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OCCURRENCE OF *N*-MALONYL-D-ALANINE IN PEA SEEDLINGS

TADASHI OGAWA, MITSURU FUKUDA AND KEI SASAOKA
The Research Institute for Food Science, Kyoto University, Kyoto (Japan)
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SUMMARY

One of the ninhydrin-negative alanine conjugates isolated from pea seedlings was identified as *N*-malonyl-D-alanine.

The identification of this conjugate was carried out by a comparison of its gas–liquid chromatographic and mass spectrometric properties, and its nuclear magnetic resonance and infrared spectra with those of synthetic *N*-malonyl-D-alanine. The alanine in the conjugate was shown to be present as the D-isomer by enzymatic and chromatographic analyses.

INTRODUCTION

In a previous paper[1], the authors reported that various amino acids were liberated by acid hydrolysis of the non-cationic fraction of pea seedling extract, and that at least 20 sub-fractions containing ninhydrin-negative conjugated amino acids could be obtained by chromatographic fractionation. Alanine was one of the most predominant amino acids in the hydrolyzate of the non-cationic fraction, and occurred in many of the sub-fractions in the form of various conjugates.

In the present paper, selective incorporation of DL-[1-^{14}C]alanine into one of the ninhydrin-negative alanine conjugates was observed. This alanine conjugate was isolated and identified as *N*-malonyl-D-alanine. Malonylation of various D-amino acids by their administration into plants has been reported[2–5]. However, malonyl-D-tryptophan[6] was the only malonylated amino acid which has so far been proved to be a natural product.

MATERIALS AND METHODS

Materials

DL-[1-^{14}C]Alanine (spec. act., 4.3 Ci/mole) was obtained from the Daiichi Pure Chemicals Co., Tokyo. NADH, beef liver catalase (EC 1.11.1.6) and lactate dehydrogenase, Type I (EC 1.1.1.27) were purchased from the Sigma Chemical Co. FAD was obtained from the Kyowa Hakko Kogyo Co. Crystalline D-amino acid oxidase (EC 1.4.3.3) was prepared from fresh pig kidney by the method of Massey *et al.*[7]. L-Leucine-*N*-carboxy anhydride was synthesized according to Leuchs[8].

* Present address: Department of Nutrition, School of Medicine, Tokushima University, Tokushima, Japan.

Pea seedlings

Pea seeds (*Pisum sativum*, var. Alaska) were sterilized with a 0.01 % solution of benzalkonium chloride and were germinated in the dark at 25 °C for 6 days, as described previously[9].

Administration of DL-[1-^{14}C]*alanine*

Decotylized pea seedlings (6-day-old, 30 pieces) were incubated in a medium containing 19.3 μmoles of DL-[1-^{14}C]alanine (83 μCi in 16.5 ml of distilled water) in the dark at 25 °C for 24 h.

Isolation of the alanine conjugate

An ethanol (75 %, v/v) extract of decotylized pea seedlings (fresh weight, 6.3 kg) was applied to a 6 cm × 35 cm column of Amberlite IR-120 B (H^+ form). The non-cationic fraction, which passed straight throught the column, was neutralized with triethylamine and then concentrated *in vacuo* below 40 °C. An aliquot of the concentrated non-cationic fraction prepared from the seedlings incubated with DL-[1-^{14}C] alanine was added to this concentrate. The radioactive alanine conjugate in this fraction acted as a marker during the isolation procedure. After this treatment, the non-cationic fraction was applied to a 5 cm × 25 cm column of Dowex 1-X4 (100–200 mesh, acetate form), and compounds were first eluted with 5 M acetic acid (1750 ml) and then with 6.5 M formic acid (1500 ml). The 6.5 M formic acid fraction which contained the desired compound was concentrated to dryness *in vacuo* below 40 °C, and was further purified by cellulose column chromatography (Avicel SF, 10 cm × 15 cm). Fractionation was carried out with *n*-butanol–acetic acid–water (4 : 1 : 1, by vol.). Fractions of 16.5 ml were collected at a flow rate of 5 min per tube. The compound was detected by radioactivity and also by the ninhydrin method described previously[1]. The pooled fraction from 1480 to 2050 ml was evaporated to dryness under reduced pressure below 40 °C. The residue was then dissolved in a small volume of distilled water and was applied to a 2.5 cm × 75 cm column of Sephadex G-15 which was eluted with distilled water. The fractions of 3 ml were collected at a flow rate of 3 min per tube. The pooled fraction from 300 to 390 ml was lyophilized. The residue was further purified by preparative paper chromatography (Whatman No. 3 filter paper, 40 cm × 40 cm, solvent system I described below). The radioactive zone on the paper chromatogram was eluted with distilled water, and the eluate was lyophilized. The lyophilized residue was dissolved in methanol. This solution was evaporated to yield crystalline material (95 mg)* which was homogeneous by paper, thin-layer and gas–liquid chromatography. This compound had the following properties: m.p. 138–140 °C (with evolution of gas), $[\alpha]_D^{28}+33°$, $[\alpha]_{300}^{28}+253°$ (*c* 0.38 in water), (Found: C, 41.70; H, 5.37; N, 8.15. Calcd for $C_6H_9O_5N_1$: C, 41.14; H, 5.18; N, 8.00 %).

Analyses

Combined gas–liquid chromatography–mass spectrometry was carried out with a Shimadzu-LKB 9000 instrument equipped with a 0.3 cm × 200 cm glass

* The concentration of this compound in decotylized pea seedlings (6-day-old) was approx. 3 mg per 100 g of fresh seedlings, as calculated from the amino acid analysis of the hydrolyzate of the 6.5 M formic acid fraction. Therefore, the recovery of this compound was 50 %.

column packed with 3 % SE-52 on chromosorb W, 60–80 mesh. The sample used for the analysis was esterified with diazomethane according to the procedure of Schlenk and Gellerman[10].

The proton magnetic resonance studies were performed with a Hitachi NMR spectrometer R-22 at 90 mHz with tetramethylsilane as the internal standard.

Infrared spectra were obtained with a Hitachi recording spectrophotometer EPI-S2.

Optical rotations were determined with a JASCO optical rotatory dispersion recorder J-5.

Quantitative amino acid analysis was carried out with a Yanagimoto LC-5S automatic amino acid analyzer.

Radioactivity measurement was performed with an Aloka TDC-201 gas-flow counter.

Malonic acid in the conjugate was detected by gas–liquid chromatography. The conjugate was hydrolyzed with 1 M HCl in a boiling water bath for 5 h. After evaporation, the malonic acid in the hydrolyzate was esterified with diazomethane[10], and the ester was analyzed by gas–liquid chromatography at 150 °C in a Shimadzu GC-1C apparatus equipped with a 0.3 cm × 270 cm column packed with 10 % DEGS on Neopak 1A, 60–80 mesh.

In paper and thin-layer chromatography, phenol–water (4 : 1, w/w; solvent system I), *n*-butanol–acetic acid–water (4 : 1 : 1, by vol.; solvent system II) and ethylacetate–acetic acid–water (3 : 1 : 1, by vol.; solvent system III) were used in most cases. Ninhydrin-negative conjugated amino acids were detected as described previously[1].

Determination of configuration

The configuration of the alanine moiety of the isolated conjugate was determined enzymatically and chromatographically.

Enzymatic determination was carried out with D-amino acid oxidase, and the formation of pyruvate was detected by the lactate dehydrogenase system[11]. The assay procedure used was as follows: the hydrolyzate of the isolated alanine conjugate (0.1–0.5 μmoles) was incubated at 20 °C in 3 ml of Tris–HCl buffer (0.2 M, pH 8.3) containing 260 μg of D-amino acid oxidase, 11 μg of catalase, 100 μg of lactate dehydrogenase, 0.01 μmole of FAD and 0.6 μmole of NADH. The decrease in absorbance at 340 nm was followed by a Hitachi-124 recording spectrophotometer. After incubation, amino acid analysis of an aliquot of the reaction mixture was carried out to confirm the disappearance of alanine.

The configuration of alanine was also determined chromatographically according to the method of Manning and Moore[12]. The hydrolyzate of the alanine conjugate (0.5–1.0 μmole) was treated with L-leucine *N*-carboxy anhydride (0.75–1.5 μmoles) in 1 ml of borate buffer (0.45 M, pH 10.2) for 2 min at 0 °C with vigorous stirring. The reaction was stopped by the addition of 0.8 ml of 1 M HCl, and an aliquot of the reaction mixture was studied by amino acid analysis.

Synthesis of N-malonyl-D-alanine

N-Malonyl-D-alanine was synthesized from the D-alanine benzylester *p*-toluenesulfonate and the potassium salt of malonate monobenzylester, which was itself

prepared from malonate dimethylester according to the method of Rosa and Neish[5]. D-Alanine benzyl ester *p*-toluene sulfonate (3 g in 50 ml of methylene dichloride) and potassium malonate monobenzylester (2 g in 2 ml of water) were mixed at 0 °C. Then, 1.8 g of dicyclohexylcarbodiimide in 30 ml of methylene dichloride were slowly added, and the mixture stirred for 20 min. After standing for 3 h at room temperature, the reaction mixture was filtered. The filtrate was concentrated *in vacuo*. The resulting oil was dissolved in 200 ml of ethylacetate, and the insoluble residue was removed by filtration. The filtrate was washed successively with 1 M HCl, 0.5 M $NaHCO_3$ and water, and the ethyl acetate layer was dried over Na_2SO_4. The solution was concentrated with a rotary evaporator, and treated with light petroleum (b.p. 30–50 °C) at 0 °C to produce crystals of *N*-malonyl-D-alanine dibenzyl ester. Recrystallization from ethyl acetate–light petroleum gave 2.4 g of crystals (yield 78 %). A study of the compound gave: m.p. 43–45 °C (Found: C, 67.95; H, 5.86; N, 4.05. Calculated for $C_{20}H_{21}O_5N_1$: C, 67.59; H, 5.96; N, 3.94 %).

A solution of *N*-malonyl-D-alanine dibenzyl ester (2.3 g) in methanol was hydrogenated with 0.5 g of palladium black for 4 h at room temperature. After hydrogenation, the solution was filtered and the filtrate was concentrated *in vacuo*. When the concentrated syrup was treated with light petroleum at 0 °C, fine crystals formed. Recrystallization from methanol–light petroleum (b.p. 30–50° C) gave 1.2 g of crystals (yield 92 %). A study of the compound gave: m.p. 139–140 °C (with evolution of gas, presumably CO_2), $[\alpha]_D^{25}$, +36 °, $[\alpha]_{300}^{25}$, +288 ° (*c* 2.5 in water), (Found: C, 40.64; H, 5.21; N, 7.81. Calculated for $C_6H_9O_5N_1$: C, 41.14; H, 5.18; N, 8.00 %).

N-Malonyl-L-alanine was synthesized by the same method ($[\alpha]_D^{28}$, −35 °, $[\alpha]_{300}^{28}$, −280 °).

RESULTS

Incorporation of DL-[1-^{14}C]*alanine into ninhydrin-negative alanine conjugates*

Decotylized pea seedlings (30 pieces) fed with DL-[1-^{14}C]alanine were extracted with 75 % ethanol, and the non-cationic fraction was prepared from this extract by use of Amberlite IR-120 B. This fraction contained approx. 33 % of the total radioactivity of the extract. The radioautogram of this fraction is shown in Fig. 1. Due to the viscous nature of this fraction the spots were diffuse in the first run. However, at least three radioactive spots could be found (Fig. 1, Spots A, B and C), with most of the radioactivity being concentrated in Spot B. This fraction was then applied to a 1 cm × 13 cm column of Dowex 1-X4 (100–200 mesh, acetate form), which was successively eluted with 3 M acetic acid (30 ml), 5 M acetic acid (30 ml) and 6.5 M formic acid (50 ml). The 6.5 M formic acid fraction contained approx. 90 % of the applied radioactivity, and alanine was the predominant amino acid in the hydrolyzate of this fraction. These results indicated that, in the non-cationic fraction, there was a strongly acidic alanine conjugate into which DL-[1-^{14}C]alanine was selectively incorporated. Therefore, large scale isolation of this compound was carried out as described in Materials and Methods.

Characterization of the alanine conjugate

The alanine conjugate isolated here gave malonic acid as well as alanine after

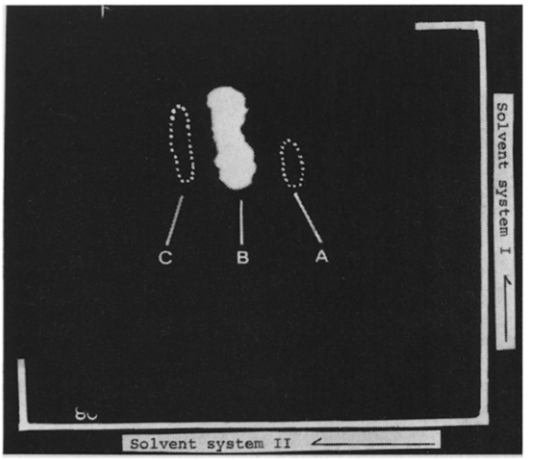

Fig. 1. Radioautogram of ninhydrin-negative alanine conjugates. An aliquot of the concentrated non-cationic fraction, prepared from the extract of decotylized pea seedlings fed with DL-[1-^{14}C]-alanine, was applied to Toyo No. 51 A filter paper (20 cm × 20 cm) and was developed first with the solvent system I and then with the solvent system II. The paper chromatogram was exposed to Fuji medical X-ray film for 48 days. Dotted lines indicate weak radioactive spots.

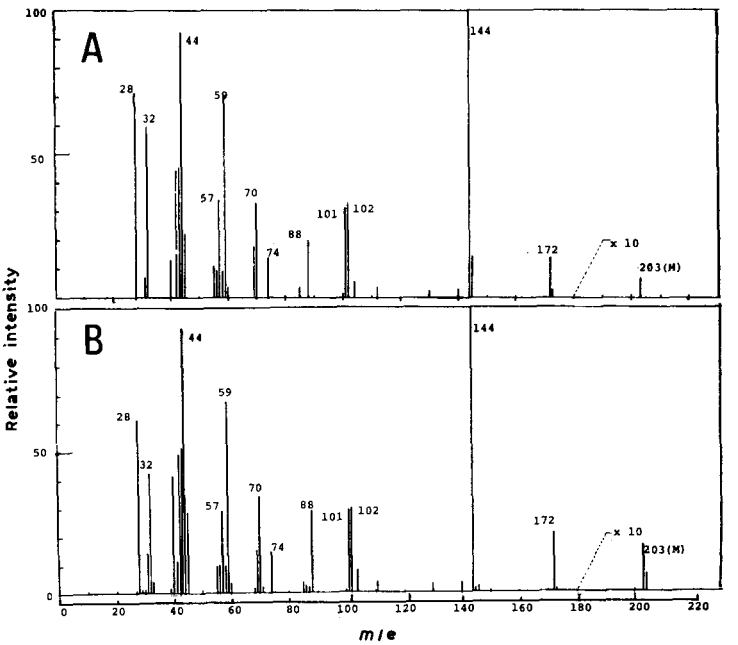

Fig. 2. Mass spectra of the dimethylesters of the isolated compound and the synthetic *N*-malonyl-D-alanine. A, isolated compound; B, synthetic *N*-malonyl-D-alanine.

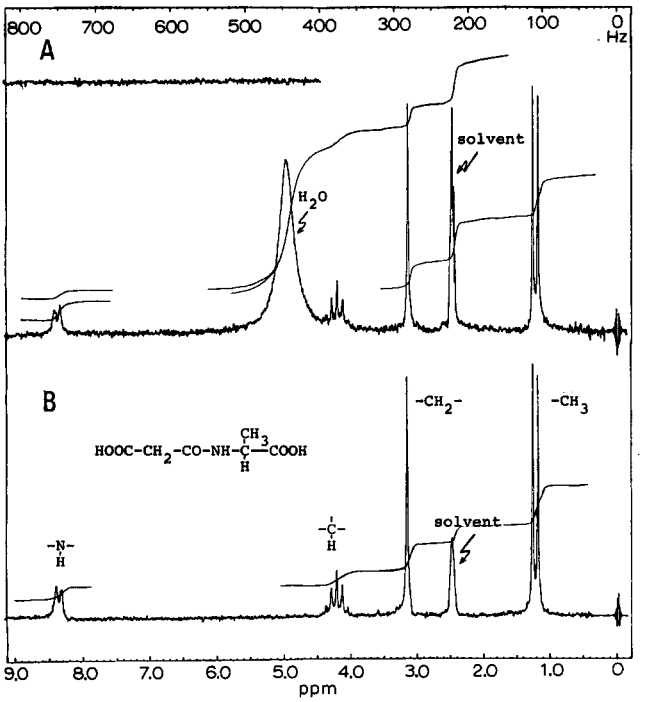

Fig. 3. Proton magnetic resonance spectra of the isolated compound and synthetic *N*-malonyl-D-alanine in deuterodimethylsulfoxide. A, isolated compound; B, synthetic *N*-malonyl-D-alanine.

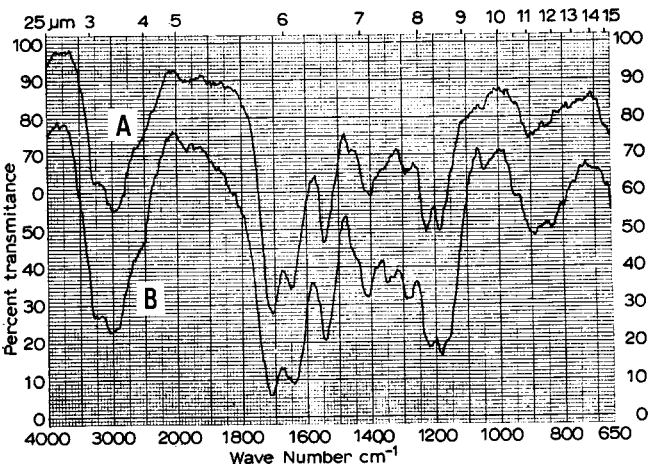

Fig. 4. Infrared spectra of the isolated compound and synthetic *N*-malonyl-D-alanine (KBr disc). A, isolated compound; B, synthetic *N*-malonyl-D-alanine.

acid hydrolysis. This result, together with that of elementary analysis, suggested that the conjugate was *N*-malonylalanine. The following experiments were carried out to demonstrate this structure.

In the combined gas–liquid chromatography–mass spectrometric analyses, both the esterified derivatives of the isolated conjugate and the synthetic *N*-malonyl-D-alanine appeared at the same retention time (5.6 min) on the column, and gave identical mass spectra as shown in Fig. 2, A and B. The small peak at *m/e* 203 corresponded to the molecular ion of *N*-malonylalanine dimethyl ester. The peaks at *m/e* 172 and 144 (base peak) suggested the losses of $-O-CH_3$ and $-\overset{O}{\overset{\|}{C}}-O-CH_3$, respectively.

The proton magnetic resonance spectrum of the isolated compound in deutrodimethylsulfoxide indicated the presence of the alanine moiety as well as the methylene group of the malonyl moiety, which appeared at 3.15 ppm as shown in Fig. 3, A. The synthetic compound gave the same results (Fig. 3, B). The spectrum of the isolated compound in deutromethanol was also identical with that of the synthetic compound in the same solvent.

TABLE I

R_F VALUES OF THE ISOLATED COMPOUND AND SYNTHETIC *N*-MALONYL-D-ALANINE

The solvent systems I, II and III are described in Materials and Methods.

	*Paper chromatography**			*Thin-layer chromatography***		
Solvent system:	*I*	*II*	*III*	*I*	*II*	*III*
Isolated compound	0.75	0.67	0.66	0.13	0.28	0.30
Synthetic *N*-malonyl-D-alanine	0.75	0.67	0.66	0.13	0.27	0.30
DL-Alanine	0.60	0.29	0.28	0.34	0.26	0.17
Malonic acid	0.54	0.63	0.73	0.17	0.37	0.39

* Toyo No. 51 A filter paper.

** Silica gel F_{254} thin-layer plate (Merck).

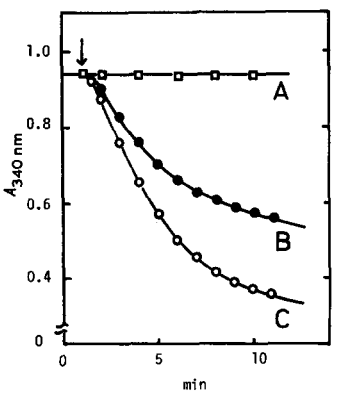

Fig. 5. Enzymatic determination of the alanine configuration. Experimental details are described in the text. The reaction was started at the point indicated. A, alanine in the hydrolyzate of synthetic *N*-malonyl-L-alanine (0.5 μmole); B, alanine in the hydrolyzate of the isolated compound (0.28 μmole); C, alanine in the hydrolyzate of synthetic *N*-malonyl-D-alanine (0.5 μmole).

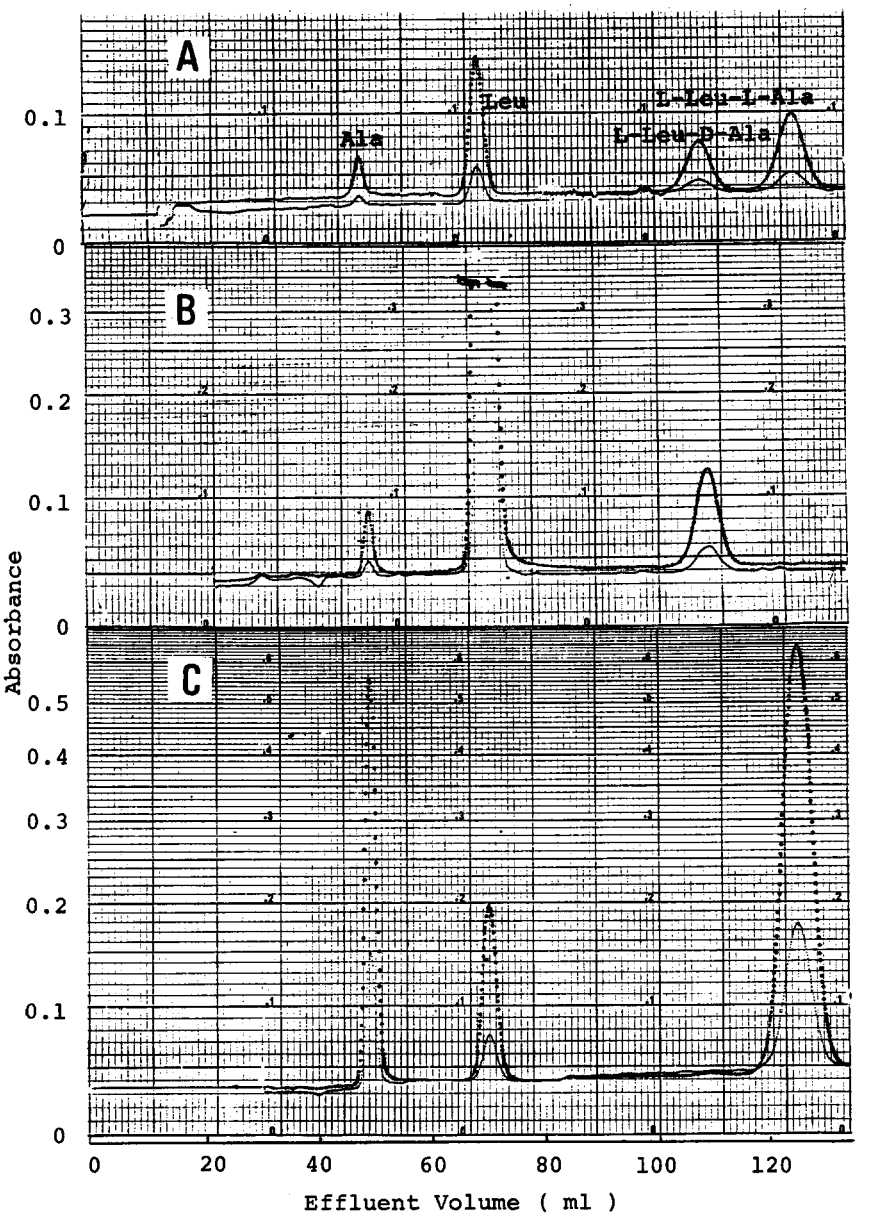

Fig. 6. Chromatographic separation of diastereoisomeric dipeptides, L-leucyl-D-alanine and L-leucyl-L-alanine. Chromatographic conditions: Bio-Rad Aminex A-4 (0.9 cm × 90 cm), sodium citrate buffer, 0.2 M, pH 4.25, 55 °C, flow rate, 80 ml/h. A, authentic materials; B, L-leucylalanine prepared from the hydrolyzate of the isolated compound; C, L-leucyl-L-alanine prepared from the hydrolyzate of synthetic *N*-malonyl-L-alanine.

The infrared spectrum of the isolated compound was as that of synthetic *N*-malonyl-D-alanine (Fig. 4, A and B).

The R_F values of the isolated and synthetic compounds were listed in Table I. The identity of the compound was further confirmed by co-chromatography in all chromatographic systems shown in the table.

These results clearly showed that the isolated compound was *N*-malonylalanine.

Configuration of the alanine moiety

To determine the configuration of alanine in the isolated conjugate, it was hydrolyzed with 6 M HCl at 105 °C for 20 h. The hydrolyzate was treated with D-amino acid oxidase, as described in Materials and Methods. The decrease in absorbance at 340 nm was observed both for the hydrolyzates of the isolated compound and for the authentic *N*-malonyl-D-alanine (Fig. 5, B and C). After 2 h, the alanine in these reaction mixtures had quantitatively (98 %) disappeared. Such a decrease in absorbance at 340 nm with the disappearance of alanine were not observed when the hydrolyzate of synthetic *N*-malonyl-L-alanine was used (Fig. 5, A). These results indicated that the alanine was in the D-configuration. This was further confirmed by chromatographic separation of the diastereoisomeric dipeptides prepared by L-leucine *N*-carboxy anhydride coupling. Under the chromatographic conditions described in Fig. 6, synthetic L-leucyl-D-alanine and L-leucyl-L-alanine were eluted at 108 and 125 ml, respectively (Fig. 6, A). The dipeptide synthesized from alanine in the hydrolyzate of the isolated compound appeared at the position of L-leucyl-D-alanine (Fig. 6, B). On the other hand, the position of the dipeptide prepared from alanine in the hydrolyzate of synthetic *N*-malonyl-L-alanine coincided with that of L-leucyl-L-alanine (Fig. 6, C). These results gave additional proof of the D-configuration of alanine in the isolated conjugate. The positive rotatory dispersion data given in Materials and Methods also supported this conclusion.

DISCUSSION

The most predominant alanine conjugate in the non-cationic fraction of the decotylized pea seedling extract was isolated and identified as *N*-malonyl-D-alanine. To our knowledge, this is the first report of its natural occurrence.

Good and Andreae[2] isolated *N*-malonyltryptophan from spinach incubated with DL-tryptophan. Zenk and Scherf[6] showed that the tryptophan in the malonyl conjugate was the D-isomer, and detected this conjugate in a number plants. *N*-Malonyl-D-methionine and *N*-malonyl-D-serine were formed in *Nicotiana rustica* after the administration of D-methionine[3] and D-serine[4], respectively. Rosa and Neish[5] observed the formation of *N*-malonylphenylalanine in barley shoots fed with D-phenylalanine. In the same paper they also reported that, when D-glutamic acid, D-alanine and other D-amino acids were administrated to barley shoots, these amino acids were malonylated. However, except for *N*-malonyl-D-tryptophan, these malonyl-D-amino acids were formed by the administration of exogenous D-amino acids, so that their natural occurrence in plants is doubtful. In the present experiments, although the radioactive alanine conjugate obtained from the seedlings incubated with DL-[1-^{14}C]-alanine was added as the marker during the isolation process, the amounts of the radioactive alanine conjugate added were calculated to be only 0.6 μmole on the basis

of radioactivity measurement, and yet the amount of *N*-malonyl-D-alanine isolated was 543 μmoles. This therefore demonstrates the endogenous occurrence of *N*-malonyl-D-alanine in pea seedlings.

Another important fact established here was that the alanine in the conjugate had the D-configuration. D-Alanine is a constituent of bacterial peptides[13], and free D-alanine has also been detected in some animals[14]. Recently, Aldag *et al.*[15] suggested that free D-alanine was present in the extract of corn roots. This suggestion, together with the findings reported in the present paper, may indicate that there is a wide spread occurrence of D-alanine in higher plants.

Studies on biosyntheses and functions of D-alanine and *N*-malonyl-D-alanine are in progress.

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