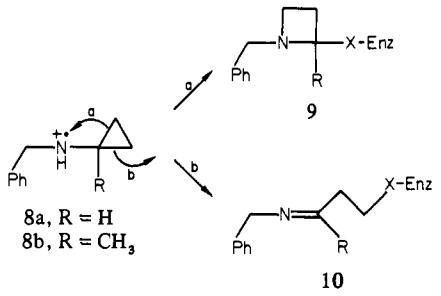
Scheme I



by a group on the enzyme. Precedent for this mode of reactivity is found in the oxidative rearrangement of N-monosubstituted 1-aminocyclopropanols to β-lactams with *t*-BuOCl followed by silver ion.[17] Path b would simply involve opening of the cyclopropyl ring and reaction with a group from the enzyme. However, while this mode of reaction is well precedented in the reaction of cyclopropyl alcohols[18] and thiols[14] by one-electron oxidants, it is doubtful that an adduct such as **10** would be sufficiently stable toward hydrolysis to account for the covalent binding of *benzylic* ^3H and ^{14}C, which is observed even after harsh acid treatment[9] of the microsomal proteins.

While **4** remains a potential intermediate in the metabolic activation of **2** by cytochrome P-450, it is also possible that both **2** and **6** are activated via aminium radical cations as suggested in Scheme I. Several additional observations are consistent with this hypothesis. First, the cyclopropyl moiety is resistant to attack by extremely strong oxidants such as RuO_4[19] or dry ozone,[20] which readily oxidize aromatic and aliphatic positions *adjacent* to a cyclopropane ring. Second, cyclopropyl ethers and thioethers are apparently not inactivators of P-450[12] despite their *potential* for metabolism to reactive cyclopropanone hemiketals or hemithioketals analogous to **4**. However, the contrasting behavior of ethers and thioethers to that of amines **2** and **6** may be understandable in terms of cation-radical intermediates. Oxygen cation radicals are much less likely to form because of the high ionization potential of ethers, and sulfur cation radicals, if formed,[21,22] may react further to give sulfoxide metabolites rather than enzyme inactivation. Finally, the very recent report that **6** is a suicide substrate for monoamine oxidase,[23] which we had independently observed,[24] lends further credence to the possible involvement of aminium cation radicals in the metabolic activation of cyclopropyl amines and possibly in the oxidative metabolism of amines in general. Studies directed toward trapping and identifying of enzymatically activated forms of **2** and **6** are presently under way in our laboratory.

Acknowledgment. We thank the NIH for financial support of this work through a predoctoral traineeship (GM-07775) to R.H.T. and Grant GM-22357.

Registry No. 2, 13324-66-8; [7-^{3}H]-**2**, 80926-12-1; [^{14}C]-**2**, 80926-13-2; **6**, 79432-92-1; [7-^{3}H]-**6**, 80926-14-3; **7**, 3378-72-1; cytochrome P-450, 9035-51-2; *N*-benzylidenecyclopropylamine, 3187-77-7; *N*-benzylidene(1-methylcyclopropyl)amine, 80926-15-4; [*carboxyl*-^{14}C]-benzoic acid, 1589-66-8; cyclopropylamine, 765-30-0.

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Mechanism of Cytochrome P-450 Inhibition by Cyclopropylamines

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Received October 5, 1981

The cytochrome P-450 enzymes are a family of heme-containing proteins involved in the metabolism of a range of substances that possess great diversity in structure. These monooxygenase enzymes catalyze several oxidative reactions that have a common unifying feature: the covalent incorporation of one atom of oxygen into the substrate molecule.[4] These enzymes are subject to mechanism-based or suicide inhibition[5] by numerous substrates as a function of the metabolism of uniquely positioned olefinic,[6] acetylenic,[7] or cyclopropylamine[8] moieties. Cyclopropylamines are also suicide inhibitors of mitochondrial monoamine oxidase[9] and of plasma amine oxidase.[5a] The mechanism-based inhibition of these distinct enzyme classes by cyclopropylamines has been postulated to be a consequence of α-hydroxylation to form a cyclopropylcarbinolamine, which reacts with enzyme-bound nucleophiles via a highly reactive iminium ion (Figure 1: **1a** → **2** → **3**).[8,9b] We present evidence here that an alternative mechanism, postulated to involve initial amine nitrogen oxidation to a radical cation, is operative for the suicide inactivation of cytochrome P-450 by cyclopropylamines.

We have investigated the in vitro mixed-function oxidative metabolism of cyclopropylbenzylamine, **1a**, and (1-methylcyclopropyl)benzylamine, **1b**, by hepatic microsomes and by a reconstituted hepatic cytochrome P-450 system, both isolated from phenobarbital-pretreated rats.[10] Cytochrome P-450 content was determined after metabolism of both compounds directly by spectral assay and indirectly by biochemical assay of enzyme activities. Cytochrome P-450 was determined by its ferrous carbonyl Soret absorbance,[11] and total heme was determined as the pyridine hemochromagen.[11,12] *d*-Benzphetamine[10] and am-

(1) Research Fellow of the Alfred P. Sloan Foundation (1981–1983).
(2) National Research Service Award Fellow supported by USPHS Training Grant ES07028.
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Table I. Destruction of Cytochrome P-450 and Associated Activities during the Metabolism of Cyclopropylamines 1a and 1b[a]

incubation system	cytochrome P-450, nmol (mg of protein)$^{-1}$	heme, nmol (mg of protein)$^{-1}$	nmol of HCHO min^{-1} (mg of protein)$^{-1}$ aminopyrine demethylase	nmol of HCHO min^{-1} (mg of protein)$^{-1}$ benzphetamine demethylase
microsomes	1.58		5.5 ± 0.2	11.0 ± 0.16
+NADPH	1.76	3.09	5.5 ± 0.3	8.9 ± 0.10
+cyclopropylamine 1a	1.65		5.1 ± 0.1	10.2 ± 0.10
+NADPH + 1a	0.48	2.37	4.0 ± 0.3	4.5 ± 0.10
+cyclopropylamine 1b	1.72		5.4 ± 0.2	10.5 ± 0.3
+NADPH + 1b	0.62	2.04	4.6 ± 0.1	6.8 ± 0.25
reconstituted cytochrome P-450 [nmol of P-450 (mL of incubation)$^{-1}$]				
+NADPH	1.56			
+NADPH + 1a	0.13			
+NADPH + 1b	0.13			

[a] The methods of assay are described in the text. Demethylase activities were determined after dialysis of incubations for 16 h against 100 volumes of 0.1 M potassium phosphate buffer (pH 7.7) at 4 °C. All incubations were carried out at 37 °C for 20 min in Teflon-sealed vials in a final volume of 1.5 mL, containing the cyclopropylamine (1.0 mM), potassium phosphate (0.1 M, pH 7.7), phenobarbital-induced rat hepatic microsomal protein (1.0 mg/mL, containing ~1.6 nmol cytochrome P-450/mg) or a reconstituted enzyme system [containing 1.0 nmol of cytochrome P-450,[10] 1.0 nmol of β-nicotinamide adenine dinucleotide phosphate (NADPH)–cytochrome P-450 reductase,[10,12] 30 μM L-α-dilauroylglyceryl-3 phosphorylcholine, and 1.0 μg of catalase/mL] and, when indicated, an NADPH-generating system containing $NADP^+$ (0.5 mM), glucose-6-phosphate dehydrogenase (1.0 IU/mL), glucose 6-phosphate (10 mM), and magnesium chloride (5 mM).

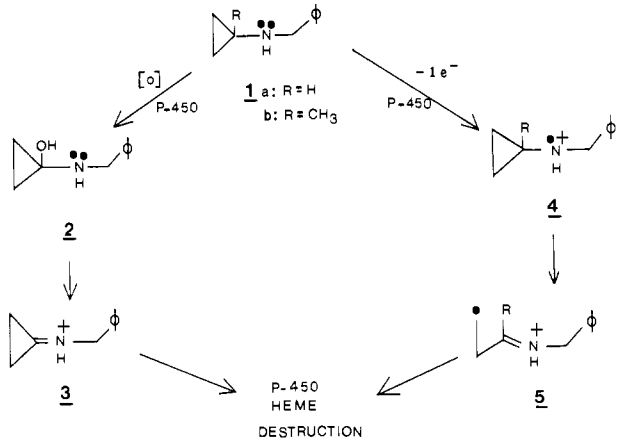

Figure 1. Mechanisms postulated for the suicide inhibition of cytochrome P-450 monooxygenases by cyclopropylamines.

inopyrine[8] *N*-demethylase activities were determined by measurement of formaldehyde formation by the method of Nash[13] (Table I). Preliminary microsomal experiments utilizing benzphetamine and aminopyrine *N*-demethylase assays according to the procedure of Hanzlik et al.[8] demonstrated substantial (~85%) inhibition of these activities for both compounds **1a** and **1b**. However, after removal of substrate by dialysis a substantial portion (~40–50%) of these activities could be recovered, suggesting that these cyclopropyl amines are *both* competitive inhibitors of benzphetamine and aminopyrine *N*-demethylase activities and suicide substrates for cytochrome P-450. Additional microsomal experiments, utilizing dialysis, established that both benzphetamine (BP) and aminopyrine (AP) *N*-demethylase activities were inhibited by cyclopropylamines **1a** (BP, ~28% of control; AP, ~51%) and **1b** (BP, ~42%; AP, ~58%) and that metabolism of the substrate was required for inhibition (Table I). In addition, cytochrome P-450 and prosthetic heme were destroyed in a process dependent upon metabolism of the amines [table: **1a** (P-450, ~27% of control; heme, ~77%) and **1b** (P-450, ~35%; heme, ~66%)]. Spectral analysis also revealed that the relative amount of cytochrome P-420, the denatured hemoprotein derived from cytochrome P-450, remained constant during the incubation period. The extent of cytochrome P-450 destruction determined via spectral analysis was greater than the corresponding loss of *N*-demethylase activities; these data reflect that in microsomes cytochrome P-450 is not rate limiting and is in excess relative to cytochrome P-450 reductase and possibly that a different isozyme selectivity is observed for enzyme destruction and for benzphetamine and aminopyrine metabolism.[4]

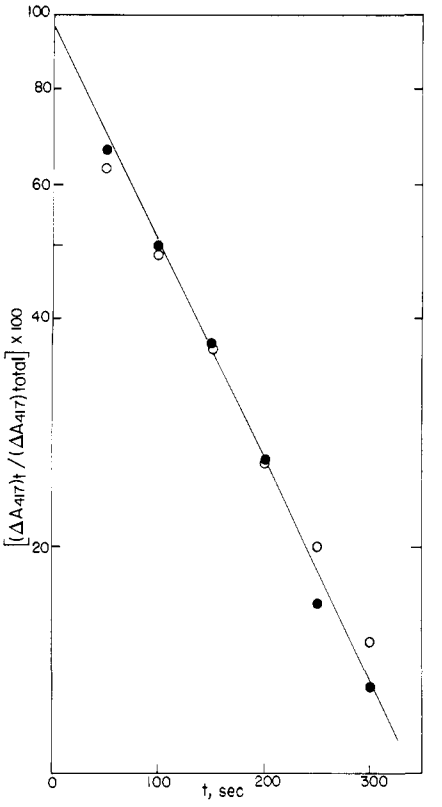

Figure 2. Destruction of cytochrome P-450 Soret absorbance during metabolism of cyclopropylamine **1b** as a function of time. The decrease in A_{417} was measured continuously in a reconstituted enzyme system containing purified cytochrome P-450 (as in Table I).[12] The destruction is expressed as A_{417} remaining compared to the total observed decrease in A_{417}.[6a] The two sets of points were obtained from separate experiments.

In order to more closely monitor the involvement of cytochrome P-450 in the initiation of mechanism-based inhibition by cyclopropylamines **1a** and **1b**, a reconstituted, purified cytochrome P-450 system was examined and shown to undergo metabolism-dependent enzyme destruction (Table I). Although cytochrome P-450 destruction was not complete during the metabolism of either amine, the rate of enzyme disappearance, determined by plotting cytochrome P-450 Soret absorption vs. time to the apparent endpoint,[6] exhibited first-order kinetics (k = 0.42 min^{-1}) for methylcyclopropylamine **1b** (Figure 2). Such kinetics are

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indicative of suicide substrate inhibition processes.[6a,14] The rate of cytochrome P-450 disappearance during the metabolism of cyclopropylamine **1a** appeared to be the superimposition of two first-order enzyme-destruction processes: an initial rapid rate (k = 0.49 min^{-1}) and a subsequent slower one (k = 0.17 min^{-1}). The rationale for this biphasic kinetic behavior is unclear at present and is the subject of continuing investigation.[15]

Partition coefficients, which illustrate the distribution of substrate nonsuicide to suicide metabolic events, were obtained for both cyclopropylamines from the rates of product disappearance determined via quantitative liquid chromatography and the rates of enzyme destruction determined for the reconstituted monooxygenase system. The partition coefficients corrected for enzyme loss for cyclopropylbenzylamine, **1a**, (~40:1) and its 1-methyl analogue **1b** (~60:1) indicated that suicide inhibition by these substrates occurred with reasonable efficiency.[16] For example, the partition coefficient for the classical cytochrome P-450 suicide substrate, 2-isopropyl-4-pentenamide (AIA), is ~230:1.[6a] Furthermore, the partition coefficients are comparable for both cyclopropylamines, consistent with related mechanisms of suicide inhibition for both.

Compelling evidence for the mechanism-based inhibition of cytochrome P-450 monooxygenases by cyclopropylamines is provided by (1) the inhibition of cytochrome P-450 dependent *N*-demethylase activities after removal of the inhibitor, (2) the disappearance of cytochrome P-450 and of prosthetic heme in a time-dependent manner, (3) the requirement for metabolism to effect enzyme inhibition, and (4) the first-order kinetics of cytochrome P-450 destruction (for **1b**) characteristic of suicide substrate inhibition.

We suggest the chemical mechanism of inhibition of cytochrome P-450 by cyclopropylamines to be a consequence of initial amine nitrogen oxidation to generate a radical cation, followed by rapid ring opening in the enzyme cavity to form a highly reactive carbon-centered radical. Such a reactive species could react with the proximate heme moiety of the enzyme (Figure 1: **1** → **4** → **5**). Clearly, a mechanism that invokes initial α hydroxylation to form a cyclopropylcarbinolamine followed by reaction with protein-bound nucleophiles via a reactive iminium ion (Figure 1: **1** → **2** → **3**) would not be valid, since both cyclopropylamine **1a** and its methyl analogue **1b** are equally efficient in suicide inhibition of the enzyme. Due to the linear relationship between cytochrome P-450 loss and heme destruction for both inhibitors (**1a** and **1b**), the principal mode of cytochrome P-450 inactivation would appear to be destruction (presumably via alkylation) of the heme prosthetic group. However, concomitant enzyme loss through protein modification cannot be discounted. It is unlikely that a cyclopropyliminium ion (**3**) would be a sufficiently potent electrophile to destroy the heme group, since other equally (or more) potent electrophiles (epoxides and alkyl and acyl halides) do not.[6,7,17]

Support for this proposed mechanism of enzyme inhibition is derived from both chemical and biochemical studies. A process analogous to the critical step in the postulated mechanism, ring opening of cyclopropylamine radical cations, has been examined by Ingold and co-workers.[18] The Ingold group found that ring opening of cyclopropylamine radicals [$(CH_2)_2CH\dot{N}R$] was too fast to measure ($k_{25\,°C} \geq 5 \times 10^8$ s^{-1}) via kinetic EPR spectroscopy and independent of the *N*-alkyl substituent. The nitrogen-centered radicals studied by Ingold et al.[18a] and the radical-cation intermediates proposed here are interrelated by simple protonation, e.g.,

$$(CH_2)_2CH\dot{N}R \overset{H^+}{\rightleftharpoons} (CH_2)_2CH^+\dot{N}HR$$

and would be anticipated to undergo related chemical transformations. It is possible that nitrogen-centered radical intermediates are in fact generated by abstraction of an amine hydrogen atom by cytochrome P-450; however, we favor enzyme-mediated electron transfer to generate the transient radical-cation species.

The cytochrome P-450 mediated oxidation of substrates containing heteroatoms has been suggested to proceed through initial oxidation of the heteroatom to a cation radical via single electron transfer. Griffin and co-workers[19] have presented experimental evidence for the formation of nitrogen-centered radical cations as intermediates in the N-dealkylation of amines by cytochrome P-450 and related enzymes. Ortiz de Montellano et al.[20] have invoked possible amine radical-cation generation to rationalize prosthetic heme–alkyl adduct formation during cytochrome P-450 mediated metabolism of 4-alkyl-1,4-dihydropyridine compounds. In addition, the rate (V_{max}) of formation of aryl sulfoxides from the corresponding sulfide has been logarithmically correlated with the one-electron oxidation potential of the sulfide suggesting (circumstantially) that oxygenation proceeds via one-electron oxidation by transfer from sulfide to the active species of the enzyme and subsequent recombination.[21]

The partition coefficients of cyclopropylamines **1a** and **1b** indicate that nonsuicide reaction processes predominate during the metabolism of these compounds. The predominance of benign metabolite generation could be a consequence of alternate metabolic processes such as aromatic ring hydroxylation or amine α-hydroxylation or of the partitioning of the postulated amine radical-cation **4** into nonsuicide products through iminium ion formation and subsequent α-hydroxylation or by *N*-oxide formation.[16] Reactions of carbon-centered free radical **5**, generated through β scission of radical cation **4**, also may not lead to heme adduct formation with high frequency. The latter rationale has been suggested to apply to the reactive intermediate capable of heme destruction generated during the metabolism of 2-isopropyl-4-pentenamide (AIA).[6a] We are currently attempting to identify the unreactive metabolites of these cyclopropylamines to resolve this issue.[16]

This postulated mechanism for the inactivation of cytochrome P-450 by cyclopropylamines **1a** and **1b** has implications for the suicide inhibition of monoamine oxidase and plasma amine oxidase. In addition, the unique chemistry of cyclopropyl compounds, illustrated by their tendency to undergo rapid cyclopropane opening when radicaloid or cationic substituents are bound to the ring is being employed to examine the hypothesis of cytochrome P-450 mediated heteroatom oxidation via single electron transfer.[19-22]

Acknowledgment. The assistance of D. E. O'Dell is gratefully acknowledged. This research was supported by USPH Grants ES01590, ES02205, ES00267, and ES02702.

Registry No. 1a, 13324-66-8; **1b**, 79432-92-1; NADPH, 53-57-6.

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(15) This biphasic kinetic behavior cannot be a function of two competing first-order suicide inhibition processes for cyclopropylamine **1a** with the purified cytochrome P-450 isozyme, since such behavior would exhibit pseudo-first-order *not* biphasic kinetics. Our tentative rationale for this kinetic behavior is that a metabolite of cyclopropylamine **1a** (e.g., benzylamine,[16] cyclopropylamine) is generated that subsequently competes effectively for metabolism. Benzylamine (competitive) and cyclopropylamine (competitive, suicide) are inhibitors of benzphetamine and aminopyrine demethylase activities for this isozyme (Macdonald, T. L.; Zirvi, K.; Guengerich, F. P., unpublished observations).

(16) A major soluble metabolite of cyclopropylbenzylamine, **1a**, has been established to be benzylamine (Hanzlik, R. P.; Tullman, R. H., personal communication).

(17) See for example: Halpert, J.; Neal, R. A. *Mol. Pharmacol.* **1981**, *17*, 427 and references therein.

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(22) See for example: Tachizawa, H.; Neal, R. A.; Macdonald, T. L. *Mol. Pharmacol.* **1982**, submitted for publication.