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IDENTIFICATION OF 1-(MALONYLAMINO)CYCLOPROPANE-1-CARBOXYLIC ACID AS A MAJOR CONJUGATE OF 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID, AN ETHYLENE PRECURSOR IN HIGHER PLANTS.

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When labeled 1-aminocyclopropane-1-carboxylic acid, an ethylene precursor, was administered to light-grown wheat leaves, it was primarily converted into a nonvolatile metabolite, which was identified as 1-(malonylamino)cyclopropane-1-carboxylic acid. The natural occurrence of this conjugate in the wilted wheat leaves was confirmed by gas chromatography-mass spectrometry.

INTRODUCTION

Ethylene is a plant hormone involved in numerous physiological responses (1). Adams and Yang (2) established the following sequence for the pathway of ethylene biosynthesis in ripening apples: Methionine → S-adenosylmethionine → ACC[1] → ethylene. When wheat leaves were subjected to wilting, ACC and ethylene synthesis increased markedly and then declined (3). Apelbaum and Yang (3) confirmed that ethylene was synthesized from ACC, as in apple, but observed that the decrease in ACC in the wilted leaves was much greater than the amount of ACC converted to ethylene. These observations suggested that ACC is metabolized to products other than ethylene (3). The present investigation was undertaken to further study the metabolism of ACC in light-grown wheat seedlings.

MATERIALS AND METHODS

Materials. Wheat seeds (*Triticum aestivum* L. cv. Anza) were germinated, grown and excised as previously described (3). Monomethyl malonate potassium salt was purchased from Tridom Chemicals, dicyclohexylcarbodiimide from Aldrich, succinic anhydride and BF_3-MeOH were from Sigma, acetic anhydride was from

[1]Abbreviation: ACC, 1-aminocyclopropane-1-carboxylic acid.

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Allied Chemicals and S-adenosyl[3,4-^{14}C]methionine was purchased from Research Products International. [2-^{14}C]ACC was prepared enzymatically from S-adenosyl[3,4-^{14}C]methionine (35 μCi/μmol) with ACC synthase isolated according to Yu et al. (4). N-Acetyl[2-^{14}C]ACC was prepared from [2-^{14}C]ACC in 150 μl pyridine and 50 μl acetic anhydride at 45°C for 1 h. N-Succinyl-[2-^{14}C]ACC was prepared similarly with succinic anhydride and pyridine. ACC methyl ester was prepared by refluxing ACC in 2 ml BF_3-MeOH for 12 h; after the solution was taken to dryness, this esterification procedure was repeated once more. N-Malonyl-ACC dimethyl ester was synthesized from monomethyl malonate (0.5 mmol) and ACC methyl ester (0.5 mmol) with excess dicyclohexylcarbodiimide (1 mmol) in 5 ml ice cold acetonitrile stirred for 12 h. After filtering dicyclohexylurea and evaporating acetonitrile, the residue was resuspended in water and the dimethyl ester was extracted into ether. N-Malonyl-ACC was obtained following saponification of its dimethyl ester with KOH. Excess of potassium was removed by passing the saponified solution through cation ion exchange resin, Dowex-50 (H^+ form).

Administration of [2-^{14}C]ACC. Radioactive ACC (1 μCi and 30 nmol in 0.3 ml water) was administered to plant tissue by placing the cut ends of leaves in tubes containing the feeding solution. Air was blown across the leaves to facilitate uptake through transpiration. After the solution was absorbed, an additional 0.1 ml of water was added. After incubation for 6 h, leaves were cut into pieces and extracted with hot 80% ethanol. The radioactive metabolite was partially purified by paper chromatography using 1-butanol:acetic acid:water (4:1:1, v/v) as the solvent and by paper electrophoresis at pH 2.2 (10% acetic acid, v/v/), pH 6.0 (pyridine:acetic acid:water, 10:15:900, v/v), pH 10.7 (0.1 M Na borate) and pH 11.8 (5% NH_4OH, w/v). After paper chromatography or electrophoresis, amino acids were visualized by spraying with ninhydrin and organic ammonium salts were visualized by spraying with Nessler's Reagent (Sigma). Additionally, paper chromatograms and electrophoretograms were cut into strips and eluted with 50% ethanol and the eluates were assayed for ACC conjugate as described below.

Determination of ACC-conjugate. Quantification of the ACC-conjugate was carried out by hydrolyzing it to ACC in 6 N HCl at 100°C for 1 h, and the resulting ACC was assayed according to the method of Lizada and Yang (5). Ethylene was determined by gas chromatography. Radioactive ethylene was determined in a liquid scintillation counter after absorption into 0.25 ml of 0.25 M $Hg(ClO_4)_2$.

Isolation of the ACC Conjugate. Wheat leaves (1 kg fresh weight) were water stressed as previously described (3). After they lost 9% fresh weight the leaves were cut into small pieces and extracted four times in 2 liters of boiling 95% ethanol. Ethanol was removed, *in vacuo*, at 38°C and the resulting aqueous solution was extracted once with 0.1 volume of chloroform, which was discarded. The concentrated aqueous solution was repeatedly slurried with 100 g polyvinylpyrrolidone. The filtrate and water washings were combined and passed, in series, through a 1.5 cm x 50 cm column of Dowex-50 (H^+ form) and a 1.5 cm x 40 cm column of Dowex-1 (OH^- form). Anions were eluted from the Dowex-1 column with 250 ml of 6 N formic acid, which was evaporated to a brown oily residue which was again slurried with polyvinylpyrrolidone until the solution was yellow. The resulting solution was mixed with the isolated radioactive ACC-conjugate and chromatographed on silica gel GF plates (20 x 20 cm, 1 mm thickness), using butanol:acetic acid:water (4:1:1, v/v) as developing solvent. The plates were developed for 15 cm and dried; this process was repeated 3 times. The zone, which contained radioactivity, was scraped off and eluted with 50% ethanol. After evaporation the eluate was methylated with diazomethane, and subjected to gas chromatography-mass spectrometry analysis with a Finnigan 3200 instrument equipped with a 0.25 mm x 30 m glass column coated with SE-54; temperature was programmed from 60°C to 280°C at a rate of 6°C/min; the ionization potential of the source was 70 eV.

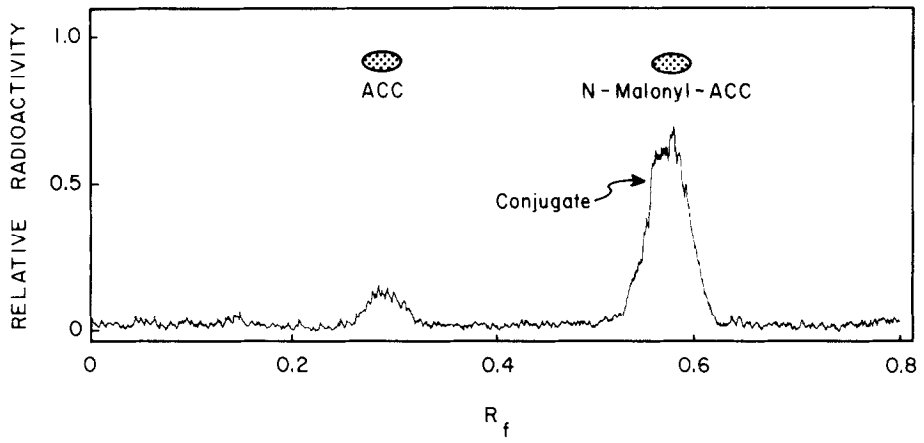

Fig. 1. Paper radiochromatogram of extracts of wheat leaves which were administered [2-^{14}C]ACC and incubated for 6 h.

RESULTS AND DISCUSSION

After light-grown wheat leaves were allowed to take up [2-^{14}C]ACC and incubated for 6 h, 60 to 70% of the label was found to be metabolized into a nonvolatile product, while 10 to 20% of the radioactivity remained as ACC, as revealed by paper chromatography (Fig. 1). Both nonstressed and water-stressed tissues readily formed this metabolite following ACC application, indicating that the enzyme system responsible for this metabolism was not induced by water stress.

Unlike ACC this metabolite was not adsorbed by cation ion exchange resin, Dowex 50 (H^+), indicating that the metabolite does not contain a free amino group. After hydrolysis in 6 N HCl at 100°C, the metabolite was converted into a compound which was indistinguishable from ACC in paper chromatography and paper electrophoresis (Table I). Furthermore, when the labeled hydrolyzed product was mixed with a large known quantity of unlabeled ACC, the labeled product and unlabeled ACC were converted with equal efficiency into ethylene by the method of Lizada and Yang (5); the unhydrolyzed metabolite was not, however, converted to ethylene. Evidence that the metabolite was an N-substituted derivative of ACC was further provided by data from paper chromatography and paper electrophoresis. On electrophoresis at pH 2.2 the derivative was neutral, and at pH above 6.0 it was anionic, indicating that the amino group is substituted. On paper chromatography the conjugate moved

Table I. Chromatographic and electrophoretic behavior of ACC conjugate isolated from wheat leaves and other related compounds

	Paper Chromatography	pH of Electrophoresis			
		2.2	6.0	10.7	11.8
	R_f	Relative Mobility[a]			
[^{14}C]Conjugate	0.60	0	1.08	1.09	1.21
N-malonyl-ACC	0.60	0	1.08	1.09	1.21
N-acetyl-[^{14}C]ACC	0.88	-	-	0.65	
N-succinyl-[^{14}C]ACC	0.70	-	-	0.90	
ACC	0.28	3.0	0	0.75	
Hydrolysate of [^{14}C]conjugate	0.28	3.0	0	0.75	

[a]Electrophoresis mobilities were relative to Nε-(2,4-dinitrophenyl)lysine for pH 2.2 and to N-(2,4-dinitrophenyl)cysteic acid for other pH's.

far ahead of ACC, as would be expected for an N-substituted ACC. Labeled N-acetyl-ACC and N-succinyl-ACC were synthesized to compare their chromatographic and electrophoretic behavior to that of the conjugate (Table I). On electrophoresis at pH 10.7, the conjugate moved far ahead of N-acetyl-ACC and slightly ahead of N-succinyl-ACC, suggesting that, like N-succinyl-ACC, the conjugate is a dianion, but has a slightly smaller mass. This prediction was consistent with the behavior of these compounds on paper chromatography. The R_f of the conjugate was considerably lower than that of N-acetyl-ACC but was only slightly lower than that of N-succinyl-ACC indicating that the conjugate resembled N-succinyl-ACC but was slightly more polar. The conjugate was thus suspected to be N-malonyl-ACC. Indeed, the labeled conjugate was found to be indistinguishable from synthetic N-malonyl-ACC on paper electrophoresis and paper chromatography (Table I).

Induction of ACC synthesis by light-grown wheat leaves in response to water stress has been reported (3). We have now shown that ACC is converted into N-malonyl-ACC in the same tissue. It is therefore expected that N-malonyl-ACC would be a natural product of water-stressed wheat leaves. Labeled N-malonyl-ACC, which had essentially no mass, was added as a marker to the extract of stressed wheat leaves so that isolation and purification of the conjugate could be followed as described in the "Materials and Methods" section. The partially purified fraction was converted into its methyl ester with diazomethane and analyzed by GC-MS for m/e peaks of 83, 100, 101 and 115,

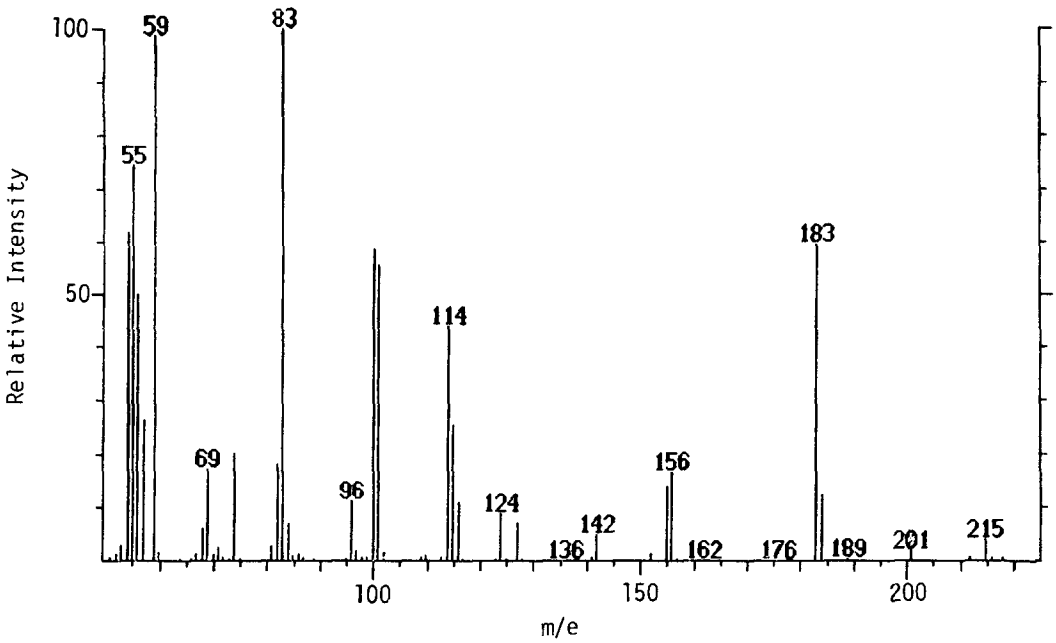

Fig. 2. Mass spectrum of a compound isolated from water-stressed wheat leaves and methylated with diazomethane. This spectrum is identical to that of synthetic dimethyl ester of N-malonyl-ACC.

which were characteristic of ACC (6) and ACC methyl ester. Only one compound, shown in Figure 2, was detected which contained these major peaks. This mass spectrum was essentially identical to that of synthetic N-malonyl-ACC methyl-ester. Thus, it was verified that wilted wheat leaves contained N-malonyl-ACC.

The occurrence of this natural product suggests the possibility that ethylene biosynthesis can be regulated through malonylation of ACC. N-Malonyl-amino acids have been previously isolated from plant tissue (7-11). The conjugate is formed primarily with the D-amino acids. It has been suggested that the physiological significance of this reaction is to inactivate D-amino acids which might otherwise be toxic to the plants (7). It is to be noted that the ACC molecule possesses no asymmetric carbon and hence lacks enantiomers. It has not yet been shown whether the enzyme responsible for malonylation of ACC is highly specific for ACC or simply specific for D-amino acids.

During the completion of this work, N. Amrhein (personal communication) informed us that they had also identified N-malonyl-ACC from etiolated buckwheat seedlings after feeding exogenous ACC. Although its structure has

not been characterized, D. O. Adams (personal communication) has also observed an N-conjugated ACC in green tomato fruit.

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