Phytochemistry, 1974, Vol. 13, pp. 965 to 971. Pergamon Press. Printed in England.

D-METHIONINE METABOLIC PATHWAYS IN BRYOPHYTA: A CHEMOTAXONOMIC EVALUATION*

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(*Revised Received* 25 *September* 1973. *Accepted* 18 *October* 1973)

Key Word Index—Bryophyta; mosses; D-methionine; comparative metabolism; chemotaxonomic evaluation.

Abstract—Metabolic pathways of L- and D-methionine-methyl-^{14}C have been studied in a wide range of mosses. The natural L-isomer is metabolized by a deamination reaction in all the Bryophyta, whereas the unnatural D-isomer follows either a deamination pathway or conjugation pathway with acetic acid and with malonic acid, respectively. The phytochemical relationship of Bryophyta with lower and higher plants based on the different D-methionine metabolic pathways is discussed.

INTRODUCTION

COMPARATIVE metabolic studies of L- and D-anomers of tryptophan,[1] methionine[2–4] serine[5] and alanine[6] in a number of plant species at all levels of evolution showed that the L-isomers, in general, are metabolized by deamination reactions, whereas the D-isomers can follow at least two different pathways, which are determined by the evolutionary stage of phyla. In vascular plants the D-isomers are acylated to the corresponding *N*-malonyl-D-conjugates while in fungi and some lichens they are converted into the *N*-acetyl-D-amino acids. In bacteria and algae, the metabolic pathways of L- and D-isomers are qualitatively identical.

Preliminary experiments with Bryophyta[2] have shown that this phylum metabolizes D-methionine by different pathways. Beside deamination and acetylation, conjugation with malonic acid was observed for the first time in several species. These results are interesting, since mosses form a transition group between lower and higher plants and are not phylogenetically and evolutionary distinctly defined.

In the present study we have investigated in more detail the metabolic pathways of D-methionine-methyl-^{14}C in phylum Bryophyta. D-Methionine was chosen for two reasons: in plants *N*-malonyl-D- and *N*-acetyl-D-methionine are formed from the precursor

* Part III in the series "Comparative Studies of L- and D-Amino Acid in Plants". For Part II see POKORNY, M., MARČENKO, E. and KEGLEVIĆ, D. (1970) *Phytochemistry* **9**, 2175.

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in considerable amounts and are metabolically inert;[2,7] and in the process of isolation *N*-malonyl-D-methionine does not decarboxylate to *N*-acetyl-D-methionine as was found with some other amino acids.[6,8,9] The results obtained show that the D-methionine metabolic pathways in mosses offer new information on the Bryophyta taxonomy and phylogeny; they indicate that mosses are a more heterogenous group than previously considered.

RESULTS

In parallel experiments, equal amounts of L- and D-methionine-methyl-^{14}C were administered to several species of Bryophyta under identical conditions. Tracers were absorbed either from the aqueous solution through the whole plant surface or in the case of axenic cultures through the risoids. In all cases plants absorbed 80–90% of the radioactivity given, and there were no essential differences with respect to the isomers

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN BRYOPHYTES AFTER ADMINISTRATION OF L- AND D-METHIONINE-$^{14}CH_3$

Plant investigated (classes, species)*	Isomer fed	Radioactivity recovered (%) Amino acid fraction	Water effluent	Dry tissue residue	Total
Hepaticae					
Aneura pinguis	—	—	—	—	—
Dum.	D	48	8	32	88
Fegatella conica	—	—	—	—	—
Corda	D	47	7	18	72
Lophozia ventricosa†	—	—	—	—	—
(Dicks) Dum.	D	60	6	20	86
Marchantia polymorpha	L	20	18	36	74
L.	D	46	9	25	80
Metzgeria furcata	—	—	—	—	—
Lindb.	D	52	7	30	89
Pellia epiphylla	L	18	18	38	74
Lindb.	D	60	5	26	91
Blassia pusilla L.	—	—	—	—	—
	D	61	9	21	91
Riccia fluitans L.	L	42	7	27	76
	D	73	11	13	97
Sphaerocarpus textanus†	L	45	5	—	—
Aust.	D	75	4	—	—
Sphaerocarpus Donellii†	—	—	—	—	—
Aust.	D	60	6	—	—
Anthocerotae					
Anthoceros laevis	L	54	14	14	82
Raddi	D	64	12	11	87
Anthoceros husnoti†	—	—	—	—	—
St.	D	69	14	—	—
*Anthoceros mandoni**	L	52	8	18	78
Stephani	D	72	10	8	90
Anthoceros punctatus	—	—	—	—	—
L.	D	68	15	10	93
Musci					
Acrocladium cuspidatum	—	—	—	—	—
L.	D	48	13	28	89

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TABLE 1 (*cont.*)

Plant investigated (classes, species)*	Isomer fed	Radioactivity recovered (%) Amino acid fraction	Water effluent	Dry tissue residue	Total
Acrocladium sp.	—	—	—	—	—
	D	41	12	23	76
Amblystegium serpens (L.) B.S.G.	—	—	—	—	—
	D	24	11	49	84
Amblystegium sp.	—	—	—	—	—
	D	65	14	—	—
Andreaea sp.	—	—	—	—	—
	D	48	14	—	—
Andreaea rothii	—	—	—	—	—
	D	71	11	11	93
Brachytecium sp.	—	—	—	—	—
	D	53	10	39	96
Bryum sp.	—	—	—	—	—
	D	47	10	39	96
Buxbaumia indusiata† Brid.	L	48	8	—	—
	D	78	6	—	—
Buxbaumia aphylla† L.	—	—	—	—	—
	D	80	6	—	—
Cinclidotus aquaticus (Lacg.) B.S.G.	—	—	—	—	—
	D	72	13	10	95
Hylocomium proliferum (L.) Lindb.	—	—	—	—	—
	D	59	13	23	95
Mnium undulatum (L.) Weis	L	38	6	28	72
	D	79	18	8	95
Mnium punctatum (L.) Hedw.	—	—	—	—	—
	D	55	6	16	77
Mnium cuspidatum Hedw.	—	—	—	—	—
	D	41	11	31	85
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
Polytrichum gracile Dicks.	—	—	—	—	—
	D	78	12	—	—
Pogonatum subrotundum (Huds.) Lindb.	—	—	—	—	—
	D	80	6	11	97
Platyhypnidium rusciforme Fleischr.	L	41	8	28	77
	D	75	5	12	92
Rhythiadelphus sp.	—	—	—	—	—
	D	35	13	48	96
Sphagnum cuspidatum†	—	—	—	—	—
	D	49	30	12	91
Sphagnum sp.	—	—	—	—	—
	D	61	18	7	86
Sphagnum medium Limpr.	—	—	—	—	—
	D	55	21	16	92
Sphagnum molluscum Bruch.	—	—	—	—	—
	D	60	18	11	88
Sphagnum palustre L.	L	34	4	34	72
	D	54	22	18	94
Sphagnum subbicolor Hampe	L	32	4	33	69
	D	58	15	9	82

* Arranged according to Smith, G. M.[10]
† Axenic cultures.
—Not tested or measured.

and plants tested. At the end of each experiment, mosses were extracted with 80% ethanol and the extracts were passed through column of Dowex 50, H^+. Fractions were then investigated by paper chromatography and electrophoresis; distribution of radioactivity is given in Table 1.

The highest amount of radioactivity was always found in the amino acid fraction: in experiments with the D-isomer, the radioactivity was essentially higher than that with the L-isomer. Regardless of the isomer applied, the largest part of radioactivity was associated with methionine, while a small part of activity was found in *S*-methylmethionine.

Fractions containing ninhydrin-negative, acidic and neutral compounds (water effluent) were less radioactive than the corresponding amino acid fractions especially those from the D-methionine experiments. In the case of L-methionine the qualitative pattern of radioactivity was identical for all species and was associated with α-hydroxy— and α-keto-γ-methylthiobutyric acid, an unidentified, neutral, thioether-positive, non-conjugated metabolite and, in several cases, with two or three acidic, thioether-positive non-conjugated compounds of low radioactivity.

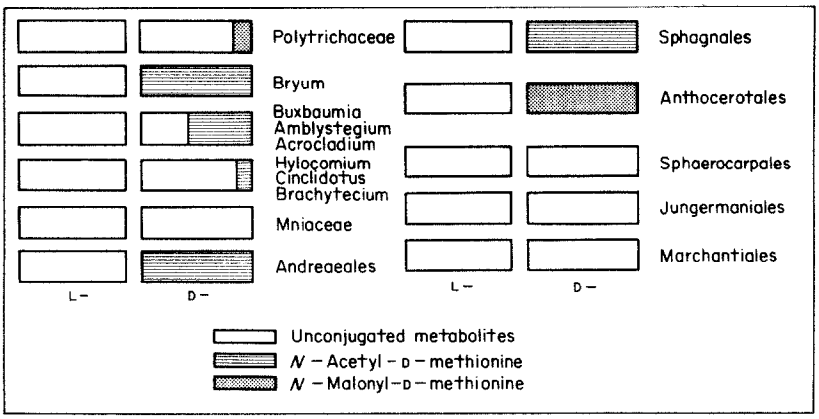

FIG. 1. METABOLIC PATHWAYS OF METHIONINE ISOMERS IN PHYLUM BRYOPHYTA.

In contrast to those of the L-isomer, the metabolites from D-methionine differed with respect to the species and classes tested (Fig. 1). The class Hepaticae and the family Mniaceae from the class Musci showed identical metabolic patterns whether fed with L- or with D-methionine: only non-conjugated radioactive metabolites were obtained. Anthocerotae was found to be the first group in the phylum Bryophyta, which showed a sharp distinction between the L- and D-isomer; with D-methionine as precursor, the entire radioactivity was detected in a conjugate which was chemically and enzymatically identified as *N*-malonyl-D-methionine. In *Anthoceros mandoni*, about 10% of water effluent radioactivity was also found in *N*-acetyl-D-methionine.

The most heterogenous class seem to be Musci. As was mentioned above, *Mnium* species form only non-conjugated metabolites. Water effluent of a great number of species contained, besides non-conjugated metabolites, the D-methionine conjugate with acetic acid. The amount of radioactivity incorporated in *N*-acetyl-D-methionine varied from species to species; in *Hylocomium*, *Cinclidotus* and *Brachythecium* species *ca* 10%, in *Buxbaumia*, *Amblystegium* and *Acrocladium* species *ca* 60% and in *Bryum*, *Sphagnum* and

Andreaea species practically 100% of water effluent radioactivity was found in this conjugate. Only mosses from family Polytrichaceae form, besides non-conjugated metabolites, a smaller amount of radioactive *N*-malonyl-D-methionine (5–10% of water effluent radioactivity).

To obtain evidence whether *N*-acetyl-D-methionine was a metabolic product of *N*-malonyl-D-methionine or an artefact formed during isolation, *Sphagnum palustre* was incubated with synthetically prepared *N*-malonyl-2-^{14}C-D-methionine.[7] After 4 days, the isolated radioactivity (94%) was still associated with *N*-malonyl-D-methionine, and no traces of *N*-acetyl-D-methionine could be observed.

Incorporation of radioactivity in the fibrous part of Bryophyta (Table 1), regardless of the isomer administered, was considerably higher than in the case of other plant phyla.[2] Generally, incorporation into fibrous part was always higher in those species which contained a great amount of radioactive *S*-methylmethionine.

DISCUSSION

The results obtained indicate several points which may be useful for chemotaxonomical purposes. The first is that, in comparison with other plant phyla, the phylum Bryophyta is the most heterogenous. The finding that in Bryophyta all the D-methionine pathways known in the plant kingdom are present, indicates that this phylum represents a transition group between the lower and higher plants. Since lower plants, in contrast to higher plants, do not qualitatively differentiate the methionine isomers, Bryophyta which metabolize L- and D-anomers identically, should be considered more primitive than those which differentiate between the two isomers. The finding that Hepaticae metabolize D-methionine via a deamination reaction indicates a similarity with the algae, and this result supports the classical phylogenetical thesis that these mosses were evolved from algae or alga-like plants.[10–15] The problem as to the algal type which should be considered as an ancestor of the present day mosses has still not been solved.[10–15] Some information about this hypothetical ancestor can be found in the fact that many sea-water algae specifically metabolize methionine to dimethyl-β-propiothetin,[2] a metabolite which was not found in the forty Bryophyta tested (Table 1). If Bryophyta, more exactly Hepaticae, had evolved from algae, their ancestor must be found among those algae which do not form dimethyl-β-propiothetin.

The class Anthocerotae seems to be the most interesting group in the phylum Bryophyta; it is very often placed into class Hepaticae because of habitual similarity.[11–14,16–18] In recent years there has been a growing tendency to place the Anthocerotae class into a taxon coordinate with Hepaticae and Musci.[10,15,19,20] This is

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now supported by the fact that the Anthocerotae are the only Bryophyta, which make a sharp distinction between the methionine isomers and metabolize D-methionine through conjugation with malonic acid. This metabolic pathway indicates a relationship between Anthocerotae and vascular plants and it is interesting that the recent paleobotanical records suggest that Anthocerotae indeed originate from the more primitive vascular plants.[21-23]

Phytochemically, the most heterogenous group within Bryophyta seems to be the class Musci. This class is usually divided into three subclasses: Sphagnidae, Andreaidae and Bryidae.[17] Sphagnidae and Andreaidae differentiate sharply between L- and D-methionine, the latter being metabolized, as the fungi, only by conjugation with acetic acid. This finding is interesting because there is no other green plant with this precise metabolic pathway.[2] Representatives from subclass Bryidae do not show any uniformity in their D-methionine metabolism; metabolic pathways indicate that within these mosses at least two phytochemically different lines have evolved. One line can be traced through the simultaneous disappearance of the deamination pathway and the successive development of the *N*-acetylation pathway. In the other line *N*-malonylation takes place partially in the morphologically most developed family Polytrichaceae,[24] and this finding supports the theory that malonylation of D-amino acids is characteristic of plants at the higher level of evolution.[1,2,5-8]

EXPERIMENTAL

Materials and methods. L-Methionine-methyl-^{14}C, 91·3 μCi/mM, $[\alpha]_D$ − 9·9° (*c* 1·2, H_2O), D-methionine-methyl-^{14}C, 286·5 μCi/mM, $[\alpha]_D$ + 9·8° (*c* 1·0, H_2O) and *N*-malonyl-2-^{14}C-D-methionine Ca salt, 291 μCi/mM, $[\alpha]_D$ − 10·3° (*c* 1·0, H_2O) were prepared as previously described.[7,25] PC was carried out on Whatman No 1 paper in: (a) *n*-BuOH–HOAc–H_2O (12 : 3 : 5), (b) *iso*-PrOH–NH_4OH–H_2O (10 : 1 : 1) and MeOH–H_2O (19 : 1). Paper electrophoresis was performed on Whatman No. 1 paper at room temp. with voltage gradient of 12 V/cm in pyridine–HOAc buffer pH 6·5. The spots were visualized with platinum reagent[26] for thioethers, sulphoxides and sulphonium compounds, HI-starch reagent for sulphoxides, ninhydrin for amino acids, 2,4-dinitrophenylhydrazine for keto acids and bromocresol green for organic acids. Radioactivity of solid samples was counted as infinite thickness and, after corrections, compared with polyethylene-^{14}C standards. Aliquots of liquid samples were measured on stainless-steel planchets, dried under a IR lamp and counted as infinite thin specimen in a mica-window GM counter. After corrections counts were compared with the precursor prepared in the same way. Chromatograms and electrophoretograms were scanned for radioactivity with an automatic GM mica-window or with a Nuclear Chicago Actigraph II.

Plant materials and incubation procedures. Most plant species were collected in their natural habitat (mostly near Zagreb, Croatia) several days before incubation. Plant material was stored on wet filter paper in Petri dishes under diffused light at room temp.[27] Young plants (500 mg) were incubated by immersing the thalli in substrate soln (0·2 mg in 5 ml H_2O). After 24 hr the thalli were taken out, washed and transferred into 5 ml fresh or in the case of peats in distilled H_2O and illuminated 12 hr daily with fluorescent lamps (3000 lx) for 3 days To axenic cultures substrate solution were added (0·2 mg in 5 ml H_2O for *ca* 500 mg of plant material) under aseptic conditions and allowed to stand 4 days as nonsterile plants.

Fractionation of plant material. At the end of each experiment, the plant material was homogenized with 80% EtOH (*ca* 10 ml), the homogenates were centrifuged and the precipitate was washed with 50% EtOH. The combined supernatants were evaporated *in vacuo* to a smaller vol. and then passed through a column (6 × 1·2 cm) of Dowex 50, H^+ (200–400 mesh) and followed by H_2O (40 ml). The H_2O effluent was neutralized and concentrated *in vacuo*. Amino acids were displaced from the column with 2N NH_4OH and H_2O. Aliquots of fractions were subjected to PC and electrophoresis. In many cases H_2O effluents were further submitted to the hydrolysis, oxidation, reduction and enzymic cleavage as described.[2]

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Acknowledgements—The author thanks Dr. D. Keglević for her help and interest in this work as well as for critical reading of the manuscript. He is also indebted to Professor Z. Pavletić, Department of Botany, Faculty of Science, University of Zagreb for the botanical identifications of the plant material and for stimulating discussions, to Dr. B. Ladešić for a sample of *N*-malonyl-2-^{14}C-D-methionine Ca salt and to Dr. M. Baslerovà, Czechoslovak Academy of Sciences, Prag, for supplying me with axenic cultures.