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The Effect of Horseradish Peroxidase on Some Organic Peroxides

Dennis Lietz

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THE EFFECT OF HORSE TRADISH PEROXIDASE
ON SOME ORGANIC PEROXIDES

By

Dennis Lietz

A thesis submitted in partial fulfillment of the requirements for the degree Master of Science at South Dakota State College of Agriculture and Mechanic Arts

June, 1958

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ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to Dr. John Tanaka for the helpful advice and guidance given him during the course of these investigations and the writing of this thesis. A vote of thanks is also due Miss Lenore Johnston for the typing of this manuscript and Mr. Edwin Reiner of Hillview, South Dakota, for his generous donation of horseradish without which these investigations could not have been conducted.

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INTRODUCTION

Horseradish peroxidase is a well known enzyme and one that is often studied. It is well suited for enzyme studies due to its availability, relative ease of preparation, and stability. Discussions of the enzyme in general can be found in any standard biochemistry or enzyme chemistry textbook. In addition, a number of excellent reviews have been published among which are those of Theorell (43) and Chance (44). The enzyme will be discussed in more detail later in this thesis.

Although a wealth of knowledge has been gained about this enzyme, very little can actually be said about the mechanism of the reaction. Much of the work that has been done has been concerned with the reaction with hydrogen peroxide. Methyl and ethyl hydroperoxides have also been studied in some detail but only a very few references can be found concerning the action of the enzyme on other organic peroxides. The references which can be found usually state whether the reaction does or does not proceed and give very little kinetic data. As a result, this study was undertaken with the hope that a knowledge of the effect of horseradish peroxidase on some of the higher organic peroxides would lead to an increased understanding of the enzyme.
REVIEW OF LITERATURE

Peroxidase was first discovered by Schonbein in 1863 (43). It has since been found to have a widespread occurrence being found in nearly all plant cells. There is very little found, however, in animal tissue. Small amounts have been found in the saliva of man, dog, cow, horse, and cat, in milk, in liver and spleen, and in leukocytes. The plant kingdom, however, provides good sources of peroxidase with the highest concentration being found in the sap of the fig tree. Second to this as a source is the root of the horseradish which is commonly used to prepare peroxidase.

Horseradish peroxidase catalyzes the oxidation of many phenols and aromatic amines in the presence of suitable peroxides. It consists of a molecule of ferriprotoporphyrin linked to a colorless protein and has a molecular weight of 44,100 (1). It is also known to act upon methyl and ethyl hydroperoxides (2) but does not show activity with benzyl peroxide (3).

The activity of the enzyme is usually determined from the amount of purpurogallin formed from the oxidation of pyrogallol (6,7,16,17,18). The activity is reported as the purpurogallin number (Purpurogallin Zahl, P.Z.). This is the number of mg. of purpurogallin formed per mg. of dry enzyme preparation in 5 minutes at 20°C. The method used was originally set forth by Willstatter and Stahl (4). It has since been modified by Sumner and Gjessing (5) whose method is now used. Other proposed methods utilizing pyrogallol are those of Barta (20) and of Gibson and Maher (21). Methods utilizing other donors have also been
proposed. Avi-Dor and Paul (22) suggest the use of mesidine while
Ishidate and Okano (23) propose the use of phenolphthalein. None of
these has come into widespread use.

Many methods have been worked out for the extraction of peroxi-
dase from horseradish (6,7,16,17,18). One of the earliest and simplest
is that of Elliott (6). It involves an ammonium sulfate fractionation
followed by dialysis, but unfortunately this easy process gives a
product of low activity. The method of Kanten and Mann (7), while
being more complex, leads to a product of much higher activity. Their
process involves ethanol-chloroform fractionation, repeated ammonium
sulfate fractionation, alcohol fractionation, and dialysis. The
crystalline enzyme has been prepared by Theorell (8,9) by use of
ammonium sulfate and alcohol fractionation in conjunction with electro-
phoresis. His crystalline preparations always showed a purpurogallin
number of about 900. This established the relationship between P.Z. and
purity since the reports of other workers had been rather inconsistent.
Willstatter and Pollinger (16) reported a preparation having a P.Z. of
3070. Elliott and Kellin (18) reported a value of 818 for their
preparation. Other values were 3400 reported by Kuhn, et al. (17),
1080 by Kanten and Mann (7), and 25.3 by Elliott (6). Thus the P.Z.
may be above or below 900 depending upon the method of preparation.

The light absorption curve of peroxidase resembles that of other
heme pigments and is very similar to that of methemoglobin. Theorell's
crystalline preparation gave absorption bands at 260, 402, 500, and 640
m\(\mu\) (8). Of the four, the band at 402 m\(\mu\) is the most significant. This
is termed the Soret band and is characteristic of the porphyrins. The position and intensity of the Soret band is changed when the porphyrin is complexed. This varies according to the ligand present (19). Thus a convenient method is afforded for study of the enzyme-substrate complexes, since the Soret bands of these complexes will differ from that of the original enzyme. The complex will have a Soret band that is lower in intensity and shifted from that of the free enzyme. With hydrogen peroxide the Soret bands appear at 407 and 417 μm (2). The first is rapidly converted to the second and can not be observed in the ordinary spectrophotometer. The second is easily observed so long as the donor is absent. With methyl hydroperoxide the bands appear at 410 and 419 μm (2).

Peroxidase is not unlike other enzymes in its reactions. It forms an enzyme-substrate complex with the peroxide. However the reaction ceases at this point unless a suitable donor molecule is present. When this condition is met, the complex reacts with the donor forming the oxidized product, the reduced substrate, and regenerating the free enzyme. The general reaction scheme is as follows:

\[ \text{En} + \text{S} = \text{EnS} \]
\[ \text{EnS} + \text{AH}_2 = \text{En} + \text{SH}_2 + \text{A} \]

Thus there are two consecutive bimolecular reactions, rather than a bimolecular reaction followed by a monomolecular decomposition of the enzyme-substrate complex.

Peroxidase is not specific for hydrogen peroxide alone.
Although it seems to react best with H$_2$O$_2$, Chance (2) has reported that it will also react with methyl hydroperoxide and ethyl hydroperoxide. He has determined the kinetics of the reactions. Dixon has reported that benzoyl peroxide is not affected by peroxidase (3).

Little is known of the actual mechanism involved in the peroxidase reaction. Recently however, the possibility of free radical intermediates has been considered. Saunders proposed a free radical mechanism to explain the oxidation of 4-methoxy-2,6-dimethyl aniline (10). A later publication discussed this further (11). He proposed the transfer of two electrons to form the diradical

![Diradical](image)

which reacts with H$_2$O$_2$ to form

![Reaction](image)

This would be very unstable and would decompose to form 2,6-dimethyl-p-benzoquinone.

![Decomposition](image)

This would react with a molecule of the original compound to give 2,6-dimethylbenzoquinone-4-(4'-methoxy-2',6'-dimethyl) anil.

![Reaction](image)

The latter was identified as a product of the reaction.

Magnetic studies of the methyl hydroperoxide-peroxidase complex
showed three unpaired electrons in complex I and two in complex II (12). This suggests reaction of enzyme with peroxide to form complex I containing two oxidation equivalents of peroxide. This reacts with the donor, relinquishing an oxidation equivalent to form complex II. Finally complex II reacts in the same fashion to regenerate the enzyme. The following resonance is suggested for complex I (12):

\[
\text{Fe}^{\text{III}}\underline{\text{O}}\underline{\text{O}}\text{-R} \leftrightarrow \text{Fe}^{\text{II}}\underline{\text{O}}\underline{\text{O}}\text{-R}
\]

And for complex II (12):

\[
\text{Fe}^{\text{III}}\underline{\text{O}} \leftrightarrow \text{Fe}^{\text{IV}}\underline{\text{O}} \leftrightarrow \text{Fe}^{\text{II}}\underline{\text{O}}
\]

The physiological role of peroxidase is as yet unknown. It seems unlikely that it is present mainly to decompose the toxic peroxides since catalase is also effective in this decomposition and does not require the donor that peroxidase does. Peroxidase has, however, been shown to act as an oxidase in the oxidation of dihydroxymaleic acid (24). In this case the dihydroxymaleic acid acts both as substrate and donor. Recent tracer studies (45) have shown that peroxidase activates molecular oxygen when a system consisting of peroxidase, dihydroxyfumarate, and oxygen catalyses the non-specific hydroxylation of salicylic acid. Whether or not either of these functions is of physiological importance has not been determined.

A non-biochemical reaction involving peroxidase is the emulsion polymerization of isoprene by Clark, et al. (13). A 50% yield of polymer was reported but no further work has apparently been done with it.
The individual reactions such as the formation of the enzyme-substrate complexes are too rapid to be followed by simple means. A method involving rapid flow technique in conjunction with a sensitive spectrophotometer has been worked out by Chance (25,26) to follow these reactions. The overall reaction can be followed by a series of P.Z. determinations (5). This is, however, quite laborious.

Peroxides can be reduced at the dropping mercury electrode (27,28,29,30,31,32). This makes the polarograph a useful tool. By setting the polarograph at a constant potential and reading the current at given time intervals, the reaction can be conveniently followed as it proceeds in the polarograph cell. This has been done by Boardman (33) to follow the reduction of cumene hydroperoxide by substances other than peroxidase and by Anderson and Estee (15) in the reaction of H₂O₂ with KI. It has also been used by Bernard (34) to follow the catalase reaction. A modification involving the polarographic determination of the donor has been used by Doskacil (14) to follow the peroxidase reaction.
Isolation of Peroxidase. The peroxidase was isolated from the root of wild horseradish by the procedure of Kenten and Mann (7). The horseradish was generously donated by Edwin Reiner of Hillsview, South Dakota.

In this procedure 5 kg. of washed roots were ground in a meat grinder. This was then mixed with 7 liters of water and macerated in a Waring Blender. The resulting mixture was kept at a temperature of 0-10°C, for 10 hours. To this mixture, 2.7 liters of a 2:1 mixture of ethanol and chloroform were added with stirring over a period of 20 minutes. The pulp was then separated from the liquid by the use of suction filtration. The filtrate was centrifuged in a Sharples centrifuge which had been adjusted to the speed which produced a clear supernatant layer. The supernatant layer was then adjusted with a Beckman pH meter, model H2, to pH 5.5 using 2N NaOH. This was then distilled under reduced pressure and at a temperature of 25 to 30°C, until approximately 2.18 liters remained. This distillation required about three days. To the remaining mixture, 545 g. of Merck Reagent grade ammonium sulfate was added. The mixture was then centrifuged at 3000 rpm for 15 minutes and the precipitate discarded. Again 545 g. of ammonium sulfate were added to the remaining liquid and the mixture allowed to stand overnight at a temperature of 0-10°C. The mixture was then centrifuged and the supernatant layer discarded. The residue was suspended in 50 ml. of 0.2M orthophosphate buffer (pH 7.0) and dialyzed from sausage casing for 3 hours against
running tap water and then overnight against 5 liters of distilled water at a temperature of 0-10°C. The mixture remaining in the dialysis bag was centrifuged and the precipitate discarded. The supernatant layer was cooled in an ice brine bath and 1.5 volumes of ethanol were added while maintaining a temperature of 0 to -5°C. The resulting mixture was allowed to stand for 30 minutes and then centrifuged. To the resulting supernatant layer was added 2.5 volumes of ethanol at the same temperature as before, the mixture allowed to stand for thirty minutes, and then centrifuged. The precipitate formed was suspended in 25 ml. of water and again centrifuged. The supernatant layer from the last centrifugation was fractionated with 1.2 volumes of ethanol in the same manner as before. The resulting supernatant layer was then fractionated with 2 volumes of ethanol. The precipitate from the last fractionation composed the enzyme preparation. The procedure was repeated three times yielding a total of about 0.5 g. of enzyme preparation. The preparation was stored in a dessicator at room temperature.

**Determination of Purpurogallin Number.** The method used was that of Sumner and Gjessing (5). This consisted of pipetting 2 ml. of a 5% pyrogallol solution, 2 ml. of 0.5M phosphate buffer adjusted to pH 6.0, 15 ml. of water, and 1 ml. of 1% H₂O₂ into a 125 ml. Erlenmeyer flask. This was then brought to 20°C. in a constant temperature water bath. When temperature equilibrium had been attained, 1 ml. of peroxidase solution was added and the flask shaken for 5 minutes. All timing was accomplished by means of a stopwatch. At the end of the 5 minutes
1 ml. of 2N H₂SO₄ was added. The purpurogallin was then extracted with 40 ml. of ether. This was diluted to a volume of 50 ml. with ether. The concentration of purpurogallin was determined colorimetrically using the Beckman Model DU spectrophotometer at a wavelength setting of 425 nm.

The enzyme solution used in this and in the kinetic studies consisted of 3.9 mg. of enzyme preparation dissolved in enough water to prepare a liter of solution. When not in use this solution was stored in the refrigerator.

Since purpurogallin does not obey Beer's Law, a standard curve of optical density versus purpurogallin concentration had to be prepared. The purpurogallin for this was prepared by the method of Evans and Dehn (35). A solution of 10 g. of pyrogallol (Merck) dissolved in a few milliliters of cold water was placed on a magnetic stirrer. To this was added, dropwise, 100 ml. of cold 8% NaIO₃ solution. The purpurogallin was filtered out and recrystallized from glacial acetic acid. The product consisted of red prisms which decomposed at 273-275°C. Known amounts of this were dissolved in ether and a standard curve prepared. The curve was linear but did not pass through the origin.

The preparation showed a P.E. of 1700. This is not unusual for an impure enzyme preparation.

Preparation of α-Propyl and Isopropyl Hydroperoxides. The procedure used was that of Williams and Mosher (36), which consisted of the reaction of alkyl methane sulfonate with 30% H₂O₂.
The n-propyl methane sulfonate was prepared by stirring a mixture of 22.9 g. of methanesulfonyl chloride (Eastman) and 12 g. of n-propyl alcohol in an ice brine bath while adding 31.6 g. of pyridine. The pyridine had previously been dried over KOH pellets. The addition of the pyridine was carried out over a period of about three and one half hours. The resulting mixture was then added to 125 ml. of ice cold 10% HCl and extracted with 75 ml. of ether. The ether solution was washed with two 20 ml. portions of water followed by 30 ml. of saturated NaHCO₃ solution. The resulting solution was dried over anhydrous K₂CO₃, filtered, and heated on the steam bath to remove the ether. The residue was distilled at aspirator pressure. The product distilled at 126°C.

A mixture of 5.52 g. of n-propyl methane sulfonate and 20 ml. methanol was cooled in an ice bath and combined with 20 g. of 30% H₂O₂ and 5 g. of 50% KOH in that order. The mixture was placed in a constant temperature bath set at 25°C for 20 hours. The mixture was then cooled in ice and slowly combined with 15 g. of 50% KOH. This was then extracted with 25 ml. of benzene. The aqueous layer was again cooled in ice, made acid with HCl, and extracted with ether. The ether layer was dried over Na₂SO₄ and distilled at atmospheric pressure. The fraction distilling from 80-90°C was collected and used in the enzymatic reactions.

Isopropyl hydroperoxide was prepared according to the procedure of Williams and Mosher (37) for the preparation of sec-alkyl hydroperoxides. It was necessary to make certain minor changes since
isopropyl methane sulfonate was apparently somewhat unstable. An attempt to dry the ether solution over $\text{K}_2\text{CO}_3$ resulted in vigorous evolution of gas. This was thought to be due to hydrolysis of the isopropyl methane sulfonate. When $\text{Na}_2\text{SO}_4$ was used as the drying agent this did not occur. Distillation of the isopropyl methane sulfonate resulted in decomposition with the formation of black, tarry substances. Because of this, heat was not applied in the distillation and only those substances were removed which would distill at aspirator pressure and room temperature. The residue was used in the synthesis of isopropyl hydroperoxide without further purification. The synthesis of the hydroperoxide was carried out in the same manner as that of the n-propyl hydroperoxide. The fraction distilling from 100 to 103°C was collected and used in the enzymatic reactions.

**Preparation of Peroxide Solutions.** Solutions of the peroxides were prepared by diluting known amounts of peroxide to 250 ml. with water. The $\text{H}_2\text{O}_2$ solution was prepared by pipetting 0.5 ml. of 30% $\text{H}_2\text{O}_2$ into a 250 ml. volumetric flask and diluting with water. The n-propyl and isopropyl hydroperoxides were weighed out and contained 1.8641 g. and 0.2433 g., respectively. All three solutions were analyzed by pipetting a 25 ml. aliquot into 150 ml. 2N $\text{H}_2\text{SO}_4$, adding three drops of ferroin indicator, and titrating to the colorless endpoint with 0.1060N $\text{Ce (SO}_4\text{)}_2$ solution which had been previously standardized against primary standard $\text{As}_2\text{O}_3$. The results of the analyses are given in Table I.
Table I. Concentrations of Peroxide Solutions

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<th>Molarity</th>
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<tr>
<td>$\text{H}_2\text{O}_2$</td>
<td>$1.40 \times 10^{-2}$</td>
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<tr>
<td>Isopropyl hydroperoxide</td>
<td>$2.14 \times 10^{-3}$</td>
</tr>
<tr>
<td>n-Propyl hydroperoxide</td>
<td>$1.56 \times 10^{-2}$</td>
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Ten ml. of the n-propyl hydroperoxide solution were diluted to 100 ml. in order to make the concentration more nearly equal that of the isopropyl hydroperoxide.

The peracetic acid solution was prepared by weighing out 6.9869 g. of Becco 40% peracetic acid and diluting to 500 ml. This solution was analyzed by the method of Greenspan and MacKellar (38). This involved addition of a 25 ml. aliquot to 150 ml. of 2N $\text{H}_2\text{SO}_4$ and sufficient cracked ice to maintain a temperature of 0-10°C. Three drops of ferroin indicator were added and the solution titrated with $\text{Ce(SO}_4\text{)}_2$ to the colorless end point. This titration determined the amount of $\text{H}_2\text{O}_2$ present. Sufficient $\text{Na}_2\text{CO}_3$ was then added to fill the flask with CO$_2$. To this was added 10 ml. of 10% KI solution. The liberated I$_2$ was titrated to the starch end point with 0.1020N $\text{Na}_2\text{S}_2\text{O}_3$, which had been previously standardized against NaIO$_3$. This gave a value for peracetic acid. The solution was found to be $2.41 \times 10^{-2}$ M in $\text{H}_2\text{O}_2$ and $7.84 \times 10^{-2}$ M in peracetic acid. In order to obtain more convenient concentrations, 10 ml. of this solution were diluted to 100 ml.

The t-butyl hydroperoxide solution was prepared by weighing out 0.2816 g. of Lucidol t-butyl hydroperoxide and diluting to one liter with water. Since this product is quite pure as purchased and since no suitable method of analysis could be worked out, no analysis was...
carried out. Based on pure hydroperoxide, the concentration would be $3.13 \times 10^{-3} \text{M}$.

**Determination of Polarograph Constants.** The height of the polarographic wave, in addition to being dependent upon the concentration of the reducible substance, is dependent upon two constants, the drop rate and the drop time. The drop rate is the rate of flow of mercury in units of weight/unit time. It varies only with the pressure of the mercury (39). The drop time is the time required for a drop of mercury to form and fall. It varies with the mercury pressure, the medium in which the drop forms, and with the applied potential (39).

The drop rate was determined by starting the mercury flowing through the capillary and collecting it as it fell. A weighing bottle was placed under the capillary just after a drop of mercury had fallen and a stopwatch was started at the same time. The mercury was allowed to drop in air for an arbitrary length of time and collected in the weighing bottle. The weighing bottle was removed and the stopwatch read just after a drop had fallen. The mercury was then weighed. The drop rate was thus found to be $1.4 \text{ mg/sec}$.

The drop time was determined with the capillary immersed in 4/15 phosphate buffer, the same medium in which the reactions were studied, and with an applied potential of 1600 millivolts, the potential at which the reactions were studied. The stopwatch was started just as a drop fell, the drops were counted, and the stopwatch was stopped just as the tenth drop fell. The drop time was found to be 4.32 seconds.
Polarography of the Peroxides. The polarographic wave was determined for each of the peroxides used. The instrument used was a Sargent Model III manually operated polarograph. The electrolysis vessel was of the H-cell type proposed by Lingane and Laitinen (40). This consists of an H-cell with a sintered glass disk in the crossarm. An agar plug saturated with KCl was placed on the sintered glass disk. The right side of the cell was occupied by a saturated calomel half cell. The left side was used as the electrolysis vessel. The dropping mercury electrode was used as the cathode and the calomel half cell served as the anode. Thus all values reported are against the saturated calomel half cell.

By means of a pipette, 25 ml. of N/15 phosphate buffer adjusted to pH 6.0 were placed in the H-cell. The phosphate buffer served not only as a buffer but also as the supporting electrolyte. To this was added 1 ml. of peroxide solution. This was then purged of oxygen by bubbling nitrogen through the solution for 15 minutes. The nitrogen had previously been purified by bubbling through alkaline Na₂SO₃ solution. The capillary was then immersed in the solution and the flow of mercury started. A stream of nitrogen was kept flowing over the top of the solution during the electrolysis. Galvanometer readings were taken at intervals of 200 millivolts applied potential. The range from 0 to 1800 millivolts was studied. Beyond this point the phosphate buffer began to show a wave. A plot of current against applied potential composed the polarogram. It was found that each of the peroxides gave a wave such that 1600 millivolts applied potential
represented a point on a limiting current plateau no matter what the peroxide might be. Thus this potential was selected for the study of the enzymatic reactions.

It was also necessary to check each of the peroxides to make certain that the concentration was linearly related to the current. This was done by adding 1 ml. of peroxide solution to 25 ml. of phosphate buffer in the electrolysis vessel. The solution was purged of oxygen, the flow of mercury started, and the current recorded with the applied potential set at 1600 millivolts. A second milliliter of peroxide solution was added and the process repeated. This was done a total of three times for each peroxide. A plot was then made of the current against the concentration for each peroxide. The relationship proved to be linear in each case.

Studies of the Enzymatic Reactions. The method used to follow the enzymatic reaction was the same in each case. Three ml. of peroxide solution were added to 25 ml. of M/15 phosphate buffer in the electrolysis vessel. To this was added 1 ml. of enzyme solution. This was then purged with nitrogen and the capillary flow begun.

Pyrogallol was used as the donor. A solution of this was prepared by dissolving a known amount of pyrogallol in 25 ml. of water. The solution was purged with nitrogen for 15 minutes and then an airtight seal was placed on the flask containing the solution.

In order to prevent the introduction of oxygen into the system a stream of nitrogen was kept flowing over the surface of the liquid in the electrolysis vessel at all times. A hypodermic syringe was used
to introduce the donor solution. One ml. of donor solution was used in each case. The stopwatch was started immediately after addition of the donor solution and galvanometer readings were taken at given time intervals. The applied potential was maintained at 1600 millivolts. A plot of current against time constituted the reaction curve.

The procedure was repeated in each case using 2 ml. of peroxide solution and 1 ml. of water rather than 3 ml. of peroxide solution. Everything else remained the same. Thus curves were obtained at two different peroxide concentrations.

A blank was run in each case using 3 ml. of peroxide solution but replacing the enzyme solution with 1 ml. of water. The procedure was the same. The temperature was maintained at 23°C in all cases by immersing the electrolysis vessel in a beaker of water.

The order of each reaction was determined by means of a fractional-life method (41) which involved the equation

\[ \log t'^{n+1} - \log t' \]

\[ n! \log a - \log a' \]

where \( n \) equals the order of the reaction, \( a \) equals the initial concentration of peroxide from the first curve, \( a' \) equals the initial concentration of peroxide from the second curve, and \( t' \) and \( t'^{n+1} \) are the corresponding times required for the given fraction of peroxide to react.

Since the concentration of pyrogallol was maintained in large excess of that of the peroxide, the order calculated was the pseudo-order with respect to peroxide.
**Determination of Soret Bands.** A more concentrated enzyme solution was used for the determination of the Soret Bands. It consisted of 3.5 mg. of enzyme preparation dissolved in sufficient water to make 100 ml. of solution. Solutions for the Soret band determinations were prepared by pipetting 1 ml. of enzyme solution, 1 ml. of peroxide solution, and 1 ml. of phosphate buffer into a 1 cm. cuvette. Solutions were also prepared consisting of 1 ml. of enzyme solution in 2 ml. of phosphate buffer and of 1 ml. of peroxide solution in 2 ml. of phosphate buffer. These were used for comparison. The optical properties were determined by the use of the Beckman Model DU spectrophotometer in the range from 380 to 430 nm.
RESULTS AND CONCLUSIONS

Enzymatic Reactions. Five reactions were studied in all. These were the reactions with hydrogen peroxide, peracetic acid, \( n \)-propyl hydroperoxide, isopropyl hydroperoxide, and \( t \)-butyl hydroperoxide. Well defined curves were obtained for all.

The reaction with hydrogen peroxide was studied mainly to establish the reliability of the method. The curves are shown in Figure 1. The blank shows a small drop at the beginning which corresponds quite well with the calculated effect due to dilution of the solution by addition of the donor. It is horizontal after this initial drop indicating that no reaction is taking place. The other two curves both show that a definite reaction is taking place. A calculation of the reaction order reveals that the reaction is zero order with respect to peroxide during the time required for about the first one fourth of the peroxide to react. From there on it is first order with respect to peroxide. This is in agreement with data published by Chance (2).

The reliability of the method having been established, studies of the other peroxides were begun. Figure 2 shows the curves for peracetic acid. The curves were corrected for the \( H_2O_2 \) impurity by plotting the curves calculated for the titrated concentration of \( H_2O_2 \) and subtracting the respective curves. The \( H_2O_2 \) curves were determined from the first order rate equation.

In this case the blank shows a slight reaction indicating that
peracetic acid slowly oxidizes pyrogallol by itself. However the addition of enzyme produces a definite increase in the rate of the reaction. The reaction is again zero order in the beginning but changes to first order after a short time.

The effect of the enzyme can also be seen in Figure 3 which shows the curves for n-propyl hydroperoxide. The blank shows very little reaction but the presence of the enzyme produces a definite reaction. The reaction is three halves order throughout with respect to peroxide.

Figure 4 illustrates the effect of the enzyme on isopropyl hydroperoxide. It is quite evident that isopropyl hydroperoxide is also acted upon by peroxidase. However the reaction in this case is second order throughout.

The results with t-butyl hydroperoxide are considerably different from those obtained with the other peroxides. The curves for t-butyl hydroperoxide are shown in Figure 5. While the blank in this case shows very little reaction, the enzyme too seems to have little effect.
Figure 1. Reaction curves for hydrogen peroxide.
Figure 2. Reaction curves for peracetic acid. (Corrected for $H_2O_2$.)

- $0.984 \times 10^{-4} M$ PERACETIC ACID
- $0.523 \times 10^{-4} M$ PERACETIC ACID
- $2.97 \times 10^{-2} M$ PYROGALLOL
- WITHOUT ENZYME
- WITH ENZYME

Note: The figure is a graph with time on the y-axis and current on the x-axis, showing the reaction curves for different concentrations of peracetic acid.
Figure 3. Reaction curves for \textit{n}-propyl hydroperoxide.
Figure 4. Reaction curves for isopropyl hydroperoxide.
Figure 5. Reaction curves for t-butyl hydroperoxide.
Soret Bands. None of the peroxides had absorption peaks in the area studied. However the enzyme and the enzyme peroxide mixtures all showed peaks.

The enzyme showed a peak at 402 m\(\mu\) which agrees with the figure given by Theorell (8). The extinction coefficient, assuming pure enzyme, is \(3.92 \times 10^4\). The enzyme-\(H_2O_2\) mixture gave a peak at 416 m\(\mu\) which agrees quite well with the figure reported by Chance (2). The extinction coefficient in this case was \(3.42 \times 10^4\). The n-propyl and isopropyl hydroperoxides gave peaks at 415 and 420 m\(\mu\) respectively with extinction coefficients of \(2.91 \times 10^4\) and \(2.78 \times 10^4\). The peracetic acid gave a very small peak at 410 m\(\mu\) but it was too small to be certain that it was a Soret band since the extinction coefficient was only \(5.06 \times 10^3\). The t-butyl hydroperoxide gave a peak at 414 m\(\mu\) with an extinction coefficient of \(2.78 \times 10^4\).

The spectrum for peracetic acid is quite informative in spite of the fact that a good Soret band wasn't obtained. The fact that no peak was obtained for the free enzyme in this mixture indicates that a reaction has taken place and a complex has formed. In all the others, a complex formation was indicated by the absence of the free enzyme peak and the presence of a Soret band.

Conclusions. It is quite evident that the polarograph affords a convenient and simple method of following the overall peroxidase reaction. The reaction can easily be followed while it proceeds. In addition only a minimum amount of reagents is necessary. This is a desirable feature since an adequate supply of enzyme is not always
Figure 6. Soret Bands

a. Hydrogen Peroxide
b. Isopropyl Hydroperoxide
c. Tertiary Butyl Hydroperoxide
d. Normal Propyl Hydroperoxide
e. Peracetic Acid
The change in reaction order with change in peroxide indicates a change in the reaction mechanism. However there is insufficient data at present to propose a mechanism. Knowledge of the reaction order with respect to hydrogen donor and the activation energy of the reaction may enable a proposal of mechanism to be made.

A change in the position and intensity of the Soret band on addition of substrate is evidence of the formation of an enzyme-substrate complex. Thus all the peroxides studied formed complexes with peroxidase. All are active complexes with the exception of t-butyl hydroperoxide which shows no activity.
DISCUSSION OF RESULTS

Peracetic Acid. Since peroxides all give waves in the region of 1600 millivolts the polarograph can not differentiate between the various peroxides that may be present in any given solution. A measure of total peroxide is obtained rather than the concentration of each individual peroxide. Since the amount of \( \text{H}_2\text{O}_2 \) impurity present in peracetic acid was known it was deemed necessary to determine whether the polarograph was measuring the reaction with peracetic acid and \( \text{H}_2\text{O}_2 \) or with \( \text{H}_2\text{O}_2 \) impurity alone. For this reason the correction for \( \text{H}_2\text{O}_2 \) was applied to the curve. It was found that a definite reaction curve remained after applying the correction. This was assumed to be due to peracetic acid.

It is conceivable that impurities such as diacetyl peroxide may also be present in the peracetic acid. Unfortunately, there is no simple method of determining this. Since the polarograph measures all peroxides, the horizontal part of the curve in the latter part of the reaction may indicate the presence of disubstituted peroxides. These are inactive insofar as the enzyme is concerned. Thus the peracetic acid may have been depleted and the reaction stopped, with a peroxide wave still present.

Isopropyl and \( \text{p-Propyl} \) Hydroperoxides. The possibility of a hydrogen peroxide impurity should be considered with these peroxides, especially since the weight values differ so from the titration values. The method of preparation, however, should prevent \( \text{H}_2\text{O}_2 \) from being present.
in a preparation of either of the peroxides. The benzene wash used in the preparation should remove all or nearly all H₂O₂ since it has been reported (42) that the ratio of the concentration of H₂O₂ in the aqueous phase to that in the organic phase in the water-benzene system is 1/200. Thus the majority of the H₂O₂ would be removed by washing with benzene.

The discrepancy in concentration values is explained by the thermal decomposition of the peroxide. These hydroperoxides undergo smooth but rapid decomposition at temperatures from 90 to 100°C. Thus the peroxide was probably decomposed in the process of distillation. The distillate contained a low concentration of peroxide as a result. This could have been prevented by the use of reduced pressure distillation.

**Tertiary Butyl Hydroperoxide.** It would appear that there is a negligible reaction with t-butyl hydroperoxide. The nature of this reaction is not known. It would appear, however, that the reaction is due to an impurity such as H₂O₂ since the curve is very nearly horizontal after the reaction has proceeded for a short time. Unfortunately, no simple method is available for the determination of H₂O₂ in the presence of t-butyl hydroperoxide. Therefore a definite statement as to the nature of the reaction cannot be made.

The t-butyl hydroperoxide appears to be the point in increasing size of alkyl group where peroxidase action ceases. Further research is necessary to verify this conclusion. In the case of t-butyl hydroperoxide, an enzyme-substrate complex is formed as evidenced by the
presence of the Soret band. If steric hindrance is preventing complete reaction it is not preventing the enzyme from reacting with the substrate. This would indicate that it prevents reaction of the complex with the hydrogen donor. This would in turn imply that a termolecular complex is necessary. Knowledge gained from future work may allow a definite statement about this to be made.
SUMMARY

It has been found that horseradish peroxidase is effective with several small organic peroxides. It reacts with peracetic acid in a first order fashion with respect to peracetic acid. This does not differ significantly from the reaction with hydrogen peroxide. With n-propyl and isopropyl hydroperoxides, however, the reaction order became three halves and second respectively. It can be concluded that the reaction mechanism for these peroxides is not the same as that for hydrogen peroxide. Further data is needed before a mechanism can be proposed. It also can be concluded that the organic groups represented in these peroxides are not sufficient to inactivate the enzyme. The t-butyl group, however, appears to somehow inactivate the enzyme since no significant reaction was observed in this case. However, an enzyme-substrate complex is formed as evidenced by the appearance of a definite Soret band. The complex is inactive but no explanation can be offered at present for this inactivity.
LITERATURE CITED


