SPECTROSCOPY OF CATALASE

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Catalase is a hemin containing enzyme. This fact was rendered very probable by the discovery of a modified hemin spectrum in purified enzyme solutions from animal and plant tissues by Zeile and Hellström (1, 2). They found that the ratio between catalytic activity and porphyrin bound iron in a given preparation remained constant in the course of fractionation and inactivation experiments. This ratio may, however, vary considerably in different preparations, even from the same type of tissue. Besides the approximate position of the absorption bands of the enzyme in the visible region, the spectrum of the cyanide and sulfo-complex and that of the pyridine hemochromogen derived from the enzyme hemin were described.

That the compound responsible for the hemin spectrum in catalase preparations is identical with the enzyme, was proven by the observation that it undergoes a cyclic spectroscopic change during the decomposition of ethyl hydrogen peroxide (3). The prosthetic group of the enzyme has been isolated in crystalline form and its identity with protohematin IX has been demonstrated by conversion into dimethyl-IX-mesoporphyrin ester (4).

The object of the present study was to gain further information concerning the absorption spectrum of the enzyme and to study the effects of certain reagents on this spectrum. It was hoped to arrive at a decision concerning the state of valency of the iron contained in the enzyme.

EXPERIMENTAL

Enzyme Preparations

Purified catalase solutions were prepared from horse liver\(^1\) in the manner described by Zeile and Hellström (1). The activity of the solutions employed in

\(^1\) The author is indebted to Messrs. Chappel Brothers, Rockford, Illinois, and to the Hill Packing Company, Topeka, Kansas, for furnishing some of the horse liver required for these preparations.

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this study, as expressed by the monomolecular velocity constant obtained under standard conditions was from \( k = 1610 \) to \( 7125 \).

The enzyme solutions are brown in incident light and brown-red in transmitted light. They show the spectrum of the enzyme in layers of 1 to 5 cm., depending on their catalytic activity. In addition to catalase, these solutions contain traces of biliverdin and hepatoflavin. These two pigments do not interfere with observations of the enzyme spectrum in the range of 650 to 400 m\( \mu \). Furthermore, the solutions contain colorless proteins and electrolytes.

**Technique**

The preliminary experiments were carried out with the aid of pocket spectrosopes (Brown, Zeiss). The scale of the Brown instrument was calibrated with the aid of a number of emission lines of the mercury arc and of impregnated carbons. The Zeiss instrument has a scale permitting direct reading of the wave-length. The use of such instruments with small dispersion makes possible the detection of indistinct or faint absorption bands which would be difficult to observe with large instruments. Well defined absorption bands were then measured more accurately with a Hilger wave-length spectrometer. In case of the comparison of two absorption spectra, they were projected simultaneously into the instruments by means of comparison prisms. The Hilger spectrometer was frequently standardized with the aid of an electric sodium burner (Zeiss) and of the mercury arc. As light sources tungsten filament lamps, ranging in intensity from 60 to 750 c.p., were used, depending on the transparency of the objects under study. The solutions were contained either in absorption cells of definite layer of thickness, ranging from 0.5 to 3.0 cm., or in a Baly cuvette graduated to a length of 5.0 cm. in millimeters. For the experiments dealing with the effect of gases on the enzyme, capillary stopper cells of 2.0 cm. thickness of the type described by Warburg et al. (5) were used. For observations in layers from 10 to 50 cm. polarimeter tubes were used.

**Position of the Absorption Bands of Catalase**

The most conspicuous absorption band of catalase solutions, upon direct examination in the visible range, is seen in the red region. Besides, two indistinct bands may be seen in the green region. Zeile and Hellström (1) have photographed the band in the red, and they have also determined the position of the two bands in the green. They give the following positions for these three bands:

- **I.** \( 650 \cdots 646 \cdots 620 \cdots 610 \);  
  \( 629 \) \( 540 \) \( 500 \) \( m\mu \)

According to these authors, the maximum of extinction in the first band is not situated symmetrically between 646 and 620, but shifted
towards the blue range, at about 629 mμ. They did not measure beyond 463 mμ.

The absorption band of greatest extinction of hematin-protein compounds is situated in the far violet region, near 400 mμ (Soret’s band). In the case of hemochromogens like cytochrome, this band may be directly observed if the red region is shut off by a suitable light filter (6). Inasmuch as the observation of a strong absorption band of catalase in this region would constitute additional proof for its hemin nature, the present author has attempted to locate this band. Direct observations in the manner described by Warburg were not successful, principally because of the great decrease of luminous intensity of the tungsten filament lamps in this range and because the spectrum of the carbon arc appears no longer continuous in this range. But when various catalase solutions were analyzed by the recording, photoelectric spectrophotometer of Hardy at the Massachusetts Institute of Technology,2 the Soret band of the enzyme was clearly recorded in diluted solutions. The peak of this band is at 409 mμ, as compared with 436 mμ for the oxygen transferring enzyme No. 1 of Warburg and Negelein (7). From these records the position of the maxima of the three bands in the visible region is taken as follows:

I. $640 - 600$; II. $550 - 530$; III. $515 - 490$ mμ

Though there is general agreement with the values given by Zeile and Hellström, slight differences exist, as shown above.

**Effect of pH on the Enzyme Spectrum**

The appearance of the enzyme spectrum is the same in the pH range from about 5 to 10. Below and above this hydrogen ion concentration, the spectrum fades and the enzyme is destroyed. The first step in this irreversible process appears to be the detachment of the prosthetic group of the enzyme from its protein carrier. In

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2 These measurements were carried out by Mr. O. Barstow, Color Measurement Laboratory, Department of Physics, Massachusetts Institute of Technology, Cambridge, Massachusetts.
SPECTROSCOPY OF CATALASE

acid solution, the protein is precipitated, in alkaline solution it is
denatured. By the latter process a new compound, exhibiting a
characteristic spectrum, is formed. It shows two absorption bands
in the green, one at approximately 575 and the other at 545 m\(\mu\).
The conversion of the enzyme into this compound is slow in 0.05
\(\text{N NaOH}\), being complete after 1 hour. In 0.125 \(\text{N NaOH}\) the transforma-
tion takes place instantaneously.

Zeile and Hellström found a similarity between the spectrum of
catalase and that of alkaline hematin. It appears to the writer that
a perhaps still closer resemblance exists between the spectra of
catalase and methemoglobin. Whereas the band of alkaline hematin
in the red is at 616 m\(\mu\) compared with 622 m\(\mu\) for catalase, the
corresponding band of methemoglobin is centered at 630 m\(\mu\) (8, 9).
Upon visual observation this band has been found between 630 and
620 m\(\mu\) (10). The similarity finds its explanation in the fact that
catalase and methemoglobin have an identical prosthetic group,
parahematin, and differ only in respect to their protein carrier (4).
It is desirable to obtain reliable criteria for distinguishing these two
substances in solution. A number of such criteria have been found
in the course of this work. One of them is the fact that the spectrum
of the enzyme is the same between pH 5 and 10. The spectrum of
methemoglobin is known to change from the neutral or acid type to
the alkaline type at pH 8.5 to 9. Whereas the main bands of neutral
or acid hemoglobin in the visible are at 630 and 500 m\(\mu\), the bands of
alkaline methemoglobin are at 589-579 and 558-535 m\(\mu\), with a faint
band near 600 m\(\mu\) (8, 10). The Soret band of methemoglobin is at
415 m\(\mu\).

Experiments on the Valency of the Enzyme Iron

The remarkable stability of the catalase spectrum against hydro-
sulfite and ferricyanide, which are known to react with all other
porphyrin iron complexes yet studied,\(^8\) prevented Zeile and Hell-
ström (1) from arriving at a definite conclusion concerning the state

\(^8\) The only other exception known to the writer is the modified cytochrome—a
component of certain bacteria. According to Fujita and Kodama (11), the band
of this hemochromogen in the red region may be abolished by ammonium sulfide
of valency of the iron in catalase. In preliminary experiments this observation could be confirmed. With ferricyanide, no change in the enzyme spectrum could be detected even after standing for 30 minutes at room temperature. In order to obtain more conclusive evidence the effect of a variety of reducing agents and of compounds, forming stable complexes with either ferric or ferrous iron, on the enzyme was studied.

Effect of Reducing Agents on Catalase

Stokes' reagent, alkaline cysteine hydrochloride solution, and hydrogen, activated by colloidal palladium, did not produce any change in the enzyme spectrum. These agents reduce methemoglobin rapidly to hemoglobin.

Freshly prepared ammonium sulfide caused the appearance of a new band at 560–590 mμ. The band in the red increased in intensity and showed a shift to 640 mμ. The solution became black-green. This phenomenon is probably due not to a reduction of the enzyme but to the formation of a compound corresponding to sulfo-catalase, which was produced by Zeile and Hellström by adding hydrogen sulfide.

Interesting observations were made when hydrazine hydrate was tried. The addition of comparatively large quantities of 50 per cent hydrazine hydrate to a neutral catalase solution of k = 2092 caused a decrease in the intensity of the enzyme band in the red and the appearance of a new band in the yellow. The final spectrum after 30 minutes was:

<p>| | | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>I. 640 – 600; II. 598 – 578; III. 550 – 540 mμ.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>622</td>
<td>588</td>
<td>545</td>
</tr>
</tbody>
</table>

The order of intensity of these bands was III, II, I. When this solution was saturated with carbon monoxide, the solution appeared or hydrazine hydrate but not by hydrosulfite. It is possible that the band, which these authors describe, is the α-band of the oxidized form of the respiratory enzyme which, according to Warburg (12), is directly visible in microorganisms of high respiratory activity.
cherry red in layers of 5 cm. The spectroscope revealed the presence of two absorption bands:

\[
\begin{align*}
\text{I.} & \quad 586 - 570 \text{ m\mu}; \\
\text{II.} & \quad 554 - 531 \text{ m\mu}.
\end{align*}
\]

Band I was stronger than band II.

At first the writer was inclined to interpret these observations as follows: The enzyme is reduced to the ferrous form by the hydrazine hydrate. The ferrous form in turn combines with carbon monoxide. Control experiments, however, which were partly suggested by Professor D. Keilin, Cambridge, to whom the author is indebted for his valuable advice, showed that such an interpretation cannot be correct. If the compound formed by the action of hydrazine hydrate were the ferrous form of the enzyme, it should be possible to reoxidize it to the original ferric form of the enzyme. Neither treatment with air nor with ferricyanide effected such a reoxidation: Ferricyanide caused a fading of the absorption bands in the visible region; air was without effect. Furthermore, the hydrazine hydrate solution proved to be so strongly alkaline that the phosphate buffer concentration of the enzyme solution was not able to prevent a shift to the alkaline side. Inasmuch as it was observed that comparatively large amounts of hydrazine hydrate had to be used to produce the new compound, it is to be concluded that the latter is not merely the reduced form of the enzyme but an hemochromogen in which hydrazine acts as the nitrogenous base. Hemochromogens form complexes with carbon monoxide.

Photodissociation of Carbon Monoxide Complexes of Catalase Derivatives

The photodissociation of hemochromogen-carbonyl complexes may be directly observed with the aid of a spectroscope and of two light sources. A light source of low intensity is arranged in line with the spectroscope and the absorption cell. Its intensity is sufficient to see the absorption spectrum of the CO-complex but not sufficiently strong to cause photodissociation. A 60 c.p. tungsten filament lamp (110 volts) is satisfactory for this purpose. The second light source is arranged at a right angle to the optical axis. It must be of high
intensity and its light must be concentrated on the side wall of the absorption cell. A 750 c.p. projection lamp (110 volts) with concentrated filament was used. A similar arrangement has been used by Warburg in a demonstration at the Conference on Biological Oxidations in Heidelberg in 1932 (13).

In preliminary experiments with CO-pyridine-hemochromogen from blood hemin it was ascertained that on observation with the weak light source, the absorption spectrum of the undissociated CO-complex was visible. When the strong light source was turned on, at first the long wave band split up into two components and then both bands shifted towards the blue. In the stationary state the spectrum of the free pyridine hemochromogen was alone visible. The process could be reversed by turning off the strong light source.

When the same experiment was tried with the carbon monoxide complex of the hydrazine derivative of catalase, no change in its spectrum could be observed upon illuminating with the strong light source. There was a suggestion that the long wave band gained a little in intensity.

If solutions showing the catalase spectrum are treated with hydro- sulfite in the presence of NaOH and pyridine, the hematin group of the enzyme is detached from the protein carrier and a pyridine hemochromogen is formed (1). This hemochromogen combines reversibly with carbon monoxide. The free hemochromogen and the CO-complex show the same absorption spectrum as the corresponding protohem derivatives prepared from blood hemin. To 10 cc. of a catalase preparation of \( k = 2092 \), 2 cc. n NaOH, 5 cc. pyridine, water to make up a volume of 20 cc., and a small amount of solid sodium hydrosulfite were added. After flushing for 5 minutes with carbon monoxide the solution assumed a pink color. It showed the bands

\[
\begin{align*}
\text{I. } & 563 - 568; & \text{II. } & 523 - 537 \text{ m\(\mu\)}; \\
& 565 \quad & 530
\end{align*}
\]

when examined in the light of the 60 c.p. lamp in the arrangement described above. When the 750 c.p. lamp was turned on, photo-dissociation was complete within about 1 second. The maxima of the bands of the free hemochromogen are at 557 and 527 m\(\mu\). Re
versal of the process began when the strong lamp was turned off; for completion of the recombination a longer period than for that of dissociation was required (about 3 seconds). The experiment shows that the pyridine hemochromogen obtained from catalase is subject to photodissociation as is also the corresponding derivative from blood hemin (15).

Effect of Carbon Monoxide on Catalase

If catalase were an hematin compound containing ferrous iron, it would be expected to form a complex with carbon monoxide. It should be mentioned, however, that cytochrome-c, which in the reduced form is a true hemochromogen, will combine with CO only in rather alkaline solutions (14). If, on the other hand, catalase in the normal state were a ferric compound, but would undergo reversible reduction in the course of its catalytic action on peroxides, such an intermediary ferrous form might be expected to combine with carbon monoxide and to accumulate in the form of this complex. In both cases CO would inhibit the enzymatic reaction in a manner similar to the inhibition of the respiratory enzyme No. 1 (Warburg (15)). Sometime ago the writer observed an inhibition of the catalase of leucocytes by carbon monoxide (16). Shortly afterward, however, this inhibition was explained in terms of an unspecific damage to the enzyme by treating the solutions with a gas. This was indicated by the observation that even hydrogen when bubbled through enzyme solutions may cause a decrease of the activity (17). The so called inhibition by CO of peroxidase, which appears to be another enzyme with a hemin group (18), has found a similar explanation (19). Recently, however, Califano (20) in a short note has reported that catalase is specifically inhibited by CO and that the inhibition may be relieved by illumination of the reaction system. This situation made a reinvestigation desirable.

Effect of CO on the Catalase Spectrum.—When a solution of catalase is saturated with carbon monoxide, no change in the enzyme spectrum is detectable. Since in some hemin compounds CO will produce merely a slight change in the position of the absorption bands or even only a change in intensity of one band (21), the spectra of solutions of catalase in air and in carbon monoxide were projected
simultaneously into the spectroscope. Both spectra appeared identical in every respect. The iron of the enzyme therefore appears not to be in the bivalent state. In other experiments, a catalase solution of \( k = 1610 \) was saturated with carbon monoxide and treated with small amounts of 30 per cent hydrogen peroxide while CO flushed the solution. There occurred a violent decomposition of the substrate; the spectrum of the enzyme remained apparently unchanged. This experiment makes it improbable that the ferrous form of the enzyme is an intermediate in the catalysis.

**Effect of CO on the Activity of the Enzyme.**—The experiments were arranged under conditions favorable for an inhibition of the enzyme reaction by carbon monoxide. A low substrate concentration was selected in order to facilitate a combination of the enzyme with CO. The experiments were carried out at low temperatures because this increases the stability of hemin complexes with CO. The rapid decrease of the dissociation constant of CO-hemochromogens and of the CO-compound of the respiratory enzyme with decrease in temperature has been demonstrated by Warburg and Negelein (22) and Kubowitz and Haas (23). The enzyme concentration was kept small compared with the carbon monoxide concentration. The experiments were performed in an atmosphere of pure CO. During the reaction with the substrate the system was flushed with CO in order to remove any oxygen formed which might interfere with the inhibition effect (20).

A chamber was constructed which was similar to the vessels used in potentiometric titrations. It was provided with in- and outlets for carbon monoxide and nitrogen, with a syphon system permitting the addition of reagents without introducing air and the removal of fluid from the vessel. A stirrer with a mercury seal and a microburette completed the equipment. The chamber was cooled by running ice water. The temperature was kept within 5 to 6.5°C. The apparatus was shielded against direct light to prevent the photo-dissociation of a carbonyl complex. The chamber was filled with 10 cc. m/15 phosphate buffer, pH 6.8, 35 cc. 0.02 N \( \text{H}_2\text{O}_2 \), and 4 cc. water. While the solution was cooled, it was saturated with pure CO. In order to avoid mechanical damage of the enzyme, the rate of gas flow was kept low (7 to 22 cc. CO per minute). A catalase
preparation of \( k = 2092 \) was diluted 1:2000.\(^4\) 1 cc. of this solution was added to the substrate-buffer system. After 10 minutes the reaction was stopped by adding 5 cc. 33 per cent \( \text{H}_2\text{SO}_4 \). The solution was withdrawn from the chamber and the amount of remaining hydrogen peroxide determined by iodometric titration. The zero value was determined in a similar mixture by adding the sulfuric acid before the enzyme. Control experiments were conducted with air and nitrogen instead of carbon monoxide. A total of 45 experiments were performed. A few results are given in Table I. Little, if any, inhibition by CO was observed in these experiments. This inhibition was smaller than that produced by flushing the solution with nitrogen. These results, then, lend no support to Califano’s claim that catalase is specifically inhibited by CO. Califano\(^5\) worked under conditions which were less conducive to an inhibition effect than those just described. He used a manometric method. Although he states that oxygen will prevent the inhibition, oxygen accumulated in his vessels during the catalysis, and it was by the amount of evolved oxygen that he measured the progress of the reaction. The temperature (20°) and the substrate concentration (0.17 \( \text{N} \)) in his experiments were much higher than in those here reported. He states that the inhibition was around 35 per cent and the reactivation by light around 15 to 20 per cent. At present no satisfactory explanation can be offered for the discrepancy in the results of Califano and of the writer.

Effect of Fluoride on the Catalase Spectrum

The experiments with oxidizing and reducing agents or with carbon monoxide allow no final decision as to whether catalase contains ferric or ferrous iron. Fluoride has a marked affinity for ferric but not for ferrous iron. It reacts with catalase to form a stable compound with a characteristic absorption spectrum.

The absorption band of the enzyme in the red is centered around 622 m\(\mu\). When neutral catalase solutions are made weakly acid by

\(^{4}\) It is advisable to use ice cold distilled water for the preparation of the diluted enzyme solution. Fresh dilutions must be prepared each day.

\(^{5}\) Professor L. Califano was kind enough to supply the writer with information concerning these details of his experiments.
adding primary potassium phosphate, the spectrum remains unchanged. When neutral sodium fluoride is added to the acid solution, the enzyme band in the red splits into two components. The new bands are better defined and thinner than the original band.

25 cc. of a catalase solution of \( k = 2092 \) were saturated with solid \( KH_2PO_4 \). 1 gm. of pure sodium fluoride was added and the solution was filtered. The solution appeared greenish in incident and orange-red in transmitted light. The long wave bands were measured in 5 cm. layer with the Hilger spectrometer:

\[
\begin{align*}
\text{I. } & 624 - 617; \\
\text{II. } & 604 - 597 \ldots 588 \text{ m\mu.}
\end{align*}
\]

Band I is better defined than band II, but the latter is stronger. Besides these bands the bands of the enzyme at 540 and 500 m\( \mu \) could be seen. The pH of the final mixture was 5.88.

TABLE I

<table>
<thead>
<tr>
<th>Experimental series No.</th>
<th>Atmosphere</th>
<th>Substrate decomposed</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Air</td>
<td>1.89 cc. 0.1 N</td>
<td>Air at rest</td>
</tr>
<tr>
<td>VI</td>
<td>Air</td>
<td>3.93</td>
<td>Air at rest</td>
</tr>
<tr>
<td></td>
<td>Nitrogen</td>
<td>1.9</td>
<td>Flushed with ( \text{N}_2 )</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>3.93</td>
<td>Air at rest</td>
</tr>
<tr>
<td>VII</td>
<td>Air</td>
<td>3.16</td>
<td>Flushed with ( \text{CO} )</td>
</tr>
<tr>
<td></td>
<td>CO</td>
<td>3.65</td>
<td>Air at rest</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>3.53</td>
<td>Flushed with ( \text{CO} )</td>
</tr>
<tr>
<td></td>
<td>CO</td>
<td>3.23</td>
<td>( \text{Air} ) at rest</td>
</tr>
<tr>
<td></td>
<td>CO</td>
<td>3.35</td>
<td>Flushed with ( \text{CO} )</td>
</tr>
<tr>
<td>VIII</td>
<td>Air</td>
<td>2.99</td>
<td>7 cc. ( \text{CO} ) per min.</td>
</tr>
<tr>
<td></td>
<td>CO</td>
<td>2.88</td>
<td>( \text{Air} ) at rest</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>3.05</td>
<td>22 cc. ( \text{CO} ) per min.</td>
</tr>
<tr>
<td>X</td>
<td>Air</td>
<td>5.0</td>
<td>26 cc. ( \text{CO} ) per min.</td>
</tr>
<tr>
<td></td>
<td>CO</td>
<td>4.6</td>
<td>( \text{Air} ) at rest</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>5.3</td>
<td>( \text{CO} ) at rest</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>5.15</td>
<td>620 600</td>
</tr>
</tbody>
</table>
The complex between catalase and fluoride is stable for several days at room temperature.

The reaction of catalase with fluoride is evidence for its constitution as a ferric compound. It also affords a simple means of differentiating the enzyme from methemoglobin. The red band of methemoglobin is not split by fluoride but merely shifted from 630 to 610 m\(\mu\) (24, 25). The exact position of the band of fluormethemoglobin depends on the pH (26).

*Effect of Nitric Oxide on the Catalase Spectrum*

Catalase reacts with NO in neutral solution. The color of the enzyme solution changes from green-brown to red. The NO compound has a characteristic spectrum with two bands in the visible range.

Catalase solution \((k = 2092)\) was placed in a capillary stopper cell. The air was displaced by pure nitrogen. Nitric oxide was prepared in the apparatus described by Warburg (27). The enzyme solution was saturated with NO and protected against oxygen by the capillary stopper. The measurement with the Hilger spectrometer had the following result:

\[
\begin{align*}
\text{I. } & 581 - 572; \\
\text{II. } & 549 - 534 \text{ m}\mu.
\end{align*}
\]

Band I is much stronger and better defined than Band II. The short wave edge of II was difficult to ascertain. There is a shadow at 610 m\(\mu\), but this is only visible with a pocket spectroscope.

The NO-catalase compound has a higher extinction than the free enzyme. 3 to 4 cm. layers of the enzyme solution are required to clearly see the absorption band in the red but the bands of the nitric oxide complex are already distinct in 0.5 cm. layer of thickness. This fact may be used to detect catalase in comparatively dilute solutions. A catalase preparation obtained from pumpkin seedlings by the method of Zeile (2) showed a low activity \((k = 10)\). Consequently the enzyme spectrum, which has been found in similar preparations of higher activity by Zeile, could not be seen directly with a pocket spectroscope in layers up to 50 cm. But after saturation with NO
the solution appeared red in 50 cm. layers and showed the spectrum of the catalase-NO complex given above. The extinction of the NO-complex is also greater than that of the fluoride-catalase compound described in the preceding section. The combination of the enzyme with NO is reversible. By removing the NO from the solution by prolonged flushing with nitrogen, the spectrum of the free enzyme was completely restored. The regenerated enzyme was active. The linkage between the enzyme and nitric oxide may also be dissolved by the substrate. When hydrogen peroxide is added to the solution of NO-catalase complex, the substrate is actively decomposed and the spectrum of the free enzyme is partially restored.

The reaction between catalase and nitric oxide cannot be used to distinguish the enzyme spectrum from that of methemoglobin. The spectrum of NO-methemoglobin is identical with the spectrum of NO-catalase, with the exception of an additional very faint band of the enzyme complex at 610 m\(\mu\). Neither complex is appreciably dissociated by illumination with a 750 c.p. tungsten lamp.

**Effect of Acetylene**

Acetylene does not combine with catalase. When a neutral enzyme solution of \(k = 3410\) was saturated with pure acetylene, the spectrum appeared unchanged. Methemoglobin, at pH 6.9, was likewise found not to combine with acetylene. The same applies to hemoglobin prepared by reduction of methemoglobin with hydrosulfite at pH 6.9. A hemoglobin complex with acetylene has been described but its existence is doubtful (28).

**Effect of Hydroxylamine**

To 9 cc. of a catalase solution of \(k = 7125\) there was added 1 cc. of 20 per cent hydroxylamine hydrochloride solution. When the spectrum of this solution in 2 cm. layer was projected into the pocket spectroscope simultaneously with that of an untreated enzyme solution, no change in the spectrum could be detected within 20 hours. A fine turbidity appeared and settled to form a colorless precipitate. The stability of the enzyme spectrum toward hydroxylamine is of interest in view of the observation that this substance inhibits the activity of the enzyme (29).
Effect of Substrates on the Enzyme Spectrum

The formation of an unstable intermediate in the catalase-mono-ethyl hydrogen peroxide reaction and its absorption spectrum consisting of two bands in the green region have recently been described (3). Attempts to observe a similar intermediate in the catalase-hydrogen peroxide reaction have so far been unsuccessful.

There is no visible change in the spectrum of the enzyme when it is adsorbed on aluminum hydroxide gel or on silicic acid particles (30). It has been shown that in these adsorbates the prosthetic group of the enzyme is free. It is available for the catalysis of hydrogen peroxide and of ethyl hydrogen peroxide. In the latter case the spectrum of the intermediate is observed. Furthermore, cyanide will produce the spectrum of the inactive catalase-HCN complex which has previously been observed in solution (1).

A few experiments were conducted in which the catalase-silicic acid adsorbates were suspended in non-aqueous solvents. It was hoped that the catalysis of hydrogen peroxide by this arrangement would be slowed down to such an extent as to render the spectrum of the hypothetic intermediate in this reaction visible. Acetone, ether, and glycerol were used for suspending the dried enzyme adsorbates. The spectrum of the enzyme appeared unchanged. On addition of ethyl hydrogen peroxide the spectral cycle already described could be observed. But with hydrogen peroxide as the substrate no change in the enzyme spectrum could be detected, although in some cases the rate of the catalysis seemed to be less than in the homogeneous system.

Although these experiments did not have the result desired, they are of interest. They suggest that water as a solvent may not be indispensable for the enzymatic catalysis. In one experiment the dry adsorbate was suspended in anhydrous ether and the hydrogen peroxide-ether solution was likewise dried over sodium sulfate, but the possibility that a small amount of water was still present in the silicic acid gel cannot be excluded.

On the Visibility of the Enzyme Spectrum in Liver Tissue

The absorption band of catalase in the red at 622 µµ (α-band) does not coincide with absorption bands of other normal constituents
of living mammalian tissues. The absorption bands of cytochrome-α and the α-band of Warburg's respiratory enzyme are situated near 600 m. The catalase concentration in the liver of some animals, e.g., horse and rat, is so high that an attempt was made to see the α-band of catalase in these tissues. The main difficulty that presented itself is the high concentration of oxyhemoglobin in the liver. Though the two main absorption bands of the blood pigment are centered at about 540 and 575 m, the latter band will stretch into the red region in high concentrations. Though it was possible to make slices of fresh horse liver of 1 cm. sufficiently transparent for spectroscopic examination by the use of carbon arc lamps (25 amperes), the absorption of the blood pigment made an observation of the enzyme band impracticable. The liver of a rat was therefore freed from blood as much as possible by perfusion with Ringer solution in situ. The whole liver, which had assumed a yellow-pink color, was placed in a 3 cm. absorption cell and examined with a pocket spectroscope in the concentrated beam of a 500 watt projection lantern. The bands of oxyhemoglobin were still visible, but no band of the enzyme in the red region could be seen. The liver was then ground up and placed in a 1 cm. absorption cell. Under these conditions, there was a faint band visible near 630 m. Upon adding neutralized sodium cyanide solution, the band disappeared, and two bands at 600–590 and 560 m appeared instead. To another sample primary potassium phosphate and sodium fluoride were added. The band in the red was shifted to about 610 m. As has been shown above, the catalase-fluoride complex has two bands in the red, one at 620 and the other at 600 m. But in crude liver extracts, where the enzyme spectrum may be observed (1), it was found that upon adding fluoride only the stronger short wave band of the two was visible. It is therefore not impossible that the band observed in liver tissue is the α-band of catalase.

DISCUSSION AND CONCLUSIONS

Catalase is resistant to oxidizing agents; e.g., ferricyanide. It is also resistant to reducing agents; e.g., catalytically activated hydro-

⁶ The writer wishes to thank Dr. Walter E. Hambourger for his assistance in this experiment.
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gen, hydrosulfite, ferrotartrate, cysteine. The hemin group of the enzyme will combine with cyanide, sulfides, nitric oxide, fluoride. It will not combine with carbon monoxide. Catalase is therefore a ferric complex. The stability of the ferric iron in the enzyme toward reducing agents is not due to the structure of the porphyrin with which it is combined. This porphyrin is the protoporphyrin of the blood pigment. In combination with globin (methemoglobin) the ferric iron is readily reduced by the same reagents which have no effect on catalase. The stability of the ferric iron in the enzyme is

| TABLE II |
|---|---|---|
| **Absorption Maxima of Catalase and Derivatives** |

<table>
<thead>
<tr>
<th>Compound</th>
<th>Position of absorption maxima</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>622; 540; 505; 409</td>
<td></td>
</tr>
<tr>
<td>HF-catalase</td>
<td>620; 600; 540; 505</td>
<td></td>
</tr>
<tr>
<td>NO-catalase</td>
<td>576; 541</td>
<td></td>
</tr>
<tr>
<td>HCN-catalase</td>
<td>589; 557</td>
<td>(1)</td>
</tr>
<tr>
<td>H2S-catalase</td>
<td>640; 580</td>
<td>(1)</td>
</tr>
<tr>
<td>C2H2O2H-catalase</td>
<td>570; 534</td>
<td>(3)</td>
</tr>
<tr>
<td>Pyridine hemochromogen, derived from catalase</td>
<td>557; 527</td>
<td>(32)</td>
</tr>
<tr>
<td>CO-pyridine hemochromogen</td>
<td>565; 530</td>
<td></td>
</tr>
<tr>
<td>Hydrazine hemochromogen, derived from catalase</td>
<td>(622); 588; 545</td>
<td></td>
</tr>
<tr>
<td>CO-hydrazine hemochromogen</td>
<td>578; 542</td>
<td></td>
</tr>
</tbody>
</table>

The spectra for which no reference is given have been determined in the present study.

The Soret band in the far violet region has so far only been determined for the unmodified enzyme. It is very probable that the derivatives of the enzyme possess maxima in the same region.

therefore due to the protein component. It may be that the type of hematin-protein linkage in catalase is the reason for this phenomenon. The suggestion of Bersin (31), that sulfur may participate in this linkage, is interesting but, as yet, has no experimental basis.

Hydrazine or pyridine and hydrosulfite convert catalase into hemochromogens containing ferrous iron. But in these hemochromogens the hematin is no longer attached to the protein. This has been replaced by the nitrogenous bases hydrazine and pyridine. Both hemochromogens combine reversibly with carbon monoxide. Photo-
dissociation has only been demonstrated in the case of the pyridine hemochromogen.

The positions of the absorption bands of catalase and its derivatives are listed in Table II.

The main absorption band (Soret's band) of hemin complexes with nitrogenous substances (nitrogen bases, proteins) is situated at the border between the visible and the ultraviolet region of the spectrum. It has now been found that the spectrum of purified liver catalase has a well defined maximum of high extinction in this range, at 409 m\(\mu\). This is further evidence for the hemin nature of the enzyme.

The writer wishes to thank Professor E. C. Dodds for providing working facilities at the Courtauld Institute of Biochemistry, where the greater part of the experiments was carried out.

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BIBLIOGRAPHY

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