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NICOTINIC ACID-*N*-GLUCOSIDE IN HETEROTROPHIC PARSLEY CELL SUSPENSION CULTURES

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Abstract—Heterotrophic cell suspension cultures of *Petroselinum hortense* rapidly convert exogenously applied nicotinic acid to nicotinic acid-*N*-β-D-glucopyranoside and not to the previously reported nicotinic acid-*N*-arabinoside. The structure of the *N*-glucoside was determined by spectroscopic techniques and comparison with synthetic material. The nicotinic acid-*N*-glucoside was also found to be an endogenous constituent of parsley cells at a level of *ca* 35–40 nmol/g fr. wt. Application of [U-^{14}C]-aspartate to parsley cell cultures led to the accumulation of labelled nicotinic acid-*N*-glucoside, which was subsequently turned over with a half life of 24 hr indicating rapid metabolism of the *N*-glucoside

INTRODUCTION

Nicotinic acid can be regarded as an important connecting link between primary and secondary metabolism in higher plants. Being a constituent of the pyridine nucleotide cycle, nicotinate is a precursor and a degradation product of the pyridine nucleotide coenzymes NAD and NADP which play a decisive role in oxidation–reduction reactions [1, 2]. On the other hand nicotinic acid is the basic unit of the pyridine alkaloids [3, 4]. Furthermore, plant cells are able to completely degrade nicotinic acid [5] or to form conjugates [6–9]. In the case of suspension cultured plant cells the rapid alternative conversion of exogenously applied nicotinic acid to either the *N*-methyl or a *N*-glycosyl conjugate has been observed [9, 10]. From heterotrophic parsley (*Petroselinum hortense*) cell suspension cultures, a glycosyl conjugate had been isolated and shown to be nicotinic acid-*N*-arabinoside [8, 11]. In this study we have now revised the structure of this nicotinate conjugate as nicotinic acid-*N*-glucoside, which had already been isolated from roots of *Nicotiana tabacum* [6]. We also report on tracer experiments in parsley cell cultures and demonstrate rapid turnover of the nicotinic acid conjugate, which is thought to be a storage form for nicotinic acid [9].

RESULTS AND DISCUSSION

*Structural elucidation of nicotinic acid-*N*-glucoside*

When the putative nicotinic acid-*N*-arabinoside formed from exogenous nicotinate by suspension cultured parsley cells [11] was used as reference material for studies on nicotinic acid metabolism in tobacco (*Nicotiana tabacum*) plants, this *N*-glycoside revealed the chromatographic properties of nicotinic acid-*N*-glucoside [12]. The latter compound has already been purified and thoroughly characterized from tobacco roots [6]. These observations caused us to reinvestigate the structure of the nicotinic acid-*N*-glycoside formed by parsley cell cultures from exogenous nicotinic acid.

Reference material was synthetic nicotinic acid-*N*-β-D-glucopyranoside, which had been prepared following published methods [13, 14] and characterized by spectroscopic techniques (see Experimental).

The relevant nicotinic acid-*N*-glycoside was isolated from batch-cultured parsley cells (728 g fr. wt) incubated with 10^{-4} M nicotinic acid for 12 hr. After ethanol extraction of the cells the glycoside was purified by ion exchange chromatography on DOWEX 1 × 8 followed by TLC in the solvents S1 (R_f = 0.14), S3 (R_f = 0.39) and S4 (R_f = 0.21). During the chromatographic separations a sample of [7-^{14}C]-nicotinic acid-*N*-glycoside from the parsley cells (see Experimental) served as reference. Crystallization of the isolated product from aqueous ethanol yielded 27 mg nicotinic acid-*N*-glycoside (mp 234–236°).

The UV and IR spectra of the isolated product and the chemically synthesized nicotinic acid-*N*-glucoside were exactly superimposable. Furthermore, the ^{1}H NMR spectra of the isolated nicotinic acid-*N*-glycoside, the synthetic nicotinic acid-*N*-glucoside and the putative nicotinic acid-*N*-arabinoside from previous studies [11] were identical. An additional signal of aliphatic protons (δ = 1.3 ppm, *d*) previously observed in the spectrum of the so called arabinoside might have led to the false conclusion that a pentose was linked to the nitrogen atom of nicotinic acid. The nicotinic acid-*N*-glycoside was also subjected to acidic and enzymatic hydrolysis followed by GC analysis for identification of the sugar moiety [15]. The gas chromatogram revealed signals for the α and β

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Table 1 Pool sizes of endogenously synthesized nicotinic acid-*N*-glucoside from parsley cell cultures cultivated on nicotinate-free medium

Period of growth (days)	Fresh weight (g/flask)*	Nicotinic acid-*N*-glucoside content (nmol/g fr wt)	(nmol/flask)
1	2 7	37 0	100
2	3 2	40 6	130
3	4 0	38 3	153
4	4 8	38 0	184
5	6 6	43 2	285
6	8 7	39 9	347
7	10 0	35 6	356
8	12 1	36.3	439
10	13 0	35 3	459
12	13 5	33 7	454
14	13 4	29 1	385

*The culture flasks were inoculated with 2 5 g fr wt

anomers of glucose as demonstrated by co-chromatography The ^{1}H NMR spectrum was consistant with that of glucose and there was no evidence for the presence of arabinose

These spectroscopic and chromatographic data prove that suspension cultured parsley cells convert nicotinic acid to nicotinic acid-*N*-glucoside and not to nicotinic acid-*N*-arabinoside

Pool sizes of endogenously synthesized nicotinic acid-N-glucoside

Results from previous experiments [11] support the assumption that nicotinic acid-*N*-glucoside is not exclusively synthesized from nicotinic acid applied to suspension cultured parsley cells, but that the conjugate is also an endogenous constituent of these cells. Therefore, the endogenous level and pool size of the nicotinic acid-*N*-glucoside was quantitatively measured over a complete growth period in cell cultures of *Petroselinum hortense* which had been cultivated on nicotinate-free medium for at least 20 passages

A new method for the purification of nicotinic acid-*N*-glucoside was devised which allowed reproducible quantitation of the conjugate requiring less starting material than previous methods. Ion exchange chromatography provided partially purified extracts from which nicotinic acid-*N*-glucoside could be quantitated by HPLC Identification was achieved by co-chromatography with the chemically synthesized *N*-glucoside and by FDMS analysis of the compound after HPLC separation

Nicotinic acid-*N*-glucoside was thus quantitated over an extended growth period of 14 days (Table 1) A nearly constant level of 35–40 nmol nicotinic acid-*N*-glucoside/g fr wt could be detected which slightly decreased when the cell culture entered the stationary phase after 12 days of growth These data prove that parsley cell cultures are able to synthesise nicotinic acid-*N*-glucoside

Metabolism of nicotinic acid-N-glucoside

Glucose is considered to be one of the most important conjugating moieties of primary and secondary plant constituents The transformation of plant metabolites to storage and detoxification products is the essential aspect of glucosyl-conjugation reactions [16, 17]

Our studies on the metabolism of nicotinate-*N*-glucoside aim at elucidating a possible reservoir function for this conjugate, from which nicotinic acid can be released for coenzyme synthesis or the observed degradation reaction of nicotinate [5] We have therefore determined the turnover [18] of the *N*-glucoside in parsley cell suspension cultures

During the logarithmic growth phase 2 22 MBq [U-^{14}C]-aspartate was administered to 65 g parsley cells in the presence of 10^{-6} M non-labelled aspartate for 10 hr After separation from the incubation medium and further subculturing, the cells were allowed to grow for 168 hr on aspartate-free medium Every 24 hr (or 48 hr) aliquots of the cells were harvested and subjected to methanol extraction Nicotinic acid-*N*-glucoside was purified and quantitated by HPLC The conjugate was collected and radioactivity determined by liquid scintillation counting The results prove the rapid incorporation of the precursor in nicotinic acid-*N*-glucoside (Fig 1A) After subculturing for 24 hr, 0 184% of the [U-^{14}C]-aspartate had been incorporated into the glucoside (sp act. 17 kBq/μmol) The dilution factor (sp act of the precursor divided by the sp act of the isolated conjugate) of [U-^{14}C]-aspartate in its incorporation into nicotinic acid-*N*-glucoside was 543 Very similar dilution factors of 600 [19] and 263 [20] were reported for the incorporation of aspartic acid into nicotine and ricinine Such small dilution factors point to a close metabolic connection between the applied precursor and the obtained product

During the incubation period of 7 days the specific radioactivity of the nicotinic acid-*N*-glucoside declined to 2 2% of its maximum value (Fig 1A). The total radioactivity of the glucoside isolated showed the same course (Fig 1B) thus indicating the rapid metabolism of nicotinic acid-*N*-glucoside A decrease of the sp act due to dilution of the labelled compound by subsequent synthesis of unlabelled glucoside would have kept the value of total radioactivity constant The parallel decrease of both specific and total radioactivity of the conjugate clearly prove a turnover of nicotinic acid-*N*-glucoside in heterotrophic parsley cells with a half life of 24 hr

The endogenous synthesis and rapid metabolism of

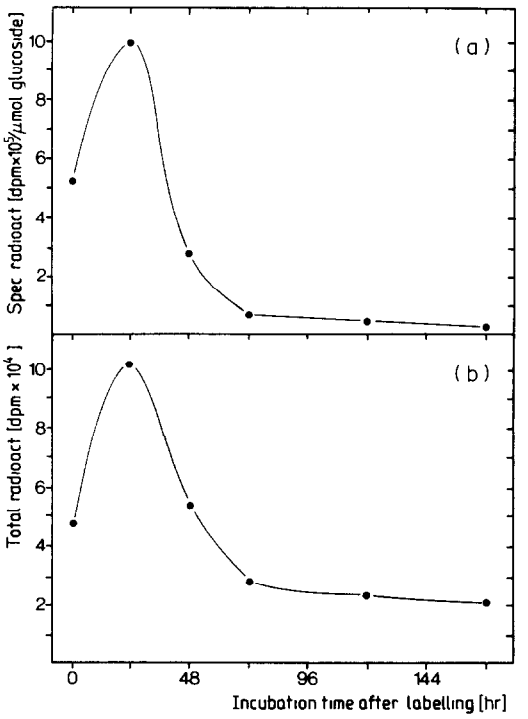

Fig. 1. Tracer experiment demonstrating the turnover of the nicotinic acid-*N*-glucoside in parsley cell suspension cultures (a): Time course of specific radioactivity of nicotinic acid-*N*-glucoside after application of (U-^{14}C)-aspartate for 10 hr (b) Time course of total radioactivity of nicotinic acid-*N*-glucoside after application of (U-^{14}C)-aspartate for 10 hr

nicotinic acid-*N*-glucoside in parsley cells lead to the assumption that this conjugate functions as a storage form for nicotinic acid for the synthesis of NAD and NADP in the pyridine nucleotide cycle [2] or degradation [5]. The role of a nicotinate detoxification product may rather be attributed to nicotinic acid glucose ester which had been found in parsley cells when exposed to nicotinic acid in concentrations higher than 10^{-5} M [3] This ester has not been observed in these studies

The turnover of nicotinic acid-*N*-glucoside is presently explained by the reversibility of the UDP-glucose–nicotinic acid-*N*-glucosyltransferase reaction [12].

In contrast to batch-propagated parsley cells, heterotrophic cell suspension cultures of Papillionaceae convert nicotinic acid to the *N*-methyl conjugate, trigonelline [10]. Application of [U-^{14}C]-aspartate to soybean (*Glycine max* L.) cell cultures led to the accumulation of labelled trigonelline, but failed to demonstrate a turnover of the *N*-methyl-conjugate (B. Upmeier and W. Barz, unpublished results). Demethylation of trigonelline seems to proceed too slowly to meet the nicotinate requirement of the cell for pyridine nucleotides. In contrast to the nicotinic acid-*N*-glucoside from cells of *Petroselinum hortense* Hoffm., trigonelline cannot be regarded as a storage form for nicotinic acid, but exhibits the properties of a typical secondary plant product.

EXPERIMENTAL

Chemicals. All chemicals for the synthesis of nicotinic acid-*N*-glucoside were purchased from Merck (Darmstadt, F R G.), Roth (Karlsruhe, F.R.G) and Schuchardt (Munich, F R G) Nicotinic acid was from Merck (Darmstadt, F.R.G), acetonitrile was obtained from Baker (Groß-Gerau, F R G) Ion exchange resins came from Serva (Heidelberg, F R G) and the putative nicotinic acid-*N*-arabinoside was from previous studies

The radiochemicals [7-^{14}C]-nicotinic acid (2 07 GBq/mmol) and L-[U-^{14}C]-aspartate (8 3 GBq/mmol) were obtained from Amersham, U K

Cell cultures Heterotrophic parsley (*Petroselinum hortense* Hoffm.) cell suspension cultures were cultivated on Gamborg B_5-medium [21] under conditions previously described [22] The application of compounds under aseptic conditions, the collection and measurement of $^{14}CO_2$ followed previous reports [23].

Thin layer chromatography Chromatographic solvents were S1: *n*-BuOH–HOAc–H_2O (6 3 1), S2 Me_2CO–H_2O (4·1); S3· Me_2CO–H_2O (7 3), S4 MeCOEt–MeOH–H_2O–HCO_2H (11 6 2 1) Chromatography was performed on silica gel plates GF_{254} (Merck, Darmstadt, F R G).

Radioactivity. All soluble samples were measured for radioactivity as previously described [11] Labelled compounds on chromatograms were located according to ref [11]

Ion exchange chromatography. Cell extracts were applied to a column (50 × 10 mm) filled with Dowex 50W × 8 (100-200 mesh, H^+-form) The column was washed with 50 ml H_2O and eluted with 50 ml 2 M NH_4OH. The NH_4OH-fraction was collected, brought to dryness and the residue was dissolved in 1 ml MeOH prior to HPLC

HPLC. A Kontron chromatograph was equipped with a Kontron UV-Vis detector (LCD 725, Kontron Analytik, Hanover, F.R G) and a Shimadzu C-R1A integrator Separation of nicotinic acid-*N*-glucoside from partially purified extracts of parsley cell cultures was performed with a LiChrosorb NH_2-column (250 × 4 mm, 5 μm, Merck, Darmstadt, F.R.G) The chromatograms were developed by isocratic elution with 80% MeCN and 20% H_2O. Product quantities were determined from the peak height or area using external reference compounds.

GC analyses were conducted with a Varian Aerograph 2440 equipped with a flame ionisation detector. The column (2.8 m) was filled with 3% OV-101 on Chromosorb W AW-DMSC (100–200 mesh) as stationary phase the temp. of the injector and the detector was 250° The gases were N_2 (70 ml/min), H_2 (30 ml/min) and air (450 ml/min) For derivatization compounds were carefully dried under vacuum and redissolved in absolute pyridine. TMSi derivatives were prepared by treatment with hexamethyl-disilazane and trimethylchlorosilane according to ref [24] or with MSTFA (pyridine–MSTFA 2 1) for 12 hr at room temp

Mass spectra FDMS analysis of chromatographically purified nicotinic acid-*N*-glucoside provided the following data MS *m/z* (rel int): 286 $(M)^+$ (34), 106 (nicotinyl) (58) and 60 ($C_2O_2H_4$) (17).

^1H NMR spectroscopy NMR spectra were recorded at 90, 270 and 100 Hz

Preparation of N-(β-D-*glucopyranosyl*)-3-*carboxypyridinium hydroxide* Under acidic conditions D-glucose and Ac_2O were converted to pentaacetylglucose which yielded acetobromoglucose after addition of HBr [25] The condensation of nicotinamide with acetobromoglucose was performed according to ref [13]. Hydrolysis of the acetyl moieties was carried out in 3% HBr at room temp. Crystallization from MeCN–*iso*PrOH (1 1) and from EtOH–*iso*PrOH (1 1) gave *N*-(β-D-glucopyranosyl)-3-carboxamido pyridinium bromide, as judged from UV and

^{1}H NMR spectra The nicotinamide conjugate was transformed to the corresponding carboxylic acid according to ref [14] and the acid was purified by ion exchange chromatography on Dowex 50 (H^+-form) The glucoside was converted to the hydroxide form by treatment with Ag_2O and H_2S Recrystallization from aq EtOH or aq Me_2CO afforded colourless needles of *N*-(β-D-glucopyranosyl)-3-carboxypyridinium hydroxide (mp 232–234°) [6]

Preparation of [7-^{14}C]-*nicotinic acid*-N-*glycoside* Dark grown cell suspension cultures of *Petroselinum hortense* were incubated with [7-^{14}C]-nicotinic acid (74 KBq per flask, 10^{-4} M nicotinic acid) for 12 hr Cells (2 × 17 g fr wt) were separated from the medium and extracted with hot 70% aq EtOH using an Ultraturrax homogenizer Cell debris were again extracted with hot H_2O and boiled for 5 min The extracts were collected, concd and subjected to TLC in S1 with the putative nicotinic acid-*N*-arabinoside [8, 11] as reference The radioactive material which co-chromatographed with the arabinoside was eluted from the silica gel with H_2O, concd and used as labelled reference for the preparative isolation of nicotinic acid-*N*-glycoside from parsley cells

Purification of the nicotinate conjugate for structural elucidation After incubation with 10^{-4} M nicotinic acid for 12 hr parsley cells (728 g fr wt) were harvested and extracted as described above The extract was treated with activated charcoal The filtrate was reduced to a few ml and the extract was subjected to ion exchange chromatography on a Dowex-1 × 8 column (300 × 28 mm, formate form) The nicotinic acid-*N*-glycoside could be removed from the resin with H_2O The H_2O fraction was brought to dryness The residue was redissolved in aq MeOH and purified by TLC in S1, S4 and S3 After elution from the silica gel 27 mg nicotinic acid-*N*-glycoside could be crystallized from aqueous EtOH (mp 232–234°)

Spectroscopic analyses The synthetic nicotinic acid-*N*-glucoside, the *N*-glycoside isolated from the parsley cells and the putative nicotinic acid-*N*-arabinoside [8, 11] showed the following spectroscopic properties The UV spectrum showed maximum absorption at 263 nm which was shifted to 335–340 nm after addition of $NaBH_4$ thus indicating the existence of a quaternary pyridinium compound [26, 27] KCN caused a maximum absorption at 313 nm The ^{1}H NMR spectrum (270 Hz, D_2O) showed four aromatic protons at δ 9 35 (1H, s, C-2 PyrH), δ 9 09 (1H, *d*, *J* = 6 Hz, C-6 PyrH), δ 9 01 (1H, *d*, *J* = 8 Hz, C-4, PyrH) and δ 8 24 (1H, *m*, C-5 PyrH) The nonaromatic part of the spectrum revealed signals between δ 4 04 and 3 65 due to the sugar protons The anomeric proton of the glucose moiety was monitored at δ 5 87 (1H, *d*, *J* = 9 Hz, C-1'H) indicating that the glucose was in the β-form Mass spectra were recorded at two different temps At 150° mainly fragments of the nicotinoyl moiety were found MS *m/z* (rel int) 123 [nicotinoyl] (100), 106 [nicotinyl] (41), 105 [nicotinyl − H] (61), 78 [pyridyl] (46), 51 (25), 50 (17) and 39 (11) Signals due to the glucose moiety were 268 $[M-18]^+$ (1) and 163 [glucopyranosyl] (1) A spectrum recorded at 230 showed the decay of glucose in more details MS *m/z* (rel int) 268 $[M-18]^+$ (2), 163 [glucopyranosyl] (18), 123 [nicotinoyl] (37), 146 [glucopyranosyl − H_2O] (26), 97 (19), 73 [$C_3O_2H_5$] (57), 60 [$C_2O_2H_4$] (50) and 57 (50) The above information indicated that *N*-(β-D-glucopyranosyl)-3-carboxypyridinium hydroxide had been successfully synthesized It served as reference during further studies on nicotinic acid conjugates from parsley cell cultures

Hydrolysis of nicotinic acid-N-*glycoside* An aliquot of nicotinic acid-*N*-glycoside was incubated with 3 M HCl for 6 hr at 100 The soln was neutralized and desalted by ion exchange chromatography on Dowex 50W × 8 (H^+-form) and Dowex-1 × 8 (formate form)

For enzymatic hydrolysis nicotinic acid-*N*-glycoside was dissolved in 50 mM citrate–phosphate buffer pH 5 5 and incubated with a β-glucosidase (1% w/v Emulsin, Sigma, Munich, F R G) for 10 hr at 37° The reaction was stopped by adding an equal vol of MeOH

Extraction procedure for nicotinic acid-N-*glucoside quantitation* Cells were harvested by filtration on a glass funnel, washed with H_2O and homogenized in a mortar with 50% aq MeOH (4 ml/g fr wt) at room temp The extract was obtained by filtration and the extraction procedure repeated twice with 80% aq MeOH The methanolic extracts were pooled and concentrated under red pres to a vol of *ca* 2 ml The pH was adjusted to pH 1 with 10 M HCl and the extract was centrifuged prior to ion exchange chromatography

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