

THE OXIDATIVE INACTIVATION OF POISON IVY ALLERGENS BY PEROXIDASE*

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INTRODUCTION

The oxidative inactivation of poison ivy allergens and related skin irritants by tyrosinase and laccase has been previously reported (1, 2, 3, 4). Both these oxidases are copper proteins and utilize molecular oxygen in the oxidation of substrates. An entirely different type of oxidase is the iron heme enzyme, peroxidase, which utilizes hydrogen peroxide in the oxidation of organic molecules. Despite this difference in properties many substrates (e.g. guaiacol, catechol, cresol, tyrosine, adrenalin) are oxidized by both peroxidase and tyrosinase. It, therefore, seemed advisable to investigate the possibility that the poison ivy allergens might be oxidatively inactivated by peroxidase as well as by tyrosinase and laccase.

EXPERIMENTAL

Peroxidase preparations

All peroxidase preparations were made from horse-radish according to the method of Elliott (5). Most of the preparations were kindly supplied by Dr. Kolmer of Eli Lilly and Co. and had a peroxidase activity of about 40 P.Z. units/mgm. (6). Commercial peroxidase (Delta Chemical Works) is also quite satisfactory for this work. In a typical experiment 0.3 mgm. peroxidase was used. Control experiments using boiled peroxidase were always run simultaneously with the experimental solutions, because it was found that the hydrogen peroxide, even in the absence of peroxidase, produced some oxidation of the poison ivy allergen. While a control containing boiled enzyme was ideal for spectroscopic studies, it was not suitable for investigations (manometric or titrimetric) in which residual peroxide was measured at the end of the reaction. This was because the peroxidase was never completely free of some catalase activity (7). When residual peroxide was to be measured, the allergens were omitted from the control solution and active peroxidase was used.

Measurement of residual peroxide

The oxidation of the poison ivy allergens by hydrogen peroxide catalyzed by peroxidase can be followed by measuring the disappearance of peroxide from the system, or by studying directly the oxidation of the allergens. For the former method both manometric and titrimetric technics for H_2O_2 determinations were used, while for the latter method spectroscopic studies and skin tests were employed.

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The manometric technic was complex and less satisfactory for many reasons than the other methods. Nevertheless, the manometric experiments showed qualitative evidence for utilization of peroxide by crude preparations of poison ivy allergens. The manometric experiments were performed at 37°C with the Barcroft differential manometers at a shaking rate of 85 strokes/min. In the experimental cup (15 ml. capacity) were placed 0.1 ml. 0.25% H_2O_2 , 0.1 ml. 10% poison ivy extract, 0.1 ml. peroxidase (0.3 mg.), 1 ml. M/20 phosphate buffer, pH 7.3, and in the side-arm 1 ml. 2% suspension MnO_2 .* The reaction was allowed to proceed for 90 min., then the oxygen was liberated from the residual peroxide by tipping the MnO_2 suspension into the main chamber from the side-arm. The experiment was run simultaneously in another manometer in duplicate except that the poison ivy allergen was omitted from the system. The difference in the amount of oxygen liberated in the two manometers represents the amount of peroxide utilized in the oxidation of the poison ivy extract. Positive results indicating oxidation by peroxidase plus H_2O_2 were obtained with commercial poison ivy allergens and with the crude extract (kiurushi) of the Japanese lac tree. In a typical experiment using Mulford poison ivy allergen the comparison of the experiment and control manometers indicated that H_2O_2 equivalent to 160 μl . oxygen had been utilized in the oxidation of the poison ivy extract.

A much more satisfactory method of measuring H_2O_2 in the system was to titrate the peroxide with 0.005 N sodium thiosulfate using starch as an indicator of the free iodine liberated. This method was difficult when the poison ivy preparation was so pigmented as to partially obscure the blue starch-iodine color. Titration studies were carried out as follows: Poison ivy extract, hydrogen peroxide, peroxidase and buffer were mixed in test tubes and allowed to incubate at 37°C for varying periods of time. Controls omitting the allergens were incubated simultaneously. At appropriate intervals the contents of an experimental and control test tube were poured into separate Erlenmeyer flasks, each containing 5 ml. of 2 N sulfuric acid. Ten ml. of 10% potassium iodide and 1 drop of 1% ammonium molybdate were added to each flask. The contents were mixed and after 3 minutes the iodine set free by the peroxide was titrated with 0.005 N sodium thiosulfate, using 0.5 ml. 1% soluble starch as an indicator. Any difference between the amounts of thiosulfate used in the control and the experimental flasks was considered an indication of hydrogen peroxide utilization.

In a typical experiment the system contained 0.2 ml. 10% Mulford poison ivy allergens, 0.2 ml. 0.2% H_2O_2 , 0.3 ml. peroxidase (1 mg./ml.) and 0.1 M phosphate buffer to bring the volume to 5 ml. Titration of these 5 ml. tubes was performed at successive time intervals. Results of such a typical experiment are presented in Table I, from which it is apparent that the peroxide is rapidly utilized in the oxidation of the poison ivy allergens catalyzed by peroxidase, but in the absence of the allergens (control tube) the peroxide is fairly stable.

Spectroscopic studies

Peroxidase plus H_2O_2 oxidized poison ivy allergens to highly colored end products which are also produced, but at a very much slower rate, by the peroxide alone. The colored solution absorbs throughout the visible spectrum, but absorbs maximally in the violet region. Results obtained with Mulford poison ivy allergens and with the pure poison ivy compound, n-pentadecylcatechol,† are presented in Fig. 1. Using color change as a measure of the extent of oxidation, the kinetics of the action of peroxidase plus H_2O_2 on the poison ivy allergens can be

* Catalase was often used instead of the MnO_2 .

† Kindly furnished by Dr. Howard S. Mason, National Institute of Health.

TABLE I

Oxidation of Mulford poison ivy allergens by peroxidase plus H_2O_2

The reaction was followed by titrating the residual H_2O_2 with 0.005 N sodium thio-sulfate. See text for details.

TIME	CONTROL	EXPERIMENTAL
min.		
0	4.95 ml. $Na_2S_2O_3$	4.92 ml. $Na_2S_2O_3$
1	4.85 ml. $Na_2S_2O_3$	3.20 ml. $Na_2S_2O_3$
2	4.85 ml. $Na_2S_2O_3$	1.95 ml. $Na_2S_2O_3$
3	5.00 ml. $Na_2S_2O_3$	1.36 ml. $Na_2S_2O_3$
4	5.00 ml. $Na_2S_2O_3$	0.70 ml. $Na_2S_2O_3$
5	4.80 ml. $Na_2S_2O_3$	0.40 ml. $Na_2S_2O_3$

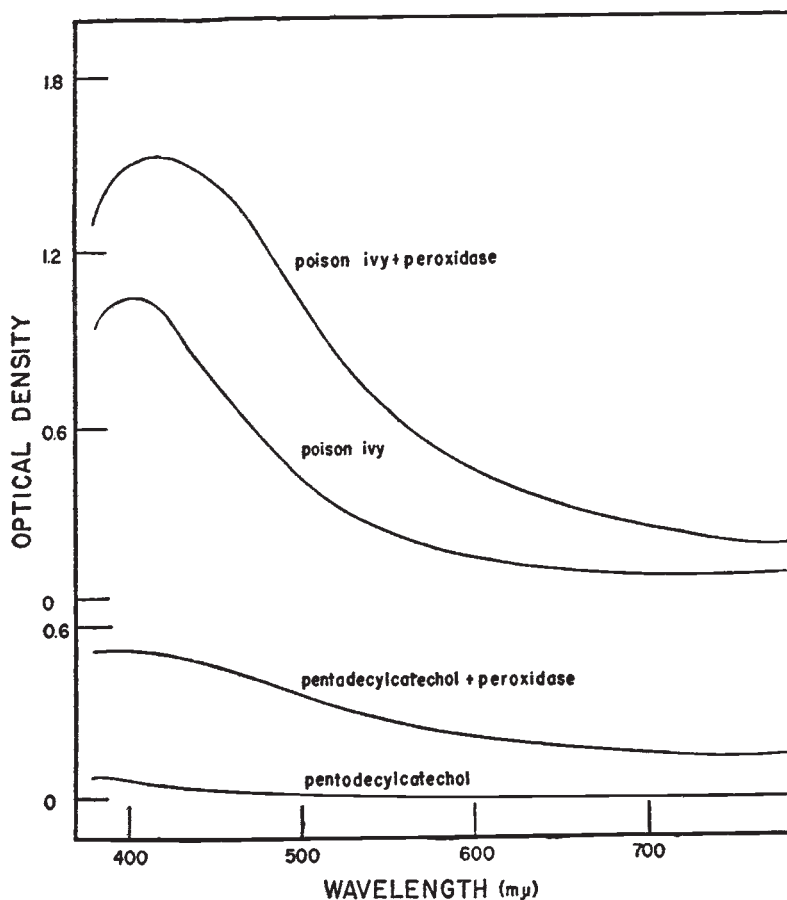


FIG. 1. Change in visible absorption spectra of poison ivy allergens after treatment for 24 hours at 37° with peroxidase. Boiled peroxidase was used in the control experiments.

Upper curves: The digest contained 0.1 ml. 10% Mulford poison ivy allergens, 0.1 ml. 0.25% H_2O_2 , 0.1 ml. (0.3 mg.) peroxidase plus 5 ml. M/20 phosphate buffer, pH 7.3.

Lower curves: The digest contained 10 ml. 50% ethanol, 0.5 mg. n-pentadecylcatechol, 0.1 ml. 0.25% H_2O_2 , plus 0.1 ml. (0.3 mgm) peroxidase; final pH, 6.0.

followed by measuring the optical density of the digest as a function of the duration of the reaction (Fig. 2). In comparison with similar studies made using tyrosinase or laccase (2, 4) the oxidation of poison ivy allergens by peroxidase occurs much more rapidly.

Since phenol, catechol and their derivatives absorb in the ultraviolet, the poison ivy allergens which are all phenolic derivatives should also show strong

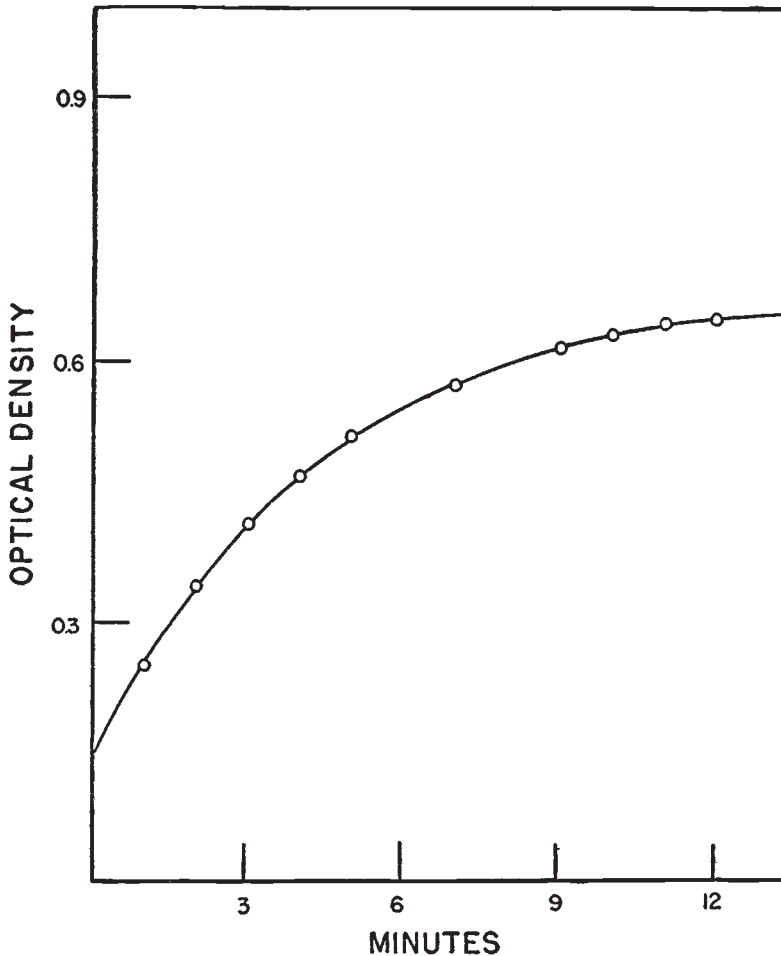


FIG. 2. Oxidation of the poison ivy allergens as measured by the increase in absorption of light of 600 $m\mu$ wavelength. The digest contained 10 ml. 0.1 M phosphate buffer pH 7.23, 0.2 ml. 10% Mulford poison ivy, 0.2 ml. 0.2% H_2O_2 and 0.015 mgm. peroxidase.

ultraviolet absorption (8). The crude poison ivy preparations were quite unsuitable for this study, since they do not form clear solutions and contain numerous impurities which also absorb in the ultraviolet. All studies in the ultraviolet were performed using the synthetic poison ivy allergen, 3-n-pentadecylcatechol.†

The reaction was carried out at pH 6.0 (0.01 M phosphate buffer) in 60% absolute alcohol. The alcohol was required to dissolve the pentadecylcatechol and was found, in independent experiments, not to inactivate the peroxidase under the conditions of the experiment. The final solution contained 0.075 mg. penta-

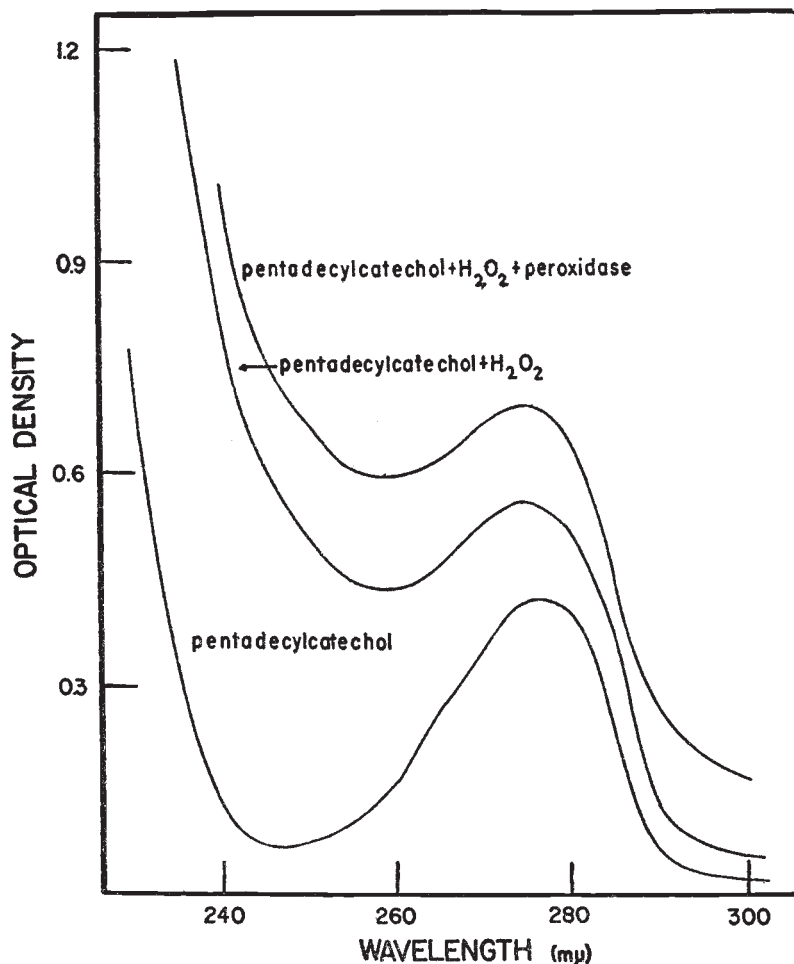


FIG. 3. Absorption in the ultraviolet after 5 hours incubation at 37° of a solution of 3-n-pentadecylcatechol (0.075 mg./ml.) dissolved in buffer-alcohol (0.01 M phosphate, pH 6.0, 60% ethanol).

Top curve: pentadecylcatechol plus 0.15 mgm. H₂O₂/ml. plus 0.05 mgm. peroxidase/ml.

Middle curve: pentadecylcatechol plus 0.15 mgm. H₂O₂/ml.

Lowest curve: pentadecylcatechol.

decylcatechol, 0.06 mg. peroxidase and 0.01 mg. H₂O₂ per ml. At the same time, a control experiment was run in which the enzyme was omitted from the system. Since H₂O₂ itself absorbs strongly in the ultraviolet (9), the absorption spectra were measured before the addition of peroxide and again at the end of the reac-

tion after all the peroxide had disappeared from the system. Typical results in an experiment run for 5 hours at 37° are shown in Fig. 3. Although there is appreciable oxidation of the pentadecylcatechol by the peroxide alone, the oxidation by peroxide plus peroxidase is much more marked.

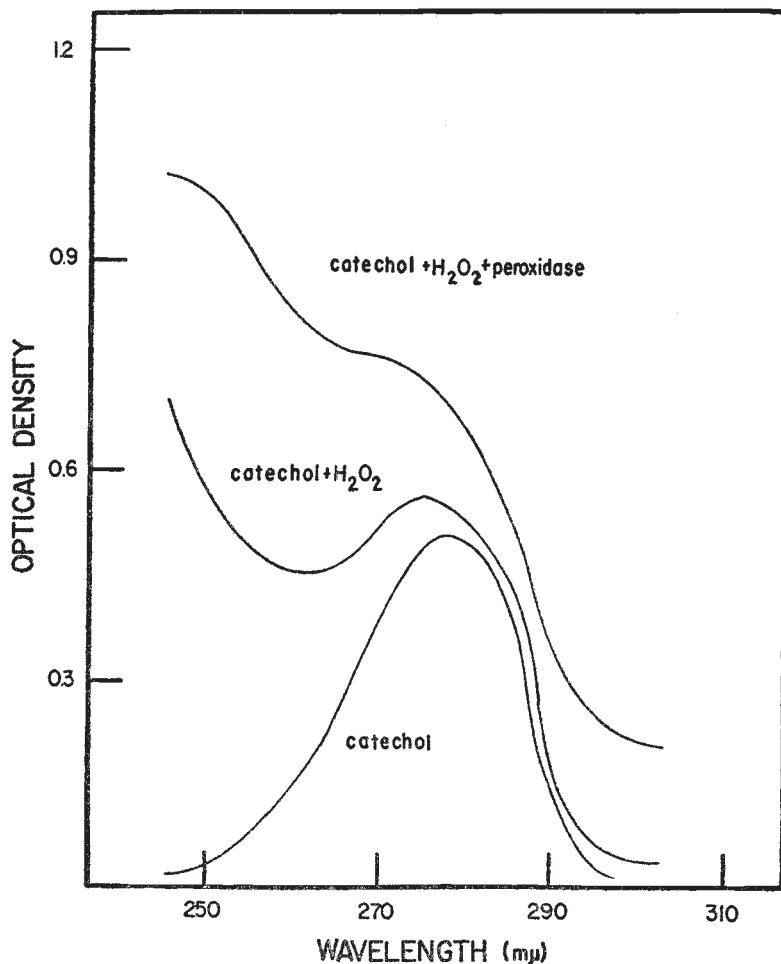


Fig. 4. Absorption in the ultraviolet after 2.5 hrs. incubation at 37° of a solution of catechol (0.0188 mg./ml.) dissolved in buffer-alcohol (0.01 M acetate, pH 5.0, 60% ethanol).

Top curve: catechol plus 0.15 mgm. H₂O₂/ml. plus 0.05 mgm. peroxidase/ml.

Middle curve: catechol plus 0.15 mgm. H₂O₂/ml.

Lowest curve: catechol.

In order to interpret these ultraviolet investigations on the action of peroxidase on pentadecylcatechol, comparable studies have been made using catechol as a substrate. This experiment was run in 60% alcohol at pH 5.0 using 0.0188 mg. catechol, 0.015 mgm. H₂O₂ and 0.05 mgm. peroxidase per ml. The reaction was allowed to proceed for 2.5 hours at 37° before the absorption spectra were

measured. The changes induced by peroxide alone, and by peroxide plus peroxidase, although more extensive, are quite comparable to those induced in a comparable system using pentadecylcatechol as a substrate.

The general close similarity of results on the oxidation of pentadecylcatechol and catechol by peroxidase to those with tyrosinase (2, 3, 10, 11, 12, 13) lends strong support to an interpretation of peroxidase oxidation of poison ivy allergens based upon the conversion of phenolic groups of the allergens to quinones, followed by an oxidative polymerization. Evidence for such polymer formation in the case of peroxidase oxidation of pentadecylcatechol is obtained from the fact that the colored oxidation products are not dialyzable through a cellophane dialysis tube. Polymerization is also evidenced by the fact that black granules gradually form in the pink solution obtained in the oxidation of pentadecylcatechol by peroxidase.

Studies on biological activity

Since the manometric, titrimetric, visible and ultraviolet spectroscopy studies all indicated a marked oxidation of poison ivy allergens by peroxidase plus peroxide, it seemed advisable to determine whether or not these oxidized poison ivy allergens had undergone any change in their ability to produce dermatitis. All the tests on skin irritation of human subjects were done using the oxidized crude poison ivy allergens, since the solutions of pentadecylcatechol used in the peroxidase studies were not sufficiently concentrated* to produce dermatitis.

The technic used was the same as that of Sizer and Prokesch (2). The skin of the forearm or leg was prepared by washing with soap and water, and the hair was usually removed with an electric shaver. Before applying to the skin, the poison ivy allergen (previously oxidized by peroxidase plus H_2O_2) was taken up in ether. A glass ring 1.75 cm. in diameter and 0.5 cm. high was held firmly against the skin. An aliquot (usually 0.1 ml.) of the ether extract was then spread on the area of skin inside the ring. The ring was removed after the solvent had evaporated. To avoid spreading by contact with clothing, the areas were covered with "band-aids." The allergen was left on the skin for 24 hours, or until dermatitis was apparent, at which time the residual allergen was removed with soap solution or alcohol and ether. Experimental and control areas were always treated identically. Attempts were made to adjust the dosage used to just above the threshold level in order to avoid a severe dermatitis, and since, at this level, differences between control and experimental dermatitis areas are most apparent.

The diagnosis of the comparative dermatitis of the control and experimental areas was always made by at least two observers. Appraisal of the severity of the dermatitis was based on the degree of edema and erythema and intensity of pruritus. The diagnosis was usually repeated on three successive days. A decision was then made as to whether the dermatitis of the experimental area (poison ivy allergen previously oxidized with peroxidase) is more or less severe than the control area (allergen not treated with peroxidase). The results of the

* For the same reason pentadecylcatechol was not suitable for manometric or titrimetric studies.

skin tests can be summarized by the statement that, in all cases, the previous oxidation of the allergens with peroxidase has resulted in an appreciable decrease in the dermatitis-producing properties of the poison ivy preparation. In most experiments, however, the peroxidase only partially destroyed the irritant properties of the allergen.

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SUMMARY

Crude preparations of the poison ivy allergens and pentadecylcatechol are rapidly oxidized and inactivated by horse-radish peroxidase plus hydrogen peroxide. Evidence for this oxidation was obtained from studies on the utilization of peroxide in the system as indicated by manometric and titrimetric technics. Evidence was also provided by the marked change in absorption of light in the visible and ultraviolet regions of the spectrum, and by the decrease in the ability of the poison ivy allergens to produce dermatitis of human skin after they had been oxidized with peroxidase plus peroxide. Some oxidation of the poison ivy allergens by peroxide alone was noted.

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