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METABOLIC PATTERNS OF L- AND D-SERINE IN HIGHER AND LOWER PLANTS*

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Abstract—L- and D-serine-3-^{14}C were fed in parallel experiments to intact *Nicotiana rustica* and after various metabolizing times the distribution of ^{14}C was investigated. With the L-precursor the majority of the label was incorporated into insoluble tissue residues, while with the D-precursor as much as 30 per cent of the label was associated with *N*-malonyl-D-serine; evidence for the structure and configuration of the conjugate is presented. A comparison between the ^{14}C content of lignin and that of the parent tissue residue revealed that a lower portion of the label was incorporated into lignin with the D- than with the L-precursor. The results of screening experiments with labelled L- and D-serine in plant species at all levels of evolution are consistent with those found in analogous experiments with D-methionine and give a further support for the taxonomic significance of the malonate-D-amino acid conjugation in plants.

INTRODUCTION

THE ISOLATION of *N*-malonyltryptophan from plant material was reported by Good and Andreae,[1] and its formation in several higher plants from D-tryptophan with retention of configuration was established by Zenk and Scherf.[2] In parallel experiments with L- and D-methionine-methyl-^{14}C it has been shown[3,4] that the majority of the D-isomer fed to the intact *Nicotiana rustica* plant is converted into *N*-malonyl-D-methionine while this pathway is completely lacking with the L-isomer. Comparative studies of L- and D-methionine metabolism in a number of plant species have further shown[5] that the metabolism of D-methionine proceeds by two essentially different ways which are determined by the evolutionary stage of the phyla involved. In lower plants the metabolic patterns of L- and D-methionine are qualitatively identical while in higher plants the D-isomer undergoes acylation, *N*-malonyl-D-methionine being practically the only metabolite of the D-isomer in vascular plants. Additional evidence for the malonyl conjugation of D-amino acids in higher plants has been recently obtained in several laboratories.[6–8]

In the present work the examination of the metabolism of D-amino acids in plants has been extended to D-serine, a bifunctional amino acid, the L-isomer of which participates in a number of key biochemical reactions. Experiments with L- and D-serine-3-^{14}C were designed with a view to determining the specificity and the extent of the *N*-malonyl conjugation and assessing the taxonomic significance of this reaction. The results obtained in intact tobacco plant and in screening experiments performed with species at all levels of

* Part II in the series "Comparative Studies of L- and D-Amino Acid Metabolism in Plants". For Part I see ref. 5.

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plant evolution corroborate those found for D-methionine metabolism and give a further support for the taxonomic significance of the malonate–D-amino acid conjugation in plant kingdom.

RESULTS AND DISCUSSION

In parallel experiments, equal amounts of L- and D-serine-3-^{14}C were fed to the intact *Nicotiana rustica* plants and the distribution of the label at various time intervals was investigated. The percentages of the recovered radioactivity are given in Table 1. The methanolic plant extracts contained at all times more activity after administration of the D-isomer than after the L-precursor. On the contrary, the amount of the label incorporated into the tissue residues from the whole plant was higher after L-serine, although the residues originating from the shoot of the 4-day D-experiments (Nos. VI and VIII) contained even more ^{14}C than those from the corresponding L-experiments (Nos. V and VII).

TABLE 1. PERCENTAGE OF ABSORBED RADIOACTIVITY IN *Nicotiana rustica* AFTER ADMINISTRATION OF L- AND D-SERINE-3-^{14}C*

Experiment	Duration of expt. days	Serine isomer fed*	Radioactivity recovered (%) Crude methanolic extract Root	Shoot	Dry plant tissue residue Root	Shoot
I	2	L-	10	6	38	11
II	2	D-	18	13	21	11
III	3	L-	13	3	35	4
IV	3	D-	22	8	20	5
V	4	L-	6	3	41	6
VI	4	D-	25	18	13	13
VII	4	L-	7	2	46	2
VIII	4	D-	20	20	22	15

* 10–11 mg/plant.

The distribution of radioactivity in methanolic plant extracts is given in Table 2. Radioactivity of the water effluent was significantly higher, after the D-precursor accounting to more than 30 per cent of the total dose absorbed by plant. In the L-experiments, the low radioactivity of the amino acid fractions was associated with glutamic and aspartic acid, methionine, alanine, proline and a neutral compound, behaving on paper chromatography as a peptide; only traces of label associated with serine could be detected. In the D-experiments, serine was by far the most radioactive component of this fraction.

In the L-experiments, the electrophoretograms of water effluent showed several small, ill-defined peaks which were not further investigated. In the D-experiments, the high activity associated with this fraction was located practically in one acidic spot which upon acid hydrolysis yielded radioactive serine and malonic acid. In the 4-day D-experiments (Nos. VI and VIII) the isolation of the acidic metabolite was performed by submitting the methanolic plant extract to continuous paper electrophoresis; in this way a very good separation from the unchanged tracer and other amino acids was achieved (Fig. 1).

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN METHANOLIC PLANT EXTRACTS AFTER ADMINISTRATION OF L- AND D-SERINE-3-^{14}C

Experiment	Duration of expt. days	Serine isomer fed	Radioactivity recovered (%)* Amino acid fraction Root	Amino acid fraction Shoot	Water effluent Root	Water effluent Shoot
I	2	L-	3	1	4	2
II	2	D-	8	5	10	8
V	4	L-	4	2	2	1
VI	4	D-	5	2	19	16
VII	4	L-	3	1	1	< 0·5
VIII	4	D-	4	4	16	15

* Expressed as percentage of total radioactivity absorbed by plant.

The metabolite of D-serine gave upon hydrazinolysis malonylhydrazide and when it was kept at 140° *in vacuo* the evolution of carbon dioxide and the formation of a new radioactive spot, identified as *N*-acetylserine, was established. The definitive proof for the metabolite structure was obtained by comparison with synthetically prepared *N*-malonyl-DL-serine: the condensation of *o*-benzyl-DL-serine ethyl ester and monoethyl malonate in the presence of dicyclohexylcarbodi-imide gave *N*-ethoxymalonyl-*o*-benzyl-DL-serine ethyl ester which was subjected to alkaline hydrolysis followed by catalytic debenzylation. The resulting crystalline *N*-malonyl-DL-serine was chromatographically and electrophoretically indistinguishable from the D-serine metabolite.

Evidence for the D-configuration of *N*-malonylserine formed in the plant was deduced from the behaviour of its decarboxylation product toward L-acylase from hog kidney: the

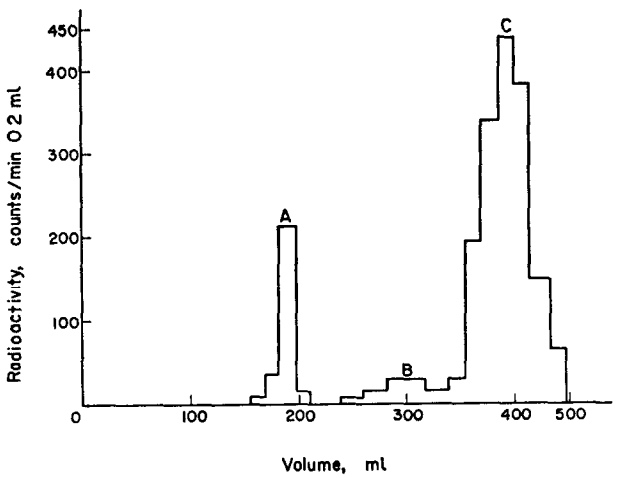

FIG. 1. SEPARATION OF *N*-MALONYL-D-SERINE BY CONTINOUS PAPER ELECTROPHORESIS IN THE METHANOLIC ROOT EXTRACT OF *Nicotiana rustica* FED D-SERINE-3-^{14}C (EXPERIMENT NO. VIII).
(A) serine; (B) glutamic and aspartic acid; (C) *N*-malonyl-D-serine.

obtained *N*-acetylserine proved not to be the substrate of this enzyme. In parallel assays with synthetically prepared *N*-malonyl-DL-serine, and *N*-acetyl-DL-, L- and D-serine it was established that under experimental conditions used neither racemization nor $N \rightarrow O$ acyl-group migration took place.

The above results clearly indicate that the tobacco plant conjugates *D*-serine into the corresponding *N*-malonyl derivative while this pathway is lacking with the L-isomer. However, when compared with the experiments[3] performed under identical conditions with D-methionine-methyl-^{14}C, the formation of *N*-malonyl-D-serine in *Nicotiana rustica* proceeds to a smaller extent than that of *N*-malonyl-D-methionine. The fact that after D-serine-3-^{14}C a significant portion of the label was incorporated into the insoluble plant material indicates that D-serine also undergoes some other metabolic pathway(s) by which the labelled β-carbon atom enters into the normal schemes of plant metabolism.

To obtain some information on the relative utilization of the β-carbon atom of L- and D-serine in transmethylation reactions, the incorporation of ^{14}C into nicotine and lignin was measured. The ^{14}C content of nicotine isolated as dipicrate salts in the 3-day experiments (Nos. III and IV) was generally very low: with the L-precursor, nicotine from the root incorporated about 0·1 and from the shoot about 0·5 per cent of the label while with the D-precursor the values were much lower. Lignins originating from the L-experiments (Nos. I and VII) incorporated a significantly higher percentage of the parent tissue activity than those obtained from the parallel D-experiments (Nos. II and VIII); the ratio of radioactivity, tissue residue–lignin being about 3·1 with the L- and 10.1 with the D-precursor. This would indicate that compounds associated with the initial D-serine activity were used to a much lower extent for the biosynthesis of lignin than those arising from the L-isomer.

The taxonomic significance of D-serine metabolic pathways was investigated in a series of screening experiments performed parallelly with L- and D-serine-3-^{14}C. In Table 3 the distribution of radioactivity in lower and higher plants after administration of labelled L- and D-serine is given. With bacteria and algae the pattern of ^{14}C incorporation differed only quantitatively with respect to the isomer applied; in the D-experiments the majority of the activity in the amino acid fraction was associated with serine, while in the L-experiments besides serine, glutamic and aspartic acid contained significant amounts of the fraction activity.

The first conjugation of D-serine with acetate was observed at the stage of fungi and with malonate at the stage of mosses; in parallel L-serine experiments neither the corresponding *N*-acetyl nor *N*-malonyl derivative could have been detected. The high ability of baker's yeast to acetylate D-amino acids has been again confirmed:[9] by far the greatest part of D-serine was metabolized into *N*-acetylserine which proved to be of D-configuration. On the other hand, in *Agaricus campestris* and *Cantharellus cibarius* as well as in the two species of lichens which had been investigated, D-serine was partly conjugated with acetic acid and partly converted into several ninhydrin-negative, acidic and neutral compounds, indistinguishable from the radioactive spots appearing in the water effluent of the parallel L-experiment

Mosses proved to be the most heterogeneous class. In *Riccia fluitans* and *Mnium undulatum* no conjugation of D-serine took place, while in *Sphagnum palustre* and *Polytrichum* sp., the activity was associated with the neutral metabolites common to the both precursors, and with *N*-acetyl- and *N*-malonyl-serine.

Experiments performed with Pteridophyta and Spermatophyta showed unequivocally

[9] M. H. ZENK and H SCHERF, *Planta* **62,** 350 (1964).

TABLE 3. DISTRIBUTION OF RADIOACTIVITY IN LOWER AND HIGHER PLANTS AFTER ADMINISTRATION OF L- AND D-SERINE-3-^{14}C

Plant investigated	Serine Isomer fed	Radioactivity recovered (%) Amino acid fraction	Water effluent	Dry tissue residue	Total
Bacteria					
Escherichia coli B.	L	3	4	80	87
	D	4	3	78	85
Xanthomonas sp.	L	12	6	54	72
	D	22	4	51	77
Algae					
*Oscillatoria animalis**	L	18	9	12	39
	D	68	4	6	78
*Euglena gracilis**	L	30	5	10	45
	D	60	2	1	63
*Chlorella vulgaris**	L	86	4	7	97
	D	96	2	1	99
Ulva lactuca	L	61	8	12	81
	D	72	6	9	87
Fungi					
Saccharomyces cerevisiae	L	28	4	16	48
	D	9	28	8	45
Agaricus campestris	L	28	6	21	55
	D	48	15	16	79
Cantharellus cibarius	L	31	4	13	48
	D	49	16	3	68
Lichens					
Cladonia sp.	L	48	6	23	57
	D	68	12	11	91
Xanthoria parietina	L	39	4	18	61
	D	58	9	9	76
Bryophyta					
Riccia fluitans	L	18	5	48	71
	D	41	3	38	82
Mnium undulatum	L	20	10	50	80
	D	68	5	18	91
*Sphagnum palustre**	L	58	15	18	91
	D	78	5	13	96
Polytrichum sp.	L	48	13	26	87
	D	71	4	20	95
Pteridophyta					
Selaginella denticulata	L	18	5	54	77
	D	55	9	25	89
Nephrolepis exaltata	L	17	6	66	89
	D	41	18	35	94
Spermatophyta					
Pinus nigra	L	21	4	58	83
	D	28	7	48	83
Phaseolus vulgaris	L	13	8	60	81
	D	12	24	54	90
Pisum sativum	L	30	8	40	78
	D	40	18	22	80
*Wolffia arrhiza**	L	39	4	26	69
	D	41	20	14	75
Zea mays	L	37	8	18	63
	D	52	18	12	82

Time of incubation: for Bacteria, *Agaricus campestris* and *Cantharellus cibarius* 1 day, for all other species 2 days.

* Axenic cultures.

that the vascular plants conjugate D-serine into *N*-malonyl-D-serine. The amino acid fractions originating from the D-experiments contained in all cases serine as the most radioactive component; exceptions were *Selaginella denticulata* which incorporated a significant amount of the water effluent activity into a neutral ninhydrin-negative metabolite, and *Pinus nigra* which formed besides *N*-malonylserine also the corresponding *N*-acetyl derivative.

In view of the taxonomic significance, the data obtained with D-serine are fully consistent with those found for D-tryptophan[9] and D-methionine. However, the malonyl conjugation of D-serine, relative to D-methionine,[5] proceeded definitely to a lower extent. These findings, as well as the fact that the tobacco shoots originating from the 4-day D-experiments were more radioactive than those from the corresponding L-experiments, suggest that the inclusion of D-serine into normal metabolic pathways proceeds successively and to a greater extent than with D-methionine. The present data are insufficient to give any information about the nature of the pathway(s) by which this conversion takes place.

EXPERIMENTAL

Materials and Methods

L-Serine-3-^{14}C, 12·8 μc/m-mole, $[\alpha]_D$ − 6·2° (*c* 1·2 in H_2O) and D-serine-3-^{14}C, 19·2 μc/m-mole, $[\alpha]_D$ +6·8° were prepared by the treatment[10] of *N*-chloroacetyl-DL-serine-3-^{14}C with hog renal acylase and used in Experiment Nos. III and IV, respectively. Identical treatment of *N*-acetyl-DL-serine-3-^{14}C as described[11] for the inactive compound gave L-serine-3-^{14}C, 120·8 μc/m-mole, $[\alpha]_D$ − 7·3° (*c* 1·3 in H_2O) and D-serine-3-^{14}C, 110·8 μc/m-mole, $[\alpha]_D$ +8·0° in better yields and of higher optical purity, these compounds were used in all other tracer experiments.

Paper chromatography was carried out on Whatman No. 1 paper in solvent systems (all by vol.): (1) *n*-BuOH–HOAc–H_2O (60:15:25); (2) *iso*-PrOH–NH_4OH–H_2O (10:1·1); (3) pyridine–aniline–H_2O (9·1:4); and (4) *tert*-BuOH–methyl ethyl ketone–HCOOH–H_2O (40:30:15:15). The spots were visualized with ninhydrin (0·2% in EtOH) for amino acids, with bromo-cresol green (0·04% in EtOH plus one drop of morpholine) for organic acids, and with ammoniacal $AgNO_3$ reagent for the hydrazines. Unidimensional paper electrophoresis was performed on Whatman No. 1 paper at room temp. with a voltage gradient of 12 V/cm in pyridine–HOAc pH 6·5. Continuous-flow paper electrophoresis (Beckman–Spinco Model CP) was made in the same buffer on Schleicher-Schuel 470 paper at room temp. (16 V/cm) for 10 hr. Fractions were collected in test tubes, and aliquots were taken for counting and for chromatography.

Radioactivity of solid samples was counted at infinite thickness and, after corrections, compared with the ^{14}C-polyethylene standards (Amersham, England). Aliquots of liquid samples were counted as infinite thin specimens, and after corrections the counts were compared with the precursor prepared in the same way. Chromatograms were scanned for radioactivity with an automatic GM mica-window scanner or with a Nuclear Chicago Actigraph II.

In the identification of the isolated *N*-malonylserine, acid hydrolysis was performed under reflux in 2 N HCl for 1 hr.; decarboxylation and hydrazinolysis of the conjugate were performed as already described[3] for the D-methionine analogue. For enzymic determinations, samples of the conjugate isolated from *Nicotiana rustica* were decarboxylated in ethylene glycol by heating the solution at 160° for 30 min, the solution was submitted to paper electrophoresis, the radioactive area corresponding to *N*-acetylserine was eluted with water, the eluate was concentrated *in vacuo* and *N*-acetylserine was incubated with acylase (N B.C., from hog kidney) at pH 7·3 and 37° for 24 hr. The mixture was deproteinized with EtOH, evaporated to dryness *in vacuo* and the residue was passed through a column of Dowex 50-X8, H^+ followed by H_2O and then by *N* NH_4OH. Aliquots of the water effluent and the amino acid fraction were checked for radioactivity, and serine was determined spectrophotometrically, in two separate experiments 6 and 10% of serine was obtained. Parallel assays with DL-*N*-acetylserine obtained by decarboxylation of *N*-malonyl-DL-serine and with L- and D-*N*-acetylserine gave 46, 94 and 6% of serine, respectively.

Plant Materials and Incubation Conditions

The growing of *Nicotiana rustica* L., the administration of the tracer through the root to the intact plant, and extraction of the homogenized plant material with methanol was performed as already described.[12]

E. coli B. and *Xanthomonas* sp. were grown in the liquid medium[13] and incubated at concentrations of

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10^7/ml with 150 μg of L- and D-serine-3-^{14}C, respectively. Incubation of *Oscillatoria animalis* (1 ml of the suspension), *Euglena gracilis* (10^5/ml) and *Chlorella vulgaris* (10^6/ml) was performed as already described[5] with the same amount of serine. *Ulva lactuca* (500 mg) was incubated after Greene[14] in 10 ml of sea water with 150 μg of the substrate. Baker's yeast (100 mg) was incubated[15] in 20 ml of 10% sucrose in H_2O with 3 mg of the substrate. Fungi *Agaricus campestris* and *Cantharellus cibarius* were incubated[5] as tissue slices (2·5 g) in 10 ml H_2O with 150 μg of the substrate.

Screening experiments with higher plants were performed with young leaves or cut shoots (500 mg) by absorption of the substrate (150 μg in 0·1 ml H_2O) through the cut area of the sample.

Fractionation of Plant Material

The MeOH extracts from intact *Nicotiana rustica* plants were concentrated *in vacuo* (10–15 ml/plant) and aliquots were treated in one of the following ways: (a) the concentrate was neutralized to pH 7 and passed followed by water through the connected columns of Dowex 50-X4 in NH_4^+ and H^+ form,[16] and the fractions containing basic amino acids, acidic and neutral amino acids, and the H_2O effluent were separately investigated; (b) the concentrate was subjected directly to continuous-flow paper chromatography; and (c) the concentrate was made strongly alkaline with NH_4OH and nicotine was isolated[17,18] as the dipicrate salt. Lignin was isolated from the insoluble tissue residue by subjecting the material to a series of solvent extractions, followed by treatment with cold and boiling H_2SO_4.[19]

The incubates of bacteria, algae and yeast were centrifuged off, the precipitates were treated with N HCl nutrient medium and 50% EtOH, the combined supernatants were neutralized, concentrated *in vacuo* and passed through a column of Dowex 50W-X4 in H^+ form, followed by H_2O; amino acids were displaced from the column with 2 N NH_4OH. Fungi and all other higher plants were homogenized in a mortar with 80% EtOH, the homogenate was centrifuged off, the precipitate washed with 50% EtOH and H_2O, and the combined supernatants were treated as described above.

Synthesis of N-*malonyl*-DL-*serine*

N-*Ethoxymalonyl*-O-*benzyl*-DL-*serine ethyl ester*. To a solution of *O*-benzyl-DL-serine ethyl ester[20] (780 mg, 3·5 m-moles) and monoethyl malonate (462 mg, 3·5 m-moles) in 8 ml tetrahydrofuran (THF), the equivalent amount of dicyclohexylcarbodi-imide (721 mg) in 4 ml THF was added, and the mixture was left to stand at room temp. overnight. Dicyclohexylurea was filtered off, the solvent was removed *in vacuo*, and the remaining oil was chromatographed on a silicagel column (77 × 1·6 cm) with Et_2O–light petroleum (1:1); the displacement of the product was followed by TLC on silica-gel plates in the same solvent. Fractions containing the pure product were pooled, evaporated *in vacuo* and the remaining viscous oil (924 mg, 78%) was crystallized from light petroleum: m.p. 61·5–62·5°. (Found: C, 60·81; H, 6·72; N, 4·41. $C_{17}H_{23}NO_6$ required: C, 60·52; H, 6·87; N, 4·15%.)

N-*Malonyl*-O-*benzyl*-DL-*serine*. The saponification of the ethyl ester (914 mg, 2·7 m-moles) was performed in 12 ml acetone with 5·4 ml *N* NaOH at room temp. under shaking for 5 hr. The solvent was removed *in vacuo*, H_2O was added to the residue, the solution was extracted with EtOAc, acidified with 2 N HCl to pH 2 and the product was extracted with EtOAc. After drying and evaporation of the solvent, the remaining viscous oil was triturated with ether whereupon it crystallized; yield: 467 mg (67%). For analysis it was recrystallized from dry EtOH–Et_2O, m.p. 153–154°. (Found: C, 55·63; H, 5·24; N, 5 12. $C_{13}H_{15}NO_6$ required: C, 55·51; H, 5·38; N, 4·98%.)

N-*Malonyl*-DL-*serine*. A solution of *N*-malonyl-*O*-benzyl-DL-serine (281 mg, 1 m-mole) in dry MeOH (15 ml) was hydrogenated over Pd/C (10% Pd, Fluka puriss., 100 mg) at room temp. and pressure. The mixture was centrifuged off, the precipitate washed with MeOH and the combined supernatants were evaporated to dryness. The remaining oil was dissolved in acetone (5 ml) and petroleum was added until turbidity persisted. On cooling crystals of *N*-malonyl-DL-serine (146 mg, 76%) deposited. They were recrystallized from dry EtOH–Et_2O m.p. 131–133°. (Found: C, 37·52; H, 4·47; N, 7·60. $C_6H_9NO_6$ required: C, 37·70; H, 4·75; N, 7·33%.) IR spectrum (KBr): 3400s (OH), 1630s (amide I) and 1540s (amide II) cm^{-1}. R_fs: solvent (1), 0·45 (after prolonged standing in water an additional acidic ninhydrin-negative spot at 0·32 appears); solvent (2), 0·07; solvent (4), 0·58.

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