OXIDATION OF PYROGALLOL TO PURPUROGALLIN
BY CRystALLINE CATALASE

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It has been recently reported that catalytic quantities of crystalline catalase, in the presence of hydrogen peroxide, can rapidly oxidize a variety of molecules larger than hydrogen peroxide and aliphatic alcohols (1). The qualitative experiments have shown that α-naphthol and p-phenylene-diamine are condensed by oxidative coupling to indophenol purple; p-aminobenzoic acid, sulfathiazole, adrenaline, ephedrine sulfate, and tyrosine were found to be coupled with catechol. Generally, amines and phenols were also found to be oxidized to colored compounds (1).

There is described here a quantitative study concerning the conversion of pyrogallol (the classical peroxidase substrate) to purpurogallin by very small quantities of crystalline catalase. It will be shown that catalase, under definite conditions and at physiological pH, can perform the functions of a peroxidase. Catalase is suitable for the preparation of pure purpurogallin in less than 2 hours.

EXPERIMENTAL

Preparation of Crystalline Catalase

The crystalline cow liver catalase used in these experiments was prepared by the method of Tauber and Petit (2). Frozen cow liver was employed instead of fresh liver. The extraction with distilled water was carried out in a Waring blender. The latter change resulted in a considerable increase in the yield of crystalline catalase. The crystals which separated during dialysis were recrystallized by dissolving in the least volume of 0.01 N sodium hydroxide. Without centrifuging, the solution was at once adjusted to pH 5.8 by the addition of 0.1 N acetic acid. A small quantity of insoluble matter was removed by centrifuging at room temperature. The clear supernatant fluid was placed in a refrigerator at 4°C. Within 3 hours the catalase began to crystallize. When crystallization was complete, the catalase was recrystallized again by this same procedure. Then the crystals were dissolved in sodium chloride-phosphate buffer, 0.2 ionic strength, at pH 7.3 (3). The activity of the enzyme was 34,000 Kat. f. (4). A concentrated stock solution in sodium chloride-
phosphate buffer of pH 7.4 was kept in the refrigerator. Dilute catalase solutions were prepared just before use for each of the following experiments by diluting an aliquot of the stock solution with distilled water. All other solutions were prepared daily. The crystalline catalase used in these experiments was found to be homogeneous by paper electrophoresis at pH 8.6 (Veronal buffer).

**Peroxidation of Pyrogallol by Catalase**

Fig. 1 demonstrates the relationships between the quantity of catalase used and purpurogallin formed as measured colorimetrically. It is seen that a reasonably satisfactory linear relationship is obtained. A correction was made in the colorimeter reading for the slight autoxidation. The purpurogallin values were taken from the calibration curve (Fig. 2) obtained with twice recrystallized purpurogallin. The Klett-Summerson
photoelectric colorimeter with green filter No. 50 (maximal transmission at 500 μ) was employed. Readings were measured against distilled water blanks.

The formation of purpurogallin was studied at pH 6.9, because at this pH autoxidation of pyrogallol, and of its oxidation products, is insignificant. At alkaline pH autoxidation is considerable. At a more acid pH (5.3) purpurogallin formation by catalase-hydrogen peroxidase is nil. Hydrogen-peroxidase decomposition, however, at pH 5.3 is intense. Thus it was necessary to conduct these experiments within these narrow limits.

Preparation of Purpurogallin by Catalase Action

Solutions—(a) 18 gm. of pyrogallol were dissolved in 500 ml. of distilled water and the pH was adjusted to 7.2 with N sodium hydroxide. (b) 120 ml. of 3 per cent hydrogen peroxide solution. (c) 1.5 mg. of twice recrystallized catalase in 15 ml. of sodium chloride-phosphate buffer of pH 7.4.

Procedure—The pyrogallol solution was placed in a 1 liter beaker and mixed with an electrically driven glass stirrer at room temperature. 5 ml. of hydrogen peroxide solution and 5 ml. of catalase solution were added. Stirring was continued for 1 minute. The remaining 115 ml. of hydrogen peroxide were added in 5 to 8 ml. portions within 50 minutes. Brief stirring was applied after each addition of materials. In the first 20 minutes no further catalase solution was added. The pH of the mixture was determined with a Beckman pH meter every few minutes and was maintained between pH 7.0 and 6.3 by the addition of N sodium hydroxide. At the end of the first 20 minutes and during the next 30 minutes 1 ml. portions of catalase solution were added every 3 minutes to the reaction mixture.

At the end of 1 hour the reaction mixture was adjusted to pH 7.0. Then the purpurogallin crystals were filtered by suction through No. 2 Whatman filter paper. The crystals were washed with distilled water and dissolved in boiling 95 per cent ethanol. The solution was filtered and the compound precipitated with 2.5 volumes of distilled water. The purpurogallin was further purified by two more precipitations from ethanol. Hot glacial acetic acid and also anisole are good solvents for purpurogallin (5). Crystals readily form from these solvents at room temperature. The yield of twice recrystallized purpurogallin was 0.6 gm.

Control—In a control of pH 6.9, without catalase, there appeared after 1 hour only a light yellow color. Purpurogallin crystals did not precipitate.

Catalase Does Not Form Purpurogallin at Acid pH—When the pyrogallol solution remained unadjusted at its original pH of 5.3, catalase and hy-
drogen peroxide did not form purpurogallin. There was, however, an intense decomposition of hydrogen peroxide under those conditions. Peroxidase on the other hand does form purpurogallin at pH 5.3 (see the experiment to follow).

**Preparation of Purpurogallin with Crude Peroxidase**

This method was similar to that employed in the catalase procedure. Crude peroxidase was prepared as follows: 75 gm. of grated horseradish roots were extracted for 5 minutes with a 5 per cent acetic acid solution. The mixture was squeezed through cloth toweling and filtered through No. 12 Whatman filter paper. The first 10 ml. of filtrate were refiltered. The pH of the clear filtrate was 3.7. This extract was practically free of catalase, since it did not decompose hydrogen peroxide. 15 ml. of the extract were used for the preparation of purpurogallin at the original pH (5.3) of the pyrogallol solution. The yield of twice recrystallized purpurogallin was 0.31 gm. per 18 gm. of pyrogallol.

**General Properties of Enzymatically Prepared Purpurogallin**

Crystals of Purpurogallin—Microscopic rosettes of purpurogallin are obtained by peroxidation of pyrogallol by crystalline catalase and recrystallization from 40 per cent ethanol. Almost identical crystals were
obtained by recrystallization from hot glacial acetic acid and from hot anisole. When peroxidase was employed as the catalyst, similar crystals were obtained. Occasionally, however, the crystals separated from dilute ethanol in the form of thin microscopic needles. The product obtained both by the action of catalase and by the action of peroxidase melted with decomposition at 256-259°. Haworth and Hobson (5) report the melting point of synthetic purpurogallin at 276° (with decomposition).

Absorption Spectrum—In Fig. 3 is shown the absorption spectrum of purpurogallin prepared by peroxidation of pyrogallol with catalase. The spectra of this product and of the product obtained by using horseradish peroxidase as the catalyst were identical with the spectrum given by the chemically synthesized purpurogallin (5).

DISCUSSION

Catalase has been classified for many years as having the specific function of decomposing the toxic hydrogen peroxide that may accumulate in tissues to water and O₂. Recently, however, Keilin and Hartree (6) made the remarkable observation that a portion of the hydrogen peroxide which forms when certain oxidases aerobically oxidize their substrates is utilized by catalase to oxidize ethanol to acetaldehyde. Oxidation by catalase and hydrogen peroxide proceeded at a slower rate. No evidence is recorded, however, to indicate that catalase can oxidize larger molecules of biological importance or any of the many types of compounds that are oxidized by peroxidase.

In these experiments pyrogallol has been chosen since it is the ideal substrate for peroxidase. The intense orange color of purpurogallin which forms when pyrogallol is oxidized by peroxidase and hydrogen peroxide may readily be measured colorimetrically.

In the reaction, catalase or peroxidase oxidizes pyrogallol (I) to its orthoquinone (II) which after further dehydrogenation and loss of carbon is coupled with 1 molecule of pyrogallol to form the insoluble end-product, purpurogallin (III).

This new structure of purpurogallin has been recently proposed by several groups of English investigators (7, 8) and has been conclusively proved by Caunt and his associates (9).

These and our earlier experiments (1) demonstrate that at physiological
pH and under the conditions described (for which typical examples were given) catalase can oxidize the usual peroxidase substrates. Many complex compounds appear in nature which can be derived from simple phenols and phenol derivatives by oxidative coupling. A striking example is the conversion of pyrogallol to purpurogallin. Thus the fact that catalase can function as a peroxidase appears to be a much more important rôle than is the destruction of hydrogen peroxide by the enzyme.

SUMMARY

Experimental evidence has been brought forward which shows that crystalline catalase can function as a peroxidase, provided the pH is not much more acid than 6.9. The reaction product of pyrogallol, purpurogallin, has been isolated and compared with the product obtained by the action of horseradish peroxidase. Both reaction products were found to be identical.

Our experiments suggest that catalase can play an important rôle in biology by its function as a peroxidase, catalyzing the formation of large molecules by coupled oxidation.

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