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# OXIDATIVE RING-COUPLING OF TYROSINE AND ITS DERIVATIVES BY PURIFIED RAT INTESTINAL PEROXIDASE

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**Abstract**—Intestinal peroxidase was shown to catalyse the oxidative ring-coupling of tyrosine, α-methyltyrosine, tyramine and morphine whereas amphetamine was not oxidized to any detectable extent. The oxidative ring-coupling reaction can be monitored by changes in absorbance spectra and the dimers formed in this way with morphine and α-methyltyrosine were identified by mass spectrometry. Intestinal peroxidase also catalysed the peroxidatic oxidation of L-DOPA and α-methyl-L-DOPA, but in this case the reaction would be expected to be more complicated and to yield a variety of possible products. The kinetic parameters for the oxidation of each of these substrates were determined. Since the products of the oxidative ring-coupling reactions may have different pharmacological properties to those of the parent compounds, these studies suggest that, in the presence of an adequate supply of metabolically produced hydrogen peroxide, the action of intestinal peroxidase may affect the behaviour and pharmacokinetics of these compounds after oral administration.

Dietary tyramine, which is present in a number of foods and beverages [1–3], can induce a hypertensive response because of its ability to be taken up by peripheral adrenergic nerves, releasing stored noradrenaline [3]. Under normal circumstances this effect is small except if large amounts of tyramine are ingested, although there may be considerable individual variability in the response [4]. This is because of the efficient presystemic metabolism of tyramine. Monoamine oxidase (EC 1.4.3.4; MAO‡) has been shown to play the major role in this metabolism and the hypertensive response of subjects treated with inhibitors of MAO, as antidepressants, can be severe and in some cases fatal, although there appears to be some individual variability in the response [1, 5]. MAO-A in the intestine appears to play the major role in this process [5–7] although MAO activity in the stomach may also contribute [8] and tyramine escaping first-pass metabolism in the gastrointestinal tract can be oxidized by MAO present in other peripheral organs.

Under conditions where peripheral MAO is inhibited tyramine, which can also be formed by the action of L-aromatic amino acid decarboxylase on tyrosine, at lower levels may contribute to the hypotensive effects of MAO inhibitors by acting as a false transmitter itself or after conversion to octopamine (see Ref. 9). The compound α-methyltyrosine can also be decarboxylated to the corresponding false transmitter amine, α-methyltyramine, which is not a substrate for MAO [10].

Because of the importance of the intestine in the first-pass metabolism of ingested tyramine we have investigated an alternative pathway of its metabolism involving the peroxidase activity associated with that tissue. Peroxidase (EC 1.11.1.7) is known to be capable of catalysing the oxidative ring-coupling of some aromatic compounds, such as that involving tyrosine shown in Fig. 1, in a reaction which probably proceeds by way of radical intermediates [11–14]. Many of these studies have involved the enzyme from horseradish and the ability of intestinal peroxidase to catalyse this reaction has not been characterized. We have reported recently the purification of rat intestinal peroxidase and its ability to effect the oxidative ring-coupling of the commonly used antioxidant BHA [15]. In the present communication we report the ability of this enzyme to catalyse the oxidative ring-coupling of tyramine, its parent amino acid tyrosine and the related compound α-methyltyrosine. Since peroxidation might affect the availability of L-DOPA administered in the therapy of Parkinson's disease the reaction with that amino acid was also studied. The drugs morphine, which might be expected to undergo peroxidative ring-coupling [16] because of its

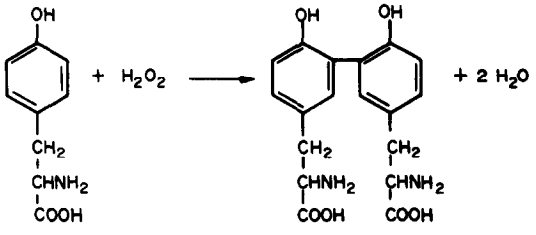


Fig. 1. The oxidative ring-coupling reaction of tyrosine.

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‡ Abbreviations: MAO, monoamine oxidase; BHA, 2-*t*-butyl-4-methoxyphenol, butylated hydroxyanisole; TFAA, trifluoroacetic acid anhydride.

structural similarity to tyrosine, and amphetamine, which might be expected to be less susceptible to such oxidation, were also investigated since this reaction might affect the responses to oral administration of these compounds.

## MATERIALS AND METHODS

Peroxidase was purified to apparent homogeneity from rat intestine by the procedure reported previously [15]. Crystalline horseradish peroxidase was obtained from the Sigma Chemical Co. (Poole, U.K.). All other chemicals were from Sigma, Merck A.G. (Darmstadt, Germany) or as reported previously [15]. The concentrations of solutions of $H_2O_2$ were determined spectrophotometrically at 240 nm using an extinction coefficient of 43.6 L/mol/cm [17]. Protein concentration was determined by the methods of Bensadoun and Weinstein [18] with bovine serum albumin as the standard.

### *Assay procedures*

All activities were determined spectrophotometrically at 37° in reaction mixtures containing, in a volume of 1.0 mL, 75 mM Tris–HCl buffer, pH 8.0, 330 $\mu$M $H_2O_2$, enzyme and substrate. The activity towards guaiacol was determined under these conditions as described previously [15], one unit of activity being defined as that catalysing the oxidation of 1 $\mu$mol of substrate in 1 min.

*Tyrosine, tyramine and α-methyltyrosine.* The oxidative ring-coupling of L-tyrosine and α-methyl-L-tyrosine was followed by measuring the increase in absorbance at 315 nm [12]. The oxidation of tyramine was followed initially by a series of repetitive scans of the absorbance spectra during the course of the reaction. From these results a wavelength of 318 nm was chosen for routine assays.

The molar extinction coefficient of di-tyrosine was determined from the changes in absorbance at 315 nm when a series of aliquots of tyrosine were added to the standard assay mixture containing an excess of $H_2O_2$ and horseradish peroxidase [19]. Although a value of 6700 L/mol/cm has been reported for the molar extinction coefficient of di-tyrosine at 310 nm [12], it is pH-dependent, and rather lower values, ranging from about 1400 to 4500 L/mol/cm, have been reported for the pH range 6.0–8.5 at this wavelength [13]. The molar extinction coefficients of di-tyrosine at 315 nm determined in the present work were 1800 at pH 7.0 and 5080 at pH 8.0. The same values were found for the synthetic di-α-methyltyrosine.

The corresponding value for di-tyramine was determined in the same way using a wavelength of 318 nm. The value for di-tyramine was also determined at completion of the reaction by measuring the $H_2O_2$ remaining from the absorbance at 240 nm [17], after absorbing the tyramine and di-tyramine from the mixture on to Dowex-50 WX-4 (equilibrated as the sodium form) [20]. Absorbance spectra of samples of the column eluates were determined to check for the presence of any tyramine or di-tyramine which had not been absorbed to the ion-exchange resin. The recovery of $H_2O_2$ was determined by adding samples to the reaction mixtures before treatment with the ion-exchange resin. Spectrophotometric determinations of the eluates from the ion-exchange resin showed complete retention of tyramine and di-tyramine, and $H_2O_2$ was quantitatively recovered. The two procedures gave consistent results with extinction coefficients of 2220 and 5080 L/mol/cm being calculated at 318 nm and at pH 7.0 and 8.0, respectively.

*DOPA, α-methyl-DOPA and amphetamine.* The concentrations of L-DOPA and α-methyl-L-DOPA in solution were monitored by determining the absorbance at 280 nm [21] and the peroxidation reaction was measured at either 306 or 482 nm. The reaction products formed in the peroxidase-catalysed oxidation of DOPA and α-methyl-DOPA are likely to be complex (see later). The overall molar extinction coefficients of the products were determined to allow quantitation of kinetic data. This was done by adding small aliquots of $H_2O_2$ (10, 12.5 and 20 nmol) to the reaction mixture containing 0.2 mM L-DOPA and determining the total change in absorbance at 306 and 482 nm. When the absorbance change had ceased the addition of a further sample of DOPA or guaiacol, to give a final or additional concentration of 0.2 mM gave no further absorbance change, indicating that there was no residual $H_2O_2$.

In the case of (+)-amphetamine, the possible reaction was investigated by monitoring the absorbance due to $H_2O_2$ at 240 nm in the reaction mixture described above and concentrations of up to 100 $\mu$M amphetamine. Repetitive determinations of the absorbance spectra of reaction mixtures containing 100 $\mu$M amphetamine were also performed.

*Morphine.* The oxidation of morphine was followed initially by a series of repetitive scans of the absorbance spectra during the course of the reaction. From these results a wavelength of 317 nm was chosen for the routine assay. The molar extinction coefficient of dimer of morphine at 317 mm was calculated using the di-morphine (pseudomorphine), synthesized from morphine by alkaline ferricyanide oxidation as described by Bentley and Duke [22].

Mass spectra of authentic and peroxidase-formed di-morphine were determined with a VG 70S spectrometer (VG analytical, Manchester, U.K.), after direct injection by a probe for solid samples. The mass spectra were recorded in the electron ionization mode at 70 eV, 200 $\mu$amp trap current, 8000 V accelerating voltage, using a DEC micro PDP 11/83 computer at a scan rate 2 sec/decade at 2000 M/$\Delta$M mass resolution (5% valley definition) over the 50–750 mass range.

### *Synthesis, purification and analysis of di-α-methyltyrosine*

Di-α-methyltyrosine was prepared as described by Amadò et al. [23] for the synthesis of di-tyrosine with some modifications. α-Methyltyrosine (1 mmol) was dissolved in 200 mL of 0.2 M sodium borate buffer, pH 9.5, containing 0.5 mmol of $H_2O_2$ and 1 mg horseradish peroxidase was added. The mixture was incubated at 37° for 12 hr and, then, concentrated in a rotatory evaporator. The concentrate was applied to a cellulose microcrystalline Avicel column

(35 × 2 cm) pre-equilibrated with 2-propanol–17% $NH_3$ (4:1 v/v) and washed with 500 mL of the same solution. The fluorescent fractions visualized under UV light and obtained by elution with 2 M $NH_3$ were collected, concentrated and dissolved in the minimum amount of 0.5 M acetic acid. This solution was applied on a Dowex 50 X-8 column (20 × 1 cm). The column was washed extensively with water and di-α-methyltyrosine was subsequently eluted with 2 M $NH_3$.

TLC analysis of di-α-methyltyrosine was performed on silica gel 60 plates; 5 × 20 cm, 0.25 mm thick (Merck, Darmstadt, Germany). The chromatograms were developed 15 cm at room temperature with *n*-propanol–25% $NH_3$ (7:3 v/v) and visualized under long wavelength UV light or after staining with Folin and Ciocaltau's phenol reagent, as described for di-tyrosine [24]. α-Methyltyrosine ($R_f = 0.48$) was positive to the phenol reagent; di-α-methyltyrosine ($R_f = 0.20$) was positive to UV light and also to the phenol reagent. The eluate from Dowex column was dried and about 1 mg was dissolved in 0.5 mL of acetonitrile and 100 μL of TFAA was added. The mixture was incubated for 0.5 hr at 80° and analysed by mass spectrometry after direct injection with a probe for solid samples. The mass spectra were recorded in the electron ionization mode at 20 eV, 50 μamp trap current, 8000 V using a scan rate 2 sec/decade at 2000 M/ΔM mass resolution (5% valley definition) over a mass range of 100–1000.

## RESULTS

### *Characterization of reaction products*

When tyrosine and tyramine were used as substrates, the respective reaction products with peroxidase and $H_2O_2$ were identified as the corresponding dimers by means of the spectral and chromatographic characteristics which were comparable to those already described for di-tyrosine and di-tyramine [11, 12].

Figure 2 shows the mass spectra of di-α-methyl-L-tyrosine prepared and purified as described above (panel A) and of the intestinal peroxidase oxidation product (panel B), both derivatized with TFAA. Because of the small amounts of material available from the reaction catalysed by intestinal peroxidase the molecular ion of the product was not detectable. However, no other fragments from the intestinal peroxidase product differed from those seen with purified di-α-methyltyrosine in the range 100–1000 $m/z$. Although it is not easy to interpret these mass spectra, the fragmentograms recorded are similar to those reported for di-tyrosine derivatized with TFAA and diazomethane [25]. From those data the ions ($m/z$) shown in Fig. 2 would correspond to 772 = $[M^+]$; 492 = $[M^+ - COCF_3 - HOOCCCH_3N-COCF_3]$; 446 = $[492^+ - (H_2O + CO)]$; 396 = $[M^+ - 2COCF_3 - OOCCCH_3NCOCF_3]$; 350 = $[396^+ - (H_2O + CO)]$. These data and those from TLC analysis and determination of the absorbance spectra show that intestinal peroxidase catalyses the oxidation of α-methyl-L-tyrosine to the corresponding ring-coupled dimer.

Figure 3 shows the mass spectrum of synthetic dimorphine (panel A) and of the intestinal peroxidase oxidation product of morphine (panel B). The corresponding structures of the ions in the fragmentograph are indicated in the legend to the Figure. The changes in the absorbance spectrum during the reaction of morphine with hydrogen peroxide in the presence of intestinal peroxidase were also consistent with the formation of di-morphine.

The spectral changes during the reaction of L-DOPA with $H_2O_2$ in the presence of peroxidase showed the appearance of absorbance peaks at 306 and 483 nm, indicating the reactions involved to be more complex than simple ring-coupling (Fig. 4). This might be expected from the greater reactivity of catechols, and the changes seen would be consistent with the formation of quinone and aminochrome derivatives and, perhaps, further polymerization products. These reactions yield complex mixtures of products which have been characterized by others [14, 26–28]. The absorbance maxima observed here would be consistent, dopachrome being the major oxidation product formed under the conditions used [29, 30]. The overall molar extinction coefficients of the products, calculated at pH 8.0 as described above, were 6480 and 2880 L/mol/cm at 306 and 483 nm, respectively. Similar results were found when α-methyl-DOPA was used. Further analysis would be necessary to determine whether any oxidative ring-coupling occurred in competition with these extremely facile competing reactions.

### *Kinetic studies*

The time-course of the spectral changes during the reaction of L-tyrosine with hydrogen peroxide in the presence of peroxidase is shown in Fig. 5. Similar results (not shown) were obtained when tyramine, α-methyl-L-tyrosine and morphine were used as substrates.

Assays of the reactions under the conditions described above showed that product formation was linear with time for at least 10–15 min and that the initial rate of reaction was proportional to the concentration of rat intestinal peroxidase added. Double-reciprocal plots of the oxidation of these substrates are shown in Fig. 6 and the kinetic parameters, determined from non-linear regression analysis of the initial rate data, are shown in Table 1.

In the absence of added peroxidase there was a significant rate of oxidation of L-DOPA by $H_2O_2$, as measured by the changes of absorbance at 306 and 483 nm. These non-enzymic rates were subtracted from those obtained after enzyme addition in the kinetic studies. At 25 μM L-DOPA the blank rate was about 22% of that obtained in the presence of 0.022 U of intestinal peroxidase but this value fell to about 5% at a DOPA concentration of 200 nM. Time-courses of the increases in absorbance at the two wavelengths showed no detectable burst or lag phases indicating that any reaction steps preceding, or between, the formation of these absorbing intermediates were not rate-limiting under the conditions used here. Initial rates of the peroxidase-catalysed reaction were proportional to enzyme

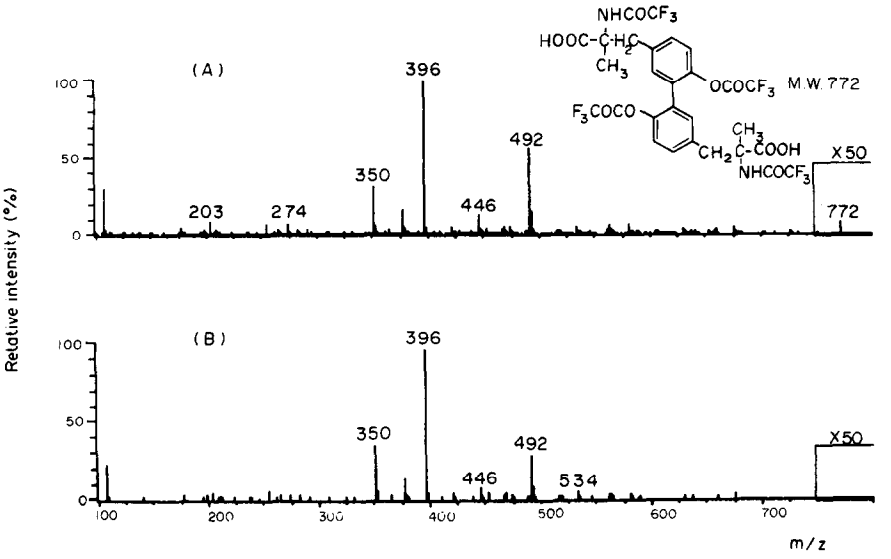


Fig. 2. Electron-impact mass spectra of di-α-methyl-L-tyrosine derivatized with TFAA. (A) Di-α-methyl-L-tyrosine prepared as described in the text; (B) sample obtained after intestinal peroxidase oxidation.

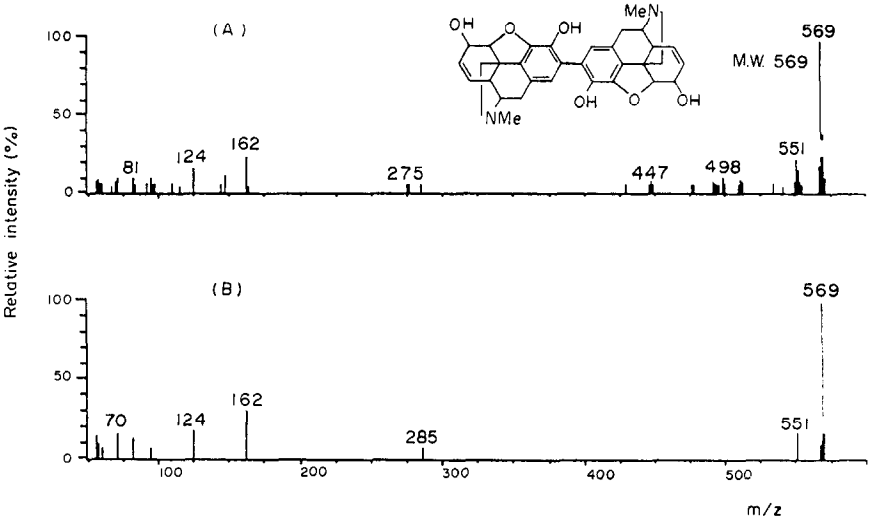


Fig. 3. Electron impact mass spectra of di-morphine. (A) Di-morphine obtained after alkaline ferricyanide oxidation; (B) sample obtained after intestinal peroxidase oxidation. The ions ($m/z$) correspond to: 569 = $[M^+]$; 551 = $[[M^+ - H_2O]$; 285 = $[M^+/2]$; 162 = $[HOC_6H_3(CH_2)_2NCH_3CH]^+$ and 124 = $[HOC_6H_3OHCH_3]^+$.

concentration when measured at either wavelength. Double-reciprocal plots of the dependence of the initial rate on the L-DOPA concentration were linear, and the same kinetic parameters were obtained whether the reaction was followed at 306 or 483 nm (Fig. 7 and Table 1). Thus, despite the complexity of the reactions involving DOPA, the overall molar extinction coefficients calculated for the products formed may be used as a basis for the spectrophotometric assay of the activity of peroxidase towards this substrate.

When the absorbance spectrum of (+)-amphetamine, at concentrations of up to 100 μM and in the presence of 330 μM $H_2O_2$ and 0.22 U of the intestinal peroxidase, was determined between 250 and 450 nm at 1-min intervals for 10 min under the standard assay conditions, no change was detected. Neither was any decrease in the $H_2O_2$ concentration,

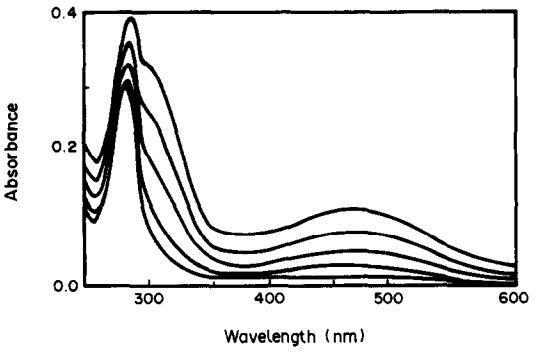


Fig. 4. Absorbance changes during the oxidation of L-DOPA by rat intestinal peroxidase. The reaction mixture contained 75 mM Tris–HCl buffer, pH 8.0, 12 mM L-DOPA, 330 $\mu$M $H_2O_2$ and 0.8 $\mu$g enzyme. Spectra were recorded at 5-min intervals. The absorbance increased with time at all wavelengths shown. Other experimental conditions were as described in the text.

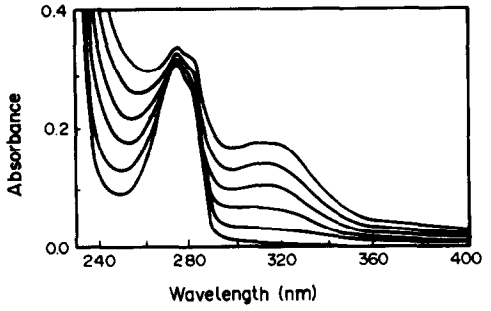


Fig. 5. Absorbance spectra during the oxidation of L-tyrosine by rat intestinal peroxidase. Spectra were recorded every 5 min. The absorbance increased with time at all wavelengths shown. Experimental conditions were as described in the text.

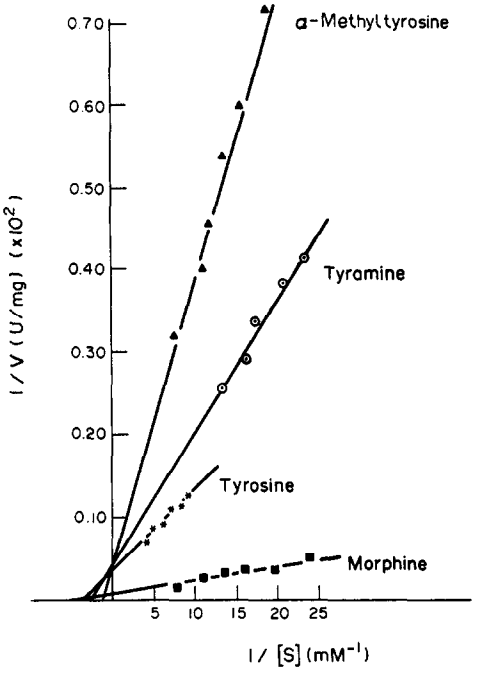


Fig. 6. Double-reciprocal plots for the oxidation of tyramine. L-tyrosine, $\alpha$-methyl-L-tyrosine and morphine by rat intestinal peroxidase. Each value is the mean of three separate determinations. Initial velocities for morphine have each been multiplied by 10 in order to allow these values to be conveniently displayed on the same scale as the other substrates. Experimental conditions are given in the text.

determined at 240 nm, detectable over a period of 20 min. Similar determinations in the presence of 5 $\mu$g horseradish peroxidase gave no detectable spectral changes. Amphetamine was found to be a poor inhibitor of the intestinal peroxidase. The presence of 1 mM amphetamine resulted in a 22% inhibition of the oxidation of 2 mM guaiacol under the conditions used here.

## DISCUSSION

The kinetic parameters for tyrosine and its decarboxylation product tyramine were comparable. The introduction of a methyl group in the $\alpha$-carbon of tyrosine reduced markedly the apparent affinity of the enzyme for this substrate while the $V_{max}$ remained unaltered. In contrast, the introduction of a second hydroxyl group in the 3-position of the phenol ring did not affect the apparent enzyme affinity but increased the $K_{cat}$ by about two orders of magnitude. In the case of $\alpha$-methyltyrosine, the apparent first-order rate constant for ES complex formation, $K_{cat}/K_m$, was about 10 times higher than for tyrosine. However, in the case of L-DOPA and $\alpha$-methyl-L-DOPA there was little difference between these values which were dominated by a much higher reactivity of the cathecol radical formed during the peroxidase oxidation [27].

The $K_m$ values obtained for tyramine, tyrosine, L-DOPA and morphine are comparable to those determined for this enzyme with BHA as the substrate [15], whereas the maximum velocities with tyramine, tyrosine and $\alpha$-methyltyrosine were all much lower than that obtained with guaiacol. Only in the cases of L-DOPA and $\alpha$-methyl-DOPA did the maximum velocities approach that obtained with guaiacol. However, as discussed above, the reactions with DOPA and its $\alpha$-methyl-derivative are more complicated because their much higher reactivities lead to the formation of quinones, aminochromes and perhaps polymerization products. Morphine gave the lowest $V_{max}$ value of the substrates examined here.

These values can be compared with $K_m$ values of 120 and 212 $\mu$M for rat intestinal MAO-A and -B, respectively, oxidizing tyramine at pH 7.2 and 37° [6]. The maximum velocities of these two forms of MAO in intestinal homogenates are 68 and 32 nmol/hr/mg protein, respectively [6]. From the purification data on the rat intestinal peroxidase [15] one can

Table 1. Kinetic parameters for the oxidative ring-coupling of some phenol derivatives by rat intestinal peroxidase

| Substrate | $K_m$ (μM) | $V_{max}$ (μmol/min/mg) | $K_{cat}$ ($min^{-1}$) | $K_{cat}/K_m$ ($min^{-1}$ $mM^{-1}$) |
|---|---|---|---|---|
| L-Tyrosine | 300 | 29.6 | $3.3 \times 10^2$ | $1.1 \times 10^3$ |
| Tyramine | 380 | 20.9 | $2.8 \times 10^2$ | $7.4 \times 10^2$ |
| L-α-methyltyrosine | 1090 | 28.9 | $1.3 \times 10^2$ | $1.2 \times 10^2$ |
| L-DOPA | 420 | 182 | $6.2 \times 10^4$ | $1.5 \times 10^5$ |
| L-α-methyl-DOPA | 423 | 139 | $9.8 \times 10^4$ | $2.3 \times 10^5$ |
| Morphine | 250 | 1.2 | $2.7 \times 10^2$ | $1.1 \times 10^3$ |
| BHA | 180 | 8.0 | $1.1 \times 10^2$ | $6.4 \times 10^{2*}$ |
| Guaiacol | 21,000 | 244 | $9.8 \times 10^3$ | $4.7 \times 10^{2*}$ |

* From Ref. 15.

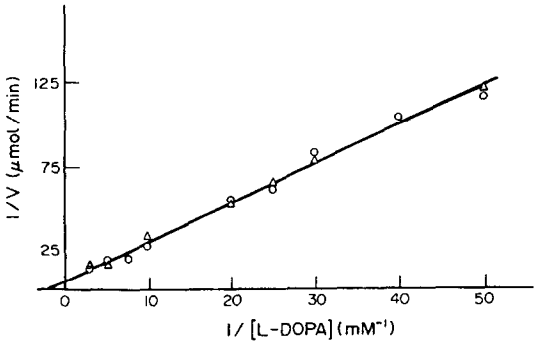


Fig. 7. Double-reciprocal plots for the oxidation of L-DOPA by rat intestinal peroxidase. (△) Initial rate measured at 483 nm; (○) initial rates measured at 306 nm. Each value is the mean of three separate determinations. Experimental conditions are given in the text.

calculate that the maximum velocity of tyramine oxidation in the crude homogenate would be about 565 nmol/mg/hr. These values suggest that intestinal peroxidase might make a contribution to tyramine metabolism under normal conditions and would have the potential to play a major role when MAO was inhibited.

The actual activity of the peroxidase *in vivo* is difficult to predict because of the complicated dependence of its activity on the ionic strength [15]. Furthermore, the activity may depend on an adequate supply of $H_2O_2$. Several metabolic processes together with the activities of resident and infiltrated lymphocytes could contribute to this. Indeed, in the absence of MAO inhibitors, one contributor might be MAO itself since $H_2O_2$ is a product formed during amine oxidation by this enzyme. With L-DOPA the apparent affinity and $V_{max}$ observed during peroxidase metabolism were greater than those reported for the L-aromatic amino acid decarboxylase present in supernatant preparations from rat kidney and brain ($K_m$ = 2 mM, $V_{max}$ = 33 and 8 nmol/mg protein/min, respectively) [31].

The peroxidation of L-DOPA, which yields a mixture of products [26–28], occurs more readily than that of tyrosine. One product of L-DOPA peroxidation would be the corresponding quinone [27]. This compound has been shown to react with sulphydryl groups of proteins to form cysteinyl-DOPA derivatives. These have been detected in brain following L-DOPA treatment and there have been speculations about their potentially damaging effects [32]. The possibility that such compounds may also be formed in the intestine as a result of L-DOPA ingestion requires direct investigation in terms of the possible damaging effects of long-term L-DOPA therapy.

Further work will be necessary before the extent of the possible pharmacological importance of these reactions can be assessed *in vivo*. They might affect both the availabilities of the compounds in the tissues after oral administration and the products might have distinct pharmacological actions. Indeed di-BHA has recently been shown to be a less potent inhibitor of the mitochondrial respiratory chain than BHA itself [33]. The possible activities of the dimers formed during the peroxidase-catalysed oxidation of BHA, morphine and tyramine are under investigation in this laboratory.

It is possible that other enzymes may also compete with intestinal MAO and peroxidase in the metabolism of the substrates considered here. Under *in vitro* conditions it has been shown that catechol derivatives are oxidized efficiently by xanthine oxidase (EC 1.2.3.2) [34]. However the significance of this *in vivo* is hard to assess, since it has been shown that in the gut, as well as in the liver and kidney, this enzyme is formed from xanthine dehydrogenase (EC 1.2.1.37) only after prolonged periods of hypoxia followed by re-oxygenation of the tissue [35–37]. Further experiments will be required to assess the abilities of these compounds to interact with the dehydrogenase.

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