Metabolism and Degradation of Nicotinic Acid in Parsley *(Petroselinum hortense)* Cell Suspension Cultures and Seedlings

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Z. Naturforsch. **41 c,** 148–157 (1986); received July 15, 1985

Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

Degradation, Cell Suspension Cultures, *Petroselinum hortense*, Nicotinic Acid, 6-Hydroxynicotinic Acid

Application of [6-^{14}C]- or [7-^{14}C]nicotinic acid to parsley cell suspension cultures led to the accumulation of labelled nicotinamide mononucleotide, nicotinamide adenine dinucleotide, nicotinamide N-riboside, nicotinamide and nicotinic acid, indicating the operation of the pyridine nucleotide cycle in these cells. As main conjugates, nicotinic acid N-glucoside and nicotinic acid glucose ester were found. For nicotinic acid degradation the following sequence is suggested: nicotinic acid → 6-hydroxynicotinic acid → 2,5-dihydroxypyridine → a C_4/C_3 unit of unknown structure → CO_2. In aseptically grown parsley seedlings nicotinic acid is also degraded to CO_2

Introduction

Investigations with plant cell suspension cultures [1–3] have demonstrated the ability of plants for the degradation of nicotinic acid (**1**) to CO_2. Catabolites of the degradative pathway in these cells have so far remained unknown. In the present time such knowledge is only available from microbial systems where several degradative pathways of nicotinate have been reported [4–8]. Animals seem to lack the ability of nicotinate catabolism but rather excrete various nicotinic acid conjugates and pyridones [9, 10]. Although microbial degradative routes for **1** all start by the initial formation of 6-hydroxynicotinic acid [4], previous experiments with this acid in plant cell cultures [3, 11] were interpreted as to indicate that 6-hydroxynicotinic acid is not involved in the degradation of **1** in plant cells. This paper provides evidence that the exogenously added 6-hydroxynicotinic acid is trapped in form of conjugates and thus withdrawn from the normal catabolic pathway of nicotinate.

We now report on more extensive studies to elucidate the catabolic sequence of nicotinic acid in parsley cell cultures. This work has yielded evidence for 6-hydroxynicotinic acid, 2,5-dihydroxypyridine and a C_3/C_4 compound as catabolites of **1** in these cells.

Materials and Methods

Cell culture

Growth and cultivation of parsley cell suspension cultures has been described [2]. Cells were subcultured every week and used in the late linear growth phase. The application of filter-sterilized substrates to cell cultures under aseptic conditions and collections of $^{14}CO_2$ followed previous reports [1, 2, 11, 32]. The same feeding procedure was used for the aseptically grown seedlings.

Aseptic seedlings

Seeds were defatted with 70% ethanol for 5 min and surface-sterilized in 5% sodium hypochloride solution for 30 min. After complete removal of the sterilizing reagent with sterile water seeds were germinated in sterile Petri dishes on moist filter paper at 20 °C for some 3 weeks. Aseptic conditions were controlled by microscopic inspection and incubation of aliquots on Merck standard nutrient agar I at 35 °C for 4 days.

Abbreviations: NMN, nicotinamide mononucleotide; NAD, nicotinamide adenine dinucleotide; TLC thin layer chromatography; HPLC, high performance liquid chromatography; MW, molecular weight; GC, gas chromatography; MS, mass spectroscopy/spectrum; fr. w., fresh weight; TMSi, trimethylsilyl; MSTFA, N-methyl-N-trimethyl-silyl-trifluoracetamide; HMDS, hexamethyl-disilazan.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/86/0100–0148 $ 01.30/0

Extraction procedure

Cells were harvested by filtration, washed with water and suspended in 80% aqueous methanol at room temperature. Subsequently, cells were homogenized using an Ultraturrax homogenizer and repeatedly extracted with methanol at less than 20 °C. Extracts obtained by centrifugation were concentrated at reduced pressure and always handled at room temperature. If necessary the extraction procedure was carried out under a nitrogen atmosphere.

Reagents

[6-^{14}C]nicotinic acid, [7-^{14}C]nicotinic acid and [6-^{14}C]6-hydroxynicotinic acid were from previous studies [3]. Nicotinic acid, nicotinamide, NMN, NAD, mono- and dihydroxypyridines were purchased from Sigma, München, Merck, Darmstadt, and Schuchardt, München.

MSTFA came from Machery-Nagel and HMDS from Merck, Darmstadt. β-glucosidase (emulsin), esterase (type I, hog liver) and alcaline phosphatase *(E. coli)* were obtained from Sigma, München. Glucoseoxidase reagent was from Boehringer, Mannheim.

2,5-Dihydroxypyridine was synthesized according to published reports [33] and crystallized three times from ethanol under a nitrogen atmosphere. The product was free from 2-hydroxy- and 2,3-dihydroxypyridine as judged by GC of the TMSi derivatives.

Nicotinamide N-riboside was prepared from NMN using alkaline phosphatase according to [34]. The product was characterized as described [35].

Paper electrophoresis

According to previous reports [23] the following buffers were used: a) potassium phosphate, 0.1 M, pH 8; b) potassium phosphate, 0.1 M pH 4, and c) formic acid (0.6 N): acetic acid (2 N) = 1:1 (v/v), pH 2.

Radioactivity

$^{14}CO_2$ and all soluble samples were measured for radioactivity as previously described [2]. Labelled compounds on chromatograms were located according to published methods [2].

Chromatographic solvents

Solvents for TLC on Si gel were:

S1: *n*-butanol : acetic acid : water, 4:1:1;
S2: acetone : water, 4:1;
S3: $CHCl_3$: methanol, 4:1;
S4: methanol : $CHCl_3$, 3:1;
S5: methanol : $CHCl_3$: water, 3:2:1;
S6: methylethylketone : methanol : acetic acid : water, 5:3:2:1;
S7: dichloromethane : methanol, 3:1;
S8: ether : methanol : *n*-hexane, 5:1:1;
S9: *iso*-propanol : water, 3:2.

Solvent for TLC on cellulose was:

S10: *n*-butanol : acetic acid : water, 4:1:1.

Gas chromatography

GC analyses were conducted with a Varian Aerograph 2440 equipped with a flame ionisation detector. Column: 3′ × 1/8″, length 1.5 m, filled with 3% OV 101 on chromosorb W/AW DMCS 80–100 mesh. Injector temp. 230 °C, detector temp. 260 °C, gas flow nitrogen 30 ml/min, temp. program from 80 ° to 250 °C with 6°/min. Detector: hydrogen gas, 30 ml/min and air, 300 ml/min.

For GC-MS coupling, a capillary column length 15 m, filled with SE 30, 3%, was used.

For derivatization compounds were carefully dried over $CaCl_2$ under vacuum. TMSi derivatives were prepared by treatment with MSTFA and HMDS for 12 h at 20 °C or at 40–60 °C for 60 min under careful exclusion of moisture using abs. acetonitrile as solvent.

The described gas chromatographic procedure was especially suitable for the separation of nicotinic acid, nicotinamide, 6-hydroxynicotinic acid, the 3 isomeric monohydroxypyridines and 2,3-, 2,4-, 2,5-, and 2,6-dihydroxypyridine.

High performance liquid chromatography

A Kontron chromatograph was used equipped with a Kontron UV/VIS detector, LCD 725, and a Shimadzu C-R1A integrator. For separation of nicotinic acid, nicotinamide, nicotinic acid N-glucoside a Si 60 Li chrosorb column (250 × 4 mm, 5 μm) was used. The solvent consisted of 70% acetonitrile, 30% water and 1% H_3PO_4, flow: 0.8 ml/min; detection at 260 nm.

For separation of nicotinic acid, 6-hydroxynicotinic acid and dihydroxypyridines a Li chrosorb RP-8 column (250 × 4 mm, 5 μm; Merck, Darmstadt) was used. A linear gradient of 0% B to 15% B in (A + B) in 20 min was applied (A: H_2O/0.5% H_3PO_4; B: 25% CH_3CN/20% CH_3COOH/55% H_2O/0.5% H_3PO_4). Flow 0.8 ml/min, detection at 260 nm.

For separation of nicotinamide, nicotinamide N-riboside and nicotinic acid N-glucoside a Li chrosorb NH_2 column (250 × 4 mm, 5 μm; Merck, Darmstadt) was used. Two different separation systems were applied, a linear gradient of 95% B to 25% B in (A + B) in 30 min (A: 3% CH_3COOH, B: CH_3CN) and a linear gradient of 100% B to 20% B in (A + B) in 40 min (A: 0.25% H_3PO_4, B: CH_3CN). Flow 0.8 ml/min, detection at 263 nm.

UV spectra

Absorption spectra were recorded with a Leitz-Unicam SP 8000 spectrophotometer in methanol or water.

Mass spectra

Mass spectrometer of Varian, model CH 7 and SM 1 were used.

GC/MS spectra

A Varian mass spectrometer, model CH 7 with databank coupled to a Varian gas chromatograph, model 1040 was used.

Structural elucidation of catabolites

The spectroscopic data which were essential for the determination of isolated catabolites or synthesized intermediates are as follows.

6-Hydroxynicotinic acid

Samples of isolated as well as of authentic material were measured as the di-TMSi derivative, $C_{12}H_{21}NO_3Si_2$, MW = 283. MS *(m/e):* 283 (M^+), 282 (M^+-H), 268 (M^+-CH_3), 224 (268-CO_2), 194 (M^+-OTMSi), 165 (282-CO_2TMSi), and 151 (268-CO_2TMSi)

2,5-Dihydroxypyridine

The di-TMSi derivative was analyzed by MS; $C_{11}H_{21}NO_2Si_2$, MW = 255. MS *(m/e):* 255 (M^+), 254 (M^+-H), 240 (M^+-CH_3), 210 (240-TMSi), 181 (254-TMSi), 168 (240-$CH_2Si(CH_3)_2$) and 112 (240-$C_5H_{10}NOSi$).

2,5-Dimethoxypyridine

$C_7H_9NO_2$, MW = 139. MS *(m/e):* 139 (M^+), 138 (M^+-H), 124 (M^+-CH_3), 109 (M^+-CH_2O), 108 (M^+-OCH_3), 96 (124-CO), 82 (124-CH_2CO), 79 (109-CH_2O), 69 (96-HCN), and 66 (108-CH_2CO).

Glycerol

Authentic material as the tri-TMSi derivative and the compound obtained from catabolite IX by silylation with MSTFA/HMDS showed the same mass spectrum.

$C_{12}H_{32}O_3Si_3$, MW = 308. MS *(m/e):* 308 (M^+), 293 (M^+-CH_3), 218 (M^+-HOTMSi), 205 (M^+-CH_2OTMSi), 191 (M^+-CO_2TMSi), 177, 163, 147, 133, 129 (218-OTMSi), 117, 103 (M^+-$C_2H_3O_2(TMSi)_2$).

Results

Metabolites of nicotinic acid

Previous work [1–3] had clearly demonstrated that batch-propagated cell suspension cultures of *Petroselinum hortense* and *Galium verum* readily oxidized both [carboxyl-^{14}C]- and [6-^{14}C]nicotinic acid to $^{14}CO_2$. As judged from the constant rates of $^{14}CO_2$ formation, decarboxylation was constantly found to precede ring cleavage in plant nicotinate catabolism [3, 12, 13]. Chromatographic analyses of cell extracts of such cells had only led to the isolation of various nicotinic acid conjugates [1, 12, 14], but had failed to detect any catabolites of nicotinate. To improve the previous studies we have now substantially increased both the amount of the investigated parsley cell culture material as well as the absolute and specific ^{14}C radioactivity of the nicotinic acid samples used in these experiments; furthermore, cell extracts were now exclusively prepared at room temperature. This extraction procedure avoided the disintegration of labile compounds during workup of cell material.

Batch-propagated, dark-grown cell suspension cultures of *Petroselinum hortense* were incubated with either [carboxyl-^{14}C]- or [6-^{14}C]nicotinic acid (10^{-5} M; 5 μCi per flask) for 72 h. During this period, $^{14}CO_2$ was again evolved at constant rates (data not shown; but see Fig. 2) yielding altogether 2.8% $^{14}CO_2$ from the carboxyl group and 2.2% from the ring position. The average rate of nicotinate degradation by these

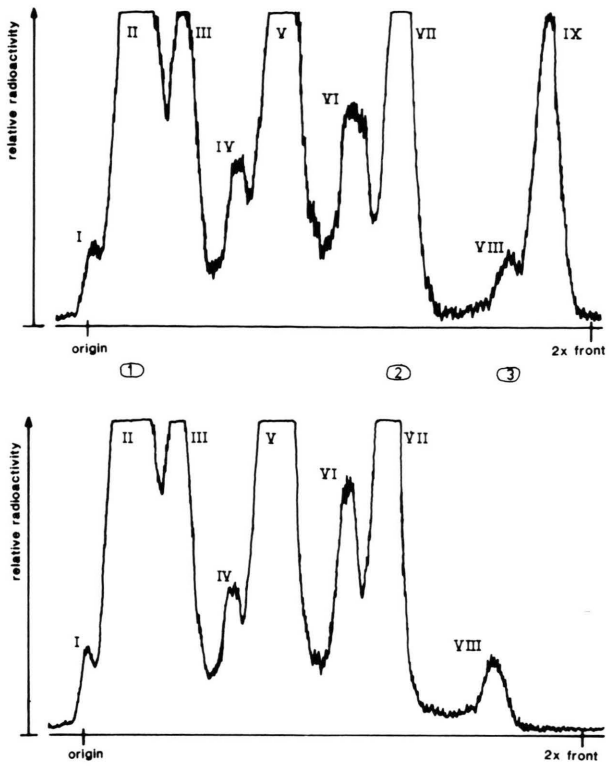

Fig. 1. Radioscans of TLC plates showing the distribution of radioactivity in extracts of parsley cell suspension cultures after application of [6-^{14}C]nicotinic acid (upper panel) and [7-^{14}C]nicotinic acid (lower panel) for 72 h. Extracts were separated by TLC, solvent S1, and plates were developed twice. Compounds of bands I to IX are discussed in the text.
Reference compounds were:

① = nicotinic acid N-glucoside;
② = nicotinic acid;
③ = 6-hydroxynicotinic acid.

parsley cell cultures can thus be estimated to be 0.2 μmol/24 h/8 g fresh weight of cells.

Extracts of the two kinds of [^{14}C]nicotinate-incubated cells, prepared at room temperature with 80% methanol and separated by TLC, yielded several metabolites (I–IX) as shown in Fig. 1. The two radioscans in Fig. 1 appeared to be identical except for band IX which represents a compound exclusively obtained from ring-labelled nicotinate. Using cochromatography of TLC, HPLC, GC, MS and other methods discussed below, most of be labelled compounds shown in Fig. 1 could be identified.

Structural elucidation of nicotinate metabolites

Band I of Fig. 1 contained NMN and NAD (ratio 2:3) as indicated by TLC (solvents S_1, S_2) and HPLC. Band II represents nicotinic acid N-β-D-glucoside (TLC, HPLC, see [14]) which is the earliest and most dominant conjugate of nicotinic acid in parsley and several other cell cultures [1–3, 12, 13]. In previous work [2, 3] this glycoside had erroneously been called nicotinic acid N-arabinoside which was later revised to the N-glucoside [14, 15].

For structural identification the ^{14}C-labelled compound of band III was isolated from 200 g of parsley cells incubated with [^{14}C]nicotinate [5×10^{-5} M] for 48 h and partially purified by TLC in solvents S1 (2 times) and S2. It turned out to be a very labile, acid sensitive derivative of nicotinamide (TLC, S3, HPLC) which could not be obtained in quantities sufficient for spectroscopic analysis. Finally, cochromatography in 6 solvents (S_1, S_2, S_4–S_6, S_{10}), HPLC with 2 different gradients and dilution analysis, all with synthetic reference material, showed that III represents nicotinamide N-riboside.

Compounds IV and VI are presently unknown, nicotinic acid-containing metabolites (acid hydrolysis, TLC in S1, S2 and S6) whereas band VII is a mixture of nicotinic acid and nicotinamide (ratio 6:1) as determined by HPLC.

Compound V purified (TLC) with solvents S1, S5 and S6 from extracts of parsley cell cultures incubated with [7-^{14}C]nicotinate (10^{-4} M; 72 h) possesses an UV spectrum (λmax in water 264 nm) essentially as nicotinic acid (λmax 260 nm). The absorption spectrum shows no bathochromic shift upon treatment with $NaBH_4$. Thus, a N-substituted nicotinic acid derivative can be excluded. Incubation of compound V with either 4 N HCl, β-glucosidase (emulsin) or an esterase (hog liver) always yielded nicotinic acid (TLC, S1, S2, S4, S5) and glucose (S11, glucose oxidase assay). Quantitative determinations established a nicotinic acid to glucose ratio of 1:0.83. Compound V is thus tentatively characterized as nicotinic acid glucose ester (1-O-nicotinoyl β-D-glucose).

Compounds VIII and IX will further be discussed below. It is, however, essential to stress that compound IX will only accumulate in labelled form after application of [6-^{14}C]nicotinic acid (Fig. 1).

In general, Fig. 1 clearly shows that in contrast to our previous work [2, 3] numerous nicotinic acid

metabolites have now been obtained from parsley cell suspension cultures. Kinetic experiments [13, 16] (data not shown) on the rate and the sequence of the formation of these nicotinic acid metabolites again revealed [1, 11] that the first and quantitatively most important metabolite is the nicotinic acid N-glucoside (compound II). One hour after beginning of incubation with [^{14}C]nicotinic acid, II represents 85% of the extractable total radioactivity of the parsley cell cultures. Within 25 h the radioactivity of II sharply decreased to some 30%, while the radioactivity in the other compounds, namely NAD, NMN, nicotinamide N-riboside, nicotinic acid and nicotinamide, rose from zero to values between 5 and 25%.

After approximately 25 h all compounds seemed to have reached steady state concentrations because the ratio of the radioactivity of all compounds as shown in Fig. 1 did not change for up to 100 h. Radioactivity in bands VIII and IX (after application of [6-^{14}C]nicotinate) and the concomitant evolution of $^{14}CO_2$ from either [7-^{14}C]- or [6-^{14}C]nicotinic acid had also reached constant rates after approx. 25 h.

Formation of compound V, the nicotinic acid glucose ester, could only be observed [13, 16] if nicotinic acid had been applied at a concentration of 10^{-5} M or higher.

In various feeding experiments as well as pulse and pulse-chase experiments [13, 16] at nicotinic acid concentrations of 5×10^{-7} to 5×10^{-6} M compound V failed to accumulate though all other bands (Fig. 1) could be demonstrated to occur and the same isolation technique had been used. These results indicate that the nicotinic acid glucose ester might function as a trapping mechanism for nicotinate if the cells are loaded with unphysiologically high concentrations of this vitamin.

Identification and metabolism of 6-hydroxynicotinic acid

The ^{14}C-labelled compound of fraction VIII (Fig. 1) cochromatographed (TLC) with 6-hydroxynicotinic acid in solvents S1, S5, and S9. Using cell extracts from incubation experiments with both [7-^{14}C]- and [6-^{14}C]nicotinate, isotope dilution analyses of fraction VIII together with non-radioactive 6-hydroxynicotinic acid as carrier were performed. Repeated TLC purification steps led to a preparation of constant specific radioactivity. Furthermore, using HPLC with 2 different elution gradients the ^{14}C-labelled compound from fraction VIII comigrated with 6-hydroxynicotinic acid.

When gaschromatographic and radiogaschromatographic analyses of the trimethylsilyl derivatives of VIII and 6-hydroxynicotinic acid were performed, identical retention values for compound and radioactivity were observed. The gaschromatographic separation of TMSi derivatives has turned out to be a very efficient system for the separation of nicotinic acid, nicotinamide, 6-hydroxynicotinic acid, the three isomeric monohydroxypyridines and the four isomeric dihydroxypyridines mentioned in the legend of Fig. 2. These gaschromatographic data are therefore especially important to determine the structural identity of compound VIII as 6-hydroxynicotinic acid.

Final proof was obtained when compound VIII isolated from a large scale incubation experiment (360 g parsley cells, 10^{-4} M nicotinate, 48 h) and repeatedly purified by TLC was analyzed as the di-TMSi derivative by GC-MS techniques. The mass spectra of compound VIII and of 6-hydroxynicotinic acid were identical in all details.

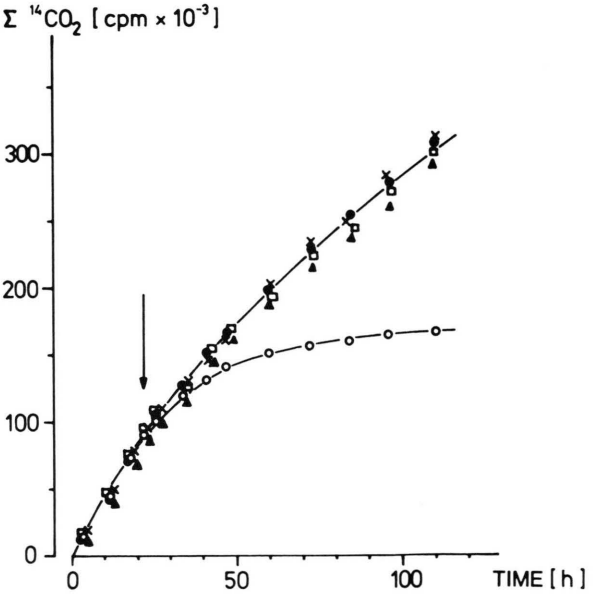

Fig. 2. Formation of $^{14}CO_2$ from [6-^{14}C]nicotinic acid (2.7×10^{-6} M) in parsley cell suspension cultures in the absence (●———●) and in the presence of either 2,3-dihydroxypyridine (×———×), 2,4-dihydroxypyridine (□———□), 2,5-dihydroxypyridine (○———○) or 2,6-dihydroxypyridine (▲———▲). Addition of dihydroxypyridines (10^{-4} M) was at a time indicated by the arrow.

All results taken together show that comparatively large amounts of free 6-hydroxynicotinic acid accumulate during nicotinate degradation in parsley cell cultures. On the other hand, earlier studies [3, 11] had shown that 6-hydroxy-[6-^{14}C]nicotinic acid, when added to parsley cultures, resulted in only very small amounts of $^{14}CO_2$. Further investigations on the metabolic fate of this hydroxycompound have now been performed by adding 6-hydroxy-[7-^{14}C]-nicotinic acid (1 μCi, 10^{-6} M) to parsley cell suspension cultures. In comparison to control flasks incubated with [7-^{14}C]nicotinic acid (7.0% of total radioactivity in $^{14}CO_2$/75 h), much less $^{14}CO_2$ (1.3% of total radioactivity in $^{14}CO_2$/75 h) could be observed. When these cells were fractionated and extracts separated by TLC in solvent S 1, very little free 6-hydroxy-[7-^{14}C]nicotinic acid (approx. 15%) could be found (see [13]); the bulk of the labelled material appeared as two compounds which upon acid hydrolysis yielded 6-hydroxynicotinic acid (TLC, HPLC).

These compounds have so far not been observed as catabolites of nicotinic acid degradation. Upon prolonged incubation of the parsley cells these conjugates of 6-hydroxynicotinic acid were seemingly not further metabolized as judged by radioscans of appropriate TLC analyses. Though these conjugates still await structural elucidation, our data are presently interpreted [15, 16] as to indicate that exogenously added 6-hydroxynicotinic acid is rapidly converted to compounds which prevent further degradation of the aglycone portion.

Evidence for 2,5-dihydroxypyridine

The successful isolation of 6-hydroxynicotinic acid as a nicotinate metabolite has led to investigations on dihydroxypyridines as subsequent catabolites. Involvement of such a compound appeared especially mandatory because nicotinate degradation in parsley cell cultures, as measured by formation of CO_2 from both position 6 and 7 strictly depended on aerobic conditions [12]. Furthermore, monohydroxypyridines such as 2-hydroxy-, 3-hydroxy- or 4-hydroxypyridine had no effect on nicotinate catabolism in parsley cell cultures [17].

The participation of 2,5-dihydroxypyridine in nicotinic acid degradation was demonstrated in a set of parallel isotope dilution experiments where the formation of $^{14}CO_2$ from [6-^{14}C]nicotinic acid (2.7×10^{-6} M) was drastically lowered by the addition of non-radioactive material of this dihydroxypyridine (Fig. 2). Addition of either one of the three isomers (2,3-dihydroxy-, 2,4-dihydroxy- or 2,6-dihydroxypyridine) resulted in $^{14}CO_2$ formation essentially as measured for the control. Further studies had also shown that the four isomeric dihydroxypyridines were all rapidly taken up into the cells as measured by disappearance from the nutrient medium.

A similarly pronounced dilution effect of 2,5-dihydroxypyridine on $^{14}CO_2$ formation from ring-labelled nicotinate (Fig. 2) was obtained when the dihydroxypyridine was added together with [6-^{14}C]-nicotinate acid (data not shown).

Due to the great sensitivity of 2,5-dihydroxypyridine to oxidation and polymerization all attempts to isolate 2,5-dihydroxy-[^{14}C]pyridine from [6-^{14}C]nicotinate-catabolizing parsley cell cultures have totally failed. However, the occurrence of 2,5-dihydroxypyridine during nicotinic acid degradation could still be demonstrated.

Parsley cell cultures were allowed to metabolize [6-^{14}C]nicotinic acid (10^{-5} M) in the presence of non-labelled 2,5-dihydroxypyridine (10^{-4} M) for some 80 h. In comparison to the control flasks (100%), only 25% of $^{14}CO_2$ was measured during this period. Finally, cells were homogenized and extracted with 85% methanol at 4 °C under a nitrogen atmosphere with the addition of further non-labelled 2,5-dihydroxypyridine. After concentration of the extract at reduced pressure exhaustive permethylation with diazomethan was carried out. Preliminary studies had shown that under these conditions 2,5-dihydroxypyridine was quantitatively converted to 2,5-dimethoxypyridine, as measured by spectroscopic analysis of the product. Subsequent chromatographic purification of the methylated [^{14}C]compounds by TLC and scanning demonstrated that [^{14}C]2,5-dimethoxypyridine of constant radioactivity had been obtained (Fig. 3).

Analysis of catabolite IX

As presented in Fig. 1, catabolite IX only accumulates in labelled form after application of [6-^{14}C]nicotinic acid. Furthermore, parsley cells which had been incubated with [6-^{14}C]nicotinic acid in the presence of excess amounts of 2,5-dihydroxypyridine (Fig. 2) failed to accumulate catabolite IX in noticeable amounts (data not shown). These results are best interpreted by assuming catabolite IX to be a

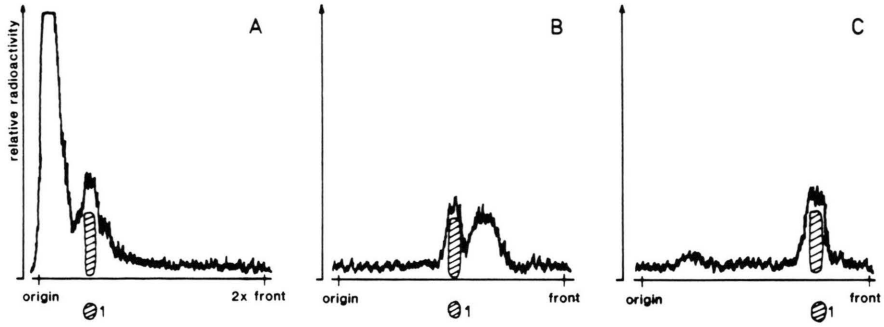

Fig. 3. Radioscan of TLC plates developed with solvents S7 (2 times) (A), S3 (B) and S8 (C). Repeated TLC chromatography led to ^{14}C-labelled 2,5-dimethoxypyridine (shaded area and reference spot ①) as obtained from 2,5-dihydroxypyridine by CH_2N_2 methylation. The 2,5-dihydroxypyridine was isolated from [6-^{14}C]nicotinate-catabolizing parsley cell cultures.

nicotinic acid degradation product which is placed in the catabolic route after loss of the carboxyl group and after 2,5-dihydroxypyridine.

Partially purified (TLC, S1, S5) catabolite IX, when subjected to electrophoresis at different pH values, appeared to be a neutral, uncharged molecule at pH 2 and an anion of increasing electrophoretic mobility between pH values 4 and 8. However, incubation of compound IX at pH 8 for a longer period of time led to decomposition, as indicated by the appearance of at least one new radioactive spot on the electropherograms.

Radiogaschromatographic investigations of catabolite IX in form of the TMSi derivative showed that only one radioactive compound was involved. The mass spectrum of this TMSi derivative (see experimental section) was undoubtedly identical with that of glycerol tri-TMSi. However, prior to silylation with MSTFA catabolite IX was not identical with glycerol because catabolite IX and the product obtained after silylation and subsequent hydrolysis (*i.e.* glycerol) greatly different in their chromatographic (TLC) and electrophoretic properties. Since the newly formed product no longer behaved as an anion between pH 4 and 8, loss of a carboxyl group during the process of silylation can be postulated.

Periodate cleavage of the ^{14}C-labelled catabolite IX and of [^{14}C]glycerol obtained from IX by silyla-

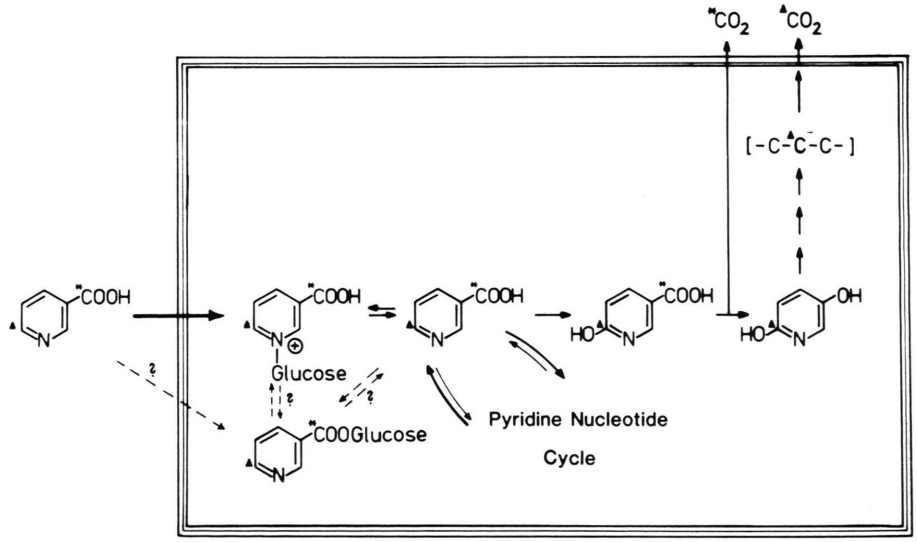

Fig. 4. Hypothetical scheme of nicotinic acid metabolism in parsley cell suspension cultures. Upon uptake by the cells exogenous nicotinate with ^{14}C label in either position 6 (▲) or 7 (★) is first converted to the N-glucoside and/or the glucose ester. Subsequently the nicotinic acid moiety may be funnelled into the pyridine nucleotide cycle or the catabolic route.

tion and hydrolysis have been performed to locate the position of label. Carbon atoms 1 and 3 of glycerol can thus be obtained as formaldehyde (or a higher aldehyde in case of a substituted glycerol) with carbon atom 2 being converted to formic acid [20]. Both compounds were readily cleaved by periodate. Contrary to expectation, some 90% of the ^{14}C-radioactivity of both compounds was found in the formic acid (central carbon atom of glycerol) fraction. This result (compare Fig. 4) possibly indicates that a molecular rearrangement occurs during formation of catabolite IX or that a cyclic compound is involved. Structural elucidation of catabolite IX, which is presently under investigation, requires the isolation of larger amounts which can be assayed by NMR spectroscopy without derivatization.

Metabolism of nicotinic acid in parsley seedlings

Studies with intact plant material on nicotinic acid metabolism in various plant species [14, 18, 19] have so far failed to demonstrate nicotinate degradation under reliably aseptic conditions.

Incubation experiments with 3-week-old parsley seedlings (2.4 g fr.w.), grown and handled under strictly controlled sterile conditions, using [7-^{14}C]nicotinic acid (10^{-5} M; 10 μCi) have now been performed. After a lag phase of some 45 h a strong and constant formation of $^{14}CO_2$ could be measured. After 100 h some 3% of the applied radioactivity had been collected in the $^{14}CO_2$. Control flasks with non-sterile plant material had produced some 30% of the total radioactivity as $^{14}CO_2$ practically without a lag phase. Extracts of these aseptically-incubated parsley seedlings were prepared and chromatographed as shown in Fig. 1. The radioscans of the TLC plates and further analyses showed the accumulation of ^{14}C-labelled NMN and NAD (band I), nicotinic acid N-glucoside (band II), nicotinamide N-riboside (band III), nicotinic acid glucose ester (band V), and the mixture of free nicotinic acid/nicotinamide (band VII) in essentially the same ratio as obtained from the cell culture extracts (see Fig. 1). 6-hydroxynicotinic acid (band VIII) as well as bands IV and VI could not be detected.

The data corroborate that nicotinic acid degradation can indeed be assumed to occur in intact plant material. However, the cell cultures appear to be superior experimental systems, because they show a much higher rate of nicotinic acid degradation as measured by $^{14}CO_2$ formation and a much better ability for the accumulation of nicotinate catabolites.

Discussion

This paper is the first report dealing with chemical structures of nicotinic acid degradation products in a plant system. Our comparative studies with parsley seedlings and cell suspension cultures have clearly shown that the latter are the more suitable experimental systems. The advantages refer especially to the rate of nicotinic acid degradation, the pronounced accumulation of catabolites, the ease of conducting trapping experiments and the reliably aseptic conditions. With regard to the accumulation of nicotinic acid conjugates which are part of the pyridine nucleotide cycle (Fig. 1, bands I, III and VII) and other nicotinate derivatives (compounds II and V), there are only slight differences between seedlings and cell suspension cultures.

Fig. 4 presents a scheme of nicotinic acid metabolism and degradation which summarizes our present knowledge. As pointed out before [2, 3, 12, 14, 15], nicotinic acid N-glucoside is the first and most prominent nicotinic acid conjugate of exogenously applied nicotinate. However, this N-glucoside is also an endogenously formed constituent of parsley cell cultures with an average concentration of 35–40 μmol/g fr.w. and a rapid rate of turnover (biological half life approx. 24 h) [21]. We consider this N-glucoside to be a reservoir form of nicotinic acid.

Turnover of nicotinic acid N-glucoside is best explained by the reversibility of the N-glucosyltransferase reaction [14, 15] which provides nicotinate for introduction in the pyridine nucleotide cycle. The above mentioned kinetic data on the rate of accumulation of NMN, NAD, nicotinamide N-riboside, nicotinic acid, and nicotinamide (see Fig. 1 and text) support the assumption that this cycle is operating in parsley cells essentially as in other plant systems ([25] K. Wagner, personal communication).

While turnover and metabolic role of nicotinic acid N-glucoside has been investigated [14, 15, 21], the metabolic fate of nicotinic acid glucose ester (compound V, Fig. 1 and 4) remains to be elucidated. Though numerous glucose esters of aromatic carboxylic acids are metabolically very active intermediates in higher plant metabolism [22], future studies will have to deal with this hitherto unknown nicotinic

acid glucose ester. This latter compound is undoubtedly different from other previously described nicotinoyl glucosides [23, 24] and demonstrates the structural diversity of nicotinic acid glucosyl conjugates. The responsible biosynthetic enzyme (see [14]) of this glucose ester as well as the metabolic relationship between compounds II and V (see Fig. 4) will be the object of future studies. These studies should also deal with the remarkable difference *as to* the concentration of nicotinate required for the formation of compounds II and V.

6-Hydroxynicotinic acid is now considered to be the first intermediate in nicotinate degradation in parsley cells, essentially as in all nicotinic acid catabolizing microorganisms [4–6]. During studies on nicotinate metabolism in tobacco leaves this compound had previously been isolated [18]. The present observation that considerable amounts of exogenously applied 6-hydroxy-^{14}C-nicotinic acid are trapped in form of conjugates readily explains the very low rate of $^{14}CO_2$ formation from this substrate ([3], and this report). Therefore, it must be assumed that non-conjugated, free 6-hydroxynicotinic acid is the essential intermediate during nicotinic acid degradation in parsley cells. The metabolic difference between exogenously applied and endogenously formed products as caused by conjugation reactions has repeatedly been observed [27].

The catabolic sequence in Fig. 4 clearly explains the experimental observation that the rate of $^{14}CO_2$ formation from [7-^{14}C]nicotinic acid has always been much higher than from [6-^{14}C]nicotinic acid. Oxidative decarboxylation of 6-hydroxynicotinic acid to yield 2,5-dihydroxypyridine with subsequent degradation to fumaric acid has been shown to occur during nicotinate degradation in *Pseudomonas fluorescens* [28–30] and other bacteria [7, 31].

In case of parsley cell cultures the first two degradative reactions appear to be identical to what has been found in the bacterial systems. However, the subsequent degradation of 2,5-dihydroxypyridine will most likely not proceed through the stage of N-formylfumaramic acid, fumaramic acid and fumaric acid as known from the bacterial sources [29]. Catabolite IX, though it no longer possesses a pyridine skeleton, is by no means identical with any of the aforementioned aliphatic acids. Except for the known C_3 fragment and the postulated loss of a carboxyl group which would render catabolite IX to be a C_4 compound, no structural suggestions are presently available. Investigations are under way to isolate catabolite IX in larger amounts which are necessary for spectroscopic and chemical methods of structural elucidation. These studies will exclusively deal with underivatized catabolite IX because of the documented sensitivity during preparation of a trimethylsilyl derivative.

Fig. 4 also documents that nicotinic acid metabolism in parsley cell cultures offers an interesting system where a catabolic route and the metabolic function of at least two glucosyl conjugates must be integrated into the regulatory patterns of the pyridine nucleotide cycle and its complex cellular compartmentation. Characterization of the various enzymes involved, determination of the pool sizes of the compounds and measurements of fluxes through the pools offer a means to elucidate the indicated regulatory pattern.

Acknowledgements

Financial support by Deutsche Forschungsgemeinschaft (Forschergruppe "Sekundäre Naturstoffe/Zellkulturen") and Fonds der Chemischen Industrie is gratefully acknowledged. We thank Prof. E. Leistner, Bonn, for performing radiogaschromatographic analyses, Dr. Matthiesen, Düsseldorf, for GC-MS measurements and Dr. Strack, Cologne, for helpful advice concerning HPLC.

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