Metabolism of Amitrole in Apple: I. Soluble Metabolites from Mature Fruits

Bernd Schneider, Manfred Stock, Gernot Schneider, Horst Robert Schütte, Klaus Schreiber
Institute of Plant Biochemistry, Weinberg 3, O-4050 Halle/S.

Angela Brauner, Ernst Ulrich Kaußmann
Bayer AG, Crop Protection Research, Environmental Research, Institute for Metabolism, W-5090 Leverkusen, Bayerwerk

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Mature fruits from apple trees contain residual amounts of radiolabel derived from soil-applied [3,5-^{14}C]amitrole. This report deals with characterization of the soluble part of this radioactivity.

By GC/MS of suitable derivatives 3-(1,2,4-triazol-1-yl)-2-aminopropionic acid was identified as a new metabolite of amitrole. Significant parts of radiolabel were incorporated into genuine plant products, indicating liberation of $^{14}CO_2$ from the applied substance followed by reassimilation. Possible pathways of metabolism of amitrole within the system soil/apple tree are discussed.

Introduction

Except for CO_2 [1], only few metabolites were identified in earlier studies on the metabolism of amitrole in plants. Amitrole may form conjugates with carbohydrates. Thus a glucose conjugate was assumed to exist [2] which after isomerization to the corresponding fructose conjugate by effect of aldolase is thought to be converted to a triose conjugate [3]. However, it cannot be excluded that the glucose conjugate is an artefact [4].

The metabolite most frequently found in different plants is 3-(3-amino-1,2,4-triazol-1-yl)-2-aminopropionic acid (aminotriazolylalanine) [5–7] whose chemical structure was determined later and termed 3-ATAL [8]. Besides aminotriazolylalanine, further metabolites with unknown structure were detected. In a recent study [9] aminotriazolylalanine, a so-called metabolite Y, and other unidentified metabolites were found. In soils, amitrole is rapidly degraded by microbial [10] and abiotic [11] processes. Degradation of the triazole ring by oxidative or radical mechanisms [12] is possible, both mechanisms yielding urea, cyanamide, and CO_2. More detailed information on this subject is given by Carter [4], Gräser [13], and Ashton and Crafts [14].

Abbreviations: amitrole, 3-amino-1,2,4-triazole; fw, fresh weight; HFAA, heptafluorobutyric anhydride; HFB, heptafluorobutyryl; LSC, liquid scintillation counting.

Reprint requests to Prof. Dr. H. R. Schütte.

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Materials and Methods

Radiochemicals and application

[3,5-^{14}C]Amitrole, prepared by Bayer AG, Wuppertal, was applied as aqueous solution in 2 doses (May, July) to the soil below 5 year old apple trees under outdoor conditions and in tubs (double-walled PVC tubs with polystyrene foam insulation were used [15], soil surface 70 × 70 cm, soil volume 0.245 m^3). The apple trees were planted to the tubs in November, 6 months before the first application of [3,5-^{14}C]amitrole and were placed outdoors in an apple plantation. Outdoor experiments (1) and (2) "Golden Delicious" (400 mg [3,5-^{14}C]amitrole, 92 MBq × mmol^{-1}); tub experiment (3) "Golden Delicious" (400 mg [3,5-^{14}C]amitrole, 61.3 MBq × mmol^{-1}); tub experiment (4) "Gloster" (400 mg [3,5-^{14}C]amitrole, 61.3 MBq × mmol^{-1}).

Extraction

After harvest in October, the mature apples were homogenized and extracted with acetonitrile at −10 °C, followed by filtration and chloroform/water partition (Fig. 1).

Ion exchange and adsorbtion chromatography

After chloroform/water partition, the aqueous phase was chromatographed over strong basic anion exchange resin (Lewatit MP 500, elution with

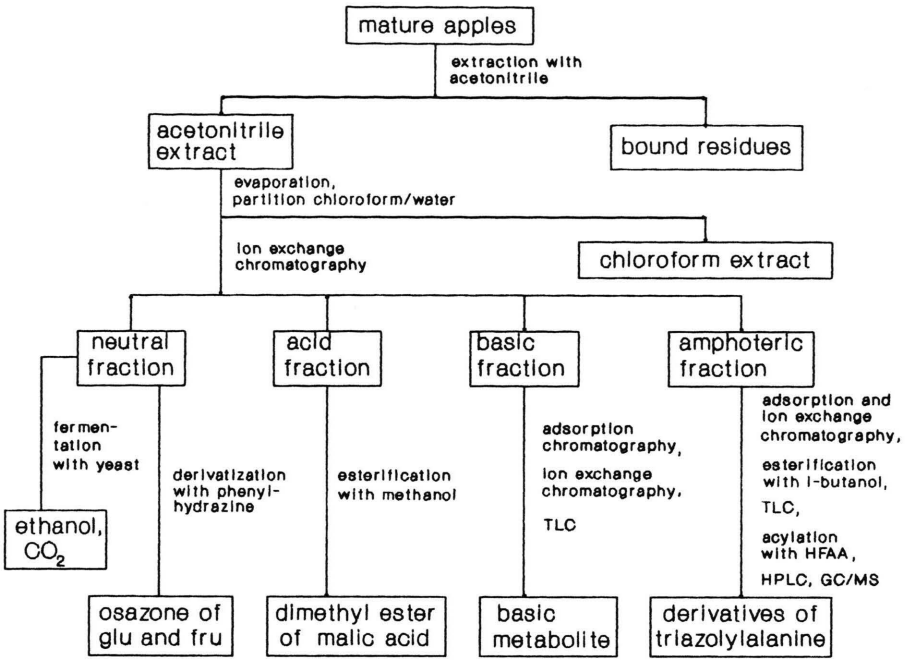

Fig. 1. Scheme of extraction and separation of metabolic fractions of mature apples after application of 400 mg [3,5-^{14}C]amitrole (61.3 MBq × mmol^{-1}) to apple trees.

5 N HCOOH) and strong acid cation exchange resin (Lewatit S 100, elution with 2 N NH_3).

The amphoteric fraction and the basic fraction were purified by adsorption chromatography on Amberlite XAD-2 (0.2–0.25 mm), followed by ion exchange chromatography of the aqueous effluence from the XAD column on Lewatit TSW 40 (elution with 1 N HCl and afterwards with 1 N NH_3). The alkaline eluate was used for derivatization.

TLC

TLC was carried out on silica gel 60 plates from Merck, Darmstadt, 2 mm thickness, with a concentration zone for preparative mode, and had a 0.25 mm thickness for the analytical mode. As solvent systems were used: 1. *n*-propanol/ethyl acetate/water (v/v/v) 60:10:30; 2. chloroform/methanol/ammonia (25%) (v/v/v) 60:9:1; 3. *i*-propanol/ammonia (25%)/water (v/v/v) 70:15:15; 4. *n*-butanol/acetic acid/water (v/v/v) 60:20:20; 5. cyclohexane/ethyl acetate/acetone (v/v/v) 50:50:25.

HPLC

HPLC was performed with a HP 1090 Liquid Chromatograph (Hewlett-Packard). Gradient 1: cyclohexane/ethyl acetate/acetone (v/v/v), silica gel Si 100 Polyol, Serva, 4.6 × 250 mm; gradient 2: acetonitrile/water (v/v), RP 8, MOS Hypersil 5 µm, 4.6 × 200 mm; isocratic elution: acetonitrile/water (v/v) 50:50; 0.6 ml/min.

Measurement of radioactivity and spectrometric methods

The radioactivity of liquid samples was determined by LSC of aliquots with a Tricarb 2260 (Packard Instruments). Solid samples (osazone, plant residues) before LSC were incinerated (OX 300, Harvey Instr. Corp.).

The TLC plates were analyzed for radioactive zones with an automatic TLC linear analyzer LB 2832 (Berthold). ^{14}C detection during HPLC was performed with a IM 2021 "Ramona".

GC/MS (EI) of the derivative of triazolylalanine was carried out with HP 5970 with GC 5880 A (Hewlett-Packard).

Derivatization

For esterification with *i*-butanol, the dry sample was refluxed for 3 h with 50 ml *i*-butanol/3 N HCl and then evaporated. This procedure was repeated 2 times and then the sample was treated with 50 ml water and 1.5 ml conc. HCl. This solution was extracted 3 times with dichloromethane, adjusted to pH 10, and extracted with dichloromethane again. This "basic" dichloromethane extract after TLC (solvent system 2) was used for acylation with HFAA: The dry sample was refluxed for 1 h with 15 ml ethyl acetate and 0.25 ml HFAA. The volume of this solution was reduced to 0.05 ml under a stream of nitrogen. This had to be very carefully executed because of the extremely high volatility of the "double" derivative.

Esterification of malic acid was performed with methanol/HCl.

Osazone: 25 ml of the neutral fraction, containing about 5 g of carbohydrates, was refluxed with a solution of 12 ml phenylhydrazine and 12 ml glacial acetic acid in 50 ml water for 30 min. The yellow osazone was filtered, washed with water, ethanol, and ether, and recrystallized 5 times from ethanol. The pure osazone (m.p. 210 °C) co-chromatographed with the authentic reference compound (solvent system 5, 2 times developed).

Enzymatic methods

For alcoholic fermentation, 50 ml of the neutral fraction were incubated with wine yeast (34 °C, pH 4). The released CO_2 was absorbed with soda lime, liberated by HCl, and absorbed with a solution of 12.5% phenylethylamine in methanol. The ethanol was distilled from the solution and collected. The radioactivities were measured by LSC.

The quantification of glucose and fructose in the neutral fraction was performed with an enzymatic UV test (Boehringer, Mannheim).

Results

In outdoor and tub experiments with [3,5-^{14}C]amitrole, applied to soil, the radioactivity of mature fruits was determined. The apples from outdoor experiment (1) contained 0.013% (16.4 Bq × g^{-1} fw) of the applied radioactivity, outdoor experiment (2) 0.034% (19.4 Bq × g^{-1} fw), tub experiment (3) 0.048% (137.1 Bq × g^{-1} fw) and tub experiment (4) 0.064% (94.5 Bq × g^{-1} fw). Fig. 2 demonstrates the distribution of radiolabel within various fractions obtained from mature apples after the extraction and purification procedure according to Fig. 1.

Significant parts of the radioactivity were found in bound residues [16]. The major portion of radiolabel (about 68–80% from experiments with "Golden Delicious" and 50% from "Gloster") were extracted with acetonitrile. From the aqueous phase, remaining after evaporation of the crude acetonitrile extract with chloroform, only small portions (1.5–5.5%) of radioactivity were extracted. The hydrophilic radioactive substances

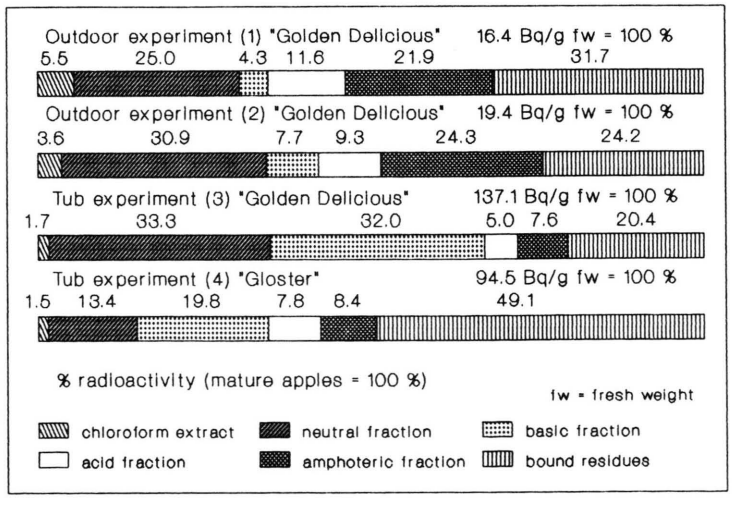

Fig. 2. Distribution of radioactivity in various fractions of mature apples after application of 400 mg [3,5-^{14}C]amitrole (61.3 MBq × $mmol^{-1}$) to apple trees.

were present within the aqueous phase. In the experiments with "Golden Delicious" after ion exchange chromatography about 25–33% of the radioactivity were found in the neutral fraction. The acid fraction contained only minor portions of about 5–12%.

Differences in the metabolism between outdoor and tub experiments were indicated by the portions of radioactivity in the basic and amphoteric fractions. Besides the neutral fraction, the amphoteric fraction with more than 20% of radiolabel was the major soluble fraction obtained from outdoor experiments, and the basic one from the tub experiments (Fig. 2).

Amphoteric fraction

3-(1,2,4-Triazol-1-yl)-2-aminopropionic acid (triazolylalanine) was the main metabolite of amitrole in the amphoteric fraction from mature apples. It occurred in free form and as conjugates, probably with carbohydrates. TLC (solvent system 1) showed the peaks of triazolylalanine (R_F 0.47) and its conjugate (R_F 0.22). The polar conjugate was not stable under the acid and alkaline conditions of ion exchange chromatography and was hydrolyzed. Amitrole itself was no more detectable.

After ion exchange chromatography, triazolylalanine was esterified with *i*-butanol. During preparative TLC (solvent system 2), the *i*-butylester (R_F 0.67) in part was transesterified to the methylester (R_F 0.48).

Acylation of the *i*-butylester and also of the methylester was performed with HFAA. Structural elucidation of the "double" derivatives after 3 times purification by means of HPLC was performed with GC/MS, confirming the structures of the derivatives of triazolylalanine as heptafluorobutyryltriazolylalanine-*i*-butylester (Fig. 3) and the corresponding methylester (*e.g.* *m/z* 366, parent peak; *m/z* 307, M–$COOCH_3$).

Basic fraction

TLC of the basic fraction showed only one radioactive peak (R_F 0.5 in solvent system 3). This basic metabolite was purified similar as aminotriazolylalanine by adsorption chromatography and ion exchange chromatography. The purification and derivatization procedure included extensive loss of the very volatile heptafluorobutyryl derivative. Therefore identification was not possible.

Acid fraction

The major natural compound in the acid fractions from mature apples was malic acid. It was identified as free acid by TLC (solvent system 4, R_F 0.3) and after esterification with methanol as the dimethylester by MS (*m/z* 162, parent peak; *m/z* 131, M–OCH_3; *m/z* 130, M–CH_3OH; *m/z* 103, M–$COOCH_3$). The purified malic acid dimethylester (distillation, $b.p._3$ 84 °C) contained all the radioactivity of the neutral fraction, indicating that reassimilated $^{14}CO_2$ was incorporated.

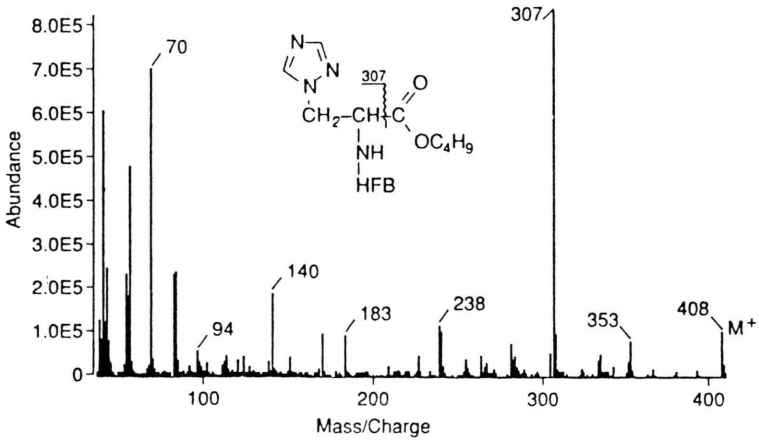

Fig. 3. Mass spectrum of triazolylalanine (heptafluorobutyryl amide, *i*-butylester) isolated from mature apples after application of 400 mg [3,5-^{14}C]amitrole (61.3 MBq × $mmol^{-1}$) to apple trees.

Neutral fraction

Various experiments to isolate definite metabolites of amitrole from the neutral fraction failed. By means of fermentation with yeast it was demonstrated that the radiolabel was associated with the carbohydrates within this fraction. CO_2 and ethanol, both liberated from carbohydrates, contained up to more than 80% of the radioactivity of the neutral fraction. Because of experimental difficulties the ratio $^{14}CO_2$:[^{14}C]ethanol was not exactly 1:2 as expected theoretically.

This result was supported by another experiment: Glucose and fructose are the major carbohydrates in mature apples [17]. The concentrations, determined for "Golden Delicious" by means of glucose/fructose test, was 19 mg glucose and 77 mg fructose, together 96 mg hexoses per g fresh weight. After ion exchange chromatography both glucose and fructose completely were present within the neutral fraction (Fig. 1). Because of their epimerism they form the same osazone. This derivative was prepared and after purification its specific radioactivity was measured (Table I, A), from which the specific radioactivity of glucose and fructose was calculated (Table I, B).

Provided that glucose and fructose were essentially the only radioactive compounds in the neutral fraction, the quotient of radioactivity of this fraction and of the contents of glucose and fructose of the apples (Table I, C) should give similar values for the specific radioactivity as measured for glucose and fructose *via* the osazone method (Table I, B).

The results in Table I show that (B) and (C), with some differences, indeed are in the same range, indicating that the whole radiolabel of the neutral fractions from outdoor and tub experiments (Fig. 2) was attached to carbohydrates and can be considered as to be reassimilated.

Discussion

Reports concerning radiolabeled natural products recycled from $^{14}CO_2$ which was liberated during catabolism of herbicides and other xenobiotic substances are rare. Under outdoor conditions reassimilation has not been expected to this extent. The long experimental period of 150 days may favour total degradation of the applied radiolabeled amitrole to $^{14}CO_2$. The metabolism within the amitrole-treated soil was not studied. A microbial and abiotic CO_2 release from soil-applied amitrole has been reported [10, 11]. Thus the liberation of $^{14}CO_2$ may proceed in soil and/or the apple tree. From the soil $^{14}CO_2$ was released to the atmosphere and reassimilated by the apple tree. $^{14}CO_2$ originated within the apple tree may be recycled directly into natural products without passing the atmosphere.

In general 3-(1,2,4-triazol-1-yl)-2-aminopropionic acid is an important metabolite of triazole-based pesticides. It was shown to be formed from O-acetyl-L-serine and triazole by cysteine synthase

Table I. Specific radioactivity in some compounds of the neutral fraction.

No.	Experiment	A Osazone [Bq × g^{-1}]	B Sum of glu and fru [Bq × g^{-1}]	C RA[a] of neutr. fract. Cont. of glu/fru [Bq × g^{-1}]
1	Outdoor experiment "Golden Delicious"	30	60	43
2	Outdoor experiment "Golden Delicious"	27	54	62
3	Tub experiment "Golden Delicious"	130	260	476
4	Tub experiment "Gloster"	70	140	132[b]

A: Measured by LSC; B: calculated from A; C: calculated from the radioactivity of the neutral fraction (Bq × g^{-1} fw, see Fig. 2) and the contents of glucose and fructose (= glu, fru) in apples (together 96 mg × g^{-1} fw).
[a] Radioactivity; [b] for "Gloster" the contents of glucose and fructose was not measured. For calculation the value of "Golden Delicious" was used.

[18]. However, to date it was not described as a metabolite of amitrole in plants. Formation of triazolylalanine as the main soluble metabolite in apple trees most probably involves microbial deamination in the soil, uptake of triazole by the apple tree, and conjugation with serine.

A second possibility yielding triazolylalanine is formation of aminotriazolylalanine by cysteine synthase in a first step, followed by deamination within the plant. This was supported by using excised sprouts from apple trees [16] and apple cell suspension cultures [19] and seems to depend on the duration of the experiment. In cell suspension cultures (7 days incubation time) only aminotriazolylalanine was found, while excised sprouts (19 days after application) contained both aminotriazolylalanine and triazolylalanine. As demonstrated in this study, mature fruits from apple trees only contained triazolylalanine after one vegetation period.

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