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DETERMINATION OF PUTRESCINE *N*-METHYLTRANSFERASE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Key Word Index—*Nicotiana tabacum*, *Datura stramonium*, Solanaceae, putrescine *N*-methyltransferase, HPLC

Abstract—A novel procedure is described for the chemical synthesis of *N*-methylputrescine, the product of the title enzyme This is obtained from putrescine by formylation followed by the reduction of the monoformylputrescine intermediate with $LiAlH_4$ An assay method for putrescine *N*-methyltransferase was developed which depends on the determination of *N*-methylputrescine in the presence of an excess of putrescine This method, which makes use of a radiolabeled substrate unnecessary, is based on dansylation of the product followed by HPLC separation on a reversed-phase column The enzyme activity of the protein peak extracted from plant material was measured after treatment by gel filtration on prepacked disposable PD 10 columns The specific enzyme activities determined in the extract from the roots of *Nicotiana tabacum* and *Datura stramonium* plants, and from a root culture of *D stramonium*, are reported With an enzyme preparation from the last root culture, K_m values for putrescine and *S*-adenosylmethionine (SAM) were determined as 0 88 mM and 0 15 mM, respectively

INTRODUCTION

The biosynthetic pathway to the tropane alkaloids and nicotine contains the route for the transformation of ornithine into *N*-methylpyrroline [1–3] The first enzyme, in the secondary metabolism domain, is putrescine *N*-methyltransferase (PMT), which has been partially purified and characterized from the roots of tobacco [4] Its activity was enhanced after decapitation of tobacco plants [5] We have initiated an investigation on the regulation of this enzyme in nicotine-producing tissue culture and on a comparison of enzyme activities in *Nicotiana* and *Datura* plants and organ cultures In order to avoid the necessity of synthesizing and applying radioactive precursors we developed an enzyme assay based on the dansylation of the enzymatic product and its separation by reversed-phase HPLC Furthermore, a novel chemical procedure was used to prepare *N*-methylputrescine Data are presented on the kinetic parameters of PMT from *Nicotiana tabacum* and *Datura stramonium* tissue

RESULTS AND DISCUSSION

Chemical synthesis of N-*methylputrescine*

The synthesis of *N*-methylputrescine is difficult, as putrescine has two equivalent amino functions and alkylation of each normally leads to di-substituted amino groups since secondary amines are more reactive than primary ones Maier *et al* [6] described the synthesis of this compound by methylation of tosylacetyl putrescine Following this procedure, we obtained an impure product in low yield The alternative synthesis described here, however, allowed large quantities of the desired compound to be synthesized from commercially available putrescine The identity and quality of the intermediates and the product were checked by ^{13}C NMR spectroscopy Assignments follow directly from the SFORD ^{13}C spectra and literature data for putrescine (Table 1)

After completion of the present work a further synthetic method was described [7] which uses the condensation of methylamine with 4-bromobutyronitrile followed by the reduction of the cyano group (For review see ref [8])

HPLC enzyme activity assay

For protein extraction from the plant material a procedure developed in our laboratory [9] was applied, which includes a purification step on prepacked disposable PD 10 gel filtration columns However, as the next step in the method requires dansylation of the product, only phosphate buffer was used For dansylation and determination of dansylmethylputrescine by reversed-phase HPLC, procedures from the literature [10] were modified with the aim of quantifying the enzymatic product in the presence of a large excess of putrescine which behaves very similarly Whereas the product of the

Table 1 ^{13}C chemical shifts (δ) of *N*-formylputrescine*, *N*-methylputrescine* and putrescine†

	RNH 5	CH_2 1	CH_2 2	CH_2 3	CH_2 4	NH_2	X
R	X	Solvent	C-1	C-2	C-3	C-4	C-5
CHO	—	CD_3OD	41 9	27 7	30 5	38 7	163 8
H	—	$CDCl_3$	42 1	31 3	31 3	42 1	—
CH_3	HCl	D_2O	48 1	22 8	23 7	38 7	32 6
H	HCl	D_2O	39 8	24 7	24 7	39 8	—

*The spectra of formylputrescine and methylputrescine were measured at ambient temperature on a Varian XL 100

†Data for putrescine were taken from Bremser *et al* [13]

enzymatic reaction is stable and can be stored frozen for a long time, the dansylated compound is stable only for about 45 min and then gradually declines in yield, hence HPLC determination was performed immediately after the derivatization

Figure 1 shows a typical HPLC separation of the dansylated methylputrescine and putrescine Detection was performed at 217 nm as the UV spectrum of dansylputrescine indicates that the molar extinction coefficient at this wavelength is about 2 5 times larger than at 250 nm

Properties of the enzyme

The enzyme is not very stable in the treated extract, at 4° the activity is reduced to 25% after 3 hr The addition of 1 mg bovine serum albumin/ml has no effect, although the addition of glucose (12 5% w/v) improves the stability In its presence the activity is reduced to only 65% after 3 hr at 4°, furthermore, the enzyme is stable for longer periods when it is quickly frozen with liquid nitrogen and kept frozen in the presence of glucose The kinetics of the enzymatic action indicates that the reaction is linear for about 1 hr The enzyme extracted from the root organ culture of *D stramonium* was used to determine the K_m values of the substrates Values of 880 μM and 150 μM were obtained for putrescine and SAM, respectively These values are only slightly higher than those reported for the enzyme from tobacco roots [4] which revealed K_m values of 400 μM (putrescine) and 110 μM (SAM) With the enzyme from the roots of *N tabacum* var Samsun we determined a K_m value of 114 μM for SAM which is nearly the same as that mentioned above for the variety Bright Yellow [4]

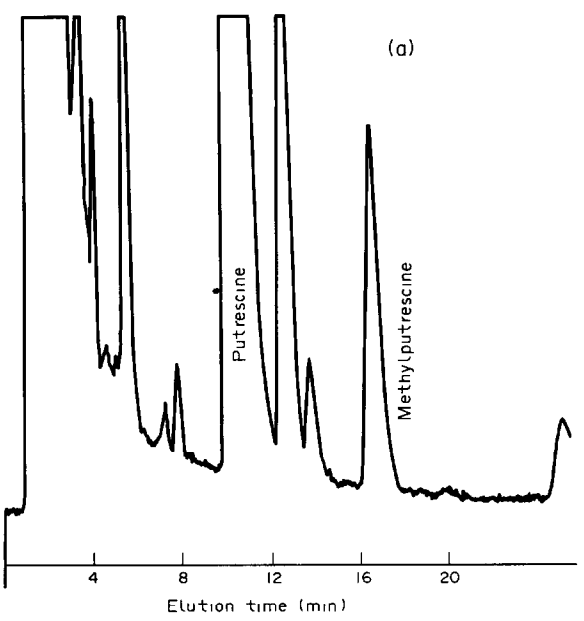

Fig 1 Elution profile on an RP 18 column for the determination of the product of the enzymatic reaction 20–100 μl sample injection, flow rate 1 5 ml/min, scale 8 m *A* at 217 nm, eluant (isocratic) 2% phosphoric acid in water adjusted to pH 5 2 with triethylamine–acetonitrile, 40 60

Specific activities in roots of different origin

The activity of PMT was determined in the extract of the roots of single plants and of root material from the organ culture Roots (1 5 g fr wt) usually from one single plant were extracted, for comparison specific activities were calculated after protein determination The specific activities determined with the roots of *N tabacum* var Samsun varied considerably from plant to plant (from 3 5 to 35 pkat/mg), yielding a mean value of 14 pkat/mg for 8 plants The deviation was much smaller and in the expected range with two parallel extractions from the root of the same plant, indicating that the variance originates in the physiology of the individual plants With the roots of *D stramonium* plants a mean value of 2 7 pkat/mg was found, whereas the root organ culture of the same species revealed an average value of 9 pkat/mg (seven determinations) with a much smaller variance than that of the plant roots

Mizusaki *et al* [5] reported specific activities of 2 5 pkat/mg for the roots of *N tabacum* var Bright Yellow which increased to 37 pkat/mg after decapitation of the plants With our variety Samsun, we did not observe an increase due to decapitation, however, the range of the specific activities determined for the eight individual plants (cf the values indicated above) is about the same as these authors determined for decapitated and non-decapitated plants One should emphasize that in the present work the specific activity of individual plants were determined, whereas Mizusaki *et al* [5] mixed the roots of several plants before extraction For the roots of *D stramonium* Mizusaki *et al* [5] reported a specific activity of 7 8 pkat/mg, while we determined a slightly higher value for the *Datura* root organ culture and a lower value for the roots of *Datura* plants

EXPERIMENTAL

Materials The chemicals used for the synthesis of *N*-methylputrescine and the silica gel TLC plates F254 were purchased from Merck, dansylchloride was obtained from Serva and SAM from Sigma *Nicotiana tabacum* var Samsun and *Datura stramonium* plants were grown in a greenhouse with natural lighting conditions Most of the young tobacco plants were kindly provided by Dr W Huth from the Institut fur Viruskrankheiten, Biologische Bundesanstalt Braunschweig The root organ culture was established according to standard procedures [11] from the roots of young seedlings and cultivated in LS-Medium with 5% sucrose at pH 5 8 They were subcultured every 5 weeks taking half of the amount of old roots

Chemical synthesis of N-*methylputrescine* Putrescine (10 ml, 110 mmol) and ethylformate (20 ml, 270 mmol) were mixed and stirred at room temp for about 2 hr During this time H_2O was added to dissolve the white ppt Evaporation under red pres resulted in 5 g of a mixture of formylputrescine, diformylputrescine and unreacted putrescine The products were analysed on silica gel TLC plates (25% aq NH_3–CH_3OH, 1 9) developed either with I_2 or a ninhydrin spray Monoformyl putrescine was assigned by its pink colour after ninhydrin staining, whereas diformylputrescine gave white spots by fluorescence quenching and revealed a brown colour with I_2 As expected, diformylputrescine ran almost with the front (R_f 0 68), unmodified putrescine was left at the starting position and monoformylputrescine ran in between these (R_f 0 23) The reaction mixture was separated on a silica gel column (500 ml) eluted with 25% aq NH_3–MeOH (1 9) The fractions containing monoformylputrescine

(1 3–2 15 l) were collected and evapd to dryness (yield 2 g, 17 mmol) A suspension of $LiAlH_4$ (1 3 g, 34 mmol) in dry THF (100 ml) was added slowly at 0° to monoformylputrescine (2 g, 17 mmol) in dry THF (100 ml) The mixture was carefully heated on a backflow cooler for 1 hr at 80° The excess $LiAlH_4$ was degraded with H_2O, the soln was filtered and the solid was washed with THF The combined filtrate was evapd to dryness *in vacuo* and the residue (1 8 g) was chromatographed on a silica gel column (200 ml) eluted with 25% aq NH_3–MeOH (1 1) The fractions were analysed on silica gel TLC plates with the same solvent Unreacted formylputrescine could be assigned by comparison with authentic material, whereas *N*-methylputrescine showed a low R_f value (0 1) as expected The respective fractions (0 38–1 1 1) were evapd, and the residue was treated with H_2O–$CHCl_3$ The product was obtained from the $CHCl_3$ phase by evaporation, dissolved in aq 1 M HCl and again evapd and dried over KOH *N*-Methylputrescine hydrochloride (12 mmol) was obtained in 70% yield relative to formylputrescine

Enzyme extraction This was performed at 0–4° in an analogous manner to that described previously [9] About 1 5 g roots, carefully washed in tap water, were suspended in 3 ml buffer A (100 mM K-Pi of pH 7 5, 5 mM EDTA, 10 mM mercaptoethanol, 0 5% Na ascorbate, 2% polyethyleneglycol 400), homogenized in a Potter–Elvehjem homogenizer and after treatment with 1 5 g XAD-4 centrifuged at 27 000 g The supernatant (2 5 ml) was applied on to a Sephadex G 25 prepacked PD 10 column equilibrated and eluted by 3 5 ml buffer B (pH 8, 50 mM K-Pi, 1 mM EDTA and 5 mM mercaptoethanol) Protein concn was determined according to Bradford [12]

Enzyme assay The reaction was performed in buffer B at 30° and started by the addition of the enzyme soln (100 μl in a total of 140 μl) The putrescine and SAM concns were 3 6 mM and 0 6 mM, respectively For the determination of the K_m values the incubation time was 15 min, for other determinations 30 min at 30° was used The reaction was stopped by heating at 100° for 1 min and the samples were frozen for the determination of the product

Reaction with dansylchloride To the enzyme incubate (140 μl), 65 mM borate–KOH buffer of pH 10 5 (460 μl) was added, and thereafter a soln of 5 4 mg dansylchloride in 1 ml of acetonitrile (300 μl) The mixture was heated for 15 min at 56° and applied directly to the HPLC column (20–100 μl)

HPLC chromatography Dansylated *N*-methylputrescine was separated on a 5-μm Merck Lichrosorb RP-18 column (25 cm × 0 4 mm), the HPLC system consisted of a LDC Constametric II pump, Rheodyne probe injector and Kontron Uvikon 730 LC The isocratic mobile phase was composed of 40 parts of an aq soln of 2% H_3PO_4 adjusted to pH 5 2 by triethylamine and 60 parts of acetonitile The column was run at 1 5 ml/min and the detection was at 217 nm

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