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The Nature of Uterine Phenol-Activated Oxidation of DPNH[1]

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Purification of uterine phenol-activated DPNH oxidase activity over a 50–100-fold range fails to separate this activity from uterine peroxidase. Phenol can be recovered unchanged from enzymic reaction mixtures after oxidation of DPNH. Uterine peroxidase reaction mixtures require phenols for oxidation of scopoletin. Omission of scopoletin from such mixtures results in enzyme destruction. Such enzyme destruction fails to occur if the phenol is also omitted from the reaction mixture. Ce^{+++} can replace Mn^{++} in the uterine DPNH oxidase system. When H_2O_2 is added to such reaction mixtures, the metal is no longer required for DPNH oxidation. When Ce^{+++} catalyzes the phenol-activated DPNH oxidase reaction, the metal undergoes oxidation to Ce^{++++}. Ce^{++++} is capable of the non-enzymic oxidation of DPNH, and addition of phenol enhances this reaction. Mixtures of DPNH and Ce^{+++} in citrate buffer generate H_2O_2 by a non-enzymic reaction. The evidence for the identity of phenol-activated DPNH oxidase and uterine peroxidase is reviewed. The role of certain non-enzymic steps in the mechanism is described.

INTRODUCTION

A phenol-activated DPNH oxidase is present in rat uterus (1, 2). Oophorectomy results in disappearance of enzymic activity which is promptly restored by the administration of estrogen (3). Administration of estrogen also stimulates the activity of uterine peroxidase (4). Lactoperoxidase and horse-radish peroxidase show DPNH oxidase activity in the presence of phenols. However, the identity of uterine peroxidase and phenol-activated DPNH oxidase was left open because reproducible enzymic peroxidation of DPNH could not be demonstrated (1). This report presents evidence for the identity of these enzymic activities. The enzyme has been partially purified, the role of the metal studied, and two non-enzymic steps in the oxidation of DPNH delineated.

EXPERIMENTAL PROCEDURE

Uterine peroxidase was assayed fluorometrically by a modification of the method of Andrae (5). The reaction mixture consisted of 0.02 *M* phosphate buffer pH 6.8, scopoletin (Mann Laboratories) 3.05×10^{-6} *M*, dichlorophenol (DCP)[4] 1.3×10^{-4} *M*, and hydrogen peroxide 4.3×10^{-5} *M* in a total volume of 1.0 ml. DPNH and scopoletin can both be used as fluorescent peroxidase substrates, but the latter was used because of its enhanced sensitivity and stability. The reaction was started by the addition of 0.1 ml. enzyme. The reaction rate was observed in an Aminco-Bowman spectrophotofluorometer. The excitation wavelength for scopoletin was 340 mμ, and its emission wavelength was 450 mμ. In order to avoid the induction period seen in an occasional sample, the

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[4] The abbreviations used are: DCP, 2,4-dichlorophenol; DEAE-cellulose, *N*,*N*-diethylaminoethylcellulose.

assay was done by determination of the time for the galvanometer needle to pass between two arbitrary points on the scale. Assays were performed in that range of enzyme concentration in which the reciprocal of this time was linear with concentration. Omission of enzyme, scopoletin, or DCP resulted in complete absence of reaction. Addition of Mn^{++} to the reaction mixture did not enhance the rate.

Phenol-activated DPNH oxidase activity was determined by a similar fluorometric technique in a reaction mixture consisting of 0.02 *M* phosphate buffer pH 6.8, DPNH 8.2×10^{-5} *M*, DCP 2.9×10^{-4} *M*, and $MnCl_2$ 1.4×10^{-4} *M* in a volume of 1.0 ml. Reaction was started by the addition of 0.1 ml. enzyme. Activity was measured in the enzyme concentration range with a linear relationship between rate and enzyme. Excitation and fluorescence wavelengths were the same for scopoletin and DPNH measurement.

The method of Andrae (5) was adapted to detect H_2O_2 in uterine oxidase reaction mixtures. To 1.0 ml. of a mixture of phosphate buffer 0.02 *M* pH 6.8, scopoletin 9.6×10^{-9} *M*, DCP 1.02×10^{-7} *M*, and 0.5 μg. horse-radish peroxidase, were added various amount of H_2O_2. The decrease in fluorescence is proportional to peroxide concentration as shown in Fig. 1. The addition of 1.0 μg./ml. catalase completely inhibited the oxidation of scopoletin. Addition of Ce^{++++} to the scopoletin mixture to a final concentration of 1.5×10^{-6} *M* caused no fall in fluorescence.

Preparation of the Uterine Enzyme

Mature Sprague-Dawley rats were injected subcutaneously with 100 μg. daily of estradiol-17β in propylene glycol for 3 days. A 20% uterine homogenate was prepared in 0.005 *M* phosphate buffer pH 7.0 containing 0.001 *M* Versene. The homogenates were centrifuged for 10 min. at $600 \times g$ at 2°. The supernatant was then frozen for 30 min. in a Dry Ice–butyl cellosolve mixture in order to render the activity soluble. The thawed liquid was centrifuged at $30{,}000 \times g$ for 10 min., and the supernatant was filtered through gauze and dialyzed for 30 min. against 0.005 *M* phosphate buffer pH 7.0 which contained 0.001 *M* Versene and 0.15 *M* NaCl. A column of DEAE-cellulose, 3.0×11.0 cm., was prepared by washing with 0.1 *M* HCl containing 1 *M* NaCl, water, 0.1 *M* NaOH, water and finally equilibrated with 0.005 *M* phosphate buffer pH 7.0 containing 0.15 *M* NaCl and 0.001 *M* Versene.

The dialyzed, solubilized enzyme was applied to the column and eluted by a gradient technique involving two mixing chambers and a reservoir. The first mixing chamber contained 140 ml. of 0.005 *M* phosphate buffer pH 7.0 with 0.15 *M* NaCl, the second, 140 ml. buffer with 0.5 *M* NaCl. The reservoir contained buffer with 1.0 *M* NaCl. Chromatography was conducted at 2° and fractions were collected every 3 min. Eluates were assayed for phenol-activated DPNH oxidase, peroxidase, DPNH cytochrome c reductase, and protein. DPNH cytochrome c reductase was assayed by the method of Mahler (6) and protein by the method of Lowry (7).

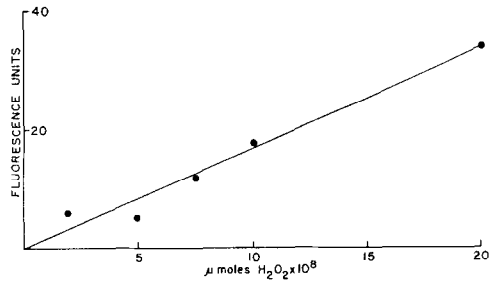

Fig. 1. Standard curve for 10^{-8} *M* H_2O_2. The reaction mixture is as described in the text. The Aminco-Bowman spectrophotofluorometer was adjusted to maximum sensitivity, and the decrease in fluorescence produced by H_2O_2 after 5.0 min. was noted.

Figure 2 shows that a 64-fold purification did not separate the peroxidase and oxidase activities. The discrepancies in the ratio of peroxidase to oxidase are thought to be due to technical factors in the assay. The lack of separation of the two activities has been noted in four experiments involving purifications of 50–100-fold. A small amount of peroxidase and phenol-activated oxidase activity was eluted with hemoglobin. Recrystallized hemoglobin was also noted to have these catalytic properties. DPNH cytochrome c reductase was well separated (tubes 35–45) from peroxidase activity. It is worthy of note that the total oxidase and peroxidase activity was greater in the purified than in the crude preparation, being 128 and 115%, respectively. This discrepancy was noted in several experiments. The purified uterine enzyme is much more stable than the crude preparation and can be stored for several months at −20° without appreciable loss of activity. No substantial purification of uterine enzyme was achieved by rechromatography on DEAE-cellulose, zonal electrophoresis, gel adsorption, protamine treatment, or salt precipitation.

RESULTS

The Role of the Activating Phenol

Previous studies (1) have indicated that under certain conditions uterine DPNH

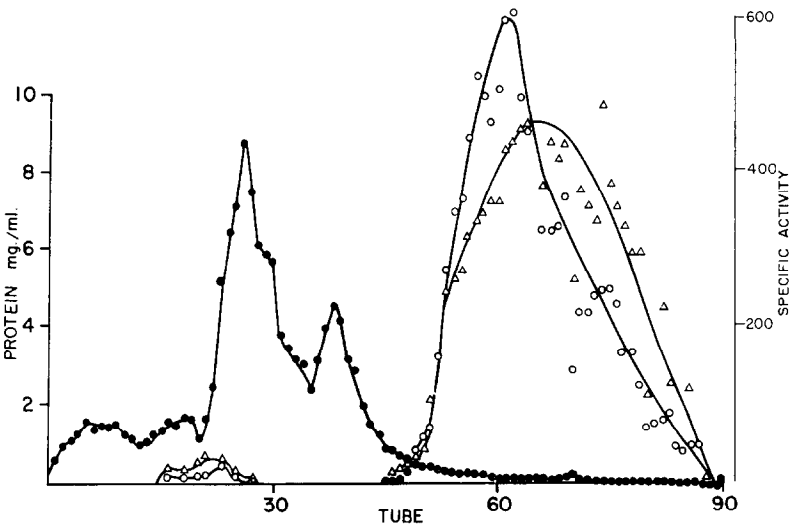

FIG. 2. Purification of uterine peroxidase. Protein mg./ml. ●—●—, peroxidase specific activity —○—○—; oxidase specific activity —△—△—.

oxidase can be activated by phenol as well as by DCP. In the phenol-activated enzymic oxidation of DPNH, phenol was not chemically altered in the presence of excess substrate. This was demonstrated in two experiments using isotopic phenol.

Experiment 1

The incubation mixture consisted of 0.19 *M* citrate buffer pH 6.3, 0.36 μmole DPNH, 0.50 μmole Ce^{+++}, 0.10 ml. uterine enzyme containing 20 μg. protein, and 0.55 μmole phenol-1-C^{14} (270,000 d./min.) in a total volume of 3.0 ml. After 50 min. all the DPNH had been oxidized, and 225.5 mg. phenol in 10 ml. water was added to the reaction mixture. The phenol was extracted with 4 × 25 ml. ethyl ether. The ether then was extracted with 4 × 25 ml. of 1 *N* NaOH. The alkaline fraction was acidified with 3 *N* sulfuric acid and extracted with 4 × 25 ml. ether. The ether extract was dried over anhydrous sodium sulfate and then evaporated. The remaining phenol was dissolved in 2.5 ml. pyridine to which was added 0.5 g. 3,5-dinitrobenzoyl chloride. The mixture was refluxed for 30 min. and then diluted with 100 ml. methylene chloride. This solution was washed 4 × 25 ml. of 1 *N* $NaHCO_3$, 2 × 25 ml. water, 4 × 25 ml. of 1 *N* HCl, and 2 × 25 ml. water, and dried over anhydrous sodium sulfate. After evaporating the solvent, the product was recrystallized from 95% ethanol.

The recrystallized phenol derivative was chromatographed on a silica gel column. A solution of methylene chloride–ligroine was used for elution, the methylene chloride concentration being increased during the chromatography. The residue from each evaporated fraction was dissolved in acetone, and the optical density of an aliquot was determined spectrophotometrically at 340 mμ by comparison with a standard. A similar aliquot was taken for counting. The remaining derivative from the tubes containing the highest specific activity was pooled and further recrystallized from 95% ethanol and from benzene to constant specific activity. Samples were assayed for radioactivity in the Packard Liquid Scintillation Counter. Quenching was corrected for by the use of an internal standard. The specific activities and the recoveries obtained are shown in Table I. All of the radioactivity in the phenol added to the incubation mixture is accounted for by the derivative.

Experiment 2

The reaction mixture contained 20 ml. of 0.02 *M* pH 6.8 phosphate buffer, 0.24 ml. of 1.5 × 10^{-3} *M* $MnCl_2$, 0.30 ml. of 0.01 *M* DPNH, 0.67 ml. of 0.01 *M* phenol, 0.10 ml. of phenol-1-C^{14} equivalent to 1 μc., and 0.5 ml. of 5% uterine homogenate. The optical density decreased from 0.85 to 0.48 corresponding to 43.5% DPNH oxidized. The phenol was worked up in a way similar to that described above except that toluene was used in place of ethyl ether. An eight-plate countercurrent separation of the phenolic fraction was carried out utilizing 10 ml. each of toluene-saturated water and water-saturated toluene. Appropriate aliquots were taken for analysis of radioactivity and of phenol. Twenty milligrams of phenol was added as carrier. Phenol was estimated by the Lowry

TABLE I

SPECIFIC ACTIVITY OF PHENYL 3,5-DINITROBENZOATE

	Disintegrations/ min./mg.	Total d./min. accounted for
Initial radioactivity in reaction mixture		270,000
First recrystallization 95% EtOH	280	194,000
Column	370	255,000
2nd recrystallization 95% EtOH	403	278,000
3rd recrystallization benzene	398	274,000
4th recrystallization benzene	390	270,000
Mother liquor of 2nd recrystallization	405	280,000

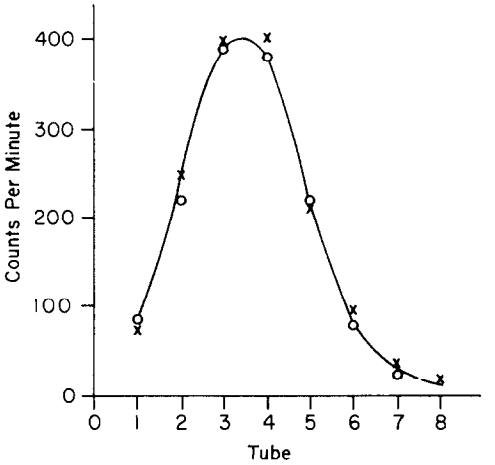

FIG. 3. Distribution of phenol-1-C^{14} after enzymic reaction. Phenol determined colorimetrically ×——× and by radioactivity —○—○. Maximum for radioactivity and color plotted to coincide.

reaction for protein (7). In Fig. 3 the maxima for phenol and radioactivity are made to coincide. It can be seen that there is excellent correspondence between the colorimetric determinations and the radioactivity in this separation. Of the total radioactivity in the reaction mixture, 89% was accounted for as phenol by this procedure.

ACTIVATION OF OTHER PEROXIDASES BY DCP

Figure 4 shows that under the conditions of the peroxidase assay there was almost no oxidation of scopoletin by either lactoperoxidase[5] or horse-radish peroxidase in the absence of DCP. Uterine peroxidase shares the property of phenol activation with well established peroxidases.

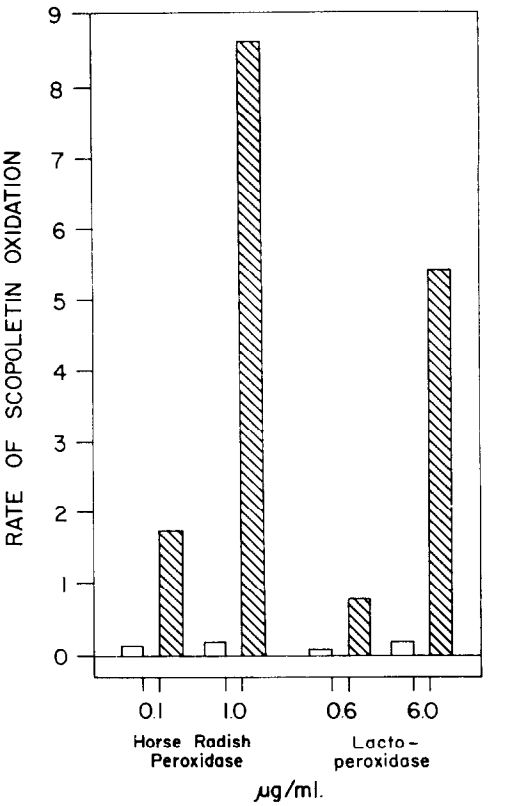

FIG. 4. The efficiency of DCP as an activator for peroxidase reaction. The scopoletin reaction mixture is as described in text, peroxidase concentration as indicated in figure. The oxidation of scopoletin by horse-radish and lactoperoxidases proceeds in the absence of DCP □ but is greatly enhanced by its presence ■. Rate expressed as the decrease in fluorescence in 1.0 min. after addition of enzyme.

EFFECT OF OMISSION OF REACTANTS FROM THE PEROXIDASE SYSTEM

Omission of any constituent of the peroxidase reaction mixture outlined above prevented scopoletin oxidation. Figure 5 shows the rates of reaction resulting from the addition of the omitted reactant after incubation for 1 hr. at 25°.

[5] We are indebted to Dr. Martin Morrison of the University of Rochester Medical School for this compound.

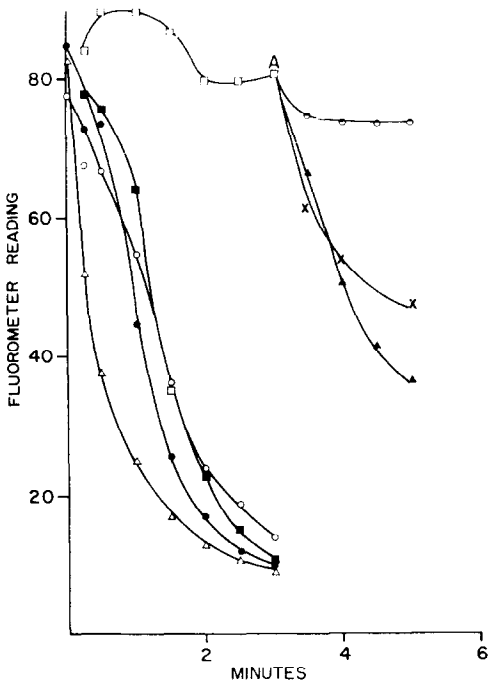

FIG. 5. The effect of delayed addition to the scopoletin system. The incubation mixture and method was the same as that described in the text for peroxidase assay. Complete system —○—○; reaction started by the addition of H_2O_2 after 60 min. preincubation of the other reactants ■—■—, the reaction started by the addition of DCP after 60 min. preincubation of the other reactants ●——●; the reaction started by the addition of scopoletin and DCP after 60 min. preincubation of the reactants —△—△—; the reaction started by the addition of scopoletin after 60 min. preincubation of the other reactants —□—□. At *A* the reaction after addition of more enzyme —▲—▲; the reaction after addition of more H_2O_2 ◓——◓; the reaction after addition of more enzyme and H_2O_2.

The reconstitution with H_2O_2, DCP, and scopoletin plus DCP to a preincubated incomplete reaction mixture gave a rate of reaction comparable to that of a complete mixture. However omission of scopoletin resulted in inactivation of the enzyme. Oxidation of scopoletin then occurred on the addition of more enzyme.

A reactive moiety presumably was generated in the presence of DCP and H_2O_2 which inactivated the enzyme unless substrate was present. This reaction has been interpreted in the discussion as an enzymic generation of phenoxy radicals by peroxide.

THE ROLE OF THE METAL

Although a number of metal ions failed to replace Mn^{++} in the phenol-activated oxidation of DPNH by uterine enzyme (1), the report by Mudd and Burris (8) that peroxidase-catalyzed aerobic oxidation of DPNH could proceed in the presence of DCP and Ce^{+++} prompted the trial of Ce^{+++} in the uterine system. Either Ce^{+++} or Ce^{++++} could replace Mn^{++} if citrate buffer pH 6.3 was used. Cerium did not catalyze the reaction as well in either Tris or phosphate buffer.

LACK OF METAL REQUIREMENT WHEN HYDROGEN PEROXIDE IS ADDED

Table II shows that if hydrogen peroxide was added to the uterine enzymic system, Mn^{++} was unnecessary but DCP was still required for the oxidation of DPNH. If H_2O_2 was not present, Mn^{++} as well as DCP was required to effect oxidation of DPNH. Identical results were obtained when Ce^{+++} was substituted for Mn^{++} in a reaction mixture containing citrate buffer.

THE OXIDATION OF Ce^{+++} DURING THE UTERINE OXIDASE REACTION

Mudd and Burris (8) demonstrated that Ce^{+++} is oxidized by H_2O_2 in the presence of horse-radish peroxidase and resor-

TABLE II

THE REQUIREMENTS OF THE DPNH OXIDASE SYSTEM

The complete incubation mixture was the oxidase mixture described in the text to which 4.3×10^{-5} *M* H_2O_2 was added. The reaction was initiated by the addition of 0.05% uterine homogenate, and incubation was for 30 min. at 30°C. Results are expressed as the percentage of DPNH oxidized.

	Per cent oxidized
Complete system	100
Complete system minus Mn^{++}	100
Complete system minus H_2O_2	67
Complete system minus H_2O_2 and Mn^{++}	0
Complete system minus DCP	0

cinol. Results entirely similar to theirs were observed with purified uterine enzyme and DCP. The formation of Ce^{++++} could be conveniently followed either at 272 mμ or at 400 mμ. The latter wavelength was preferred in mixtures containing DPNH since only Ce^{++++} absorbed. Figure 6 shows that the absorption at 400 mμ increased during the oxidation of DPNH in the presence of uterine peroxidase, DCP, and Ce^{+++}. There was a much smaller increase in absorption at 400 mμ in the absence of Ce^{+++}, and this increase did not occur when DCP was omitted from the reaction mixture. The color generated in the absence of Ce^{+++} was easily extracted with toluene, while the extra color generated in the presence of Ce^{+++} was not extracted. The addition of HNO_3 to a final concentration of 8.0 *M* permitted ether extraction of the Ce^{++++} in accordance with the observation of Wylie (9). The generation of Ce^{++++} from Ce^{+++} in the oxidase reaction mixture could also be followed by the starch iodide reaction. Aliquots of 0.1 ml. were removed during DPNH oxidation. These aliquots were treated with 0.025 μg. catalase in 25 μl. water. After 5 min. 0.05 ml. of 0.1% potassium iodide, 0.02 ml. of 1% starch, and 0.02 ml. of 6 *N* H_2SO_4 were added. The color was developed for 30 min., the volume made up to 1.0 ml., and the absorption at 600 mμ determined. Preliminary tests showed that the blue color from hydrogen peroxide was entirely prevented by catalase treatment. However, catalase had no effect on the color produced by Ce^{++++}. No blue color was produced by the reaction mixture at zero time, but during the reaction 8–12% of the Ce^{+++} was oxidized to the higher valence. When Ce^{+++} was omitted from the oxidase mixture no blue color was formed.

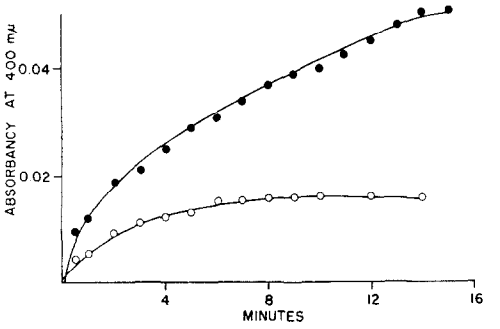

FIG. 6. Increase in absorbancy at 400 mμ. Curve —○—○ shows the increase in absorbancy when uterine peroxidase is added to a system containing citrate buffer 0.19 *M* pH 6.2, DCP 0.5 μmole, H_2O_2 0.5 μmole in 3.0 ml. Curve —●—● shows the increase when uterine peroxidase, 50 μg. is added to a system containing citrate buffer 0.19 *M* pH 6.2, DCP 0.5 μmole, H_2O_2 0.5 μmole, Ce^{+++} 0.5 μmole in 3.0 ml. The small optical density changes involved were amplified with the aid of a Gilford density recorder operating at a full-scale deflection of 0.20 optical density unit.

NON-ENZYMIC OXIDATION OF DPNH BY Ce^{++++}

Ce^{++++} is generated during enzymic oxidation of DPNH by mixtures of uterine peroxidase, DCP, and Ce^{+++} in citrate buffer. The effect of DCP on the non-enzymic oxidation of DPNH by Ce^{++++} was then studied. Figure 7 shows the enhanced rate of non-enyzmic oxidation of DPNH by Ce^{++++} in citrate buffer pH 6.3 when DCP was present. The reaction was carried out in an atmosphere of nitrogen to avoid a small amount of autoxidation of DPNH in the presence of metal and phenol. DPNH plus Ce^{++++} utilization was determined at 340 mμ where both absorbed, Ce^{++++} utilization at 400 mμ where only Ce^{++++} absorbed. The phenol-activated non-enzymic reaction followed the expected consumption of two moles of Ce^{++++} for each equivalent of DPNH oxidized. The figure demonstrates that the rate, but not the final extent of reaction is influenced by the phenol. The product of DPNH oxidation by Ce^{++++} was shown to be DPN by the use of alcohol dehydrogenase under conditions previously described (1). In the absence of DPNH there was very slow reduction of Ce^{++++} by the phenol. The non-enzymic phenol effect was not noted in Tris or phosphate buffer. In an incubation mixture identical to that used in Fig. 7 except that Ce^{+++} was substituted for Ce^{++++}, there was no reaction in comparable phenol concentration and time. When the concentration of DCP was increased to 0.01 *M*, 5×10^{-4} *M* Ce^{+++}

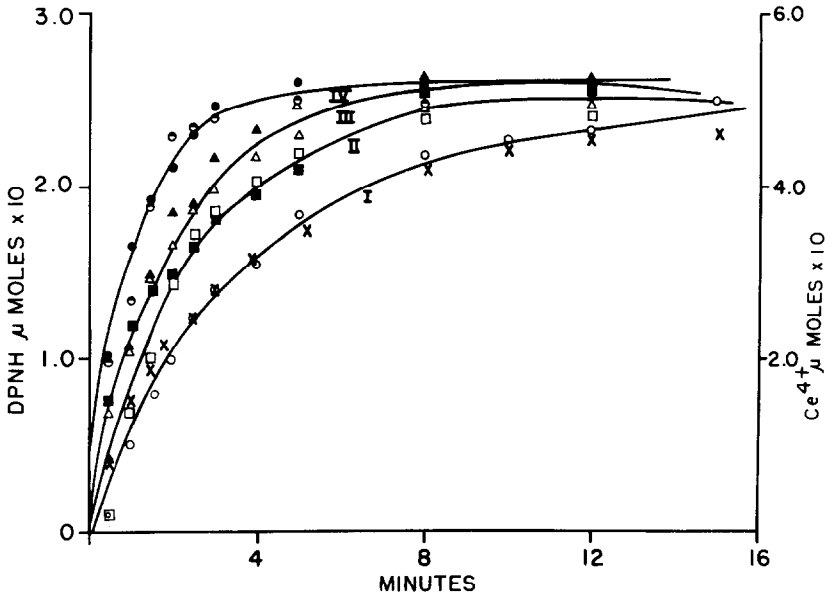

FIG. 7. Non-enzymic DCP-catalyzed consumption of Ce^{++++} and DPNH. The incubation mixture consisted of 0.19 *M* citrate buffer pH 6.2, 0.33 μmole DPNH, and DCP as indicated, in a volume of 3.0 ml. at 29°C. The reaction was started by the addition of 0.5 μmole $Ce(SO_4)_2$ in a volume of 5 μl. Curve 1 shows the oxidation of DPNH —○—○— by Ce^{++++} —×—× in the absence of DCP. Curve 2 shows the oxidation of DPNH ■——■ by Ce^{++++} —□—□ in the presence of 0.25 μ mole DCP. Curve 3 shows the oxidation of DPNH —▲—▲ by Ce^{++++} —△—△ in the presence of 0.5 μmole DCP. Curve 4 shows the oxidation of DPNH —●—● by Ce^{++++} — ◓ — in the presence of 1.0 μmole DCP.

catalyzed the aerobic oxidation of DPNH in citrate buffer. This latter reaction is under further study. When uterine peroxidase was added to the reaction mixtures illustrated in Fig. 7, containing Ce^{++++}, the DCP-catalyzed oxidation of DPNH was many times faster than that obtained in the illustrated non-enzymic reaction. The addition of 0.01–1.0 μmole H_2O_2 to the non-enzymic reaction illustrated in Fig. 7 did not alter the rate of DPNH oxidation either in the presence or in the absence of phenol. The addition of catalase (Mann Laboratories) 100 μg./ml. to the non-enzymic incubation mixture illustrated in Fig. 7 did not affect the initial rate. Under these conditions the phenol-activated enzymic reaction catalyzed by Ce^{+++} was inhibited. 2,4,6-Trichlorophenol was as effective as DCP in the non-enzymic reaction. As has been previously shown, it is also as effective as DCP in the enzymic reaction.

Effect of Mn^{++} and Ce^{+++} on Hydrogen Peroxide Production

The observation that hydrogen peroxide could be omitted from uterine peroxidase incubation mixtures if Mn^{++} (or Ce^{+++}) were added suggested that these metals were concerned with generation of hydrogen peroxide. A non-enzymic generation of H_2O_2 dependent on these metals was found. It was observed that the addition of 10^{-4} *M* Ce^{+++} to 10^{-4} *M* DPNH in citrate buffer at pH 6.3 promptly resulted in mixtures that gave a positive starch iodide test. The prior addition of catalase to such mixtures prevented the positive starch test. These observations caused us to suspect the accumulation of H_2O_2 in such mixtures. The modification of the method of Andrae described above was employed to determine the level of H_2O_2 produced. Varying aliquots of the non-enzymic peroxide generating system were added to 0.10 vol. of 1 *N*

HCl. Appropriate aliquots were then added to the scopoletin peroxidase reaction mixture, described for peroxide analysis under the *Methods* section, and the hydrogen peroxide content was determined fluorometrically. Specificity of high order was achieved by the omission of peroxidase, or by the addition of catalase to the analytical incubation mixture. Both enzymic maneuvers completely prevented the oxidation of scopoletin by hydrogen peroxide. Table III shows that up to 10% of the added DPNH was autoxidized to effect production of H_2O_2. Under aerobic conditions maximal concentrations were achieved with Ce^{+++} in 1 min. No hydrogen peroxide was formed under anaerobic conditions. DCP did not appear to influence this reaction of DPNH, Ce^{+++}, and oxygen. H_2O_2 has also been detected in mixtures containing Mn^{++} and DPNH in phosphate buffer pH 6.8, and this work is under further study.

An oxidase reaction mixture consisting of phosphate buffer 0.02 *M* pH 6.8, $MnCl_2$ 1.2×10^{-5} *M*, DCP 8.3×10^{-5} *M*, 8×10^{-5} *M* DPNH, and 100 μg. of purified uterine peroxidase in a final volume of 3.5 ml. was incubated at 26.5°C., and 0.20-ml. aliquots were removed at appropriate times and added to 20 μl. of 1 *N* HCl to destroy DPNH. Appropriate aliquots were added to the scopoletin assay mixture for the estimation of H_2O_2, outlined under *Methods*, and the decrease in fluorescence was proportional to the volume added. Neither oxidase reaction mixture prior to the addition of uterine enzyme nor enzyme alone showed any trace of hydrogen peroxide. Figure 8 shows the H_2O_2 concentration found at various stages of the reaction. The concentration of H_2O_2 in the oxidase incubation mixture rose to a maximum of somewhat less than 10^{-8} *M* when about half of the DPNH was consumed, and then declined. No decrease in fluorescence was observed when horse-radish peroxidase was omitted from the assay mixture, and addition of catalase to a final concentration of 1.0 μg./ml. completely inhibited the oxidation of scopoletin.

TABLE III

NONENZYMIC PRODUCTION OF HYDROGEN PEROXIDE

Nonenzymic production of H_2O_2 upon incubation of the indicated amount of DPNH with 0.10 μmole Ce^{+++} in 1.0 ml. of 0.2 *M* citrate buffer pH 6.3. Reaction is complete within 1 min.

DPNH	H_2O_2
µmoles	*µmoles*
1.3	0.05
0.58	0.025
0.11	0.015
0.059	0.005
0.008	0.0003

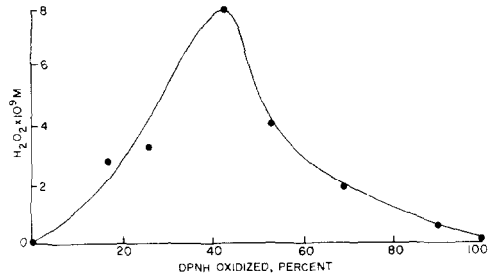

FIG. 8. Hydrogen peroxide production during an oxidase reaction. Twenty-five-microliter samples throughout the oxidase reaction were assayed as described, and the decrease in fluorescence was noted.

DISCUSSION

The demonstration of hydrogen peroxide in the DPNH oxidase reaction mixture removes the distinction between uterine oxidase and peroxidase. The failure to separate phenol-activated DPNH oxidase from uterine peroxidase over a 50–100-fold purification is consistent with the identity of the two enzymic activities. The resistance of the chromatographed material to further purification is unexplained, and further efforts in this direction continue. The replacement of the Mn^{++} or Ce^{+++} requirement for the oxidase system by hydrogen peroxide also supports this identity.

The over-all oxidase and peroxidase reactions are discussed below with respect to the following reactions:

$$DPNH + O_2 + H^+ \xrightarrow{Ce^{+++}} DPN^+ + H_2O_2 \quad (1)$$

$$H_2O_2 + 2ROH \rightarrow 2RO\cdot + 2H_2O \quad (2)$$

$$H^+ + Ce^{+++} + RO\cdot \rightleftharpoons Ce^{++++} + ROH \quad (3)$$

$$2RO\cdot + DPNH + H^+ \rightarrow DPN^+ + 2ROH \quad (4)$$

where ROH represents the phenol and RO· the phenoxy radical. Reaction (1) has been demonstrated to occur non-enzymically. The initial rate of H_2O_2 production in this reaction is rapid, and presumably H_2O_2 generated by such a non-enzymic process can be utilized by the peroxidase. Evidence for the non-enzymic reaction (1) includes the demonstration of H_2O_2 production by the starch iodide reaction and the enzymic peroxidation of scopoletin. Hydrogen peroxide was not found if the reaction was allowed to occur anaerobically. The concomitant formation of DPN could be demonstrated by means of alcohol dehydrogenase. It was not possible to prove stoichiometric formation of DPN because of the small amount of peroxide present. No explanation is available for the failure of the reaction to go to completion. The control of peroxide formation by this process is under further study. Presumably the non-enzymic generation of H_2O_2 from DPNH is pertinent to the observations of Klebanoff (10) that peroxidase oxidation of DPNH in the presence of phenol and the absence of added peroxide permits the oxidation of such substrates as epinephrine. Reaction (2), the enzymic production of free radicals from the phenol, has been suggested by Akazawa and Conn (11) and Williams-Ashman *et al.* (12). The inactivation of uterine enzyme by the peroxidase assay mixture when scopoletin is omitted but not when either scopoletin + DCP, or DCP alone is omitted from the complete reaction mixture, indicates that a very reactive substance is generated from the phenol. This substance is not a quinone, since 2,4,6-trichlorophenol also serves as a phenolic catalyst. The complete regeneration of the phenol from the enzymic reaction mixtures is compatible with the role of a free radical like RO· in the presumably non-enzymic reaction (4). Considerable effort to isolate radical-scavenging products such as diphenyl phenols or ethers was completely unrewarding. The isolation of such products would provide stronger evidence for reaction (2). Reaction (3) in the forward direction is consistent with the enzymic formation of Ce^{++++} observed during the oxidase reaction, presumably by a mechanism analogous to the oxidation of Mn^{++} by horse-radish peroxidase in the presence of phenols and H_2O_2 as observed by Kenten and Mann (13). Mudd and Burris also observed that mixtures of horse-radish peroxidase, phenol, and hydrogen peroxide could oxidize Ce^{+++} (8). Reaction (3) in the backward direction serves to explain the observed phenol catalysis of DPNH oxidation by Ce^{++++} in the absence of enzyme. The reaction generates phenoxy radicals which can then oxidize DPNH anaerobically. Reaction (4) is consistent with the quantitative regeneration of phenol observed, the rapid oxidation of DPNH to DPN proven by alcohol dehydrogenase for non-enzymic oxidation in this paper, and for enzymic oxidation in prior work (1). The free radical oxidation of DPNH would presumably be non-enzymic.

The above sequence suggests a possible mechanism for the peroxidase reaction and serves to emphasize the role of non-enzymic steps in this reaction.

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