(K_i) were calculated from the replot of slope vs. inhibitor concentration from double-reciprocal plots. The thymidine concentrations used in these studies ranged from 0.3 to 2.3 μM and that of the inhibitor from three- to eightfold that of their K_i.

Acknowledgment. We are grateful to Mrs. J. J. Lee and Mrs. E. Lentz for their technical assistance. This investigation was supported by U.S. Public Health Service Research Grant CA-05262 from the National Cancer Institute. G.T.S. was supported in part by the USPHS Cancer Center Support Grant 1-PO1-CA-16359. We also acknowledge the support of Southern New England High Field NMR Facility made possible by a grant from the Biotechnology Resource Program of the National Institutes of Health (1-PO7-PR798).

Trapping of Metabolically Generated Electrophilic Species with Cyanide Ion: Metabolism of 1-Benzylpyrrolidine

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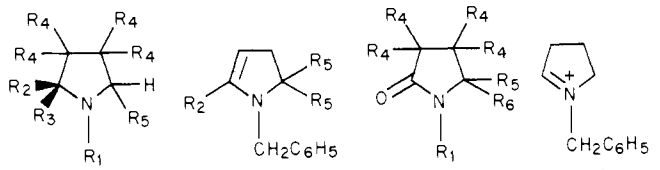
Incubations of 1-benzylpyrrolidine (**4**) and specifically deuterium-labeled analogues of **4** with rabbit liver microsomal preparations in the presence of cyanide ion have led to the characterization of 1-benzyl-2-cyanopyrrolidine (**13**), *cis*- and *trans*-1-benzyl-2,5-dicyanopyrrolidine (**14a** and **14b**, respectively), and 1-benzyl-5-cyano-2-pyrrolidinone (**15**). The cyano adducts of the amine are thought to result from nucleophilic attack by cyanide ion on metabolically generated iminium species. The cyanolactam may be produced by mixed function oxidation of the dicyano compounds. Incubations of tritium-labeled 1-benzylpyrrolidine with rabbit liver microsomal preparations led to the reduced nicotinamide adenine dinucleotide phosphate dependent incorporation of the label into the macromolecular fraction isolated from the postincubates. Although the level of incorporation was low compared to the amount of cyano adducts formed, it is comparable to that reported for other metabolically activated cytotoxic agents. Attempts to identify the possible arene oxide rearrangement product 1-(4-hydroxybenzyl)pyrrolidine (**24**) as a metabolite of **4** were unsuccessful. The results have prompted us to postulate that metabolically generated iminium ions are capable of alkylating nucleophilic functionalities present on microsomal macromolecules.

The toxicity of a variety of lipophilic xenobiotics is thought to involve oxidative metabolism of the parent compound to reactive electrophiles which alkylate nucleophilic functionalities present on macromolecules.[1] Previous studies by us[2] and others[3] have shown that the tobacco alkaloid nicotine (**1**) is oxidatively metabolized by hepatic microsomal preparations to reactive intermediates, which in the presence of cyanide ion are converted to the isolable cyano adducts **2** and **3**. In an attempt to further characterize the metabolic pathway leading to the formation of such electrophilic intermediates, we have examined with the aid of specifically deuterium-labeled compounds and GC–EIMS the rabbit liver microsomal metabolism of the model *tert*-amine 1-benzylpyrrolidine (**4**) in the presence and absence of cyanide ion. Additionally, we have attempted to evaluate the possible toxicological significance of electrophilic metabolic intermediates derived from *tert*-amines by examining the metabolically dependent formation of covalent adducts between tritium-labeled 1-benzylpyrrolidine and microsomal macromolecules.

The GC analysis of the base fraction isolated from 1-benzylpyrrolidine postincubates showed the presence of two major and one minor metabolite (Figure 1a). In the absence of NADPH or when boiled microsomes were used, only the starting substrate **4** was observed in the GC tracing. The structure assignments (see below) and GC–EIMS characterizations of these compounds and unmetabolized 1-benzylpyrrolidine are summarized in Table I.

The GC–EIMS spectra of all four compounds are dominated by the tropylium ion ($C_6H_5CH_2^+$) at m/e 91. The appearance of this fragment ion in these spectra, as well as in the mass spectra of the cyano adducts discussed below, indicates that the benzylic carbon atom of 1-benzylpyrrolidine is not altered in these metabolically generated products. The possibility that substituent rearrangements might accompany the fragmentations was



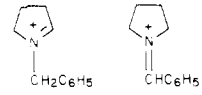
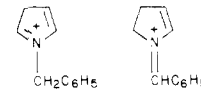
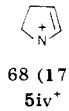
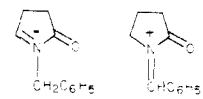
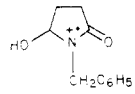
1-4, 8, 9, 13, 14, and 16 — 5 — 6, 7, and 15 — 10

	R_1	R_2	R_3	R_4	R_5	R_6
1	CH_3	H	3-C_5H_4N	H	H	
2	CH_2CN	H	3-C_5H_4N	H	H	
3	CH_3	H	3-C_5H_4N	H	CN	
4	$CH_2C_6H_5$	H	H	H	H	
4-α,α-d_2	$CD_2C_6H_5$	H	H	H	H	
4-2,2-d_2	$CH_2C_6H_5$	^2H	^2H	H	H	
4-d_4	$CH_2C_6H_5$	H	H	^2H	H	
4-α,α-t_2	$CT_2C_6H_5$	H	H	H	H	
5		H			H	
5-d_1		^2H			H	
5-d_2		H			^2H	
6	$CH_2C_6H_5$			H	H	H
6-α,α-d_2	$CD_2C_6H_5$			H	H	H
6-5,5-d_2	$CH_2C_6H_5$			H	^2H	^2H
6-d_4	$CH_2C_6H_5$			^2H	H	H
7	$CH_2C_6H_5$			H	OH	H
7-d_1	$CH_2C_6H_5$			H	OH	^2H
8	$C(=O)C_6H_5$	H	H	H	H	
9	$CH_2C_6H_5$	OH	H	H	H	
13	$CH_2C_6H_5$	CN	H	H	H	
13-d_1	$CH_2C_6H_5$	CN	^2H	H	H	
13-d_2	$CH_2C_6H_5$	^2H	^2H	H	CN	
14a, 14b	$CH_2C_6H_5$	CN	H	H	CN	
14a-d_1, 14b-d_1	$CH_2C_6H_5$	CN	^2H	H	CN	
15	$CH_2C_6H_5$			H	CN	H
15-d_1	$CH_2C_6H_5$			H	CN	^2H
16	$CHCNC_6H_5$	H	H	H	H	

(1) Nelson, S. D.; Boyd, M. R.; Mitchell, J. R. *ACS Symp. Ser.* **1977** *no. 44*, 155–185. Miller, J. A. *Cancer Res.* **1970**, *30*, 559.
(2) Nguyen, T. L.; Gruenke, L. D.; Castagnoli, N., Jr. *J Med. Chem.* **1976**, *19*, 1168; Nguyen, T. L.; Gruenke, L. D.; Castagnoli, N., Jr. *Ibid.* **1979**, *22*, 259.
(3) Murphy, P. *J. Biol. Chem.* **1973**, *248*, 2796.

0022-2623/80/1823-0133$01.00/0

Table I. GC-EIMS Characterization of Products Obtained from the Metabolism of 1-Benzylpyrrolidine

metabolite M⁺, %	retention time, min	approx. % formation	major GC-EIMS fragment ions: *m/e*, %
 161 (26) **4⁺**	1.6		$C_6H_5CH_2^+$, 91 (100);  160 (31) **4i⁺** **4ii⁺**;  84 (68) **4iii⁺**;  70 (59) **4iv⁺**
 159 (22) **5⁺**	2.0	71	 158 (8) **5i⁺** **5ii⁺**;  157 (5) **5iii⁺**;  68 (17) **5iv⁺**
 175 (70) **6⁺**	10.2	26	 174 (9) **6i⁺** **6ii⁺**;  146 (37) **6iii⁺**
 191 (1) **7⁺**	13.5	3	 173 (32) **7i⁺**

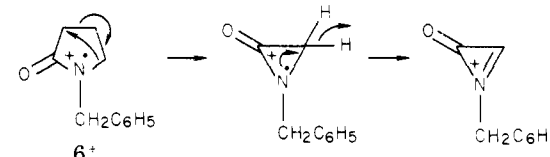
ruled out, since the corresponding metabolites derived from 1-benzylpyrrolidine-α,α-d_2 [**4**-α,α-d_2; prepared by $LiAlD_4$ reduction of 1-benzoylpyrrolidine (**8**)] displayed a strong tropylium ion ($C_6H_5CD_2^+$) at *m/e* 93. Attempts to characterize benzylic carbon–nitrogen cleavage products (pyrrolidine, benzyl alcohol, benzaldehyde, and benzoic acid) by GC analysis against authentic compounds also were unsuccessful.

The GC–EIMS of substrate **4** displayed in addition to the tropylium ion a strong parent ion at M^+ 161 and the benzylic C–N cleavage fragment **4iv⁺** at *m/e* 70 (Table I). In this case (as well as with related compounds), loss of the proton α to nitrogen generated the fragment ions **4i⁺** and **4ii⁺** (which were distinguishable with **4**-α,α-d_2 as substrate), complicating deuterium content measurements in the parent ion mass region. A third ion (**4iii⁺**) resulting from α-carbon cleavage appeared at *m/e* 84.

The metabolite eluting just after 1-benzylpyrrolidine displayed a parent ion at M^+ 159. This information plus the fragmentation data listed in Table I led to the tentative structure assignment of 1-benzyl-2-pyrroline (**5**) for this compound. In order to confirm this assignment, as well as the assignment of other metabolically derived compounds encountered in this study, we examined the metabolism of 1-benzylpyrrolidine-2,2-d_2 (**4**-2,2-d_2), prepared by $LiAlD_4$ reduction of 1-benzyl-2-pyrrolidinone (**6**). The mass spectrum of metabolite **5** derived from **4**-2,2-d_2 showed the expected cluster of ions at masses 158, 159, 160, and 161, due to oxidative attack at C-2 (to yield **5**-d_1, M^+ 160) and C-5 (to yield **5**-d_2, M^+ 161). The fragment ions corresponding to the deuterated analogues of **5i⁺**, **5ii⁺**, and **5iii⁺** also were present. The deuterated fragment ions corresponding to **5iv⁺** shifted from *m/e* 68 to *m/e* 69 and 70. If one excludes the unlikely possibility of multiple migrations of the double bond, these data confirm the α,β location of the double bond of **5**, since the 3-pyrroline structure would be expected to retain both deuterium atoms originally present in the substrate molecule.

The identification of **5** does not necessarily mean that the microsomal enzymes form this compound directly. It is generally agreed than oxidative metabolism of amines leads to unstable carbinolamine intermediates[4] which in the case of cyclic compounds may be further metabolized, perhaps via open-chain aminoaldehydes, to lactams by soluble aldehyde oxidases.[5] In the present case, the proposed carbinolamine intermediate **9**, if extractable from the incubation mixture, would be expected to undergo thermal dehydration to **5** upon GC analysis. An alternative chemical pathway for **9** would involve ionization to the conjugate acid of **5**, namely, the pyrrolinium species **10**. Since the incubation mixtures are worked-up at pH 10, **10** would be converted to the free base **5** under our conditions.

Scheme I



6⁺ **6iii⁺**

The second major metabolite characterized from incubations of **4** was 1-benzyl-2-pyrrolidinone (**6**). This metabolite had GC and GC–EIMS characteristics identical with authentic **6**. The strong fragment ion at *m/e* 146 has been assigned structure **6iii⁺** on the basis of its nominal mass. This fragment ion shifts to *m/e* 148 in the GC–EIMS of metabolite **6**-α,α-d_2 (M^+ 177), derived from **4**-α,α-d_2. On the other hand, **6iii⁺** appears exclusively at *m/e* 146 in the GC–EIMS of the mixture of **6** and 1-benzyl-2-pyrrolidinone-5,5-d_2 (**6**-5,5-d_2, M^+ 177) derived from **4**-2,2-d_2 as substrate. The fragmentation to form **6iii⁺** therefore appears to involve loss of the C-4 and C-5 methylene groups plus one additional proton. This question was further examined with the aid of 1-benzylpyrrolidine-3,3,4,4-d_4 (**4**-d_4), prepared by base-catalyzed deuterium exchange at C-3 and C-4 of 1-benzylsuccinimide followed by AlH_3 reduction. Consistent with our interpretation, the fragment ion corresponding to **6iii⁺** derived from metabolite **6**-d_4 (M^+ 179) following incubation of **4**-d_4

(4) Testa, B.; Jenner, P. "Drug Metabolism: Chemical and Biochemical Aspects", Marcel Dekker: New York, 1976; pp 83–97.
(5) Hucker, H. B.; Gillette, J. R.; Brodie, B. B. *J. Pharmacol. Exp. Ther.* **1960**, *129*, 94.

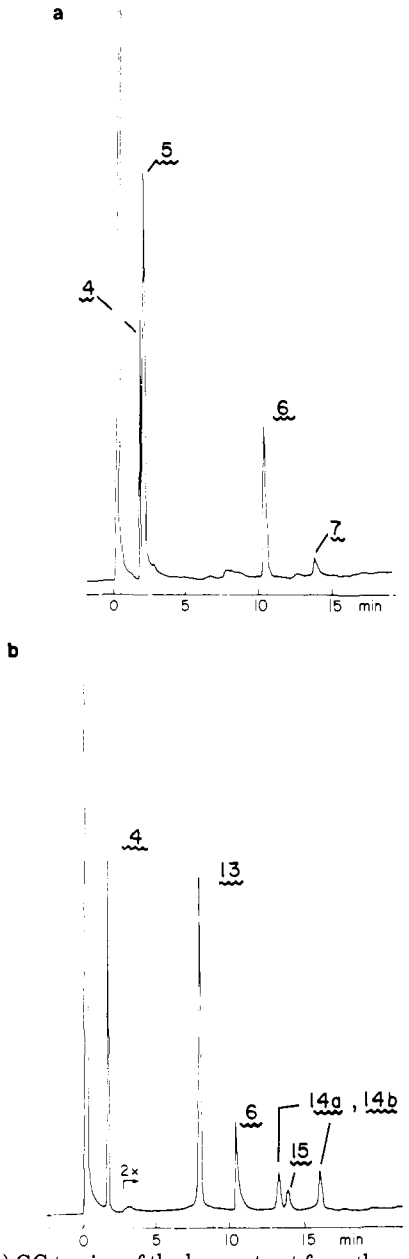
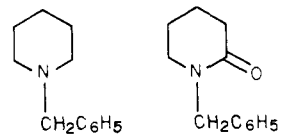


Figure 1. (a) GC tracing of the base extract from the postincubate of **4**. (b) GC tracing of the base extract from the postincubate of **4** in the presence of cyanide.

appeared at m/e 147. Therefore, one deuterium atom of 4-d_4 (presumably at C-3) is retained in the fragment. A plausible sequence to account for this unusual fragmentation process is outlined in Scheme I.

A minor metabolite observed in the microsomal incubations of 1-benzylpyrrolidine was assigned the structure 1-benzyl-5-hydroxy-2-pyrrolidinone (**7**). The GC–EIMS displayed a weak parent ion at M^+ 191 (1-benzyl-2-pyrrolidinone + oxygen) and a fairly strong fragment ion at m/e 173 (**7i**$^+$) generated from the loss of water. The location of the newly introduced oxygen atom was established with 4-2,2-d_2 as substrate. The appearance of two parent ions at M^+ 191 and 192 [derived from **7** and 1-benzyl-5-hydroxy-2-pyrrolidinone-5-d_1 (**7**-d_1), respectively] and two corresponding fragment ions at m/e 173 and 174 require the hydroxyl group to be located at C-5. If the two oxygen atoms were located at positions other than C-2 and C-5, one of the metabolites should retain both deuterium atoms originally present in 4-2,2-d_2.

Since an appreciation of the quantitative aspects associated with the C-2 metabolic oxidation of **4** was important in our planned cyanide ion trapping experiments, we developed a quantitative GC assay for 1-benzylpyrrolidine (retention time 1.6 min) and 1-benzyl-2-pyrrolidinone (retention time 10.2 min). The homologues 1-benzylpiperidine (**11**, retention time 2.4 min) and 1-benzyl-2-



11 **12**

piperidone (**12**, retention time 12.1 min) served as internal standards. With the aid of this assay, we examined the influence of soluble enzyme systems on the metabolism of **4** by comparing metabolic profiles obtained from 10000*g* hepatic homogenate supernatant fractions with the washed 100000*g* microsomal fractions. The results show that the amount of lactam **6** formed by the 10000*g* supernatant fraction (93% of total metabolites) is four to five times greater than that generated by the 100000*g* preparation (21%). Yet, the level of substrate metabolism is virtually equal in both hepatic fractions. These findings are consistent with the proposal that the soluble aldehyde oxidase[5] is at least in part responsible for the further oxidation of the initially formed 2-electron oxidation product, presumably the carbinolamine **9**, to the lactam. The absence of detectable amounts of enamine **5** in 10000*g* incubates of **4** also supports the role of soluble enzymes in the overall metabolism of cyclic amines to lactams. Since our interests focused on cyanide trapping of iminium ions, subsequent experiments were performed with 100000*g* microsomal preparations.

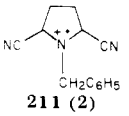
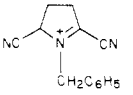
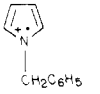
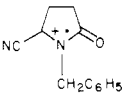
A typical GC tracing obtained from extracts of 1-benzylpyrrolidine–sodium cyanide coincubations is shown in Figure 1b. In addition to recovered 1-benzylpyrrolidine and 1-benzyl-2-pyrrolidinone, four new peaks appeared. The GC and GC–EIMS characteristics of these compounds are summarized in Table II.

Quantitatively, the most important metabolite proved to be 1-benzyl-2-cyanopyrrolidine (**13**). The GC–EIMS displayed a parent ion at M^+ 186 which requires the substitution of one proton of the substrate with a cyano group. The facile loss of HCN to form the fragment ion **13ii**$^+$ plus the presence of the tropylium ion (m/e 91) as the base peak eliminated on mass spectral grounds the isomeric α-cyano structure, α-1-pyrrolidinylphenylacetonitrile (**16**). The GC (retention time 7.4 min) and GC–EIMS characteristics of synthetic **16** [M^+ 186 (42), m/e 185 (43), 160 (8), 159 (9), 116 (72), 109 (72), 91 (35), 70 (100)] prepared from the reaction of pyrrolidine, benzaldehyde, and sodium cyanide[6] did not correspond to any of the products isolated from the 1-benzylpyrrolidine–sodium cyanide coincubation mixtures.

The location of the cyano group was established with the aid of 1-benzylpyrrolidine-2,2-d_2. The GC–EIMS of the monocyano product isolated from the coincubation of this deuterated substrate and sodium cyanide displayed two weak parent ions at M^+ 187 and 188 [1-benzyl-2-cyanopyrrolidine-2-d_1 (**13**-d_1) and 1-benzyl-5-cyanopyrrolidine-2,2-d_2 (**13**-d_2), respectively] and two fragment ions at m/e 160 and 161 generated by the loss of HCN from the d_1 and d_2 metabolites, respectively (cf. **13ii**$^+$, Table II). Final proof of structure was based on comparison of the GC and GC–EIMS characteristics of the metabolically derived product with those of the fully characterized synthetic 1-benzyl-2-cyanopyrrolidine which

(6) Wunderlich, H. H. *Pharmazie* **1954**, *8*, 15.

Table II. GC–EIMS Characterization of Products Obtained from the Metabolism of 1-Benzylpyrrolidine in the Presence of Cyanide

cyano adduct M^+, %	retention time, min	% formation (of total metabolites)	major GC-EIMS fragment ions: *m/e*, %
			$C_6H_5CH_2^+$, 91 (100)
 186 (4) **13**$^+$	7.6	65	 185 (4) **13i**$^+$  159 (25) **13ii**$^+$
 211 (2) **14a**$^+$, **14b**$^+$	12.8, 15.6	8, 7	 210 (2) **14i**$^+$  157 (13) **14ii**$^+$
			$C_6H_5C{\equiv}NH^+$ 104 (49)
 200 (47) **15**$^+$	13.4	4	 173 (3) **15i**$^+$  146 (62) **15ii**$^+$

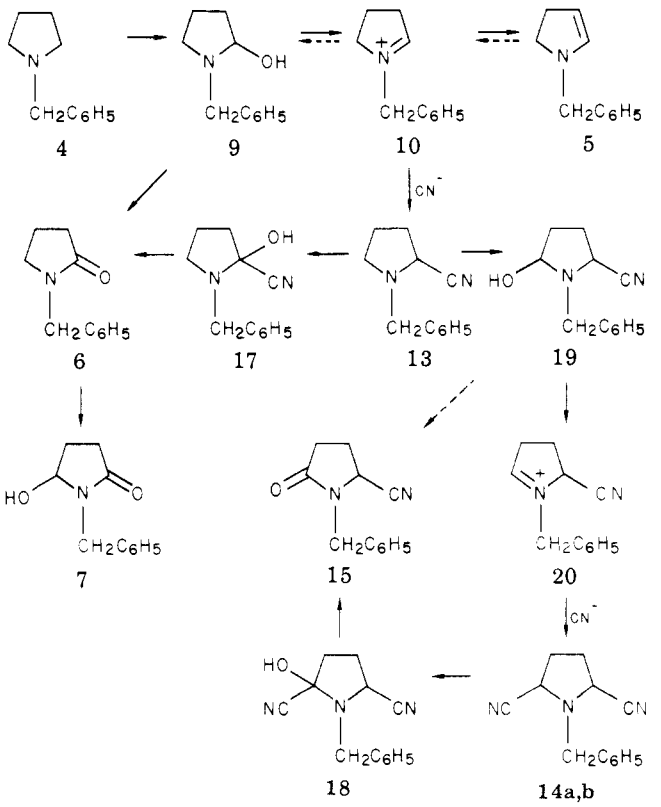
was prepared by the oxidation of 1-benzylpyrrolidine with mercuric acetate, followed by treatment of the intermediate **10** with sodium cyanide.[7]

GC–EIMS analysis of two of the remaining three new peaks gave identical mass spectra, which suggested they were geometric isomers. The parent ions appeared at M^+ 211 (introduction of two cyano groups); the only significant fragment ion appeared at *m/e* 157. These products were shown to be the cis and trans isomers of 1-benzyl-2,5-dicyanopyrrolidine (**14a** and **14b**), since the corresponding metabolites (**14a**-d_1 and **14b**-d_1) isolated from incubations of **4**-2,2-d_2 both retained one deuterium atom [observed in their parent ions (M^+ 212) and the fragment ion (*m/e* 158, cf. **14ii**$^+$)]. The only assignments consistent with the retention of only one deuterium atom in these structures require the introduction of the cyano groups at C-2 and C-5. The equal intensities of the two GC peaks for **14a** and **14b** (Figure 1b) indicate that neither deuterium isotope effects nor stereochemical effects influence the formation of **14a** and **14b**.

The final product characterized in these incubations has been assigned the structure 1-benzyl-5-cyano-2-pyrrolidinone (**15**). This assignment is based on the nominal masses of the parent ion at M^+ 200 and the expected fragment ions **15i**$^+$ and **15ii**$^+$. Incubation of substrate **4**-2,2-d_2 led to the two expected products **15** and 1-benzyl-5-cyano-2-pyrrolidinone-5-d_1 (**15**-d_1, M^+ 201). Fragment ions derived from **15** and **15**-d_1 appear at *m/e* 173 and 174 (cf **15i**$^+$) and at *m/e* 146 (**15ii**$^+$). The presence of only deuterium-free **15ii**$^+$ is consistent with the formation of this ion by the fragmentation process discussed for 1-benzyl-2-pyrrolidinone.

Evidence will be presented below to support a metabolic pathway in which the cyano adducts **13**, **14a**, and **14b** result from cyanide ion attack on reactive iminium intermediates that are likely to be formed by ionization of precursor carbinolamines. The same carbinolamines may be substrates for lactam formation. On the other hand, the formation of lactams **6** and **15** in the incubations containing cyanide ion also could result from mixed function oxidation at the carbon atoms bearing the cyano group to form the corresponding cyanohydrins **17** and **18**,

Scheme II. Proposed Pathways to the Various Compounds Characterized from Microsomal Incubates of 1-Benzylpyrrolidine in the Presence and Absence of Sodium Cyanide



respectively, which would be expected to breakdown spontaneously to the lactams (see Scheme II). This question was addressed by examining the metabolism of 1-benzyl-2-cyanopyrrolidine (**13**) in the absence of cyanide ion and of 1-benzyl-2-pyrrolidinone (**6**) in the presence of cyanide ion. No cyanolactam was formed from **6**. On the other hand, the cyanopyrrolidine was extensively metabolized, with lactam **6** being the major product (48% of total metabolites).

We were somewhat surprised to find in addition to **6** significant amounts of the dicyano compounds **14a** (20%),

(7) Leonard, N. J.; Cook, A. G. *J. Am. Chem. Soc.* **1959**, *81*, 5627.

Table III. Effect of Cyanide Ion Concentration on the Formation of 13 from Microsomal Incubations of 4 (1.0 mM)

NaCN, mM	0	0.2	0.4	0.8	1.0	2.0	5.0	10.0
% 4 metabolized	70	78	58	68	61	61	53	51
cyano adduct 13 formed, % of total metabolites		7	25	40	40	36	20	17

Table IV. Formation of 1-Benzyl-2-cyanopyrrolidine (13) After Addition of Sodium Cyanide to a 1-h Postincubate of 1-Benzylpyrrolidine (4)

min after addition of sodium cyanide	0	15	30	45	60
cyano adduct 13 formed, % of 4 metabolized		49	70	71	60

14b (17%), and the cyanolactam **15** (15%). The remarkable trapping efficiency observed with the metabolically generated cyanide ion suggested that the concentration of cyanide ion used in earlier experiments (10 mM) was unnecessarily high. Therefore, we carried out a series of experiments to determine the optimal cyanide ion concentration for the formation of **13** from 1-benzylpyrrolidine (1 mM) incubations. The results, which are summarized in Table III, establish that maximum trapping efficiency is achieved at a concentration of 1 mM inorganic cyanide and that higher concentrations inhibit 1-benzylpyrrolidine metabolism and also lead to lower yields of **13**. Although 1 mM NaCN is still considerably higher than the estimated maximum cyanide ion concentration achieved in incubations of **13** (<0.2 mM), membrane barriers not encountered by metabolically generated cyanide ion from **13** may limit the access to metabolic sites of added inorganic cyanide.

It is apparent from the preceding discussion that 1-benzylpyrrolidine undergoes an NADPH-dependent microsomal oxidation to generate an intermediate species which in the presence of cyanide ion are converted to isolable cyano adducts. The monocyano adduct **13** may be further metabolized to the lactam **6**, or, by oxidative attack at C-5, it may form a second electrophilic intermediate that leads to the *cis*- and *trans*-dicyano adducts **14a** and **14b**. Further metabolism of **14a** and/or **14b** may result in the cyanolactam **15**. The biotransformation scheme (Scheme II) attempts to summarize these pathways.

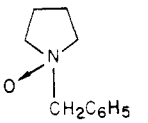
The key steps in the postulated cyanation reactions are the ionizations of the carbinolamines **9** and **19** to the iminium ions **10** and **20**.[8] Although we have no direct evidence for the presence of these intermediates, they provide a reasonable rationalization for the formation of the isolated cyano adducts. The fact that the enamine **5** was detected only in those incubations not containing cyanide ion suggests that the iminium ion **10** is a precursor to both the enamine **5** and the monocyano adduct **13**.

The possible involvement of an N-oxidation metabolic pathway in the formation of these cyano adducts was examined with the aid of 1-benzylpyrrolidine *N*-oxide (**21**) synthesized by the *m*-chloroperoxybenzoic acid oxidation of **4**.[8] Gas chromatographic analysis of the extract obtained from the postincubate of **21** with the 100000*g* microsomal fraction in the presence of inorganic cyanide showed no trace of the cyano adducts **13**, **14**, or **15**. In addition, attempts to characterize a metabolic product corresponding to **21** from the incubation of **4** with the 100000*g* microsomal fraction failed.

Table V. Incorporation[a] of 4-α,α-t_2 into Microsomal Macromolecules

washing procedure	with NADPH	without NADPH
$HClO_4$/EtOH	0.17	0.02
	0.19	0.02
EtOH	0.29	0.04
	0.36	0.05

[a] In nmol/mg of protein.



21

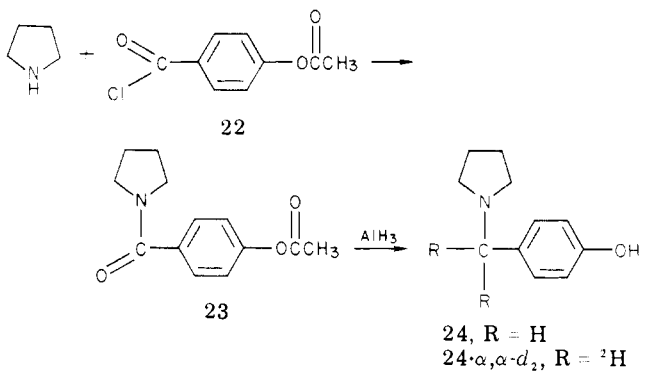
In an effort to further characterize the pathway leading to **13**, 1-benzylpyrrolidine was incubated with microsomes and NADPH in the absence of cyanide ion for 1 h, then sodium cyanide (10 mM) was added, and the extent of cyano adduct **13** formation was measured over time. A previous experiment had established that 30 min after the addition of NADPH no further metabolism of substrate occurred.

Inexplicably, the GC tracing of an aliquot of the incubation mixture analyzed at the end of the 1-h preincubation period but before the addition of sodium cyanide did not show the enamine product. On the other hand, the amount of lactam **6** formed accounted for only 15–20% of the total amine metabolized, leaving unaccounted 80–85% of the metabolites formed. Formation of cyano adduct **13** was monitored following addition of sodium cyanide to this mixture (Table IV). After 30 min, 70% of the 1-benzylpyrrolidine metabolized could be accounted for as cyano adduct **13**. It is clear, therefore, that mixed function oxidation of this amine generates a relatively stable species (carbinolamine?) which undergoes conversion to an electrophilic intermediate (iminium ion?) that is trapped by cyanide ion.

The capability of the proposed metabolically generated iminium intermediates to alkylate macromolecules was tested on microsomal protein-binding studies with radiolabeled 1-benzylpyrrolidine. Since products resulting from metabolic oxidation at the benzylic carbon of **4** were not detected, the tritium label was introduced at the benzylic position. The radiolabeled substrate, 1-benzylpyrrolidine-α,α-t_2 (**4**-α,α-t_2, 1.97 mCi/mmol) was synthesized by $LiAlT_4$ reduction of 1-benzoylpyrrolidine (**8**). After the incubation of **4**-α,α-t_2 with rabbit liver microsomal preparations, the postincubates were repeatedly homogenized with ethanolic $HClO_4$ or EtOH to remove any noncovalently bound metabolites or substrate. The results (Table V) show that the binding of **4**-α,α-t_2 to the microsomal macromolecular fraction was seven to eight times greater in incubations performed in the presence of NADPH than in the absence of NADPH. The level of radiolabel incorporated into the macromolecular fraction was low compared to the amounts of cyano adducts iso-

(8) An alternative pathway to these cyano products suggested by a reviewer involves ring opening of the carbinolamine intermediates to the corresponding amino aldehydes, followed by cyanohydrin formation and cyclization to the final products. Although this pathway cannot be excluded at this time, we favor the ionic process since (a) *N*-(cyanomethyl)nornicotine, a noncyclic α-cyanoamine, is formed under analogous conditions from nicotine[2] and (b) the dehydrative cyclization reaction proposed in this alternative pathway is likely to be a high-energy process that would proceed slowly at 37 °C.

Scheme III



lated from the sodium cyanide coincubates. However, the level of incorporation found in these experiments is comparable to that reported in the literature for other metabolically activated cytotoxic xenobiotics.[10] Since incorporation of the label is dependent on the presence of NADPH, the species binding to macromolecular microsomal components are likely to be formed by the mixed function oxidation of the substrate. Preliminary experiments indicate that sodium cyanide does not block this binding. Further characterization of the molecular events associated with this binding will be required to assess more accurately the significance of iminium ion intermediates as potential alkylating agents. Additionally, we plan to examine reactions of suspected iminium intermediates with model nucleophiles in an attempt to characterize the stability of possible adducts with macromolecules.

Metabolic aromatic oxidation to an arene oxide intermediate is a second pathway that might lead to macromolecular binding of substrate. Since arene oxide formation would be expected to produce the corresponding para-phenolic metabolite, we decided to examine microsomal postincubates of 4-α,α-d_2 for the presence of 1-(4-hydroxybenzyl)pyrrolidine-α,α-d_2 (**24**-α,α-d_2). The deuterium-free internal standard **24** was synthesized by AlH_3 reduction of 1-(4-acetoxybenzoyl)pyrrolidine (**23**), which in turn was prepared by acetylation of pyrrolidine with *p*-acetoxybenzoyl chloride (**22**) (Scheme III).

Postincubates of 4-α,α-d_2 (1 mM) were first treated with 10 μg of **24** and then extracted with ethyl acetate (at pH 9). Following derivatization with BSTFA, GC–EIMS analysis was performed. Both the parent ion (M^+ 249) and base peak (*m/e* 179) of the internal standard were readily detected by multichannel averaged narrow scans. The intensities of the ions at masses 251 and 181 for the proposed metabolite **24**-α,α-d_2 were indistinguishable from those observed with pure labeled **24**. We estimate that less than 0.05% of the substrate is present as the *p*-hydroxy compound in the postincubate. Based on these results, it appears unlikely that arene oxide formation is responsible for the observed macromolecular binding of tritium-labeled **4**.

Experimental Section

All synthetic reactions were carried out under a nitrogen atmosphere. Nuclear magnetic resonance (NMR) spectra were obtained on a Perkin-Elmer R12B or a Varian FT-80 instrument. Chemical shifts are reported in parts per million downfield from internal tetramethylsilane (Me_4Si). Infrared (IR) spectra were taken on a Perkin-Elmer 337 grating IR spectrophotometer. Elemental analyses were done by the Microanalytical Laboratory of the University of California, Berkeley. Gas chromatography (GC) was performed on a Varian Aerograph Series 2100 gas chromatograph with an H_2 flame-ionization detector. A U-shape 2 m × 2 mm i.d. glass column was packed with 3% OV-25 on acid-washed DMCS Chromosorb W (mesh 100/120). GC–EIMS was performed on an Infotronics 2400 gas chromatograph interfaced to an AEI MS-12 mass spectrometer. Multichannel averaged narrow scans were performed on a 10 m × 0.325 mm i.d. glass capillary column coated with 0.1% SE-52 coupled directly to a Hitachi M-52 GC–MS. Scintillation counting was performed on a Packard Tri-Carb Model 3375 liquid scintillation spectrophotometer.

1-Benzylpyrrolidine-2,2-d_2 (4-*2,2*-d_2). To a stirred solution of $LiAlD_4$ (0.01 mol) in 15 mL of freshly distilled ether at 0 °C was added dropwise 1-benzyl-2-pyrrolidinone (**6**, 0.01 mol, Aldrich) in ether. The reaction mixture was heated under reflux for 8 h and stirred overnight at room temperature. After the reaction mixture was cooled to 0 °C, 0.4 mL of H_2O, 0.4 mL of 15% NaOH, and 2 mL of H_2O were added sequentially, and the resulting mixture was stirred for 30 min. The white precipitate was filtered and washed with ether. The combined ether washes and filtrate were dried over Na_2SO_4 and distilled (bulb to bulb) to give a clear liquid in 40% yield: bp 50 °C (0.15 mm); NMR ($CDCl_3$) δ 1.6–1.9 (m, 4 H), 2.3–2.7 (m, 2 H), 3.5 (s, 2 H), 7–7.5 (m, 5 H); EIMS 163 (M^+, 39%), 91 (100%), 86 (69%), 72 (35%); (*m/e* 86/$\sum$ *m/e* 84–86) × 100 = 97% deuteration ($d_2/d_1/d_0$, 97:1:1).

1-Benzylpyrrolidine-*3,3,4,4*-d_4 (4-*3,3,4,4*-d_4). Benzylamine (0.1 mol) was added to a stirred solution of succinic anhydride (0.1 mol) in toluene. A white precipitate appeared after a few minutes. The solution was heated under reflux for 4 days with a Dean–Stark trap. The clear solution upon cooling yielded a white precipitate of *N*-benzylsuccinamic acid melting at 138 °C (lit.[11] mp 138). The precipitate was filtered and the filtrate evaporated to dryness, leaving a yellow residue which was crystallized from hot $CHCl_3$/hexane to give a white solid in 40% yield: mp 99–102 (lit.[12] mp 103 °C).

The 1-benzylsuccinimide (0.03 mol) was dissolved in anhydrous pyridine, 20 mL of 99.8% D_2O (1 mol) was added, and the solution was heated under reflux. The exchange reaction was followed by NMR with fresh D_2O added repeatedly. After 30 days, the reaction mixture was evaporated to dryness to provide a yellow solid, which was recrystallized repeatedly from hot $CHCl_3$/hexane to provide a product melting sharply at 103 °C: CIMS MH^+ 194.

To a stirred solution of $LiAlH_4$ (6 mmol) in dry THF at 0 °C was added slowly 100% H_2SO_4 (3 mmol). To this solution was then added slowly with stirring at 0 °C 1-benzylsuccinimide-d_4 (1.5 mmol) and the mixture was heated under reflux overnight. To the cool reaction mixture was added 0.25 mL of H_2O, 0.25 mL of 15% NaOH, and 0.75 mL of H_2O. The solution was stirred at 0 °C for 15 min. The resulting precipitate was filtered and washed with THF, and the combined THF washes and filtrate were dried over Na_2SO_4 and evaporated to a clear oil. The oil was distilled to yield 50 mg of a clear liquid: bp 70 °C (0.25 mm); EIMS 165 (M^+, 38%), 91 (100%), 88 (57%), 74 (39%); (*m/e* 165/$\sum$ *m/e* 161–165) × 100 = 90% deuteration ($d_4/d_3/d_2/d_1/d_0$, 89:3:6:2:1).

1-Benzylpyrrolidine-α,α-d_2 (4-α,α-d_2). To a stirred solution of $LiAlD_4$ (12.5 mmol) in 25 mL of anhydrous ethyl ether was added 1-benzoylpyrrolidine (10 mmol) at 0 °C. The solution was allowed to warm to room temperature, heated under reflux for 4 h, and then stirred at room temperature overnight. The reaction mixture was cooled to 0 °C and 0.5 mL of H_2O, 0.5 mL of 15% NaOH, and 1.5 mL of H_2O were slowly added sequentially. The resulting precipitate was filtered and washed with ether, and the combined washes and filtrate were dried over Na_2SO_4 and rotary evaporated to a clear oil. The oil was distilled to give a clear liquid in 81% yield: bp 70 °C (0.25 mm); NMR ($CDCl_3$) δ 1.5–2.0 (m, 4 H), 2.2–2.7 (m, 4 H), 7.0–7.5 (s, 5 H); EIMS 163 (M^+, 36%), 93 (100%), 86 (54%), 70 (40%); (*m/e* 86/$\sum$*m/e* 84–86) × 100 = 93% deuteration ($d_2/d_1/d_0$, 93:3:4).

(9) Castagnoli, N. J.; Craig, J. C.; Melikian, A. P.; Roy, S. K. *Tetrahedron* **1970**, *26*, 4319.
(10) Potter, W. Z.; Davis, D. C.; Mitchell, J. R.; Jollow, D. J.; Gillette, J. R.; Brodie, B. B. *J. Pharmacol. Exp. Ther.* **1978**, *187*, 203. Nelson, S. D.; Mitchell, J. R.; Snodgrass, W. R.; Timbrell, J. A.; Corcoran, G. B. *Science* **1976**, *193*, 901.
(11) Pressman, D.; Bryden, J. H.; Pauling, L. *J. Am. Chem. Soc.* **1948**, *70*, 1352.

1-Benzylpyrrolidine-α,α-t_2 (4-α,α-t_2). To a stirred solution of $LiAlH_4$ (0.2 g, 0.5 mmol) in 15 mL of ether at 0 °C was added 1-benzoylpyrrolidine (0.875 g, 5 mmol) in 5 mL of ether. The solution was stirred at room temperature for 2 h, followed by the addition of $LiAlT_4$ (0.8 mg, 240 mCi/mmol, New England Nuclear) at 0 °C. The solution was stirred with heating for 2 h and allowed to stir overnight at room temperature. More $LiAlH_4$ (0.18 g, 4.5 mmol) was added to the reaction mixture, and the solution was allowed to stir for 3 h at room temperature. After the solution was cooled, 0.2 mL of H_2O, 0.2 mL of 15% NaOH, and 0.6 mL of H_2O were added sequentially, and the resulting white precipitate was filtered and washed with ether. The combined ether extracts and washes were dried over Na_2SO_4 and rotary evaporated to a clear oil which was distilled (bulb to bulb) to give the product in 36% yield. The specific activity (scintillation counting) was 1.97 mCi/mmol.

1-Benzyl-2-cyanopyrrolidine (13). To a solution of mercuric acetate (0.2 mol) dissolved in 150 mL of 5% aqueous CH_3COOH was added 0.05 mol of 1-benzylpyrrolidine (Aldrich). The reaction mixture was stirred at 50 °C for 7 h, and the resulting mercurous acetate precipitate was filtered and washed with 5% aqueous CH_3COOH. The combined washes and filtrate were saturated with H_2S at 0 °C, and the resulting black precipitate was filtered. The pH of the filtrate was adjusted to 4–5 with saturated $KHCO_3$ solution. NaCN (0.25 mol) was then added and the reaction mixture stirred overnight at room temperature. The solution was then made alkaline (pH 8) with saturated $KHCO_3$ solution and extracted with ether. The ether extracts were dried over Na_2SO_4 and rotary evaporated to give a gold-colored oil. The oil was column chromatographed on 53 g of silica gel (40–140 mesh) with acetone as the developing solvent. The desired fraction (by GLC) was dried over Na_2SO_4 and evaporated to a gold-colored oil, which upon distillation (bulb to bulb) gave a colorless liquid in 27% yield: bp 75–80 °C (0.25 mm); NMR ($CDCl_3$) δ 1.6–2.3 (m, 4 H), 2.3–3.2 (m, 2 H), 3.5–3.9 (m, 3 H), 7.1–8.5 (m, 5 H); IR (neat) 2240 cm^{-1} (C≡N); EIMS 186 (M^+, 4%), 159 (28%), 91 (100%). Anal. ($C_{12}H_{14}N_2$) C, H, N.

1-(4-Acetoxybenzoyl)pyrrolidine (23). Pyrrolidine (80 mmol) and 4-acetoxybenzoyl chloride (40 mmol) were stirred overnight in 25 mL of $CHCl_3$ at room temperature. The reaction mixture was washed twice with 0.1 N HCl, dried over K_2CO_3, and evaporated to a yellow oil. The oil was distilled (bulb to bulb) to 155 °C (0.025 mm) to give a clear oil. The oil later crystallized to a white solid, which was recrystallized from benzene/hexane: mp 90–95 °C; NMR ($CDCl_3$) δ 1.9 (m, 4 H), 2.3 (s, 3 H), 3.4–3.7 (m, 4 H), 7.0–7.6 (m, 4 H); EIMS 233 (M^+, 49%), 191 (43%), 121 (100%). Anal. ($C_{13}H_{15}NO_3$) C, H, N.

1-(4-Hydroxybenzyl)pyrrolidine (24). To a stirred solution of $LiAlH_4$ (15 mmol) in dry THF at 0 °C was added slowly 100% H_2SO_4 (7.5 mmol). The amide **23** (5 mmol) was then slowly added to the stirred AlH_3 solution at 0 °C. The reaction mixture was stirred under reflux for 24 h and at room temperature for an additional 18 h. The reaction mixture was treated with 0.6 mL of H_2O and acidified with 1 N HCl. The resulting precipitate was filtered and the filtrate was evaporated to a gel. The gel was dissolved in H_2O and the pH of the solution was adjusted to 9 with 0.1 N NaOH. The aqueous solution was extracted with $CHCl_3$, and the $CHCl_3$ extracts were dried over K_2CO_3 and evaporated to a yellow oil which later crystallized. The crystals were sublimed at 80 °C (0.01 mm) to give a white crystalline product: mp 107–110; NMR ($CDCl_3$) δ 1.75 (m, 4 H), 2.5–2.7 (m, 4 H), 3.5 (s, 2 H), 6.6–7.3 (m, 4 H); CIMS MH^+ 178. Anal. ($C_{11}H_{15}NO$) C, H, N.

Liver Preparations. Most incubations were performed with rabbit liver 100000*g* microsomal pellet preparations. Preparation of the microsomal pellet was done at 0–4 °C in a cold room. Male Dutch rabbits 6 months to 1 year old were stunned and decapitated. The livers were excised and rinsed in cold isotonic (1.15%) KCl. The liver tissue (12.5 g) was homogenized in 25 mL of cold 0.2 M, pH 7.4, Tris-HCl buffer using a Potter-Elvehjem Teflon pestle homogenizer. The homogenates were centrifuged at 10000*g* for 20 min in a Sorvall RC-2 refrigerated centrifuge at 0–4 °C to yield the 10000*g* supernatant fraction. The supernatant fractions were recentrifuged at 100000*g* for 1 h at 0–4 °C in a Spinco Model L refrigerated centrifuge to obtain the 100000*g* microsomal pellet. The sediment (microsomal pellet) was resuspended in the amount of cold 0.2 M, pH 7.4, Tris-HCl buffer to make 1 mL of solution correspond to 0.5 g of liver. The resuspended pellet was then recentrifuged at 100000*g* for 1 h at 0–4 °C. The resultant pellet was then resuspended as described above for use in incubations. A Lowry assay was done to determine microsomal protein concentration.[12]

Incubations. All substrates were synthesized or purchased as previously described. NADPH was purchased from Sigma Chemical Co.

A typical incubate (5.0 mL total volume) contained 4 mL of microsomal fraction (0.5 g of liver/mL), $MgSO_4$ (15 mM), 1.0 mg of the desired substrate, and NADPH (4 mg added every 20 min unless indicated). Incubations in the presence of sodium cyanide contained 1.0 mM or 10 mM NaCN (in H_2O). Incubations were done at 37 °C in air for 1 h in a metabolic shaker. In preincubation experiments, NADPH was added at time 0, and incubations were allowed to proceed for 1 h before the addition of NaCN. At the end of the incubation period, the incubates were chilled in ice and the internal standards were added (0.5 mg of 1-benzylpiperidine and 0.2 mg of 1-benzyl-2-piperidone). Analysis of the phenol **24**-α,α-d_2 employed 10 μg of **24** as internal standard. The incubates were made basic with solid $NaHCO_3$ and then extracted with fresh diethyl ether (2 × 12 mL). Analysis for **24** involved extraction with ethyl acetate at pH 9. The ether extracts were dried over Na_2SO_4 and were reduced in volume to 0.3 mL under a N_2 stream with gentle heating (28–32 °C) and then analyzed by GC or GC–EIMS. The GC column oven temperature was held at 125 °C for 2 min 40 s and then programmed at 6 °C/min to 250 °C. Quantitation of GC peaks was achieved by measuring peak heights.

In the protein-binding studies, the incubations were conducted as described above. After the incubation period, the reactions were terminated by chilling in ice and by adding either 1 M ethanolic $HClO_4$ or EtOH. The resulting suspension was centrifuged and the supernatant removed. The protein pellet was homogenized with the aid of a Potter-Elvehjem Teflon pestle homogenizer in 5 mL of either 0.4 N ethanolic $HClO_4$ or EtOH and recentrifuged. This washing process was repeated seven times. The pellet was then dissolved in 2 N NaOH, Aquasol scintillation fluid (New England Nuclear) was added, and the mixture was counted in a liquid scintillation counter.

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(12) Weast, R. C., Ed. "Handbook of Chemistry and Physics", 55th ed., CRC Press: Cleveland, 1974; p C-495.

(13) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.