Microbial Degradation of Nicotine-N'-oxide I Degradation Products[†]

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Bacteria degrading nicotine-N'-oxide were isolated from tobacco leaves and soil of tobacco field. The bacterial metabolites from nicotine-N'-oxide were identified to be N'-methylmyosmine and 4'-keto-4'-(3-pyridyl)butyric acid, and four unidentified compounds were present. Accumulation of N'-methylmyosmine in the cultured medium reached 43%, in quantity of the initial substrate on the third day of culture. On the other hand, nicotine was far more slowly degraded by the same bacteria, and the first metabolite was identified to be 6-hydroxynicotine and the second to be 6-hydroxy-N'-methylmyosmine, suggesting that nicotine and its oxide are metabolized in different pathways.

In connection with studies on tobacco alkaloids, numerous investigations have been made on the biochemical and chemical decomposition of nicotine. Frankenberg and co-workers¹⁻³⁾ showed that during fermentation of the leaves, considerable amounts of the nicotine contained in cigar tobacco leaves transformed into other 3-substituted pyridine compounds such as nicotine-N'-oxide, 3-pyridylmethylketone, cotinine *etc.* The greater part of those 3-substituted pyridine compounds was nicotine-N'-oxide. Nicotine-N'-oxide is easily produced from nicotine by air oxidation,⁴⁾ by hydrogen peroxide⁵⁾ and UV irradiation.⁶⁾ In mammals, nicotine is known to undergo quick oxidization into nicotine-N'-oxide besides cotinine formation.⁷⁻¹⁰⁾ Frankenberg had excluded nicotine-N'-oxide from the degradation pathway of nicotine in the fermentation of tobacco leaves.¹¹⁾ In the microbial oxidation the oxide was also proved not to be involved as an intermediate,^{12,13)} though it had been postulated as an intermediate.⁵⁾ However, considering the findings that the oxide is exothermally transformed by acylation reagents into pseudoxynicotine¹⁴⁾ which is

easy to degrade and that iron-tartrate complex catalytically splits tertiary amine-N'-oxide into secondary amine and aldehyde,¹⁵⁾ a part of 3-pyridyl compounds present in fermented tobacco leaves presumably is derived from nicotine *via* the oxide by some reaction. On the other hand, the oxide is very likely to be degraded in soil and on tobacco leaves by microorganisms, but no microorganism degrading the oxide has ever been reported. We searched the oxide-degrading microorganisms and found that such microorganisms were abundant in the sources mentioned above. Microbial pathways of nicotine degradation have been well established in a number of works. They are summarized into the following two pathways: 1) nicotine is first oxidized into 6-hydroxynicotine, and 2) it is first dehydrogenated at pyrrolidine moiety into N'-methylmyosmine, and successively degraded into 4'-keto-4'-(3-pyridyl)butyric acid. This report deals with a microbial degradation pathway of nicotine-N'-oxide in comparison with that of nicotine.

MATERIALS AND METHODS

Microorganisms and cultural conditions. The microorganisms degrading nicotine-N'-oxide were isolated by the enrichment culture technique from the fermented cigar tobacco leaves (var. Nambu) and from

[†] One of the series of studies on "Microbial Degradation of Tobacco Constituents."

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the soils of the tobacco field in Iwate prefecture of Northern Japan in the tobacco growing season. Eleven strains were isolated, and all of them were bacteria. The colonies of one strain were white and those of others were yellow on nutrient agar plates. All strains were identified as *Arthrobacter globiformis*. *Arthrobacter globiformis* JTS-0006 (Nox 2) which most actively degraded the oxide was employed in the experiment, since the metabolic products of all the strains isolated were similar on paperchromatogram. Culture medium reported by Wada *et al.*,¹⁷⁾ containing 2g of nicotine-N'-oxide or nicotine; 1 g, KH_2PO_4 ; 0.5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.3 ml of 1% FeSO_4 solution; 0.3 ml of 1% CaCl_2 solution; trace of MnSO_4 in 1 liter of distilled water adjusted to pH 6.4 was used and 1.8% agar was added when necessary. The cultures were carried out at 28°C aerobically by shaking or aeration.

Chemicals. Commercially purchased *l*-nicotine (I) was used after distillation. *l*-Nicotine-N'-oxide (mp 170~171°C, V) was prepared by the method of Pinner and Wolfenstein.¹⁷⁾ The oxide should be a diastereoisomeric mixture of R, *S*-*cis* and S, *S*-*trans*-nicotine-N'-oxide. Pseudooxynicotine-2HCl (decomp. 196~197°C, VII) was prepared by the method of Kisaki *et al.*¹⁴⁾ 4'-Keto-4'-(3-pyridyl)butyric acid (mp 161~162°C, VIII) was synthesized by the method of Castle and Burger.¹⁸⁾ *dl*-Hydroxynicotine (mp 121~122°C, II) was synthesized by the method of Tschitschibabin and Kirssanov.¹⁹⁾ 6-Hydroxypseudooxynicotine-2HCl (mp 133~135°C, IV) was synthesized with bacterial enzyme by the method of Hochstein and Rittenberg.²⁰⁾

Chromatography. Chromatography was carried out ascendingly by the cellulose (Avicel SF, Funakoshi) thin-layer method and descendingly with Whatman No. 1 paper impregnated with 0.2 N sodium acetate in the solvent system of the upper layer of *tert*-amyl alcohol and 0.2 N sodium acetate (pH 5.6) mixture (1/1, v/v). Degradation products were located with *p*-aminobenzoic acid cyanogenbromide (Koenig reagent) and Dragendorff reagent.

Isolation of metabolite G. Strain JTS-0006 grown on a nutrient agar (Difco) slant was inoculated into 5 liters of medium in a 10-liter glass jar fermenter and cultured under gentle aeration for three days. The cultured broth was filtered through celite filter aid (Johns Manvil 545) on a Buchner funnel, and the filtrate was adjusted to pH 3.5 with HCl and concentrated under vacuum at 45°C to 500 ml. The concentrate was passed through filter paper to remove mineral and purple substance. The filtrate was readjusted to pH 8.5 with NaOH and subjected to continuous diethyl-ether extraction with 1 N HCl added in advance to the ether reservoir flask. The extraction was continued for three days. Upon concentration of the content of the

FIG. 1. High-speed Liquid Chromatogram of Nicotine-N'-oxide Medium Cultured with *A. globiformis* JTS-0006.

The strain grown on a nutrient agar slant at 28°C for 24 hr was inoculated into the synthetic liquid medium containing 0.2% nicotine-N'-oxide at a concentration of 10^8 cells/ml. 100 ml of it in a 500 ml-flask was cultured by rotary shaking (200 rpm) at 28°C for 4 days.

The broth was acidified with HCl and mixed with methanol, and then the denaturated protein and deposits were removed by centrifugation. A Waters Associates Model ALC-202 analyzer with μ -bondapak C18 column and UV (254 nm) detector was used, and the solvent system used was 50% methanol/water (v/v). Peak C represents the substrate.

FIG. 2. High-speed Liquid Chromatogram of the Nicotine Medium Cultured with *A. globiformis* JTS-0006.

Chromatography was conducted in the same way as described in the legend of Fig. 1.

The nicotine medium was cultured for 21 days. Peak M represents the substrate.

reservoir flask to dryness under vacuum, yellow crystals remained. Upon recrystallization from methanol three times to a constant melting point (decomp. 196~197°C) 812 mg of white colorless crystal was obtained. This compound gave peak G in the chromatogram of liquid chromatography shown in Fig. 1.

Isolation of metabolite B. Twenty ml of four-day culture broth of nicotine-N'-oxide was diluted with an equal volume of methanol and centrifuged to remove precipitate. The supernatant was dividedly chromato-

graphed under the conditions described in Fig. 1, and the fractions corresponding to peak B on the chromatogram were collected. The combined eluate was concentrated and subjected to liquid chromatography twice to be further purified. After concentration of the collected fraction to dryness *in vacuo*, white solid powder remained. Upon recrystallization twice from methanol in a capillary tube, *ca.* 1 mg of colorless needles was obtained (mp 160~162°C).

Isolation of metabolite H. Strain JTS-0006 grown on a nutrient agar slant was inoculated to 100 ml of the medium containing 0.2% nicotine in a 500-ml flask and cultured for 15 days under shaking. An equal volume of methanol was added to the cultured medium, and the mixture was centrifuged to exclude bacteria and deposited salts. Further isolation of metabolite H was accomplished by liquid chromatography in a similar manner to metabolite B. The fraction corresponding to metabolite H gave a colorless jelly-like residue when dried under vacuum. The residue was dissolved in a small amount of benzene, *n*-hexane was added until the solution became slightly turbid and the mixture was kept in a refrigerator overnight. The crystallized precipitates were further recrystallized as described above to yield *ca.* 1 mg of white colorless powder that melted at 120~123°C.

Isolation of metabolite I. Metabolite I was collected at the same time when metabolite H was collected by liquid chromatography. After the fraction corresponding to metabolite I was acidified by HCl until the pH value became below 2, the solution gave dull white powder when dried under vacuum. The residue was dissolved in a small amount of hot absolute ethyl alcohol, and into the alcohol solution was added anhydrous ethylether until a faint turbidity appeared. The solution was cooled in the refrigerator overnight. The crystalline needles were further recrystallized as described above to yield *ca.* 2 mg of white crystalline needles that melted at 133~135°C.

RESULTS

Identification of pseudooxynicotine

Metabolite G was readily colored green to reddish in an alkaline solution. It reacted positive by the Koenig reagent and 2,4-dinitrophenylhydrazine in acidic solution, suggesting the presence of a pyridine ring and carbonyl. The spectra were: UV $\lambda_{\text{max}}^{0.3N\text{HCl}}$ nm (ϵ): 223 (5970), 263.5 (5298) and $\lambda_{\text{min}}^{0.3N\text{HCl}}$ nm: 242 (pyridine ring with conjugated C=O); $\lambda_{\text{max}}^{0.05N\text{NaOH}}$ nm (ϵ): 233.5 (6268), (pyridine ring with conjugated double bond); IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹:

1690 (C=O), 1990 and 1600 (pyridinium). *Anal.* Found: C, 47.76; H, 6.31; N, 11.03; O, 6.75; Cl, 28.54. *Calcd.* for C₁₀H₁₂N₂O-2HCl, C, 48.21; H, 5.66; N, 11.24; O, 6.24; Cl, 28.46%. No considerable change in elemental composition was observed as compared with the starting nicotine-N'-oxide. These findings suggested that the hydrochloride of compound G was pseudooxynicotine (1'-N-methyl-4'-keto-4'-(3-pyridyl)butylamine) dihydrochloride. The IR spectrum of synthesized pseudooxynicotine dihydrochloride was identical to that of the metabolite, and its melting point was not altered on admixing with a synthesized one.

Identification of 4'-keto-4'-(3-pyridyl)butylic acid

Metabolite B reacted positively to the Koenig reagent, suggesting the presence of pyridine ring, and showed acidic character with BTB indicator. Its spectra were: UV $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ nm (ϵ): 223 (9600), 263.5 (8350) and $\lambda_{\text{min}}^{\text{H}_2\text{O}}$ nm: 242, (pyridine ring with conjugated C=O); IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 1727 (COOH), 1685 (C=O), 810 and 720 (3-pyridyl). Final structural confirmation of metabolite B was accomplished by comparing it with synthetic 4'-keto-4'-(3-pyridyl)-butylic acid. Their IR spectra were completely identical, and their melting points did not change upon admixture.

Identification of 6-hydroxynicotine

The spectra of metabolite H were: UV $\lambda_{\text{max}}^{0.3N\text{HCl}}$ nm (ϵ): 297 (6400) (pyridone); IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 1660, 1610, 1550, 1230 and 1160 (α -pyridone). Final structural confirmation of metabolite H was accomplished by comparing it with synthesized 6-hydroxynicotine. Their UV and IR spectra were completely identical, and their melting points did not change upon admixture.

Identification of 6-hydroxypseudooxynicotine

The spectra of metabolite I were: UV $\lambda_{\text{max}}^{0.1N\text{HCl}}$ nm (ϵ): 289 (13,500) (pyridone with conjugated C=O), $\lambda_{\text{max}}^{0.1N\text{NaOH}}$ nm (ϵ): 310 (15,600), $\lambda_{\text{max}}^{\text{pH } 10.6, \text{ M/15phosphate buffer}}$ nm (ϵ): 328 (21,000) (pyridone with conjugated double

bond)²⁸⁾; IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 1640, 1605, 1545, 1250 and 1140 (α -pyridone), 1660 (C=O), 3030 (dimeric >NH and C=O). These findings suggested that metabolite I was 6-hydroxypseudooxynicotine. The UV and IR spectra of the metabolite and synthesized 6-hydroxypseudooxynicotine-dihydrochloride were completely identical (Fig. 3) and their melting points did not change upon admixture.

FIG. 3. Infrared Absorption Spectra of Metabolite I and 6-Hydroxypseudooxynicotine.

Both metabolite I and 6-hydroxypseudooxynicotine were measured as 2HCl salts in KBr pellets. —, metabolite I; ----, 6-hydroxypseudooxynicotine synthesized by enzyme.

Metabolism of nicotine-N'-oxide and nicotine

Strain JTS-0006 was able to grow well on nicotine-N'-oxide but poorly on nicotine as sole carbon and nitrogen source. No growth factors were required. Typical liquid chromatograms of cultured broths of nicotine-N'-oxide and nicotine are shown in Figs. 1 and 2, respectively. In a four-day culture of nicotine-N'-oxide, pseudooxynicotine (peak G), 4'-keto-4'-(3-pyridyl)butyric acid (peak B) and four unidentified compounds (peaks A, D, E, and F) were detected as UV absorptive metabolic products, but no 6-hydroxynicotine or 6-hydroxypseudooxynicotine was detected in the broth. Degradation of nicotine-N'-oxide reached its maximum on the second day, and 97.5% of the initial substrate was consumed (Fig. 4). Accumulation of the pseudooxynicotine reached its maximum (43% of the initial substrate) on the third day. During

FIG. 4. Degradation of Nicotine-N'-oxide by Strain JTS-0006.

The upper figures represent bacterial concentrations, and the lower ones, concentrations of the substrate (—) and metabolite G (-----) in the medium with the lapse of time. The culture conditions were the same as described in the legend of Fig. 1. The bacterial concentrations were measured by the plate dilution method. The substrate and product were quantitatively assayed from the peak area in liquid chromatogram. Calibration curves were made with authentic substrates.

the four-day culture the bacterial concentration increased from 1.0×10^8 cells/ml to 2.4×10^9 cells/ml. JTS-0006 produced a purple colored substance which gradually turned brown as the culture was continued. The colored substance collected by filtration on the filter paper could not be dissolved in methanol, ethanol, ether, chloroform or ethyl acetate, and irreversibly changed to a reddish color in a strong alkaline solution. The pigment is assumed to be a similar product to what has been reported in a number of studies on nicotine degradation by microorganism.^{5,12,22,23)}

Nicotine was slowly degraded, 17% by the fifth day and 58% the twelfth day, by strain JTS-0006 (Fig. 5). The culture broth of nicotine showed a new absorption around 290 nm, which is a partial proof of formation of pyridone wheareas no such noticeable absorption was observed in the culture of nicotine-N'-oxide. In a twenty-one-day culture of nicotine, 6-hydroxynicotine (peak H), 6-

FIG. 5. Degradation of Nicotine by Srtain JTS-0006. The upper figures represent bacterial concentrations and the lower ones, the substrate concentrations. The culture conditions were the same as described in the legend of Fig. 2. The assay methods were the same as described in the legend of Fig. 4.

hydroxypseudooxynicotine (peak I) and unidentified compounds (peak J, K and L) were detected as UV-absorptive metabolic products.

DISCUSSION

Those isolated metabolites from nicotine-N'-oxide are the same ones as those reported by Wada and Yamasaki¹⁶⁾ in microbial degradation of nicotine. It is probable that nicotine-N'-oxide is first reduced back to nicotine and then oxidized to pseudooxynicotine in pathway 2) reported by them. The reduction back to the amine from the amine oxide has been established as a common metabolic route in mammals,^{9,10)} and pyridine oxide and trimethylamine oxide were known to be reduced

to the corresponding bases by microorganisms.^{24,25)} Reduction of nicotine-N'-oxide to nicotine has been shown in the gastro-intestinal tract in man after over-administration.²⁶⁾ When nicotine was fed to the same organism, the cultured broth afforded 6-hydroxypseudooxynicotine but not pseudooxynicotine as seen in Fig. 2. These findings suggest that in this microorganism nicotine-N'-oxide is directly converted into pseudooxynicotine and nicotine and nicotine is metabolized in a different route, pathway 1), by first hydroxylation of pyridine moiety. Isolation of 4'-keto-4'-(3-pyridyl)butylic which is not hydroxylated in pyridine ring further suggests that metabolisms of nicotine-N'-oxide and nicotine are initiated in different pathways as summarized in Fig. 6. Concerning the mechanism of oxidative cleavage of the pyrrolidine ring of nicotine, it has been suggested by Fish *et al.*²⁷⁾ and the subsequent kinetic studies of Ziegler and Pettit²⁸⁾ that in the liver microsome the initial oxidative attack occurs on the nitrogen atom of the tertiary amine to yield an intermediate N-oxide and this oxygenated intermediate is rearranged into α -hydroxylated tertiary amine and dealkylated by a second enzyme system to give a secondary amine and an aldehyde. However, unlike those system, the microorganisms which give pseudooxynicotine as first metabolite of nicotine were shown not to involve nicotine-N'-oxide in the pathway of nicotine metabolism.¹³⁾ The cleavage of the C₂'-N bond of pyrrolidine of nicotine-N'-oxide, namely the formation of pseudooxynicotine, is supposed to occur *via* 2'-(3-pyridyl)-2'-hydroxy-1'-N-methylpyrrolidine and the following dehydrated form, N'-methylmyosmine which has been estab-

FIG. 6. Degradation Sequence of Nicotine and Nicotine-N'-oxide by *A. globiformis* JTS-0006.

lished to be a dehydrated species of pseudooxynicotine, in an analogous manner to the above. Pseudooxynicotine in the cultured broth in pH range 6.5~8.5 shows the same absorption spectrum as that in a 0.05 N NaOH solution. The true metabolic species seems to be N'-methylmyosmine.

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