Studies on Horseradish Peroxidase

IX. KINETICS OF THE OXIDATION OF p-CRESOL BY COMPOUND II*

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SUMMARY

The kinetics of the oxidation of p-cresol by Compound II of horseradish peroxidase has been studied by the stopped flow technique at an ionic strength of 0.11 from pH 2 to 11. In acid solution the reaction is kinetically first order in p-cresol, but in the alkaline region a saturation effect attributable to complex formation is observed. At very high pH an additional second order reaction between p-cresol and the complex is detectable. The existence of the complex is confirmed by spectrophotometric determination of its dissociation constant. Since this constant and the overall second order rate constant do not depend on any common ionizing group in the enzyme, it is argued that the complex is unproductive in nature. Use of the diffusion-controlled limit shows that p-cresol reacts in the unionized form over the experimental pH range, and that electrostatic interactions are therefore not responsible for the degree of complexity of the pH log rate profile.

Horseradish peroxidase (EC 1.11.1.7, donor-H₂O₂ oxido-reductase) catalyzes the oxidation of a wide variety of compounds by hydrogen peroxide. The early work of Chance (1) and George (2) established that the reaction normally proceeds by the following general mechanism

\[
\text{HRP} + \text{H}_2\text{O}_2 \rightarrow \text{HRP-I}
\]

\[
\text{HRP-I} + \text{AH} \rightarrow \text{HRP-II} + \text{AH}^+.
\]

\[
\text{HRP-II} + \text{AH}_2 \rightarrow \text{HRP} + \text{AH} + 2 \text{AH}^+ \rightarrow \text{products}
\]

where HRP represents the native enzyme, HRP-I and HRP-II are the oxidized forms of the enzyme referred to as Compounds I and II, respectively, and AH₂ is the reducing substrate. Previous work on this enzyme up to 1966 has been reviewed by Brill (3).

As part of a general program designed to obtain some insight into the detailed mechanism of each step in the above scheme, kinetic studies over a range of pH have been carried out in this laboratory on the oxidation of ferrocyanide by Compounds I and II (4) and of iodide by Compound II (5). In order to test whether the more complex nature of the pH rate profile exhibited by the former substrate in the Compound II reaction resulted from its higher electronic charge, it was considered desirable to investigate the behavior of a neutral organic molecule. p-Cresol was chosen because of the marked efficiency of Compound II in oxidizing phenolic substances, and because of its resemblance to tyrosine while having fewer acid-base sites than the amino acid itself. Tyrosine is of interest in connection with the peroxidase-catalyzed iodination mechanism, in which it shows a small degree of stereospecificity (6) and which is probably related to the biosynthesis of the thyroid hormone (7).

The present kinetic studies demonstrated the formation of a complex between Compound II and p-cresol, which was confirmed spectrophotometrically. They also suggested that the complex is unproductive in nature, and that electrostatic interactions are not responsible for the form of the pH rate profile.

EXPERIMENTAL PROCEDURE

Materials—Horseradish peroxidase was obtained from Boehringer Mannheim as a highly purified suspension in aqueous hydrogen peroxide. The early work of Chance (1) and George (2) established that the reaction normally proceeds by the following general mechanism:

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EXPERIMENTAL PROCEDURE

Materials—Horseradish peroxidase was obtained from Boehringer Mannheim as a highly purified suspension in aqueous ammonium sulfate and was dialyzed and filtered before use. The ratio of the absorbances at 403 and 280 nm (P.N.) was never less than 3.0. Solutions of the enzyme were stored in the cold at concentrations of about 10⁻⁷ M and were diluted immediately before use. Enzyme concentrations were determined spectrophotometrically at 403 nm, using a molar absorptivity of 9.1 × 10⁴ M⁻¹ cm⁻¹ (8).

Practical grade p-cresol was obtained from Eastman Organic Chemicals and was purified by sublimation (m.p. 33°, literature 35°). Its aqueous solution had maximum molar absorptivities of 1.09 × 10⁵ M⁻¹ cm⁻¹ at 277 nm in the presence of 0.01 M perehloric acid and 2.58 × 10⁴ M⁻¹ cm⁻¹ at 296 nm in 0.01 M sodium hydroxide. These compare favorably with the maximum values of 1.71 × 10⁵ M⁻¹ cm⁻¹ at 277 nm and 2.55 × 10⁴ M⁻¹ cm⁻¹ at 295 nm, respectively, found for a sample of 99.96% purity (9). All other substances were of reagent grade and were used without further purification. Hydrogen peroxide, 30% by weight obtained from the Fisher Scientific Co., was stored in...
the cold as an approximately $5 \times 10^{-3}$ M solution and diluted before use; its concentration was checked periodically by the method of Ovenston and Rees (10). Water was distilled from alkaline potassium permanganate and then redistilled.

**Kinetic Experiments**—Investigations were carried out using the stopped flow apparatus described previously (4). In a typical experiment a $2 \times 10^{-4}$ M solution of the enzyme in aqueous potassium nitrate was partly converted to Compound II immediately before use by adding 0.8 times the number of moles of hydrogen peroxide from a Hamilton microliter syringe. Spectrophotometric tests showed that most of the resulting Compound I was reduced to Compound II within a few minutes by impurities associated with the nitrate, while the very rapid reaction between Compound I and p-cresol (1) could be relied upon to complete this process within the dead time of the instrument. The alternative method of preparation (4) in which the prior conversion of Compound I to Compound II was assisted by the addition of a small amount of p-cresol was shown to give rise to identical kinetic behavior. Solutions of p-cresol were prepared by weighing and the concentrations were checked spectrophotometrically at 277 nm. Potassium nitrate and the appropriate buffer were added to these a short time before the reaction, together with enough enzyme to give a final concentration of about $10^{-4}$ M; this served to catalyze the removal of small amounts of oxidizing impurities which would otherwise interfere with the reaction. The final reaction mixture entering the observation chamber consistently had an ionic strength of 0.11, of which 0.01 was contributed by the buffer. At high pH the p-cresol made a contribution towards both the ionic strength and the buffering capacity, and the former was allowed for by suitable adjustment of the amount of added nitrate.

The reduction of Compound II to the native enzyme was monitored at 426 nm by following the amplified photomultiplier voltage against time trace on a 564 B Tektronix storage oscilloscope. The data were recorded either in the form of a four-figure digital print-out at 30 equally spaced intervals of time or as readings taken visually from a photograph of the trace. The two methods were shown to yield rise to identical rate constants. All reactions were conducted at 25°C, and had half-times ranging from about 5 milliseconds to several seconds. The use of initial concentrations of Compound II typically below $10^{-4}$ M enabled pseudo first order conditions to be achieved by keeping the p-cresol in at least a 10-fold excess over Compound II. The resulting absorbance changes were normally less than 0.04, and could hence be regarded as proportional to the observed voltage changes. An average rate constant with standard deviation was obtained from 6 to 10 traces for each set of conditions. After reaction the solutions were collected for pH measurements, which was carried out with an Orion digital pH meter in conjunction with a Fisher combination electrode.

Preliminary attempts to investigate the kinetics in the steady state were abandoned because of the very sparing solubility of the reaction products.

**Initial Absorbance of Compound II**—In order to check for the formation of a complex between p-cresol and Compound II, experiments were devised to measure the dependence of the total amplitude of the reaction trace on the p-cresol concentration. Since the concentration of Compound II in the storage syringe varied slowly with time and from one experiment to another, this was achieved by a direct comparison method. A given sample of Compound II was allowed to react in the stopped flow apparatus first with a dilute stock solution of p-cresol at pH 9.84 and ionic strength 0.11, then with a solution containing a higher concentration of the reducing substrate under the same conditions, and finally with the original dilute stock solution. For each solution the amplitudes of about six reaction traces were read off the photographic records, and the three average values compared. A decrease of 3 to 10% occurred between the first and last figures as a result of the slow spontaneous decomposition of Compound II, and the mean of these two was therefore used to compare with the average amplitude observed for the middle test solution.

Separate tests showed that at the concentration of enzyme used in these experiments the absolute final absorbances at 426 nm were independent of the concentration of p-cresol.

**pKₐ of p-Cresol**—The degree of dissociation of p-cresol at the same salt concentrations as were used in the kinetic experiments was measured spectrophotometrically, using a Cary 14 spectrophotometer with the cell block maintained at 250°C. The pH was maintained by a carbonate-bicarbonate buffer, which was also added to the reference solution, and was measured for each sample directly in the cuvette by the use of a Fisher combination microprobe electrode. Absorbance measurements were made at 295 nm, close to the maximum in the difference spectrum for the acidic and basic forms. Eight solutions were studied over the pH range 9.35 to 11.12, and readings were also taken for solutions 0.01 M in perchloric acid and sodium hydroxide.

**RESULTS**

**pKₐ of p-Cresol**—The effective pKₐ of p-cresol under the conditions of this work may be defined in terms of concentrations and the operational pH scale by the relation

$$pK_a = \log \frac{[ArOH]}{[ArO^-]} + pH$$

Nonlinear least squares analysis of the spectrophotometric data, using the measured value of 60.1 M⁻¹ cm⁻¹ for the molar absorptivity in acid solution, gave $pK_a = 10.12 \pm 0.01$. The largest deviation from the best fit curve was of 2%, in absorbance, and the predicted molar absorptivity of the anion was $2.47 \times 10^5$ M⁻¹ cm⁻¹ compared with $2.53 \times 10^5$ M⁻¹ cm⁻¹ obtained by direct experimental measurement. This value of $pK_a$ is in reasonable agreement with the figure 10.16 obtained by the use of Davies' equation (11) together with the value at zero ionic strength found by Chen and Laidler (12) and Herington and Kynaston (9).

**Order of Reaction in Compound II and p-Cresol**—All reaction traces were simple exponential curves, showing the reaction to be kinetically first order in Compound II. A typical trace is shown in Fig. 1. The results were analyzed by a nonlinear least squares method with equal weighting of all voltage readings. A number of experiments were also conducted at pH 9.89 employing absorbance changes of up to 0.1 (with an appropriate transformation of voltage into absorbance) in order to obtain a more precise measure of the degree of first order behavior. Table 1 shows the constancy of the first order rate constants obtained in such a series of reactions with a given p-cresol concentration and varying initial concentrations of Compound II. A typical semilogarithmic plot obtained in one such run is reproduced in Fig. 2, and is linear over at least four half-lives.
Fig. 1. Oscilloscope trace of voltage versus time for the reaction of Compound II with p-cresol at pH 6.83 observed at 426 nm. The initial concentrations of Compound II and p-cresol were 8 $\times$ 10$^{-3}$ M and 3.57 $\times$ 10$^{-5}$ M, respectively. Each division of the abscissa scale corresponds to 20 milliseconds.

![Oscilloscope trace of voltage versus time](image)

### FIG. 2. Semilogarithmic plot of residual absorbance $(A_t - A_0)$ against time in the reaction of Compound II with p-cresol at pH 9.89. The initial p-cresol concentration was 8.0 $\times$ 10$^{-4}$ M.

![Semilogarithmic plot](image)

### TABLE I

<table>
<thead>
<tr>
<th>$\Delta A$</th>
<th>$k_{obs}$ (s$^{-1}$)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.081</td>
<td>12.5 $\pm$ 0.3$^b$</td>
</tr>
<tr>
<td>0.071</td>
<td>12.0 $\pm$ 0.3</td>
</tr>
<tr>
<td>0.054</td>
<td>12.5 $\pm$ 0.3</td>
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<td>14.2 $\pm$ 0.7</td>
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<td>11.7 $\pm$ 0.2</td>
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<tr>
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<td>12.2 $\pm$ 0.4</td>
</tr>
<tr>
<td>0.028</td>
<td>12.1 $\pm$ 0.4</td>
</tr>
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<td>0.026</td>
<td>11.8 $\pm$ 0.4</td>
</tr>
<tr>
<td>0.025</td>
<td>11.9 $\pm$ 0.4</td>
</tr>
<tr>
<td>0.024</td>
<td>12.3 $\pm$ 0.4</td>
</tr>
<tr>
<td>0.014</td>
<td>10.7 $\pm$ 0.3</td>
</tr>
<tr>
<td>0.013</td>
<td>12.4 $\pm$ 1.0</td>
</tr>
<tr>
<td>0.012</td>
<td>11.3 $\pm$ 0.8</td>
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</table>

$^a$ Obtained from nonlinear least squares analysis of voltage versus time trace at pH 9.89 and constant initial p-cresol concentration 8.0 $\times$ 10$^{-4}$ M. Mean $K_{obs} = 12.1 \pm 0.8$ s$^{-1}$.

$^b$ Internal standard deviation.

The manner in which the observed first order rate constant depended on the concentration of p-cresol varied with the pH region of the study. In the acid region a simple linear dependence was observed,

$$k_{obs} = k_o[S]/K_m$$  \hspace{1cm} (2)

where $k_{obs}$ is the experimental first order rate constant, $k_o/K_m$ is a second order rate constant, and $[S]$ is the p-cresol concentration. This is shown in Fig. 3, in which the fastest rate, which corresponds to a half-life somewhat smaller than the dead time of the instrument, may be subject to a slight systematic error arising from the finite mixing time, and so has not been included in the analysis.

In the region immediately to the alkaline side of neutrality the rate showed a leveling off at high p-cresol concentrations. This behavior can be described by a simple binding interaction between the two reactants according to Equation 3,

$$k_{obs} = \frac{k_o[S]}{K_m + [S]}$$  \hspace{1cm} (3)

where $K_m$ is a Michaelis constant, and a typical set of data is represented by a Lineweaver-Burk plot in Fig. 4. When $K_m$ is large compared with the maximum value of $[S]$ that can be used, the plot of $k_{obs}$ versus p-cresol becomes effectively linear with slope $k_o/K_m$, as observed at lower pH. Since the experimental rate constants in excess of 150 s$^{-1}$ were scattered and cannot be regarded as being completely free of systematic error, it was difficult to obtain reliable values of $K_m$ in the immediate vicinity of pH 7. A $K_m$ value of 1.5 $\times$ 10$^{-2}$ M corresponds to about a 10% deviation from linear behavior when $k_{obs} = 150$ s$^{-1}$, and must therefore be regarded as the maximum measurable value in this region of pH.
Table II

<table>
<thead>
<tr>
<th>pH</th>
<th>$10^4 \times$ concentration range of p-cresol</th>
<th>Buffer</th>
<th>Equation used for analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.29</td>
<td>0.2-0.4</td>
<td>CI</td>
<td>2</td>
</tr>
<tr>
<td>2.63</td>
<td>0.1-1.0</td>
<td>CI</td>
<td>2</td>
</tr>
<tr>
<td>3.05</td>
<td>0.2-0.7</td>
<td>CI</td>
<td>2</td>
</tr>
<tr>
<td>3.91</td>
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<td>A</td>
<td>2</td>
</tr>
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<td>A</td>
<td>2</td>
</tr>
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<td>0.1-0.4</td>
<td>A</td>
<td>2</td>
</tr>
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<td>2</td>
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<tr>
<td>6.00</td>
<td>0.1-1.8</td>
<td>P</td>
<td>3</td>
</tr>
<tr>
<td>6.18</td>
<td>0.1-0.4</td>
<td>A</td>
<td>2</td>
</tr>
<tr>
<td>6.37</td>
<td>0.1-0.4</td>
<td>P</td>
<td>2</td>
</tr>
<tr>
<td>6.63</td>
<td>0.1-1.8</td>
<td>P</td>
<td>3</td>
</tr>
<tr>
<td>6.83</td>
<td>0.1-0.4</td>
<td>P</td>
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</tr>
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<td>6.86</td>
<td>0.1-1.4</td>
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</tr>
<tr>
<td>7.32</td>
<td>0.1-2.1</td>
<td>P</td>
<td>3</td>
</tr>
<tr>
<td>7.68</td>
<td>0.1-5.0</td>
<td>P</td>
<td>3</td>
</tr>
<tr>
<td>7.95</td>
<td>0.1-3.6</td>
<td>P</td>
<td>3</td>
</tr>
<tr>
<td>8.11</td>
<td>0.1-1.5</td>
<td>P</td>
<td>3</td>
</tr>
<tr>
<td>8.51</td>
<td>0.1-3.2</td>
<td>C</td>
<td>3</td>
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<tr>
<td>8.65</td>
<td>0.1-1.6</td>
<td>C</td>
<td>3</td>
</tr>
<tr>
<td>8.70</td>
<td>0.1-2.3</td>
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<td>3</td>
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<tr>
<td>8.83</td>
<td>0.1-8.0</td>
<td>C</td>
<td>3</td>
</tr>
<tr>
<td>8.85</td>
<td>0.1-2.0</td>
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</tr>
<tr>
<td>9.09</td>
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<td>3</td>
</tr>
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<td>9.32</td>
<td>0.2-14.0</td>
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</tr>
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<td>9.69</td>
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<td>9.82</td>
<td>0.4-4.5</td>
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<td>4</td>
</tr>
<tr>
<td>9.84</td>
<td>0.1-11.0</td>
<td>C</td>
<td>4</td>
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<tr>
<td>9.89</td>
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<td>C</td>
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<td>C</td>
<td>4</td>
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<td>10.15</td>
<td>0.4-18.3</td>
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<td>3</td>
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<tr>
<td>10.34</td>
<td>0.6-44.3</td>
<td>C</td>
<td>4</td>
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<tr>
<td>10.37</td>
<td>0.2-84.7</td>
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<td>4</td>
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<td>10.38</td>
<td>0.7-71.3</td>
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<td>4</td>
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<td>2.0-10.0</td>
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<tr>
<td>10.79</td>
<td>2.4-120.0</td>
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<td>4</td>
</tr>
<tr>
<td>10.83</td>
<td>2.4-120.0</td>
<td>CI</td>
<td>4</td>
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<tr>
<td>11.08</td>
<td>3.2-160.0</td>
<td>C</td>
<td>2</td>
</tr>
<tr>
<td>11.20</td>
<td>5.5-111.0</td>
<td>C</td>
<td>2</td>
</tr>
</tbody>
</table>

*Buffer key: CI, citrate; A, acetate; P, phosphate; C, carbonate; GL, glycine.

\[
\frac{k_{\text{obs}}}{K_n} = \frac{k_\text{d}[S]}{K_n} + \frac{k_3K_m[S]}{K_m + [S]} \tag{5}
\]

At still higher pH the reactions slowed down considerably, while $k_{\text{obs}}$ failed to show the approach to a constant value at high substrate concentrations which would be expected from Equation 3. A typical plot is shown in Fig. 5, and can be represented by the inclusion of a further bimolecular reaction between p-cresol and the complex with a second order rate constant $k_3$ as in Equation 4

\[
k_{\text{obs}} = \frac{k_d[S]}{K_n} + k_3K_m[S] 
\]

The mechanistic significance of these parameters will be discussed below. If Equation 4 is put into the form it can readily be seen that the simpler behavior observed at only slightly alkaline pH is due to the small magnitude of the ratio $k_3K_m/k_2$. At the upper limit of the pH range of the study this ratio approaches unity, giving the essentially linear lower plot in Fig. 5, from which no value of $K_m$ can be obtained.

The buffer system and concentration range of p-cresol used, as well as the equation by which the results were analyzed, are shown in Table II as a function of pH. The use of several buffer systems rules out any significant specific effect on the
parameters from this source. The nonlinear least squares program was used in analyzing the data by Equations 3 and 4 primarily because it readily permitted weighting of the experimental \( k_{\text{obs}} \) values according to their measured individual standard deviations. In addition, very similar values of \( K_m \) and \( k_2/K_m \) were obtained by a linear best fit to the Lineweaver-Burk plot, and similar values of \( k_a \) were obtained from the linear relation between \([S]\) and the deviations of \( k_{\text{obs}} \) from an extrapolation of the same plot. In borderline cases the results were analyzed in the interests of accuracy using the more complex of the two equations found to be appropriate at adjacent pH levels, but the resulting \( K_m \) or \( k_2 \) was inevitably too poorly defined to be of any value. For some of the experiments at alkaline pH instrumental problems prevented the use of a sufficient number of \( p \)-cresol concentrations high enough to yield reliable figures for either \( K_m \) or \( k_1 \), but in such cases both Equations 3 and 4 gave identical values of \( k_2/K_m \), which have therefore been included in Fig. 6.

For the slower runs at high pH small spontaneous rate contributions associated with the presence of reducing impurities in the buffer and never exceeding 0.3 s\(^{-1} \) were observed. At medium and low pH on the other hand small negative intercepts were obtained as a result of the prereaction of a portion of the rather small amounts of \( p \)-cresol used with traces of oxidizing impurities. Allowance was made for these effects in the analysis, although little difference was made to the resulting parameters. Below pH 4 a second reaction was observed, probably attributable to denaturation of the native enzyme, but even at pH 2.29 this was at least 20 times slower than the reaction of interest and so had a fairly small effect on the apparent first order rate constants.

Dependence of Kinetic Parameters on pH—The logarithms of \( k_2/K_m \) and \( 1/K_m \) are plotted against pH in Fig. 6 along with their standard deviations. The values of \( K_m \) are less well defined at high pH as a result of the increase in the ratio \( k_2K_m/k_2 \), and at neutral pH because of the particularly high rates of the reactions under conditions of significant binding. Consequently the data only justify analysis in terms of a simple pH maximum resulting from an enzyme ionization at neutral pH and the acid dissociation of the substrate. A weighted nonlinear least squares treatment allowed the data to be fitted to the equation

\[
K_m = K'_m(1 + [H^+]^*/[K]) (1 + K_s/[H^+])
\]

where \( K'_m \) is a pH-independent Michaelis constant, and \( K_1 \) and \( K_2 \) are acid dissociation constants of Compound II and \( p \)-cresol, respectively. The best fit curve is shown in Fig. 6, and is defined by the parameters in Table III. The value of \( pK_s \) agrees with that determined above within experimental error. The significance of these results is discussed below.

The plot of \( \log k_2/K_m \) versus pH in Fig. 6 is quite well defined, and can be seen with the aid of Dixon's rules to depend on the acid dissociation of the substrate at pH 10 and the ionization of three kinetically important enzyme groups having \( pK_a \) values of around 2, 6, and 8. The minimum reaction scheme consistent with the data is therefore that given in Scheme 1, in which \( SH \) and \( S \) represent \( p \)-cresol and its anion, respectively, \( E, EH, EH_2 \), and \( EH_3 \) are the four kinetically distinct states of protonation of Compound II, the \( K \) values are acid dissociation constants, and the \( k \) values are bimolecular rate constants.

\[
\begin{align*}
K_{\text{obs}} &= K'_m(1 + [H^+]^*/[K]) (1 + K_s/[H^+]) \quad (6) \\
K_m &= K'_m(1 + [H^+]^*/[K]) (1 + K_s/[H^+]) \quad (6)
\end{align*}
\]

The corresponding kinetic expression takes the form:

\[
k_3 \frac{K_m}{K} = \frac{[k_3[H^+]^*/K] + [K_s/K][k_2/K]}{[K_s/K][k_2/K] + [K_s/K][k_2/K] + k_1}
\]

\[
\begin{align*}
\text{Scheme 1} \\
S &\xrightarrow{k_3} \text{products} \\
SH &\xrightarrow{k_2} \text{products} \\
EH_2 &\xrightarrow{[K_s]} S \\
E &\xrightarrow{[K]} S \\
K_4 &\xrightarrow{k_1} S \\
K_3 &\xrightarrow{k_2} \text{products} \\
K_2 &\xrightarrow{k_3} \text{products} \\
\end{align*}
\]
such ionizations may therefore be read off the plot by an extension of Dixon's rules, in the same way as the pK₄ values of the enzyme or enzyme-substrate complex. In the present case a single kinetically important pK₄₊ is observable at about pH 5, so that the reaction may be represented by Scheme 2, which leads without further algebraic manipulation to Equation 8. This equation, in which k₄/K₄ is a pH-independent second order rate constant,

$$k_4 = \frac{1 + K_4[H^+]}{K_m[H^+]}$$

beams an obvious resemblance to that for the pH variation of the dissociation constant of an enzyme-substrate complex, a feature which is due to the close analogy between the formation of such a complex and that of the transition state. The best fit values of the parameters are shown in the third column of Table IV. This method of analysis is introduced here since, as shown below, it leads to a number of useful insights into the mechanism of the reaction.

The value of pK₄ obtained from the log k₄/K₄ versus pH profile is significantly different from the spectrophotometric result. A second analysis with pK₄ = 10.12 as an invariable parameter produced only small changes in the other values, as shown in the last column of Table IV, but the resulting theoretical curve was found to lie below all five points at highest pH by amounts several times larger than their standard deviations, confirming that the difference is statistically significant. A possible explanation will be discussed below.

Reliable values of k₄ are recorded in Table V along with their standard deviations, and appear to fall off approximately as the first power of the hydrogen ion activity. Although quantitative data are not available, the fact that the results at lower pH can be represented adequately by Equation 3 is concordant with the reasonable proposal that k₄ reaches a plateau below pH 10, associated with the acid dissociation of the substrate.

**Spectrophotometric Evidence for Binding**—The difference in absorbance between Compound II and the native enzyme at a given wavelength can be described by an effective molar absorptivity Δε, given by

$$Δε = \frac{K_DΔα + [S]Δα_0}{K_D + [S]}$$

where K_D is the spectrophotometrically measured dissociation constant of the complex, Δα₀ is the difference in molar absorptivity between Compound II and the enzyme, and Δα that between the complex and enzyme. The amplitude ratios, measured as described above and plotted against log [S] in
DISCUSSION

As pointed out by Chance (15), the observation of apparent saturation kinetics may not necessarily be due to the formation of an enzyme-substrate complex, and it is therefore desirable to exclude other kinetic explanations. In the present case a possible alternative cause of the observed behavior is competition between unchanged p-cresol and the initially formed radical (or any other product species) for reaction with Compound II, but a number of pieces of evidence suggest that this does not occur. In the first place the reaction would not be strictly first order in Compound II, contrary to observation. Secondly, identification of the three major products of the reaction, even under conditions in which towards the end of the reaction the concentration of unchanged substrate had fallen to zero (16), showed that in only one product had more than 1 electron been removed per molecule of reactant. The attempt of Cormier and Pritchard (17) to argue from titration data showing luminol to function as a 2-electron donor that the radical must be further oxidized by a 2nd molecule of Compound II is unconvincing, since the data could equally well be explained by radical disproportionation. In fact for other organic substrates it has been shown by quantitative electron spin resonance spectroscopy that the radicals react only with each other (18). In the present case the quantitative agreement between the kinetic and spectrophotometric measurements of the dissociation constant provides additional positive evidence that complex formation is actually responsible for the observed kinetic behavior.

As first pointed out by Henri (19), the formation of either a productive or an unproductive complex can give rise to the kinetic behavior summarized by Equation 3. In either case, as is shown in the "Appendix," the observation of strict first order kinetics in the reduction of Compound II to the native enzyme is evidence for the existence of a true equilibrium. This implies that at neutral pH the second order rate constant for the combination reaction must be at least $10^8$ M$^{-1}$ s$^{-1}$. At no pH could the kinetics of this reaction be observed on the stopped flow apparatus.

A general reaction scheme which can represent the experimental results while taking into account the above kinetic ambiguity is depicted below,

$$
\text{S} \rightarrow \text{HRP-II} + \text{S} \\
\text{HRP-II} + \text{S} \rightarrow \text{products}
$$

At a given pH $K_p$ and $K_u$ are the dissociation constants of the productive and unproductive complexes, respectively, $k_u$ is a first order rate constant, and $k_0$ and $k_u$ are second order rate constants. No single-step termolecular process has been included, since the over-all rate of reaction makes this unlikely. The above scheme gives rise to the expression

$$k_{obs} = \left( \frac{k_u}{K_p} + \frac{k_u}{K_u} \right) \frac{[S] + \frac{k_u}{K_u} [S]^2}{1 + \left( \frac{1}{K_u} + \frac{1}{K_p} \right) [S]}$$

which shows the same dependence on $[S]$ as does Equation 4. Similarly if $\Delta \varepsilon_p$ and $\Delta \varepsilon_u$ are the differences in molar absorptivity between the productive and unproductive complexes, respectively, and the native enzyme, Equation 9 becomes

$$\Delta \varepsilon = \frac{\Delta \varepsilon_p + \left( \frac{1}{K_u} + \frac{1}{K_p} \right) [S] \left( \frac{\Delta \varepsilon_p K_p + \Delta \varepsilon_u K_u}{K_u + K_p} \right)}{1 + \left( \frac{1}{K_u} + \frac{1}{K_p} \right) [S]}$$

Thus both the kinetically and spectrophotometrically measured quantities $K_m$ and $K_B$ are complex and equal to the harmonic mean of $K_u$ and $K_p$.

In order to gain some insight into the nature of the mechanism of the reaction, it is highly desirable to determine which of the two complexes predominates. The inherently unlikely possibility that both are important is further discounted by the simple form of the pH dependence of $K_m$. A direct decision
can be made in principle by studying the rate of production of HRP in the initial stages of the reaction, as has been pointed out by Viale (20) for the analogous steady state situation, but the very fast rate of complex formation rules this out in the present case. However, a number of indirect sources of evidence suggest that the complex is unproductive. The chief of these is the observation that the enzyme pKa (pK) which causes the degree of complex formation to fall off below pH 7.3 appears to have no effect on the over-all second order rate constant. When, as in the present case, the complex is in true equilibrium with the substrate and enzyme, a plot of log 1/Km against pH in general may show regions of negative curvature (i.e. convex upwards) which are indicative of the pKa values of those groups on the enzyme and substrate which are affected by the binding, as well as regions of positive curvature arising from the ionizing behavior of the complex. The plot of log k2/Km against pH normally reveals the influence of the same enzyme and substrate groups, and in addition may be sensitive to other groups in the enzyme which although not involved in the binding take part in the catalytic process. The appearance of the same enzyme groups in the 1/Km and k2/Km profiles shows that the enzyme binds the substrate at the same site as it reacts with it; the complex is therefore productive and an examination of its properties provides useful insights into the catalytic behavior of the enzyme. In the present case, on the other hand, the group of pKa 7.3 (pK2) which is involved in binding equilibrium appears to have no effect on the second order rate constant, an observation which can only be explained if the active site for the oxidation of p-cresol is different from the site on the enzyme at which binding occurs. The complex may accordingly be described as unproductive, and an understanding of the complexing equilibrium is of no direct use in the search for an understanding of the mechanism of oxidation.

The fact that ferric ions form complexes with phenols, as shown by the experiments of Milburn (21) and the well known qualitative test for these compounds, might have led one to expect the formation of a productive heme-bound complex in the reaction with Compound II. However, recent work in this laboratory has shown that cyanide fails to inhibit the oxidation of ferrocyanide by Compound II, the binding of other potential ligands to the iron atom in this enzyme complex is unlikely. In addition, it would be difficult to explain the fair degree of stability of the complex to oxidative decomposition if it consisted of iron-bound p-cresol. Because of the quite large change in absorbance at 426 nm upon complex formation, the p-cresol is probably bound either close to the porphyrin ring or in such a way that the environment of the ring system is altered.

Previous work with either horseradish peroxidase or related enzymes has failed to reveal the formation of a complex between any simple reducible substrate and the primary or secondary enzyme compounds. However, Chance found that the oxidation of ferrocyanochrome c by Compound II of horseradish peroxidase exhibited saturation kinetics (22), and kinetic evidence has also been found for the formation of an analogous complex in the oxidation of ferrocyanochrome c by cytochrome c peroxidase (23, 24). The existence of the additional reaction pathway characterized by k3 has a number of parallels in other enzyme systems, notably in the hydrolysis of acetylglucosyl methyl ester and p-nitrophenylacetate by a-chymotrypsin (25, 26). In those cases the assumption that the enzyme-substrate complex was productive in nature led the authors to describe the phenomenon as substrate-induced activation, i.e. it was envisaged that the interaction of the 2nd substrate molecule with the complex served to accelerate its decomposition to products. In the present case the complex is incapable of productive decomposition, although it still retains some residual ability to oxidize a 2nd molecule of p-cresol; binding of the substrate therefore inhibits the reaction by interfering in some way with the oxidizing ability of the enzyme. Activation by substrate binding will occur when k3Km/k2 > 1, and extrapolation of the pH rate profiles for k3 and Km/k2 suggests that this should happen above about pH 11. Equation 5 shows that a plot of kobs versus [S] would then be expected to exhibit an initial upward curvature at [S] < Km, and in fact such behavior has recently been observed in this laboratory in the reaction of Compound II of lactoperoxidase with iodide ion (27).

Turning now to the pH dependence of the kinetic parameters, the simplest explanation of the Km curve is in terms of a hydrogen-bonding interaction between p-cresol and a basic group in the enzyme. Either protonation of this group or ionization of the substrate leads to a reduction in the efficiency of binding. The nature of the group is a matter for speculation, although its pKa is suggestive of histidine.

In order to gain any understanding of the origin of the catalytic efficiency of the enzyme it is necessary to search for a similarly detailed interpretation of the observed variation of log k2/Km with pH. This is particularly difficult in the present case because of the complicated nature of the pH profile and the fact that the apparent occurrence of complex formation away from the active site prevents the usual division of the catalytically important groups into those involved in binding the substrate in a position suitable for its oxidation and those required for the catalytic step itself. In the following discussion this problem is tackled as far as possible in conventional ways, but reference is made to the concept of transition state acid dissociation constants where this affords additional insights. Since an understanding of the reactivity and selectivity behavior of Compound II must ultimately depend on a description of the activated complex, there may be some advantage in an approach which deals directly in terms of transition state parameters.

From the first column of results in Table IV it appears that two of the terms in the numerator of Equation 7 account for the observed rate. In order to decide which of the kinetically equivalent pathways included in each of these terms is responsible for the reaction it is necessary to make use of the maximum value which can be placed on the rate of a diffusion-controlled process. This has been estimated (28) to lie between 1010 and 1012 M-1 s-1 for the interaction of a macromolecule with a small species such as p-cresol. Since Ks/K2 = 1.26 × 10-8 and Ks/K3 = 3.16 × 10-4, it follows that even for the maximum...
diffusion-controlled limit the predominant pathways are those described by $k_f$ and $k_a$. The protonated forms of Compound II existing in the pH ranges 2.2 to 5.7 and 5.7 to 8.6 would appear to react with the neutral form of the substrate. Two assumptions are implicit in these conclusions: (a) that no protonated form of the reactants existing outside the pH range of the study is kinetically important within this range, and (b) that for example in the reactive species $E_H$, the protonated groups are those of $pK_a$ 8.6 and 5.7 and not alternative pairs such as those of $pK_a$ 8.0 and 2.2.

A rigorous discussion with explicit consideration of the above possibilities would require further expansion of Scheme 1 and Equation 7, but this can be achieved more easily by the use of the alternative approach based on Scheme 2 and Equation 8. The reaction can be seen to proceed through two transition states, one of which is important below $\text{pH} = pK_{a} = 5.4$, whereas the other predominates at neutral and high pH and contains 1 less proton in the region of the active site. Over the whole of the pH range corresponding to each of these transition states it is necessary that each ionizing group of the enzyme and substrate be in a state of ionization which allows it to exist in sufficient concentration to account for the observed rate. For example, at pH 4 the observed second order rate constant is close to $5 \times 10^3$ M$^{-1}$ s$^{-1}$ and the fraction of the substrate in the anionic form is $K_{a}/[H^+] < 10^{-4}$, so that if the c-pthalate ion were the reactive species the true second order rate constant would have to be greater than $5 \times 10^3$ M$^{-1}$ s$^{-1}$, which exceeds the diffusion-controlled limit. Thus the low pH transition state is formed by reaction of neutral $p$-cresol. At high pH the anion is present in sufficient concentration to account for the observed rate, but ceases to be so below pH 6, so that a similar conclusion must also apply to the pathway operative above pH 5.4. The same method can be used for each of the kinetically important enzyme groups, including any which may have $pK_a$ values outside the range of the study. Thus, taking the upper limit for a diffusion-controlled reaction, it is possible to exclude from the reaction at low pH the basic form of the group of $pK_a$ 8.6 or of any other group of $pK_a > 11$ as well as the acidic form of any group of $pK_a < 1.4$. If the diffusion-controlled limit is stricter by little more than an order of magnitude, it becomes possible to exclude the basic form of the group of $pK_a$ 5.7 and the acidic form of the group of $pK_a$ 2.2, which leaves as the only allowed pathway below pH 5.4 a reaction between neutral $p$-cresol and the form of Compound II as it exists between pH 2.2 and 5.7. A similar line of argument leads to the conclusion that the reaction at medium and high pH involves the neutral $p$-cresol molecule and the form of Compound II existing between pH 5.7 and 8.6. These conclusions, which are independent of the assumptions mentioned above, have been reached without recourse to complex reaction schemes or the unwieldy algebraic expressions derived from them.

Although interesting in itself, the identification of the reactive species does not lead directly to a description of the state of protonation of the various groups in the transition state unless it can be assumed that no proton transfers occur after collision of the reactants but before the transition state is reached. Such a possibility is difficult to exclude in all circumstances, but is unlikely in the present case since it would be difficult to understand, for example, why even at pH 11 collisions between the $p$-cresol anion and the enzyme are ineffective if the activated complex contained the substrate species in its ionized state. A similar point may be made for the various enzyme groups, allowing a reasonable picture to be formed of the state of protonation of the kinetically important groups in the two transition states. In both cases these contain the neutral substrate molecule, the group of $pK_a$ 2.2 in its basic form, and that of $pK_a$ 8.6 in its acidic form. In the low pH transition state the group of $pK_a$ 5.7 is protonated, whereas in the transition state for the reaction occurring above pH 5.4 this group is in its unprotonated form.

Although only one transition state acid dissociation constant is apparent over the range of pH covered, all ionizing groups in both the substrate and Compound II must be capable of both protonation and deprotonation in the activated complex at sufficient extremes of pH. If the $pK_{a}$ value of a particular group is higher than its ground state $pK_a$, protonation speeds the reaction, whereas if these lie in the opposite direction the reaction is retarded. The difference between the $pK_a$ of a group in the enzyme or substrate and its $pK_{a}^+$ value in the transition state is a measure of the extent to which protonation influences the kinetics. At a sufficient distance from the active site $pK_a = pK_{a}^+$ and the rate of reaction is insensitive to the state of ionization. These considerations suggest an alternative way of discussing the structure of the transition state in the light of the experimental pH rate profile, which although more inductive in nature can be applied even when the rates are insufficient for the criterion of the diffusion-controlled maximum to be used.

In the present case the $pK_a$ of 2.2 is most probably associated with an initial reversible step in the acid-catalyzed splitting of the porphyrin ring from the apoprotein, perhaps by protonation of the acceptor atom of a hydrogen bond; protonation causes a decrease in rate and $pK_{a}^+ < pK_a$. A similar falling off in the rate of binding of cyanide to the native enzyme (30) occurs at pH 4, below which separation of the prosthetic group is also observed (31). The $pK_a$ of 5.7 is notable for its closeness to the $pK_{a}^+$ value of 5.4 ($pK_{a}^+$), and it is therefore tempting to regard these as arising from the ionization of the same group. Protonation of this group, which probably lies close to the active site but is not directly involved in the catalytic process, exerts a small retarding effect on the reaction. Since at sufficiently high and low pH the pH rate profile must in principle become horizontal, both the remaining acid dissociation of the enzyme at pH 8.6 and that of the substrate can now only be paired with the two transition state ionizations expected above pH 11, and both groups must therefore be protonated in the activated complex. Although this method of searching for a reasonable description of the state of ionization of the various kinetically important groups in the transition state is based on a tentative interpretation of the acid portion of the experimental profile, the fact that it leads to the same conclusions as those derived above from application of the criterion of the diffusion-controlled limit adds further weight to the picture developed here.

The discrepancy between the kinetically and spectrophotometrically measured $pK_a$ of $p$-cresol, if not due to some unforeseen source of systematic error, may be attributed to a weak interaction between substrate and protein at the active site. If this causes a slight strengthening in the acidity of some group of $pK_a \approx 10$ on forming the transition state, the resulting pH log rate profile would show a small shift towards
high pH in this region, as is observed. Some steric interference by the substrate with the stability of a hydrogen bond or the formation of such a bond between the phenolic group of the substrate and the phenolic oxygen of a tyrosine residue with a consequent small increase in the acidity of the tyrosine proton could both produce such a shift. In any event, since the effect is small its part in the over all catalytic process must be a minor one.

Although somewhat less familiar, the use of the concept of transition state acid dissociation constants in the above discussion has permitted a simple and rigorous discussion of the experimental results in the light of the diffusion-controlled limit, as well as providing an alternative method by which the same conclusions may be reached. A simple sigmoid or bell-shaped pH rate profile presents few problems in interpretation, but for the more complicated behavior shown in Fig. 6 the rapid multiplication of the number of kinetically indistinguishable pathways makes the approach presented here the more economical. For example it is difficult to see any other way in which the apparently anomalous value of $K_S$ obtained from the kinetic analysis could be explained without introducing further complications into Scheme 1 and Equation 7. It is hoped that these advantages are sufficient to justify the adoption of this somewhat unfamiliar approach.

Since the unionized form of p-cresol has been shown to be the only reactive form, the present results provide no support for the view that the magnitude of the charge on the substrate determines the complexity of the pH log rate profile, as would be expected if the interactions were mainly electrostatic. However, the conclusions of Roman et al. (5) that the simple pH dependence of the rate of oxidation of iodide may be explained by either the operation of a different mechanism or the lack of steric interactions between the protein and a monatomic substrate in the activated complex are still valid in the light of the behavior found here for p-cresol. In the following paper the present conclusions concerning the structure of the transition state will be combined with further experimental evidence in an attempt to distinguish between these two possibilities and provide a description of the intimate mechanism of the reaction.

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APPENDIX

Proof that the Complex Exists in True Equilibrium with Compound II

If the complex is productive the reaction scheme is

$$
\begin{align*}
\text{HRP-II} & \xrightarrow{k_{12}} \text{S-HRP-II} \xrightarrow{k_{31}} \text{HRP} \\
\text{HRP-II} & \xrightarrow{k_{13}} \text{S-HRP-II} \xrightarrow{k_{32}} \text{HRP}
\end{align*}
$$

in which $k_{12} = k'_{12}[S]$. Since only a single "turnover" is observed and in the general case the concentration of the complex S-HRP-II need not be small relative to that of the free Compound II, the steady state assumption is not valid. In the rigorous treatment (32) the concentration of native enzyme formed from an initial concentration [HRP-II]$_0$ of Compound II is given as a function of time by the expression

$$
[\text{HRP}] = [\text{HRP-II}]_0 \left[ \frac{k_{12} k_{32} e^{\lambda_1 t} + k_{13} k_{31} e^{\lambda_2 t}}{\lambda_1 (\lambda_1 - \lambda_2) - \lambda_2 (\lambda_2 - \lambda_1)} \right]
$$

(12)
in which $\lambda_1 = \frac{1}{2}(p + q)$, $\lambda_2 = \frac{1}{2}(p - q)$, $p = k_{13} + k_{31} + k_{32}$, and $q = (p^2 - 4 k_{12} k_{32})^{1/2}$. Equation 12 only reduces to a simple exponential function within a given time scale when $\lambda_1 \gg \lambda_2$, the observed exponential decay then being described by $\lambda_2$. This occurs when $4 k_{12} k_{32} < p^2$, which may in turn be achieved under one of the following conditions: (a) $k_{12} < k_{23}$ and (b) $k_{12} < k_{23}$ for both of which $\lambda_1 \approx k_{23} + k_{31} / (1 + k_{23} / k_{12})$, (c) $k_{12} < k_{23}$ and (d) $k_{12} < k_{23}$ for both of which $\lambda_1 \approx k_{12} + k_{31} / (1 + k_{31} / k_{12})$. The expressions for $\lambda_1$ and $\lambda_2$ have been derived from the relations $\lambda_1 \approx p$ and $\lambda_2 \approx k_{12} k_{32} / p$, which are true when $\lambda_2 \gg \lambda_1$. Conditions a and b predict a first order dependence on the p-cresol concentration and therefore give no information about the permitted relative magnitudes of the rate constants when the complex is present in significant concentrations. Conditions c and d on the other hand show that the reaction will be first order in [HRP-II] even when [HRP-II] $\approx$ [S-HRP-II] (i.e. $k_{12} \approx k_{32}$) provided that $k_{23} \ll k_{31}$. Any productive complex must therefore exist in true equilibrium with free Compound II and p-cresol.

Similar expressions for $\lambda_1$ and $\lambda_2$ apply in the case of an unproductive complex

$$
\text{S-HRP-II} \xrightarrow{k_{12}} \text{HRP-II} \xrightarrow{k_{31}} \text{HRP}
$$

with the exception that now $k_{11} = k'_{11}[S]$ and $k_{22} = k'_{22}[S]$. Conditions a and b can be rejected since they predict that the observed first order rate constant should always be independent of the concentration of p-cresol. Conditions c and d show that the reaction will be first order in [HRP-II] even when [HRP-II] $\approx$ [S-HRP-II] provided that $k_{23} \ll k_{31}$, so that the complex and free Compound II must again be in true equilibrium.

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Studies on Horseradish Peroxidase: IX. KINETICS OF THE OXIDATION OF p-CRESOL BY COMPOUND II
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