Enzymatic Oxidation of Nicotine to Nicotine Δ¹(5') Iminium Ion

A NEWLY DISCOVERED INTERMEDIATE IN THE METABOLISM OF NICOTINE

(Received for publication, October 30, 1972)

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SUMMARY

Oxidation of nicotine in the presence of rabbit liver 10,000 g supernatant, NADPH, and cyanide results in the production of 5'-cyanonicotine. NaBD₄, reduction of the reaction mixture from a similar incubation of nicotine, NADPH, and supernatant results in the production of deuteronicotine having a single deuterium in the pyrrolidine ring. Oxidation of nicotine to cotinine under ¹⁸O₂ did not lead to incorporation of ¹⁸O in the cotinine produced. These results are explained by the formation of nicotine Δ¹(5') iminium ion as an intermediate in the degradation of nicotine. The formation of the iminium ion is catalyzed by an enzyme system having the properties of a mixed function oxidase. The reaction requires NADPH and O₂ and is inhibited by carbon monoxide.

It is proposed the nicotine Δ¹(5') iminium ion is formed by loss of water from 5'-hydroxy nicotine. The iminium ion formed from nicotine represents a nicotine analogue with a markedly altered chemical reactivity and must therefore be considered in studies on the physiological action of nicotine.

In order to more clearly define the enzymatic steps involved in the production of cotinine from nicotine we have re-examined the oxidation of nicotine by rabbit liver preparations in vitro. When nicotine was incubated with reconstituted rabbit liver 10,000 g supernatant in the presence of an NADPH generating system and 0.01 M KCN, the major product isolated was identified as 5'-cyanonicotine. It is postulated that this compound is produced by the reaction of the nicotine Δ¹(5') iminium ion with cyanide.

MATERIALS

All cofactors were purchased from Boehringer Mannheim. Nicotine was obtained from Eastman Kodak Co., Rochester, New York. ¹⁸O₂ was purchased from Miles Laboratories, Inc., Elkhart, Indiana. NaBD₄ was a product of Alfa Inorganics, Beverly, Massachusetts.

METHODS

In Vitro Incubations—Livers were obtained from rabbits that were pretreated with phenobarbital for 1 week prior to sacrifice (0.2 mg per ml in drinking water). Rabbit liver microsomes were prepared by differential centrifugation of 20% liver homogenates prepared in 0.25 M sucrose (4). The 100,000 g pellet was resuspended in the original volume of 0.05 M phosphate buffer pH 7.4. Reconstituted 10,000 g supernatant was prepared by the addition of 1 ml of 100,000 g supernatant fraction to 2 ml of resuspended microsomes.

NADPH was prepared in situ by the addition of 0.5 mM NADP⁺, 5 mM α-ketoglutarate, 0.01 mM MnCl₂, and 0.1 mg of isocitrate dehydrogenase (pig heart, 2 units per mg, Boehringer Mannheim). Incubations were performed at 37°C for a maximum of 30 min using 1 mM nicotine as the substrate. The reaction rates were linear with respect to cotinine or nicotine Δ¹(5') iminium ion formation during this time period. The reaction rates were also linear with respect to enzyme concentration throughout the concentration range used in these studies.

Extraction Procedures—(a) In the initial experiments, extractions were performed essentially as described by Hucker et al. (3). Incubations were transferred to 30 ml of 1.5% isoamyl alcohol-heptane. The samples were mixed for 30 min on a rotary mixer, and then the heptane layer was drawn off.

A 15-ml portion of the heptane layer was then mixed with 10

1 N-Methyl-2-(3-pyridyl)-5-pyrrolidone.

2 A preliminary account of this work was presented in the Proceedings of the Second International Symposium on Microsomes and Drug Oxidation, July 28-30, Stanford, California, 1972.
mixture was extracted as described in Extraction Procedure.

The reaction was performed with an LKB-9000 GC-MS combination using a 3% UC-W98 column as described under "Methods." The mass ion was at 187 with prominent peaks at 160, corresponding to the loss of HCN, and at 109, corresponding to the N-methyl cyanopyrroli dine fragment. The 100 MHz nuclear magnetic resonance spectrum contained 4 multiplets in the region between 3.0 and 4.5 ppm. Since none of the multiplets appeared to be coupled to one another, the cyano group is assigned to position 5' rather than position 2'.

RESULTS AND DISCUSSION

Gas chromatographic analysis of the extracts obtained after incubation of nicotine with reconstituted rabbit liver 10,000 x g supernatant fraction in the presence of 0.01 M cyanide indicated

The presence of a product having a gas chromatographic retention time of 7.6 min. Mass spectrometric analysis of this product indicated that the molecular weight was 187 (Fig. 1). This corresponds to the displacement of a proton of nicotine by a cyano group. The fragmentation pattern indicated that the cyano group was present in the pyrrolidine ring.

Chemical oxidation of nicotine followed by reaction with cyanide yielded 5'-cyanonicotine. Comparison of the mass spectra of the chemical product and the incubation product indicated that the two compounds were identical.

There are two likely explanations for the production of cyanonicotine during the enzymatic oxidation of nicotine and in the presence of cyanide. These are shown in Equations 1 and 2.

1. $$\text{N}$$

2. $$\text{N}$$

WHERE Z = PYRIDYL

In order to distinguish between these two mechanisms, an experiment was performed wherein the nicotine-microsomal reaction mixture was treated with NaBD₄. If the reaction proceeds through a stable iminium ion it should be reduced by the NaBD₄ and yield deuteronicotine. The mass spectrum of nicotine isolated from NaBD₄ treated reaction mixture is shown in Fig. 2A. A comparison of this spectrum with the mass spec-

![Fig. 1 (left). Mass spectrum of 5'-cyanonicotine. The spectrum was obtained using the extract from an incubation of 1 mM nicotine with reconstituted rabbit liver 10,000 x g supernatant in the presence of 0.5 mM NADPH and 0.01 M KCN. Analysis was performed with an LKB-9000 GC-MS combination using a 3% UC-W98 column as described under "Methods."](http://www.jbc.org/)

![Fig. 2 (center and right). A, mass spectrum of nicotine obtained after treatment of a nicotine incubation mixture with NaBD₄. Nicotine (1 mM) was incubated with rabbit liver microsomes in the presence of 0.5 mM NADPH for 20 min at 37°C. The reaction mixture was extracted as described in Extraction Procedure b of the "Methods" section in order to remove unchanged nicotine. The aqueous layer was then treated with 1 mM NaBD₄ and incubated at 37°C for 30 min. The reaction mixture was re-extracted with CH₂Cl₂. The CH₂Cl₂ extract was concentrated and analyzed on an LKB-9000 GC-MS combination using a 3% UC-W98 column as described under "Methods." B, mass spectrum of nicotine obtained from a control incubation of nicotine, NADPH, and rabbit liver microsomes. The conditions used were identical with those described in the legend of A with the exception that NaBD₄ was not added.](http://www.jbc.org/)
trum of nicotine shown in Fig. 2B clearly shows that NaBD₄ reduction resulted in the production of deuteronicotine. The mass ion at 165 shows the presence of 1 deuterium in the nicotine molecule, while the fragment at 85 is attributed to the pyridoline ring containing a single deuterium atom.

This result indicates the presence of the nicotine iminium ion as a discrete entity in the enzymatic reaction medium. In light of the known chemical reactivity of iminium salts (6) to nucleophiles such as cyanide it seems likely that the cyanonicotine also was produced from the nicotine iminium intermediate. We have, therefore, used the production of cyanonicotine as a measure of the oxidation of nicotine to nicotine Δ¹(α') iminium ion.

The effects of various cofactors on the production of nicotine iminium ion are shown in Table I. NADPH is the preferred cofactor for the reaction. NADH is approximately one-third as effective, while NAD⁺ or NADP⁺ is completely devoid of activity.

The effects of changes in oxygen concentration and carbon monoxide on the oxidation of nicotine to either nicotine Δ¹(α') iminium ion or to cotinine are shown in Table II. There is an absolute requirement for O₂ for either cotinine or iminium ion production. Carbon monoxide inhibits both reactions but is a more effective inhibitor of the production of cotinine. This may be due to the fact that there are two oxidative steps involved in the production of cotinine and only one for the production of the nicotine iminium ion.

The sensitivity to CO and the requirements for O₂ and NADPH all indicate that the production of nicotine iminium ion is catalyzed by a mixed function oxidase. The most likely pathway for the production of this intermediate is via the formation of 5'-hydroxynicotine. 5'-Hydroxynicotine could participate in the formation of the nicotine iminium ion. A reaction sequence involving these intermediates is shown in Equation 3. Since none of the intermediates are available it is not possible, at this time, to determine the various equilibrium constants involved in these reactions. If the equilibrium rates are rapid relative to the irreversible steps designated K₁, K₂, and K₃ in Equation 3, then it should be possible to increase the rate of formation of one product at the expense of the other. This seems to be the case when cyanide is added to a microsomal incubation containing nicotine and NADPH. Fig. 3 shows the effect of cyanide concentration on cotinine and cyanonicotine formation. The decline in cotinine formation parallels the increase in cyanonicotine formation. This supports the hypothesis that they share a common intermediate.

Mixed function oxidases are characterized by the incorpora-

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**Table I**

| Pyridine nucleotide                  | Concentration | Cyanonicotine a | Cotinine |
|--------------------------------------|---------------|-----------------|----------|
| 1. NADPH                             | 0.5 mM        | 0               | 0        |
| 2. NAD                               | 0.5 mM        | 0               | 0        |
| 3. NADH                              | 0.5 mM        | 24 ± 1          | 0        |
| 4. NADPH generating system           | 0.5 mM        | 84 ± 10         | 0        |
| 5. NADPH generating system + NADH    | 1.0 mM        | 108 ± 7         | 0        |
| 6. NADPH generating system + NADH, - KCN | 0.5 mM  | 112 ± 5         | 0        |
| 7. No additions                      | 0.5 mM        | 0               | 0        |
| 8. NADPH generating system + NADH, - KCN | 0.5 mM  | 0               | 0        |

a The formation of nicotine iminium ion has been quantitated by gas chromatographic analysis of the cyanonicotine formed by performing the oxidation in the presence of KCN.

**Table II**

| Gas phase | [CN] | Cyanonicotine a | Cotinine |
|-----------|------|-----------------|----------|
| 1. O₂/N₂ 5/95 | 5 mM | 122 ± 4         | 0        |
| 2. O₂/N₂ 5/95 | 0    | 0               | 117 ± 8  |
| 3. O₂/CO/N₂ 5/10/85 | 5 mM | 99 ± 11         | 0        |
| 4. O₂/CO/N₂ 5/10/85 | 0    | 0               | 61 ± 7   |
| 5. CO 100% | 5 mM | 0               | 0        |
| 6. CO 100% | 0    | 0               | 0        |
| 7. N₂ 100% | 5 mM | 0               | 0        |
| 8. N₂ 100% | 0    | 0               | 0        |
| 9. Air     | 5 mM | 132 ± 12        | 0        |
| 10. Air    | 0    | 0               | 150 ± 5  |

a The formation of nicotine iminium ion has been quantitated by gas chromatographic analysis of the cyanonicotine formed by performing the oxidation in the presence of KCN.
The formation of a carbinolamine during the oxidation of substituted amines has been postulated for a wide variety of dealkylation reactions (9). Carbinolamines can break down to give an aldehyde or ketone plus an amine or they can lose water to form an imine or iminium ion. While there are many examples of the former pathway, there are only a limited number of examples of the latter due, presumably, to the instability of the intermediate. An imine has recently been proposed as an intermediate in the oxidation of amphetamine to amphetamine oxime (10, 11). The instability of this imine has prevented its direct isolation. Breck and Trager (12) have proposed the formation of an imine during the metabolism of lidocaine. The imine, which would normally break down to acetaldehyde and an amine, is thought to react intramolecularly to form a cyclic addition compound.

The stability of imines and iminium ions is increased in heterocyclic systems (13). Five and six member ring systems contain an imine or iminium moiety can be readily prepared chemically. A stable cyclic imine has been isolated as a metabolite of medazepam by Schwartz and Kolis (14). These authors were also able to isolate 2-hydroxy medazepam proving the existence of a carbinolamine intermediate in the metabolism of medazepam.

In summary, evidence has been obtained that nicotine A1'c5') iminium ion via direct dehydrogenation, the properties of the enzymatic reaction make this pathway less likely. The fact that the reaction is inhibited by carbon monoxide and relatively insensitive to cyanide indicates that the enzyme is not similar to the fatty acid desaturase found in rat liver microsomes (8).

Although it is not possible to rule out formation of nicotine Δ′(5) iminium ion via direct dehydrogenation, the properties of the enzymatic reaction make this pathway less likely. The fact that the reaction is inhibited by carbon monoxide and relatively insensitive to cyanide indicates that the enzyme is not similar to the fatty acid desaturase found in rat liver microsomes (8).

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The formation of an iminium ion during the oxidation of nicotine in vitro raises important questions concerning its role in vivo. The iminium ion has a chemical reactivity towards nucleophiles not present in the parent nicotine. At the same time it carries a positive charge and thereby could presumably bind at nicotine receptor sites.

It has been reported that a metabolite of nicotine can block the lethal effects of nicotine (15). These authors postulated that the aminocarbinol, 5’-hydroxynicotine, might perform this function. The iminium ion must also be considered as a possible blocker of nicotine receptor sites. Studies on the physiological properties of the nicotine iminium ion are currently in the progress.

Acknowledgments—I would like to thank T. L. Williams and J. R. Bernstein for assistance in these experiments. I would also like to express my appreciation to Dr. R. E. McMahon for assistance in the 1802 experiments and for helpful discussions of this work.

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*J. Biol. Chem.* 1973, 248:2796-2800.

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