

Glutaric Acid as a Catabolite of Nicotinic Acid in Parsley Cell Suspension Cultures

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A degradation product of nicotinic acid representing the pyridine carbon skeleton was isolated and purified from parsley cell suspension cultures after incubation with [6-¹⁴C]nicotinic acid for 70 h. The catabolite was identified as glutaric acid by means of spectroscopic (GC-MS and ¹H NMR) and chromatographic (TLC, HPLC) techniques. Glutaric acid when applied to parsley cell cultures was readily degraded to CO₂ but intermediate products could not be identified.

Introduction

The degradation of nicotinic acid in plant cell suspension cultures has repeatedly been observed [1–4] but a complete degradative pathway for the ring carbon skeleton of nicotinate has so far not been reported. Especially the mechanism of the opening of the pyridine ring remains to be elucidated. Nicotinic acid degradation has extensively been studied in various microorganisms and various complete degradative sequences are known [5–7].

In a previous study [8] it was demonstrated that parsley (*Petroselinum hortense*) cell suspension cultures transiently accumulate 9 labelled metabolites from [6-¹⁴C]nicotinic acid whereas only 8 metabolites were found after application of [7-¹⁴C]nicotinic acid. Eight metabolites obtained from the two differently labelled substrates were identical and their structures have been elucidated [4]. However, one metabolite only obtained from ring labelled nicotinic acid and previously designated “metabolite IX” remained to be elucidated. In the catabolic sequence postulated for nicotinate in parsley cell suspension cultures [4],

this metabolite IX could be unequivocally located after 6-hydroxynicotinic acid and 2,5-dihydroxypyridine. These results indicated that metabolite IX lacked the carboxyl group of nicotinate but still contained the ring carbon skeleton of nicotinic acid.

We now report on the purification and structural elucidation of metabolite IX by means of spectroscopic and chromatographic analyses. Metabolite IX could unequivocally be identified as glutaric acid.

Materials and Methods

Cell cultures

Growth and cultivation of parsley cell suspension cultures, the application of sterilized substrates and the apparatus for the radiorespirometric experiments have previously been reported [2, 9].

Extraction procedure

Cells were harvested by filtration, washed with water and suspended in 80% aqueous methanol at 4 °C. Cells were ground in a mortar three times and the combined extracts were concentrated at reduced pressure.

Reagents

[6-¹⁴C]Nicotinic acid (1.67 GBq/mmol) and [1,4-¹⁴C]succinic acid (4.11 GBq/mmol) were purchased from Amersham, whereas [1-¹⁴C]glutaric acid (1.92 GBq/mmol) was from CEA. All other chemicals were purchased from Aldrich (Steinheim), Baker (Groß-Gerau), Merck (Darmstadt), Roth (Karlsruhe) and Sigma (München).

Abbreviations: Glt, glutaric acid; 2-MG, 2-methylene-glutaric acid; Nic, nicotinic acid; 6-OH-Nic, 6-hydroxynicotinic acid; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; GC, gas chromatography; MW, molecular weight; MS, mass spectroscopy/spectrum; NMR, nuclear magnetic resonance; UV, ultraviolet; S, solvent system for TLC; sg, silica gel; c, cellulose; p, polyamide; H, HPLC separation system.

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Syntheses

Diazomethane [10], 2-methyleneglutaric acid [11] and glutaric acid diphenacyldiester [12] were synthesized according to published methods. The latter two substances were also identified by MS- and ^1H NMR spectra.

The localization of labelled carbon in $[1-^{14}\text{C}]$ -glutaric acid and in metabolite IX from the parsley cells was determined by using the Schmidt degradation procedure [13].

Radioactivity

Measurements of radioactivity in CO_2 and in soluble samples were carried out as previously described [9]. Labelled compounds on TLC chromatograms were located according to published reports [14]. For the determination of radioactivity in HPLC eluates the HPLC radioactivity monitor "Ramona 5" (Raytest, Straubenhardt) was used with a 400 μl glass scintillator cell.

Thin layer chromatography

Analytical TLC separations were performed on either silica gel (sg; 0.2 mm layer), cellulose (c; 0.1 mm layer) or polyamide (p; 0.15 mm layer) 20 \times 20 cm plates (Merck, Darmstadt). For preparative TLC silica gel glass plates with 0.5 or 2 mm layers were used.

The solvents for TLC were:

- S 1: *n*-butanol:acetic acid:water, 4:1:1 (sg)
- S 2: acetone:water, 4:1 (sg)
- S 3: methanol: CHCl_3 :water, 3:2:1 (sg)
- S 4: methylethylketone:methanol:acetic acid:water, 5:3:2:1 (sg)
- S 5: *n*-propylacetic acid:formic acid:water, 11:5:3 (sg, c)
- S 6: *n*-butanol:*n*-propanol:water:1 M NH_3 , 25:50:15:1 (sg)
- S 7: *n*-propanol:water:acetic acid, 2:2:1 (sg)
- S 8: acetic acid ethylester:methylethylketone:formic acid:water, 5:3:1:1 (sg, c)
- S 9: CHCl_3 :acetic acid ethylester:formic acid, 5:4:1 (sg, c)
- S 10: ethanol:water:25% NH_3 , 78:9.5:12.5 (sg, c, p)
- S 11: benzene:methanol:acetic acid, 79:14:7 (sg)
- S 12: acetonitrile:acetic acid ethylester:formic acid, 82:9:9 (sg, p)
- S 13: ethanol:25% NH_3 :water, 8:2:1 (sg)
- S 14: acetonitrile:acetic acid ethylester, 9:1 (sg)

High performance liquid chromatography

A Latek chromatograph was used equipped with a pump P400, UV detector SPD2A, Rheodyne injector 7125, Shimadzu CR3A-integrator and a Gynkothek gradient former MB250.

H1: For the separation of nicotinic acid, 6-hydroxynicotinic acid and glutaric acid a Merck LiChrosorb NH_2 -column (250 \times 4 mm, 7 μm) was used. After 10 min of isocratic elution at 95% B a linear gradient elution followed which ranged from 95 to 25% B in (A + B) in 40 min (A: 3% acetic acid, B: CH_3CN); flow 0.8 ml/min, detection at 260 nm.

For analyzing glutaric acid diphenacyldiester two different separation systems were used. H2: A Merck LiChrosorb RP 18-column (250 \times 4 mm, 7 μm) with a linear gradient elution from 30 to 70% B in (A + B) in 30 min (A: H_2O , B: CH_3CN); flow 0.8 ml/min, detection at 241 nm. H3: A Merck LiChrosorb Si60-column (250 \times 4 mm, 7 μm) with CHCl_3 /*n*-hexane 1:4 as eluent; flow 1 ml/min, detection at 254 nm.

For the separation of succinic, glutaric and other dicarboxylic acids two other separation systems were used. H4: Two Waters $\mu\text{Bondapak}$ C18-columns (300 \times 3.9 mm) operating in series with an eluent consisting of 1 ml H_3PO_4 , 249 ml water and 5 ml CH_3CN ; flow 0.675 ml/min, detection at 210 nm. H5: A Waters $\mu\text{Bondapak}$ NH_2 -column (300 \times 3.9 mm) with linear gradient elution from 90 to 50% B in (A + B) in 35 min (A: 3% acetic acid, B: CH_3CN); flow 0.8 ml/min.

Sephadex LH-20

For the isolation of metabolite IX a Sephadex LH-20-column (320 \times 40 mm; Pharmacia) was used with 80% aqueous methanol as eluent.

UV spectra

Absorption spectra were recorded with a Kontron Uvikon 810 spectrophotometer in ethanol or acetonitrile.

Mass spectra

A Varian MAT44S equipped with a Varian DATA188 and a SM1b was used. Chemical ionization was performed with ammonium.

GC/MS spectra

A Hewlett Packard mass spectrometer HP 5970 (MSD coupled with GC 5880A) was used. The GC separations were conducted with a DB-5-column (ICT, length 15 m, interior diameter 0.25 mm); gas flow 4 psi helium. The gas for chemical ionization was 2-methylpropane. Derivatization of compounds was performed with diazomethane in methanol at room temperature.

¹H NMR spectra

¹H NMR spectra were recorded with a Bruker AC300 instrument or a Bruker WM-300 MHz NMR spectrometer.

Structural elucidation of compounds

Compounds were elucidated according to the following spectra.

Glutaric acid

¹H NMR (CD₃OD) δ [ppm] = 1.9 (quintette, ³J = 5.25 Hz; 2H, H-3); 2.3 (t, ³J = 5.25 Hz; 4H, H-2 and H-4).

MS: Samples of isolated metabolite IX as well as of glutaric acid were measured after methylation with diazomethane.

C₇H₁₂O₄, MW 160; MS (*m/z*) 129 (30%, M⁺-OCH₃), 128 (20%, 129-H), 101 (34%, M⁺-COOCH₃), 100 (47%, 101-H), 87 (12%, M⁺-C₂H₄COOCH₃), 74 (8%), 59 (100%, M⁺-C₃H₆COOCH₃), 55 (43%), 42 (45%, 87-OCH₃).

After chemical ionization with 2-methylpropane: (*m/z*) 161 (100%, [M + H]⁺).

2-Methyleneglutaric acid

¹H NMR (CD₃OD) δ [ppm] = 2.4 (t, ³J = 5.25 Hz; 2H, H-4); 2.6 (dd, ⁴J = 2.0 Hz; ³J = 5.25 Hz; 2H, H-3); 5.5 (dd, ⁴J = 2.0 Hz; ²J = 1.9 Hz; 1H, H-1' *trans*); 6.0 (d, ²J = 1.9 Hz; 1H, H-1' *cis*).

MS: Samples of the isolated metabolite IX and of 2-methyleneglutaric acid were measured after methylation with diazomethane.

C₈H₁₂O₄, MW 172; MS (*m/z*) 141 (38%, M⁺-OCH₃), 140 (59%, 141-H), 113 (49%, M⁺-COOCH₃), 112 (100%, 113-H), 97 (17%, 112-CH₃), 82 (35%, M⁺-COOCH₃-OCH₃), 81

(51%, 112-OCH₃), 59 (82%, M⁺-C₄H₆COOCH₃), 54 (33%, M⁺-COOCH₃-COOCH₃).

After chemical ionization with 2-methylpropane: (*m/z*) 173 (100%, [M + H]⁺).

Glutaric acid diphenacyldiester

UV absorption spectrum (CH₃CN) λ_{max} 241 nm, sh 280 nm.

¹H NMR (CDCl₃) δ [ppm] = 2.12 (quintette, 2H, H-3); 2.64 (t, 4H, H-2 and H-4); 5.34 (s, 4H, H-2' and H-2''); 7.50, 7.57, 7.68 (mc, 10H, aromatic phenyl protons).

MS: C₂₁H₂₀O₆, MW 368; MS (*m/z*) 369 (<1%, [M + H]⁺), 351 (<1%, [M + H]⁺-H₂O), 333 (<1%, [M + H]⁺-2H₂O), 233 (76%, M-OCH₂-CO-C₆H₅), 205 (3%, 233-CO), 119 (73%, 205-C₃H₆CO₂), 105 (100%, M-CH₂-O-CO-C₃H₆-CO-O-CH₂-CO-C₆H₅), 91 (55%, C₆H₅-CH₂), 77 (100%, C₆H₅), 65 (17%), 55 (51%).

After chemical ionization with ammonium: (*m/z*) 386 (100%, [M + H]⁺ + NH₃).

Results*Purification of metabolite IX*

For the successful isolation of metabolite IX in amounts sufficient for spectroscopic and chemical investigations 120 parsley cell suspension cultures were incubated with [6-¹⁴C]nicotinic acid (296 KBq, 5 × 10⁻⁴ M) for altogether 70 h. The cells were then extracted with methanol and the extract separated by TLC (sg, S1). As demonstrated in Fig. 1 and in accordance with previous results [8] metabolite IX could well be separated from the various other radioactive compounds. After extraction of metabolite IX from the silica gel with 80% methanol, the eluate was further purified using a Sephadex LH-20-column. Fractions containing metabolite IX were pooled and again subjected to TLC (sg, S8). As a final purification step a HPLC procedure (H1) was used.

After HPLC chromatography metabolite IX could be obtained in a yield of approximately 230 μg as indicated by the specific radioactivity of the applied nicotinic acid and the measured radioactivity in the purified product. The purification procedure and the radioactivity obtained in the various fractions are summarized in Table I.

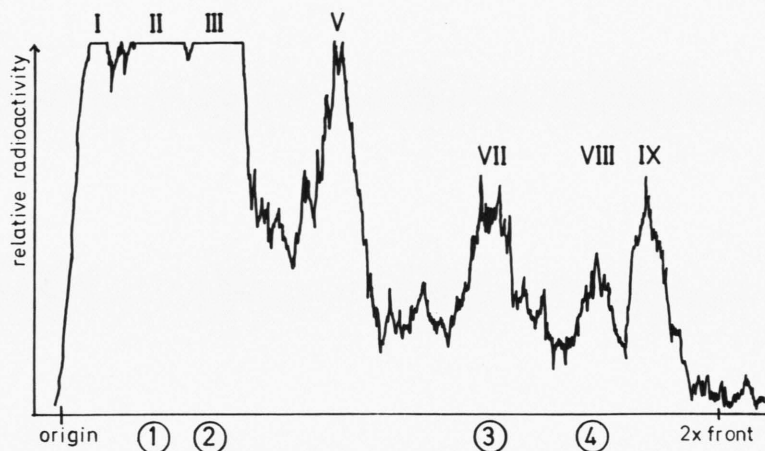


Fig. 1. Radioscan of a TLC plate showing the distribution of radioactivity in extracts of parsley cell suspension cultures after application of [6-¹⁴C]nicotinic acid. Extracts were separated on silica gel in S1 and plates were developed twice. Reference compounds were: ① nicotinic acid N-glucoside; ② nicotinamide N-riboside; ③ nicotinic acid; ④ 6-hydroxynicotinic acid.

Table I. Purification scheme of metabolite IX isolated from parsley cell suspensions.

	Total radio-activity [KBq]	Percent of radio-activity isolated
[6- ¹⁴ C]nicotinate applied	296	100
Methanol extract	243	82.7
TLC (sg, S1)	7.8	2.6
LH-20	1.9	0.6
TLC (sg, S8)	0.45	0.2
HPLC	0.28	0.1

Identification of metabolite IX as glutaric acid

After methylation with diazomethane the purified sample of metabolite IX was subjected to thorough GC- and MS-analyses. The spectra obtained (see Materials and Methods) indicated that the isolated sample consisted of two compounds with glutaric acid as the main product and 2-methyleneglutaric acid as a minor component. This statement is based on the following data.

The mass spectra of the two compounds isolated from the parsley cell suspension cultures and the mass spectra obtained with synthesized reference compounds were completely identical. Furthermore, the molecular mass of the two dimethyl esters of the acids from the cell suspension cultures were corroborated during MS-analyses using chemical ionization with 2-methylpropane as a reactant gas. The ¹H NMR spectra obtained with the isolated compounds and with the reference materials substantiated that

both glutaric acid and 2-methyleneglutaric acid had been obtained during the purification procedure shown in Table I.

During an intensive thin layer chromatographic comparison using [1-¹⁴C]glutaric acid as a marker compound it could be demonstrated that only the glutaric acid isolated from the parsley cell suspension cultures carried the ¹⁴C-label. In all TLC systems applied (S1–S14) scanning of the thin layer plates for radioactivity showed that only glutaric acid was radioactive. Furthermore, in two HPLC systems (H1 and H4) it was found by co-chromatography that the ¹⁴C-labelled metabolite IX was identical with glutaric acid. When the diphenacylesters of metabolite IX and of synthetic glutaric acid were subjected to HPLC analysis (H2 and H3) identity of the products was clearly substantiated. These data unequivocally demonstrate that metabolite IX is identical with glutaric acid and that the isolated 2-methyleneglutaric acid can be excluded as a catabolite of nicotinic acid in parsley cell suspensions. The occurrence of glutaric acid in nicotinate degradation in parsley cells was further demonstrated by an isotope dilution experiment. The formation of ¹⁴CO₂ from [6-¹⁴C]nicotinic acid in parsley cell suspension cultures was substantially reduced when exogenous glutaric acid was simultaneously applied to the culture medium (Fig. 2). The dilution effect caused by exogenous glutaric acid could also be seen in the altered accumulation of labelled metabolite IX from [6-¹⁴C]nicotinic acid. A thin layer chromatographic comparison of the cell extracts of parallel experiments carried out with or without exogenous glutaric

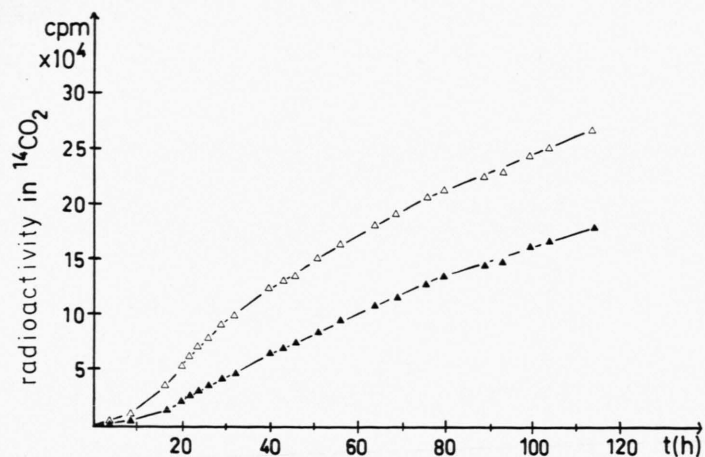


Fig. 2. Formation of $^{14}\text{CO}_2$ from $[6\text{-}^{14}\text{C}]$ nicotinic acid in parsley cell suspension cultures in the absence ($\Delta\text{---}\Delta$) and in the presence of exogenous glutaric acid ($\blacktriangle\text{---}\blacktriangle$) $[10^{-5}\text{ M}]$.

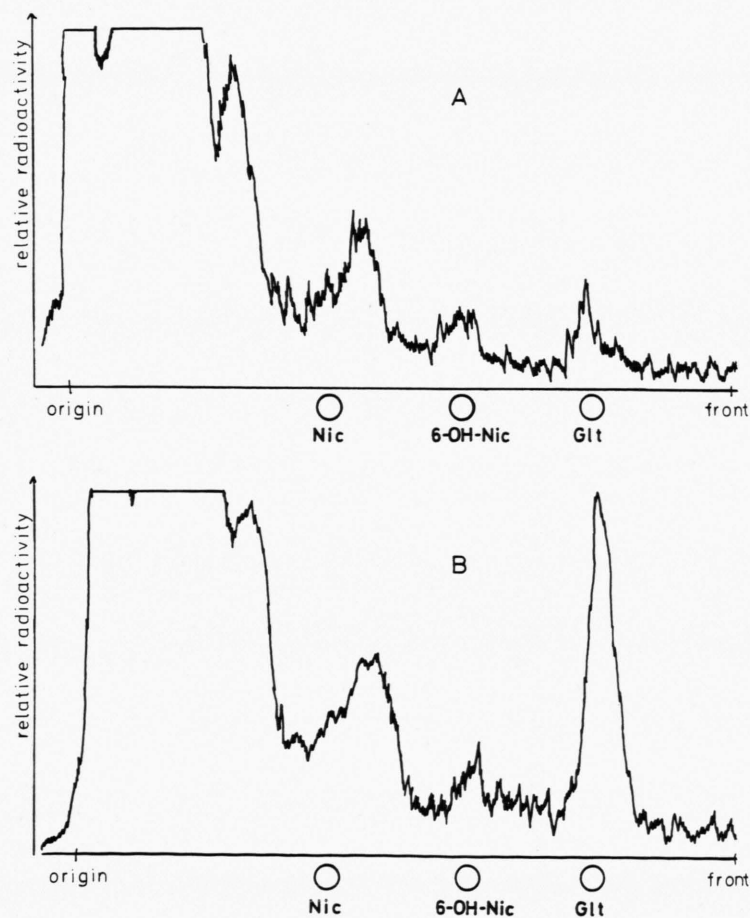


Fig. 3. Radioscans of TLC plates showing the distribution of radioactivity in extracts of parsley cell suspension cultures after incubation with $[6\text{-}^{14}\text{C}]$ nicotinic acid in the absence (graph A) and in the presence (graph B) of exogenous glutaric acid. Extracts were separated on silica gel in S12. Exogenous glutarate $[10^{-5}\text{ M}]$ leads to a much higher accumulation of radioactivity in Glt, *i.e.* metabolite IX.

acid (Fig. 3) revealed that the amount of radioactivity in metabolite IX was much higher when the cells degraded [6- ^{14}C]nicotinic acid in the presence of exogenous glutaric acid.

In order to obtain further insight into the mechanism of nicotinic acid degradation in parsley cell suspension cultures the ^{14}C -label in metabolite IX was localized by chemical degradation. When the sample of ^{14}C -glutaric acid was subjected to Schmidt degradation it could be shown that the radioactivity was exclusively found in the carboxyl groups of glutarate. Since glutarate is a symmetrical molecule it must be assumed that [1- ^{14}C]glutaric acid was formed from [6- ^{14}C]nicotinic acid.

Metabolism of glutaric acid

After the successful demonstration of glutaric acid as a catabolite of nicotinic acid in parsley cell suspension cultures experiments were carried out to elucidate the subsequent metabolism of glutarate.

[1- ^{14}C]Glutaric acid (37 KBq/flask; 10^{-5} M) was applied to parsley cell suspension cultures at day 7 of the growth cycle. The compound was rapidly taken up from the growth medium and during a 72 h incubation substantial $^{14}\text{CO}_2$ -formation was observed after a lag-phase of some 12 h. During this experiment more than 40% of the applied radioactivity appeared in CO_2 . Extracts of such cells were subjected to HPLC analysis (H4) and the distribution of radioactivity in the HPLC eluate was monitored. In addition to glutaric acid at least 4 other radioactive compounds were detected which appeared to be aliphatic carboxylic acids as judged from their chromatographic behaviour.

During subsequent isotope dilution analyses using various carboxylic acids, succinate caused a significant decrease in the formation of $^{14}\text{CO}_2$ from [1- ^{14}C]glutaric acid. This observation led to the assumption that succinate could be a metabolite of glutaric acid in the parsley cell suspension cultures. However, despite intensive efforts to identify succinate as a labelled catabolite of [1- ^{14}C]glutarate, our TLC- and

HPLC analyses of the cell extracts of labelled catabolites failed to demonstrate either succinate or any other aliphatic carboxylic acid as a degradation product of glutaric acid. Future experiments on the metabolism of glutaric acid in parsley cell suspension cultures will have to demonstrate the exact route of degradation of this C_5 -acid.

Discussion

The successful isolation of glutaric acid as a degradation product of nicotinic acid represents the first report for a plant system on an aliphatic compound as a catabolite of the pyridine ring skeleton. The present knowledge of nicotinate catabolism in parsley cell cultures is depicted in Fig. 4.

In microorganisms glutarate has been demonstrated as a lysine catabolite in *Pseudomonas* [15, 16], various yeasts or fungi [17]. *Nocardia* species degrade pyridine [18] via glutarate and *Pseudomonas fluorescens* catabolizes 1-methylpyridinium iodide [19] to glutarate. The degradative sequence of 4-phenylbutyric acid in *Phenylobacterium immobilis* [20] has also been shown to include glutarate.

In our previous report [4] 6-hydroxynicotinic acid and 2,5-dihydroxypyridine have been described as nicotinate catabolites in parsley cell cultures. 6-Hydroxynicotinic acid seems to be the first product formed during nicotinate degradation in all microbial systems [5, 21]. The subsequent oxidative decarboxylation yielding 2,5-dihydroxypyridine is only known to occur in aerobic microorganisms such as *Pseudomonas fluorescens* [6, 22, 23]. However, in these microbial degradative pathways of nicotinic acid glutaric acid has not been found to occur but rather fumaric acid. This latter compound is most likely not a major catabolite of nicotinate in the parsley cells.

During anaerobic degradation of nicotinic acid by *Clostridium barkeri* [7] 2-methyleneglutaric acid has been demonstrated as an intermediate which is further metabolized to propionate and pyruvate. The 2-methyleneglutaric acid co-isolated with glutarate from the parsley cell suspension cultures must, how-

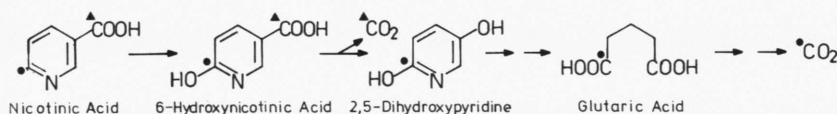


Fig. 4. Established pathway of nicotinic acid degradation in parsley cell suspension cultures.

ever, be excluded as a nicotinate catabolite because it did not carry radioactivity from the [6-¹⁴C]nicotinic acid used as a substrate. Furthermore, if 2-methyleneglutaric acid would be an intermediate in nicotinate degradation in the parsley cells it should also have appeared in labelled form after application of [7-¹⁴C]nicotinic acid; this has so far never been observed [8, 24, 25]. The source of the 2-methyleneglutarate in parsley cells remains to be determined.

After the structural elucidation of metabolite IX we must withdraw previous statements [8] claiming this catabolite IX to be a glycerol derivative. The earlier report was obviously based on insufficient purification procedures for metabolite IX though our observations [8] on the electrophoretic mobility of this catabolite indicating a carboxylic acid (anion between pH 4 to 8, neutral molecule at pH 2; glutaric acid pK_{S1} 4.34, pK_{S2} 5.42) match the data obtained in this study. The localization of the labelled carbon atom derived from [6-¹⁴C]nicotinic acid in a carboxyl group of glutarate leads to the assumption that the pyridine ring may have been hydrolytically opened at

the pyridone carbon atom 2 of 2,5-dihydroxypyridine. Future studies must then reveal the mechanism of elimination of the nitrogen atom from carbon 6. Furthermore, it is decisive to clarify whether the isolated glutarate still contains the complete and un-rearranged C₅-carbon chain of 2,5-dihydroxypyridine. Investigations on nicotinate catabolism in parsley cell cultures using [2-¹⁴C]- or [3-¹⁴C]nicotinic acid with subsequent stepwise chemical degradation of the isolated glutarate will reveal how this dicarboxylic acid is formed from 2,5-dihydroxypyridine. Similarly, glutarate metabolism in parsley cells requires elucidation of the aliphatic acids derived from this nicotinate catabolite.

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