiments with 0.01 M phosphate buffers in the range pH 6 to 8 and 0.05 M glycine buffers of pH 9 to 11 (Experiment 570). (25°C.)

![Graph of decrease in steady state concentration of catalase-hydrogen peroxide complex](image2)

**Fig. 2.** The decrease in the steady state concentration of the catalase-hydrogen peroxide complex in alkaline solutions, 0.54 μM horse liver catalase, 400 μM hydrogen peroxide, 2 mM ethanol, 0.05 M glycine buffer (Experiment 402b).

concentration of the complex decreases in alkaline solutions, indicating that the amount of active enzyme or the speed of formation of the complex (\( k_1 \)) has decreased. And some decrease in the latter has been observed (roughly 8-fold at pH 12 compared to pH 7). Thus the effect of the heme linkage is probably upon \( k_1 \).
A partial explanation of these effects is given by our theory of catalase action (11).

\[ E + S \xrightarrow{k_1} ES \]  
\[ ES + S \xrightarrow{k_1'} E + P \]

In the steady state,

\[ p = \frac{e}{1 + \frac{k_1'}{k_1}} \]

where \( p = ES, \ e - p = E, \) the enzyme concentration, and \( x = S, \) the substrate concentration. Thus a decrease of \( k_1 \) or \( e \) would result in a decrease of \( p, \) as in Fig. 2. The over-all activity \( (dx/dt) \) is also proportional to \( p. \)

\[ \frac{dx}{dt} = -2k_1'xp \]

However, the decline of over-all activity of Fig. 1 in alkaline solutions is somewhat more rapid than that of \( p \) in Fig. 2 and a more detailed explanation is required.

**Effect of pH upon Kinetics of Catalase-Methyl Hydrogen Peroxide**—More accurate studies of the effect of pH upon the binding of catalase and peroxide according to Equation 3 are given in Fig. 3 and show a remarkably small effect; in the acid region nearly constant values are obtained until protein denaturation occurs. In the alkaline region, the activity probably is also terminated by the splitting of catalase, but the similarity of the
spectra of the peroxide complex and split catalase causes some difficulty in the interpretation of the experimental data. However, in the broad region of catalase stability the velocity of combination with methyl hydrogen peroxide is constant. This result is in accord with our data of Fig. 1 in the acid region but differs in the alkaline region; the loss of activity with hydrogen peroxide occurs at a lower pH than with methyl hydrogen peroxide.

*Secondary Catalase-Methyl Hydrogen Peroxide Complex*—The primary green complex is slowly transformed into a red secondary complex in neutral solutions, but Fig. 4 shows that this reaction is accelerated by hydrogen ions below pH 5. It is unlikely that this is caused by a simple heme-linked dissociation, because the logarithmic plot (Curve B) has a slope of 0.3 instead of 1.0. A similar effect probably occurs with the catalase-hydrogen peroxide complex (8). Since the secondary red complexes are inactive, these data illustrate how much more rapidly catalase activity is lost in acid than in neutral solutions. In fact we have observed spectrophotometrically the formation of the secondary complex upon addition of a solution of hydrogen peroxide to strong catalase at pH 2.2.

*Effect of pH upon “Spontaneous” Decomposition of Catalase-Hydrogen Peroxide Complex*—The catalase-hydrogen peroxide complex is not stable, but decomposes in about 30 seconds at pH 7, possibly caused by some donor molecule contained in the catalase preparation. The effect of pH upon the speed of this reaction is shown in Fig. 5, and an abrupt increase
occurs below pH 5. In this case the logarithmic plot is linear and of unity slope, suggesting that the donor molecule has a pK of about 5 or that the complex itself reacts with hydrogen ions below pH 5. This reaction apparently has a different pH dependence from that with alcohol or formic acid, and this difference suggests that the mechanism of the "spontaneous" decomposition involves more than a simple donor reaction.
Effect of pH upon Activity of Catalase-Hydrogen Peroxide Complex towards Hydrogen Donors. Ethanol—By means of the spectrophotometric technique (3), the reaction of the complex with ethanol is measured at various values of pH, and the reaction velocity constant, $k_4$, is calculated as described previously (12). As shown by Fig. 6, the lack of pH effect is remarkable: grossly there is no change of $k_4$ from pH 4.3 to 12.0. In detail, there is a small increase of activity, 14 per cent in a change of 108
EFFECTS OF PH ON CATALASE KINETICS

in hydrogen ion concentration. There is no participation of hydrogen or hydroxyl ions directly or indirectly through heme linkages in this reaction.

Nitrous and Formic Acids—Ethanol does not ionize under these conditions, but nitrous and formic acid are mainly ionized at neutral pH (pK 3.4 and 3.76 respectively), and in the range pH 5 to 8 the free acid concentration would decrease by about 1000-fold. Interestingly enough the value of $k_4$ computed on the basis of the total acid plus anion concentration decreases exactly 1000-fold over this pH range, as shown by the data of Fig. 7, in which logarithmic coordinates are used. Thus the reaction of the complex is with the undissociated acid, not the anion. The true reaction velocity constant ($k_4$) is computed from $k'_4$ when pH > pK.

$$\log k_4 = \log k'_4 + \text{pH} - \text{pK}$$

$$k_4 = 1.0 \times 10^6 \text{ M}^{-1} \times \text{sec}^{-1}$$

for formic acid and $$k_4 = 1.4 \times 10^7 \text{ M}^{-1} \times \text{sec}^{-1}$$

for nitrous acid at 24°C. This reaction is much more rapid than that with alcohols and is comparable to the speed of combination of enzyme and substrate.

A special study of this reaction has been carried out in acid solutions in which the value of pH approaches the pK of formic acid. Considerable attention has been paid to experimental detail in order to avoid an error

$^2$ No inhibition of catalase (2) by these formate concentrations occurs at these values of pH.
due to the rapid spontaneous decomposition of the complex (see Fig. 5) or to the circumstances described previously (12). Results for both ethanol and formic acid are plotted in Fig. 8. Since \( k_4 \) is computed as in Equation 8, constant values are to be expected and are found. At the low values of pH, formate combines with catalase hematin (\( K \), the equilibrium constant, is about \( 10^{-4} \) at pH 4.3 (2)) and some inhibition may have occurred.

Detection of Formic Acid Formed from Carbon Monoxide—We had previously detected formic acid formed from bubbling carbon monoxide through catalase solutions (13), but have been unable to reproduce such large effects. In acid solutions very dilute formic acid is detectable by the method used above and a summary of the tests is given in Table I. A solution equilibrated several days with CO gave an effect equivalent to about 6 \( \mu M \) formic acid. The previous results therefore represent a gross exaggeration of the effect and were probably caused by formic acid from the carbon monoxide generator entrained through an inadequate washing system. Such a reaction is indeed possible on thermodynamic grounds; the equilibrium constant is 6.90 at 25° for the reaction \( HCOOH \rightarrow H_2O + CO \) (14), saturated carbon monoxide containing about 8 \( mM \) formic acid at equilibrium.

### TABLE I

*Effect upon \( k_a \) of Bubbling Carbon Monoxide through Solution of Catalase*

<table>
<thead>
<tr>
<th>Time after starting bubbling CO, min.</th>
<th>0</th>
<th>7</th>
<th>22</th>
<th>40</th>
<th>Several days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recorder deflection corresponding to catalase hydrogen peroxide, mm.</td>
<td>50</td>
<td>52</td>
<td>58</td>
<td>60</td>
<td>85</td>
</tr>
<tr>
<td>Time for half decomposition of catalase-hydrogen peroxide, sec.</td>
<td>5.0</td>
<td>5.5</td>
<td>4.2</td>
<td>4.0</td>
<td>1.8</td>
</tr>
</tbody>
</table>

\[
k_a' = \begin{cases} \frac{0.69}{et_f}, \text{sec}^{-1} & \text{for } k_a \neq 0 \\ 0.14 & \text{if } k_a = 0 \end{cases}\]

\[
a_0 = \frac{k_a' - k_a}{0.4}, \mu M^* \]

* The apparent value of \( k_a \) is \( 0.4 \times 10^6 \text{M}^{-1} \times \text{sec}^{-1} \) at this value of pH. \( a_0 \) is the donor concentration.

† The CO-saturated solution was diluted 10-fold; hence the actual concentration was 6 \( \mu M \).

SUMMARY

1. The effect of pH upon the velocity of decomposition of hydrogen peroxide in the presence of catalase has been measured over the range 3.0 to 11.4. The activity is constant in the region pH 4 to 8.5. Below pH
4, the decrease of activity is caused in part by the formation of the secondary inactive catalase-hydrogen peroxide compound. The decrease of activity of catalase in acid solutions is considerably less under these experimental conditions than in Agner and Theorell's earlier study. There is very little experimental evidence for the existence of a heme linkage in the acid region that has significance in the enzymatic activity. Above pH 8.5, the activity decreases slowly and falls to about 20 per cent at pH 11.4. This decrease of activity is paralleled by a decrease in the steady state concentration of the primary catalase-hydrogen peroxide complex in alkaline solutions. This decrease is caused, in part at least, by a decrease in the velocity constant for the formation of the catalase-hydrogen peroxide complex. The experimental results suggest that a heme linkage responsible for the rapid combination of enzyme and substrate is dissociated in alkaline solutions.

2. The effect of pH upon the velocity of combination of catalase and methyl hydrogen peroxide has been measured over the range 2.2 to 12.5. No effect of significance to the enzymatic activity is found in the range pH 2.2 to 12.0, and the changes in the reaction velocity beyond this range are largely attributed to the denaturation of catalase. No evidence of the pH dependence of the formation of the hydrogen peroxide compound is found with methyl hydrogen peroxide. Nor does the ionization of methyl hydrogen peroxide in alkaline solutions appear to alter the reaction velocity.

3. The effect of pH upon the reaction of the catalase-hydrogen peroxide complex with ethanol has been studied in the range pH 4.3 to 12.0, and no effect of significance in the enzymatic activity has been found. Thus there is no participation of hydrogen or hydroxyl ions in this reaction.

4. The velocity of the reaction of catalase-hydrogen peroxide with formate or nitrite increases 10-fold for each unit decrease of pH in the range 5 to 8, and it is concluded that only the free acids react with the peroxide complex. On this basis, the true velocity constants for the reaction with catalase-hydrogen peroxide with formic acid or nitrous acid are $1.0 \times 10^6$ and $1.4 \times 10^7 \text{ M}^{-1} \times \text{sec.}^{-1}$ respectively. These values are much larger than the values for the reactions with alcohols and approach the velocity constant for the combination of enzyme and substrate.

5. The velocity constant for the reaction of catalase-hydrogen peroxide with formic acid (computed on the basis of free formic acid) is relatively constant in the region pH 4.3 to 5, and under these conditions less than 1 $\mu$M formic acid gives a readily measurable reaction.

6. A definite and related effect of pH has been studied in the transition from the primary to the secondary catalase-methyl hydrogen peroxide complex and in the spontaneous decomposition of the primary hydrogen
peroxide complex. Both of these reactions are accelerated by hydrogen ions below pH 5, and the latter reaction shows a 10-fold increase of rate per pH unit in the region pH 3.3 to 4.8. It is very likely that a heme-linked group of catalase is responsible for this effect. The pK of such a group would lie at about pH 5.

7. The formation of formic acid from carbon monoxide in the presence of catalase has been reinvestigated, and the previous results indicate a gross exaggeration of the effect; very small quantities of formic acid are formed. Thus the reaction of catalase with formic acid does not provide a complete explanation of the conversion of carbon monoxide to carbon dioxide in vivo, although catalase could indeed be responsible for the oxidation of formate to carbon dioxide in the presence of hydrogen peroxide.

BIBLIOGRAPHY
14. Randall, M., in International critical tables of numerical data, physics, chemistry and technology, New York, 7, 244 (1930).
EFFECT OF pH UPON THE REACTION KINETICS OF THE ENZYME-SUBSTRATE COMPOUNDS OF CATALASE
Britton Chance


Access the most updated version of this article at http://www.jbc.org/content/194/2/471.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/194/2/471.citation.full.html#ref-list-1