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# Oxidation of *p*-Aminophenol Catalyzed by Horseradish Peroxidase and Prostaglandin Synthase

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SUMMARY

*p*-Aminophenol oxidation is catalyzed by horseradish peroxidase. ESR spectroscopy demonstrates the formation of the *p*-aminophenoxy free radical, the one-electron oxidation product of *p*-aminophenol. The same radical is formed by alkaline autoxidation of this compound. The ultimate products of *p*-aminophenol oxidation are mainly polymeric; however, indophenol was isolated in low yield. Oxidation of *p*-aminophenol is also catalyzed by the hydroperoxidase activity of prostaglandin synthase, using ram seminal microsomal preparations as the enzyme source.

## INTRODUCTION

*p*-Aminophenol is a nephrotoxic agent, causing renal necrosis and inhibition of renal microsomal and mitochondrial enzyme activities in the rat (1). In addition, *p*-aminophenol is a potent teratogen in the hamster (2). *p*-Aminophenol may be formed *in vivo* by the metabolism of related xenobiotics. Aniline is oxidized to *p*-aminophenol by the cytochrome P-450 system (3) and by a hemoglobin-dependent system in erythrocytes (4). The widely used analgesic acetaminophen is converted to *p*-aminophenol by deacetylation in kidney slices and homogenates (5). In addition, *p*-aminophenol is a component of auburn, red-brown, and similar permanent hair dyes (6). Activation of *p*-aminophenol to a reactive intermediate is suggested by the observation of covalent binding of radiolabeled compound to kidney and liver protein, and depletion of renal glutathione content (1). The nature of the enzyme system responsible for the activation of *p*-aminophenol *in vivo* is unclear. However, the lack of consistent effects of inhibitors and inducers of the microsomal cytochrome P-450 system on *p*-aminophenol nephrotoxicity and protein binding led to the suggestion that other pathways are involved (7). Elucidation of the chemistry of *p*-aminophenol oxidation is germane to the study of the mechanism of action of this agent.

*p*-Aminophenol is oxidized very readily by peroxidases, such as HRP[4] (8). Mammalian tissues, including renal medulla, contain a microsomal enzyme system, prostaglandin endoperoxide synthase, which possesses hydroperoxidase activity (9). Aromatic amine xenobiotics, including *p*-aminophenol, are oxidized by this system (10–12). In this paper, we examine the oxidation of *p*-aminophenol by HRP and prostaglandin hydroperoxidase, using ram seminal vesicle microsomal preparations as the source of the latter enzyme.

## MATERIALS AND METHODS

*p*-Aminophenol·HCl and indophenol were obtained from Eastman Organic Chemicals (Rochester, N. Y.). HRP (Type VI) was purchased from Sigma Chemical Company (St. Louis, Mo.). Hydrogen peroxide, 30%, was purchased from Fisher Scientific Company (Pittsburgh, Pa.). All other chemicals were reagent-grade.

Ram seminal vesicle microsomes were prepared as described (13). Arachidonic acid was purchased from Nu-Chek-Prep, Inc. (Elysian, Minn.), and was stored in the dark at −60°. Indomethacin was purchased from Sigma Chemical Company.

ESR measurements were made on a Varian E-104 spectrometer equipped with a $TM_{110}$ cavity and an aqueous flat cell. Spectra were obtained at room temperature. TLC was performed on Whatman silica gel plates, type LK5DF, in a solvent system of ethyl acetate/*n*-hexane (50:50). Mass spectra were obtained using direct-probe insertion and electron ionization.

The buffers used were as follows: HRP incubation, 0.1 M acetate buffer (pH 5.0) or 0.1 M phosphate buffer (pH 6.6); ram seminal vesicle microsomal incubations, 0.1 M phosphate buffer (pH 6.95).

Indophenol was isolated from the HRP-catalyzed oxidation of *p*-aminophenol as follows. *p*-Aminophenol (1 ml, 0.25 M) and hydrogen peroxide (0.25 ml, 1 M) were added to 10 ml of acetate buffer (pH 5). HRP (50 μg) was added. The solution turned purple, then red-brown. The aqueous mixture was extracted with 10 ml of ethyl acetate, and the resulting orange organic layer was washed with distilled water, dried with anhydrous sodium sulfate,

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[4] The abbreviations used are: HRP, horseradish peroxidase; TLC, thin-layer chromatography.

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filtered, and evaporated at reduced pressure. The residue was dissolved in a small volume of ethyl acetate and applied to a preparative TLC plate. The plate was developed as described above, and the orange band corresponding to indophenol was scraped and eluted with ethyl acetate. The absorption spectrum of the resulting solution was recorded, and the recovery of indophenol was calculated based on the extinction coefficient of the authentic standard. A control incubation mixture (no hydrogen peroxide) was spiked with authentic indophenol and extracted with ethyl acetate. Extraction was judged to be 100%, since no detectable colored material remained in the aqueous phase. Losses during workup were no more than 20%; the reported yield (see below) is uncorrected.

## RESULTS

*p*-Aminophenol is subject to base-catalyzed auto-oxidation and, at neutral pH, solutions turn yellow-brown over a period of a few hours (14). We studied auto-oxidizing solutions of *p*-aminophenol at pH 10 using ESR spectroscopy, and observed a strong, well-resolved, multiline signal (Fig. 1A). Computer simulation of the hyperfine pattern confirmed the identification of the paramagnetic species as the *p*-aminophenoxy radical (Fig. 1B). This radical is the one-electron oxidation product of *p*-aminophenol and has been studied previously by ESR using chemical (15) and radiolytic (16) oxidation techniques. The assignment of the amino *N* and *H* hyperfine splitting constants was confirmed by a simulation of the spectrum of the deuterium-exchanged radical, which agreed well with the observed spectrum generated in $^2H_2O$ (Fig. 1C and D).

At pH 6.6, no detectable signal was observed in freshly prepared solutions of *p*-aminophenol. Oxidation with HRP and $H_2O_2$ gave a short-lived ESR spectrum similar to that measured during alkaline oxidation (Fig. 2A). The signal was dependent on the presence of both HRP and $H_2O_2$ (Fig. 2B and C). The enzymatic oxidation of *p*-aminophenol yielded a red-brown precipitate.

The oxidation of *p*-aminophenol is also catalyzed by the prostaglandin synthase/arachidonic acid system. In this case, no hydroperoxide is added to the system; prostaglandin synthase catalyzes both the oxidation of arachidonic acid to prostaglandin $G_2$, an organic hydroperoxide, and the reduction of the latter compound to prostaglandin $H_2$ by a peroxidase activity. Addition of arachidonic acid to an incubation mixture of *p*-aminophenol and ram seminal vesicle microsomal protein at pH 6.95 gave an ESR spectrum identical with that observed with HRP and $H_2O_2$ (Fig. 3A). No ESR signal was observed if arachidonic acid or ram seminal vesicle microsomal protein was omitted (Fig. 3B and C). Incubation of the microsomal protein with the prostaglandin synthase inhibitor, indomethacin, at 4° for 5 min prior to addition of substrates inhibited the oxidation of *p*-aminophenol almost completely (Fig. 3D). At higher protein concentration, a stronger ESR signal was observed (Fig. 3E).

Oxidation of *p*-aminophenol by the HRP/$H_2O_2$ system under acidic conditions (pH 5) gave a transient purple color which soon turned red-brown. ESR revealed the presence of the *p*-aminophenoxy radical, as at neutral pH.

Attempts to isolate the initial purple product by extraction with organic solvents or lyophilization were unsuccessful. Extraction of the red-brown product with ethyl acetate yielded an orange-brown organic layer, while much brown material and precipitate remained in the aqueous phase. The organic layer was dried and concentrated by evaporation at reduced pressure. TLC revealed the presence of a single orange product ($R_F$ = 0.55) and a considerable amount of dark brown residue at low $R_F$. The orange product was recovered from preparative TLC plates. Addition of base gave a brilliant blue, water-soluble product, which could be converted back to the original orange compound with acid. This suggested that the product was indophenol; this was verified by comparison with authentic indophenol (co-chromatography on TLC, and identical mass spectra).

The yield of indophenol was measured as described under Materials and Methods. The recovery of indophenol was about 2 μmoles, from 250 μmoles of aminophenol. Since 2 moles of *p*-aminophenol are needed, theoretically, to produce 1 mole of indophenol, this corresponds to a yield of about 2%. Apparently, most of the product consists of unidentified polymeric material. A similar yield of indophenol was obtained from solutions of *p*-aminophenol allowed to auto-oxidize at pH 10, and then acidified and extracted with ethyl acetate.

## DISCUSSION

Aromatic compounds bearing electron-donating substituents (such as phenol, aniline, and their derivatives) are oxidized by the HRP/$H_2O_2$ system. Spectrophotometric studies demonstrate the conversion of HRP-Compound I to Compound II by these substrates (8). This suggests that the substrates are oxidized to free radicals, according to the well-known scheme:

$$\text{HRP} + H_2O_2 \rightarrow \text{HRP-Compound I}$$
$$\text{HRP-Compound I} + AH_2 \rightarrow \text{HRP-Compound II} + AH^{\cdot}$$
$$\text{HRP-Compound II} + AH_2 \rightarrow \text{HRP} + AH^{\cdot}$$

The formation of substrate-derived free radicals has been demonstrated directly by ESR spectroscopy, for certain phenols (17) and aromatic amines (18, 19). The stable products that have been isolated from peroxidase/substrate incubations are formed from the reactions of the initial radical products with each other or with the unaltered substrate molecule or from the further oxidation of the radical. These products can be very complex and are typically colored compounds formed from two, three, or more molecules of substrate. Mann and Saunders (20) studied the oxidation of aniline by HRP/$H_2O_2$. The initial color observed was blue-violet; this rapidly turned to brown precipitate. The brown solid was a complex mixture including induline, pseudo-mauveine, aniline black, and unidentified products.

In this paper, we have shown that the initial product of *p*-aminophenol oxidation in a variety of systems is the *p*-aminophenoxy free radical. Oxidation or disproportionation of this radical would yield *p*-benzoquinoneimine. The formation of indoaniline and indamine dimers was interpreted as evidence for the transient existence of *p*-

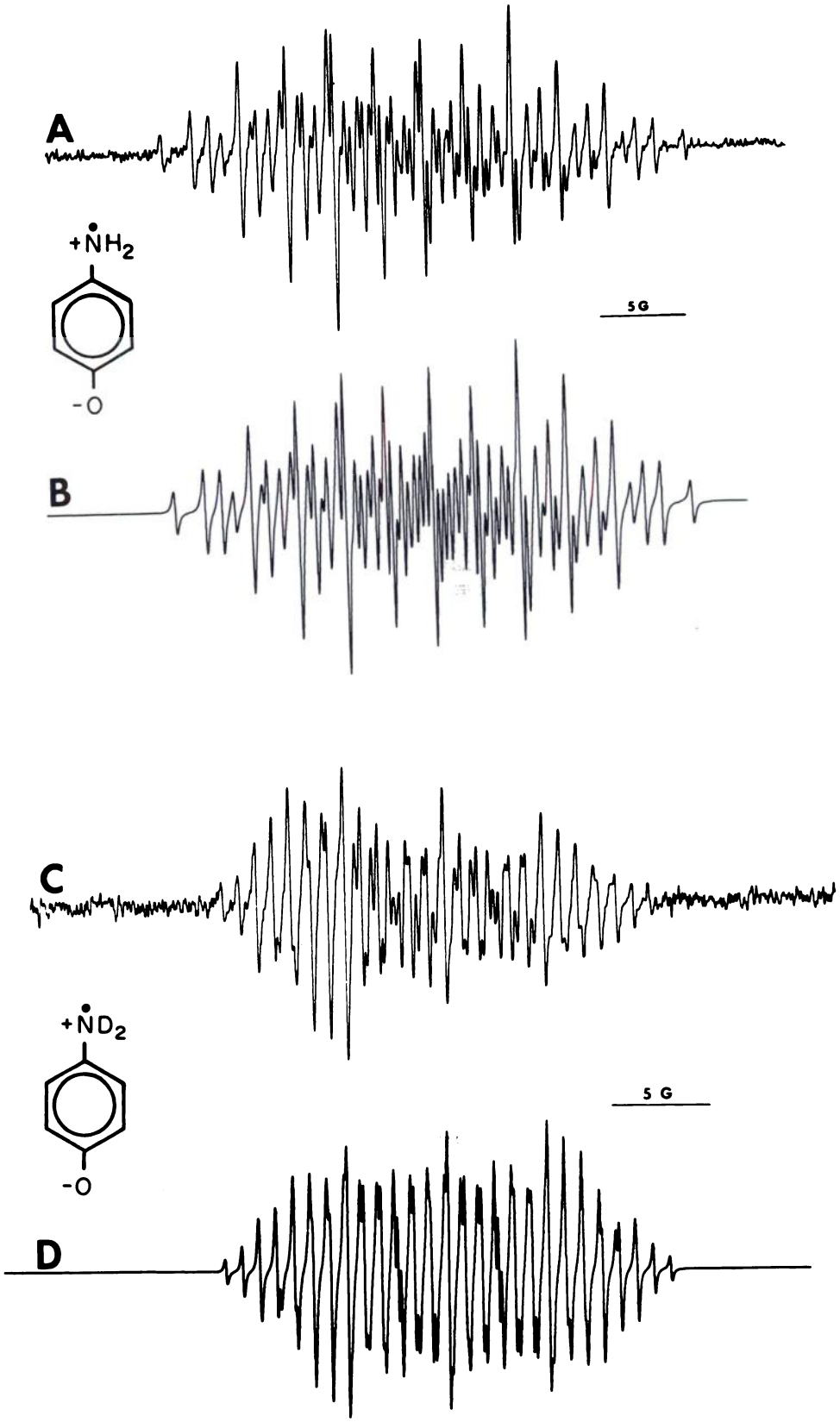



FIG. 1. *Oxidation of p-aminophenol at alkaline pH*

A and B, $H_2O$ ESR spectrum.

A. The reaction mixture contained 1 ml of $H_2O$, 50 μl of *p*-aminophenol·HCl (0.5 M), and 35 μl of NaOH (1.0 M), to give a pH of 10.2. Instrumental conditions were as follows: magnetic field, 3367 G; scan range, 50 G; modulation amplitude, 0.1 G; gain, 8000; power, 20 mW. Scan time was 4 min, time constant 0.128 sec.

B. Simulation of spectrum in A. Hyperfine splitting constants were as follows: $a^N = 5.21$ G; $a^H_{NH_2} = 5.55$ G; $a^H_{2,6} = 2.77$ G; $a^H_{3,5} = 1.77$ G. Lorentzian peak-to-peak linewidth = 0.21 G.

C and D. $^2H_2O$ ESR spectrum.

C. Conditions were as in A, except that $^2H_2O$ and $NaO^2H$ were used, and the spectrometer gain was 12,500.

D. Computer simulation. Hyperfine splitting constants were as in B, except that $a^{^2H}_{NH_2} = 0.852$ G.

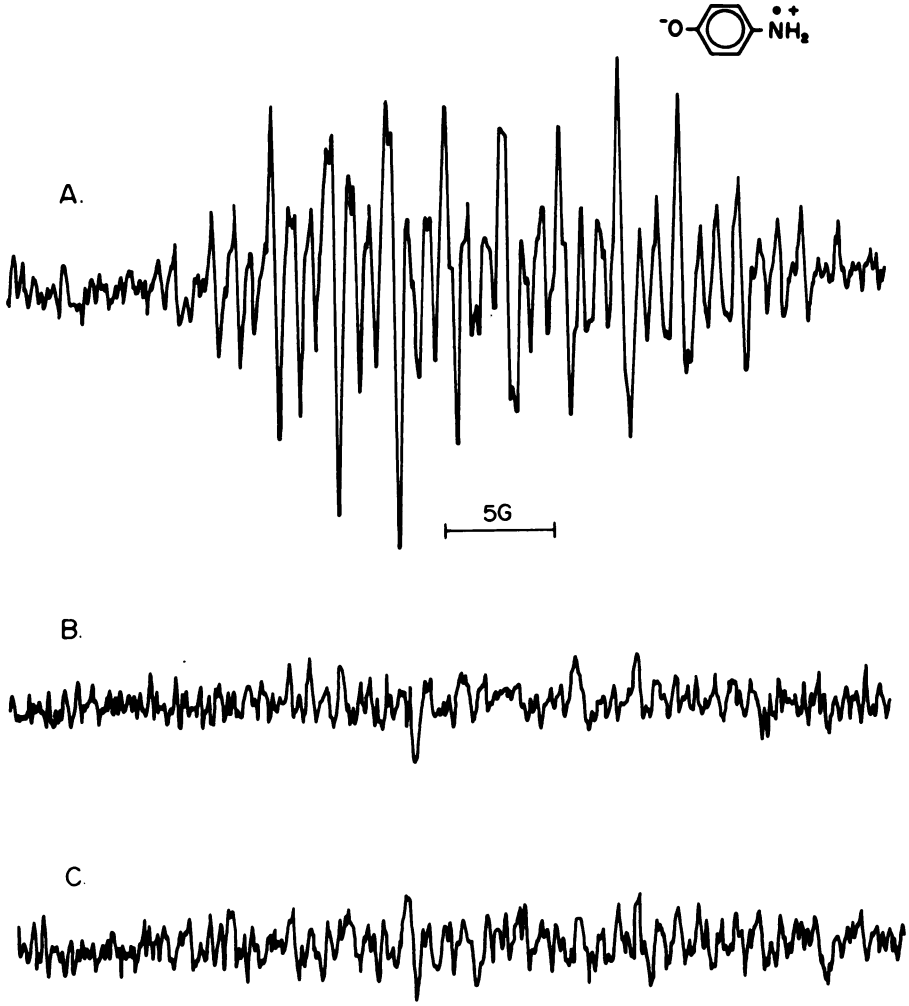


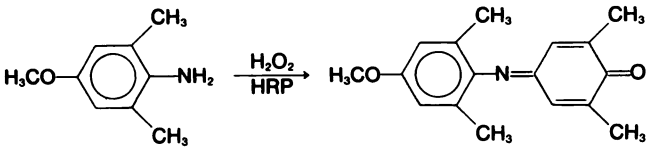

FIG. 2. *Oxidation of p-aminophenol catalyzed by HRP*

A. The reaction mixture contained *p*-aminophenol (10 mM), $H_2O_2$ (2.5 mM), and HRP (100 ng/ml) in 0.1 M phosphate buffer (pH 6.6). B. Enzyme omitted. C. $H_2O_2$ omitted.

Instrumental conditions were as follows: magnetic field, 3363.4 G; scan range, 50 G; modulation amplitude, 0.16 G; gain, $8 \times 10^4$; power, 20 mW. Scan time was 16 min, time constant 1.0 sec.

benzoquinoneimine and *p*-benzoquinone diimine during the alkaline oxidation of *p*-phenylenediamine (21). However, both of these species deaminate readily (22), and are too short-lived to be observed directly during the oxidation of aromatic amines (21). The inhibition of oxidative metabolism of liver homogenates by aged solutions of *p*-aminophenol (14) and the teratogenicity of such solutions (2) are probably not due to the presence of *p*-benzoquinoneimine. Such preparations are complex mixtures of oxidation products of the starting material.

The mechanism of formation of indophenol (albeit in low yield) during the oxidation of *p*-aminophenol is unclear. Possible routes include the reaction of *p*-benzoquinoneimine and *p*-aminophenol with loss of ammonia, or the reaction of *p*-benzoquinone and *p*-aminophenol with loss of water. Addition of reagent-grade *p*-benzoquinone to *p*-aminophenol gave products similar to those formed by the enzymatic oxidation of *p*-aminophenol, including indophenol (data not shown). Analogous reactions have been reported by Saunders (23). For example, the peroxidase-catalyzed oxidation of 4-methoxy-2,6-dimethylaniline gave a purple product identified as 2,6-dimethylbenzoquinone-4-(4-methoxy-2′,6′-dimethyl)-anil:




The amine and methoxy groups were eliminated as ammonia and methanol, respectively (24). The oxidation of *o*-aminophenol by cytochrome *c*/cytochrome oxidase gave the product 2-amino-3*H*-isophenoxazine-3-one, presumably *via* an *o*-quinoneimine intermediate (25).

Many aromatic amines, including *p*-aminophenol (11), benzidine (10, 26, 27), and acetaminophen (12, 28, 29), are oxidized by the prostaglandin synthase/arachidonic acid system. The metabolites include reactive species capable of binding to macromolecules, but, in most cases, their chemical nature is unknown. In this paper, we have

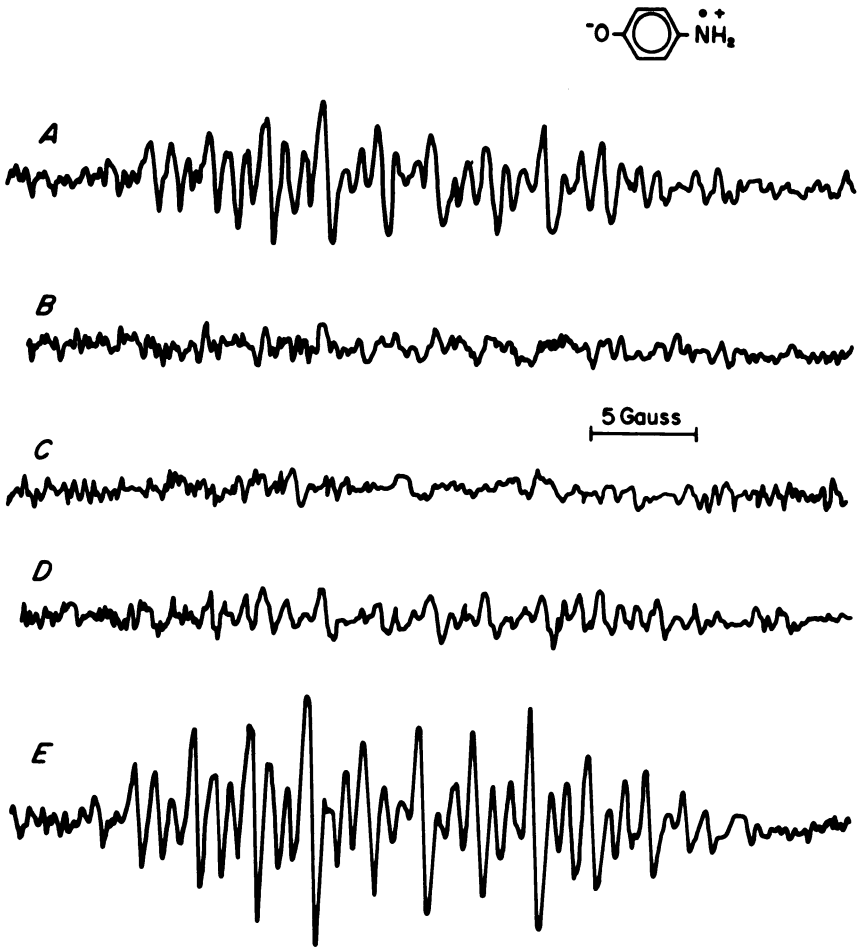



FIG. 3. *Oxidation of p-aminophenol catalyzed by prostaglandin synthase*

A. The reaction mixture contained *p*-aminophenol (0.5 mM), arachidonic acid (0.4 mM), and ram seminal vesicle microsomal protein (50 μg/ml) in 0.1 M phosphate buffer (pH 6.9). B. Arachidonic acid omitted. C. Microsomal preparation omitted. D. Microsomal preparation was preincubated with indomethacin (100 μM) for 5 min before addition of arachidonic acid. E. As in A, but microsomal protein, 200 μg/ml.

Instrumental conditions were as follows: magnetic field, 3364 G; scan range, 50 G; modulation amplitude, 0.41 G; gain, $3.2 \times 10^4$; power, 20 mW. Scan time was 8 min, time constant 0.5 sec.

identified the initial product of the peroxidase-catalyzed oxidation of *p*-aminophenol using ESR spectroscopy. The *p*-aminophenoxy free radical or, more probably, the two-electron oxidation product, *p*-benzoquinoneimine, may be responsible for binding to macromolecules. In the case of *ortho*-aminophenols, Nagasawa *et al.* (30) suggested that "*o*-quinoneimines might ... bind free functional groups of protein side chains. ... " Since the free radical and the quinoneimine can coexist in rapid equilibrium, we cannot distinguish between effects due to one or the other species. Subsequent non-enzymatic reactions of these species lead to a complex pattern of products. Indophenol was isolated in low yield. Under physiological conditions, it is possible that the intermediates of *p*-aminophenol oxidation would react with endogenous compounds to give an even more complex spectrum of metabolites.

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