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N-METHYLPUTRESCINE OXIDASE FROM TOBACCO ROOTS*

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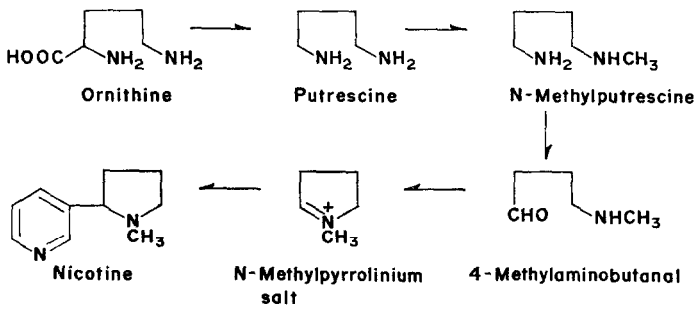
Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; nicotine biosynthesis; *N*-methylputrescine oxidase; enzyme.

Abstract—*N*-Methylputrescine oxidase, catalyzing the oxidative deamination of the primary amino group of *N*-methylputrescine, was demonstrated in roots of tobacco plants, and purified 150-fold. *N*-Methylpyrrolinium salt was identified as the reaction product by comparison with the authentic compound. The enzyme had a pH optimum at 8·0 and the K_m value for *N*-methylputrescine was $4{\cdot}5 \times 10^{-4}$ M. Putrescine and cadaverine were also oxidized. Activity of the enzyme was strongly inhibited by carbonyl reagents, thiol inhibitors and diethyldithiocarbamate, but hydrazine derivatives were without significant effect on the enzyme activity. The enzyme was localized exclusively in the roots and its activity markedly increased on decapitation of the shoots. The demonstration of *N*-methylputrescine oxidase, in addition to putrescine *N*-methyltransferase, provides evidence that the biosynthetic route of nicotine involves *N*-methylputrescine and 4-methylaminobutanal (*N*-methylpyrrolinium salt) as intermediates.

INTRODUCTION

IT HAS been shown by feeding experiments with isotopically labelled compounds that *N*-methylpyrrolidine ring of nicotine was synthesized from ornithine via *N*-methylpyrrolinium salt as illustrated in Scheme 1.[1,2] To elucidate the mechanism of the reaction it is necessary to show the presence of enzymes responsible for the biosynthetic steps.



SCHEME 1. BIOSYNTHETIC PATHWAY OF *N*-METHYLPYRROLIDINE RING OF NICOTINE.

We previously demonstrated the occurrence in tobacco roots of putrescine *N*-methyltransferase, an enzyme catalyzing the formation of *N*-methylputrescine from putrescine and *S*-adenosyl-L-methionine and some properties of the partially purified enzyme.[3]

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[2] S. MIZUSAKI, T. KISAKI and E. TAMAKI, *Plant Physiol.* **43,** 93 (1968).
[3] S. MIZUSAKI, Y. TANABE, M. NOGUCHI and E. TAMAKI, *Plant Cell Physiol.* **12,** 633 (1971).

Discovery of putrescine *N*-methyltransferase suggested that the biosynthetic route of nicotine involves *N*-methylputrescine as an intermediate and *N*-methylputrescine may serve as a direct precursor of 4-methylaminobutanal. However, the enzymic synthesis of 4-methylaminobutanal from *N*-methylputrescine by a cell free preparation of tobacco roots does not appear to have been demonstrated previously.

The present paper describes the partial purification of *N*-methylputrescine oxidase from extracts of tobacco roots and some of its properties. The enzyme catalyzes the oxidative deamination of the primary amino group of *N*-methylputrescine to give 4-methylaminobutanal.

RESULTS

Purification of N-*Methylputrescine Oxidase*

A summary of enzyme purification and the yield in each step is given in Table 1. The specific activity at the final stage was increased approximately 150-fold with a 38% enzyme recovery as compared with the initial extract. The increase in the total enzymic activity

TABLE 1. SUMMARY OF PURIFICATION OF *N*-METHYLPUTRESCINE OXIDASE FROM TOBACCO ROOTS

Purification step	Vol. (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (fold)	Yield (%)
Crude extract	1640	1281	23 944	18·7	1	100
Ammonium sulfate fraction	205	564	31 945	56·7	3	133·2
DEAE-cellulose chromatography	172	116	27 110	233·7	12·5	113·2
Sephadex G200 gel filtration	75	32	18 225	569·5	30·5	76·1
DEAE-cellulose chromatography	15	3·2	9099	2843·4	152	38

The activity was assayed under standard conditions. One unit equals 1 nmol of *N*-methylpyrrolinium salt formed in 30 min.

after dialysis of the 0–65% $(NH_4)_2SO_4$ precipitate might have been due to the removal of inhibitors. Using DEAE-cellulose chromatography in step 3 the bulk of the protein was removed by washing the column with the buffer used for equilibration of the column and the enzyme was then eluted with 0·2 M NaCl in the same buffer. In Sephadex G200 gel filtration the enzyme was eluted at approx. 1·6 times the void volume, whereas the bulk of the protein applied to the column was eluted much closer to the void volume. At the final step the enzyme fractions obtained from Sephadex G200 gel filtration was subjected to DEAE-cellulose chromatography. *N*-Methylputrescine oxidase was eluted by NaCl in concentrations about 0·2 to 0·25 M in the same buffer used for equilibration. The *N*-methylputrescine oxidase preparation in each step became inactivated in the absence of a sulfhydryl compound; 2-mercaptoethanol has been used routinely at 10 mM concentration.

pH Optimum

The effect of pH on the rate of oxidation of *N*-methylputrescine by the enzyme was examined using both Tris and phosphate buffers. It was found to have a maximal activity at pH 8·0. Activity was half proximal at pH 7·2 and 8·7.

Michaelis Constant

The effect of the concentration of *N*-methylputrescine on the reaction velocity was determined. Under the standard assay conditions, enzyme activity was linear with time and proportional to protein concentration. The K_m value for the substrate was calculated by plotting the Lineweaver–Burk plots and was found to be $4{\cdot}5 \times 10^{-4}$ M.

Substrate Specificity

The rate of oxidation of several amines by the enzyme was tested by measuring the oxygen uptake at pH 8·0. The enzyme oxidized putrescine and cadaverine at a rate which was 40% that found for *N*-methylputrescine. Histamine, spermine, tyramine, *n*-hexylamine and phenethylamine were not oxidized by the enzyme.

Effect of Inhibitors

Effects of inhibitors on the enzyme activity are shown in Table 2. The enzyme was inhibited strongly by carbonyl reagents and thiol inhibitors at 10^{-4} M concentration. Sodium diethyldithiocarbamate, which is usually considered to be a specific inhibitor of copper containing enzymes[4–6] produced complete inhibition of the enzyme at 10^{-3} M concentration, but EDTA did not affect enzyme activity. Aminoguanidine, which is one

TABLE 2. EFFECT OF INHIBITORS ON *N*-METHYLPUTRESCINE OXIDASE ACTIVITY

Inhibitor	Concn (mM)	Inhibition (%)
Hydroxylamine	0·1	92
Hydrazine	0·1	86
Potassium cyanide	1·0	92
Diethyldithiocarbamate	1·0	100
EDTA	1·0	0
p-Chloromercuribenzoate	1·0	77
Mercuric chloride	0·1	87
Aminoguanidine	1·0	16
Isoniazid	1·0	0
Iproniazid	1·0	8

The reaction conditions were as described in the Experimental except that the chemical indicated was added to the mixture.

of the most potent inhibitors of diamine oxidase,[7] caused only 16% inhibition and hydrazine derivatives had no effect on the enzyme activity at 10^{-3} M concentration. Nicotine and related compounds such as nornicotine, pseudo-oxynicotine and myosmine were without significant effect at 10^{-3} M concentration. To test the essential nature of copper in maintaining enzymic activity, the enzyme solution was dialyzed against Tris-HCl containing 10 mM sodium diethyldithiocarbamate for 5 hr, and then against Tris-HCl to remove the

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[5] J. M. HILL and P. J. G. MANN, *Biochem. J.* **91,** 171 (1964).
[6] B. MONDOVI, G. ROTILIO, M. T. COSTA, A. FINAZZI-AGRO, E. CHIANCONE, R. E. HANSEN and H. BEINERT, *J. Biol. Chem.* **242,** 1160 (1967).
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excess of diethyldithiocarbamate overnight. The enzyme activity of the dialyzed preparation was depressed, but the addition of $CuSO_4$ at 10^{-6} M concentration restored 90% of the original activity (Table 3). Addition of Zn was without effect.

TABLE 3. EFFECT OF CUPRIC ION ON DIETHYLDITHIOCARBAMATE-TREATED ENZYME

Treatment	Enzyme activity (units) Concn of $CuSO_4$ (M) 0	10^{-7}	10^{-6}	10^{-5}
Control*	43·2			
Diethyldithiocarbamate†	5·2	23·5	38·7	27·0

The reaction conditions were as described in the Experimental except that $CuSO_4$ was added at various concentrations. One unit equals 1 nmol of *N*-methylpyrrolinium salt formed in 30 min.

* The enzyme solution was dialysed for 20 hr against 0·025 M Tris-HCl, pH 7·4.

† The enzyme solution was dialysed for 5 hr against 0·025 M Tris-HCl, pH 7·4 containing 10 mM of diethyldithiocarbamate and then 0·025 M Tris-HCl for 15 hr.

Formation of N-*Methylpyrrolinium Salt*

Incubation of the partially purified enzyme with *N*-methylputrescine-$^{14}CH_3$ resulted in the formation of a radioactive compound, which was chromatographically indistinguishable from authentic *N*-methylpyrrolinium chloride in 4 different solvent systems.[2] The elution positions of the reaction product and the authentic *N*-methylpyrrolinium salt on an amino acid analyzer and a flow monitor system were identical.

Distribution of Enzyme Activity in Tobacco Plants

The tissue extract was applied to a Sephadex G25 column and the eluted protein fraction was used as enzyme solution. The reaction mixture consisted of 50 μmol Tris-HCl, (pH 8·0), 0·5 μmol *N*-methylputrescine-$^{14}CH_3$ ($9{\cdot}4 \times 10^4$ dpm) and 0·5 ml of the enzyme solution in a final volume of 1·05 ml. One unit then equalled 1 nmol of *N*-methylpyrrolinium salt formed in 30 min. In roots of intact plants 8·2 units of activity were found per mg protein while in roots of decapitated plants 21·6 units per mg protein were found. On the other hand, no enzyme activity was detected in extracts from leaves and from callus tissues which were cultured in the liquid medium of Linsmaier and Skoog[8] supplemented with 2,4-dichlorophenoxyacetic acid (0·2 mg/l.).

DISCUSSION

The experiments described in this paper have shown that tobacco roots contain an amine oxidase having a high substrate specificity for *N*-methylputrescine. We propose the term of *N*-methylputrescine oxidase for this enzyme. According to the classification of amine oxidases proposed by Blaschko *et al.*,[9] the enzyme isolated from tobacco roots belongs to the group of diamine oxidases with regard to its substrate specificity and its

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behavior toward carbonyl reagents. The enzyme activity is completely inhibited by dialysis of the preparation against the buffer containing diethyldithiocarbamate, but the activity is restored by the addition of $CuSO_4$ at 10^{-6} M concentration. These results suggest that the presence of copper is essential for enzymic activity. Mann,[4] and Hill and Mann[5] reported that the content of copper in a diamine oxidase preparation from pea seedlings increased during purification and $CuSO_4$ restored the activity of the enzyme after dialysis against diethyldithiocarbamate. Mondovi *et al.*[6] obtained similar results with a highly purified preparation of pig-kidney diamine oxidase.

The oxidation product of *N*-methylputrescine by the enzyme was proved to be *N*-methylpyrrolinium salt and formation of the other radioactive compounds was not observed. Oxidative deamination of the primary amino group of *N*-methylputrescine formed 4-methylaminobutanal which undergoes spontaneous ring closure to give the *N*-methylpyrrolinium salt. Experiments reported in our previous paper[2,10] showed that *N*-methylpyrrolinium salt, which is produced from ornithine or putrescine when these compounds are fed to tobacco roots, serves as an efficient precursor of nicotine. Leete[1] also reported that the administration of *N*-methylpyrrolinium chloride-^{14}C to tobacco plants yields radioactive nicotine. The demonstration of *N*-methylputrescine oxidase in tobacco roots provides definitive evidence that the biosynthetic route of nicotine involves *N*-methylputrescine as an intermediate. In support of this, recently we have demonstrated the occurrence in tobacco roots of putrescine *N*-methyltransferase, which catalyses the formation of *N*-methylputrescine from putrescine and adenosylmethionine.[3] Schütte, Maier and Mothes[11] reported that *N*-methylputrescine is incorporated into *N*-methylpyrrolidine ring of nicotine when fed to tobacco roots. Thus, the biosynthetic pathway of *N*-methylpyrrolinium salt from ornithine involves at least three enzymes, namely: ornithine decarboxylase; putrescine *N*-methyltransferase: and *N*-methylputrescine oxidase. The last step seems to be a non-enzymic reaction. *N*-methylputrescine oxidase is localized exclusively in the roots, where active nicotine biosynthesis occurs, and the level of the enzyme activity markedly increases by topping the shoots of the plants. Similar results have been obtained with putrescine *N*-methyltransferase.[3] These results indicate that both the enzymes are specifically concerned with the biosynthesis of nicotine. The demonstration of putrescine *N*-methyltransferase and *N*-methylputrescine oxidase in tobacco roots provides the foundation for enzymic studies on the control of nicotine biosynthesis.

EXPERIMENTAL

Plant material. Tobacco plants (*Nicotiana tabacum* L. 'Bright Yellow') were grown hydroponically in a green-house for 4 weeks in summer and then decapitated leaving 16–15 leaves as described previously.[3] One day after decapitation, roots were collected from the plants.

Purification of enzyme. All operations were performed in a cold room at 4°. The roots (600 g) were macerated in a cooled blender with 1·2 l. of extracting solution containing 0·05 M Tris-HCl, pH 7·4, 0·01 M 2-mercaptoethanol, 0·005 M EDTA, 0·5% sodium ascorbate and 1% polyethyleneglycol 400. The macerate was filtered through nylon cloth and the filtrate was clarified by centrifugation at 10 000 *g* for 15 min. Solid ammonium sulfate was added to the supernatant to give 65% saturation. After standing for 1 hr, the precipitate was resuspended in 200 ml of 0·01 M Tris-HCl, pH 7·4 containing 0·01 M 2-mercaptoethanol and 0·005 M EDTA and 0·5% sodium ascorbate and dialysed overnight against 0·01 M Tris-HCl, pH 7·4 containing 0·01 M mercaptoethanol and 0·001 M EDTA. The dialyzed solution was applied to a DEAE-cellulose column (2·5 × 15 cm) equilibrated with the buffer used for dialysis and the column was washed with 300 ml of the same buffer, which was sufficient to remove all unadsorbed proteins. The enzyme was then eluted by 0·2 M NaCl in the same buffer. Fractions exhibiting enzyme activity were pooled and the protein

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[11] H. E. Schütte, M. Maier and K. Mothes, *Acta Biochem. Polon.* **13**, 401 (1966).

was concentrated by adding solid ammonium sulfate to 60% saturation. The precipitate obtained was dissolved in 3 ml of 0·025 M Tris-HCl, pH 7·4 containing 0·01 M mercaptoethanol, 0·001 M EDTA and 0·2 M NaCl, which was used to equilibrate a Sephadex G200 column (2·8 × 90 cm). The protein was allowed to flow into the bottom of a Sephadex G200 column and elution achieved by allowing the same buffer to percolate up the column at a rate of 15 ml/hr and fractions of 10 ml were collected. Fractions from tube 18 to 24 were pooled and solid ammonium sulfate was added to the combined eluate to give 60% saturation. The precipitate was dissolved in 10 ml of 0·01 M Tris-HCl, pH 7·4 containing 0·01 M mercaptoethanol and 0·001 M EDTA and dialyzed overnight against the same buffer. The dialyzed solution was applied to a DEAE-cellulose column (1 × 12 cm) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of NaCl (0·0–0·4 M in the above buffer) in a total volume of 200 ml and fractions of 2 ml were collected. Fractions (tube 36–42) exhibiting high enzymic activity were combined and solid ammonium sulfate was added to the solution to 60% saturation. The suspension was stored at 0° until required. Under these conditions activity of *N*-methylputrescine oxidase showed no loss for 1 week. An aliquot of the suspension was dialysed against 0·01 M Tris-HCl, pH 7·4 containing 0·01 M mercaptoethanol or treated with a Sephadex G 25 column prior to use for all further experiments involving the properties of the enzyme.

Enzyme assay. The activity of *N*-methylputrescine oxidase was determined by measuring the radioactivity of 1-methyl-2-cyanopyrrolidine-$^{14}CH_3$ converted from *N*-methylpyrrolinium-$^{14}CH_3$ salt, a reaction product of the incubation, in the presence of excess KCN. Aqueous KCN reacts quickly and completely with *N*-methylpyrrolinium salt to give 1-methyl-2-cyanopyrrolidine.[12] The reaction mixture consisted of 20 μmol of Tris-HCl, pH 8·0, 0·4 μmol of *N*-methylputrescine-$^{14}CH_3$ (2·35 × 10^5 dpm/μM), 50 μg of catalase and enzyme solution in a final vol. of 0·25 ml. After 30 min of incubation at 30°, the reaction was stopped by the addition of 0·2 ml of 1% KCN solution and 5 ml of toluene. The tube was stoppered and shaken vigorously. One ml of the toluene phase was transferred to a counting vial and radioactivity was measured after the addition of 10 ml of a toluene scintillator (0·01% 1,4-bis[2(4-methyl-5-phenyloxazolyl)]-benzene and 0·4% 2,5-diphenyloxazole) in a liquid scintillation spectrometer. More than 95% of 1-methyl-2-cyanopyrrolidine passed into the toluene phase in one extraction; N-methylputrescine was not extracted by toluene. In some cases the enzymic activity was assayed by measuring the radioactivity of *N*-methylpyrrolinium salt formed from *N*-methylputrescine using an amino acid analyser and a flow monitor system as described previously.[2] One unit of enzyme activity is defined as the amount of enzyme which catalyzes the formation of 1 nmol of *N*-methyl pyrrolinium salt in 30 min under standard experimental conditions.

Reagents and analytical procedures. Methyl iodide-^{14}C was purchased from Daiichi Pure Chemical Co. Japan. *N*-Methylputrescine was prepared using the method of Maier *et al.*[13] All other chemicals were commercially available. Protein was estimated according to the method of Lowry *et al.*[14] Oxygen uptake was measured at 30° by a Beckman oxygen analyser in a reaction vessel containing 3 ml of reaction mixture; 200 μmol of Tris-HCl (pH 8), 1 μmol of substrate, 100 μg of catalase and enzyme solution. PC was carried out with the following solvents:[2] *n*-BuOH–HOAc–H_2O (4:1:5); *n*-BuOH–*n*-PrOH–0·1N HCl (1:1:1); *ter*-BuOH–HCOOH–H_2O (5:1:1) and *n*-BuOH saturated with H_2O.

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