Phytochemistry, 1970, Vol. 9, pp. 2175 to 2188. Pergamon Press. Printed in England.

COMPARATIVE STUDIES OF L- AND D-METHIONINE METABOLISM IN LOWER AND HIGHER PLANTS

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(*Received* 6 *January* 1970)

Abstract—The fate of L- and D-methionine-methyl-^{14}C has been studied in a number of plant species at all levels of plant evolution. The ^{14}C distribution in ethanolic plant extracts and dry tissue residues shows that L-methionine, in general, is metabolized in the same way in lower and higher plants whereas the D-isomer follows two essentially different pathways which are determined by the evolutionary stage of the phyla involved. In bacteria, algae, liverworts and some lichens the metabolic patterns of L- and D-methionine are qualitatively identical: the major part of acidic fraction activity is associated with α-hydroxy- and α-keto-γ-methylthiobutyric acid. Higher plants acylate D-methionine with retention of configuration; by far the greater number convert the D-isomer exclusively into the *N*-malonyl conjugate, while some of them form the corresponding *N*-acetyl derivative also. Fungi and some lichens, and mosses and club mosses represent two transitional phyla in which besides deamination, acetylation and malonylation also takes place. None of the species tested showed the ability to malonylate L-methionine, while baker's yeast metabolized the L-isomer partly into the acetyl conjugate which proved to be of D-configuration. The results tend to show the scope and specificity of malonylation reaction for all D-amino acids in the plant kingdom.

INTRODUCTION

IN RECENT years several reports in the literature have been dealing with the formation of the conjugates between D-amino acids and malonate in plants. *N*-Malonyltryptophan was isolated by Good and Andreae[1] from the spinach leaves incubated in an aqueous solution of DL-tryptophan. Subsequent work of Zenk *et al.*[2-4] has shown that *N*-malonyl-D-tryptophan occurs as a natural product in a number of higher plants and that it is formed upon administration of D-tryptophan whereas this is not the case when L-tryptophan is used. Our studies on the metabolism of the optical isomers of methionine in intact *Nicotiana rustica* have shown[5] that, under identical experimental conditions, the majority of the D-isomer underwent malonic acid conjugation, while this pathway was completely lacking with its enantiomorph; the *N*-malonyl-methionine formed proved to be of D-configuration.[6] Rosa and Neish[7] established that feeding of barley shoots with L- and D-isomers of phenylalanine gives the corresponding *N*-malonyl derivative only in the case of the D-isomer. Malonyl conjugation of the D-isomer has been further observed in similar tests performed with L- and D-anomers of

* This work forms part of a thesis to be submitted by M.P. to the University of Zagreb in partial fulfilment of the requirements for the Ph.D. degree.

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[1] N. E. GOOD and W. A. ANDREAE, *Plant Physiol.* **32**, 561 (1957).
[2] M. H. ZENK and H. SCHERF, *Biochem. Biophys. Acta* **71**, 737 (1963).
[3] M. H. ZENK and H. SCHERF, *Planta* **62**, 350 (1964).
[4] M. H. ZENK and J. H. SCHMITT, *Biochem. Z.* **342**, 54 (1965).
[5] D. KEGLEVIĆ, B. LADEŠIĆ and M. POKORNY, *Arch. Biochem. Biophys.* **124**, 443 (1968).
[6] B. LADEŠIĆ, M. POKORNY and D. KEGLEVIĆ (in press).
[7] N. ROSA and A. C. NEISH, *Can. J. Biochem.* **46**, 797 (1968).

seven amino acids in all of the vascular plants tested. In their study on translocation of L- and D-phenylalanine in *Vicia faba*, Eschrich and Hartmann[8] found that, in parenchyma, the D-isomer is acylated with malonic acid to the phloem-immobile *N*-malonyl-D-phenylalanine. Moreover, evidence is given[9] that the tobacco plant metabolizes D-β-methionine (D-β-amino-γ-methylthiobutyric acid), a structurally and sterically unnatural amino acid, into the corresponding *N*-malonyl conjugate; the latter is not formed in tobacco plant fed with L-β-methionine.

Malonylation of D-amino acids has been observed only in higher plants, however, and with the exception of bacteria and moulds, very few studies have been done with D-amino acids in lower plants. Zenk and Scherf[3] studied the metabolism of D-tryptophan in a number of plant species and found that vascular plants convert D-tryptophan to *N*-malonyl derivative while certain fungi form the corresponding *N*-acetyl conjugate, and some bacteria reacemize it.

The aim of the present study was to get more information about the taxonomic significance of the D-amino acid metabolic pathway in plant kingdom, particularly with respect to the scope and specificity of the D-amino acid–malonate acylation reaction. The results obtained with ^{14}C labelled L- and D-methionine in a number of species, at all levels of plant evolution, show that the metabolism of D-methionine in lower plants differs essentially from that in higher plants, and suggest that the correlation between metabolic pathways of D-amino acids and the species in which they proceed could add new information to plant taxonomy.

RESULTS

L- and D-methionine-methyl-^{14}C were administered under identical conditions, in parallel experiments, to several species of each class of plant kingdom, except Myxomycetes and Psilophyta. Plant extracts were passed through Dowex H^+ and the fractions were submitted to chromatographic and electrophoretic separation.

In Table 1 the distribution of radioactivity in bacteria, algae, fungi and lichens is given. Regardless of the isomer applied, by far the largest part of activity in amino acid fractions is associated with methionine and its sulphoxide; however, the latter should be considered more as an artifact arising during the isolation procedure than as a true metabolite. The remaining, mostly very small portion of activity is associated with *S*-methylmethionine.

Fractions containing the ninhydrin-negative, acidic and neutral compounds are, in general, less radioactive than the corresponding amino acid fraction. Incorporation of radioactivity into the fibrous part of the plant is for the most part considerably higher after the administration of L-methionine. Hydrolysis of the dry tissue residues yielded methionine as the preponderant radioactive spot; regardless of the isomer fed, it was of L-configuration.

The results obtained with liverworts, mosses and vascular plants are summarized in Table 2. The ^{14}C content of amino acid fractions and the incorporation pattern do not differ essentially from those found in lower plants. However, in contrast to the latter, the organic acid fractions from higher plants are considerably more radioactive after feeding the D-isomer and the difference between the two anomers increases with the stage of evolution of the species investigated. Hydrolysates of the tissue residues obtained from higher plants fed with the L-isomer contain methionine as the major radioactive spot. On the contrary, methionine

[8] W. ESCHRICH and T. HARTMANN, *Planta* 85, 213 (1969).
[9] B. LADEŠIĆ and D. KEGLEVIĆ, *Phytochem.* 8, 51 (1969).

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN LOWER PLANTS AFTER ADMINISTRATION OF L- AND D-METHIONINE-METHYL-^{14}C

Plant investigated (classes, genus and species)*	Isomer fed	Radioactivity recovered (%)			
		Amino acid fraction	Organic acid fraction	Dry tissue residue	Total
BACTERIA					
Escherichia coli B	L	23	25	18	66
	D	54	7	16	77
Escherichia coli K_{12} meth$^-$	L	14	14	17	45
	D	43	4	13	60
Epiphytic bacteria A†	L	64	15	—	—
	D	83	11	—	—
Epiphytic bacteria B†	L	68	12	—	—
	D	83	8	—	—
Epiphytic bacteria C†	L	70	11	—	—
	D	83	8	—	—
CYANOPHYCEAE					
Anabaena doliolum Bharadwaja‡	L	53	9	5	67
	D	70	7	5	82
Fisherella mucicola (Thuret) Gomont‡	L	21	17	10	48
	D	50	21	6	77
Nostoc spp.	L	21	13	5	39
	D	26	14	5	45
Oscillatoria animails Agardh‡	L	17	19	3	39
	D	28	18	3	49
EUGLENOPHYCEAE					
Euglena gracilis "Z" strain‡	L	2	26	4	32
	D	20	13	4	37
PYRROPHYCEAE					
Ceratium and *Peridinium* spp.§	L	50	7	—	—
	D	90	1	—	—
CHRYSOPHYCEAE					
Navicula spp.	L	13	13	—	—
	D	29	26	—	—
Nitzschia spp.	L	27	33	—	—
	D	38	34	—	—
Synedra spp.‡	L	7	5	—	—
	D	18	6	—	—
XANTHOPHYCEAE					
Vaucheria spp.	L	35	10	6	51
	D	80	5	2	87
CHLOROPHYCEAE					
Ankistrodesmus falcatus (Corda) Ralfs.‡	L	32	1	7	40
	D	83	1	3	87
Chlamydomonas reinhardii Dangeard‡	L	10	46	—	—
	D	90	2	—	—
Chlorelugai vulgaris Beyerinck‡	L	29	1	6	36
	D	88	0	2	90
Scenedesmus quadricauda (Turpin) Brébisson‡	L	30	14	5	49
	D	74	3	1	78
Udotea petiolata (Turra) Börges	L	15	25	34	74
	D	60	12	13	85
Ulva lactuca L.	L	25	17	35	77
	D	47	10	27	84
PHAEOPHYCEAE					
Cystoseira abrotanifolia Ag.	L	16	21	27	64
	D	47	12	17	76

TABLE 1—*cont.*

Plant investigated (classes, genus and species)*	Isomer fed	Radioactivity recovered (%) Amino acid fraction	Organic acid fraction	Dry tissue residue	Total
Dilophus spp.	L	11	11	59	81
	D	27	13	42	82
Fucus virsoides J. Ag.	L	19	23	35	77
	D	40	15	24	79
Padina pavonia (L.) Gail	L	21	21	42	84
	D	41	16	34	91
RHODOPHYCEAE					
Ceramium spp.	L	11	11	65	87
	D	38	12	40	90
Laurentia obtusa (Huds.) Lam.	L	22	28	37	87
	D	63	10	13	86
Rhodymenia spp.	L	30	21	14	65
	D	66	6	7	79
Vidalia volubilis (L.) J. Ag.	L	31	20	31	82
	D	64	4	14	82
PHYCOMYCETES					
Rhisopus nigricans Ehr.	L	13	13	50	76
	D	33	14	39	86
ASCOMYCETES					
Saccharomyces cerevisiae Hansen.	L	12	19	20	51
	D	18	56	9	83
Schizosaccharomyces pombe A-7 Strain‡	L	82	2	—	—
	D	98	0	—	—
Beauveria bassiana (Bals.) Vuill‡	L	16	8	66	90
	D	60	3	34	97
BASIDIOMYCETES					
Agaricus campestris L. ex Fraction	L	60	6	10	76
	D	70	6	7	83
Boletus edulis Bull. ex Fraction	L	12	12	8	32
	D	49	4	6	59
Cantharellus cibarius Fraction	L	15	5	49	69
	D	42	24	18	84
Rhodophyllus mammosus (Fraction) Quél.	L	58	9	8	75
	D	91	3	1	95
LICHENS					
Cladonia spp.	L	18	18	30	66
	D	30	32	15	77
Parmelia spp.	L	12	10	24	46
	D	30	16	12	58
Usnea barbata (L.) Körb.	L	10	9	32	51
	D	40	3	18	61
Xanthoria parietina (L.) Beltram.	L	12	8	16	36
	D	38	13	8	59

Time of incubation: for Bacteria and Basidiomycetes 1 day, for Asomycetes 3 days, and for all other classes 2 days.

* Arranged according to E. Strasburger.[10]

† Epiphytic bacteria: A, rod-shaped cells, yellow colonies; B, rod-shaped cells, pale colonies; C, coccoid cells, yellow colonies.

‡ Axenic cultures.

§ The content of phytoplankton sample.

[10] E. STRASBURGER, *Lehrbuch der Botanik für Hochschulen* (edited by D. DENFFER, W. SCHUMACHER, K. MÄGDEFRAU and F. FIRBAS), 29th edition, Fisher Verlag, Stuttgart (1967).

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN HIGHER PLANTS AFTER ADMINISTRATION OF L- AND D-METHIONINE METHYL-^{14}C

Plant investigated (classes, genus and species)*	Isomer fed†	Radioactivity recovered (%) Amino acid fraction	Organic acid fraction	Dry tissue residue	Total
HEPATICAE					
Riccia fluitans L.	L	42	7	27	76
	D	73	11	13	97
Marchantia polymorpha L.	L	20	18	36	74
	D	46	9	25	80
MUSCI					
Mnium undulatum (L.) Weis	—	—	—	—	—
	D	79	8	8	95
Polytrichum attenuatum Menz.	L	26	7	22	55
	D	66	16	13	95
Polytrichum commune L.	L	32	21	22	75
	D	80	5	13	98
Sphagnum palustre L.	L	34	4	34	72
	D	54	22	18	94
LYCOPODIINAE					
Lycopodium clavatum L.	—	—	—	—	—
	D	45	31	4	80
Selaginella denticulata (L.) Lk.	L	40	15	18	73
	D	40	50	7	97
Selaginella exaltata (L.) Lk.	—	—	—	—	—
	D	50	26	6	82
EQUISETINAE					
Equisetum hiemale L.	L	28	11	24	63
	D	25	29	16	70
Equisetum arvense L.	—	—	—	—	—
	D	50	17	11	78
FILICINAE					
Adiantum capillus Veneris L.	—	—	—	—	—
	D	66	11	4	81
Asplenium trichomanes L.	—	—	—	—	—
	D	40	22	8	70
Marsilia quadrifolia L.	—	—	—	—	—
	D	46	26	7	79
Nephrolepis exaltata (L.) Schott	—	—	—	—	—
	D	49	18	8	75
Polypodium vulgare L.	L	21	6	40	67
	D	15	38	21	74
Polystichum setiferum (Forsk.) Wojnar	—	—	—	—	—
	D	51	30	—	—
Pteridium aquilinum (L.) Kuhn	L	44	13	19	76
	D	35	40	10	85
Salvinia natans (L.) All.	—	—	—	—	—
	D	70	16	4	90
Scolopendrium vulgare L.	—	—	—	—	—
	D	24	60	6	90
CYCADINAE					
Cycas revoluta Thunb.	L	30	5	36	71
	D	47	25	18	90
Cycas circinalis L.	—	—	—	—	—
	D	55	16	18	89
GINKGOINAE					
Ginkgo biloba L.	L	35	14	23	72
	D	52	26	15	93

TABLE 2—*cont.*

Plant investigated (classes, genus and species)*	Isomer fed†	Radioactivity recovered (%) Amino acid fraction	Organic acid fraction	Dry tissue residue	Total
CONIFERAE					
Abies alba Mill.	—	—	—	—	—
	D	45	37	6	88
Araucaria spp.	—	—	—	—	—
	D	66	21	—	—
Cedrus atlantica Manetti	L	26	13	30	69
	D	30	34	20	84
Picea excelsa (Lam.) Lk.	—	—	—	—	—
	D	62	6	—	—
Pinus nigra Arnold	—	—	—	—	—
	D	15	50	14	79
Sequoiadendron giganteum Buchh.	—	—	—	—	—
	D	50	30	12	92
Taxus baccata L.	—	—	—	—	—
	D	36	20	—	—
Thuja occidentalis L.	—	—	—	—	—
	D	58	18	6	82
GNETINAE					
Ephedra campylopoda C. A. Mey.	L	17	7	30	54
	D	30	34	8	72
Ephedra nebrodensis Host.	—	—	—	—	—
	D	31	41	7	79
DICOTYLEDONEAE					
Begonia rex Putz.	—	—	—	—	—
	D	40	27	—	—
Carpinus betulus L.	L	42	7	18	67
	D	33	51	8	92
Hedera helix L.	—	—	—	—	—
	D	54	46	—	—
Helleborus niger L.	—	—	—	—	—
	D	21	60	4	85
Laportea gigas Wedd.	—	—	—	—	—
	D	40	51	—	—
Nicotiana rustica L.‡	L	62	9	18	89
	D	18	71	3	92
Nicotiana tabacum L.	—	—	—	—	—
	D	74	16	1	91
Pastinaca sativa L.	—	—	—	—	—
	D	29	44	12	85
Phaseolus vulgaris L.	—	—	—	—	—
	D	45	42	4	91
Prunus laurocerasus L.	—	—	—	—	—
	D	35	45	11	91
Raphanus sativus L.	L	21	10	35	66
	D	31	45	9	85
Sibiraea croatica Deg.	—	—	—	—	—
	D	26	40	13	79
Urtica dioica L.	—	—	—	—	—
	D	31	34	11	76
MONOCOTYLEDONEAE					
Allium sativum L.	L	28	1	6	35
	D	77	6	3	86

TABLE 2—*cont.*

Plant investigated (classes, genus and species)*	Isomer fed†	Radioactivity recovered (%) Amino acid fraction	Organic acid fraction	Dry tissue residue	Total
Avena sativa L.	—	—	—	—	—
	D	61	21	—	—
Colchicum autumnale L.	L	20	10	41	71
	D	33	42	19	94
Galanthus nivalis L.	—	—	—	—	—
	D	50	21	12	83
Elodea canadensis Rich.	—	—	—	—	—
	D	85	9	3	97
Rhoeo discolor (L'Herit) Hance ex Walp.	—	—	—	—	—
	D	57	36	—	—
Wolffia arrhiza (L.) Wimm.‡	L	31	23	17	71
	D	39	45	6	90
Zea mays L.	—	—	—	—	—
	D	69	10	—	—
Zebrina pendula Schnizl.	—	—	—	—	—
	D	50	22	—	—

Time of incubation: for Dicotyledoneae and Monocotyledoneae 2 days and for all other classes 4 days.
* Arranged according to E. Strasburger.[10]
† A— means "not tested".
‡ Axenic cultures.

originating from the tissue residues of plants given the D-anomer proved to be completely inactive; during acid hydrolysis the greatest part of radioactivity disappeared while a small portion remained associated with the insoluble part of the cell-wall constituents.

In Table 3 are listed plant species whose ^{14}C distribution in amino acid fraction differs distinctly from the general incorporation pattern. Among the lower plants tested, *S*-methylmethionine appears as one of the major metabolites of L- and D-methionine only in the sea lettuce, *Ulva lactuca*, while in higher plants, representatives of several classes form considerable amounts of this compound from L-methionine. Five species of seaweeds, among ten species tested, metabolize labelled L-methionine into dimethyl-β-propiothetin; with the D-isomer this compound is formed by three species in small but still significant amounts.

Regardless of the phyla investigated, the radioactivity of acidic fractions obtained from species fed with L-methionine-methyl-^{14}C is, in general, associated with α-hydroxy- and α-keto-γ-methylthiobutyric acids. Bacteria, algae, fungi and lichens, contain two additional acidic thioether-positive compounds, mostly of very low activities (acidic metabolites *A* and *B*); one of them has been identified as β-methylthiopropionic acid. Liverworts, mosses, club mosses and some algae and lichens contain, in addition, a neutral thioether-positive compound of low radioactivity (neutral metabolite *C*) which has not been identified. Fungi produce another metabolite of low activity (neutral metabolite *D*) which has been recognized as 3-methylthiopropan-1-ol. The R_f values of the isolated metabolites are given in Table 4.

The separation of the above ninhydrin-negative metabolites proved to be rather difficult, partly due to very similar R_f values and electrophoretic mobilities, and partly because during the isolation procedure the oxidation to the sulphoxide stage took place. Since the corresponding sulphoxides give a better resolution, the mixture of the thioether-positive metabo-

TABLE 3. RADIOACTIVITY OF *S*-METHYLMETHIONINE AND DIMETHYL-β-PROPIOTHETIN FOUND IN THE AMINO ACID FRACTION OF THE VARIOUS PLANT SPECIES FED WITH L-AND D-METHIONINE-METHYL-^{14}C

Species investigated	Isomer fed	Radioactivity (%)*†	
		S-Methylmethionine	Dimethyl-β-propiothetin
Escherichia coli K_{12} meth$^-$	L	10	—
	D	4	—
Ulva lactuca	L	33	56
	D	18	33
Padina pavonia	L	—	45
	D	—	—
Dilophus spp.	L	—	7
	D	—	2
Vidalia volubilis	L	—	48
	D	—	33
Ceramium spp.	L	—	48
	D	—	28
Marchantia polymorpha	L	51	—
	D	18	—
Polytrichum commune	L	21	—
	D	5	—
Equisetum hiemale	L	43	—
	D	16	—
Cedrus atlantica	L	18	—
	D	2	—
Ephedra campylopoda	L	22	—
	D	3	—
Raphanus sativus	L	24	—
	D	7	—
Wolffia arrhiza	L	18	—
	D	2	—

* Expressed as percentage of amino acid fraction radioactivity.
† Indicates that the metabolite was not formed in detectable amounts.

TABLE 4. R_f VALUES OF THE METHIONINE METABOLITES AND THEIR SULPHOXIDES*

	Solvents		
	1	2	3
α-Hydroxy-γ-methylthiobutyric acid	0·85 (0·50)	0·50 (0·33)	0·70 (0·50)
α-Keto-γ-methylthiobutyric acid	0·68 (0·50)	0·56 (0·33)	0·70 (0·50)
Acidic metabolite *A*	0·90 (0·60)	0·57 (0·35)	0·70 (0·60)
β-Methylthiopropionic acid	0·89 (0·60)	0·58 (0·36)	0·70 (0·61)
Acidic metabolite *B*	0·93 (0·75)	0·67 (0·46)	0·81 (0·70)
Neutral metabolite *C*	0·40 (0·20)	0·15 (0·10)	0·40 (0·30)
Neutral metabolite *D*	0·90 (0·65)	0·95 (0·69)	0·98 (0·85)
3-Methylthiopropan-1-ol	0·91 (0·66)	0·95 (0·69)	0·98 (0·85)
N-Acetylmethionine	0·85 (0·50)	0·56 (0·35)	0·70 (0·50)
N-Malonylmethionine	0·75 (0·33)	0·20 (0·10)	0·60 (0·39)

* R_f values of sulphoxides are given in parenthesis.

lites was oxidized, the resulting sulphoxides were resolved by paper chromatography (Table 4) and then reduced to the starting thioethers.

It should be emphasized that after administration of labelled L-methionine neither *N*-acetyl nor *N*-malonyl-methionine could have been detected. The only exception among

TABLE 5. METABOLITES OF D-METHIONINE-METHYL-^{14}C FORMED BY THE DIFFERENT CLASSES OF PLANTS*

Classes†	Number of species tested	Conjugated		Non-conjugated‡	
		N-Malonyl-D-methionine	*N*-Acetyl-D-methionine	Acidic	Neutral
BACTERIA	5	—	—	+	—
CYANOPHYCEAE	4	—	—	+	—
EUGLENOPHYCEAE	1	—	—	+	—
PYRROPHYCEAE	§	—	—	+	—
CHRYSOPHYCEAE	3	—	—	+	—
XANTHOPHYCEAE	1	—	—	+	—
CHLOROPHYCEAE	6	—	—	+	+
PHAEOPHYCEAE	4	—	—	+	+
RHODOPHYCEAE	4	—	—	+	+
PHYCOMYCETES	1	—	+	+	+
ASCOMYCETES	3	—	+	+	+
BASIDIOMYCETES	4	—	+	+	+
LICHENS	4				
Cladonia spp.		—	+	+	+
Parmelia spp.		—	+	+	+
Usnea barbata		—	—	+	+
Xanthoria parietina		—	—	+	+
HEPATICAE	2	—	—	+	+
MUSCI	5				
Brachytecium spp.		—	—	+	+
Mnium undulatum		—	—	+	+
Polytrichum attenuatum		+	—	+	+
Polytrichum commune		+	—	+	+
Sphagnum palustre		—‖	+	+	—
LYCOPODIINAE	3	+	—	—	+
EQUISETINAE	2	+	—	—	—
FILICINAE	9	+	—	—	—
CYCADINAE	2	+	—	—	—
GINKGOINAE	1	+	—	—	—
CONIFERAE	8				
Abies alba		+	+	—	—
Araucaria spp.		+	—	—	—
Cedrus atlantica		+	+	—	—
Picea excelsa		+	+	—	—
Pinus nigra		+	+	—	—
Sequoiadendron giganteum		+	—	—	—
Taxus baccata		+	—	—	—
Thuja occidentalis		+	—	—	—
GNETINAE	2	+	—	—	—
DICOTYLEDONEAE	13	+	—	—	—
MONOCOTYLEDONEAE	9	+	—	—	—

* + Indicates formation of the corresponding metabolite and — indicates that it was not formed in detectable amounts.

† In the case of differences within the class, the species investigated are tabulated.

‡ See text for full chemical name of these acidic and neutral metabolites.

§ The content of phytoplankton sample.

‖ Traces of *N*-malonylmethionine were detectable after the first and second day of incubation.

all the species tested is the baker's yeast where about 10% of the acidic fraction activity was associated with *N*-acetyl-methionine. The isolated conjugate proved to be of D-configuration. In a second experiment, a heavy overloading of the yeast (10 g) with L-methionine-methyl-^{14}C (1 g) raised the content of *N*-acetyl-D-methionine to about 30% of the fraction activity.

Contrary to the L-isomer, the metabolites of D-methionine differ substantially with respect to the phyla investigated (Table 5). Bacteria, algae, liverworts and some lichens show practically identical qualitative pattern of radioactive metabolites for both anomers. Moreover, in *Euglena gracilis* and *Fisherella mucicola*, as after administration of L-methionine, α-keto-γ-methylthiobutyric acid and β-methylthiopropionic acid, respectively, are the major metabolites.

The first conjugation of D-methionine with acetate starts with fungi. This group of plants and two species of lichens, *Cladonia* spp. and *Parmelia* spp., metabolize the D-isomer by both pathways.

With the class of horsetails begins plants which make a sharp distinction between the L- and D-isomer. The first conjugation with malonate appears at the stage of mosses, which, with respect to the variety of metabolites formed, represent the most heterogenous class surveyed. From Lycopodiinae upwards all classes have the malonylation reaction. In vascular plants, *N*-malonyl-D-methionine is practically the only metabolite; exceptions are several species of coniferous plants which form both the *N*-acetyl and *N*-malonyl conjugate.

Evidence that *N*-malonylation of D-amino acids in vascular plants proceeds without any participation of epiphytic bacteria on plant surfaces was obtained in experiments with axenic cultures of intact *Nicotiana rustica* and *Wolffia arrhiza* (Table 2). In both cases the rate of formation and the amount of *N*-malonyl-D-methionine were identical to those obtained with the corresponding non-sterile plants. In addition, when bacterial flora from hydrocultured tobacco roots was incubated with D-methionine-methyl-^{14}C no *N*-malonyl-methionine could be detected (Tables 1 and 5).

The fact that in lower plants D-methionine follows the metabolic pathway of L-methionine at the slower rate, indicates that it has been previously converted to the L-form. Cooper[11] found that methionine-requiring strains of *Escherichia coli* grow on D-methionine and that the rate of growth is slightly slower than on L-methionine. We incubated in parallel experiments *E. coli* meth$^-$ mutant[12] with L- and D-methionine-methyl-^{14}C, respectively. From Fig. 1 it can be seen that the intensive growth on D-methionine starts at the time when the culture grown on L-methionine has already reached the stationary phase; after 24 hr there is no more difference in the mass of bacteria. Kinetic measurements established that the beginning of the growth coincides with the disappearance of radioactivity from the media. After 24 hr the incubation mixtures were worked up; regardless of the isomer fed, methionine in the amino acid fraction and in the tissue hydrolysate proved to be of L-configuration.

DISCUSSION

The data obtained in this study point to several facts. One of the most obvious is the evidence that the metabolism of D-methionine in plants proceeds by two essentially different ways which are determined by the evolutionary stage of the plant. In lower plants, the identity of the L- and D-methionine metabolites, the lower rate of their formation from the D-isomer, as well as the fact that after administration of the D-anomer the radioactive methionine incorporated in the plant tissue is of L-configuration, strongly indicate that a

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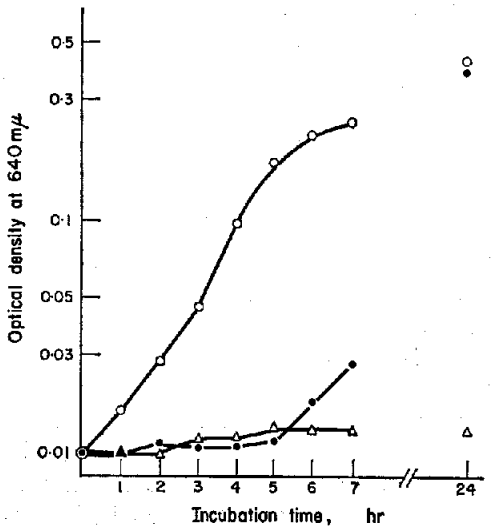

FIG. 1. GROWTH OF *Escherichia coli* K_{12} METH⁻ ON L- AND D-METHIONINE-METHYL-^{14}C.
Cells were inoculated into fresh liquid mineral medium (20 ml) containing 1·2 mg of the substrate.
△ Control; ● D-methionine; ○ L-methionine.

part of D-methionine is converted into the L-form. This conversion proceeds presumably through oxidative deamination by D-amino acid oxidase, followed by L-specific reamination, and/or, at least in some classes, through the enzymic racemization of the isomer. In most of the higher plants these pathways are lacking, and D-methionine is acylated under retention of configuration; by far the greatest part of higher plants metabolize D-methionine exclusively into the *N*-malonyl conjugate.

Fungi and some lichens represent a transition group in which deamination products and the acetate conjugate are formed; the latter is particularly predominant in baker's yeast and *Cantharellus cibarius*. The high acetylating ability of these species has been already observed[13,14] with D-tryptophan, and an enzyme D-acetyltransferase has been isolated from yeast by Zenk and Schmitt.[15,16] Although liverworts are classified into higher plants, they still metabolize D-methionine in the same way as the L-form, while mosses and club mosses represent a transition group among the higher plants. The fact that Pinaceae form *N*-acetyl-D-methionine too, indicates that acetylation should not be considered as a metabolic pathway of D-amino acids attributive only to fungi.

The labelling pattern of L-methionine metabolites, in general, fits well into the already known schemes. The observation that *S*-methylmethionine appears practically in every species and in some higher plants even in considerable amounts, corroborates the study of Splittstoesser and Mazelis[17] who indicate the general distribution and a metabolic function of this compound in higher plants.

Greene[18] has established that methionine is an efficient precursor of dimethyl-β-propio-

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[18] R. C. GREENE, *J. Biol. Chem.* **237**, 2251 (1962).

thetin in alga *Ulva lactuca;* this has now been confirmed on further four species of marine algae. However, the present results do not support the suggestion[18] that β-methylthiopropionic acid arising from the oxidation of methionine may be an intermediate in the biosynthesis of dimethyl-β-propiothetin. We found β-methylthiopropionic acid as a minor metabolite of methionine in a number of lower plants but not in marine algae; moreover, the freshwater alga *Fisherella mucicola,* the only species tested which metabolizes methionine almost exclusively into β-methylthiopropionic acid, does not contain dimethyl-β-propiothetin.

It is known[19] that some moulds and fungi are able to convert methionine to 3-methylthiopropan-1-ol (methionol); in mammals the formation of this metabolite is considered as a pathway which comes in function when the main pathways of methionine are overloaded or defect.[19] We have not observed such a phenomenon in plants, and the present results indicate 3-methylthiopropan-1-ol as a compound associated exclusively with the fungal metabolism of methionine.

The finding that the baker's yeast metabolizes L-methionine partly into *N*-acetyl-D-methionine seems to be of particular interest and could be explained as the result of an enzymic racemization followed by D-stereospecific acetylation. Zenk[3] in his study on L- and D-tryptophan metabolism has already observed a racemase activity in yeast.

The results obtained in different laboratories[2–9] leave little doubt that malonylation represents a general method of metabolism of D-amino acids in plants. Hence, the present study tends to indicate for all D-amino acids the scope and limitation of this metabolic pathway in plant kingdom.

EXPERIMENTAL

Materials and Methods

L-Methionine-methyl-^{14}C, 91·3 μc/m-mole, $[\alpha]_D$ −9·9° (*ca.* 1·2 in water) and D-methionine-methyl-^{14}C, 286·5 μc/m-mole, $[\alpha]_D$ +9·8° (*ca.* 1·0 in water) were prepared as already described.[20] The sodium salt of α-keto-γ-methylthiobutyric acid was prepared after Yamade and Sakurai.[21] 3-Methylthiopropan-1-ol was synthesized after Hurd and Gershbein.[22]

β-Methylthiopropionic acid. The procedure given[23] for the corresponding ammonium salt was followed. The oily acid was obtained analytically pure after distillation at 80–85°/1 mm Hg. (Found: C, 39·82; H, 6·51; S, 26·92. $C_4H_8O_2S$ required: C, 39·98; H, 6·71; S, 26·68%.)

α-Hydroxy-γ-methylthiobutyric acid Ca-salt was prepared after the procedure[24] given for the corresponding Zn-salt, except that for diazotation $NaNO_2$ was used. To the aqueous solution of the crude acid, equivalent amount of $CaCO_3$ was added, the mixture was shaken for 2 hr, filtered and the filtrate evaporated *in vacuo*; the residue was crystallized from 50% ethanol giving the Ca-salt containing 1 H_2O as a white powder; yield: 28%. (Found: C, 33·80; H, 5·62; Ca, 11·60. $C_{10}H_{18}O_6S_2Ca$, 1 H_2O required: C, 33·69; H, 5·66; Ca, 11·24%.)

Paper chromatography was carried out on Whatman No. 1 and 3 MM paper in solvent systems (all by vol.): (1) *n*-BuOH–acetic acid–H_2O (60:15:25); (2) *iso*-propanol–NH_3–H_2O (10:1:1); (3) *tert*-BuOH–methyl ethyl ketone–NH_3–H_2O (40:30:10:20); (4) *tevt*-BuOH–methylethyl ketone–formic acid–H_2O (40:30:15:15); (5) MeOH–H_2O (95:5); and phenol–NH_3–H_2O (160:1:40). The spots were visualized with platinum reagent for thioethers, sulphoxides and sulphonium compounds, with the HI-starch reagent for sulphoxides, with 0·2% ninhydrin in EtOH for amino acids, with 0·5% 2,4-dinitro-phenylhydrazine in 2 N HCl for keto acids, and with 0·04% bromocresol green in 96% EtOH (plus 1 drop of morpholine) for organic acids. Paper electrophoresis was performed on Whatman No. 1 paper at room temp. with a voltage gradient of 12 V/cm in pyridine–acetic acid, pH 6·5.

Acid hydrolysis of radioactive metabolites and of dry tissue residues was performed in 6 N HCl in a sealed Carius tube at 95° for 3 hr. The reduction of sulphoxide-positive peaks was performed with thioglycollic acid after Barnsley.[25] Isolation and identification of dimethyl-β-propiothetin was performed after Greene.[18]

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Enzymatic reactions were performed with acylase (N.B.C., from hog kidney), L-amino acid oxidase (Koch and Light, from *Crotalus adamanteus*), and D-amino acid oxidase (Fluka, pract., from pig kidney) in the standard manners.

Radioactivity of solid samples was counted at infinite thickness and, after corrections, compared with the ^{14}C-polyethylene standards (Amersham, Bucks.). Aliquots of liquid samples were plated in triplicate on stainless-steel planchets, dried under an i.r. lamp and counted as infinite thin specimens in a gas-flow or mica-window GM counter. Chromatograms were scanned for radioactivity with an automatic GM mica-window scanner or with a Nuclear Chicago Actigraph II.

Plant Materials and Incubation Conditions

E. coli B. and *E. coli* K_{-12} meth$^-$ (optimal conc. of methionine for the growth 60 μg/ml) were grown on the liquid mineral medium as already described[12] and incubated at 37° with L- and D-methionine-methyl-^{14}C, respectively. Epiphytic bacteria from hydrocultured tobacco plant roots were isolated by streaking drops of diluted medium in which the plants have grown on yeast extract–glucose agar plates; conditions of incubation were identical with those for the whole plant.

Cyanophyceae were grown in mineral media described previously in the literature: *Anabaena doliolum* and *Fisherella mucicola* after Allen and Arnon,[26] *Nostoc* spp. after Perminova[27] and *Oscillatoria animalis* after Sager and Granick.[28] The incubation (0·2 mg of the substrate) was carried out with 2–3 ml of a concentrated algal suspension in 20–50 ml of the corresponding nutrient media.

Euglenophyceae, Chrysophyceae and Chlorophyceae were grown basically in the mineral medium[28] to which in the case of Euglenophyceae vitamins B_1 (0·1 mg/ml) and B_{12} (4 μg/l.), and in the case of Chrysophyceae traces of silica gel were added. In all cases 10^5 cells/ml in 20 ml of the nutrient medium were left for 2 days and then incubated with 0·2 mg of the substrate. Xanthophyceae were grown in soil H_2O medium and incubated as described for Cyanophyceae.

Samples of phytoplankton and marine Chlorophyceae, Phaeophyceae and Rhodophyceae were collected in the Adriatic Sea near Rovinj (Istria). Marine algae were incubated after Greene;[18] 0·2 mg of the substrate was given to 500 mg of the algal material.

Phycomycetes (*Rhizopus nigricans*) was grown in a rich organic medium.[29] Baker's yeast (100 mg) was incubated: (*a*) in 20 ml of 0·3% yeast extract, 0·5% peptone and 3% saccharose with 4 mg of the substrate; each day 200 mg of saccharose was added to the medium; (*b*) in 10% saccharose in H_2O[30] to which the appropriate amount of the substrate was added. Basidiomycetes were incubated as tissue slices (1 g) in 10 ml H_2O with 0·1 mg of the substrate as already described.[31]

All higher plants used were obtained either from the Botanical Garden, Faculty of Science, University of Zagreb, or were cultivated in our laboratory from seeds, or were commercially available. In general, the leaves or the young cut shoots (500 mg) were incubated with 0·2 mg of the substrate dissolved in H_2O; H_2O was added to keep the level of the feeding solution constant.

Fractionation of Plant Material

At the end of the experiment, the incubates of bacteria, unicellular algae and Ascomycetes were centrifuged off, the precipitate was treated in turn with N HCl, nutrient medium and 50% EtOH and dried at room temp. to constant weight. The combined supernatants were neutralized with N NH_4OH, concentrated *in vacuo* to about 5 ml, passed through a column (6 × 1·2 cm) of Dowex 50W-X4 H^+ (200–400 mesh) and followed by H_2O (40–50 ml). The acidic H_2O effluent was either neutralized and evaporated *in vacuo* to a smaller volume or extracted several times with ethyl acetate; by the latter procedure the corresponding sulphoxides remained in the water layer. Aliquots of the ethyl acetate extracts were subjected either to paper chromatography and electrophoresis or were treated with 2,4-dinitrophenylhydrazine according to Pohloudek-Fabini and Papke.[32] Amino acids were displaced from the column with 2 N NH_4OH, radioactive fractions were pooled, concentrated *in vacuo* and subjected to paper chromatography and electrophoresis.

Marine algae and all higher plants were homogenized in a mortar with 80% EtOH, the homogenate was centrifuged, the precipitate was washed with 50% EtOH and H_2O, and the combined supernatants were treated as described above.

The separation of thioether-positive, ninhydrin-negative metabolites was performed as follows: to the

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aqueous solution of the metabolites, hydrogen peroxide was added until the concentration was about 3%, the mixture was left for 1 hr at room temp. and then submitted to chromatography and electrophoresis. Radioactive peaks were eluted with 50% EtOH, and the separated sulphoxides were reduced to the corresponding thioethers with thioglycollic acid.[25] Since *N*-acetylmethionine and α-hydroxy-γ-methylthiobutyric acid, as well as their sulphoxides, show identical chromatographic and electrophoretic behaviour, for definitive identification the radioactive peaks corresponding to these two metabolites have to be submitted also to the acid hydrolysis.

Acknowledgements—The authors thank Professor Z. Devidé, Department of Botany, Faculty of Science, University of Zagreb, for making available facilities for the cultivation of higher plants in this Institute and for also critically reading the manuscript. We thank Mrs. Jasna Ban, M.Sc., for the preparation of cultures of *E. coli*, Dr. B. S. Srivastava for supplying us with blue-green algae, Mrs. Marjana Krsnik-Rasol, B.Sc., for axenic cultures of *Nicotiana rustica* and *Wolffia arrhiza*, and Dr. S. Kveder for providing facilities for the work at the Centre for Marine Research, Rovinj.