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Mechanisms of Activation of Phenacetin to Reactive Metabolites by Cytochrome P-450: A Theoretical Study Involving Radical Intermediates

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SUMMARY

The cytochrome P-450-mediated activation of phenacetin (PHEN) to reactive intermediates by two hypothetical mechanisms has been studied by use of SV 6-31G *ab initio* energy and spin distribution calculations. In our calculations, the cytochrome P-450 enzyme system has been substituted by a singlet oxygen atom in order to reduce the computational efforts and to fulfill the requirements as to spin conservation. Both mechanisms are based on the currently increasingly accepted view that radical intermediates, formed via sequential one-electron steps, play a crucial role in the metabolic activation of substrates by cytochrome P-450. The first pathway is proposed to involve an initial abstraction of an electron and a proton from the α-methylene carbon atom in the ethoxy side chain and can explain the *O*-deethylation products paracetamol and acetaldehyde. In the second pathway, an initial abstraction of an electron and a proton from the nitrogen atom in the acetylamino side chain is proposed. The calculated spin densities of the formed nitrogen radical indicate that the unpaired electron is primarily localized at the nitrogen atom and to a smaller extent at the *ortho*- and *para*-carbon atoms relative to the acetylamino group. Radical recombination reactions between a hydroxyl radical and the spin delocalization-radicalized reactive centers of the nitrogen radical can explain the formation of the metabolites *N*-hydroxy-PHEN, 2-hydroxy-PHEN, and the arylating metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI), which forms a 3-(*S*-glutathionyl)paracetamol conjugate in the presence of glutathione. NAPQI is proposed to be formed via intermediate formation of a hemiketal. Proposals are made for the decomposition of this hemiketal into NAPQI that are consistent with currently available experimental data on ^{14}C- and ^{18}O-labeled PHEN.

PHEN (Fig. 1), a structural analogue of paracetamol, is widely used as a mild antipyretic and analgesic drug. High doses of PHEN have been shown to cause hepatic necrosis in hamsters, due to arylating and alkylating metabolites (1, 2). In addition, PHEN has been reported to cause renal damage and methemoglobinemia in humans (3) and experimental animals (4). The renal toxicity of PHEN is suggested to result from hydrolysis of PHEN, by amidases, to *p*-phenetidine and subsequent oxidation of this aromatic amine. Prostaglandin H synthase, present in high concentrations in the kidney, has been shown to convert *p*-phenetidine to reactive *N*-(4-ethoxyphenyl)-*p*-benzoquinone imine and diimine metabolites, which are held responsible for the renal toxicity of PHEN (5, 6). On the other hand, the hepatic cytochrome P-450-containing mixed function oxidase enzyme system is thought to be responsible for the hepatic injury caused by PHEN (6). Previous studies have suggested that the hepatic cytochrome P-450 system oxidatively metabolized PHEN via three major pathways (1, 2, 7–10). These pathways include oxidative *O*-deethylation to paracetamol, *N*-oxygenation to *N*-hydroxy-PHEN, and the formation of an arylating metabolite, which in the presence of glutathione forms a 3-(*S*-glutathionyl)paracetamol conjugate. In addition, 2-hydroxy-PHEN has been detected as a minor metabolite of PHEN in humans and rats (11).

Oxidative *O*-deethylation of PHEN to form acetaldehyde and paracetamol and further metabolism of paracetamol constitute the major pathway for reactive metabolite formation from PHEN by cytochrome P-450 *in vivo* (7, 8). In both rabbit (9) and hamster liver microsomal systems (4), the mechanism of *O*-deethylation of PHEN was shown to occur through the formation of an unstable hemiketal at the α-methylene carbon atom, which decomposes rapidly into paracetamol and acetaldehyde (Fig. 1, route A). However, PHEN was also shown to be oxidized by cytochrome P-450 to *N*-hydroxy-PHEN (10), which in turn can be converted to *N*-*O*-sulfate and *N*-*O*-glucuronide conjugates, both of which decompose spontaneously into reactive metabolites (12) (Fig. 1, route B). Fur-

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ABBREVIATIONS: PHEN, phenacetin; NAPQI, *N*-acetyl-*p*-benzoquinone imine; GAMESS, general atomic and molecular electronic structure system; UHF, unrestricted Hartree-Fock.

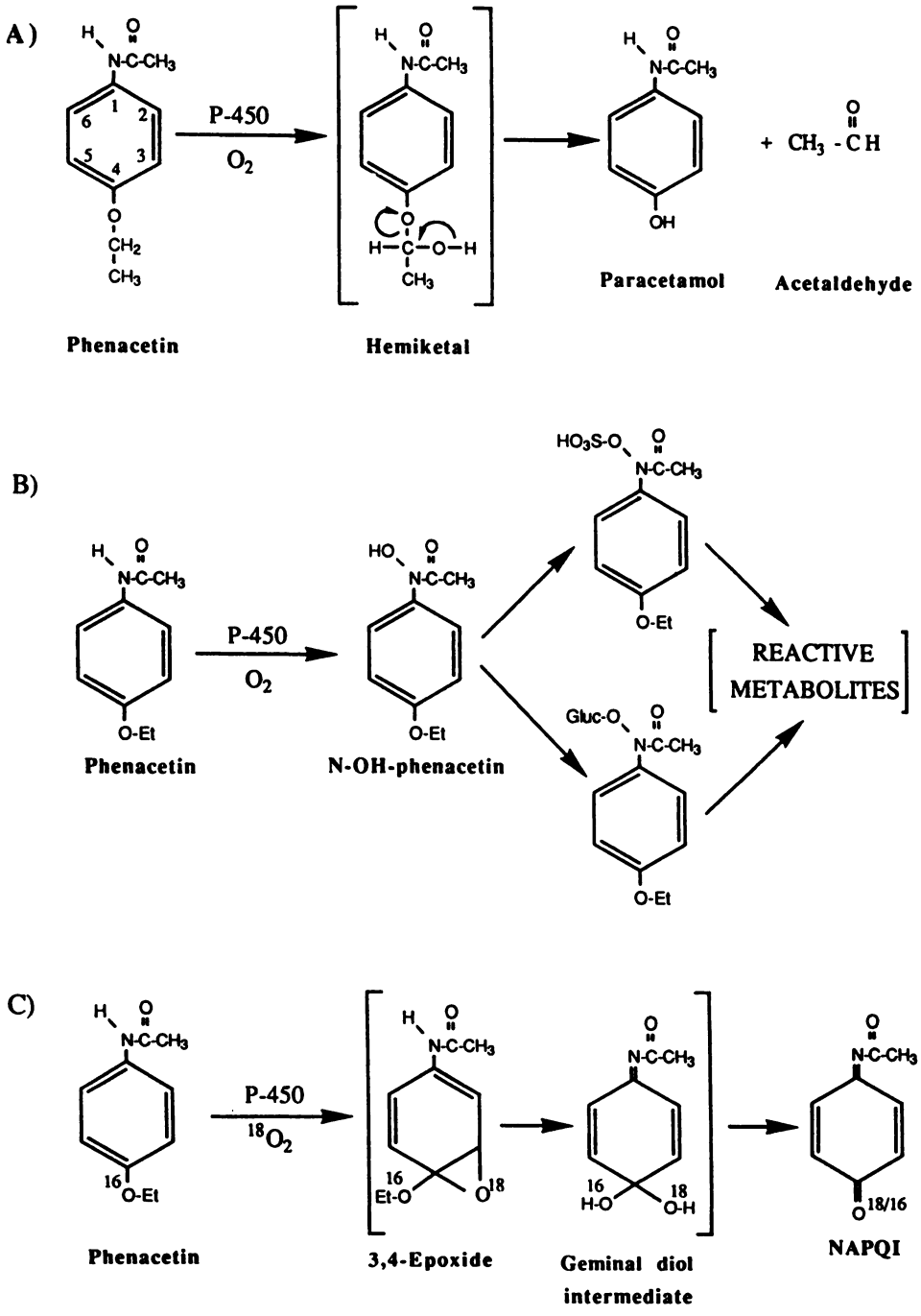


Fig. 1. Mechanisms of cytochrome P-450-mediated biotransformation of PHEN to reactive metabolites proposed in the literature. Route A, mechanism of oxidative *O*-deethylation of PHEN via formation of an unstable hemiketal that decomposes to paracetamol and acetaldehyde (4, 9). Route B, oxidative metabolism of PHEN to *N*-hydroxy-PHEN, which can be converted to *N*-*O*-sulfate and *N*-*O*-glucuronide conjugates, both of which decompose spontaneously to reactive metabolites (12). Route C, the formation of NAPQI directly from PHEN. Initially, a 3,4-epoxide intermediate is formed, which is converted to a symmetrical geminal diol intermediate. One of the hydroxyl groups is then randomly lost during dehydration of this intermediate, resulting in approximately 50% incorporation of molecular oxygen into the formed NAPQI metabolite (7, 13). The compounds in brackets are proposed intermediates.

thermore, PHEN was shown to be converted by cytochrome P-450 to an arylating metabolite, probably NAPQI, which reacts with glutathione to form a 3-(*S*-glutathionyl)paracetamol conjugate (1, 7). Although both paracetamol and PHEN form 3-(*S*-glutathionyl)paracetamol in microsomal incubations, the conjugates must arise from different intermediates, because in the presence of ^{18}O-labeled molecular oxygen the phenolic oxygen was totally retained in the conjugate arising from paracetamol, whereas approximately 50% of the oxygen in the phenolic group of the conjugate arising from PHEN appeared to originate from ^{18}O-labeled molecular oxygen (7, 13). The partial incorporation of atmospheric oxygen in the 3-(*S*-glutathionyl)paracetamol conjugate formed from PHEN was suggested to occur via a 3,4-epoxide intermediate (7, 13) (Fig. 1, route C). This intermediate could then be converted to a symmetrical geminal diol intermediate at position 4 of the ring. One of the hydroxyl groups would then randomly be lost during dehydration of this intermediate to form NAPQI and subsequently a 3-(*S*-glutathionyl)paracetamol conjugate. In addition to the above mentioned metabolic pathways involving oxidation at the α-C carbon atom, the C_3-C_4 carbon atoms, and the nitrogen atom, PHEN was also shown to be converted by cytochrome P-450 to 2-hydroxy-PHEN (11).

The relative importance *in vivo* of the above mentioned pathways by which PHEN is converted to chemically reactive metabolites has been established in hamsters (1, 7) and humans (11). From these studies it can be concluded that *in vivo* most of the reactive metabolites of PHEN arise from oxidative

deethylation to paracetamol and acetaldehyde, followed by further metabolism of paracetamol. However, *in vitro* in hamster liver microsomes it was shown that extensive covalent binding takes place, whereas only a small fraction of PHEN is converted to paracetamol at the end of the 10-min incubation period (13). Therefore, *in vitro* the pathways involving the formation of *N*-hydroxy-PHEN, the formation of 2-hydroxy-PHEN, and the formation of NAPQI directly from PHEN (Fig. 1, routes B and C) seem to be more important than the *O*-deethylation pathway (Fig. 1, route A).

In general, the mechanism of metabolic activation of substrates by cytochrome P-450 appears to consist of sequential one-electron steps, rather than a single concerted transfer of the activated reactive oxygen of cytochrome P-450 to the substrates (14, 15). The radical intermediates formed via these one-electron steps can (before or after rearrangement of the radical) recombine with the activated oxygen species of cytochrome P-450 to yield oxygenated metabolites or undergo alternative reactions associated with radical species (14). In a previous theoretical study on the metabolic activation of paracetamol by cytochrome P-450 (16), we proposed a hypothetical mechanism of activation via initial hydrogen abstraction (abstraction of an electron and a proton) from the phenolic oxygen, yielding a phenoxy radical. This initial hydrogen abstraction was calculated to be favored by 30.1 kcal/mol over an initial hydrogen abstraction from the nitrogen atom of paracetamol. Assuming that radical rearrangement of the phenoxy radical takes place via delocalization of the unpaired electron, we were able to explain the occurrence of all the observed oxidized metabolites of paracetamol via one uniform oxidation mechanism (16). *Ab initio* SV 6-31G energy calculations pointed to the most likely mechanistic pathway for bioactivation of paracetamol by cytochrome P-450 and, interestingly, the *ab initio* results are in agreement with recent NMR longitudinal relaxation rate measurements suggesting that the orientation of the phenolic group of paracetamol towards the heme iron atom of cytochrome P-450 plays an essential role in the isoenzyme-selective oxidative bioactivation of paracetamol to NAPQI (17). We hypothesized (16) that the formation of different oxidative metabolites from substrates by cytochrome P-450 might more generally be explained via initial one-electron steps and subsequent rearrangement of the radical intermediates. To investigate the validity of this hypothesis we studied the oxidative metabolism of PHEN, a structural analogue of paracetamol. Until now, it has not been possible to detect experimentally as yet elusive intermediates, free radical or others, that might be formed in the active site pocket of cytochrome P-450 during the transformation of PHEN to its metabolites. In such cases a theoretical study might be useful. However, the current theoretical calculations are limited in the sense that they can only account for the electronic aspects of cytochrome P-450-mediated metabolism. Neither an influence of the environment of the active site on the metabolic profile nor a possible regioselective metabolism of the substrate due to a specific orientation within the active site are accounted for in the approach used.

In the current theoretical study, we use *ab initio* energy and spin distribution calculations to support a new hypothetical mechanism for oxidative activation of PHEN by cytochrome P-450. This mechanism explains the formation of all the above mentioned oxidative metabolites of PHEN, considering only two different pathways; the initial step involves a hydrogen abstraction either from the α-methylene carbon atom of the ethoxy side chain (Fig. 2) or from the nitrogen atom in the acetylamino side chain (Fig. 3). The formation of paracetamol is explained via the former pathway, whereas the formation of *N*-hydroxy-PHEN, 2-hydroxy-PHEN, and the arylating metabolite of PHEN, NAPQI, are all explained via the latter pathway.

Experimental Procedures

The *ab initio* calculations carried out in this study were performed at the linear combination of atomic orbitals-molecular orbital-self-consistent field level by using the STO-3G (18) minimal basis set for the geometry optimizations and a SV 6-31G (19) basis set for the subsequent self-consistent field energy calculations. The quantum chemical program package GAMESS (20–22) was used on the Cyber 995 and the Cyber 205 of the Academic Computer Centre of Amsterdam (SARA).

In a previous theoretical study on the metabolic activation of paracetamol by cytochrome P-450 (16), it was shown that a flat "conjugated" geometry of paracetamol is energetically favored over a geometry in which the acetylamino side chain is rotated in a perpendicular orientation relative to the benzene ring. Therefore, flat conjugated geometries of the parent compound PHEN, intermediates, and products were taken as starting points for geometry optimizations. These geometries were optimized by variation of all bond distances, bond angles, and torsion angles using the STO-3G minimal basis set. For the geometry optimization of radical species, the UHF formalism was used, which assumes different orbitals for different spins. For the optimized geometries, SV 6-31G self-consistent field energies were calculated and it was investigated whether these *ab initio* results could support the hypothetical mechanisms shown in Fig. 2 and 3.

As to the spin distribution of the α-methylene carbon atom and the acetylamino nitrogen radical, it is known that, in UHF calculations, admixture of higher spin states may distort the results, leading to too negative energies and unrealistic spin densities (23). Therefore, SV 6-31G restricted Hartree-Fock spin distributions were calculated for the UHF geometries of the radicals. The Mulliken spin distributions were expressed as the difference between the α- and β-spins of the respective radicals (Fig. 4).

A cytochrome P-450 enzyme reaction is supposed to consist of a transfer of an activated oxygen atom from the enzyme to the enzyme-bound substrate via sequential one-electron steps. The reactive oxygen atom of the proposed biologically active ferric-oxene state of cytochrome P-450 is a triplet (^{3}P)O species. We used a simplified model system for our calculations by substituting the cytochrome P-450 enzyme complex with an oxygen atom. This was done in order to reduce the computational efforts and should not be construed as implying that the activated enzyme-bound oxygen is a free atomic oxygen. Therefore, and in order to comply with the law of conservation of spin momentum, a singlet oxygen species had to be used in our calculations. Apart from this, the energy difference between a singlet and a triplet oxygen is too small to influence the energy calculations of the proposed hypothetical mechanism of activation of PHEN.

Results

The cytochrome P-450-containing mixed function oxidase enzyme system has been shown to oxidize PHEN to several oxygenated and oxidated metabolites, notably paracetamol, *N*-hydroxy-PHEN, NAPQI, and, to a minor extent, 2-hydroxy-PHEN. Assuming a sequential one-electron step mechanism for oxygenation or oxidation by cytochrome P-450, the formation of these metabolites is explained by considering two different hypothetical pathways, as depicted in Figs. 2 and 3. Fig.

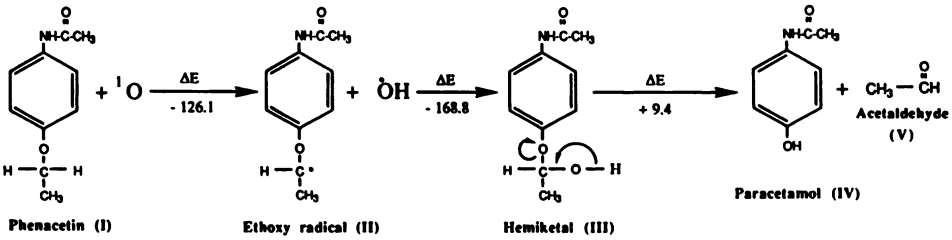


Fig. 2. Hypothetical ethoxy radical pathway for the *O*-deethylation of PHEN. An initial hydrogen abstraction is assumed to take place at the α-methylene carbon atom in the ethoxy side chain of PHEN. After a radical recombination reaction between a hydroxyl radical and the α-methylene carbon radical, the formed hemiketal III decomposes to paracetamol and acetaldehyde. ΔE (kcal/mol) is the calculated energy difference between reactants, intermediates, and products. The cytochrome P-450 enzyme complex has been substituted by a singlet oxygen species.

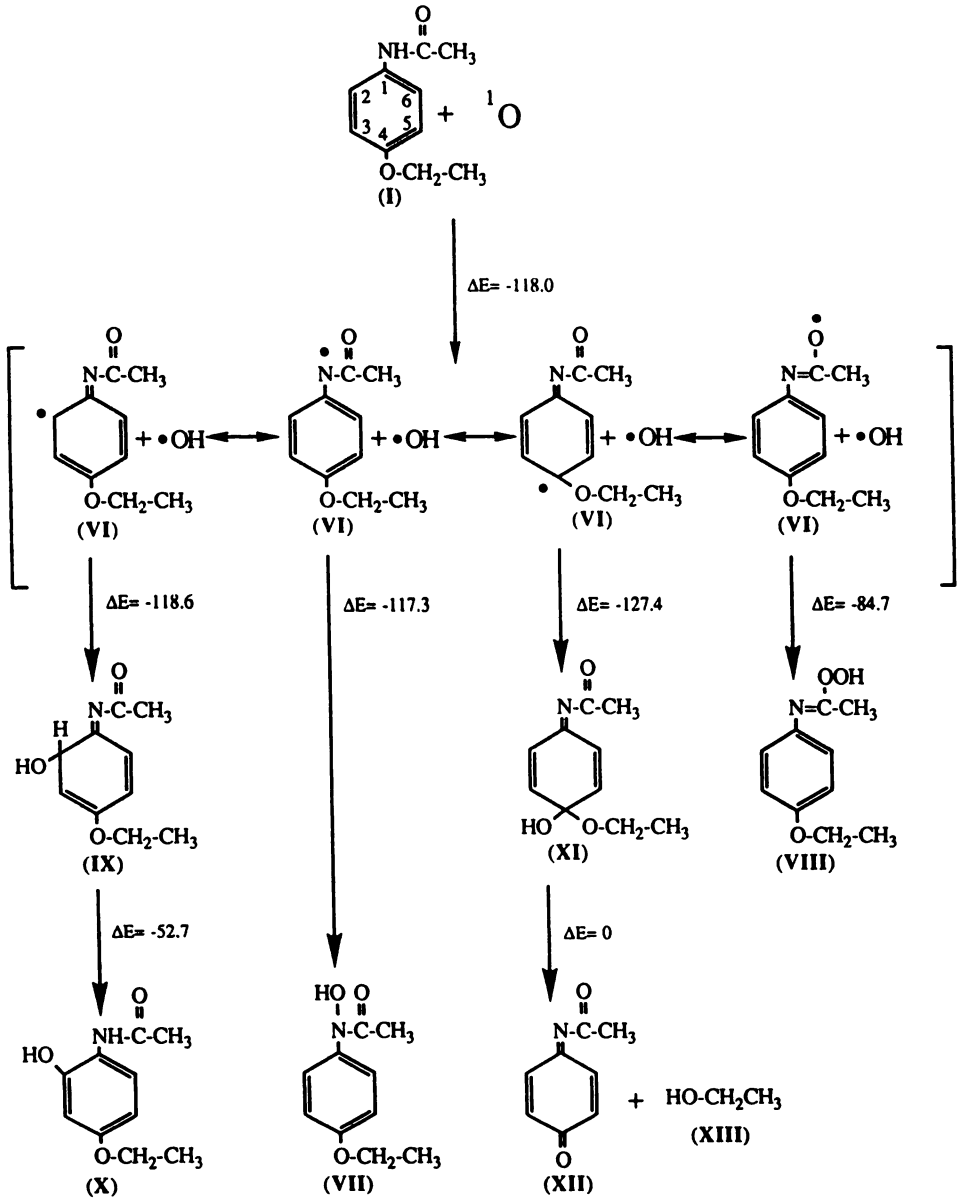


Fig. 3. Hypothetical nitrogen radical pathway. An initial hydrogen abstraction is assumed to take place at the nitrogen atom in the acetylamino side chain of PHEN. Possible subsequent radical recombination and rearrangement reactions are indicated. ΔE (kcal/mol) is the calculated energy difference between reactants, intermediates, and products. The cytochrome P-450 enzyme complex has been substituted by a singlet oxygen species.

2 describes a mechanism in which a hydrogen abstraction (i.e., abstraction of an electron and a proton) from the α-methylene carbon atom in the ethoxy side chain of PHEN is assumed to be the initial step. A second hypothetical pathway, in which initially a hydrogen atom is abstracted from the nitrogen atom in the acetylamino side chain of PHEN, is depicted in Fig. 3. In both mechanisms, the abstracted hydrogen atom is supposed to react with the enzyme-bound reactive oxygen species to yield a hydroxyl radical and an α-methylene carbon radical or a nitrogen radical of PHEN. Possible subsequent radical recombination, rearrangement, and/or decomposition reactions are considered.

Ethoxy radical pathway. A hydrogen abstraction from the α-methylene carbon atom in the ethoxy side chain of PHEN

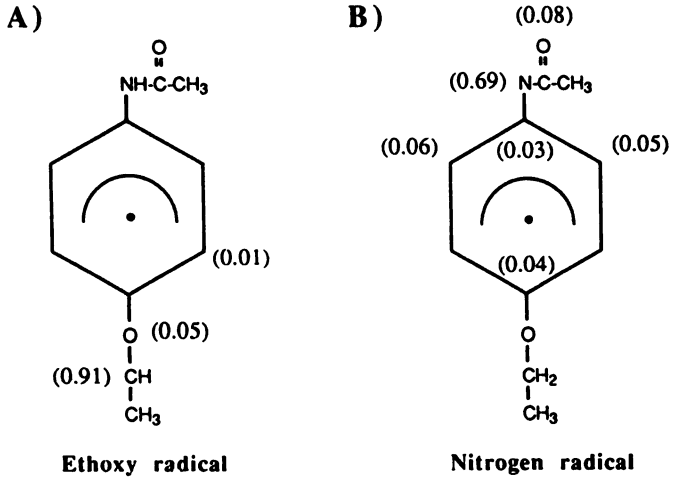


Fig. 4. Mulliken spin distributions of the ethoxy radical (A) and the nitrogen radical (B) of PHEN. Spin distributions are expressed as the difference in spin between the α- and β-spins of the respective radicals.

gives rise to a hydroxyl radical and an α-methylene carbon radical (Fig. 2, **II**) (ΔE = −126.1 kcal/mol). The calculated Mulliken spin distribution of this carbon-centered radical is shown in Fig. 4A. Apparently, the unpaired electron is predominantly localized at the α-methylene carbon atom. A recombination reaction between the hydroxyl radical and the radicalized α-methylene carbon results in an α-carbon hemiketal (Fig. 2, **III**) (ΔE = −168.8 kcal/mol). The hemiketal is expected to decompose rapidly into the deethylated product paracetamol and acetaldehyde (Fig. 2, **IV** and **V**, respectively) ΔE = +9.4 kcal/mol).

Nitrogen radical pathway. As depicted in Fig. 3, a hydrogen abstraction from the nitrogen atom in the acetylamino side chain of PHEN (**I**) yields a hydroxyl radical and the corresponding nitrogen radical (Fig. 3, **VI**) (ΔE = −118.0 kcal/mol). The calculated Mulliken spin distribution of this nitrogen radical, as shown in Fig. 4B, indicates that the unpaired electron is primarily localized at the nitrogen atom, to a minor extent at the ring carbon atoms in the *ortho* and *para* positions relative to the acetylamino group, and at the carbonyl oxygen in the acetylamino side chain of PHEN.

A recombination reaction between the nitrogen radical and the hydroxyl radical yields *N*-hydroxy-PHEN (Fig. 3, **VII**), a well known metabolite of PHEN. This reaction is energetically favored (ΔE = −117.3 kcal/mol).

A radical recombination reaction between the hydroxyl radical and one of the radicalized ring carbon atoms at the *ortho* position relative to the acetylamino group gives rise to intermediate **IX** (Fig. 3) (ΔE = −118.6 kcal/mol). This intermediate gains an additional amount of energy by rearranging to the stable 2-hydroxy-PHEN (Fig. 3, **X**), which is known to be one of the (minor) metabolites of PHEN.

When the hydroxyl radical and the radicalized ring carbon atom at the *para* position relative to the acetylamino group recombine, hemiketal **XI** (Fig. 3) is formed (ΔE = −127.4 kcal/mol). Hemiketal **XI** is proposed to decompose to NAPQI and ethanol (ΔE = 0 kcal/mol).

A peroxide intermediate (Fig. 3, **VIII**) is formed when the hydroxyl radical and the radicalized carbonyl oxygen in the acetylamino side chain recombine. This radical recombination reaction is energetically unfavorable (ΔE = −84.7 kcal/mol), when compared with a radical recombination reaction between the hydroxyl radical and the other radicalized centers of the nitrogen radical (ΔE = −117.3, −118.6, and −127.4 kcal/mol).

Comparison of the ethoxy and nitrogen radical pathway reveals that an initial hydrogen abstraction from the α-methylene carbon atom in the ethoxy side chain of PHEN (Fig. 2) is favored by 8.1 kcal/mol over an initial hydrogen abstraction from the nitrogen atom in the acetylamino side chain (Fig. 3).

Discussion

The hepatic cytochrome P-450-containing mixed function oxidase system is involved in the metabolism of a wide variety of exogenous and endogenous compounds. The main biological function of cytochrome P-450 under aerobic conditions and in the presence of NADPH is monooxygenation, i.e., the addition of one oxygen atom originating from molecular oxygen into the substrates, leading to hydroxylation, heteroatom oxygenation, or epoxidation reactions. According to recent views, these monooxygenation reactions appear to involve sequential one-electron steps, rather than a single concerted mechanism of incorporation of an activated oxygen atom into the substrates (14–16). The initial step in the metabolic activation of substrates by cytochrome P-450 consists of either an one-electron oxidation (abstraction of an electron) or a hydrogen abstraction (abstraction of an electron and a proton). After the initial one-electron step, the oxidized substrate (before or after rearrangement of the formed radical) can either recombine with the activated oxygen species to yield oxygenated products or undergo a second one-electron step leading to dehydrogenation. In the case of PHEN, we assume that, after initial abstraction of a hydrogen atom (or an electron and a proton) from either the α-methylene carbon atom or the nitrogen atom in the acetylamino side chain, the radical formed is stable enough to rearrange, so that different radicalized centers are theoretically able to react with the activated oxygen species of cytochrome P-450.

It has been established now that multiple forms of cytochrome P-450 with different but overlapping substrate specificities exist. In the case of PHEN, the 3-methylcholanthrene-inducible isoenzymes (cytochromes P-450c and P-450d) are the most active in both the *N*-hydroxylation and *O*-deethylation of PHEN, whereas the phenobarbital-inducible isoenzymes (cytochromes P-450b and P-450e) also have a considerable activity in these processes (10, 13, 24). Furthermore, the 3-methylcholanthrene-inducible isoenzymes seem to lower the covalent binding of PHEN to microsomal proteins, whereas the phenobarbital-inducible isoenzymes seem to stimulate the formation of arylating metabolites from PHEN. (13). This diffuse pattern of isoenzyme-selective metabolic activation of PHEN and the serious limitations of extrapolating the isoenzyme selectivity determined in one tissue to other tissues (25) make it difficult to get a fully consistent picture of the available experimental data on the isoenzyme selectivity of PHEN. As a consequence of this and because of the limitations of the current approach, only qualitative aspects of the mechanism of activation of PHEN by cytochrome P-450 are discussed. Therefore, it is not possible to use the results of the present theoretical calculations to predict the relative amounts and the relative rates of biotransformation of products of PHEN.

The results of the present *ab initio* calculations reveal that the ethoxy radical, formed via initial hydrogen abstraction from

the α-methylene carbon atom in the ethoxy side chain, is energetically 8.1 kcal/mol more stable than the corresponding nitrogen radical, formed via initial hydrogen abstraction from the nitrogen atom. This energy difference is relatively small and, thus, theoretically both radicals might probably be formed during the metabolic activation of PHEN by cytochrome P-450.

Ethoxy Radical Pathway

Our *ab initio* calculations of the spin distribution of the ethoxy radical (Fig. 4A) indicate that the unpaired electron stays primarily localized at the α-methylene carbon atom of PHEN. Oxidation of PHEN via an intermediate ethoxy radical can explain the formation of the deethylated product paracetamol (Fig. 2). In conformity with this mechanism, it was shown that the rate of deethylation of PHEN to paracetamol in both hamster and rabbit liver microsomal systems was substantially decreased by substituting the hydrogen atoms in the α-methylene position of the ethoxy group by deuterium; this rate was not significantly decreased by substituting hydrogen for deuterium in the β-methylene group of the ethoxy side chain (4, 9).

Nitrogen Radical Pathway

Oxidation of PHEN via initial formation of a nitrogen radical can explain the formation of the metabolites *N*-hydroxy-PHEN, 2-hydroxy-PHEN, and an arylating metabolite, NAPQI, forming a 3-(*S*-glutathionyl)paracetamol conjugate in the presence of glutathione. The *ab initio* Mulliken spin distribution, as shown in Fig. 4B, indicates that the unpaired electron of the nitrogen radical is primarily localized at the nitrogen atom, to a smaller extent at the *ortho*- and *para*-carbon atoms relative to the acetylamino group, and at the carbonyl oxygen in the acetylamino side chain.

***N*-Hydroxy-PHEN.** This *N*-oxygenated metabolite of PHEN (Fig. 3, **VII**) is suggested to be formed via a radical recombination reaction between a hydroxyl radical and the radicalized nitrogen atom of PHEN (ΔE = −117.3 kcal/mol). *N*-Hydroxylation is a well known metabolic route for acetylarylamines (25) and has been reported by several authors (10, 11). *N*-Hydroxy-PHEN is rather stable; it can be converted to *N*-*O*-sulfate and *N*-*O*-glucuronate derivatives and subsequently to a chemically reactive intermediate that can bind to proteins covalently (12) (Fig. 1B).

2-Hydroxy-PHEN. The formation of 2-hydroxy-PHEN (Fig. 3, **X**) (ΔE = −118.6 kcal/mol), which is detected as a minor metabolite in both humans and experimental animals (11), can be explained via delocalization of the unpaired electron of the nitrogen radical towards the *ortho*-carbon atoms relative to the acetylamino group, subsequent recombination with a hydroxyl radical, formation of intermediate **IX**, and further rearrangement of this intermediate to 2-hydroxy-PHEN (Fig. 3).

NAPQI. In the case in which the unpaired electron of the nitrogen radical is delocalized towards the ring carbon at the *para* position relative to the acetylamino group, a recombination reaction between a hydroxyl radical and the radicalized carbon atom yields hemiketal **XI** (Fig. 3). This reaction (ΔE = −127.4 kcal/mol) is energetically favored over the corresponding reactions between a hydroxyl radical and the other radicalized reactive centers of the nitrogen radical. Hemiketal **XI** is expected to rapidly decompose into the arylating metabolite NAPQI (Fig. 3, **XII**). The formation of NAPQI according to the pathway suggested in Fig. 3 is consistent with findings of Hinson *et al.* (13), who showed that the reactive metabolite of PHEN is not simply generated by deethylation of PHEN to paracetamol and further metabolism of paracetamol to NAPQI. The results showed that the rate of covalent binding of PHEN to microsomal proteins was greater than that of paracetamol in a hamster liver microsomal system, whereas only a fraction of PHEN was converted to paracetamol within the same incubation time. The low velocity of covalent binding of NAPQI, formed via initial deethylation of PHEN and subsequent metabolism of paracetamol to NAPQI, may be explained by assuming a competition for the binding site of cytochrome P-450 between PHEN, present in high levels, and the deethylated product paracetamol (8).

Peroxide intermediate. In the case in which the unpaired electron of the nitrogen radical is delocalized towards the carbonyl oxygen in the acetylamino side chain, a radical recombination reaction between a hydroxyl radical and the radicalized carbonyl oxygen would yield a peroxide (Fig. 3, **VIII**). However, the formation of this peroxide, which is an unknown metabolite of PHEN, is energetically unfavored (ΔE = −84.7 kcal/mol) when compared with the radical recombination reactions between a hydroxyl radical and the other radicalized positions of the nitrogen radical (ΔE = −117.3, −118.6, and −127.4 kcal/mol).

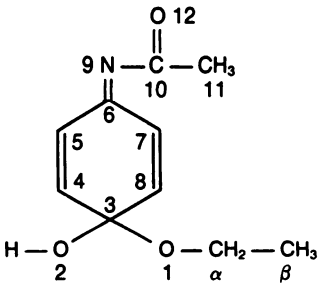
The above proposed nitrogen radical pathway can thus explain the formation of the known metabolites *N*-hydroxy-PHEN, 2-hydroxy-PHEN, and NAPQI by one uniform mechanism of oxidation of PHEN, i.e., via initial hydrogen abstraction from the acetylamino nitrogen atom.

Mechanism of Decomposition of Hemiketal XI

The *ab initio* calculated Mulliken SV 6-31G charge distribution of hemiketal **XI** reveals that the sum of the net atomic charges of both the hydroxyl group and ethoxy group of hemiketal **XI** are almost identical (Table 1) (charge = −0.29 and −0.31, respectively). This leads us to propose a new simplified hypothetical mechanism for the decomposition of hemiketal **XI**, both with (Fig. 5, routes A and B) and without (Fig. 5,

TABLE 1

Ab initio calculated Mulliken net atomic charge distribution of hemiketal **XI**



C_α	0.00	C_8	−0.14	$H_{\beta\text{-}3}$	+0.18
C_β	−0.45	N_9	−0.60	H_2	+0.42
O_1	−0.71	C_{10}	+0.60	H_4	+0.76
O_2	−0.71	C_{11}	−0.51	H_5	+0.74
C_3	+0.43	O_{12}	−0.54	H_7	+0.76
C_4	−0.13	$H_{\alpha\text{-}1}$	+0.16	H_8	+0.78
C_5	−0.13	$H_{\alpha\text{-}2}$	+0.17	$H_{11\text{-}1}$	+0.19
C_6	+0.22	$H_{\beta\text{-}1}$	+0.16	$H_{11\text{-}2}$	+0.20
C_7	−0.13	$H_{\beta\text{-}2}$	+0.17	$H_{11\text{-}3}$	+0.21

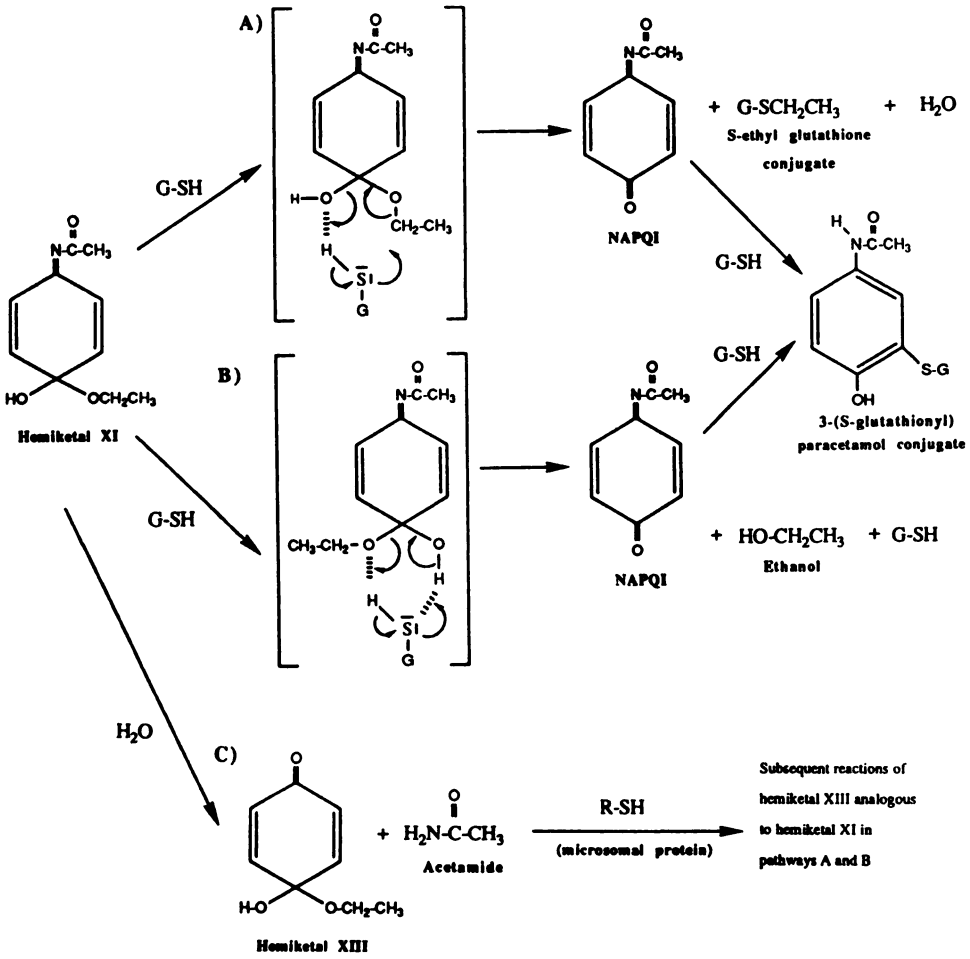


Fig. 5. Hypothetical mechanism for the decomposition of hemiketal **XI** in the presence (routes A and B) and absence (route C) of glutathione (GSH). Route A, formation of an assembly in which the hydroxyl and ethoxy group of hemiketal **XI** together with the nucleophilic -SH group of glutathione form a six-membered ring intermediate state, leading to NAPQI, H_2O, and a *S*-ethyl glutathione conjugate. The formed NAPQI reacts with a second molecule of glutathione, giving rise to 3-(*S*-glutathionyl)paracetamol, with 100% retention of the oxygen atom from the ethoxy side chain of PHEN in the 3-(*S*-glutathionyl)paracetamol conjugate. Route B, formation of an assembly in which the hydroxyl and ethoxy group of hemiketal **XI** together with the -SH group of glutathione form a six-membered ring intermediate state, leading to ethanol and NAPQI. The quinone imine interacts with glutathione in formation of a 3-(*S*-glutathionyl)paracetamol conjugate with 0% retention of the oxygen atom from the ethoxy side chain of phenacetin in the formed conjugate. Route C, hydrolysis of hemiketal **XI** at the aromatic imine ring carbon atom with formation of acetamide and hemiketal **XIII**. Hemiketal **XIII** is proposed to react with -SH groups of microsomal proteins in a similar way to hemiketal **XI** reacting with glutathione (routes A and B).

route C) glutathione as a nucleophile or a general acid/base catalyst in the incubation mixtures, which is consistent with the available *in vitro* experimental data (1, 7, 13).

Glutathione is present. In this case we propose that hemiketal **XI** interacts with glutathione in two different ways (Fig. 5, routes A and B). In pathway A the nucleophilic sulfur atom of glutathione interacts with the positively charged α-methylene group of hemiketal **XI**, whereas at the same time a hydrogen bond is formed between the sulfur hydrogen atom of glutathione and the negatively charged hydroxyl oxygen atom of hemiketal **XI**. These interactions result in an assembly in which the hydroxyl and ethoxy group of hemiketal **XI**, together with the -SH group of glutathione, form a six-membered ring intermediate state (Fig. 5A). This leads to simultaneous formation of NAPQI, H_2O, and *S*-ethyl glutathione. NAPQI then reacts with a second molecule of glutathione, giving rise to 3-(*S*-glutathionyl)paracetamol. This pathway implies a 100% retention of the oxygen atom from the ethoxy side chain of PHEN in the 3-(*S*-glutathionyl)paracetamol conjugate and, in addition, formation of a *S*-ethyl glutathione conjugate, as detected by Nelson *et al.* (1).

In pathway B of Fig. 5 we propose that the sulfur hydrogen atom of glutathione forms a hydrogen bond with the ethoxy oxygen atom of hemiketal **XI**, whereas at the same time a hydrogen bond is formed between the hydroxyl hydrogen atom of hemiketal **XI** and the sulfur atom of glutathione. These interactions also result in the formation of a six-membered ring intermediate state in an assembly comparable to the one in pathway A of Fig. 5, in this case ultimately leading to simultaneous formation of NAPQI and ethanol. NAPQI is supposed to rapidly form a 3-(*S*-glutathionyl)paracetamol conjugate, with 100% incorporation of an oxygen atom from molecular oxygen into the glutathione conjugate. Via this route, no *S*-ethyl glutathione conjugate is formed. An attack of the nucleophilic sulfur atom of glutathione at the positively charged ring carbon atom at position 3 (Table 1) seems unlikely because of steric hindrance around this sp^3-hybridized aromatic hemiketal carbon atom (1).

The above hypothetical mechanism for the decomposition of hemiketal **XI** via pathways A and B (Fig. 5) results in a partial incorporation of an oxygen atom from molecular oxygen into the 3-(*S*-glutathionyl)paracetamol conjugate. In glutathione trapping experiments in a hamster liver microsomal system in the presence of an $^{18}O_2$ atmosphere, Hinson *et al.* (13) found that the arylating metabolite from PHEN incorporates approximately 50% ^{18}O-labeled oxygen into the phenolic oxygen of the 3-(*S*-glutathionyl)paracetamol conjugate. In addition, when [*p*-^{18}O]PHEN was oxidized in a hamster liver microsomal system *in vitro* in the presence of glutathione, there was approximately a 50% loss of ^{18}O label in the resulting 3-(*S*-glutathionyl)paracetamol conjugate (7). This suggests that hemiketal **XI** can decompose via pathways A and B in Fig. 5 to an equal extent. Further support for this hypothesis was obtained in the absence of glutathione (see below); the ^{14}C label in the ethyl

group of PHEN was incorporated in microsomal proteins to about one half the extent to which the ^{14}C-labeled aromatic ring was bound to microsomal protein.

Furthermore, our hypothetical mechanism is also consistent with experimental data of Nelson *et al.* (1), who found that, in the presence of glutathione, a *S*-ethyl glutathione conjugate is formed from PHEN in a hamster liver microsomal system.

Glutathione is absent. In the absence of glutathione, hemiketal **XI** (Fig. 3) is hypothesized to be susceptible to hydrolysis, i.e., to addition of H_2O to the positively charged aromatic imine ring carbon atom, with subsequent formation of acetamide and hemiketal **XIII** (Fig. 5, route C), as suggested by Nelson *et al.* (1). Hemiketal **XIII** is then proposed to react with -SH groups of microsomal proteins in a way similar to that shown for hemiketal **XI** with glutathione in pathways A and B of Fig. 5. The putative formation of hemiketal **XIII** is in agreement with the lack of covalent binding of the ^{14}C-labeled acetyl group of PHEN to microsomal protein and the substantial formation of acetamide (1). Furthermore, using *ring*-^{14}C- and *ethyl*-^{14}C-labeled PHEN, Nelson *et al.* (1) found that the ^{14}C-labeled ethyl group of PHEN, in the absence of glutathione, is covalently bound to microsomal protein to about one-half of the extent to which the ^{14}C-labeled aromatic ring is bound. This suggests that hemiketal **XIII** can react with microsomal protein similarly to the reactions of hemiketal **XI** with glutathione via routes A and B in Fig. 5 in a 1:1 ratio. The *ring*-^{14}C-label is covalently bound to microsomal proteins after decomposition of hemiketal **XIII**, analogous to pathways A and B in Fig. 5 [with hemiketal **XIII** instead of hemiketal **XI** and -SH groups of microsomal proteins (RSH) instead of glutathione], whereas the *ethyl*-^{14}C-label is covalently bound only after decomposition of hemiketal **XIII**, analogous to pathway A in Fig. 5.

A mechanism involving 3,4-epoxidation of PHEN was previously proposed to be involved in the *in vitro* formation of the 3-(*S*-glutathionyl)paracetamol conjugate by cytochrome P-450 and in the 50% incorporation of an oxygen atom from molecular oxygen into this conjugate (1, 7, 13) (Fig. 1). The 3,4-epoxide of PHEN was suggested to rearrange to a symmetrical geminal diol intermediate, which would rapidly dehydrate randomly under the formation of NAPQI (7, 13). However, the lack of covalent binding of [*acetyl*-^{14}C]PHEN to microsomal protein and, in addition, the findings that [*ethyl*-^{14}C]PHEN and [*ring*-^{14}C]PHEN are covalently bound to microsomal protein in a 1:2 ratio (1) are not accounted for by the 3,4-epoxidation pathway. Furthermore, the 3,4-epoxide intermediate of PHEN also would be expected to rearrange in significant amounts to 3-hydroxy-PHEN, a metabolite of PHEN that only has been detected in trace amounts (11). Although no further theoretical or experimental evidence is presented to support the proposed alternative mechanism of decomposition of hemiketals **XI** and **XIII** via pathways A and B in Fig. 5, at present it remains the most likely alternative explaining all the available experimental data, including those with ^{18}O- and ^{14}C-labeled PHEN.

In conclusion, based on theoretical calculations, the formation of arylating and alkylating metabolites from PHEN by cytochrome P-450 is proposed to be initiated by hydrogen abstraction either from the α-methylene carbon atom in the ethoxy side chain (Fig. 2) or from the nitrogen atom in the acetylamino side chain (Fig. 3) and subsequent decomposition of the formed hemiketal **XI** (Figs. 5 and 6). *O*-Deethylation of PHEN is explained via initial hydrogen abstraction from the α-methylene carbon atom in the ethoxy side chain and subsequent decomposition of the formed hemiketal (Fig. 2, **III**) to paracetamol and acetaldehyde. The formation of *N*-hydroxy-PHEN, 2-hydroxy-PHEN, and NAPQI is explained via initial hydrogen abstraction from the nitrogen atom in the acetylamino side chain and subsequent radical delocalization, recombination, and rearrangement reactions (Fig. 3). Furthermore, based on charge distribution calculations, a hypothetical mechanism is proposed explaining the decomposition of hemiketal **XI** to NAPQI without intermediacy of a previously proposed 3,4-PHEN epoxide. This hypothetical mechanism in principle explains all available experimental data with ^{14}C-radiolabeled and ^{18}O-labeled PHEN (1, 7, 13).

The above mechanisms of oxidation and metabolic activation of PHEN by cytochrome P-450 are in conformity with an increasingly accepted general mechanism of oxidation or oxygenation of substrates by cytochrome P-450, involving sequential one-electron steps. Rearrangements of intermediate substrate radicals, formed after an initial hydrogen abstraction, and recombination reactions with a hydroxyl radical apparently can explain the formation of different oxidized metabolites of the substrate. Whether the presented quantum chemical approach involving *ab initio* energy and spin distribution calculations is more generally applicable to cytochrome P-450-mediated biotransformations remains to be further established.

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