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Metabolic Activation and Toxicity of Acetaminophen and Related Analogs

A Theoretical Study

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SUMMARY

Reaction thermodynamics have been calculated for an oxene model for cytochrome P-450 oxidations of four related arylamines: aniline, *p*-hydroxyaniline, acetanilide, and acetaminophen, by both radical and nonradical mechanisms, using a semiempirical molecular orbital method (modified neglect of differential overlap). The results indicate that for both *p*-hydroxyaniline and acetaminophen, a recently proposed peroxidase-like mechanism leading directly to *p*-benzoquinoneimines via radical intermediates is thermodynamically favored over *N*-hydroxylamine formation by H abstraction or addition rearrangement. These studies also provide a detailed characterization of three candidate species for the toxic reactive intermediate of acetaminophen: 1) *p*-benzoquinoneimines, 2) the radical intermediate formed by H abstraction from the nitrogen, and 3) the radical intermediate formed by H abstraction from the phenol. Calculated electron and spin densities indicate that the radical formed by H abstraction from the phenol oxygen does not remain localized on the oxygen, but is primarily a semiquinone aryl radical with significant unpaired spin density on the ring carbon atoms, particularly on C-3 and C-5. This result is consistent with the hyperfine splitting pattern observed for a transient radical species in a hydroxyl radical-mediated chemical oxidation of acetaminophen. The radical formed by H abstraction from the nitrogen also delocalizes on the ring carbons, but to a lesser extent and at the 2- and 4-positions. A closed shell mechanism of N oxidation of arylamines appears to lead directly to the hydroxylamines with less likelihood of precursor reactive intermediates. Toxic species could then be formed by loss of H_2O from the hydroxylamines.

INTRODUCTION

Acetaminophen (*p*-hydroxyacetanilide) (Scheme IA, *1*) widely used as an analgesic, is known to cause liver necrosis both in animals and man (1, 2). A toxic intermediate is thought to be formed via N oxidation by cytochrome P-450 (3) but the exact nature of the reactive intermediate and the pathway to its formation is a subject of continued controversy.

One plausible path to a postulated reactive intermediate, NAPQ[1] (*3*), is via *N*-hydroxyacetaminophen as shown in Scheme IA. Evidence for this scheme comes from a variety of studies (4–8). *N*-Hydroxyacetaminophen, *2*, has been synthesized and shown to exhibit chemical and toxicological behavior consistent with its postulated role as an intermediate in Scheme IA.

The nonenzymatic decomposition of *N*-hydroxyacetaminophen by first order dehydration to NAPQ has also been demonstrated (9) and preliminary toxicological studies of the chemically synthesized NAPQ have been reported (10). As shown in Scheme IB, loss of a proton can occur from either the phenolic OH or the *N*-hydroxy-OH. However, only the former process leads to NAPQ. The current consensus is that, once formed, NAPQ is either directly responsible for toxicity by reacting with tissue nucleophiles or, as shown in Scheme IC, it forms a more reactive intermediate (*4a*) by reacting with acetaminophen (10).

There is, however, a growing body of evidence (9, 11, 12) to suggest that either the decomposition of *N*-hydroxyacetaminophen occurs extremely rapidly or that *N*-hydroxyacetaminophen is not a metabolite of acetaminophen. Thus, a number of alternative mechanisms of transformation of acetaminophen leading to formation

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[1] The abbreviations used are: NAPQ, *N*-acetyl-*p*-benzoquinoneimine; PQ, *p*-benzoquinoneimine; LUMO, lowest energy unoccupied molecular orbital; HOMO, highest energy occupied molecular orbital; HRP, horseradish peroxidase; MNDO, modified neglect of differential overlap; UHF, unrestricted Hartree-Fock; RHF, restricted Hartree-Fock; ESR, electron spin resonance.

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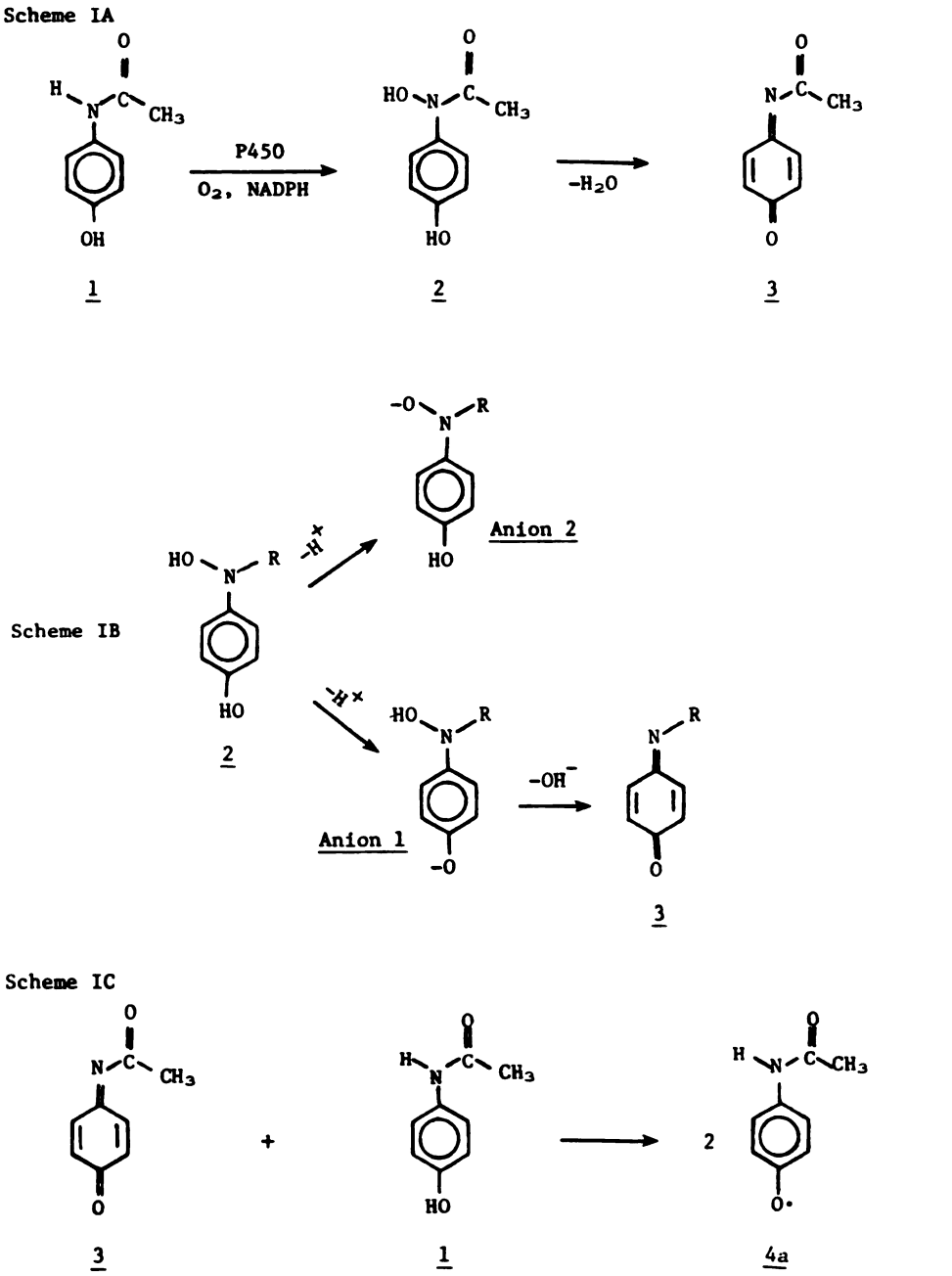
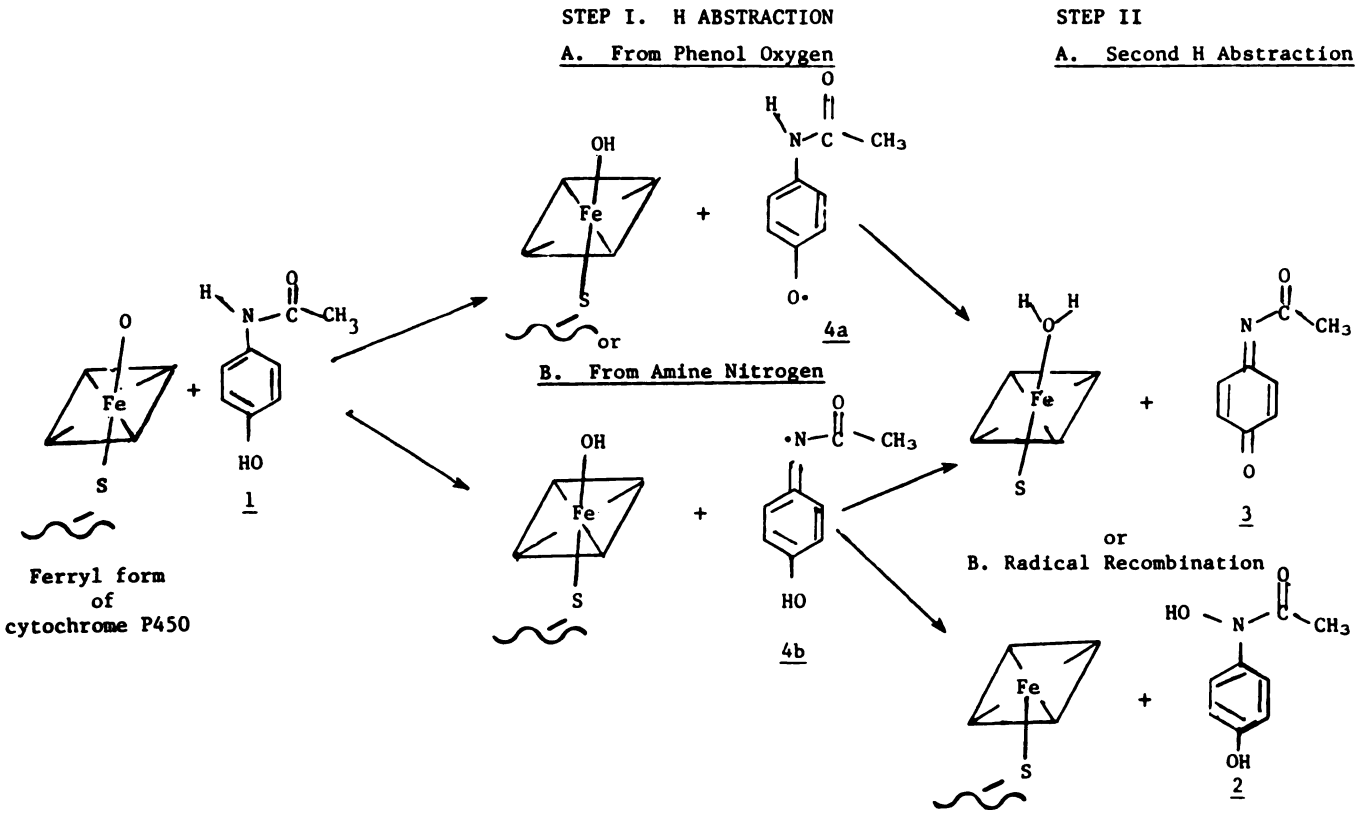


SCHEME I. *Proposed pathway to formation of toxic species from acetaminophen via N-hydroxyacetaminophen*

of NAPQ, but not involving *N*-hydroxyacetaminophen, have recently been postulated.

Among these "direct" pathways is one shown in Schemes II and III. It is based upon a recent suggestion (13) that cytochrome P-450 could be acting like a HRP in its oxidation of acetaminophen (9, 13). As shown in Scheme II, this pathway consists of two sequential H atom abstractions from the N and O atoms of acetaminophen by the reactive oxygen atom of the putative P-450 "ferryl" state. Radical intermediates formed in step I can undergo a second HRP-like H abstraction leading to species *3*. Alternatively, the amine radical can also recombine with the OH· radical forming the hydroxylamine *2* in a more typical cytochrome P-450 pathway. The requirement for both a phenol group and an amine hydrogen for maximum covalent binding of radiolabeled substrate to a microsomal preparation makes this scheme an enticing possibility. However, no direct evidence exists for this P-450-mediated pathway. While an acetaminophen radical was detected in the HRP-H_2O_2-mediated metabolism and tentatively identified by ESR behavior in chemical oxidation of acetaminophen to be an aryl carbon radical (13), no radical species of any kind was detected in the normal P-450-mediated metabolism of acetaminophen. Moreover, while some evidence exists for H abstractions from N and C atoms (14–19), no such H abstraction from phenol oxygens has been demonstrated for any phenol substrates of P-450.

A simplified model system for these transformations is shown in Scheme III. In this scheme, all the alternative



SCHEME II. *Direct pathway proposed for formation of toxic species from acetaminophen*

transformations of the parent compound shown in Scheme II are retained, but an oxene, (^{3}P)O species has been substituted for the proposed biologically active ferryl state of cytochrome P-450. It is the thermodynamics of this scheme which we propose to investigate in this study.

As indicated in Scheme III, three competing initial steps in the oxidation process are included: 1) H abstraction from the phenol oxygen leading to radical intermediate *4a*; 2) H abstraction from the nitrogen leading to radical intermediate *4b*; and 3) addition of the oxygen to the nitrogen leading to intermediate *4c*. Four possible subsequent reactions are also depicted. These include a second H abstraction from intermediates 4a and 4b, both leading to the quinone product 3; a radical recombination by intermediate 4b and a rearrangement of the intermediate 4c, both leading to the *N*-hydroxylamine product *2*. Intermediate *4c*, a triplet biradical species, must undergo spin state crossing to a singlet state of comparable energy in order to form product *2*. Scheme III, then, represents three plausible types of radical mechanisms of arylamine oxidations by cytochrome P-450 involving H abstractions from oxygen and nitrogen atoms and an addition-rearrangement mechanism.

In addition to the radical mechanisms shown in Scheme III, it is also possible, as shown in Scheme IV, that a nonradical (singlet) oxygen species is involved in P-450 N oxidations (20, 21). In this study, we have also investigated the thermodynamics of both the concerted (Scheme IVA) and two-step pathways (Scheme IVB).

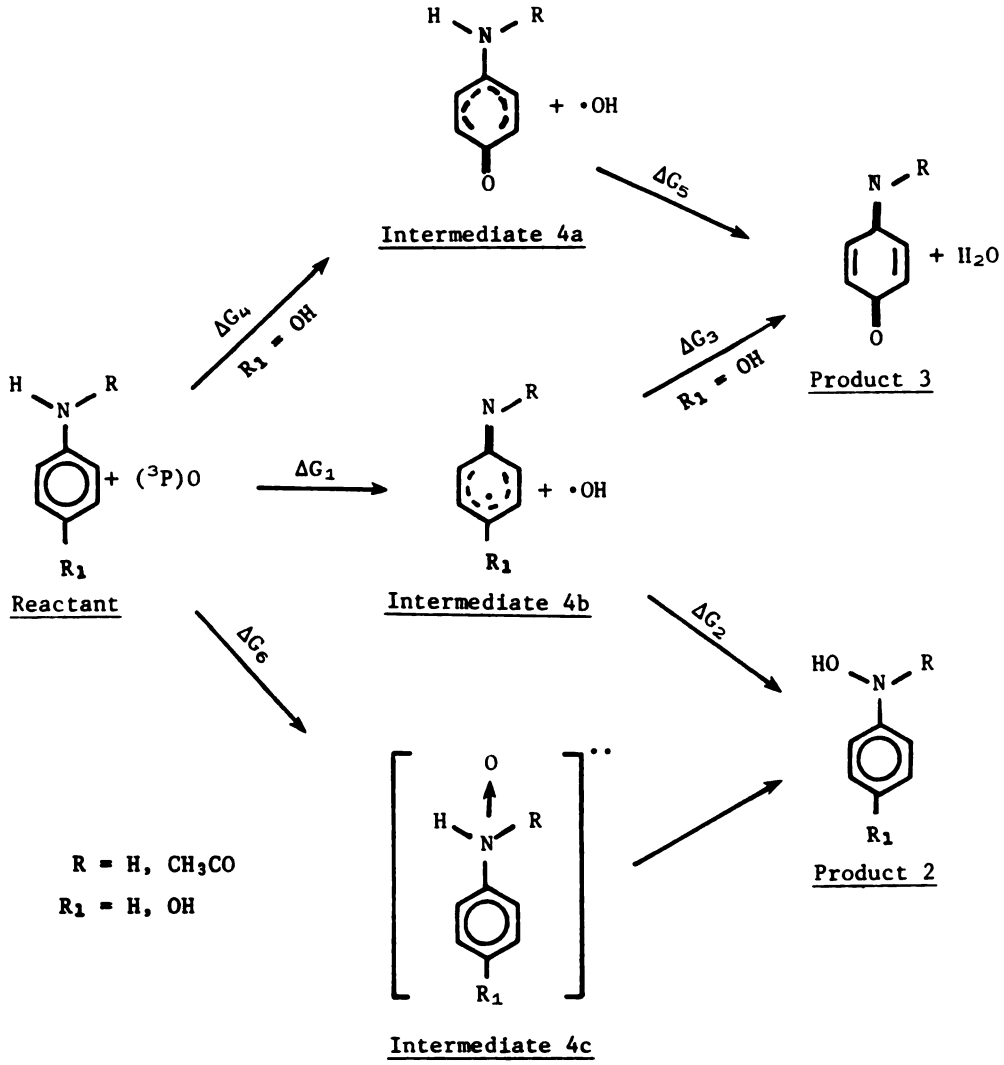
Finally, we have addressed the feasibility of formation of *p*-benzoquinoneimine by dehydration of the *N*-hydroxy products (Scheme IB) in a reaction between NAPQ and acetaminophen and the formation of reactive intermediate *4a* by Scheme IC.

In particular, using the semiempirical molecular orbital method, MNDO (22, 23),[2] we have calculated and compared the free energies of the reactions shown in Schemes IB, IC, III, and IV for aniline, *p*-OH aniline, acetanilide, and acetaminophen and characterized the spin and electron distribution of key postulated radical intermediates and products.

METHOD AND PROCEDURE

In the study reported here, we have used a revised version[2] of the semiempirical all valence electron molecular orbital method MNDO (22, 23) to calculate geometry-optimized enthalpies of formation for the four substrates studied and all stable intermediate, reactants, and products formed by them as indicated in Schemes IB, IC, III, and IV. This program has been carefully parameterized to give accurate enthalpies of formation at 298° K and geometries for a variety of organic molecules. A microwave structure of aniline (24) was incorporated into the starting geometry for all optimizations. For the acetanilide geometry, a H atom was replaced by a $COCH_3$ group and standard bond

[2] These programs were originally developed in the laboratory of Professor M. J. S. Dewar in collaboration with W. Thiel, G. P. Ford, M. McKee, D. Nelson, S. Olivella, and H. Rzepa. They were obtained from the National Resource for Computational Chemistry at Lawrence Berkeley Laboratories and included MNDO and two additional programs to calculate vibrational and rotational partition functions and perform statistical mechanical calculations of thermodynamic properties. The original programs have been modified to increase their efficiency and combined into one program in our laboratory by Dr. Dale Spangler.



SCHEME III. *Model for direct pathway proposed for formation of toxic species from acetaminophen*

lengths and angles used as an initial geometry for this group. For geometry optimization of radical species, we employed a UHF formalism which assumes different orbitals for different spins. The combined use of MNDO with the UHF formalism results in an underestimation of individual heats of formation, an error which partially cancels in determining reaction thermodynamics involving radical reactants and products. However, to prevent spin contamination, the unpaired spin distribution of these radicals was calculated by the half-electron method or RHF procedure in MNDO (22, 23). In this method, HOMO is half occupied by the spin-unpaired electron and the spin density is equal to the electron density distribution in this orbital.

Enthalpies of reaction were obtained as differences between enthalpies of formation for reactants and products,

$$\Delta H_r = \sum_{i}^{\text{products}} \Delta H_f(i) - \sum_{j}^{\text{reactants}} \Delta H_f(j)$$

and entropies of reaction were calculated from entropy differences between reactants and products:

$$\Delta S_r = \sum_{i}^{\text{products}} S_i - \sum_{j}^{\text{reactants}} S_j$$

The Gibbs free energy of reaction at 298°

$$\Delta G_r = \Delta H_r - T\Delta S_r$$

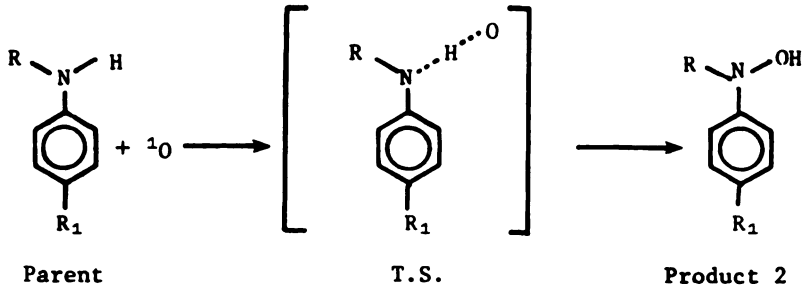
was then obtained from calculated values of ΔH_r and ΔS_r. In addition to energy-optimized geometries, net atomic charges, spin distributions, and orbital energies were obtained for each species investigated. These properties could be relevant to the proposed electrophilic activity of reactive intermediates formed during the oxidation of the arylamines.

RESULTS

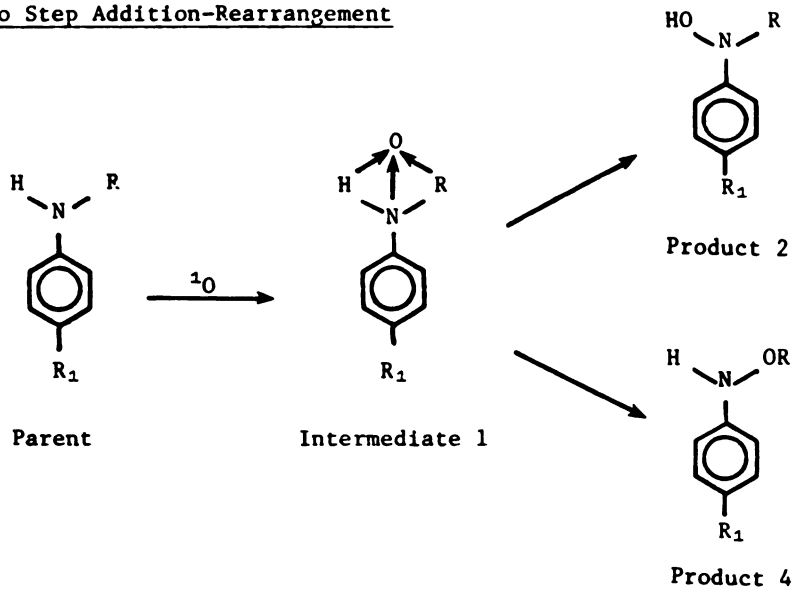
Description of parent compounds. In the optimized structures of the four parent arylamines, aniline, *p*-hydroxyaniline, acetanilide, and acetaminophen, the amine group in aniline and *p*-hydroxyaniline is tetrahedral and pyramidal. The HNC bond angles are 111°; there is a 120° difference in the torsion angles $\tau_{H_1NC_1C_1}$ and $\tau_{H_2NC_1C_2}$, and a 28° angle between the NH_2 and phenyl planes. This value is in good agreement with the microwave structure value (24) and is somewhat smaller than a minimum basis set *ab initio* results (25). With this geometry, the nitrogen-lone pair of electrons can participate in the π electron system of the benzene ring.

By contrast, in the optimized geometry of acetanilide and acetaminophen, the plane of the amide group, i.e., the H-N-C-O plane is rotated nearly perpendicular to the benzene ring ($\tau_{CNC_1C_2}$ = 90–95°). In this geometry, the

A. Concerted "Insertion" Reaction



B. Two Step Addition-Rearrangement



SCHEME IV. *Proposed N oxidation of acetaminophen by closed shell oxygen species*

π electrons of the amide group are nearly perpendicular to the π electron system of the benzene ring, and hence the two π systems, are independent. The amide nitrogen is not totally sp^2 hybridized. The difference in torsion angles between $\tau_{H_NNC_1C_2}$ and $\tau_{CNC_1C_2}$ is 150° rather than 180° and the <HNC$_1$ bond angle is 113° while the <CNC$_1$ bond angle is 124°. The X-ray structure of acetaminophen (26) has the amide group about 21° out of planarity with the benzene ring. Deviations from planarity in aromatic amides were found in other cases (27). It is unreasonable to argue that the perpendicular structure predicted by MNDO is the only one to be expected in the gas phase. However, a calculation of the rotational barrier with optimization at each restricted amide dihedral angle is required to quantitatively weigh the population of this conformation.

The net atomic charges, π electron densities, and bond overlap density of the parent compounds calculated by a Mulliken population analysis are given in Fig. 1. When normalized to the charge distribution calculated by MNDO for benzene ($q_c = -0.06$, $q_H = +0.06$; one electron in every carbon π orbital), we see that the amine group in aniline is electron donating at the *ortho* and *para* position and is electron withdrawing at the *meta* position both in terms of net atomic charge and in terms of π electron densities. By contrast, since the amide group in its optimized geometry interacts much less with the benzene ring, both the π and total electron densities are essentially unaltered in acetanilide.

In the *p*-hydroxy aniline, the *ortho* electron-donating effects of the −OH and NH_2 groups are in conflict, since the *ortho* position of one is the *meta* position of the other group. The position of maximum π and total electron density is C-5, *ortho* to the OH and *meta* to the NH_2.

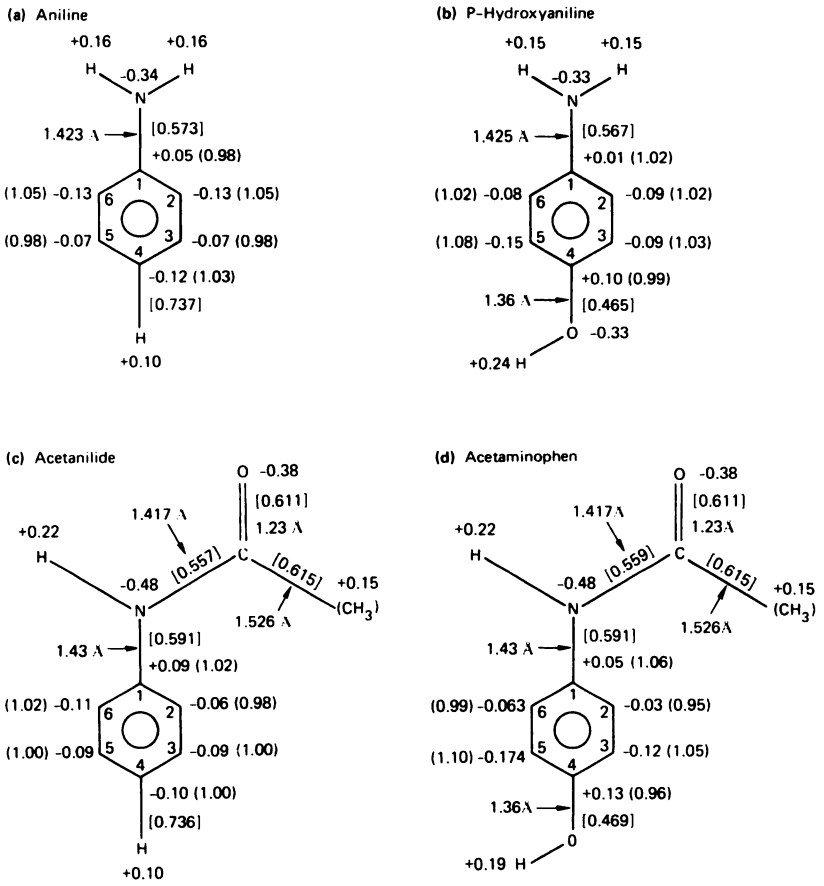


FIG. 1. *Calculated net atomic charges (±q), π electron densities (ρ), and bond overlap densities (ρ_{AB}) for four parent compounds*

TABLE 1

Calculated thermochemical quantities for reactants in arylamine oxidations by ^1O and $(^3P)O$

	$(^3P)O$	^1O	Aniline	*p*-OH aniline	Acetanilide	Acetaminophen
$\Delta H_f°$(kcal/mol)	(59.56)[a]	112.83 (105.0)[a]	21.65 (20.8)[a]	−25.88	−16.42	−64.29
$S°$(cal/mol-deg)	(38.50)[a]	(37.40)[a]	76.22	82.47	96.52	102.52

[a] Experimental values in parentheses.

This position is even more activated in acetaminophen with a net charge of −0.174 and a π electron density of 1.10e.

The bond overlap density between the nitrogen and ring carbon is somewhat enhanced in going from amine to amide 0.57 → 0.59 and seems unaffected by the *p*-OH group. The bond overlap density between the phenolic oxygen and the ring carbon is less than the nitrogen, i.e., ~0.47e, and is about equal for aniline and acetanilide. The lack of double bond character of the amide N-C bond is manifested in the fact that although it has a somewhat smaller bond length $r_{N\text{-}C} = 1.417$ Å, it also has a somewhat smaller bond overlap density 0.557e than for the N-ring carbon bond.

Reaction thermodynamics. Table 1 gives the calculated enthalpies of formation and entropies for arylamine substrate reactants involved in oxidation by ^1O and $(^3P)O$ models for the P-450 ferryl oxygen. As indicated in Table 1, the calculated heat of formation of aniline is in excellent agreement with the experimental value.

Tables 2 and 3, respectively, give the calculated enthalpies of formations and entropies for intermediates and products of oxidation of the substrates by $(^3P)O$ as shown in Scheme III and by ^1O as shown in Scheme IV.

Table 4 gives the calculated enthalpies of formation and entropies for the two possible anions *1* and *2* formed by loss of a proton from *N*-hydroxy-*p*-hydroxyaniline and *N*-hydroxyacetaminophen (Scheme IB).

Tables 5 and 6 summarize the calculated reaction thermodynamics for N oxidation of the arylamines by triplet oxygen (Scheme III) and by singlet oxygen (Scheme IV), respectively.

Table 7 gives the calculated reaction thermodynamics for formation of a proposed radical intermediate from *N*-acetyl-*p*-benzoquinoneimine and acetaminophen (Scheme IC).

Reactive intermediates. Figs. 2 and 3 summarize electronic and spin distributions of three types of putative reactive intermediates. In Fig. 2 are the net atomic charges, total π electron densities, and π electron density

TABLE 2

Calculated thermochemical quantities for intermediates and products for N oxudation of four arylamines by (^{3}P)O (Scheme III)

ΔH_f° in kcal/mol; S° in cal/mol-deg.

Parent	Intermediate *4a*		Intermediate *4b*		Intermediate *4c*		Product 3 (*p*-benzoquinone)	
	ΔH_f°	S°	ΔH_f°	S°	ΔH_f°	S°	ΔH_f°	S°
Aniline			41.75	80.50	92.48[a]			
p-OH aniline	−5.30	81.67	−7.57	81.06			15.96	80.50
Acetanilide			1.41	95.88				
Acetaminophen	−42.30	101.97	−47.95	103.20			−24.45	101.29

·OH		H_2O	
ΔH_f°	S°	ΔH_f°	S°
0.23 (9.4)[b]	43.8 (43.9)[b]	−60.94 (−57.8)[b]	46.32

[a] In this triplet state, unpaired spins are primarily on the ring carbon atoms.

[b] Experimental values in parentheses.

TABLE 3

Calculated thermochemical quantities for intermediates and products for N oxidation of four aromatic amines by ^{1}O (Scheme IV)

ΔH_f° in kcal/mol; S° in cal/mol-deg.

Parent		Intermediate 1		Product 1		Product 2	
R	R_1	ΔH_f°	S°	ΔH_f°	S°	ΔH_f°	S°
H	H	57.33	82.89	9.76	83.39		
H	OH	9.16	88.81	−38.37	88.83		
$COCH_3$	H	30.69	100.44	−23.93	100.32	−24.07	102.22
$COCH_3$	OH	−17.36	106.38	−72.04	105.98	−74.45	109.22

and energies of LUMO for the two *p*-benzoquinoneimines, candidate reactive intermediates of *p*-hydroxyaniline and acetaminophen. Fig. 3 gives the calculated spin distribution on the radical intermediates *4a* and *4b* formed by hydrogen atom abstraction from the oxygen and the nitrogen atoms, respectively, of the parent compounds studied. The results reported are for the RHF method of MNDO. In this method, HOMO contains the spin-unpaired electron. This method avoids spin contamination and the unpaired spin density distribution is the electron density distribution in HOMO.

DISCUSSION

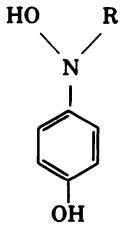
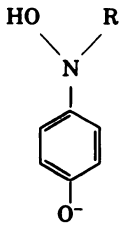
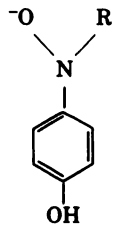
Reaction thermodynamics. The calculated reaction thermodynamics of model enzymatic N oxidation by a closed shell oxygen species (Scheme IV, Table VI) suggest that both steps of the addition-rearrangement reaction of singlet oxygen with the four arylamines studied are highly exothermic processes that would yield *N*-acetoxy as well as *N*-hydroxy products.

If such nonradical mechanisms are involved in P-450 oxidations, then the reactive species leading to toxic effects is most likely formed from *N*-hydroxy or *N*-acetoxy products. There have been no reports of the latter products and their detection could help resolve whether a singlet mechanism is a plausible one for N oxidation by P-450. However, previous mechanistic studies for simple model oxidations of aliphatic, allylic hydroxylations and alkene epoxidations together with experimental isotope effects and suicide substrate adduct

TABLE 4

Calculated thermochemical quantities for anion formation by proton abstraction from N-hydroxy-p-hydroxyaniline and N-hydroxy acetaminophen (Scheme IB)

ΔH_f° in kcal/mol; S° in cal/mol-deg.

Product 2	Anion 1		Anion 2	
				
R	ΔH_f°	S°	ΔH_f°	S°
H	−59.98	86.94	−35.76	86.31
$COCH_3$	−94.70	105.50	−76.56	105.56

formation argue against such a closed shell mechanism (14–19).

If, as is now favored, the reactive oxygen species in cytochrome P-450 has radical character, a H abstraction mechanism or oxygen addition mechanism is possible (Scheme III). The enthalpy of formation of the intermediates *4a* and *4b* from the former and *4c* from the latter initial reaction with aniline is given in Table 2. We note that the enthalpy of formation for the radical species formed by H abstraction (intermediate 4b) is much more favorable than the triplet *N*-oxide (intermediate 4c), in which the unpaired spin is largely on the ring carbon atoms. This result indicates that, by a thermodynamic criterion, a H abstraction mechanism would be favored over an addition-rearrangement. Consequently, we have explored the reaction thermodynamics of that pathway (Scheme III) in detail.

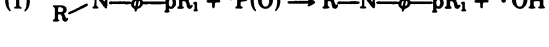
The results, summarized in Table 5, indicate that direct formation of *p*-benzoquinoneimines from two sequential H abstractions; i.e., the "HRP-like" pathway is thermodynamically favored over hydroxylamine formation. If the first step in P-450 oxidations of arylamines is H abstraction from the nitrogen, then, comparing ΔG_2 and ΔG_3, we see that H abstraction from the phenol oxygen leading directly to the *p*-benzoquinoneimine product is favored over the formation of hydroxylamine by 19 kcal/mol for *p*-hydroxyaniline and by 26 kcal/mol for acetaminophen. Moreover, the alternative pathway to direct *p*-benzoquinoneimine formation via initial H abstraction from the phenol also seems competitive. Differences in ΔG_1, H abstraction from the nitrogen, and ΔG_4, H abstraction from the phenol, favor the former by 1.7 kcal/mol for *p*-OH aniline and 6 kcal/mol for acetaminophen. If H abstraction from the phenol does occur first, the subsequent H abstraction from the amine hydrogen (ΔG_5) is about equally exothermic. These results support the plausibility of an HRP-like direct pathway to *N*-acetyl-*p*-benzoquinoneimine formation from acetaminophen without the intermediacy of a *N*-hydroxy metabolite. They also indicate that the same pathway is possible for *p*-OH aniline which is also known to be toxic.

Such thermodynamic criteria do not rule out the radical recombination reaction to hydroxylamine and hydroxamic acid intermediates. The efficacy of these inter-

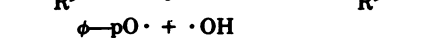
TABLE 5

Calculated reaction thermodynamics for pathway III: oxidation of arylamines by (^{3}P)O

ΔH_r, ΔG_r, kcal/mol; ΔS_r, cal/mol-deg.

(1) 

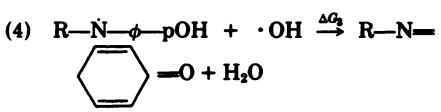
R	R_1	ΔH_r	ΔS_r	ΔG_1
H	H	−39.23	9.57	−42.10
H	OH	−41.02	3.88	−42.18
$COCH_3$	H	−41.50	4.66	−42.90
$COCH_3$	OH	−42.99	5.98	−44.78

(2) 

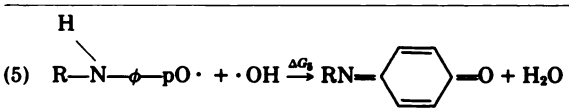
ΔH_r	ΔS_r	ΔG_4
−38.75	4.49	−40.09
37.33	4.75	−38.76

(3) 

R	R_1	ΔH_r	ΔS_r	ΔG_2
H	H	−32.22	−40.90	−20.02
H	OH	−31.03	−36.02	−20.29
$COCH_3$	H	−25.56	−39.36	−13.82
$COCH_3$	OH	−24.32	−41.01	−12.09

(4) 

ΔH_r	ΔS_r	ΔG_1
−37.64	1.96	−38.22
−37.67	0.61	−37.85

(5) 

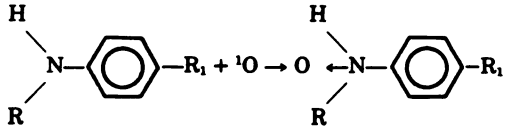
R	R_1	ΔH_r	ΔS_r	ΔG_5
H	OH	−39.91	1.35	−40.32
$COCH_3$	OH	−43.32	1.84	−43.87

TABLE 6

Calculated reaction thermodynamics for pathway IV: oxidation of arylamines by ^1O

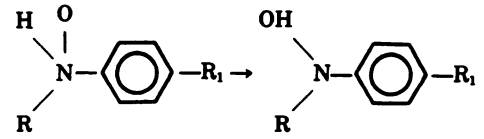
ΔH_r, ΔG_r, kcal/mol; ΔS_r, cal/mol-deg.

A. Intermediate *N*-oxide formation



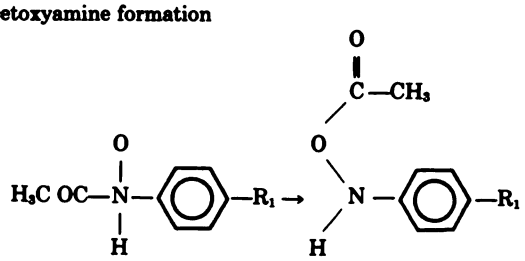
R	R_1	ΔH_r	ΔS_r	ΔG_r
H	H	−77.15	−30.73	−67.98
H	OH	−77.78	−31.06	−68.53
$COCH_3$	H	−65.72	−33.48	−55.74
$COCH_3$	OH	−65.90	−33.54	−56.90

B. Hydroxylamine formation



R_1	R_1	ΔH_r	ΔS_r	ΔG_r
H	H	−47.54	+0.50	−47.60
H	OH	−47.54	+0.03	−47.55
$COCH_3$	H	−54.62	−0.12	−54.58
$COCH_3$	OH	−54.68	−0.40	−54.56

C. Acetoxyamine formation

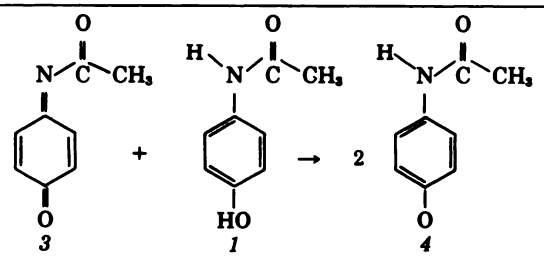


R_1	ΔH_r	ΔS_r	ΔG_r
H	−54.76	+1.78	−55.29
OH	−57.09	+2.83	−57.94

TABLE 7

Calculated reaction thermodynamics for formation of radical intermediate from N-acetyl-p-benzoquinoneimine and acetaminophen (Scheme IC)

ΔH_f, ΔH_r, kcal/mol; $S°$ in cal/mol-deg.



	3	*1*	*4*
ΔH_f	−24.45	−64.29	−42.30
$S°$	101.29	102.51	101.97
ΔH_r	ΔH_r = 4.15	ΔS_r = 0.13	ΔG_r = 4.01

mediates to form *p*-benzoquinoneimine products would depend on the rates of the dehydration reactions.

The reactivity of the *p*-hydroxy hydroxylamines do point to their further transformation to quinoneimine products. As shown in Table 4, proton abstraction from the C-OH, a possible first step in dehydration, is favored by 18-19 kcal/mol over proton abstraction from the N-OH. Only the favorable anion *1* can lose OH^- to form the *p*-benzoquinoneimine.

The relative ease of loss of proton to which these relative energies apply is related to the gas phase proton affinities of the anions, rather than to the solution pK_a values of hydroxamic acids and phenols. In general, pK_a values are macroscopic quantities, intimately involving the effect of bulk water. The calculated quantity is most relevant to the mechanism of quinoneimine products if they are formed in or near the hydrophobic substrate-binding site of cytochrome P-450 rather than in solution.

As shown in Scheme IC, it has also been proposed that *N*-acetyl-*p*-benzoquinoneimine reacts with acetaminophen to form the radical species *4a*. Table 7 gives the calculated thermodynamics for the reaction in Scheme IC indicating it to be modestly endothermic by 4 kcal/mol. Thus, both enzymatic and nonenzymatic pathways to this reactive intermediate are plausible.

Candidate toxic intermediates. Formation of three types of putative reactive intermediates that could be responsible for toxicity have been investigated in this study: 1) *p*-benzoquinoneimines, 2) radicals formed by H abstraction from the nitrogen of the parent compounds, and 3) radicals formed by H abstraction from the phenolic oxygen of the *p*-hydroxy analogs.

If, as suggested, the *p*-benzoquinoneimines are the electrophilic reactive intermediates which cause toxicity by attack at nucleophilic sites of tissue macromolecules, then incipient covalent bond formation could be initiated by electron transfer from the nucleophile to their LUMO. In such "overlap-controlled" reactions (28), the nature and energy of the empty, electron-accepting orbitals (LUMOs) are a relevant measure of electrophilicity. As shown in Fig. 2B, for *p*-benzoquinoneimine and the *N*-acetyl analog, the LUMO is a π^* orbital with a negative energy indicative of potential electrophilicity of both analogs, but somewhat enhanced in the quinoneimine from acetaminophen. The π electron distribution in LUMO, particularly for acetaminophen, while showing only small variations, is consistent with observed thioadduct formation at the C-3 position (29, 30).

In contrast to overlap-controlled reactions, the total net atomic charges could be indicators of electrophilicity if reactions are "charge controlled" (26). As shown in Fig. 2A, by this criterion, only the ring carbon atoms directly attached to the O and N substituents have positive charges and would be electrophilic in a charge-controlled reaction. While there is some evidence of adduct formation at C-3, there is none for formation at the C-1 or C-4 positions. Thus, an overlap rather than an electrostatic criterion for electrophilicity appears to be a more reasonable indicator of the reactivity of the *p*-benzoquinoneimines. The nature of LUMO also suggests

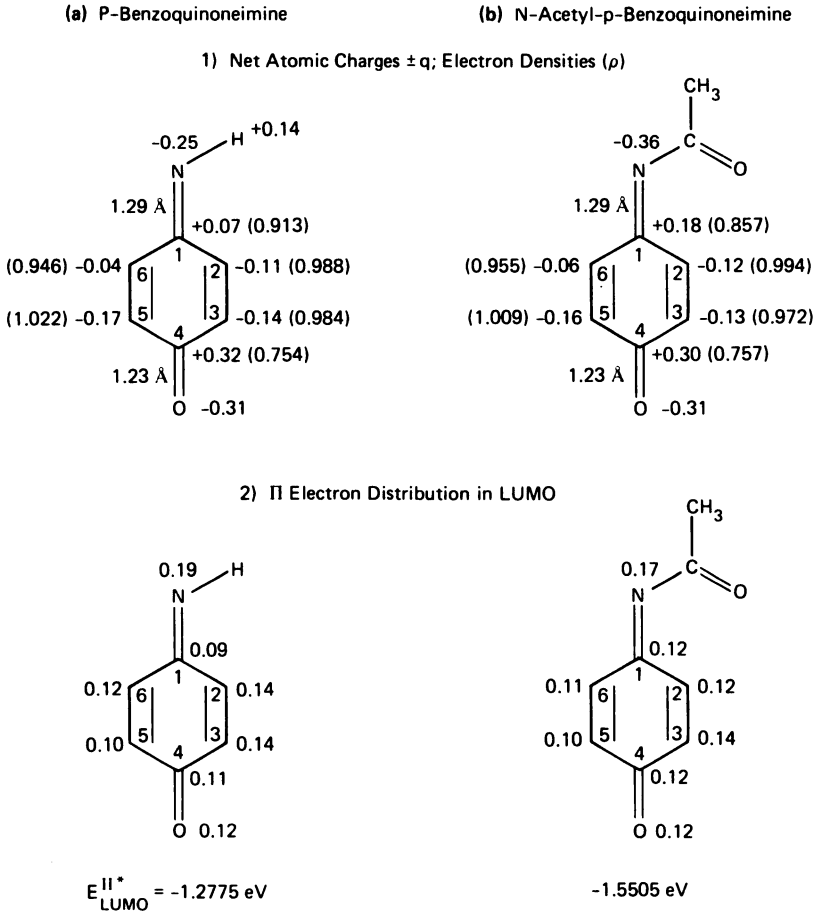
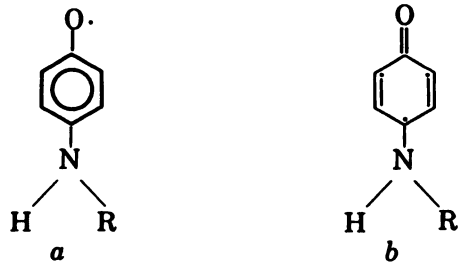


FIG. 2. *Calculated charge distributions for (a) p-benzoquinoneimine and (b) N-acetyl-p-benzoquinoneimine 1*, net atomic charges ± *q* and π electron densities (ρ). *2*, π electron distributions and energies of LUMO.

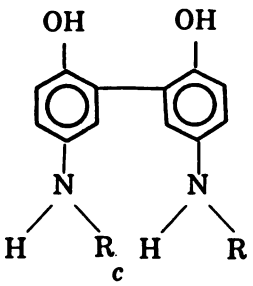
a π approach of a nucleophile to a preferred site of covalent adduct formation.

Calculated spin densities for the radicals formed by H abstraction from the nitrogen and oxygen atoms (Fig. 3) strongly suggest that they are the intermediates spin-trapped in the hydroxyl radical-mediated oxidation of acetaminophen (13).

As shown in Fig. 3, the radical formed by H abstraction from the phenol oxygen of *p*-OH aniline and acetaminophen is not an oxy radical. Instead, the oxygen atom retains only 12–13% of the unpaired spin density and the remainder of the unpaired spin becomes delocalized on the ring carbon atoms, making the radicals primarily aryl carbon radicals. This result is consistent with and explains the radical species reported trapped in the HRP-H_2O_2-mediated transformations of acetaminophen (13). The electron spin resonance of this species was interpreted as due to an aryl radical but the origin of such a radical was unclear. The unpaired spin distribution on the aromatic ring, is mainly at the C-1, C-3, and C-5 positions with little or none at the C-2, C-4, and C-6 positions. Moreover, the O—C-4 bond length is considerable shortened to that of a C═O double bond. Thus, the radical formed from H abstraction from the phenol oxygen is not an oxy radical *a*, but a semiquinone aryl radical *b*.



This description of the radical is not only consistent with observed ESR behavior but makes it a very plausible precursor to the known dimeric products *c* of phenol oxidations by HRP (31), e.g.,



The spin distribution also indicates that it is a species capable of producing toxicity by adduct formation at positions (3, 5) with tissue macromolecules. Formation

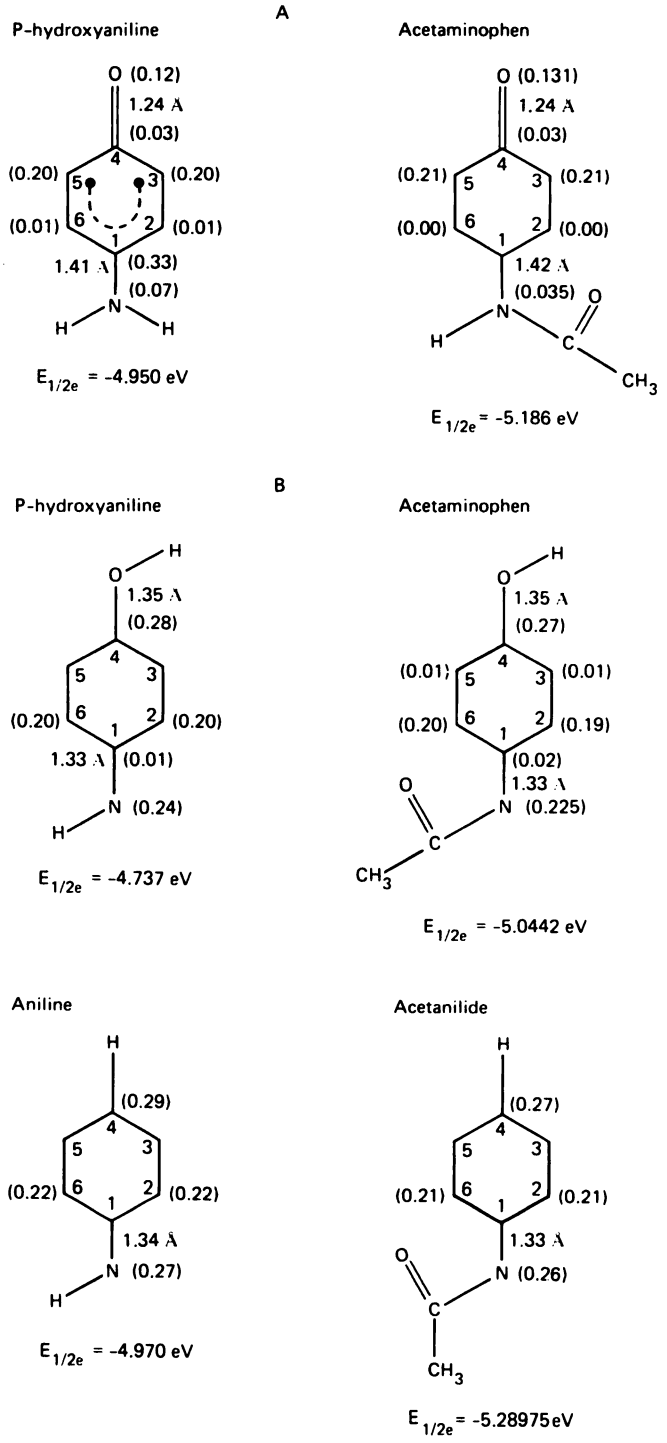


FIG. 3. *Unpaired spin density distribution*

A, radicals formed by H abstraction from oxygen of parent compounds B, radicals formed by H abstraction from nitrogen of parent compounds.

of this intermediate is consistent with the observation that glutathione forms an adduct with a reactive intermediate of acetaminophen at the 3-position (29, 30). Such a reaction can occur between the acetaminophen radical and the parent glutathione with concerted loss of a H atom or between the acetaminophen radical and a thiyl radical subsequent to its loss of an H atom. In either the one-step or two-step reaction, unpaired spin density on the electrophilic acetaminophen radical at the site of covalent bond formation should enhance this reaction.

As shown in Fig. 3, the radical formed by H abstraction from the nitrogen is also delocalized onto the aryl ring but to a lesser extent than in the phenol radical. Moreover, the C-N bond is not appreciably shortened by H abstraction from the nitrogen. The unpaired spin is on the ring C atoms but in this radical is mainly at positions 2, 4, and 6. Dimerization or adduct formation with tissue nucleophiles would then be expected at the 2- and 6-positions.

CONCLUSIONS

Reaction thermodynamics for a two-step H abstraction by a radical oxygen species have been calculated for acetaminophen and *p*-hydroxy aniline. For both analogs, this HRP-like mechanism leads directly to *p*-benzoquinoneimines via radical intermediates and is calculated to be thermodynamically favorable compared to the calculated thermodynamics for *N*-hydroxylamine formation by H abstraction or addition rearrangement.

The aryl radicals formed by H abstraction from oxygen or nitrogen can: 1) be potentially toxic intermediates by interaction with nearby radicals; 2) go on to form *p*-benzoquinoneimines, another possible toxic intermediate; or 3) because of proximity and steric constraints (i.e., a radical cage), recombine with the enzyme to form enzyme-bound recombination products.

A closed shell mechanism of N oxidation of arylamines appears to lead directly to the hydroxylamines with less likelihood of precursor-reactive intermediates. Toxic species would then most likely be formed by loss of H_2O from the hydroxylamines.

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