THE EFFECT OF pH UPON THE EQUILIBRIA OF CATALASE COMPOUNDS*

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In the preceding study of the effect of pH upon catalase activity (1), the heme-linked hydroxyl group of Agner and Theorell (2) was not found to be necessary for the enzymatic activity, and we have therefore reinvestigated the experimental evidence for this heme linkage from both equilibrium and kinetic studies. And since many of our views of the heme linkages of catalase are based upon analogies with ferrihemoglobin and ferrimyoglobin, similar studies have been made of their heme linkages.

Physical methods, such as spectroscopy, reveal no change in the catalase spectrum caused by hydrogen ions alone in the region over which the catalase protein is stable; if catalase-heme linkages exist, they are not operative spectroscopically in the free enzyme above pH 3 (2). The method used here is to measure the effect of pH upon the kinetics and equilibria of the compounds of catalase with weak and strong acids. In this paper data on hydrazoic, hydrocyanic, formic, hydrofluoric, and acetic acids are presented over as wide a range of pH as practicable. A detailed study of the effect of pH upon the kinetics and equilibrium of the reactions of cyanide with ferrohemoglobin and ferrimyoglobin is described.

The interpretation of the studies of heme linkages in catalases and peroxidases has closely followed the pattern set by Coryell, Stitt, and Pauling (3) in their studies of the ferrihemoglobin compounds with fluoride and cyanide ions, etc., in which no effect of pH was found in fluoride binding and a pH effect in cyanide binding was assumed. Thus the ferrihemoglobin reactions were written as follows:

\[ \text{Hb}^+ + \text{F}^- \leftrightarrow \text{HbF} \] (1)
\[ \text{Hb}^+ + \text{HCN} \leftrightarrow \text{HbCN} + \text{H}^+ \] (2)

When catalases and peroxidases were found to show pH-sensitive fluoride binding, Thorocell (4), following Pauling’s reasoning, postulated that a

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hydroxyl group was bound to the iron atom of these enzymes and that they combine with fluoride ions as follows:

\[ \text{FeOH} + \text{F} \rightleftharpoons \text{FeF} + \text{OH}^- \] (3)

Chance (5) found no effect of pH upon cyanide binding in the region of neutrality and, following the same pattern, wrote the reaction thus:

\[ \text{FeOH} + \text{HCN} \rightleftharpoons \text{FeCN} + \text{H}_2\text{O} \] (4)

Similar experimental results were found in the binding of peroxides (6).

But it has not yet been demonstrated that the binding of fluoride becomes independent of pH or that the binding of cyanide becomes pH-dependent in acid solutions in which the heme-linked hydroxyl group would be dissociated. In fact, some data show no pH dependence of cyanide binding at low pH (5). This critical region at low pH is covered in considerable detail by these experiments.

**Catalase Preparations**—Catalases were prepared from horse and from human erythrocytes by Dr. A. C. Maehly, according to the methods of Bonnichsen (7) and Herbert and Pinsent (8), and showed similar spectrophotometric and activity constants. The ferrihemoglobin was prepared according to the method of Keilin and Hartree (9), and the ferrimyoglobin was a pure sample prepared by Dr. M. Besnak (10), to whom many thanks are due. These preparations were dialyzed to remove interfering anions. Fresh catalase preparations were used; older ones were occasionally found to be less resistant to low values of pH.

**Method**—Our experimental method is well illustrated by Fig. 1. Very dilute catalase is used to avoid the necessity for large buffer concentrations. Direct recording of the results is achieved by a sensitive spectrophotometer (11), and, for small optical density changes, the concentration of the catalase compounds is read directly from the record on a linear scale. Microliter additions of strong solutions of the reactants make dilution corrections negligible. The value of the pH is measured directly in the cuvette of the spectrophotometer by using a “baby stomach electrode” manufactured by the National Technical Laboratories.

**Computation of Results**—The apparent dissociation constant is computed from the experimental data in all cases on the basis of the simplest possible reaction. For example, for the equilibrium of catalase and fluoride, the apparent dissociation constant is computed

\[ K' = \frac{[\text{catalase}][\text{total fluoride}]}{[\text{catalase fluoride}]} \] (5)

Then, according to the postulated reaction mechanism, the values of \( K' \) are amended to account for a pH dependence of the reactants.
Spectroscopic Data—The existence of compounds of catalase with azide and fluoride was discovered by Keilin and Hartree (12), while those with formate and acetate were found by Agner and Theorell (2). Keilin and Hartree have recently published spectra of the azide and fluoride compounds (13). The spectra of the formate, acetate, and azide compounds are shown in Fig. 2; the Soret band of the enzyme is shifted several millimicrons towards the visible region of the spectrum, and its extinction coefficient is essentially unchanged. Even at 420 mμ the change of extinction coefficient is small and highly accurate titrations are difficult. The shift of the Soret band on combination with fluoride is not shown in Fig. 2, but is even more difficult to measure; we find a value of $\Delta \epsilon_{420} = +9 \text{ cm}^{-1} \times \text{mm}^{-1}$, in contrast to Keilin and Hartree (13), and have made our measurements at this wave-length.

Titrations with Stronger Acids—In order to avoid interaction with buffer anions, the titration reagent itself is used as a buffer in the case of acetate. With formic and hydrazoic acids the catalase is buffered with dilute lactate solutions which have a very low affinity for catalase. In the case of fluoride, lactate buffers were also used, but the titrations were made with a sodium fluoride-hydrofluoric acid solution of pH 4.0.

Lactic acid is most satisfactory for acidification of catalase because it gives a warning of its improper use by causing a shift of the catalase spectrum. Phosphoric and sulfuric acids have been found to alter the titration curves for acetate, even though no shift of the catalase spectrum occurs: a 2-fold increase was caused by only 230 μM sulfuric acid at pH 3.8.
EFFECTS OF pH ON CATALASE EQUILIBRIA

The results of the titrations are given in Fig. 3 and are in good accord with the effects found previously by Agner and Theorell (2); the negative logarithm of the dissociation constants (pK') of the catalase compounds increases linearly with decreasing pH until, in the range below pH 4.5, a slower increase is noted. But our more extensive data will permit a rather different interpretation of the results, as discussed below.

Titration with Weak Acid—A detailed study of the effect of pH upon the reaction of catalase and cyanide is given by the data of Fig. 4, which show that there is no systematic effect of pH upon this reaction in the range pH 3.1 to 5.8 (and thence to pH 7.0 in view of the earlier data (5)). The average value of the dissociation constant for the human blood catalase compound is somewhat larger than that found for the horse blood catalase compound, $7 \times 10^{-6}$ compared to $4 \times 10^{-6}$ m. It is reasonable to attribute this to a species difference.

Hydrocyanic acid is an excellent substance for the study of pH effects in the acid region, because the pH of the acid (see Table I) lies several pH units below the region of interest, and hence no measurable effects could be caused by changes in the concentration of the hydrocyanic acid with
Fig. 3. The effect of pH upon the dissociation constants of the compounds of catalase found in the presence of azide, formate, fluoride, and acetate. The negative logarithms of the dissociation constants ($pK'$) are plotted. In all curves, ◇ represents points of Agner and Theorell (2). The conditions in these experiments were as follows: acetate (□) 0.9 μM human blood catalase, $\lambda = 420$, titrated with acetate buffers (Experiment 661d); fluoride (○) 1.55 μM human blood catalase, $\lambda = 450$, acidified with 2 to 67 mM lactate buffers, depending upon the pH; titrated with NaF-HF solution, pH 4.0 (Experiment 663b); formate (⊘) 0.9 μM human blood catalase, $\lambda = 420$ mμ, acidified with acetic acid or acetate buffers (0.1 to 1.0 mM), titrated with formate buffers (Experiment 661a); azide (□, ▽) 0.27 μM human blood catalase, $\lambda = 413$ mμ, acidified with 2 to 20 mM lactate buffers, depending upon the pH, titrated with sodium azide solution acidified with lactic acid to pH 4.0 (Experiments 664a, 664b). (25°C.)
pH in the region 3.1 to 7.0. The lack of an effect of pH upon pK in the region in which Agner and Theorell previously concluded a heme-linked hydroxyl group to be dissociated (see Equation 6) is remarkable. Ac-

\[
\text{FeOH} \rightleftharpoons \text{Fe}^+ + \text{OH}^- \quad (6)
\]

\[
\text{Fe}^+ + \text{HCN} \xrightleftharpoons[k_1]{k_2} \text{FeCN} + \text{H}^+ \quad (7)
\]

Fig. 4. The effect of pH upon the dissociation constant of the compounds of catalase with the free acids: acetic, hydrofluoric, hydrocyanic, formic, and hydrazoic. In each case the values of pK' of Fig. 3 are recomputed to the basis of the free acid concentration by adding log \((\frac{K}{[H^+]} + 1)\). In the cyanide titration, 1.1 \(\mu\)M human blood catalase, \(\lambda = 425 \text{ m}\mu\); catalase solutions acidified with lactic acid or lactate buffers (2 to 5 mM) (Experiment 662c). A hypothetical curve representing the effect of a heme-linked hydroxyl group of pK 3.8 is included for comparison.

According to their conclusions, the value of pK should begin to decrease at about pH 3.8 and take on a linear dependence upon decreasing pH thereafter as indicated by Equation 7.
Correlation of Data Obtained in Titrations with Weaker and Stronger Acids—The dissociation of some of the acids used in the titrations of Fig. 3 changes markedly in the pH range studied. The values of pH are given in Table I; acetic, formic, and hydrazoic acids pass through their pH in the range studied, and the pH of hydrofluoric acid lies only slightly below the range studied. Since the values of the dissociation constant have been computed on the basis of total reactant (acid plus anion), a clearer picture of the pH effect would be obtained if the dissociation constants were recomputed on the basis of only one species, acid or anion. The dissociation constants measured in Fig. 3 are recomputed on the basis of the free acid and are plotted together with the data on hydrocyanic acid in Fig. 4.

**Table I**

**Dissociation Constants of Acids Used in Catalase Titrations (from International Critical Tables)**

<table>
<thead>
<tr>
<th>Acid</th>
<th>Acetic acid</th>
<th>Hydrofluoric acid</th>
<th>Formic acid</th>
<th>Hydrazoic acid</th>
<th>Hydrocyanic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K$</td>
<td>$1.7 \times 10^{-5}$</td>
<td>$7.2 \times 10^{-4}$</td>
<td>$1.7 \times 10^{-4}$</td>
<td>$1.9 \times 10^{-5}$</td>
<td>$7.2 \times 10^{-10}$</td>
</tr>
<tr>
<td>$pK$</td>
<td>4.77</td>
<td>3.14</td>
<td>3.72</td>
<td>4.72</td>
<td>9.14</td>
</tr>
</tbody>
</table>

The actual concentration of free acid is given by the law of mass action in terms of the dissociation constant $K$.

$$\frac{(\text{Total acid})}{(\text{Free acid})} = \frac{K}{[H^+] + 1} \quad (8)$$

The values of $pK'$ of Fig. 3 are readily normalized to the basis of free acid by adding $\log \left( \frac{K}{[H^+] + 1} \right)$, and the results are plotted for the various values of pH in Fig. 4.

We are now in a position to look for a systematic decrease along a 45° slope in the values of $pK$, which could be attributable to the $pK$ of a heme-linked hydroxyl group. In the case of acetic and hydrazoic acids, the value of $pK$ increases somewhat, while, in the case of formic and hydrofluoric acids, a slope of approximately zero is obtained. In no case do the data acquire the characteristics of the theoretical curve for a heme-linked hydroxyl group of $pK$ 3.8, although the data do not disprove the existence of a heme-linked hydroxyl group having a value of $pK$ lower than approximately pH 3.

The values of dissociation constants are given in Table II.

Kinetic Studies on Velocity of Formation and Dissociation of Catalase Formate—In a catalase possessing a hydroxyl group, one distinctive effect
would be the second order reaction of hydroxyl ions with the anion compound (FeA).

\[
\text{FeA} + \text{OH}^- \xrightleftharpoons[k_2]{k_1} \text{FeOH} + \text{A}^-
\]  

Thus the velocity of dissociation of the anion compound would be directly proportional to the hydroxyl ion concentration. The velocity of combination would be independent of the values of the pH greater than the pK of the acid used. On the 45° portions of the curves of Fig. 3, the speed of combination of catalase and anion should be unaffected by pH, while the speed of dissociation would increase with pH.

Only in the case of catalase formate are the velocities of formation and dissociation measurable with present apparatus, and then barely satisfactory results are obtained, owing to the small changes of extinction coefficient.

The results of a series of experiments are presented in Tables III and IV. As the pH increased, it was found that the speed of the combination reaction (see Table III) decreases roughly 10-fold per pH unit when the velocity constants are computed on the basis of total formate. The velocity constants are recomputed on the basis of free formic acid according to Equation 8 and are seen to decrease only 5-fold for a 320-fold variation of the hydrogen ion concentration. And this 5-fold variation may be attributed in part to the rather large experimental error.

The similarity of the speed of combination of formic acid with catalase hematin iron and with the catalase-hydrogen peroxide complex at the "donor spot" (1) is remarkable.

The velocity constant for the dissociation of the compound is measured by mixing a lightly buffered catalase formate solution at pH 4.4 with a more alkaline buffer of a higher concentration. This abrupt change of pH causes the dissociation to occur, and the measured values are given in Table IV. In this case, there is no significant pH dependence.

As a check of the reliability of the results, the ratio of the two velocity constants (computed on the basis of free formic acid) is given in Table III (last column) and is seen to agree roughly with the dissociation constant obtained in titration studies \( (6 \times 10^{-6}, \text{see Table II}) \). Thus an excellent over-all check against a possible artifact is provided.

The results of these kinetic experiments are not in accord with the mechanism indicated by Equation 9, and a complete discussion of the results is given below.

**Effect of High Values of pH upon Dissociation Constant of Catalase Cyanide**—Extensive studies of the effect of pH upon the equilibrium of the compounds of catalase with the stronger acids on the acid side of their
pK are difficult to carry out. But hydrocyanic acid, having pK 9.14, is suitable for studies above and below this value. The effect of pH upon the equilibrium constant is given in Fig. 5. The curve follows, to a first approximation, the theoretical curve for the dissociation of the hydrocyanic acid molecule, although there are some deviations that are not

1 Hydrosulfuric acid, pK 7.0, would also be suitable for such studies, although the stability of hydrosulfide solutions is not all that could be desired.

2 The effect of pH found here is larger than that found in the spot check at pH 7.0 and 9.3, mentioned in a previous paper (5).
understood at present. These data support the conclusion that the hydrocyanic acid molecule reacts with catalase.

Effect of pH upon Reactions of Strong and Weak Acids with Ferrihemoglobin and Ferrimyoglobin Cyanide

Table V

Effect of pH upon Dissociation Constant and Rate of Formation of Ferrihemoglobin and Ferrimyoglobin Cyanide

\[
\text{[Ferrihemoglobin]} = 0.6 \mu M, \quad \text{[ferrimyoglobin]} = 0.4 \mu M, \quad 0.01 \text{ M phosphate buffers except for pH 5.1, which is 0.01 M citrate (Experiments 674a, 674b). (25\degree C.)}
\]

<table>
<thead>
<tr>
<th>pH</th>
<th>Dissociation constant</th>
<th>Velocity constant for formation</th>
<th>Velocity constant for dissociation</th>
<th>Dissociation constant</th>
<th>Velocity constant for formation</th>
<th>Velocity constant for dissociation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experimental</td>
<td>Experimental</td>
<td>Computed</td>
<td>Experimental</td>
<td>Experimental</td>
<td>Computed</td>
</tr>
<tr>
<td>7.0</td>
<td>4.5</td>
<td>263</td>
<td>1.2</td>
<td>1.4</td>
<td>323</td>
<td>0.45</td>
</tr>
<tr>
<td>6.0</td>
<td>4.6</td>
<td>223</td>
<td>1.1</td>
<td>9</td>
<td>170</td>
<td>1.5</td>
</tr>
<tr>
<td>5.1</td>
<td>22</td>
<td>96</td>
<td>2.1</td>
<td>34</td>
<td>123</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Hemoglobin and Ferrimyoglobin—There is little question that fluoride ion reacts with ferrihemoglobin in accordance with Equation 1 (3, 4). We have, however, reinvestigated the mechanism proposed by Coryell, Stitt, and Pauling for the binding of cyanide according to Equation 2. In neutral or acid solutions the reaction of Equation 2 would be represented by Equation 7, and acidification should cause the dissociation of the cyanide compound by increasing the value of \(k_2\). No such effect was found. Thus
could only be measured indirectly from the product of the dissociation constant $K$, determined by cyanide titration, and the velocity constant for the formation of the cyanide compound, $k_1$, measured in kinetic studies. The results are shown in Table V. The value of $k_2$ for ferrihemoglobin cyanide is nearly constant from pH 5.1 to 7.0. For ferrimyoglobin, a 10-fold change is observed, but Equation 7 requires a 100-fold change in both cases and therefore Equation 7 does not represent the reaction of these pigments with cyanide.

These kinetic studies of the effect of pH upon $k_1$ afford a positive indication that the hydrocyanic acid molecule is bound without the ejection of a hydrogen ion. Fig. 6 shows that the speed of combination of ferrimyoglobin declines as the concentration of hydrocyanic acid is diminished by dissociation of the molecule into cyanide ion. These blood pigments act like catalase in their reaction with cyanide, but not with fluoride.

The explanation of the disagreement of our results and those obtained by Coryell, Stitt, and Pauling (3) is clear from a study of their data which shows a titration at only one value of pH (4.77). The pH dependence of the reaction must have been inferred (incorrectly) from their fluoride studies.

The reaction kinetics were measured in the presence of a large excess of cyanide (600 μM) and hence the speed of the forward reaction greatly exceeded that of the reverse reaction. The competition between hydroxyl ions (pK 8.84 (14)) and cyanide should cause no significant error in the rate measurement.
DISCUSSION

The following equations are to be considered as possible explanations of the interaction of hemoproteins and molecules or ions.

\[
\begin{align*}
\text{Equation 10:} & \quad \text{FeH}_2\text{O} + \text{HA} & \leftrightarrow & \text{FeA}^- + \text{H}_2\text{O}^+ \\
\text{Equation 11:} & \quad \text{FeH}_2\text{O} + \text{A}^- & \leftrightarrow & \text{FeA}^- + \text{H}_2\text{O} \\
\text{Equation 12:} & \quad \text{FeH}_2\text{O} + \text{HA} & \leftrightarrow & \text{FeHA} + \text{H}_2\text{O} \\
\text{Equation 13:} & \quad \text{FeOH} + \text{HA} & \leftrightarrow & \text{FeA} + \text{H}_2\text{O} \\
\text{Equation 14:} & \quad \text{FeOH} + \text{A}^- & \leftrightarrow & \text{FeA} + \text{OH}^- \\
\text{Equation 15:} & \quad \text{FeOH} + \text{HA} & \leftrightarrow & \text{FeHA} + \text{OH}^- 
\end{align*}
\]

Equations 10, 11, and 12 represent the reactions possible between the hydrated iron atom of the hemoprotein and the molecule (HA) or the anion (A\textsuperscript{-}) of the acid. In Equations 13, 14, and 15, the heme-linked hydroxyl group of the hemoprotein is taken into consideration. Catalase exhibits no pH effect in binding acids according to Equations 10 and 15, which require a pH effect, and exhibits a large pH effect in binding anions according to Equation 11, which gives no pH effect. Thus Equations 10, 11, and 15 are inapplicable. Equation 12 correctly expresses the result of these studies with a minimum of hypothesis, but Equations 13 and 14 must also be considered, because Theorell has postulated the existence of the heme-linked hydroxyl group. Actual proof of the existence of the hydroxyl group is lacking, since our titration data show no inflection that can be attributed to this group. But we are ready to admit that the pK of this group lies in a pH region that is inaccessible because of the denaturation of the catalase protein.

On the basis of kinetic studies of the effect of pH upon the combination of catalase and formic acid, Equation 14 is incorrect and only Equations 12 and 13 remain for consideration. They also explain the experimental results on the combination of cyanide and ferrihemoglobin or ferrimyoglobin.

On the other hand, the reaction of these two blood pigments with fluoride follows Equation 11, an apparent inconsistency. But complete consistency in the pattern of hemoprotein reactions may not exist; there is no reason why the reaction of the blood pigments with fluoride should be identical to that of catalase with fluoride.\textsuperscript{4} Nor do we know that the structures of the compounds that are formed need be identical. A further elucidation of the exact nature of the reactions of hemoproteins depends largely upon a more detailed knowledge of the nature of their compounds; kinetic and equilibrium studies have served best to clarify, but not to establish with finality, the possibilities of chemical structures.

\textsuperscript{4} Theorell's recent magnetic susceptibility studies of metmyoglobin fluoride show a rather complex pH dependence not found in catalase (14).
SUMMARY

1. The equilibrium of catalase and formic, hydrofluoric, hydrazoic, acetic, and hydrocyanic acids is unaffected by pH in the range 3.5 to 5.5.

2. The equilibrium of catalase and hydrocyanic acid depends upon the free hydrocyanic acid concentration in the range pH 7.5 to 10.

3. The velocity of formation of catalase formate depends upon the formic acid concentration, and the velocity of dissociation does not depend upon the value of pH in the range 4.1 to 6.6. Thus catalase reacts with the formic acid molecule and not the anion. By analogy, the reactions are with hydrofluoric, hydrazoic, and acetic acids. It has previously been concluded that catalase reacts with the hydrocyanic acid and with the hydrogen peroxide molecules. The reaction with strong and weak acids follows the same course.

4. None of the studies of the effect of pH upon the reaction of catalase with acids has yet been carried far enough into the acid region (without protein denaturation) to prove the existence of the heme-linked hydroxyl group. Nor is the existence of this group disproved.

5. Two mechanisms are under consideration for the reaction of catalase with strong and weak acids.

\[ \text{FeH}_2\text{O} + \text{HA} \rightarrow \text{FeHA} + \text{H}_2\text{O} \]

\[ \text{FeOH} + \text{HA} \rightarrow \text{FeA} + \text{H}_2\text{O} \]

6. The dissociation constants of human blood catalase compounds computed according to the above equations are as follows: hydrofluoric acid \(4 \times 10^{-5}\), formic acid \(6 \times 10^{-6}\), hydrocyanic acid \(7 \times 10^{-6}\), hydrazoic acid \(3 \times 10^{-6}\), acetic acid \(9 \times 10^{-3}\) (the last two values are correct at pH 3.8).\(^5\)

7. The evidence for the conclusion of Coryell, Stitt, and Pauling that ferrihemoglobin reacts with cyanide ion is inadequate, since the increase of the dissociation velocity with decreasing values of pH is much less than 10-fold per unit pH, as required by their mechanism. The effect of pH upon the velocity constant for the formation of ferrimyoglobin cyanide suggests that the free acid reacts as in the case of catalase.

8. Formic acid combines with catalase hematin iron at the same speed with which it reacts with the catalase-peroxide complexes.

BIBLIOGRAPHY


\(^5\) The value for hydrofluoric acid is \(10^{-5}\) if the presence HF\(_2\)\(^-\) is taken into account (16).
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Britton Chance


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