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THE OXIDATION OF *p*-PHENETIDINE BY HORSERADISH PEROXIDASE AND PROSTAGLANDIN SYNTHASE AND THE FATE OF GLUTATHIONE DURING SUCH OXIDATIONS

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Abstract—The oxidation of *p*-phenetidine by horseradish peroxidase and prostaglandin synthase was investigated. The existence of a free radical intermediate formed during enzymatic oxidation was supported by a ratio of hydrogen peroxide: *p*-phenetidine consumed of 1 : 2 in the horseradish peroxidase system. Furthermore in both enzyme systems a rapid oxidation of added glutathione was observed and in the presence of the thiol there was a decreased removal of *p*-phenetidine. This suggests the reduction of a *p*-phenetidine radical by glutathione generating *p*-phenetidine and a thiyl radical. The latter react with oxygen and a rapid oxygen uptake was observed during enzymic oxidation in the presence of thiols. That *p*-phenetidine radicals were produced during horseradish peroxidase catalyzed oxidation of *p*-phenetidine was supported by experiments using the spin probe OXANOH. This was oxidized to its stable free radical form (OXANO˙) in an enzyme- and substrate-dependent reaction and the EPR signal obtained was not decreased by SOD (80 μg/ml) or benzoate (10–100 mM). TLC characteristics of the products of the oxidation of *p*-phenetidine by both enzymes were almost identical inferring a similar mechanism of oxidation. Two of the metabolites were characterized by mass spectrometry and by comparison with reference compounds prepared by chemical oxidation. One metabolite was identified as 4,4′-diethoxyazobenzene, which further supports a radical mechanism, and the other was a *p*-phenetidine trimer which could exist in both oxidized and reduced forms. On the basis of these observations a mechanism for the oxidation of *p*-phenetidine and the fate of glutathione during such oxidations is proposed.

There are several examples of xenobiotics which are metabolized by peroxidases such as horseradish peroxidase (HRP)[2] and prostaglandin synthase (PGS)§ [1, 2]. With some compounds, such as certain aromatic amines, these reactions can result in the formation of reactive metabolites which may bind to protein and nucleic acids [3–5] and induce mutations in bacteria [6–8]. Since PGS activity is relatively high in certain extrahepatic tissues such as the lung, kidney and gastrointestinal tract [9], it has been suggested that this enzyme may catalyze the metabolic activation of compounds which exhibit extrahepatic toxicity and carcinogenicity [4].

Phenacetin is an analgesic and antipyretic drug which is recognized to be responsible for several toxic effects in the kidney and lower urinary tract [10–12]. The mechanism of this toxicity is not well understood but as PGS activity is high in the kidney a PGS-catalyzed activation has been suggested to be involved [13]. Phenacetin itself is not a substrate for PGS but *p*-phenetidine and acetaminophen, major metabolites of phenacetin both *in vitro* [14] and *in vivo* [15], are [13]. We have previously shown that HRP and PGS in ram seminal vesicle microsomes (RSVM) are able to metabolize both acetaminophen and *p*-phenetidine to species which covalently bind to protein [13, 16]. Peroxidase-catalyzed activation of *p*-phenetidine however, unlike that of paracetamol, results in the production of species which bind to DNA and induce DNA strand breaks in cultured human fibroblasts [13].

The aim of this study was to investigate the mechanisms of the HRP- and PGS-catalyzed oxidation of *p*-phenetidine and in particular to determine if *p*-phenetidine free radicals, electron deficient species which may interact with cellular nucleophiles such as reduced glutathione (GSH), were produced in these reactions. In addition the isolation, and characterization of some of the products of the peroxidase-catalyzed oxidation of *p*-phenetidine are described and the relevance of these findings to the production of reactive species in such reactions is discussed.

MATERIALS AND METHODS

Arachidonic acid (AA), GSH and oxidized glutathione grade III (GSSG), superoxide dismutase (SOD), HRP type VI (250 U/mg), catalase (3400 U/mg) and 5,5-dimethyl-l-pyrroline-N-oxide (DMPO) were purchased from Sigma Chemical Co. (St. Louis,

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§ Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; AA, arachidonic acid; PGS, prostaglandin synthase (EC 1.14.99.1.); HRP, horseradish peroxidase (EC 1.11.1.7); H_2O_2, hydrogen peroxide; MS, mass spectrometry; RSVM, ram seminal vesicle microsomes; OXANOH, 2-ethyl-1-hydroxy-2,5,5-trimethyl-3-oxazolidine; OXANO˙, 2-ethyl-2,5,5-trimethyl-3-oxazolidinoxyl; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; SOD, superoxide dismutase.

MO). Thin layer chromatography (TLC) plates (Silica gel 60, 0.25 and 2 mm) were obtained from Merck (Darmstadt, W. Germany). Nucleosil (C_{18} 10 μ) was obtained from Macherey–Nagel & Co., Düren, W. Germany and PIC-B7 ion pairing reagent was purchased from Waters Associates (Milford, MA). [^{3}H]AA (127 Ci/mmole) was obtained from the Radiochemical Centre, Amersham, England. [^{14}C]-*p*-phenetidine hydrochloride was synthesized as before [16]. All other chemicals and solvents were of analytical grade and purchased from local commercial suppliers. 2-ethyl-2,5,5-trimethyl-3-oxazolidinoxyl (OXANO·) was a gift from Dr. L. I. Olsson, Pharmacia Fine Chemicals AB (Uppsala, Sweden) and its reduced form (OXANOH) was prepared as described previously [17].

Incubation of p-*phenetidine with HRP/H_2O_2.* A standard incubation mixture (1 ml) contained 0.5 mM *p*-phenetidine hydrochloride, 0.2 μg/ml HRP and 1 mM hydrogen peroxide (H_2O_2) in 1 ml of phosphate buffer 100 mM (pH 8) containing EDTA 1 mM. Reactions were performed at 25°, initiated by the addition of H_2O_2 and terminated by the addition of catalase (50 μl, 1500 U/ml).

For measurement of *p*-phenetidine consumption, limiting concentrations of H_2O_2 were used (0–0.2 mM). That these concentrations were limiting was established for each value by determination of time-courses of *p*-phenetidine disappearance. From these results an incubation time of 15 min was chosen for experiments, including those conducted in the presence of thiols.

Source of PGS activity. RSVM was prepared as described previously [18]. Protein was determined by the method of Lowry [19].

Incubation of p-*phenetidine with PGS/AA.* A standard incubation mixture (1 ml) contained *p*-phenetidine hydrochloride 0.05 mM, 1 mg RSVM in phosphate buffer 100 mM (pH 8) containing EDTA 1 mM, and the reactions were initiated by the addition of AA 0.05 mM. Reactions were performed at 25° and in some cases contained GSH 1 mM.

Measurement of p-*phenetidine consumption.* This was demonstrated essentially as described previously for the estimation of phenacetin in the presence of its metabolites [20].

A spectral Physics 3500 liquid chromatograph was used equipped with a C_{18} column (10 μm, Nucleosil) and a u.v. detector ($\lambda = 223$ nm). The mobile phase was CH_3CN:H_2O:PIC-B7:30:70:1,6 and was eluted isocratically at a flow rate of 1 ml/min. The sample (20 μl) was injected by the use of a WISP 710B. The limit of detection of *p*-phenetidine was 0.2 μg/ml, the coefficient of variation of peak height of six repeated injections of a standard was $< 3\%$ of the mean, and all calibration lines had correlation coefficients of $r^2 > 0.99$.

Oxygen uptake. Oxygen uptake was monitored at 25° with a Clark electrode (Yellow Springs Instrument Co., Yellow Springs, OH). The reaction mixture contained 0.1 mM *p*-phenetidine hydrochloride 0.5 μg/ml HRP, 1 mM of various thiols in a final volume of 2 ml of phosphate buffer 100 mM (pH 8) containing 1 mM EDTA. Oxygen uptake rates were calculated from the linear part of the recorder graph.

Preparation of reference compounds. p-Phenetidine was oxidized with potassium ferricyanide ($K_3[Fe(CN_6)]$) in a two phase system to obtain reference oxidation products for comparative mass spectrometry (MS) and NMR studies.

Freshly distilled *p*-phenetidine (1.9 g, 0.014 mole) dissolved in dichloromethane (20 ml) was mixed with an aqueous solution (80 ml) containing $K_3[Fe(CN_6)]$ (17 g, 0.05 mole) and saturated with Na_2CO_3. The mixture was stirred for 10 min, the organic phase was then separated, dried ($MgSO_4$) and evaporated under dry nitrogen. The residue was redissolved in $CHCl_3$ (10 ml) filtered and subjected to TLC analysis (silica 0.25 mm plates, $CHCl_3$:MeOH, 19:1) which revealed the presence of more than 10 coloured oxidation products. The solution was concentrated and passed through a silica gel column (30 g) which was eluted with $CHCl_3$ and $CHCl_3$:MeOH mixtures (up to 20% MeOH). 13 fractions (20 ml) were collected and subjected to TLC analysis. Two fractions had identical TLC characteristics to two of the major *p*-phenetidine metabolites. The first of these (fractions 1–3) produced crystals on cooling, which after recrystallization from petroleum ether yielded yellow needles (m.pt. 155–157°). The second fraction (no. 6) was further purified by preparative TLC (silica 2 mm plates, $CHCl_3$:MeOH, 19:1), extracted from the gel with $CHCl_3$. Drying by evaporation yielded crystalline material (m.pt. 195°). The two crystalline compounds were further characterized by MS and NMR.

Isolation of metabolites. Standard incubation mixtures were extracted with water-saturated ethyl acetate and the organic layer was evaporated to dryness. The residues were redissolved in a small volume of methanol and aliquots were applied to silica TLC plates which were developed in chloroform:methanol 19:1. However, experiments using the HRP/H_2O_2 system showed that the extraction itself yielded additional products. Therefore aliquots (100 μl) from a standard incubation mixture were mixed with catalase (10 μl, 1500 U/ml), and subjected to TLC analysis without prior extraction. Only those compounds which appeared on the TLC plates both with and without extraction are described in the metabolite pattern shown in Table 2.

Further TLC analysis of metabolites to check purity was performed on silica plates using mobile phases of A/ Toluene: Ether 1:1 and B/ Isopropanol:MeOH 1:1. These studies indicated that the yellow band contained two yellow compounds (system A—major component $R_f = \cdot 69$, minor component $R_f = \cdot 53$) and that the material at the origin was a single compound (system B).

Bulk incubations (100 ml) were performed using the HRP/H_2O_2 system to prepare metabolites for MS. After extraction with ethyl acetate (250 ml) and concentration, preparative TLC plates (silica 2 mm, $CHCl_3$:MeOH, 19:1) were used for initial separations. The metabolites, dependent on polarity, were then extracted from the gel with either ethyl acetate or methanol, subjected to further purification on 0.25 mm plates using the same solvent system, extracted from the gel once more and the solvent removed by evaporation under a stream of nitrogen.

Quantitation of metabolites. This was performed by using [^{14}C]-*p*-phenetidine hydrochloride in stan-

dard incubation mixtures. The gel containing the metabolites was removed from TLC plates, added to 5 ml of Instagel (Packard) and counted in a Rackbeta scintillation counter for two minutes.

Measurement of arachidonic acid consumption. Incubations containing [^{3}H] AA were used and reactions were terminated by extraction with ethyl acetate (2 × 2 ml). The extracts were blown down to dryness under a stream of nitrogen, redissolved in methanol and TLC plates (0.25 mm) were developed in chloroform/methanol/acetic acid/water 180/16/2/1.6 (v/v). The plates were stained immediately with iodine vapour and the spot corresponding to [^{3}H] AA was removed and radioactivity determined as described.

Measurement of GSH, GSSG and water soluble conjugates. In the case of the PGS/AA incubations (1 ml) containing [^{14}C]-*p*-phenetidine and GSH the reactions were stopped by the addition of trichloroacetic acid (50 μl, 50% w/v). In the case of incubations containing HRP/H_2O_2, reactions were stopped with catalase (50 μl, 1500 U/ml) left to stand for 15 sec and then GSH (2.5 mM) was added and left to react for 60 sec. Aliquots (0.5 ml) of the reaction mixtures were then either derivatized for the determination of GSH and GSSG using HPLC as described previously [21], or extracted with ethyl acetate (2 ml) and the radioactivity in the aqueous phase counted after the addition of Instagel (5 ml). For estimation of GSSG formation control incubations were performed without substrate to correct for non-enzymatic oxidation of GSH to GSSG which occurs particularly in the case of the HRP/H_2O_2 catalyzed reaction. It was assumed that the residual aqueous radioactivity represented water soluble glutathione conjugates of *p*-phenetidine metabolites.

EPR studies. These were performed using a Varian E line spectrometer in the presence or absence of the spin trap DMPO (100 mM) or the presence or absence of the spin probe OXANOH (1 mM). Studies were performed using a standard incubation mixture with 100 KHz field modulation. The spin probe OXANOH can be oxidized to its stable free radical form by other free radicals. The resultant OXANO$^\cdot$ can easily be detected using EPR spectroscopy.

Mass spectral analysis. Mass spectra were recorded on either an LKB 9000 mass spectrometer or a Varian MAT 311 A mass spectrometer connected to a Varian SS200 data system. The samples were introduced using either a direct inlet (DI) probe or a gas chromatograph (Varian 1400 Aerograph) which was equipped with a BP-10 25 m × 0.3 mm in WCOT (Hewlett–Packard) column. The column and injector temperatures were as stated in 'Results'. A flow rate of 2 ml min^{-1} was used with a split ratio of 1:5. The mass spectra were recorded at ionizing voltages ranging from 70 to 85 eV. The accelerating voltages were 3.5 kV (LKB) and 2.2 kV (Varian) and the trap currents 60 μA (LKB) and 1000 μA (Varian) respectively. The ion source temperatures were held at approximately 150° (both cases).

NMR spectra. ^{1}H NMR spectra were recorded on a Jeol FX 90Q spectrometer using C^2HCl_3 solutions and with tetramethylsilane as the internal marker. Chemical shifts are expressed in ppm (γ).

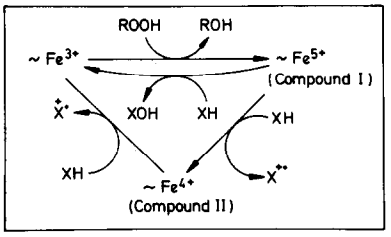

Fig. 1. Mechanisms of peroxidase-catalyzed oxidations.

RESULTS

The peroxidase-catalyzed metabolism of *p*-phenetidine to species which bind to DNA and protein has been suggested to proceed via an initial formation of a *p*-phenetidine free radical [13]. Our observations indicate the presence of a *p*-phenetidine free radical during the peroxidase-catalyzed metabolism of *p*-phenetidine and these are summarized.

In a peroxidase-mediated reaction the enzyme undergoes a divalent oxidation by a peroxide co-factor. The reduction of this oxidized enzyme (Compound I) may occur by a divalent mechanism or by two successive univalent reductions (see Fig. 1). The latter mechanism results in the generation of substrate-derived free radicals and if this is the sole route of reduction a ratio of substrate consumed to peroxide co-factor used of 2/1 would be expected. This was shown to be the case for the HRP/H_2O_2 catalyzed oxidation of *p*-phenetidine (Fig. 2), where the disappearance of the amine substrate was meas-

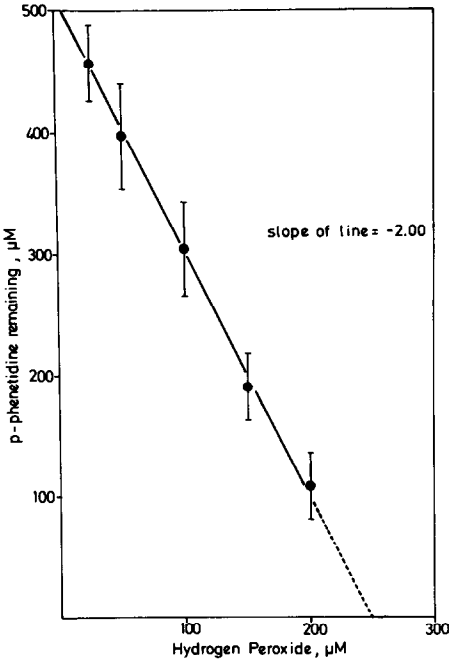

Fig. 2. Stoichiometry of *p*-phenetidine and H_2O_2 removal during the HRP/H_2O_2 catalyzed oxidation of *p*-phenetidine. Incubations were performed as described in 'Materials and Methods'. Data represent means ± S.D. of at least four determinations.

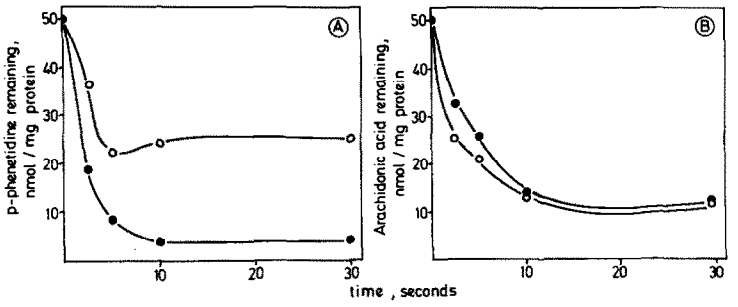

Fig. 3. Removal of *p*-phenetidine (A) and arachidonic acid (B) in the presence (○) or absence (●) of GSH during the PGS/AA-catalyzed oxidation of *p*-phenetidine. Incubations were performed as described in 'Materials and Methods'. Data represent means of two different experiments.

ured in reactions which contained limiting concentrations of H_2O_2.

During the PGS/AA (Fig. 3A) catalyzed oxidation of *p*-phenetidine the inclusion of GSH in the reaction caused a decrease in the amount of *p*-phenetidine consumed. The decreased removal of *p*-phenetidine was not accompanied by a decrease in the utilization of arachidonic acid (Fig. 3B) which suggests that GSH does not inhibit *p*-phenetidine removal by inhibition of PGS activity. A 40% inhibition of *p*-phenetidine (0.5 mM) removal by GSH (0.5 mM) was also observed in the HRP/H_2O_2 system using an excess of peroxide (1 mM) to ensure that the inhibition was not due to removal of the peroxide cofactor, because of reaction with GSH. These results inferred that the reduction in *p*-phenetidine usage may represent a regeneration of *p*-phenetidine. If this was the case then the reduction of the *p*-phenetidine radical by GSH would result in the production of a thiyl radical which would be expected to dimerize to form GSSG (see Fig. 12). The oxidation of GSH to GSSG was considerably higher than glutathione conjugate formation in the PGS/AA catalyzed reaction (Fig. 4). Of particular relevance is that in the HRP/H_2O_2 catalyzed oxidation of *p*-phenetidine the formation of GSSG (after subtraction of GSSG formed in control incubations without substrate) was much higher than glutathione conjugate formation when GSH was added at early time points but this relationship was inverted when GSH was added at later time points (Fig. 5). This suggested that an early product of the peroxidase-mediated oxidation of *p*-phenetidine was involved in the production of GSSG and that the major proportion of conjugates formed were a result of the reaction of GSH with secondary oxidation products of *p*-phenetidine.

Attempts to observe the putative *p*-phenetidine radical directly using EPR spectroscopy were unsuccessful and the inclusion of the spin trap DMPO did not result in the detection of a paramagnetic signal. That *p*-phenetidine radicals were produced in this system was shown by use of the spin probe OXANOH. This can be readily oxidized to its stable free radical form in the presence of other radicals. An EPR signal corresponding to OXANO˙ was observed during the HRP catalyzed oxidation of *p*-phenetidine which was substrate- and enzyme-dependent and which was not reduced by SOD (80 μg/ml) or Benzoate (10–100 mM) (Fig. 6).

When oxygen uptake during the HRP/H_2O_2 catalyzed oxidation of *p*-phenetidine was investigated no oxygen uptake could be measured unless thiols were present in the reaction mixture (Table 1). In the absence of *p*-phenetidine no oxygen uptake was observed.

The oxidation of *p*-phenetidine by PGS/AA and HRP/H_2O_2 resulted in the formation of several intensely coloured products which were separated by

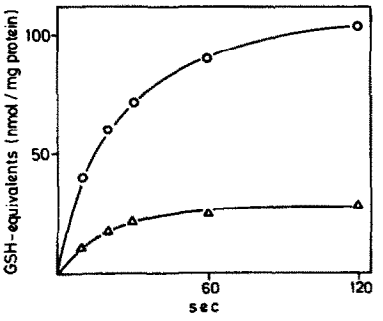

Fig. 4. The formation of water-soluble conjugates (△) and GSSG (○) during the PGS/AA catalyzed oxidation of *p*-phenetidine in the presence of GSH. Incubations were performed as described in 'Materials and Methods'. Data represent one experiment typical of three.

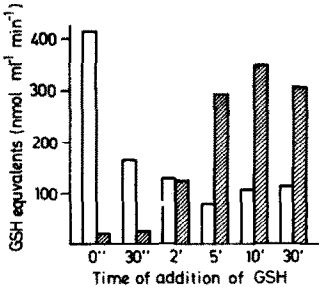

Fig. 5. The formation of water-soluble conjugates (▨) and GSSG (□) during the HRP/H_2O_2 catalyzed oxidation of *p*-phenetidine. Reactions contained *p*-phenetidine 0.5 mM, HRP 0.2 μg/ml, H_2O_2 1 mM were stopped with catalase at various times, GSH (2 mM) was then added (see 'Materials and Methods'). GSH conjugate and GSSG formation was determined as described in 'Materials and Methods'.

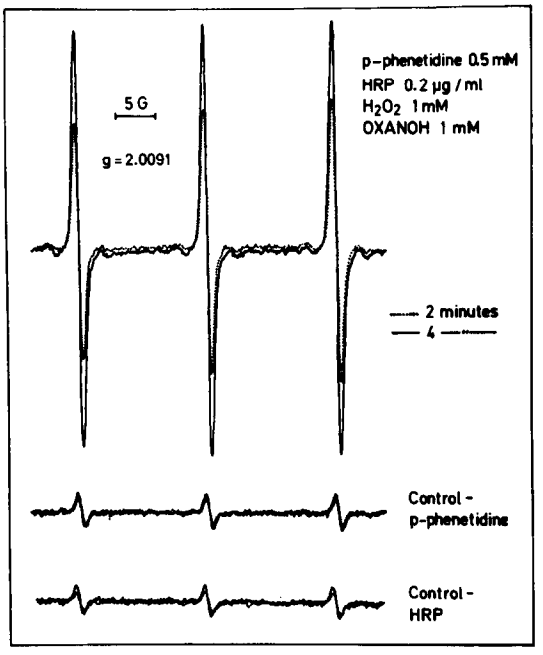

Fig. 6. EPR spectra obtained during the HRP/H_2O_2 catalyzed oxidation of *p*-phenetidine in the presence of the spin probe OXANOH.

TLC. The metabolite pattern produced by the two peroxidases was very similar (Table 2) and suggests a common mechanism of oxidation of *p*-phenetidine in the two systems. The time course of appearance of the various metabolites was investigated using the HRP/H_2O_2 system and the results are summarized in Fig. 7. It can be seen that quantitatively the most important metabolite is the orange compound (R_f 0.65) whereas other major metabolites are the red compound (R_f 0.10) and the yellow band (0.70).

By comparison with the products obtained from potassium ferricyanide oxidation of *p*-phenetidine, the major component of the yellow band (R_f 0.70) was shown to be a di-azo compound (m/z = 270, Fig. 8). The mass spectrum shown in Fig. 8 is identical to that of the yellow crystals recovered from the K_3 Fe(CN)$_6$ oxidation. Moreover, the NMR spectrum

Table 1. Thiol-dependent O_2 uptake rates during the HRP/H_2O_2-catalyzed oxidation of *p*-phenetidine*

System	nmole O_2/min/µg HRP
Complete	293
HRP	40
H_2O_2	0
GSH	0
p-Phenetidine	0
GSH + GSSG	0
GSH + N-acetylcysteine	255
GSH + cysteine	367

Incubations were performed as described in 'Materials and Methods' and data represent means of two experiments.

* Final concentration of thiols was 1 mM.

Table 2. Characteristics of *p*-phenetidine metabolites formed in prostaglandin synthase and horseradish peroxidase catalyzed reactions

	RSVM/AA		HRP/H_2O_2	
Metabolite	R_f	Colour	R_f	Colour
7	0.74	Pink	—	—
6*	0.70	Yellow	0.70	Yellow
5	0.65	Orange	0.65	Orange
4	0.56	Purple	0.56	Purple
3	0.25	Brown	0.25	Brown
2	0.10	Red	0.10	Red
1	0	Origin	0	Origin

Incubations were performed as described in 'Materials and Methods'.

* A mixture of two yellow metabolites.

of the latter (not shown) is in full agreement with the di-azo structure and the synthetic material had the same melting point as that previously reported [22] for the di-azo compound.

The mass spectrum of the red compound (Fig. 9) isolated from the HRP/H_2O_2 catalyzed oxidation of *p*-phenetidine is indicative of the presence of a '*p*-phenetidine trimer', as a conceivable molecular ion of m/z 407 should correspond to the combination of 3 *p*-phenetidine units with loss of 4 hydrogens (3 × 137−4 = 407). The structure proposed for the red metabolite (R_f 0.10) is shown in Fig. 10 together with assignments for the fragmentation observed. Thus two consecutive aromatization steps with concomitant loss of two times H_2 account for the for-

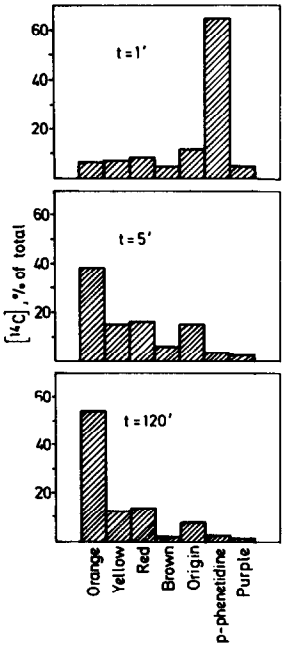

Fig. 7. Time course of metabolite formation during the HRP/H_2O_2 catalyzed oxidation of *p*-phenetidine. Incubations were performed as described in 'Materials and Methods'.

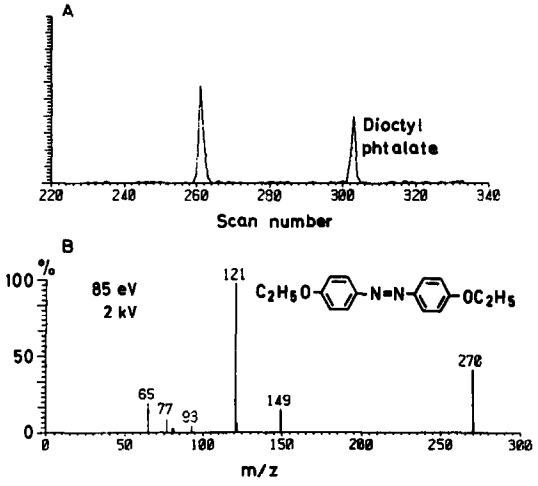

Fig. 8. GC–MS analysis of the content in the yellow band (R_f 0.70 cf. Table 2) formed in the horseradish peroxidase catalyzed oxidation of *p*-phenetidine. (A) Total ion current profile of the GC-effluent. (B) Mass spectrum of Scan 261 compatible with the diazo-structure. GC–MS analysis was performed as described in 'Materials and Methods'.

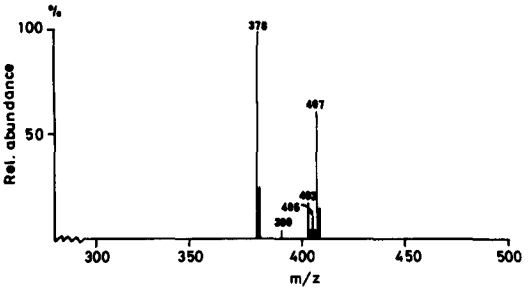

Fig. 9. Direct inlet EI mass spectrum of the content in the red band (R_f 0.10 cf. Table 2) formed in the horseradish peroxidase catalyzed oxidation of *p*-phenetidine. For experimental details see 'Materials and Methods'.

mation of the two radical ions (m/z = 405, m/z = 403). Direct loss of $^{\cdot}C_2H_5$ will produce the base peak ion at m/z = 378, while loss of ammonia accounts for the formation of the small but distinct radical ion at m/z = 390. An NMR spectrum obtained after the red compound had been repeatedly purified is shown in Fig. 11. The spectrum is in full agreement with the proposed diimine structure. The structure shown in Fig. 10 is a fully reduced form of the compound and thus would not be expected to be coloured red. However, if a two electron oxidation of this material occurred an extensively conjugated and presumably coloured product would be formed (m/z = 405 − loss of H_2 in Fig. 10). A red compound with this structure was isolated after potassium ferricyanide oxidation of *p*-phenetidine. Consistent with this hypothesis decolorization of red material occurred upon addition of dithionite whilst air oxidation of the reduced material regenerated the red colour. These data suggest that the red band isolated from the HRP/H_2O_2 reaction (R_f 0.10) contains species which are easily interconvertible by redox processes.

DISCUSSION

Peroxidase-catalyzed reactions are generally considered to occur via the production of substrate-derived free radicals and a one electron oxidized form of the peroxidase enzyme (see Fig. 1). Despite this the formation of substrate-derived free radicals has only been demonstrated in a few cases, i.e. acetaminophen [23], aminopyrine [24], phenidone

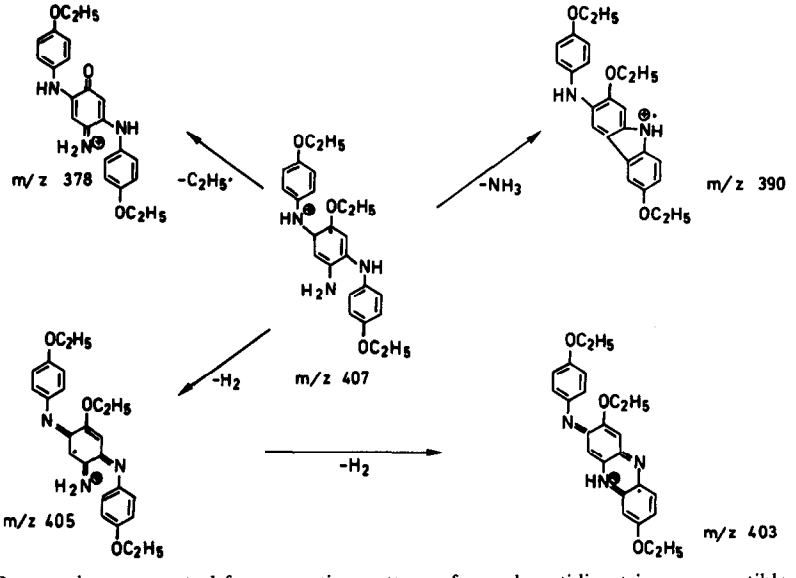

Fig. 10. Proposed mass spectral fragmentation pattern of a *p*-phenetidine trimer compatible with the mass spectrum shown in Fig. 9.

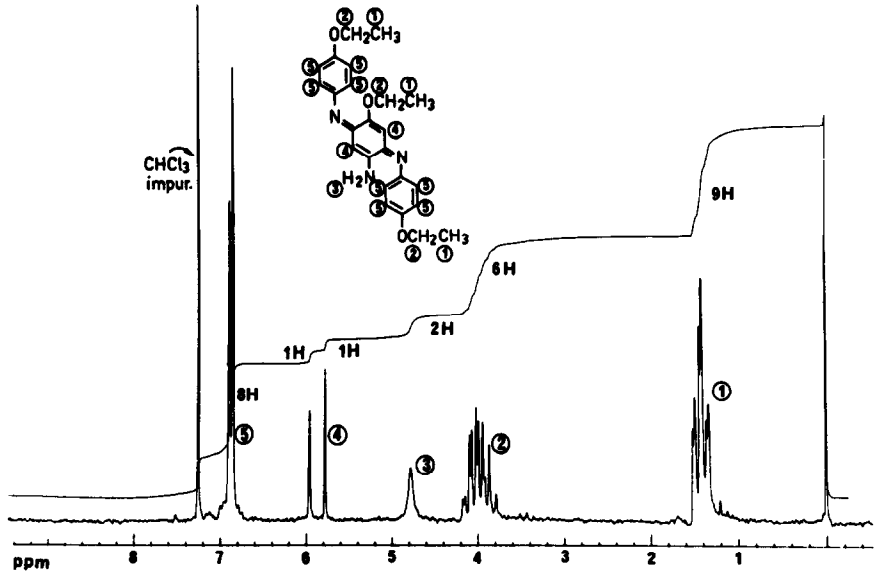

Fig. 11. ^{2}H-NMR-spectrum of the red product isolated after potassium ferricyanide oxidation of *p*-phenetidine. For experimental particulars see 'Materials and Methods'.

[25], benzidine [26], 3,5,3′,5′-tetramethylbenzidine [27], acridine derivatives [28] and *p*-aminophenol [29]. There are, however, exceptions to this mechanism and the peroxidation of iodide and sulfite have been shown to be devoid of one electron character [30–32]. Investigations of the oxidation of aromatic amines by peroxidases have suggested that a single mechanism cannot explain all such oxidations (summarized in ref. 1). The mechanisms proposed involve either dehydrogenation, which results in the production of substrate free radicals centred on the amine nitrogen atom [33, 34], or the production of an activated complex of hydrogen peroxide and substrate which depends essentially upon ionic interactions [35, 36].

In the case of the peroxidase-catalyzed oxidation of *p*-phenetidine our experimental data suggests that a *p*-phenetidine free radical is formed. The first area of evidence which supports the formation of the radical concerns the stoichiometry of the HRP/H_2O_2 catalyzed oxidation of *p*-phenetidine (Fig. 2). In particular the observation that the ratio between phenetidine consumed and hydrogen peroxide used in the HRP/H_2O_2 catalyzed oxidation of *p*-phenetidine was 2 : 1 supports a mechanism which involves two successive one electron reductions of an oxidized enzyme complex (Fig. 1).

The strongest indication that *p*-phenetidine radicals were produced during the HRP/H_2O_2 catalyzed oxidation of *p*-phenetidine was obtained using EPR spectroscopy. Attempts to observe the radical directly using EPR spectroscopy or in conjunction with the spin trap DMPO were unsuccessful. In the presence of the spin probe OXANOH, however, the HRP/H_2O_2 catalyzed oxidation of *p*-phenetidine resulted in the generation of an EPR signal typical of OXANO$^\cdot$. As the intensity of this signal was not decreased by SOD or a hydroxyl radical scavenger we concluded that *p*-phenetidine radicals were responsible for the oxidation of the hydroxylamine spin probe to its stable free radical form. It seems probable that the proposed radical produced would be a cation radical localized on the nitrogen atom (Fig. 12). Indeed such a radical intermediate has been shown to be produced during the HRP/H_2O_2 catalyzed oxidation of *p*-aminophenol [29]. This compound is very closely related to *p*-phenetidine. However, the *p*-aminophenol radical can undergo greater resonance stabilization due to the para-hydroxyl group and thus the steady state concentration of the aminophenol free radical would be expected to be considerably higher than that of the radical derived from *p*-phenetidine and thus facilitate direct observation. This study demonstrates the potential importance of the spin probe OXANOH as a means of demonstrating, albeit indirectly, the production of free radicals which cannot be observed by conventional EPR/spin trapping methods.

The fate of thiols such as GSH during peroxidase-catalyzed oxidations is of potential toxicological relevance and in this case yielded further evidence

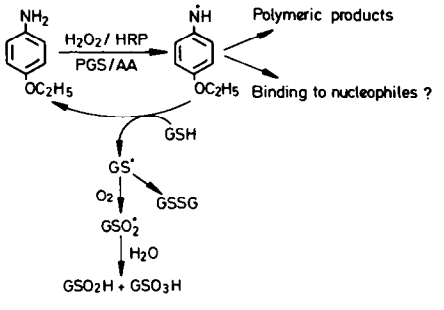

Fig. 12. Proposed scheme for the peroxidase-catalyzed oxidation of *p*-phenetidine in the presence of glutathione.

which supported the generation of a radical during the peroxidase-catalyzed oxidation of *p*-phenetidine. The results presented (Figs. 3–5) suggest that GSH can serve as an electron donor to a *p*-phenetidine radical which results in the formation of a glutathione thiyl radical which readily undergoes dimerization to form GSSG (see Fig. 12). Other radicals such as those derived from chlorpromazine have been shown to be reduced by GSH in a similar manner [37]. The presence of thiols in incubations containing HRP/H_2O_2 and *p*-phenetidine caused extensive oxygen uptake (Table 1). Similar behaviour has been observed in our laboratories using aminopyrine [38] and acetaminophen [39] as substrates, both of which have been shown to form free radical intermediates by EPR spectroscopy during peroxidase-catalyzed reactions [23, 24]. The *p*-phenetidine radical itself does not combine directly with oxygen as in the absence of thiols no oxygen up-take occurred. These results suggest therefore the interaction of a thiyl radical with molecular oxygen. Such reactions of glutathione-derived thiyl radicals are well documented [40, 41]. They give rise to unidentified oxygen-containing products which have been suggested to be sulfinic and sulfonic acid derivatives of the thiol, which are formed according to equations (1)–(2) [42].

$$GS^{\cdot} + O_2 \rightarrow GSO_2^{\cdot}, \quad (1)$$

$$2GSO_2^{\cdot} + H_2O \rightarrow GSO_2H + GSO_3H. \quad (2)$$

We consider the oxygen uptake observed in this study to be an intriguing aspect of radical production and as this reaction may have physiological relevance we are currently investigating the mechanisms underlying this oxygen uptake. Preliminary experiments have shown that glutathione sulfonic acid (GSO_3H) is not formed during the peroxidase catalyzed oxidation of *p*-phenetidine in the presence of GSH but the identity of the glutathione derived intermediates formed during this reaction is still under investigation.

The broad spectrum of coloured products formed in the peroxidase-catalyzed metabolism of *p*-phenetidine is typical of a radical mechanism of oxidation [1, 43]. The similarity of the products formed during the HRP/H_2O_2 and PGS/AA catalyzed oxidations of *p*-phenetidine infer a similar mechanism of oxidation of the compound in the two systems. This has recently been proposed for the aromatic amine benzidine [44], but some compounds such as 5-nitrofurans are metabolized by PGS/AA but are not substrates for HRP/H_2O_2 catalyzed biotransformation [45]. Two of the major products of the HRP/H_2O_2 catalyzed, and by inference the PGS/AA catalyzed, oxidation of *p*-phenetidine have been characterized as 4,4′-diethoxyazobenzene (Fig. 8) and a '*p*-phenetidine trimer' (Figs. 9, 10). The latter compound because of its diimine structure was found to be easily interconvertible into its reduced or oxidized forms by redox processes. The structure of the oxidized form of the trimer is analogous to a previously characterized compound—Bandrowski's base [46, 47].

In summary we present evidence for the production of a substrate derived free radical during the peroxidase-catalyzed oxidation of *p*-phenetidine and characterize some of the secondary oxidation products. In addition the fate of glutathione during peroxidase-catalyzed oxidation of *p*-phenetidine is described. The proposed mechanism involves the interaction of glutathione with at least two different reactive species produced during peroxidase-catalyzed oxidations: with the *p*-phenetidine radical to generate a thiyl radical and *p*-phenetidine, and with secondary oxidation products of *p*-phenetidine to form conjugates.

Further studies regarding the structures of the products of the HRP/H_2O_2 and PGS/AA catalyzed oxidation of *p*-phenetidine, the identity of reactive intermediate(s) and the toxicological consequences of their production are presently in progress.

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