THE PRIMARY AND SECONDARY COMPOUNDS OF CATALASE AND METHYL OR ETHYL HYDROGEN PEROXIDE

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In this and following papers, the properties of two new catalytically active enzyme-substrate compounds of catalase are given. Paper I gives spectroscopic data, Paper II and III, data on kinetics and activity, and, in Paper IV, the reactions of these enzyme-substrate compounds with hydrogen peroxide are discussed.

The red catalase ethyl hydrogen peroxide compound studied by Stern (1) is characterized by two sharp bands in the green region of the spectrum and requires about 0.3 M ethyl hydrogen peroxide for maximum spectroscopic effects. Stern's compound is, however, a secondary product of the reaction of enzyme and substrate. The primary reaction product is a green compound which has a single diffuse band in the red region of the spectrum and requires only a very small excess of ethyl hydrogen peroxide for maximum spectroscopic effects (\(5 \times 10^{-4}\) M). Catalase is here shown to form completely analogous primary and secondary compounds with methyl hydrogen peroxide (2). These primary (I) and secondary (II) compounds resemble the green and red peroxidase hydrogen peroxide compounds (see Theorell (3)).

The spectra of the primary compounds of catalase with these alkyl hydrogen peroxides are related to that of the catalase-hydrogen peroxide complex (4) and give an indication of the spectrum of the latter compound in regions for which data have not yet been obtained.

Preparations—Bonnichsen's (5) catalase preparations were used in these experiments, and many thanks are due him. Ethyl hydrogen peroxide† was prepared and standardized as described by Stern (1). According to his tests, a pure material gave the same results, and since the concentrations employed here are about 0.001 to 0.0001 of those he employed, the effect of an impurity would be smaller. Methyl hydrogen peroxide was prepared as described by Reiche and Hitz (6). The distillate was about

* This is Paper 3 of a series on catalases and peroxidases.
† Credit is due Miss Peri Kara for this preparation.
0.3 M. The concentrations were determined by titration as described by Stern (1) but more readily by the spectrophotometric method (see Reiche (7)).

Visual Spectroscopy of Catalase Alkyl Hydrogen Peroxides—Stern (1) found absorption bands at 535.5 and 580 μm for his compound of catalase and ethyl hydrogen peroxide. In his experiments there was always an appreciable delay in the formation of the red compound; it was preceded by a greenish color which was attributed by him to "concomitant pigments in the enzyme solutions." A repetition of Stern's visual tests with pure catalase and either methyl or ethyl hydrogen peroxide shows that a diffuse absorption band appears at 670 μm and is responsible for the transient green color of the catalase alkyl hydrogen peroxide solutions. Using a 5 to 10 μM catalase solution in a 10 cm. tube, one sees the band at 670 μm very clearly for about 4 seconds upon the addition of 40 μM of methyl hydrogen peroxide and less distinctly for about 20 seconds upon the addition of 160 μM of ethyl hydrogen peroxide. At the end of these times, the band at 670 μm disappears, and that at 630 μm reappears. With stronger catalase, the band at 670 μm appears to extend from 640 μm towards 700 μm. On addition of larger amounts of the alkyl hydrogen peroxides, the green compounds are converted into the red compounds, which are stable for several minutes. The red methyl and ethyl hydrogen peroxide compounds of catalase have the same visible absorption spectrum, judged from the hand spectroscope, and their bands lie at 536 and 572 μm, at approximately the positions found previously by Stern.

Stern's red compound is therefore a secondary compound which is preceded by a primary green compound, as in the case of the red and green peroxidase-hydrogen peroxide compounds (3).

Relation between Spectral Shifts in Visible and in Soret Region—The photosensitivity and the changes of extinction coefficient are so small at 670 and 580 μm that kinetic studies with the rapid flow apparatus are uneconomical of enzyme solution, and use of the Soret region is always preferable. Therefore a series of experiments has been carried out in a 1.33 cm. cuvette to show the correspondence between the spectral shifts in the visible and Soret regions. The capillary cuvette of the flow apparatus (8) is replaced by a pair of 1.33 cm. cuvettes. One cuvette is filled with 1.13 μM of catalase solution (5 cc.), and upon addition of 0.05 cc. of 0.3 M ethyl hydrogen peroxide and stirring, the transmission changes are recorded directly, as shown in Fig. 1. The constant transmission of the catalase solution is followed by a sharp spike, indicating the moment at which the ethyl hydrogen peroxide is stirred in. Then the kinetics of the intermediate compound are recorded. The sensitivity is adjusted appropriately for each record. In records B and D, the transmission changes...
are larger than a few per cent and are not, therefore, directly proportional to the concentration of the intermediate compound. In record A at 650 m\(\mu\), the green compound rapidly forms directly from catalase, and in a few minutes disappears. At 580 m\(\mu\) there is very little, if any, evidence of a rapid reaction; only the slow formation of the relatively stable red compound at about the same rate as the green compound disappeared at 650 m\(\mu\). Thus it is shown that the green compound, in accordance with the visual tests, forms first and is slowly converted into the red compound. Similar records were obtained at 423 and 435 m\(\mu\). At the wave-lengths

![Image of a diagram showing the relationship between the spectral shifts in the formation of catalase ethyl hydrogen peroxide I and II in the visible region and in the region of the Soret band of catalase. The units of \(\epsilon\) are cm\(^{-1}\) x mm\(^{-1}\). The wave-lengths and sensitivities (amperes per scale division) used in the four records are as follows: record A, 650 m\(\mu\), 6 \times 10\(^{-10}\); B, 423 m\(\mu\), 6.2 \times 10\(^{-10}\); C, 580 m\(\mu\), 1.3 \times 10\(^{-10}\); D, 435 m\(\mu\), 7.3 \times 10\(^{-10}\). The spacing between two of the heavier lines represents 10 scale divisions. An open 1.33 cm. cuvette was used in these experiments. The spike indicates the moment at which the ethyl hydrogen peroxide was stirred into the solution. The final ethyl hydrogen peroxide concentration was 3 mM. 1.13 \(\mu\)M of horse liver catalase, pH 7.0, 0.01 M phosphate (Experiment 145d).

650 and 423 m\(\mu\), there are isosbestic points (wave-lengths of equal extinction coefficient) for catalase and compound II, and at 580 and 435 m\(\mu\), there are isosbestic points for catalase and compound I. Thus the kinetics of these two compounds can be independently recorded at these points. The kinetics of compound I are usually recorded at 405 m\(\mu\), because small concentrations of substrate give negligible amounts of compound II before compound I decomposes into free catalase (9).

Soret Bands of Primary and Secondary Compounds—As the data of Fig. 1 show, the secondary compounds are stable for several minutes when
dilute (\(\sim 1 \mu M\)) catalase solutions are used, and the Soret band can be measured directly in the Beckman spectrophotometer, as shown in Fig. 2, Curves IIa and IIb. To insure that compound I has been completely converted into compound II, the spectrum is measured after the initial change of optical density at 421 m\(\mu\) upon addition of the peroxide has de-

Fig. 2. The Soret bands of the primary and secondary catalase-alkyl hydrogen peroxide complexes. The curve following the circles is the Soret band of catalase. Curves IIa and IIb are the Soret bands of catalase methyl hydrogen peroxide II and catalase ethyl hydrogen peroxide II respectively. The Soret band of the primary catalase-alkyl hydrogen peroxide complex is given in Curve I and is obtained by subtracting from Curve IIa the changes of extinction coefficient found in the rapid flow apparatus corresponding to the conversion of compound I to II. Curve IIa, 0.64 \(\mu M\) horse erythrocyte catalase, 300 \(\mu M\) of methyl hydrogen peroxide (Experiment 139). Curve IIb, 0.57 \(\mu M\) of catalase, 5, 10, 100 \(\mu M\) of ethyl hydrogen peroxide. Curve I (\(\triangle\)) 0.66 \(\mu M\) of horse erythrocyte catalase, 100 \(\mu M\) of methyl hydrogen peroxide; and (\(\square\)) 200 \(\mu M\) of ethyl hydrogen peroxide (Experiments 127b and 130a). All curves, pH 6.5, 0.01 M phosphate buffer.

creased to zero. Also the density at 435 m\(\mu\) is checked before and after the measurement to insure that complex II has not decomposed.

It has been found with both horse radish peroxidase and lactoperoxidase (10) that the extinction coefficient of the secondary complexes is the same, regardless of whether methyl or ethyl hydrogen peroxide is used. Here the shapes of Curves IIa and IIb are similar, but the extinction coefficient of catalase ethyl hydrogen peroxide II as measured in the
Beckman spectrophotometer is usually found to be slightly less (ε_{425} = 232 cm^{-1} \times \text{mm}^{-1}) than that of the methyl hydrogen peroxide complex (ε_{425} = 242 cm^{-1} \times \text{mm}^{-1}). Since 10 to 100 times more ethyl hydrogen peroxide than methyl hydrogen peroxide are required to stabilize the ethyl hydrogen peroxide complex for the duration of the experiment, some catalase is probably destroyed when ethyl hydrogen peroxide is used. The data for methyl hydrogen peroxide are therefore considered to be more accurate.

In the ordinary spectrophotometer, the conversion of complex I into complex II begins before satisfactory measurements can be made (see Fig. 1). Therefore, the rapid flow apparatus is used to measure the change of optical density from complex I to complex II as illustrated by Figs. 3 and 8 of Chance (9). The values obtained in such experiments are identical within the experimental error for the methyl and ethyl hydrogen peroxide complexes, as Fig. 2 shows. These changes of optical density are converted into changes of extinction coefficient as before (4), except that here the optical density change at 435 μm (where complex I and catalase have isosbestic points) is used to calculate the scale factor for the conversion from change of optical density to change of extinction coefficient at the other wave-lengths. By subtracting these changes of extinction coefficient from Curve II{\alpha}, Curve I is obtained.

If Curve I is constructed by applying the changes of extinction coefficient from catalase to complex I to the catalase spectrum, the curve lies above that given in Fig. 2 in the region of 405 μm. The method described above is considered to be more accurate because the changes of extinction coefficient between Curves II and I are smaller and the shapes of the curves are more alike than are the catalase spectrum and Curve I. Therefore, errors caused by excessive spectral interval have a small effect upon the values of extinction coefficient of Curve I.

In experiments with horse liver catalase, the Soret bands of the secondary complexes of methyl and ethyl hydrogen peroxide were found to be nearly identical. The maximum extinction coefficient (ε_{425} = 204 cm^{-1} \times \text{mm}^{-1}) is less than that obtained with horse erythrocyte catalase in Fig. 2 (ε_{425} = 242 cm^{-1} \times \text{mm}^{-1}) in accordance with studies of the cyanide complex (11).

DISCUSSION

The primary catalase-alkyl hydrogen peroxide complexes are now identified as greenish colored complexes which precede the red secondary complexes, the general effect closely resembling that found by Theorell for horse radish peroxidase and hydrogen peroxide (3). As observed in the hand spectroscope, the addition of alkyl hydrogen peroxide to catalase
gives a large decrease of absorption in the green region and the formation of a diffuse band at 670 m\(\mu\). A similar band of the primary catalase-hydrogen peroxide complex has been demonstrated spectrophotometrically (2).

The Soret bands of the primary complexes of catalase with methyl or ethyl hydrogen peroxide are the same but are much less intense than the Soret band of catalase hydrogen peroxide. The Soret bands of the primary peroxidase-peroxide complexes are identical, regardless of whether hydrogen peroxide or alkyl hydrogen peroxide is attached to peroxidase hematin. It is reasonable to conclude that the decrease of extinction coefficient of the Soret bands of the catalase-peroxide complexes per hematin iron group bound to peroxide is identical for hydrogen peroxides and alkyl hydrogen peroxides. Thus the difference between the intensity of the Soret bands of the primary catalase-hydrogen peroxide and catalase-alkyl hydrogen peroxide complexes is caused by a difference in the number of hematin groups bound to peroxide.

The Soret bands of the primary complexes of catalase and methyl or ethyl hydrogen peroxide are about half the intensity of the Soret band of the free enzyme and are of a shape generally similar to that of the free enzyme. No other known compound of catalase has a similar Soret band. In fact, a Soret band of this type is obtained only when the porphyrin ring is opened, as in the degradation of hemoglobin (12) or when the bile pigment content of catalase is increased (11), for example, by treatment with a large excess of hydrogen peroxide (13). The decrease of extinction coefficient at 405 m\(\mu\) obtained on formation of the primary alkyl hydrogen peroxide complex \(\Delta e_{405} = 45 \text{ cm}^{-1} \times \text{mm}^{-1}\) per hematin iron bound to peroxide) roughly equals the difference between a three- and four-hematin catalase \(\Delta e_{405} \approx 380 - 340 = 40 \text{ cm}^{-1} \times \text{mm}^{-1}\) for one hematin converted to bile pigment). But the peroxides combine directly with the iron atom of catalase and not to the methine bridges of the porphyrin ring (14). Speculation as to whether the porphyrin ring is actually oxidized on formation of the primary complex by electron transfer from the iron-peroxide complex and is then reduced on reaction with the reducing substrate or acceptor affords very interesting possibilities but is premature at this time.

The visible absorption bands of the secondary catalase-alkyl hydrogen peroxide complexes lie at 536 and 572 m\(\mu\), at approximately the positions found by Stern (1). Recently the secondary catalase-hydrogen peroxide complex has been found, and it has the same visible absorption bands. Whereas the Soret bands of the peroxidase-alkyl hydrogen peroxide complexes are identical (10), the extinction coefficient of catalase ethyl hydrogen peroxide II was found to be slightly less than that of catalase methyl.
hydrogen peroxide II. This is probably caused by slight destruction of the enzyme by the large excess of ethyl hydrogen peroxide.

The valence of the iron in the primary catalase-alkyl hydrogen peroxide complexes is established as ferric because of the lack of any carbon monoxide inhibition of the oxidation of alcohols (9). Also, cyanide has been shown to compete with peroxide for the iron atoms of catalase hematin (14).

The secondary catalase-peroxide complexes are not enzymatically active, and their valence is not indicated by these activity tests. However,

![Fig. 3. The Soret bands of catalase cyanide and azide catalase.](image)

the relationships between absorption spectra and iron bonding, which were established by Theorell (15) and recently summarized by Hartree (16), are applicable here. Catalase cyanide has been shown to be a ferric iron compound with covalent bonds by magnetic susceptibility tests (17). Its visible and Soret bands (compare Figs. 2 and 3) resemble those of the secondary catalase-peroxide complexes. By analogy, these secondary catalase-peroxide complexes are also ferric compounds with covalent bonds.

No measurements have yet been made of the magnetic susceptibility of the primary catalase-peroxide complexes, and spectral analogies are weak because there is no compound of catalase which has a similar spectrum.
Azide catalase is colored green and has been found to be a ferric compound with ionic bonds (17). But there is a very remote similarity between the Soret bands of azide catalase and the primary catalase-peroxide complexes, as Figs. 2 and 3 clearly show. Only by analogy with the primary peroxidase-peroxide complexes can the bond type of the primary catalase-peroxide complexes be established as ionic. The experimental data for the bond type of the primary peroxidase-peroxide complexes is, however, weak (10).

The formation of a covalent iron-peroxide bond does not necessarily explain the slow conversion of the primary to the secondary catalase-peroxide complexes; such a shift can occur in peroxidase as rapidly as peroxide combines with the iron atom to form the primary complex (10). Nor is it true that covalent compounds of hematins form very slowly; the covalent catalase (18) and peroxidase (19) cyanides form fairly rapidly.

Evidence is now accumulating for the importance and generality of the green primary peroxide complexes of presumably ionic bonds in catalysis by catalase, horseradish peroxidase (10), and lactoperoxidase (10). On the other hand, it is not yet established that cytochrome c peroxidase forms such a complex (20), but the ability of the horseradish peroxidase to oxidize ferrocytochrome c (10) indicates that the reaction mechanism is very similar in these two cases. Verdoperoxidase has been observed to form a green complex but no red complex (21). No green complex of methemoglobin with peroxides has yet been observed.

**SUMMARY**

1. Catalase forms primary and secondary enzyme-substrate compounds with methyl or ethyl hydrogen peroxide.

2. The primary complexes are green and have a diffuse absorption band starting at 670 mμ. The secondary complexes are red and have visible bands at 536 and 572 mμ in approximately the positions found by Stern in his work on catalase ethyl hydrogen peroxide II.

3. These complexes contain ferric iron according to the lack of carbon monoxide inhibition and spectral analogy. In the secondary complexes, the iron is probably bound by covalent bonds according to spectral analogy with the covalent cyanide compound. In the primary complexes, the iron is probably bound by ionic bonds according to spectral analogy with the primary peroxidase-peroxide complexes.

4. In the primary catalase-alkyl hydrogen peroxide complexes, the Soret bands are similar in shape to that of the free enzyme but are shifted towards the visible region of the spectrum by several millimicrons. At 405 mμ, the decrease of extinction coefficient is the same for ethyl and methyl hydrogen peroxide, about 180 cm.−1 X mm−1 for an erythrocyte
catalase or about 45 cm.\textsuperscript{−1} \times mm\textsuperscript{−1} per hematin iron group bound to peroxide.

5. The Soret band of the secondary catalase-methyl hydrogen peroxide complex resembles that of catalase cyanide and has a maximum value, $e_{422} = 242$ cm.\textsuperscript{−1} \times mm\textsuperscript{−1}. The Soret band of the secondary catalase-ethyl hydrogen peroxide complex was found to be slightly smaller, owing probably to catalase destruction.

6. It is probable that the reaction of all catalases and peroxidases with peroxides involves the primary formation of the same type of complex. The exact nature of this complex is not known; the spectroscopic data suggest that changes in the porphyrin ring of the hematin group may be involved.

**BIBLIOGRAPHY**

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