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THE INTERACTION OF 5-HYDROXYTRYPTAMINE AND RELATED HYDROXYINDOLES WITH HORSERADISH AND MAMMALIAN PEROXIDASE SYSTEMS*

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Abstract—5-Hydroxytryptamine (5-HT) was found to be a competitive inhibitor with respect to guaiacol, and a non-competitive inhibitor with respect to H_2O_2 in a guaiacol–H_2O_2–horseradish peroxidase system. Other indolic compounds possessing a hydroxyl group in the C5 position behaved similarly to 5-HT, but those devoid of a nuclear substituted hydroxyl group were poor inhibitors. 5-HT altered the spectral characteristics of peroxidase, a shift from 417 to 399 nm occurring when an equimolar ratio of 5-HT and H_2O_2 is present. During such interaction 5HT fluorescence is lost and a yellow-brown product is formed. Attempts to characterize this product were only partially successful but it is suggested that it may possibly possess a dimeric structure. The formation of this product from ^{14}C 5-HT could be demonstrated in disrupted mammalian spleen preparations but not in spleen slices or brain synaptosomes.

The capacity of the peroxidase system to oxidize phenolic and indolic compounds has been known for many years, and a number of the oxidation products have been characterized [1, 2].

Purified horseradish peroxidase can catalyse the aerobic oxidation of indolylacetic, indolylpropionic and indolylbutyric acids [3–10]. These reactions involve the same uptake of oxygen and evolution of carbon dioxide per molecule of indolyl derivative as those catalysed by the indolylacetic acid "oxidase" systems found in various fungi and higher plants [4, 5, 10].

5-Hydroxytryptamine has been reported [11] to interfere with the production in the presence of peroxidase, of a fluorescent product from the reaction between homovanillic acid and H_2O_2.

We have recently demonstrated that 5-hydroxytryptamine (5-HT) and related indolic compounds interfere with commonly used assay systems for glucose, which depend on peroxidase to couple the hydrogen peroxide generated by the action of glucose oxidase, with a suitable hydrogen donor to give a chromogenic product [12].

The present work analyses the cause and structural dependence of this interference and suggests the possibility that the peroxidative degradation of 5-HT results in the formation of a coloured dimeric product. The *in vitro* formation of a similar product by mammalian peroxidase preparations has also been demonstrated.

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MATERIALS AND METHODS

Animals. Locally bred albino rats (Wistar strain 140–170 g) and albino mice (25–30 g) were used. They were kept under controlled conditions, fed on M.R.C. cube diet No. 41 and allowed water *ad lib.*

Enzymes. The following commercial preparations were obtained from Boehringer & Soehne, Mannheim: peroxidase (donor–H_2O_2 oxidoreductase; EC 1.11.1.7) from horseradish; catalase (H_2O_2 H_2O_2 oxidoreductase; EC 1.11.1.6) from beef liver; D-amino acid oxidase (D-amino acid–O_2 oxidoreductase; EC 1.4.3.3) from pig kidney; glucose oxidase (β-D-glucose–O_2 oxidoreductase; EC 1.1.3.4) of fungal origin; glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate; NADP oxidoreductase; EC 1.1.1.49) from yeast; glutamic-pyruvic transaminase (L-alanine–2-oxoglutarate aminotransferase; EC 2.6.1.2) from pig heart; hexokinase (ATP–D-hexose 6-phosphotransferase; EC 2.7.1.1) from yeast; lactate dehydrogenase (L-lactate–NAD oxidoreductase; EC 1.1.1.27) from rabbit muscle; uricase (urate–O_2 oxidoreductase; EC 1.7.3.3) from pig liver; xanthine oxidase (xanthine–O_2 oxidoreductase; EC 1.2.3.2) from milk.

Chemicals. There were obtained as follows: 5-hydroxytryptamine as the creatinine sulphate and bimaleinate salts—Koch-Light Laboratories Ltd, Colnbrook, Bucks; 5-hydroxytryptamine oxalate—Sigma Chemical Co., Missouri, U.S.A. All 5-HT salts were stored dessicated at 0–4° in the dark. Since solutions of 5-HT are somewhat unstable, particularly under alkaline conditions, fresh solutions were always prepared for each experiment and stored in the dark at 0–4° prior to use. All other indoles—R. Emanuel, Alper-

ton, Middlesex. Hydrogen peroxide, 30% (w/v)—British Drug Houses Ltd, Poole, Dorset; this was stored at 0–4° and periodically checked by titration against 0·1 N potassium permanganate. All other chemicals were the best grade commercially available, double glass distilled water being used for all solutions.

Radioactive compounds. These were obtained as follows: 3′-^{14}C–5-hydroxytryptamine (creatinine sulphate salt: 58 mCi/m-mole)—Radiochemical Centre, Amersham; 3′-^{14}C–5-hydroxytryptamine (binoxalate salt: 18 mCi/m-mole)—NEN Chemicals GmbH, Frankfurt, stored at −30° prior to use.

Enzyme assays: Peroxidase. The method was based on that of Chance and Maehly [13] which utilizes guaiacol as the hydrogen donor. The change in absorbance at 436 nm was measured with a Hilger Uvispek H700 spectrophotometer fitted with a Gilford Absorbancy Converter the volumes and final concentrations present in the 1 cm cuvette (thermostated at 25°) were as follows: total volume 3·0 ml, made up of 2·6 ml 0·1 M potassium phosphate buffer pH 7·0 (86·7 μM), 0·1 ml guaiacol (1·1 ml/l. = 330 μM), 0·2 ml hydrogen peroxide (0·04 ml 30% w/v per l. = 23·2 μM), 0·1 ml enzyme (usually 10 μg/ml = 0·18 activity units). Peroxidative oxidation of guaiacol followed at 436 nm for a minimum of 60 sec and the linear part of the curve used to calculate the initial reaction velocity. From this and using a value of 6·4 cm^2/μmole (436 nm) for the extinction coefficient of the oxidation product of guaiacol, the activity was calculated in terms of μmoles guaiacol oxidized/min per mg enzyme.

In those experiments involving 5-hydroxytryptamine (5-HT) and other indole analogues etc., these substances were diluted with potassium phosphate buffer to give a final concentration in the cuvette from 10^{-7} to 10^{-3} M. The results were expressed by plotting the per cent inhibition of the peroxidase activity against $\log_{10}$ of the indole concentration and the I_{50} and I_{20} determined for each inhibitor. The nature of the inhibitory effects observed was investigated by means of double reciprocal plots, two sets of experiments being required, one at a fixed hydrogen peroxide concentration, the other at a fixed guaiacol concentration. The conditions for these were designed (as far as possible) to incorporate the suggestions of Cleland [14].

The reversibility of peroxidase inhibition by 5-HT or an indole analogue was tested as follows: a fixed concentration (10 μg/ml) of peroxidase was preincubated at 25° for up to 6 hr with varying inhibitor concentrations (0, 2×10^{-3} M or 5×10^{-3} M) and 0·1 ml samples removed at various time intervals and assayed for peroxidase activity. After 6 hr the test and control enzyme solutions were dialysed at 4° against 0·1 M phosphate buffer (pH 7·0) for 2, 4 or 20 hr with frequent changes of buffer, and then assayed for peroxidase activity.

Catalase. In order to study the possible effect of 5-HT various methods were tried, including those of Cohen *et al.* [15] Goldstein [16] Luck [17] and Bonnichsen *et al.* [18] but they suffered either from insufficient sensitivity or interference from indoles present. Finally the iodometric method for determining the residual H_2O_2 (modified after Bonnichsen *et al.* [18]) was shown to be unaffected by the presence of 5-hydroxyindoles ($\leqslant 10^{-3}$ M).

For the estimation of catalase activity in sub-cellular fractions of liver, spleen and brain the spectrophotometric method of Luck [17] was used. The rate of decrease in absorbance at 240 nm was followed and (using a value of 0·036 cm^2/μmole for the extinction coefficient of H_2O_2 at 240 nm) the enzyme activity in terms of mmoles H_2O_2 destroyed/min per mg protein was determined.

Xanthine oxidase. The assay method was based on that of Praetorius and Poulsen [19], the rate of formation of uric acid being measured by the increase in absorbance at 293 nm. Using the extinction coefficient for uric acid at 293 nm of 12·5 cm^2/μmole the enzymic activity in μmoles uric acid formed/min per mg enzyme was calculated.

Uricase. Based on method of Praetorius [20]. Activity expressed as μmoles uric acid consumed/min per mg enzyme.

D-*Amino acid oxidase.* Assayed manometrically with Gilson differential respirometer using method of Boulander and Osteux [21] with D-alanine (10 μmoles/ml) as substrate.

Glucose oxidase. Manometric method of Kusai *et al.* [22].

Cytochrome oxidase. Method based on that of Smith [23] which measures the oxidation of reduced cytochrome *c* at 550 nm.

A value of 21·0 cm^2/μmole, for the extinction coefficient of cytochrome *c* at 550 nm was used to calculate the activity in terms of μmoles cytochrome *c* oxidized/min per mg protein.

Acid phosphatase. Method was based on that of Plummer [24], activity expressed as nmoles phenol liberated/min per mg protein.

Protein concentration. Method was that of Gornall *et al.* [25], bovine serum albumin (Armour Pharmaceuticals Co. Ltd.) being used as a standard.

Spectrum of peroxidase. The absorption spectrum of peroxidase was determined over the range 350–700 nm. Recordings made from 1 ml silica cells (1 cm light path) containing 0·7 ml of stock enzyme solution (70 μg). The position and size of Soret peaks at 399 and 417 nm was determined in the presence of various fixed concentrations (0–10^{-4} M) of 5-HT and varying concentrations (10^{-6} to 10^{-3} M) of H_2O_2. When the effect of other indoles was studied the H_2O_2 concentration was fixed at $2{\cdot}32 \times 10^{-5}$ M.

Fluorimetric analysis of 5-HT. The method was based on that of Udenfriend [26], the fluorescence of aqueous solutions of 5-HT being measured with an Aminco–Bowman spectrophotofluorimeter. An activation wavelength of 295 nm was used and the emitted fluorescence at a wavelength of 350 nm determined. The loss of fluorescence in the initial 2-min per-

iod of the enzymic reaction was compared by determining the difference between control and test cells, the results being expressed in arbitrary fluorescence units. Reaction system was contained in 3 ml 0·1 M phosphate buffer pH 7·0 and maintained at 25°.

Preparation and characterization of peroxidative products from 5-HT. Preparation method involved dissolving 100 mg of 5-HT (creatinine sulphate salt) and 2 μCi of ^{14}C–5-HT in 10 ml of 0·05 M phosphate buffer (precooled—pH 7·0) to give a 5-HT concentration of $2{\cdot}5 \times 10^{-2}$ M 0·2 ml of horseradish peroxidase (10 mg/ml) was then added and mixture kept on ice.

Four ml of a precooled aqueous solution of H_2O_2 ($4{\cdot}4 \times 10^{-2}$ M) was slowly added dropwise to the 5-HT–enzyme mixture with gentle shaking. (For column chromatography only, the mixture was concentrated to about 3 ml by rotary evaporation under reduced pressure at 40° in the dark, and then stored on ice until required).

Thin layer chromatography [27] on silica gel G plates (20 × 20 cm) was used to characterize the components of the preparative mixture produced above, and also to determine the effects of varying the molar proportions of the reactants (see Results section). The solvent system used contained ethylacetate–isopropanol 25% ammonia (45:35:30) by volume. Plates developed at 20° in darkened room, dried in air and viewed under ultraviolet light to detect any fluorescent areas. To further locate the separated components the radioactive areas were visualized by radioautography, using Kodak Blue Brand X-ray film, an exposure time of 14 days and developing and fixing with Kodak DX80 developer and FX40 fixer diluted 1:5 parts H_2O by volume.

The radioactive components were quantitized by scraping off the areas shown up by radioautography into scintillation vials containing 10 ml of "Cab-o-sil" (Koch-Light Ltd.), 10 ml of scintillation fluid containing 6 g 2,5-bis [5′-*t*-butyl-benzoazyl (2′)]-thiopene (BBOT)/l. of toluene was then added to form a thixotropic mixture, ^{14}C hexadecane being used as an internal standard.

Various colorimetric spray reagents were also employed to aid identification of the separated components as follows: (1) Ehrlich's reagent (*p*-dimethylaminobenzaldehyde, 10% w/v in conc. HCl/acetone (1:4 v/v). 5-Hydroxyindoles give a blue coloration (see Jepson [27]). (2) ninhydrin (0·2% in acetone) gives brownish purple with 5-HT. (3) α-nitroso-β-naphthol (0·1% in 95% ethanol). Gives violet chromophore with 5-hydroxyindoles after warming for 2 min at 50° with an equal volume of nitrous acid (see Renson *et al.* [28]). (4) Acidic diazo-reagent [27] specific for 6-hydroxyindoles giving a brilliant red colour. (5) Ferric chloride (60% w/v) containing 0·1% conc. HCl gives coloured complexes with phenols. (6) 2,4-Dinitrophenylhydrazine reagent was prepared by dissolving 0·5 g in 80 ml of conc. HCl, 100 ml H_2O added while mixture heated on a water bath, cooled and a further 120 ml H_2O added.

To separate the components of the preparative system various gel chromatographic procedures were tried including Sephadex G-15, LH-20 and LH-20 plus formamide. The LH-20 system gave a better result than G-15 Sephadex. A column (70 × 1·5 cm) of Sephadex LH-20 was equilibrated with 50% ethanol–25% ammonia (4:1 v/v) and adjusted to give a flow rate of approximately 8 ml/hr, 2 ml fractions being collected at 4° in the dark. Each fraction was analysed for (i) radioactivity by adding 0·05 ml to 10 ml BBOT-toluene–methanol scintillation mixture (6 g BBOT:900 ml toluene:100 ml methanol), ^{14}C hexadecane being used as an internal standard; (ii) the I_{300} and I_{400} of each fraction was determined (Unicam SP 800), the fraction occurring at the maximum of each peak being analysed by TLC as described above.

Further chromatography at 4° in the dark of the peak representing the peroxidative product (see Fig. 6) using a 10 × 3 cm column of Biorad Lab. resin AG 11A8 equilibrated with 50% ethanol (25 ml/hr: 2 ml fractions), followed by evaporation of major peak fractions under reduced pressure (40° in the dark), and vacuum dessication yielded a light-brown amorphous solid.

Preparation and assay of mammalian peroxidase. Rat spleen peroxidase was prepared according to the method of Neufeld *et al.* [29]. The peroxidase activity of the dialysed, resuspended pellet (75,000 *g*) was assayed spectrophotometrically at 25° using guaiacol as before [13], 0·1 mM 2,4-dichlorophenol being incorporated into the assay system to specifically inhibit endogenous catalase activity [30]. In experiments involving the incubation of 5-HT with this spleen peroxidase preparation, the enzyme was preincubated with 1 mM dichlorophenol prior to use. For most experiments 5-HT oxalate or creatinine sulphate (3×10^{-3} M) containing 3-^{14}C–5-HT (1 μCi) was incubated with the dialysed fraction for 10 min at 25° in the presence of various H_2O_2 concentrations. The mixture was then analysed by TLC as previously described.

Preparation of subcellular fractions: (1) *rat spleen.* A 20% homogenate of the spleens from five female rats was prepared in 0·25 M sucrose containing 0·1 mM EDTA, centrifuged (600 *g*, 10 min), the supernatant (S1) removed, the pellet resuspended in the sucrose medium (equivalent to the volume S1) and recentrifuged (600 *g*, 10 min). The supernatant (S2) was removed and combined with the supernatant S1. The combined supernatants were centrifuged (8000 *g*, 10 min) and supernatant S3 discarded. The pellet was resuspended in a volume of sucrose—EDTA equivalent to S3, recentrifuged (8000 *g*, 10 min), supernatant S4 removed and the residual mitochondrial pellet resuspended in a small volume of 0·25 M sucrose–EDTA.

The supernatants S3 and S4 were combined and centrifuged (15,000 *g*, 10 min), supernatant S5 removed and the pellet resuspended in a small volume of 0·25 M sucrose to form the lysosomal fraction. The supernatant S5 was centrifuged (100,000 *g*, 60 min), on a

Spinco Ultracentrifuge using a 40 rotor. The supernatant S6 i.e. the soluble fraction was removed and the residue resuspended in a small volume of sucrose (0·25 M) to form the microsomal fraction. All these operations were performed at 0–4°, and the various subcellular fractions stored on ice until required for analysis.

Each fraction was characterized by means of known marker enzymes. The enzyme assays for cytochrome oxidase (marker enzyme for mitochondria), and acid phosphatase (marker enzyme for lysosomes) were performed as described previously. The marker enzyme for the soluble fraction was lactic dehydrogenase which was assayed spectrophotometrically by the method of Bergmeyer *et al.* [31] enzyme activity being expressed in terms of the change in absorbancy at 340 nm/min per mg protein. The protein content of each fraction was also determined [25].

(2) *Rat liver peroxisomes.* Preparation by a modification of the method of Leighton *et al.* [32].

Of the crude mitochondrial fraction 0·8 ml was added to each of three cellulose nitrate centrifuge tubes (5 × 1 cm) containing 4·6 ml of a continuous sucrose gradient (density 1·17–1·26) and centrifuged (100,000 *g*, 170 min, Spinco L ultracentrifuge SW39 rotor). After centrifugation the various bands were carefully removed using a syringe and their volumes measured. Protein and enzyme analysis were carried out on each band as described previously.

(3) *Rat brain synaptosomes.* Prepared according to the method of De Robertis *et al.* [33].

After centrifugation the various fractions were carefully separated using a syringe and their volumes measured. Each fraction was then analysed for (a) protein concentration [25], (b) peroxidase activity as described previously (c) 5-HT content [26], the presence of 5-HT being used as a marker substance for synaptosomes.

RESULTS

Effects of 5-HT on the guaiacol–H_2O_2–horseradish peroxidase system. Previous studies [12] indicated that the ability of chromogenic hydrogen donors to react in standard H_2O_2–peroxidase assay system was reduced by 5-HT. We observed that 5-HT caused approximately 50 per cent inhibition of chromogen production from *o*-dianisidine; the ammonium salt of 2,2′-azino-di[3-ethyl-benzthiazoline-6-sulphonic acid] (ABTS); and from guaiacol at 5-HT concentrations of $7·5 \times 10^{-6}$ M; 6×10^{-6} M; and $2·5 \times 10^{-5}$ M respectively.

Further studies were made of the nature of this inhibitory effect of 5-HT using the standard Chance and Maehly assay [13] which utilizes guaiacol as the hydrogen donor, and from kinetic experiments involving double reciprocal plots [34] values for the $K_{m(app)}$ and $V_{max.}$ determined. It must be emphasized that since the $K_{m(app)}$ etc. of peroxidase is known to vary with hydrogen donor concentration the values which follow only apply to the specific conditions used.

The data for varying concentrations of H_2O_2 gave values for the $K_{m(app)}$ and $V_{max.}$ of $1·77 \pm 0·14 \times 10^{-5}$ M and $1·59 \pm 0·05$ m-moles guaiacol oxidized/min per mg peroxidase when $3·3 \times 10^{-4}$ M guaiacol was present. On the addition of $1·0 \times 10^{-5}$ M 5-HT the values obtained were $2·21 \pm 0·25 \times 10^{-5}$ M and $1·30 \pm 0·06$ respectively. Further increasing the 5-HT concentration to $2·0 \times 10^{-5}$ M gave values for $K_{m(app)}$ and $V_{max.}$ of $2·22 \pm 0·31 \times 10^{-5}$ M and $0·86 \pm 0·05$. These results indicate that 5-HT is a non-competitive inhibitor of the guaiacol–H_2O_2–peroxidase system with respect to H_2O_2. When the concentration of guaiacol was the variable parameter (the H_2O_2 concentration being fixed at $2·32 \times 10^{-5}$ M), in the absence of 5-HT the $K_{m(app)}$ was $0·34 \pm 0·04 \times 10^{-3}$ M and $V_{max.}$ was $1·71 \pm 0·06$ m-moles guaiacol oxidized/min per mg peroxidase. The addition of $1·0 \times 10^{-5}$ M 5-HT gave values of $0·61 \pm 0·07 \times 10^{-3}$ M and $1·55 \pm 0·06$ while at $1·0 \times 10^{-4}$ M 5-HT the $K_{m(app)}$ was $2·99 \pm 0·42 \times 10^{-3}$ M and the $V_{max.}$ $1·26 \pm 0·13$. These findings demonstrate that 5-HT is a competitive inhibitor with respect to guaiacol in the H_2O_2–peroxidase system. The reversibility of the 5-HT inhibition was studied (see Methods section) and the results indicated that the 5-HT inhibition of the guaiacol–H_2O_2–peroxidase system does not increase with time and progressively disappears on subsequent dialysis, about 90 per cent of original activity being restored after 20 hr, allowing for the slow inactivation of peroxidase under control conditions in the absence of added 5-HT.

Other experiments were carried out to compare the relative affinities of guaiacol, ABTS, *o*-dianisidine and 5-HT when acting as hydrogen donors in the horseradish peroxidase system. The results were as follows, the first value given relates to a constant initial H_2O_2 concentration ($3·3 \times 10^{-4}$ M) the second to a constant initial hydrogen donor concentration ($2·3 \times 10^{-5}$ M guaiacol and ABTS; $2·3 \times 10^{-6}$ M *o*-dianisidine; $2·3 \times 10^{-4}$ M 5-HT); $K_{m(app)}$ guaiacol $2·00 \pm 0·11 \times 10^{-5}$ M, $3·40 \pm 0·19 \times 10^{-4}$ M; ABTS $0·43 \pm 0·02 \times 10^{-5}$ M, $3·72 \pm 0·62 \times 10^{-4}$ M; *o*-dianisidine $2·87 \pm 0·40 \times 10^{-4}$ M, $10·11 \pm 0·99 \times 10^{-4}$ M; 5-HT $1·45 \pm 0·10 \times 10^{-4}$ M, $4·19 \pm 0·36 \times 10^{-4}$ M. Both plots involving 5-HT showed deviation from linearility at high values of [S] indicating the inhibitory effect of high concentrations of either H_2O_2 or 5-HT. It must again be emphasized that the comparative values quoted only apply to the specific conditions used since the $K_{m(app)}$ of peroxidase is known to vary with hydrogen donor concentration.

Structural requirements for interference of horseradish peroxidase system by indolic compounds. The effects of various compounds, including 5-hydroxyindoles, non-hydroxylated indoles, hydroxylated non-indolic compounds etc. on the Chance and Maehly assay system [13] are summarized in Table 1. These results show that indolic compounds possessing a hydroxyl group at C5 position are good inhibitors of peroxidase–H_2O_2–guaiacol reaction, in general exhibiting an I_{50} of approximately 2×10^{-5} M. Those indolic compounds

Table 1. The effects of various indolic and other hydroxylated compounds on the horseradish peroxidase–H_2O_2–guaiacol system

Compound	I_{50} (M)	I_{20} (M)
5-OH Tryptamine (creatinine sulphate salt)	2×10^{-5}	$3{\cdot}5 \times 10^{-6}$
5-OH Tryptamine (bimaleinate salt)	2×10^{-5}	3×10^{-6}
5-OH Indole	$0{\cdot}9 \times 10^{-5}$	4×10^{-6}
5-OH-N_w-Methyl tryptamine	2×10^{-5}	6×10^{-6}
5-OH-N_w-Dimethyl tryptamine oxalate (bufotenine)	2×10^{-5}	4×10^{-6}
5-OH-Indolyl acetic acid	2×10^{-5}	5×10^{-6}
5-OH-DL-Tryptophan	2×10^{-5}	6×10^{-6}
5-OH-D-Tryptophan	$2{\cdot}5 \times 10^{-5}$	6×10^{-6}
5-OH-Indole-2-carboxylic acid	6×10^{-5}	6×10^{-6}
Tryptophol	—	5×10^{-4}
Indole	—	1×10^{-3}
N-Acetyl-5-methoxy tryptamine (melatonin)	—	1×10^{-3}
L-Tryptophan	—	1×10^{-3}
Tryptamine	—	1×10^{-3}
Dopamine (3-OH tyramine)	1×10^{-5}	3×10^{-6}
5-OH-Quinoline	—	1×10^{-3}
8-OH-Quinoline	—	1×10^{-3}
2-OH-Purine	—	1×10^{-3}
Hydroxylamine	9×10^{-4}	6×10^{-4}
Potassium cyanide	7×10^{-6}	2×10^{-6}

Assay system as in Methods section. Molar concentrations of inhibitors giving 20% (I_{20}) and 50% (I_{50}) inhibition of $\Delta E_{436\,nm}$ with $2{\cdot}3 \times 10^{-5}$ M H_2O_2 and $3{\cdot}3 \times 10^{-4}$ M guaiacol.

devoid of a nuclear substituted hydroxyl group are relatively poor inhibitors of the peroxidase reaction, requiring millimolar concentrations to elicit 20 per cent inhibition. Other hydroxylated non-indolic compounds e.g. 8-OH quinoline, 5-OH quinoline, 2-OH purine etc. were poor inhibitors, although dopamine (3-OH tyramine) was strongly inhibitory (I_{50} $1{\cdot}0 \times 10^{-5}$ M) as was cyanide which was included for comparison. The monoamine oxidase inhibitors iproniazid (I_{20} 4×10^{-4} M), amino guanidine sulphate (I_{20} 2×10^{-3} M) were poor inhibitors, while isoniazid has no effect.

Further experiments with various 5-hydroxyindoles indicated that with the exception of 5-OH indole and 5-OH indole-2-carboxylic acid all the compounds studied were reversible non-competitive inhibitors with respect to the oxidant hydrogen peroxide. With 5-OH indole and 5-OH indole-2-carboxylic acid however the absorbance of the brown reaction product(s) slowly decreased during the spectrophotometric assay. This effect became more apparent as the indole concentration increased so that these compounds gave atypical double reciprocal plots.

Further studies on the nature of the 5-HT–H_2O_2-horseradish peroxidase interaction. The experiments described in this section may be subdivided as follows: (a) the changes in the absorption spectrum of horseradish peroxidase during its interaction with 5-HT and other 5-hydroxyindoles, (b) the stoichiometry of this interaction and (c) the loss of 5-HT fluorescence.

(a) Studies on the absorption spectral changes of peroxidase occurring during the interaction of horseradish peroxidase–H_2O_2 system with 5-hydroxyindoles.

5-Hydroxytryptamine was shown to alter the spectral characteristics of ferric chloride but not ferrous chloride, and since all peroxidases contain a ferric protohaemin as a prosthetic group the effect of 5-HT on the absorption spectrum of peroxidase was examined.

The visible absorption spectrum of peroxidase (shown in Fig. 1) is characterized by an intense Soret peak at approximately 399 nm (curve A), whereas in the presence of low concentrations of hydrogen peroxide ($< 10^{-4}$ M) the Soret peak shifts to approximately 417 nm (curve C) with a diminution in peak

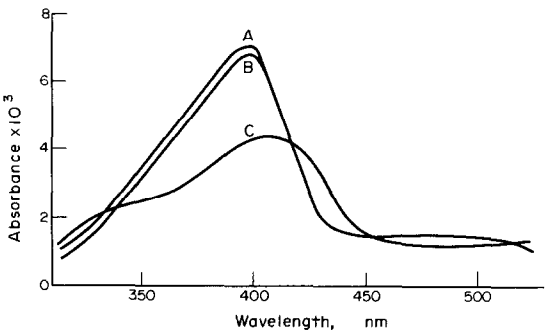

Fig. 1. The absorption spectra of horseradish peroxidase showing the Soret peak region in the absence and presence of H_2O_2 and 5-HT. Curve A is horseradish peroxidase in phosphate buffer (70 μg); curve B is horseradish peroxidase + H_2O_2 (1×10^{-5} M) + 5-HT (3×10^{-5} M); curve C is horseradish peroxidase + H_2O_2 (1×10^{-5} M). Note shift from 399 to 417 nm when H_2O_2 added reversed in presence of excess 5-HT.

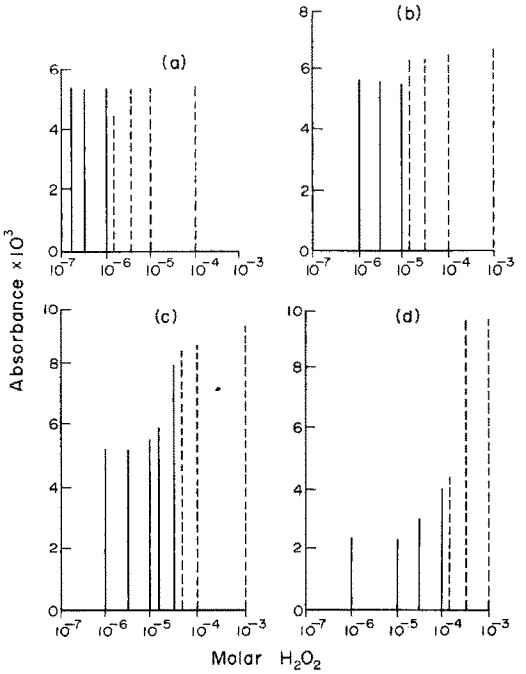

Fig. 2. The effect of varying hydrogen peroxide concentration (10^{-7} to 10^{-3} M) on Soret peak intensity and wavelength of the horseradish peroxidase–H_2O_2–5-HT system at various fixed concentrations of 5-HT. The solid line represents the Soret peak intensity at 399 nm i.e. horseradish peroxidase alone and the broken line represents the Soret peak intensity at 417 nm i.e. horseradish peroxidase in the presence of H_2O_2. The fixed concentrations of 5-HT are: curve a 10^{-6} M; curve b 10^{-5} M; curve c 5×10^{-5} M and curve d 10^{-4} M. Note reversal of Soret peak position when stoichiometry between H_2O_2 and 5-HT is 1:1.

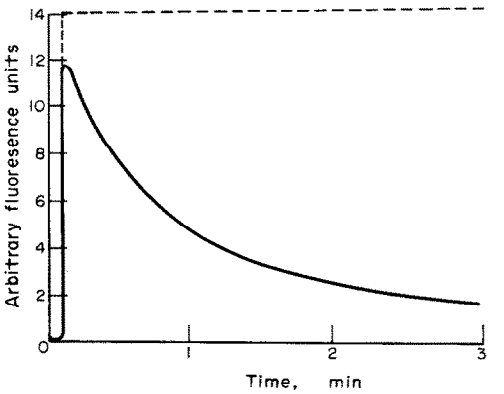

Fig. 3. 5-Hydroxytryptamine (10^{-4} M) fluorescence against time in minutes in the presence of the horseradish peroxidase (1 μg/ml) and H_2O_2 (5×10^{-5} M) system is shown by the solid curve. The broken curve shows the appropriate control solutions of 5-HT + enzyme and 5-HT + H_2O_2. The fluorescence was followed as λex 295 nm and λem 350 nm and expressed in arbitrary fluorescence units. At zero time horseradish peroxidase was quickly added and the fluorimeter shutter closed. Note rapid loss of 5-HT fluorescence.

height intensity i.e. indicating the formation of peroxidase–H_2O_2 II complex [35]. In addition minor peaks are present at 555 and 645 nm (not shown in Fig. 1). When 5-HT (3×10^{-5} M) was added to the peroxidase–hydrogen peroxide mixture (curve B) the position of the Soret peak returned to 399 nm suggesting that 5-HT reacts with the peroxidase–H_2O_2 II complex and H_2O_2 is destroyed in the course of the reaction.

(b) Stoichiometry of horseradish peroxidase–H_2O_2–5-HT interaction.

The precise stoichiometry between 5-HT and H_2O_2 in the presence of peroxidase was determined by measuring the position and intensity of the Soret peak at various fixed 5-HT concentrations 10^{-6} to 10^{-4} M in the presence of varying concentrations of H_2O_2 10^{-6} to 10^{-3} M. Figure 2 demonstrates that the spectral shift of the Soret peak position from 417 to 399 nm occurs when the added molar H_2O_2 concentration equals the molar 5-HT present i.e. a 1:1 relationship exists between 5-HT and H_2O_2 in the peroxidative oxidation of this amine.

Similar experiments with 5-hydroxyindole, 5-hydroxyindolyl acetic acid, 5-hydroxy-DL-tryptophan, 5-hydroxyindole carboxylic acid and 5-hydroxy-N_w-dimethyl tryptamine (bufotenine) also suggested a similar 1:1 ratio.

(c) Fluorescence changes during the interaction of 5-hydroxyindoles with the horseradish peroxidase H_2O_2 system.

In a preliminary experiment (Fig. 3) the fluorescence at ($\lambda ex^{295} \lambda em^{350}$ nm) (5×10^{-5} M) was recorded continuously for 3 min (Aminco Bowman Spectrophotofluorimeter). A 50 per cent loss of fluorescence occurred within 35 sec and over 85 per cent within 180 sec when 5-HT was present, there being little change for at least 100 min in the fluorescence of the appropriate control mixtures of 5-HT and enzyme and 5-HT and H_2O_2.

Confirmation that the loss of fluorescence was associated with the disappearance of 5-HT was obtained by thin layer chromatography of reaction mixture containing 3-^{14}C 5-HT (see next section and Fig. 5 for further details), and also by the demonstration of the simultaneous loss of biological activity in terms of the activity of the reaction mixture to contract a rat fundus strip, a well established assay for 5-HT activity.

Further experiments which measured the loss of 5-HT fluorescence in the initial period of 120 sec at various fixed 5-HT concentrations (10^{-4} to 10^{-6} M) and H_2O_2 concentrations (10^{-3} to 10^{-7} M) indicated that the minimal concentration of H_2O_2 necessary to produce the greatest loss of fluorescence is equivalent to the concentration of added 5-HT (Fig. 4). They thus confirm the 1:1 ratio between 5-HT and H_2O_2 deduced from the spectral shift experiments reported above.

Before attempting to isolate and characterize the products of this reaction further qualitative studies

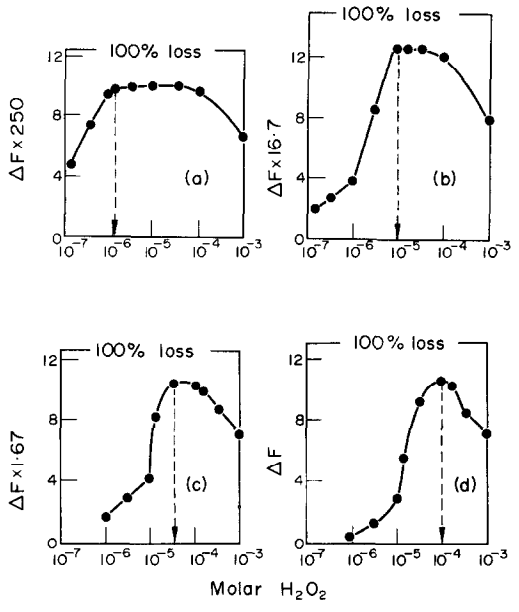

Fig. 4. The loss of 5-HT fluorescence (λex 295 nm; λem 350 nm) in the horseradish peroxidase (0·33 μg/ml)–H_2O_2 (10^{-7}–10^{-3} M) 5-HT system at various fixed concentrations of 5-HT. The broken arrow represents the minimal concentration of H_2O_2 required to produce the greatest loss of 5-HT fluorescence (ΔF) expressed in arbitrary fluorescence units. The fixed concentrations of 5-HT are: curve a 10^{-6} M; curve b 10^{-5} M; curve c 5×10^{-5} M and curve d 10^{-4} M. Note 1:1 stoichiometry of H_2O_2:5-HT when maximum fluorescence loss occurs.

were made. Firstly no requirement for oxygen could be demonstrated—judged by comparing the appearance and fluorescence of the reaction mixture (peroxidase 30 μg:H_2O_2 1–2 × 10^{-4} M:5-HT 1 × 10^{-4} all in 0·1 M phosphate buffer pH 7·0) following incubation at 25° for 30 min (Gilson Differential Respirometer) under 1·0 atmospheric oxygen or nitrogen, the flasks having been previously flushed for 15 min prior to initiating the reaction.

Other 5-hydroxyindoles gave the results shown in Table 2 when incubated under similar conditions. Thus indolic compounds possessing a hydroxyl group at the C5 position produce mainly yellow chromophores which darken in the presence of excess H_2O_2.

Compounds such as tryptophan and tryptophol which lack a C5 hydroxyl group did not produce chromophores. Indole itself appeared somewhat anomalous in giving a pale pink chromophore, but it is known to be oxidized by peroxidase–H_2O_2 systems to the trimer 2,2′-bis (3-indolyl)-indoxyl (yellow) as well as indoxyl red [1].

In another experiment the peroxidase inhibitory activity was shown not to be related to the yellow "5-HT" chromophore. A range of 5-HT concentrations (10^{-7} to 10^{-4} M) were preincubated for 30 min in the presence of peroxidase and a slight excess of H_2O_2, any residual H_2O_2 destroyed with catalase (0·01 ml, 7800 units) which is in turn inhibited after 10 min by 2,4-dichlorophenol (final concn 10^{-3} M). Each solution was then tested for its peroxidase activity suitable controls having been preincubated etc. in a similar manner. Only the highest concentration of 5-HT (10^{-4} M) which was reacted exhibited any inhibitory activity (~ 20 per cent) and this can probably be accounted for by the presence of some unreacted 5-HT, a possibility strengthened by it also being the only sample to retain any fluorescence at 350 nm when excited at 295 nm. The residual fluorescence value obtained was equivalent to 3·5 × 10^{-6} M 5-HT, in reasonable agreement with ~ 20 per cent inhibitory activity observed above.

The effect of 5-HT on other enzymes involved in H_2O_2 interactions. Catalase and the aerobic oxidases xanthine oxidase, glucose oxidase, D-amino acid oxidase and uricase were assayed (see Methods section) in the

Table 2. Chromophores produced with various hydroxyindoles by the horseradish peroxidase–hydrogen peroxide system

Indolic compound	Chromophore produced in presence of	
	Equimolar H_2O_2	Excess H_2O_2
5-Hydroxytryptamine (creatinine sulphate)	yellow	yellow–brown
5-Hydroxytryptamine (oxalate)	yellow	yellow–brown
5-Hydroxyindole	yellow	orange–yellow
5-Hydroxytryptophan	yellow	orange–yellow
5-Hydroxy-N_w-methyl tryptamine	yellow	pale brown
5-Hydroxyindole-2-carboxylic acid	yellow	orange brown
5-Hydroxyindole-3-acetic acid	yellow	yellow–brown
N-Acetyl-5-methoxy tryptamine	yellow–brown	brown
5-Hydroxy-2-oxindole	brown	dark brown
Tryptophol	no coloration	no coloration
DL-Tryptophan	no coloration	no coloration
Indole	pale pink	pale pink

Concentration of indole 1 × 10^{-4} M, reaction at pH 7·0 in 0·1 M phosphate buffer. No reaction observed in controls.

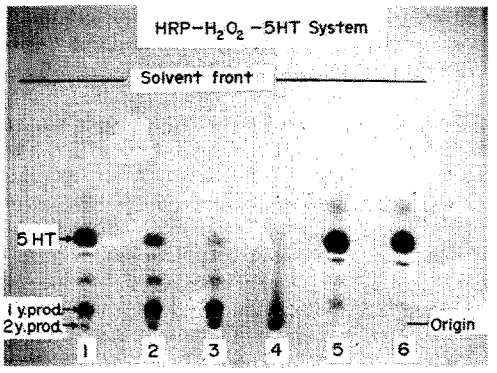

Fig. 5. Autoradiogram of TLC separation of a horseradish peroxidase (HRP)–H_2O_2–^{14}C-3'-5-HT (0·5 μCi)/unlabelled 5-HT *in vitro* system. The solvent system was ethyl acetate–isopropanol–25% NH_3 (45:35:20 by vol.). "1 y prod." represents primary yellow product R_f 0·09, "2 y prod." represents secondary brown product R_f 0·00 and 5-HT is shown at R_f 0·42. 1 = HRP (1 mg/ml) + H_2O_2 1·8 mM + 5-HT 2·9 mM; 2 = HRP + H_2O_2 3·6 mM + 5-HT 2·9 mM; 3 = HRP + H_2O_2 5·4 mM + 5-HT 2·9 mM; 4 = HRP + H_2O_2 22 mM + 5-HT 2·9 mM; 5 = H_2O_2 8·8 mM + 5-HT 2·9 mM and 6 = HRP + 5-HT 2·9 mM. Note progressive loss of 5-HT spot from 1 to 4; significant production of primary yellow product in 1–3; progressive appearance of secondary brown product from 1 to 4.

presence of 5-HT concentrations ranging from 10^{-6} to 10^{-3} M. No significant effect of 5-HT on the activity of any of these enzymes was observed and it was also demonstrated by fluorimetric measurements that none of these enzyme systems could significantly catalyse the breakdown of 5-HT.

Separation and attempted characterization of 5-HT–H_2O_2–peroxidase reaction products. As has been noted previously during the 5-HT–H_2O_2–peroxidase reaction generated a yellowish chromophore. Since this coloration did not occur in the control mixtures, containing only two of the three reactants, it was thought to represent the initial product of the reaction, and attempts made to isolate and characterize it.

The complete enzyme system (in 1 ml) of HRP (1 mg/ml)–H_2O_2 ($1·25 \times 10^{-3}$ M)–5-HT oxalate ($1·25 \times 10^{-3}$ M) containing 1 μCi of 3'-^{14}C-5-HT was incubated at pH 7 and 25° for 10 min and then chilled in ice. Other mixtures containing either excess H_2O_2 (2×10^{-2} M); HRP and 5-HT only or H_2O_2 and 5-HT only were similarly incubated, the H_2O_2 being added slowly dropwise to minimize local concentration effects. Samples (50 μl) of the reaction mixtures were spotted on silica gel G TLC plates and chromotographed (in the dark), the radioactive areas were visualized by autoradiography and their radioactivity estimated (see Methods section). Figure 5 shows the effect of varying hydrogen peroxide concentrations (1·8 to 22·0 $\times 10^{-3}$ M). At low H_2O_2 concentrations the yellow radioactive product (R_f 0·09; purple–blue fluorescence in u.v. light) is the initial product. This yellow product is decreased in amount as the H_2O_2 concentration is raised, being replaced by a brown melanin-like radioactive product (R_f 0·00; quenches in u.v. light). At the highest H_2O_2 concentration used the 5-HT (R_f 0·42; orange fluorescence in u.v. light) is completely metabolized. The initial product is also produced to some extent (~5 per cent) in the 5-HT–H_2O_2 control, and the minor radioactive sports (R_f's 0·31, 0·51, and 0·59) were found to be contaminants initially present in the 5-HT. Attempts were made to isolate the primary yellow product directly from the enzyme reaction mixture by a modified organic extraction method [36], but problems related to the instability of the yellow compound during recrystallization precluded the possibility of obtaining a pure sample.

Gel chromatography using Sephadex G15 equilibrated with 0·02 M ammonium bicarbonate or Sephadex LH-20 equilibrated with 50% ethanol 25% ammonia (4:1 v/v) gave better results and a separation achieved with Sephadex LH-20 is shown in Fig. 6. Six peaks were obtained, which by means of evidence based on TLC and absorption spectra were identified as follows: peak 1 = peroxidase; peak 2 = brown melanin-like product (R_f 0·00); peak 3 = primary yellow product (R_f 0·09–0·10); peak 4 = minor 5-HT

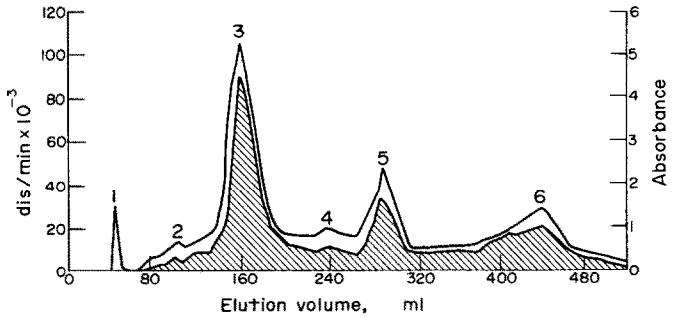

Fig. 6. Elution profile of a horseradish peroxidase–H_2O_2–^{14}C 5-HT/unlabelled 5-HT system using Sephadex LH20. Elution medium 50% ethanol/25% ammonia (4:1 v/v). The shaded curve represents ^{14}C radioactivity in dis/min $\times 10^3$ while the unshaded curve represents absorbancy measured at 300 nm. Peak 1 absorbancy is measured at 400 nm. Identities of peaks are: 1 = peroxidase, 2 = secondary brown product, 3 = primary yellow product, 4 and 6 = minor 5-HT contaminants, 5 = 5-HT.

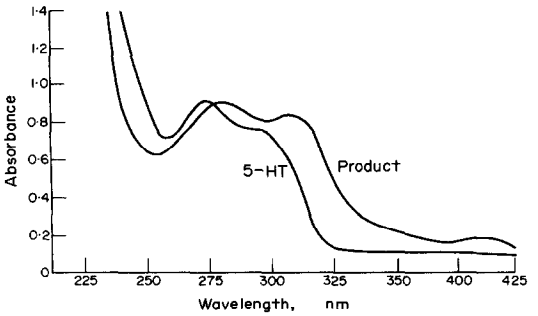

Fig. 7. Absorption spectra of 5-HT and its horseradish peroxidase catalysed primary yellow product using SP800 spectrophotometer. The product possessed two major peaks at 277 and 302 nm and a minor peak at 410 nm. 5-HT exhibits a characteristic spectrum with a single peak at 275 nm and a shoulder at 290 nm.

contaminant (R_f 0·30); peak 5 = 5-HT (R_f 0·42) peak 6 = 5-HT contaminant (R_f 0·50). The absorption spectra of peak 3 (primary yellow product) and peak 5 (5-HT) are shown in Fig. 7.

The combined fractions from the middle two-thirds of peak 3 were taken and reduced to a small volume under reduced pressure at 40° in the dark and dried *in vacuo*. Even under these conditions the ease of conversion of the primary yellow product to the brown melanin-like material prevented a successful molecular weight determination by mass spectroscopy. An approximation of the molecular weight was attempted utilizing the inverse linear relationship between K_{av} (partition coefficient between liquid phase and gel phase) and $\log_{10}$ molecular weight within the fractionation range of the gel. Since some adsorption of 5-HT and its peroxidative product occurred [37] ($K_{av} > 1$) a direct comparison of the calculated K_{av} values for 5-HT and its unknown product was made. This gave a value of 3·45 for 5-HT and 1·64 for the yellow product suggesting molecular weight of product is 2·1 times that of 5-HT.

Further qualitative tests made on peak 3 material from the Sephadex LH-20 columns, with the 5-HT peak 5 acting as a control, suggest that the primary product probably possesses the following characteristics:

A basic indole nucleus (positive reaction with Ehrlich's reagent in the cold); possesses the $—CH_2.CH_2.NH_2$ side chain at C3 (ninhydrin test positive; retention of ^{14}C from 3'-^{14}C–5-HT); does not apparently possess a hydroxyl group at C5 (α-nitroso-β-naphthol test negative).

Evidence for the peroxidative degradation of 5-HT in mammalian tissue. All the experiments reported above involve horseradish peroxidase, those which follow were designed to determine if the peroxidative degradation of 5-HT is also characteristic of animal tissues.

In vivo—radioactively labelled ^{14}C 5-HT (1 μCi) was injected intravenously into mice and a variety of tissue extracts analysed by TLC. Typically the labelled 5-HT disappeared rapidly (~ 55 per cent in 2 min) from the blood and radioactivity accumulated in the liver and spleen within 2 min, and in the kidneys by 10 min. No radioactivity was detected in the brain thus confirming the findings of Axelrod and Inscoe [38]. Apart from the presence of an indolic spot (Ehrlich test positive) with an R_f of 0·95 in spleen no detectable amounts of any 5-HT metabolites were found.

In vitro—since rat spleen contains high levels of peroxidase 6·4 units/mg dry wt [29]) and 5-HT (2·90 μg/mg wet wt [39]) it was selected for studying possible peroxidase—5-HT interactions in mammals.

Rat spleen peroxidase activity prepared by the method of Neufeld *et al.* [29], was measured in the presence and absence of 5-HT by the standard guaiacol assay used previously [13] except that 10^{-3} M 2,4-dichlorophenol was introduced into the system to specifically inhibit catalase. These experiments demonstrated that 5-HT also has a similar inhibitory action on rat spleen peroxidase (I_{50} 2×10^{-5} M 5-HT) to that on the horseradish enzyme, being a competitive inhibitor with respect to guaiacol and a non-competitive inhibitor with respect to H_2O_2. Addition of the monoamine oxidase inhibitor iproniazid (10^{-4} M) did not affect the inhibitory action of 5-HT suggesting that effect is due to 5-HT and not its deamination product 5-hydroxyindoleacetic acid.

Further experiments studied the peroxidative degradation of 5-HT by rat spleen preparations. The system comprised 0·3 ml of a dialysed rat spleen peroxidase preparation; 1×10^{-3} M 2,4-dichlorophenol; 3×10^{-3} M ^{14}C–5-HT oxalate (1·0 μCi); and $1{\cdot}46 \times 10^{-3}$ M H_2O_2 in a total volume of 0·6 ml 0·1 M phosphate buffer pH 7·0 at 37°. After ten minutes 25 μl samples were chromatographed on silica gel plates, the separated compounds located (a) by viewing under ultra violet light and (b) with Ehrlich's reagent, scraped off the plate and their ^{14}C content determined. In the control samples (i.e. 5-HT + peroxidase and 5-HT + H_2O_2) no detectable breakdown of 5-HT occurred, but when the complete 5-HT–peroxidase–H_2O_2 system was present over 25 per cent of the ^{14}C–5-HT was converted to a product with a R_f of 0·10. This material possessed similar chromatographic and fluorimetric properties to the primary product described previously for the horseradish peroxidase system, and also gave similar reactions with Ehrlich's and ninhydrin reagents. Two other components at R_f 0·04 and 0·0 were also produced but in much smaller amounts.

Attempts to repeat these findings using rat spleen slices instead of disrupted preparations were unsuccessful although uptake experiments showed that nearly 30 per cent of the labelled 5-HT had been taken up by the slices in a 30 min incubation period. On analysis of both the spleen slice and the medium after incubation, apart from a fast moving spot (R_f 0·95) present in both control (no spleen slice) and experimental samples, and which proved to be an extraction artifact, only unchanged ^{14}C–5-HT was detected.

Further studies on subcellular fractions of rat spleen and liver showed that the majority of the peroxidase activity is present in the lysosomal (37 per cent) and mitochondrial (44 per cent) fractions of spleen while none could be detected in the liver fractions. The crude spleen mitochondrial fraction was further separated by density gradient centrifugation (see Methods section for details) to give three sub-fractions designated α, β, γ. The α sub-fraction (small amount of brownish material at density 1·178–1·185; significant cytochrome oxidase activity) could represent "light mitochondrial" material and contained 10 per cent of peroxidase activity; the β sub-fraction (pale brown material at density 1·193–1·209; high cytochrome oxidase activity) representing the "true mitochondrial" material contained 17 per cent of peroxidase activity while the γ sub-fraction (red-brown pellet at density 1·225–1·230; high acid phosphatase activity) comprising the "lysosomal" material contained over 70 per cent of the peroxidase activity. Catalase activity on the other hand was relatively evenly distributed between the α, β and γ fractions. In similar experiments to those described previously, the γ fraction from rat spleen catalysed the formation of the primary product from 5-HT when H_2O_2 was present, the catalase activity being inhibited by the addition of 10^{-3} M 2,4-dichlorophenol.

Obviously the possibility existed that this γ fraction could contain peroxisome-like particles similar to those found in liver and kidney by De Duve and Baudhuin [40]. However experiments involving the intraperitoneal injection of Triton WR-1339 and the preparation method of Leighton *et al.* [32] failed to show the presence of such particles in rat spleen.

Finally whole brain rat synaptosome preparations were assayed for peroxidase activity but only a very low activity was detected in the gray synaptosomal fraction at density 1·140–1·170, none being found in an off-white fraction at density 1·030–1·108 or in the light brown pellet at density 1·190. Attempts to demonstrate the peroxidative degradation of 5-HT by the synaptosomal fraction were unsuccessful.

DISCUSSION

The results reported clearly establish the structural requirements for the interaction of indolic compounds with peroxidase systems. Indoles substituted with a hydroxyl group at C5 inhibited the peroxidase catalysed oxidation of guaiacol, the i_{50} being about 2×10^{-5} M (Table 1). Non-hydroxylated indoles on the other hand were only slightly inhibitory even at concentrations of 10^{-3} M.

Evidence derived from kinetic studies of both the horseradish and mammalian peroxidase–H_2O_2–guaiacol systems indicates that 5-HT behaved as a competitive inhibitor with respect to guaiacol, and as a non-competitive inhibitor with respect to H_2O_2. The inhibition of the guaiacol reaction could be abolished by increasing the guaiacol concentration suggesting that 5-HT might act as a competing hydrogen donor in the peroxidase system. Indole itself has been reported to act as a hydrogen donor in the horseradish peroxidase–H_2O_2 system to produce a dimer (indoxyl red) and a trimer (2,2′-bis (3-indolyl)-indoxyl) as major products [1] while homovanillic acid and *p*-hydroxyphenyl-acetic acid have also been shown to form dimers in the presence of peroxidase [2]. In our experiments however 10^{-3} M indole only inhibited the peroxidase–H_2O_2–guaiacol system by 20 per cent indicating that the presence of a C5 hydroxyl group greatly promotes the ability of the indole nucleus to act as a hydrogen donor in the peroxidase system, while melatonin (*N*-acetyl-5-methoxy tryptamine) was a poor inhibitor, suggesting that the hydrogen atom of the C5 hydroxyl may play an integral part in the peroxidative interaction with 5-hydroxyindoles. It is possible of course that the presence of the *N*-acetyl group could affect in the same way the ability of melatonin to act as an effective hydrogen donor in this system. Such results correlate with the previous report [11] that adrenaline, noradrenaline and 5-HT interfere with the ability of homovanillic acid to react with an H_2O_2–peroxidase system. Obviously it would be of interest to test indole derivatives with hydroxyl groups in the C4, C6 or C7 positions but such compounds were not available to us.

Except for minor variations involving 5-hydroxyindole and 5-hydroxyindole-2-carboxylic acid all the 5-hydroxyindoles tested behaved like non-competitive inhibitors with respect to H_2O_2 in the horseradish peroxidase–H_2O_2–guaiacol system. A possible explanation for these observations is that in a peroxidase–H_2O_2–guaiacol system containing a fixed inhibitory concentration of a 5-hydroxyindole, an increase in the H_2O_2 concentration simply provides an increased availability of oxidant for both the 5-hydroxyindole and guaiacol to act as hydrogen donors in the peroxidase system. Such a situation would be characterized by the production of double reciprocal plots typical of non-competitive inhibition with respect to H_2O_2.

The evidence from the changes in absorption spectra and loss of fluorescence (see Figs. 1–4) clearly establishes the enzymic nature of the interaction between 5-HT and the peroxidase system and that 5-HT is converted with loss of fluorescence to a yellow biologically inactive compound. In the absence of H_2O_2 the Soret peak of peroxidase is found at 399 nm shifting to 417 nm on the addition of H_2O_2 [35]. When a concentration of 5-HT greater than that of the H_2O_2 present is added to the peroxidase–H_2O_2 system, the Soret peak immediately shifts back from 417 to 399 nm (see Fig. 2). A probable explanation for such a spectral "shift" is that H_2O_2 is consumed during the enzymic degradation of 5-HT.

Edelhoch and Wilchek [41] observed that the fluorescence of tryptophyl residues in peptides was quenched by the conversion of phenolic groupings into the phenolate anions and we suggest an analogous conversion of the C5 hydroxyl into a C5 ($-O^-$) anion

might account for the loss in fluorescence during the interaction of peroxidase with 5-HT.

At least four reaction products were detected in the peroxidase–H_2O_2–5-HT system but only the initial yellow product was considered to be of possible physiological significance. Since its instability precluded exact characterization we can only make suggestions as to its structure. From the gel chromatography experiments the molecular weight of this product appears to be approximately twice that of 5-HT. This is in agreement with the fact that indoles are known to dimerize [1, 42] and also that the majority of known hydrogen donors in the peroxidase–H_2O_2 system tend to form dimeric or polymeric products [1, 2, 9, 43]. Of those phenolic compounds known to dimerize in a peroxidase system [1, 2, 43], the dimeric structures previously suggested for the peroxidative products involve direct dehydrogenative coupling of the aromatic ring and retain the original monomeric hydroxyl groups. However, since we were unable to demonstrate the presence of a C5 hydroxyl group in our "dimeric" yellow product it is possible that it may possess a different structure. For example, a dimer could well be formed from two 5-HT moieties bonded through the oxygen atoms of their C5 hydroxyl groups instead of via dehydrogenative coupling of the aromatic nucleus.

The formation of coloured compounds from 5-HT is well documented and a number of enzyme systems have been implicated including monoamine oxidase [44] ceruloplasmin [45], hydroxyindole oxidase [46] and cytochrome oxidase [47] as well as the chemical systems silver nitrate/Cu^{2+} [45]. Of the enzyme mediated reactions those involving hydroxyindole oxidase and cytochrome oxidase show oxygen dependence and therefore differ from the system under discussion. The monoamine oxidase reaction also involves changes in the side chain due to the oxidative deamination and any polymer of the product is unlikely to be similar to the dimer postulated.

Eriksen *et al.* [45] suggest that one of the ceruloplasmin reaction products is a dimer formed by dehydrogenative coupling of the benzene ring moieties of two molecules of 5-HT. Although the visible spectrum of this compound was not given, the ultraviolet spectrum is basically very similar to that of the primary yellow peroxidase reaction product, apart from the latter relatively lower intensity at 305 nm (see Fig. 7). Other differences are the presence of a C5 hydroxyl group in Eriksen *et al.* structure [45], and that neither 5-hydroxytryptophan nor 5-hydroxyindole were effective substrates for the ceruloplasmin system.

Blum and Ling [48] studied the pseudo-peroxidative degradation of 5-hydroxyindoles and found that incubation of 5-HT of bufotenine with denatured oxyhaemoglobin resulted in the formation of pink products of unknown structure. Structural determinations proved unsuccessful owing to their instability but these authors postulated they could be derived from the quinone derivative of 5,6-dihydroxytryptamine.

Thus 5-hydroxyindoles can undergo peroxidative degradation the coloured products varying with the enzyme concerned. No coloured products or 5-HT degradation could be demonstrated with catalase or the aerobic dehydrogenases uricase, glucose oxidase, D-amino acid oxidase or xanthine oxidase.

In view of the possibly novel nature of the 5-HT H_2O_2–peroxidase reaction product the question of its possible physiological significance must be considered. Although we have demonstrated its formation by horseradish peroxidase and by mammalian peroxidase preparations from rat spleen, attempts to show the formation of the primary yellow product by spleen slices, as distinct from disrupted spleen preparations, were unsuccessful. The spleen peroxidative activity was shown to be predominately in the mitochondrial and lysosomal fractions and further sub-fractionation of the "crude mitochondrial fraction" indicated the peroxidase activity was mainly associated with a lysosomal sub-fraction.

One problem with the localization of enzymic activity in sub-cellular fractions is the possibility of contamination with material, either bound to, or possessing a similar buoyant density to the fraction under investigation. Thus the peroxidase activity present in lysosomal fraction may originate from elsewhere in the cell, indeed it has been suggested that the peroxidase activity found in spleen may result from the verdoperoxidase present in the leucocyte population characteristic of this tissue [29].

Lysosomal preparations may also contain particles of a peroxisomal nature, but no such particles could be separated from rat spleen, using method of Leighton *et al.* [32]. Rat liver peroxisomes appeared to be devoid of detectable peroxidase activity although containing a considerable proportion of the total catalase activity, did not convert 5-HT to the yellow product. Finally synaptosomal preparations known to contain 5-HT, were also tested for their peroxidative activity against exogenous 5-HT and H_2O_2 but no activity could be detected.

Thus our investigations have not provided any convincing evidence to suggest that the peroxidase catalysed degradation of 5-HT is an important route under normal physiological conditions. It is possible however that under abnormal conditions such a pathway might assume greater significance. For example the accepted major route for 5-HT catabolism is via monoamine oxidase, but under circumstances where this enzyme is inhibited, the peroxidative reaction might play a more prominent role. Another possibility is that in tumours known to possess both high levels of peroxidase and 5-HT [49, 50], the peroxidative process may be operative.

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REFERENCES

1. B. C. Saunders, A. G. Holmes-Siedle and B. P. Stark, in *Peroxidase*, pp. 25, 186. Butterworths, London (1964).
2. G. C. Guilbault, P. J. Brignac and M. Zimmer, *Anal. Chem.* **40,** 190 (1968).
3. W. A. Gortner and M. Kent, *J. biol. Chem.* **204,** 593 (1953).
4. R. H. Kenten, *Biochem. J.* **59,** 110 (1955).
5. R. H. Kenten, *Biochem. J.* **61,** 353 (1955).
6. P. M. Ray and M. V. Thimann, *Science, N.Y.* **122,** 187, (1955).
7. Y. W. Tang and J. Bonner, *Archs Biochem.* **13,** 11 (1947).
8. P. M. Ray, *Ann. Rev. Plant Physiol.* **9,** 81 (1958).
9. H. S. Mason, *Advan. Enzymol.* **19,** 79 (1957).
10. A. C. Wageknecht and R. H. Burris, *Archs Biochem.* **25,** 30 (1950).
11. S. H. Snyder and E. D. Hendley, in *Methods of Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. 17B, p. 741. Academic Press, New York (1971).
12. D. R. Nelson and A. K. Huggins, *Anal. Biochem.* **59,** 46 (1974).
13. B. Chance and A. C. Maehly, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. 2, p. 764. Academic Press, New York (1955).
14. W. W. Cleland, in *Advances in Enzymology* (Ed. F. F. Nord), Vol. 29, p. 1. Interscience, New York (1967).
15. G. Cohen, D. Demdiec and J. Marcus, *Anal. Biochem.* **34,** 30 (1970).
16. D. B. Goldstein, *Anal. Biochem.* **24,** 431 (1968).
17. H. Luck, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), p. 885. Academic Press, New York (1963).
18. R. K. Bonnichsen, B. Chance and H. Theorell, *Acta Chem. Scand.* **1,** 685 (1947).
19. E. Praetorius and H. Poulsen, *Scand. J. clin. Lab. Invest.* **5,** 273 (1953).
20. E. Praetorius, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), p. 500. Academic Press, New York (1963).
21. P. Boulanger and R. Osteux, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), p. 367. Academic Press, New York (1963).
22. K. Kusai, I. Sekuzo, B. Hagihara, K. Okunuki, S. Yamauchi and M. Nakai, *Biochim. Biophys. Acta* **40,** 555 (1960).
23. L. Smith, *Archs Biochem. Biophys.* **50,** 285 (1954).
24. D. T. Plummer, in *An Introduction to Practical Biochemistry* p. 325. McGraw-Hill, London (1971).
25. A. G. Gornall, C. J. Bardawill and M. M. David, *J. biol. Chem.* **177,** 751 (1949).
26. S. Udenfriend, H. Weissbach and B. B. Brodie, in *Methods of Biochemical Analysis* (Ed. D. Glick), Vol. 6, p. 95. Interscience, New York (1958).
27. J. B. Jepson, in *Chromatographic and Electrophoretic Techniques* (Ed. I. Smith), Vol. 1, p. 183. Heinemann, London (1960).
28. J. Renson, H. Weissbach and S. Udenfriend, *J. biol. Chem.* **237,** 2261 (1962).
29. H. A. Neufeld, A. N. Levay, F. V. Lucas, A. P. Martin and E. Stotz, *J. biol. Chem.* **233,** 209 (1958).
30. P. L. Goldacre and A. W. Galston, *Archs Biochem. Biophys.* **43,** 169 (1953).
31. H. U. Bergmeyer, E. Bernt and B. Hess, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), p. 736. Academic Press, New York (1963).
32. F. Leighton, B. Poole, H. Beaufay, P. Baudhuin, J. W. Coffey, S. Fowler and C. de Duve, *J. cell. Biol.* **37,** 482 (1968).
33. E. De Robertis, A. Pellegrino De Iraldi, G. Rodriguez De Lores Arnaiz and L. Salganicoff, *J. Neurochem.* **9,** 23 (1962).
34. H. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56,** 658 (1934).
35. D. Keilin and T. Mann, *Proc. Roy. Soc.* **122B,** 119 (1937).
36. D. F. Bogdanski, A. Pletscher, B. B. Brodie and S. Udenfriend, *J. Pharmac. exp. Ther.* **117,** 82 (1956).
37. M. Wilk, J. Rochlitz and H. Bende, *J. Chromatog.* **24,** 414 (1966).
38. J. Axelrod and J. K. Inscoe, *J. Pharmac. exp. Ther.* **141,** 161 (1963).
39. V. Erspamer, in *Handbook of Experimental Pharmacology* (Eds. O. Eichler and A. Farah), Vol. 19, p. 132. Springer, Berlin (1966).
40. C. de Duve and P. Baudhuin, *Physiol. Rev.* **46,** 323 (1966).
41. H. Edelhoch and M. Wilchek, *Biochemistry* **6,** 547 (1967).
42. G. F. Smith, *Advan. Heterocyclic Chem.* **2,** 287 (1963).
43. G. S. Bayse, A. W. Michaels and M. Morrison, *Biochim. biophys. Acta* **284,** 34 (1972).
44. H. Blaschko and K. Hellmann, *J. Physiol.* **122,** 419 (1953).
45. N. Eriksen, G. M. Martin and E. P. Benditt, *J. biol. Chem.* **235,** 1662 (1960).
46. H. Blaschko and W. G. Levine, *Br. J. Pharmac.* **15,** 625 (1960).
47. L. J. Weber and A. Horita, *Life Sci.* **2,** 44 (1963).
48. J. J. Blum and N. S. Ling, *Biochem. J.* **73,** 530 (1959).
49. W. Lovenberg and K. Engelman, in *Methods of Biochemical Analysis* (Ed. D. Glick), suppl. vol., p. 1. Wiley–Interscience, New York (1971).
50. H. A. Neufeld, F. V. Lucas, A. P. Martin and E. Stotz, *Cancer Res.* **15,** 550 (1955).