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# METABOLISM OF LUNULARIC ACID IN LIVERWORTS

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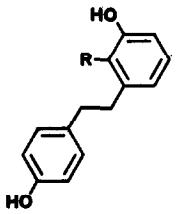
**Key Word Index**—*Conocephalum conicum*; *Lunularia cruciata*; liverwort; lunularic acid; lunularin; lunularic acid decarboxylase.

**Abstract**—The presence, in *Conocephalum conicum*, of the enzymes phenylalanine ammonia lyase, *trans*-cinnamic acid-4-hydroxylase and lunularic acid decarboxylase has been demonstrated. Tracer studies showed that phenylalanine was more readily incorporated into lunularic acid in *Conocephalum conicum* and into hydrangenol in *Hydrangea* than was tyrosine. Lunularic acid-[$^{14}$C] fed to *Conocephalum* thalli was rapidly converted into a methanol-insoluble substance.

## INTRODUCTION

Thalli of the liverwort *Lunularia cruciata* readily incorporate $^{14}$C-labelled phenylalanine, acetate and hydrangenol into the liverwort growth inhibitor lunularic acid (1) [1]. Lunularic acid was further metabolized by thallus tips of *Lunularia cruciata* to the equally ubiquitous [2] liverwort bibenzyl, lunularin (2) [3]. The enzyme catalyzing this reaction has recently been demonstrated in cell-free extracts of the liverworts *Conocephalum conicum* and *L. cruciata* [4]. It was suggested that light led to increased lunularic acid levels by a phytochrome mediated increase in the enzymes involved in its synthesis, rather than a change in the rate of its further metabolism. The results of additional investigations into the metabolism of lunularic acid in liverworts are now presented.




(1), R = COOH
(2), R = H

## RESULTS AND DISCUSSION

As a preliminatry step towards the study of the light-controlled synthesis of lunularic acid an attempt was made to detect the enzymes thought to be involved in the pathway [1]. Although phenylalanine ammonia lyase (PAL) and cinnamic acid 4-hydroxylase activity were detected in cell-free preparations from *C. conicum* and *Marchantia polymorpha*, the levels of these enzymes were too low for quantitative estimation by GLC analysis of the reaction products to be practical. PAL activity had previously been reported from *M. polymorpha* [5] and *L. cruciata* [3]. No tyrosine ammonia lyase activity could be detected in *Marchantia* by a radioactive tracer technique [5], nor was it detected by GLC analysis of the reaction products in the present investigation. A high level of lunularic acid decarboxylase activity was present in crude extracts of *C. conicum*, *L. cruciata*, *M. polymorpha* and *Preissia quadrata*. Optimum activity was found at 30° and pH 5.5. EDTA at $10^{-3}$ M caused 70–75% inhibition of lunularic acid decarboxylase activity *in vitro*, but the $Na_2$ and NaFe salts were less effective. Treatment of gemmalings of *M. polymorpha* and *L. cruciata* with EDTA and its $Na_2$ salt at $10^{-3}$ M caused an increase in the surface area of the gemmalings compared to controls over a 7 day incubation period. Treatment of *Conocephalum* thalli with EDTA, either in the light or in the dark, did not affect lunularic acid levels. The mechanism by which EDTA affects the growth of these liverworts is not clear, but it is unlikely to be mediated by lunularic acid. A spectrophotometric method for the assay of purified preparations of lunularic acid decarboxylase was developed.

In order to confirm the theory that the synthesis of lunularic acid and hydrangenol proceeds via PAL rather than via tyrosine ammonia lyase to *p*-coumaric acid, thallus tips of *C. conicum* and washed root segments of *Hydrangea macrophylla* were incubated with L-3-phenylalanine-[U-$^{14}$C] or L-tyrosine-[U-$^{14}$C]. The results are presented in Table 1. Much greater incorporation of phenylalanine than of that from tyrosine was achieved in both *Hydrangea* and *Conocephalum*, the difference being a factor of *ca* 2 in the case of *Hydrangea* and of *ca* 11 in the case of *Conocephalum*. The incorporation of phenylalanine into lunularic acid in *Conocephalum* is very high and indicates a rapid turnover rate for lunularic acid metabolism. In another experiment an incorporation rate of 7% in 20 hr was recorded.

In an experiment following the time-course of incorporation of phenylalanine into lunularic acid, *Conocephalum* thallus tips, 10 mm long, were immersed in nutrient solution containing L-3-phenylalanine-[U-$^{14}$C] for 2 hr and then transferred to 'cold' nutrient solution.

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Table 1. The incorporation of phenylalanine and tyrosine into hydrangenol, hydrangenol glucoside and lunularic acid

| Compound isolated | Incubation time (hr) | % Incorporation Tyrosine | Phenylalanine |
|---|---|---|---|
| *Hydrangea* | | | |
| Hydrangenol | 21 | 0.27 | 0.52 |
| Hydrangenol glucoside | 21 | 2.14 | 4.76 |
| *Conocephalum* | | | |
| Lunularic acid | 16 | 1.16 | 12.50 |

The results of the analysis of radioactivity recovered in lunularic acid and lunularin after different incubation periods are given in Fig. 1. Similar results were obtained when thallus tips were incubated for 144 hr with L-3-phenylalanine-[U-$^{14}$C] (Fig. 2). Maximum incorporation into lunularic acid was achieved after 10–15 hr, but even after 144 hr there remained a large amount of extractable, labelled lunularic acid. This contrasts sharply with the behaviour of exogenously supplied $^{14}$C-labelled lunularic acid. Pryce [3] had found a rapid decline in total recoverable radioactivity when *Lunularia* thalli were incubated for 8 hr with $^{14}$C-lunularic acid and samples extracted at intervals with methanol. A similar experiment was set up with *Conocephalum* thallus tips in which the disappearance of total radioactivity in lunularic acid extracted from thalli and nutrient solution was followed (Fig. 3). Almost all of the radioactivity had disappeared from the extractable lunularic acid after 23 hr. From the data presented by Pryce [3] it would seem that radioactivity in the *Lunularia* thalli remained reasonably constant whilst activity in the nutrient solution diminished rapidly.

The fate of this lost radioactivity was determined by incubating thallus tips of *Conocephalum* and *Lunularia* in an airtight container for 24 hr in the light with lunularic acid labelled universally in its phenylpropanoid moeity. $CO_2$ was absorbed onto KOH soaked glass fibre discs separated from the nutrient solution by a special container. The analysis of the distribution of radioactivity in methanol extracts, residue and carbon dioxide (Table 2) showed that 80% of the radioactivity had been incorporated into the methanol insoluble residue. TLC radioautography showed that the radioactivity remaining in the strong acid fraction and nutrient solution was mainly due to residual $^{14}$C-lunularic acid. A time-course study of the incorporation of lunularic acid-[$^{14}$C] into the insoluble material is shown in Fig. 4, in which it can be seen that incorporation was rapid and that radioactivity in the residue remained high over at least 8 days. Again the activity in extractable lunularic acid virtually disappeared with 24 hr.

The radioactivity remained in the insoluble residue after refluxing methanol-extracted thalli for several hours in 2N methanol HCl, 1% NaCl solution or N $H_2SO_4$. It could not be solubilized by treatment for 16 hr at 30° with cellulase, pectinase, macerozyme or moist DMSO, PMF or dichloroethane–ethanol (2:1). Treatment with N NaOH released 12% of the radioactivity into solution and a preliminary experiment indicated

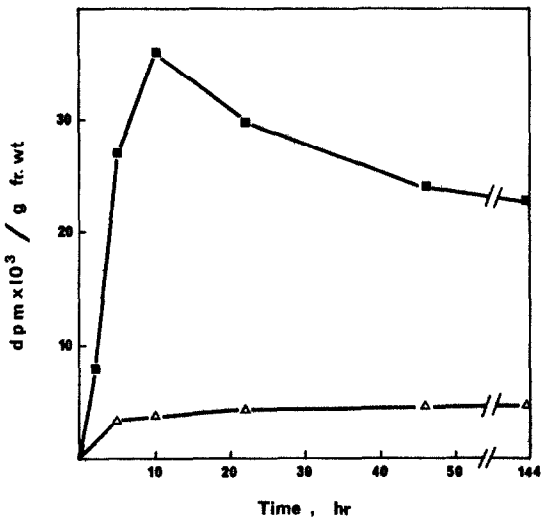


Fig. 2. Radioactivity in lunularic acid (■) and lunularin (△) following incubation of *Conocephalum conicum* thalli with L-3-phenylalanine-[U-$^{14}$C] for 144 hr.

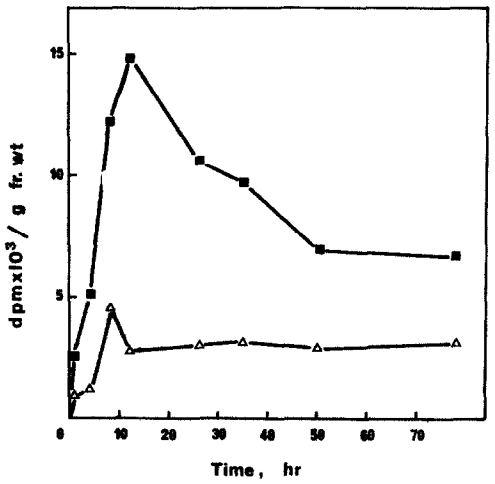


Fig. 1. Radioactivity in lunularic acid (■) and lunularin (△) following incubation of *Conocephalum conicum* thalli with L-3-phenylalanine-[U-$^{14}$C] for 2 hr.

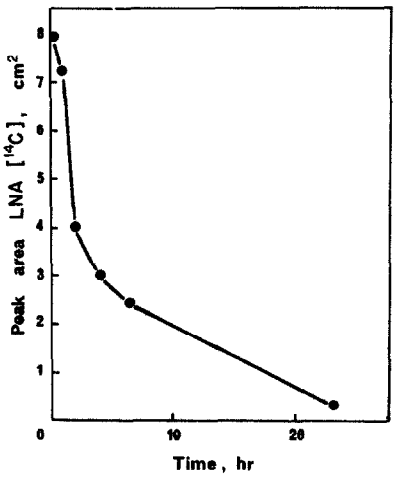


Fig. 3. Loss of radioactivity in lunularic acid fed to *Conocephalum conicum* thalli.

Table 2. Metabolism of lunularic acid [$^{14}$C] by *Lunularia and Conocephalum*. Distribution of radioactivity in various fractions after incubation of thallus tips in the light for 24 hr with lunularic acid [$^{14}$C]

| Fraction | d.p.m. *Lunularia* | *Conocephalum* |
|---|---|---|
| Methanol extract | | |
| neutral | 566 | 706 |
| strong acids | 4830 | 4510 |
| weak acids | 930 | 960 |
| aqueous | 2820 | 3800 |
| $CO_2$ evolved | 857 | 1030 |
| Nutrient solution | 2250 | 3370 |
| Residue | 41600 | 33800 |
| Total | 53900 | 48200 |

that complete solubility could be achieved by nitrobenzene oxidation. Further investigations are being carried out to determine the nature of the insoluble product.

Investigations of bryophyte lignins have produced conflicting results. Although Siegel [6], for example, had demonstrated the presence of lignin in the large Australian polytrichalian mosses *Dawsonia* and *Dendroligotrichum*, Erickson and Miksche [7] have suggested alternative structures for phenolic bryophyte cell wall materials. The insoluble product of lunularic acid metabolism may be associated with such materials or it may, for example, be bound to proteins within the cell. It is not yet known how lunularic acid or its metabolic products are distributed within the cells of liverworts. However, the disparity between the metabolism of exogenously supplied lunularic acid-[$^{14}$C] and lunularic-[$^{14}$C]acid biosynthesized from phenylalanine-[$^{14}$C] suggests some degree of compartmentation of lunularic acid metabolism and raises the question of how much of the endogenous lunularic acid is converted into the insoluble product. It might be, for example, that the rapid removal of exogenously supplied lunularic acid represents a mechanism for the protection of the liverwort from excessive inhibitor levels.

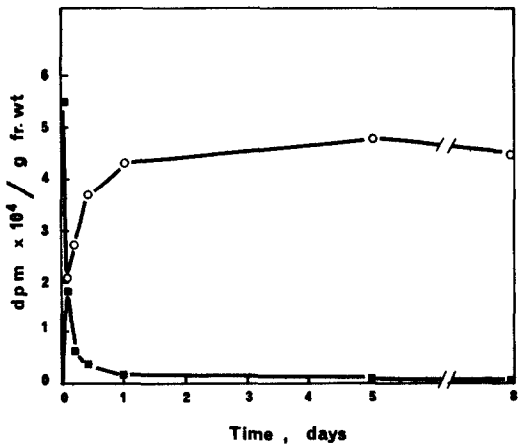


Fig. 4. Distribution of radioactivity in lunularic acid (■) and the methanol-insoluble residue (○) after incubation of *Conocephalum conicum* thallus tips with lunularic acid [$^{14}$C].

Table 3. The effect of light on the incorporation of phenylalanine into lunularic acid in *Conocephalum*

| Treatment | dpm in lunularic acid Expt 1† | Expt 2‡ |
|---|---|---|
| Darkness | 1620 | 3760 |
| Light (3000 lx) | 3120 | 8060 |
| Red light break | 1300 | 4150 |
| Far-red light break | 1800 | 4960 |

*10 hr incubation period. The thallus tips were kept in the dark for 24 hr before addition of the phenylalanine-[$^{14}$C] and exposure to 3 min light breaks or continuous light. 40 nCi of phenylalanine-[$^{14}$C] was used for each treatment; †0.5 g thalli; ‡1 g thalli.

The effect of narrow wavebands of red and far-red light on the incorporation of phenylalanine-[$^{14}$C] into lunularic acid was studied in the apparatus described by Wilson and Schwabe [8]. The results of two experiments with *Conocephalum* thallus tips are presented in Table 3. Although there was a large difference in incorporation between thalli incubated either in the dark or at 3000 lx, the red and far-red treatments had relatively little effect on incorporation. Incubation in light or darkness did not greatly affect the conversion of lunularic acid-[$^{14}$C] to its insoluble metabolic product (Table 4). The smaller degree of incorporation compared to previous experiments is due to the use of only 0.5 g of thallus tips in this instance.

The role of phytochrome in the control of lunularic acid biosynthesis requires further investigation. It is apparent from previous experiments [2], however, that light intensity is an important factor in determining lunularic acid levels in liverworts in the natural environment.

## EXPERIMENTAL

Details of extraction and chromatography have been described elsewhere [2].

*Radioactivity measurements.* Radioactivity was measured in a liquid scintillation counter. KOH-soaked glass fibre discs used to absorb $CO_2$ were dried under an IR lamp, cut into pieces and placed in scintillation vials to which the standard toluene-based POPOP/PPO scintillation fluid was added. Residues from the extraction of liverwort material with organic solvents were dried, weighed and ground to a powder with a pestle and mortar. Samples of this powder (5–10 mg) were pyrolysed in a sample combustion system in which the resulting $CO_2$ was absorbed by 2-phenylethylamine. A PBP based scintillator fluid was added to the vials and the radioactivity of the sample measured in the liquid scintillation counter. The preparation of lunularic acid [$^{14}$C] was carried out as described previously [2].

Table 4. The effect of light on the incorporation of lunularic acid [$^{14}$C] into the methanol insoluble material in *Conocephalum*

| Compound | dpm Expt. 1 Light* | Dark | Expt 2 Light | Dark |
|---|---|---|---|---|
| Lunularic acid | 10600 | 6730 | 10300 | 6680 |
| Residue | 30100 | 35660 | 30400 | 23730 |

0.5 g of thallus tips incubated for 16 hr; *1500 lx, warm white fluorescent lights; 22.5 nCi of lunularic acid [$^{14}$C] was used for each treatment.

*PAL assay*. Thallus tips of *Conocephalum* were macerated at 0–4° in a top-drive homogenizer. After filtration through beds of acid-washed sand, $Al_2O_3$ and Polyclar AT, and centrifugation at 15000 *g* for 15 min, the supernatant was concentrated and desalted with dry Sephadex G-25. Aliquots of the enzyme preparation (0.2 ml) were incubated for 5 hr at 30° with 0.5 ml of 0.1 M phenylalanine and 0.3 ml of 0.1 M borate buffer pH 9. After acidification to pH 2 with conc HCl the *trans*-cinnamic acid was extracted into $Et_2O$, purified by TLC on Si gel developed in EtOAc–$CHCl_3$–HOAc (15:5:1), silylated with BSA and detected by GLC on 1% OV-17 or 3% SE-30 columns. The level of PAL activity detected in *C. conicum* extracts was less than 1 nmol cinnamate produced/mg protein/hr.

*Cinnamic acid-4-hydroxylase assay*. Samples (100 μl) of the enzyme prepn were incubated at 27° in the dark for 16 hr with *trans*-cinnamic acid (300 μmol), mercaptoethanol (150 μmol), glu-6-phosphate (200 μmol), glu-6-phosphate dehydrogenase (0.2 units. Koch-Light) and NADP tetraNa salt (870 μg) in 2 ml of 0.2 M Pi buffer pH 7.6. After acidification and extraction into $Et_2O$ the *p*-coumaric acid was separated from unreacted *trans*-cinnamic acid by TLC on steamed Si gel G–cellulose (1:1) plates developed in toluene–Et formate–$HCO_2H$(5:4:1) [9]. After silylation, GLC was again used to detect the reaction product. The activity of *trans*-cinnamic acid 4-hydroxylase in *Conocephalum* extracts was less than 0.2 nmol of coumaric acid produced/mg protein/hr.

*Lunularic acid decarboxylase assay*. The crude enzyme prepn described above was further purified as described in ref. [4] and made up to a standard soln containing 1 mg protein/ml. 50 μl of the preparation were incubated with 50 μg of lunularic acid in 1 ml of 0.1 M acetate buffer pH 5.5 at 30° for 90 min. GLC analysis of the reaction mixture was carried out as previously described [4], except that the TMSi rather than the methylated derivatives were employed.

For the spectrophotometric assay the quantities in the reaction mixture were ×3 and 5 min after the addition of the enzyme prepn the $A_{300}$ of the reaction mixture (pH 5.5) was recorded, and again at the end of the 90 min incubation period. The decrease in $A_{300}$ caused by the decarboxylation of the A ring of lunularic acid was used as a measure of enzyme activity. In other expts the reaction mixture was acidified to pH 2.5 with conc HCl and the lunularic acid and lunularin extracted into $Et_2O$. After addiction of a drop of 2 M methanolic HCl the $Et_2O$ was made up to 5 ml and the $A_{305}$ compared to that of controls. This assay could only be used on purified prepns of lunularic acid decarboxylase because of interference by UV-absorbing substances in crude extracts. Extraction of the lunularic acid and lunularin into $Et_2O$ gave more consistent results than direct measurement of the A of the reaction mixture. The spectrophotometric assay was more convenient than the GLC method for preparations with high levels of lunularic acid decarboxylase, but was not as sensitive to low levels of this enzyme.

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