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Metabolism of Aromatic Amines by Prostaglandin H Synthase

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The metabolism of aromatic amines by the peroxidase activity of prostaglandin H synthase (PHS) has been studied in this laboratory by use of two model compounds, the carcinogenic primary amine 2-aminofluorene (2-AF) and the substituted amine aminopyrine (AP). 2-AF is oxidized by PHS to 2, 2-azobisfluorene, 2-aminodifluorenylamine, 2-nitrofluorene, polymeric material, and products covalently bound to macromolecules. In the presence of phenolic compounds, 2-AF oxidation results in the formation of amine/phenol adducts. The data are consistent with a one-electron mechanism of 2-AF oxidation by PHS; furthermore, an *N*-hydroxy intermediate is not involved in 2-AF metabolism by PHS. PHS also catalyzes the binding of 2-AF to DNA *in vitro*. Unique 2-AF/DNA adducts were isolated and are distinct from the *N*-(deoxyguanosin-8-yl)-2-AF adduct formed from the reaction of *N*-hydroxy-2-AF with DNA. These new adducts represent a marker unique to peroxidative activation of 2-AF.

AP is oxidized by the peroxidase activity of PHS to the cation radical, with one molecule of hydroperoxy fatty acid reduced for every two molecules of AP free radical formed. The decay of the AP radical follows second order kinetics, supporting the proposed mechanism in which the AP radical disproportionates to an iminium cation, followed by hydrolysis of this species to the demethylated amine and formaldehyde. In the presence of glutathione, the cation radical is reduced to the parent amine, resulting in the formation of the glutathione thiyl radical. It thus appears that both primary and substituted aromatic amines may undergo one-electron oxidation by PHS.

Introduction

A large number of structurally diverse xenobiotics may serve as reducing cofactors for the peroxidase activity of prostaglandin H synthase (PHS). Comprehensive reviews summarizing these reactions have appeared elsewhere (*1,2*). Aromatic amines are generally very efficient cofactors for the reduction of peroxides by peroxidase (*3*). The focus of the work described here was to determine the mechanism by which compounds from two classes of aromatic amines, primary (2-aminofluorene) and tertiary (aminopyrine), are oxidized by PHS.

2-Aminofluorene (2-AF) is a model arylamine bladder carcinogen, known to undergo *N*-hydroxylation by the cytochrome P-450-dependent mixed-function oxidase system of hepatic microsomes (*4*). Metabolic *N*-hydroxylation is thought to be an obligatory step in the activation of primary arylamines to their ultimate carcinogenic form (*5*). Recent investigations, however, have focused not only on target tissue activation (e.g., the bladder) of these compounds (*6,7*) but also on alternative mechanisms of metabolic activation (*8*). Our original work with 2-AF suggested that the oxidation of this compound by PHS occurred through a one-electron mechanism, and that an *N*-hydroxy intermediate was not involved (*9*). Since 2-AF is also activated to a potent mutagen by PHS in the Ames test (*10*), further work was carried out to determine the metabolic pathway of 2-AF peroxidation (*11*). In addition, the DNA adducts formed from PHS-dependent oxidation of 2-AF were studied, and found to be unique from that adduct formed by reaction of *N*-hydroxy-2-AF with DNA (*12*).

A preliminary study on the metabolism of a variety of *N*-alkyl xenobiotics by PHS indicated that aminopyrine (AP) was among those compounds that underwent *N*-demethylation (*13*). Subsequently, the aminopyrine cation radical was identified as the initial product of PHS-dependent oxidation (*14*). Our most recent studies have centered on the formation of this radical as well as the subsequent reactions of the radical (*15*).

2-Aminofluorene

Preliminary studies of 2-AF peroxidation were carried out at pH 7.8 with the use of PHS obtained from ram seminal vesicle microsomes (*9*). Oxidation was in-

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itiated by arachidonic acid, H_2O_2, or a lipid hydroperoxide; indomethacin inhibited the arachidonic acid-dependent reaction but not the peroxide-dependent reactions. These results indicated that the peroxidase activity of PHS was responsible for 2-AF oxidation. The time course of the reaction was extremely rapid, reaching conclusion in less than 20 sec. Organic extractable products were isolated and identified as 2,2'-azobisfluorene and 2-nitrofluorene. In addition, 2-AF was metabolized to water-soluble polymeric material and products that covalently bound to tissue macromolecules. The reaction conditions and results are summarized in Table 1. A comparison was made between the PHS-dependent oxidation of 2-AF and that of two other well-characterized peroxidases, horseradish peroxidase and chloroperoxidase. The former enzyme yielded a product profile qualitatively identical to that obtained with PHS, while in the latter system, 2-nitrosofluorene was the primary oxidation product. These results are also summarized in Table 1. We were unable to detect *N*-hydroxy-2-AF or 2-nitrosofluorene at any point during 2-AF oxidation by PHS, as measured spectrophotometrically or colorimetrically using two different trapping reagents. Furthermore, the stable condensation product of these two compounds, azoxyfluorene, was not detected under any conditions. The tentative conclusion was that 2-AF was oxidized by PHS via a free-radical mechanism, and that an *N*-hydroxy intermediate was not involved.

Subsequent studies were designed to further test the hypothesis that the PHS-dependent oxidation of 2-AF occurs through a one-electron mechanism, and that the reactive products generated are free-radical in nature rather than *N*-oxygenated (*11*). Oxygen uptake studies indicated that, although 2-AF metabolism was extensive, no molecular oxygen was utilized during 2-AF peroxidation. When the reaction was carried out at pH 5.0 rather than 7.0, slightly greater metabolism was observed, and the development of a blue color was readily seen. The addition of ascorbic acid completely quenched the blue chromophore, and the primary product was subsequently isolated and identified as a nitrogen to ring 2-AF dimer, 2-aminodifluorenylamine. We concluded that the blue color was a 2-AF diimine, existing in acid-base equilibrium with a nitrenium ion. The reaction mechanism and structures are summarized in Figure 1; the metabolism data comparing product formation at pH 5.0 and 7.0 are shown in Table 2. The qualitative difference in product formation at the two pH values is probably due to the deprotonation of the amine radical at neutral pH, resulting in N-N coupling to yield azofluorene. At pH 5.0, however, N-C coupling predominates because of the charged cation radicals.

When a phenolic compound, either 2-*tert*-butyl-4-methoxyphenol (BHA) or 2,6-dimethylphenol (DMP), was included in 2-AF reaction mixtures, a bright pink or violet color rapidly developed upon 2-AF oxidation. The colored compounds were isolated and identified as amine/phenolic adducts, the structures of which are shown in Figure 2. For each phenol, there were two adducts formed, with the nitrogen of either 2-AF or 2-aminodifluorenylamine coupled to the *para* position of the phenol (loss of $-OCH_3$ with BHA). In contrast, acid hydrolysis of *N*-hydroxy-2-AF to yield the nitrenium ion, in the presence of phenol, also resulted in adduct formation, but only at times >2 hr and in very limited yield. The peroxidase-catalyzed adduct formation, however, was rapid (<2 min) and extensive. Peroxidase-dependent oxidation of *N*-hydroxy-2-AF in the presence of a phenol also failed to yield adducts. A pathway showing a free-radical mechanism for amine/phenol adduct formation is shown in Figure 3.

Chemical oxidation of 2-AF with the one-electron oxidants potassium ferricyanide and Fenton's reagent resulted in a product profile qualitatively similar to that obtained with PHS. Inclusion of BHA in the chemical system also resulted in amine/phenol adduct formation, similar to that obtained in the peroxidase systems. Our data strongly suggest that 2-AF is oxidized by PHS via a one electron mechanism, and that an *N*-hydroxy-2-AF intermediate is not involved. The hypothesis was made that the reactive species involved in the PHS-dependent mutagenesis of 2-AF is a free radical.

The binding of reactive electrophiles to DNA appears to be a critical event in the initiation of chemical carcinogenesis. Since the oxidation of 2-AF by PHS results in a unique reactive intermediate, it was presumed that unique DNA adducts would be formed as well. Addi-

Table 1. Oxidation of 2-AF by peroxidases.

Reaction mixture	Organic extractable, nmole/reaction		Nonorganic extractable, nmole/reaction	
	Azofluorene	2-Nitrofluorene	Water-soluble	covalently bound
PHS + AA[a]	7.33	5.00	3.00	7.93
PHS + H_2O_2[b]	5.92	5.19	3.61	11.96
HRP + H_2O_2[c]	6.79	2.81	82.20	
CPO + H_2O_2[d]	14.56 (2-nitrosofluorene) 26.38 (oligomers)		38.21	

[a] Reaction conditions: 25 mM potassium phosphate buffer, pH 7.8, 0.8 mg ram seminal vesicle microsomal protein, 100 nmole [^{3}H]2-AF, and 200 nmole arachidonic acid, in 2 mL.

[b] Same as in footnote a, with 200 nmole H_2O_2 instead of arachidonic acid.

[c] 25 mM potassium phosphate buffer, pH 7.0, 20 μg horseradish peroxidase, 100 nmole [^{3}H]2-AF, and 200 nmole H_2O_2, in 2 mL.

[d] 25 mM sodium acetate buffer, pH 5.0, 20 μg chloroperoxidase, 100 nmole [^{3}H]2-AF, and 200 nmole H_2O_2, in 2 mL.

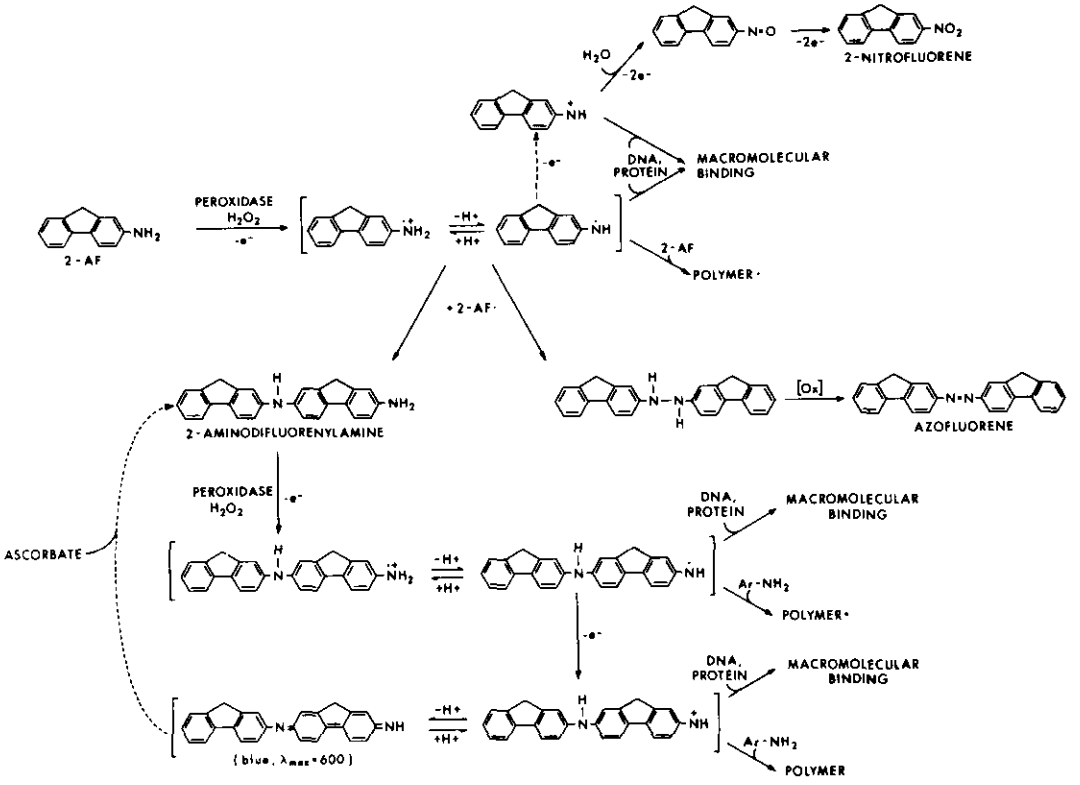


FIGURE 1. Proposed pathway for the oxidation of 2-AF by PHS. Ar-NH_2 = either 2-AF or 2-aminodifluorenylamine.

tional studies were therefore carried out to determine the nature of the DNA adducts formed as a result of PHS-dependent 2-AF oxidation (*12*). As in the metabolism studies, binding of 2-AF to DNA was dependent upon the peroxidase activity of PHS and required a peroxide substrate. As seen in Table 3, PHS also catalyzed the binding of 2-AF to tRNA and two homopolymers, polyguanylic acid, and polycytidylic acid. Little or no binding was detected with polyadenylic acid and polyuridylic acid.

Modification of DNA by *N*-hydroxy-2-AF under mildly acidic conditions yielded a single 2-AF/nucleoside adduct, identified as *N*-(deoxyguanosin-8-yl)-2-AF (C8-dG-AF) (*16,17*). Enzymatic hydrolysis of DNA modified by 2-AF activated in the PHS system, however, liberated 2-AF/nucleoside adducts that differed considerably from C8-dG-AF in chromatographic and extraction properties. HPLC chromatograms of 2-AF/DNA adducts derived from the PHS and horseradish peroxidase systems are compared to C8-dG-AF in Figure 4. Additional evidence suggests that the peroxidase-derived adducts may possess a negative charge at neutral pH. Structural identification of these unique adducts is now in progress. These adducts represent a marker unique to the peroxidative activation of 2-AF, and may therefore be used as a differential endpoint with which to assess the relative roles of *N*-hydroxylation and peroxidation in the metabolic activation of 2-AF in cell culture and in target tissues *in vivo*.

In summary, the studies described above provide

Table 2. Peroxidase-dependent 2-AF oxidation products.

Reaction mixture	Organic extractable, nmole/reaction			Nonorganic extractable, nmole/reaction	
	Azofluorene	2-ADFA	2-Nitrofluorene	Water-soluble	Covalently bound
PHS, pH 5.0[a]	17.4	22.7	7.3	9.3	23.0
PHS, pH 7.0[b]	12.2	9.2	11.2	6.3	16.9
HRP, pH 5.0[c]	10.7	121.0	21.6	32.1	
HRP, pH 7.0[d]	125.5	20.0	4.0	41.6	

[a] Reaction conditions: 25 mM sodium acetate buffer, pH 5.0, 1.0 mg solubilized PHS, 250 nmole [^{3}H]2-AF, and 500 nmole H_2O_2, in 5 mL.
[b] Same as above in 25 mM potassium phosphate buffer, pH 7.0.
[c] 25 mM sodium acetate buffer, pH 5.0, 5.0 μg horseradish peroxidase, 250 nmole [^{3}H]2-AF, and 500 nmole H_2O_2, in 5 mL.
[d] Same as above, in 25 mM potassium phosphate buffer, pH 7.0.

strong evidence that PHS catalyzes the oxidation of 2-AF via a one-electron mechanism. Furthermore, a free radical, or free radical-derived product, appears to be the reactive intermediate responsible for mutagenicity and DNA binding catalyzed by PHS. *N*-hydroxylation may therefore not be an obligatory step in the activation of arylamines to carcinogenic species.

Aminopyrine

Early studies indicated that a variety of secondary and tertiary amines were substrates for the peroxidase activity of PHS, and underwent oxidative *N*-demethylation (*13*). Aminopyrine (AP), aminocarb, and *N*-methylaniline were among the best substrates, while *S*-alkyl and *O*-alkyl compounds such as methiocarb and 7-ethoxyresorufin were not substrates for PHS (Table 4).

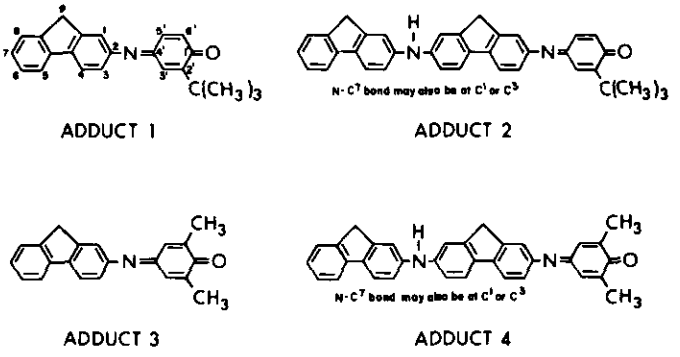


FIGURE 2. Proposed structures of 2-AF/phenolic adducts. Adducts 1 and 2 are from reaction with BHA; adducts 3 and 4 are from reaction with DMP.

In addition to the PHS activity of ram seminal vesicle microsomes, that of guinea pig lung, mouse lung, and rabbit kidney medulla also catalyzed *N*-demethylation.

The mechanism of peroxidase-dependent *N*-demethylation appears to be free-radical in nature, as opposed to the oxygen-insertion mechanism proposed for the cytochrome P-450-dependent monooxygenase. The mechanism of PHS-dependent *N*-demethylation of amines was investigated by using AP as a model substrate. A transient blue-colored AP free-radical species was detected using electron spin resonance (ESR) spectroscopy (Fig. 5), and subsequently identified as the cation radical of the parent compound (*14*). Radical formation was dependent upon the peroxidase activity of PHS, and could be initiated with arachidonic acid, 15-hydroperoxyarachidonic acid, or *tert*-butyl hydroperoxide. Free-radical formation corresponded closely with formaldehyde formation, in terms of substrate dependence, cofactor concentration, and time course.

Table 3. PHS-dependent binding of 2-AF to nucleic acids.

Nucleic acid	[^{3}H]2-AF bound, pmole/mg DNA
DNA[a]	25.7
tRNA[b]	287.8
Polyguanylic acid[c]	166.2
Polycytidylic acid	35.4
Polyadenylic acid	5.0
Polyuridylic acid	<2.0

[a] Reaction conditions: 0.8 mg ram seminal vesicle microsomal protein, 200 nmole arachidonic acid, 100 nmole [^{3}H]2-AF, and 2 mg calf thymus DNA in 2 mL of 100 mM sodium phosphate buffer, pH 7.8.
[b] Same reaction conditions as above with 2 mg tRNA substituted for DNA.
[c] Same reaction conditions as above with 2 mg homopolymer instead of DNA.

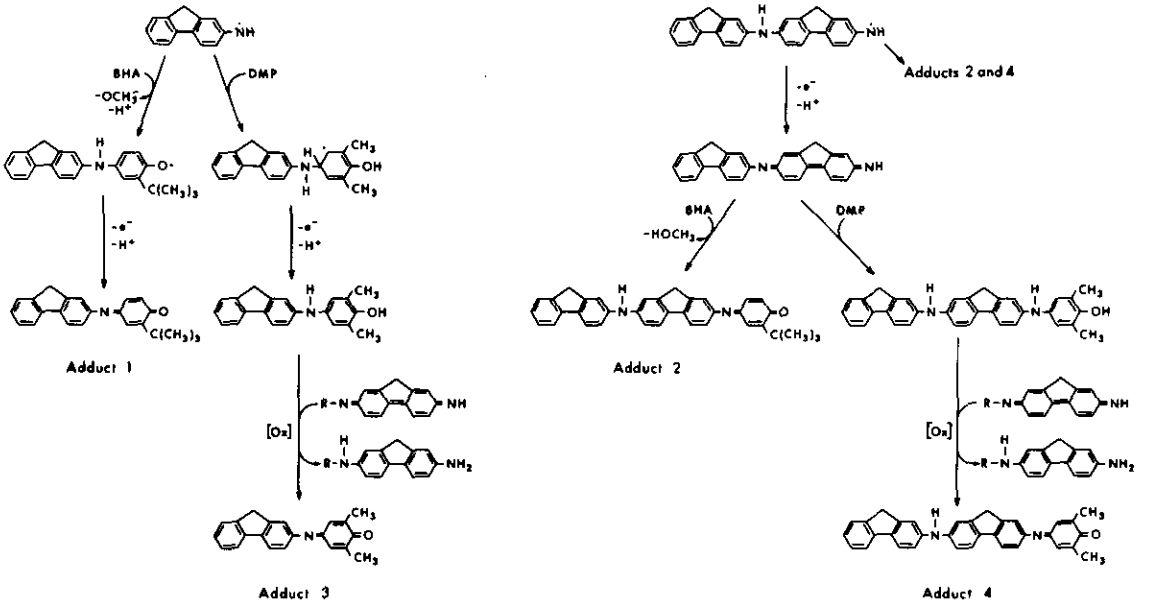
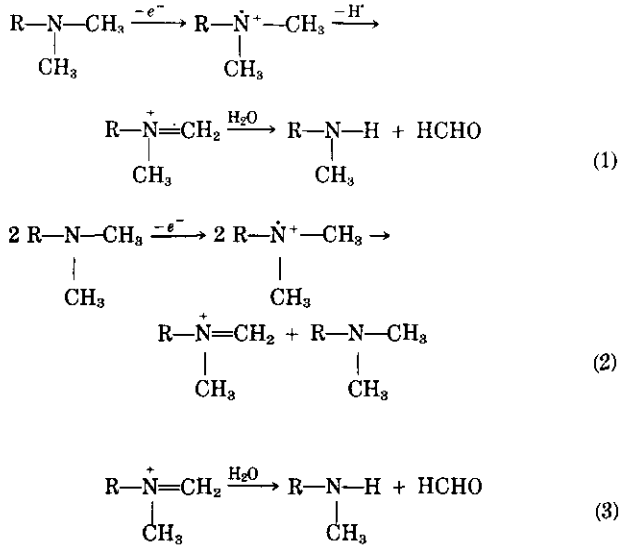


FIGURE 3. Proposed pathway for the formation of phenolic adducts by 2-AF radical products, R = fluorene.

Several mechanisms for the *N*-demethylation of aromatic amines by peroxidase have been proposed, using AP as a model substrate. Using horseradish peroxidase, it was proposed that AP undergoes one-electron oxidation to a cation free radical, which is then further oxidized to an iminium cation (*18*). Hydrolysis of the iminium cation yields the monomethyl amine and formaldehyde as depicted in Eq. (1).



(1)

(2)

(3)

Table 4. *N*-Demethylation of xenobiotics by PHS.

Compound	Rate of *N*-demethylation, nmole HCHO formed/min/mg protein[a]
Monomethyl-substituted	
N-Methylaniline	40.3
N-Methyl-*p*-chloroaniline	33.5
N-Methyl-*o*-nitroaniline	ND[b]
N-Methyl-*p*-nitroaniline	ND
N-Methyl-*o*-toluidine	22.4
N-Methyl-*m*-toluidine	39.9
N-Methyl-*p*-toluidine	10.7
N-Methyl-*o*-phenylenediamine	3.5
N-Methyl-*p*-phenylenediamine	16.5
N-Methyl-*n*-nitrosoaniline	5.9
Dimethyl-substituted	
N,N,-Dimethylaniline	35.3
N,N,-Dimethylaminobenzoic acid	13.5
N,N,-Dimethyl-*p*-nitroaniline	6.0
N,N,-Dimethyl-*p*-phenylenediamine	10.2
Miscellaneous	
Aminopyrine	53.8
Aminocarb	56.4
Benzphetamine	5.2
p-Nitroanisole	ND
1,2,4-Trimethoxybenzene	ND
Methiocarb	ND
7-Ethoxycoumarin	ND
7-Ethoxyresorufin	ND

[a] Reaction conditions: 66 mM Tris-HCl buffer, pH 7.4, 0.2 mg ram seminal vesicle microsomal protein, 15 μmole semicarbazide, 10 μmole $MgCl_2$, 2 μmole substrate, and 0.4 μmole arachidonic acid in 2 mL.
[b] Not detectable.

We have proposed a different mechanism for AP demethylation by PHS, in which AP is oxidized to a free-radical cation, which then disproportionates to the iminium cation and AP. The iminium cation is subsequently hydrolyzed to the monomethyl amine and formaldehyde as shown in Eqs. (2) and (3).

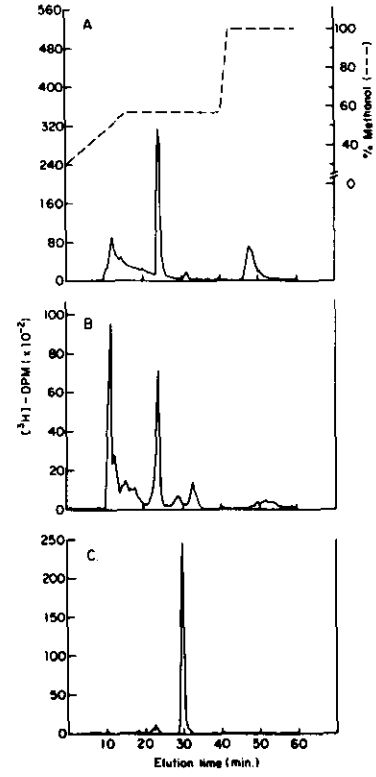


FIGURE 4. HPLC chromatograms of 2-AF/DNA adducts: (*A*) adducts formed in PHS/arachidonic acid system; (*B*) adducts formed in horseradish peroxidase/H_2O_2 system; (*C*) C8-dG-AF. Chromatography was performed using an Altex Ultrasphere ODS column (5 μm; 0.46 × 25 cm). Adducts were eluted using a methanol/water gradient of 30 to 50% methanol over 15 min, followed by 57% methanol for 25 min. Flow rate was 1 mL/min.

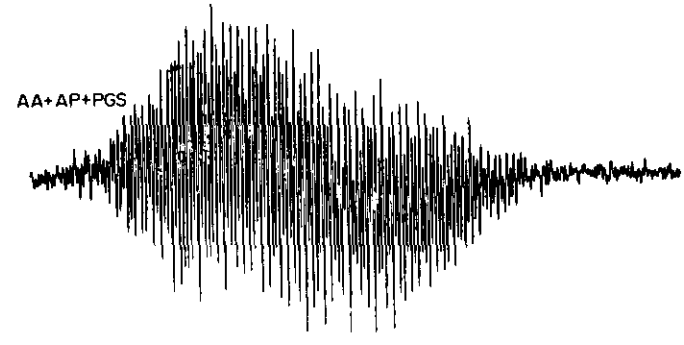


FIGURE 5. ESR spectrum of AP free radical. Reaction mixtures contained 2 mg of ram seminal vesicle microsomal protein, 0.8 μmole of arachidonic acid (AA), and 2 μmole of AP in 2 mL of 100 mM potassium phosphate buffer, pH 7.4. Spectrum was recorded at room temperature approximately 30 sec after mixing.

Our proposal is based on the finding that the steady-state concentration of the radical is linearly related to the square root of the enzyme concentration (*14*), which is consistent with a bimolecular decay. We have also measured the decay of the AP free radical directly, taking advantage of the very rapid AP oxidation by PHS and subsequent inactivation of the enzyme by 15-hydroperoxyarachidonic acid (*15*). The AP free-radical decay obeyed second-order kinetics, indicating a bimolecular decay consistent with the proposed disproportionation mechanism.

Most recent experiments were designed to further elucidate the mechanism of PHS-dependent *N*-demethylation of AP and to determine subsequent reactions of the AP radical (*15*). Experiments carried out with highly purified PHS confirmed that the peroxidase activity of PHS is responsible for AP oxidation (*15*). When this reaction was initiated with [^{14}C]15-hydroperoxyarachidonic acid, the corresponding alcohol was isolated and identified. Using this procedure, we determined that two molecules of AP were oxidized for every molecule of [^{14}C]15-hydroperoxyarachidonic acid reduced. This stoichiometry of 2:1 is expected for a peroxidase-mediated reaction.

Glutathione (GSH) included in reaction mixtures rapidly reduced the AP free radical to AP, with concomitant oxidation of GSH to the thiyl radical (GS$^{\cdot}$). This radical was identified from the spectrum of its adduct with the spin trap DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide) (Fig. 6). Previous work by Moldeus et al. (*19*) had shown that GSH was oxidized to GSSG by either

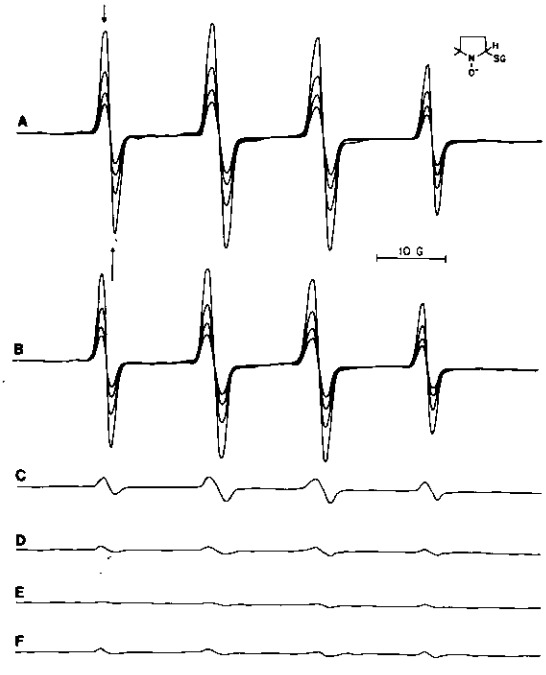


FIGURE 6. ESR spectra of the GSH thiyl radical adduct: (*A*) reaction mixture consisting of 0.5 mg solubilized PHS, 1 μmole AP, 100 nmole 15-hydroperoxyarachidonic acid, 1 μmole GSH, and 60 μL DMPO in 2 mL of 33 mM HEPES buffer, pH 7.8; (*B*) identical to (*A*) except that GSH was added after a 10 sec delay. (*C*) identical to (*A*), except AP was omitted. (*D*) identical to (*B*) except AP was omitted; (*E*) identical to (*A*) except 15-hydroperoxyarachidonic acid was omitted; (*F*) identical to (*A*), except that the enzyme was boiled for 5 min.

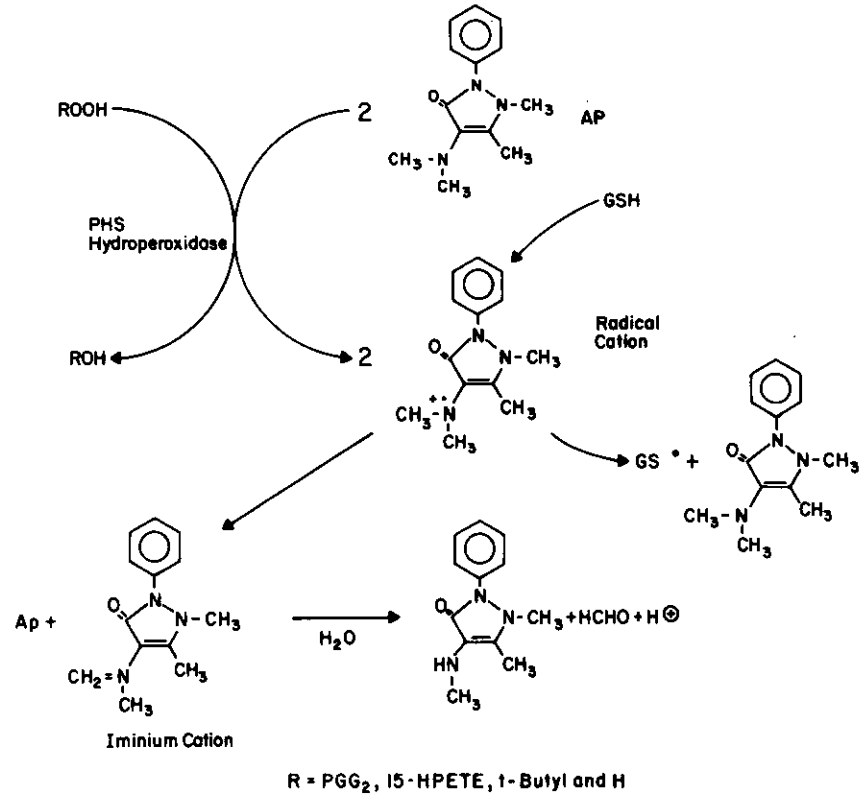


FIGURE 7. Proposed mechanism for the oxidation of AP by PHS.

$$AP^{+\cdot} + GSH \rightarrow AP + GS^{\cdot} \quad (4)$$

$$2\ GS^{\cdot} \rightarrow GSSG \quad (5)$$

the AP or dimethylaniline cation radical. This reaction was presumed to proceed via the $GS^{\cdot}$ radical. Our data thus support the hypothesis (*19*) that amine cation free radicals generated by intact cells which contain GSH may result in the formation of $GS^{\cdot}$.

In summary, the experimental data indicate that AP is oxidized by the peroxidase activity of PHS to the cation free radical. This radical then disproportionates to yield the iminium cation and parent amine. Hydrolysis of the iminium cation yields the demethylated amine and formaldehyde. This pathway is summarized in Figure 7.

Conclusions

The data presented above indicate that both primary aromatic amines and alkyl-substituted amine compounds may serve as reducing cofactors for the peroxidase activity of PHS. The metabolism of 2-AF and AP was studied and evidence was presented indicating that both compounds undergo one electron oxidation to free-radical intermediates. In the case of 2-AF, the radical may react with itself to form stable products (primarily azofluorene or 2-aminodifluorenylamine, depending on the pH of the reaction mixture), or react with nucleophiles (phenols or DNA) to form adducts. The DNA adducts are unique to the PHS system and may represent a marker with which to assess the relative contribution of peroxidase-dependent metabolism of 2-AF *in vivo*. It is predicted that a free radical is the mutagenic intermediate in the PHS-dependent oxidation of 2-AF.

In the case of AP, the radical may disproportionate to form formaldehyde and the parent amine, or react with GSH to form the thiyl radical and parent amine. This latter reaction may represent a detoxification mechanism for the reactive amine compound. Thiyl radicals themselves may exert a toxic effect, however, representing an indirect mechanism for PHS-mediated xenobiotic toxicity.

The possible target tissue relevance of these reactions *in vivo* remains a subject for future research. The isolation of unique DNA adducts and the subsequent reaction products of thiyl radicals ($GS^{\cdot}$) are but two ways that the PHS-dependent oxidation of xenobiotics may be assessed *in vivo*.

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