NICOTINE METABOLISM IN MAMMALS

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SUMMARY

Over the past decade studies on nicotine metabolism have been advanced by use of new methods involving enzyme purification, preparation of specific antibodies and HPLC. In *in vivo* experiments of nicotine metabolism, a number of new metabolites have been identified, and most major and minor metabolites have been quantitatively determined, which make it now possible to investigate the contribution of each pathway to overall nicotine metabolism. In *in vitro* experiments, *C*-oxidation and *N*-oxidation have been precisely characterized. Despite extensive investigations, metabolic regulation of nicotine metabolism still remains unclear.

I. INTRODUCTION

Nicotine, a major constituent of tobacco, exerts a number of physiological effects involving the central and peripheral nervous systems, the cardiovascular system and endocrine system. Distribution, metabolism and excretion of nicotine have attracted notice in connection with the physiological effects of nicotine. Since tobacco contains carcinogens such as benzopyrene, metabolic relations between the carcinogens and nicotine have also been given attention. For these reasons, there have been many studies on the distribution, metabolism and excretion of nicotine, and a number of reviews have focused mainly on the fundamental pharmacological problems, that is, absorption, distribution, metabolic pathways and excretion of nicotine /1-6/. More recently, new biochemical and immunochemical methods have allowed considerable progress on the elucidation of the enzymatic processes of nicotine metabolism and made it possible to answer some important questions on nicotine metabolism. First, studies on cytochrome P-450 and FAD-containing monooxygenase (EC 1.14.13.8) have made remarkable progress, which have provided new methods in biochemical and immunochemical analysis of nicotine metabolism. Before the 1980s, most studies on nicotine metabolism depended on the isolation and the identification of nicotine metabolites and the determination of kinetic parameters of the enzyme reactions by crude systems involving microsomes or a post-mitochondrial supernatant. Recently,

biochemical and immunochemical methods have been applied to the precise analysis of the enzymatic process of nicotine metabolism and have produced important information on the participation of cytochrome P-450 and FAD-containing monooxygenase in microsomal nicotine metabolism. Secondly, methods for determination of nicotine and its metabolites have advanced. For example, in previous experiments, two major metabolites, cotinine and nicotine-1'-N-oxide, were selectively extracted with organic solvents or separated by thin layer chromatography or paper chromatography. Recently these two metabolites have been separated and determined simultaneously with HPLC. In addition, many other kinds of nicotine metabolites have also been determined by this method, which makes it possible to investigate more precisely *in vivo* nicotine metabolism. GC-MS and gas chromatography equipped with a nitrogen detector are also available for the qualitative and quantitative analysis of nicotine metabolism. This review first describes the pathways of nicotine metabolism and then details recent progress specially related to cytochrome P-450- and FAD-containing monooxygenase-dependent reactions. Since nicotine exerts complex physiological effects, this review pays much attention to the differences between the findings obtained from *in vivo* and *in vitro* experiments.

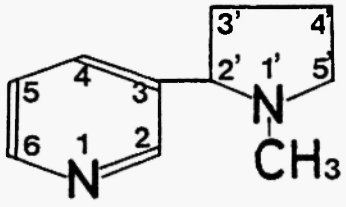

Fig. 1: Numbering of nicotine molecule

II. METABOLIC PATHWAYS OF NICOTINE

The numbering of the nicotine molecule used in this review is shown in Figure 1. The metabolism of nicotine is complicated, and

the important pathways are presented in Figures 2 and 3. To facilitate understanding, the pathways are considered as four parts: (a) C-oxidation and the subsequent conversion of 5'-hydroxy-nicotine into 3-pyridylacetic acid, (b) formation of nornicotine, demethylcotinine, hydroxycotinine and γ-(3-pyridyl)-γ-methylaminobutyric acid, and the conversion of these metabolites into intermediates in the pathway of 3-pyridylacetic acid formation as mentioned above, (c) *N*-oxidation, (d) *N*-methylation.

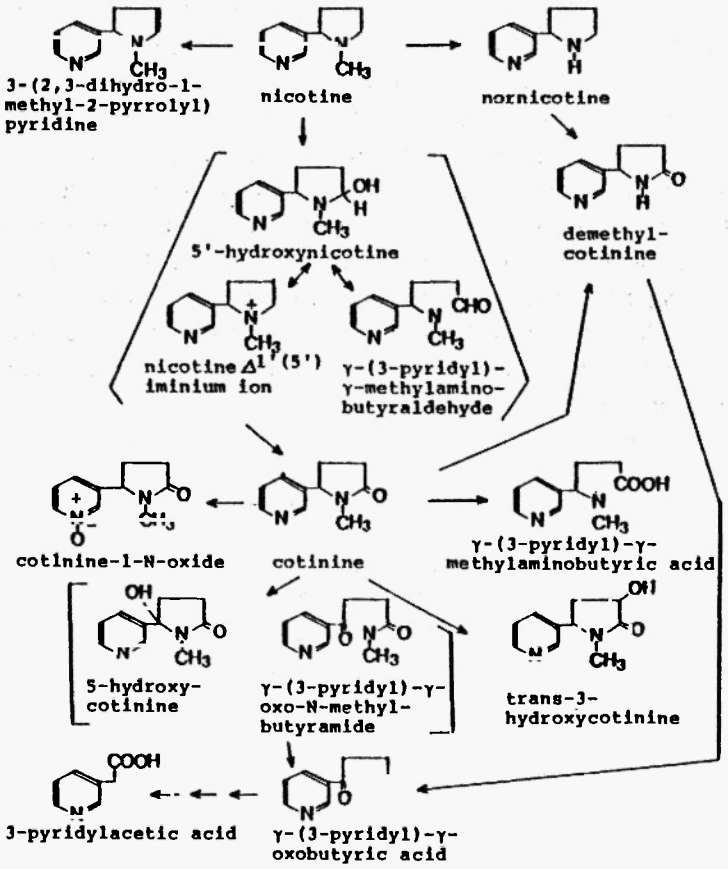

Fig. 2: Metabolic routes for the metabolism of nicotine in mammals (1). C-oxidation

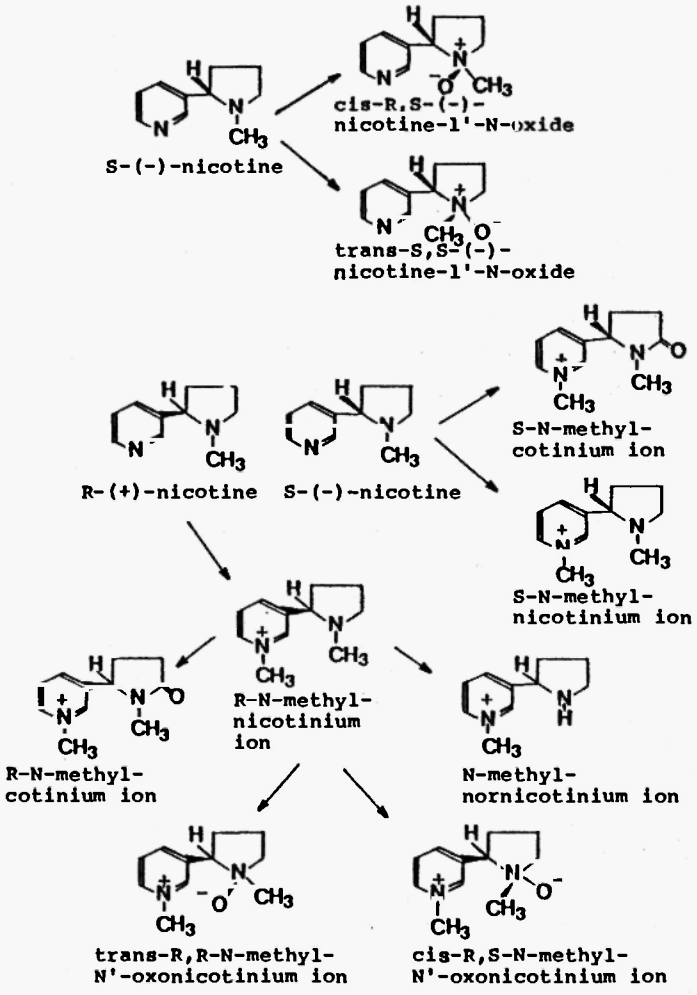

Fig. 3: Metabolic routes for the metabolism of nicotine in mammals (2). N-oxidation and N-methylation

2.1 C-Oxidation and the subsequent conversion of 5'-hydroxy-nicotine into 3-pyridylacetic acid

Our knowledge concerning the biotransformation of nicotine to 3-pyridylacetic acid is largely due to the pioneer work of McKennis and coworkers and Hucker and coworkers in the early sixties. Coti-

nine was first isolated and identified as a urinary metabolite after administration of (-)-nicotine to dogs /7,8/ and then detected as a major urinary metabolite of man /9/, rabbit /10/, mouse /11/, rat /11/, and cat /12/. In addition to the *in vivo* experiments, cotinine was shown to be a major metabolite of nicotine in lung and kidney as well as liver, using a postmitochondrial supernatant /13/ and tissue slices /14/. Hucker *et al.* presented the first study on the enzymatic conversion of nicotine to cotinine and proposed the following pathway: hydroxylation of nicotine by microsomal enzymes and conversion to the corresponding aldehyde and, finally, production of cotinine by a cytosolic enzyme /15/. Murphy reported that the nicotine iminium ion was formed by loss of water from 5'-hydroxynicotine and converted to cotinine /16/. Brandange and Lindblom have investigated the conversion of the nicotine iminium ion to cotinine by cytosolic aldehyde oxidase, and stressed that this iminium ion serves as a substrate for aldehyde oxidase /17/. Furthermore, Gorrod and Hibberd have confirmed the formation of cotinine from nicotine iminium ion by *in vitro* and *in vivo* experiments /18,19/.

γ-(3-pyridyl)-γ-oxo-*N*-methylbutyramide was first isolated and identified as a urinary metabolite after administration of (-)-cotinine to dogs /20,21/, and then detected in urine of humans /22/. This amide was also isolated from the urine of dogs after administration of ^{14}C-(-)-nicotine /23/. In *in vitro* experiments, liver and kidney slices of mice converted ^{14}C-(-)-nicotine into this amide /14/. Later, this amide was reported to be formed from ^{14}C-(-)-nicotine and ^{14}C-(-)-cotinine by a 10,000 x g supernatant of mouse livers /24/.

A number of intermediates in the metabolism of γ-(3-pyridyl)-γ-oxo-*N*-methylbutyramide to 3-pyridylacetic acid were isolated and identified as urinary metabolites after administration of (-)-cotinine, (-)-demethylcotinine and other metabolites to animals /23,25-27/.

3-Pyridylacetic acid was isolated and identified as a urinary metabolite after administration of (-)-cotinine to dogs /28/, humans /27/, and mice /29/. Furthermore, other intermediates, demethylcotinine and γ-(3-pyridyl)-γ-oxobutyric acid, were reported to be converted to 3-pyridylacetic acid after administration of these intermediates to animals /26,27/. On the other hand, there has been only one *in vitro* study concerning the formation of 3-pyridylacetic

acid /13/. When a 18,500 x g supernatant of rabbit liver was incubated with ^{14}C-(-)-nicotine in the presence of NADPH and O_2, a substance corresponding to 3-pyridylacetic acid was detected on paper chromatograms. On the other hand, Stalhandske reported that after incubation of (-)-cotinine with a 10,000 x g supernatant of mouse liver, about 95% of added cotinine was recovered unchanged from chloroform extracts of the reaction mixture, showing a low metabolism of cotinine /24/. McKennis *et al.* have also suggested additional routes in the metabolism of nicotine to 3-pyridylacetic acid /30/. All these results suggest conversion of cotinine into 3-pyridylacetic acid proceeds much more slowly than that of nicotine into cotinine.

2.2 Formation of nornicotine, demethylcotinine, trans-3-hydroxycotinine and γ-(3-pyridyl)-γ-methylaminobutyric acid

Nornicotine, demethylcotinine and 3-hydroxycotinine were isolated and identified as urinary metabolites after administration of (-)-nicotine or (-)-cotinine to humans /9,22/, dogs /31,32/, rats /11,23/, mice /11,29/, rabbits /33/ and cats /12/. A post-mitochrondrial supernatant of rabbit livers was also reported to convert ^{14}C-(-)-nicotine to demethylcotinine and nornicotine /13,33/. However, there have been conflicting reports concerning the *in vitro* formation of nornicotine, that is, it has been reported that nornicotine was not formed from nicotine by a 9000 x g supernatant of rabbit liver /15/. Recently, formation of nornicotine has not been found with either microsomes or a postmitochondrial supernatant of hamster liver /34/.

3-Hydroxycotinine was reported to be formed from ^{14}C-(-)-nicotine by mouse liver slices /14/. Using a 10.000 x g supernatant of mouse liver Stalhandske showed that nicotine-methyl-^{14}C was converted into 3-hydroxycotinine, cotinine and γ-(3-pyridyl)-γ-oxo-*N*-methylbutyramide, whereas ^{14}C-(-)-cotinine was converted into 3-hydroxycotinine, demethylcotinine and γ-(3-pyridyl)-γ-oxo-*N*-methylbutyramide /24/. Castognoli and his coworkers determined the structure of 3-hydroxycotinine in the urine of monkey as *trans*-3-hydroxycotinine /35,36/. Recently they have also shown that 5-hydroxycotinine formed from (-)-cotinine leads to a product which

consists of a tautomeric mixture of the hydroxylactam and keto amide (γ-(3-pyridyl)-γ-oxo-*N*-methylbutyramide) /37/. Papadopoulos reported that a 9000 x g supernatant of rabbit liver converted nornicotine into demethylcotinine, suggesting that demethylcotinine can be formed from nicotine via nornicotine as well as cotinine /33/.

McKennis *et al.* reported that cotinine was formed non-enzymatically *in vitro* from γ-(3-pyridyl)-γ-methylaminobutyric acid /7,8/, whereas Hucker *et al.* indicated that little or no γ-(pyridyl)-γ-methylaminobutyric acid was converted to cotinine by microsomes of rabbit liver /15,38/.

2.3 N-Oxidation

Nicotine-1'-*N*-oxide was first isolated and identified from the reaction mixture after incubation of a 9000 x g supernatant of rabbit liver with (-)-nicotine, NADPH and O_2 /13,39/. Booth and Boyland showed that nicotine was converted to isomers of nicotine-1'-*N*-oxide by a 2650 x g supernatant of guinea pig liver and the ratio of two isomers produced by the reaction varied with tissues and species /40,41/. Nicotine-1'-*N*-oxide was also detected as a urinary metabolite in rabbit /33/, cat /12/ and man /42,43/. From *in vitro* and *in vivo* experiments, nicotine-1'-*N*-oxide has been shown to back-convert to nicotine /41,44-47/.

2.4 N-Methylation

McKennis *et al.* first reported that isomethylnicotinium ion (methylnicotinium ion, Fig. 3) and cotinine methonium ion (*N*-methyl-cotinium ion) were formed as urinary metabolites after administration of (-)-nicotine and (-)-cotinine to dogs /48/. Recently, Crooks and coworkers have shown that nicotine *N*-methylation in guinea pigs is a stereospecific reaction involving only the (+)-enantiomer of nicotine, and a cytosolic azaheterocycle-*N*-methyl transferase catalyzes this methylation /49-52/. The *N*-methylcotinium and *N*-methylnornicotinium ions were isolated and identified as urinary metabolites after administration of R-(+)-nicotine or R-(+)-*N*-methylnicotinium acetate to guinea pigs /53,54/. Fur-

thermore, *in vivo* experiments have shown that both R-(+)-nicotine and R-(+)-methylnicotinium acetate were converted into isomers of the *N*-methyl-*N'*-oxonicotinium ion in guinea pigs /55/. After administration of [^{3}H]-S-(-)cotinine to guinea pigs, *S*-*N*-methylcotinium ion was not detected in the urine /56/. Studies on species and tissue differences in *N*-methylation of nicotine would seem to be necessary.

III. ENZYMES PARTICIPATING IN THE MAIN PATHWAYS OF NICOTINE METABOLISM

3.1 Cotinine formation

In early studies on nicotine metabolism, the participation of cytochrome P-450 in the conversion of nicotine into cotinine has been suggested by the following experiments:

1) the conversion of nicotine into cotinine requires microsomes, NADPH and O_2 /24,43,57,58/.
2) liver microsomal cytochrome produces a nicotine-induced type 2 spectral change /58,59,60/.
3) SKF-525A, a potent inhibitor of cytochrome P-450, inhibits the conversion of nicotine into cotinine /15,24,58/.
4) CO also inhibits cotinine formation /16,57/.

Furthermore, Hill *et al.* have proposed that cytochrome P-450 and FAD-containing monooxygenase participate in the formation of cotinine and nicotine-1'-N-oxide, respectively, on the basis of comparative studies on metabolism of nicotine and cyclophosphamide /57/. By use of several kinds of metabolic inhibitors, Gorrod *et al.* also indicated that metabolic *N*- and *C*-oxidation of nicotine were mediated by different processes /58/. However, the non-availability of inhibitors to distinguish completely between cytochrome P-450 and FAD-containing monooxygenases /61/ has delayed the confirmation of their suggestion.

Recently, using purified cytochrome P-450 and antibodies prepared against cytochrome P-450 and NADPH-cytochrome P-450 reductase (fP_2), we have presented clear evidence for the participation of P-450 in microsomal nicotine oxidation as shown below /62-65/. Nicotine metabolism is known to be enhanced after pheno-

barbital (PB) pretreatment /66-68/, and PB-inducible cytochrome P-450 (PB-cytochrome P-450) purified from liver microsomes of rats and guinea pigs produces a nicotine-induced difference spectral change and catalyzes nicotine *C*-oxidation in a reconstituted system /62,63/. Specific antibodies prepared against PB-cytochrome P-450 inhibited liver microsomal nicotine *C*-oxidation of PB-pretreated guinea pig by about 30% /63/. In addition, specific antibodies prepared against fP_2 inhibited nicotine *C*-oxidation by PB-pretreated guinea pig liver microsomes by about 70% /64/. From these results, we have concluded that PB-cytochrome P-450 participates in liver microsomal nicotine *C*-oxidation in PB-pretreated guinea pigs. On the other hand, there is still no direct evidence for involvement of constitutive form(s) of cytochrome P-450 in nicotine oxidation of untreated animals. However, using antibody against fP_2, we have demonstrated clearly that microsomal electron transport systems participate in the conversion of nicotine into cotinine. Antibody against fP_2 inhibited nicotine *C*-oxidation by untreated guinea pig liver microsomes by about 45% /64/, and prevented completely the formation of cotinine /65/. In this case, the formation of nicotine-1'-*N*-oxide was not inhibited by the antibody. Furthermore, metyrapone, n-octylamine and α-naphthoflavone, which are known to be potent inhibitors of cytochrome P-450, also inhibited the formation of cotinine but not of nicotine-1'-*N*-oxide /65/. The results of our studies, along with those of others, indicate the participation of cytochrome P-450 in the conversion of nicotine into cotinine in normal animals. Which types of cytochrome P-450 participate in cotinine formation, other than PB-cytochrome P-450, remains unknown, but a number of experiments suggest that the affinity of cytochrome P-450 for nicotine varies with cytochrome P-450 isozymes. Indeed, the Km value of *C*-oxidation for nicotine was reported to be 5.6 and 1.9 mM in liver microsomes of hamsters and mice, respectively /34,57/ whereas spectral dissociation constants (Ks) of liver microsomes for nicotine were 0.39 and 0.3 mM in monkeys and dogs, respectively /60/. The Ks value of PB-cytochrome P-450 of rat liver was found to be 0.16 mM for nicotine /62/. α-Naphthoflavone has been suggested to inhibit selectively microsomal nicotine *C*-oxidation by PB-cytochrome P-450 and constitutive form(s) of the enzyme, whereas administration of 3-

methylcholanthrene (3-MC) or β-naphthoflavone (β-NF) to rats resulted in a decrease of nicotine oxidation by cytochrome P-450 (P-448), suggesting that cytochrome P-448 induced by these chemicals does not participate in nicotine oxidation /67/. Recently SKF-525A has been shown not to inhibit microsomal nicotine *C*-oxidation by hamster liver /34/. This shows that other types of cytochrome P-450 may also be involved in nicotine *C*-oxidation since inhibition of cytochrome P-450 by effectors varies with species.

Hucker *et al.* reported that 5'-hydroxynicotine, a product of cytochrome P-450-dependent nicotine oxidation, was so unstable as to convert nonenzymatically to the corresponding aldehyde, which was identified qualitatively /15/.The intermediate formed non-enzymatically from 5'-hydroxynicotine has been reported to convert to cotinine in the presence of the cytosolic enzyme /15,43,57/. In these studies, cyanide ion, an inhibitor of aldehyde oxidase, prevented the cotinine formation /15,57/, and purified aldehyde oxidase substituted for the cytosolic enzyme in the cotinine formation /57/. Therefore, aldehyde oxidase is probably involved in cotinine formation, though no *in vivo* evidence for the participation of aldehyde oxidase in cotinine formation has been obtained. The product formed from 5'-hydroxynicotine, i.e., nicotine iminium ion, has been identified as an intermediate in the conversion of nicotine into cotinine /16,17/ and determined by the formation of cyanonicotine /16,34/, which has been applied to the determination of the rate of microsomal *C*-oxidation /16,34/. Based on a kinetic study of the enzymatic conversion of nicotine iminium ion to cotinine, Brandange and Lindblom have shown that aldehyde oxidase, partially purified from rabbit liver, has a high affinity for nicotine iminium ion /17/. In this report, they have proposed that "iminium oxidase" is a more adequate name than "aldehyde oxidase". Gorrod and Hibberd have reported that 140,000 x g supernatant of guinea pig, rat, mouse, hamster livers catalyzes the formation of cotinine from nicotine iminium ion and the intraperitoneal administration of the ion to animals results in the formation of cotinine /18,19/.

3.2 Nicotine-1'-N-oxide formation

There have been a number of previous studies on the enzyme(s) participating in the formation of nicotine-1'-*N*-oxide /34,39-41, 58, 65-71/. Cysteamine and methimazole, substrates for the FAD-containing monooxygenase, strongly inhibited the microsomal conversion of nicotine to nicotine-1'-*N*-oxide, whereas antibodies against fP_2, SKF-525A and metyrapone had little or no effect on the formation of nicotine-1'-*N*-oxide /34,58,65,69/. In the presence of antibodies against fP_2, Tynes and Hodgson demonstrated microsomal nicotine *N*-oxidation by mouse liver, lung and kidney and pig liver /71/. The FAD-containing monooxygenase isolated from pig liver was earlier reported to catalyze nicotine N-oxidation /72/, but the N-oxide metabolite was not unequivocally identified. These results strongly suggest that conversion of nicotine to nicotine-1'-*N*-oxide is catalyzed by the FAD-containing monooxygenase; a very recent study has identified the reaction products of S-(-) and R-(+)-nicotine by purified pig liver FAD-containing monooxygenase /101/ (see later this section).

The Km value of nicotine-1'-*N*-oxide formation from (-)-nicotine has been found to be much higher in liver from hamsters /33/ than mouse liver /71/. Although nicotine has been shown to be converted to nicotine-1'-*N*-oxide by liver, lung and kidney homogenates of mouse /57/, the specific activity of microsomal nicotine oxidation in the presence of antibodies against fP_2 is higher in mouse liver and lung than in mouse kidney /71/. Recently, large species and tissue differences have been found in the activities, substrate specificities and immunochemical properties of FAD-containing monooxygenase /73-76/, suggesting the existence of multiple forms of this flavin-monooxygenase.

Booth and Boyland reported that (-)-nicotine is oxidized to two optically-active stereoisomers of nicotine-1'-*N*-oxide by microsomes in the presence of NADPH and O_2, and the laevo-rotatory and dextro-rotatory isomers were the isomers with the methyl and pyridyl groups in cis and trans positions, respectively /40,41/. The ratio of cis- and trans-nicotine-1'-*N*-oxide formed from S-(-)-nicotine was found to be different from that formed from R-(+)-nicotine /69/. The ratio of the amounts of two isomers of nicotine-1'-*N*-oxide also varies with tissues and species /41,70/.

N-Oxidation of nicotine isomers by purified pig liver FAD-containing monooxygenase has recently been reported by Damani *et al.* /107/; these studies revealed a clear stereoselectivity in the formation of the diastereomeric *N'*-oxides. In contrast to results with microsomal preparations, S-(-)-nicotine did not exhibit stereoselectivity in the formation of cis-1'R, 2'S- and trans-1'S, 2'S products with the purified enzyme. However, with R-(+)-nicotine, only the trans-1'R, 2'R *N*-oxide was formed. The concentration of each isomer required for half maximal activity differs significantly, and access of S-(-)-nicotine to the active site appears to be more restricted than for R-(+)-nicotine as judged by the observed Km values (Km = 181μM and 70μM, for the -(-) and-(+) isomers, respectively). This preliminary study demonstrates that detailed studies on the stereochemical outcome of the *N*-oxidation of prochiral tertiary amines to chiral *N*-oxides by the flavin-monooxygenase are warranted.

IV. RELATIVE CONTRIBUTION OF *C*- AND *N*-OXIDATION TO MONOOXYGENASE MEDIATED NICOTINE METABOLISM

C- and *N*-Oxidation are two main pathways of nicotine metabolism and therefore the relative contributions of the two pathways to overall nicotine metabolism has been estimated from *in vivo* and *in vitro* experiments.

4.1 In vivo experiments

Beckett et al. examined assay conditions for cotinine and nicotine-1'-*N*-oxide in urine, and showed that the urinary excretion of nicotine-1'-*N*-oxide in 24 hr from smokers was about half that of cotinine /42/. Petersen et al. reported that in mice the rapid metabolism of nicotine resulted in maximum blood concentrations of cotinine and nicotine-1'-*N*-oxide in 10 and 15 min, respectively, and the maximum concentration of cotinine was about 9 fold higher than that of nicotine-1'-*N*-oxide /77/. The maximum concentration of cotinine in livers was also found to be considerably higher than that of nicotine-1'-*N*-oxide. The half life of cotinine in blood was about 1.3 fold longer than that of nicotine-1'-*N*-oxide. In this case,

large strain differences in the half life and maximum concentration of cotinine were found. These results suggest that *C*-oxidation of nicotine is more active than *N*-oxidation, although *C*-oxidized metabolites other than cotinine were not determined.

A recent study by Crooks and coworkers has examined the enantioselective metabolism during continuous administration of S-(-)- and R-(+)-nicotine isomers in guinea pigs /108/. S-(-)-Nicotine afforded oxidative metabolites only, whereas the R-(+)-isomer formed both oxidative and *N*-methylated metabolites. Interestingly, 3-hydroxycotinine and nicotine-1'-*N*-oxide were major urinary metabolites of both enantiomers, accounting for 28% and 43% of 24 hr dose respectively for the S-(-)-isomer, and 13% and 22% of 24 hr dose respectively for R-(+)-isomer. This is in contrast to many other reports of cotinine being a 'major' metabolite of nicotine. In the study of Crooks and coworkers /108/, cotinine and nornicotine were very minor urinary metabolites. The major *N*-methylated metabolite of R-(+)-nicotine was *N*-methylnicotinium ion, although *N*-methylcotininium ion and *N*-methylnornicotinium ion were also identified as urinary metabolites.

4.2 In vitro experiments

Cotinine was found to comprise about 60% of the nicotine that disappeared after nicotine oxidation by a 9000 x g supernatant of rabbit liver /15/, whereas a 2650 x g supernatant of guinea pig liver catalyzed nicotine oxidation to nicotine-1'-*N*-oxide about 4 times greater than to cotinine /41/. Jenner et al. reported the species differences in *C*- and *N*-oxidation of nicotine metabolism as follows: more cotinine was produced than nicotine-1'-*N*-oxide by 10,000 x g supernatants of rabbit, guinea pig, hamster and rat livers, but both nicotine metabolites were formed equally by the supernatant of mouse livers /70/. McCoy *et al.* have determined by HPLC the amounts of 5'-cyanonicotine and nicotine-1'-*N*-oxide after nicotine oxidation in the presence of cyanide ion /34/. By this method, they found that the ratio of *N*-oxidation to *C*-oxidation by microsomes of hamster liver ranged from 1.2 to 2.0.

In general, three methods have been available for estimating the rate of *C*-oxidation of *in vitro* microsomal nicotine oxidation in the

presence of cytosol or cytosolic aldehyde oxidase. The first is to determine the amounts of cotinine and precursors of cotinine, that is, nicotine iminium ion, '-hydroxynicotine and the corresponding aldehyde. In this case, since it is rather difficult to determine the precursors of cotinine, 5'-cyanonicotine produced by microsomal nicotine oxidation in the presence of cyanide ion has been determined /16,34,68/. This method is supported by the fact that in microsomal nicotine oxidation by hamster liver, an unidentified nicotine metabolite formed in the absence of cyanide ion disappears by the addition of cyanide ion, accompanied with the formation of 5'-cyano-nicotine /32/. Furthermore, the rate of 5'-cyanonicotine formation in the presence of cyanide ion was shown to be greater than that of cotinine formation in the absence of cyanide ion, showing that most of the precursors of cotinine may convert to 5'-cyanonicotine. Murphy also showed that the decrease in the cotinine formation paralleled the increase in the cyanonicotine formation /16/.

The second method for estimating the rate of *C*-oxidation is to determine cotinine formation in the presence of sufficient aldehyde oxidase to convert all the precursors of cotinine into cotinine /17,57,65/. This method was first examined by Hill *et al.*, who demonstrated that an unidentified nicotine metabolite produced by microsomal nicotine oxidation disappeared on the addition of cytosolic aldehyde oxidase with the appearance of cotinine /57/.

The third method is to determine microsomal nicotine oxidation in the presence of a specific antibody prepared against fP_2 /64,71/. The decrease in activity produced by the antibody is considered to correspond to *C*-oxidation of nicotine metabolism. Since many kinds of drugs oxidized by FAD-containing monooxygenase are also oxidized by cytochrome P-450, antibody against fP_2 is currently used to estimate the relative contribution of FAD-containing monooxygenase and cytochrome P-450 /71,79-82/. By this method, it has been estimated that about 45% and 70% of liver microsomal nicotine oxidation depends on *C*-oxidation in untreated and PB-treated guinea pigs, respectively /64/. The relative contribution of cytochrome P-450 and FAD-containing monooxygenase to the metabolism of common substrates also has been investigated by use of metyrapone and heat treatment /80-82/. However, since inhibition of cytochrome P-450 by effectors varies with the isozyme

of cytochrome P-450, antibodies against fP_2 seems to be a more useful tool than effectors of cytochrome P-450. Up to now, an inhibitor which inhibits completely *C*-oxidation of nicotine without affecting N-oxidation has not been found.

V. REGULATION OF NICOTINE METABOLISM

Numerous studies have shown that some cytochrome P-450s are induced by a large number of drugs, xenobiotics and carcinogens, most of which are substrates of the enzyme, and this process is influenced by age and sex. In such cases, drug administration leads to enhancement of not only its own metabolism but also the metabolism of other drugs, which is due to overlapping specificities of substrates and inducers of cytochrome P-450. FAD-containing monooxygenases also have broad substrate specificities, but regulation is completely different from cytochrome P-450 /73/. Activities of not only cytochrome P-450 but also FAD-containing monooxygenase were reported to change with age and sex /73/. Therefore, nicotine metabolism can be changeable in response to drug administration, age and sex. To study the regulation of nicotine metabolism, it is necessary to investigate the relationship between nicotine and other drug metabolic reactions and to analyze the factors which affect nicotine metabolism. Unfortunately, despite extensive investigation, there are apparently conflicting reports concerning induction of nicotine metabolism, and the regulation mechanisms of nicotine metabolism remain unclear. In this section, therefore, observations on the regulation of nicotine metabolism are summarized and problems involved in such studies are discussed.

5.1 Effects of nicotine administration on nicotine and other drug metabolism

When male rats were injected intraperitoneally with 4 mg/kg of nicotine four times a day for two or three days, nicotine oxidation by a 12,500 x g supernatant of the livers was not found to be significantly increased, but *N*-dealkylation of ethylmorphine and *O*-dealkylation of norcodeine were increased /83/. Stalhandske and

Slanina investigated the effects of nicotine administration to male mice on the conversion of nicotine to cotinine by a 10,000 x g supernatant /84/. In this report, intraperitoneal injection of nicotine at 5 mg/kg, three times a day for three days decreased by about 50% the cotinine formation, whereas administration of the drug at 24.5 and 27.8 mg/kg/day in drinking water did not affect significantly cotinine formation. Nakashima et al. administered intraperitoneally 1 mg/kg of nicotine, five times a day, for 10 days to a number of animals and determined the activity of nicotine oxidase by a 10,000 x g supernatant of the livers /85/. They showed that nicotine administration resulted in a slight increase of nicotine oxidase activity in dogs but no change of the enzyme activities in mice (ddy, C57BL/6J, DBA/2J and BDF strains), rats, guinea pigs and rabbits. From these reports, no pronounced induction of nicotine metabolism has been observed.

To understand these results, two important factors must be considered: first, nicotine causes a number of physiological effects which may lead to changes in nicotine metabolism. It is well known that administration of large doses of nicotine results in toxic effects or the production of stress as suggested by Ruddon and Cohen /83,86/. Therefore, different effects of nicotine administration may be mediated by not only nicotine itself but also factors produced by nicotine administration. This problem must be further investigated in connection with effects of nicotine administration on cytochrome *P*-450 and FAD-containing monooxygenase. Secondly, it is necessary to take into consideration the rapid metabolism and excretion of nicotine /11,12/. To elevate tissue concentration of nicotine or reduce stress by nicotine administration, nicotine has been injected repeatedly throughout the day or administered in drinking water. However, rapid metabolism and excretion of nicotine may lead to a low concentration of the drug in the liver, which may make it difficult to characterize nicotine as a potent inducer of cytochrome P-450. Species differences in rates of metabolism and excretion of nicotine may lead to different effects on induction of enzymes participating in nicotine metabolism. The effects of nicotine on other drug metabolic pathways are further complicated as shown below.

Yamamoto et al. showed that a single intraperitoneal injection of 40 mg/kg of nicotine to rats resulted in the increase of acetyl-

aminofluorene (AAF) hydroxylase and 3,4-benzopyrene (BP) hydroxylase activities, but daily injections of 40 mg/kg of nicotine for two to three days decreased AAF hydroxylase activity to less than control /87/. Cotinine is also reported to increase AAF hydroxylase and BP hydroxlase activities /87/. In contrast, Weber et al. showed that a single intraperitoneal injection of 25 mg/kg of nicotine decreased BP hydroxylase activity in lungs, livers and small intestines, and the rate of biliary excretion of metabolites of BP also decreased /88/. Similarly, administration of nicotine at 1 mg/kg, five times a day for 10 days, to rats, was found to decrease AAF and BP hydroxylase activities /85/. On the other hand, liver microsomal *N*-demethylation of amidopyrine, morphine and pethidine in female rats was found to increase 33, 100 and 164%, respectively, when nicotine was given orally to rats at a dose of 10 mg/kg twice a day for 28 days /89/. Furthermore, activities of phenacetin *O*-dealkylase and aniline hydroxylase were found to increase after oral nicotine administration at a dose of 10 mg/kg twice a day for 6 and 21 days to female rats /90/. Meprobamate metabolism was reported to be increased remarkably by nicotine administration /91/. In this case, when male mice were administered nicotine (average 2.28 mg/kg/day) in the drinking water for two days, meprobamate metabolism increased 4 fold and this elevation was prevented by ethionine, an inhibitor of protein systhesis. From these reports, it is possible that nicotine serves as an inducer of cytochrome P-450 and the route of nicotine administration plays an important role in studies on the effects of nicotine administration on drug metabolism. When administration of nicotine leads to a marked increase of drug metabolism, changes in P-450 isozymes are expected.

5.2 Effects of drug administration on nicotine metabolism

Yamamoto *et al.* first reported that nicotine oxidation in a 9000 x g supernatant of mouse livers increased after administration of PB, AAF and 3-MC /87/. Stalhandske showed an increase of nicotine metabolism *in vitro* and *in vivo* after administration of PB to mice /66/. They showed that nicotine concentrations were lower in livers of PB-pretreated mice than in livers of control mice, whereas cotinine concentrations in livers of PB-pretreated mice were higher

than in controls during the initial period after nicotine injection. In this case, administration of PB increased cotinine formation by the 10,000 x g liver supernatant. Furthermore, administration of PB resulted in an increase in the intraperitoneal LD_{50} value, but no change of the intravenous LD_{50} value. Recent reports also have shown the increase of liver microsomal nicotine oxidation in rat /67/, guinea pig /63,64/ and hamster /71/ following administration of PB. In hamster, administration of PB increased the formation of 5'-cyanonicotine but not nicotine-1'-oxide. We found that administration of PB did not increase the specific activity of FAD-containing monooxygenase. Antibodies prepared against PB-P-450 inhibited liver microsomal nicotine *C*-oxidation in PB-pretreated and untreated guinea pigs by about 30% and less than 5%, respectively /63/. These reports show that PB-cytochrome P-450 was partially involved in microsomal nicotine *C*-oxidation in PB-pretreated animals, but only minimally so in untreated animals.

β-NF and 3-MC are known to be inducers of cytochrome P-448, in contrast to the early report by Yamamoto et al. /87/, a recent report showed that after administration of 3-MC and β-NF to rats, the specific activity of liver nicotine oxidase remained unchanged, whereas the activity per unit of P-448 decreased /67/.

When rats received ethanol at doses of 4 g/kg/day for 7 days followed by 8 g/kg/day for 5.5 days, cotinine concentrations in plasma were found to be higher during its formation phase and lower during its elimination phase in ethanol-pretreated rats than in controls /92/. On the basis of a pharmacokinetic analysis of cotinine formation, the apparent volume of distribution of cotinine and its rate of formation were found to increase significantly. On the other hand, when hamsters were given free access to 18.5% ethanolic water for 28 days, it has been shown that the formation of nicotine-1'-*N*-oxide by liver microsomes increased but the formation of 5'-cyanonicotine remains unchanged /68/. To resolve this apparent discrepancy, further investigations involving the determination of the rate limiting step of the conversion of nicotine into cotinine are necessary. Although there have been several studies on the induction of nicotine metabolism by smoking /43,93,94/, the specific chemicals in tobacco which induce nicotine metabolism have not been determined.

5.3 Age and sex differences

Age and sex differences in cytochrome P-450 are well known. FAD-containing monooxygenase activity is also reported to change with age and is regulated by sex hormones /73/. There are several reports concerning age and sex differences in nicotine metabolism /43,95-97/. The foetal livers of mice on the day before delivery were shown to metabolize slightly the conversion of nicotine into cotinine /95/. In comparative studies on metabolism of nicotine in livers of foetal, newborn and young mice, only cotinine was found to be produced by liver slices from two days before birth and up to the third day of postnatal life and the formation of cotinine increased rapidly between the third and fourth weeks /96/. Furthermore, using thin-layer chromatography of chloroform-methanol extracts after nicotine oxidation by the liver slices, they found that the livers of the two week old mice produced all the metabolites observed in adult animals. Klein and Gorrod determined cotinine and nicotine-1'-N-oxide in the urine of 85 healthy cigarette smokers /97/. The amount of cotinine excreted was found to decrease in relation to nicotine-1'-N-oxide for older compared with younger smokers, but no significant difference in the ratio of cotinine to nicotine-1'-N-oxide was observed between males and females. Beckett et al. showed that male non-smokers excreted more cotinine than female non-smokers /43/. In the 1980s, further studies on age and sex differences in nicotine metabolism have not been reported to date.

VI. NEW ASPECTS OF STUDIES ON IN VIVO NICOTINE METABOLISM

Recently, HPLC has been used for the separation of nicotine and its metabolites /49,94,98-102,108/ and this has made it possible to carry out quantitative studies on many kinds of nicotine metabolites in urine and serum. By radiometric HPLC, Kyerematen et al. have investigated the kinetic properties of nicotine and 12 of its metabolites in rat plasma, showing that cotinine-1-*N*-oxide and allohydroxydemethylcotinine have half lives longer than those of cotinine /103/. In addition to the major nicotine metabolites, they have determined γ-(3-pyridyl)-γ-oxo-*N*-methylbutyramide, γ-(3-pyridyl)-

γ-oxobutyric acid and 3-pyridylacetic acid, indicating an appreciable conversion of nicotine to 3-pyridyl-acetic acid. After administration of [^{3}H]-S-(-)-cotinine to guinea pigs, Cundy and Crooks have quantitated cotinine and 7 of its potential urinary metabolites and showed that 3-hydroxycotinine accounted for 57% of the total radioactivity in the urine and no formation of S-*N*-methylcotinium ion was found /57/. Trans-3-hydroxycotinine has also been found as a main nicotine metabolite in the urine of smokers /104,105/, and in guinea pigs /108/. On the other hand, the amounts of nicotine-1'-*N*-oxide, cotinine, cotinine-1-*N*-oxide and nornicotine have been found to be higher than that of trans-3-hydroxynicotine in rat urine /102,103/. This different observation may reflect species difference of nicotine metabolism. HPLC methods allow the rapid simultaneous determination of nicotine and its metabolites in biological fluids and is, therefore, suitable for studies on complicated *in vivo* nicotine metabolism. Recently, Mattammal *et al.* have reported that peroxidation of nicotine by prostaglandin H synthetase leads to the formation of 3-(2,3-dihydro-1-methyl-2-pyrrolyl)pyridine /106/. This metabolite has been isolated in urine from rabbits administered nicotine and from a male cigarette smoker using HPLC.

VII. CONCLUSIONS AND FUTURE PROSPECTS

A number of recent studies show that cytochrome P-450 and FAD-containing monooxygenase participate in the conversion of nicotine into cotinine and nicotine-1'-*N*-oxide, respectively. Administration of nicotine and other drugs have been found to effect nicotine metabolism, probably via effects on cytochrome P-450- and/or FAD-containing monooxygenase-dependent reactions. Although new methods employed in these studies make it possible to analyze the enzymatic process, little is presently established about mechanisms of the regulation by which nicotine metabolism is enhanced or reduced. To understand the regulatory mechanisms of nicotine metabolism, two important problems must be resolved. First, it is not yet established which forms of cytochrome P-450 participate in nicotine metabolism with untreated and drug-pretreated animals. If the cytochrome P-450(s) participating in nicotine

metabolism is (are) elucidated in more detail, sex and age differences in microsomal nicotine oxidation and metabolic relations between nicotine and other drugs may be characterized more clearly. Secondly, there is no experimental evidence for hormonal regulation of nicotine metabolism. Since administration of nicotine causes changes in a number of hormones, it is necessary to investigate the hormonal control of nicotine metabolism. Additionally, the enzymes involved in nicotine metabolism other than cytochrome P-450 and FAD-containing monooxygenase need to be examined. Based on these physiological and biochemical researches on nicotine metabolism, interactions of nicotine and other constituents of tobacco smoke may be expected to be studied more precisely.

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