Metabolism of Anthranilic Acid in Plant Cell Suspension Cultures

Johannes Köster, Monika Ohm, and Wolfgang Barz

Lehrstuhl für Biochemie der Pflanzen der Universität Münster

Cell suspension cultures of some 12 plants were investigated for anthranilic acid metabolism. Rapid uptake of substrate is accompanied by partial excretion of anthranilic acid-N-glucoside and followed by predominant conversion into tryptophan. Ring cleavage reactions of anthranilate could not be observed but peroxidatic polymerisation occurred to a high percentage. Anthranilic acid-N-glucoside is not permanently stored by the cell cultures.

Introduction

In microorganisms and plants anthranilic acid occupies a central position as intermediate in tryptophan biosynthesis [1, 2] and in the formation of various classes of alcaloids [3, 4] and antibiotics [5]. Furthermore, animals and fungi use anthranilate [6] or 3-hydroxyanthranilate [4] derived from tryptophan as substrate for nicotinic acid biosynthesis. Though various studies with plants [7 — 11] and plant cell cultures [12 — 15] report the efficient conversion of anthranilate into tryptophan or anthranilic acid glucoside nothing is known neither on the degradation of this acid in plant cells nor on its oxidative incorporation into insoluble polymeric structures [16]. This lack of information is in contrast to both the substantial knowledge on microbial degradation of anthranilate [17 — 19] and the previously observed capability of plant cells for the degradation of various aromatic compounds [22].

We now report experiments carried out to measure ring fission reactions of anthranilic acid and also its polymerisation into polymers. We hereby determined the possible advantages of plant cell cultures for the eventual elucidation of anthranilate catabolism. This seemed mandatory in view of the sensitivity of anthranilate or its hydroxy derivatives for oxidative reactions [20, 21]. Therefore, rate of conversion of anthranilic acid into tryptophan versus ring fission versus incorporation into polymeric structures [16] or conjugates [22] were of special interest.

Experimental

Cell cultures and feeding experiments

Cultivation of cell suspension cultures, application of autoclaved (20 min, 120 °C) substrate, collection of $^{14}CO_2$, fractionation of cells, measurement of soluble and insoluble material for radioactivity and determination of radioactivity on chromatograms was accomplished as previously described [23 — 26].

Cell cultures were used 7 days after inoculation of cells into fresh medium and carefully checked for sterility.

Materials

[Carboxyl-^{14}C]anthranilic acid (spec. radioact. 48 mCi/mmol) and [U-^{14}C]aniline (0.116 mCi/mmol) were obtained from the Isotopendienst West, Frankfurt.

[Ring-U-^{14}C]anthranilic acid was prepared from [U-^{14}C]aniline as described in [27] *via* [ring-^{14}C]isatine [28] (1 mmol) using 6 mmol conc. H_2SO_4. [Ring-U-^{14}C]anthranilic acid (spec. radioactivity 0.113 mCi/mmol) was checked for purity in systems S 1, S 3, S 4, S 5, and S 6 on Whatman 3 MM paper.

Hog-liver esterase (E.C. 3.1.1.1), β-glucosidase (E.C. 3.2.1.21) and glucose-oxidase/peroxidase standard for glucose determination were obtained from Boehringer, Mannheim. Horseradish peroxidase was purchased from Roth, Karlsruhe.

Chromatography

TLC on silicagel or PC were carried out with the following solvent systems

S 1: ethylacetate : water : acetic acid = 8 : 1 : 1,
S 2: *n*-butanol : acetic acid : water = 4 : 1 : 1,
S 3: toluene : ethylformate : formic acid = 5 : 4 : 1,

S 4: ethylacetate : methyl ethyl ketone : water : formic acid = 5 : 3 : 1 : 1,

S 5: isopropanol : 25% eq. ammonia : water = 8 : 1 : 1,

S 6: *n*-butanol sat. with water.

Experiments with peroxidase

Oxidation of [carboxyl-^{14}C]anthranilate (10^{-5} M) and [ring-^{14}C]anthranilate (10^{-4} M) were treated with horseradish peroxidase and H_2O_2 (0.8 M) in 0.05 M acetate buffer (pH 5.0) according to Ref. [24]. [14]CO_2 was collected as described [24] and samples drawn every 10 min for 2 h were chromatographed in S 1 after the enzyme reaction had been stopped with acetic acid.

Hydrolyses

The purified anthranilic acid conjugate (S 2, S 1) was either hydrolyzed with 5% HCl or with esterase and β-glucosidase, respectively according to established procedures [29]. Protein in insoluble cell material was hydrolyzed with 6 N HCl in the standard way [25].

Results and Discussion

Uptake of anthranilic acid by cell cultures

Organic substrates are in general well taken up by plant cell cultures, but negative examples [22] require the determination of rate of uptake in each case. As an example of such studies with anthranilate Fig. 1 demonstrates the effect of substrate concentration on rate of uptake by soybean cells. Though quantitative uptake is eventually reached the results are unusual because an early phase of rapid uptake is followed by a transient increase of radioactivity in the medium.

Chromatographic analysis (TLC in S 1) of aliquots of the nutrient medium during the first 60 min of uptake revealed that the cells excreted some anthranilate metabolites of which the anthranilic acid glucoside was especially prominent. Comparative studies with [ring-U-^{14}C]- and [carboxyl-^{14}C] anthranilate revealed that some of the excreted material no longer contained the carboxyl group. The formation of some polymeric material due to peroxidases in the medium could also be observed (see below). While most conjugates are intracellularly stored by cultured plant cells [22] without any efflux such observations as shown in Fig. 1

have only occasionally been made with some steroids [30], lumichrome [31], hydroxypyridines [32] and NAD [33]. The rational and the biochemical basis of these "leaking" reactions in comparison to the "normal" unidirectionally accumulating processes are not known.
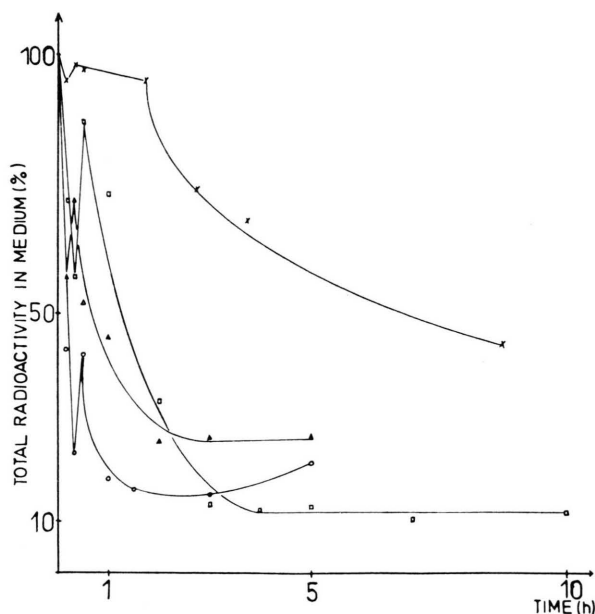

Fig. 1. Uptake of [ring-U-^{14}C]anthranilic acid in soybean cell suspension cultures as measured by decrease of radioactivity in the nutrient medium. Substrate concentration varied from 5×10^{-4} M ($\times - \times$), 1×10^{-4} M ($\square - \square$), 5×10^{-5} M ($\triangle - \triangle$) to 10^{-5} M ($\bigcirc - \bigcirc$).

Experiments with labelled anthranilic acid

Conversion of anthranilic acid into tryptophan is accompanied by loss of the carboxyl group [3]. Various experiments with [carboxyl-^{14}C]anthranilate (10^{-5} M) in cell suspension cultures demonstrated (Fig. 2) the very rapid formation of labelled CO_2 which amounted to $50-75\%$ of the total radioactivity and ceased after approx. 5 h. When soybean cell cultures were incubated with [carboxyl-^{14}C] anthranilate under anaerobic conditions for three h [23] the rate of [14]CO_2 formation remained linear but fell to approx. one third of the value shown in Fig. 2. After reentry of air normal rate of decarboxylation could be observed. This decrease of anthranilate decarboxylation during anaerobiosis led to the assumption that some oxygen requiring reactions might be involved in the rapid processes shown in Fig. 2. This could also be expected because the
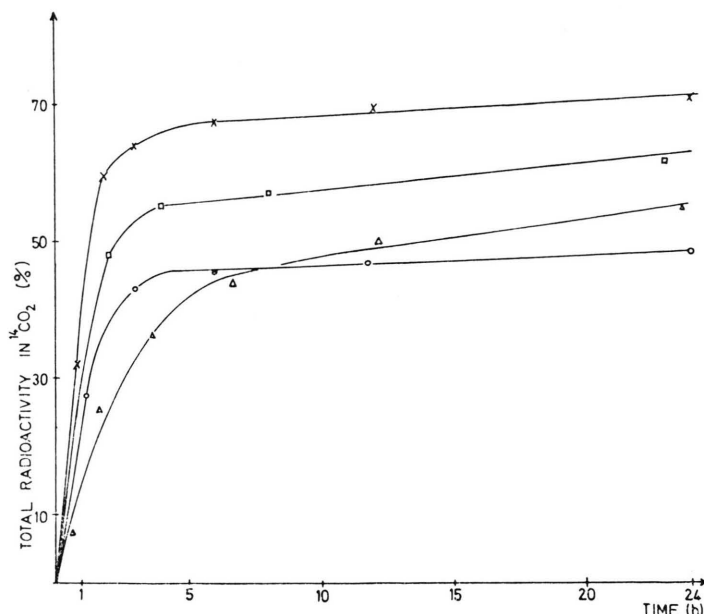
Fig. 2. Formation of $^{14}CO_2$ from [carboxyl-^{14}C]anthranilic acid in cell suspension cultures of soybean ($\times - \times$), mungbean ($\bigcirc - \bigcirc$), parsley ($\triangle - \triangle$ and garbanzo bean ($\square - \square$). Incubations were carried out with 1 μCi per flask, identical amounts of cells and the results were repeated at least twice.

oxygen-requiring, peroxidatic decarboxylation of *p*-hydroxybenzoic acids in cell cultures [24] has led to curves similar to those in Fig. 2 which were characterized by an almost immediate beginning of CO_2-formation. Cell-free experiments with horse radish and soybean peroxidases under established procedures [24] were therefore conducted but they all failed to show any peroxidatic decarboxylation of [carboxyl-^{14}C]anthranilic acid. Similar experiments with [ring-U-^{14}C]anthranilic acid and horse-radish peroxidase also failed to produce any $^{14}CO_2$. Therefore, peroxidases should be eliminated as anthranilate degrading enzymes such as recently determined to be involved in catechol catabolism [34]. The experiments with peroxidases did, however, show (TLC in S 1 and S 2) that the polymeric material as found in the nutrient media after anthranilate application (see above) and as extracted from the cells (Fig. 3) or as found in the insoluble cell residues (Table I) has its origin in peroxidatic reactions.

That the very rapid and strong $^{14}CO_2$ formation from [carboxyl-^{14}C]anthranilic acid (Fig. 2) is almost exclusively due to tryptophan synthesis was corroborated by experiments with [ring-U-^{14}C] anthranilate (10^{-4} M). Thus rate of conversion of anthranilic acid into the amino acid and all other detectable metabolites was measured and typical results as obtained with the soybean cell suspension

Table I. Percent distribution of radioactivity in CO_2 and insoluble cell residue in plant cell suspension cultures after application of [ring-U-^{14}C]anthranilic acid.

Cell culture	Total radioactivity [%] in		$^{14}CO_2$ [%]
	insoluble cell residue	$^{14}CO_2$	g fresh weight
Petroselinum hortense	18	0.18	0.015
Phaseolus aureus	36	0.55	0.05
Chenopodium rubrum	13	0.2	0.02
Glycine max	20	0.17	0.04
Cicer arietinum	6.5	0.25	0.03
Ruta graveolens	15	0.25	0.03
Daucus carota	11	0.19	0.03
Nicotiana tabacum	15	0.24	0.02
Nicotiana glauca	11.4	0.13	0.02
Sinapis alba	19	0.25	0.03
Galium verum	22	0.4	0.08
Triticum aestivum	25	0.6—1.3	0.4—0.9

culture are shown in Fig. 3. Decrease of anthranilic acid in the cell extracts is exactly mirrored by appearance of label in tryptophan and this reaction is much more rapid than formation of anthranilic acid-N-glucoside. The latter compound is not permanently stored (comp. Ref. [22]) but gradually funnelled into anthranilate metabolism. The only transient accumulation of the glucoside became even more evident when cells are preincubated with tryptophan (5×10^{-4} M) before anthranilic acid
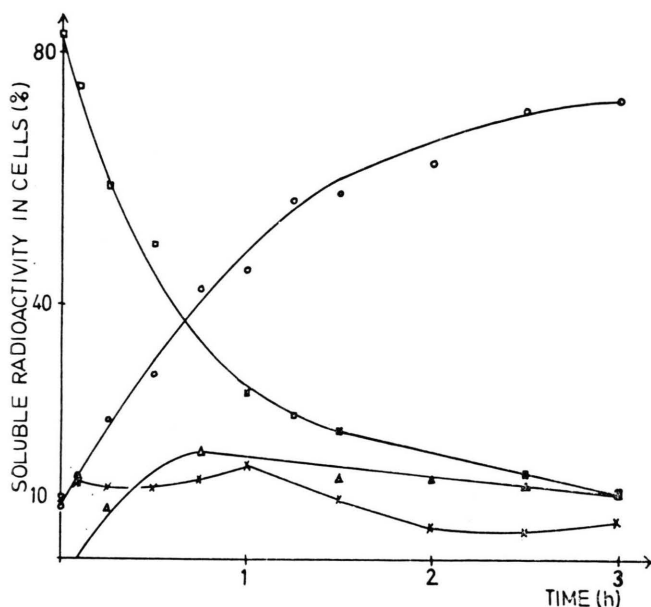
Fig. 3. Distribution of radioactivity in extracts of soybean cell suspension cultures after application of [ring-U-^{14}C] anthranilic acid. Label was found in anthranilate ($\square-\square$), tryptophane ($\bigcirc-\bigcirc$), anthranilate-N-glucoside ($\triangle-\triangle$) and polymeric material ($\times-\times$).

(1×10^{-4} M) was applied. Then, up to 40% of the total radioactivity appeared in the glucoside within 2 h and this value again fell to some 15% after another 6 h. Apart from some polymeric material which remained at the origin of chromatograms (PC in S 1) no other labelled compounds than those mentioned in Fig. 3 could be found in several TLC systems. Conflicting data in the literature as to whether the main anthranilate conjugate is the glucose ester [7 – 9] or the N-glucoside [13, 14] forced us to elucidate the structure of the metabolite found in our cell cultures. Material obtained from incubations with [ring-U-^{14}C]anthranilic acid was purified by TLC in S 1 and S 2. Acid hydrolysis yielded anthranilate (TLC in S 1 and S 2) and glucose (enzymatic determination). Quantitative enzymic hydrolysis of aliquots of the conjugate by β-glucosidase but not by hog-liver esterase (see

Experimental) showed that anthranilic acid-N-glucoside had been isolated from the cell suspension cultures.

Except for the anthranilate-derived benzene ring of tryptophan [35], no evidence for anthranilic acid ring fission reactions has so far been obtained in plant systems. Therefore, studies with [ring-U-^{14}C] anthranilic acid (10^{-4} M) in some 12 cell suspension cultures were conducted to determine ring fission by measuring $^{14}CO_2$-formation. The data in Table I, however, document that except for the wheat cell culture the values from the others are negligibly small. In view of the high values obtained in plant cell suspension cultures with such well degradable compounds as tyrosine and homogentisic acid [36] or nicotinic acid [37] these results document that anthranilate is essentially used for biosynthetic purposes. Catabolites of anthranilate were not observed by chromatographic analyses of cell extracts though they could have been expected to partly accumulate in conjugated form [26].

The very high incorporation (Table I) of [ring-^{14}C]anthranilic acid into insoluble cell residues must mainly be explained by peroxidatic reactions (see above and Ref. [25]) and not by [^{14}C]tryptophane-containing protein. This became evident by unsuccessful attempts to remove such labelled protein by hydrolysis with 6 N HCl. Insignificant amounts of radioactivity could only be solubilized by this treatment leading to the known fibrous residual material previously shown to contain peroxidase-catalyzed phenolic polymers [25]. This type of reaction seemed to proceed especially well with such cultures as *Ph. aureus* or *T. aestivum* which were previously shown to possess higher peroxidase levels [25].

In summary, the data demonstrate that in plant cell cultures anthranilic acid is essentially a biosynthetic intermediate which is practically not funnelled into degradative pathways.

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