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THE PEROXIDASE-CATALYZED OXIDATION OF TYROSINE

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SUMMARY

The horseradish peroxidase- and lactoperoxidase-(donor: H_2O_2 oxidoreductase, EC 1.11.1.7) catalyzed oxidation of tyrosine has been studied. Kinetics of the oxidation catalyzed by these enzymes were followed both spectrophotometrically and fluorometrically. Oxidation of tyrosine by both enzymes has a pH optimum at 8.2.

Most interesting was the finding that both enzymes demonstrate stereospecificity in the oxidative coupling reaction for tyrosine isomers. Lactoperoxidase couples the L-isomer more readily than the D-isomer, in contrast horseradish peroxidase oxidizes the D-isomer more readily. The apparent second order rate constants for lactoperoxidase catalyzed oxidation are $1.03 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ for L-tyrosine and $6.89 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ for D-tyrosine. The values for horseradish peroxidase are $7.53 \cdot 10^2$ $M^{-1} \cdot s^{-1}$ for L-tyrosine and $1.55 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ for D-tyrosine.

The products of these enzyme-catalyzed reactions were characterized by chromatographic, spectral and fluorimetric analysis. The product formed under the initial rate conditions was the tyrosine dimer linked in *o,o*-biphenyl linkage. Extensive oxidation results in the production of trityrosine, thyronine and insoluble yellow to brown colored products.

INTRODUCTION

The peroxidases (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) will catalyze the oxidation of a large number of substrates[1]. The compounds oxidized *via* such reactions are usually termed electron donors. The peroxidases have been considered a uniform group of enzymes with a low degree of specificity for the electron donor. Recent work, however, has shown that lactoperoxidase differs from horseradish peroxidase not only in its structure[2], but also in the reactions which these two enzymes will catalyze[3–5]. For example, although the literature previously indicated that both enzymes will catalyze iodination, in fact, only lactoperoxidase does. Both enzymes will catalyze the oxidation of iodide to iodine. The iodine reacts spontaneously with phenolic compounds to yield iodinated compounds. Lactoperoxidase, however, catalyzes the direct iodination of tyrosine not *via* I_2 while horseradish peroxidase catalyzes the oxidation of iodide and the iodination is the result of the reaction between I_2 and the phenol.

Thus, these peroxidases are neither identical with respect to their structure nor the reactions they will catalyze.

Among the potential electron donors in mammalian systems, probably the most important is tyrosine or its derivatives. Peroxidase-catalyzed oxidation of tyrosine residues may be involved in the biosynthesis of thyroxine, melanin and the cross linkage of proteins. An analysis of the products of horseradish peroxidase-catalyzed oxidation of tyrosine showed that the chief product is the tyrosine dimer, dityrosine in *o,o*-biphenyl linkage[6,7]. Other investigations[7–13] of horseradish peroxidase-catalyzed reactions of tyrosine have also been primarily concerned with the isolation and identification of the products of the reaction.

It was the objective in the present investigation to study the overall kinetics of the oxidation of tyrosine catalyzed by horseradish peroxidase and to investigate the products and kinetics of the reaction catalyzed by lactoperoxidase. In the course of these studies the stereospecific oxidation of tyrosine has been noted for the first time.

EXPERIMENTAL PROCEDURE

Lactoperoxidase was isolated and purified as previously reported[14,15], and the concentration was determined at 412 nm using a millimolar extinction coefficient of 114 (ref. 16). Horseradish peroxidase, Type VII-L, was obtained from Sigma Chemical Company. The concentration was determined at 403 nm using a millimolar extinction coefficient of 91 (ref. 1).

All chemicals were reagent grade. L-Tyrosine was obtained from CalBiochem. D-tyrosine was obtained from CalBiochem or Sigma Chemical Company. L-[^{14}C]-Tyrosine, uniformly labeled, with a specific activity of about 400 mCi/mmole was obtained from either New England Nuclear Corporation or Schwarz BioResearch. H_2O_2 concentration was measured at 230 nm using a molar extinction coefficient of 72.4 (ref. 17).

The various spectra were obtained by use of a Cary Model 14 recording spectrophotometer. Fluorescence measurements were made on a Hitachi Perkin Elmer Model MPF-2A or on a Farrand MK-1 spectrofluorimeter. All spectral measurements were made at 25 °C in cuvettes with a 1-cm light path.

Dityrosine formation was followed on a Gilford recording spectrophotometer. The increase in absorbance at 315 nm was used as an index of dityrosine production. It was not necessary to correct for the initial tyrosine present, since its contribution at 315 nm to the difference spectrum is less than 1%. Dityrosine production could also be followed by the increase in fluorescence emission at 405 nm using a wavelength of 285 or 315 nm for activation. Except where indicated the assay mixture contained 2.7 mM tyrosine, 0.1 mM H_2O_2, and 7.4 nM lactoperoxidase or 65.2 nM or 32.8 nM horseradish peroxidase, 0.05 M phosphate buffer, pH 8.2. At pH values of 6.0 and below 0.05 M acetate buffer was used. In some assays 1.0 mM EDTA was included but had no effect on the reaction rate.

Thin-layer chromatography. Ascending thin-layer chromatography was carried out on MN cellulose powder 300 (Machery-Nagel) precoated plastic sheets. The plates were obtained from Brinkmann Instruments, Inc. Solvent systems employed were: 1-butanol–acetic acid–water (4:1:1, by vol.)[18], isopropanol–ammonia–water (8:1:1, by vol.)[18]. Tyrosine and reaction derivatives were detected by fluorescence or by spraying

with diazotized sulfanilic acid[19] or 0.5% ninhydrin in ethanol. In experiments employing [^{14}C]tyrosine radioautography was employed. Thin-layer chromatograph plates were kept in close contact with Kodak Medical X-ray film (Blue Band, tinted Estar safety base, BB-14) for several days. The position of the radioactive tyrosine or oxidized derivatives was then localized by development of the film.

Column chromatography. The separation of peroxidase-catalyzed oxidation products of tyrosine was also performed by chromatography on a 1 cm × 40 cm cellulose phosphate column according to the method of Andersen[20], except that 0.2 M acetic acid containing 0.2 M NaCl was used to elute the dityrosine. Fractions were monitored by ultraviolet absorbance and by fluorescence, following dilution into neutral pH buffer.

RESULTS

The oxidation of tyrosine is catalyzed by both lactoperoxidase and horseradish peroxidase. A number of reports have indicated that *o,o*-dityrosine is a product of the reaction. In order to establish that this was true, the experimental difference spectrum observed with lactoperoxidase-catalyzed oxidation of L-tyrosine is compared in Fig. 1 with the difference spectrum calculated from the data of Andersen[20] and Lehrer[10] assuming the stoichiometry of 2 tyrosine → *o,o*-dityrosine. Results are given at a pH value of 7.0. The experimental spectrum has higher absorbance in the wavelength range of 290–300 nm, while there is good agreement at wavelengths above 310 nm.

Since dityrosine ionizes resulting in changes in absorbance in the pH range used in these studies, the extinction coefficient at 315 nm of dityrosine for the pH range 5.5–9.0 was calculated from the data of Andersen[20] and is shown in Fig. 2. In order to

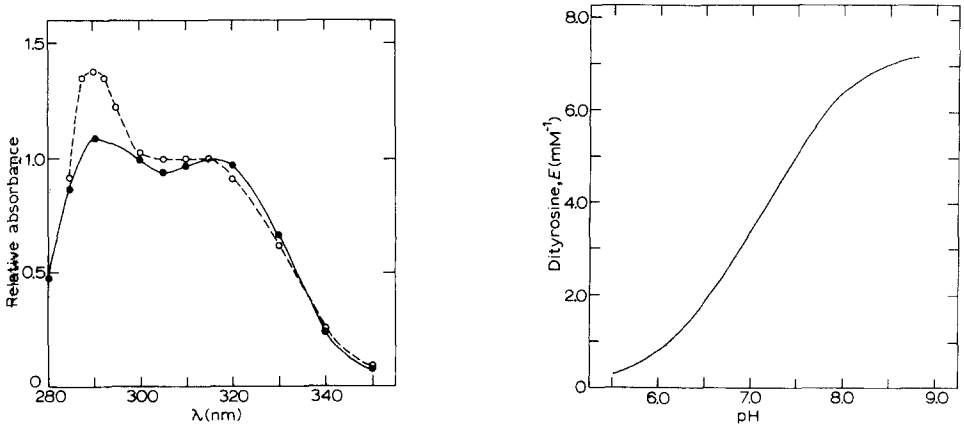

Fig. 1. Calculated and experimental difference spectrum of dityrosine *versus* tyrosine. The calculated spectrum is obtained from the data of Andersen[20] and Lehrer[10], and is based on the stoichiometry of 2 tyrosine → dityrosine. For the experimental difference spectrum, each cuvette contained 1.36 mM L-tyrosine and 16.7 nM lactoperoxidase in 3.0 ml of 0.05 M buffer at the indicated pH. The difference spectra exhibited no further change 2.0 min after the addition of 7.85 μM H_2O_2 to the reference cuvette. The spectra were matched at 315 nm to give the relative absorbance shown. ●—●, calculated; ○—○, experimental.

Fig. 2. The difference millimolar extinction for dityrosine at 315 nm as a function of pH. Extinction coefficient values for dityrosine were calculated from the data of Andersen[20].

TABLE I

TOTAL DITYROSINE FORMATION AS A FUNCTION OF pH

Difference spectra were obtained on a Cary Model 14 employing the 0.1-absorbance slidewire. Both cuvettes contained 1.36 mM L-tyrosine and 16.7 nM lactoperoxidase in 0.05 M phosphate buffer. The reaction was initiated by addition of 7.85 μM H_2O_2 to the reference cuvette. Scans were made until no further change was observed. Dityrosine concentration was calculated from the values in Fig. 2.

pH	$\Delta A_{315\ nm}$	*Dityrosine (μM)*
6.0	0.005	6.72
6.55	0.011	5.24
7.0	0.017	4.71
7.5	0.029	5.56
8.2	0.036	5.37
8.5	0.036	5.19
9.0	0.039	5.34

establish that the same stoichiometry and products are produced over the pH range studied, the reaction was performed under identical conditions except for the pH. Results of experimental reactions carried out over this pH range are given in Table I. As shown in Table I the approximate stoichiometry of H_2O_2 used to dityrosine produced is 1.0:0.73 and the amount of dityrosine produced is constant within experimental error over the range pH 6.0 to 9.0.

The product of the reaction was also characterized by chromatography on a thin-layer plate. The reaction mixture contained a fluorescent, ninhydrin-positive, sulfanilic acid-positive product having the R_F values appropriate for dityrosine.

As shown in Fig. 1 there is a maximum in the difference absorption spectrum at 315 nm. Therefore, the kinetics of dityrosine formation could be followed at this wavelength on a Gilford recording spectrophotometer.

Although the rate of dityrosine production was a function of both the enzyme and tyrosine concentration, the data in Fig. 3 show that the initial rate of dityrosine

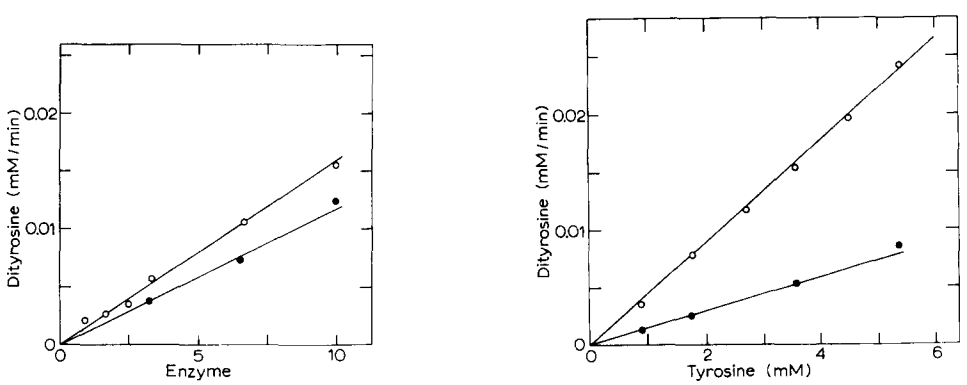

Fig. 3. Rate of dityrosine formation as a function of enzyme concentration. Dityrosine was assayed according to the procedure outlined in the Experimental Section.)Peroxidase concentrations were varied as shown. ○—○, $\cdot 10^9$ M lactoperoxidase; ●—●, $\cdot 10^8$ M horseradish peroxidase.

Fig. 4. Rate of dityrosine formation as a function of tyrosine concentration. Dityrosine was assayed as indicated in the Experimental Section except for the variation in L-tyrosine. ○—○ 7.4 nM lactoperoxidase; ●—●, 32.8 nM horseradish peroxidase.

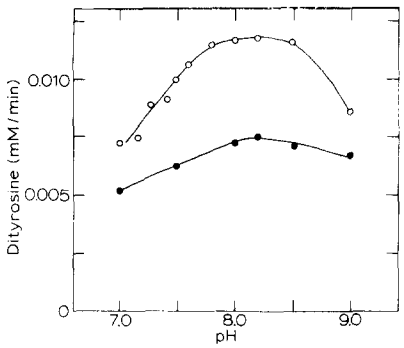

Fig. 5. Rate of dityrosine formation as a function of pH. Dityrosine was assayed as indicated in the Experimental Section except for the variation of pH values. ○—○, 7.4 nM lactoperoxidase; ●—●, 65.2 nM horseradish peroxidase.

production was a linear function of enzyme concentration for both lactoperoxidase and horseradish peroxidase at a constant tyrosine concentration. However, dityrosine production was also pseudo first-order with respect to tyrosine concentration at a constant enzyme concentration as shown in Fig. 4. The effect of pH on the initial rate is shown in Fig. 5. There is an optimum at pH 8.2 for oxidation of L-tyrosine by both peroxidases.

Lineweaver–Burk plots[21] were used to investigate the effect of variation in H_2O_2 concentration on the rate of dityrosine production. The data are given in Fig. 6. Two different tyrosine concentrations of both the D- and the L-isomers were investigated with each peroxidase. A series of parallel lines was obtained with the apparent K_m value for peroxide increasing with increase in V.

The rate at which tyrosine isomers were oxidized was also examined. Table II gives the apparent second order rate constants for D- and L-tyrosine in the dityrosine reaction for both lactoperoxidase and horseradish peroxidase. Dityrosine production was followed with time both by absorbance increases at 315 nm and by increase in fluorescence emission at 405 nm upon activation at 285 nm. Both methods give identical results. The oxidation of L-tyrosine is catalyzed more readily than D- by lactoperoxidase. Horseradish peroxidase also catalyzes the oxidative coupling of the

TABLE II

COMPARISON OF APPARENT RATE CONSTANTS OF OXIDATION OF TYROSINE ISOMERS

The second order rate constants were calculated according to the integrated equation[34], and expressed for molar peroxidase concentration. The rate of tyrosine oxidation was measured as outlined in Experimental Section. The final concentrations employed in the assay were 2.7 mM tyrosine, 0.1 mM H_2O_2, and 7.4 nM lactoperoxidase or 65.2 nM horseradish peroxidase for L-tyrosine and 32.8 nM horseradish peroxidase for D-tyrosine in a final volume of 3 ml in 0.05 M phosphate buffer, pH 8.2.

Enzyme	$k(M^{-1} \cdot s^{-1})$		
	L-*Tyrosine*	D-*Tyrosine*	H_2O_2
Lactoperoxidase	10 300	6890	$5.55 \cdot 10^6$
Horseradish peroxidase	753	1550	$3.05 \cdot 10^6$

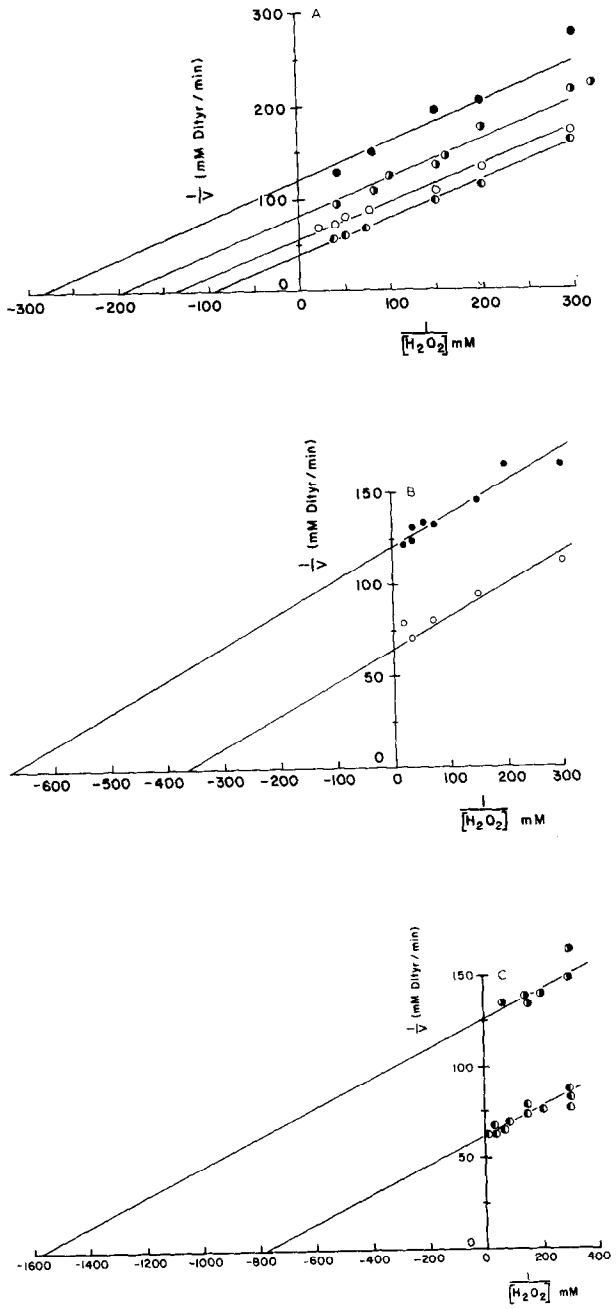

Fig. 6. Double-reciprocal plots of dityrosine formation *versus* H_2O_2 concentration. The data were obtained at pH 8.2, using various concentrations of H_2O_2 and the indicated amount of the tyrosine isomers. (A) 7.4 nM lactoperoxidase: ●—●, 2.7 mM D-tyrosine; ◑—◑, 2.7 mM L-tyrosine; ○—○, 5.4 mM D-tyrosine, ◐—◐, 5.4 mM L-tyrosine. (B) 32.6 nM horseradish peroxidase: ●—●, 2.7 mM D-tyrosine; ○—○, 4.5 mM D-tyrosine. (C) 65.2 nM horseradish peroxidase: ◐—◐, 5.4 mM L-tyrosine; ◑—◑, 2.7 mM L-tyrosine.

isomers of tyrosine at different rates but with this enzyme the D-isomer is coupled more readily.

Fluorescence characteristics of dityrosine were also investigated. An unbuffered 100 ml reaction mixture was prepared containing 1.35 mM L-tyrosine, 16.0 nM lactoperoxidase, and 0.22 mM H_2O_2. After 15 min at 25 °C the solution was adjusted to pH 2.0, applied to a phosphocellulose column, and chromatographed as indicated under Experimental Procedure. Dityrosine was obtained free of tyrosine and diluted into appropriate buffers to obtain solutions at different pH values. The fluorescence emission obtained at 405 nm upon excitation at 315 nm is compared in Fig. 7 to literature values over the pH range of 2.0–11.0. The fluorescence is expressed as percentage relative to that at pH 7.0. There is very good agreement of the literature and experimental values.

DISCUSSION

The peroxidase-catalyzed oxidation of phenolic compounds has been observed by numerous investigators since 1900[1]. However, in only a few of these studies have products of the oxidation been investigated. Westerfeld and Lowe[22] isolated and identified three of the products obtained from horseradish peroxidase-catalyzed oxidation of *p*-cresol. The products were identical to those obtained by Pummerer *et al.*[23] from ferricyanide oxidation of *p*-cresol.

Mayrargue-Kodja *et al.*[24] observed polymerization of tyrosine and thyronine catalyzed by horseradish peroxidase. Gross and Sizer[7] reported the horseradish peroxidase-catalyzed oxidation of tyramine, tyrosine and certain tyrosine derivatives to fluorescent products. The chief product from either tyramine or tyrosine was demonstrated to be the dimer in *o,o*-biphenyl linkage. Guilbault *et al.*[8] observed the horseradish peroxidase-catalyzed oxidation of tyrosine, tyramine and certain hydroxyphenyl acids to fluorescent products whose spectral characteristics were tabulated.

Compounds such as those derived by peroxidase-catalyzed oxidation of tyrosine have been shown to be present in biological systems. The first report of natural occurrence of dityrosine was made by Andersen in 1964[18]. He isolated two fluorescent amino acids, di- and trityrosine, from hydrolysates of resilin, a structural protein of arthropod elastic ligaments.

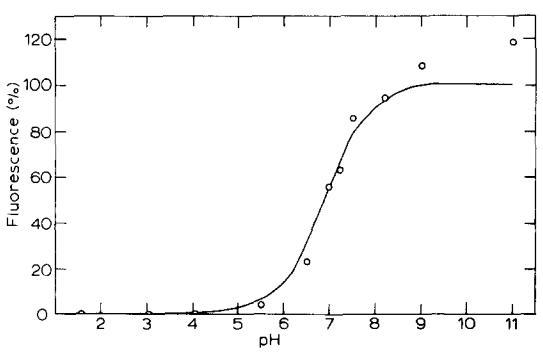

Fig. 7. Relative fluorescence of dityrosine as a function of pH. The curve represents data from refs 10 and 20; ○, experimental as outlined in the text.

Subsequently, LaBella *et al.*[25–27] found dityrosine in chick aortic elastic hydrolysates and in some preparations of highly purified collagens. Tyrosine was shown to serve as a precursor. Furthermore, soluble collagens incubated with horseradish peroxidase and peroxide exhibited rigid gel formation and significant dityrosine production.

The product of lactoperoxidase-catalyzed oxidation of tyrosine under initial rate conditions appears to be chiefly dityrosine. This conclusion is based on the fact that only dityrosine is identified from initial rate reactions by thin-layer or column chromatography. The experimental difference spectra obtained at pH 8.2 have a constant ratio of $A_{315\ nm}/A_{290\ nm}$ of 1.65 only under the initial conditions employed when a few percent of the tyrosine has been oxidized. The same difference spectrum was obtained at pH 8.2 for the oxidation product of both D- and L-tyrosine catalyzed by either lactoperoxidase or horseradish peroxidase. It is evident from Fig. 1 that some product or intermediate other than dityrosine contributes to the absorbance from 290 to 300 nm. However, this component is not fluorescent, since the excitation spectrum of the lactoperoxidase produced dityrosine completely agrees with the literature data. The amount of dityrosine produced with a given concentration of peroxide is constant over the pH range 6.0–9.0.

On a preparative scale, when excess peroxide and lactoperoxidase were used, products other than dityrosine such as trityrosine and thyronine were also found by thin-layer chromatography. Extensive enzymatic oxidation of labeled tyrosine at pH 8.2 also results in insoluble radioactive yellow to brown colored products which are not eluted from a phosphocellulose column and which do not migrate in the thin-layer chromatography solvents. These products may be confused for melanin pigments[28] which can be produced by peroxidase-catalyzed oxidation of dihydroxyphenylalanine.

The reaction mechanism for catalysis by peroxidase probably proceeds by the Chance–George classical scheme[1]. In this mechanism the rate limiting step is usually the reaction between the electron donor and peroxidase Compound II, and is often[1,29–31] proportional to donor concentration as noted in these studies.

Despite the numerous investigations of the peroxidase-catalyzed oxidative coupling of tyrosine the stereospecificity with regard to the tyrosine residues has not previously been noted. Most investigations of peroxidase-catalyzed reactions have indicated that these reactions have a very low degree of specificity. This led to the view of these reactions as being simple redox reactions between the prosthetic group of the peroxidase Compound I and II and the electron donors. The present investigations clearly indicate that this reaction is more complex and the specificity indicates that there is a binding site for tyrosine which can distinguish between the D- and L-isomers. The degree of stereospecificity of the peroxidases in the case of oxidation of tyrosine isomers is not as high as in some enzyme-catalyzed reactions. This is probably due to the fact that the asymmetric center is removed from the site of enzyme action[32]. It is interesting to note that in the case of lactoperoxidase D-tyrosine is iodinated more readily than the L-isomer while the opposite is true in oxidative coupling; the L-isomer is coupled more readily than the D-isomer.

Horseradish peroxidase does not catalyze iodination directly, but does catalyze the oxidative coupling of the phenols and at pH 8.2 the stereospecificity is the opposite of that shown by lactoperoxidase (Table II).

The most likely mechanism for the oxidative coupling is *via* a free radical reac-

tion. The stereospecificity observed in the enzyme catalyzed reaction indicates that tyrosine complexes with the enzyme. Critchlow and Dunford[33] have obtained evidence that *p*-cresol forms a complex with horseradish peroxidase, and have proposed that the phenol binds first before undergoing oxidation. The coupling reaction differs from the iodination reaction because in the iodination reactions iodide atoms are probably oxidized and then interact with the enzyme complexed tyrosine.

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