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CHARACTERIZATION OF HAMSTER LIVER NICOTINE METABOLISM

I. RELATIVE RATES OF MICROSOMAL C AND N OXIDATION

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Abstract—A high pressure liquid chromatographic procedure has been developed for the determination of the two principal N- and C-oxidation products of nicotine in hamster liver subcellular fractions. Advantage was taken of the fact that cyanide ion forms a stable adduct with the microsomal metabolite that is the precursor of cotinine. The rate and extent as well as the sensitivity of inhibition were similar for cotinine, the 5′-cyanonicotine adduct, and an as yet unidentified microsomal metabolite which is presumed to be the initial microsomal metabolite on the pathway to cotinine formation. The rates of nicotine-*N*′-oxidase and nicotine-5′-hydroxylase activities exhibited differential response to inhibitors as well as differential susceptibility to proteolytic digestion. Data are presented which indicate that low levels of nornicotine contamination in stock nicotine resulted in the artifactual formation of methylcyanonornicotine adduct. No evidence consistent with the formation of nornicotine by isolated mircosomes was obtained.

The metabolism of the tobacco alkaloid nicotine is quite complex and at least eighteen metabolites have been identified [1]. The principal metabolites are thought to be cotinine[3], nornicotine[2] and nicotine-*N*′-oxide[4] (Fig. 1). Cotinine is not a primary microsomal product but involves the enzymatic conversion of either 5′-hydroxynicotine[3a] or nicotine-1′,5′-iminium ion[3b] to cotinine by a cytosolic aldehyde oxidase [2, 3]. Significant rates of cotinine formation from nicotine can be demonstrated only with liver post-mitochondrial supernatant fractions or with purified microsomes in the presence of purified aldehyde oxidase [4–6]. The formation of nornicotine[2] from nicotine has been suggested by several groups [7, 8]. Convincing evidence for the participation of the methyl iminium ion[2b] as an intermediate in the formation of nornicotine has been presented by Nguyen *et al.* [9, 10] using rabbit liver post-mitochondrial supernatant fractions. The formation of nicotine-*N*′-oxide[4] from nicotine has been shown to be catalyzed by microsomal amine oxidase [11, 12]. In addition, Gorrod and coworkers [13] have shown that both microsomes and post-microsomal supernatant fractions can catalyze the reduction of nicotine-*N*′-oxide to nicotine in the absence of oxygen.

The purpose of these investigations was to establish conditions for the direct determination of the principal microsomal metabolites of nicotine and

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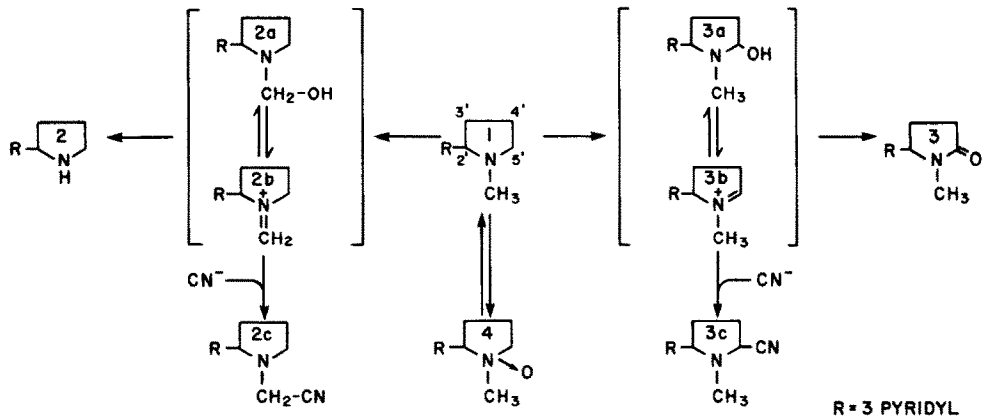

Fig. 1. Primary nicotine metabolites: nicotine[1]; methylhydroxynornicotine[2a]; nicotine methyl iminium ion[2b]; methyl-cyanonornicotine[2c]; nornicotine[2]; 5′-hydroxynicotine[3a]; nicotine-1′,5′-iminium ion[3b]; 5′-cyanonicotine[3c]; cotinine[3]; and nicotine-*N*′-oxide[4].

to study the effects of inhibitors of mixed-function oxidase activity on the individual pathways of nicotine metabolism.

MATERIALS AND METHODS

Chemicals

Bromelain, papain, cytochrome *c* (Type III), cysteamine, potassium cyanide, nicotinamide adenine dinucleotide phosphate (monosodium salt), nicotinamide, Tricine (*N*-tris-[hydroxymethyl]-methyl glycine), Tris (tris-[hydroxymethyl]-aminomethane), glucose-6-phosphate (monosodium salt), and glucose-6-phosphate dehydrogenase (Type XII) were obtained from the Sigma Chemical Co., St. Louis, MO; (*S*)-(−)-nicotine, *m*-chloroperoxybenzoic acid and bromine were obtained from the Aldrich Chemical Co., Milwaukee, WI; nornicotine from Research Plus, Inc., Bayonne, NJ; titanous chloride (20%) from Fisher Scientific, Pittsburgh, PA; cimetidine (Tagamet) and SKF-525A were obtained from Smith Kline & French, Philadalphia, PA; and sucrose (special enzyme grade) from Schwarz/Mann, Orangeburg, NY.

Animals and treatment

Six-week-old male LVG Syrian golden hamsters (*Mesocricetus auratus*) were obtained from the Charles River Breeding Laboratories, Lakeview Colony, Newfield, NJ. Animals were housed two per cage in regulation stainless steel cages and were given free access to NIH-07 lab chow (Ziegler Brothers, Gardners, PA) and water. Animals were killed by decapitation at 13 weeks of age.

Subcellular fractionation and biochemical analysis

Livers were rapidly removed, weighed, minced, rinsed three times with 10 vol. of ice-cold SET (0.3 M sucrose, 0.5 mM EDTA, and 5 mM Tricine, pH 7.4), and suspended in 9 vol. of ice-cold SET. Homogenization and subcellular fractionation were performed as described previously [14]. NADPH–cytochrome *c* reductase activity was determined by the method of Phillips and Langdon [15]; cytochrome P-450 content according to the method of McLean and Day [16], using an extinction coefficient of 91 mM^{-1} for $A_{450-490\,nm}$; and cytochrome b_5 content as described by Omura and Sato [17], using an extinction coefficient of 125 mM^{-1} for $A_{425-409\,nm}$. Protein was determined by the method of Lowry *et al.* [18].

In vitro *nicotine metabolism*

The standard assay conditions for determination of the rates of nicotine N- and C-oxidation consisted of: 20 μmoles nicotinamide, 1 μmole $NADP^+$, 5 μmoles glucose-6-phosphate, 5 units glucose-6-phosphate dehydrogenase, 6 μmoles $MgCl_2$, 12 or 30 μmoles nicotine, 1 μmole potassium cyanide, and 40 μmoles Tricine, pH 7.4, in a final volume of 1.0 ml. Reactions were initiated by the addition of either microsomes or post-mitochondrial supernatant fractions (0.3 to 3 mg protein). Following incubation for 30 min at 37° in a Dubnoff metabolic shaking incubator, reactions were quenched by the addition of 1.0 ml of ice-cold acetone. After removal of protein by centrifugation, a 1.0-ml aliquot of the supernatant fraction was extracted with 5.0 ml chloroform. Five milliliters of the chloroform extract was evaporated under a stream of nitrogen, and the residue was redissolved in 0.10 ml methanol. Extraction efficiency for 5′-cyanonicotine was 96 ± 2%. Nicotine-*N*′-oxide was quantitatively retained (91 ± 1%) in the aqueous phase following chloroform extraction. Using these conditions, the rates of nicotine-*N*′-oxide and 5′-cyanonicotine formation were linear with time and protein concentration.

Protease pretreatment

The procedure employed was a modification of Gum and Strobel [19] for treatment of microsomes with steapsin. Eight milligrams of microsomal protein was incubated in a final volume of 2 ml (0.1 M potassium phosphate, 0.14 M sucrose, and 5.4 mM EDTA, pH 7.4) for 18 hr at 4° in the presence of bromelain (420 μg) or papain (24 μg). This amount of protease corresponds to 0.45 units of activity. Protease-treated and untreated samples were diluted 3-fold with SET and microsomes were reisolated by ultracentrifugation.

High pressure liquid chromatographic conditions

HPLC analyses. These analyses were performed using either a Varian series 5000 high pressure liquid chromatograph equipped with a Varian UV 100 variable wavelength detector and a Rheodyne injector or a Gilson HPLC gradient system composed of two model 302 pumps, an 802B manometric module, an 811 mixer, an ISCO V4 variable wavelength detector and a Rheodyne injector.

*Analysis of nicotine-*N′*-oxide.* Aliquots (30 μl) of the aqueous extracts were separated on a 30 cm × 0.4 cm Varian MicroPak MCH-10 analytical column using isocratic elution at 70% methanol, 30% water. The water was adjusted to pH 9.5–9.7 by the addition of 5 drops of concentrated ammonium hydroxide per liter. Flow rate was 2 ml/min, and column effluents were monitored at 254 nm. Nicotine-*N*′-oxide eluted at 5.3 min and nicotine at 9.7 min. Failure to include ammonium hydroxide in water resulted in loss of resolution and prolonged retention times (> 20 min) for nicotine.

Analysis of C-oxidized metabolites. Aliquots (20 μl) of the chloroform extracts were separated on a 30 cm × 0.4 cm Varian MicroPak MCH-100 analytical column by gradient elution. Reservoir A was water adjusted to pH 9.5–9.7 by the addition of 5 drops of concentrated ammonium hydroxide per liter. Reservoir B was 100% methanol. Gradient conditions were: 0–10 min, 20% B; 10–20 min, 20–35% B; 20–30 min, 35–70% B; and 30–33 min, 70–80% B. Flow rate was 2.0 ml/min, and column effluents were monitored at 254 nm. Metabolites eluted in the following order: cotinine (19.2 min), methylcyanonicotine (27.2 min), nornicotine (29.2 min), 5′-cyanonicotine (30.5 min), and nicotine (35.0 min).

Chemical preparations

Commercially available nicotine was partially purified by vacuum steam distillation as described by Swain *et al.* [20]. The distillate was stored at room

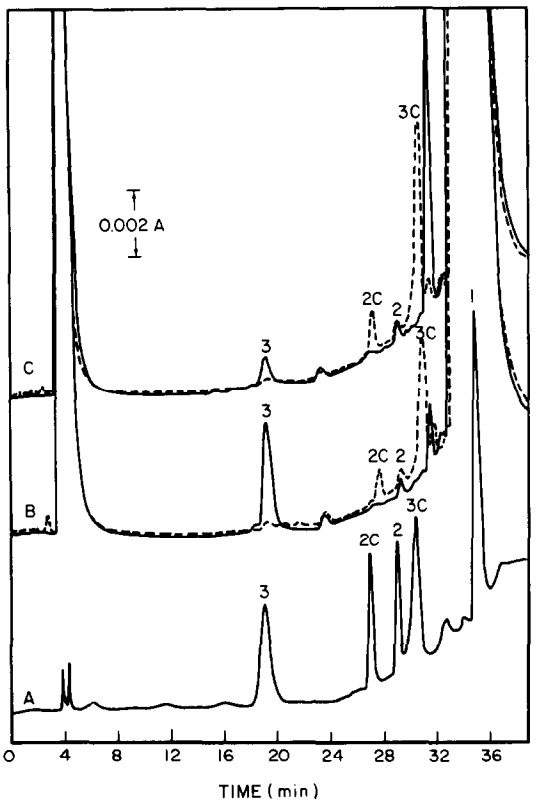

Fig. 2. High pressure liquid chromatographic separations of chloroform-extractable metabolites. Trace A: Standard metabolite profile; 3.2 nmoles cotinine[3], 5.0 nmoles methyl-cyanonicotine[2c], 5.4 nmoles nornicotine[2], 5.5 nmoles 5′-cyanonicotine[3c], and 6.5 nmoles nicotine[1]. Trace B: Post-mitochondrial supernatant incubation in the absence (solid line) and presence (dotted line) of 1 mM KCN. Trace C: Microsomal incubation in the absence (solid line) and presence (dotted line) of 1 mM KCN.

temperature under reduced pressure. Immediately prior to use in metabolic studies, 1 g of the distillate which still contained traces of nornicotine was further purified by passage through a 30 cm × 1 cm column of neutral alumina (Fisher Scientific), using benzene as the eluant. Fractions (0.5 ml) were collected and analyzed for nornicotine by high pressure liquid chromatography. Nicotine fractions free of nornicotine were pooled, evaporated under nitrogen, and used immediately. *Cotinine* was synthesized in the sequential bromination and reduction of nicotine as described by Bowman and McKennis [21]. *Nicotine-N′-oxide* was prepared by oxidation of nicotine with *m*-chloroperoxybenzoic acid as described by Craig and Purushothaman [22]. *5′-Cyanonicotine* was prepared by photochemical oxidation of nicotine in the presence of KCN, methylene blue and sodium lactate as described by Hubert-Brierre *et al.* [23]. *Methyl cyanonornicotine* was prepared by reaction of nornicotine, formaldehyde and KCN as described by Nguyen *et al.* [9].

RESULTS

Our preliminary studies took advantage of the observations of Murphy who showed that cyanide ion reacts with the initial microsomal oxidation product which is a precursor of cotinine to form a stable adduct which he identified as 5′-cyanonicotine [3] and those of Nguyen *et al.* who identified both 5′-cyano- and methyl-cyanonornicotine in rabbit post-mitochondrial supernatant fractions incubated in the presence of cyanide ion [9, 10].

A typical high pressure liquid chromatogram of the principal chloroform-extractable metabolites (cotinine[3] and nornicotine[2]), as well as the 5′-cyano[3c] and methyl-cyano[2c] adducts of nicotine, is shown in Fig. 2A. Under the conditions employed, good resolution of all four metabolites from nicotine[1] was achieved. Inclusion of KCN in complete reaction mixtures resulted in the appearance of two peaks in the chloroform-extractable fraction. The first peak comigrated with methyl-cyanonornicotine[2c] while the second comigrated with 5′-cyanonicotine[3c]. Since the chemical synthesis of methyl-cyanonornicotine involved the reaction of nornicotine, KCN and formaldehyde under relatively mild conditions [9], the possibility existed that the formation of methyl-cyanonornicotine was occurring not via the trapping of the enzymatically generated methyl iminium ion[2b] but rather as a consequence of the reaction of nornicotine, which was present in commercially available nicotine as a contaminant, added KCN, and biologically generated formaldehyde. To assess this possibility, commercially available nicotine was subjected to a two-step purification procedure which involved vacuum distillation followed by adsorption chromatography using neutral alumina. As can be seen in Table 1, this purification procedure reduced the nornicotine contaminant 45-fold and decreased

Table 1. Influence of nornicotine concentration on rates of formation of microsomal nicotine metabolites

Nicotine preparations (mM)	Nornicotine (μM)	Methyl-cyano nornicotine	5′-Cyanonicotine (nmoles/min/mg/protein)	Nicotine-*N′*-oxide
A 12	72	1.21 ± 0.11	1.90 ± 0.13	2.31 ± 0.14
B 12	12	0.25 ± 0.03	1.78 ± 0.07	3.08 ± 0.39
C 12	1.6	0.17 ± 0.01	1.91 ± 0.18	2.20 ± 0.08

Assays were performed as described in Materials and Methods in the presence of 1 mM KCN. Nicotine preparations employed were: (A) unpurified; (B) steam distilled; and (C) steam distilled and column purified. Values are means ± S.D. for four separate preparations.

the rate of formation of methyl-cyanonornicotine over 7-fold. In contrast, little change in the rate of formation of either 5′-cyanonicotine or nicotine-*N*′-oxide was observed. In addition, no net formation of nornicotine was observed with either microsomes or post-mitochondrial supernatant fractions in the absence of KCN (data not shown). We conclude therefore that, under our current assay conditions, hamster microsomes do not demethylate nicotine to nornicotine to any significant extent.

When post-mitochondrial supernatant fractions were incubated under complete assay conditions, in the absence of KCN, cotinine and an unknown metabolite which had a retention time of 31.5 min were detected in the chloroform-extractable fraction (Fig. 2B). The addition of KCN caused both peaks to disappear, and a new peak that migrated slightly faster than the unknown peak and comigrated with 5′-cyanonicotine was observed (Fig. 2B).

The effect of inclusion of KCN on the rates of cotinine and 5′-cyanonicotine formation by hamster liver post-mitochondrial supernatant fractions is presented in Table 2. In the absence of KCN, the rate of cotinine formation was 18% lower than the rate of 5′-cyanonicotine formation in the presence of KCN. We have observed that, in the absence of KCN, variable amounts (8–30%) of the unknown metabolite will be detected, suggesting that the level of aldehyde oxidase is rate limiting when hamster post-mitochondrial supernatant fractions are used as the source of enzyme activity.

When isolated microsomes were incubated in the absence of KCN, a small amount of cotinine was formed but a larger amount of the unknown peak was detected (Fig. 2C). Inclusion of KCN in microsomal reaction mixtures caused the disappearance of both cotinine and the unknown peak with the appearance of a new peak which comigrated with 5′-cyanonicotine. It should be noted in Fig. 2B and 2C that a trace of nornicotine was still present and that in the presence of KCN a small amount of the methyl-cyanonornicotine was still observed.

Nicotine-*N*′-oxide was quantitatively retained in the aqueous fraction following chloroform extraction. Gorrod and coworkers [24] have demonstrated that both *cis* and *trans* nicotine-*N*′-oxide are formed biologically and that the relative amount of each isomer is dependent on the species examined. Chromatographic elution at 70% methanol of the aqueous fraction resulted in the appearance of a single symmetrical peak which comigrated with synthesized nicotine-*N*′-oxide (Fig. 3). Reduction of either the synthesized standard and the biologically generated material with titanium trichloride as described by Beckett *et al.* [25] caused the disappearance of the *N*′-oxide and the appearance of nicotine. Consistent with the observation of Gorrod and coworkers [26], hamster post-mitochondrial supernatant fractions and microsomes produced predominantly one form of the *N*′-oxide, which they identified as (1′*R*;2′*S*)-*cis* nicotine-*N*′-oxide.

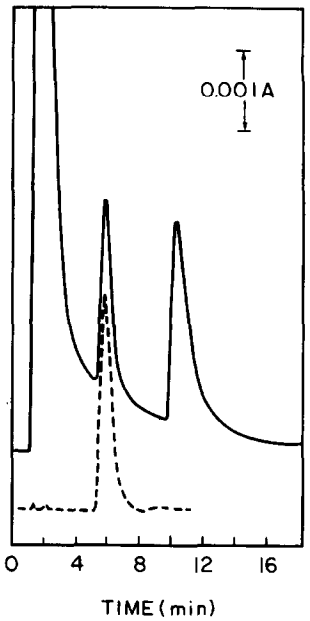

Fig. 3. High pressure liquid chromatographic separation of extracted aqueous sample. Solid line is extracted aqueous sample from microsomal incubation showing nicotine-*N*′-oxide (5 min) and residual nicotine (11 min). Dashed line is 2.8 nmoles nicotine-*N*′-oxide.

Table 2. Relative rates of cotinine and 5′-cyanonicotine formation by hamster post-mitochondrial supernatant fractions

Product formed	Specific activity (nmoles/min/mg protein)
Cotinine	0.46 ± 0.05
5′-Cyanonicotine	0.56 ± 0.03
Nicotine-*N*′-oxide	0.31 ± 0.07

Rates of cotinine and nicotine *N*′-oxide were determined in the absence of KCN while rates of 5′-cyanonicotine were determined in the presence of 1 mM KCN. Nicotine concentration was 30 mM. Values are means ± S.D. for three separate preparations.

The effects of variations in nicotine concentration on the rates of formation of 5′-cyanonicotine and nicotine-*N*′-oxide by hamster microsomes in the presence of KCN are shown in Fig. 4. The rates of formation of both metabolites showed good saturation kinetics with optimal rates occurring at substrate concentrations greater than 30 mM. A similar response was observed for the unknown metabolite (data not shown). Kinetic constants for the rates of formation of the unknown metabolite, 5′-cyanonicotine and nicotine-*N*′-oxide are summarized in Table 3. K_m values for the unknown metabolite, 5′-cyanonicotine and nicotine-*N*′-oxide were similar. Neither the K_m nor the V_{max} for nicotine-*N*′-oxidase was affected by the addition of KCN.

The effects of several inhibitors of MFO activity on the rates of product formation have been examined using both post-mitochondrial supernatant fractions and microsomes in the presence and absence of KCN. The results are summarized in Table 4. The

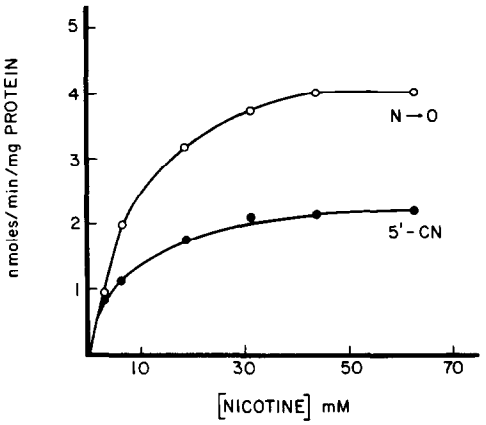

Fig. 4. Effect of nicotine concentration on rates of N'-oxidation and 5'-hydroxylation by isolated hamster liver microsomes. Key: nicotine-*N'*-oxidase activity (○——○); and nicotine-5'-hydroxylase activity (●——●). All values are means of duplicate determinations.

rate of cotinine formation catalyzed by post-mitochondrial supernatant fractions was inhibited markedly by the addition of KCN. The rate of formation of the unknown metabolite catalyzed by microsomes was also decreased. Microsomal rates of nicotine-*N'*-oxidation were not affected. Inclusion of 1 mM cimetidine resulted in partial inhibition of cotinine formation by post-mitochondrial supernatant fractions as well as microsomal rates of formation of both the unknown metabolite and 5'-cyanonicotine. Cimetidine had no effect on microsomal nicotine-*N'*-oxidase activity. In contrast to the data obtained by Gorrod *et al.* who demonstrated inhibition of cotinine formation by SKF-525A using rat post-mitochondrial supernatant fractions [27], SKF-525A had little effect on the rate of formation by hamster subcellular fractions of any of the four metabolites. Cysteamine is thought to be a physiological substrate for microsomal amine oxidase [27, 28]. Inclusion of 1 mM cysteamine inhibited nicotine-*N'*-oxidase activity but had little effect on the rates of formation of the other three metabolites.

Pretreatment of microsomes with protease at low temperatures has been shown to result in selective release of NADPH–cytochrome *c* reductase and cytochrome b_5 while not releasing or destroying cytochrome P-450 to any significant extent [19, 29, 30]. The effect of protease pretreatment of hamster liver microsomes is presented in Table 5. Compared to untreated control preparations, total protein recovery was 68 and 37%, respectively, for bromelain- and papain-pretreated microsomes, indicating extensive loss of microsomal protein. Both protease pretreatments markedly decreased NADPH–cytochrome *c* reductase specific activity (> 80%) and cytochrome b_5 specific content (> 75%). The specific content of cytochrome P-450 remained unchanged in bromelain-pretreated microsomes, whereas a small (20%) increase was seen in papain-pretreated microsomes. Bromelain pretreatment resulted in a 30% decrease in nicotine-*n'*-oxidase specific activity, while nicotine-5'-hydroxylase specific activity was decreased 70%. Papain pretreatment caused only an 18% decrease in nicotine-*N'*-oxidase specific activity, whereas 5'-hydroxylation was decreased 41%. The fact that exposure of hamster microsomes to proteases causes a greater decrease in 5'-hydroxylase than *N'*-oxidase activity suggests that the two activities are associated with separate pathways.

Table 3. Comparison of kinetic parameters for microsomal nicotine metabolite formation

	K_m (mM)	V_{max} (nmoles/min/mg)
Unknown (2)	5.6 ± 0.8	
5'-Cyanonicotine (3)	6.3 ± 1.2	2.63 ± 0.36
Nicotine-*N'*-oxide (4)	6.6 ± 2.0	3.46 ± 0.14

Numbers in parentheses are the number of preparations. Values are means ± S.D.

DISCUSSION

It has been over 25 years since Hucker *et al.* [2] first demonstrated the site of *in vitro* metabolism of nicotine. Despite widespread human exposure to this pharmacologically active alkaloid, many of the basic properties of its *in vitro* metabolism are still poorly understood. Nicotine is quite susceptible to both

Table 4. Effect of selected inhibitors on *in vitro* nicotine metabolism

	Cotinine	5'-Cyano nicotine (% of control)	Unknown	Nicotine-*N'*-oxide
Control	100 (0.38)	—	100	100 (2.24)
+ KCN (1 mM)	5	100 (1.82)	4	94
+ Cimetidine (1 mM)	60	69	69	94
+ SKF-525A (1 mM)	95	100	88	97
+ Cysteamine (1 mM)	91	93	90	56

Rates of cotinine formation were determined with post-mitochondrial supernatant fractions in the absence of KCN. Rates of 5'-cyanonicotine, nicotine *N'*-oxide and the unknown metabolites were determined with microsomes. Numbers in parentheses are the control rates of product formation expressed as nmoles/min/mg protein. Nicotine concentration was 12 mM.

Table 5. Effect of protease pretreatment on microsomal proteins and nicotine metabolite formation

	Protein recovery (%)	NADPH–cytochrome *c* reductase (nmoles/min/mg)	Cytochrome b_5 (nmoles/mg)	Cytochrome P-450 (nmoles/mg)	Nicotine-*N'*-oxidase (nmoles/min/mg)	Nicotine-5'-hydroxylase (nmoles/min/mg)
Control	100	115	0.62	1.22	3.60	2.44
Bromelain	68	19	0.08	1.26	2.41	0.75
Papain	37	14	0.16	1.53	2.98	1.42

Microsomes were incubated for 18 hr at 4* in the presence and absence of protease as described under Materials and Methods. Nicotine concentration was 30 mM.

light and air oxidation which results in formation of variable amounts of nicotine-*N'*-oxide, nornicotine, cotinine and numerous other oxidation products [23]. As a result, most studies have relied on measurement of nicotine disappearance rather than measuring the individual rates of product formation. Unfortunately, assays which are based on the quantitation of nicotine disappearance will either underestimate or overlook entirely subtle changes in the flux of nicotine through individual pathways. For example, the recent study of Nakayama *et al.* [31] has shown that phenobarbitol pretreatment results in enhanced rates of nicotine disappearance, whereas 3-methylcholanthrene or β-naphthaflavone pretreatments are without effect. Ethanol pretreatment of F-344 rats has been shown to increase the total plasma clearance of cotinine [32], suggesting inducer associated increases in mixed-function oxygenase activity. It is quite possible that modifiers of drug metabolism will have differential effects on the individual pathways of nicotine metabolism.

Microsomal nicotine metabolism may involve at least three and possibly four distinct pathways (N'-oxidation, 5'-hydroxylation, N'-demethylation, and 2'-hydroxylation). The first pathway involves microsomal amine oxidase [11, 12, 24, 26], while the latter three are catalyzed by the mixed-function oxygenase electron transport system [2, 3, 6, 9, 10, 27]. Of the four possible pathways, good evidence is available for the occurrence of the first two in man [5, 25, 33, 34]. The N-demethylation of nicotine has not been a consistent observation [1]. We are aware of no experimental evidence for the existence of a microsomal 2'-hydroxylation pathway for nicotine metabolism. However, Hecht and his associates [35–37] have provided extensive evidence for the microsomal 2'-hydroxylation of the tobacco specific carcinogen, *N'*-nitrosonornicotine (NNN). In view of the structural similarities between nicotine and NNN, it seems reasonable to expect that a similar pathway may exist for the metabolism of nicotine.

Because the cotinine formation requires the participation of a second enzymatic activity following initial cytochrome P-450 associated 5'-hydroxylation, estimates of either rate or extent of cotinine formation must result in an underestimation of the true rate of microsomal catalysis. The appearance of variable amounts of the unknown metabolite in post-mitochondrial supernatant incubations in the absence of KCN indicates that conversion to cotinine is not 100% in hamster post-mitochondrial supernatant fractions. This suggests that simply determining cotinine levels underestimates the true microsomal rate of 5'-hydroxylation. In addition, nothing is known about the effect of modifiers of mixed-function oxygenase activity on aldehyde oxidase activity. Clearly, modulation of this second enzyme could have pronounced effects on the apparent rate of cotinine formation. By performing assays in the presence of a strong nucleophile such as KCN, a much clearer estimate of the true rate of 5'-hydroxylation of nicotine is possible. In addition, formation of a stable metabolite allows for the direct estimation of microsomal catalysis. We gave much thought to the possibility of utilizing purified aldehyde oxidase as a reagent instead of KCN to determine microsomal *c* hydroxylation in these experiments, but the apparent instability of the enzyme in purified form seemed to preclude its use on a routine basis [38–40].

Our observation that the rate of formation of methyl-cyanonornicotine was decreased dramatically as the level of the contaminating nornicotine was decreased indicates the potential for artifactual formation of nicotine metabolites in biological systems. As a result, we have found it necessary to purify nicotine preparations immediately before use in order to decrease the possibility of chemical synthesis of otherwise reasonable biological metabolites. While several aspects of the formation of methyl-cyanonornicotine are intriguing, for example, the source of formaldehyde, it is clear that its formation in hamster microsomes does not occur to any significant extent via the nicotine methyl iminium ion.

The unknown metabolite shares with cotinine and 5'-cyanonicotine the sensitivity to inhibition by cimetidine and, like cotinine, disappears upon the addition of KCN. Its kinetics of formation are similar to 5'-cyanonicotine. These data, albeit circumstantial, are consistent with this metabolite being an intermediate in the formation of cotinine from nicotine. The unknown metabolite appears to be stable to storage in either aqueous reaction mixtures or chloroform extracts which have been reconstituted in methanol. This would seem to indicate that it is neither the 5'-hydroxynicotine or the nicotine-1',5'-iminium ion[3b] since both of these compounds are known to be quite unstable [2, 4, 41], but rather may be 4-(3-pyridyl)-4-methylamino butyraldehyde, the open chain form of 5'-hydroxynicotine [2].

Our results indicate that the microsomal rate of nicotine-*N'*-oxidation is greater than the rate of 5'-

hydroxylation. The ratio of N'-oxidation to 5'-hydroxylation varied from 1.2:1 to 2:1 from preparation to preparation. In contrast, 5'-hydroxylation is favored over N'-oxidation in hamster post-mitochondrial supernatant fractions. Jenner and coworkers [24–26, 42] have presented evidence which shows that cotinine formation (5'-hydroxylation) is greater than nicotine-N'-oxide formation in post-mitochondrial supernatant fractions from several species.

The reasons for differences in the ratio of N and C oxidation between microsomes and post-mitochondrial supernatant fractions are as yet unknown. It is possible that post-mitochondrial supernatant fractions contain a substrate for the N-oxidase which would act as an inhibitor of N-oxide formation. Isolation of microsomes would remove the competing substrate and result in greater relative rates of N'-oxide formation. Alternatively, it is possible that the reduction of metabolically generated nicotine-N'-oxide back to nicotine is occurring in post-mitochondrial supernatant fractions, resulting in lower relative rates of N'-oxidase. These two possibilities are currently under investigation.

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