

## On the Biosynthesis of Lichen Substances

### Part 3. Lichen Acids as Products of a Symbiosis

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A biosynthetic method for the production of labelled lichen acids from whole lichens is described. These phenolic acids become labelled as a result of the photosynthetic incorporation of  $^{14}\text{CO}_2$  by the algal partner which results in a subsequent flow of reduced carbon compounds to the fungus where the phenolic monomers are produced. Exposure to light intensities of 4000 lux resulted in the incorporation of labelled carbon into gyrophoric acid in *Umbilicaria pustulata*. This incorporation occurred in times as short as one minute of exposure in the presence of the labelled gas. Degradation of gyrophoric acid obtained by  $^{14}\text{CO}_2$  fixation during 30 min of light period showed uniform labelling in all of the carbon atoms.

One of the major fields of interest in the study of lichens is in the physiological interplay between the organisms forming the lichen symbiosis. While it has long been understood that the algal partner provides the fungal partner with organic nutrients resulting from the photosynthetic activities of the alga, few experimental data on this subject have appeared in the literature.<sup>1-4</sup> In particular, the question of whether fixed carbon moves in one or several predominant forms from alga to the fungus has only begun to be resolved. Recent work by Smith and his students<sup>2,5,6</sup> has indicated that in lichens containing algae of the genus *Trebouxia* (which are found in almost all lichens producing polyketides) the primary product reaching the fungus is a pentose or pentitol, but that the primary product may be different in different lichen algae. The means by which the photosynthate is transported is in some doubt since the actual physical relationship of the fungi and algae range from penetration of the algal cell wall by fungal haustoria in some lichens to a state of hyphal entrapment in others.

In this situation, biosynthetic as well as rate studies are impeded by the fact that it is often difficult to determine whether compounds isolated are of fungal or algal origin, and what biosynthetic interconversions have occurred. On the other hand, the phenolic monomers of lichen acids such as the tri-

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depside gyrophoric acid, (consisting of three ordered orsellinic acid moieties joined by ester bonds) are almost certainly synthesized by the fungal symbiont, since orsellinic acid is found in free living fungi<sup>7</sup> and in isolated lichen fungi<sup>8</sup> as well, but has not been found in algal cultures (Fox, unpublished). The appearance of radioactivity in these compounds can therefore serve as a final measure of carbon flow in the symbiosis, while the amounts and the rate at which it appears indicate the degree of integration between the two systems.

To permit studies of this nature, a simple and efficient technique has been worked out that will render such compounds labelled by using intact lichen thalli. These radioactive substances can be used in the search for catabolic lichen enzymes, biosynthetic studies, and in experiments in which the metabolic fate of lichen acids or their components is of interest.

### EXPERIMENTAL

<sup>14</sup>CO<sub>2</sub> fixation by *Umbilicaria pustulata* and isolation of labelled gyrophoric acid. Specimens of the lichen were collected the day before use and allowed to air dry overnight in the dark. 3 g of clean unbroken lichen thalli were used as the experimental sample. The thalli were immersed in distilled water until they became soft and pliable (2–3 min) after which they were blotted with filter paper and placed upper surface down in the bottom parts of plastic Petri dishes. When the entire surface of the dish was covered, another layer of lichens was placed on the first with the upper surface up thus furnishing a double layer of lichens, each with upper surface facing out. The Petri dishes were of the conventional disposable type (90 mm in diameter) with a hole drilled at one edge of the tops to accommodate a tightly fitted vaccine stopper. A small plastic cup was fitted to the inner side of the tops directly under the stopper. This cup had several holes drilled in its side so that when the cup was filled with sand saturated with bicarbonate solution and perchloric acid was added to the sand, the gas generated would freely pass into the rest of the dish. After the lichen thalli had been arranged in the bottom of the dish, the top with cup assembly was sealed to the bottom half by means of modelling clay pressed as a ridge around the inner surface of the lid.

The sand in the cup was charged with 100 μl of sodium bicarbonate solution containing 200 μC of <sup>14</sup>C (26.7 mC/mmmole) and the system was closed. A partial vacuum was produced by inserting a hypodermic needle attached to an aspirator through the vaccine stopper. An excess of perchloric acid was then added through the needle followed by allowing air to rush in thereby sweeping the generated gas throughout the vessel and returning the system to atmospheric pressure.

After the gas was generated, the dishes were illuminated from both sides by standing them on edge between two banks of three ordinary fluorescent 20 W tubes which produced about 4000 lux at either surface of the dish. The dishes were maintained at a temperature of 20° by forced air cooling. To stop the reaction, the thalli were dropped from the dish directly into 300 ml of boiling acetone, and were further extracted by two changes of 300 ml of boiling acetone followed by extraction with 200 ml of cold solvent overnight. The combined acetone extract was evaporated to dryness on a steam bath.

Small portions of each extract were chromatographed on Whatman No. 1 filter paper strips in a system composed of: ethyl methyl ketone:H<sub>2</sub>O:diethylamine = 921:77:2 (v/v/v).<sup>9</sup> The papers were scanned in a strip counter and those with higher activities revealed a radioactive peak at *R<sub>F</sub>* 0.67 which was identified as gyrophoric acid. Free orsellinic acid was not detected in these preparations. The major portion of the extracts was hydrolyzed with cold conc. sulfuric acid according to the procedure of Fujii and Osumi<sup>10</sup> to form orsellinic acid. The orsellinic acid was then recrystallized to constant activity from 30 % (v/v) acetic acid in water. A portion of orsellinic acid prepared by this method showed activity only in association with the orsellinic acid peak in two chromatographic systems.

The extracted lichen thalli were finely ground in a mortar and dried to constant weight at 100°. The orsellinic acid and the ground thalli were each submitted to wet

combustion by the van Slyke-Folch method and the resulting carbon dioxide was trapped as barium carbonate.

*Degradation of labelled gyrophoric acid.* Gyrophoric acid was rendered labelled by the petri dish method described above. The 3 g of thallus were given 200  $\mu\text{C}$  of  $^{14}\text{CO}_2$  with a light exposure of 30 min. This was followed by a dark period of 20 h. After isolation, the gyrophoric acid was crystallized from acetone, hydrolyzed with cold conc. sulfuric acid, and the resultant orsellinic acid recrystallized to constant activity. Yield: 140 mg. To this, 140 mg of non-labelled orsellinic acid was added as carrier. Degradation of the orsellinic acid followed in detail the method previously reported by Mosbach.<sup>11</sup>

*Measurement of radioactivities.* The barium carbonate obtained from degradations and combustions was counted in a liquid scintillation counter suspended in a gel of carboxil in a toluene solution of 2,5-diphenyloxazol.

### RESULTS AND DISCUSSION

As can be seen from Table 1, radioactive  $\text{CO}_2$  is fixed rapidly and efficiently by the photosynthetic activity of the lichen *Umbilicaria pustulata*. The assimila-

Table 1. Incorporation of fixed  $^{14}\text{CO}_2$  into thallus and gyrophoric acid (cpm/mg of carbon).

Fixation by dark control was 1200 cpm/mg C of thallus after 24 h incubation.				
Light period (min)	1	5	30	240
Thallus	4690	24 000	100 000	123 000
Gyrophoric acid	80	120	1 100	1 275
Following a dark period of 24 h:				
Thallus		28 000	78 000	87 000
Gyrophoric acid		580	1 650	6 900

tion of  $^{14}\text{CO}_2$  in the dark amounts to only about 1 % of that observed after four hours exposure to light. Almost the total administered can be accounted for as being incorporated into organic thallus constituents at the end of four hours light, the remainder being lost in the isolation of gyrophoric acid. After a dark period there is a drop in total thallus activity, presumably due to respiration. When there is only a light period, about 0.1 % of the  $^{14}\text{CO}_2$  incorporated is found in gyrophoric acid, but given an adequate period of metabolism, the amount increases to about 1 % of the total.

From these experiments, it becomes possible to produce lichen acids with specific activities of at least  $6 \times 10^6$  cpm/mmole, and given longer time and higher specific activity of the  $^{14}\text{CO}_2$  administered, even higher activities are possible. The use of carbon dioxide in the gaseous form allows the use of barium carbonate for generation of the gas and effects a considerable economy in the cost of producing the labelled compounds.

Using this procedure, the depsides evernic acid, atranorin and chlor-atranorin have been produced from *Evernia prunastri* as have the depsidones physodic and physodalic acid from *Parmelia physodes* and the dibenzofuran derivative usnic acid from *Cladonia sylvatica*.

The appearance of the label in gyrophoric acid after short assimilation times down to one minute is surprising. One possible and at present entirely hypotheti-

Table 2. Distribution of radioactivity from fixed  $^{14}\text{CO}_2$  in orsellinic acid obtained from gyrophoric acid.

Sample	cpm/mg C
Orsellinic acid	630
Carboxyl carbon	650
Methyl group	620
Carbon atom 2 adjacent to methyl group	615
Kuhn-Roth carbon dioxide	630

cal explanation is that since pentoses are among the earliest products of the photosynthetic reactions in algae, and since pentoses appear to be the first and principal products in lichen associations, there is a rapid translocation of pentose to the fungus where an enzyme similar to phosphoketolase enzyme in bacteria<sup>12</sup> would result in the formation of an acetate unit and a three carbon fragment. This possibility is now under investigation.

The incorporation of  $^{14}\text{CO}_2$  into the thallus (about 30  $\mu\text{g/g}\cdot\text{h}$ ) occurs at such a rate as to bring into question the reported slow growth of lichens in nature. It should be emphasized that the conditions reported here probably never occur in nature, and that in spite of fairly rapid photosynthesis, the limiting factor in lichen growth is still probably in the supply of nitrogen, and/or water.

Table 2 shows the results of the degradation of gyrophoric acid labelled by a 30 min exposure to light followed by a dark period of 20 h. Since all carbon atoms show uniform labelling it is obvious that under the given experimental conditions randomization of activity has taken place. In order to reveal pool sizes, shorter exposure periods will have to be used.

After this manuscript had been submitted a report has appeared by Drew and Smith in *New Phytologist* 66 (1967) 379 on somewhat similar experiments in which however the times of incorporation of labelled material were longer. We feel the slight difference obtained to our results, which in part already have been presented at the IUPAC meeting, Stockholm, Abstracts, Mosbach, K. and Fox, C. (1966) 152, to reflect different instrumentation and labelling conditions rather than any real inconsistency.

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