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Relationship between Carbohydrate Movement and the Symbiosis in Lichens with Green Algae

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Summary. When isolated in pure culture, four genera of lichen algae were able to produce the polyol which is known to move from the alga to the fungus in lichens with these algae. This conclusion corrects earlier suggestions that the mobile polyol is only formed by the alga in the lichen thallus. *Stichococcus* produced sorbitol and it is therefore suggested that, in lichens with this alga, sorbitol moves between the symbionts. *Hyalococcus* and *Stichococcus* had a similar pattern of incorporation of $H^{14}CO_3^-$ in the light, suggesting a close relationship between these algae which are only separated now on morphological grounds.

The pattern of incorporation of $H^{14}CO_3^-$ in the light into *Cladonia cristatella* and its alga (*Trebouxia erici*) in culture indicates that in the cultured algae more ^{14}C was incorporated into ethanol insoluble substances and lipids and less into ribitol than in the lichen. The pattern in a joint culture of the alga and the fungus of *C. cristatella* was approximately intermediate between that of the lichen and the alga. However, only a small amount of ^{14}C fixed by the alga reached the fungus in the joint culture, and it is therefore suggested that the presence of the fungus without morphological differentiation into a lichen thallus is not sufficient to promote the alga to release carbohydrate.

Introduction

It is well established that carbohydrate moves from alga to fungus in lichens (Smith *et al.*, 1969). The movement is characterised by the transfer of a single simple carbohydrate which, in the case of green algal symbionts, is a polyol. Richardson *et al.* (1968) showed that the identity of the polyol is determined by the genus of alga in the thallus. Thus, in lichens that contain *Trebouxia*, *Myrmecia* or *Coccomyxa*, ribitol moves between the symbionts. In those with *Hyalococcus* or *Trentepohlia*, it is sorbitol or erythritol respectively.

The algal symbiont can be isolated rapidly from the lichen by differential centrifugation of thallus homogenates. Such isolates of *Trebouxia*, *Coccomyxa* and *Hyalococcus* continue to release a small amount of the mobile carbohydrate into the medium (Richardson and Smith, 1968; Richardson *et al.* 1968; Green, 1970). After culturing for a few hours to a few days, the alga stops releasing the polyol completely and the pattern of carbon assimilation changes with a large proportion of the photosyn-

thetic products appearing in insoluble substances. In the case of *Trebouxia*, Richardson and Smith (1968) compared the directly isolated alga with a form which had been grown in culture for some time. Exposure of the former to $H^{14}CO^-$ in the light resulted in nearly all the ^{14}C being incorporated into ribitol. In the latter, little ^{14}C was found in ribitol and most of it had become incorporated into other substances (e.g. sucrose). [Komiya and Shibata (1971) however found in a similar experiment that the cultured alga from *Ramalina crassa* (Trebouxia) incorporated a substantial amount of ^{14}C into ribitol but they did not indicate what proportion this was of the total ^{14}C fixed.] In the case of *Coccomyxa*, the directly isolated alga fixed almost as much ^{14}C into ribitol as in other substances, but the cultured alga incorporated no ^{14}C into ribitol (Richardson *et al.*, 1968). In view of these results, Smith *et al.* (1969) suggested that polyols might be 'special' carbohydrates which are produced by the alga only in response to the symbiosis. However, the algae had been cultured in a medium which contained glucose because growth is otherwise very slow. It is possible that the metabolism of the algae was affected by growing them with glucose and so the results obtained from cultured algae should be regarded with caution.

The purpose of this study was, firstly, to ascertain whether or not the alga, when grown, retains the ability to form the polyol which moves between the symbionts in the lichen secondly, to find out what differences there are in the incorporation of carbon between the alga in the symbiotic state and the alga in isolation; and, finally, to examine a joint culture of the alga and the fungus of *C. cristatella* in this regard.

Materials and Methods

1. Materials

Lichen: Cladonia cristatella was collected dry from Cape Cod, Massachusetts, U.S.A., in July 1969, used July–August 1969, and kept moist for 3–4 days at 3000 lux and 18° C before use. The sample size was 10 mature podetia with apothecia removed.

Algae: Coccomyxa sp. isolated from *Botrydina vulgaris* by H. Heikkilä; *Trentepohlia* sp. (from *Pyrenula nitida*), *Trebouxia erici* (from *Cladonia cristatella*), *Hyalococcus dermatocarponis* (from *Dermatocarpon miniatum*), *Stichococcus mirabilis* (from *Staurothele clopima*), and *Stichococcus diplosphaera* (from *Endocarpon pusillum*) isolated by V. Ahmadjian. The algae were cultured in Bold's medium (Ahmadjian, 1967). The length and conditions of culture are given below in the results. Algal sample size was measured as wet-packed volume after one minute centrifugation at $250 \times g$. Additional details on the culture of *Coccomyxa* and *Stichococcus* isolates used in this study are in Heikkila and Kallio (1966) and Ahmadjian and Heikkilä (1970).

2. Methods

a) Experimental Procedure. The lichen samples were floated on 1–2 ml of medium containing 10^{-2} Molar MES (2-(N-moropholino ethane) sulphonic acid)

adjusted to pH 6.5 with NaOH and shaken at 250 cycles/min at a few mm amplitude in 15 ml centrifuge tubes with screw on caps under 4000 lux illumination at 18°C. Media were separated from the lichen by sieving through a strainer and from the algae by centrifugation.

b) Analysis. Lichen material was killed by adding 4 ml of 95% boiling ethanol to the lichen in 1 ml of buffer. Algal samples were not separated from media but sufficient boiling ethanol was added to give an 80% solution. All material was extracted by boiling 1 min, allowing to cool, then reextracting residue with 2 ml of 80% ethanol twice. Extracts were combined and made up to 10 ml. For deionisation of extracts, about 0.25 g of Dowex-1 ion-exchange resin (chloride form) was added to 1 ml of 80% ethanol extract and shaken for one hour. For preparation of the lipid fraction, 0.5 ml of 80% ethanol extract was evaporated down in a 5 ml test tube and then the lipid was removed with 1–2 ml of petroleum ether three times and the lots were added together. The isoluble fraction of the lichen was homogenised in a blender to make a suspension.

(i) Counting. Packard Tricarb Liquid Scintillation spectrometer model 3003 was used. The scintillant was 5% Omnifluor (98% P.P.O. and 2% Bis M.S.B— supplied by New England Nuclear Inc.) in 80% toluene and 20% ethanol (v/v). For liquid samples, 0.05 ml (0.1 ml for lipid fraction) was added to the vial and counted for 1–10 min. For insoluble samples, 0.05 ml of extracted algal suspension and the whole of the extracted lichen homogenate were suspended in the scintillant with Thixotrophic gel powder ('CAB-O-SIL') (by G. L. Cabot Inc. obtained from Packard).

(ii) Chromatography. Whatman paper No. 1 was used. The solvent was ethyl methyl ketone (butan-2-one), glacial acetic acid and water saturated with boric acid in the ratio 9:1:1 (v/v/v) (Rees and Reynolds, 1958). The running time was 20 hours. Detection of carbohydrates was by alkaline silver nitrate after splitting borate complexes with hydrofluoric acid (Lewis and Smith, 1967). Distribution of radioactivity along chromatograms was found by cutting the paper into 2 cm pieces and counting each piece in a liquid scintillation vial without elution.

Results

The Formation of Polyols by Algal Symbionts in Culture

Algae isolated from lichens were grown on Bold's mineral medium supplemented in some cases with glucose. The occurrence of polyols in the algae was investigated by paper chromatography of extracts. Polyols were detected by their reaction to alkaline silver nitrate, or, in experiments using ^{14}C, by radioactivity of chromatograms. The chromatographic mobility of the carbohydrates was compared with the mobility of authentic polyol markers. All the algae examined could form the polyol mobile in the original lichen (Table 1).

The presence of glucose in the culture medium prevented the formation of ribitol by *Coccomyxa* but not the formation of polyols by the other algae. *Coccomyxa* grown in the presence of glucose tended to bleach but not in the absence of glucose. No such bleaching effect was noted in *Trebouxia erici.*

Carbohydrate movement has never been studied in lichens containing the alga *Stichococcus.* However, the fact that ^{14}C with a mobility of

Table 1. The ability of alga to produce polyol in culture

Alga	Polyol known to move in lichens with this type of alga (Richardson, Hill, and Smith, 1968)	Presence or absence of glucose in culture medium	Occurrence of polyol in alga
Trebouxia erici	ribitol	+	ribitol[a,b]
		−	ribitol[a,b]
Coccomyxa sp.	ribitol	+	no ribitol[a]
		−	ribitol[b]
Hyalococcus dermatocarponis	sorbitol	+	sorbitol[b]
Stichococcus mirabilis	?	+	sorbitol[b]
Stichococcus diplosphaera	?	+	sorbitol[b]
Trentepholia	erythritol	−	erythritol[a]

Detection of polyol on chromatogram. [a] by reaction to $AgNO_3$. [b] by location of ^{14}C.

Table 2. The incorporation of $H^{14}CO_3^-$ by *Stichococcus* spp. and *Hyalococcus*

Alga	1 hr $H^{14}CO_3^-$		1 hr $H^{14}CO_3^-$ followed by 24 hr in buffer alone	
	Total ^{14}C fixed cpm in 1000s	% of total ^{14}C in insoluble	Total ^{14}C fixed cpm in 1000s	% of total ^{14}C in insoluble
Stichococcus mirabilis	3601	25.6	4139	37.2
Stichococcus diplosphaera	3894	42.6	5033	65.2
Hyalococcus dermatocarponis	3624	46.5	4725	63.2

Experiment 5 (4.8.69). Algae cultured for 1 week on Bold's mineral medium and soil extract + glucose. *Hyalococcus* 0.07 ml wet pack vol. *Stichococcus mirabilis* 0.03 ml. *Stichococcus diplosphaera* 0.05 ml. Suspended in 2 ml of buffer containing 1 μ Ci $H^{14}CO_3^-$. One ml was removed after 1 hr and run into 4 ml boiling ethanol. The other 1 ml centrifuged and taken up in 1 ml buffer only. After incubation for 24 hrs it was extracted.

sorbitol was found on the chromatograms of extracts of this alga suggests very strongly that sorbitol may be the mobile carbohydrate in these lichens, since sorbitol is known to move between the symbionts in *Dermatocarpon miniatum* (Richardson *et al.*, 1968). During photosynthesis in the presence of $H^{14}CO_3^-$ both species of *Stichococcus* and *Hyalococcus dermatocarponis* (the symbiont of *Dermatocarpon miniatum*) incorporated ^{14}C slowly into sorbitol (Fig. 1). After one hour incubation

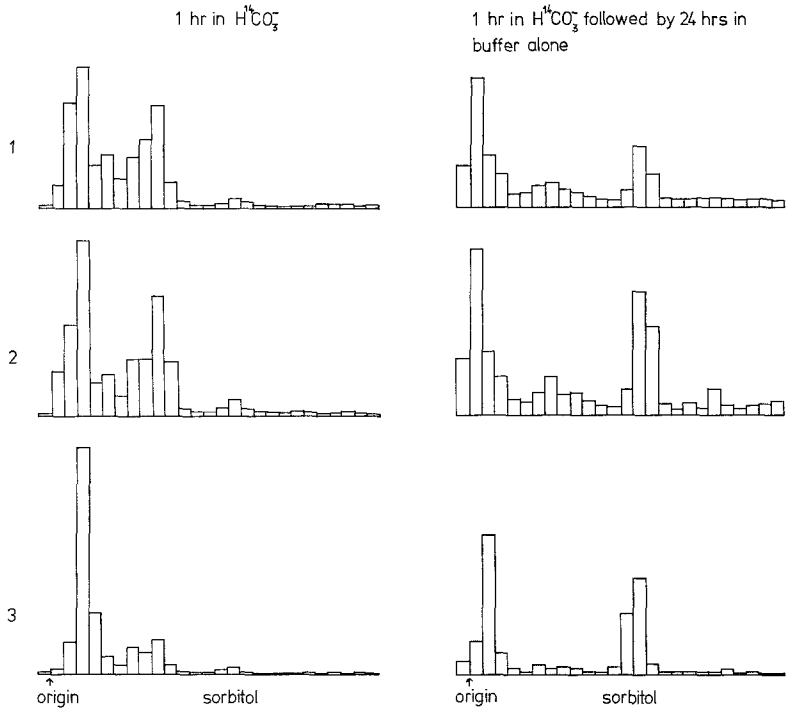

Fig. 1. Distribution of ^{14}C on chromatograms of extracts of *Stichococcus* spp. and *Hyalococcus* after exposure to $H^{14}CO^-$. *1 Hyalococcus dermatocarponis; 2 Stichococcus diplosphaera; 3 Stichococcus mirabilis.* Experiment 5 (see Table 2)

with $H^{14}CO_3^-$, almost no ^{14}C-sorbitol was formed. However, significant amounts of ^{14}C were found in sorbitol 24 hours later. Hill (1970) exposed the intact thallus of *D. miniatum* to $H^{14}CO_3^-$ and found that ^{14}C first appeared in sorbitol after 10 mins incubation. This contrasts with lichens which contain *Trebouxia* in which ^{14}C-ribitol is first detected after only 2 mins.

Comparison of $H^{14}CO_3$-Incorporation into Cladonia cristatella *with that into its Alga in Culture*

Samples of *Cladonia cristatella* and a culture of its alga (*Trebouxia erici*) were exposed to $H^{14}CO_3^-$ for one hour and then transferred to media without ^{14}C for either four or twenty-four hours. Estimates were made of the ^{14}C in the 80% ethanol insoluble fraction (which includes proteins and poly-saccharides), lipids, acids and the medium (Tables 3 and 4). Less ^{14}C was incorporated into the insoluble fraction and lipids in the lichen than in the alga. Incorporation into the acid fraction was essen-

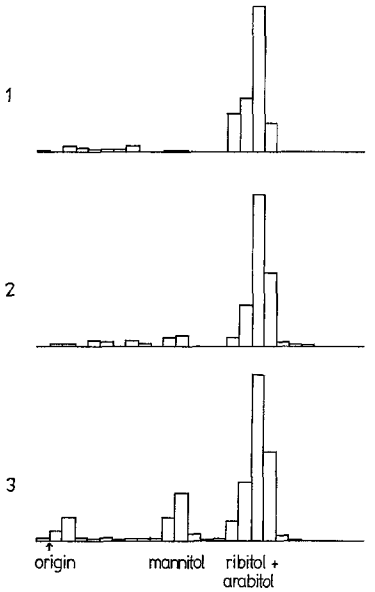

Fig. 2

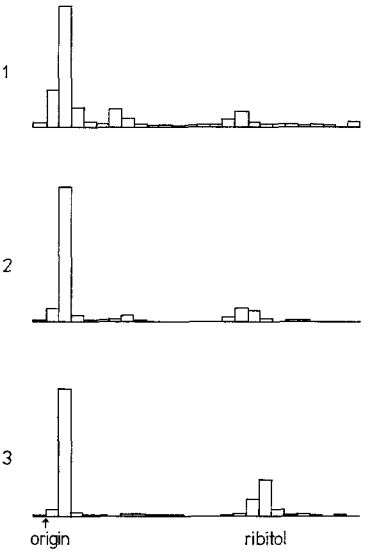

Fig. 3

Fig. 2. The distribution of ^{14}C on chromatograms of extracts of *Cladonia cristatella*. *1* 1 hr in $H^{14}CO^-$; *2* 1 hr in $H^{14}CO_3^-$ followed by 4 hrs in buffer alone; *3* 1 hr in $H^{14}CO_3^-$ followed by 24 hrs in buffer alone. Experiment 6 (see Table 3).

Fig. 3. The distribution of ^{14}C on chromatograms of extracts of *Trebouxia erici*. *1* 1 hr in $H^{14}CO_3^-$; *2* 1 hr in $H^{14}CO_3^-$ followed by 4 hrs in buffer alone; *3* 1 hr in $H^{14}CO_3^-$ followed by 24 hrs in buffer alone. Experiment 8 (see Table 4)

tially similar in the two cases except that somewhat more ^{14}C was found in the alga immediately after the pulse. Chromatography of the ethanol extracts (Figs. 2 and 3) showed that in the lichen most of the ^{14}C was found in the position of ribitol and arabitol (a fungal product in lichens), immediately after the pulse, and in the form of ^{14}C mannitol as well twenty-four hours later. The ^{14}C in mannitol represented about 15% of the total ^{14}C fixed. The formation of ^{14}C-mannitol indicated that ^{14}C had moved to the fungus, as mannitol is only formed by the fungus in lichens. In the cultured alga relatively little ^{14}C was in the form of ribitol. Most of the ^{14}C remained near the origin of the chromatograms, a position characteristic of sugar phosphates, amino and organic acids and oligosaccharides. The overall results of these experiments indicate that during exposure of $H^{14}CO_3^-$ in the light in the lichen, as compared with the cultured alga, more ^{14}C is incorporated into ribitol and less into lipids, insoluble fraction and other metabolites.

An "inhibition" experiment (Hill and Smith, 1972) confirmed that ribitol moves between the symbionts in this lichen as it does in other

Table 3. The incorporation of $H^{14}CO_3^-$ by *Cladonia cristatella*

Sample		Total ^{14}C fixed (cpm in 1000s)	% of total ^{14}C in			
			insoluble	medium	lipid	acids
Pulse only	1	4560	8.2	—	1.7	25.7
	2	3921	6.0	—	4.7	24.9
Pulse + 4 hrs	3	5528	5.8	—	1.5	27.3
	4	3363	4.5	—	1.3	32.8
Pulse + 24 hrs	5	4023	11.0	6.0	1.7	23.8
	6	5774	3.0	9.5	4.6	29.8

Experiment 6. (7/8/69). Samples of 10 podetia (without apothecia) were incubated for 1 hr in 1 ml of buffer containing 10 μ Ci $H^{14}CO_3^-$. Some of them were then transferred to 1 ml of buffer alone for 4 or 24 hrs.

Table 4. The incorporation of $H^{14}CO_3^-$ by *Trebouxia erici* in culture

Sample		Total ^{14}C fixed (cpm in 1000s)	% of total ^{14}C in			
			insoluble	medium	lipid	acids
Pulse only	1	2690	20.0	—	14.5	42.2
	2	2482	15.9	—	17.1	49.6
Pulse + 4 hrs	3	3985	29.7	0.3	12.9	25.9
	4	4145	28.3	0.2	13.1	30.1
Pulse + 24 hrs	5	1900	16.9	0.8	10.5	30.4
	6	3005	18.9	1.3	8.9	26.7

Experiment 8 (12.8.69). Algae grown for 6 weeks on Bold's mineral medium + glucose. Samples of 0.017 ml wet pack volume were incubated in 1 ml of buffer containing 10 μ Ci $H^{14}CO_3^-$ for 1 hr. Some of them were then resuspended in 1 ml of buffer alone for 4 or 24 hrs.

lichens with *Trebouxia*. Very little carbon was liberated into the medium in the culture of *Trebouxia*. Appreciable release of ribitol by the alga occurs only in the lichen.

The Incorporation of $H^{14}CO_3^-$ into a Joint Culture of the Fungus and Alga Isolated from Cladonia cristatella

An experiment similar to the two described above was conducted with a culture of the fungus and the alga of *Cladonia cristatella* growing together in the same culture. The two symbionts of *Cladonia cristatella* were inoculated onto nutrient agar and after four months' culturing they had formed a compact, hard mass of fungus and alga, which did not resemble the thallus of *C. cristatella* in form or structure. (A photo-

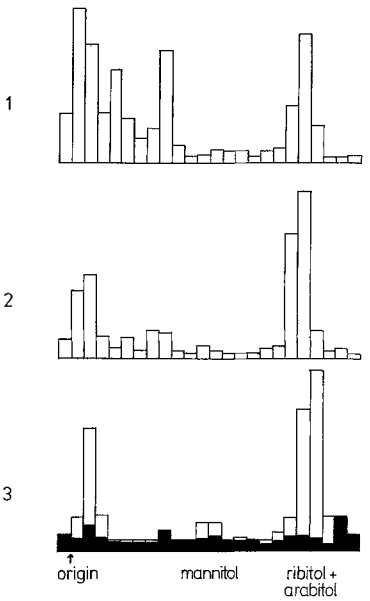

Fig. 4. Distribution of ^{14}C on chromatogram of extracts of joint culture. *1* 1 hr in $H^{14}CO_3^-$ *2* 1 hr in $H^{14}CO_3^-$ followed by 4 hr in buffer alone *3* 1 hr in $H^{14}CO_3^-$ followed by 24 hrs in buffer alone. The solid black refers to a chromatogram of a dark control. Experiment 4 (see Table 5)

Table 5. The incorporation of $H^{14}CO_3^-$ by a joint culture of alga (*Trebouxia erici*) and fungus of *Cladonia cristatella*

Sample	Total ^{14}C fixed (cpm in 1000's)	% of total ^{14}C			
		in-soluble	medium	lipid	acids
1) Pulse only	1354	29.0	—	3.5	28.9
2) (dark)	141	—	—	—	—
3) Pulse + 4 hrs	1197	24.8	—	4.6	15.5
4) (dark)	105	—	—	—	—
5) Pulse + 24 hrs	883	26.5	—	4.4	22.1
6) (dark)	40	—	—	—	—

Experiment 4 (4.8.69). Mycelium and alga were cultured together for 4 months in 4% malt yeast agar. The dual colony was cut into 6 equal portions by eye. Each portion was incubated for 1 hr in 1 ml of buffer containing 10 μ Ci $H^{14}CO_3^-$. Some samples were then transferred to 1 ml of buffer alone. For dark controls, the tubes were wrapped in aluminium foil.

graph of this culture is reproduced by Hill, 1970). The distribution of ^{14}C after exposure to $H^{14}CO_3^-$ in the previous experiment was analysed (Table 5 and Fig. 4). The results are compared with those of the cultured alga and the lichen. The amount of ^{14}C which was incorporated into the insoluble fraction was about the same as, or more than, that in the alga, but the amount in the lipid fraction was more like that of the lichen. The chromatograms of the ethanol extracts indicated that a large proportion of the ^{14}C was incorporated into the substances near the origin as well as into ribitol. Thus it appears as if this joint culture is intermediate between the cultured alga and the lichen in the pattern of $H^{14}CO_3^-$ incorporation. A significant point is that 24 hours after the pulse some ^{14}C (about 4% of the total ^{14}C fixed) occurred in the position of mannitol on the chromatograms. As this is over and above the ^{14}C incorporated in the dark, it indicates that the ^{14}C-mannitol was not formed by dark fixation by the fungus but that the ^{14}C had been transferred from the alga to the fungus.

Discussion

The formation of the polyol which is mobile in the lichens is not dependent on the symbiotic state of the alga because the alga in axenic culture can synthesise the mobile carbohydrate. It is reasonable to suggest that this conclusion applies to all green-algal symbionts. This invalidates the suggestion by Smith *et al.*, (1969) that the mobile polyol is only formed by the alga when it is in the symbiotic state.

Stichococcus spp. and *Hyalococcus* were very similar in their pattern of $H^{14}CO_3^-$ incorporation. The only difference was the smaller amount of ^{14}C found in the insoluble fraction in *Hyalococcus* (Table 2). The striking similarity indicates a close relationship between these two algal genera which are now separated only on the ability of *Hyalococcus* to form aplanospores; however, these could not be found by one of us (V.A.) (Ahmadjian and Heikkilä, 1970). At present the taxonomy of unicellular algae takes little account of physiological and phytochemical data and it is possible that the occurrence of polyols may be a useful character.

The results of the second section of the 'Results' confirmed and extended for *C. cristatella* what Richardson and Smith (1968) found for *Xanthoria aureola*. These are that the pattern of fixation of $H^{14}CO_3^-$ in the light into the alga in culture, as compared with the alga in the symbiotic state, is characterised by the incorporation of more ^{14}C into insoluble substances and soluble substances other than ribitol. Among other factors, the nutrient status of the alga may be particularly important with regard to the movement of carbon to the fungus. Given sufficient inorganic nutrients, an alga will incorporate carbon into the com-

ponents of new cells, e.g. proteins, polysaccharides and nucleic acids (ethanol insoluble substances) and pigments, lipids and intermediates in biosynthetic pathways (mostly ethanol soluble substances). If an alga has a limited supply of nutrients, the cells will not tend to grow and divide and as a result most of the carbon would be incorporated into carbohydrate (for instance, ribitol). In many lichens few nutrients are available to the alga and excess carbohydrate is used to supply the fungus. If the lichen is subjected to a nutrient rich environment, it is likely, therefore, that the alga would tend to use carbon fixed during photosynthesis for growth rather than for the manufacture of carbohydrate. The symbiosis would then break down. In fact Scott (1960) found that if thalli of *Peltigera praetextata* were kept in a nitrogen rich medium the alga (*Nostoc*) grew out of the thallus allowing the symbiosis to break down. This did not occur if low levels of nitrogen were used. One of us (D.J.H.) found that if thalli of *Xanthoria aureola* were kept in the laboratory and irrigated daily with weak Bristol's medium (and allowed to dry out in the light between times) the alga (*Trebouxia*) grew out of the thalli especially in places where damage had occurred. Perhaps it is reasonable to suggest then that the movement of carbohydrate from the alga to the fungus is dependent on the lichen being in an oligotrophic environment.

In resynthesis experiments, it is well established that when the two symbionts are cultured together on nutrient-rich media that sustain their independent growth, lichenization does not occur (Ahmadjian, 1967), while dual inocculation on nutrient-poor media which cannot support independent growth results in physical contact between the symbionts similar to that found in the lichen thallus. In the joint culture used in the present study, the nutrient medium (4% malt/yeast agar) had supported independent growth, although the two symbionts had grown in a single mass. The results using this culture suggest that some modification of the metabolism of the alga had occurred. This modification was a diversion of photosynthetic products into carbohydrate, particularly the mobile one, from lipid and insoluble substances. It is not clear how it may have been caused. The movement of a small amount of ^{14}C from the alga to the fungus, although significant, may not have been due to lichenization. Rather, the fungus might well have taken up minute amounts of substances which are normally released by algae when grown in culture (Fogg, 1962; and Table 4) without the fungus stimulating the alga to release any ribitol. The present experiments do not give weight to the theory that release of carbohydrate is caused by the fungus producing a substance capable of causing the plasmalemma of the alga to become permeable to ribitol, unless such a substance were only released when the fungus is in a lichenized state. If a joint culture

is grown on a medium unable to support independent growth, the two organisms normally become more closely associated. The alga would then have more limited growth and hence be able to produce more carbohydrate which could be available to the fungus. The fungus relying on the alga as a source of reduced carbon could possibly cause the alga to release carbohydrate (ribitol). Such alga behaviour would be equivalent to that in the lichen thallus. In future synthesis experiments the pattern of $H^{14}CO_3^-$ incorporation in the light would be a useful guide to the development of the symbiosis.

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