

STUDIES IN THE BIOLOGY AND PHYSIOLOGY OF LICHENS

WITH SPECIAL REFERENCE TO XANTHORIA PARIETINA (L.) Th. Fr.

ABSTRACT

The work described in this thesis had several main objects which are summarised as follows:

1. To study the carbohydrate movement from alga to fungus during photosynthesis in the lichen Xanthoria aureola (Ach.) Erichs. (since this work commenced the variety of Xanthoria parietina used in the experiments has been recognised as a separate species and is referred to by this new name).

Previous research on this aspect of the physiology of lichens has been largely limited to Peltigera polydactyla. This lichen contains the blue-green alga Nostoc which produces glucose during photosynthesis that passes to the fungus where it is

rapidly converted into mannitol. Xanthoria aureola was selected for the experiments in this investigation because it contains a green alga, Trebouxia, which is the commonest algal symbiont found in temperate lichens.

2. To study the carbohydrate movement between the symbionts of other lichens to determine the extent to which the pattern found in Peltigera polydactyla and Xanthoria aureola occurred in other species.
3. To study some general aspects of the carbohydrate metabolism of Xanthoria aureola such as the changes which occur during starvation, the uptake of carbohydrates and the variation in sugar alcohol content at various times of the year.
4. To study a number of ecological and taxonomic aspects of the biology of Xanthoria parietina sensu lato by transplanting thalli of different varieties between inland and sea shore habitats.

1. Carbohydrate movement from alga to fungus in Xanthoria aureola

a. Products of photosynthesis in the intact thallus.

Before investigating the role of the alga and fungus in the symbiosis, it was necessary to study photosynthesis in the complete thallus. When

samples of this lichen were floated in the light on aqueous ^{14}C sodium bicarbonate solutions, ^{14}C was at first incorporated in substantial amounts into the sugar alcohol ribitol which had not previously been reported in lichens. After several hours ^{14}C also began to accumulate in mannitol and a small amount was also found in arabitol. It was noted that less than 10% of the fixed ^{14}C occurred in the ethanol-insoluble fraction over a 24 hour period, and in experiments where the redistribution of a pulse of ^{14}C -ribitol was studied, it was found that this became largely converted into ^{14}C -mannitol and ^{14}C -arabitol over a 48 hour period.

b. Studies on the isolated components of the lichen

i. The alga. The alga of Xanthoria aureola, which is a species of Trebouxia, was successfully isolated and maintained in pure culture. Since there was the possibility that the behaviour of the alga in pure culture is different from that in the lichen, a technique was developed for isolating quantities of the alga direct from the thallus. This was done by gently homogenising thallus lobes and subjecting the homogenate to a regime of low speed differential centrifugation so as to produce a moderately pure suspension of living intact algal cells.

It was demonstrated that both the cultured and directly isolated alga produced ^{14}C -ribitol and ^{14}C -sucrose within the cells during photosynthesis on ^{14}C -sodium bicarbonate solution. However when the alga had been in pure culture for some time, less ^{14}C was incorporated

into carbohydrates, especially ribitol, than when the alga was directly isolated from the thallus. Both kinds of algal preparation were found to release carbohydrate, mainly in the form of ribitol, into the medium. However they did this only slowly, and the cultured alga appeared to release substances less readily than the directly isolated alga.

ii. The fungus. This was also isolated into pure culture from fungal ascospores and was subsequently grown in liquid culture. The lichen fungus of Xanthoria aureola was found to grow on a range of sugars and sugar alcohols. It was able to convert ^{14}C -glucose rapidly into ^{14}C -mannitol. Under certain cultural conditions it was found also to contain arabitol, confirming that this is a fungal product.

c. Attempts to inhibit the movement of carbohydrates between the symbionts

If ^{12}C -pentitols were present in the medium during photosynthesis by Xanthoria aureola on ^{14}C -sodium bicarbonate solutions, then ^{14}C -ribitol appeared in the medium and little ^{14}C -mannitol was formed within the thallus. This was explained in terms of the ^{14}C -ribitol produced by the alga being unable to compete with the high concentration (1 or 2%) of non radioactive pentitols at the fungal uptake sites, so that the entry of ^{14}C -ribitol into fungus was prevented and it therefore diffused out into the medium. It was only possible to obtain this inhibition with pentitols. Other sugars or sugar alcohols were unable

to prevent carbohydrate movement, suggesting that the fungal uptake mechanisms had a high specificity for pentitols. These results suggested that carbon moved between the symbionts principally in the form of ribitol.

d. The rate of movement of carbohydrate between the symbionts

In Peltigera polydactyla it had been shown by earlier workers that ^{14}C fixed by the alga moves to the fungal medulla within thirty minutes from the start of photosynthesis on ^{14}C -sodium bicarbonate. In the type of inhibition experiment described above, at least 25% of the total fixed ^{14}C was released from samples of Peltigera into the medium within three hours if it contained ^{12}C -glucose. It was therefore concluded that movement of carbon between the symbionts was rapid. In analogous inhibition experiments carried out here using Xanthoria aureola, it took as long as twenty four hours for a similar proportion of the total fixed ^{14}C to be released into the medium when it contained ^{12}C -ribitol, and this suggested that the movement of carbohydrates between the symbionts in this species was much slower. Further experiments were devised in which the rate of transfer of a pulse of ^{14}C from alga to fungus was estimated both in Peltigera polydactyla and Xanthoria aureola under identical conditions. These confirmed that movement of carbohydrate was much slower in the latter lichen.

2. Studies of carbohydrate movement in other lichens

a. Lichens containing blue-green algae

Three lichens were examined, two contained Nostoc (Sticta fuliginosa, Lobaria scrobiculata) and the third, Rivularia (Lichina pygmaea). They all showed similarities with Peltigera polydactyla in that glucose appeared to move between the symbionts and this was converted into mannitol by the fungal part of the thallus. There was evidence that the movement of carbohydrate from alga to fungus was rapid in the species containing Nostoc but slower in Lichina pygmaea.

b. Other lichens containing green algae

Lichens containing four genera of green algae (including Trebouxia) were studied and it was remarkable that in each case ^{14}C initially accumulated in sugar alcohols, during photosynthesis. The compounds ribitol, erythritol and sorbitol appeared to be the respective photosynthetic products of the algae Trebouxia (and Coccomyxa), Trentepohlia and Myrmecia. These sugar alcohols were converted into mannitol by the fungal part of the lichen and to a lesser extent into arabitol and volemitol in certain species.

In the lichens containing Trebouxia that were studied (Parmelia saxatilis, Lecanora conizaecoides, Umbilicaria pustulata, Lobaria laetevirens, Lobaria pulmonaria) movement between the symbionts appeared to occur at a similar rate to that found in Xanthoria aureola and little arabitol was formed. In the lichens containing

Coccomyxa (Solorina saccata, Peltigera aphthosa) the movement of carbohydrates was more rapid and a much larger proportion of the fixed carbon was found in arabitol after twenty four hours photosynthesis. Similarly the transfer of carbohydrate from alga to fungus was faster in Dermatocarpon miniatum, which contains Myrmecia, than in Xanthoria aureola and this lichen also differed in that substantial amounts of volemitol accumulated in the thallus. However the slowest rate of movement of carbohydrates between the symbionts was observed in those lichens containing Trentepohlia (Roccella fuciformis, R. phycopsis, Lecanactis stenhamarii, Gyalecta cupularis).

c. Lichens symbiotic with more than one alga

Three lichens were examined in which a green alga was symbiotic in the greater part of the thallus but where there are limited areas within the thallus (or outgrowths from it) where the fungus is symbiotic with a blue-green alga such as Nostoc. These areas, called cephalodia, have usually been assumed to be important in the nitrogen metabolism of the thallus. The pattern of carbohydrate movement between the fungus and its two symbionts was studied to try and find out if it was the fungus or alga which controlled the pattern of carbohydrate transfer in the thallus.

It was possible to excise the cephalodia of one species (Lobaria amplissima) and in this case the pattern of carbohydrate

movement from alga to fungus in the cephalodia was closely similar to that described for Peltigera polydactyla which also contains Nostoc. In the rest of the thallus, which contained Trebouxia, it was similar to that found in other lichens containing this alga. When samples of the other two lichens (Solorina saccata, Peltigera apthosa) in which the cephalodia could not be excised, were placed in the light on solutions of ^{14}C -sodium bicarbonate to which had been added ^{12}C -ribitol or ^{12}C -glucose, fixed ^{14}C appeared in the medium. This was either in the form of ^{14}C -ribitol or ^{14}C -glucose depending on which sugar had been used. If a mixture of the two sugars was placed in the medium, then both ^{14}C -glucose and ^{14}C -ribitol were found therein. Thus some ^{14}C -glucose appeared to be moving from the cephalodia to the fungal part of the thallus and this indicates that they may contribute to the carbohydrate nutrition of the thallus, particularly in Solorina saccata, where they are quite large in proportion to the rest of the thallus.

These results suggest that the type of alga symbiotic in the thallus has an important role in dictating the pattern of carbohydrate movement in the thallus. This was confirmed by studies on the directly isolated algae and by an investigation (described in Appendix A) into four species of the genus Lobaria which contain closely related fungi symbiotic with either Nostoc or Trebouxia. The two patterns of carbohydrate transference observed were therefore described as the 'Nostoc' and 'Trebouxia' patterns.

3. Absorption and loss of carbohydrates

Samples of Xanthoria aureola were found to absorb both pentitols and mannitol rapidly at 18°C which is in agreement with other studies on the uptake of substance from solution by lichens. On starvation in the dark at the same temperature, the content of pentitols (initially about 3% of the dry weight) in Xanthoria aureola fell rapidly and these compounds were not detectable after four days. By contrast, there was at first a rise in mannitol content (initially also about 3% dry weight), presumably due to conversion of pentitols into mannitol, followed by a slow fall. Mannitol was detectable in samples starved under these conditions for fourteen days. Some measurements were made on the seasonal variation in the total sugar alcohol content of this lichen and the results suggested that the sugar alcohol content fell in winter but rose rapidly in early spring.

4. The Transplantation of lichen thalli

Xanthoria parietina sensu lato now comprises two species, Xanthoria parietina and Xanthoria aureola. The former is separated into two varieties, var parietina and var ectanea. Lichen thalli were transplanted between Devon and Oxfordshire to gain further information about the exact taxonomic status of the species and varieties. The material of Xanthoria aureola from Oxfordshire was

planted on the rocks near Slapton in Devon whilst the material from that habitat, Xanthoria parietina var ectanea, was transplanted to the habitat of Xanthoria aureola at Wytham in Oxfordshire.* No method for transplanting saxicolous lichens had apparently been published, so one was developed using resin glue. This proved successful as the thalli survived and grew.

On preliminary evidence it was found that Xanthoria parietina and Xanthoria aureola were distinct species. However on transplanting X. parietina var ectanea to Oxfordshire, the lobe width increased so that it could not be distinguished from X. parietina var parietina.

The experiments also suggest that the amount of the yellow pigment, parietin, in these lichens is largely controlled by the environment. It was found that on transplanting thalli between the two habitats, the parietin content changed to that of control transplants within six months. It has been suggested that the amount of parietin developed is related to the light intensity. The Devon transplants had some four times more parietin than those from Oxfordshire. Since all the thalli grew in unshaded habitats it seems that light may not be the only factor responsible for the development of this pigment.

ERRATUM

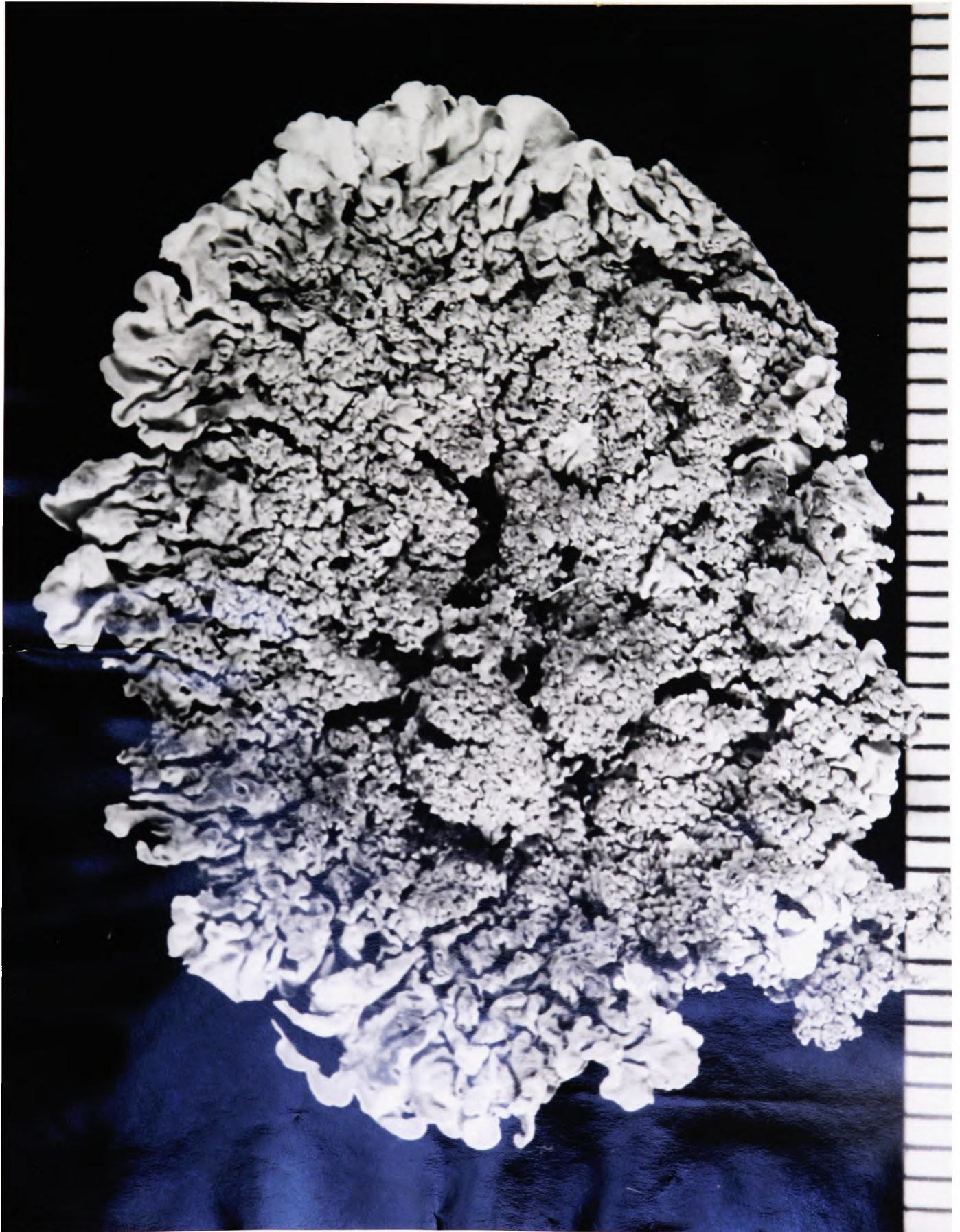
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THROUGHOUT THIS READ BERKSHIRE IN PLACE OF OXFORDSHIRE.

Xanthoria aureola (Ach.) Erichs.

(= Xanthoria parietina var. aureola)

one division of the scale = 1 mm.



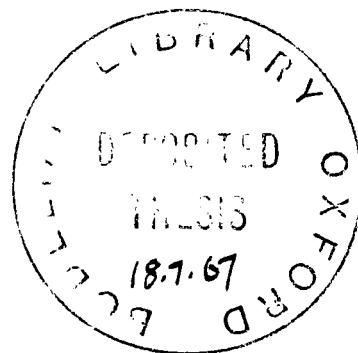
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by

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CHAPTER I

GENERAL INTRODUCTION

Lichens are one of the outstanding examples of symbiosis. In this group of plants, two organisms, an alga and a fungus, become closely associated and function as a single unit in nature. Both the interrelationships between the symbionts and the physiological properties of the complete thallus have been the subject of various studies which are briefly summarised as follows.

1. Studies on the isolated symbionts

Using standard microbiological techniques it is normally possible to obtain both lichen fungus and lichen alga in pure culture, though occasionally this proves very difficult (Scott 1957). The nutritional requirements of such isolates have been extensively studied, though it is by no means certain that they have the same physiological characteristics in culture as they do within the thallus.

a. The fungus.

Almost all lichen fungi belong to the class Ascomycetes and resemble related free living saprophytic forms in being able to live on a wide variety of carbohydrate and nitrogen sources (Ahmadjian 1966). However there are a number of respects in which they differ from these fungi: (a) they are extremely slow growing; (b) they are nearly all deficient for biotin and thiamine; (c) they grow better at 18 - 21°C than at 25°C; and (d) they hardly ever form conidia or other reproductive structures in culture.

b. The alga

Ahmadjian (1966) reports that there are twelve genera of blue-green algae and twenty genera of green algae symbiotic in lichens. In the British Isles, only about 8 - 9% of the lichen flora contain blue-green algae, the rest containing a green alga which in the majority of cases is a species of Trebouxia.

i. Blue-green algae. The commonest alga of this class found in lichens is Nostoc. Little is known of the cultural requirements of symbiotic strains of this lichen but Drew (1966) found that his isolates from Peltigera polydactyla were capable of growing on media without either carbohydrate sources or combined nitrogen. He found that the symbiotic strain of Nostoc differed from the free-living Nostoc muscorum in lacking heterocysts, in the production of aseriate structures rather than typical nostocacean filaments, and in the

greater production of mucilaginous sheaths in culture. Geitler (1933) and Zacharias (1900) also observed that the symbiotic blue-green algae had few or no cyanophycin granules. These are possibly a form of protein reserve (Fritsch 1959). It was further shown (Drew 1966) that the main product of photosynthesis of Nostoc immediately after isolation from the lichen thallus was glucose, most of which was excreted into the medium. However after only two days in culture, little free glucose was produced or liberated. Instead a compound believed to be a polysaccharide was excreted into the medium while within the cells more carbon was incorporated into amino acids and insoluble material.

ii. Green algae. The commonest alga in temperate lichens is Trebouxia while in tropical and subtropical areas of the world Trentepohlia is more frequently encountered.

Trebouxia is a unicellular alga which is seldom found in the free living state (Degelius 1964). It is very slow growing in culture and has been observed from the earliest studies (Beijerinck 1890) to require carbohydrate in the medium for good growth. Most species seem to grow better on organic nitrogen sources, but only if a suitable sugar such as glucose is also present in the medium (Ahmadjian 1966). Trebouxia sp can grow quite satisfactorily on media in complete darkness if they contain both sugar and peptone, and Mish (1953) has reported that this alga could be freeze dried

successfully. For optimum growth rather low light intensities are required (Ahmadjian 1966) since high light intensities lead to loss of pigmentation by the cells especially when the medium does not contain added carbohydrate (Ahmadjian 1959). Studies have recently been made on the photosynthetic products of isolates of the alga from Xanthoria parietina by Bednar and Smith (1966). Their results are described in the Chapter III.

Trentepohlia occurs as the algal symbiont in many crustaceous lichens. It is a filamentous alga and in culture in high light intensities the colonies are usually orange due to haematochrome which occurs in fat globules around the chloroplast (Fritsch 1961). When shaded and in some lichen thalli, the pigment is almost lacking; it is thought to be a form of food reserve as it disappears if the alga is placed in the dark. The ends of the filaments are covered with a cap of pectose which West and Hood (1911) think is excreted from within the cells. The soluble carbohydrates of Trentepohlia have been studied by Tischer (1936) who showed that this alga contained erythritol.

2. Attempts at resynthesis of the lichen

During the last century there were a number of reports of successful resynthesis of lichens in culture from the constituent alga and fungus; for example Reess (1871) with Collema sp, Stahl (1877) with Endocarpon sp, Bonnier (1889) with Xanthoria sp, and

Möller (1887) with Calicium sp. Many attempts have since been made to repeat their results with partial or no success. As a result, doubts have been raised by many authors (e.g. Quispel 1943; Ahmadjian 1962) as to the purity of the cultures of the isolated symbionts and whether the apparent resynthesis could have resulted from airborne contamination. Some of the published illustrations by these early workers of the resynthesised structures do not resemble very closely the complete lichen growing naturally nor do they show mature reproductive structures.

More recently, Thomas (1939) claimed complete synthesis in sterile culture on elder pith of the lichen Cladonia pyxidata. Upright growing structures (podetia) bearing cups (scyphae) that in nature develop the fruiting structures (apothecia) were formed. However he was not able to repeat this result with a further 800 cultures.

Within the last decade Ahmdjian (1962, 1963, 1966) has attempted to resynthesise the crustaceous lichen Acarospora fuscata on agar media and rock chips. He found that on minimal agar media and rock chips the fungus was only able to survive when the alga was present. A pseudoparenchyma was formed round the alga by the fungus but no typical thallus was developed, the upper cortex being absent. Also, no reproductive structures were formed. There was some evidence that the maintenance of the established symbiosis

depended upon variation in humidity, a suggestion made previously by Thomas (1939) and Quispel (1943). Henrisect (1945) tried to resynthesise various crustaceous lichens belonging to the Graptidiales which contain Trentepohlia. Whilst he too managed to get a plectenchymatous association and saw haustoria, he did not observe the production of apothecia in any cultures. Ahmadjian (1966) had more success with Cladonia cristatella; podetia were formed in response to the joint effect of drying and nutrient poor conditions, and these bore pycnidia and apothecia but did not contain asci.

Scott (1960) has used a different approach. He has tried to maintain the symbiosis in discs of Peltigera praetextata kept in illuminated culture tubes. He discovered that variations in nutrient supply, light and moisture upset the symbiotic balance. Anderson and Ahmadjian (1962) using Scott's technique were able to get development of podetia from discs of Cladonia coniocrea on which the initial stages of apothecia were found.

Thus a complete synthesis has not been achieved to date but some of the problems of maintaining the symbiosis are now known.

3. Physiology of the thallus

Many of the studies on the physiology of lichens have been directed at explaining the ecological occurrence of a particular

species. For example Reid (1960) studied the resistance of lichens to drought and found that the aquatic Verrucaria elaeomeleana was permanently damaged after twenty four hours in such conditions whilst Rhizocarpon geographicum, which lived on rocks subject to isolation, withstood six months drought without damage. In general, he found that lichens suffered most during the rewetting after drought and that the frequency of rewetting was more important than the actual length of the dry conditions.

Studies on the photosynthesis of lichens by Reid (1960) and Stålfelt (1939) showed a decline in photosynthetic rate above an optimum water content but Butin (1954) found that the maximum rate was maintained up to 100% saturation. Stålfelt and Butin determined compensation points for photosynthesis of a number of lichens. These varied from 400 lux for Usnea dasypoga to 2000 lux for Ramalina fraxinea. Barkman (1958) has concluded that their results show a fairly good relationship to habitat preference. Thus Ramalina fraxinea grows only in the light whilst Usnea dasypoga will tolerate shade.

Recently, the effects of pollution on the occurrence of lichens in the vicinity of industrial centres has merited attention. Pearson & Skye (1965) and Rao & Le Blanc (1966) have demonstrated that sulphur dioxide leads to abnormal photosynthetic patterns and damage to the chloroplasts of lichen algae. They suggest that this compound may

play a major role in causing death of lichens in such areas. Certainly, Gilbert (1965) found that the geographical distribution of lichens in the industrial Tyne valley suggested that they were particularly sensitive to sulphur dioxide. Smith (1962) concluded that lichens have very efficient power of absorption of substances from solution, and suggested that this was one of the reasons for the sensitivity of lichens to atmospheric pollution.

Examination of the chemistry of the whole lichen thallus has led to the identification of a range of 'lichen acids' (Asahina and Shibata 1945), antibiotics (Fujikawa et al 1957, Virtanen and Kilpiö 1957) and enzymes (Moissejeva 1961). In addition, the structure of the insoluble polysaccharides in a few species (Peat et al 1957) and the identity of some soluble carbohydrates of a number of other lichens have been determined (Lindberg et al 1952, 53, 55, 64) but very little is known about the amino acid or protein content of lichens. Ramakrishnan and Subramainian (1965a & b) have reported some very high protein contents; for example, Peltigera canina (22%) and Roccella montagnei (14%); but these require confirming. They also found the latter lichen, which is used for cattle fodder, was rich in β -carotene, ergosterol and riboflavin.

4. The physiology of the symbiosis

Since lichens appear to behave as autotrophic plants, it is usually assumed that substances produced by the alga during photosynthesis must pass to the fungus. Therefore a study of the carbohydrate metabolism of the thallus is an obvious starting point in an investigation of the symbiosis and physiology of lichens. Such studies have been rendered much easier by the availability of ^{14}C -labelled compounds and the development of tracer techniques.

The first experiments on the carbohydrate metabolism of lichens using these techniques were carried out by Smith (1961). He showed that discs of Peltigera polydactyla when photosynthesising on solutions of ^{14}C -sodium bicarbonate, incorporated ^{14}C into organic compounds, mainly mannitol. At first these occurred in the algal layer but later radioactive compounds were found in the medulla of the lichen. Smith (1963) also showed that absorbed glucose ^{14}C was rapidly converted into ^{14}C -mannitol in the thallus. Smith and Drew (1964) studied the transfer of fixed ^{14}C from algal layer to fungal medulla in Peltigera polydactyla. They found that ^{14}C moved into the medulla from the algal layer within thirty minutes. This movement continued at a steady rate equivalent to 40 to 50 per cent of the total rate of ^{14}C fixation and could also continue at a reduced rate in the dark for some time. Drew (1966) demonstrated that the blue-green alga Nostoc within the thallus produced glucose during

photosynthesis and that this passed quickly to the fungal medulla where it was converted into mannitol.

5. The present study

The studies of Smith and Drew have outlined the pattern of carbohydrate transfer between the alga and fungus of a lichen which contains a blue-green alga. However the majority of lichens contain green algae as symbionts and hence it was desirable to study the physiology and carbohydrate metabolism of such lichens to see if the results obtained from experiments on Peltigera polydactyla could be applied to them.

The objects of the present study may therefore be summarised as follows:

a. The physiology of the symbiosis in Xanthoria aureola

- i. The identification of the main products of photosynthesis.
- ii. An investigation of the form in which carbohydrate fixed by the alga passes to the fungus and the rate at which this occurs.
- iii. An investigation of photosynthesis by the isolated alga with special reference to the subsequent release of fixed carbohydrate.
- iv. The identification of the soluble carbohydrates of the isolated fungus of this lichen.

b. Experiments using other lichens

i. The identification of the carbohydrates produced during photosynthesis by a range of lichens.

ii. An examination of the form in which carbohydrates move to the fungal parts of these lichens and the rate at which this occurs.

c. Other aspects of the biology of Xanthoria

i. The examination of the effects of starvation and seasonal variation on the sugar alcohol content of Xanthoria aureola.

ii. A study of the effect of transplanting different varieties of Xanthoria parietina sensu lato between different habitats to try and determine the taxonomic status of the varieties.

CHAPTER II

MATERIALS AND METHODS

1. Materials - Xanthoria aureola

a. Habitat

This lichen grew extensively on a south-facing tiled roof in the farmyard of the University Farm, Wytham, Oxfordshire (Figure 74).

b. Collection

In early experiments whole thalli were scraped off the roof, cleaned and sorted into samples of approximately 100 mg fresh weight. However, the central part of the thalli was so encrusted with dirt that it was not possible to use that part of the lichen at all. It was therefore more convenient to collect only the marginal lobes by making a cut some 5 mm from the thallus margin and removing that part of it with a scalpel. Whenever the roof was dry, water was thrown on to it prior to collection so that the marginal lobes could be removed easily and without damage.

c. Sampling

Harley and Smith (1956) developed a method for sampling Peltigera polydactyla thalli on a surface area basis by punching discs 7 mm in

diameter from healthy lobes. In Xanthoria aureola, the lobes are both narrow and convoluted so it is not possible to sample in this way. The problems associated with sampling thalli of this lichen are illustrated in Figure 1. This shows that the surface of the thallus is very uneven and also demonstrates that in vertical section the lichen is wedge shaped, being thick at the centre of the thallus and thin at the margin. There are also proliferations at the centre of the thallus, so that the proportion of algal layer to overall surface area is not constant.

In order to try to determine the best parameter for sampling this thallus, 100 mg fresh weight samples were weighed out and their surface area estimated. The samples were then placed in the light with carrier free ^{14}C -sodium bicarbonate for six hours (see next section for details). The total ^{14}C fixed was determined and the results expressed on the basis of either surface area, fresh weight, or dry weight after extraction in ethanol. Analysis of the results suggested that sampling on a fresh weight basis was most satisfactory (Figure 2) so this method was used throughout all experiments.

2. Materials - other lichens

The other lichens used in this study were collected from a variety of places and habitats which are cited in Appendix B. The method of sampling was either similar to that described for Xanthoria

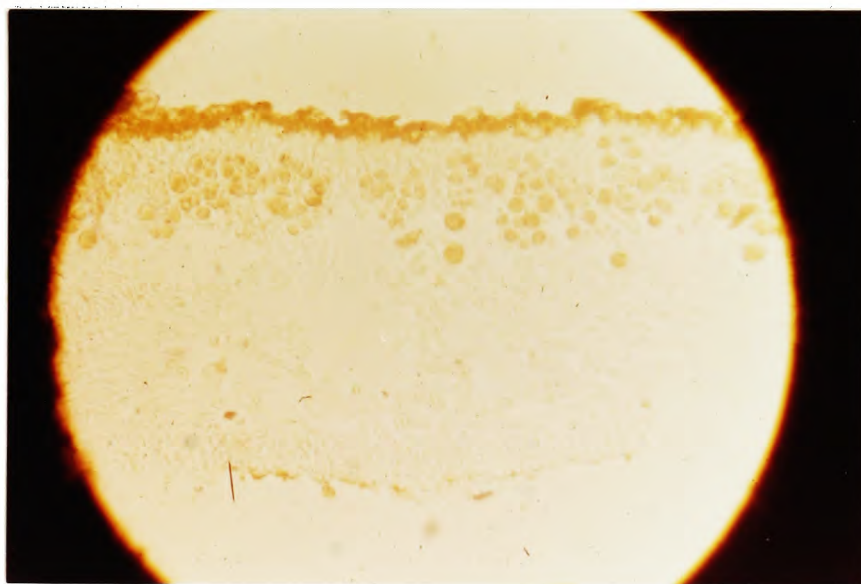
Figure 1

The anatomy and morphology of Xanthoria aureola

- a. vertical section of the thallus
- b. diagrammatic representation of a section
of half a thallus
- c. a close up photograph of thallii of
Xanthoria aureola growing on the roof
of the University Farm, Wytham.

15.

a.



upper cortex

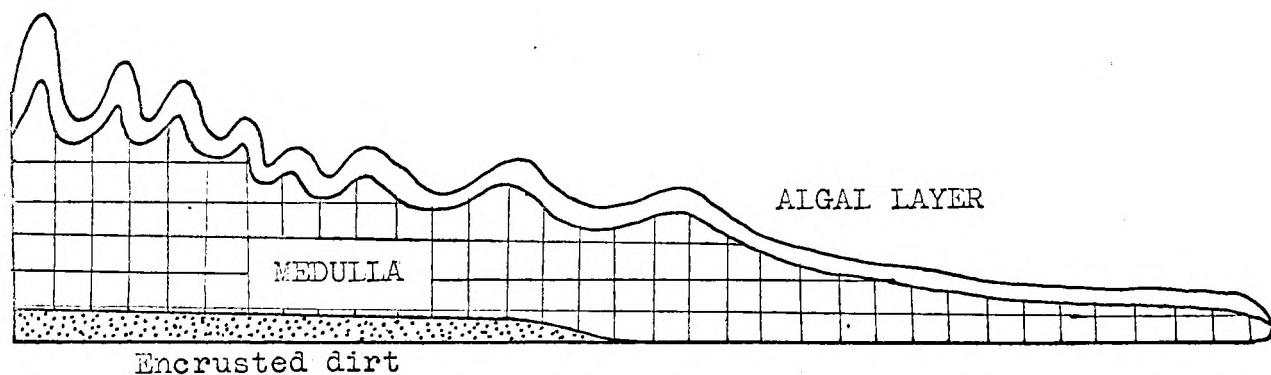
algal layer

medulla

lower cortex

15 μ .

b.



THALLUS CENTRE

INTERMEDIATE AREA

MARGINAL LOBE

c.



Figure 2

A comparison of the coefficient of variation of measurements of ^{14}C fixation made on a dry weight, fresh weight and surface area basis. Five samples of Xanthoria aureola were used per treatment.

$$\text{(Coefficient of variation = } \frac{\text{standard deviation}}{\text{mean}} \times 100)$$

Fraction	Fresh weight	Dry weight	Surface area
Soluble	8.2	9.6	14.4
Insoluble	11.8	14.1	19.4
Medium	32.6	34.1	68.4
Total	8.1	9.4	14.3

Material: 100 mg fresh weight per sample
 Period of photosynthesis: 6 hours
 Temperature: 18°C
 Light: 500 fc
 Vessels: 2 x 1" specimen tubes
 Radioactivity: 10 μc NaH $^{14}\text{CO}_3$
 Medium: 3 ml distilled water

aureola or similar to that used for Peltigera polydactyla. Details are given in Chapters VII to IX in which experiments on these lichens are described. These lichens were used in experiments as soon after collection as possible. They were kept in transit in a moist condition in sealed polythene bags. Normally the time between collection and experiment was less than 36 hours.

3. Experimental conditions

a. Conditions for photosynthesis

Experiments were carried out in an illuminated shaking water bath at 18°C (room temperature) or 5°C (cold room) with a light intensity of 500 foot candles provided by four 40 watt daylight fluorescent tubes.

In early experiments, samples were placed in 50 ml conical flasks but these reduced the incident light and were awkward for manipulating small thallus lobes. Beakers (10 ml) with small glass squares were next tried but it was felt that the large air/liquid surface might lead to significant loss of ¹⁴C carbon dioxide from solutions of ¹⁴C-sodium bicarbonate. Finally 2" x 1" specimen tubes were used and these were sealed from the atmosphere with 1" glass cover-slips kept in position by vaseline on the lip of the specimen tube.

The standard procedure was as follows: samples of any lichen material was placed in a specimen tube with 3 ml of distilled water, the samples floating with the upper cortex towards the light. In

some species (eg Roccella) which had an algal layer on both surfaces, a piece of aluminium foil was placed under each tube to reflect the light. A quantity of ^{14}C -sodium bicarbonate (usually 10 μc in 0.1 or 1.0 ml of water) was added to the tube which was sealed with a cover-slip and clipped into the illuminated bath.

b. Use of carrier ^{12}C -bicarbonate

One of the problems associated with experiments lasting over longer periods was that lichen material fixed a large proportion of the added ^{14}C -sodium bicarbonate in the first few hours which resulted in a non linear rate of fixation over a 12 or 24 hour experiment. In order to try to obtain a linear rate of fixation, increasing amounts of carrier bicarbonate were added to the medium. However even with 1 mg per ml of carrier, fixation was not linear for times in excess of 12 hours at 18°C using Xanthoria aureola (Figure 3). In experiments described in Chapter VI, where lichen thalli were induced to release fixed ^{14}C to the medium by placing them in solutions of various carbohydrates, it was found that this release was reduced if large amounts of carrier ^{12}C -bicarbonate were present. The reasons for this remained obscure until work on the isolated alga showed that the release of fixed ^{14}C was much reduced at high pH and the pH of a solution containing 1 mg per ml of sodium bicarbonate proved to be 7.8 to 8.2.

It would have been possible to incorporate large amounts of carrier bicarbonate and to control the pH with buffer but there were

Figure 3

Total fixation of ^{14}C by samples of Xanthoria aureola and Peltigera polydactyla during photosynthesis on solutions of ^{14}C -sodium bicarbonate to which had been added 1 mg per ml of carrier ^{12}C -sodium bicarbonate.

Experiment: 58

100 mg fresh weight Xanthoria per sample

7, 7 mm discs of Peltigera per sample

Period of photosynthesis: up to 24 hours

Temperature: 5 and 18°C

Light Intensity: 500 foot candles

Vessels: 2 x 1" specimen tubes

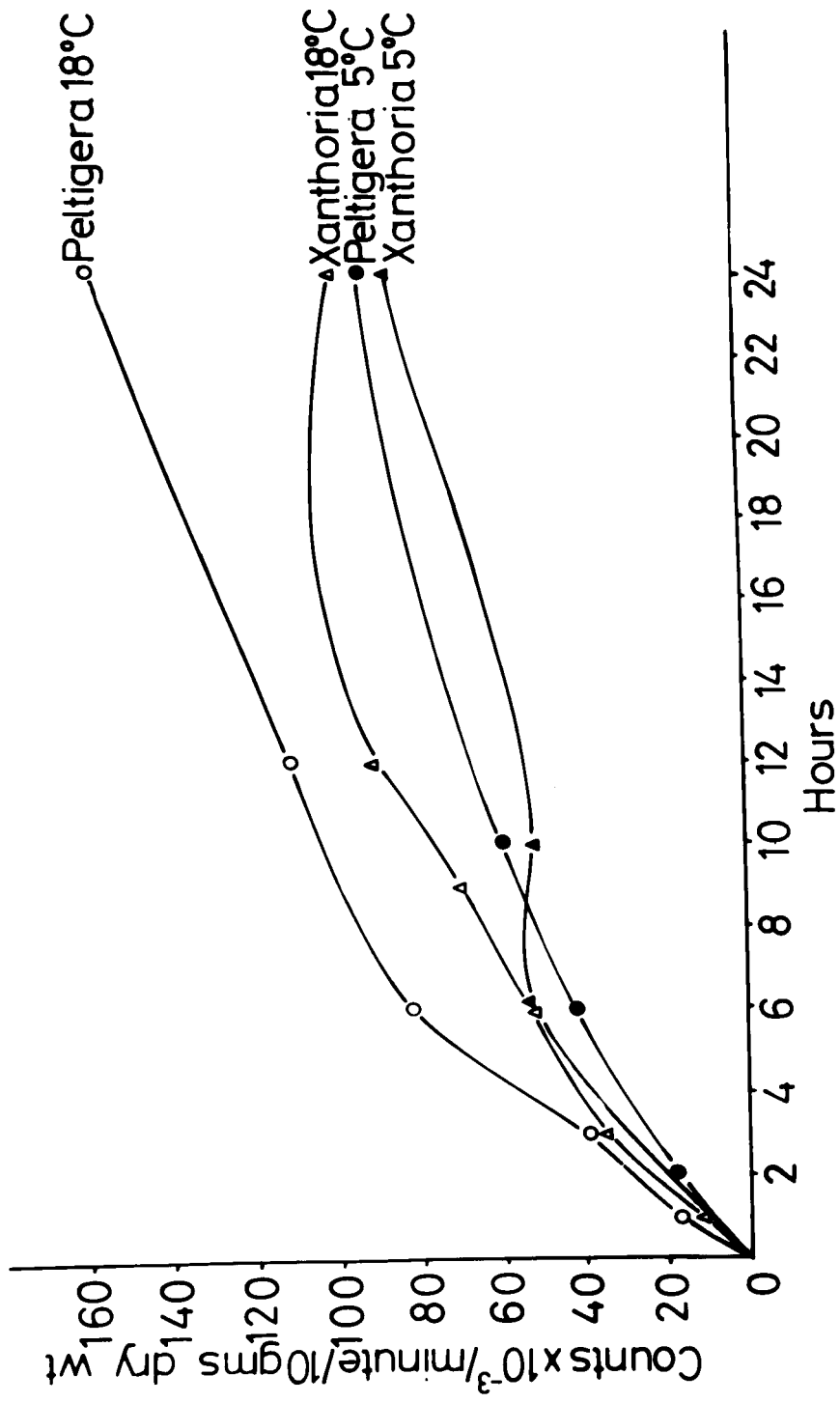
Volume: 3 ml

Activity: 10 μc per sample $\text{NaH}^{14}\text{CO}_3$

Solutes: 2% glucose or ribitol

Carrier: 1 mg per ml $\text{NaH}^{12}\text{CO}_3$

Date: 1.3.66.



two disadvantages to this. Firstly the components of most buffers are known to have some effect on the metabolism of the plants immersed in them, and secondly high concentrations of carbonate ions can affect certain aspects of photosynthesis such as glycollic acid production.

It was therefore decided to use carrier-free aqueous ^{14}C -sodium bicarbonate solutions in sealed vessels. In some of the earlier experiments carrier bicarbonate was used; in such cases the amount used is indicated on the relevant figure with other experimental details.

4. Extraction procedure

Each sample to be analysed was dropped into a McCartney bottle containing 5 ml of 80% ethanol which was raised to boiling point by placing it in a boiling water bath. Samples were then cooled and left in stoppered tubes at room temperature for at least 24 hours. Each was then given two further five minute extractions with 5 ml aliquots of 80% ethanol and the three extracts and washings were combined and made up to 25 ml with 80% ethanol. This comprised the soluble extract. It was found that further extractions with 80% ethanol removed no further radioactivity from the samples. Since sugar phosphates were not routinely analysed, a final extraction with 20% ethanol or water was not considered necessary.

The residue from the above extractions was the ethanol-insoluble fraction (referred to in text as the insoluble fraction) and was kept under 80% ethanol in screw-capped bottles.

5. Preparation of samples for chromatography

a. The insoluble fraction

Extracted washed lobes of lichen were hydrolysed under reflux with 5 ml of 1.5N sulphuric acid (Lewis and Harley 1965). This was neutralised with barium carbonate and the filtrate deionised. Alternatively ground and dried lichen were hydrolysed using the technique of Saeman et al (1954), for the complete hydrolysis of cellulose. The extracted ground lichen (300 mg) was warmed for one hour at 30°C with 3 ml of 72% sulphuric acid. The resulting hydrolysate was transferred to a 250 ml flask and 84 ml of water added. The flask was autoclaved for one hour at 15 lbs per sq. inch, cooled and 25 g of amberlite 1R4B resin added to remove the acid. The resin was filtered off and the volume of filtrate reduced to about 4 ml in a vacuum rotary evaporator at 37°C.

b. The soluble fraction

Aliquots of extracts were placed in 2 oz porcelain crucibles and evaporated to near dryness under an infra-red lamp. The crucibles were placed in a Petri dish of cold water and a fan was used to maintain a draft, ^{THIS} and to accelerate the evaporation and minimise

deleterious effects on the extract due to overheating. If there was reason to avoid this, samples were evaporated in a cold air current overnight. Where quantitative transfer was desirable, the near dry extract was washed from the crucible with a series of 0.1 ml quantities of warm distilled water which were summed and made up to known volume. Amounts (usually 10 μ l) were then transferred to chromatograms using microcap disposable pipettes.

When it was necessary to deionise the extracts, the evaporated residues were redissolved in warm distilled water and then shaken in a flask or test tube with a mixture of amberlite 1R 120 and 1R 45 resins for 30 minutes. In cases where pigments also had to be completely removed, as in the purification of sugars, the extracts were cleared using the method of Somogyi (1954). A volume of 0.3N barium hydroxide was added to the extract such that 1 ml of barium hydroxide was equivalent to about 5 mg fresh weight of the lichen from which the extract was prepared. To this was added an equal volume of approximately 5% zinc sulphate so adjusted as to neutralise the excess barium hydroxide. The precipitate was centrifuged and the clear filtrate deionised as above.

c. The medium

The medium was stored in a deep freeze. Before use it was melted and quickly evaporated to a small volume under infra-red lamps in a draft, and then aliquots transferred to chromatograms.

6. Chromatography

a. Paper

Descending paper chromatography was routinely used for the separation of compounds. Whatman no 1 paper was used except where heavy loading of the paper was required, in which case Whatman no 3MM paper was employed. Washed papers were only used for chromatography of extracts in the picric acid solvent.

b. Solvents

Figure 4 gives a list of the solvents and the corresponding abbreviations used in the text. Both one and two dimensional chromatograms were run. All chromatograms were run at room temperature (18°C) except those developed in the picric acid solvent which were run in a cabinet carefully controlled at 24°C.

Two dimensional chromatograms were run in butanol:propionic acid:water and then in phenol:water. Another system consisted of ethyl acetate:acetic acid:water in the first dimension and ethyl methyl ketone:acetic acid:water saturated with boric acid in the second dimension.

c. Detection reagents

A large number of these were tried, especially in attempts to detect sugar alcohols on chromatograms run in boric acid solvents or after electrophoresis in basic lead acetate solutions. All these methods are reviewed by Lewis and Smith (1967). Figure 5 gives a list of the methods which proved most useful and which were routinely used.

Figure 4

25.

Solvent	Abbreviation in Text	Compounds separated	reference
Ethyl acetate/Acetic acid 14 : 3 Water : 3	EA/AA/aq	sugars	Smith (1960)
Ethyl methyl ketone/ 9: Acetic acid/Water sat. 1: with boric acid : 1	EMK/AA/Baq	Polyols & sugars	Rees & Reynolds (1958)
n Butanol/Acetic acid 12 : 3 water : 5	Bu/AA/aq	organic acids sugars	Smith 1960
Picric acid/ n Butanol 2.2g. : 80 ml. water : 20 ml.	Picric acid solvent	Total soluble extracts	Haynes & Isherwood (1949)
Phenol/water 500g. : 125 ml.	Phenol/water	"	Smith 1960
n Butanol/Propionic acid water	Bu/PrA/aq	"	"

Figure 5

Substance detected	Reagent	Colour of spots	Reference
All sugars reducing sugars	alkaline silver nitrate aniline phosphate enhanced by uv light	black spots pink, yellow, brown	Trevelyan et al. 1950 Frahn & Mills 1958
Sugar alcohols	*1 Alkaline silver nitrate Bromo cresol purple * Potassium permanganate/ chromium trioxide	black spots white spots, blue blackground white spots on brown background	Trevelyan et al. 1950 Bradfield & Flood 1958 Frahn & Mills 1958 (Modified by using 2.5% $KMnO_4$ in place of 0.5%)
Amino acids	Ninhydrin/acetic acid/ collidine	blue to purple	Levy & Chung 1953
Organic acids	Acridine enhanced by UV light Aniline Xylose	yellow on white background brown spots on yellow background	Smith 1960 "

* Used on chromatograms run in boric acid

* Used after breaking boric sugar complex with HF Britton 1959.

7. Electrophoresis

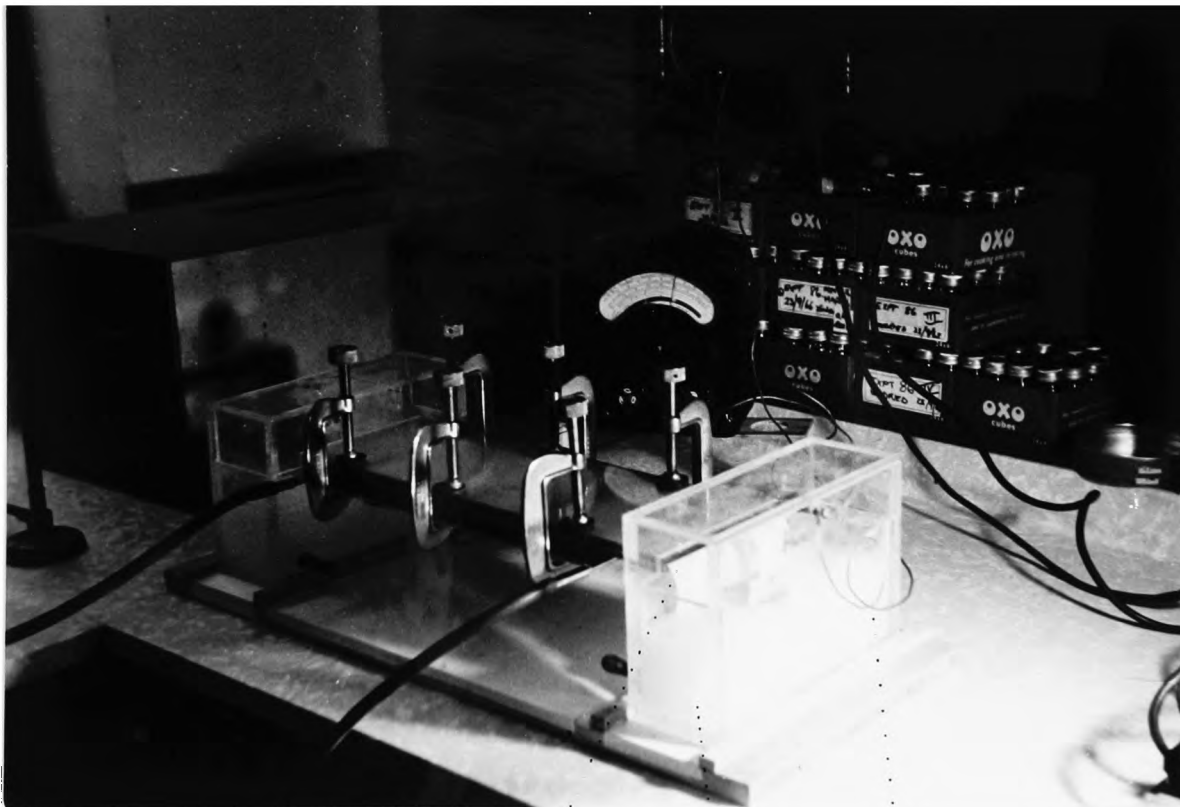
Paper ionophoresis was also used to separate sugar alcohols and particularly pentitols. The apparatus described by Frahn and Mills (1959) was constructed (Figure 6) and basic lead acetate (58 g A.R. per litre distilled water) proved the most useful electrolyte. The troughs at each end of the apparatus were filled with electrolyte. The paper (Whatman no 4) strip was quickly passed through a solution of the electrolyte and then blotted dry between two sheets of chromatography paper using only the weight of a rubber paper hanging roller. The paper strip was then placed in the apparatus, a polythene mask was laid on to the moistened strip to protect it from atmospheric carbon dioxide, and extracts inoculated through a series of 0.4 mm holes. The apparatus was clamped and runs were made for up to three hours, at a potential difference of 800 volts.

Authentic markers were used to compare with unknown compounds and R_{ribose} values calculated using a marker mixture of ribose and the non-migrating compound caffeine. The paper was dried at 100°C and spots developed with potassium permanganate:chromium trioxide reagent modified by using 2.5% potassium permanganate instead of 0.5% as recommended by Frahn and Mills (1959).

Figure 6

The electrophoresis apparatus used for
the separation of pentitols

Duration of run: 3 hours
Voltage: 800 volts
Temperature: 5°C = water cooled
Electrolyte: basic lead acetate
Paper: Whatman No. 4



w

s

e

p

w = water for metal cooling plate
s = paper strip held between two sheets of plate glass
e = electrolyte
p = power supply (800 volts)

8. Radiochemical techniques

a. Counting

i. The soluble extract. 0.2 ml of the extracts were transferred to aluminium planchettes which were ringed with silicone grease to prevent the creep of the alcoholic extracts up the sides. The extracts were then dried down under infra-red lamps with a fan to accelerate evaporation. The samples were counted with a thin end-window Geiger counter fitted with automatic sample change and print out units. This had an efficiency of 2-4% from ^{14}C -labelled samples.

ii. The media. The media in which photosynthesis had occurred ~~were~~ ^{were} pipetted on to planchettes but where this contained a high proportion of dissolved sugar the planchettes were previously etched with sodium hydroxide solution so that the aqueous extract spread out over the maximum area. Aliquots of the medium (0.2 ml) were placed on the planchettes with one drop of 0.1N hydrochloric acid which removed any remaining ^{14}C -sodium bicarbonate.

iii. The insoluble fraction. This fraction was treated in two ways. Firstly the lichen could be hydrolysed using 1.5N sulphuric acid (Lewis and Harley 1965) and the hydrolysate counted as above. Secondly the thallus lobes could be counted directly by placing dried extracted thallus lobes on double sided sellotape on the bottom of planchettes. Since the self absorption of lichen material is related to its cross sectional density (which is about 5 mg/sq. cm), Drew

(1966) estimated that this direct count only produced a reduction of about 10% in the total count. This was confirmed using Xanthoria aureola and was the technique routinely used for this lichen.

However some lichens such as Lecanora conizaecoides and Roccella fuciformis which possessed particularly tough thalli showed considerable reduction (up to 50%) if the insoluble fraction was counted by the direct counting technique.

b. Scanning

After chromatograms were developed, they were cut into strips and scanned on the apparatus described by Loughman and Martin (1957). By measuring the areas under the peaks on the scan, it was possible to estimate approximately the proportion of activity in each labelled compound.

c. Autoradiography

This was used to detect spots of very low activity. The chromatogram strips were covered with Melinex polyester film (25^s gauge) to prevent spoilage of the emulsion by residual solvents such as picric acid and phenol. Kodirex X-ray plates were then placed in contact with chromatograms for up to 21 days and then developed in the usual way. Chromatograms were aligned with the film, either by stabbing both with a sharp needle or by placing spots of radioactive ink upon the chromatogram before placing the film in contact with it.

9. Optical rotation of polyols

Polyols within a particular series, eg hexitols or pentitols, have closely similar physical and chemical properties because the molecules vary only in the position of the hydroxyl groups to one another. They thus present problems in identification. The optical rotation of polyols is normally very small but Gernez (1891) discovered that the optical rotation of these compounds is enhanced in molybdate solutions. Using this method it is possible to discover whether a polyol is in an active or meso state and this may help in the identification of an unknown polyol.

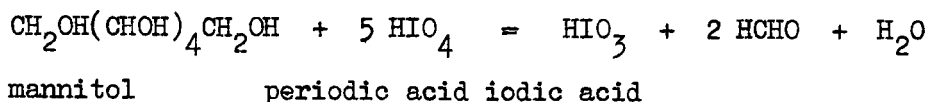
The procedure used to measure the optical rotation of the unknown polyols from Xanthoria was that of Richmeyer and Hudson (1951). Known polyols were dissolved (0.1 g in 25 ml) in ammonium molybdate tetrahydrate (25 g dissolved in 500 ml distilled water). The optical rotation of the dissolved polyol was then measured on an ELT, NLP automatic polarimeter (Type 143a). An aliquot of the solution (4 ml) was then acidified with N sulphuric acid (1 ml) and the optical rotation again measured. This was also done with a solution of the unknown polyol. The concentration of the unknown polyol was determined by the spectrophotometric method of Lewis and Harley (1965) and the optical rotation worked out using the following formula:

$$\alpha D = \frac{I \times 48}{C \times L}$$

C = polyol concentration mg/ml
 L = length of cell in cm
 I = reading

10. The estimation of sugar alcohol content of lichens

The commonest method used for the quantitative estimation of sugar alcohols depends on their reaction with periodic acid.



Cameron et al (1948) found that acidified potassium iodide released iodine quantitatively from periodic and iodic acids. This could be titrated against N/10 sodium thiosulphate solution using starch indicator. This method proved satisfactory for the routine examination of mannitol in seaweeds but it was found that reducing sugars also reacted with periodic acid, though more slowly. Therefore the time between the addition of periodic acid and acidified potassium iodide (which stopped the reaction) was adjusted to exactly one minute. Drew (1966) modified this method for the micro determination of mannitol in samples of the lichen Peltigera polydactyla. He used M/400 periodic acid and N/800 sodium thiosulphate solution. This method proved unsuitable for the estimation of polyols in Xanthoria aureola as great difficulty was experienced in getting a good end-point. The titrated solution contained parietin and hence the end-point was a change from pale yellow to pale green. Attempts to remove the pigment with charcoal or resin without also removing the sugars failed. However it was possible to use this method on eluates from chromatograms.

Agranoff et al (1958) designed a spectrophotometric method for the determination of inositol which is based on the fact that periodic acid has a strong absorption peak at 260 μ . They first converted all simple sugars to their acidic forms with boiling barium hydroxide and then reacted their cooled deionised extract with periodic acid for 30 minutes and measured the absorption at 260 μ . This enabled them to estimate glycerol because periodic acid does not react with inositol under these conditions. They then incubated the mixture at 65°C for two hours when inositol reacted. The mixture was again cooled and the absorbance again determined.

Lewis and Harley (1965) employed the spectrophotometric method of Agranoff et al but made use of the report of Cameron et al (1948) that glucose and other sugars were not readily oxidised under acid conditions whereas the oxidation of mannitol was almost complete within one minute at room temperature. Therefore one ml of test solution containing 10 to 100 μ g of mannitol was mixed with one ml of M acetate buffer pH 4.5 and then one ml of sodium periodate (approx 750 mg/l) was added. The absorbance was measured and the reduction in absorbance compared with a water control.

This method was adopted in estimating the sugar alcohol content of Xanthoria aureola. A Zeiss PMQ II spectrophotometer was used and the water blank replaced by one containing one ml of extract, one ml of buffer and one ml of distilled water. This was necessary because

the crude extracts used had a considerable absorbance at 260 m μ . One ml of test solution was added to a 3 ml cuvette, followed by one ml of buffer, and then one ml of sodium periodate solution was quickly added and the cuvette inverted ten times. It was not actively shaken since this resulted in the formation of small bubbles which affected the absorbance. The tube was placed in the spectrophotometer and the absorbance measured against the blank exactly one minute from the beginning of the addition of the periodate solution.

One problem which arose in the case of Xanthoria aureola was that this lichen contains three sugar alcohols, mannitol, arabitol and ribitol. Mannitol and arabitol react rapidly with periodic acid, about 90% oxidation occurring within one minute. However ribitol reacts much more slowly, only 55% being oxidised in this time (Figure 7). Thus in the starvation experiments described in Chapter X, the total sugar alcohol content of the extracts is expressed in mannitol equivalents which may be an underestimate if a significant amount of ribitol was present.

Lewis and Smith (1967) found that in cases where solutions contained only small amounts of free sugars it is possible to let the reaction proceed for two minutes. In some later experiments, for example in the estimation of sugar alcohol content in relation to seasonal change (Chapter X), this procedure was adopted.

Figure 7

The rate of reaction of various sugar alcohols with periodic acid measured with a spectrophotometer.
μg of sugar alcohol plotted against absorbance.

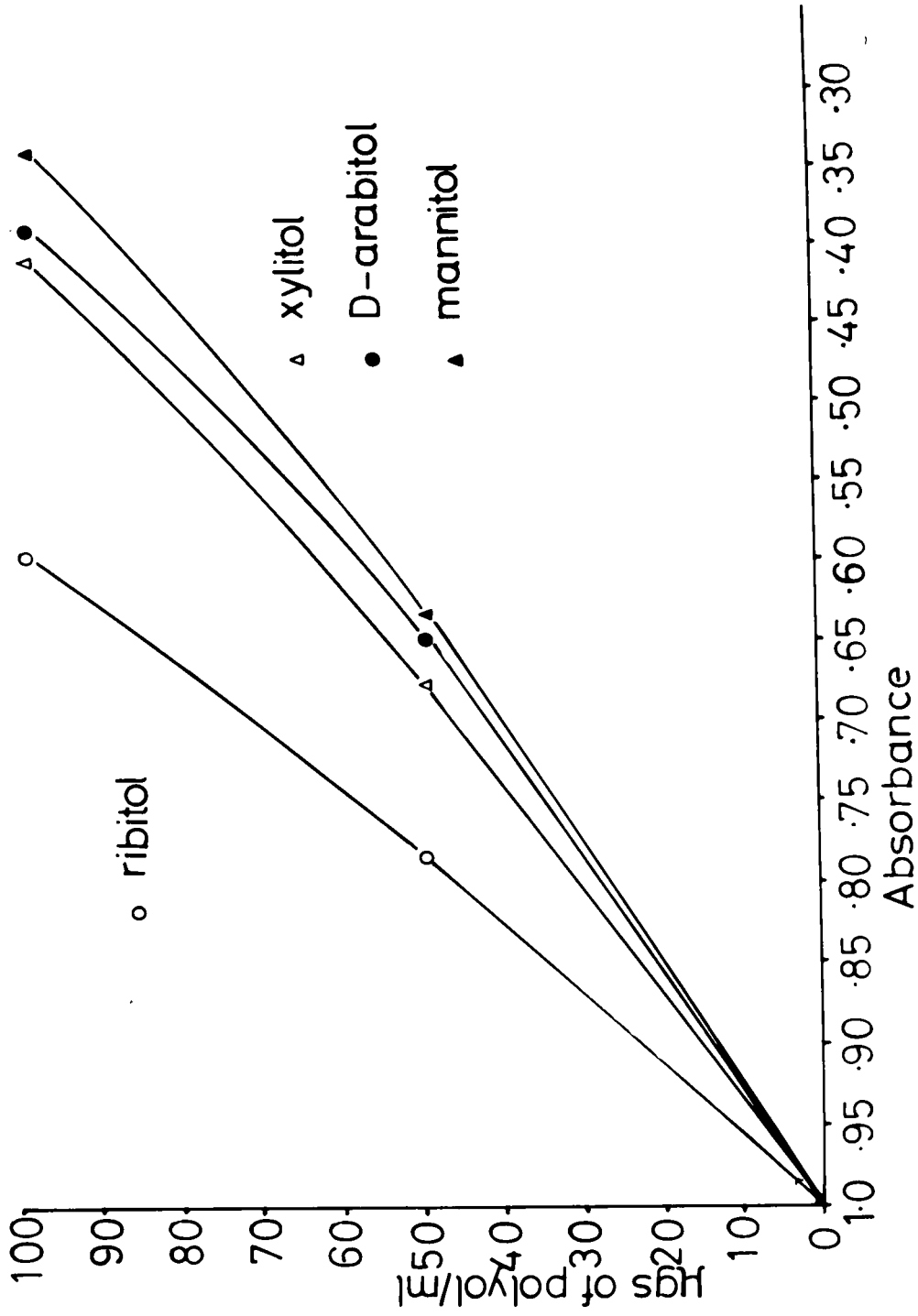
0, 50, 100 μg of polyol per ml test solution

Oxidation time: one minute

Temperature: 18°C

Experiment: 24

Date: 20.6.65.



11. Estimation of parietin

This pigment occurs in the lichen family Caloplacaceae (which includes Xanthoria) and in some strains of Aspergillus glaucus (Raistrick 1932); a probably identical pigment occurred in the cultured fungus Bulgaria inquinans (Moreau and Moreau 1937). The formula was determined by Oesterle and Johann (1910) (see Chapter XI, p. 258).

Cuthbert (1931) made the first attempts at quantitative estimation, using the fact that an aqueous solution of parietin is yellow but if alkali is added it turns purple. He extracted the ground lichen with ether and added aqueous potassium hydroxide solution. This left the chlorophyll in the ether layer and the parietin reacted with the potassium hydroxide in the aqueous layer. The resulting purple colour was compared with permanent colour standards made from a mixture of methyl purple and aqueous eosin because the purple parietin compound slowly precipitates out of solution, and could not be used for this purpose.

Scott (1965 and personal communication) extracted known weights of thalli with acetone and measured the optical density of the extract with an absorptiometer and compared the readings with standard solutions of pure parietin.

Hill (1966) used a similar technique to Cuthbert, but found that diethyl ether was an unsuitable solvent for extraction because

it partitioned badly with aqueous potassium hydroxide solution, and this affected absorption at 500 μ . Higher ethers such as isopropyl ether contained peroxides which converted parietin into a brown colour. These peroxides could be removed by shaking with ferrous sulphate, but then it was found that this ether did not extract parietin well. The bulk of the pigment was therefore extracted with chloroform which, since it partitioned badly, could not be used alone, and the remainder extracted by the ether. The chloroform-ether mixture then was found to partition satisfactorily.

In this study an attempt was made to develop a method for the routine estimation of parietin in 80% ethanolic extracts because this was the standard way in which lichen was killed and extracted in all experiments.

The difficulty of measuring parietin directly as in Scott's method was that chlorophyll absorbs near the maximum absorption of parietin. This could be overcome by converting parietin into the purple form with aqueous potassium hydroxide. Initially it was found that this results in a certain amount of precipitation but it was discovered that this could be prevented by using ethanolic potassium hydroxide. The results were still not satisfactory. Figure 8 shows that there were other compounds in the crude extract which absorbed at 510 μ before addition of potassium hydroxide though pure parietin without alkali has no absorption at this wavelength.

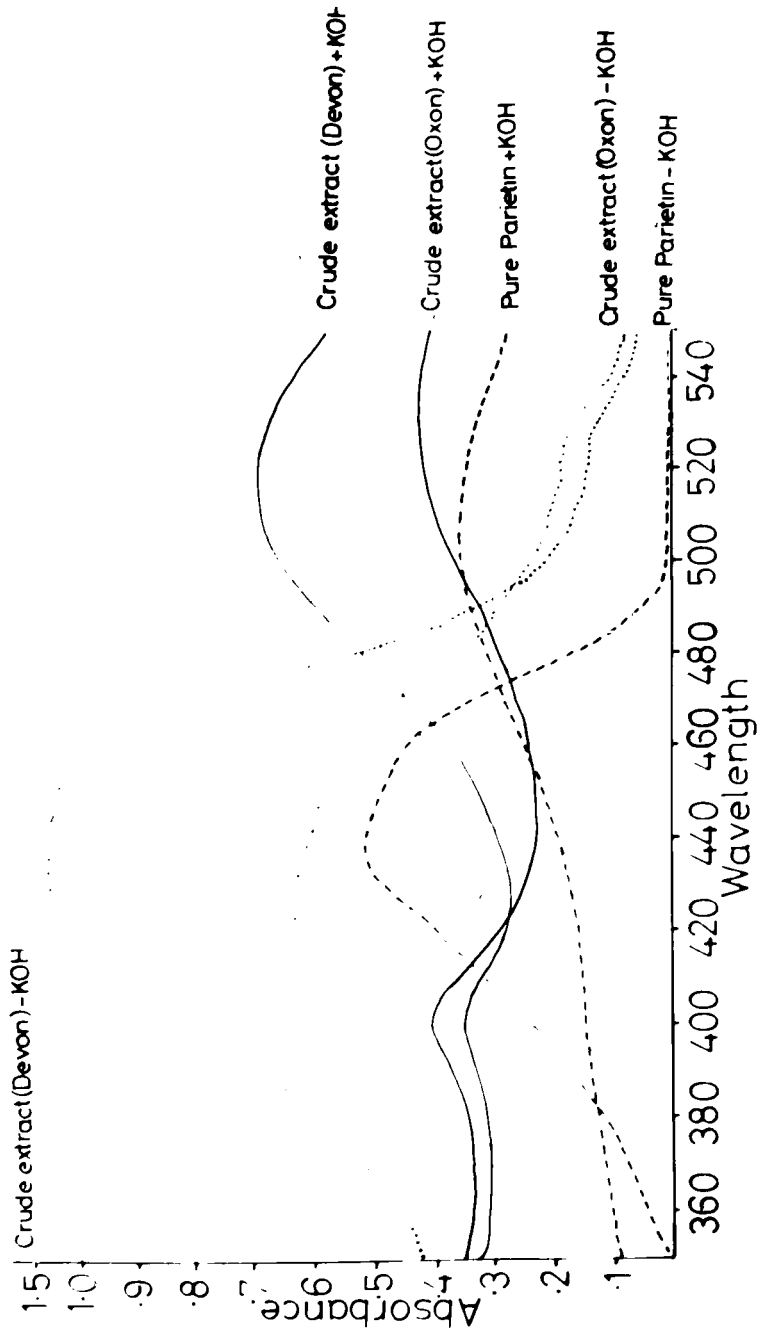
Figure 8

The absorbance of solutions of parietin with or without added potassium hydroxide solution at different wavelengths.

Absorbance measured with an Optika recording spectrophotometer

Parietin solutions approximately 50 $\mu\text{g/ml}$

Date: 12.11.66.



A technique was therefore devised in which the absorbance at 510 $m\mu$ of a test solution containing parietin made purple with ethanolic potassium hydroxide was compared with one in the yellow form. Hence all the compounds other than parietin cancelled each other out in the two solutions (i.e. if it was assumed that there were no compounds other than parietin which changed their absorption after addition of potassium hydroxide). Curves of the form shown in Figure 9 were produced on the recording spectrophotometer but for measuring a large number of samples a Zeiss PMQ II spectrophotometer was employed.

i. Procedure. Exactly 0.4 ml of a test solution in 80% ethanol from a 25 ml extract of 200 mg fresh weight of lichen was placed in a cuvette. This was diluted with 1.6 ml of 80% ethanol and then one ml of 0.5N ethanolic potassium hydroxide was added. A control was made from 0.4 ml of extract with 2.6 ml of 80% ethanol. Using a Zeiss spectrophotometer at 510 $m\mu$, the yellow solution was set to zero absorbance and the absorption of the red solution measured. Alternatively the red solution could be set to zero and the absorbance of the yellow solution determined at 435 $m\mu$, but this was less accurate as a greater slit width had to be used owing to the large absorbance of chlorophyll at this wavelength.

ii. Preparation of standard solutions. Since parietin is only sparingly soluble in 80% ethanol and slowly precipitates out of

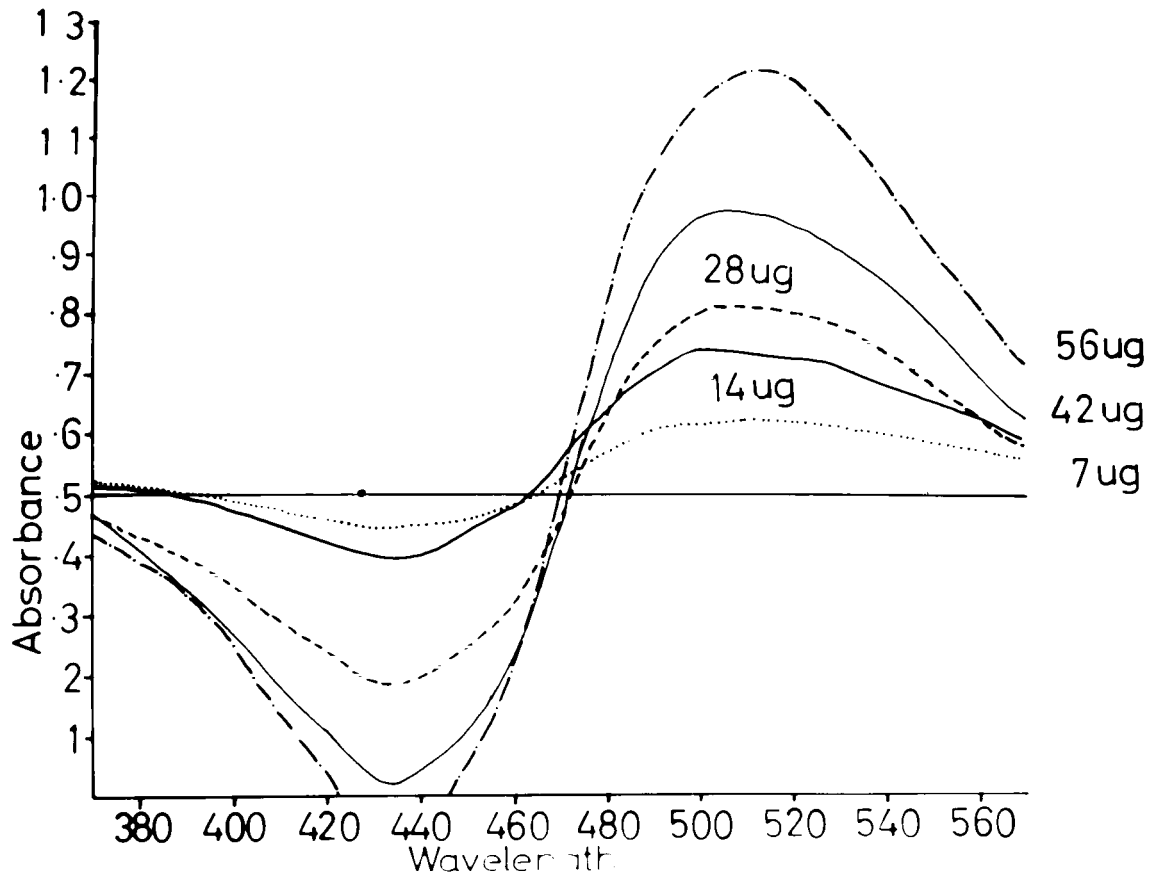
Figure 9

The absorbance of a parietin solution containing potassium hydroxide measured against a parietin solution without added alkali.

Absorbance measured with an Optika recording spectrophotometer.

Parietin solutions: 7 - 56 μg per ml

Date: 12.1.66.



concentrated solution, parietin standards were made up in chloroform. Aliquots of these (0.4 ml) were added to 1.6 ml of 80% ethanol and one ml of 0.5N ethanolic potassium hydroxide. Pure parietin was prepared by extracting Xanthoria aureola with acetone and recrystallising the pigment from glacial acetic acid (Asahina and Shibata 1945).

SECTION A

The Physiology of the Symbiosis in Xanthoria

CHAPTER IIIINTRODUCTION TO THE STUDY OF XANTHORIA PARIETINA

The first descriptions of Xanthoria parietina were made by Colonna (1606). Perhaps because of its conspicuous colour and abundance, it has been used for a variety of physiological and ecological studies. Amongst the earliest of these are the experiments by De Candolle (1798) on water uptake, Bonnier (1889) on symbiosis and Jumelle (1890) on photosynthesis and respiration. However many problems with regard to the taxonomy, ecology and physiology of this species remain unsolved.

1. Taxonomy

Until recently, three varieties of Xanthoria parietina were recognised in the British Isles (Smith 1921). These were: var. parietina, with numerous apothecia, broad marginal lobes and no proliferations in the centre of the thallus; var. ectanea, which is similar to the preceding variety but has very narrow lobes and a distinct maritime habitat; and var. aureola, which has peg-like proliferations in the centre of the thallus and apothecia with

coronate margins. These Apothecia are less numerous than in Xanthoria parietina var. parietina.

The var. aureola has been separated as distinct species, Xanthoria aureola (James 1965). This is the lichen used in the experiments described in Section A of this thesis and which grows on the roof of the Oxford University Experimental Farm at Wytham, Oxfordshire. It is referred to hereafter as Xanthoria aureola.

The other population of Xanthoria studied grew close to the sea near Slapton, Devon. This is referable to Xanthoria parietina var. ectanea. Experiments designed to discover the exact taxonomic status of these two lichens are described in Chapter XI .

2. Distribution

The world distribution of Xanthoria parietina sensu lato is often said to be cosmopolitan though it has not been collected in Malaysia. In the southern hemisphere it occurs in New Zealand, South Africa and South America. In New Zealand, Murray (1960) thought it may well have been introduced as it rarely grows far from settlements and usually on fruit trees. This is similar to the well authenticated case of the introduction of Lecanora conizaeoides into New Zealand also on fruit trees. In Africa, Xanthoria parietina occurs at low altitude in the north and Cape Province, but on the equator, in Kenya, it is only found at high altitude (Maas Gesteranus 1955).

In Europe this lichen rarely grows above 1500m but can occur well inland, whilst in North America it grows only near the sea shore except in one locality on the Connecticut river (Hale 1955).

It is not known if these apparent anomalies in distribution and habitat preference can be explained by different varieties or subspecies having different ecological requirements; for instance it is not clear whether any of the above records should be referred to Xanthoria aureola.

3. Ecology

In the British Isles, Xanthoria parietina sensu lato grows in at least three different habitats.

a. Farm roofs and roadside trees

Its distribution in this situation is thought to be due to nitrogenous material, but the species cannot be very nitrophilous as it is replaced on bird perches by Candelaria concolour and Candelariella vitellina. Evidence that nitrogenous material does stimulate its occurrence is cited by Skye (1965) who found that a chemical plant at Koping, Sweden, which released nitrogenous compounds greatly stimulated the spread of Xanthoria parietina.

b. Limestone rocks, asbestos and mortar

In these habitats it may be the calcium and/or phosphate in the substrate which explains the occurrence of the lichen. Brightman

(personal communication) found this species growing on pine trees in Suffolk, an unusual habitat as it is normally restricted to bark richer in nutrients. These trees were found to be close to a lime works and were periodically covered with dust from the factory.

c. Maritime distribution

Xanthoria parietina sensu lato and especially var ectanea grows just above high tide level all round the coast of Britain. It may be that sodium phosphates or chlorides cause the plant to be so abundant in this habitat. Maas Gesteranus (1955) found that the only habitat for Xanthoria parietina in central Africa was near soda lakes. However experimental evidence to show which factors control the occurrence of this lichen in this or any other habitat is still lacking.

One biological factor that may affect the occurrence of this species is the presence of mites of the family Oribatidae which feed on the thallus and especially the apothecia. Oribata parmeliae occurs exclusively on Xanthoria parietina inland whilst Scutovertes maculatus eats this and other lichens on the sea shore (Michael 1884). The population of Xanthoria aureola growing on the roof of the University Farm, Wytham, normally has a large number of mites on the thalli but it is not known to what extent they cause damage.

4. The isolated symbionts

Both symbionts of Xanthoria parietina sensu lato have been isolated a number of times by other workers, but it is not known whether any of the isolates were from material now referred to as Xanthoria aureola.

a. The alga

This is a unicellular green alga belonging to the genus Trebouxia and classified by Fritsch (1960) in the order Chlorococcales. Most studies on the isolates of Trebouxia from this lichen have dealt with its growth and nutrition in culture. These have shown that, like other symbiotic algae of this genus, it requires added sugars for optimum growth (Quispel 1943), a temperature of 12 - 15°C (Thomas 1939) and organic nitrogen sources (Ahmadjian 1966). Recently di Benedetto and Furnari (1962) have shown that indole acetic acid simulates the growth of Trebouxia albulescens, isolated from Xanthoria parietina, and the free living Trebouxia humicola.

Some studies comparing the pigments of three symbiotic strains of Trebouxia isolated from Xanthoria parietina with a free living strain have shown that they all contain similar pigments, but the symbiotic forms contained more carotenoids, more chlorophyll and less β -carotene (De Nicola and di Benedetto 1962).

Very few investigations have been carried out on the photosynthetic products of these algae. Bednar and Smith investigated

photosynthesis by pure cultures of Trebouxia isolated from Xanthoria by Quispel and Ahmadjian. When the algae were incubated in ^{14}C -sodium bicarbonate solution, ^{14}C became incorporated into a wide range of compounds including amino acids, organic acids, sugar phosphates and carbohydrates. They found that a number of compounds were excreted into the medium including traces of amino acids and organic acids. The two compounds excreted with the greatest amount of ^{14}C were found to be a polysaccharide and a compound running in the pentose/pentitol region on two dimensional chromatograms. No major differences were detected in the behaviour of the two cultures.

b. The fungus

Like other lichen fungi, the isolates from Xanthoria parietina are very slow growing and do not produce asexual reproductive structures in culture. (There is one report, by Tomaselli et al (1963), of the production of conidia; however Ahmadjian (1966) states that they are not true reproductive structures but part of the typical morphology of this lichen fungus in culture.)

Cultural studies have shown that the fungus can utilise a wide range of carbon sources. In liquid medium a culture of Xanthoria parietina fungus used a greater number of nitrogenous sources, both organic and inorganic, when the carbon source was pectin rather than sucrose. The same fungus on agar showed a preference for organic nitrogen sources (Ende 1950).

Tomaselli (1963) confirmed the work of Thomas (1936, 1939) in demonstrating that the fungus can produce the pigment parietin in pure culture. He also found it produced another anthraquinone and a water soluble substance which fluoresces blue-green in ultra-violet light.

No studies on the nature of soluble carbohydrates of the mycelium of the isolated fungus appear to have been published.

5. Physiology of the complete thallus

De Candolle (1798) carried out the earliest experiments on Xanthoria parietina. He observed the uptake of aqueous solutions of cochineal by this and other lichens. Jumelle (1890, 1892) was perhaps the first to make extensive comparative studies of water uptake by a range of species including Xanthoria parietina. He found that lichens of the Collemaceae absorbed up to ten times their dry weight of water but other foliose lichens far less, for instance Xanthoria parietina absorbed only 1.71 times its own dry weight.

Jumelle (1890) was also amongst the first to study photosynthesis and respiration in lichens. For instance he showed that samples (0.44 g dry weight) of Xanthoria parietina absorbed 0.2 cc of carbon dioxide and gave off 0.19 cc oxygen at 15°C in two hours with diffuse light and a saturated atmosphere. Under similar conditions in the dark the lichen produced 1.05 cc of carbon dioxide. Tohler (1925)

demonstrated the dependence of assimilation and respiration on temperature and also showed that lichens could carry on these processes at temperatures below 0°C. He found that samples of Xanthoria parietina collected in December assimilated less carbon dioxide and respired less at 2000 lux than samples collected in February at 400 lux. The results may be correlated with the observations of Paulson and Hastings (1920) that the algae multiply within the thallus during the spring.

Recently Pearson and Skye (1965) showed that thalli kept constantly moist at 15°C for several days showed abnormal photosynthetic patterns, whereas those samples of Xanthoria parietina which had been initially moistened, kept illuminated but not covered (so that they dried up slowly) for three days at this temperature and then placed in another illuminated 'Thalotron' at 27°C for a further three days before being returned to the lower temperature and remoistened, retained the normal photosynthetic pattern for six months in this cycle.

Skye also showed that this and other lichens had abnormal photosynthetic patterns when exposed to various concentrations of sulphur dioxide gas. Rao and Le Blanc (1966) investigated the effect of sulphur dioxide on lichen thalli and found that Xanthoria parietina and Xanthoria fallax, if exposed to 5 ppm of sulphur dioxide for twenty four hours, showed abnormalities and brown spots in the

chloroplasts of the algae within the thallus. They suggested that the action of the sulphur dioxide was to cause the formation of phaeophytin from chlorophyll and hence upset photosynthesis by the alga. The lichens in industrial areas may hence starve to death.

6. The physiology of the symbiosis

Bednar and Smith (1966) found that most of the carbon fixed during photosynthesis accumulated in sugar alcohols and relatively little was incorporated into the insoluble compounds at first. They showed that two kinds of sugar alcohols were involved - mannitol and a pentitol. During the first six hours photosynthesis, much more carbon accumulated in pentitol than mannitol.

The level of ^{14}C in metabolic intermediates, after short exposure to ^{14}C -sodium bicarbonate solution in the light, became more or less steady and remained so for several hours. Bednar and Smith concluded from this that the flow of carbon from alga to fungus was relatively rapid and efficient.

They were unable to make a precise identification of the pentitol but found it did not correspond exactly with arabitol which had been identified in *Xanthoria* sp by Pueyo (1960) and Lindberg et al (1953).

CHAPTER IVPHOTOSYNTHESIS BY THE COMPLETE THALLUS1. Short term photosynthesis

Bednar and Smith (1966) studied the photosynthesis of Xanthoria aureola in solutions of ^{14}C -sodium bicarbonate. During the first twenty five minutes photosynthesis, they found that the incorporation of ^{14}C into insoluble compounds was less than 0.5% of the total. Compounds such as PGA, hexose monophosphates and organic acids achieved a steady state after 9 to 15 minutes but there was a small rise in the amount of radioactivity in amino acids over this time which was probably due to dark fixation.

Most of the photosynthetically fixed carbon was incorporated into a five carbon sugar alcohol (pentitol) and a small amount into mannitol. Photosynthesis for periods up to six hours showed rather more radioactivity was incorporated into mannitol.

2. Identity of the main products of photosynthesis

Bednar and Smith found that three soluble carbohydrates became labelled to a significant extent during photosynthesis on ^{14}C -sodium

bicarbonate solution. These were identified as mannitol, a ' β -glycoside' and a 'pentitol'.

a. Mannitol

The mannitol was identified by Bednar and Smith by one and two dimensional paper chromatography in various solvent systems. These results were confirmed and agree with those of Lindberg et al (1953) who found this compound in sixty five species of lichens; indeed Lewis and Smith (1967) suggest that it may be universally present in this group of plants.

b. The glycoside

Bednar and Smith provisionally identified this compound as a β -glycoside as it could be hydrolysed with a β -glucosidase enzyme to give glucose and another compound with a rather higher Rf value when chromatogrammed in phenol/water solvent systems. In this study the ^{14}C -labelled glycoside was separated from the other soluble carbohydrates by paper chromatography in EA/AA/aq solvent. The disaccharides were eluted and treated in three ways based on the methods of Bell (1955):

1. Eluate hydrolysed in 1.5N H_2SO_4 for 2 hours at 100°C
2. Eluate hydrolysed in 0.02N H_2SO_4 for 20 minutes at 100°C
3. Eluate added to distilled water and left at room temperature for 2 hours.

If the disaccharides were hydrolysed by the dilute as well as the stronger acid, it would indicate that a hexuloside or pentafuranoside

was present. After hydrolysis the tubes were cooled in ice and the contents shaken with 1 ml of a 10% solution of methyl dioctylamine in chloroform. This was repeated after the upper aqueous layer had been drawn off into a new tube. This effectively removes acid from the solutions (Lester Smith and Page 1948) and was easier than the traditional barium carbonate neutralisation where small volumes of hydrolysate were concerned. It was in fact found that the more dilute acid did completely hydrolyse the 'glycoside' which then separated into two components when chromatogrammed in EMK/AA/Baq solvent. Both components were equally labelled and had a chromatographic mobility identical to glucose and fructose (Figure 10). This suggested that the 'glycoside' was sucrose. This was confirmed by co-chromatography with marker compounds.

c. The pentitol

Pueyo (1960) & Lindberg et al (1953) showed that Xanthoria spp contain arabitol. However, Bednar and Smith using a series of solvent systems found that the ^{14}C -pentitol produced by Xanthoria aureola did not exactly correspond with arabitol. The following techniques were used to identify the pentitol.

i. Paper chromatography. Ribitol, xylitol and arabitol have slightly different mobilities in EMK/AA/Baq solvent (Figure 11). Autoradiography showed that the ^{14}C -pentitol from Xanthoria aureola corresponded to ribitol. However when the chromatograms were sprayed with alkaline silver nitrate reagent, the spot which developed extended over the whole pentitol area.

Figure 10

The hydrolysis of 'the β -glycoside' in Xanthoria aureola

The disaccharide region of chromatograms of the soluble fraction of samples of Xanthoria aureola which had photosynthesised on solutions of ^{14}C -sodium bicarbonate, eluted, hydrolysed, and run in EMK/AA/Baq solvent.

Conditions for hydrolysis

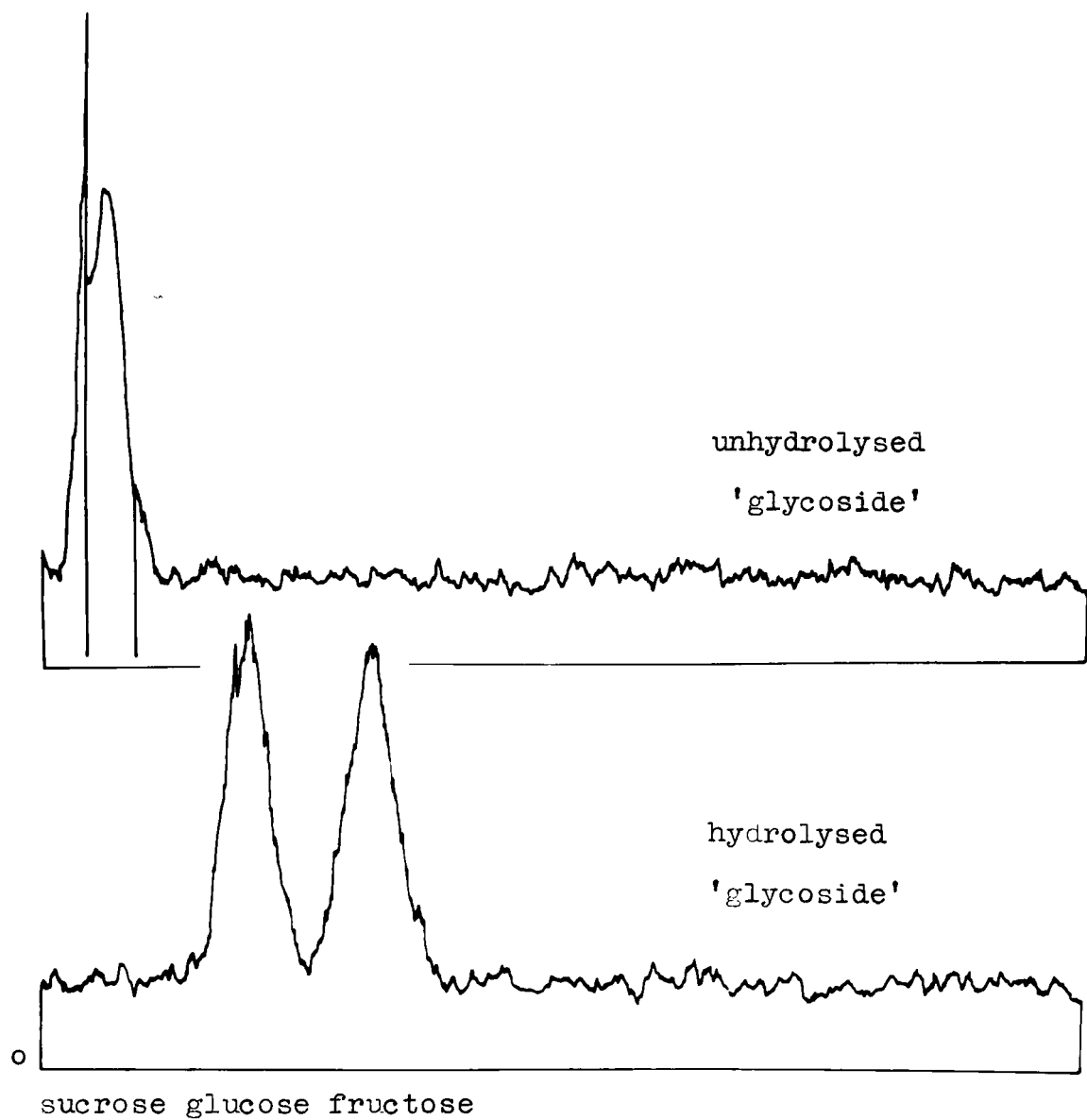
Acid: 0.02N sulphuric acid

Time of hydrolysis: 20 minutes

Temperature: 100°C

Experiment: 31

Date: 15.7.66.



o = origin

Figure 11

The separation of marker compounds on Whatman No. 1 paper using EMK/AA/Baq solvent. The position of the sugar alcohols and sugars determined by using the alkaline silver nitrate reagent after treatment with hydrofluoric acid.

Paper: Whatman No. 1

Time: 30 hours

Temperature: 18°C

trehalose
mannitol-
glucoside

galactose

mannose

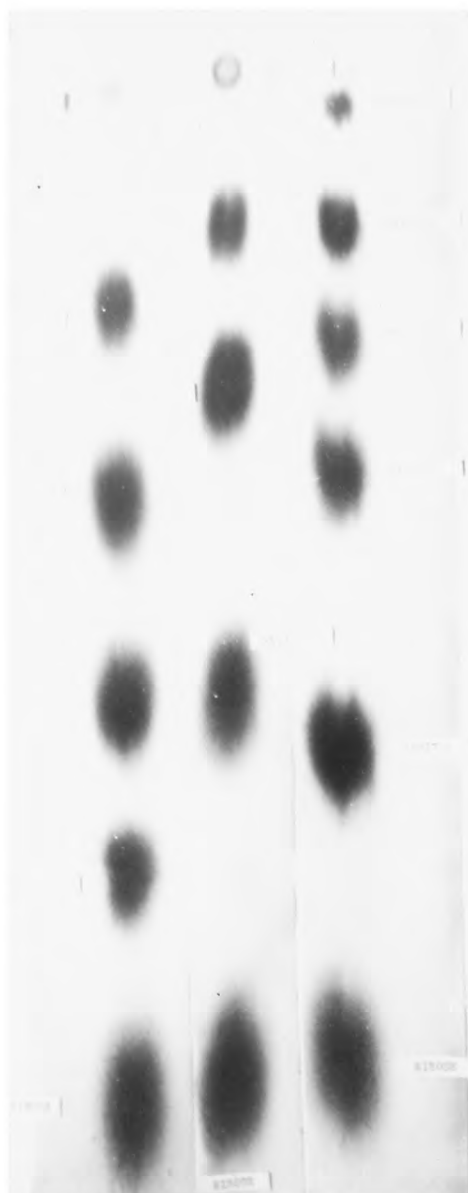
arabinose

xylose

arabitol

erythritol

ribose



origin

sucrose

glucose

fructose

mannitol

xylitol

ribitol

ribose

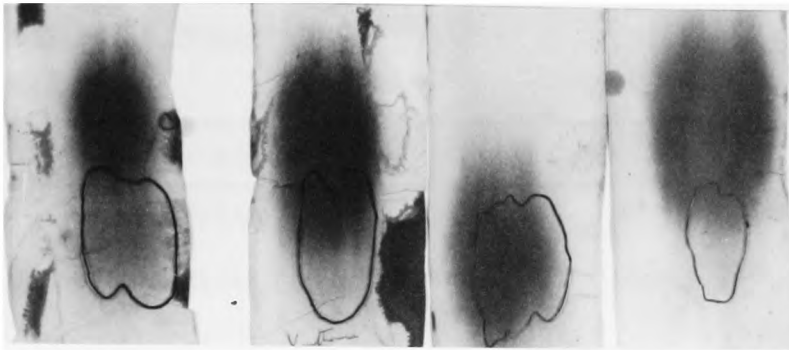
ii. Isotope dilution technique. A small amount of the ^{14}C -pentitol extracted from Xanthoria aureola was placed on the origins of paper chromatograms with a greater amount of pure non-radioactive ribitol or xylitol or arabitol. Autoradiographs were made of the chromatograms after running these in EMK/AA/Baq solvent. The chromatograms were then developed with alkaline silver nitrate reagent after preliminary treatment with HF. Comparison of the autoradiographs and chromatograms indicated that the pentitol which became labelled after three hours photosynthesis was ribitol (Figure 12).

iii. Optical rotation. This technique was used to try and confirm the above identification because ribitol has almost no optical rotation in acidified ammonium molybdate whereas that of arabitol is considerable. A mass extract of Xanthoria aureola (100 g fresh weight) was made in 80% ethanol, cleared and deionised. The sugars were separated on Whatman 3MM paper using EA/AA/aq solvent. The pentitol region was eluted and the optical rotation of the eluate measured in acidified ammonium molybdate (Methods section, p. 32). The results given in Figure 13 showed either that the pentitol was not ribitol or that the extract was contaminated with some other substance. Since other workers had demonstrated that lichens contain arabitol by such techniques as mixed melting points with pure compounds it seemed possible that Xanthoria aureola might contain arabitol in addition to ribitol.

Figure 12

Isotope dilution of the ^{14}C -pentitols found in samples of Xanthoria aureola and the cultured Trebouxia sp after three hours photosynthesis. The limits of the radioactive areas on the autoradiograph have been outlined and superimposed on the chromatogram (which has been developed with ethanolic silver nitrate so that the sugar alcohols appear as black spots).

Date: 31.5.65.



arabitol

ribitol

XANTHORIA

X

X

ALGA

+

+

+

+

ARABITOL

XYLITOL

RIBITOL

ARABITOL

Figure 13

Optical rotation of polyols in acidified ammonium molybdate compared with that of the unidentified pentitol isolated from Xanthoria aureola

Substance	Optical Rotation
D-arabitol	+154
L-arabitol	-137 (130)
xylitol	-0.11
ribitol	+0.75
erythritol	+0.56
mannitol	+144 (141)
volemitol	(+109)
Unknown pentitol	+125

() - figures from Richmeyer & Hudson (1951)

Optical rotation calculated on the formula -

$$D = \frac{I \times 48}{C \times L}$$

C = Concentration of polyol in mg/ml
 L = Length of cell in cm
 I = Reading

iv. Electrophoresis. Frahn and Mills (1959) reported that excellent separations of pentitols were possible using paper electrophoresis. The eluate of the pentitol region of chromatograms of Xanthoria aureola extracts were electrophorised with pure marker compounds using an apparatus similar to that described by Frahn and Mills (1959). Basic lead acetate proved the best electrolyte.

The electrophoretogram was scanned using the automatic chromatogram scanner (Loughman and Martin 1957) and then developed with modified potassium permanganate:chromium trioxide spray. The results indicated that the pentitol area resolved into two spots, one corresponding to marker ribitol and the other to marker arabitol (Figure 14). It was evident that after photosynthesis for three hours arabitol did not incorporate detectable amounts of ^{14}C ; only ribitol became labelled.

Thus, there are two pentitols in Xanthoria but only one of these, ribitol, incorporates ^{14}C during photosynthesis for short periods. This explains why earlier attempts to identify the pentitol in this lichen had given results which were difficult to interpret.

3. Photosynthesis over longer periods

Smith and Bednar found that most of the ^{14}C fixed in photosynthesis appeared first in pentitol and that the amount in mannitol was initially small although it increased over a six hour period.

Figure 14

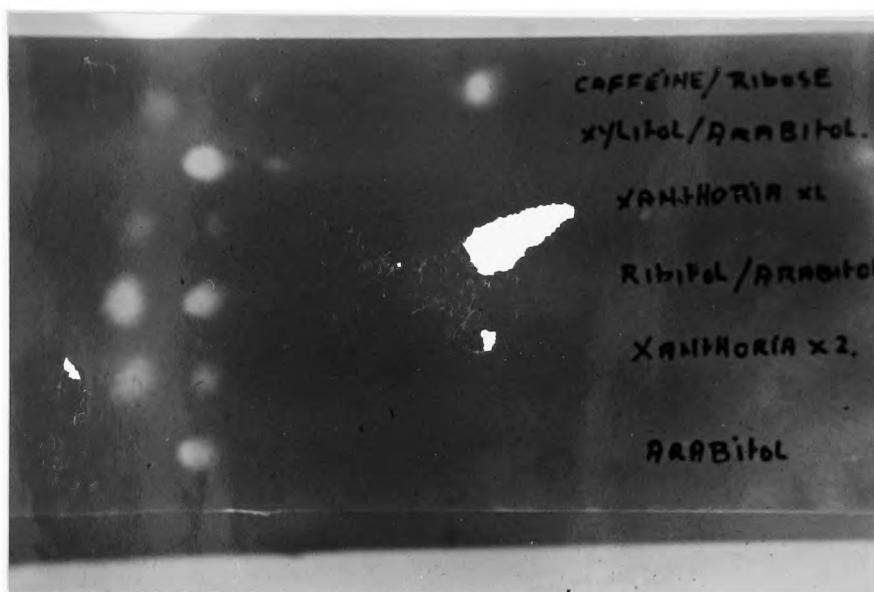
Electrophoretogram of the separated pentitols from Xanthoria aureola, developed with modified permanganate/chromium trioxide reagent.

Electrolyte: basic lead acetate

Time of run: 3 hours

Voltage: 800 V

Date: 9.11.65.



R A X

R = ribitol

A = arabitol

X = xylitol

Xanthoria x 1 = 1 drop of pentitol solution extracted
from Xanthoria aureola

Xanthoria x 2 = 2 drops of pentitol solution extracted
from Xanthoria aureola

These observations were confirmed in preliminary experiments. Lobes of Xanthoria aureola were allowed to photosynthesise on ^{14}C -sodium bicarbonate solution for 24 hours. More radioactivity was then found in mannitol than in the pentitol and up to 10% of the total fixation appeared in the insoluble fraction.

In order to study these changes in more detail, samples of lichen were placed on ^{14}C -sodium bicarbonate solution in the light for two hours. The thallus lobes were removed, thoroughly washed and replaced in the light in a medium of distilled water with no radioactivity present. At intervals from 4 to 48 hours, samples were removed, killed in 80% ethanol and analysed. The results are shown in Figure 15.

It may be seen that after four hours most of the radioactivity appeared in the pentitols while there was only a small amount in mannitol. As time progresses, the total amount of ^{14}C -mannitol rises with a corresponding fall in pentitols. After 48 hours the activity in both compounds declines presumably because the carbohydrates are respired and the ^{14}C lost to the atmosphere. (The vessels in which photosynthesis occurred were not sealed so as to reduce re-fixation of $^{14}\text{CO}_2$ from respiration.)

Figure 15 also shows that there was a slow rise in the amount of activity present in the insoluble compounds during the experiment. Close examination of the pentitol region of the scans indicates

Figure 15

The redistribution of ^{14}C in the soluble fraction of samples of Xanthoria aureola during photosynthesis on solutions of ^{14}C -sodium bicarbonate.

Material: 200 mg fresh weight samples Xanthoria aureola

Time: 2 hours on ^{14}C -sodium bicarbonate solution,
remainder on distilled water

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes without lids

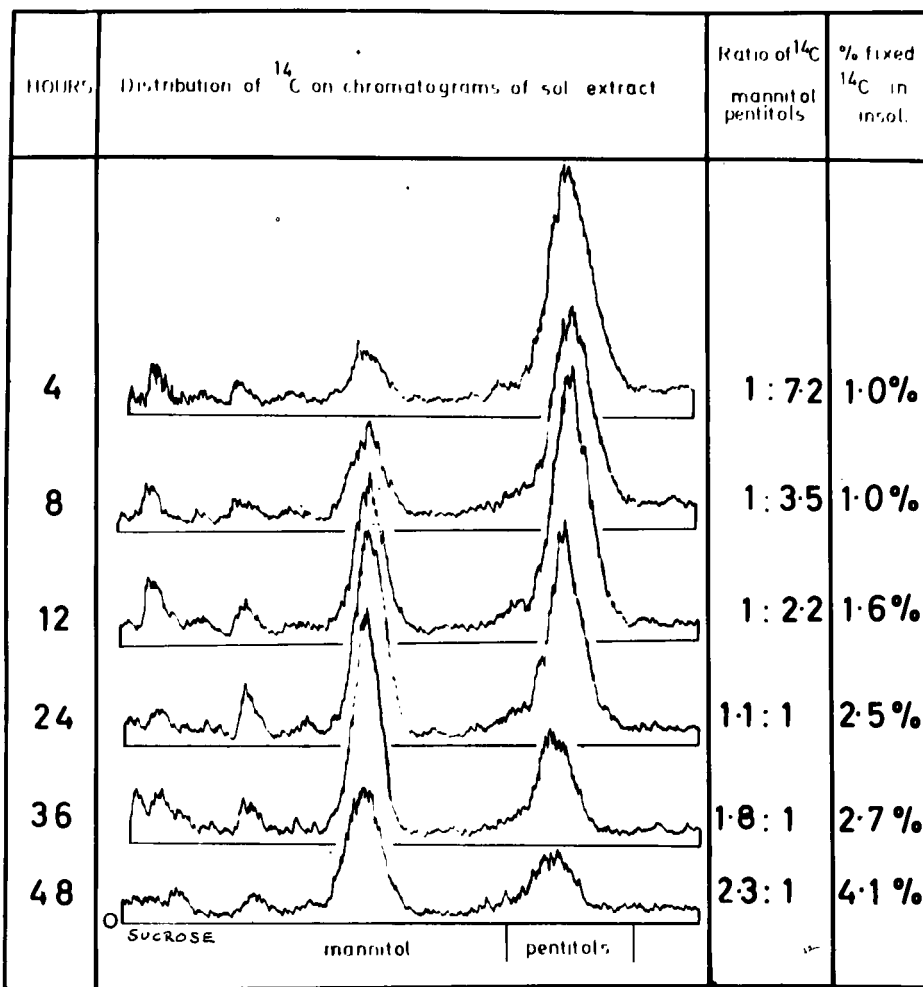
Radioactivity: 20 μc $\text{NaH}^{14}\text{CO}_3$ per sample

Medium: 3 ml distilled water

Carrier: none

Experiment: 54

Date: 14.2.66.



Redistribution of fixed ^{14}C in soluble extracts of
Xanthoria aureola after preliminary exposure to
Natl $^{14}\text{CO}_2$ in the light.

o = origin

that there was a slight change in the position of the peak, especially in the 36 and 48 hour samples indicating incorporation of ^{14}C into arabitol. There is a small but significant amount of radioactivity in sucrose, but this is no longer evident after 12 hours.

CHAPTER VTHE ISOLATED SYMBIANTS

(a) The Alga

1. Introduction

The algal symbiont of Xanthoria parietina sensu lato belongs to the genus Trebouxia, a member of the Chlorophyta. At present the taxonomy of this genus, which is the commonest algal symbiont of lichens, is confused. If consideration is restricted to those forms of Trebouxia isolated from Xanthoria, it is nevertheless found that a variety of specific and subspecific epithets **have** been given by various authors from different parts of the world.

These taxa have been erected on a mixture of cultural and morphological criteria that involve the use of chemically undefined media and anatomical characteristics which are difficult to determine and poorly described. For example, Ahmadjian (1960) recently divided the genus into two main groups on the basis of the position assumed by the chloroplast during its bipartitions. Further subdivisions

were made on whether or not the motile zoospores produced under certain conditions could fuse and act as gametes. Thus it is by no means easy to determine an unknown isolate of this genus.

The situation with regard to isolates from Xanthoria parietina sensu lato may be summarised as follows. Atari (1901) isolated an alga from this lichen and named it Chlorococcum infusorium. Treboux (1912) however considered that it should be called Cystococcus humicola. This name was adopted by Waren (1920) and Jaag (1929). Quispel (1943) made further isolates of the alga from Xanthoria parietina and after reviewing the work on the taxonomy of Cystococcus from lichens states 'I gave up trying to identify my algae with the species described by Waren and Jaag, therefore I will mention them only as Xanthoria-Cystococcus'. Nevertheless Quispel's isolate in the Cambridge Collection of Algae and Protozoa is listed under the name Cystococcus humicola. Werner (1954) followed the custom of a number of earlier authors in naming the algal symbiont after the lichen in which it occurred, thus giving the name Cystococcus xanthoriae parietinae; furthermore he described three subspecies and four varieties all based on cultural characteristics.

Although de Puymaly revised the nomenclature in 1924, it is only recently that his revisions have become generally accepted in that symbiotic forms are now regarded as belonging to the genus Trebouxia. Of the other forms, he found that Cystococcus humicola Naeg. was a species of Chlorococcum Fr.,

the Cystococcus Hedland belonged to Priasiola and Cystococcus Gerneck was really a Dictyococcus Gerneck.

Ahmadjian (1960) criticised earlier workers for using the growth characteristics of lichen algae in culture as criteria for primary separations in their taxonomy. He described four Trebouxia species, using as his criteria the form of the chloroplast during bipartition and the presence or absence of sexual fusion in clonal cultures as already mentioned, as well as cell size. His isolate of Xanthoria parietina sensu lato was assigned to one of these, Trebouxia decolorans, a new species, which was further characterised by a tendency to bleach when exposed to direct light.

De Nicola and di Benedetto (1962) isolated three strains of Trebouxia from Xanthoria parietina. Two of these they identified as T. decolorans but they proposed that the third should be a new species T. albulescens nob. since it differs in the possession of an unknown pigment, and has distinct morphological and cultural characteristics. However these authors refer to a free living species of Trebouxia which they obtained from the Czechoslovak Academy at Prague as T. humicola (Naeg.) Treboux and it is implicit that they regard this as a different species to that found in lichens in general. It is probable that this alga is Chlorococcum humicola (sensu Puymaly) and not a Trebouxia at all.

Di Dio (1961) complicates matters further by stating that European strains of Trebouxia from Xanthoria parietina do not produce

zoospores in culture. However, Quispel (1943) describes zoospore formation in his isolates, and the isolates made from Xanthoria aureola used in the experiments described here also formed zoospores. Quispel found that only very young cultures produced these motile stages whilst in the case of the algae from Xanthoria aureola, freezing overnight seemed to stimulate zoospore production. Presumably their formation is sensitive to cultural conditions and since one of the criteria required in the identification of some species is to observe fusion of zoospores, the uncertainty of their being produced makes certain identification of isolates extremely difficult.

As a result of the shortcomings in the taxonomy of these unicellular algae, it would seem best not to give a formal specific epithet to the alga isolated from Xanthoria aureola. Instead this isolate and the others, used in the experiments described here, are referred to as strains.

2. The origin and isolation of the strains examined

a. 'The Quispel strain'

Cambridge Culture Collection no. 219/d was originally isolated from Xanthoria parietina in Holland by Quispel, using the following technique. The thallus was washed with sterile water and then ground up. An algal cell with some adhering hyphae was transferred to a

hanging drop with a small glass capillary. This was repeated and then the cell placed on agar media and illuminated by light from a north facing window. One in ten of his isolations was successful, the others died or were contaminated.

b. 'The Ahmadjian strain'

Indiana Culture Collection no. 901 was isolated by Ahmadjian in the U.S.A. Single algal cells from a suspension of ground up thallus fragments were picked up with a micropipette and transferred through several drops of water on a slide, then placed in nutrient medium.

c. 'The Oxford strain'

Cambridge Culture Collection no. 219/2 was isolated from Xanthoria aureola growing at Wytham, Oxfordshire. Since the method used by Ahmadjian is rather difficult because cells tend to get damaged or lost when being transferred from drop to drop of water, a new technique was devised.

Washed marginal lobes of Xanthoria aureola (200 mg fw) were ground up in a pestle and mortar with a little sterile distilled water. The suspension was centrifuged for 10 seconds at 500 rpm (approx 60 g). This precipitated the heavier thallus fragments and most of the hyphae. The supernatant was transferred to a new sterile tube and centrifuged for five minutes at 3000 rpm (approx 375 g). The cell fragments were then discarded and the whole cells resuspended

in sterile water. This process was repeated twice more which resulted in a suspension largely free of bacteria and other cell debris.

A drop of this suspension was then placed in agar medium in a Petri dish and spread out to about one inch diameter with a sterile glass rod. The dish was left for a short time to allow the liquid to be absorbed by the agar. Using a micromanipulator, single algal cells with a small piece of adhering hypha were picked up with a fine glass loop. These were transferred to the centre of 2 mm cores which had been cut in the agar by an agar cutter in a different part of the plate to that on which the drop of suspension had been placed.

Each core with a single algal cell in its centre was then placed in a McCartney bottle containing Trebouxia agar (Star 1964). If bacteria or yeasts were present they became evident within the first few days on this rich medium. Half the inoculated bottles did not develop any growth presumably due to the death of the algal cell as a result of injury during manipulation. A further 25% of the cultures became contaminated with bacteria or yeasts and were discarded. The algal colonies became visible after about six weeks growth at 18°C 400 - 500 fc.

d. 'The directly isolated alga'

Drew (1966) had shown that both the products of photosynthesis and the rate of excretion of glucose by the Nostoc symbiont of Peltigera polydactyla changed after this had been in culture for some time.

Therefore a suspension of algal cells relatively free of fungal hyphae and bacteria was prepared in a similar manner to the initial stages of the isolation of the 'Oxford Strain'. A photomicrograph of a drop of suspension of the 'directly isolated alga' gives some idea of the amount of contamination by fungal fragments (Figure 16). It may be seen that the process of grinding and differential centrifugation frees the algal preparation of all but very short lengths of fungal hyphae. These did not appear capable of manufacturing detectable amounts of ^{14}C -mannitol from ^{14}C compounds liberated into the medium by the photosynthesising alga.

3. Preparation of cultured strains of alga for experiments

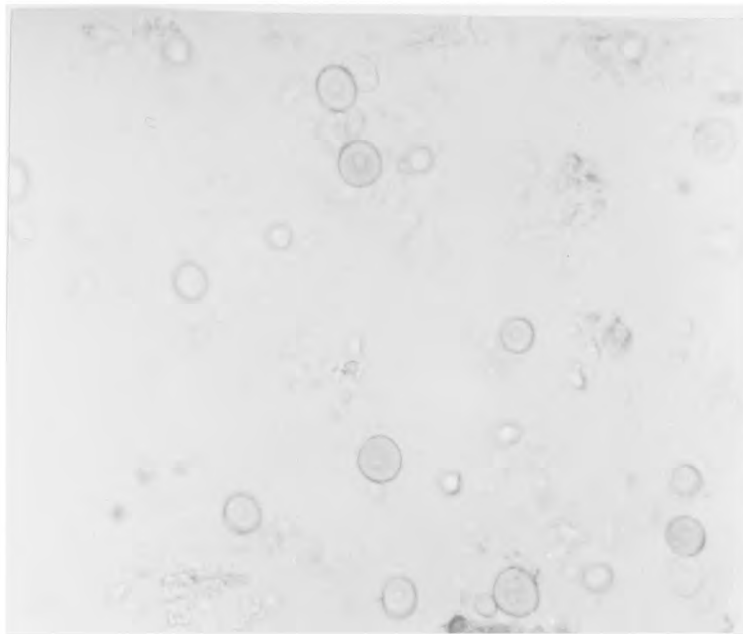
Cells from stock cultures of the strain required were transferred to 10 ml of Trebouxia medium in 50 ml conical flasks using a wire loop. These were shaken at 18°C with an illumination of 250 fc for 14 days. The cells were centrifuged, washed with distilled water and then used immediately in the experiments described below.

4. Photosynthesis by the algae

Bednar and Smith (1965) used the 'Ahmadjian' and 'Quispel' strains to study the photosynthetic products of the alga. After one hour of photosynthesis on ^{14}C -sodium bicarbonate solution they found that ^{14}C was incorporated into a wide range of compounds including

Figure 16

- (a) A photomicrograph of a preparation of algae which had been directly isolated from the thallus of Xanthoria aureola by a process of grinding the thallus and centrifugation.
- (b) Trebouxia sp growing in the lichen (250 fc) on solid media which contains glucose and peptone.

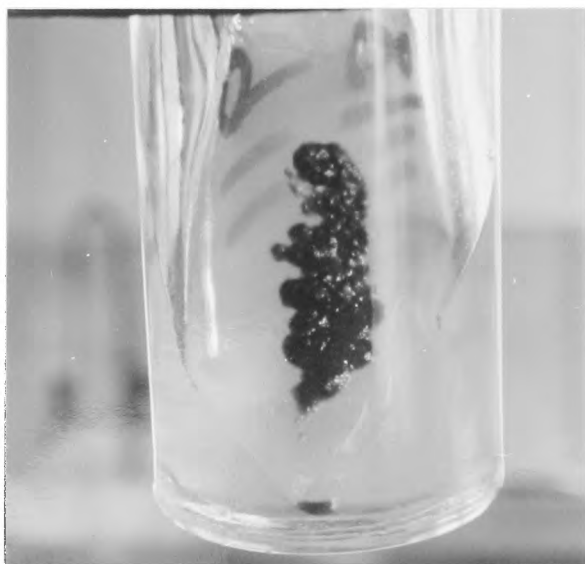


82.

scale

—
40μ.

Directly isolated algal cells



Trebouxia
decolorans



Trebouxia
albulescens

amino acids, organic acids, sugar phosphates and carbohydrates. In both strains of alga several of these were excreted in small amounts into the medium. In two dimensional chromatography of the medium using phenol water:butanol propionic acid solvents, the greatest amount of activity was in two spots. The first remained on the baseline and, on hydrolysis, yielded hexose units which suggested that it was a soluble polysaccharide. The second spot ran in the pentose: pentitol area of the chromatograms.

These results were confirmed in the experiments described here except that less radioactivity appeared on the baseline. Instead there was a spot which became labelled in the disaccharide region of the chromatogram, in a position similar to that of sucrose. The differences may have been due to the fact that Bednar and Smith cultured their algae on one of the media (no 32) of Zehnder and Gorham (1960) whereas in this study the algae were grown on Trebouxia medium.

In some experiments a large amount of the fixed ^{14}C appeared in amino and organic acids which was probably due to using dense algal suspensions so that substantial dark fixation occurred.

The 'Oxford Strain' showed a similar photosynthetic pattern to the other two strains except that a smaller proportion of the total fixed ^{14}C appeared in carbohydrates and more was found in organic and amino acids.

5. The identity of the soluble carbohydrates produced during photosynthesis

The soluble carbohydrates into which ^{14}C was incorporated during photosynthesis on solutions of carrier-free ^{14}C -sodium bicarbonate were as follows:

a. The soluble polysaccharide

This compound appeared in significant amounts in the experiments of Bednar and Smith and, as previously noted, these authors found that on hydrolysis it yielded hexose units. Since it was only found in appreciable amounts in one experiment described here, attempts at a more precise identification were not made.

b. Sucrose

This was identified in the following way. The eluates of the disaccharide region of chromatograms of the soluble extract were hydrolysed with sulphuric acid. Two concentrations of acid were used (1.5N at 100°C for 2 hours and 0.02N at 100°C for 20 minutes). Both concentrations hydrolysed the eluted disaccharide completely. The fact that the very dilute concentration was able to effect hydrolysis proved that the compound was a hexuloside or pentofuranoside (Bell 1955). Chromatography of the two moisties produced showed that they corresponded to glucose and fructose, and that they were also equally labelled. The unhydrolysed eluate had a mobility identical to that of authentic sucrose in EA/AA/aq and EMK/AA/Baq solvents and hence it was identified as this compound.

c. Ribitol

Bednar and Smith found a soluble carbohydrate in the medium in which the algae had photosynthesised. On two dimensional chromatograms this compound occupied a similar position to pentoses and pentitols. In this study a similar compound was found in the alcohol soluble extracts of the algal cells (Figure 17).

This substance had the following characteristics: (a) it was a neutral compound which reacted with silver nitrate on chromatograms; (b) it did not react with aniline phosphate which suggested it was not a pentose or reducing sugar; (c) if it was eluted from chromatograms of crude extracts run in EA/AA/aq and then rechromatogrammed in neutral solvents, it reacted with bromocresol purple, indicating that it was a sugar alcohol; (d) the R_{glucose} of this compound on chromatograms run in EMK/AA/Baq solvent was 2.6, i.e. that of pentitols.

Using the isotope dilution technique described in the previous chapter, (page 63), it was found that the ^{14}C -labelled compound eluted from chromatograms of the soluble extract ran ahead of arabitol and xylitol but coincided with the ^{12}C -ribitol spot (Figure 18).

On examining the media in which either the directly isolated alga or the cultured alga had photosynthesised, a ^{14}C spot with the mobility on chromatograms of authentic ribitol was found. This was also confirmed by the isotope dilution method.

Figure 17

Autoradiograph of two dimensional chromatograms of the soluble extract and media of cultured algae, indicating that ^{14}C compounds are released by the alga into the medium in which photosynthesis had occurred. The upper pair of autoradiographs shows the undeionised medium and soluble fractions developed in one direction with phenol water and in the other with Bu/PrA/aq solvents. The lower pair of autoradiographs shows the deionised soluble fraction and medium developed in one direction with EA/AA/aq and in the other with EMK/AA/Baq solvents.

Material: 'Quispel strain' cultured Trebouxia

Period of photosynthesis: one hour

Temperature: 18°C

Light: 500 fc

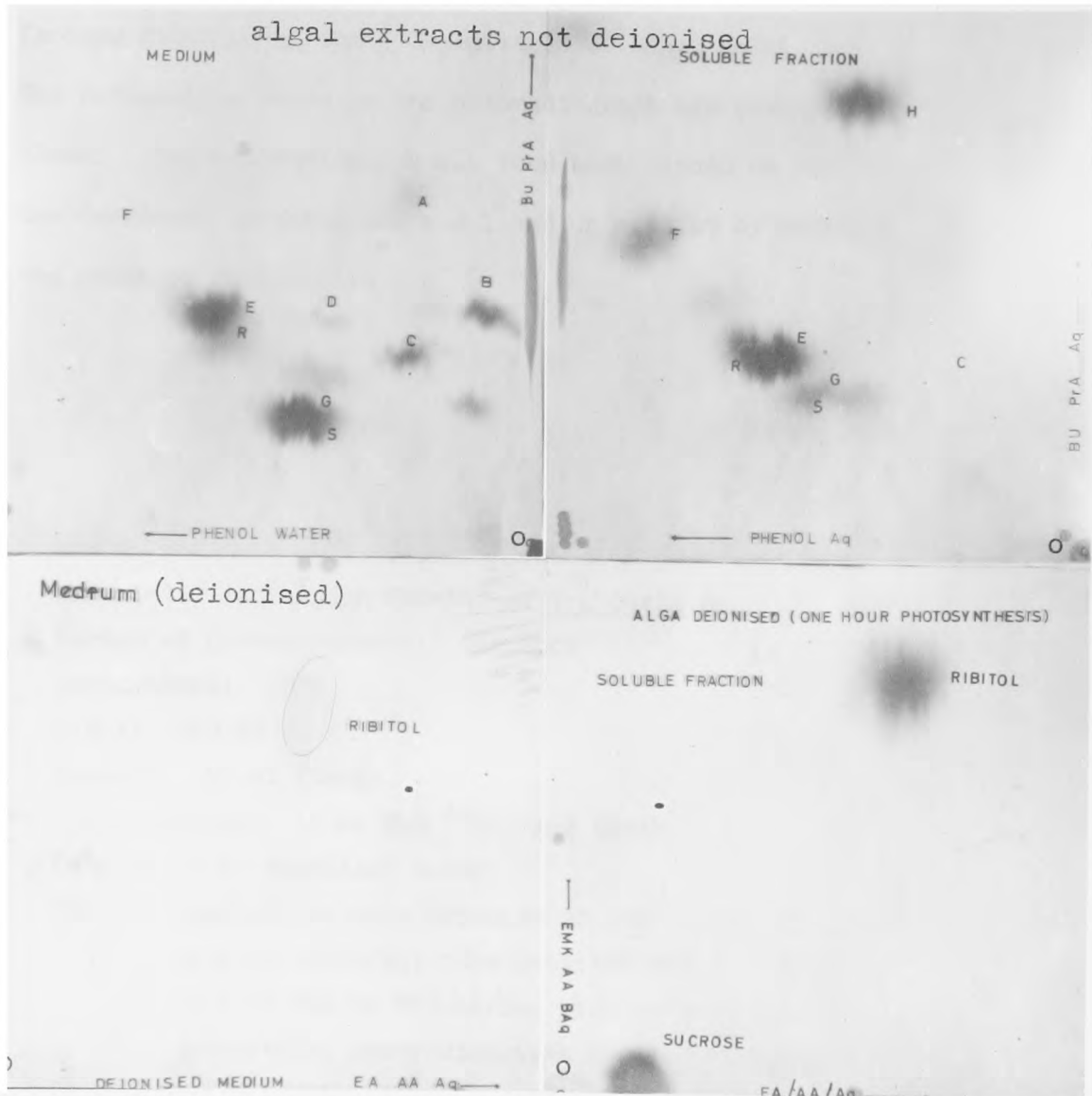
Vessels: 50 ml flasks

Radioactivity: 20 μc per flask

Medium: 5 ml distilled water

Experiment: 0

Date: 14.1.65.



A = ? malonate
 B = " citrate
 C = " aspartate
 D = -lutamate
 E = alanine
 F = valine
 G = serine & Glycin 2.
 H = unknown acid

o = origin of 2 D chromatograms

R = ribitol
 S = sucrose

Figure 18

Isotope dilution of the ^{14}C -pentitols of algal extracts. The radioactive areas on the autoradiograph are outlined in black. The autoradiograph has then been placed on top of the developed chromatogram and lined up with it by means of the spots of radioactive ink.

Material: 'Ahmadjian Strain' of Trebouxia sp

Period of photosynthesis: one hour

Temperature: 18°C

Light: 500 fc

Vessels: 50 ml flasks

Radioactivity: 10 μc NaH $^{14}\text{CO}_3$ per flask

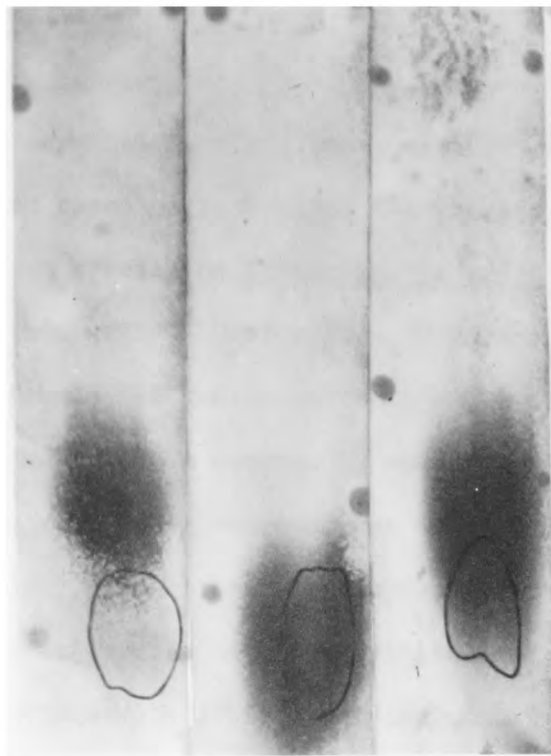
Medium: 5 ml distilled water

Chromatography: soluble fraction of cell initially run in EA/AA/aq; the pentitol region eluted and re-run in EMK/AA/Baq with authentic marker pentitols, non-radioactive marker pentitols.

Development reagent: Alkaline silver nitrate after treatment with hydrofluoric acid

Experiment: 15

Date: 14.4.65.



arabitol

ribitol

ALGA	A	A
+	+	+
ARABITOL	RIBITOL	XYLITOL

d. Conclusions

It therefore appears that Trebouxia can produce sucrose and ribitol during photosynthesis. This was confirmed in a paper by Maruo et al (1965) which came to light after the present study was complete. They used two species of Trebouxia, T. erici and T. glomerata, and incubated them with $^{14}\text{CO}_2$ for 20 - 30 days at 20 - 25°C. After this time they found two radioactive compounds in the medium. One of them was identified as sucrose by co-chromatography and acid hydrolysis. The other compound was identified as ribitol by paper chromatography, paper electrophoresis, periodic acid oxidation, recrystallisation with authentic ribitol and finally by the enzymatic method with ribitol dehydrogenase. Although sucrose was not found in appreciable amounts in the media of most of the experiments described here, it must be noted that they were carried out for a much shorter period than those of Maruo et al.

6. Release of ^{14}C by the alga

It is usually assumed that in a lichen the alga supplies the entire thallus with carbohydrates produced in photosynthesis. Direct evidence of a rapid and substantial transference of photosynthetically fixed carbon from alga to fungus in Peltigera polydactyla has been provided by Drew and Smith (1966) and Drew (1966). It was calculated that during photosynthesis at room

temperature, 40% of the total carbon fixed in the algal cells moved to the fungal medulla.

It follows that in the thallus, products of photosynthesis must be able to pass out of the algal cell in substantial amounts and that this must be one of the key features of the symbiosis. In the case of Peltigera polydactyla, Drew presented preliminary evidence that the directly isolated Nostoc from the thallus could release larger amounts of fixed carbon from the cells than algae which had been in culture for a period of time.

The experiments described in this section were designed to study the phenomenon of excretion by the Trebouxia, the symbiotic alga of Xanthoria aureola.

a. Differences between the cultured and directly isolated alga
Both the 'Oxford strain' and the 'directly isolated alga' produce ^{14}C -ribitol and ^{14}C -sucrose within the algal cells after photosynthesis in ^{14}C -sodium bicarbonate solution, although the cultured alga fixes a greater proportion of ^{14}C into amino and organic acids (Figure 19).

Both strains of the alga released ^{14}C -ribitol into the media, but the directly isolated alga released a much larger proportion of the total ^{14}C fixed over a period of 24 hours (Figure 20). This shows that some of the products are liberated much more readily by the directly isolated alga than by the cultured form.

Figure 19

A comparison of the distribution of ^{14}C in the soluble fractions of the directly isolated and cultured algae Trebouxia after 24 hours photosynthesis on solutions of ^{14}C -sodium bicarbonate. Scans on chromatograms run in EMK/AA/Baq solvent for 24 hours.

Material: 0.2 ml wet packed volume of 'Oxford strain'
of Trebouxia and directly isolated cells

Temperature: 18°C

Period of photosynthesis: one hour

Light: 500 fc

Vessels: 2 x 1" specimen tubes

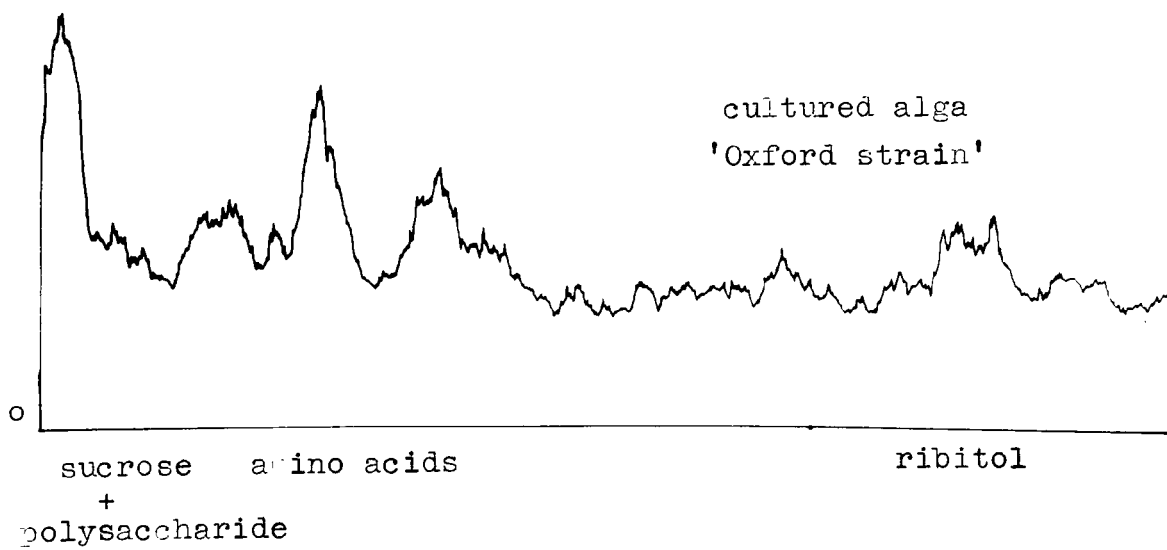
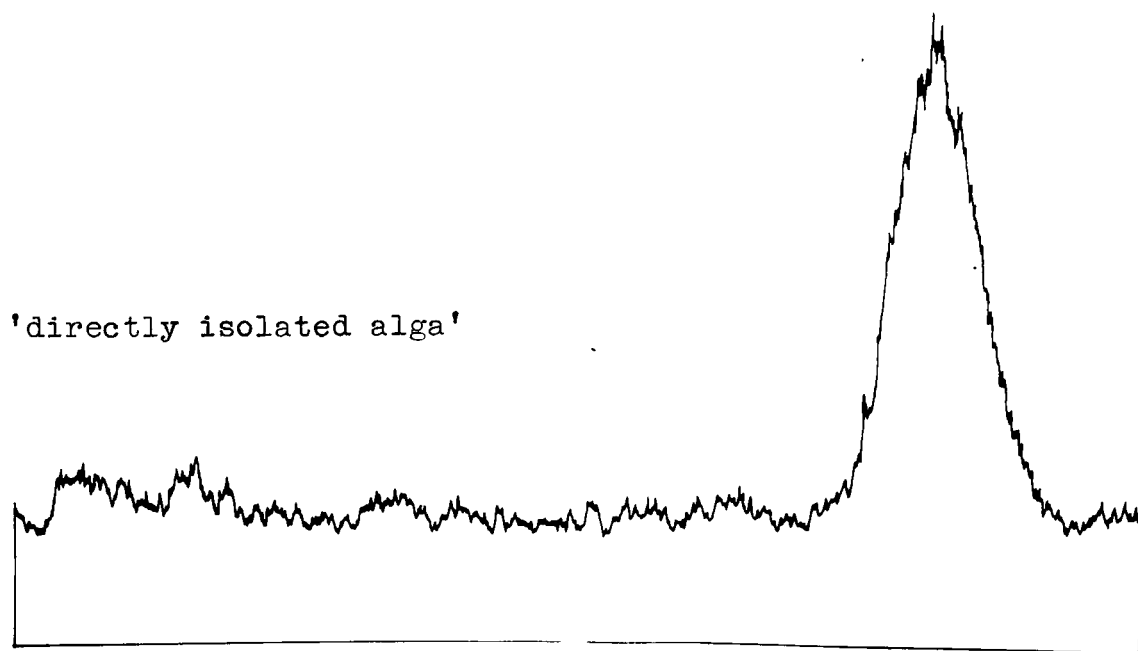
Radioactivity: 20 μc per sample

Medium: 3 ml distilled water

Experiment: 66

Solvent:

Date: 20.4.66.



o = origin

Figure 20

A comparison of the release of fixed ^{14}C by the cultured and directly isolated strains of Trebouxia. Values in Table as counts per minute $\times 10^{-3}$.

	Fraction			Total ^{14}C fixed	% Total ^{14}C in medium fraction	% Total ^{14}C in insoluble fraction
	soluble	insoluble	medium			
Directly isolated	298	106	106	510 510	20.8 20.8	20.8 20.8
Cultured	855	198	13	1066 1066	1.24	18.9
Directly isolated + parietin	145	28	62	235	26.2	
Cultured + parietin	321	65	7	392	1.66	

Material: directly isolated algae + cultured 'Oxford Strain'

Period of photosynthesis: 24 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: $10 \mu\text{c NaH } ^{14}\text{CO}_3$

Medium: 2 ml distilled water, 200 μg per ml $\text{NaH } ^{12}\text{J}_3$

Experiment: 47

Date: 1.12.65.

b. Progress with time of release from the directly isolated cells

Preliminary experiments showed that the alga released very little fixed ^{14}C during the first hours photosynthesis (Figure 21). However, over a 24 hour period the directly isolated alga excreted some 20% of the total fixed ^{14}C , while the cultured alga only released about 1%. These results were similar to those of Drew (1966) in that the directly isolated Nostoc symbiont of Peltigera polydactyla released more fixed ^{14}C than the cultured strain, but Nostoc differs from Trebouxia because it releases over 20% of the fixed ^{14}C in a three hour period.

In order to study the progress of excretion of this fixed ^{14}C in more detail, samples of algae were pipetted into carrier-free ^{14}C -sodium bicarbonate solutions and placed in the light. After intervals from 3 to 24 hours samples were removed, the algae centrifuged and killed. The amount of fixed ^{14}C remaining in the medium was determined and expressed as a percentage of total fixation. These results may be seen in Figure 22 and show that there is considerable variation between samples killed at each time. However a general trend is apparent which indicates that the alga excretes ^{14}C in increasing amounts, especially after 24 hours.

c. The effect of parietin on excretion

Follman (1960) suggested that lichen acids may affect the permeability of the algal membranes within the thallus so that transfer of substances between the two organisms is facilitated. It was thought

Figure 21

The release of fixed ^{14}C by cells of the cultured and directly isolated strains of Trebouxia during 3 hours photosynthesis. Values for ^{14}C fixation given as counts per minute $\times 10^{-3}$.

	Fraction			Total ^{14}C Fixation	% Total ^{14}C in Medium	% Total ^{14}C in Insoluble
	Soluble	Insoluble	Medium			
Directly isolated alga	112.3	5.4	0.12	118.0	0.10	4.6
Cultured alga	81.5	47.0	0.09	128.6	0.07	36.7

Material: Directly isolated algae and "Oxford strain"

Period of photosynthesis: 3 hours

Temperature: 18°C

Light: 500 fc

Vessels: 50 ml conical flasks

Radioactivity: 10 μc $\text{NaH}^{14}\text{CO}_3$ per flask

Medium: 6 ml distilled water

Experiment: 46

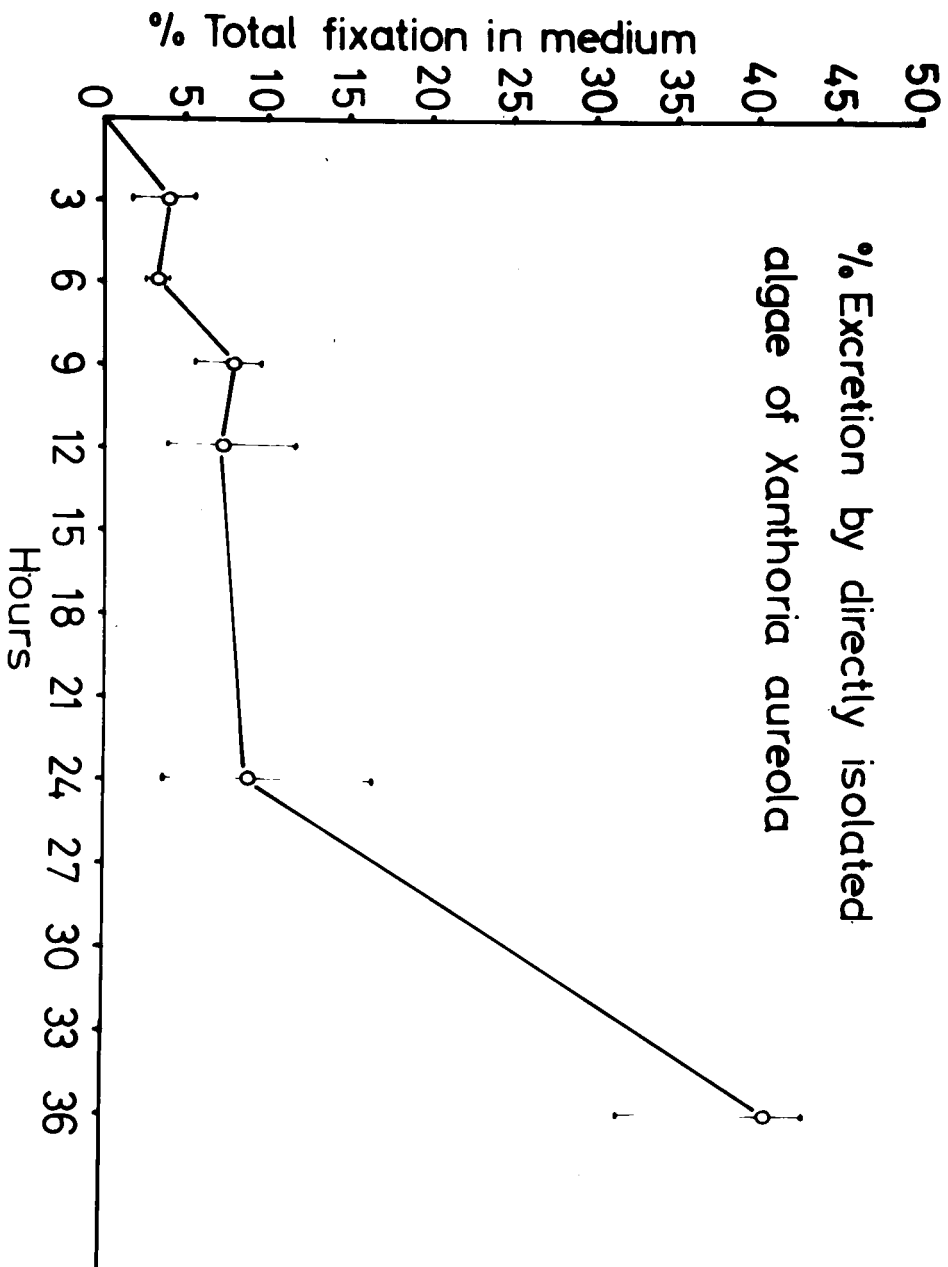
Date: 20.11.65.

Figure 22

Progress of the release of fixed ^{14}C into the medium by
'directly isolated' algae during photosynthesis on solutions
of ^{14}C -sodium bicarbonate.

Limits of variability between samples shown as
mean shown as O.

Material: 0.2 cm wet packed volume of algae per sample
Period of photosynthesis: 3 to 36 hours
Temperature: 18°C
Light: 500 fc
Vessels: 2 x 1" specimen tubes
Radioactivity: 10 μc $\text{NaH}^{14}\text{CO}_3$
Medium: 3 ml distilled water
Experiment: 87
Date: 5.10.66.



possible that parietin might have a similar effect. Therefore pure parietin, which is very sparingly soluble in water, was added to the medium of algal samples photosynthesising on solutions of ^{14}C -sodium bicarbonate. Over a 24 hour period, parietin did not appreciably increase the amount of ^{14}C compounds which appeared in the medium (Figure 20).

d. The effect of pH on excretion

Muscatine (1965) showed that pH affected the excretion of carbohydrates from zoochlorellae directly isolated from Chlorohydra viridissima.

He isolated these algae by a centrifugation technique similar to that used for the direct isolation of Trebouxia cells. The zoochlorellae were washed and suspended in McIlvaine citrate/phosphate buffer (1921) (0.14 to 0.2M) of the desired pH within the range 4.5 to 8.1. Samples were placed in the light (1500 fc) at 26°C and ^{14}C -sodium bicarbonate added for 30 minutes. He found that the symbiotic algae of Chlorohydra viridissima excreted up to 85% of the total fixed ^{14}C at pH 4.5 after 30 minutes photosynthesis. As the pH rose, the alga excreted less until at pH 7.5 only 6% of the fixed ^{14}C was found in the medium. However at pH 8.1 there was another rise with 23% being excreted. Total fixation was greatest at pH 6-7 falling to half the maximum values at high (8.1) and low (4.5) pH.

The products excreted into the medium were glycollic acid, maltose and glucose. At pH 6-7 one third of the excreted radioactivity was in the form of glycollic acid, but under other pH conditions maltose and glucose predominated. He suggested that most of the glucose was produced in the medium by the action of a maltase enzyme from the Hydra cells as exhaustively washed algal suspensions excreted a high proportion of maltose.

A similar experiment was set up using the directly isolated alga of Xanthoria aureola. The same buffer, amount of radioactivity and final volume of solution (20 μ c in 2 ml medium) were used so as to make the results as comparable as possible. However, a lower light intensity, 500 fc, was employed as Ahmadjian (1966) had shown that high light intensities caused bleaching in many symbiotic strains of Trebouxia. Also, the lower temperature of 18°C was used in preference to 26°C, and a longer time of photosynthesis was tried because earlier experiments on Trebouxia indicated that fixed ^{14}C was liberated rather slowly.

After eighteen hours photosynthesis a greater percentage of the total fixed ^{14}C was excreted at low than at high pH (Figure 23), although the amount rose slightly from pH 7 to pH 8. After three hours (Figure 24) the pattern was similar except that the proportion of total fixed ^{14}C excreted fell steadily all the way from pH 3 to pH 8. Unlike Muscatine's results, the net total fixation by the

Figure 23

The effect of pH on the release of fixed ^{14}C by the directly isolated alga.* Values for ^{14}C fixation given as counts per minute $\times 10^{-3}$.

pH	Fraction			Total ^{14}C fixed	% ^{14}C in medium
	soluble	insoluble	medium		
3	116	112	99	326	30.3
4	245	69	72	386	18.6
5	330	157	20	508	4.0
6	428	209	23	660	3.5
7	1116	304	29	1449	2.0
8	1386	492	131	2009	6.5
Control Distilled water (pH 5.8)	767	270	35	1071	3.3

Material: 0.5 cm wet packed volume algae per sample

* Period of photosynthesis: 18 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: 20 μc $\text{NaH}^{14}\text{CO}_3$

Medium: 3 ml distilled water buffered with McIlvaine buffer

Experiment: 73

Date: 5.6.66.

Figure 24

The release of fixed ^{14}C by the directly isolated algae of Xanthoria aureola after a three hour period of photosynthesis.

pH	% ^{14}C in insoluble fraction	% ^{14}C in medium
3	34	16.9
4	18	7.5
5	31	1.3
6	32	1.6
7	21	0.8
8	25	0.7
Control Distilled water (pH 5.8)	25	1.1

Details as figure 23

directly isolated Trebouxia cells rose from pH 3 to 8, after both 3 and 18 hours photosynthesis. The soluble and medium fractions were examined chromatographically with the following results.

i. The soluble extract. One dimensional paper chromatography was carried out in picric acid, bu/AA/aq and FMK/AA/Baq solvents. This showed that most of the fixed ^{14}C accumulated into carbohydrates (Figure 25) which were predominantly ribitol and sucrose. Glycollic acid could not be detected in the cell extracts. It was found that a greater percentage of the ^{14}C activity occurred in sucrose than in ribitol at low pH (Figure 26) whilst the reverse was true at high pH. From pH 4 to 6 there were approximately equal amounts of the two labelled sugars present.

ii. The medium. Experiments described earlier in this chapter showed that when the medium was distilled water, ribitol was the only carbohydrate released into the medium in significant amounts. It was found that in the presence of the buffer used, sucrose as well as ribitol appeared in the medium, and also in some cases a small amount of the soluble polysaccharide found by Bednar and Smith (1966). In one case a small peak of radioactivity was apparent on scans of the chromatograms with an Rf similar to glycollic acid, but this was not confirmed as the spot did not develop with acridine.

The proportions of ribitol to sucrose varied with pH (Figure 26). Ribitol constituted 85 - 90% of the excreted compounds at pH 3 but only 19 - 26% at pH 7. At pH 8 there was a rise in the amount of

Figure 25

The effect of pH on the photosynthetic fixation of ^{14}C by the 'directly isolated alga' from Xanthoria aureola. The soluble fraction chromatogrammed and autoradiographs made which show that most ^{14}C was fixed into sugars ~~or~~ sugar alcohols.

Material: 0.5 ml wet packed volume directly isolated
alga per sample

Period of photosynthesis: 3 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

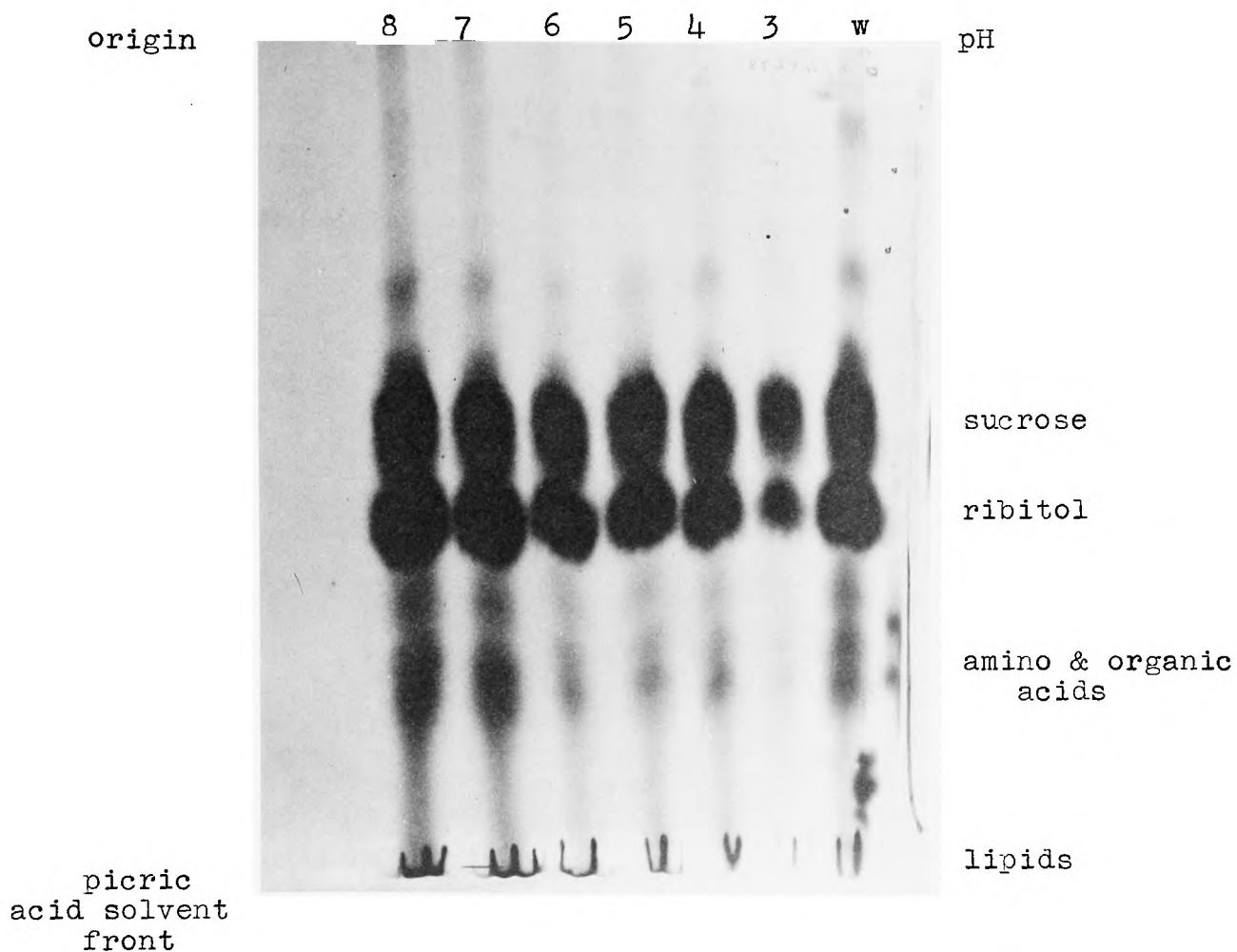
Radioactivity: 20 μc per sample $\text{NaH } ^{14}\text{CO}_3$

Medium: 3 ml distilled water buffered with McIlvaine
buffer

Experiment: 73

Solvent: Picric acid

Date: 5.6.66.



The distribution of ^{14}C carbohydrate in the soluble extracts and in the media of directly isolated Trebouxia cells photosynthesising on buffered distilled water. The distribution represented as a percentage of the total ^{14}C carbohydrate fixed in either the soluble or medium fractions.

pH	Soluble Fraction		Medium		
	sucrose	ribitol	polysaccharide	sucrose	ribitol
3 Hours					
3	62.3	37.7	0.8	13.5	85.7
4	51.0	49.0	2.6	13.3	84.1
5	48.5	51.5	4.8	28.6	66.7
6	44.9	55.1	3.1	30.6	66.3
7	38.0	62.0	0.9	72.4	26.6
8	30.2	69.8	1.08	24.9	74.1
24 Hours					
3	-	-	0.45	3.2	90.3
4	-	-	0.96	5.1	93.9
5	-	-	1.61	12.1	86.3
6	-	-	1.45	75.4	23.2
7	-	-	0.65	80.4	18.9
8	-	-	2.14	50.6	47.2

Material: 0.5 ml wet packed volume of directly isolated algae per sample

Period of photosynthesis: 3 and 24 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: 20 μc per sample

Medium: 3 ml distilled water buffered with McIlvaine buffer

Experiment: 73

Date: 5.6.66.

ribitol to 47 - 74% of the total. This roughly correlates with the situation inside the cells where the greatest amount of ribitol occurs under conditions in which sucrose is mainly excreted, and vice versa. It is not known why excretion into buffered media is different from excretion into distilled water media at similar pH. It should be noted that the buffers were used at rather a high concentration (0.14 to 0.2M) in order to have conditions comparable to those of Muscatine. The components of the buffer may also have affected the metabolism of the cells and further work on the effect of pH should involve the use of other buffers at lower concentration.

7. Conclusions

A number of conclusions may be drawn from the experiments described in this chapter. Because only a few experiments have been carried out, these conclusions should be regarded as tentative and only indicating where future studies might profitably be undertaken.

1. Ribitol, sucrose and possibly a soluble polysaccharide form the major soluble carbohydrate products of photosynthesis, by Trebouxia strains isolated from Xanthoria parietina sensu lato.

2. Cultured strains of the alga tend to fix a higher proportion of ^{14}C into amino and organic acids as compared with the directly isolated algae.

3. All strains of algae tested liberated a proportion of the fixed ^{14}C into the medium during photosynthesis. This was mainly in the form of ^{14}C -ribitol, when the medium was distilled water, though lesser amounts of amino acids, sucrose or soluble polysaccharides were liberated under certain conditions.

4. The cultured and directly isolated alga excreted little of the fixed ^{14}C during the first three hours photosynthesis, but after 24 hours the directly isolated alga excreted considerably more than the cultured forms.

5. Over the course of a 24 hour period the directly isolated alga Trebouxia released progressively more ^{14}C into the medium but there was some variation between replicate samples.

6. Parietin had little effect on the excretion of organic compounds by the alga.

7. pH affected the excretion of fixed ^{14}C by the directly isolated alga. Low pH enhanced excretion and at this pH ribitol was principally excreted. At high pH, sucrose was liberated into the medium in greater amounts than ribitol.

8. It is still not known what causes the alga within the lichen thallus, or when directly isolated, to excrete fixed carbon. The likely possibilities would seem to be one of the following:
(a) the presence of lichen acids lowers the pH in the vicinity of

the algal cells which may have an increased permeability under acid conditions; (b) the fungus excretes some factor that affects the permeability of the algal cell; and (c) the fungus excretes a substance which affects the ability of the algal cells to utilise excreted ribitol for cell wall synthesis.

CHAPTER VTHE ISOLATED SYMBIANTS

(b) The Fungus

1. Introduction

A study of the isolated fungus of a lichen should help to determine: (a) which compounds in the thallus are of fungal origin; (b) whether the fungus can utilise the substances which are believed to move to it from the alga; and (c) if the fungus is deficient for any growth factors.

Lichen fungi are extremely slow growing in culture. For instance, Quispel (1943) obtained only 35.1 mg dry weight of Xanthoria parietina fungus in 25 ml of enriched Czapek Dox medium after three months growth. Therefore it is not as easy to make cultural and nutritional studies on these fungi as on many free-living species.

2. Morphology of the isolated fungus

The fungal component of the lichen Xanthoria parietina sensu lato has been isolated by Tobler (1909), Werner (1925), Bartusch (1932), Lange de la Camp (1933), Quispel (1943), Ende (1950), Thomas (1929), Tomaselli (1963) and Ahmadjian (1963 Pers. Comm.). It is not known whether the lichen material used by any of these workers was specifically referable to Xanthoria aureola.

All the isolates were slow growing and tended to form hard convoluted colonies. The best descriptions of the fungal component are given by Werner (1925). He found that the aerial hyphae on the periphery of the colonies consisted of chains of small round cells whose ends grew out to form long fringe-like threads. The thallus was stratified and hard and these aerial hyphae were only visible on young colonies. Tomaselli et al (1963) have published photographs of the isolated fungus which confirm these descriptions.

The isolates from Xanthoria aureola had a similar morphology on solid media and had the typical aerial hyphae described by earlier workers. In liquid media the colonies were convoluted and spherical (Figure 27).

3. Isolation of the fungus of Xanthoria aureola

Isolation of the fungal component of lichens may either be achieved by dissecting out fungal hyphae from the mature thallus

Figure 27.

The morphology of the isolated fungus of Xanthoria aureola growing in liquid culture on Bianchi medium.

Material: Xanthoria aureola fungus

Time: 8 weeks

Temperature: 18°C

Vessels: 50 ml flasks

Medium: Bianchi medium.

11.3.



with a micromanipulator or by growing the ascospores. It is difficult using the first method to be sure that a para-symbiont of lichen parasite has not been isolated, whilst in the second one cannot be sure that a monokaryotic culture will behave biochemically in an identical way to the heterokaryotic condition which might well occur in the complete thallus.

Isolation from lichen ascospores is carried out by suspending a single ascocarp of the lichen above a Petri dish of nutrient agar. The ascocarp is stuck to the Petri dish lid on a small piece of moist cotton wool with a little petroleum jelly. The spores which are ejected under these moist conditions fall on to the agar below and there germinate. There are a number of variations to this technique (Richardson 1964) but the one described here proved quite successful in the case of Xanthoria aureola.

The spores of Xanthoria aureola proved capable of germination and further growth in late summer, autumn, winter and spring, which supports the view of Kofler and Bouzon (1960) that the spores could germinate at all times of the year rather than that of Werner (1930) who thought that there was a seasonal variation in the viability of the spores. However the ascospores of Xanthoria aureola took up to 96 hours to germinate at 18°C which is much longer than the time of 24 hours observed by Kofler and Bouzon for Xanthoria parietina sensu lato. Once the spores had germinated they were picked off with a fine needle under a binocular microscope, placed on malt/yeast extract agar and left at 18°C until visible colonies developed.

4. Cultural studies on the isolated fungus

In order to study the physiology of the fungus it is necessary to grow considerable quantities in liquid media which is as chemically defined as possible.

a. Vitamin requirements of the isolates

Bianchi (1964) carried out a number of nutritional studies on the ascomycete fungus Neurospora sitophylla. Bianchi's medium was used here as a basis for the cultural studies rather than the semi-natural media (malt/yeast extract agar) which had been widely used for growing lichen fungi (Ahmadjian 1962).

10 ml of Bianchi medium were placed in each of ten 50 ml flasks, which were divided into six pairs; each pair being treated as follows.

1. Bianchi medium + no additions
2. " + 10 μ g biotin + 5 μ g thiamine
3. " + biotin, thiamine, + 0.1 g proteose peptone
4. " + complete vitamin mix
5. " + vitamin mix + 0.1 g. proteose peptone
6. Malt/yeast extract medium (Lilly and Barnet 1951)

The flasks were autoclaved and inoculated with an amount of Xanthoria aureola fungus as big as a small pin head. This was the minimum size that could be used because the fungus grows on solid media as a hard convoluted colony and it is difficult to remove a smaller inoculum from it. One flask from each pair was placed on a

constant shaking bath at 18°C and the other placed in an incubator at the same temperature. After five weeks the flasks were inspected.

In still culture practically no growth had occurred in any medium. In shake culture the basal medium showed no growth and the malt/yeast extract medium very little. Apparently it was not a good liquid medium though satisfactory for growing stock cultures when solidified with agar. All other treatments showed approximately equal growth; the size of the colonies being some 20 times that of the original inoculum. In subsequent experiments, therefore, the basal medium was used, supplemented only with biotin and thiamine.

b. Utilisation of various sugars

An experiment was carried out to see which sugars could be used to support the growth of the fungal component of Xanthoria aureola in culture. Bianchi medium was made up without glucose and sucrose and 10 ml of it added to each of twenty-four 50 ml flasks. To each set of six flasks a test sugar (ribitol, mannitol, sucrose or glucose) was added in amounts of 0.1 g per flask. The medi^{um} was autoclaved and inoculated with fungus. After eight weeks growth in shake culture at 18°C, the dry weights of the fungus were determined.

The results of two experiments are given in Figures 28a & b. It should be stressed that since the results for each sugar are so variable, it is not possible to conclude from this experiment that any one sugar is significantly better at supporting the growth of the

Figure 28a

The amount of Xanthoria fungus (in mg dry weight) produced during growth in shake culture on media supplemented with the following sugars:

	glucose	sucrose	mannitol	ribitol
	68	119	46	123
	118	22	42	57
	34	10	66	153
	148	82	142	123
	140	110	30	141
	112	108	71	60
Total	620	451	397	657

Material: Xanthoria aureola fungus

Time: 8 weeks

Temperature: 18°C

Vessels: six 50 ml flasks per treatment

Medium: Bianchi media with 1% added sugar (10 ml)

Experiment: 64

Date: 17.3.66.

Figure 28b

The amount of Xanthoria fungus (in mg ^{Dry} weight) produced during growth in shake culture on media supplemented with the following sugars:

	mannitol	glucose	ribitol	basal medium	10% glucose**
	19.4	17.8	1.8	1.8	12.5
	11.8	15.0	6.2	0.8	6.1
	18.0	17.4	2.0	0.7	7.3
	22.0	17.6	8.2	0.3	0.8
	17.5	9.1	5.0	0.4	-
	(82.8)*	8.6	7.0	0.8	-
Average	17.7	14.3	5.0	0.8	6.7

*Yellow pigment developed in medium - turned purple on adding alkali. This flask not included in average.

**A 10% glucose medium was used in one case as a high concentration of glucose was reported to enhance the production of parietin. This was not confirmed.

Material: Xanthoria fungus

Time: 8 weeks

Temperature: 5°C - two weeks, 35°C - one day, remainder 18°C

Vessels: six 50 ml flasks per treatment

Radioactivity: none

Medium: Bianchi media with added sugar (10 ml)

Experiment: 90

Date: 19.12.66.

fungus. However it is of interest that the fungus is able to grow in media containing ribitol, since evidence given in the previous chapters suggested that ribitol was the main carbohydrate released from the algal cells.

5. Carbohydrates of the mycelium

Chromatography of the soluble extracts of the isolated fungus grown in Bianchi medium at 18°C indicated that there were only two soluble carbohydrates present in significant amounts. These were mannitol and glucose. These results were interesting since it had been suggested from work on the complete thallus that arabitol was a fungal product.

The fungus was then cultured on a series of media (Figure 28b) and then the soluble carbohydrates of the extracted fungus were chromatogrammed. Figure 29 shows that arabitol was produced when the fungus was growing on ribitol and also in small amounts when it was growing on a high concentration of glucose (10%). Reasons for these results are suggested in the discussion (Chapter XII).

6. Utilisation of ^{14}C -exogenous sugars

a. ^{14}C -glucose

The isolated fungus was grown on Bianchi medium supplemented with biotin and thiamine. 100 mg fresh weight of fungus was supplied with

Figure 29

Chromatography of the ethanol-soluble fractions of Xanthoria
fungus grown on various media.

Details as Figure 28

Solvent: EMK/AA/Baq

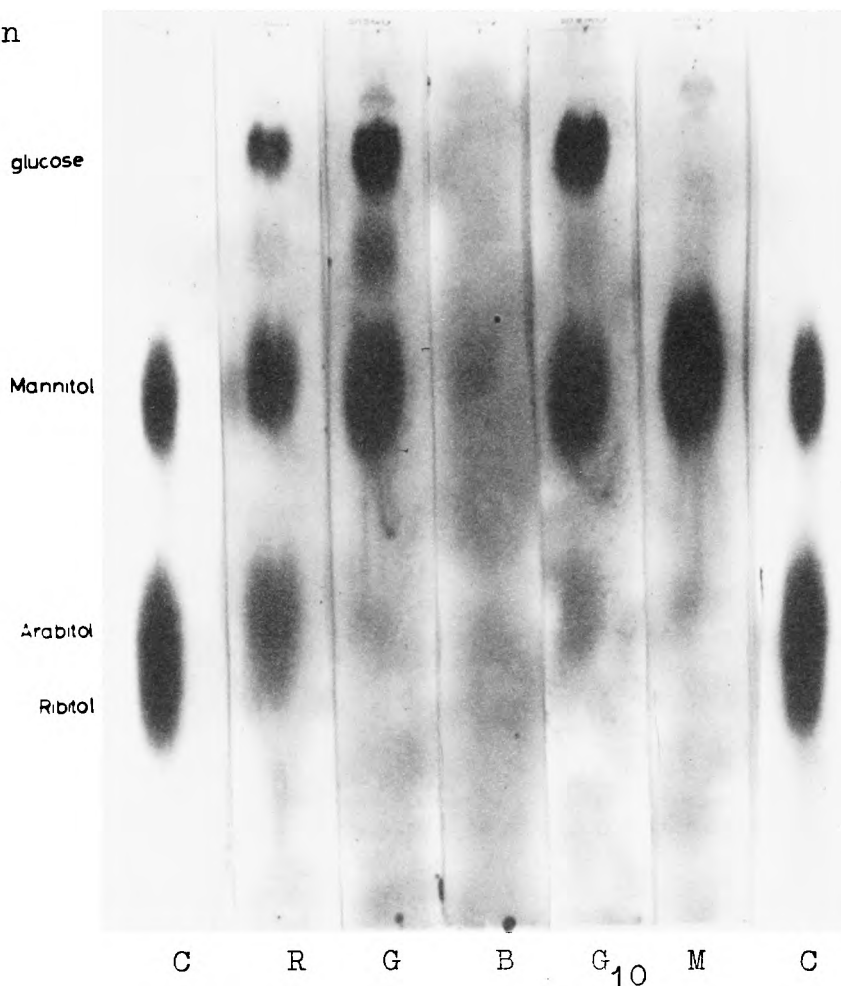
Paper: 3 mm

Development reagent: Alkaline silver nitrate
after treatment with hydrofluoric acid

Time: 30 hours

Temperature: 18°C

origin



C = marker mixture

R = medium supplemented with ribitol

G = medium supplemented with glucose

B = basal medium without added sugar

M = medium supplemented with mannitol

C = marker mixture (mannitol, arabitol, ribitol)

G₁₀ = medium supplemented with 10% w/v glucose} 1% w/v added
carbohydrate

5 μ c carrier-free glucose. After one hour the soluble sugars were extracted with 80% ethanol and chromatogrammed in EMK/AA/Baq solvent. It was found that 82.1% of this fraction was in the form of mannitol, 11.9% remained as glucose and 6% was in the form of a disaccharide. No ^{14}C -pentitols could be detected.

b. ^{14}C -ribitol

In a further experiment an extract of ^{14}C -ribitol was fed to 100 mg fresh weight of fungus. This ^{14}C -ribitol was prepared from lobes of Xanthoria aureola which had photosynthesised on ^{14}C -sodium bicarbonate for three hours. Thus it did not contain detectable amounts of ^{14}C -arabitol and only a small amount of ^{14}C -mannitol. This latter was removed by paper chromatography.

The ribitol was chromatogrammed before feeding to the fungus and was estimated, by measuring the area under the peaks of scans, to contain 2% ^{14}C -mannitol. After the fungus had been fed with this ^{14}C -ribitol for 60 hours at 18°C, the soluble sugars were extracted and chromatogrammed. The area under the peaks in this chromatogram scan showed that 31% of the ^{14}C in the extract was in the form of mannitol and the rest as ^{14}C -pentitols. Unfortunately it was not possible to determine at that time whether or not arabitol was present.

7. Pigment production

Tobler (1909) stated that the isolated fungus of Xanthoria parietina did not produce parietin in pure culture. However, Thomas (1936,39) found that a few strains of his isolates from Xanthoria parietina sensu lato produced this pigment. Moreau (1937) discovered that the fungus Bulgaria inquinans produced an apparently identical pigment but only when there was abundant glucose in the medium.

None of the isolates of Xanthoria aureola produced parietin under the conditions used. However some isolates made a purple diffusible pigment both when grown on malt/yeast extract agar and in liquid Bianchi medium. This pigment was only apparent after some weeks growth but eventually attained quite a high concentration in the culture medium (Figure 30).

This purple pigment was concentrated by extraction with chloroform and chromatogrammed in petrol ether : methyl alcohol (Shibata et al 1950) after which the paper was sprayed with 0.5% magnesium acetate in methyl alcohol, then heated at 90°C for five minutes. No coloured spots were developed which suggested that the pigment was not an anthraquinone or one of its derivatives. Hence there was no evidence that this pigment was a precursor of parietin.

In a recent experiment (Figure 28) one flask out of six grown on Bianchi medium supplemented with mannitol, produced a yellow pigment. It was similar to parietin in that it was converted into

Figure 30

The production of a purple pigment by some strains of the fungus of Xanthoria aureola grown on Bianchi medium and malt/yeast extract agar.

Material: Xanthoria aureola fungus

Time: 4 weeks

Temperature: 18°C

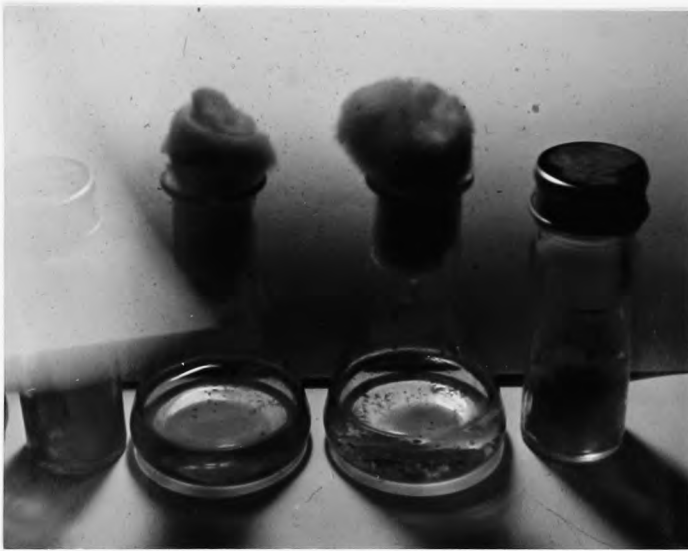
Vessels: 50 ml flasks

Medium: Bianchi medium, shake culture

Experiment: 64a

Date: 17.3.66.

- A Pigment producing isolate grown on solid media
- B Pigment producing isolate grown on liquid media
- C Isolate which did not produce a purple pigment in liquid media
- D Isolate which did not produce a purple pigment on solid media



D

B

C

A

a purple colour by aqueous potassium hydroxide. It has not been characterised further, nor is it known what caused the sudden appearance of either the yellow or the red pigment in cultures of the isolated fungus.

Isolates of the fungal components of Xanthoria parietina sent from America (by Ahmadjian 1965) and from Italy (by Tomaselli 1965) were grown under identical conditions to the isolates from Xanthoria aureola. They had a similar morphology but neither produced parietin or the purple pigment.

CHAPTER VIMOVEMENT OF CARBOHYDRATES BETWEEN THE SYMBIONTS1. Introduction

Experimental evidence for the movement of carbohydrate from the alga to the fungus of lichens was first found in Peltigera polydactyla (Smith 1961). Discs of the lichen were allowed to photosynthesise on ^{14}C -sodium bicarbonate solution and then dissected into two parts, an algal zone composed of the upper cortex together with the algal layer and the medulla which was purely of fungal origin. It was shown that ^{14}C moved quite rapidly from the algal zone to the medulla where it accumulated as mannitol.

It was not possible with this type of experiment to discover the form in which the carbohydrate moved out of the algal cells into the hyphae immediately adjacent to them. Drew (1966) found that the 'directly isolated' alga of Peltigera polydactyla excreted ^{14}C -glucose during photosynthesis on ^{14}C -sodium bicarbonate solution so that it seemed likely that this was the form in which carbohydrate might move in the thallus.

Experiments were therefore carried out by Drew in which ^{12}C -glucose was added with the ^{14}C -sodium bicarbonate to the medium in which lichen discs were photosynthesising. He found that ^{14}C -glucose appeared in the medium in which photosynthesis had occurred and that little ^{14}C -mannitol accumulated within the thallus. These results could not be obtained by including other sugars or polyols in the medium. The only other compounds having the same effect as glucose were the derivatives 2-deoxyglucose and 3-methyl glucose.

The results were explained as follows. During photosynthesis on ^{14}C -sodium bicarbonate, the alga normally excretes ^{14}C -glucose within the thallus which is quickly absorbed by the fungus. If the medium in which the discs are photosynthesising contains a high concentration of ^{12}C -glucose, then this will compete with the relatively small concentration of ^{14}C -glucose for uptake by the fungus. Hence the ^{14}C -glucose produced by the alga will not be taken up by the fungus but will diffuse into the medium and there accumulate. As a result little ^{14}C -mannitol will be formed in the thallus.

In some preliminary investigations into a number of lichens with Trebouxia as algal symbiont, Drew was unable to get a similar inhibition of ^{14}C transfer with any of a range of sugars including glucose.

2. Inhibition of carbohydrate movement in Xanthoria

The products of photosynthesis of the complete thallus and of the cultured symbiotic alga indicated that ribitol was the first substance into which appreciable amounts of ^{14}C accumulated. Preliminary experiments were therefore carried out in which 2% ^{12}C -ribitol was added to the media containing ^{14}C -sodium bicarbonate. It was found that in three hours, less than two per cent of the total fixed ^{14}C appeared in the medium. This contrasted with the situation in Peltigera polydactyla where at least 25% of the total fixed ^{14}C appeared in the medium in three hours if one or two per cent ^{12}C -glucose was present. This suggested that (a) the wrong compound was being used to inhibit carbohydrate transfer or (b) the experimental conditions were in some way not right; or (c) that carbohydrate transfer in Xanthoria operated in a different way to that in Peltigera.

Experiments with the directly isolated alga described in the previous chapter have shown that substantial amounts of carbohydrates were not excreted until 24 hours after photosynthesis had begun. It was therefore decided to carry out experiments on the inhibition of ^{14}C transfer over a longer period.

Samples of Xanthoria aureola were allowed to photosynthesise on ^{14}C -sodium bicarbonate and then placed in the dark on 2% sugar alcohols for 24 hours. On analysis it was found that 4 to 8% of

the fixed ^{14}C appeared in the medium when that contained pentitols but only 0.8 and 0.3% when that was a solution of mannitol or distilled water (Figure 31). It was also found that ^{14}C -mannitol formation in the thallus tissues was considerably reduced on media containing pentitols.

It was thought that a more effective inhibition of carbohydrate transfer might occur if pentitols were included in the medium right from the start of the experiment. Also it was considered better to carry out the whole experiment in the light so that less of the fixed ^{14}C was respired by the cells. Experiments were then carried out with a wide range of possible inhibitors of carbohydrate transfer over a period of 24 hours. The results (Figure 32) indicate that only pentitols are capable of inhibiting transfer of carbohydrates in Xanthoria aureola. Thus after 24 hours on ribitol some 23% of the total fixed ^{14}C appeared in the medium and almost no ^{14}C -mannitol was found in the thallus (Figure 33).

3. Comparison between carbohydrate transfer in Peltigera and Xanthoria

Smith (1961) and Drew (1966) had been able to show that carbohydrate transfer from alga to fungus in Peltigera polydactyla was rapid.

Bednar and Smith (1966) had also suggested that transfer was rapid in Xanthoria aureola because the levels of ^{14}C in metabolites, presumably common to both symbionts (such as PGA) achieved a constant

Figure 31

The release of fixed ^{14}C into the medium by samples of Xanthoria aureola which had been fed an initial pulse of ^{14}C and then placed in darkness on 2% solution of various sugars. Values for ^{14}C fixation given as counts per minute $\times 10^{-3}$.

Sugar	Fraction			Total fixed ^{14}C	% ^{14}C in medium	mannitol/ pentitol ratio**
	Soluble	Insoluble	Medium			
Arabitol	70	4.3	2.8	77	3.63	1:40
Mannitol*	114	5.3	0.9	120	0.75	1:60
Xylitol	46	2.9	4.5	53	8.45	1:50
Ribitol	65	2.8	3.4	72	4.79	1:14
Water only	78	3.2	0.2	81	0.28	1:4

*Twice the amount of $\text{NaH}^{14}\text{CO}_3$ added.

** The ratio of ^{14}C mannitol to ^{14}C pentitol in the chromatogrammed soluble fraction estimated by measuring the area under the peaks on scans of these chromatograms.

Material: 100 mg fresh weight per sample

Period of photosynthesis: 2 hours

Period in darkness: 22 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: $5\ \mu\text{e}$ $\text{NaH}^{14}\text{CO}_3$

Medium: 3 ml distilled water + 2% sugars

Experiment: 19

Date: 19.5.65

Figure 32

The release of fixed ^{14}C into the medium by samples of Xanthoria aureola during photosynthesis in the presence of various sugars. Values for ^{14}C fixation given as counts per minute $\times 10^{-3}$.

Sugar	Total fixation	% ^{14}C in medium
Arabitol	71.1	20.2
Ribitol	77.4	23.6
Xylitol	64.1	10.5
Mannitol	58.3	3.6
Ribose	70.0	0.73
Trehalose	57.2	1.67
Glucose*	93.1	1.80
Water control	75.1	1.14

*Values from another similar experiment

Material: 100 mg fresh weight per sample

Period of photosynthesis: 24 hours

Temperature: 20°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: 10 μC $\text{NaH}^{14}\text{CO}_3$ per sample

Medium: 3 ml distilled water, 100 μg per ml $\text{NaH}^{12}\text{CO}_3$ + 1% sugars

Experiment: 45

Date: 15.11.65.

Figure 33

The distribution of radioactivity in the soluble fraction of samples of Xanthoria aureola placed in the light on solutions of ^{14}C -sodium bicarbonate to which 1% sugars had been added. Scans of chromatograms developed in EMK/AA/Baq.

Material: 100 mg fresh weight samples

Period of photosynthesis: 24 hours

Temperature: 20°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

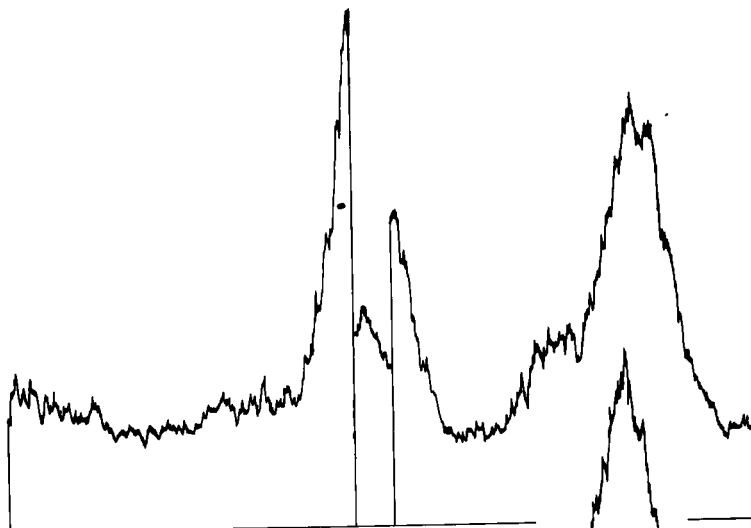
Radioactivity: 10 μc NaH $^{14}\text{CO}_3$

Medium: 3 ml distilled water, 100 μg carrier NaH $^{12}\text{CO}_3$
per ml, 1% sugars

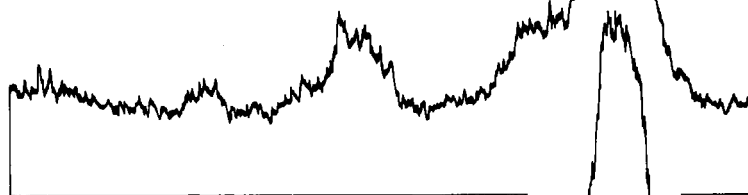
Experiment: 45

Date: 15.11.65.

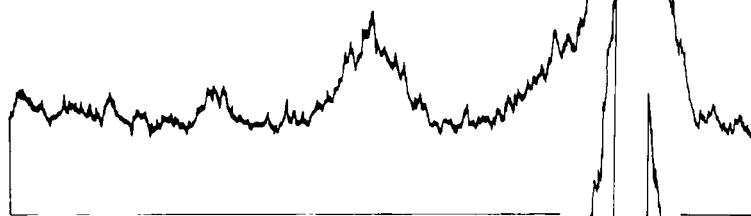
water



mannitol



sugars
(mono & di-
saccharides)



pentitols



mannitol

pentitols

o = origin

level within 15 minutes. However it was not possible to get direct proof of the rate of transfer as the morphology of Xanthoria is such that the algal layer could not be dissected from the medulla as had been done in Peltigera.

It was thought that the conditions used in this study might be slightly different from those used either by Drew or by Smith and Bednar and that this might explain the apparently slow movement of carbohydrate from alga to fungus.

A comparative experiment was therefore set up using identical apparatus and radiochemical techniques. Discs of Peltigera, 7 mm in diameter, and 100 mg fresh weight samples of Xanthoria were allowed to photosynthesise on ^{14}C -sodium bicarbonate solutions in the presence of glucose and ribitol respectively. After 1, 3, 6, 9, 12, and 24 hours, samples were removed from the illuminated bath, killed in 80% ethanol and then analysed to see how much radioactivity was in the soluble and insoluble fractions. The amount of fixed ^{14}C remaining in the medium was also determined.

The results of this experiment are shown graphically in Figure 34. They confirm the earlier results of Drew in respect of the rapid exudation of fixed ^{14}C into the medium in the case of Peltigera where the medium contained ^{12}C -glucose. They also suggest that the transfer of carbohydrate between the symbionts is much slower in Xanthoria than in Peltigera.

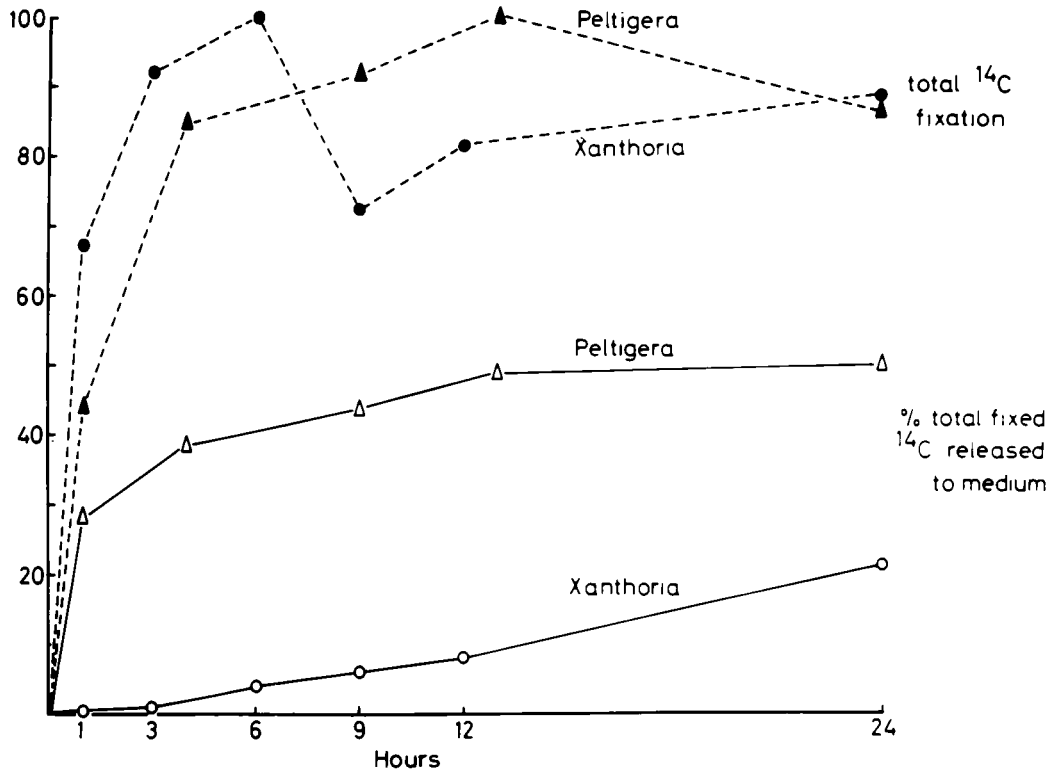
Figure 34

The release of fixed ^{14}C by Peltigera polydactyla (in 1% glucose) and by Xanthoria aureola (in 1% ribitol solutions) compared in the same experiment.

Maximum fixation by samples of Peltigera and Xanthoria plotted as 100 units on the vertical axis of the graph.

Material: 100 mg fresh weight of Xanthoria per sample
10, 7 mm discs of Peltigera per sample
Period of photosynthesis: 24 hours
Temperature: 18°C
Light: 500 fc
Vessels: 2 x 1" specimen tubes
Radioactivity: 10 μC $\text{NaH}^{14}\text{CO}_3$ per sample
Medium: 3 ml distilled water + 1% glucose or ribitol
Experiment: 42
Date: 26.10.65.

Release of fixed ^{14}C by *Peltigera* (in glucose) & *Xanthoria* (in ribitol) compared in the same experiment



4. Rate of carbohydrate transfer in Xanthoria

The foregoing experiments lead to the conclusions that the algal symbiont within the thallus leaks ^{14}C slowly though perhaps in increasing amounts as time elapses. Further evidence for the slow rate of transfer was obtained from the following experiment.

Samples of Xanthoria aureola were allowed to photosynthesise for two hours on ^{14}C -sodium bicarbonate solution. They were then washed with distilled water and allowed to continue photosynthesis in distilled water without ^{14}C -sodium bicarbonate. After increasing time intervals, samples were removed from the distilled water and placed on 2% ribitol in the light when it was assumed that the residual ^{14}C available for transfer would diffuse into the medium. At the end of 24 hours all the samples were killed in 80% ethanol and the amount of radioactivity in both media and tissues was ascertained.

The total amount of ^{14}C available for transfer from alga to fungus was assumed to approximate to the amount released by the tissues of samples transferred immediately from ^{14}C -bicarbonate to ^{12}C -ribitol media. The amount of ^{14}C moving from alga to fungus during a particular time on distilled water will be equivalent to the total amount available for transfer minus the amount released subsequently in ^{12}C -ribitol. In this way it is possible to build up a picture of the rate at which an initial 'pulse' of ^{14}C moves from alga to fungus and this is represented graphically in Figure 35.

Figure 35

A comparison of the rate of transfer of a pulse of ^{14}C from alga to fungus in Xanthoria aureola and Peltigera polydactyla.

Material: 100 mg fresh weight of Xanthoria per sample

10, 7 mm discs of Peltigera per sample

Period of photosynthesis: 24 hours

Temperature: 18°C

Light: 500 fc

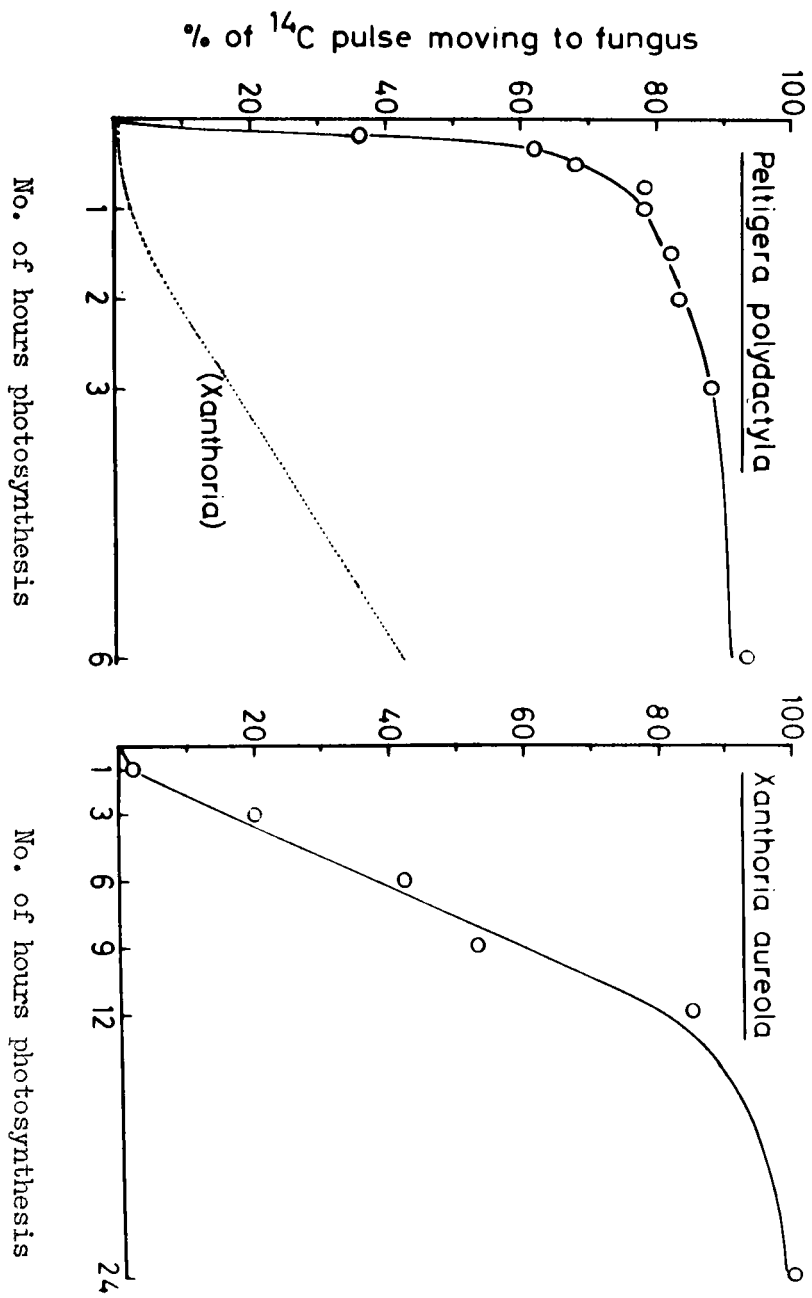
Vessels: 2 x 1" specimen tubes

Radioactivity: 10 μc $\text{NaH}^{14}\text{CO}_3$ per sample

Medium: 3 ml distilled water + 1% glucose or ribitol

Experiment: 42

Date: 26.10.65.



A similar experiment subsequently carried out with Peltigera polydactyla is also shown.

These results provide clear evidence that the rate of carbohydrate transfer in Xanthoria aureola is much slower than in Peltigera polydactyla. Thus the results in Figure 36 show that even 12 hours after the initial pulse of ^{14}C fixation it is still possible for samples of Xanthoria to release ^{14}C when transferred to ^{12}C -ribitol, indicating that transfer of the pulse of activity was not complete.

Another feature of interest which arises from this experiment is that arabitol only incorporates ^{14}C when ^{14}C -mannitol is formed in the thallus (Figure 37). This suggests that it is a fungal product, possibly involved in the conversion of ribitol into mannitol.

In conclusion, it therefore appears that the assumption by Bednar and Smith that the transfer of carbohydrate from alga to fungus was rapid is not correct. The reasons for their conclusions are given in the discussion (Chapter XII).

5. The effects of pH on carbohydrate transfer

Experiments on the isolated alga showed that at low pH the alga excreted ribitol into the medium in which photosynthesis occurred. As the pH rose the alga excreted more and more sucrose in addition to ribitol, although, as emphasised before, it is not clear to what

Figure 36

The release of fixed ^{14}C by samples of Xanthoria aureola under the following conditions: all samples initially incubated for one hour on $\text{NaH}^{14}\text{CO}_3$ solution and then kept for varying lengths of time on distilled water before transfer to 2% ribitol. Total period on distilled water and ribitol for all samples was 24 hours. The amount of activity in the medium represented as a percentage of the total fixed ^{14}C .

No. of hours on distilled water	% Total fixed ^{14}C released into ribitol media		Ratio of ^{14}C mannitol to ^{14}C pentitol in tissues	
	18°C	5°C	5°C	18°C
0	20.61	12.93	1:590	1:92
1	20.97	13.87	1:111	1:104
3	17.37	9.15	1:440	1:54
6	14.35	17.33	1:20	1:10
9	12.34	15.46	1:13	1:6
12	5.47	8.83	1:12	1:4

Material: 100 mg FW per sample
 Period of photosynthesis: 24 hours
 Temperature: 5 & 18°C
 Light: 500 fc
 Vessels: 2 x 1" specimen tubes
 Radioactivity: 20 μC $\text{NaH}^{14}\text{CO}_3$
 Medium: 3 ml as above
 Experiment: 67
 Date: 27.4.66.

Figure 37

Autoradiographs of chromatograms of the soluble fraction in Experiment 67 (Figure 35). This indicates that ^{14}C -arabitol only accumulates ^{14}C when mannitol also becomes labelled.

Material: 100 mg fresh weight per sample

Period of photosynthesis: 24 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: 10 μc $\text{NaH}^{14}\text{CO}_3$ per sample

Medium: 2 ml $\text{NaH}^{14}\text{CO}_3$ in distilled water for one hour,
0-23 hours distilled water and 23-0 hours 2%
ribitol

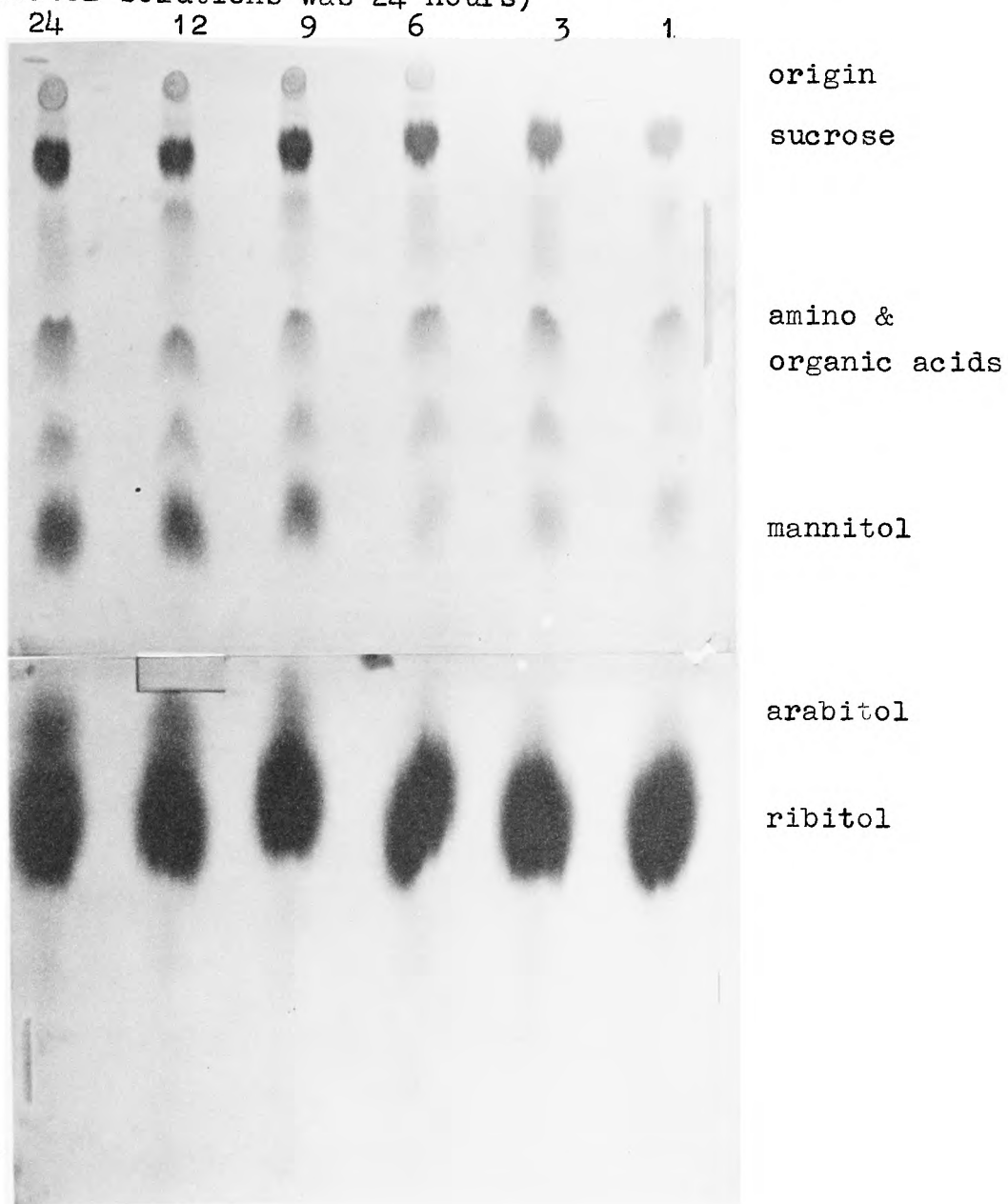
Experiment: 67

Solvent: EMK/AA/Baq

Paper: Whatman no 1

Date: 27.4.66.

number of hours on distilled water before transfer
to 2% ribitol (total length of time on distilled water
and ribitol solutions was 24 hours)



extent these effects were controlled by pH or due to the nature and rather high concentration of the buffer used.

Similar experiments were, however, carried out on the complete thallus to determine: (a) if sucrose could inhibit carbohydrate transfer, especially at high pH (i.e. the conditions under which the directly isolated algae excreted sucrose); (b) what effect pH had on the ability of ribitol to inhibit carbohydrate transfer; and (c) what effect low pH had on photosynthesis in the thallus without added solutes. The buffer used was the same as that employed on studies on the isolated alga and suffers from the disadvantages mentioned in that section, but the same conditions were applied in these experiments so that the results would be more nearly comparable.

a. Inhibition with sucrose

Samples of thallus lobes were placed in 4 ml of 0.1 - 0.2 M McIlvaine phosphate citrate buffer at pH values from about 3 to 8. A quantity of sucrose (1% w/v) and 10 μ c ^{14}C sodium bicarbonate was added to each sample. The samples were placed in the light for 24 hours. After this time the amount of radioactivity in the medium, soluble and insoluble fractions was determined. The extracts were chromatogrammed and autoradiographs made.

The results (Figure 38) show that most ^{14}C is excreted into

Figure 38

The release of fixed ^{14}C by samples of Xanthoria aureola photosynthesising on solutions of ^{14}C -sodium bicarbonate in the presence of 1% sucrose buffered with McIlvaine buffer.

Values in table as counts per minute $\times 10^{-3}$.

pH	Total fixed ^{14}C	% Total ^{14}C in medium	% Total ^{14}C in insoluble fraction
2.7	359	38.6	9.5
3.1	130	20.6	5.5
3.7	78	9.9	5.2
4.5	130	4.7	4.2
6.3	286	5.7	2.1
7.8	549	6.7	4.8
Distilled water unbuffered (pH 5.8)	343	1.4	9.6

Material: 100 mg fresh weight per sample

Period of photosynthesis: 24 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: 10 μC NaH $^{14}\text{CO}_3$ per sample

Medium: 4 ml 1% sucrose buffered

Experiment: 85

Date: 5.9.66.

the medium at very low pH. As the pH rises, less is excreted but at pH 7.8 there is a slight rise. Chromatographic analysis of the medium showed that most of the fixed ^{14}C released at low pH was in the form of ribitol, and hardly any in the form of sucrose. In the intermediate pH ranges little of the ^{14}C that was liberated was carbohydrate. In fact ^{14}C sucrose only becomes the predominant carbohydrate in the medium at high pH values when only a small percentage of the total fixed ^{14}C is excreted. (Figure 39)

These results thus suggest that sucrose is not the main form in which carbohydrates move from the alga to the fungus except possibly at high pH. Under these conditions it has already been shown that sucrose excretion was promoted in the directly isolated alga. It must be stressed that it is not known whether the effects observed in this experiment are due solely to the sucrose or to the nature and high concentration of the buffer used. Certainly the fact that no ^{14}C sucrose was detected in media containing no buffer implies that it does not have an important role in carbohydrate transfer between the symbionts of Xanthoria aureola.

Analysis of the soluble fraction showed that ^{14}C sucrose was found within the thallus at all pH ranges. ^{14}C mannitol was most apparent at intermediate pH ranges while the amount of ^{14}C ribitol appeared greatest at high pH when least ^{14}C mannitol was produced

Figure 39

Autoradiographs of one dimensional chromatograms of the medium of Experiment 85 (Figure 38) in which sucrose was used to try and inhibit transfer between the symbionts at various pH. ^{14}C -Ribitol together with a small amount of ^{14}C -sucrose was released at low pH but at high pH a greater proportion of ^{14}C -sucrose was released (though at high pH much less total ^{14}C is excreted than at low pH).

Material: 100 mg fresh weight per sample of Xanthoria aureola

Time: 30 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: 10 μc $\text{NaH}^{14}\text{CO}_3$ per sample

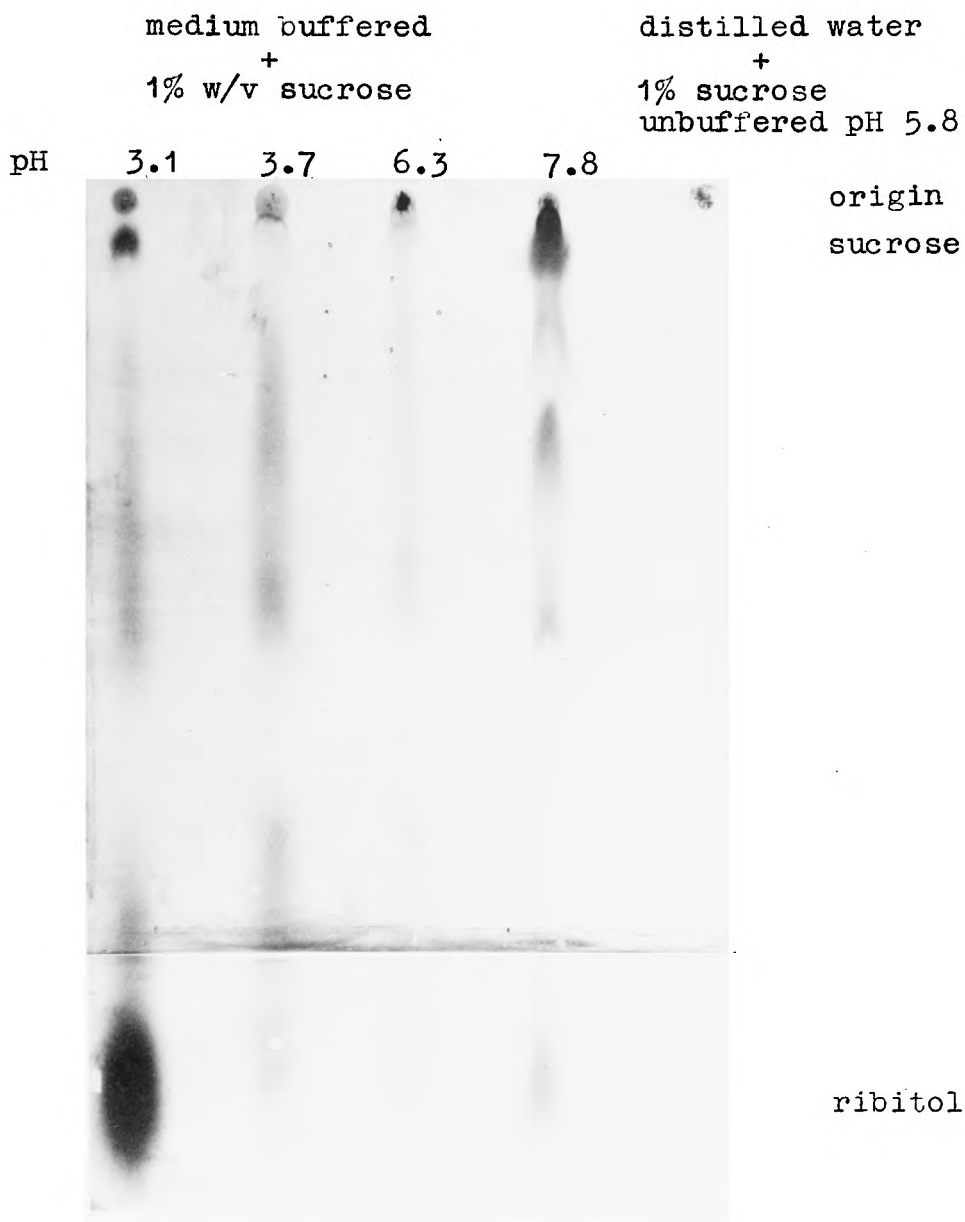
Medium: 4 ml 1% sucrose buffered

Experiment: 85

Paper: 3 mm

Date: 5.9.66.

148a.



when the medium contained unbuffered distilled water to which 1% sucrose had been added, there was almost no detectable ^{14}C liberated into the medium.

(Figure 40). The fact that little ^{14}C -mannitol is formed at high pH may imply that little ^{14}C -ribitol was able to pass to the fungus and that it was being retained within the algal cells. This would agree with previous observations on excretion of fixed ^{14}C by the directly isolated alga at high pH. (Page 106,107) However, it is also possible that high pH may inhibit the endogenous conversion of ribitol to mannitol in the fungal component of the lichen.

b. Inhibition with ribitol

A similar experiment was carried out using ribitol as the inhibiting substance. The results again indicate that more ^{14}C was released from the tissues at low than at high pH. Over a period of ten hours 14% of the total fixed ^{14}C was excreted at the lowest pH (2.7) as compared with 11% on distilled water and 2% at high pH (7.8). Thus high pH either reduces the ability of the ribitol to inhibit carbohydrate transfer or the release by the algal cells is less at this pH.

Chromatographic analysis of the medium showed that ^{14}C was released as ribitol and no sucrose could be detected. The soluble fractions contained little ^{14}C -mannitol at any pH. Thus the entry of ^{14}C -ribitol into the fungus was being prevented in all cases by the ^{12}C -ribitol. At low pH significant amounts of ^{14}C -ribitol were

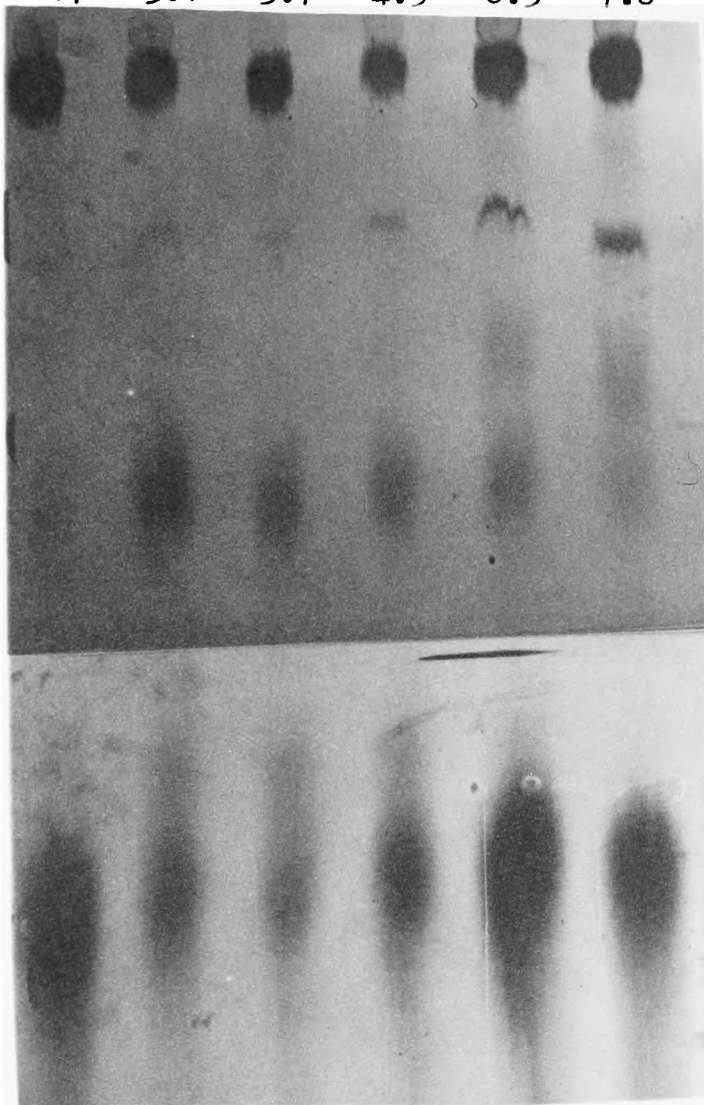
Figure 40

Autoradiographs of the soluble fraction of samples of Xanthoria used in experiment 85 (Figures 38, 39). At high and low pH little ^{14}C -mannitol or ^{14}C -arabitol is formed.

Details as Figure 39
Solvent: EMK/AA/Baq
Time: 30 hours
Paper: No 1

pH

2.7 3.1 3.7 4.5 6.3 7.8



origin
sucrose

amino acids

mannitol

arabitol

ribitol

found in the medium indicating that the algae were able to release ^{14}C under these conditions but at high pH little fixed ^{14}C appeared in the medium suggesting that it was retained within the algal cells. Analysis of the soluble fraction confirmed that large amounts of ^{14}C -ribitol ~~were~~ ^{were} present in the thallus (presumably in the algal layer).

c. The effects of low pH on carbohydrate transfer

Earlier experiments have shown that ^{12}C -ribitol is able to inhibit movement of ^{14}C from alga to fungus in Xanthoria aureola but except at low pH ^{12}C -sucrose could not. Furthermore when sucrose was present in media buffered to low pH, although carbohydrate was released, it was as ^{14}C -ribitol and not ^{14}C -sucrose. This suggested that it might be the low pH and not the sucrose which was preventing fungal uptake and conversion of the ^{14}C -ribitol released by the alga.

An experiment was therefore carried out to investigate the effects of low pH, ribitol and sucrose. Samples of thallus lobes were placed on the following media:

1. Distilled water (pH 5.8 with no added buffer)
2. " " " + 1% ^{12}C -ribitol (pH 5.8)
3. " " " + 1% ^{12}C -sucrose (pH 5.8)
4. " " " buffered to pH 3.0 with phosphate citrate buffer

Chromatography of the soluble fractions showed that ^{14}C -mannitol was produced in samples floated on distilled water and on 1% ^{12}C -sucrose, suggesting that in both cases ^{14}C entered the fungus and confirming that sucrose had little or no effect on carbohydrate transfer in distilled water (Figure 41). However when the medium contained either 1% ^{12}C -ribitol or water buffered to pH 3.0 ^{14}C -mannitol formation was inhibited.

Thus when ^{12}C -ribitol is present it competes successfully at the fungal uptake sites with the ^{14}C -ribitol whereas on media buffered to low pH, it appears that the fungus is unable to take up or convert the ^{14}C -ribitol released by the algal cells. Therefore in both cases, little ^{14}C -mannitol is formed and ^{14}C -ribitol appeared in the medium. It is relevant to note that Harley & Smith (1956) found that the uptake of carbohydrates such as sucrose and glucose, by Peltigera polydactyla, was considerably reduced at low pH.

d. Conclusions on the effect of pH on carbohydrate transfer

Although the exact effect of the nature and rather high concentration of the buffers used in these experiments is not known, the following tentative conclusions may be drawn.

- (i) At low pH values, carbohydrate transfer between the symbionts seems to be much reduced due to the low pH inhibiting

Figure 41

Autoradiographs of one-dimensional chromatogram of the soluble fraction of samples of Xanthoria in which movement between the symbionts was inhibited with various treatment. ^{14}C -Mannitol is only found (a) when ^{12}C -ribitol is not present as an inhibitor, (b) when the samples are not at very low pH.

Experiment 85b

Details as previous figures

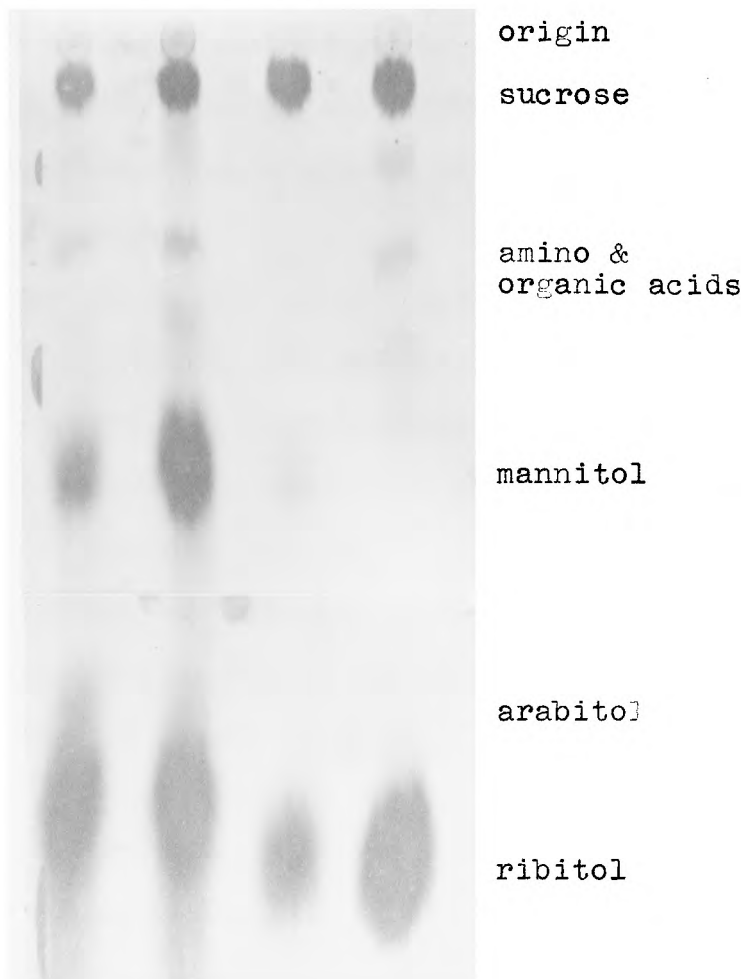
Medium a. sucrose (1%) in distilled water
b. distilled water only
c. distilled water buffered at pH 3.0
d. distilled water + 1% ribitol

Solvent: EMK/AA/Baq

Time: 30 hours

Paper: No I

sucrose	water	ribitol
+	+	+
D.W.	D.W. pH 3	D.W.



sucrose + D.W. = distilled water (unbuffered) + 1% w/v sucrose

D.W. = distilled water (unbuffered)

water + pH 3 = water buffered to pH 3.0 - no added sugars

ribitol + D.W. = distilled water(unbuffered) + 1% ribitol

carbohydrate uptake by the fungus. Low pH also appears to stimulate the release of carbohydrate in the complete thallus as it did in experiments on the isolated alga.

- (ii) At high pH values, carbohydrate transfer between the symbionts is also much reduced but in this case it appears that the high pH is reducing the ability of the algae to release carbohydrate (again confirming previous observations on the isolated alga).
- (iii) At intermediate pH values, carbohydrate transfer between the symbionts is probably optimal and the main carbohydrate involved is ribitol. Although sucrose occurs in the cells at all pH values, it only appears to be important in carbohydrate transfer at high pH.
- (iv) On distilled water, all evidence shows that sucrose does not move between the symbionts and that it is ribitol which passes from alga to fungus in Xanthoria aureola under these conditions.
-

SECTION B

Experiments using other Lichen Genera

CHAPTER VIISTUDIES ON LICHENS CONTAINING BLUE - GREEN ALGAE1. Introduction

There are several genera of blue-green algae which are symbiotic in lichens found in the British Isles: Scytonema (e.g. Placynthium sp), Stigonema (e.g. Ephebe sp), Gleocapsa (e.g. Synalissa sp), Nostoc (e.g. Peltigera sp), and Rivularia (e.g. Lichina sp).

Only the last two algae occur in lichens which are common and of sufficiently large size for reasonably easy manipulation in the laboratory. Nostoc sp occurs in Peltigera polydactyla whose physiology has been studied from a number of aspects by Smyth (1934), Harley & Smith (1956), Smith (1963), Smith & Drew (1965), Drew (1966) and Drew & Smith (1966). Drew (1966) investigated the carbohydrate metabolism of this species in detail and found that the algal symbiont produced glucose which moved rapidly to the fungus and was there converted into mannitol. When discs of the lichen photosynthesised on a solution of ^{14}C -sodium bicarbonate, the movement of ^{14}C -glucose from alga to fungus could be inhibited by including ^{12}C -glucose in the medium. Under similar conditions, inhibition of

^{14}C movement was apparent when samples of P. horizontalis, P. canina and Collema auriculatum which also contain Nostoc, photosynthesised in media containing ^{12}C -glucose.

2. Other species containing Nostoc

A preliminary experiment was carried out with species of the genus Lobaria. One of these, L. scrobiculata, contained Nostoc, while the other two, L. laetivirens and L. pulmonaria, contained Trebouxia. It was found that movement of ^{14}C could only be inhibited by including ^{12}C -glucose in the medium in the species symbiotic with Nostoc. Thus it seemed similar to the other species containing Nostoc studied by Drew.

The experiment was repeated as the material used in the first experiment was collected in Ireland and had been in transit for several days. In this second experiment, a further species containing Nostoc was examined, Sticta fuliginosa. The results of this experiment are described in Appendix A and show that all the species containing Nostoc had a carbohydrate movement pattern similar to Peltigera polydactyla.

3. Species containing Rivularia

This alga is symbiotic in the genus Lichina which occurs on the sea shore just above low tide level. There are two British species,

both fruticose, the larger, Lichina pygmaea, is up to three cm tall and was used in this study.

A recent analysis of the carbohydrates of Lichina pygmaea has been published by Lestang Laisne (1966). She found that the predominant ethanol-soluble carbohydrate was mannitol accompanied by smaller amounts of glucosan, mannosido-mannitol, glycerol and simple sugars, especially glucose (Figure 42).

She was unable to detect volemitol which had previously been recorded for this species by Pueyo (1963) and for Lichina confinis, by Shibata (1963). The mannosido-mannitol had a similar Rf to volemitol and it may be that the previous workers misidentified volemitol for this reason.

In this study, lichen material was collected from South Devon and experiments carried out to see whether the pattern of carbohydrate transfer was similar to that found in Peltigera polydactyla and the other species containing Nostoc mentioned above.

Samples of washed thalli (100 mg, fresh weight) were placed in the light in solutions of ^{14}C -sodium bicarbonate with and without 3% sodium chloride. This was to simulate conditions of coverage of the lichen by sea water at high tide or by fresh water when exposed. In both cases ^{14}C became incorporated into mannitol within three hours from the start of photosynthesis. It was also found that glucose and a spot remaining close to the baseline on chromatograms

Analysis of the sugars, sugar alcohols, mucilages etc. in
Lichina pygmeae, Rivularia bullata and an Ascomycete Fungus;
 abstracted from a paper by Lestang Laisne (1966)

Blue-green alga <u>Rivularia bullata</u>	Free living pyrenomycete fungus	Lichen	
		<u>Lichina pygmeae</u>	amount present per 100g dry wt
-	-	glycerol	30-40 mg
-	-	simple sugars: glucose fructose galactose arabinose ?xylose	20 mg
	mannitol	mannitol	3.5-6 g
		mannosido- mannitol	40 mg
trehalose	trehalose	-	
- ?	- glycogen	glucosan glycogen	1 g approx. 100 mg
mucilages, non-sulphuryl, containing: glucose arabinose uronic acid	mucilages, non-sulphuryl, containing: simple sugars (mannose) uronic acid (glucuronic)	mucilages, non-sulphuryl, a series of polyoside mucilages: mannogalactans xylogalactans ?arabinogalactans manno-xylo- galactan glucuronic polyoside complex manno-xylo-arabino gluco-galactan glucuronic	20 g (Total)

also became labelled with ^{14}C . This was probably the glucosan identified by Lestang Laisne. No significant amount of radioactivity was found in the region occupied by mannitol glycosides or heptitols (see Figure 43).

One interesting feature was that on distilled water some 30% of the total ^{14}C fixed accumulated in the ethanol-insoluble fraction whereas on 3% salt solution there was only about 6% in this fraction (Figure 44). Under conditions of reduced salinity it may be that ^{14}C is quickly incorporated into the mucilages which Lestang Laisne found so abundant. This is by no means certain but the effects of salinity on photosynthesis in this species are clearly worthy of further investigation.

When samples of Lichina pygmaea were placed in the light on ^{14}C -sodium bicarbonate solution containing 1% glucose, it was found that about 3% of the total fixed ^{14}C was liberated into the medium in three hours (Figure 44). Although this was a low value compared to similar experiments with Peltigera polydactyla under similar conditions, it was more than was liberated when distilled water or 1% ribitol was present in the medium.

In a further experiment the length of photosynthesis was extended to 24 hours and a lower temperature of 5°C was also tried. The results of Figure 44 clearly show that glucose was a better inhibitor than the other compounds tried. It was also found that

Figure 43

The release of fixed ^{14}C into the medium by samples of Lichina pygmaea during photosynthesis on solutions of ^{14}C sodium bicarbonate in the presence of various sugars. * Values for ^{14}C fixation given as counts per minute $\times 10^{-3}$.

3% NaCl in medium

Treatment	Total ^{14}C fixation	% ^{14}C in medium	% ^{14}C in insoluble fraction
Water	431	0.71	7.2
glucose	364	1.11	8.9
ribitol	596	0.46	5.7

Distilled water + sugars only in medium

Treatment	Total ^{14}C fixation	% ^{14}C in medium	% ^{14}C in insoluble fraction
Water	362	1.20	38.7
glucose	222	3.84	29.8
ribitol	331	0.85	29.4

Material: 200 mg fresh weight per sample

* Period of photosynthesis: 3 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: $10 \mu\text{C NaH}^{14}\text{CO}_3$

Medium: 3 ml distilled water, $50 \mu\text{g NaH}^{12}\text{CO}_3$ + 2% sugars, 3% NaCl

Experiment: 32

Date: 10.9.65.

Figure 44

The release of fixed ^{14}C into the medium by samples of Lichina pygmaea photosynthesising on solutions of ^{14}C -sodium bicarbonate in the presence of various sugars. *
 Fixation as counts per minute
 $\times 10^{-3}$.

Sugar	Total ^{14}C fixed	% ^{14}C in medium	% ^{14}C in insoluble fraction
erythritol	301	2.37	39.5
arabitol	298	2.75	38.3
glucose	289	19.96	45.9
water	301	2.48	37.1

Material: 200 mg fresh weight per sample

*Period of photosynthesis: 24 hours

Temperature: 5°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: $10 \mu\text{c NaH } ^{14}\text{CO}_3$

Medium: 3 ml distilled water + 2% sugars

Experiment: 62

Date: 15.3.66.

in the presence of this sugar very little ^{14}C -mannitol was produced within the thallus (Figure 45) and that ^{14}C -glucose accumulated in the medium in which photosynthesis had occurred. As in Peltigera polydactyla, if ^{14}C -glucose was fed to the thallus in the dark, it was completely converted into ^{14}C -mannitol within three hours (Figure 46).

4. Conclusions

Lichens containing two genera of blue-green algae have been studied. In both it is apparent that glucose is the principal form in which carbohydrate moves from alga to fungus.

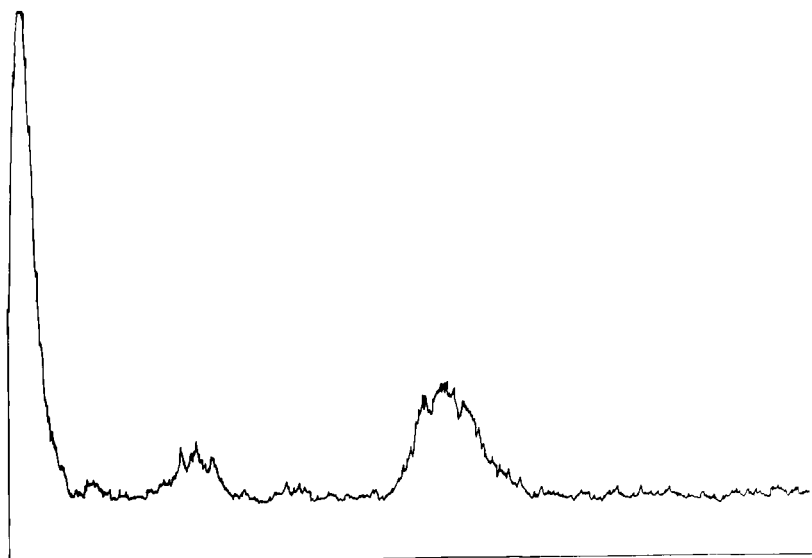
Using the technique of inhibiting the movement of ^{14}C -glucose by incorporating ^{12}C -glucose in the medium during photosynthesis, it is seen that the rate of transfer is much slower in Lichina pygmaea than in the species containing Nostoc. In all the latter lichens at least 20% of the total fixed ^{14}C was released into the medium within three hours under the above conditions as compared with only 3% in the case of Lichina pygmaea.

Figure 45

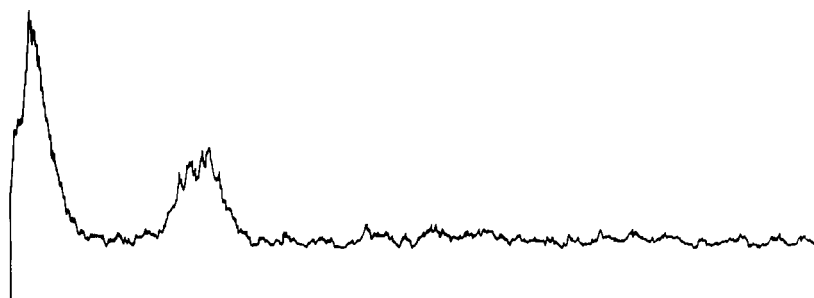
Scans of chromatograms of the ethanol-soluble fraction of Lichina pygmaea showing the effect of glucose on the distribution of ^{14}C .

Material: 200 mg fresh weight per sample
Period of photosynthesis: 24 hours
Temperature: 5°C
Light: 500 fc
Vessels: 2 x 1" specimen tubes
Radioactivity: 10 μc $\text{NaH}^{14}\text{CO}_3$ per sample
Medium: 3 ml distilled water + 2% glucose
Experiment: 62
Solvent: EMK/AA/Baq
Date: 15.3.66.

soluble
fraction
of samples
in
distilled
water

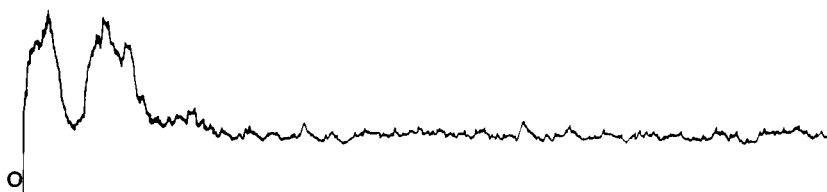


soluble
fraction
of samples
in
1% glucose



poly-
saccharide glucose mannitol

* medium of
samples in
1%
glucose



poly- glucose
saccharide

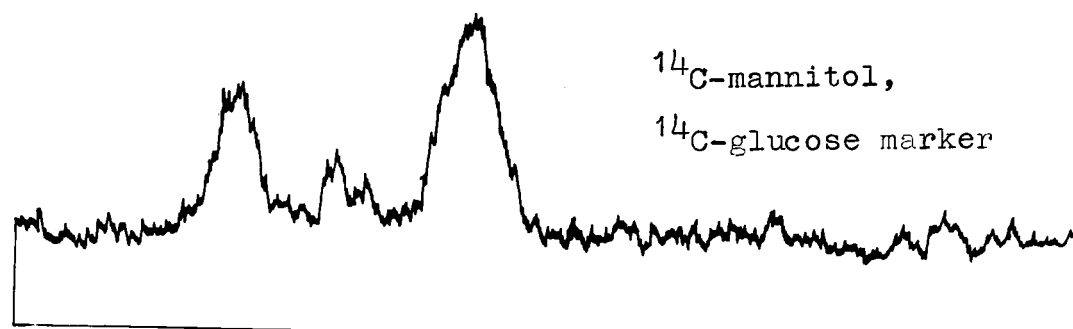
* separate chromatogram run for shorter period
on whatman 3MM paper instead of whatman no 1.

o = origin

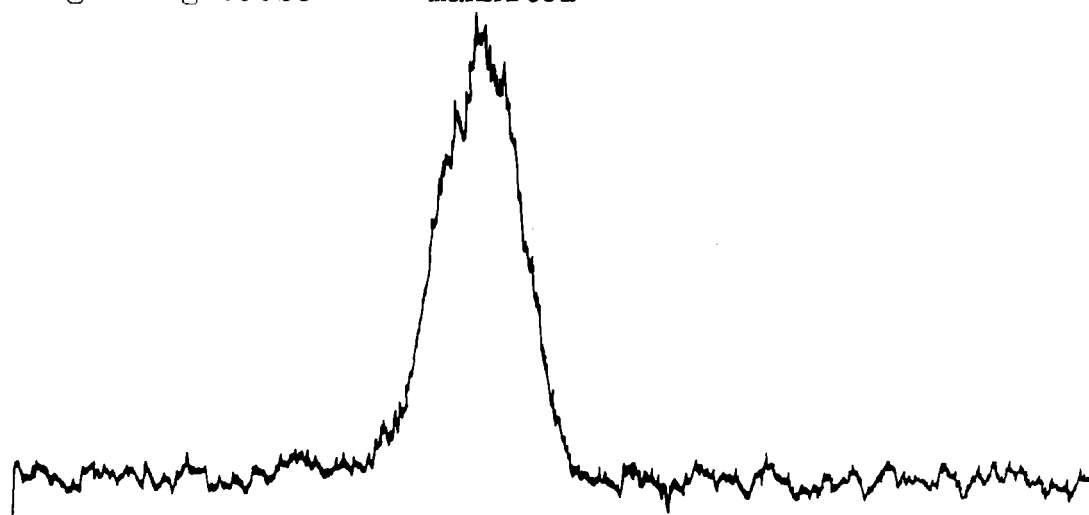
Figure 46

Scans of the chromatogrammed soluble fraction of samples of Lichina pygmaea which were incubated with ^{14}C -glucose.

Material: 200 mg fresh weight per sample
Period of photosynthesis: 3 hours
Temperature: 18°C
Light: 500 fc
Vessels: 2 x 1" specimen tubes
Radioactivity: 2 μC ^{14}C -glucose
Medium: distilled water
Experiment: 34
Solvent: EMK/AA/Baq
Date: 11.9.65.

 ^{14}C -mannitol, ^{14}C -glucose marker

origin glucose * mannitol



origin glucose mannitol

* small peak was an impurity in the ^{14}C -mannitol supplied.

CHAPTER VIIISTUDIES ON OTHER LICHENS WITH GREEN ALGAL SYMBIONTS1. Lichens which contain Trebouxia

The carbohydrate physiology of Xanthoria aureola was described in detail in the preceding chapters. To see how far the pattern found in that species was generally applicable to other lichens containing Trebouxia, three further species were briefly studied. These were Lecanora conizaeoides, a crustose species; Parmelia saxatilis and Umbilicaria pustulata which are both foliose.

The first species proved difficult to sample as it consists of a rough fragile crust growing on twigs and branches of trees. The thallus was scraped off the host bark with the back of a scalpel and the small areolate crumbs of thallus then washed in distilled water and sampled by fresh weight. Parmelia saxatilis was treated in exactly the same way as Xanthoria aureola by sampling marginal thallus lobes. In Umbilicaria pustulata, 7 mm discs were cut out of the thallus in a similar manner to that used for Peltigera polydactyla.

All three species proved similar to Xanthoria in that ribitol was the compound which first accumulates most of the fixed ^{14}C , when samples were placed in the light on ^{14}C -sodium bicarbonate solutions. Pentitols could inhibit transfer of this ^{14}C -ribitol from alga to fungus resulting in the appearance of ^{14}C -ribitol in the medium. Unfortunately a number of these experiments were carried out over periods much shorter than 24 hours. In such cases, as in Xanthoria aureola, the amount of ^{14}C -ribitol released into the medium in the presence of ^{12}C -ribitol was small (Figures 47,48,49). The identification of the pentitol in the medium as ribitol was confirmed in exactly the same way as that described earlier for Xanthoria aureola.

2. Lichens symbiotic with Trentepohlia

Trentepohlia is more frequently encountered as the symbiont of tropical and subtropical lichens. In Britain most of the species containing this alga are crustaceous but one genus, Roccella, which is found on coastal rocks in south west England, is fruticose.

Two species of this genus were examined. It was already known that these lichens contained erythritol, arabitol and mannitol (Lindberg et al 1955). Work on the free-living Trentepohlia by Tischer (1936) had also shown that this alga

Figure 47

The release of fixed ^{14}C into the medium by samples of Lecanora conizaeoides during photosynthesis in the presence of various sugars. * Values for ^{14}C fixation given as counts per minute $\times 10^{-3}$.

Sugar	Total fixation	% insoluble	% medium
arabitol	351	2.24	0.78
ribitol	314	2.53	1.70
xylitol	240	2.80	1.15
glucose	225	3.32	0.80
water	468	2.32	0.34

Material: 400 mg fresh weight

* Period of photosynthesis: 3 hours

Temperature: 22°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: 10 μc per sample $\text{NaH}^{14}\text{CO}_3$

Medium: 3 ml distilled water + 50 μg per ml $\text{NaH}^{12}\text{CO}_3$

Experiment: 40

Date: 18.10.65.

Figure 48

The release of fixed ^{14}C into the medium by samples of Parmelia saxatilis during photosynthesis in the presence of various sugars.*
 Values for ^{14}C fixation given as counts per minute $\times 10^{-3}$.

Sugar	Total fixation	% insoluble	% medium
arabitol	217	5.7	0.76
ribitol	320	10.8	4.55
xylitol	336	8.2	4.11
glucose	267	6.8	0.21
water	556	12.0	0.13

Material: 100 mg washed lobes per sample

*~~See~~ Period of photosynthesis: 3 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: $10 \mu\text{c NaH}^{14}\text{CO}_3$ per sample

Medium: 3 ml distilled water, $50 \mu\text{g NaH}^{12}\text{CO}_3$
 + 1% sugars

Experiment: 39

Date: 18.10.65.

Figure 49

The release of fixed ^{14}C into the medium by samples of Umbilicaria pustulata during photosynthesis in the presence of various sugars.

Values for ^{14}C fixation given as counts per minute $\times 10^{-3}$.

Sugar	Total fixation	% medium	% insoluble
<u>6 hours</u>			
arabitol	38	2.26	1.29
ribitol	48	6.32	0.95
mannitol	37	0.48	1.61
glucose	34	0.40	2.00
water	35	0.22	1.25
<u>24 hours</u>			
ribitol	873	44.3	3.4
glucose	571	1.79	9.1
water	1075	4.09	3.5

Material: 10, 7 mm discs per sample

Period of photosynthesis: 6 and 24 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: 10 μc $\text{NaH}^{14}\text{CO}_3$ per sample

Medium: 3 ml distilled water

Experiment: 26

Date: 26.7.65.

contained erythritol. Thus it was not surprising when discs of Roccella fuciformis, floated in the light on ^{14}C -sodium bicarbonate solution, fixed ^{14}C into this compound.

a. Inhibition of carbohydrate transfer

It therefore seemed probable that the alga produced erythritol within the thallus and this passed to the fungus where it was converted into mannitol and arabitol. Attempts were made to inhibit this movement by including ^{12}C -erythritol in the medium. For comparison, ^{12}C -ribitol and ^{12}C -glucose were also tried as inhibitors. After three hours in the presence of these sugars and ^{14}C -bicarbonate, less than one per cent of the total fixed ^{14}C had been liberated into the medium.

Since it was possible that the transfer rate was slow as in Xanthoria aureola, the duration of the experiment was lengthened to 24 hours. Also samples of Roccella phycopsis as well as Roccella fuciformis were used to see whether the two species varied in rate of carbohydrate movement. In both species some 5-6% of the total fixed ^{14}C appeared in media containing erythritol after 24 hours photosynthesis, but much less radioactivity was found when other sugars were present (Figures 50 and 51).

In the above experiments there is a wide variation in total fixation between replicate samples. A further experiment was therefore done in which a form of subjective sampling was introduced to minimise this. After 5 mm discs had been cut, they were

Figure 50

The release of fixed ^{14}C into the medium by samples of Roccella fuciformis and Roccella phycopsis during photosynthesis in the presence of various sugars.* Values for ^{14}C fixation given as counts per minute $\times 10^{-3}$.

Sugar	<u>Roccella phycopsis</u>			<u>Roccella fuciformis</u>		
	Total fixation	% insoluble	% medium	Total fixation	% insoluble	% medium
ribitol	204	9.0	0.62	176	3.5	1.16
	513	5.1	0.63	357	3.7	1.01
erythritol	378	5.0	6.1	202	4.1	5.90
	237	5.3	5.1	371	2.7	3.71
glucose	472	6.9	2.02	248	3.2	1.15
	500	5.4	2.48	599	1.97	0.99
water	367	5.4	0.47	418	1.3	1.81
	311	5.6	0.43	936	1.4	1.39

Material: 20, 1 cm lengths Roccella phycopsis, 10, 7 mm discs Roccella fuciformis, per sample

*Period of photosynthesis: 24 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: 10 μC NaH $^{14}\text{CO}_3$ per sample

Medium: 3 ml distilled water, 200 μg ml NaH $^{12}\text{CO}_3$ + 2% sugars w/v

Experiment: 51

Date: 3.1.65.

Figure 51

The release of fixed ^{14}C into the medium by samples of Rocella fuciformis during photosynthesis in the presence of various sugars.

Values for ^{14}C fixation given as counts per minute $\times 10^{-3}$.

Sugar	Total soluble fixation	% medium
erythrose	68	3.3
	91	3.5
erythritol	564	11.6
	256	11.0
ribose	147	10.4
	318	9.3
ribitol	317	1.5
	114	1.0
galactose	378	0.9
	165	2.8
glucose	159	4.5
	158	3.5
glycollic acid	178	69.7
	252	60.2
glycerol	410	2.0
	128	1.9
water	701	1.2
	511	1.4

Material: 10, 5 mm discs per sample

Time: 24 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 2" specimen tubes

Radioactivity: 10 μc $\text{NaH}^{14}\text{CO}_3$ per sample

Medium: 3 ml distilled water + 1% sugars

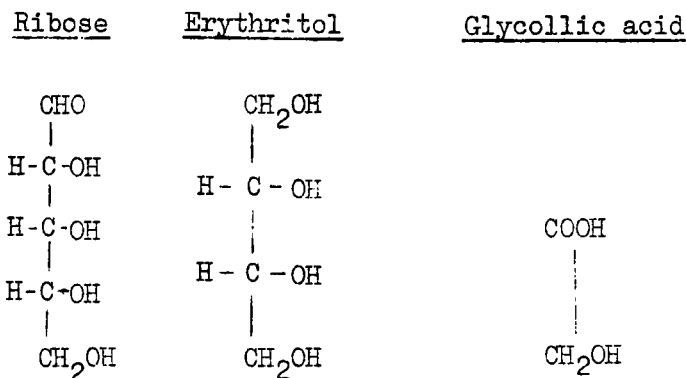
Experiment: 80

Date: 12.7.66.

sorted into thick and thin discs in Petri dishes containing moist filter paper. Discs of intermediate thickness as well as damaged or discoloured discs were discarded. Five thick and five thin discs were then used in each sample and attempts to inhibit carbohydrate transfer tried with a whole range of substances.

The results (Figure 51) show that erythritol as expected was the sugar that caused the most release of fixed ^{14}C into the medium. Some 11% of the total was excreted which was more than in earlier experiments but can be accounted for by the reduced disc size so allowing easier movement of sugars in and out of the tough thallus.

However, two other compounds, ribose and glycollic acid, caused the release of substantial amounts of ^{14}C into the medium. In the first case the most reasonable explanation would seem to be that there is a degree of molecular similarity between ribose and erythritol.



In the case of glycollic acid the pH of a 1% solution was 2.78. It therefore seemed possible that this low pH may have been having an

inhibitory effect on the fungal uptake mechanism or stimulating release of carbohydrate by the alga, or both. The fact that glycollic acid was not the compound passing between the symbionts was indicated by an examination of the fixed ^{14}C in the medium. This was found to be mainly in the form of ^{14}C -erythritol though some other ^{14}C compounds were also liberated when ^{12}C -glycollic acid was in the medium, but none of them was glycollic acid (Figure 52).

The effect of pH on carbohydrate transfer in Roccella fuciformis was therefore examined in more detail in a series of experiments described in the next section.

b. The effect of pH on the pattern of carbohydrate movement in Roccella

i. Effect of pH alone. Discs were placed in the light on distilled water buffered with McIlvaine phosphate-citrate buffer 0.14 to 0.2M and containing ^{14}C -sodium bicarbonate. After 24 hours, the amount of radioactivity in the medium was measured. The results (Figure 54) show that very little fixed ^{14}C was excreted into the medium at low pH.

ii. Effect of pH with added 1% erythritol. A similar experiment to the above was carried out except that erythritol was added to the medium. The results (Figure 55) show that a greater percentage of the total fixed ^{14}C is liberated into the medium at low pH when erythritol is present than when it is absent.

Figure 52

Autoradiographs of chromatograms of the media of samples of Roccella fuciformis after photosynthesis in the presence of erythritol, ribose or glycollic acid.

Also shown are autoradiographs of chromatograms of the media of samples of Peltigera aphthosa after photosynthesis in the presence of either ribitol or glucose.

Material: 10, 5 mm discs per sample Roccella fuciformis,
10, 7 mm discs of Peltigera aphthosa

Period of photosynthesis: 24 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: 10 μ c NaH ¹⁴CO₃ per sample

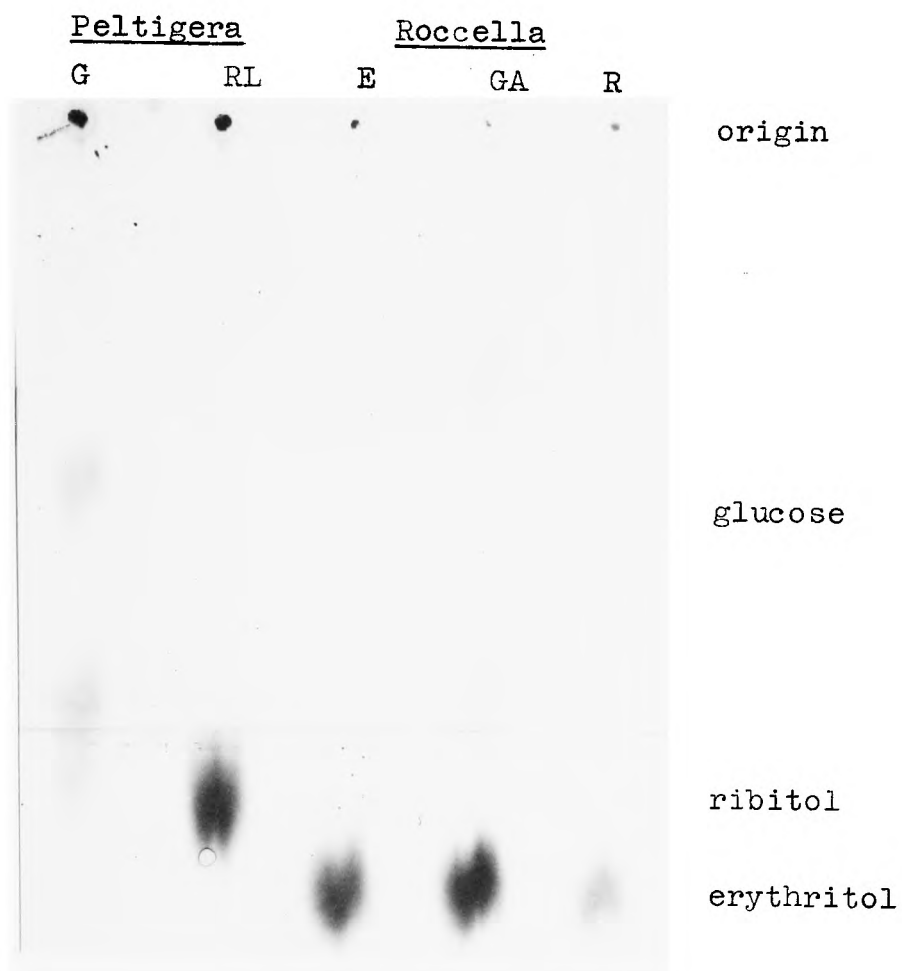
Medium: 3 ml distilled water with 1% added sugars

Experiment: 80

Solvent: EMK/AA/Baq

Paper: 3MM

Date: 12.7.66.



G = glucose
RL = ribitol

E = erythritol
GA = glycollic acid
R = ribose

Figure 54

The effect of pH on the release of fixed ^{14}C by discs of Roccella fuciformis. Values for ^{14}C fixation given as counts per minute $\times 10^{-3}$.

pH	Total fixation	% ^{14}C in medium	% ^{14}C in medulla	% ^{14}C in cortices soluble + insoluble
2.6	67	0.4	2.81	96.8
3.2	115	0.3	3.03	96.6
3.7	276	0.9	3.26	96.7
4.4	319	0.5	2.06	97.5
6.4	99	4.3	1.24	94.5
7.8	429	6.2	1.16	92.5

Material: 10, 5 mm discs per sample

Period of photosynthesis: 24 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: 10 μc per sample $\text{NaH}^{14}\text{CO}_3$

Medium: 3 ml distilled water buffered McIlvaine buffer

Experiment: 84b

Date: 1.9.66.

Figure 55

The effect of pH and added ^{12}C -erythritol on the release of ^{14}C by discs of Rocella fuciformis. * Values for ^{14}C fixation given as counts per minute $\times 10^{-3}$.

pH	Total fixation	% ^{14}C in medium	% ^{14}C in insoluble
2.6	446	16.3	2.5
3.2	598	8.1	2.8
4.4	586	6.7	2.3
6.4	742	11.3	1.8
7.8	392	0.7	1.3

Material: 10, 5 mm discs of Rocella fuciformis per sample

* Period of photosynthesis: 12 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: 10 μc $\text{NaH}^{14}\text{CO}_3$ per sample

Medium: 4 ml distilled water + 2% erythritol

Experiment: 84a

Date: 1.9.66.

In both cases the fixed ^{14}C is found in the medium almost exclusively as ^{14}C -erythritol, which contrasts with the situation when glycollic acid is present in the medium. Under the latter conditions erythritol is the principal substance released, but other ^{14}C compounds are present in significant amounts (Figure 53).

The mode of action of glycollic acid is still not clear; the above results show that they cannot be completely explained in terms of a pH effect.

c. The movement of carbohydrate from algal layer to medulla

The above experiments suggested that the rate of movement of fixed ^{14}C in this genus was very slow. To try and obtain further proof of this and to see what effect pH had on the movement of ^{14}C from algal layer to medulla the following experiments were carried out.

Discs of Rocella fuciformis were placed in the light on ^{14}C -sodium bicarbonate solution. After 3 and 24 hours, the discs were removed, washed in ice-cold water and then the upper and lower cortices and algal layers dissected from the medulla. The algal layers and medulla were killed and stored separately in 80% ethanol. The amount of ^{14}C in each tissue was determined and the results (Figure 56) show that less than 4% of the total fixed ^{14}C found its way to the medulla even after 24 hours. These results confirmed those in a previously described experiment (Figure 54) which showed that very little fixed ^{14}C passed from algal layer to medulla in media containing buffer approximately pH 3 - 8.

Figure 53

Autoradiographs of the chromatograms of media of Roccella fuciformis after photosynthesis in distilled water media containing 1% erythritol or 1% glycollic acid, or in buffered media containing 1% erythritol. This shows that except in the case where glycollic acid was present in the medium, the ^{14}C liberated was only in the form of ^{14}C -erythritol. Even where glycollic acid was present in the medium no ^{14}C -glycollic acid was released therein.

Material: 10.5 mm discs Roccella

Period of photosynthesis: 12 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: 10 μc per sample $\text{NaH } ^{14}\text{CO}_3$

Medium: 3 ml distilled water + McIlvaine buffer +
1% sugars

Experiment: 84

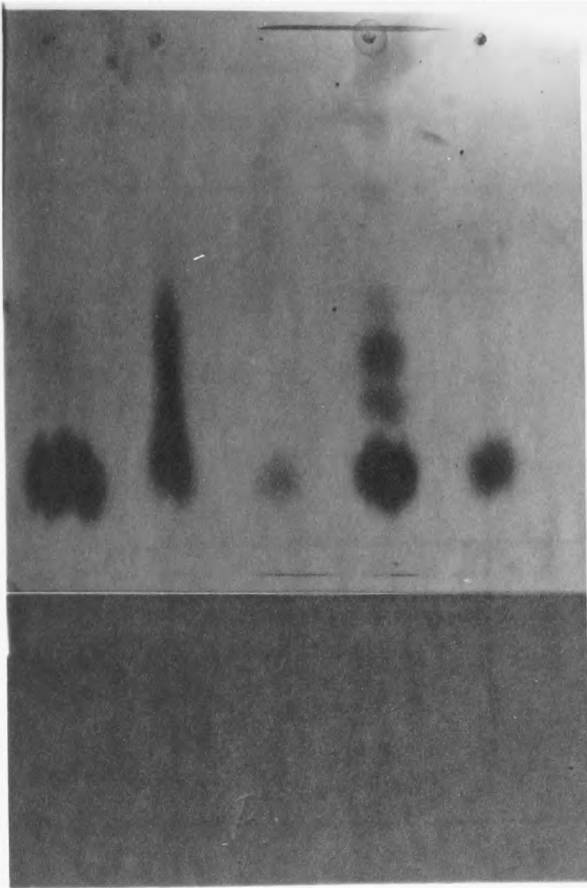
Solvent: Bu/AA/aq

Paper: 3MM

Date: 1.9.66.

distilled water
buffered to pH
2.3 3.2 4.4

distilled water(unbuffered pH 5.8)
+ +
GA E



origin

erythritol

glycollic acid

autoradiograph

marker chromatogram

GA = glycollic acid

E = erythritol

Figure 56

The distribution of fixed ^{14}C after 3 and 24 hours photosynthesis in samples of Roccella fuciformis. Per cent total fixation in each part of the thallus and in each fraction.

	% soluble	% insoluble	% medium
<u>3 hours</u>			
upper & lower algal layers	91.4 91.6	3.2 3.5	2.0 2.1
medulla	3.4 3.5	0.07 0.07	
<u>24 hours</u>			
upper & lower algal layers	88.9 88.8	5.2 5.2	2.8 2.0
medulla	3.0 3.8	0.14 0.15	

Material: 5, 5 mm discs

Period of photosynthesis: 3 and 24 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: 10 μc $\text{NaH}^{14}\text{CO}_3$ per sample

Medium: 3 ml distilled water

Experiment: 81

Date: 12.7.66.

d. Other species containing Trentepohlia

Gylecta cupularis and Lecanactis stenhamerii were briefly studied.

Both are crustaceous species living on limestone. The rock was chipped off by hammer and chisel with the adhering lichen, cut into small pieces with a rock cutting machine and then the lichen washed in distilled water.

Chips of rock were placed in the light in solutions of ^{14}C -sodium bicarbonate in the presence of ^{12}C -erythritol. It was found that fixed ^{14}C was liberated into the medium in both lichens and chromatograms showed that this ^{14}C was in the form of erythritol.

In all the lichens containing Trentepohlia only small amounts of ^{14}C -mannitol were produced after 24 hours photosynthesis (Figure 57) which suggested that either transfer of fixed carbon or conversion of the ^{14}C -erythritol into mannitol was slow. However, these lichens were able to convert ^{14}C -glucose into ^{14}C -mannitol within three hours and hence not all aspects of the metabolism may be slow as the foregoing experiments seem to indicate.

3. Species containing Myrmecia

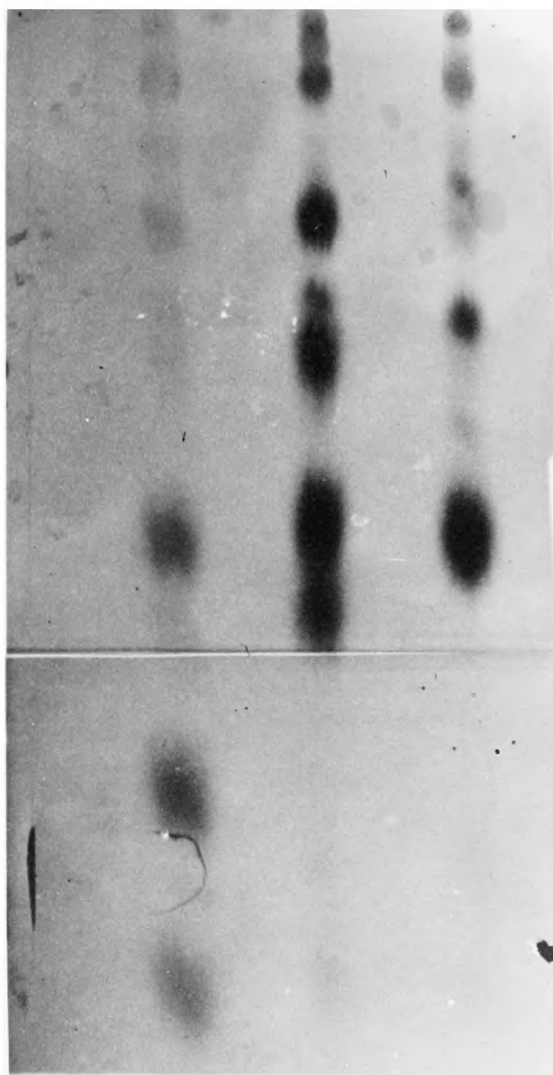
This alga is symbiotic in some species of the genus Dermatocarpon (Ahmadjian 1958). Some of the species of this genus are of interest because they contain volemitol, a seven carbon sugar alcohol (Lindberg 1955).

Figure 57

Autoradiographs of chromatograms of the soluble extract of
Gylecta cupularis and Dermatocarpon miniatum.

Material: 5, 7 mm discs Dermatocarpon
Period of Photosynthesis: 24 hours
Temperature: 18°C
Light: 500 fc
Vessels: 2 x 1" specimen tubes
Radioactivity: 10 µc NaH ¹⁴CO₃ per sample
Medium: 3 ml distilled water
Experiment: 75
Solvent: EMK/AA/Baq
Paper: no. 1
Date: 14.6.66.

Gylecta Dermatocarpon Solorina
cupularis miniatum saccata



origin
? trehalose
sucrose

glucose

? fructose
volemitol

mannitol
unknown

arabitol

erythritol

a. Photosynthesis by the thallus

The physiology of Dermatocarpon miniatum was therefore examined, but unfortunately it is found only on limestone and in small localised areas. As a result, only a few experiments could be carried out. The thallus was sampled by punching 7 mm discs from the thallus in a manner similar to that described earlier for Peltigera polydactyla.

In the first experiment samples were incubated in the light on a solution of ^{14}C -sodium bicarbonate. The ethanol-soluble components were examined by one dimensional chromatography in EMK/AA/Baq solvent. A number of ^{14}C -labelled compounds were found which were provisionally identified as sucrose, glucose and volemitol. However, much of the radioactivity was incorporated into an unknown spot which ran just ahead of mannitol (Figure 57).

b. Inhibition of carbohydrate transfer by various sugars

Very little fixed ^{14}C was liberated into the medium over a three hour period of photosynthesis when ^{12}C -glucose was present (Figure 58). In a further experiment, lasting 24 hours, glucose was found to be a better inhibitor of carbohydrate transfer than ribitol. However, even after this time, only 7% of the total fixed ^{14}C appeared in media containing ^{12}C -glucose.

In the same experiment the lichen discs were washed with ice-cold distilled water and the algal layer dissected from the medulla. The amount of ^{14}C in the medulla and algal layers was then determined

Figure 58

The release of fixed ^{14}C into the medium by samples of Dermatocarpon miniatum and Dermatocarpon fluviatile during photosynthesis in the presence of glucose.* Values for ^{14}C fixation given as counts per minute $\times 10^{-3}$.

Dermatocarpon miniatum

	Fixation	% ^{14}C in insoluble	% ^{14}C in medium
water	918	1.08	0.27
glucose	270	0.73	1.52

Dermatocarpon fluviatile

water	127	1.00	1.09
glucose	55	3.77	1.91

Material: subjectively sampled (95-128 mg fresh weight)

*Period of photosynthesis: $3\frac{1}{4}$ hours

Temperature: 18°C

Light: 500 fc

Vessels: 50 ml conical flasks

Radioactivity: 7 ml distilled water + 2% glucose

Medium: 10 μC $\text{NaH}^{14}\text{CO}_3$ per sample

Experiment: 27, 30

Date: 17.8.65.

separately. Figure 59 shows that the control discs on distilled water contained some 20% of the total fixed ^{14}C in the medulla, while those on glucose contained 15.4%. Since the presence of glucose caused only 7% of the total fixed ^{14}C to be released to the medium, it would seem that glucose was not a very successful inhibitor of carbohydrate transfer. For example, in Peltigera polydactyla, Drew (1966) found that ^{12}C -glucose almost completely inhibited transfer of ^{14}C to the medulla.

Two other sugars were also tried, sedoheptulose and erythrose. The former was used because the lichen contain volemitol (which is a reduction product of sedoheptulose) while erythrose was tried because there were indications of a carbohydrate with a high Rf being formed in photosynthesis. The results of the experiment are shown in Figure 60 which illustrates that erythrose was effective in that 41% of the total fixed ^{14}C appeared in the medium. However, one dimensional paper chromatography of the medium indicated that the ^{14}C was not in the form of erythrose but had an R_f glucose similar to the unknown substance shown in Figure 57, i.e. it ran just ahead of mannitol (Figure 61). It is probable that this is the same compound as that found in the thallus but this has not been confirmed as no more material of this species has been found.

c. Conclusions

Dermatocarpon miniatum seems to have a carbohydrate pattern distinct from that found in any of the lichens studied.

Figure 59

The distribution of fixed ^{14}C between algal layer and medulla after 24 hours photosynthesis in samples of Dermatocarpus miniatum in the presence of various sugars. Per cent total fixation given for each part of the thallus and for each fraction.

		Soluble	Insoluble	Medium
ribitol	algal layer	71.7	10.9	1.5
	medulla	11.0	5.0	
glucose	algal layer	70.6	6.2	7.7
	medulla	13.4	2.0	
water	algal layer	76.2	2.3	1.6
	medulla	18.3	1.1	

Material: 5, 7 mm discs per sample

Period of photosynthesis: 24 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: 10 μC $\text{NaH}^{14}\text{CO}_3$ per sample

Medium: 3 ml distilled water + 1% sugars

Experiment: 75

Date: 14.6.66.

Figure 60

The release of fixed ^{14}C by samples of Dermatocarpon miniatum during photosynthesis in the presence of sedoheptulose, erythrose or ribitol.* Values for ^{14}C fixation given as counts per minute $\times 10^{-3}$.

	Total fixation	% ^{14}C in insoluble fraction	% ^{14}C in medium
ribitol	437	1.8	2.6
water	1050	1.1	1.8
erythrose	536	1.5	41.7
sedoheptulose	433	7.5	1.40

Material: 10, 7 mm discs

* Period of photosynthesis: 24 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: 10 μc per sample $\text{NaH } ^{14}\text{CO}_3$

Medium: 3 ml distilled water + 1% sugars

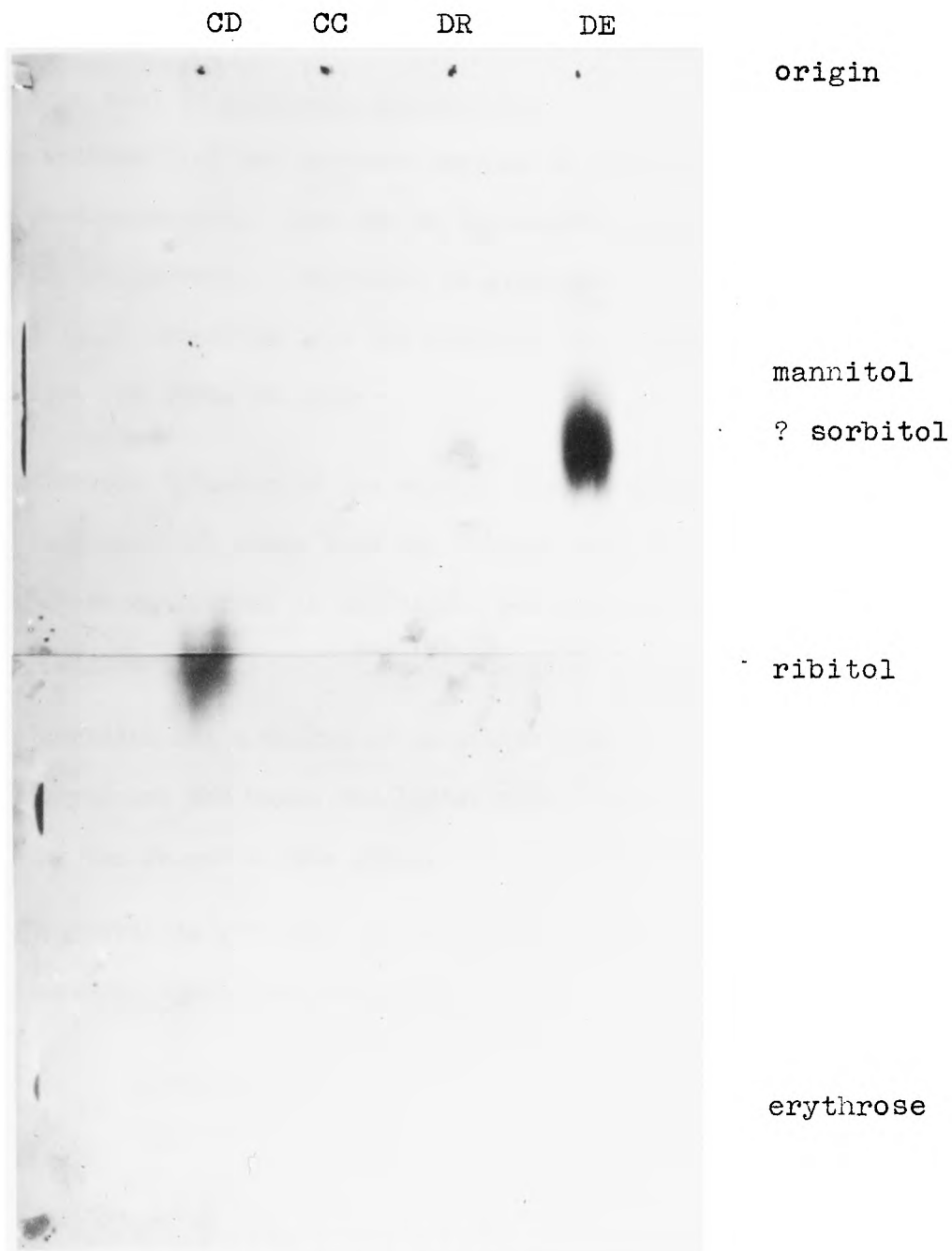
Experiment: 75

Date: 14.6.66.

Figure 61

Autoradiographs of chromatograms of the media of samples of Dermatocarpon miniatum which had photosynthesised on solutions of ^{14}C -sodium bicarbonate to which had been added 1% erythrose. (Also shown is the medium in which directly isolated and cultured cells of Coccomyxa had photosynthesised.)

Material: 10, 7 mm discs
Period of photosynthesis: 12 hours
Temperature: 18°C
Light: 500 fc
Vessels: 2 x 1" specimen tubes
Radioactivity: 10 μc per sample $\text{NaH}^{14}\text{CO}_3$
Medium: 3 ml distilled water + 1% sugars
Experiment:
Solvent: EMK/AA/Baq
Paper: 3MM
Date: 14.6.66.



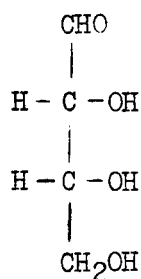
CD = directly isolated Coccomyxa
CC = cultured Coccomyxa
DR = Dermatocarpon miniatum + ribitol
DE = Dermatocarpon miniatum + erythrose

Carbohydrate movement from the algal layer to the medulla of the thallus appears to be more rapid than that found in Roccella but slower than that in Peltigera polydactyla.

An unidentified carbohydrate appears to become labelled with ^{14}C during photosynthesis. This has an R_g slightly greater than mannitol in EMK/AA/Baq solvent. Erythrose is apparently able to cause this compound to be liberated into the medium. This unknown compound might be sorbitol for three reasons:

- a. isotope dilution of the soluble extract with sorbitol and mannitol shows that the unknown substance co-chromatograms in EMK/AA/Baq solvent with sorbitol (Figure 62);
- b. sorbitol has a degree of molecular similarity to erythrose and hence the latter might inhibit uptake by the fungus in its stead;
- c. sorbitol is probably the commonest hexitol found in nature, apart from mannitol.

Erythrose



Sorbitol

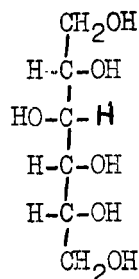


Figure 62

Isotope dilution of the soluble extracts of Dermatocarpon miniatum with sorbitol and mannitol, and glucose.

d+M = ^{14}C -soluble extract of Dermatocarpon diluted with ^{12}C -mannitol
 D+G = " " " " " ^{12}C -glucose
 D+S = " " " " " ^{12}C -sorbitol

The autoradiograph occupies the centre of the figure on which the radioactive areas appear as dark spots. The chromatograms from which the autoradiograph was made are placed on either side. These have been developed with alkaline silver nitrate so that the carbohydrates appear as black spots. It may be seen that the radioactive areas and the black spots of the marker sugar alcohols closely correspond.

Material: 10, 7 mm discs

Period of photosynthesis: 36 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: 10 μc per sample $\text{NaH}^{14}\text{CO}_3$

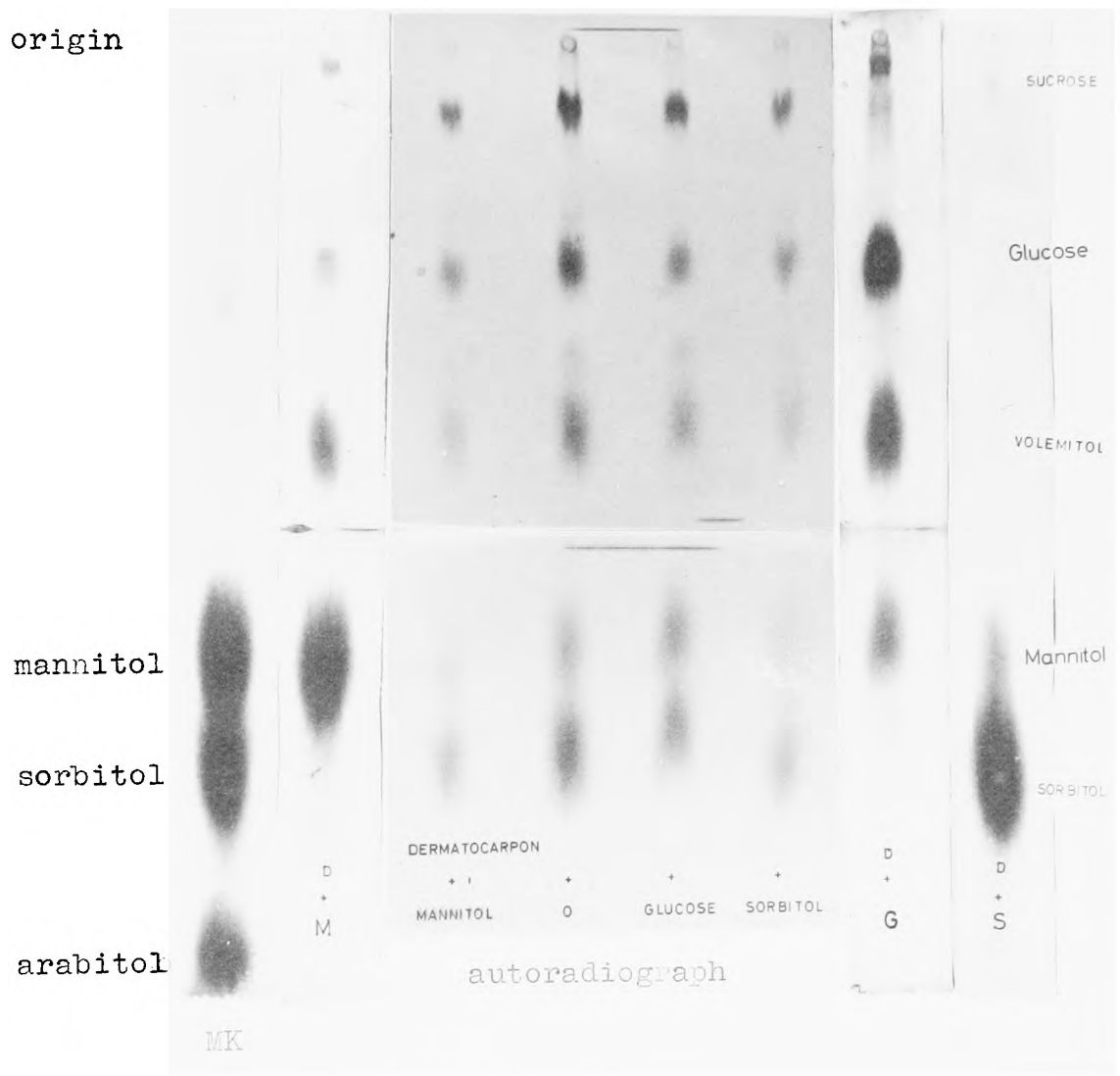
Medium: 3 ml distilled water + 1% sugars

Experiment:

Solvent: EMK/AA/Baq

Paper: Whatman no. 1

Date: 14.6.66.



MK = marker chromatogram
 (mannitol, sorbitol, arabitol)

CHAPTER IXSTUDIES ON LICHENS CONTAINING MORE THAN ONE ALGA1. Introduction

A number of lichens contain two different types of algae. In these cases a green alga is symbiotic in the greater part of the thallus, while a blue-green alga either occupies delimited small areas within the thallus or occurs as outgrowths from the surface. These structures containing blue-green algae are called cephalodia. It has been suggested that they may enable the whole thallus to benefit from atmospheric nitrogen fixed by the blue-green alga (Ozenda 1963) although no proof of this is yet available.

Cephalodia are usually regarded as specialised structures that result from the lichen fungus surrounding and becoming symbiotic with a blue-green alga. Although this is the generally accepted mode of origin of cephalodia, not all authors agree with this. Thus Hale (1961) states that 'they are in reality very tiny lichen thalli containing blue-green algae different from the alga of the host. Cephalodia apparently originate from soredia of foreign lichens which land on the appropriate host and send out rhizoidal hyphae and

eventually become an integral part of the host plant'. Hale gives no details to support his view but the evidence against his hypothesis is of two types:

a. Circumstantial evidence

Cephalodia only occur in a few species of lichens but in these cases they are constantly and abundantly present. If their origin were from foreign soredia, one would expect a more random distribution and a greater variability in abundance. Furthermore, lichens containing blue-green algae hardly ever form soredia and the one or two species which do are rare in the British Isles and certainly have a more limited distribution than lichens which bear cephalodia. Finally there is the fact that cephalodia never bear apothecia or other reproductive structures which suggests that they are indeed one fungus symbiotic with two different algae.

b. Developmental studies

The development of cephalodia has been examined by Babikoff (1878), Schneider (1897), Sernander (1907) and more recently by Moreau and Moreau (1919-1921) and Abbayes (1951).

Three lichens bearing cephalodia were examined in the present study; Peltigera aphthosa, Lobaria amplissima and Solorina saccata. A brief description of the structure and the mode of origin of the cephalodia in each species follows, based on the observations of

the authors mentioned above.

Peltigera apthosa. The cortical hyphae in this species grow out of the upper surface of the thallus into fine hair-like projections. Within these are found minute colonies of Nostoc which stimulate the hyphae to multiply, increase in size and gradually form a complete cortex over the algal cells, so forming minute cephalodia.

Solorina saccata. Blue-green algal cells are surrounded as above but this usually occurs on the lower surface of the lichen. The algae are then drawn into the interior of the thallus. Here they increase in size, but Moreau found that eventually many of the blue-green algal cells within the cephalodia died.

In Lobaria amplissima the cephalodia are first formed on the under surface of the thallus, gradually grow upwards and emerge through the upper surface where they form small fruticose thalli. Very occasionally, for example in New Zealand, these cephalodia are found without the rest of the thallus and have been assigned to a genus Dendriscoaulon. It is thought that such plants may be formed by the meeting of germinating fungal spores with filaments of blue-green algae (Ozenda 1963).

Thus all these investigations indicate that cephalodia originate from the association of the lichen fungus with a few

blue-green algal filaments. There is hence considerable interest in comparing the pattern of carbohydrate transfer in cephalodia with that in the parent thallus, because assuming that the fungus is the same in both cases it will be possible to assess the importance of the alga in determining the various features of carbohydrate transfer.

2. Studies on the carbohydrate metabolism of *Lobaria amplissima*

This is the only species of the genus which has cephalodia. The other species either contain only Trebouxia or only Nostoc sp.

The pattern of carbohydrate movement between the symbionts was examined in all British species of Lobaria and the results are given in the paper which forms appendix A of this thesis.

The results indicated that the pattern of carbohydrate transfer of the species containing Trebouxia was similar to that of Xanthoria aureola, whereas those species that were symbiotic with Nostoc, including the cephalodia of Lobaria amplissima, had a pattern of carbohydrate movement closely comparable to Peltigera polydactyla.

3. Studies on the carbohydrate metabolism of *Solorina saccata* and *Peltigera aphthosa*

In these lichens the overall thallus contains Coccomyxa sp; but there are also internal cephalodia containing Nostoc. These could not be excised and examined separately as had been done with the cephalodia of Lobaria amplissima.

a. Solorina saccata

After 24 hours photosynthesis on a solution of ^{14}C -sodium bicarbonate, most of the ^{14}C was incorporated into mannitol (Figure 63). Studies on the isolated Coccomyxa, which had been in culture some years, indicated that the alga formed glucose and sucrose as photosynthetic products. Hence, it was thought that the pattern of carbohydrate movement in this species would be similar to that in Peltigera polydactyla with both the Coccomyxa and Nostoc producing ^{14}C -glucose which would pass to the fungus and there be converted into ^{14}C -mannitol.

However, attempts to inhibit transfer of ^{14}C -carbohydrate with ^{12}C -glucose showed that only a small amount of ^{14}C -glucose was liberated into the medium and there was only a slight reduction in the amount of ^{14}C -mannitol found in the thallus (Figure 63).

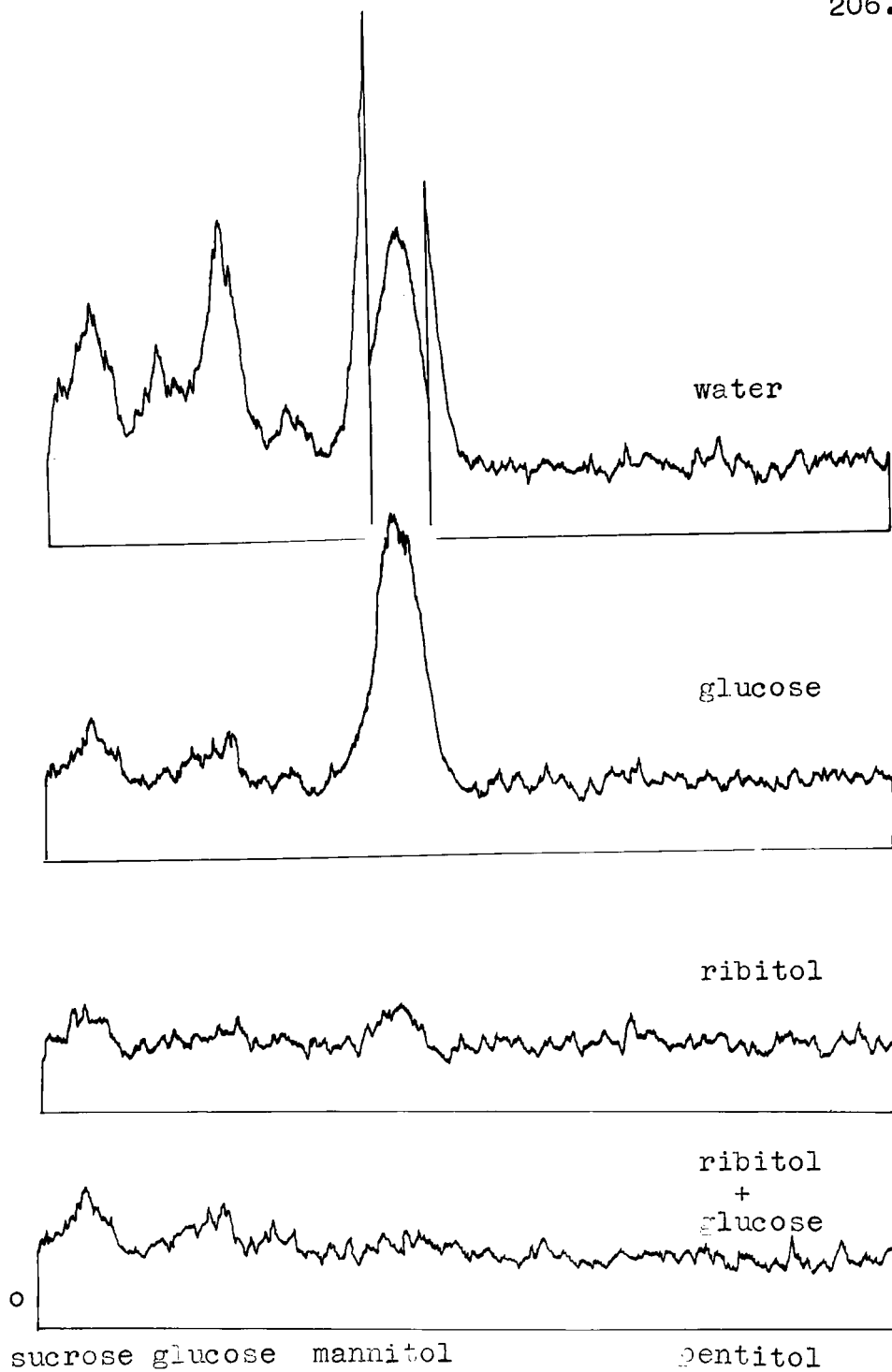
When ^{12}C -ribitol was included in the medium, there was a very considerable reduction in the amount of ^{14}C -mannitol built up and ^{14}C -ribitol was released into the medium (Figure 64).

This suggested by analogy with Lobaria amplissima that Coccomyxa was producing ^{14}C -ribitol and the Nostoc ^{14}C -glucose. To try and inhibit carbohydrate movement from both algae, a mixture of ^{12}C -glucose and ^{12}C -ribitol was included in the medium. In this case considerable quantities of both ^{14}C -ribitol and ^{14}C -glucose appeared in the medium (Figure 64), and no ^{14}C -mannitol was found in the thallus (Figure 63). In fact very little ████████ radioactivity remained in the soluble carbohydrates within the thallus after 24 hours.

Figure 63

Scans of chromatograms of the soluble fraction of samples of Solorina saccata, after photosynthesis, in the presence of various sugars.

Material: 10, 7 mm discs per sample
Period of photosynthesis: 24 hours
Temperature: 18°C
Light: 500 fc
Vessels: 2 x 1" specimen tubes
Radioactivity: 10 μ c per sample
Medium: 3 ml distilled water \pm 1% sugars
Experiment:
Solvent: EMK/AA/Baq
Date: 15.6.65.

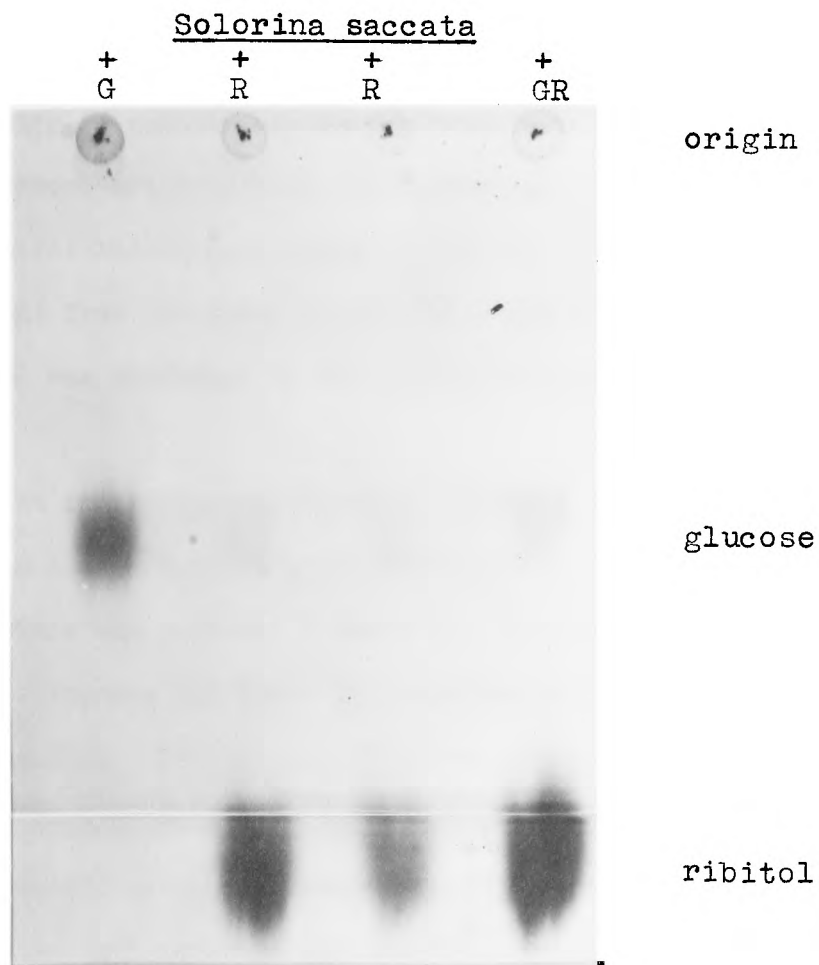


o = origin

Figure 64

Autoradiographs of chromatograms of media on which samples of Solorina saccata had photosynthesised in the presence of ^{14}C sodium bicarbonate and various sugars.

Material: 10, 7 mm discs per sample
Period of photosynthesis: 24 hours
Temperature: 18°C
Light: 500 fc
Vessels: 2 x 1" specimen tubes
Radioactivity: 10 μc per sample
Medium: 3 ml distilled water \pm 1% sugars
Experiment:
Solvent: EMK/AA/Baq
Paper: no 3MM



G = glucose

R = ribitol

GR = glucose + ribitol

b. Peltigera aphthosa

Though structurally similar to Solorina saccata, this species shows a number of points of contrast in physiological behaviour. After three hours photosynthesis on a solution of ^{14}C -sodium bicarbonate, the radioactivity is principally incorporated into ribitol and mannitol. However, after 24 hours photosynthesis there was very little ^{14}C -ribitol but an appreciable amount of ^{14}C -arabitol formed. This was evident from the scans of chromatograms run in EA/AA/Baq (Figure 65) and was confirmed by the isotope dilution method (Figure 66).

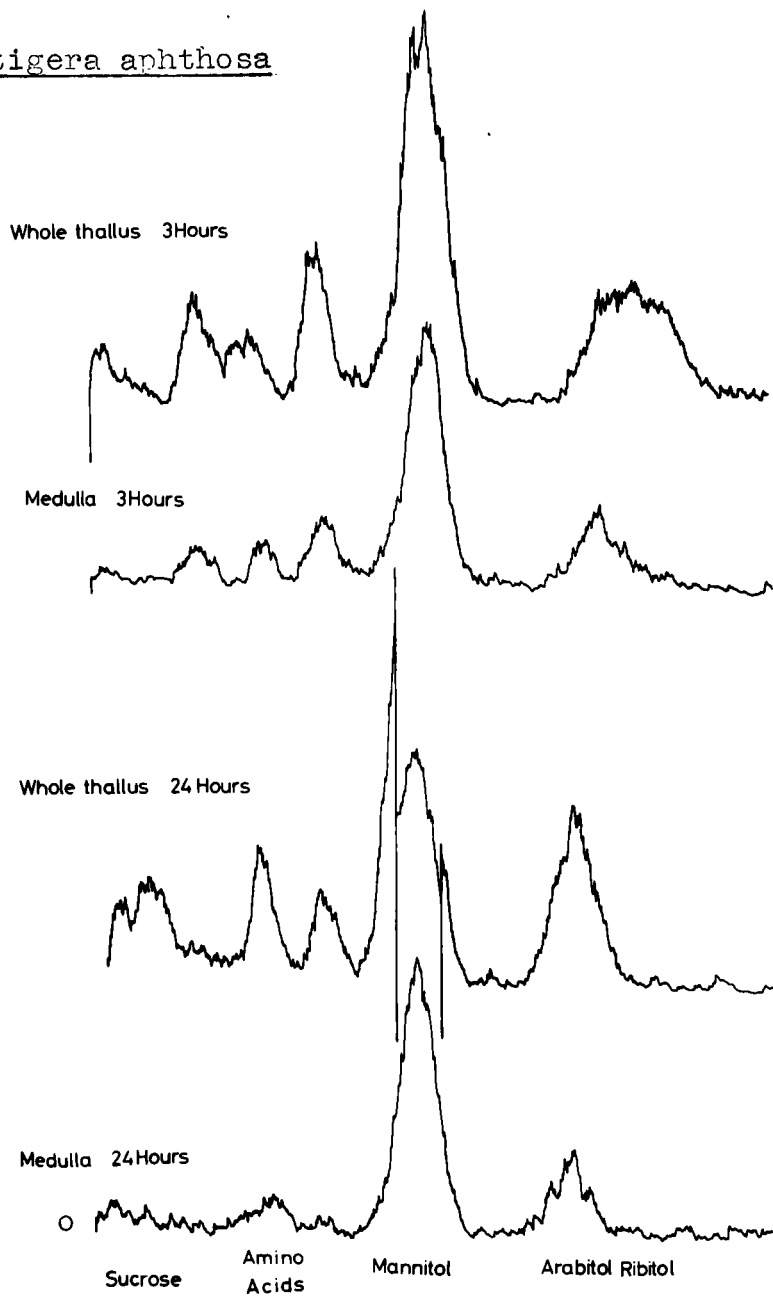
Attempts at inhibiting carbohydrate transfer showed that ^{12}C -glucose was less effective as an inhibitor in this species than in Solorina. This was probably because the cephalodia are much smaller in this species and hence the relative amount of Nostoc in the samples was less. Ribitol was effective in preventing the formation of ^{14}C -mannitol within the thallus and it also caused some 12% ^{14}C -ribitol to be liberated into the medium within three hours, suggesting that carbohydrate transfer was again much faster than in Xanthoria aureola (Figure 67). It is also of interest that a much larger proportion of fixed ^{14}C appeared in the insoluble fraction under these conditions.

Since ^{14}C -arabitol appeared in the thallus after some hours photosynthesis, and since the work on the cultured alga of this species did not show that ribitol was a photosynthetic product of

Figure 65

Scans of chromatograms of the soluble fraction of samples of Peltigera aphthosa after photosynthesis in the presence of ^{14}C sodium bicarbonate for 3 and 24 hours. The discs of the thallus were dissected into an algal layer and a medulla.

Material: 10, 7 mm discs per sample
Period of photosynthesis: 3 and 24 hours
Temperature: 18°C
Light: 500 fc
Vessels: 2 x 1" specimen tubes
Radioactivity: 10 μc $\text{NaH}^{14}\text{CO}_3$ per sample
Medium: 3 ml distilled water
Experiment: 78
Solvent: EMK/AA/Baq
Date: 5.7.66.

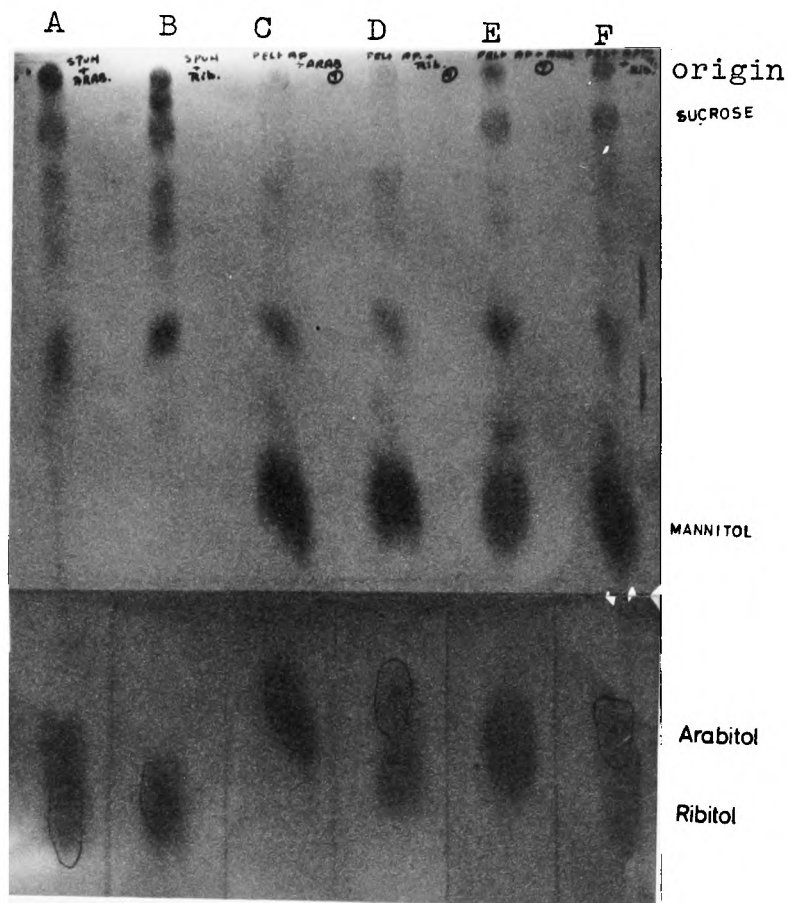
Peltigera apthosa

o = origin

Figure 66

Isotope dilution of the soluble fraction of Peltigera aphthosa with arabitol and ribitol and also of the directly isolated alga Coccomyxa.

Material: 10, 7 mm discs per sample
Period of photosynthesis: 3 and 24 hours
Temperature: 18°C
Light: 500 fc
Vessels: 2 x 1" specimen tubes
Radioactivity: 10 μc NaH $^{14}\text{CO}_3$ per sample
Medium: 3 ml distilled water
Experiment:
Solvent: EMK/AA/Baq
Time: 30 hours



A = directly isolated *Coccomyxa*; ^{14}C soluble fraction + ^{12}C -arabitol
 B = " " " ; ^{14}C " " + ^{12}C -ribitol
 C & E = *Peltigera aphthosa*; ^{14}C soluble fraction + ^{12}C -arabitol
 D & F = " " ; " " " + ^{12}C -ribitol

The radioactive areas in the pentitol region of the autoradiograph have been outlined in black. The autoradiograph has then been placed on top of the chromatogram on which the ^{12}C pentitols have been developed with alkaline silver nitrate. It may be seen that in 'B' the radioactive pentitol corresponds with ribitol but in 'C & E' it corresponds with arabitol.

Figure 67

The release of fixed ^{14}C into the medium by samples of Peltigera
aphthosa during photosynthesis in the presence of various sugars.
Values for ^{14}C fixation given as counts per minute $\times 10^{-3}$.

Sugar	Total fixation	% insoluble	% medium
<u>3 hours</u>			
water	506	29	4.67
ribitol	485	31	12.65
glucose	438	30	3.30
ribitol + glucose	224	33	9.18
<u>24 hours</u>			
water	544	34	4.72
ribitol	496	40	29.29
glucose	392	41	8.1
glucose + ribitol	145	39	26.28

Material: 10, 7 mm discs per sample

Period of photosynthesis: 3 and 24 hours

Temperature: 18°C

Light: 500 fc

Vessels: 2 x 1" specimen tubes

Radioactivity: 10 μc $\text{NaH}^{14}\text{CO}_3$ per sample

Medium: 3 ml distilled water + 1% sugars

Experiment:

Date:

Coccomyxa, an experiment was carried out on the directly isolated alga of Peltigera aphthosa using techniques of low speed differential centrifugation of homogenates. It proved possible to make a very pure preparation of this alga free from Nostoc and fungal fragments of the thallus because the Coccomyxa was very small and remains in suspension after the other elements have been precipitated by centrifuging at approximately 250 g for 2-5 minutes.

Photosynthesis by the directly isolated alga on ^{14}C -sodium bicarbonate solution showed that ^{14}C -ribitol was formed and that this was excreted into the medium within three hours. A culture of Coccomyxa from Peltigera aphthosa (Bednar and Holm-Hansen 1964) did not produce ribitol or excrete it on to the medium. Instead the principal products ^{SUCROSE and} formed appeared to be organic acids (Figure 68).

It seemed that the directly isolated alga released more of the products of photosynthesis into the medium than the cultured form, a feature previously noted here for Trebouxia as well as for Nostoc (Drew 1966) from Peltigera polydactyla.

4. Conclusions

These studies show that Coccomyxa, like Trebouxia, releases ribitol when it is symbiotic in the thallus. However, in both Solorina saccata and Peltigera aphthosa the rate of carbohydrate transfer is considerably faster than in any of the species studied

Figure 68

Scans of chromatograms of the soluble fraction of the cultured and directly isolated alga Coccomyxa from Peltigera aphthosa. Also shown is the proportion of ^{14}C fixation that was released into the medium and incorporated into the insoluble fraction. Values for ^{14}C fixation given as counts per minute $\times 10^{-3}$.

	Total fixation	% insoluble	% medium
Directly isolated	832	6.4	20.0
Cultured	746	18.9	10.2

Material: 0.2 cm wet packed volume of algae per sample

Period of photosynthesis: 3 hours

Temperature: 18°C

Light: 500 fc

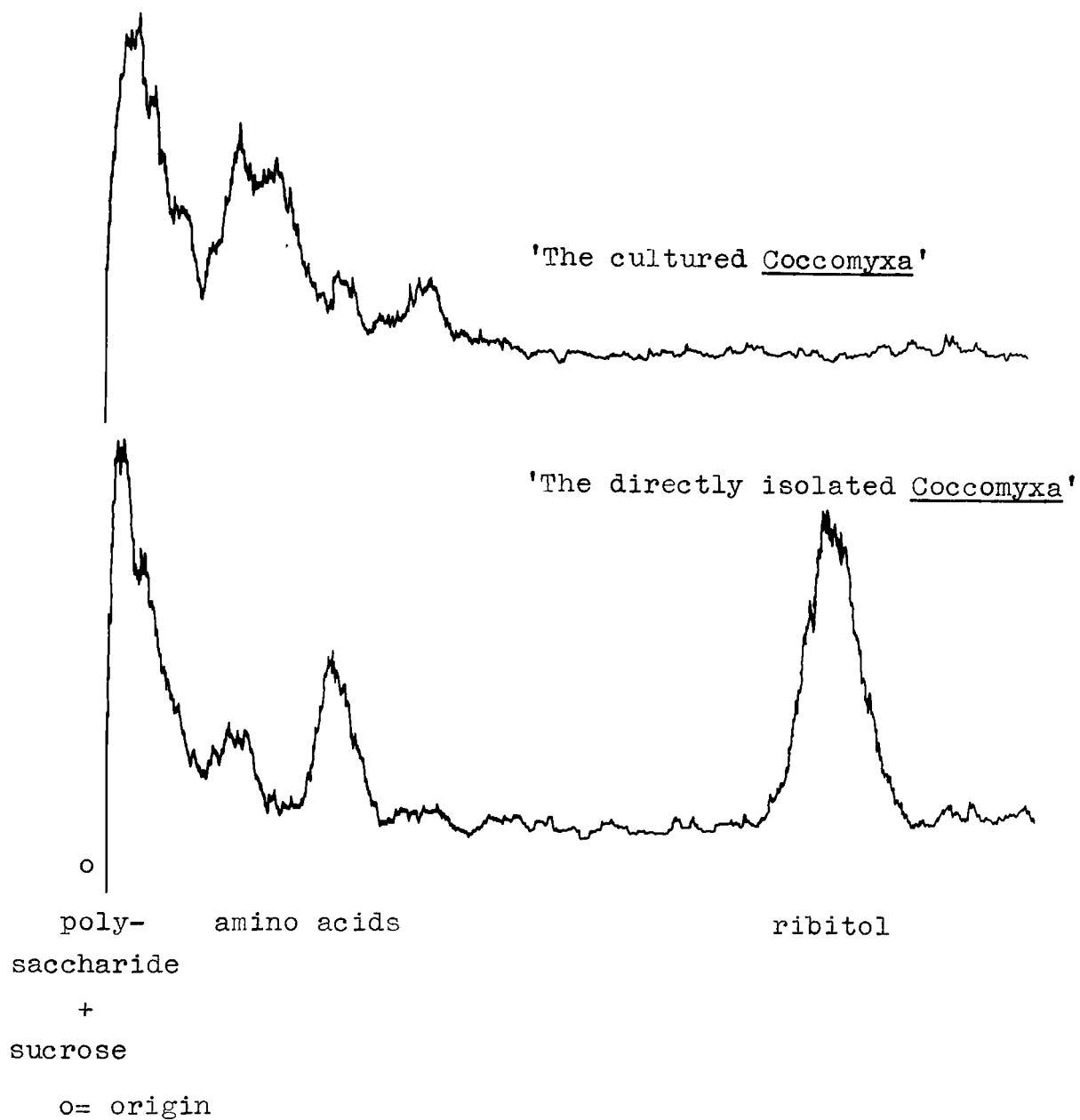
Vessels: 2 x 1" specimen tubes

Radioactivity: 20 μc $\text{NaH}^{14}\text{CO}_3$ per sample

Medium: 2.5 ml distilled water

Experiment: 79

Date: 7.7.66.



which contained Trebouxia. This is further evidence for the predominant role of the alga in determining the pattern of carbohydrate transfer.

It is also of interest that in the cephalodia, fixed ^{14}C passes from alga to fungus. These structures have traditionally been regarded as sources of nitrogen; however, these studies show that, in addition, they may supply a small amount of carbohydrate to the thallus.

SECTION C

Other Aspects of the Biology of Xanthoria

CHAPTER XABSORPTION AND LOSS OF CARBOHYDRATES IN LICHENS1. Introduction

Harley & Smith (1966), and Smith (1960a,b,c; 1961,1963), showed that discs of the lichen Peltigera polydactyla had very efficient mechanisms for the accumulation of phosphates, amino acids and sugars from aqueous solutions. Thus, samples of this lichen could absorb more than 25% of their own dry weight of glucose from a 1% solution in 24 hours at 25°C. Preliminary studies on Parmelia physodes, Peltigera rufescens and Cladonia sp showed them to have similar efficient uptake mechanisms.

The previous chapters have indicated the important role of sugar alcohols in the carbohydrate metabolism of various lichens and yet little is known of the uptake of these substances, their loss from the thallus on starvation, or the variation in content at different seasons of the year.

2. Uptake of sugar alcohols by lichens

Drew (1966) showed that Peltigera polydactyla could absorb mannitol at approximately half the rate of glucose. More recently, Smith and Ozin (unpublished) confirmed that this lichen could absorb mannitol but found that it could not take up ribitol in appreciable amounts. By contrast, Roccella fuciformis, which contains arabitol, erythritol and mannitol in the thallus, could take up considerable quantities of ribitol as well as these other sugar alcohols.

Since ribitol has been shown to be produced by the alga in Xanthoria aureola, its uptake was compared with that of the other pentitols and mannitol.

Marginal lobes of Xanthoria aureola were incubated in the dark at 18°C in 1% solutions of D-arabitol, xylitol, ribitol and mannitol. After 0, 3, 6 and 20 hours, small aliquots of the medium were withdrawn, diluted and the concentration of sugar alcohol determined by the method of Lewis and Harley (1965). The results (Figure 69) show that all the compounds were taken up rapidly and at similar rates. Approximately 50% of the amount of polyol that was absorbed in 20 hours was taken up in the first three hours incubation.

These results show that the lichen is capable of absorbing ribitol. The fact that xylitol can be absorbed even though it does not occur in lichens agrees with the observation that this pentitol, as well as arabitol and ribitol, can inhibit the movement of ^{14}C -ribitol between the symbionts.

Figure 69

The amount of sugar alcohols in mg absorbed by 100 mg fresh weight of Xanthoria in the dark, measured by the method of Harley and Lewis (1965). Values in table given as mg. of sugar alcohol absorbed by the lichen.

Dark absorption at 18°C (hours)	Arabitol	Ribitol	Xylitol	Mannitol
0	0	0	0	0
3	6.5	6.5	6.9	6.1
6	8.66	8.12	8.66	8.21
20	12.28	14.04	12.76	12.20

Material: 100 mg fresh weight samples of Xanthoria aureola

Time: Up to 20 hours

Temperature: 18°C

Light: None

Vessels: 2 x 1" specimen tubes

Radioactivity: None

Medium: 2 ml 1% sugar solutions

Experiment: 24

Date: 20.6.65.

3. The effects of starvation on the polyol content of lichens

Smith (1963) found that reducing sugars, although present in small amounts in Peltigera polydactyla, were reduced by 40% after starvation on distilled water for six days at 25°C. Drew (1966) showed that the content of mannitol, which is the major soluble carbohydrate in Peltigera polydactyla, fell by 40% during the first 48 hours starvation under similar conditions, but thereafter declined very slowly.

a. Starvation in Xanthoria aureola

Samples of thallus (200 mg fresh weight) were washed in sterile water and incubated in the dark in covered Petri dishes (Figure 70) on steripads moistened with sterile distilled water. The Petri dishes were kept at 18°C in the dark. One sample was removed each day, killed and extracted with 80% ethanol and the total sugar alcohols estimated as mannitol equivalents. The extracts were chromatogrammed, mannitol and pentitols eluted and the amount of each again determined using the spectrophotometric method of Harley and Lewis. Since it was not possible to separate arabitol and ribitol satisfactorily by chromatography they were estimated together.

The results (Figure 71) show that the amount of pentitols within the thallus quickly falls during starvation at 18°C, and after 48 hours they could not be detected. Mannitol declines more slowly, but it may be seen that there is an initial rise in mannitol content

Figure 70

200 mg fresh weight samples of Xanthoria aureola in sterile Petri dishes on 'steripads' moistened with sterile distilled water prior to starvation in the dark.

Marginal lobes washed in sterile distilled water weighed out into 200 mg samples.

Starvation temperature 18°C

223a.

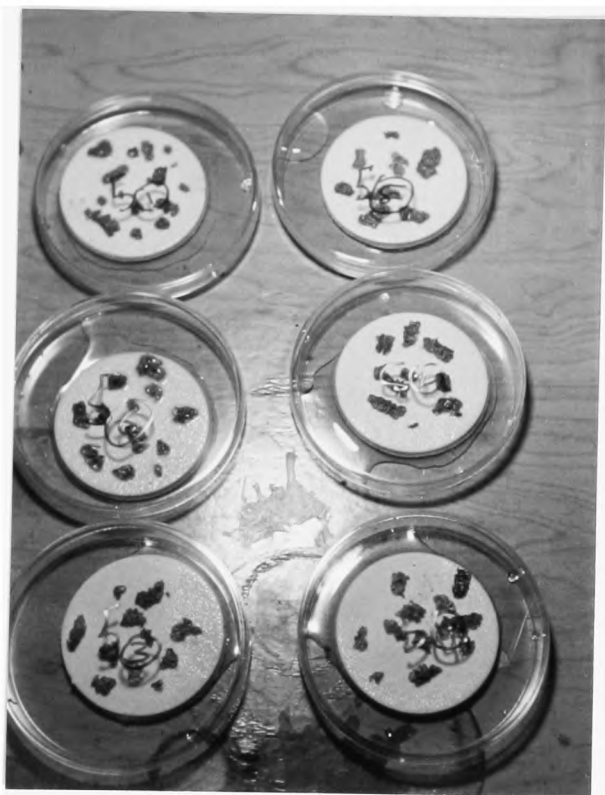


Figure 71

The effect of starvation on the sugar alcohol content of samples of Xanthoria aureola. All sugar alcohol measurements as mannitol equivalents.

Material: 200 mg fresh weight per sample

Time: 0-14 days

Temperature: 18°C

Light: none

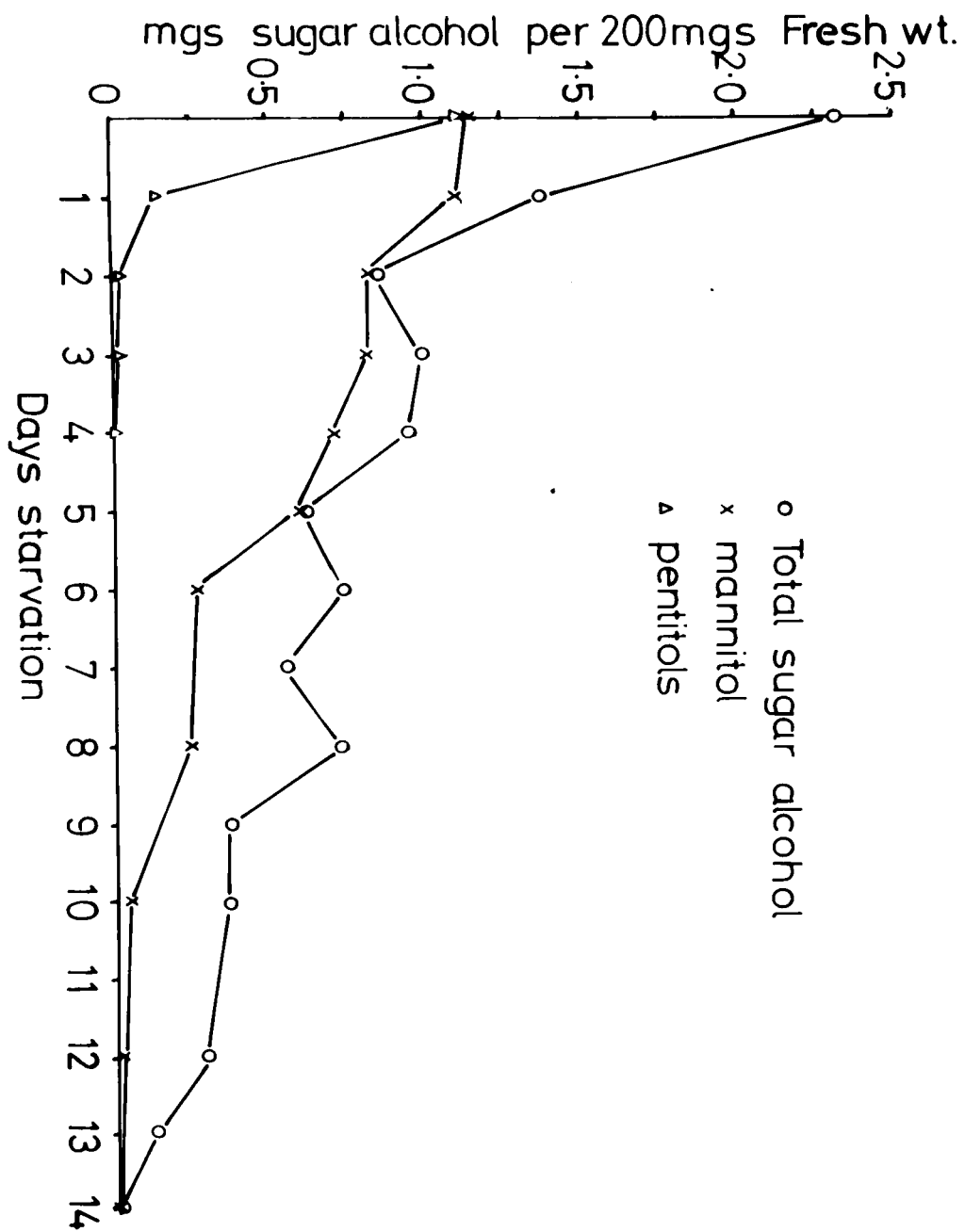
Vessels: Petri dishes

Radioactivity: none

Medium: sterile distilled water

Experiment: K

Date: 8.1.65.



which may be explained by the conversion of ribitol into this compound within the thallus. The loss of sugar alcohols was found to be much less rapid at low temperature.

b. Starvation in *Lecanora conizaeoides*

This lichen was collected in Bagley Wood, Oxfordshire, and a similar starvation experiment was carried out to that described in the previous section. The total sugar alcohols could not be estimated on the crude extract in this species because of substances which precipitated out of aqueous solution at low pH. Therefore each extract was chromatogrammed, the pentitol and mannitol areas were eluted, and the polyol content estimated in the eluate by the method of Lewis and Harley (1965).

Initially *Lecanora conizaeoides* contained much more pentitol than mannitol. As in *Xanthoria* the pentitol content fell and was accompanied by a rise in mannitol level. Unlike *Xanthoria* this rise in mannitol level continued for several days. Figure 72 shows that the overall reduction in polyol content took place much more slowly than in *Xanthoria aureola* and this may be related to the fact that this lichen grew on fallen logs under conditions which are often damp and shaded. This species is also able to survive on stone walls in polluted areas but specimens from such a habitat were not examined.

Figure 72

The effect of starvation on the sugar alcohol content of Lecanora conizaeoides. All sugar alcohol measurements as mannitol equivalents.

Material: 400 mg fresh weight per sample

Time: 0-8 days

Temperature: 18°C

Light: none

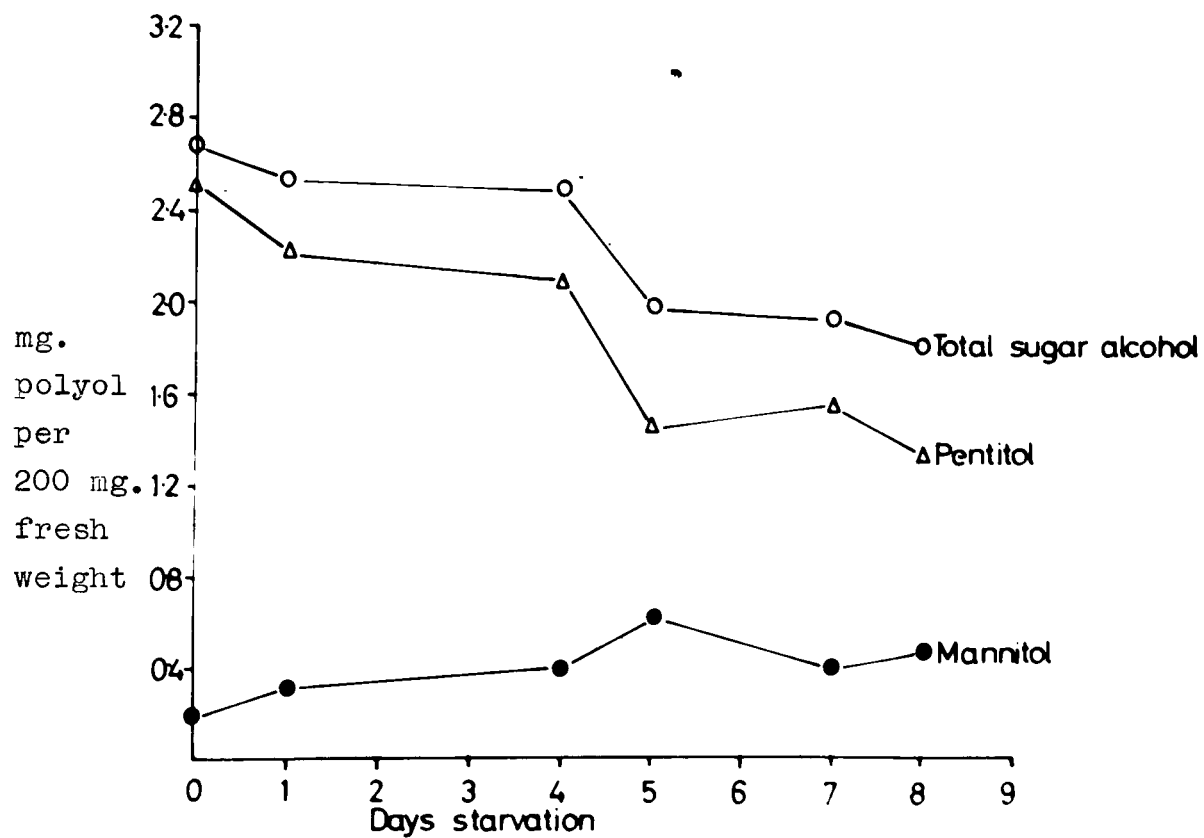
Vessels: 2 x 1" specimen tubes

Radioactivity: none

Medium: sterile distilled water

Experiment: 5

Date: 15.1.66.



3. Seasonal variation in polyol content in lichens

Recent studies have indicated that the sugar alcohol content of lichens varies at different seasons of the year. Pueyo (1960) and Lestang Laisne (1966) determined the sugar alcohol content of Lichina pygmaea and other lichens in four collections, made in spring, summer, autumn and winter. They found that there was a high polyol content in autumn and this fell to a low level in winter.

Smith and Ozin (unpublished) made a detailed study of the mannitol content of Peltigera polydactyla by collecting samples of the lichen each month for several years. Their results showed that values are at their highest in autumn and lowest in winter, as in the other lichens studied.

In order to determine whether such a variation occurred in Xanthoria aureola, 4 to 7 (usually 5) 200 mg samples of lichen were collected at the beginning of each month, killed and stored in 80% ethanol. After 18 months the total sugar alcohol contents of each sample were determined by the spectrophotometric method of Lewis and Harley (1965). The sugar alcohol content was then calculated on the basis of fresh weight and extracted dry weight. On both criteria a closely similar picture emerged.

The results shown in Figure 73 suggest that there was indeed a low winter value but in this species the highest polyol contents were

Figure 73

Seasonal variation in the sugar alcohol content of Xanthoria
aureola.

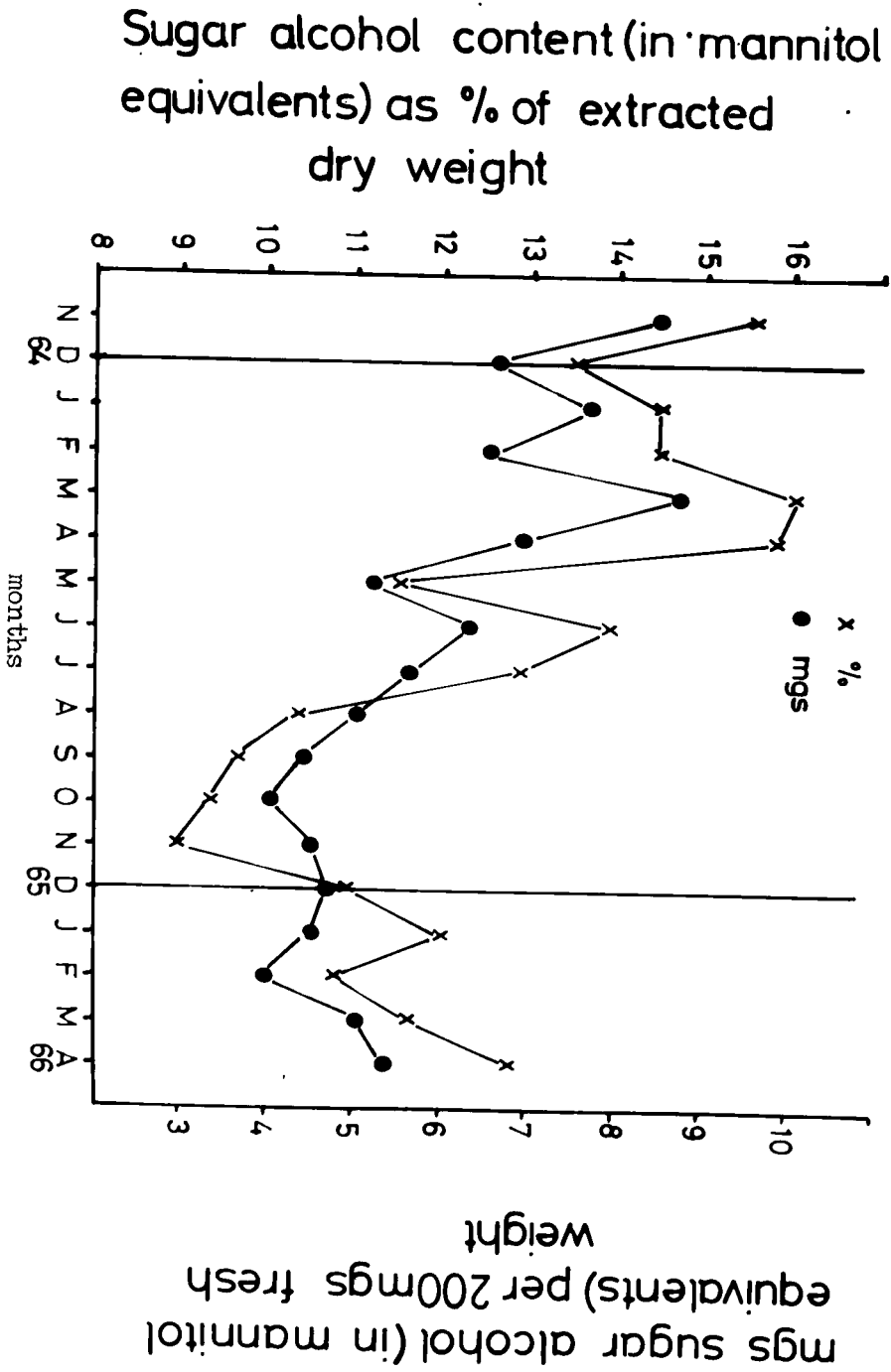
Material: 200 mg fresh weight collected, washed and
immediately killed in 80% ethanol.

Time: Oct 64 - May 66.

Experiment: 85

Date: 11.66.

Seasonal variation in sugar alcohol content in *Xanthoria aureola*



recorded in early spring. The results for each year were not exactly similar so that an overall pattern of seasonal variation in sugar alcohol contents has not yet emerged.

4. Conclusions

The above experiments indicate the importance of using as experimental material thalli which have been freshly collected and not stored or slowly dried, as under such conditions appreciable falls in polyol content, and perhaps other metabolites, can occur within 24 hours. They also demonstrate the importance of recording the time of year when an experiment was carried out especially when estimates of sugars and sugar alcohols are being made.

CHAPTER XITHE TRANSPLANTATION OF LICHEN THALLI

Some experiments were carried out to investigate the possibility of transplanting lichen thalli of Xanthoria sp between different habitats. The results of these experiments have now been described in a paper which has been accepted for publication in The Lichenologist. The text of this paper is given below, together with some monotone plates which form part of the article. In order to give more information, Figures 74, 75, 83, 84 and 85 are also included although they do not form part of the article. The references cited in this paper are included in the main bibliography.

THE TRANSPLANTATION OF LICHEN THALLI

TO SOLVE SOME TAXONOMIC PROBLEMS IN XANTHORIA PARIETINA (L.) Th. Fr.

by

D.H.S. Richardson

Department of Agriculture, University of Oxford

Introduction

This paper reports the results of some preliminary experiments to determine the extent to which differences in the morphology and other characteristics of Xanthoria parietina sensu lato (Smith 1918) were determined by habitat.

Xanthoria parietina sensu lato has recently been recognised (James 1965) as comprising two species in the British Isles, X. parietina (L.) Th. Fr. and X. aureola (Ach.) Ericha. The former is separated into two varieties, var parietina and var ectanea (Ach.) Kickx. The species and varieties differ in a number of respects such as the detailed thallus morphology, the abundance of apothecia and often in their parietin content.

Thus X. aureola has broad thallus lobes, peg-like proliferations in the centre of the thallus and few apothecia with a coronate margin. X. parietina var parietina has no such proliferations in the centre of the thallus, numerous apothecia which lack the coronate

margin and broad lobes; var ectanea is similar to the above but has very narrow lobes.

These lichens also tend to occur in distinct habitats.

X. aureola predominates in habitats such as roofs of farm buildings, especially in the east of Britain, X. parietina var ectanea on the sea shore and X. parietina var parietina on tree trunks and less abundantly in the habitats occupied by the other species or variety.

Since lichens cannot yet be grown under controlled conditions in the laboratory, it was thought that the best way to determine the effects of habitat on the morphology, etc., of these lichens was to transplant thalli between two different habitats.

Transplantation of thalli

Successful transplantation of corticolous thalli under controlled conditions has been achieved by Brodo (1961). His technique consisted of excising a core of tree bark on which a specimen of lichen was growing and taking this to a different area but similar habitat. The core was fixed in a prepared hole in the new host tree by means of grafting wax. Brodo found that the wax had little effect on the continued growth of the lichen but bleeding of the host tree was often a problem with crustaceous species.

Le Blanc and Rao (1966) have used a similar technique to transplant lichens to industrial areas in order to study the effects

of sulphur dioxide on them. Most of the lichens had died after one year, Bacidia chlorococca and Parmelia sulcata were most tolerant of a polluted atmosphere.

Hale (1954) removed clumps of soil (200 cm²) with intact lichen thalli from Baffin Island to North Western Connecticut. After two years, one lichen Cetraria islandica (L.) Acl., had made new growth, two other species of this genus survived but the other lichens had died.

None of the above workers designed their experiments to solve specific taxonomic problems, though their techniques obviously have potential in this field.

No attempts at transplanting saxicolous lichens under controlled conditions seem to have been published. The possibility of transplanting thalli of Xanthoria parietina sensu lato on portions of substratum was considered, but it was thought that the substrate might contain substances which would affect the microhabitat of the transplanted lichen for a long time. For example sea shore rocks are usually subject to salt spray while tiles from farm roofs are rich in nitrogenous material. Both these substances have been thought to affect the occurrence of these lichens and hence it would be better to transplant thalli without the attached substrate.

Materials and Methods

Collection and transplantation of lichen material

Plants of Xanthoria parietina var ectanea were carefully removed from a marked south-facing rock above Black Cove, Signal House Point, Slapton, Devon (Figure 74a) using the back of a scalpel. The thalli were placed in a polythene bag and taken to the University Farm, Wytham, Oxfordshire (Figure 74b). At Wytham, tiles of the south facing roof in the farmyard were scraped clean of all lichen growth, and the thalli from Devon were then stuck in position on these tiles using 'Araldite'. The time between collection of thalli and transplanting was in all cases twenty four hours. Araldite was chosen as it is a resin glue and therefore did not give off volatile organic solvents on hardening which might harm the transplants.

At the same time as these transplants were fixed to the roof, thalli were of X. aureola lifted from other tiles on the roof, randomly selected, and transplanted next to the sea shore lichens on the clean tiles to act as controls.

Thalli of X. aureola were similarly transferred to the sea shore from Oxford and again control transplants made.

Measurement of growth rate

Observations were made over an eighteen month period. Only one thallus out of 15 (10 from an original experiment 5 from a

Figure 74

- A. The habitat from which Xanthoria aureola was collected at the University Farm, Wytham, Oxfordshire.
- B. The habitat from which Xanthoria parietina var ectanea was collected at Black Cove, Signal House Point, near Slapton, Devon.



second experiment) transplanted from the sea shore died during this time. Immediately after transplanting, a tracing of each thallus was made on tracing paper and the underside of each tile marked. After eighteen months the thalli were again traced and both sets of tracings transferred to opaque cards. These were fed through the leaf measuring apparatus of Martin (1966 unpublished) which consists of a leaf scanning device connected to a scaler (Figure 75). This is capable of measuring the area of any shape with an error of less than ten per cent. In this way the area of each thallus before and after eighteen months growth was determined.

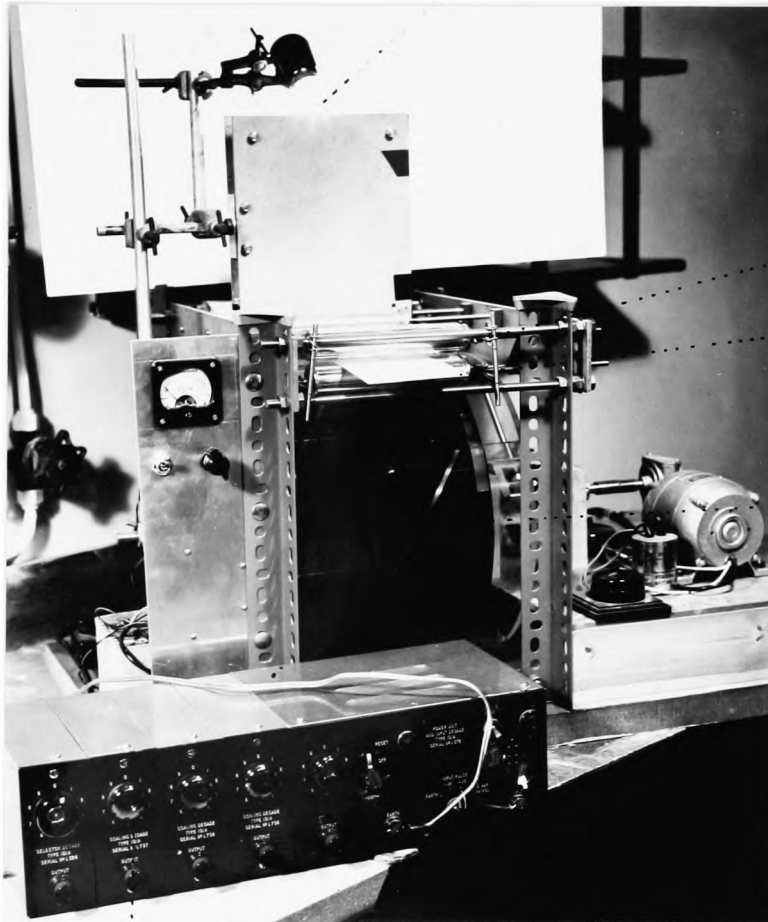
Measurement of parietin

At the beginning of the experiment the parietin content of transplants from the sea shore and of thalli from the Oxfordshire roof was measured spectrophotometrically. The following procedure, which is based on that of Outhbert (1931) and is similar in principle to that used by Hill and Woolhouse (1966), was employed. Samples of lichen (200 mg fresh weight) were extracted three times with 80% ethanol at 80°C. The extracts were pooled and made up to 25 ml. An aliquot (0.4 ml) was placed in a three ml cuvette. This was diluted with 80% ethanol (1.6 ml) and then one ml of 0.5N ethanolic potassium hydroxide solution was added. A control consisting of 0.4 ml of extract with 2.6 ml of 80% ethanol was also prepared.

Figure 75

The apparatus of Martin (1966 unpublished) which was used to determine the area of lichen thalli. The leaf (or thallus) is carried through the apparatus on celluloid belts. It is scanned as it passes beneath the light source by means of the revolving drum on which is a transparent strip. The impulses from photoreceptors in the centre of the drum are recorded by the scalar.

light source



celluloid
belts

specimen

scanning
device

scalar

Using a Zeiss PMQ 2 spectrophotometer at 510 μ , the control yellow solution was set to zero absorbance and the absorption of the alkaline red solution measured. In this way substances other than parietin which absorb at 510 μ did not affect the determination of the parietin content.

The amount of parietin was determined by reference to standard solutions containing known amounts of pure parietin in chloroform (as parietin is only sparingly soluble in 80% ethanol and slowly precipitates out of concentrated solutions). Though this method proved quite satisfactory it has been suggested (Bradshaw, personal communication) that in future more accurate spectrophotometric determinations could be made by measuring the sharp absorption peak of parietin in the ultra-violet region of the spectrum.

Results

Growth rate

The growth of control transplants from Oxford on to cleaned tiles at Oxford was compared with the growth of small, medium and large thalli from the sea shore.

Figure 76 shows that in terms of absolute increase there was no significant difference between the growth on the roof of Wytham and sea shore thalli. It confirmed that the transplants were able to grow and thrive after being moved. The area of the thalli was also

Figure 76

Growth of thalli of Xanthoria aureola and X. parietina var ectanea on the roof at University Farm, Wytham.

Origin	Wytham (<i>X. aureola</i>)	small thalli Devon	medium thalli Devon	large thalli Devon
Average absolute increase in area over 18 months in <u>sq. cm.</u>	4.10 \pm 1.23	3.38 \pm 1.17	1.58 \pm 1.54	4.07 \pm 1.23
Average initial area in <u>sq. cm.</u>	0.93	0.66	0.99	4.46
Increase in mm of the radius in 18 months	7.23	6.56	3.46	4.33

Increase in the area of each thallus determined, and analysed by analysis of variance.

expressed in terms of a perfectly round colony. Though thalli are seldom of this shape it gives a more easily comprehensible idea of the growth rate of a thallus. The results show that the radius of transplanted thalli increased by 3 - 7 mm during the eighteen month experiment. It is also seen that the smaller thalli increase in radius faster than the larger, indicating that growth rate may initially be quite fast but may slow down later as has been suggested by Abbayes (1951).

Morphology

The material collected from Devon would now be classified as X. parietina var parietina and that from Wytham as X. aureola. There has been some disagreement as to whether these are distinct species or only varieties. In order to provide further information on this point, measurements were made of the widths of the thallus lobes, number of apothecia and the presence of proliferations on the thallus in all transplants and controls.

Material transplanted to Wytham (Oxfordshire). After eighteen months growth the lobe width of the sea shore material had increased in every transplanted thallus. On average this increase was from 0.8 to 2.4 mm. This change in morphology may be seen in Figures 77 and 78. Figure 79a is a transplant of X. parietina var ectanea; Figures 79b and 80a show such a transplant after 6 and 18 months growth in the Oxford habitat. Figure 80b, by contrast, is a control transplant of

Figure 77

The detailed morphology of the thallus lobes:

- A. in a transplant of Xanthoria parietina var ectanea immediately after removal from the Devon sea shore habitat.
- B. in a transplant of Xanthoria parietina var ectanea after 18 months growth in the Oxfordshire habitat.

Each division on the scale = 1 mm.

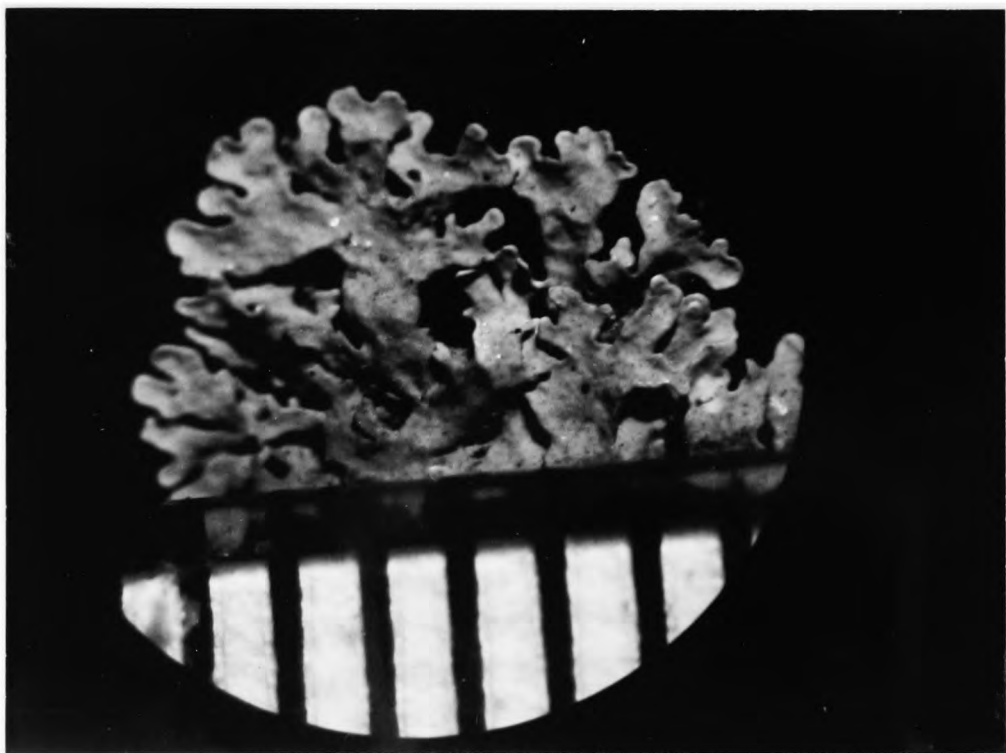


Figure 78

A. The detailed morphology of the thallus lobes of a plant of Xanthoria aureola growing in the Oxfordshire habitat.

B. Morphology of the complete thallus of a plant of Xanthoria parietina var parietina growing in the Devon sea shore habitat.

Each division on the scale = 1 mm.

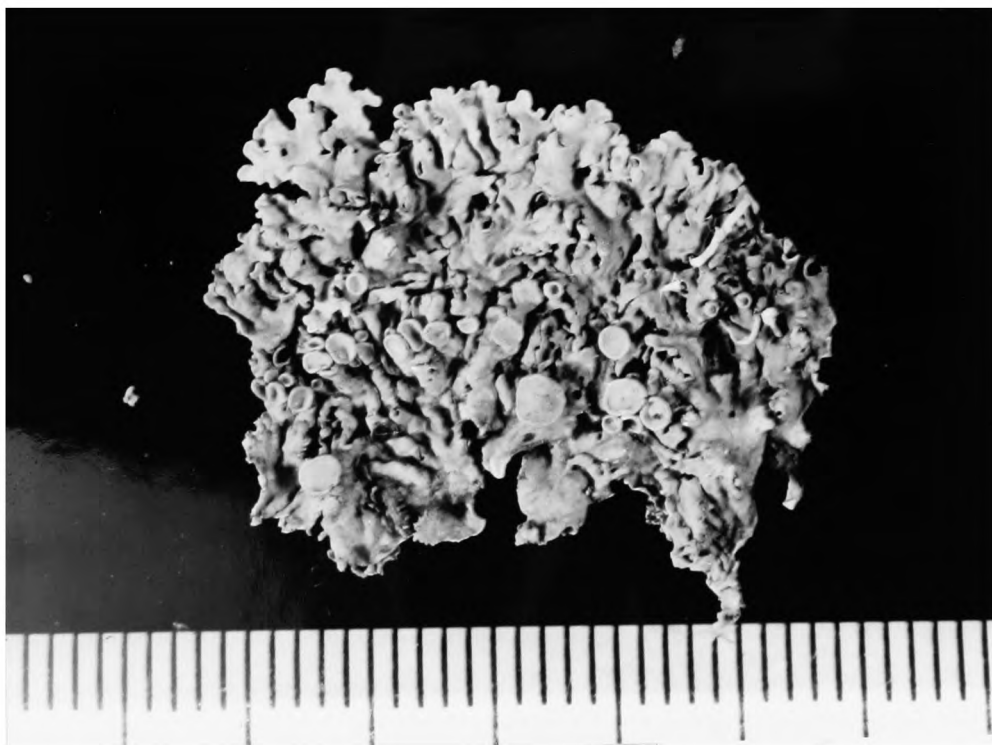
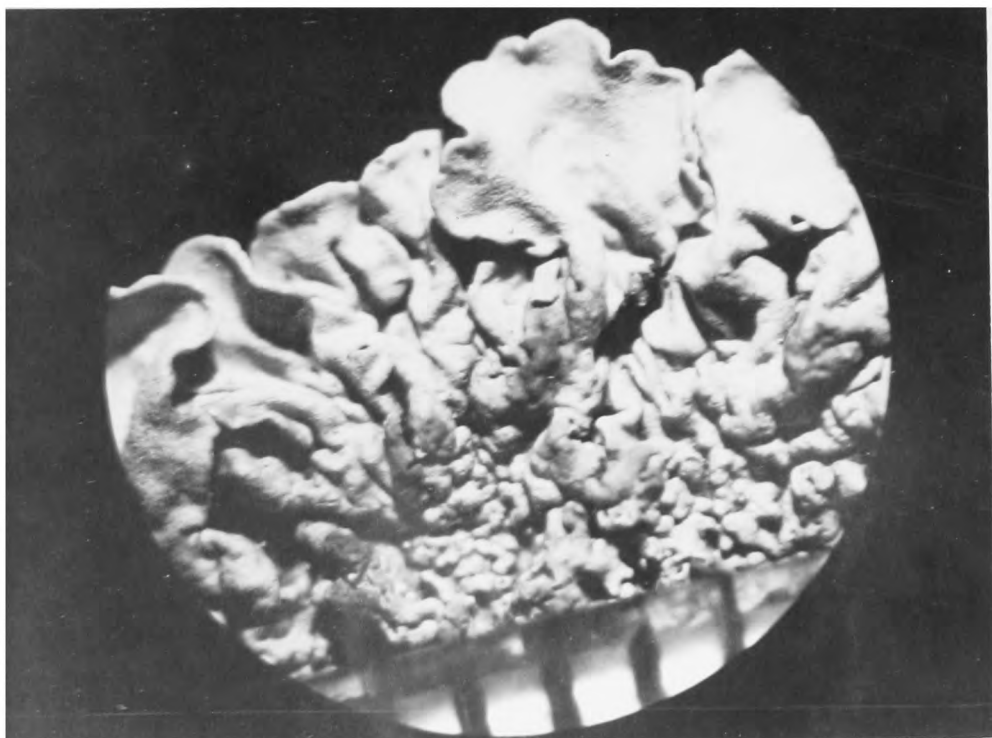


Figure 79

The morphology of the whole thallus of:

A. a transplant of Xanthoria parietina var ectanea immediately after its removal from the Devon habitat.

B. a transplant of Xanthoria parietina var ectanea after six months growth in the Oxfordshire habitat.

Each division on the scale = 1 mm.

251.

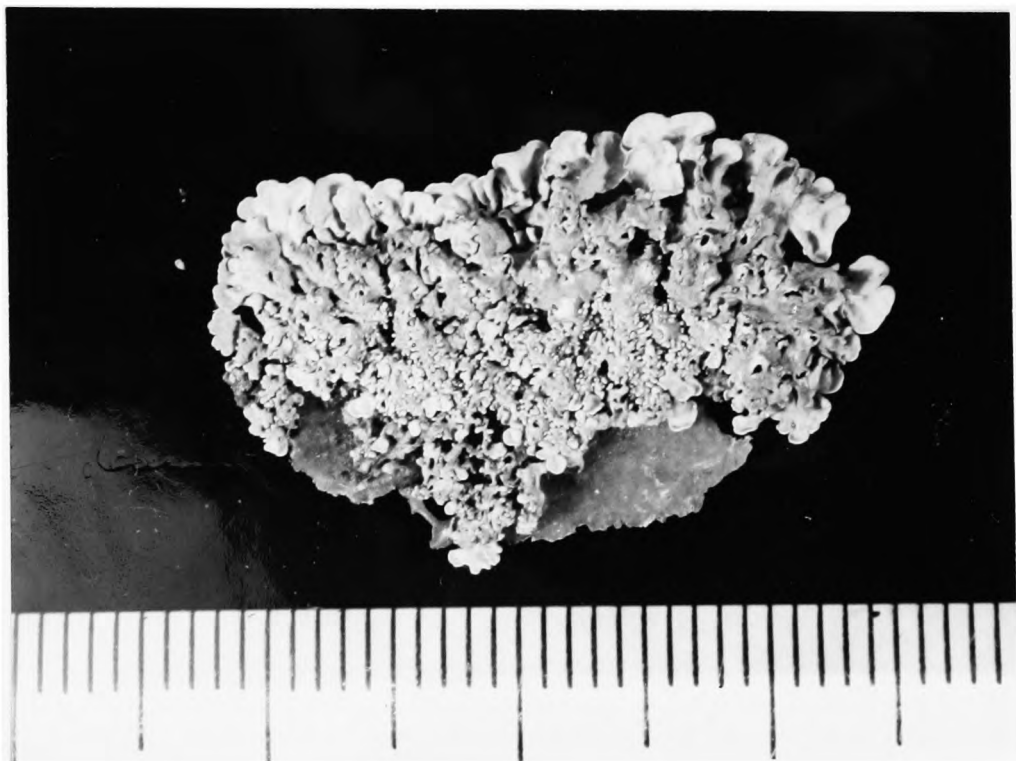
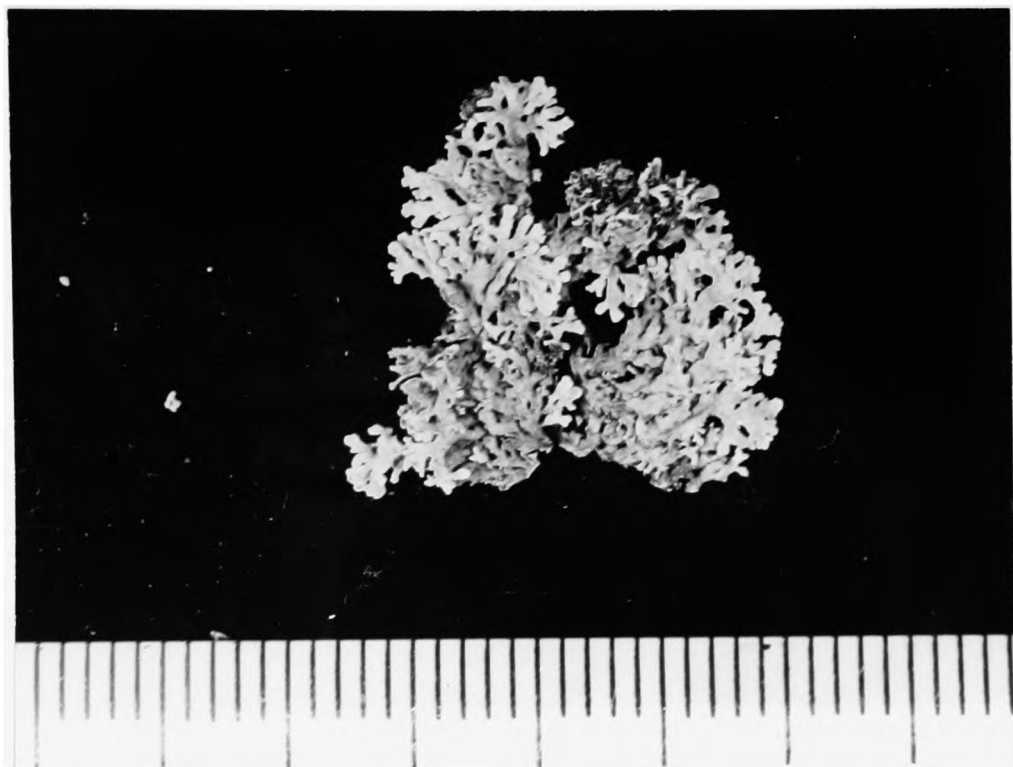


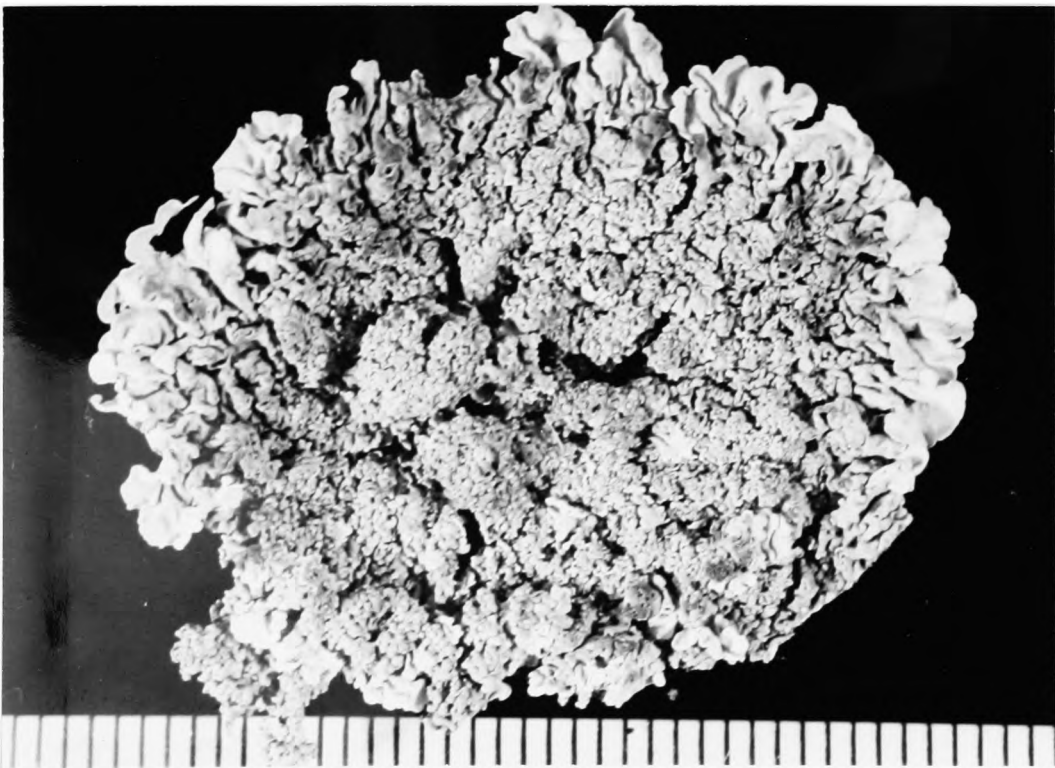
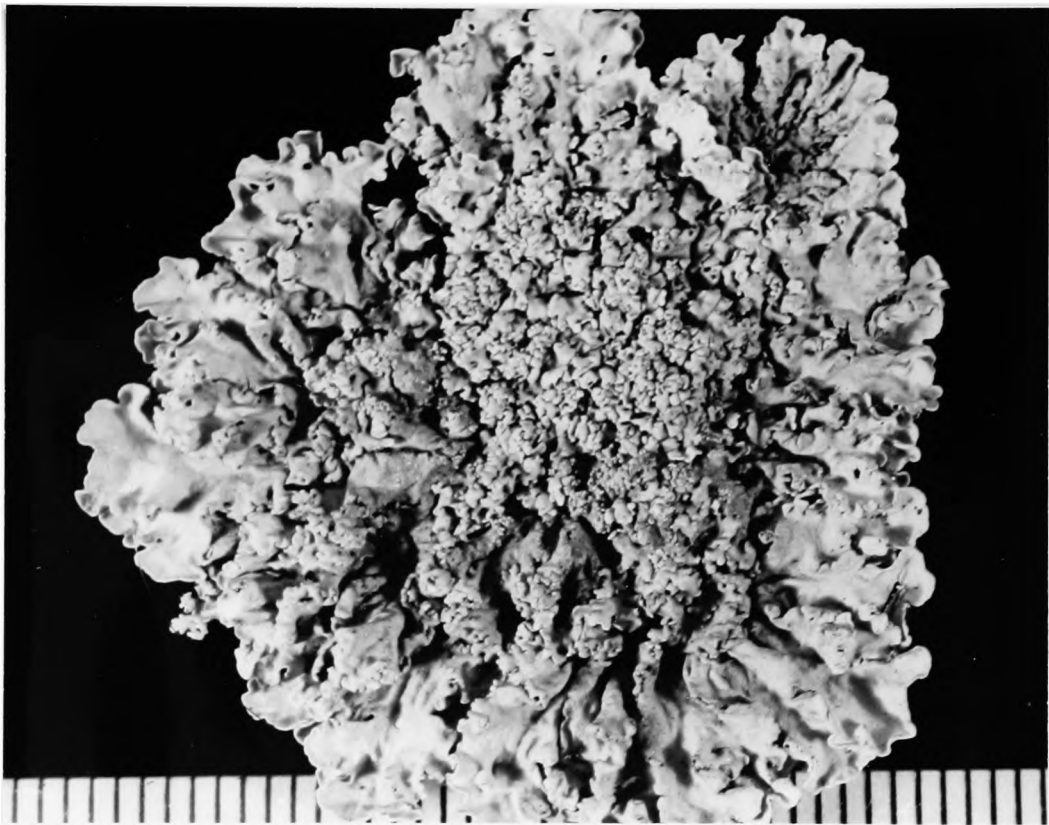
Figure 80

The morphology of the complete thallus of:

A. a transplant of Xanthoria parietina var ectanea after
18 months growth in the Oxfordshire habitat.

B. control plants of Xanthoria aureola growing in the
Oxfordshire habitat.

Each division on the scale = 1 mm.



X. aureola illustrating lobe width and the typical proliferations at the centre of the thallus in this species.

Those thalli which had apothecia on transplanting still possessed them after 18 months but none of the thalli which initially lacked apothecia subsequently developed any. However, the control plants on the Oxford roof did not form apothecia, indicating that perhaps a longer experimental time was desirable. The morphology of the centre of the thallus of X. parietina var ectanea did not change with respect to the development of peg-like proliferations, though many small lobes were produced in this area.

Material transplanted to Devon. Thalli transplanted in the opposite direction did not develop narrow thallus lobes, nor were abundant apothecia produced. The proliferations in the centre of the thalli of X. aureola have not been lost. It remains in morphology typical X. aureola, although as the next section describes, the colour has changed.

Parietin content

Parietin is the bright orange pigment found in lichens of the family Caloplacaceae. The amount of parietin in any species varies considerably; Cuthbert (1931) found that very exposed and shaded plants of Teloschistes flavicans had less pigment than those plants not growing in such habitats. X. parietina sensu lato can vary from very deep orange to almost grey in colour.

Xanthoria aureola often appears paler in colour than X. parietina var ectanea and the material of the latter species from Devon was found to have some three times more parietin than material of X. aureola from Oxford (Figure 81). Scott (1965) thought that high light intensity was responsible for high parietin production which he and Rao and Le Blanc (1965) have explained in terms of the pigment protecting the algal cells within the lichen from intense radiations. Since both lichens grew on unshaded south facing habitats, it was difficult to believe that the light intensity differed so greatly. It was therefore supposed that the difference in parietin content might be genetically controlled at least to some extent, especially as Neelakantan (1965) reports parietinic acid in Xanthoria aureola but not in X. parietina (Figure 82).

In order to investigate this possibility, transplants and controls were inspected and observation made on the parietin content. At the start of the experiment the material from Oxford was visibly paler in colour than the transplants from the sea shore (Figure 83). However, after six months on the Oxford roof the sea shore transplants were indistinguishable in colour from the plants of X. aureola (Figure 84). Similar transplants in the opposite direction showed that the sea shore material was initially far deeper in colour, but after six months the parietin content of the X. aureola transplants had increased so that they were the same colour as the native population (Figure 85). It must be noted

Figure 81

Parietin content of the thalli from Oxfordshire and Devon expressed as mg per 200 mg fresh weight of lichen.

Month	Devon	Oxfordshire
January	1.56 ± 0.2	0.60, 0.50, 0.50
March	1.84 "	-
August	1.83 "	-
September	1.69 "	-

PARIETIN AND ITS DERIVATIVES

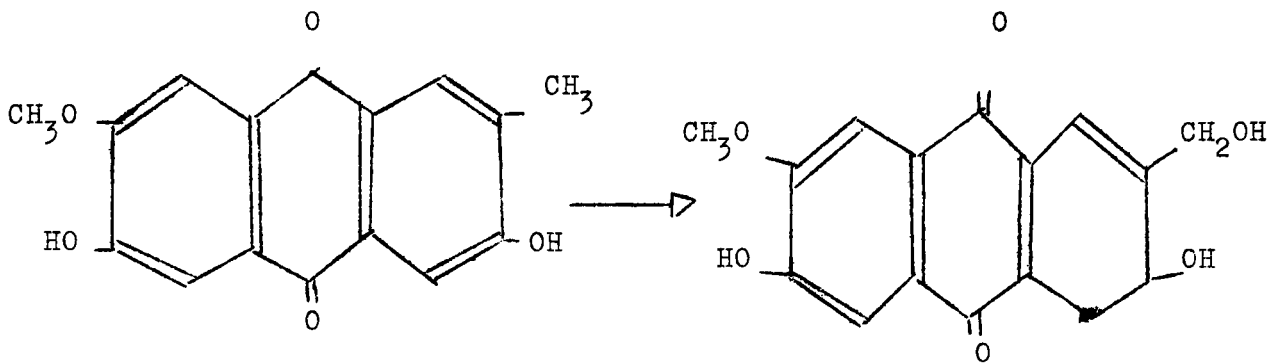
(Neelakantan 1965)

parietin

teloschistin

Xanthoria parietina

Teloschistes flavicans



Parietinic acid

Fallacinal

Xanthoria aureola

Xanthoria fallax

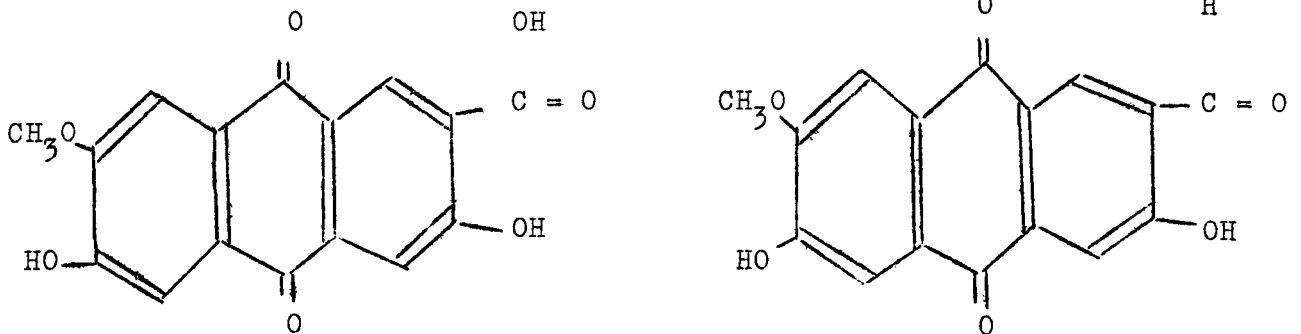


Figure 83

Transplants (A,B) of Xanthoria parietina var ectanea from the sea shore showing that they have a much greater parietin content than the control thalli (C) of Xanthoria aureola.



Figure 84

The growth of transplants of Xanthoria parietina var ectanea after 3 and 6 months in the Oxfordshire habitat. In each photograph the centre plant is a control transplant of Xanthoria aureola (i.e., 6 in upper picture, 3 in lower picture). After this time in the Oxfordshire habitat the transplants and controls were not visibly different in colour.

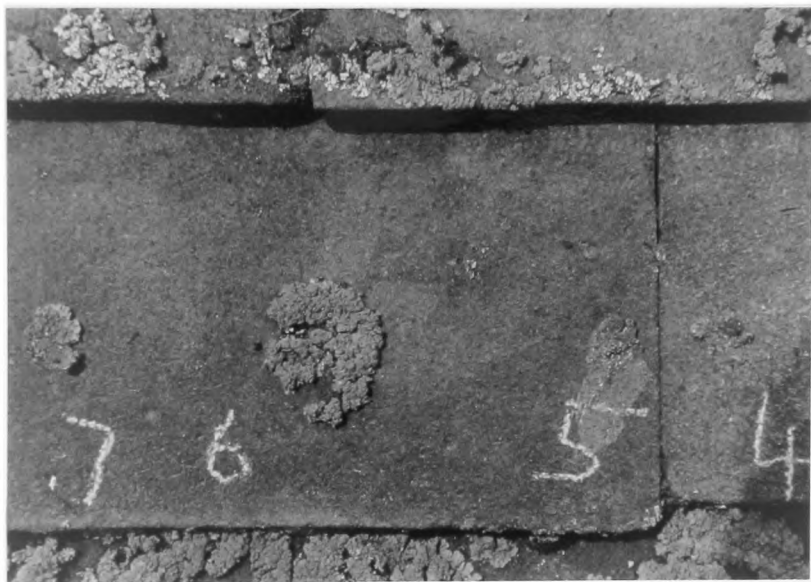


Figure 85

Showing that transplants of Xanthoria aureola from Oxfordshire to the sea shore change in parietin content to that of the surrounding thalli within six months.

B



A . . .

. . . C

. . . D

B



A . . .

. . . C

D

that these were visible comparisons and the spectrophotometric determination was not made as the experiment is still in progress.

Conclusions

The technique developed for transplanting saxicolous lichens with resin glue has proved very successful. In this study, only one out of 25 thalli which were moved failed to survive and grow. The remainder showed in some cases changes both in morphology and parietin content.

The main taxonomic conclusions are that the morphology of the two species, X. parietina and X. aureola, would appear on preliminary evidence to be distinct. However, on transplanting X. parietina var ectanea to Oxfordshire, the lobe width increased so that it could not be distinguished from X. parietina var parietina.

It is suggested that the extent of parietin production is largely controlled by the environment but that light may not be the only factor involved. The presence of atmospheric pollution, for instance, may be causing low parietin production in specimens of Xanthoria growing in the Oxford habitat.

The technique developed for transplanting saxicolous lichens together with those of Brodo (1961) and Hale (1954) for corticolous and terricolous lichens should be especially valuable in determining the exact taxonomic status of other critical species and chemical strains within such genera as Ramalina, Physcia and Parmelia.

CHAPTER XIIDISCUSSION1. Xanthoria aureola

The experiments described in Chapters IV and V showed that ribitol and sucrose were the main photosynthetic products of Trebouxia, the algal symbiont of Xanthoria aureola. Ribitol appeared to be the main carbohydrate which moved from the alga to the fungus where it was converted into mannitol and a small amount of arabitol. These results contrasted with the situation found in Peltigera polydactyla, by Drew (1966), in which the glucose produced by the alga of this lichen moved to the fungus and was converted into mannitol within thirty minutes. A number of aspects of the mechanism of carbohydrate transfer between the symbionts of Xanthoria aureola are therefore worthy of detailed consideration and these are discussed below.

a. Rate of transfer of carbohydrate

The experiments described in Chapter VI suggested that the movement of ribitol from alga to fungus in Xanthoria aureola was slow over a 24 hour period. However Bednar and Smith (1966) supposed the

movement to be relatively rapid and efficient. These two views can be reconciled when it is realised that the experiments of these latter authors were essentially short term. They thought that by comparison with Peltigera polydactyla, sugar alcohols were fungal products. Thus, when they observed that pentitols became rapidly and heavily labelled with ^{14}C after a few minutes photosynthesis on ^{14}C -sodium bicarbonate solutions, they believed that some substance such as pentose moved quickly from the alga to the fungus. They thought that this substance was quickly converted into a pentitol by the fungus and finally to mannitol. This hypothesis of rapid transfer was strengthened by the observation that the amount of ^{14}C in metabolic intermediates within the thallus, presumably common to both symbionts, reached a steady state after 9 - 15 minutes.

However, studies on the isolated alga described in Chapter v have indicated that ^{14}C is incorporated into ribitol during photosynthesis, and that it is this which moves to the fungus. Thus in the experiments of Bednar and Smith, ^{14}C -ribitol was produced within the alga and this had hardly started to move across to the fungus within the three hour duration of their longest experiments. Therefore their observations that the ^{14}C in metabolic intermediates (such as PGA, sugar phosphates and organic acids) had reached a steady state only showed that photosynthesis by the alga had attained an approximately steady state condition within fifteen minutes.

Although the results of experiments described here show that the movement of ^{14}C was much slower than Bednar and Smith had supposed, and slower than in Peltigera polydactyla, it is difficult to give an absolute value for the rate of transfer. Overall it took about 24 hours for 25% of the fixed ^{14}C to pass from the alga to the fungus within the thallus of Xanthoria aureola, as compared with three hours in the case of Peltigera polydactyla. However, reference to Figures 34 and 35 shows that initially the rate of movement of ^{14}C from alga to fungus is slow but gradually accelerates to a more or less steady rate after six hours. If this steady rate is the actual speed at which carbon flows from the alga to the fungus in plants growing on the tiled roof the rate of carbohydrate transfer may be faster than the results from laboratory experiments would suggest.

b. Excretion by the alga

Why does the alga release carbohydrate? It is found that Trebouxia as well as Nostoc and Coccomyxa release far less soluble carbohydrate after being in pure culture for some time, and they also fix proportionally much less carbon into these carbohydrates. There seem to be two possibilities why these algae should release carbohydrate within the lichen thallus.

In the first instance, it may be that the lichen fungus excretes some substance which increases the permeability of the algal cells. Follman (1960) suggested that lichen acids perform

this function. They might possess these special properties or simply lower the pH around the algal cells. There is some evidence, in this study, that low pH enhances release of fixed ^{14}C by the alga. However, one must note that within the lichen thallus there is not a simple change in overall permeability since only one of the many products of ^{14}C fixation is released from the algal cells in large amounts. (Thus both ribitol and sucrose become labelled with ^{14}C inside Trebouxia cells during photosynthesis, but only ribitol appears to move from the alga to the fungus.) Muscatine (personal communication, 1966) has found, in studies on Zooxanthellae of corals and Tridacna, that the 'directly isolated' algae release a large proportion of fixed ^{14}C (as glycerol), but if the algae are well washed, they release almost nothing. When host tissue homogenate is added back the cells leak up to 40% of the photosynthate. He suggests that there is some compound, as yet unidentified but tentatively termed a 'glycerol permease', which is produced by the coelenterate tissue and causes the alga to release carbohydrate. It is possible that a similar system is operative in lichens.

In the second instance the fungus may modify the algal cell wall metabolism in such a way that soluble compounds normally destined to become structural components of the cell wall are released and are thus available for absorption by the fungus.

This may well be the case in Peltigera because in that lichen the Nostoc lacks the thick gelatinous wall which it develops in culture (Drew 1966) and which contains glucose polymers. Similarly in culture Trebouxia is reported to produce a mucilaginous coat (Ahmadjian 1959) and Coccomyxa produces quantities of slime. These slime coats have not yet been analysed to see if ribitol is one of their constituents. However, ribitol has not so far been identified as a cell wall component of plants although in some bacterial cell walls it occurs as part of techoic acid.

With regard to the release of substances by the alga, it may be apposite to discuss fungal haustoria in lichens. Geitler (1963) and Danilov (1910) have observed haustoria in the algal cells of numerous lichens. This made the latter author conclude that the lichen fungi were parasitic on the algae. Moore and McAlear (1960), using an electron microscope, found haustoria making direct contact with the algal protoplast in Cladonia cristatella and Lecidea sp. However not all authors are convinced of the widespread nature of these structures; for instance Mameli (1920) examined many species of some 25 genera and only found seven lichens with haustoria. One of these was Xanthoria parietina but in this case the haustorium scarcely penetrated the algal membrane. Paulson and Hastings (1920) were never able to demonstrate the penetration of algal cells in this species and found the algae in a healthy condition.

Furthermore they were undergoing abundant multiplication in early Spring by means of autospores. If indeed the algal cells are penetrated by haustoria in Xanthoria aureola, it is more difficult to explain how externally supplied ^{12}C -ribitol could prevent the movement of ^{14}C -ribitol from alga to fungus. However, it might be possible that the enzyme systems of the alga and fungus involved in carbohydrate transfer are in more or less direct contact at the haustorium and that the ^{12}C -ribitol saturates one step in the transfer so halting the flow of ^{14}C -carbon between the symbionts. This might result in ^{14}C -ribitol flooding out into the medium at the algal-haustorial interface. However, evidence from electron micrographs of the sectioned thallus failed to show haustoria (Bednar and Juniper, unpublished, 1965), and the fact that it is possible to isolate intact algal cells without visible haustoria by a process of grinding and centrifugation, suggests that they are not a major feature of the symbiosis in Xanthoria aureola.

c. The lichen fungus of Xanthoria aureola

This was grown in pure culture from ascospores and found to be deficient for biotin and thiamine. If the fungus is deficient for these factors in the complete thallus, they may be provided either by the habitat which is rich in bird dung and nitrogenous dust or by the alga. In connection with the latter suggestion, Bednar and Holm-Hansen (1964) found that a Coccomyxa symbiont originally isolated

from Peltigera aphthosa excreted significantly greater amounts of biotin than a free-living Chlorella. It is not known if other lichen algae are similar, nor is there experimental evidence that vitamins do move from alga to fungus in Xanthoria.

One problem associated with the study of the carbohydrates of the isolated fungus was that arabitol could not be detected when it was grown on media containing glucose even though work on the complete thallus had suggested that this compound was purely of fungal origin. Lewis and Smith (1967) collected evidence which suggested that the pathway of conversion of ribitol into mannitol in plants was via arabitol. The isolated fungus was therefore grown on media with added ribitol and the soluble carbohydrates extracted from the fungus and chromatogrammed. The results showed that arabitol was present, confirming previous reports that glucose can be converted into mannitol without arabitol being involved. However, traces of arabitol were also found in the fungus growing on media rich in glucose (10% w/v) and the reason suggested for this is that the production of all polyols is stimulated by high sugar contents in the medium.

2. Other lichens

The studies on Peltigera polydactyla and Xanthoria aureola showed two different patterns of carbohydrate transfer. In the

first lichen, transfer was relatively rapid and in the form of glucose whilst in the second it was slow and in the form of ribitol. Some twenty other species of lichen have been investigated to determine the extent to which these patterns of carbohydrate transfer are of common occurrence and to discover whether the rate of transfer between the symbionts was principally by the overall growth of the thallus.

a. The role of the symbionts in the pattern of carbohydrate transfer

As mentioned above, there are differences in the rate and form in which carbohydrates move between the symbionts in Xanthoria aureola and Peltigera polydactyla. Since both the fungi and algae of these lichens are very different and they live in distinct habitats, it is not easy to assess the influence of the individual symbionts on carbohydrate transfer.

Studies were therefore carried out on lichens of the genus Lobaria, all collected from the same habitat. These lichens contained closely related fungi symbiotic with either Nostoc or Trebouxia; furthermore one species, Lobaria amplissima, contained both algae. The results of these experiments, described in Appendix A, suggested that the rate of carbohydrate transfer was governed by the alga rather than by the fungus. Thus all the species containing Nostoc showed a rapid carbohydrate transfer pattern similar to Peltigera and the species containing Trebouxia, a slow carbohydrate

transfer pattern similar to Xanthoria. In Lobaria amplissima, where the same fungus is symbiotic with two algae, the part of the thallus symbiotic with Nostoc exhibited rapid carbohydrate transfer from alga to fungus in the form of glucose whilst in the part symbiotic with Trebouxia, transfer was slow and in the form of ribitol.

The results of investigations on all the lichens examined in this study together with data from Drew (1966) are summarised in Figure 86. These lend support to the general conclusion that the form as well as the rate of carbohydrate transference is controlled by the algal component. Thus the lichens symbiotic with Coccomyxa and Myrmecia have an intermediate rate of transfer whilst those containing Trentepohlia exhibit a particularly slow rate of carbohydrate movement. In cases where the same compound is released by two different algae, the rate appears to be related to the type of alga rather than to the type of compound. For example it may be seen that in both lichens containing Nostoc and Rivularia, glucose moves from alga to fungus. In the first case movement is rapid whilst in the second it is slow. A similar situation occurs in lichens containing Coccomyxa and Trebouxia, where both release ribitol but the former is more rapid than the latter.

b. Studies on the directly isolated alga

Further evidence that it is the type of alga which principally controls the rate of carbohydrate transfer was gained from studies

Carbohydrate movement from alga to fungus in various lichen species

Lichen species	Algal symbiont	Carbohydrates moving from alga to fungus	*Rate of carbohydrate transference
Peltigera polydactyla P. canina P. horizontalis Lobaria scrobiculata Sticta fuliginosa Collema auriculatum	Nostoc	Glucose	Fast
Lichina pygmaea	Rivularia	Glucose	Slow
Xanthoria aureola Lecanora conizaeoides Parmelia saxatilis Umbilicaria pustulata Lobaria pulmonaria L. laetevirens	Trebouxia	Ribitol	Slow
L. amplissima	Trebouxia (Nostoc in cephalodia)	Ribitol (glucose)	Slow (fast)
Peltigera apthosa Solorina saccata	Coccomyxa (Nostoc in cephalodia)	Ribitol (glucose)	Intermediate (fast)
Rocella fuciformis R. phycopsis Gyalecta cupularis Lecanactis stenhamarii	Trentepohlia	Erythritol	Very slow
Dermatocarpon miniatum D. fluviatile	Myrmecia	? Sorbitol	? Slow

*Rate of transference estimated as the time taken for 25% of the total fixed ^{14}C to appear in the medium when it contained 1% of the ^{12}C carbohydrate which moved from alga to fungus.

Fast rate = approximately 3 hours

Slow " = " " 24 hours

Very slow = more than 24 hours.

on algae directly isolated from the lichen by differential centrifugation of a thallus homogenate. Thus, there was a close correlation between the rate of transfer in the thallus and the rate at which directly isolated algae release fixed ^{14}C . For example in Peltigera polydactyla, which has a 'fast' rate of transfer, the directly isolated Nostoc could release 50% of the ^{14}C fixed during 3 hours photosynthesis (Drew 1966). Peltigera apthosa was found here to have an 'intermediate' rate of transfer, the Coccomyxa isolated from it released 25% of the ^{14}C fixed to 3 hours. In Xanthoria aureola, with 'slow' transfer, the Trebouxia took 24 hours to release 25% of the ^{14}C . Unfortunately it was not possible to isolate Trentepohlia from lichens as the species containing this lichen were so tough. However, one would expect it to release ^{14}C even more slowly than Trebouxia.

c. The relationship between the rate of transference and growth rate

Since many of the lichens containing Nostoc (e.g. Peltigeraceae) appear to be faster growing than most of those symbiotic with Trebouxia, it is possible that there is some correlation between growth rate and the rate of carbohydrate transfer. Unfortunately little is known about the growth rate of the lichens whose carbohydrate physiology has been investigated here. It has been observed that rates of both growth and carbohydrate transfer are slower in Xanthoria aureola than in Peltigera polydactyla. In other lichens

containing Trebouxia, for instance Lobaria laetevirens, there seems to be a slightly faster growth rate and carbohydrate transfer rate than in Xanthoria aureola, but detailed studies on the growth rate would have to be carried out to see how important this factor was. In the crustaceous lichens containing Trebouxia (e.g. Rhizocarpon geographicum), whose slow growth rate is well documented, no information is available on the rate of carbohydrate transfer between its symbionts. Thus the extent of the correlation between these two factors, if there is one, is not yet known and it is not clear whether the growth rate would control or would be the result of the rate of carbohydrate transfer between the symbionts.

d. The nature of the transferred carbohydrate

Table 86 indicates that in the lichens which contained the blue-green algae Nostoc and Rivularia, the substance which moves from alga to fungus was glucose. By contrast, in the lichens which were symbiotic with green algae, the substance which passed to the fungus appeared in all cases examined to be a sugar alcohol. Thus in Trebouxia and Coccomyxa lichens it was ribitol, in Trentepohlia lichens erythritol and in Myrmecia lichens it was a compound with a chromatographic mobility identical to sorbitol.

Free glucose does not seem to occur in appreciable amounts in the cultured strains of either Nostoc or Rivularia (Drew 1966, Lestang Laisne 1966), although it is abundant in these algae when they are in symbiosis. It was suggested earlier that this difference

might result from fungus modifying algal metabolism in the lichen so that glucose is prevented from polymerising to form part of the gelatinous sheaths which are found in the cultured algae but not in the directly isolated algae.

The sugar alcohols which are transferred in lichens containing green algae have not been isolated from other free living genera of green algae nor are they known as cell wall constituents in any organisms other than bacteria. It is therefore possible that lichen fungi can only form successful symbiotic associations with those green algae which either produce sugar alcohols or can be induced to do so.

e. The role of sugar alcohols in lichens

At the present time it is only possible to speculate on the reasons for the variety and abundance of sugar alcohols in lichens. In Beech mycorrhizas, Lewis and Harley (1965) have suggested that the presence of mannitol in the fungal part of the association enabled a one-way transfer system from host to fungus to operate. They found that sucrose passed from the host root to the encircling fungal sheath where it was converted into mannitol and trehalose. Both the uninfected and host root were unable to utilise either mannitol or trehalose, and they therefore developed the theory that the formation of these compounds by the fungus made possible: (a) the maintenance of a concentration gradient of sucrose into the fungal sheath; and (b) the one-way system of carbohydrate transfer mentioned above.

Lewis and Harley presented evidence to show that such one-way systems may operate in a number of other parasitic and symbiotic associations.

In lichens, such a system could be envisaged in the case of Peltigera polydactyla, where the alga produces glucose which moves to the fungus where it is converted into mannitol. It is possible that mannitol is unavailable to the alga, since Drew (1966) found that the Nostoc isolated from the lichen could not utilise it. In lichens such as Xanthoria parietina and Peltigera aphthosa, it is more difficult to suppose that their algae, which release ribitol, could not absorb one of the fungal products, arabitol, since they are both pentitols. However it is not yet known whether the alga can utilise this compound.

There are other possible advantages to the thallus in containing sugar alcohols apart from the possible participation in one-way systems of carbohydrate transfer. Lewis and Smith (1967) have reviewed the present knowledge of the distribution, physiology and metabolism of these compounds which have been found in nearly all fungi, the brown algae, certain other algae and in many families of higher plants. In general, Lewis and Smith conclude that the possession of sugar alcohols may confer one or more of the following advantages on an organism: (a) the storage of reducing power, since sugar alcohols are in a more reduced state than either simple sugars or polysaccharides; (b) they enable the plant to

maintain a high osmotic pressure and it is suggested that high sugar alcohol contents may prevent the plant suffering from water deficit; (c) these compounds can play a part in coenzyme control as the inter-conversion of sugar alcohols with their corresponding sugars is accompanied by oxido/reduction of NAD or NADP.

3. Other aspects of the symbiosis

The term symbiosis was proposed by De Bary (1879) and he defined it as the living together of dissimilar organisms. This definition is very broad, but the symbioses studied in this thesis are those in which both partners are in close anatomical contact and neither is probably capable of an autonomous existence in nature. They have often been assumed to benefit one another in the association.

In the present study the fungal part of these lichens has been shown to receive carbohydrates from the alga. It is not known if other substances pass between the symbionts, but it seems likely in view of the variety of morphological and behavioural modifications which are manifested when the alga and fungus come into symbiotic association. There is some indirect evidence that vitamins such as biotin move from the alga to the fungus. (Thus, lichen fungi are known to be biotin deficient in culture, while Bednar and Holm-Hansen (1964), as mentioned above, demonstrated that a Coccomyxa sp isolated from a lichen excreted biotin.) It is possible that the

alga in return receives compounds excreted by the fungus. These might be nutrients, or growth promoting substances. For example, indole acetic acid has been shown to stimulate the growth of certain lichen algae in pure culture (di Benedetto and Furnari 1962) and it is known that certain free-living fungi can excrete IAA (Went and Thimann 1937). No studies appear to have been made on lichen fungi to see if they also excrete IAA.

Since supplying ribitcls and vitamins to the isolated fungus of Xanthoria aureola does not result in the formation of fruiting structures or structures at all similar to the thallus morphology found in nature, it is evident that there are other, as yet undiscovered, interactions between the symbionts.

It is also important to point out that not all associations described under the term lichen are of the same type. Some are rather loose fungal-algal associations on tree bark, others, such as the crustaceous corticolous lichen, Pyrenula nitida, have been implicated in die back of hazel twigs. There are also lichens which are only sometimes found with algae, for example Leptorhaphis tremulae, and other forms which grow closely adherent to rocks at an extremely slow rate.

Virtually nothing is known about the physiology of the lichens mentioned above and it is not clear to what extent the results of experiments described in this thesis can be extended to them. Since there are a very large number of these crustaceous lichens, it would be of great interest to discover how similar or different their physiology was from the larger lichens used in the present study.

Carbohydrate movement from alga to fungus in lichens of the genusLobaria*

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There are many symbiotic associations between autotrophic green plants and heterotrophic organisms. It is usually assumed that carbohydrates and perhaps other substances move from the green plant to the other partner, but this has seldom been shown experimentally.

Lichens provide an excellent example of a symbiosis in which this process can be studied. In the two species so far investigated, Peltigera polydactyla^{1,2} and Xanthoria aureola³, a number of differences have been found in the form and rate at which carbohydrate moves from alga to fungus. Because both the algae as well as the fungi of these lichens are different from one another, it is not easy to assess which of the symbionts has the greater influence on the pattern of carbohydrate transfer. Furthermore, the two lichens live in different habitats.

This paper describes a study of the four British species of Lobaria, all collected from the same habitat. In this genus the fungi are all closely related to each other but the algae belong either to Nostoc (as in P. polydactyla) or to Trebouxia (as in X. aureola). Also included in the investigations was a fifth species, Sticta

*This paper has been accepted for publication by Nature (1967)

fuliginosa, which contains Nostoc and belongs to the same family as Lobaria. A comparative study of these lichens should therefore indicate more clearly the influence of the alga on the way in which carbohydrates move between the symbionts.

The two patterns of carbohydrate transfer already determined in lichens may be summarised as follows. In Peltigera polydactyla, glucose moves out of the Nostoc cells, during photosynthesis in the thallus, and is absorbed by the fungus. Here it is immediately converted to mannitol which then accumulates. Thus, if material of this lichen is incubated on an aqueous solution of ^{14}C -sodium bicarbonate for 45 minutes in the light, over 60% of the fixed ^{14}C accumulates in the thallus as mannitol and only about 10% is found in the ethanol-insoluble material. Movement of ^{14}C -glucose from alga to fungus can be prevented if 1% ^{12}C -glucose is included in the medium. Under such conditions, the ^{14}C -glucose released by the algal cells competes unsuccessfully at the fungal uptake sites with the high concentration of ^{12}C -glucose and so diffuses into the medium. Consequently no ^{14}C -mannitol accumulates in the thallus but large amounts of ^{14}C -glucose are found in the medium. Other hexoses such as fructose, mannose and galactose cannot produce these effects suggesting that the fungal uptake mechanism has a high specificity for glucose. The only compounds examined other than glucose which could prevent movement of ^{14}C from alga to fungus were the glucose derivatives, 2-deoxy-glucose and 3-methyl-glucose.

Xanthoria aureola shows a number of points of contrast. It

contains the green alga Trebouxia which is the commonest symbiont of temperate lichens. The main product of photosynthesis of this alga in the lichen thallus is ribitol. After moving out of the algal cells this compound is absorbed by the fungus which converts it into arabitol and mannitol. In a similar manner to Peltigera polydactyla, movement of photosynthetically produced ^{14}C -ribitol from alga to fungus can be inhibited by adding 1% ^{12}C -ribitol to the medium, resulting in accumulation of ^{14}C -ribitol in the medium. Under these circumstances, little ^{14}C -arabitol or ^{14}C -mannitol is formed in the thallus. Both ^{12}C -arabitol and ^{12}C -xylitol were also able to inhibit this movement of ^{14}C from alga to fungus but various hexoses, hexitols and pentoses could not, suggesting that in this case the fungal uptake mechanism had a high specificity for pentitols. Although preliminary studies had earlier indicated that carbohydrate transfer between the symbionts is rapid⁴, it is now known that this is not correct. In fact, the movement of ^{14}C from alga to fungus in X. aureola is much slower during a 24 hour period than it is in P. polydactyla when they are subjected to similar experimental conditions.

Comparison of the four Lobaria species and Sticta fuliginosa

Of the five lichens investigated, Lobaria pulmonaria and L. laetevirens contain Trebouxia, while L. scrobiculata and Sticta fuliginosa contain Nostoc. In the fifth species, Lobaria amplissima, the thallus contains Trebouxia but also bears fruticose outgrowths containing Nostoc, which are termed cephalodia. It has been stated

that the cephalodia of lichens originate from the soredia of foreign species landing on the thalli of the hosts⁵, but it is more generally assumed that the fungus in a cephalodium is identical to that in the thallus and continuous with it. This belief is supported by the results of developmental studies⁶, by the rarity of lichens capable of producing soredia containing Nostoc, by the constancy and abundance of cephalodia on those species which bear them, and by the fact that cephalodia never form apothecia or other reproductive bodies.

All the lichens used in these experiments were growing on beech near Port Appin, Argyll (17/916461), and we would like to thank Mr. D. Walkinshaw for kindly collecting them for us.

In order to study the movement of carbohydrates from alga to fungus in each species, the thalli were sampled into discs 7 mm in diameter (5 mm diameter for L. amplissima) in a manner similar to that described for Peltigera polydactyla^{7,8}. The cephalodia could not be sampled as discs so they were excised from the thallus and assorted into samples each of 200 mg fresh weight. All samples were incubated in closed vessels, in the light, on aqueous media containing ¹⁴C-sodium bicarbonate. In an attempt to inhibit movement of the fixed ¹⁴C between the symbionts, either 1% ¹²C-glucose or 1% ¹²C-ribitol was included in the media of some of the samples of each species. At the end of the period of photosynthesis, measurements were made of the ¹⁴C present in the medium, the ethanol-soluble and the ethanol-insoluble fraction of each sample (after prior removal of residual ¹⁴C-sodium bicarbonate). The ethanol-soluble fractions were analysed by paper

chromatography using ethyl methyl ketone - acetic acid - water saturated boric acid (9 : 1 : 1)⁹ which gives a good separation of the polyols from one another and from related sugars. After development of the chromatograms, the position of radioactive compounds was determined by autoradiography and by scanning the strips on an automatic scanner¹⁰. The position of non-radioactive markers and other compounds was determined by developing with silver nitrate¹¹ after dipping in hydrogen fluoride¹².

To obtain a broad comparison of the rate of ¹⁴C movements between the symbionts, samples were incubated for 3 and 24 hours at room temperature (18°C) and for 24 hours at 5°C, a temperature more often occurring in the Scottish habitats from which the lichens were collected. The results of the comparison between the various Lobaria species and Sticta fuliginosa are shown in Table 1. Fig. 1 illustrates scans of chromatograms of the ethanol soluble extracts of two representative species, L. amplissima thallus (Trebouxia) and L. scrobiculata (Nostoc).

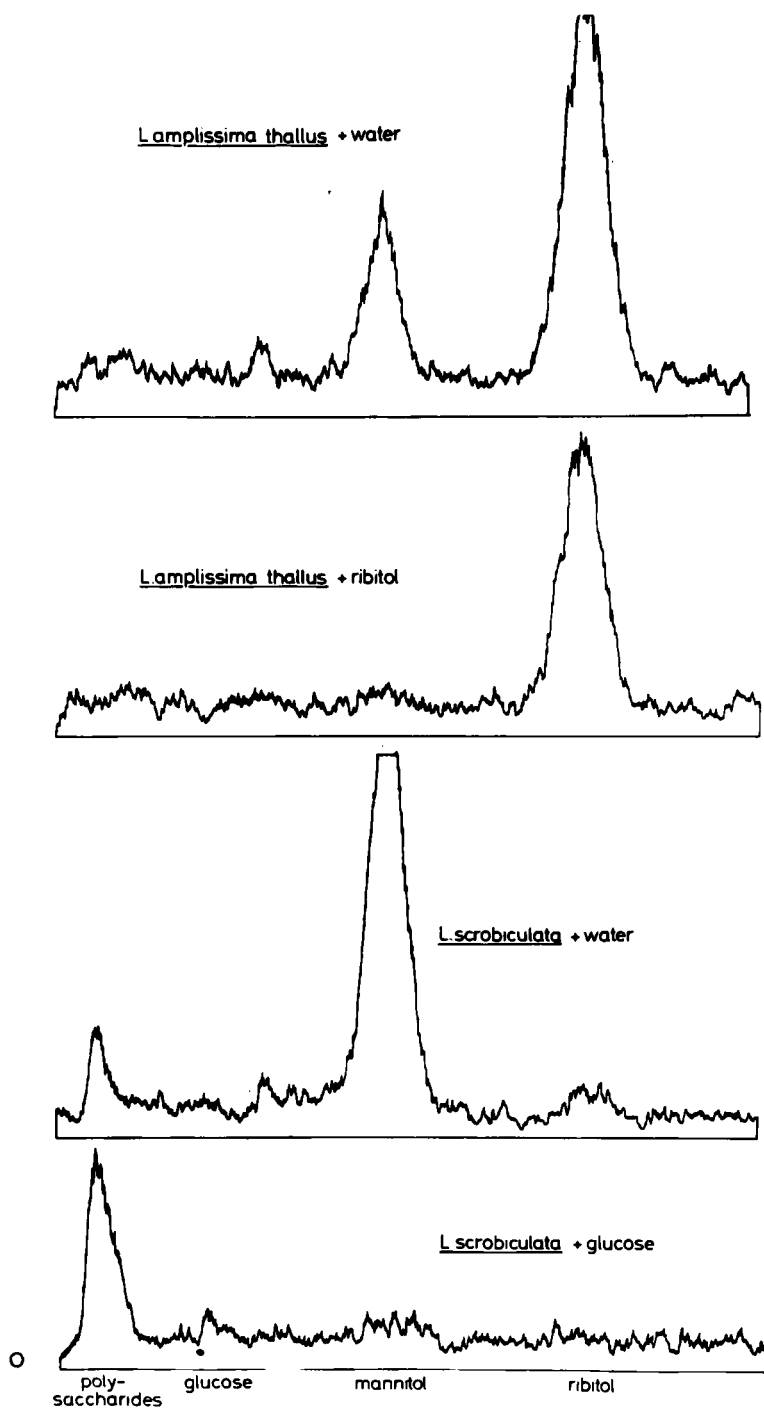
Table 1 and Figure 1 clearly illustrate the role of the algal component in determining the pattern of flow of carbohydrate between the symbionts. All the lichen material containing Nostoc showed the following features: (a) during photosynthesis on water the main compound in which ¹⁴C accumulated was mannitol; (b) on media containing glucose, there was a considerable flow of ¹⁴C out of the discs into the medium; (c) on media containing glucose little ¹⁴C-mannitol accumulated in the thallus but small amounts of ¹⁴C-glucose occurred together with greater amounts of an unidentified polysaccharide;

Table 1. Release of photosynthetically fixed ^{14}C to medium by various lichens in the presence of ^{12}C -glucose and ^{12}C -ribitol (results expressed as per cent in medium of total soluble fixed ^{14}C).

	<u>L.</u> <u>pulmonaria</u>	<u>L.</u> <u>laetivirens</u>	<u>L.</u> <u>amplissima</u> (thallus)	<u>L.</u> <u>amplissima</u> (cephalodia)	<u>L.</u> <u>scrobiculata</u>	<u>Sticta</u> <u>fuliginosa</u>
	<u>Trebouxia</u>	<u>Trebouxia</u>	<u>Trebouxia</u>	<u>Nostoc</u>	<u>Nostoc</u>	<u>Nostoc</u>
3hr/18°C						
Water	0.5	0.2	1.6	2.3	0.5	0.4
Glucose	0.4	0.2	0.6	22.6	21.3	15.9
Ribitol	1.8	1.7	1.7	1.3	0.6	0.4
24hr/18°C						
Water	0.6	0.2	0.9	2.3	0.7	0.9
Glucose	2.8	1.1	4.2	56.0	56.8	62.7
Ribitol	34.7	45.1	39.1	15.3	1.4	3.2
24hr/5°C						
Water	1.1	0.2	1.4	1.2	0.4	0.9
Glucose	1.0	0.3	1.6	39.5	35.8	31.6
Ribitol	11.4	12.6	11.6	2.5	0.7	0.9

All material as samples of 5 x 7 mm thallus discs except L. amplissima thalli (5 x 5 mm discs with no cephalodia) and cephalodia (200 mg fresh weight). All samples incubated in shaker bath on 3.05 ml distilled water with 5 μC carrier-free $\text{NaH}^{14}\text{CO}_3$ in 2" x 1" specimen tubes covered with 1" diameter cover glasses sealed on with vaseline. Light intensity 500 foot candles; controls showed dark fixation to be no greater than 5% light fixation, and no significant release of fixed ^{14}C from lichen material in any medium in the dark. Net total ^{14}C Fixation on water over 24 hours was approximately 15% greater at 5°C than at 18°C (mean of all species). Glucose and ribitol as 1% (w/v) aqueous solutions.

Fig. 1. Chromatogram scans showing distribution of radioactivity in ethanol-soluble extracts of L. amplissima thallus and L. scrobiculata after incubation for 24 hours in the light at 18°C in aqueous $\text{NaH}^{14}\text{CO}_3$ in the presence and absence of non-radioactive carbohydrates. Solvent system: ethyl methyl ketone - acetic acid - water saturated with boric acid (9 : 1 : 1). Other experimental conditions as in Table 1.



o = origin

(d) the presence of ribitol in the medium had no detectable influence, except in the case of the cephalodia of L. amplissima where there were some small effects due to fragments of thallus containing Trebouxia remaining attached to the cephalodia. All these features resemble very closely those reported for Peltigera polydactyla^{1,2}, hence it may be assumed that the pattern of carbohydrate movement between the symbionts is also very similar.

All the lichen material containing Trebouxia was characterised by: (a) after 3 hours photosynthesis on water most of the fixed ¹⁴C accumulated in ribitol, but after 24 hours some ¹⁴C appeared in mannitol and a very small amount in arabitol, especially in L. pulmonaria; (b) during photosynthesis on ribitol media, appreciable amounts of ¹⁴C diffused out of the discs after 24 hours, though very little after 3 hours; (c) on ribitol media, little ¹⁴C-mannitol or ¹⁴C-arabitol accumulated even after 24 hours at room temperature; (d) the presence of glucose in the medium had no effect other than a reduction in the total amount of ¹⁴C fixed, an effect also noted in material containing Nostoc. Again, all these characteristics closely follow those reported for Xanthoria aureola³. It therefore appears that the contrasting pattern of carbohydrate movement within the thalli of Peltigera^{*} polydactyla and X. aureola may be determined primarily by the nature of the algal component.

A particularly interesting species which indicates the importance of the algal component is Lobaria amplissima, which contains Trebouxia in the thallus and Nostoc in the cephalodia. The pattern of carbohydrate

movement between the symbionts of the thallus resembles that found in Xanthoria while in the cephalodia the pattern is comparable with Peltigera.

In all lichens, there was relatively little incorporation of ^{14}C into ethanol-insoluble material. For Lobaria species it was not more than 18% of the total fixed ^{14}C after 24 hours, and for Sticta fuliginosa no greater than 30%.

Carbohydrate movements within the fungal component

Little is known about the movement of carbohydrate within the fungal component from the photosynthesising region to other parts of the thallus. Using a dissection technique⁷ this movement from the algal layer to the underlying area has been studied in Peltigera polydactyla^{8,13} where it was shown that carbon fixed in photosynthesis can move into the medulla, presumably along the fungal hyphae, within 30 minutes and that this movement continues at a steady rate equivalent to 40% to 50% of total fixation. Unfortunately, Xanthoria aureola is not amenable to dissection so that comparable studies could not be carried out on this Trebouxia-containing lichen. However it was found that two of the Lobaria species used in the experiments described here could be dissected: L. scrobiculata (Nostoc) and L. amplissima (Trebouxia). The technique for dissecting the latter was the same as for Peltigera polydactyla¹³, viz., making an incision just below the algal layer so as to separate two tissue regions. In L. scrobiculata the algal layer was very fragile

and uneven so a different technique had to be devised. Here, the algal layer was chipped off with a scalpel as many small fragments, so giving a more exact separation of the medulla from the rest of the thallus but at the expense of destroying the algal layer. Comparisons therefore had to be made between medulla and control undissected discs, and not between two tissue regions as could be done with L. amplissima. The results of dissection experiments with these two species are shown in Tables 2 & 3.

Table 2 shows that L. scrobiculata resembles P. polydactyla in that substantial amounts of fixed ^{14}C can move into the medulla within 3 hours and that such movement can be almost completely inhibited by the presence of ^{12}C -glucose in the medium. Table 3 shows that by contrast, movement of fixed ^{14}C into the medulla of L. amplissima is much slower. Little movement was detected after three hours, in agreement with Table 1. However, even after 24 hours, movement is comparatively small although more than 40% of the fixed ^{14}C is released from the discs in the presence of ribitol, implying that substantial transfer of carbon to the fungus within the algal layer occurs during this time. Extracts of medullae of L. amplissima show that after 24 hours the principal radioactive compound detectable is mannitol, no ^{14}C -ribitol is present even though this is abundant in the algal layer. This raises the question of whether ribitol can be translocated in the fungal hyphae. If it cannot be translocated, then the slow movement of ^{14}C into the medulla of L. amplissima, may

Table 2. Movement of photosynthetically fixed ^{14}C into medulla of Lobaria scrobiculata (results expressed as per cent of total soluble ^{14}C).

		Water	1% Glucose
3 hrs	^{14}C in medulla	40.1%	8.0%
	^{14}C in medium	0.4%	25.0%
24 hrs	^{14}C in medulla	37.4%	4.0%
	^{14}C in medium	0.8%	68.0%

Each sample = 8 x 7 mm discs with 4 discs undissected to measure total fixation and 4 discs dissected to obtain medullae free of algae. Experimental conditions as in Table 1. Results corrected so that values for medulla represent ^{14}C in excess of dark fixation.

Table 3. Movement of photosynthetically fixed ^{14}C into medulla of Lobaria amplissima (results expressed as per cent of total soluble ^{14}C).

		Water	1% Ribitol
3 hrs	^{14}C in medulla	2.9%	2.1%
	^{14}C in medium	0.5%	1.7%
24 hrs	^{14}C in medulla	7.5%	4.9%
	^{14}C in medium	0.9%	40.5%

Experimental conditions as in Table 1. Results corrected so that values for medulla represent ^{14}C in excess of dark fixation.

reflect the fact that conversion of ribitol to mannitol in the thallus is slow (e.g., c.f. Fig. 1). It is therefore quite possible that lichens containing Nostoc may have a further difference from those containing Trebouxia in the rate at which carbohydrate is distributed from the photosynthetic region to other parts of the thallus.

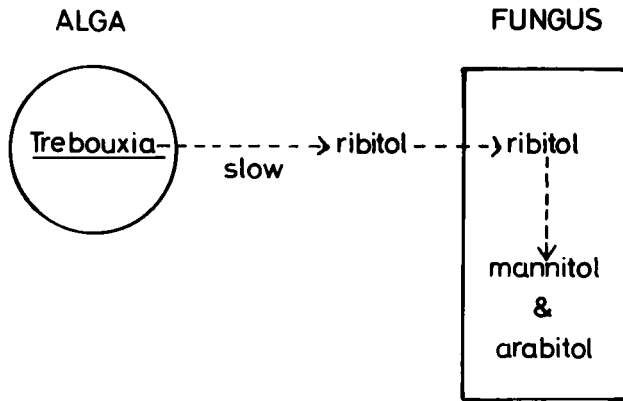
Discussion

Fig. 2 summarises the main differences between lichens with Nostoc and those with Trebouxia. It is believed that the alga plays a dominant role in dictating certain features of carbohydrate transfer between the symbionts, and therefore the two systems are termed the 'Nostoc' and 'Trebouxia' patterns.

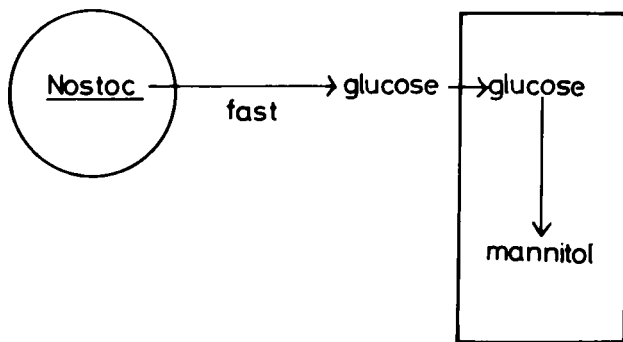
There are, however, some similarities between the two patterns. Data in Table 1 suggest that in both cases, carbohydrate movement between the symbionts is temperature sensitive, as a smaller proportion of the fixed ^{14}C is released to the medium in 24 hours at 5°C than at 18°C when inhibiting carbohydrates are present. Although it may appear from Table 1 that the 'Trebouxia' pattern is more temperature sensitive than the 'Nostoc' pattern, more detailed experiments would be required to demonstrate this conclusively. The main point of similarity is that in both patterns, the principal destination of carbohydrates in the fungus is initially into sugar alcohols, especially mannitol, rather than ethanol insoluble material.

Fig. 2 Patterns of carbohydrate movement from alga to fungus in lichens containing Trebouxia and Nostoc.

Trebouxia Pattern



Nostoc Pattern



Nostoc and Trebouxia are two of the commonest symbionts of lichens. Investigations currently in progress into lichens containing other algae, such as Trentepohlia and Coccomyxa, show that other patterns of carbohydrate transfer exist, each again appearing to be characteristic for the alga concerned.

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APPENDIX BI Citation of Specimens

Dermatocarpon miniatum (L.) Mann On Limestone.

- a. Kinsale **S**ummer Cove, Cork, Ireland (V.C.H4), 22.8.65. DHSR
- b. Muckross Abbey, Killarney, Kerry, Ireland (V.C.H2), 14.8.65. DHSR
- c. Nr. Roselle Manor, Jersey, Channel Islands (V.C.C), 14.4.66. DHSR
- d. Islam Park, nr. Dovedale, Derbyshire (V.C.57), 13.6.66. DHSR

Gylecta cupularis (Ehrh.) Schaer. On Limestone.

Dovedale, Derbyshire (V.C.57), 14.6.66. DHSR

Lecanactis Stenhammarii (Fr.) Arnold On Limestone.

- a. Berry Head, nr. Torquay, Devon (V.C.3), 14.3.66. DHSR
- b. Lathkilldale, Derbyshire (V.C.57), 13.6.66. DHSR

Lecanora conizaeoides Nyl. ex Cromb. On fallen logs.

- a. Bagley Wood, nr. Oxford (V.C.23), 8.1.66. DHSR
- b. Nr. Sheldon, Derbyshire (V.C.57), 18.10.65. DHSR

Lichina pygmaea (Lightf.) C. Ag. Hornblend Chloritic Schist.

Prawle Point, nr. Slapton, Devon (V.C.3), 10.9.65, 15.3.66. DHSR

Lobaria amplissima (Scop.) Forss. On frondose trees.

- a. Nr. Newton Stewart, Kirkud. Scotland (V.C.73), 30.7.65. DHSR
- b. On Acer, Muckross Abbey, Killarney, Ireland (V.C.H2), 14.8.65. DHSR
- c. On Fagus, Port Appin, Argyll, Scotland (V.C.98), 5.66. DW

Lobaria laetevirens (Lightf.) Zahlbr. On frondose trees.

a. On Quercus, Weir Bridge, Killarney, Ireland (V.C.H2), 14.8.65. DHSR

b. On Fagus, Port Appin, Argyll, Scotland (V.C.98), 5.66. DW

Lobaria pulmonaria Hoffm. On frondose trees.

a. On Quercus, nr. Newton Stewart, Scotland (V.C.73), 30.7.65. DHSR

b. On Fagus, Port Appin, Argyll, Scotland (V.C.98), 5.66. DW

Lobaria scrobiculata (Scop.) DC. On frondose trees.

a. Fallen Quercus, Weir Bridge, Killarney (V.C.H2), 14.8.65. DHSR

b. On Fagus, Port Appin, Argyll, Scotland (V.C.98), 5.66. DW

Parmelia saxatilis (L.) Ach. On Millstone Grit.

Nr. Sheldon, Derbyshire (V.C.57), 18.10.65. DHSR

Peltigera polydactyla (Neck.) Hoffm. On ground.

Cumnor Hill, nr. Oxford (V.C.23), 18.10.65, 4.11.65. DCS

Peltigera aphthosa (L.) Willd. On Mosses.

Scotland (V.C.), 5.7.66. DW

Roccella fuciformis DC. On Hornblend Chloritic Schist.

Prawle Point, nr. Slapton, Devon (V.C.3), 10.9.65. 12.7.66. DHSR

Roccella phycopsis (Ach.) Ach. On Hornblend Chloritic Schist.

Prawle Point, nr. Slapton, Devon (V.C.3), 10.9.65, 3.1.66. DHSR

Solorina saccata (L.) Ach. On Limestone Scree.

Dovedale, Derbyshire (V.C.57), 14.6.66. DHSR

Sticta fuliginosa (Dicks.) Ach. On fagus.

Port Appin, Argyll, Scotland (V.C.98), 5.66.

Umbilicaria pustulata (L.) Hoffm. On Granite.

Sharp Tor, Dartmoor, Devon (V.C.3), 26.7.65. DHSR

Xanthoria aureola (Ach.) Erichs. On Tiles.

Roof of the University farm, Wytham, Oxfordshire (V.C.23)

1964-1966. DHSR

Xanthoria parietina var ectanea(Ach.) Kickx On Hornblend Chloritic
Schist.

Above Black Cove, nr. Signal House Point, Slapton, Devon.

(V.C.3), 1965. DHSR

Collectors:

DCS - Dr. D.C. Smith

DW - Mr. D. Walkinshaw

DHSR - D.H.S. Richardson.

II Citation of Culturesa. The Isolated Fungi

Xanthoria aureola C.M.I. No 112697 (Commonwealth Mycological Institute), R. 202. Wytham, Oxfordshire. October 1964.

D. Richardson.

Xanthoria aureola 2nd isolate, C.M.I. No 112702, R. 209. Wytham, Oxfordshire. February 1965. (Pigment producing isolate) D.H.S.R.

Xanthoria parietina C.M.I. No 112696,

R. 210. U.S.A. V. Ahmadjian.

Xanthoria parietina C.M.I. No 112698, R. 204. Lathkilldale,

Derbyshire. October 1965. D. Richardson.

Xanthoria parietina C.M.I. No 112704, R. 212. Italy. R. Tomaselli.

b. The Isolated Algae

Trebouxia 'Quispel strain' (Cystococcus humicola (Naeg.) Treboux)

C. 219/1d (Cambridge Culture collection). Xanthoria parietina, Holland. A. Quispel circa 1940.

Trebouxia 'Ahmadjian strain' (Trebouxia decolorans Ahmadjian)

Indiana 901 (Culture collection, Indiana University, U.S.A.)

Xanthoria parietina, U.S.A. V. Ahmadjian circa 1960.

Trebouxia 'Oxford strain' Wytham, Oxfordshire.

C. 219/2, R. 207. Xanthoria aureola, U.K. D. Richardson. February 1965.

Coccomyxa sp Wisconsin. R. 204. Peltigera aphthosa, U.S.A.

T. Bednar, 1963.

APPENDIX CMedia Used

(a) Culturing the Mycobiont

Malt Extract, Yeast Extract Agar

malt extract	20 g.
yeast extract	2 g.
agar	20 g.
distilled water	1000 ml.

Adjust pH to 6.0-6.3, steam, filter if necessary and autoclave at 10 lbs. for 20 minutes. Recheck pH.

Bianchi Medium

NH_4 tartrate	5.0 g.	
NH_4NO_3	1.0 g.	
KH_2PO_4	1.0 g.	
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g.	
NaCl	0.1 g.	
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.1 g.	distilled water
biotin	4.0 ug.	1 Litre
thiamine	0.5 mg.	
trace element soln.	1.0 ml.	
sucrose	10.0 g.	

Adjust pH to 5.5-5.8, autoclave 15 lbs. for 15 minutes.

(b) Culturing the Phycobiont

Trebouxia Medium

Bristol's solution (given below)	850 ml.
soil water supernatant (" ")	140 ml.
proteose peptone	10 g.
glucose	20 g.
agar if required	15 g.

Autoclave at 15 lbs. for 15 minutes.

Bristol's Solution

NaNO_3	10.0 g.
CaCl_2	1.0 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	3.0 g.
K_2HPO_4	3.0 g.
KH_2PO_4	7.0 g.
NaCl	1.0 g.

Six stock solutions are made up in 400 ml. each containing the salts in the amounts listed above. 10 ml. of each stock solution added to 940 ml. of distilled water. To this is added 1 drop of 1.0% FeCl_3 solution, and 2 ml. of minor element solution.

(Trelease & Trelease Am. J. Bot. 22:520-542., 1935)

Soil Water Supernatant

100 g. soil added to 500 ml. hot distilled water. Left to cool and filtered through whatman No. 4 paper.

Gorhams Medium (No 32) modified E.A. Drew.

NaNO_3	42.5 g.	
K_2HPO_4	6.0 g.	
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	6.25 g.	
$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$	2.5 g.	
Na_2CO_3	1.0 g.	} buffer solution omitted for cultural work.
$\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$	5.0 g.	

sodium nitrate omitted for culture of Nostoc sp

Six stock solutions prepared in 500 ml. distilled water; each containing the salts in the amounts listed above. 10 ml. of each stock solution added to 940 ml. distilled water. Autoclaved at 15 lbs. for 15 minutes.

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