The Oxidation of Tyramine, Tyrosine, and Related Compounds by Peroxidase*

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Horse-radish peroxidase in the presence of hydrogen peroxide has been reported to act directly upon tyrosine, tryptophan, and cystine, but no reaction products have thus far been identified (1). Tyrosine is of special interest since it has been suggested (1, 2) that the inactivation of certain biologically active proteins by peroxidase depends in part upon oxidation of tyrosyl groups present in the protein. In this investigation, the first reaction product of tyramine, horse-radish peroxidase, and hydrogen peroxide has been shown to be 2,2′-dihydroxy-5,5′-bis(3-ethylamino) diphenyl (1), referred to as dityramine. Tyrosine undergoes a similar oxidation to form ditryrosine as indicated by chromatographic, ultraviolet absorption and fluorescence data. Both products are chemically analogous to di-p-cresol (2,2′-di-hydroxy-5,5′-dimethyldiphenyl) isolated by Westerfeld and Lowe (3) from the reaction of p-cresol, peroxidase, and hydrogen peroxide. Prolonged action by peroxidase upon tyrosine or tyrosine yields further reaction products, including a possible triphenyl derivative, and a brown amorphous pigment as an end product.

Tyramine → (Compounds I, II, and III) → Pigment

Other phenolic substances related to tyramine have been found also to undergo oxidation to diphenyl derivatives.

EXPERIMENTAL

A 1.0-gm. sample of tyramine hydrochloride was dissolved in 910 ml. of water, and 50 ml. of 0.1 per cent hydrogen peroxide were added, followed by 40 gm. of horse-radish peroxidase (Lilly, activity = 72 P.Z. units) dissolved in 40 ml. of water. The pH was adjusted to 9.2 with 6 N NaOH, and the mixture was incubated for 8 to 12 hours at 37°. A brown pigment formed, 200 mg., which was filtered. Chromatograms of the mixture and of the products of a similar reaction with tyrosine demonstrated the presence of new ninhydrin-positive fluorescent substances (Fig. 1). The slightly acidified, filtered reaction mixture was evaporated nearly to dryness, treated with 1 gm. of Darco G-60 charcoal, refiltered, and brought to dryness in intimate mixture with 3 gm. of Whatman No. 1 cellulose powder. Then 200 mg. of this mixture were placed on a cellulose powder column (41 × 1 cm.) and eluted with a solvent consisting of n-butanol, formic acid (88 per cent), and water, (80:10:10). Fractions examined at 275 rnp with a Beckman DU spectrophotometer showed a separation pattern similar to the paper chromatogram (Fig. 1).

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The fractions containing Compound I were combined, and each 100-ml. aliquot was shaken twice with 25 ml. of water. Dissolved butanol was removed by shaking the aqueous extract twice with 25 ml. of ether. The residual ether was evaporated under reduced pressure and the final aqueous extract was lyophilized or kept for the preparation of chemical derivatives.

Identification of Compound I—The lyophilized extract yielded a white powder (Compound I-HCl) which rapidly turned brown in air and was very hygroscopic. Compound I-HCl was found to be similar to tyramine hydrochloride in solubility, and also gave positive ninhydrin and Millon’s tests. The melting point was 210-235° with decomposition, and the fluorescence under ultraviolet light was blue. The ultraviolet absorption spectrum (Fig. 2) showed a maximum at 284 mg. (acid) and 316 mg. (basic) with molecular extinction coefficients of 4510 and 5790, respectively.

Compound I-HCl was purified by dissolving it in absolute ethanol, precipitating it with ether, and repeating this procedure several times.

Analysis of Compound I-HCl (C9H9NOCl)
Calculated: C 55.66, H 6.42, N 8.11, Cl 20.54
Found: C 55.42, H 6.69, N 7.85, Cl 20.26

Tetrabenzoate (Compound IV)—The O,O′,N,N′-tetrabenzoic derivative (Fig. 3) of ditryrosine was prepared by the Schotten-Baumann method by shaking 100 ml. of aqueous extract with 0.5 ml. of benzoyl chloride and 11 ml. of 2 N NaOH in the cold for 10 minutes, and then allowing the mixture to stand for 15 minutes at room temperature. For purification, the crude substance was passed through an alumina column with ethyl acetate as solvent, and the ultraviolet absorbing material was recovered. Recrystallization from 75 per cent ethanol produced long, white, translucent needles; m.p. 196.5° (corrected). The tetrabenzoate was insoluble in water, moderately soluble in methanol and ethanol, and readily soluble in acetone, ethyl acetate, chloroform, and ether.

Analysis of tetrabenzoate (C19H14N2O6)
Calculated: C 76.73, H 5.27, N 4.67, mol. wt. 689
Found: C 76.77, H 5.46, N 4.03, mol. wt. 785 (Rast)

Methyl Sulfate Derivative (Compound V)—To 50 ml. of aqueous extract of Compound I cooled to 0°, 6 gm. of NaOH in 10 ml. of water were added followed by the dropwise addition of 12 ml. of dimethyl sulfate during a period of 8 to 10 hours with constant stirring. The reaction mixture was gently shaken with 650 mg. of Darco G-00 charcoal and allowed to stand 10 minutes. The
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FIG. 1. Descending paper chromatograms of peroxidase-tyramine (A) and peroxidase-tyrosine (B) systems. Rf values are given for tyramine (Ta) and tyrosine (To) spots. Solvents employed: for (1), n-butanol-formic acid (88 per cent) water, (75: 10:15); for (2), n butanol acetic acid water, (55:15:30). All spots were ninhydrin positive, and except for Ta and To which absorbed ultraviolet light, all showed blue fluorescence under ultraviolet light.

FIG. 2. The ultraviolet absorption spectra of Compound II in water as a function of pH.

FIG. 3. Derivatives of Compound I and degradation leading to proof of structure. Bz = benzoyl group.

charcoal was centrifuged, gently washed three times with 10 ml. of water, and then eluted with four 10-ml. portions of absolute ethanol. The ethanol was evaporated to dryness and the derivative recrystallized from hot absolute ethanol. Hygroscopic, translucent, cigar-shaped crystals were obtained; m.p. 225° (corrected). The solubility characteristics were found to be similar to those of tyramine hydrochloride. The methyl sulfate derivative (Fig. 3) fluoresced under ultraviolet light. The ultraviolet absorption maximum was 283 mp and the molecular extinction coefficient was 6070.

Analysis of methyl sulfate derivative (C_{26}H_{41}N_{8}S_{10}O_{18})
Calculated: C 51.30, H 7.29, N 4.60, S 10.53, CH$_3$O 10.20
Found: C 51.16, H 7.52, N 4.27, S 10.35, CH$_3$O 11.03

Oxidation of Methyl Sulfate Derivative and Methylation—The methyl sulfate derivative (Compound V) was subjected to oxidation by refluxing 25 mg. of Compound V with 100 mg. of KMnO$_4$ in 2 ml. of water together with 0.5 ml. of 0.1 N NaOH for 1.5 hours, and then adding a few milligrams of NaHSO$_3$ to decolorize the solution. The resulting dicarboxylic acid (Compound VI) (see Fig. 3) was purified by repeated solution in 0.1 N NaOH and precipitation with a few drops of 6 N H$_2$SO$_4$. Upon drying, a few milligrams of a white amorphous compound were obtained; m.p. 300° (decomposition point). The acid was dissolved in a few drops of methanol and methylated by addition of diazomethane in ether (10 mg. per ml.) until evolution of nitrogen gas ceased. The ether was evaporated, and the derivative recrystallized several times from the ethanol-water mixture until white, rhombic crystals were obtained; m.p. 173° (corrected). The compound, 2,2'-dimethoxy-5,5'-dicarboxymethoxydiphenyl, (VII) (see Fig. 3), was insoluble in water, readily soluble in alcohol, acetone and ether, and fluoresced under ultraviolet light. Syn-
thesis of Compound VII has been reported by Sugii (4) and Gilman et al. (5).

Analysis of methyl ester (Compound VII) \((\text{C}_{11} \text{H}_{18} \text{O}_6)\)

Calculated: C 65.45, H 5.49

Found: C 65.89, H 5.49

Separation of Compound II-HCl and Tyramine Pigment—Compound II-HCl was isolated in the same manner as described for Compound I-HCl. Compound II-HCl was a water soluble, hygroscopic compound unstable in air and in alkaline solution. The melting point was indeterminate with decomposition. Compound II-HCl produced a yellow color with Millon’s reagent and was ninhydrin-positive. It fluoresced under ultraviolet light, its ultraviolet absorption spectrum (Fig. 4) was similar to that of Compound I. No crystalline benzoyl derivatives were obtained in several attempts. On the other hand, it was possible to methylate Compound II by the procedure used with Compound I. The few milligrams of product were purified by solution in ethanol followed by precipitation with ether. The methylated derivative is characterized by a strong absorption maximum at 283 \(\text{m}_{\text{p}}\) and a minimum at 260 \(\text{m}_{\text{p}}\). Elementary analyses of this compound, although consistent with a terphenyl structure for Compound II, are inconclusive because the sample was contaminated with salt. From the properties described, it is possible that Compound II may have a terphenyl structure analogous to the tri-\(p\)-cresol compound described by Westerfeld and Lowe (3) in the reaction of peroxidase with \(p\)-cresol.

The tyramine-peroxidase reaction also produced 200 mg. of a brown pigment which precipitated irreversibly in the \(\text{pH}\) range 8.5 to 10.5. After hydrolysis of the pigment with 6 N HCl, chromatography revealed that the hydrolysate contained Compounds I and II as indicated by \(R_F\) values and fluorescence data. The pigment was not homogeneous as was revealed by analyses of two different samples.

Sample a: C 59.17, H 6.32, N 7.32, O 27.19

Sample b: C 65.52, H 5.95, N 8.68, O 19.85

Tyramine: C 70.04, H 8.02, N 10.21, O 11.73

The increased oxygen content as compared with tyramine indicated that aerobic oxidation occurs during the polymerization, since more oxygen was present in the pigment than could be accounted for by the hydrogen peroxide supplied initially. The nitrogen lost was not detectable as free ammonia. The high proportion of oxygen in the pigment apparently results from a nonenzymatic autoxidation which occurs during the polymerization. This view is supported by the observation that dihydroxyphenylalanine, free from peroxidase and peroxide, undergoes a rapid autoxidation to a brown pigment when exposed to air. The pigment produced in the tyramine-peroxidase system seemed to be composed of monomers similar to Compounds I and II, as well as more highly oxidized substances, all of which were tightly bound into a nonhomogeneous polymer.

Action of Peroxidase on Compounds Similar to Tyrosine—To determine the general nature and specificity of diphenyl formation by peroxidase, various substrates related to tyrosine were used. The reaction conditions described earlier were used with a 10-fold increase in peroxidase level. After incubation, the mixtures were chromatographed and the major products examined for fluorescence (Table I). With the exception of diiodotyrosine and methyl salicylate, all substrates with free phenolic groups reacted to yield fluorescent products, suggesting diphenyl formation. In all cases, the major products moved more slowly than the original substrates on the chromatograms.

Dihydric phenols follow a different pathway of oxidation with peroxidase. \(o\)-Quinones are formed which undergo polymerization (6).

The tyrosyl groups in bovine fibrinogen, insulin, and pepsin were found to be unreactive with external tyrosine or tyramine in the presence of peroxidase and hydrogen peroxide, nor could one tyrosyl group form a diphenyl linkage with another tyrosyl group in the protein.

**DISCUSSION**

Studies by Pummerer et al. (7), of the oxidation of phenols with various inorganic electron-abstracting agents showed that inter-
mediate free radicals were involved in the reaction. Free radical formation has long been associated with the formation of diphenyl compounds (8). The mode of peroxidase-dityramine formation which is suggested in Fig. 5 is in agreement with the Pummerer and Rieche (9) reaction sequence for the formation of di-β-naphthol from the β-naphthoxy radical in aqueous solution. Chance's and Ferguson's (10) interpretation of the peroxidase reaction mechanism also requires the presence of a free substrate radical.

The finding that peroxidase oxidizes tyramine and tyrosine to diphenyl compounds, with the likelihood that free radical intermediates are important, lends support to Johnson's and Tewkesbury's (11) hypothesis of the formation of thyroxine from diiodotyrosine. A recent hypothesis concerning the biosynthesis of morphine alkaloids requires an enzyme capable of forming free radicals from phenolic precursors (12). In view of the widespread distribution of peroxidase in plants, it is of interest to consider whether magnolol, 2,2'-dihydroxy-5,5'-diallyldiphenyl, isolated from the bark of Magnolia officinalis (4), is produced by peroxidase action. Dityrosine formation in blood is a possibility since Knox (13) has shown that myeloperoxidase can utilize the hydrogen peroxide produced by flavoproteins in the presence of catalase. The possible role of peroxidase in the oxidation of certain metabolites (especially tyrosine and tryptophan) has been pointed out by Gross (14) and Saunders (15).

**SUMMARY**

Dityramine hydrochloride, 2,2'-dihydroxy-5,5'-bis(β-ethylamino) diphenyl hydrochloride, was isolated as the chief product of the reaction of horse-radish peroxidase and hydrogen peroxide with tyramine. A second compound which was isolated showed characteristics which suggested that it might be the terphenyl derivative, trityramine hydrochloride. A brown pigment, which separated from the reaction mixture, was shown to contain dityramine and more extensively oxidized and polymerized derivatives, and to be nonhomogeneous. Tyrosine undergoes a similar oxidation to dityrosine and oxidized polymers, in the presence of peroxidase. Peroxidase oxidizes a variety of monophenolic compounds, but does not form diphenyl linkages in situ with the tyrosyl groups of proteins. Dihydric phenols are converted by peroxidase to quinones, which then undergo oxidative polymerization.

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