STUDIES ON THE PHYSIOLOGY OF LICHENS WITH SPECIAL REFERENCE TO PELTIGERA PRAETEXTATA (FLK.) VAIN.

Thesis presented by

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for

the degree of Doctor of Philosophy of

the University of Glasgow

December, 1956

STUDIES ON THE PHYSIOLOGY OF LICHENS WITH SPECIAL REFERENCE TO PELTIGERA PRAETEXTATA (FLK.) VAIN.

Summary of thesis presented by George D. Scott, B.Sc., for the degree of Doctor of Philosophy of the University of Glasgow.

Part I comprises an investigation of several lichens for fixation of elemental nitrogen. Three lichens, Collema granosum (Scop.) Schaer., Leptogium lichenoides (L.) Zahlbr., and Peltigera praetextata (Flk.) Vain., each containing blue-green algae, were examined by a technique using the heavy isotope of nitrogen, ^{15}N. All were shown to fix atmospheric nitrogen. Evidence is provided to show that the fixation should be attributed to the algal symbiont (Nostoc) of these lichens. In the case of Leptogium and Peltigera, calculation of the total fixation of nitrogen over a period of five days, shows that the rate of fixation (approx. 0.05 mg. nitrogen per gm. fresh weight of lichen) was too high to be attributed to any nitrogen-fixing agent (e.g. Azotobacter) other than the Nostoc.

Confirmation of the fixation of nitrogen by Peltigera praetextata was obtained by a study of the growth, in nitrogen-free mineral solution, of discs of this lichen. It is also shown, by this method of culture, that nitrogen fixed by the algal symbiont is transferred to the associated fungal hyphae.

Application of the isotopic test to two lichens containing green algae, Cladonia impexa Harm. f. laxiuscula (Del.) Sandst. and Lobaria pulmonaria (L.) Hoffm., gave no evidence of fixation

of nitrogen. It is considered, on the basis of these tests, that the triple symbiosis theory of Henckel and Yuzhakova (1936) can not be universally applied to lichens.

In Part II is described an investigation of the growth, in culture, of discs of Peltigera praetextata. Experiments are described which relate to the effect, on growth of discs, of the concentration of nutrients in the culture medium, and of the moisture content of the discs. Data for the rate of growth, under greenhouse conditions, of an entire plant of this species are given. This is shown to be higher than the rate of growth of discs of the lichen in culture.

A physiological investigation of the formation of isidia on P. praetextata, and on other species of the genus, leads to the view that these structures are initiated by a light stimulus acting on the algal symbiont. An isidioid growth, somewhat similar to that commonly found on P. praetextata, has been induced to form on P. horizontalis, a species on which these structures are not found in Nature. Considerable evidence is produced, both from culture work and from examination of herbarium material, to support the view that the isidia of P. praetextata are potential reproductive organs.

The isolation of the algal symbiont of P. praetextata, in bacteria-free culture, is described in Part III. Three isolation techniques were used. The isolated alga was irradiated with ultra violet light to produce a bacteria-free culture. Various stages in the development of the algal symbiont in culture are

described and illustrated. The alga is shown to be capable of heterotrophic growth, with the continued formation of chlorophyll, using glucose as the source of energy.

Part IV comprises an investigation of the characteristics of dissemination and germination of ascospores of Peltigera praetextata. An aqueous extract of Nostoc, isolated from P. praetextata, alone provided a suitable medium for spore germination. A detailed investigation of the causes of spore rupture in various synthetic media and in media containing agar, showed that this effect was due to an excess of vitamins in these media. The addition of thiamin (100 gamma/l.) or biotin (10 gamma/ to vitamin-free media produced a similar effect. The advantages of silica-gel as a substitute for agar are discussed.

Tabulation of the data recorded for 83 apothecia of Peltigera praetextata, used in experiments over a period of three years, shows that spores are ejected from the apothecia throughout the year. At no season are they incapable of germinating.

Consideration of the data obtained regarding spore rupture leads to the view that a large percentage of spores ejected from apothecia to the soil surrounding the plant, must succumb to this effect and hence be unable to germinate.

The continued use of the term "gonidia" in the description of lichen-algae is deprecated. The term "phycobiont" is proposed, to replace "gonidium" and synonymous terms such as "algal symbiont", "algal host" and "lichen-alga". The term "mycobiont" is proposed, to replace the terms "fungal symbiont" and "lichen-fungus".

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PREFACE

The work described in this thesis was carried out in the Department of Botany of the University of Glasgow. Among the many to whom acknowledgement is due, the author wishes particularly to express his gratitude to Professor John Walton and to Dr. George Bond. Both, by their constant interest and advice in the problems arising out of this work, have done much to ensure its completion.

To Dr. R.I.Reed, of the Department of Chemistry of this University, acknowledgement is made for carrying out the mass spectrometer analyses.

Part of this work was undertaken with the help of a research grant donated by the Carnegie Trust for the Universities of Scotland. To the officers of that Trust, and to the Principal and Deans' Committee of the Senatus Academicus of the University of Glasgow, who supplied a grant for a visit to Uppsala University, the author proffers his grateful thanks.

To Professor J. Axel Nannfeldt and Dr. Rolf Santesson, of the Institute of Systematic Botany of the University of Uppsala, the author extends his thanks for unstinted hospitality and help during his visit to the Institute.

Dr. Eilif Dahl, formerly of the Department of Botany of the University of Oslo, and Professor Henry Des Abbayes, of the University of Rennes, identified specimens of lichens. Mr. Peter W. James, formerly of the Department of Botany of the University of Liverpool, supplied frequent gatherings of fresh material of Peltigera, and Mr. W. Anderson, of the Department of Botany of the University of Glasgow, was responsible for the majority of the photographic illustrations. Much appreciation of their help is expressed.

Finally, the author records a special debt of gratitude to the Librarian and staff of the Library of the University of Glasgow for their unfailing efforts to meet the many demands placed upon them for periodicals and books which, so often, had to be obtained from other libraries.

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CONTENTS

PART II. A study of the growth of species of Peltigera, with special reference to the formation of isidia.

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GENERAL INTRODUCTION

No one can fail to be impressed by the diversity of form and remarkable tenacity of life exhibited by those composite organisms - lichens - which, by their very nature, have long presented a challenge to the ingenuity of biologist and chemist alike. From the arid desert to the icy wastes of the polar regions, from the shores of the saltest seas to the silent mountain heights, scarcely a rock or tree exists that does not bear witness to the

" Ten thousand forms minute
Of velvet moss or lichen,..." *

Their capacity to endure the greatest extremes of climate has been commented upon by many a scientist and explorer, and lately there has developed a theory (not entirely free from the fantasy of fertile minds) of a hypothetical plant life on the planet Mars, having an organisation akin to that of lichens on Earth (STRUGHOLD, 1954). But aside from such speculations, the many unique features revealed by the association of alga and fungus, whether this is believed to be an instance of true symbiosis or

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* From, W. Lauder Lindsay : A Popular History of British Lichens. London, 1856.

of parasitism by one organism upon the other, can never cease to be a source of fruitful research.

Lichens have been recognised since the times of the earliest lichenologists as plants having the capacity to exist under extremely inhospitable conditions. They are to be found as the last representatives of plant life in the mountains far above the limit of other vegetation; in the polar regions they are recorded from situations in which it is scarcely conceivable that plant life could exist. They frequently occur among the first colonisers of virgin rock, and two species of Stereocaulon are noteworthy for their rapid invasion of lava flows in the active volcanic regions of the world.

To what extent the ability to exist in such situations is due to the singular morphological or physiological characteristics of the lichens, has been the subject of long discussion. There is no doubt that the mere association of alga and fungus, quite apart from the resultant physiological peculiarities, was in the beginning of the lichen synthesis the operative factor in the united advance of the two organisms into regions devoid of plant life. The process of evolution however, has brought with it the development of several physiological features, for instance, the production of "lichen acids" and various pigments which have been

assigned diverse functions in the maintenance of the symbiotic state. Whether features such as these are conditional upon the association of alga and fungus, or whether either one of the symbionts retains the attribute in isolated culture, is still far from certain. Conflicting reports appear in the literature regarding the production of the lichen acid parietin; it is said on the one hand that this is strictly a product of the symbiosis, and on the other hand that the fungus, in culture, is capable of producing the acid (see QUISPEL, 1943-45).

It is not without good reason that lichenologists of preceding generations were prone to regard the lichen as an entity distinct from either alga or fungus. This tradition has been slow in dying, principally on account of the many features which, superficially, appear to be unique to the lichen symbiosis. In recent years however, there has developed a new outlook with the introduction by THOMAS (1939) of a scheme of classification based on the characteristics of the fungal symbiont only. This scheme is unfortunately in danger of being carried to extremes. To cite one instance only, it is proposed by CIFERRI and TOMASELLI (1953) that the fungal symbionts of Usnea and of Lichina be classified together under the generic name Usneomyces. The advantages of this new systematics of lichenized fungi are evident chiefly in

the field of experimental taxonomy, and should, if judiciously applied, be instrumental in creating a better understanding of the affinities of genera and species which, under the present systematics, appear to be in no way related.

Parallel with the development of the new systematics there has arisen a renewed interest in the isolation and study of the symbionts in pure culture - a significant contribution in this respect being the work of QUISPEL (1943-45). Much of this type of work has as its basis, the elucidation of problems concerning taxonomy and the production of lichen acids having antibiotic properties. Such investigations, while serving their own ends, also provide valuable contributions to the study of the lichen symbiosis, and it is to these that we look for future developments which might unseal the many doors barring the way to the synthesis, as a routine laboratory task, of lichens from pure cultures of the appropriate algae and fungi. In the meantime however, many fundamental problems remain unsolved. Not only is there little information available regarding the nutrient requirements of alga and fungus in the state of symbiosis, but an even greater gap appears in our knowledge of the part played by moisture in the successful creation and maintenance of the proper balance between the symbionts.

While it is agreed that most lichens can get by with, and indeed there is evidence to suggest that they demand, sub-optimal levels of nutrients (in respect to the requirements of alga or fungus in pure culture), this generalisation is little short of the sum total of present day information regarding lichen nutrition. In the absence of data of this nature, it is scarcely surprising that attempts to synthesise lichens from cultures of the symbionts have so frequently been unsuccessful. The extensive literature on lichenology reveals singularly few contributions dealing with the determination of optimal cultural conditions for lichens. Even during the most active period of physiological investigation of lichens - at the turn of the century - this most important aspect seems to have been altogether by-passed, or at best, its omission excused on the grounds of the notoriously slow growth rate of these organisms.

We are favoured however, by the fortuitous occurrence of a species, Peltigera praetextata (Flk.) Vain., which shows every promise of being a profitable subject of physiological study. This lichen, for so long the centre of controversy regarding its taxonomic status, is of widespread distribution throughout the world, and in Scotland it is perhaps the most common representative of the genus. The principal specific characteristic by

which it is distinguished from the parent species, Peltigera canina, is the development in the majority of specimens, of numerous isidioid outgrowths along the margins of the lobes and around cracks and wounds in the upper cortex. It is not unusual to find specimens so completely covered by these isidia that the original form of the thallus can scarcely be distinguished. Various interpretations have been made of the function of isidia, ranging from structures which bear pycnidia, to organs which serve to increase the photosynthetic and transpiration area of the thallus. No one however, has yet succeeded in defining the inherent quality of P. praetextata which, under particular environmental conditions, finds expression in the production of isidia.

Fertile specimens of P. praetextata are of common occurrence, but while it is believed that the frequency of apothecia bears some relation to the incidence of isidia (which are themselves potential reproductive organs), little is known of the part played by the ascospores in reproduction of the species. Like those of other lichens, the apothecia of P. praetextata remain active for several years and can withstand prolonged periods of desiccation without damage to the ascospores.

The algal symbiont (a species of Nostoc) occupies a layer immediately beneath the cortex. In this position

the alga can receive adequate light through the cortical cells, mineral salts and water by absorption from the underlying hyphal tissues, and gases through the air passages in the medulla. No air spaces or pores appear in the cortex.

In Peltigera, as in all lichens, many intricacies of the relationship between alga and fungus remain obscure. Although there is now fairly general agreement that the association is not one of true symbiosis involving the exchange of nutrients otherwise unavailable, there is less agreement on the precise nature of the benefit accruing to the alga partaking in the association. There is no doubt that the alga is enabled, by its association with the fungus, to attain a wider distribution than is possible for the free-living alga, but does this fact constitute sufficient reason for describing the association as a case of symbiosis? No satisfactory answer can be given to this question until more information is available regarding the interchange of metabolites between the two organisms. In this connection, a welcome addition to our knowledge of the carbohydrate metabolism of Peltigera has just been published by HARLEY and SMITH (1956).

The obvious dependence of the fungus upon the alga for its supply (in part at least) of carbohydrate, and the fact that the algal associate is an autonomous organism,

suggests that the association is an instance of facultative parasitism. This is particularly so in the case of lichens containing blue-green algae, where there is every reason to believe that nitrogenous compounds (elaborated from atmospheric nitrogen fixed by the alga), as well as carbohydrates, are supplied to the fungus. A similar transfer of nitrogen is said to occur in lichens containing green algae, but in this case the seat of fixation is supposed to be the bacterium Azotobacter (HENCKEL and YUZHAKOVA, 1936). The theory that this bacterium is of general occurrence in lichens adds considerable complication to the system of interchange of metabolites which has been proposed, principally by QUISPEL (1943-45). There is however, no conclusive evidence that either alga or fungus benefits directly from the association with the Azotobacter.

In the early times of lichenology the algae in lichens, then considered to be reproductive organs of the fungus, were termed gonidia by WALLROTH (1825). This name has persisted in lichenological works and although it is now somewhat of a classic term, its continued use in the description of lichen-algae is undesirable. For this reason, present-day authors tend to use the terms "algal host" or "algal symbiont", depending on the views held on the nature of the alga-fungus association.

From the purely descriptive point of view we lack

convenient terms by which to designate the participants in the association; terms which would not imply any particular type of association and which would avoid confusion when reference is also made to the free-living organisms, or to epiphytic and parasitic species in lichens. It is thus proposed that the terms "phycobiont" and "mycobiont" be used to describe the lichen-algae and fungi respectively. These terms could also find wider application than in the field of lichenology. An alga, for instance, taking part in any association or symbiosis could be adequately described as a phycobiont. Instances such as the association of blue-green algae with Cycas, Gunnera and Blasia may be mentioned. Similarly, the term mycobiont would be applicable to any fungus, saprophytic, parasitic or truly symbiotic, which is associated with another plant or animal. Thus, usage of the two terms at once indicates that the alga or fungus takes part in an association with some other organism.

In using the term symbiosis, it is intended that nothing more should be conveyed than the association of the two organisms taking part in the formation of the lichen. Reference is occasionally made to "green lichens" and to "blue-green lichens". In the former, the phycobiont is a member of the Chlorophyceae, and in the latter, of the Myxophyceae. The use of numerous other lichenological

terms is in accordance with their accepted meaning.

The four parts of this thesis comprise, I) an investigation of the fixation of elemental nitrogen by several lichens, and of the transfer of fixed nitrogen to the mycobiont of Peltigera praetextata; II) a series of experiments on the growth of P. praetextata under semi-natural conditions and in culture, and a study of the formation and development of isidia of this species; III) the isolation, in bacteria-free culture, of the phycobiont of P. praetextata for use in experiments described in Part IV, and observations on the growth of the phycobiont in culture; IV) an investigation of the dissemination and germination of the ascospores of P. praetextata.

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PART I

An investigation of elemental nitrogen fixation by lichens.

INTRODUCTION

The experience of the higher-plant physiologist has led several authors within the past thirty years to suggest the possibility that certain lichens may be able to utilise the nitrogen of the atmosphere. CENGIA SAMBO (1923, 1925) pioneered this work with the introduction of the theory of poly-symbiosis in lichens. The bacterium Azotobacter was said by this author to be present in the cephalodia of Peltigera aphthosa. The principal phycobiont of this lichen is a unicellular chlorophyceous alga, while the cephalodia contain a species of Nostoc. Thus the nitrogen-fixing Azotobacter in symbiotic union with the Nostoc of the cephalodia, created a state of poly-symbiosis in this lichen.

In 1924, DARBISHIRE suggested that the cephalodia of P. aphthosa may perhaps fix nitrogen on account of the presence of Nostoc, and this suggestion was later reiterated by GOEBEL (1926). The review of nitrogen fixation by FOGG (1947) in which he states, " Nitrogen fixation may well be important in enabling some of these composite organisms to live on bare rock and in other similarly inhospitable situations ", was followed by the isolation of the Nostoc strain of Collema tenax by HENRIKSSON (1951),

who showed that the algæ, in bacteria-free culture, could fix atmospheric nitrogen.

The probability of nitrogen fixation by lichens containing blue-green algæ has thus become accepted by suggestion and by indirect evidence, but without direct experimental proof.

Concurrently with these speculations on nitrogen fixations in lichens, we see the development in Russia of the triple symbiosis theory of HENCKEL and YUZHAKOVA (1936). Five epiphytic green lichens were examined and found to contain Azotobacter which, in isolation, was shown to fix nitrogen. These investigations were furthered in later years by HENCKEL (1938) and by ISKINA (1938) whose work confirmed the presence of the bacterium in other ecological groups of lichens, including the genus Cladonia. In the same year, ZAKHAROVA (1938) isolated Azotobacter from ten lichens including the blue-green lichen Peltigera aenina, and showed that growth, and hence nitrogen fixation on mannitol agar was considerably enhanced by the addition of "bios" to the medium. On this piece of evidence rests Zakharova's theory that bios secreted by the phycobiont regulates, by its effect on bacterium and fungus, the stability of the lichen symbiosis.

Two important works however, contradict the findings

of the Russian investigators. Miss Walenkamp (QUISPEL, 1943-45) attempted to isolate Azotobacter or other nitrogen-fixing bacteria from numerous lichens growing in the vicinity of Leyden, but found no evidence of such bacteria. Some years later, KRASILNIKOV (1949) examined forty-three Russian lichens and concluded that in none of these was Azotobacter present.

Few European investigators have accepted the Russian theory of the triple symbiosis, and among the principal dissentients may be mentioned QUISPEL (l.c.), SCHAEDE (1948) and TOBLER (1953). Recently however, METCALFE (1955, personal communication) has isolated Azotobacter from the interior of numerous species of British lichens. It is worthy of note that he records instances of the presence of the bacterium in certain lichens but its absence from the soil on which the species were growing.

There is thus seen to be a lack of direct evidence that nitrogen fixation, either by blue-green algae or by the "third symbiont" Azotobacter, is associated with the lichen symbiosis. It is the purpose of this section of the work to investigate the extent of nitrogen fixation in the two groups of lichens, the "greens" and the "blue-greens".

LICHENS CONTAINING BLUE-GREEN ALGAE

The greater part of the blue-green lichens are ground-inhabiting species with, usually, distinct calcicolous tendencies. The principal phycobiont of these lichens is the nitrogen-fixing alga Nostoc, but others in the families Rivulariaceae, Scytonemataceae and Stigonemataceae, for which there are also reports of nitrogen fixation (WILSON and BURRIS, 1953), are of frequent occurrence.

Two distinct groups can be recognised in the blue-green lichens; the homoiomerous type in which the phycobiont is evenly distributed throughout the thallus, as in Collema, and the heteromerous type in which the phycobiont occupies a more or less well-defined sub-cortical position, as in the genera Peltigera and Sticta. Intermediates also occur which show an outer envelope of fungal tissue, the cortex, surrounding an inner tissue of alga and fungus. This type is characteristic of the genus Leptogium, but is also seen in some of the more highly differentiated Collemas.

For the following investigations, one species of each of the types, homoiomerous, intermediate and heteromerous, were chosen. These were, respectively, Collema granosum (Scop.) Schaer., Leptogium lichenoides (L.) Zahlbr., and Peltigera praetextata (Flk.) Vain. The three species form

a series in which can be recognised the decreasing importance of the phycobiont (Nostoc) in determining the external morphology.

The occurrence of nitrogen fixation was investigated by using the heavy isotope of nitrogen, $^{15}N_2$, and also by a disc culture method.

Tests for nitrogen fixation using isotopic nitrogen

Material and methods

The material for these tests was obtained from various sites in the vicinity of Glasgow and in the south-west of Scotland, generally a day or two before testing. Part of the material of Collema granosum however, was collected eight weeks in advance and maintained until required for the test, in aerated culture vessels (see Fig. 5, Part II). In addition, some of the Peltigera material tested had been grown in the greenhouse for periods varying from ten weeks to nearly ten months.

So far as was possible, only the cleanest parts of the lichen thalli were selected. Despite this precaution, soil particles adhered to much of the material, in particular to the rhizinous regions of the Peltigera thalli. Care was taken to remove all such soil and other debris by

repeated brushing and washing in sterile distilled water. The cleaned material was placed, until required for the tests some hours later, in deep Petri dishes lined with several layers of filter paper saturated with a nitrogen-free mineral solution. This was a modification of the solution used by BRISTOL (1920) for the culture of soil algae. The modified solution (HENRIKSSON, 1951) has the following composition:

K_2HPO_4	0.50 gm.
$NaNO_3$	0.50 gm.
$MgSO_4.7H_2O$	0.15 gm.
CaCl	0.05 gm.
NaCl	0.05 gm.
Ferric citrate	0.01 gm.
Citric acid	0.01 gm.
Distilled water	1000 ml.

The method employed to test for fixation of nitrogen was a minor modification of that used by BOND, FLETCHER and FERGUSON (1954). Fig. 1 shows the type of apparatus used to surround the lichen specimens with an atmosphere containing known amounts of ^{15}N, O_2, CO_2 and A, where employed. The system consisted of a manifold constructed of thick-walled capillary tubing having attachment points, provided with stopcocks, for three pairs of specimen

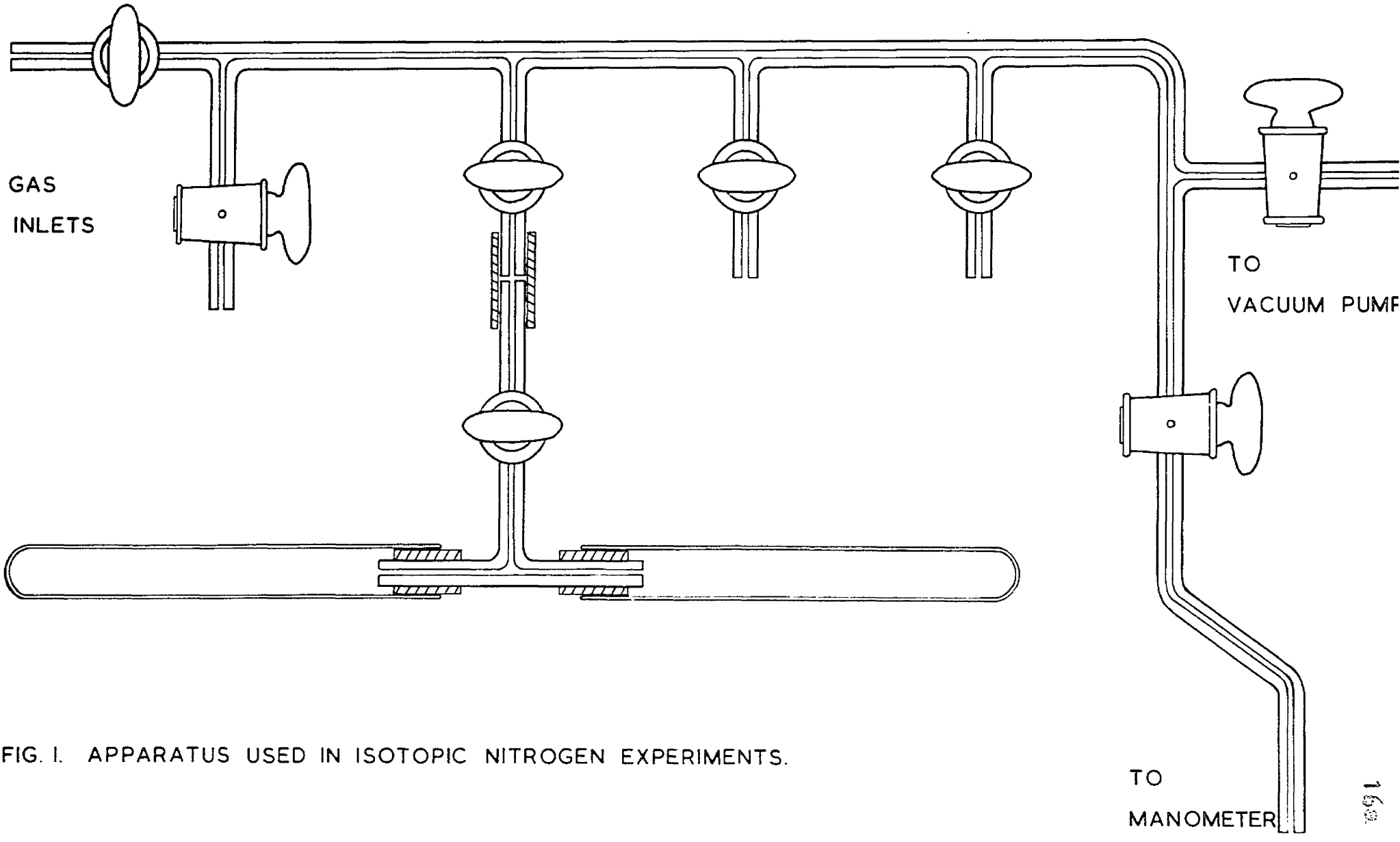

FIG. I. APPARATUS USED IN ISOTOPIC NITROGEN EXPERIMENTS.

containers, vacuum pump, mercury manometer and gas burettes.

Suitable amounts of the lichen material were placed in test tubes or in stout glass bottles together with measured volumes of sand or glass beads to act as fillers, and nitrogen-free mineral solution. The containers were attached to the manifold in pairs via T-joints supplied with stopcocks and the system tested for gas-tightness by partial evacuation. In order to replace the air in the system by a mixture of known proportions of gases, the manifold was evacuated to a predetermined degree and the gases allowed to enter, either separately or as a previously equilibrated mixture, until atmospheric pressure was restored.

Carbon dioxide and argon for the tests were obtained from cylinders, and oxygen by the action of a yeast suspension on hydrogen peroxide. Ammonium nitrate with 36.2 atom per cent ^{15}N in the ammonium radical was used as the source of isotopic nitrogen. Sufficient of the enriched nitrate to give the required volume of gaseous nitrogen was dissolved in a little distilled water and the solution run into a nitrometer. The nitrogen was released by the action of sodium hypobromite, then transferred to a gas burette containing 5% sulphuric acid and 20% sodium sulphate, and carefully washed to absorb any ammonia containing excess ^{15}N. Thereafter the

enriched gaseous nitrogen was introduced to the manifold in the quantity required. The remaining gases used in each test were transferred to the manifold from gas burettes.

In those tests in which a previously equilibrated gas mixture was used, the container and T-joint assemblies were detached from the manifold immediately after adding the gas mixture. A period of two or more hours was allowed for equilibration before removal of the containers in tests in which the gases were added separately. In all cases, the containers were kept under greenhouse conditions for periods of five or seven days according to the species of lichen tested, before removal of the material for conversion of the combined nitrogen to ammonia by the Kjeldahl process. A one-tenth part of each of the Kjeldahl distillates was titrated against standard NaOH to obtain an estimate of the total nitrogen content. The entire volume of each was then evaporated down to a small volume (3 ml.) and analysed for ^{15}N content by a Metropolitan Vickers Mass spectrometer, type MS 2 (see acknowledgements). Samples of each of the lichens, not exposed to ^{15}N, were also analysed to obtain control values for the natural ^{15}N content.

Experimental detail

Since a minimum of 1 mg. nitrogen per sample was required for mass spectrometric analysis, it was necessary to obtain values for the moisture content, dry matter content and total nitrogen content of material of the three species in order to determine the fresh weight requirement of each sample.

The moisture content of many lichens, and in particular those with blue-green phycobionts, responds quickly to changes in atmospheric humidity. Difficulty is thus experienced in the determination of fresh weights unless some standardised method is rigidly adhered to. In this work, samples of each of the lichens were immersed for some minutes in distilled water at room temperature. On removal, the material was lightly pressed between several layers of filter paper to remove surface moisture, then immediately weighed in covered watch glasses.

It is emphasised that the values obtained (Table 1) represent the maximum moisture-holding capacity which, in all cases, greatly exceeds the moisture content of the material in its natural state. Measurement of the moisture content at a constant relative humidity, for example that obtaining over a saturated solution of sodium chloride, is desirable where comparative data between species is required.

In the present instance however, although the values obtained are somewhat arbitrary, they are sufficient for the purpose required and are of advantage in that they can be determined with the minimum of delay.

All dry weights were determined after the material had been dried at 95°C. for a minimum of twelve hours. The total nitrogen content of the material was determined by the Kjeldahl process. The mean values for each of the three species are shown in Table 2. Calculation of the minimum fresh weight per sample, to ensure a total nitrogen content of at least 1 mg., was made from the data of Tables 1 and 2. The relevant data are shown in Table 3.

Table 1. Moisture content, at saturation point, of Collema granosum, Leptogium lichenoides and Peltigera praetextata.

Lichen	Fresh wt. gm.	Dry wt. gm.	Mean per cent moisture content
Collema granosum	2.11	0.08	96.0%
	2.77	0.11	
	2.16	0.10	
	2.33	0.09	
Leptogium lichenoides	1.87	0.56	70.7%
	1.79	0.52	
	1.81	0.51	
Peltigera praetextata	0.85	0.25	71.3%
	0.85	0.23	
	0.81	0.24	

Table 2. Mean per cent nitrogen content of Collema granosum, Leptogium lichenoides and Peltigera praetextata.

Lichen	Sample dry wt. mg.	Absolute N-content mg.	Mean N-content as % of dry wt.
Collema granosum	154.5	6.08	
	192.5	7.51	3.9
	256.5	10.01	
Leptogium lichenoides	555.5	27.09	5.0
	516.0	26.28	
Peltigera praetextata	246.0	12.54	
	230.0	11.03	
	243.5	10.48	4.8
	396.5	19.43	
	473.5	22.34	

Table 3. Minimum permissible fresh weight per sample of Collema granosum, Leptogium lichenoides and Peltigera praetextata for mass spectrometric analysis.

Lichen	N-content %	Dry wt. necessary to contain 1 mg. N	Moisture content %	Minimum fresh wt. per sample mg.
Collema granosum	3.9	25.65	96	510
Leptogium lichenoides	5.0	20.00	71	69
Peltigera praetextata	4.8	20.85	71	72

For most of the samples tested it proved possible to use more than the minimum amount of material. This permitted of the division of the Kjeldahl distillates into two or more parts of equal volume for separate mass spectrometric analysis. Each species was tested separately, the details of which are given below.

Experiment 1. *Collema granosum*

Prepared material of this lichen was divided into six parts, each of approximately 2.5 gm. fresh weight. Each sample was placed in a 15 ml. glass specimen tube together with 2 ml. nitrogen-free mineral solution. A mixture of gases comprising 96% nitrogen (containing 36.2 atom per cent ^{15}N) and 4% CO_2 was prepared in a gas burette and the latter attached to the manifold. The system was evacuated to 1/2 atmosphere and the gas mixture allowed to enter until atmospheric pressure was restored. The lichen material was thus surrounded by an atmosphere containing 10% O_2, 2% CO_2, the remainder being nitrogen containing approximately 20 atom per cent ^{15}N. Exposure to this gas mixture commenced on 15 July and continued for five days under greenhouse conditions. During the exposure the mean temperature was 19°C.

Experiment 2. *Leptogium lichenoides*

Six samples of mean fresh weight 1.2 gm. were used for this test. Each specimen tube (similar to those used for Expt. 1) contained, in addition to the lichen material, 5 ml. of sterile sand saturated with nitrogen-free mineral solution. The sand was added to reduce the gas space in the containers to permit of a saving in ^{15}N. The material was exposed on 22 September to a gas atmosphere similar to that of the previous experiment. Exposure continued for seven days at a mean temperature of 21°C.

Experiment 3. *Peltigera praetextata*

Lobes of the material were arranged with the upper cortex facing outwards in six 20 ml. test tubes. The mean fresh weight per tube was 0.95 gm. In addition, each tube contained 2 ml. of sterile sand saturated with nitrogen-free mineral solution. The manifold and containers were charged with a previously equilibrated gas mixture comprising 40% N_2 (containing 20 atom per cent ^{15}N), 20% O_2, 5% CO_2 and 35% A. A considerable reduction was thus made in the amount of nitrogen used. This was thought unlikely to influence the amount of any fixation obtained, since the pN_2 at which the half-maximum rate of fixation by *Nostoc muscorum* occurs is given as 0.02 atmosphere (BURRIS and WILSON, 1946).

The enriched nitrogen for this test was prepared from a mixture of five parts ammonium nitrate (with 36.2 atom per cent ^{15}N in the ammonium radical) and four parts of ordinary nitrate. A stoppered glass container of sufficient volume to just accommodate the required amount of gas mixture was attached to the manifold and fully evacuated. Each of the four gases was allowed to enter the system to a predetermined level on the mercury manometer, marked according to the respective amounts required. The gas container was left attached to the manifold for a few hours to ensure equilibration of the gas mixture. The test tubes containing the lichen samples were then attached to the manifold. The system was fully evacuated and charged with the gas mixture to restoration of atmospheric pressure. The tubes were immediately detached and sealed, no further period of equilibration being necessary. Exposure to the isotopic nitrogen commenced on 19 September and was continued for five days at a mean temperature of 14.5°C. with a range of 10 to 26°C.

Data obtained from the isotopic tests

Data for nitrogen content of each of the samples of the three lichens, determined after Kjeldahl distillation, are shown in Table 4, and the mass spectrometric data for the three tests in Table 5.

Table 4. Nitrogen content of test samples of Collema granosum, Leptogium lichenoides and Peltigera praetextata. (All weights in mg.)

Sample	Collema granosum	Leptogium lichenoides	Peltigera praetextata
1	3.2	9.0	10.6
2	5.6	9.7	11.9
3	3.2	8.6	11.2
4	3.2	8.0	7.1
5	3.2	8.3	10.9
6	-	10.0	12.3

In addition to the control values obtained by analysis of material of the three lichens not exposed to ^{15}N, use has been made of a number of controls obtained using diverse plant material analysed by the same mass spectrometer. The mean of the fifty values available is 0.372 atom per cent with a standard deviation of 0.012 (BOND, 1956). Thus the value 0.372 + (2 x 0.012) = 0.396,

was considered necessary to provide significant evidence of enrichment of the test material with ^{15}N. On this basis, the spectrometric data show that fixation of nitrogen is associated with each of the three species.

Table 5. Data obtained from ^{15}N tests of Collema crenatum, Lentogium lichenoides and Peltigera praetextata.

	^{15}N content, atom per cent		
	Collema crenatum	Lentogium lichenoides	Peltigera praetextata
Exposed to N_2 with 20 atom per cent ^{15}N	0.397	0.583	0.571
	0.408	0.597	0.596
	0.406	0.487	0.535
	0.454	0.589	0.395
	0.447	0.506	0.403
	-	0.567	0.431
Controls	0.377	0.371	0.376
	-	0.373	-

Tests for nitrogen fixation using disc cultures

Peltigera praetextata, being of large thalloid structure, is an eminently suitable material from which to cut uniform discs. In addition to the comparatively high normal growth rate, isidia* appear on the thallus of this species where it has been cut or damaged in other ways. Thus thallus discs produce a peripheral growth of isidia which, together with the normal growth, is of sufficient magnitude to obtain significant results from experiments of a comparatively short duration.

Material and methods

Material of Peltigera praetextata, of similar origin to that used for the isotopic nitrogen test, was carefully cleaned and washed in sterile distilled water. Discs of 8 mm. diameter were cut from the lobes in such a way that they included part of the intact thallus margin, so far as the supply of material would allow. The rhizinae were removed and the discs washed in sterile distilled water. Fourteen discs were arranged on double layers of filter paper (6 x 2 ins.) lining each of a number of large test

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* This term is used, for the moment, solely on account of its acceptance in the literature. The validity of the term "isidium" as applied to these traumatic outgrowths is discussed in Part II.

tubes (Fig. 2). To each tube was added 25 ml. nitrogen-free Bristol solution. The filter paper strips were thus saturated with the culture solution when the tubes were placed at a slope of 30° to the horizontal. Illumination was effected by means of fluorescent lighting of approximately 150 foot candles intensity. The mean temperature throughout the period of culture was 20°C., and the daily period of illumination of the order of twelve hours. Evaporation, with consequent concentration of the culture solution in the tubes, was offset by periodic additions of sterile distilled water.

In three such experiments two types of filter paper were used. A coarse-grain paper was used for two of those which comprised, respectively, three and six tubes, each with fourteen discs. For the third, which comprised six tubes, the discs were cultured on Whatman No.1 paper.

The discs of each experiment were removed from the tubes after fourteen weeks' growth and dried at 95°C. Dry weights were obtained, and the discs analysed for nitrogen content by the Kjeldahl process.

Data obtained from disc cultures

The extent of growth of the discs was determined in terms of increase in dry weight and in nitrogen content.

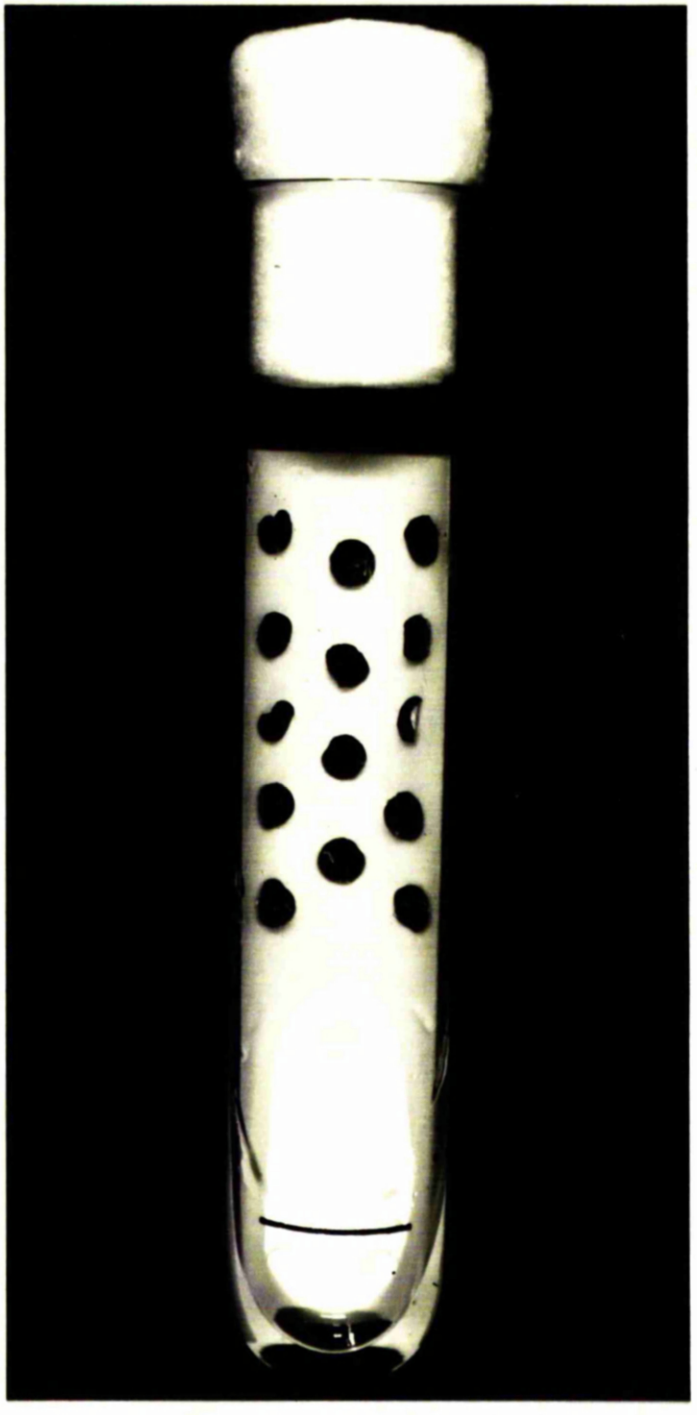

Fig. 2. One of the lichen disc culture tubes showing the fourteen 8 mm. discs arranged on the filter paper lining the tube.

x $\frac{2}{3}$

Since initial values could not be obtained for the actual discs used in the experiments, these were determined from samples of discs of the same plants from which the experimental discs were cut. For Expt. 1, two samples, each of 50 discs, served to provide the relevant data. For Expts. 2 and 3, three samples, each of 42 discs, were used.

Preliminary experiments had established that over a fourteen-week growth period a sufficiently large increase in weight of the discs occurred to render unimportant the rather high variation found in the dry weights of the initial samples. Table 6 shows the magnitude of this variation in initial dry weights, together with the mean dry weights of fourteen discs.

Table 6. Variation in initial dry weight of samples of discs of *Poltigera praetextata*.

Expt.	No. of discs per sample	Dry weight mg.	Mean dry wt. of 14 discs mg.
1	50	108	30.8
	50	112	
2	42	125	
&	42	130	41.6
3	42	119	

Table 2 shows the mean per cent nitrogen content of the species to be 4.8%. The five determinations from which this mean was calculated were made from material which was used both for the ^{15}N tests and for the present disc experiments. Thus the initial nitrogen content of fourteen discs, determined using this mean and the dry weight data of Table 6, was considered to give a satisfactory representation of the nitrogen content of each set of discs used in the experiments.

Table 7. Increases (mg.) in dry weight and in nitrogen content of discs of Poltigera praetextata cultured for fourteen weeks with nitrogen-free Bristol solution.

Expt.[R]	Mean initial values for 14 discs		Mean final values for 14 discs		Mean increase	
	Dry wt.	N-cont.	Dry wt.	N-cont.	Dry wt.	N-cont.
1	30.8	1.5	70.2	3.2	39.4	1.7
2	41.6	2.0	105.8	4.8	64.2	2.8
3	41.6	2.0	84.5	3.9	42.9	1.9

[R] Coarse-grain filter paper was used in Expts 1 and 2 and Whatman No.1 paper in Expt. 3.

Table 7 details the increases in dry weight and in nitrogen content of the discs at the end of the period of culture. Day to day observation showed that little symbiotic growth took place during the first two or three weeks. During this initial lag period however, hyphae of the medulla were seen to grow out round the circumference of the discs to form a shining white tomentum. The beginning of new symbiotic growth was marked by the appearance of large numbers of small nodules on the cut edges of the discs. Further increase in size was characterised by three distinct forms of growth. In those discs which included part of the intact margin of the thallus, rapid asymmetric expansion took place in this region. Intercalary growth contributed to the symmetrical increase in diameter of the discs and in addition, all showed growth along the cut edges and surface cracks with the formation of the nodules referred to above, later to become isidia. In the latter part of the duration of the experiments, many of the discs showed a few of the isidia becoming considerably larger than their neighbours, with the development of rhizinae and medullary air spaces (Fig. 3). These continued to increase in size to become miniature replicas of the parent thallus while the remainder of the isidia retained the structure typical of the early stage of thallus formation.

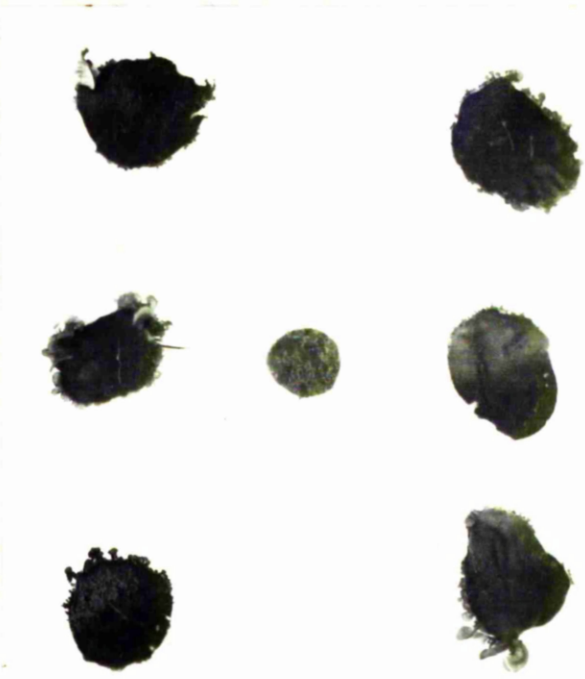

Fig. 3. Six typical discs of Peltigera praetextata as they appear after fourteen weeks' growth. The small centre disc represents the original size. Isidia at various stages of development, and asymmetric growth at regions coincident with the intact thallus margin, are evident.

x 1

At the end of the fourteen-week period there were no indications of senescence of any part, and no significant alteration was apparent in the ratio of phycobiont to mycobiont in the discs. These facts indicated that the normal symbiotic relationship between the two organisms had been maintained during growth of the discs in culture. Reference to Table 7 shows that there has likewise been no significant change in the per cent nitrogen content of the discs. In terms of absolute nitrogen content however, considerable increases are apparent. In two of the three experiments the increase exceeded the initial nitrogen content. Similarly the dry weights of all the sets of discs have been at least doubled.

These experiments thus confirm the findings of the ^{15}N test in respect to nitrogen fixation by Peltigera praetextata. The possibility of nitrogen being obtained from sources other than by fixation during disc culture will be discussed later.

LICHENS CONTAINING GREEN ALGAE

The Russian theory of the triple symbiosis implies that *Azotobacter* is present in all lichens and thus that nitrogen fixation is of general occurrence in this group of plants. The recent work of METCALFE (1955) has shown that this association may be more than purely incidental (see Introduction). It appears from his work that the *Azotobacter* may be disseminated with the lichen diaspores. One must not however, presuppose on this account that the bacterium is necessary for the maintenance of the stability of the symbiosis, nor indeed that it contributes in any way to the symbiosis.

In view of the continuing uncertainty regarding this aspect of lichenology, the isotopic nitrogen test has been applied to two green lichens belonging to widely separate ecological groups.

Cladonia impexa Harm. is a typical species of acid heath. It is of frequent occurrence as the dominant of the rather poor type of lichen heath represented in Scotland. METCALFE (*l.c.*) states that he has isolated *Azotobacter* from material of this species. The forma *laxiuscula* (Del.) Sandst. of *Cladonia impexa* was used in the isotopic test.

Lobaria pulmonaria (L.) Hoffm. is a corticolous species of mainly oceanic distribution in Britain. It is a very abundant species, particularly in the west and north of Scotland. This species, as Stieta pulmonacea (L.) Scheer., was one of the first from which HENCKEL and YUZHAKOVA (1936) isolated Azotobacter. A positive result was again obtained by HENCKEL (1938) in his re-examination of previous work. The species also figures in KRASILNIKOV's (1949) list of lichens from which he failed to isolate the bacterium.

Material and methods

Material of the two species was selected from typical sites within their main distribution areas. Cladonia impexa was obtained from a Polytrichum - Calluna community on Flanders Moss, Stirlingshire, and Lobaria pulmonaria from a fairly open stand of Quercus to the north of Oban, Argyll. The Cladonia material was collected sixteen days in advance and kept in the greenhouse in sterilised glass-covered flower pots. Material of Lobaria was collected two days before the test and kept in the sterile glass container used for collection.

On the day selected for exposure to the isotope, the lichen material was cleaned, washed in sterile distilled water and placed, until required, in Petri dishes lined with filter paper saturated with nitrogen-free mineral

solution. The experimental method was similar to that described for the blue-green lichens, but with modifications in the method of enclosure and in the gas atmosphere used.

Experimental detail

Data for moisture content, dry matter content and total nitrogen content of samples of the two species were obtained by the procedure previously described. Tables 8 and 9 respectively, show the mean per cent moisture and nitrogen contents. On the basis of these determinations, the minimum permissible amount of material per sample was calculated as illustrated in Table 10.

Table 8. Mean per cent moisture content, at saturation point, of material of *Cladonia impexa* and of *Lobaria pulmonaria*.

Species	Fresh wt. gm.	Dry wt. gm.	Mean % moisture content
Cladonia impexa	1.017 0.880	0.748 0.411	53.0
Lobaria pulmonaria	0.636 0.810	0.266 0.343	58.0

Table 9. Mean per cent nitrogen content of material of *Cladonia impexa* and of *Loharia pulmonaria*.

Species	Sample dry weight mg.	Absolute N-content mg.	Mean N-content as % of dry wt.
Cladonia impexa	730.0	2.71	0.36
	994.5	3.42	
Loharia pulmonaria	266.0	5.55	2.10
	343.0	7.38	

Table 10. Minimum permissible fresh weights per sample of material of *Cladonia impexa* and of *Loharia pulmonaria* for ^{15}N tests.

Species	N-content %	Dry weight necessary to contain mg.	Moisture content %	Minimum fresh wt. per sample mg.
Cladonia impexa	0.36	277.7	53.0	591.0
Loharia pulmonaria	2.10	47.6	58.0	113.4

The two species were separately examined for fixation of nitrogen as detailed below.

Experiment 1. Cladonia impexa

Table 10 shows, for this lichen, a minimum fresh weight of 591 mg. per sample. To provide for the division of each sample into two equal parts after Kjeldahl distillation, quantities of 1.28 gm. were used. Six such samples were enclosed, with 1 ml. sterile sand saturated with nitrogen-free mineral solution, in test tubes of 20 ml. capacity which were attached to the gas manifold in pairs. The system was evacuated to 1/3 atmosphere to allow of the introduction of ^{15}N, O_2 and CO_2 in sufficient quantity to give a final atmosphere comprising 75% N_2 (containing 20 atom per cent ^{15}N), 20% O_2 and 5% CO_2. After equilibration of the gases, the sample tubes were detached from the manifold and sealed. Exposure to the ^{15}N atmosphere commenced on 30 August and continued for five days at a mean temperature of 18.5°C., with a range of 11 to 27°C.

Experiment 2. Lobaria pulmonaria

In testing this lichen for nitrogen fixation the previously adopted procedure was modified so that the material could be exposed to the isotope as a single sample. This was made of sufficient size (1.8 gm. fresh weight) such that, on division into three equal parts after the exposure,

each would contain the minimum requirement of not less than 1 mg. total nitrogen (Table 10). The material was exposed in a flat-sided medicine bottle to a gas atmosphere similar

praetextata viz., 40% N_2 (containing 20 atom per cent ^{15}N), praetextata viz., 40% N_2 (containing 20 atom per cent ^{15}N), 20% O_2, 5% CO_2 and 35% A. This gas mixture was prepared and equilibrated in advance. The manifold with attached specimen container was fully evacuated and the gas mixture allowed to enter until atmospheric pressure was restored.

The experiment was started on 10 August and exposure continued for six days at a mean temperature of 25°C, with a daytime range of 19 to 28°C. The sample, on removal from the container, was surface dried between filter papers and divided into three parts of approximately equal weight for separate analysis for ^{15}N content.

Data obtained from the isotopic tests

Table 11 shows the nitrogen content of each of the samples of the two lichens, determined after Kjeldahl distillation. The results of the mass spectrometric analyses are shown in Table 12. None of those figures shows a significant increase over the natural ^{15}N content, determined as for the tests of the blue-green lichens. Thus within the fine limits of accuracy of this method of detecting nitrogen fixation, it is concluded that no such

fixation has taken place in the material tested.

Table 11. Nitrogen content of test samples of Cladonia impexa and of Loberia pulmonaria. (All weights in mg.)

Sample	Cladonia impexa	Loberia pulmonaria
1	5.4	4.5
2	7.5	5.8
3	5.5	5.2
4	4.8	-
5	6.0	-
6	7.8	-

Table 12. Data obtained from ^{15}N tests of Cladonia impexa and of Loberia pulmonaria.

	^{15}N content, atom per cent	
	Cladonia impexa	Loberia pulmonaria
Exposed to N_2	0.372	0.345
	0.370	0.394
with 20 atom	0.375	0.390
	0.381	-
per cent ^{15}N	0.375	-
	0.385	-
Controls	0.370	0.381

DISCUSSION OF DATA OBTAINED

The data of Table 5 show for the three blue-green lichens a significant increase in ^{15}N content of all but one of the samples. Accepting the finding of BURRIS and MILLER (1941) that there is no difference between the rate of biological fixation of the heavy isotope of nitrogen and that of ordinary nitrogen, and that there is no interchange of gaseous ^{15}N with ^{14}N already combined in the plant tissues, it is possible to calculate the mean total fixation of ^{14}N and ^{15}N which took place during the periods of the tests. These values, from the data of Tables 4 and 5 are, for Collema granosum - 0.002 mg. per gm. fresh weight, for Leptogium lichenoides - 0.06 mg. per gm., and for Peltigera praetextata - 0.05 mg. per gm. Reduced to the common basis of fixation per gm. dry weight of thallus per five days, these become 0.05 mg., 0.146 mg., and 0.18 mg. respectively.

Since Nostoc is the phycobiont of each of the three species, it is presumed that the fixation observed has been carried out by the Nostoc. There is however, considerable evidence (see Introduction) that many lichens contain Azotobacter or other bacteria capable of fixing nitrogen in the non-symbiotic state. While there appear

to be no published records of the isolation of Azotobacter from any of the three lichens of this investigation, the probability that the observed fixation was due to Azotobacter must not be overlooked.

The data of BURK (1930) show that in order to fix 1 mg. of nitrogen, at least 100 mg. of glucose or equivalent amount of alternative substrate is required by Azotobacter. Thus in the case of a single sample of Lobogium or of Peltigera, were the observed fixation to be attributed to Azotobacter, the minimum requirement of carbohydrate (as glucose) would be 5 mg. Further, this would be accompanied by a minimum of 0.5 mg. dry weight increase in bacterial cell substance. The former requirement effectively eliminates the possibility of the fixation having been carried out by surface contaminant Azotobacter; it is not a feature of the lichen symbiosis that such amounts of carbohydrate are available at the surface of the thallus. But this argument does not apply to Azotobacter in the interior of the thallus. The bacterial cells are stated to be present in the immediate vicinity of the phycobiont cells and thus in a position to receive perhaps fairly substantial amounts of carbohydrate.

Microscopic examination of sections of these lichens, stained with Boehmer's haematoxylin (McLEAN and COOK, 1952),

revealed that neither Azotobacter nor any others of the varied bacterial flora, were present in quantities remotely approaching 0.5 mg. dry weight per sample. Thus although it was not possible to eliminate bacteria from the test material, it is evident that the major part, if not all, of the fixation should be attributed to the phycobiont of these lichens.

More recent data than that of Burk shows that the efficiency of fixation by Azotobacter may reach the high level of 15-20 mg. nitrogen per gm. sucrose (WILSON and BURRIS, 1953). The appropriate ammendment does not however, invalidate the preceding argument (as published by BOND and SCOTT, 1955). The amount of carbohydrate (as sucrose) required for the fixation of 0.05 mg. nitrogen would be not less than 2.5 mg. In a lichen sample of 0.95 gm. fresh weight (Peltigera) this amount seems unduly large to be made available to any Azotobacter cells on the surface of the thallus, particularly on account of the four to five cell-thickness of the cortex intervening between the phycobiontal layer and the surface.

The assumption is justifiable, on the evidence presented that nitrogen fixation may be common to all lichens or lichen organs (e.g. cephalodia) which contain Nostoc. This reasoning lends weight to the suggestion by GOEBEL (1926) that the cephalodia of lichens such as Solorina

saccata, Peltigera aphthosa and Lobaria laciniata (although in this case the "cephalodia" are now recognised as a separate lichen), function in the fixation of nitrogen.

The present-day conception of the total lichen species known is of the order of 15,000, taking into account the multitude of taxonomic works which have appeared since the completion of ZAHLBRUCKNER's Catalogus Lichenum Universalis (1922-40). The proportion of blue-green lichens varies quite widely in different regions of the world, but is in no part known to constitute more than about 20% of the total species. In the British Isles this figure is about 9% of the 1467 species (according to WATSON, 1953), and of this total, about 7% contain Nostoc as the principal phycobiont. None of these lichens containing Nostoc is of high frequency except in a few specific areas such as on ground overlying highly calcareous rock in regions of high humidity. It is thus doubtful whether fixation of nitrogen, by the majority of blue-green lichens, is of material significance other than, as FOGG (1947) suggests, to the nutrition of the lichens themselves.

The ability of certain blue-green algae to colonise bare rock surfaces has been attributed to their property of nitrogen fixation (FOGG, l.c.). A similar faculty, being now established for some blue-green lichens, indicates that such lichens may also be enabled to exist in situations

deficient in nitrogen. In this connection it seems to be not without significance that the two species, Peltigera aphthosa and Nephroma arcticum, which are of exceptionally high frequency in the lower arctic regions, are both abundantly furnished with cephalodia containing Nostoc. It is insufficient however, to rely solely on the evidence of fixation of nitrogen by the Nostoc of such lichens in attempting to draw conclusions regarding the significance of their distribution in nitrogen deficient areas. It must be established, on a factual basis, that part at least of the nitrogen fixed by the phycobiont is made available in acceptable form to the mycobiont. Only in this way can it be concluded that nitrogen fixation is of benefit to the symbiosis as a complete entity.

The experiments with discs of Peltigera praetextata illustrate one instance in which nitrogen fixed from the atmosphere is taken up by the mycobiont. The mean increase in nitrogen content of fourteen discs (Table 7) amounts to 2.1 mg. No nitrogen was supplied in the culture solution, but three possible alternative sources require consideration, (i) absorption of ammonia from the atmosphere, (ii) uptake of combined nitrogen from the filter paper in the culture tubes, and (iii) fixation of atmospheric nitrogen by the Nostoc of the discs. Ammonia is said to be the main source of nitrogen of that well-defined group of lichens including

Caloplaca elegans, Ramalina polymorpha, Physcia caesia etc., which inhabit cliffs and birds' resting stones in the mountains (NIENBURG, 1919). In these situations however, the localised concentrations of ammonia in the atmosphere and dissolved in rain water, are much higher than in normal laboratory air. Table 13 shows the results of several determinations of the nitrogen content of filter paper similar to that used in the experiments. The total nitrogen content of two strips of coarse-grain paper is seen to be less than one tenth of the increase in nitrogen content of fourteen discs of Peltigera praetextata (Table 7). Since sources (i) and (ii), either separately or in combination, give no evidence of providing the nitrogen requirement of the discs, it is concluded that this has been obtained by fixation of atmospheric nitrogen by the phycobiont.

It has been stated that no significant change was apparent in the phycobiont : mycobiont ratio of the lichen discs at the end of the fourteen-week culture period. For this ratio to remain stable, new fungal growth associated with uptake of nitrogen must have occurred in the discs. The P/M ratio for Peltigera praetextata was determined by microscopic examination of sections of the thallus. Table 14 shows the means of twenty measurements of each of the various layers. No account was taken of the rhizinae nor of the fine hyphae between the phycobiont colonies.

In the estimation of the P/M ratio from these values it was assumed that the densities of the phycobiont and mycobiont were equal. This allowed of the direct translation of depth measurements into units of volume.

Table 14. Depth, in microns, of the morphologically distinguishable layers of the thallus of Peltigera praetextata.

Cortex	Phycobiontal layer	Medulla	Medulla and veins
39	78	141	318
63	90	159	283
51	69	159	283
42	66	124	336
39	66	124	336
45	75	159	265
45	75	177	247
45	90	124	283
51	75	159	265
45	75	177	265
39	75	141	300
45	45	106	354
45	75	106	212
45	75	159	300
45	75	212	336
45	90	141	283
45	66	177	265
45	81	124	283
48	75	159	247
39	75	106	265
45	75	147	287

Mean vein depth, by difference = 140 micr.

Estimation of the total fungal material was complicated by the irregular reticulum of veins on the under surface. The extent of this coverage was judged to be 0.4 of the total surface (Table 15). Thus the amount of vein material, when converted to units of volume, is only four tenths that of the value for vein depth, i.e. 56 units. The total volume of fungal material is therefore:

45(cortex) + 147(medulla) + 56(veins) = 248 units.

Volume of algal material = 75 units.

P/M ratio = 75:248.

Table 15. Extent of vein coverage of Peltigera praetextata.

Width of thallus surface measured mm.	Vein depth mm.
14	6
16	6
14	6
17	7
15	6
76	31

Vein coverage = 31/76 = 0.4 of surface

Discrepancies occur in assuming that the phycobiontal layer is a regular zone fully occupied by the alga and that the medulla is a compact hyphal tissue. In fact, as can be seen from Fig. 4, the phycobiont cells occur in compact groups, the spaces between which are occupied by hyphae of the mycobiont. Also, there is a fairly extensive network of air spaces in the medullary and veinous tissues. These discrepancies however, tend to cancel each other out, so that the ratio 75:248, or approximately 1:3, is considered to be a reasonably accurate representation. The ratio determined in this manner is somewhat closer than the value of 1:4 indicated by the work of DARBISHIRE (1926) for the same species.

The increase in dry weight of fourteen discs of Expt. 3 was 42.9 mg. (Table 7). On the basis of the P/M ratio of 1:3, approximately one quarter (10.7 mg.) of this increase is considered to be due to new algal growth, and the remainder (32.2 mg.) to new fungal growth. Assuming the nitrogen content of the Nostoc to be as high as 10%, the amount of nitrogen in the new algal growth would be slightly more than 1 mg. Since the total increase in nitrogen content of the discs was 1.9 mg., it is deduced that 0.9 mg. nitrogen was present in the 32.2 mg. of new fungal growth. This low estimate is fifteen times the amount of combined nitrogen present in the Whatman filter paper used in the

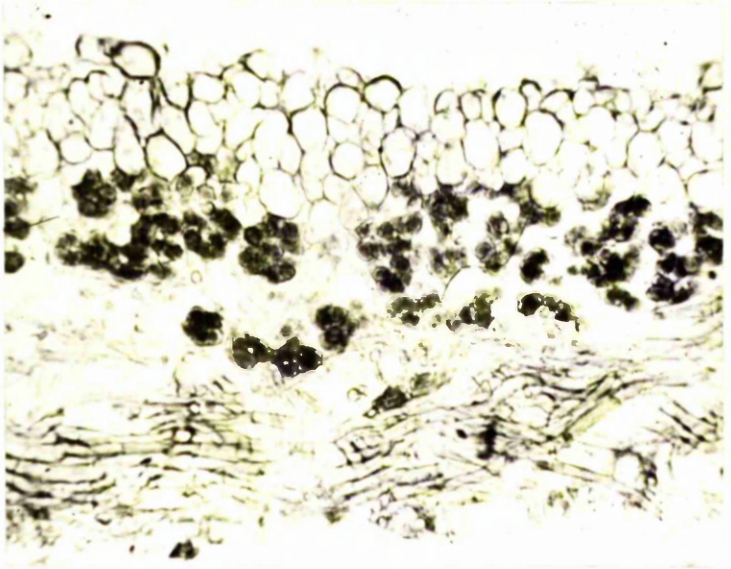

Fig. 4. Photomicrograph of the thallus of Peltigera praetextata in transverse section. The phycobiontal layer appears as a series of distinct colonies, each surrounded by a gelatinous sheath. The colonies are separated from each other by fungal tissue and air spaces.

x 400 approx.

tubes of Expt. 3. The indication is thus that fixed nitrogen has been transferred from the phycobiont to the mycobiont of the lichen discs.

No experimental evidence is available regarding the mechanism of the transfer of fixed nitrogen within the discs. In view of the numerous reports of the excretion by blue-green algae, of nitrogenous products of metabolism (FOGG, 1953) it is possible however, that there is in the lichen thallus, straightforward absorption of such excreted compounds. Of special interest in this connection is the report by HENRIKSSON (1951) of the excretion of from 19 to 28% of the nitrogen fixed by Nostoc isolated from Collema tenax. An alternative method of transfer is by means of haustorial connections between mycobiont and phycobiont, but reports of such in the blue-green lichens are scant. No connections could be found in Peltigera praetextata by DARBISHIRE (1926). Their occurrence in Collema has been reviewed by DEGELIUS (1954) who, from his own investigation of material from various European countries, concludes that haustoria " in a rather wide sense ", are present in several species.

Comparison of the total fixation by Peltigera praetextata during the ^{15}N test and in disc culture shows that this was much more active in the latter case (2.3 mg. per gm. dry

weight per five days; the corresponding figure for the isotopic test being 0.18 mg.). Of the factors regarded as contributing to this discrepancy, not the least is the wounding of the tissues with consequent growth stimulus, incurred in cutting discs from the thalli. The Nostoc colonies, on being exposed, soon form new growth leading to the formation of isidia in which fixation is likely to be more active than in the parent thallus. Observations on the disc cultures revealed that there was a lag period before the beginning of obvious new symbiotic growth. There would thus be little activity of the phycobiont during this initial period. Since active growth of blue-green algae is a pre-requisite of nitrogen fixation (FOGG, 1947), the amount of nitrogen fixed by the Nostoc in the first few days of culture would be small, but would increase with the formation of new growth. Thus, in the isotopic test, the whole duration of which fell within this initial lag period, a low fixation rate would be expected. Contributing also, is a marked temperature difference, this being some 6°C. lower in the ^{15}N test than in the disc experiments.

The data of Table 12 give no evidence that nitrogen fixation is associated with either lichen tested. Thus although the triple symbiosis theory is said to provide

an explanation of the abundance of green lichens in nitrogen deficient areas (HENCKEL and YUZHAKOVA, 1936), it appears that, for some species at least, the participation of the third symbiont is not obligatory.

It is still far from certain whether Azotobacter is of constant occurrence in green lichens. No one has yet carried out a detailed analysis of specific plants and of their substrata over a wide area of their distribution. Approaches have been made towards this ideal, but much remains to be done before a general statement regarding the prevalance of Azotobacter in lichens can be committed to paper.

Although it has been shown, for two lichens, that the symbiosis is apparently complete without the participation of any nitrogen-fixing agent, it cannot be denied that Azotobacter, if present, may contribute in small measure to the nitrogen metabolism of the lichen. SCHAEDE (1948) however, regards this as highly improbable when he says, " Wenn aber Azotobacter an der Symbiose beteiligt sein soll, müsste er schon in ansehnlicher Menge in den Flechten nachgewiesen werden; des Vorhandensein einzelner Bakterien hat keine Bedeutung."

Instances of an apparent symbiosis between Azotobacter and free-living algae were reported in 1925 by LIPMAN and TEAKLE (Chlorella) and in 1926 by ISSATSCHENKO (on the

mucilaginous surface of Laminaria and Fucus). Further, STEPHENSON (1949) writes of Azotobacter and Clostridium, " They are stated to be absent from virgin soils and from soils at high altitudes except where algae are present." Thus it is probable that the presence of Azotobacter in lichens is no more that a specialised case of a facultative association of the bacterium with the symbiotic alga.

Many of the earlier works on the isolation of the lichen symbionts, and on the synthesis of lichens, are subject to criticism regarding freedom of the cultures from bacteria. Recent work by Henriksson (reported in DEGELIUS, 1954) shows however, that the mycobionts of several species of Collema can be grown in culture only in the presence of " special bacteria ". Without these, the germ tubes of the ascospores soon stop growing. According to Mrs. Henriksson (personal communication), neither of the two species of bacteria found to be effective in this respect is identifiable as an Azotobacter. Thus it may be that bacteria perform some, as yet unsuspected, function in the lichen symbiosis, and this fact may possibly account for the success of some of the earlier works on lichen synthesis which can only be described as little short of remarkable.

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SUMMARY OF PART I

1. Three blue-green lichens, *Collema granosum*, *Leptogium lichenoides* and *Peltigera praetextata* have been shown, by an isotopic technique, to fix atmospheric nitrogen.
2. The phycobiont (*Nostoc*) of these lichens is considered to be the nitrogen-fixing agent. It is shown that the fixation rate of *Leptogium* and of *Peltigera* is too high for this to be attributed to *Azotobacter*.
3. Fixation of nitrogen by *Peltigera praetextata* is confirmed by a study of the growth of discs of the lichen in nitrogen-free culture. Evidence is presented to show that some of the fixed nitrogen is taken up by the mycobiont.
4. Two green lichens, *Cladonia impexa* and *Lobaria pulmonaria* provide no evidence, using the isotopic technique, that atmospheric nitrogen is fixed by a "third symbiont".
5. Doubt is cast upon the application to all lichens of the triple symbiosis theory. It is not denied however, that *Azotobacter*, if present, may contribute small amounts of fixed nitrogen to the symbiosis.
6. It is considered that bacteria, apart from known nitrogen-fixers, may benefit the lichen symbiosis, possibly by contributing growth substances during the initial stages in the formation of the symbiosis.

PART II

A study of the growth of species of *Peltigera*, with special reference to the formation of isidia.

INTRODUCTION

STRATO (1921) was perhaps the first to culture Peltigera, although this work was limited to placing thalli of Peltigera "canina" on soil or clay covered with a bell jar. Strato's material was cultured to illustrate that isidia were produced at places where the thallus had been wounded and were, on this account, to be described as regeneration growths. Other workers in this field, e.g. BITTER (1904), MOREAU (1919), LINKOLA (1922), DU RIETZ (1924), DARBISHIRE (1926), THOMSON (1948) and LINDAHL (1953) have each made contributions to the elucidation of the anatomical and physiological nature of these isidia, but only Darbishire has made use of *in vitro* culture of the lichen. In his very complete anatomical investigation of P. praetextata, he experimented with small fragments of the thallus in moist Petri dishes, and with isidia separated from the thallus. His conclusions from this work however, were of minor importance and did not materially contribute to our knowledge of the isidia.

Most investigations have been made in an attempt to substantiate the validity of P. praetextata as a species distinct from P. canina or from P. rufescens, but various conclusions have also been drawn regarding the function

of isidia. LINKOLA (l.c.) took the view that there were "true isidia" and "wound isidia", and distinguished between the two on anatomical grounds. DARBISHIRE (l.c.), whose investigations were concerned with "wound isidia", describes their main purpose as being that of increasing the photosynthetic and transpiration area of the thallus. THOMSON (l.c.) regards the isidia of Peltigera praetextata as "regeneration phenomena" which are of no value in the separation of the plant as a distinct species. He proposes the name Peltigera canina f. innovans (Korb.) Thoms. for the P. praetextata of Vainio. LINDAHL (l.c.) has recently experimented with material in the field and concludes that only P. praetextata, among the five species he tested, formed isidia after incisions in the cortex had been made with a knife. Although only eight of his thirteen experimental plants of P. praetextata formed isidia, he considers this to be a "good species".

To what extent the formation of isidia is dependent on the environment, or is a genetically determined factor, has never been fully investigated. It appears however, from the writings of the above-mentioned authors, that isidia often indicate a persistently high moisture content of the lichen.

While discs of Peltigera can be satisfactorily cultured

if supplied with a suitable nutrient solution which need not contain nitrogen (Part I), there is considerable risk of contamination by bacteria, green algae and animal micro-organisms. It is exceedingly difficult to obtain adequately clean material for culture, and thus any experiments involving long-term culture with organic nutrients, however desirable they may be, are ruled out for the present.

The investigations to be described were prompted firstly by the need for data regarding the optimum conditions for growth of P. *praetextata* in culture. Secondly it was felt that a physiological approach to the "isidia question" would perhaps throw new light on these structures as seen in the genus *Peltigera*.

MATERIAL AND METHODS

Most of the lichen material was obtained from a disused limestone quarry on Springkell Estate in Dumfries-shire. This quarry is on a belt of carboniferous limestone which stretches from the Solway Firth to the eastern border of the county. It is particularly rich in species of blue-green lichens. Further material was obtained from Campsie Glen and from Fin Glen in the Campsie Hills, Stirlingshire. Collections were usually made a day or so before use. In some cases however, specimens were collected a few weeks in advance and maintained on soil in culture chambers through which a current of moist air was passed (Fig. 5). Other specimens were planted out in flower pots in the greenhouse.

Unicellular and filamentous algae are usually to be found adhering firmly to the cortical cells of Peltigera species. Several methods were tried to free the thalli of these, but none was found to give consistent results. BOGUSCH (1944) describes a method for the sterilisation of the surface of lichen thalli by immersion in 2M $MgSO_4$ or KCl for fifteen seconds, followed by rinsing in sterile water. A number of trials of this method showed however, that even a two-minute immersion was insufficient.

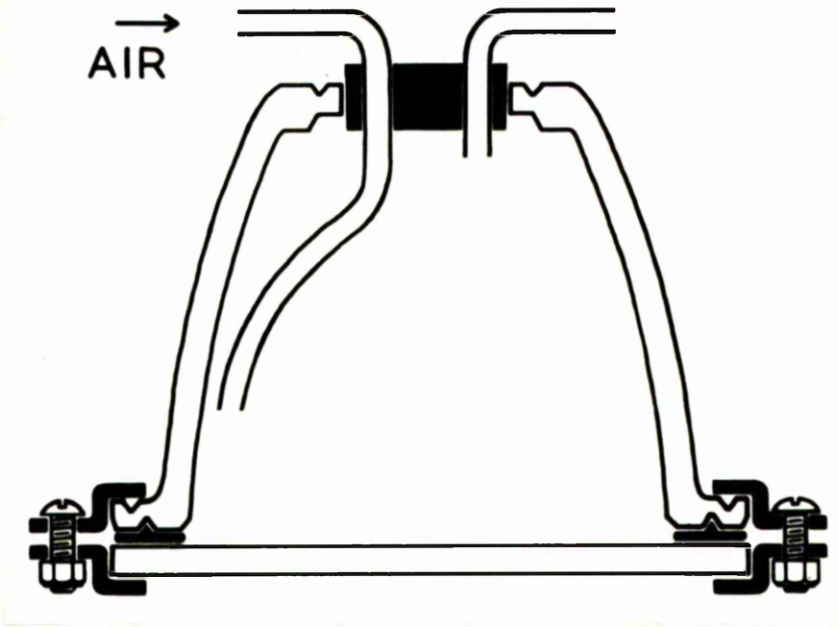

Fig. 5. Diagram of lichen culture chamber. This consists of a glass vessel of the bell jar type, supplied with air entry and exit. The glass base plate and plastic seal are clamped with two aluminium rings and screws. The lichen material is placed on a layer of moist soil on the base plate.

x 1/2 approx.

Longer periods were more satisfactory but, more often than not, the phycobiont was also killed. In the absence of a suitable chemical steriliser, recourse was made to the simple technique of careful brushing of both surfaces of the thalli and prolonged agitation in several changes of sterile distilled water.

The principal method of culture of discs has been described in Part I. In addition, DARBISHIRE's (1926) method of culture in Petri dishes lined with filter paper was used. The nutrient solutions used in this investigation were the modified Bristol solution (detailed in Part I) and Chu No. 10 mineral solution (CHU, 1942), modified by GERLOFF et al.(1950). The composition of the latter is as follows:

$Ca(NO_3)_2$	0.040 gm.
K_2HPO_4	0.010 gm.
$MgSO_4 \cdot 7H_2O$	0.025 gm.
Na_2CO_3	0.020 gm.
Na_2SiO_3	0.025 gm.
Ferric citrate	0.003 gm.
Citric acid	0.003 gm.
Distilled water	1000 ml.

EXPERIMENTAL DETAIL

1. Growth of Peltigera discs related to the nitrogen content of the medium.

It has been shown (Part I) that discs of P. praetextata can be cultured in nitrogen-free mineral solution. For further studies it was felt that a higher growth rate than that previously obtained would be desirable. Thus two experiments were set up to determine the effect on growth of adding nitrogen, in the form of sodium nitrate, to Bristol solution. The material for these was collected from the Dumfries-shire site in September 1954 and also in January 1955. After the material was cleaned and washed in sterile water, the rhizinae were removed and discs of 8 mm. diameter cut from the thalli with a cork borer. Samples of these discs were dried at 95°C to obtain mean values for the initial dry weight. Fifteen cultures (each containing fourteen discs) were set up for each experiment, as described in Part I (see Fig. 2), and supplied with 25 ml. Bristol solution containing graded concentrations of sodium nitrate. Each nitrate concentration, 0; 0.05; 0.25; 0.5 and 1 gm./l., was replicated three times. The tubes were placed at a slope of 30° to the horizontal under a bank of fluorescent lights giving 150 f.c. at a distance of six inches (Fig. 6).

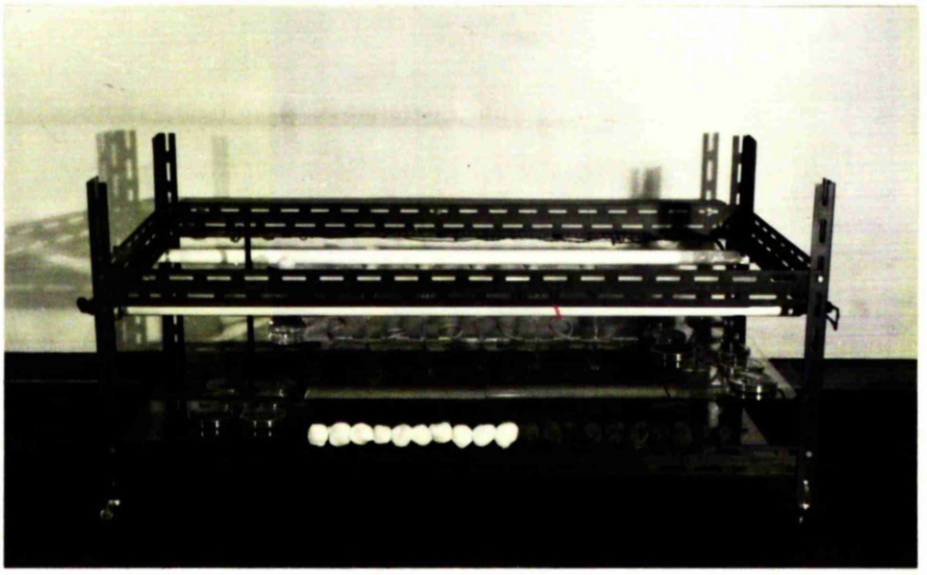

Fig. 6. General view of the arrangement for culturing Peltigera discs under fluorescent lighting.

The culture solution was maintained at the original concentration by periodic additions of sterile distilled water. After fourteen weeks in culture, the discs were removed from the tubes and dried at 95°C. for the determination of dry weights. The majority of the discs showed a prolific development of isidia and rhizinae. Some difficulty was experienced in the removal of the discs due to the growth of the rhizinae into the filter paper. Some loss of material inevitably resulted in this process.

The data of the two experiments are collected in Table 16. The mean initial dry weight of fourteen discs of Expt. 1 was 30.8 mg., and of Expt. 2, 41.5 mg.

Table 16. Mean increase in dry weight of fourteen discs of Peltigera praetextata supplied with five different levels of sodium nitrate in Bristol solution.

Nitrate level gm./l.	Mean increase in dry weight mg.	
	Expt. 1	Expt. 2
0.00	39.4	64.2
0.05	44.7	50.8
0.25	56.4	65.5
0.50	39.4	45.1
1.00	12.9	10.8

The amount of new growth produced depends on the frequency of cracks in the cortex of the discs. The extent of cracking in disc samples is impossible to predict and thus, as in Expt. 2, the effect of increasing the nitrogen supply has been partially obscured. But, although the data obtained are somewhat inconsistent, it is evident that the greatest increase in dry weight has occurred at the nitrate level of 0.25 gm. per litre.

At all concentrations of nitrate, some of the discs showed outgrowth of the phycobiont. This was particularly noticeable in the lowermost discs of each tube, regardless of the nitrate level. Similarly, the amount of isidial growth appeared to be related to the position of the discs in the tubes. Those situated in the centre of the group of discs showed the greatest growth. Above the 0.25 gm./l. level, the position was somewhat obscured by the presence of green algae, but in most cases the majority of the discs survived for only a few weeks. Here, the symbiosis broke down completely and was followed by a copious outgrowth of the phycobiont. It is thus indicated by these experiments that, while the growth rate of discs is increased by the addition of nitrate to the 0.25 gm. per litre level, the degree of moisture to which the discs are subjected is a limiting factor in growth.

2. Growth of Peltigera discs related to the potassium phosphate content of the medium.

Concurrently with the preceding experiments, the effect of variation of the level of potassium phosphate on the growth of discs was determined. Nitrogen-free Bristol solution was used with five levels of phosphate, 0; 0.05; 0.25; 0.5 and 1.0 gm. per litre. Discs were obtained from the material used for Expt. 1 above, the same mean initial dry weight being used. Triplicate cultures of fourteen discs on double layers of filter paper in large test tubes were set up for each of the five phosphate concentrations. Dry weights of the discs were determined after fourteen weeks' growth under conditions similar to those of the previous experiments. The increases in dry weight of the discs are shown in Table 17. The initial dry weight of fourteen discs was 30.8 mg.

These data show that, under the conditions of the experiment, the optimum level of phosphate is 0.25 gm./l. Discs not supplied with the phosphate showed little or no growth and most began to lose colour soon after the cultures were set up. At the 0.05 gm./l. level, most of the discs produced isidia and in some of these, outgrowth of the phycobiont was evident. All the discs at the 0.25 gm./l. level showed a prolific development of isidia. Only one

disc showed a tendency towards breakdown of the symbiosis by outgrowth of the phycobiont. At higher concentrations of phosphate, few of the discs formed new growth. The majority soon died off and resultant bacterial action has evidently been responsible for the losses in dry weight recorded in Table 17.

Table 17. Mean increase in dry weight of fourteen discs of Peltigera praetextata supplied with five different levels of potassium phosphate in Bristol solution.

Phosphate level gm./l.	Mean increase in dry weight mg.
0.00	9.4
0.05	12.4
0.25	27.5
0.50	Loss in weight
1.00	

The level of potassium phosphate, as well as of sodium nitrate, in the medium is thus seen to influence the maintenance of a true balance between growth of the two symbionts of Peltigera in culture. It must be stressed

however, that these results were obtained with impure cultures and the overall picture may well be radically altered when recourse can be made to a pure culture of this lichen. In the meantime, the results presented illustrate that the selection of a particular concentration of nutrients is not, of itself, sufficient to ensure a properly balanced symbiotic growth. The moisture content of the lichen is perhaps of equal importance.

3. Growth of Peltigera discs related to their moisture content.

The importance of moisture content is illustrated by experiments in which discs of Peltigera praetextata were cultured on glass wool. First experiments of this nature were made with fragments of the thallus on a thick layer of glass wool saturated with Bristol solution. The cultures were illuminated under fluorescent lights but, after a few weeks, none survived. It was implied from this experiment that some substance, possibly a nutrilite, was lacking in the glass wool. Disc cultures were then set up on glass wool in test tubes. Again the material died off within a few weeks. A further similar experiment was set up, but in this case the culture tubes were

supplied with outlets at the base. T-joints were attached to these and the tubes connected to a 500 ml. reservoir (Fig. 7). Nitrogen-free Bristol solution was here contained and could be run into or withdrawn from the tubes at will. The large volume of solution in the reservoir eliminated the necessity of frequent dilution to offset concentration of the medium by evaporation. In addition to the glass wool cultures, two control cultures of fourteen discs each, were set up on filter paper in tubes attached to a separate reservoir. During the course of the experiment, which was conducted under continuous fluorescent lighting, the level of the solution in the tubes was raised during each alternate period of 24 hours. The culture solution in one of the tubes with glass wool was however, kept at a constant level throughout the seven-week culture period.

It soon became apparent that the moisture content of the discs provided the key to the lack of success in the earlier experiments. After three weeks, isidia were evident round the edges and on cracks on the surface of all discs except those which were constantly saturated. The latter produced little new growth and several of the discs died off. Table 18 shows the increases in dry weight of the fourteen discs of each culture after seven weeks'

Fig. 7. Part of the fluorescent light bank showing five culture tubes attached to the reservoir.

growth. The initial mean dry weight of fourteen discs was 45.8 mg.

Whilst there has been little increase in weight of the discs on the constantly saturated glass wool, the increases in dry weight of the discs on the intermittently moistened glass wool compare favourably with those of discs on filter paper. It is indicated however, by the slower growth rate of discs on glass wool, that the physical nature of the substrate must be considered as an important factor in maintaining the discs at the optimum moisture content.

Table 18. Increase in dry weight of discs of Peltigera praetextata cultured on glass wool and on filter paper intermittently supplied with N-free Bristol solution.

Substrate	Culture No.	Increase in dry wt. of fourteen discs mg.
Glass wool	1	49.7
" "	2	47.7
" "	3*	23.7
Filter paper	4	65.2
" "	5	64.7

* The nutrient solution was kept at a constant level in this culture.

4. Observations on the growth rate of Peltigera praetextata under greenhouse conditions.

In November 1953 a young plant of P. praetextata was collected from the Dumfries-shire site and planted out on peaty soil in a flower pot in the greenhouse. The pot was placed in a shallow vessel in which water was maintained at a level just below that of the soil in the inner pot. The whole was covered with a transparent plastic dome. At the date of collection the plant consisted of several lobes each less than one centimetre long. The growth of two of these lobes was followed from 20 April to 20 November 1954 by taking monthly photographs. These were taken with a centimetre scale placed between the two lichen lobes to ensure the same degree of reduction in each case. Figs. 8 and 9 represent the plant as at 20 April and at 20 November. In August, the two lobes (A and B, Fig. 8) were transplanted closer together in order that both would appear in the same photograph.

The increase in surface area during each month was taken as a measure of the growth rate of the lobes. It was not possible to obtain accurate data for this increase since the majority of the new-formed lobes became deeply concave due to intercalary growth; so much so that in some cases the margins of the lobes took up a backwards-facing position.

Fig. 8. Plant of Peltigera praetextata as at 20 April 1954. Growth of the two lobes A and B was recorded for a seven-month period.

x 7/8

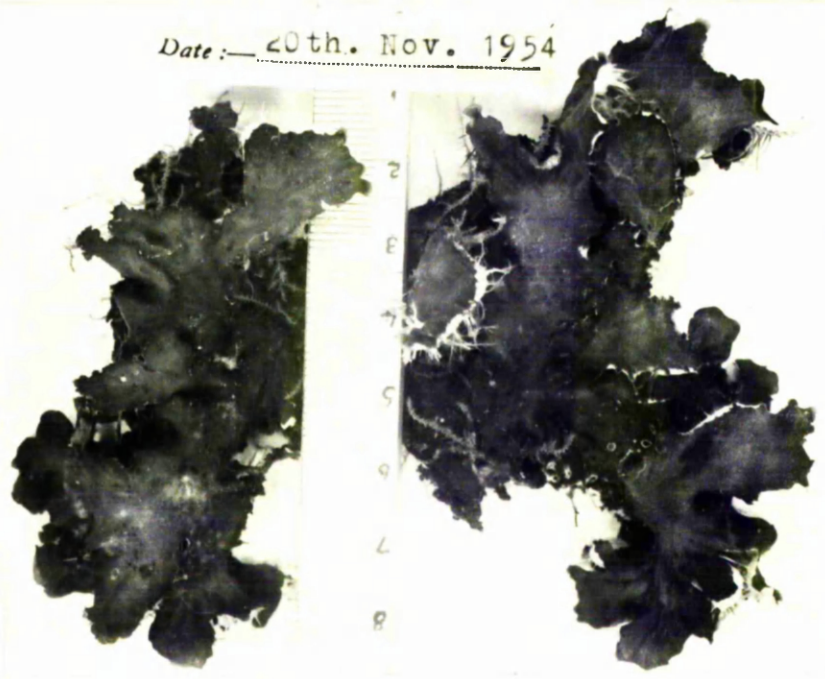

Fig. 9. The same plant seven months later.

x 7/8

The two lobes on each of the monthly photographs were outlined with Indian ink then treated with a thiocarbamide print bleach to remove the photographic image. The eight outlines of one of the lobes were transferred to graph paper (Fig. 10) and the increase in surface area estimated for each period of one month (Table 12).

The conditions under which this plant was grown were such that the intensity of sunlight, and thus the temperature were the only factors capable of significant variation. Enclosure of the plant within the plastic dome and the constant supply of water ensured that it did not dry out at any time. Thus at no period was the moisture content of the lichen a limiting factor in the rate of growth.

The absolute increases in surface area suggest that the period July to September was most favourable to growth, but the increase per unit area of thallus is seen to be at a maximum in the period April to May. Meristematic growth is however, proportional to the circumference of the lobes and intercalary growth proportional to the area, so that the increase per unit area will show a decrease as the thallus becomes larger.

During the seven-month period the thallus grew to eight times the original size as determined by the surface area. This rate of growth is exceptional among the foliose lichens,

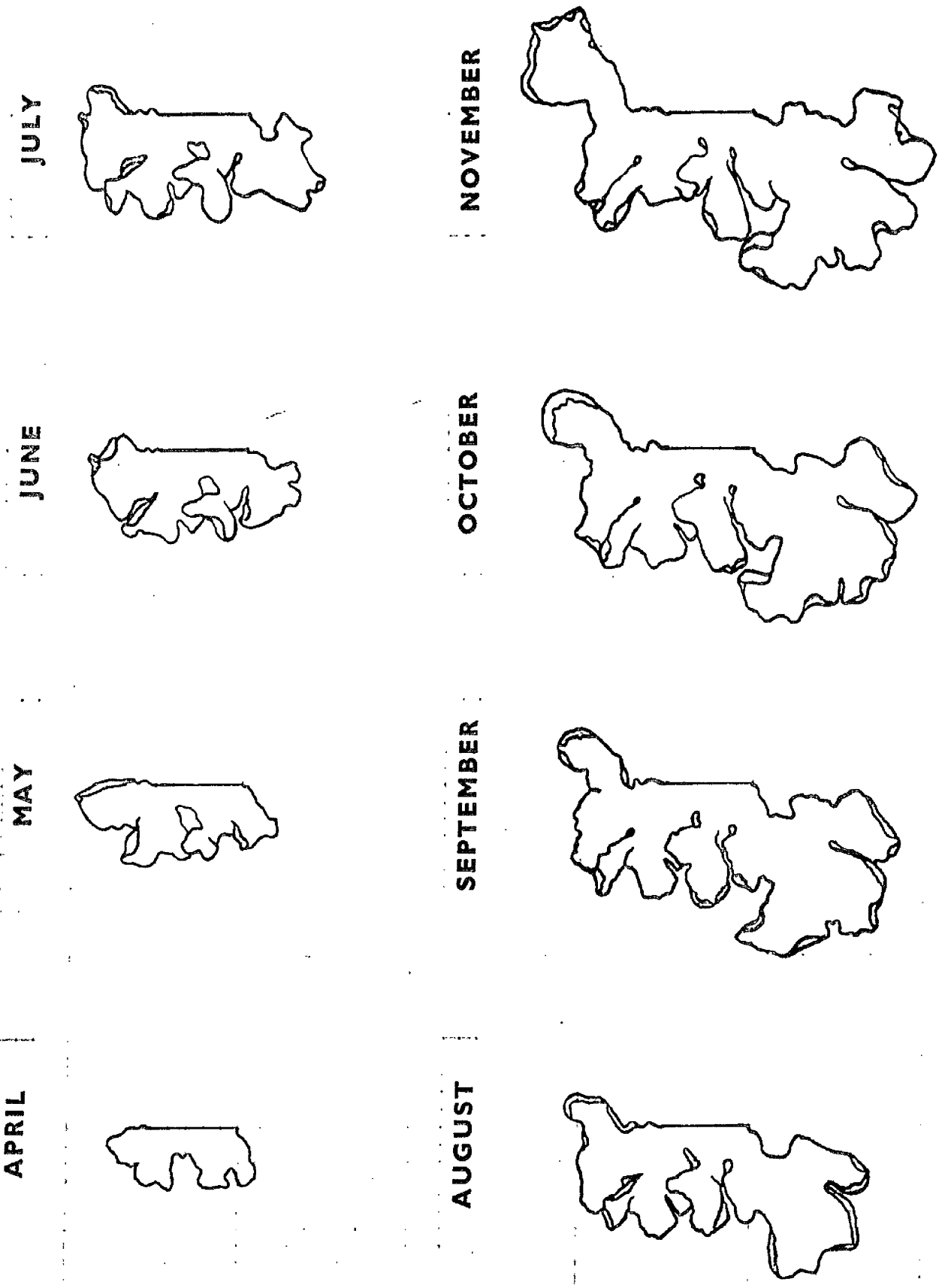

Fig. 10. Outlines of a lobe (A, Fig. 8) of Peltigera praetextata showing the increase in size in seven months.

the majority of which do not even double their size in a similar period. But the data obtained for this plant do not necessarily indicate the growth rate under natural conditions, even though this species favours shaded situations in which the humidity is fairly high for a good proportion of the sunlight hours.

Table 19. Increase in surface area, over a period of seven months, of a plant of Peltigera praetextata grown in the greenhouse.

Month	Surface area sq. ins.	Increase in surface area sq. ins.	Increase per unit surface area sq. ins.
April	0.25		
		- - - - - 0.23	0.92
May	0.48		
		- - - - - 0.12	0.25
June	0.60		
		- - - - - 0.21	0.35
July	0.81		
		- - - - - 0.41	0.51
August	1.22		
		- - - - - 0.36	0.30
September	1.58		
		- - - - - 0.20	0.13
October	1.78		
		- - - - - 0.22	0.12
November	2.00		

Fig. 11. The under surface of a lobe of Peltigera praetextata, grown in the greenhouse, showing outgrowth of the phycobiont amongst the veins.

x 2 approx.

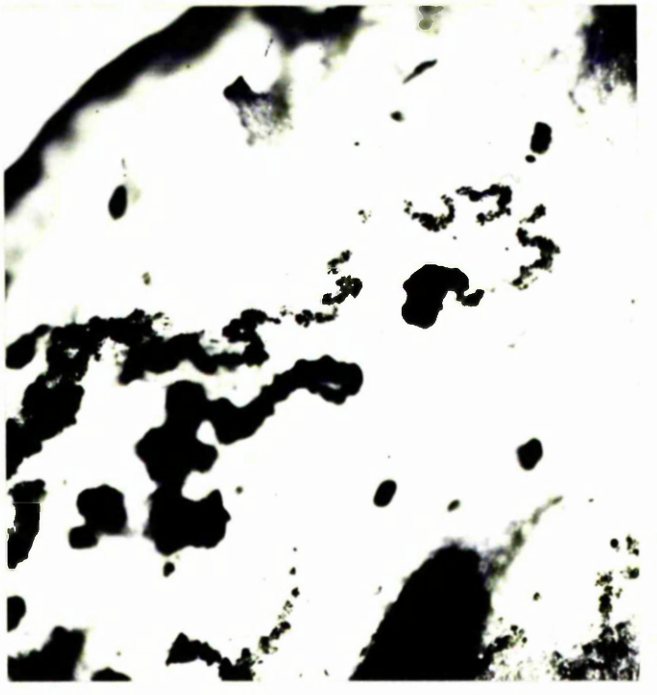

Fig. 12. An enlargement of part of Fig. 11.

x 18 approx.

A noticeable feature of growth of the specimen in the greenhouse was the tendency for the thallus to remain thin and for colonies of the phycobiont to appear on the under surface of the thallus. This condition is shown by Figs. 11 and 12. Evidently the continuous moisture and exposure to strong sunlight has upset the balance of symbiotic growth in favour of the phycobiont.

5. The effect of light intensity on the growth of Peltigera praetextata.

Most species of Peltigera can be regarded as shade plants. This term however, is applied in a totally different manner than to higher plants. For lichens, the intensity of light is a decisive factor in the preservation of the balance between growth of the two symbionts. A notable feature of the lichen symbiosis is instanced by the production of a brown pigment in the cortex of Peltigera. This is commonly regarded to be a protection against excessive insolation of the phycobiontal layer. Undoubtedly this explanation is consistent with the viewpoint that the phycobiont, as an individual, derives benefit from the symbiosis, but it is important to realise that protection against excessive insolation also serves to control the growth rate of the phycobiont and thus to prevent the

breakdown of the symbiosis.

The efficiency of control of the passage of light by pigment formation in the cortex, is illustrated by an experiment using Peltigera praetextata. The plant chosen was growing in an exposed situation, but part of the thallus was covered by a dead beech leaf. The covered part was of a light grey colour, the remainder being dark brown. One of the lobes which was partly covered by the leaf was removed and allowed to dry out. Two holes were punched in a square of thick paper such that when placed over the lichen lobe, one of the holes would be above the brown area, and the other above the grey. A photographic paper was placed behind the lobe and the whole exposed to a strong light for a few minutes. On developing the paper, there appeared the outline of the lobe in white with two circles, one dark in colour corresponding to the grey part of the lobe and the other light grey corresponding to the brown exposed area (Fig. 13). Since the pigmented area of the thallus was the thinner apical region, the difference in the amount of light passing through the two parts was attributed to the presence of the brown pigment. Thus even when plants are exposed to strong sunlight there will be little chance of overgrowth of the phycobiont. This is further guarded against by the tendency of the cortical cells to thicken when exposed to sunlight for

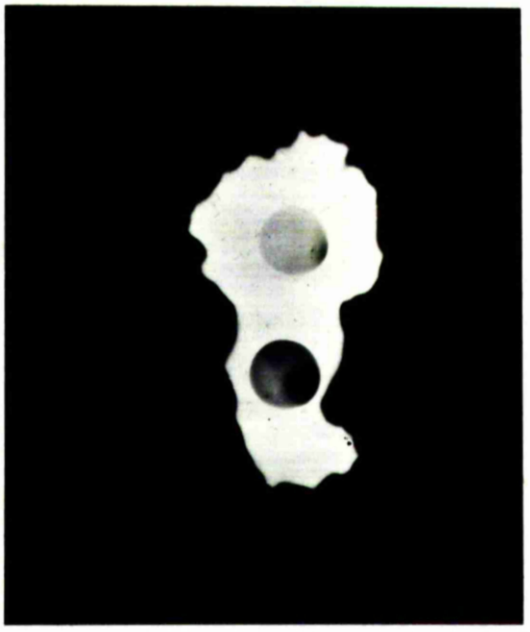

Fig. 13. Illustration of the passage of light through the pigmented and non-pigmented parts of a lobe of Peltigera praetextata. The upper circle appears over the brown area of the thallus.

x 1

long periods (SMITH, 1921).

During long exposure, drying out of the thallus is, of itself, an effective means of reducing the penetration of light to the phycobiontal layer. The cortical cells of Peltigera have thickened anticlinal walls but the periclinal walls remain thin (Fig. 14). Moisture loss brings about a contraction of the cortical cells thereby greatly reducing the area of the transverse walls through which the incident light passes. Fig. 15 illustrates the difference in the amount of light able to pass through the cortex of Peltigera horizontalis in the moist and dry states. A small square of the cortex was carefully removed from a piece of the thallus and placed on a glass plate. An enlarged image of the moistened cortex was obtained with a vertical photographic enlarger and a timed exposure made on bromide paper. A second identical exposure of the dried cortex was made on a previously masked part of the same paper. The image to the left of Fig. 15, which is a contact print of the original, represents the dry cortex. In this state, considerably less light has been able to pass through the cortical cells.

The light controlling mechanism is however, radically upset when discs of Peltigera are cultured in the laboratory. Here, the phycobiontal layer is freely exposed round the edges of the discs so that there is

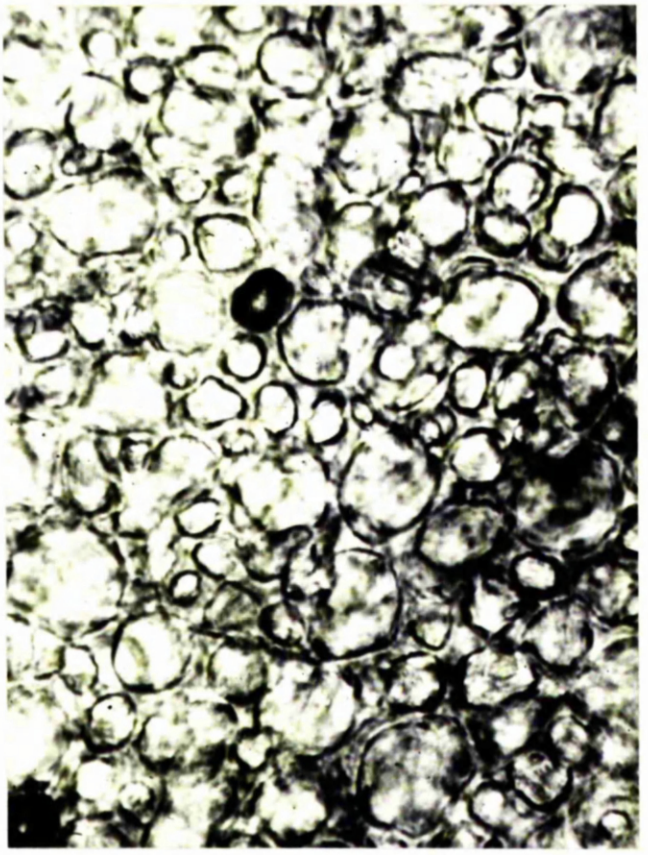

Fig. 14. Photomicrograph of the surface cells of the cortex of Peltigera horizontalis showing the thickenings of the anticlinal walls.

x 750 approx.

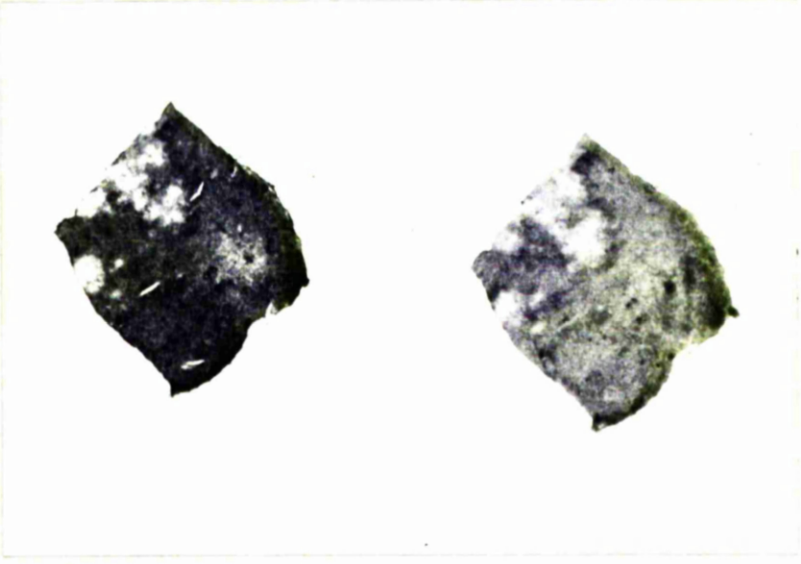

Fig. 15. Illustration of the passage of light through the moist and dry cortex of Peltigera horizontalis. The image to the left represents the dry cortex.

x 10

effectively no means of light control. There is thus a strong tendency towards outgrowth of the phycobiont in these parts. Similarly, the origin of isidia on the disc margins and on thallus parts which have been damaged may be attributed to the localised increase in light reaching the phycobiont cells. Light is thus the stimulus, the response being either outgrowth of the phycobiont or the formation of isidia.

This provides illustration of one of the fundamental features of the lichen symbiosis - the rigorous dependence of the growth of the one organism upon the growth rate of the other. In the case of Peltigera, isidia are considered to originate by an increase in the growth rate of exposed groups of the phycobiont. The increased supply of carbohydrates and possibly also of nutrilites, stimulates the mycobiont to keep pace with the new growth, thus leading to the formation of isidia. This train of events occurs when a change takes place (in the incident light) which acts on the phycobiont only. Thus it seems that, although the mycobiont is morphologically the dominant partner in lichens such as Peltigera, it is not the initial perceptor in the formation of isidia.

The theme of the foregoing paragraphs has been the importance of light in the development of the lichen thallus. Two experiments with Peltigera discs illustrate

the rapid decline in growth rate with lowering of the light intensity. In the first experiment, six discs of 8 mm. diameter were placed on filter paper strips in each of five test tubes. Half-strength Bristol solution was added to each tube. One tube was exposed to a light intensity of 150 f.c. By suitable shading, the remaining tubes were exposed, one to each light intensity of 90, 45, and 20 f.c., and one to darkness. Fig. 16 shows the state of the discs after eleven weeks' growth (those cultured in darkness soon died off and have not been included). Discs at 20 f.c. have formed no new growth, the majority of the phycobiont cells having lost colour. At 45 f.c. a very limited amount of growth is seen, while at 90 f.c. all the discs show at least the development of "regeneration nodules" (DARBISHIRE, 1926) round the margins. At this light intensity there was also a slight increase in the diameter of the discs. The most striking growth occurred at 150 f.c. One disc in particular increased considerably in diameter, while the others showed a good development of isidia.

The second experiment involved the culture of pairs of discs, one partly overlapping the other. Four such pairs were cultured for fourteen weeks on filter paper supplied with quarter-strength nitrogen-free Bristol solution. At

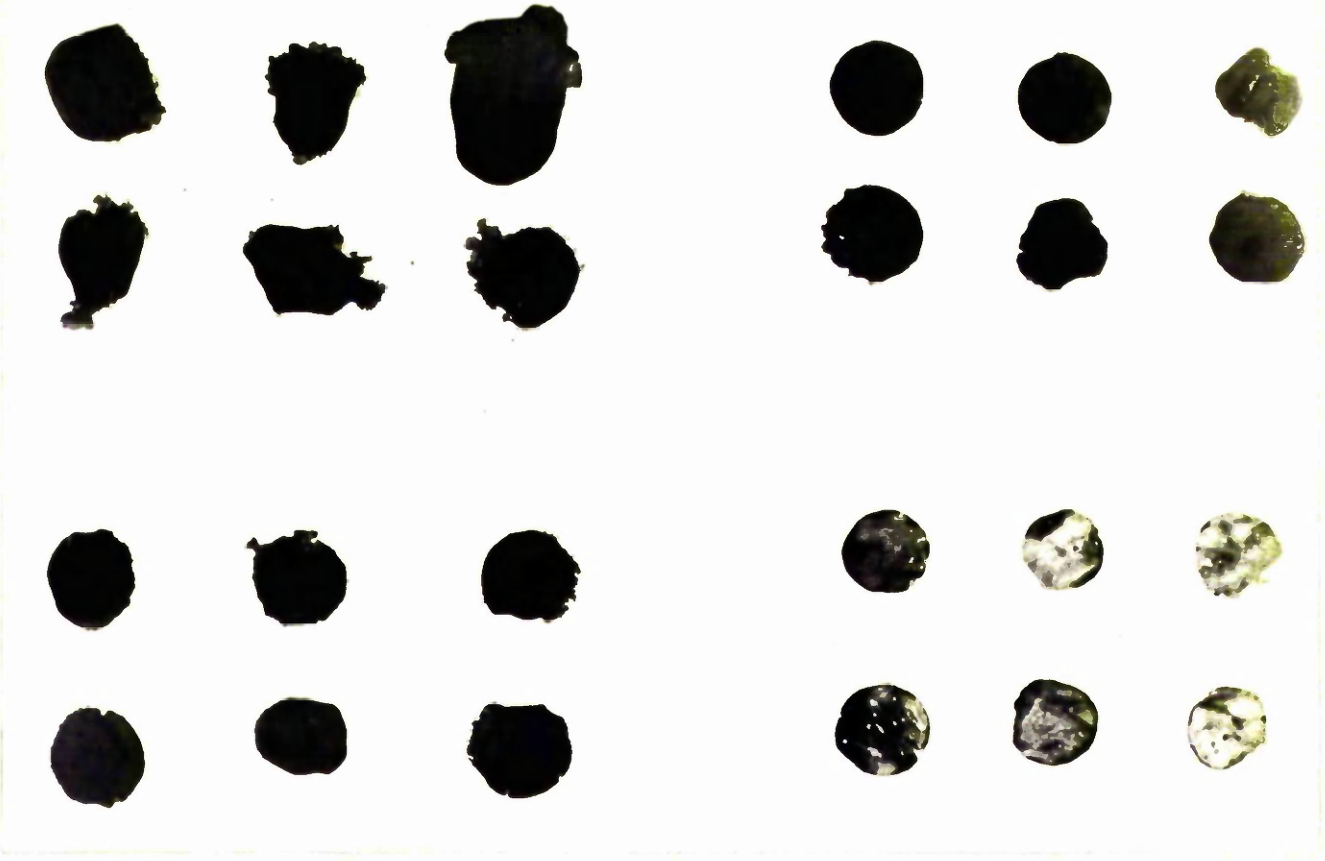

Fig. 16. Discs of Peltigera praetextata cultured at four light intensities. Upper left - 150 f.c. Lower left - 90 f.c. Upper right - 45 f.c. Lower right - 20 f.c.

x $\frac{5}{4}$

Fig. 17. Four discs of Peltigera praetextata which were partly overlapped by others for fourteen weeks on filter paper supplied with nitrogen-free Bristol solution. The lower portions of the discs were obscured from light by the overlying discs and are seen to be free of isidia.

x $\frac{3}{2}$

the end of the period, the disc-pairs were separated and the underlying discs photographed. Fig. 17 shows that isidia were not formed on the covered parts of the discs. Growth of the mycobiont is thus seen to be entirely dependent upon the phycobiont receiving an adequate supply of light. There seems to be no provision for translocation of organic nutrients from one part of the thallus to another.

6. Observations on the further growth of isidia.

LINKOLA (1922), DARBISHIRE (1926) and others, have said that isidia of Peltigera praetextata may be capable of giving rise to new individuals on separation from the parent plant. The possibility of the independent existence of isidia is illustrated by Figs. 18 and 19. The discs were cultured for four months floating on nitrogen-free Chu No. 10 solution then transferred to filter paper saturated with the same solution. During the next few months, parts of the isidial growth were frequently separated from the discs and placed elsewhere on the paper. Some also became detached by the occasional drying out and re-moistening of the discs and filter paper.

Many of the isidia established themselves as independent individuals, but under the conditions of this experiment, growth followed the pattern of repeated branchings rather

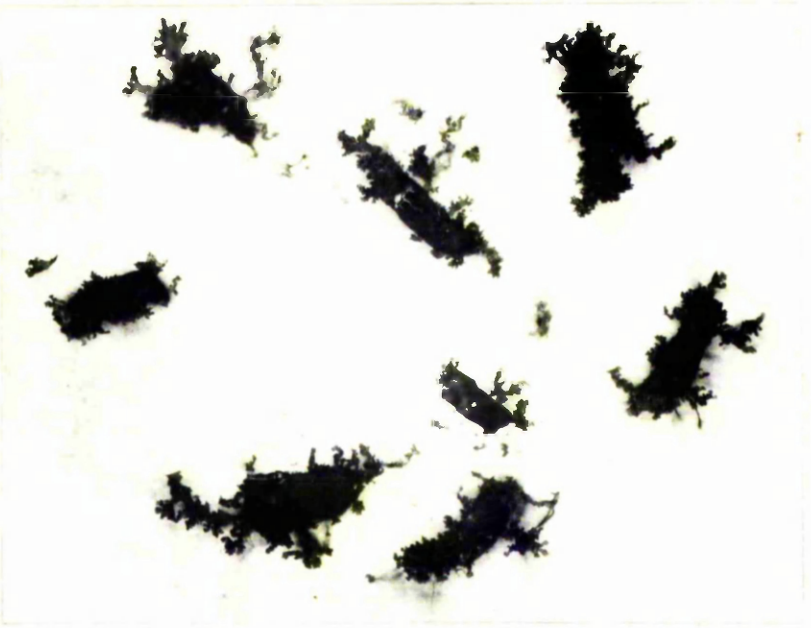

Fig. 18. Discs of Peltigera praetextata cultured for several months on filter paper supplied with Chu.No. 10 solution. Many of the isidioid growths have become separated from the discs. All were firmly attached by rhizinae to the filter paper.

x $\frac{3}{2}$ approx.

Fig. 19. An enlargement of part of Fig. 18.

x 4 approx.

than the production of a replica of the parent thallus. In some cases however, it is evident that intercalary growth has taken place leading to the broadening of the older parts. These show stages in the transition from the juvenile form to the rhizinous condition typical of the fully mature thallus. Not until the latter form is established throughout the whole structure can these separated isidia be regarded as new Peltigera plants.

It is a general rule that plants growing in open situations - those characterised by a fairly thick leathery thallus with a deep brown pigmentation - bear isidia of a similar texture. The latter, according to LINKOLA (1922) are of limited growth and are usually of simple outline. On the other hand, plants growing in deep shade and which thus spend a greater proportion of their lifetime in the moist condition, are characterised by a thinner thallus with no pigmentation, and deeply dissected isidia with branchings often of the fourth and fifth order. In such situations the isidia tend to be larger, but since there is less frequent expansion and contraction of the thallus due to changes in moisture content, these plants are commonly found with few isidia.

Some illustration of the localised effect of moisture on the further growth of isidia is given by Figs. 20 to 22.

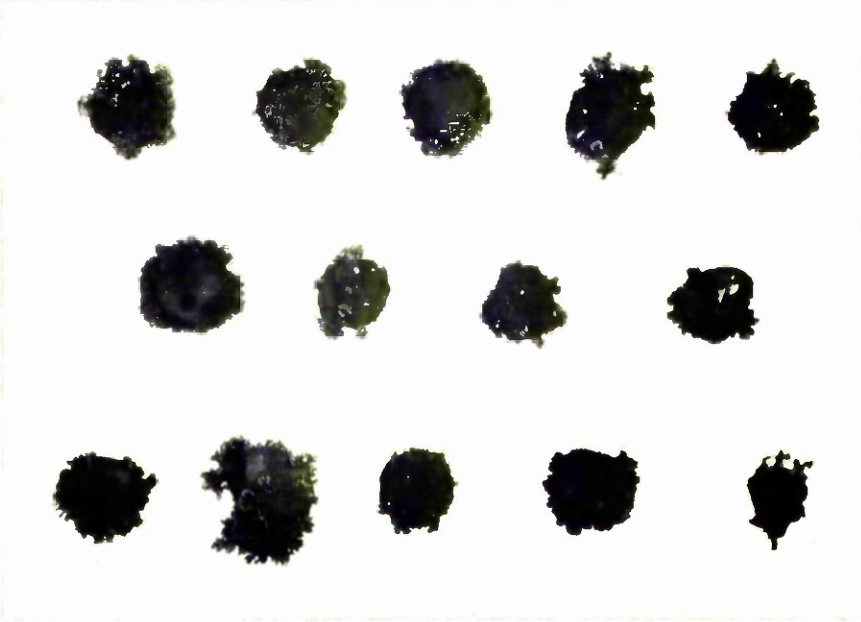

Fig. 20. Discs of Peltigera praetextata cultured for fourteen weeks on filter paper supplied with nitrogen-free Bristol solution. These show two distinct types of isidial growth.

x 1

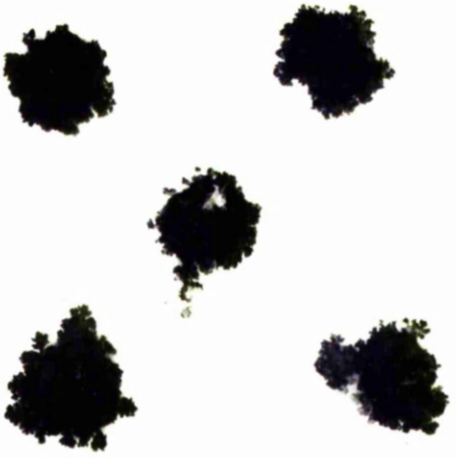

Fig. 21. Discs of Peltigera praetextata cultured for ten months on filter paper constantly saturated with nitrogen-free Chu No. 10 solution. Much-branched isidia have developed on the surface as well as round the edges of the discs.

x 1

Fig. 22. An enlargement of one of the discs of Fig. 21.

x 3

Fig. 20 shows fourteen discs which were cultured for fourteen weeks on filter paper supplied with quarter-strength nitrogen-free Bristol solution. On these, particularly the disc to the extreme right of the central row of four, can be seen large simple lobes growing on the disc surface, while the isidia protruding from the edges of the discs are much more dissected. This difference is an indication of variation in the available moisture at different parts of the discs. Most of the discs, after a few days in culture, assume a convex form so that the edges are in close contact with the filter paper while the centre is raised perhaps three to four millimetres. Moisture is thus not so readily available to those parts.

Fig. 21 shows five discs cultured in a Petri dish on filter paper saturated with nitrogen-free Chu No. 10 solution. After the discs had been in culture for a few weeks, the surface of each was lacerated with a scalpel to stimulate the formation of isidial growth. Fig. 22, an enlargement of one of the discs of Fig. 21, shows the form of the isidia after ten months' culture under constant saturation with culture solution. Even on the surface of the discs the isidia show intricate branching such as can be clearly seen in the case of those round the edges.

The 15 mm. disc illustrated in Figs. 23 and 24 was cultured initially for eight weeks floating on nitrogen-free Chu No. 10 solution in a large test tube. During this time, growth of the phycobiont exceeded that of the mycobiont, but many small regeneration nodules were formed round the edge. The disc was then transferred to a Petri dish lined with several layers of filter paper moistened with the same solution. During the next sixteen weeks, the filter paper and disc were frequently allowed to dry out for several days at a time. Fig. 23, taken after a total culture period of twenty four weeks, shows the development of several large, simply outlined lobes in addition to many small outgrowths of the usual isidioid type. Nitrogen-free Bristol solution was then substituted for Chu No. 10, and after fourteen weeks the disc was again photographed (Fig. 24). Of prime interest is the marked increase of one of the new lobes. This, had the disc been allowed to continue growth, would undoubtedly have superseded the parent disc which can be seen to be in a state of partial decay.

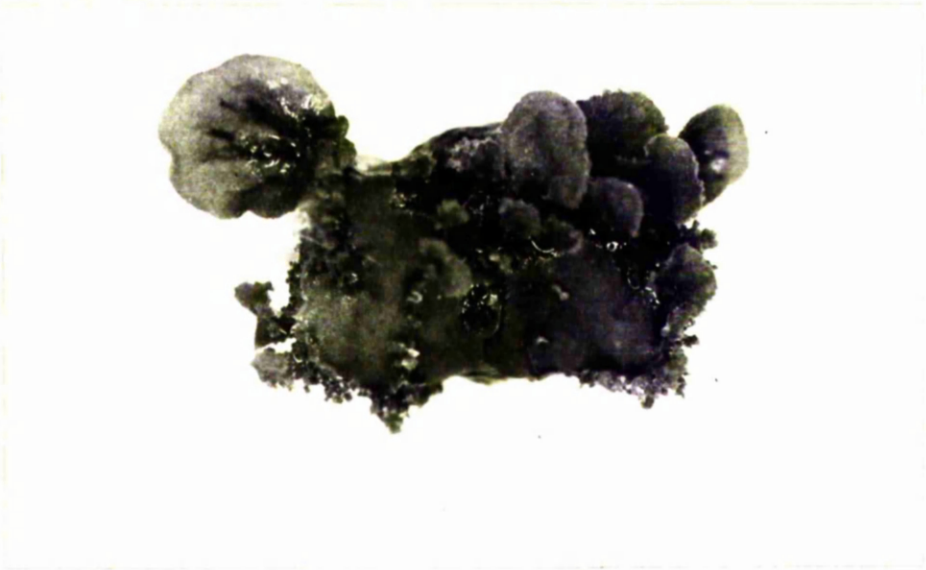

Fig. 23. Disc of Peltigera praetextata after 24 weeks' culture, showing the development of isidia into young thalli.

x 3

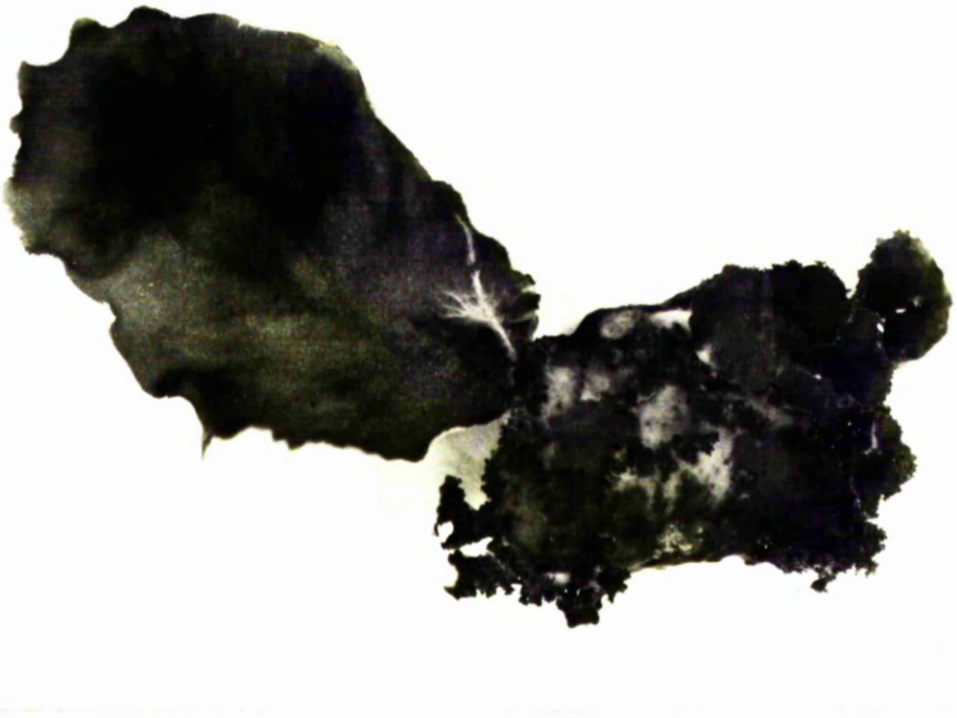

Fig. 24. The same disc fourteen weeks later. The parent disc has begun to decay and growth is being continued by one of the "isidia".

x 3

7. Observations on the development of artificially produced isidia of Peltigera praetextata.

The development of isidia of P. praetextata has been followed by (among others) LINKOLA (1922) and by DARBISHIRE (1926). Linkola figures several of the later stages of both "true isidia" and of "wound isidia". The latter are stated to originate as "...kleines gonidienreiches Knäuel..." from the wounded phycobiontal layer. Darbishire however, gives a more detailed account of how hyphae from the medullary layer grow out to invest the groups of the phycobiont where the thallus has been injured. He considers that the initial response to the wound stimulus is perceived by the medullary hyphae. This is followed by the growth of the phycobiont colonies between the outgrown hyphae.

It is unfortunate that Darbishire did not succeed in obtaining isidial growth on P. praetextata in culture. In his 1926 paper he states, " I have carried out numerous experiments with material (of P. praetextata) in Petri dishes. Small square pieces have been kept under moist conditions for a year. Others have merely had incisions made to varying depths. Typical isidia are not developed as a rule, least of all when the margin or edge has been completely removed. Small round bodies are formed which

for convenience I have called 'regeneration nodules'. These differ from normal isidia by being more loosely attached to the thallus....". Had he but realised that these "regeneration nodules" were in fact the precursors of isidia, there is no doubt that the present-day knowledge of these structures would have been materially advanced. His lack of success in this field was probably due to his failure to supply the material in Petri dishes with suitable nutrients. Indeed, no mention of any supply of nutrients is made.

Little can be added to the very full account of the naturally occurring isidia given by Darbishire, but it is instructive to note some of the developmental stages in the formation of isidia round the edges of discs of Peltigera praetextata cultured in the laboratory. It must be emphasised however, that the description to be given does not necessarily apply to isidia formed in Nature from cracks in the cortex of the thallus. There is every indication indeed that the two types are distinct results of differing environmental conditions.

When Peltigera discs are placed on filter paper or other substrate and supplied with a nutrient solution (Bristol solution in these investigations), either the phycobiont will grow out from the disc margins and spreed freely over the substrate as a non-symbiotic alga, or both

partners will maintain at least a semblance of the normal symbiotic association and produce isidia. Which of these will result has been shown to depend on several factors of which the most important are perhaps the concentration of the nutrient solution and the moisture content of the discs and substrate. In any case, the rapid outgrowth of the medullary hyphae from the cut edges of the discs is the first noticeable sign of new growth (Fig. 25). Darbishire mentions the observance of this growth as a test to determine whether the lichen material is living, but it should not be taken as an indication of the normal rate of growth of this tissue. The outgrowth becomes visible to the naked eye within two or three days of cutting the discs (that shown in Fig. 25 has a maximum length of 500 microns), and is obviously a short-lived response to mechanical injury. Meanwhile the marginal groups of the phycobiont have begun to increase in size by division of the cells within the common gelatinous sheath. Some of these enlarged groups are seen in Fig. 26. At this stage no evidence could be seen of any connection between the medullary hyphae and these groups.

With further development of the phycobiont, there is seen the formation of an organised cortex which originates from a loose investment of hyphae surrounding the groups. In Fig. 27 the associated hyphae can be seen especially

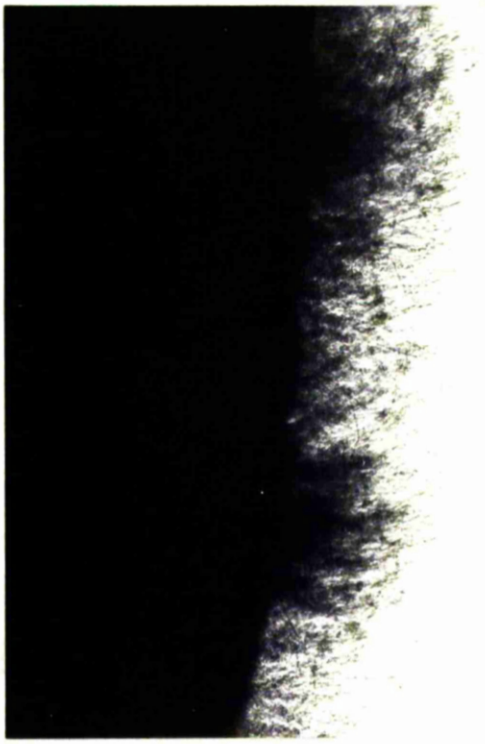

Fig. 25. The edge of a disc of Peltigera praetextata after fifteen days in culture, showing the outgrowth of medullary hyphae. A length of 500 microns was reached after four days.

x 40

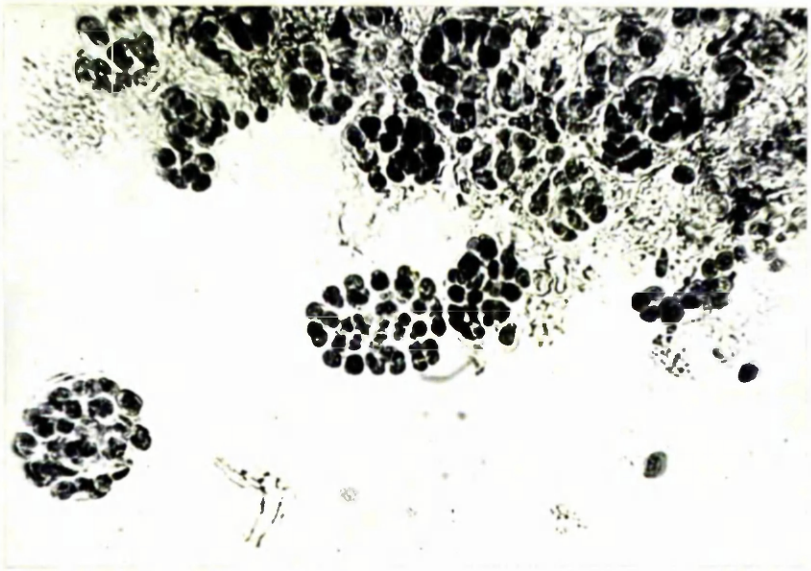

Fig. 26. An early stage in the formation of isidia of Peltigera praetextata, showing the enlarged phycobiontal groups at the margin of a disc.

x 250 approx.

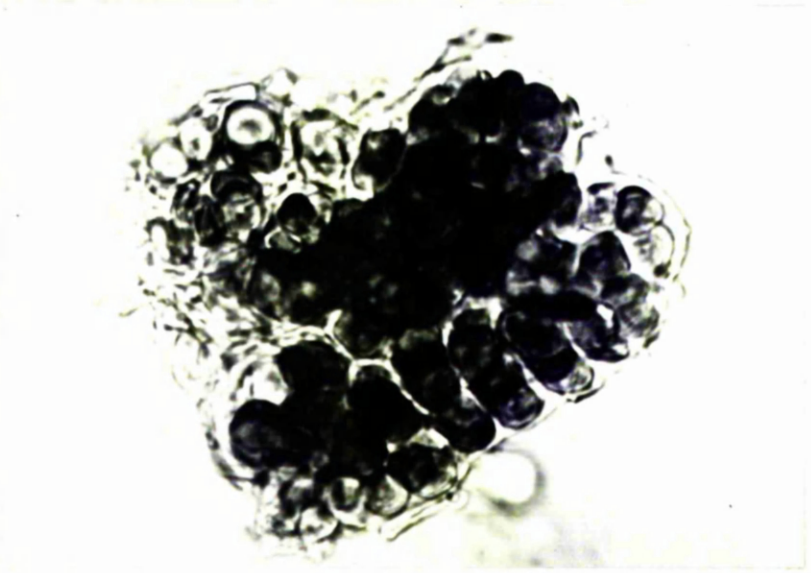

Fig. 27. Later stage in the formation of isidia. Hyphae have begun to invest the phycobiontal groups which now show a regular transverse arrangement of the cells.

x 825 approx.

near the base of the nodule. This particular structure has arisen from two adjacent phycobiontal groups which have partially coalesced to give a bi-lobed nodule. It is of interest to note the regular transverse rows of Nostoc cells in this nodule - a feature of the free growth of the phycobiont in culture. The cortex becomes defined as a single layer of cells surrounding the now greatly enlarged phycobiontal groups. Fig. 28 shows one such "isidium" with the well-defined cortical layer in sectional view. At this stage the regular arrangement of the Nostoc cells has become totally obscured. The effects of such growth are still evident however, in that the isidium is now a flattened structure with the beginnings of the formation of four secondary lobes. Eventually the stage represented by Fig. 29 is reached. This is a silhouette of a young, but fully differentiated, isidium which was removed from the parent disc. Numerous hyphae can be seen at the constricted base; some of these are rhizinal primordia while others formed the junction with the disc.

There are points in this description at variance with that given by DARBISHIRE (1926). In the first instance, it is established that isidia do arise when the margin of the thallus has been completely removed. Reference to Fig. 18 shows how prolific this growth may be. Secondly, it seems for artificially produced isidia at any rate,

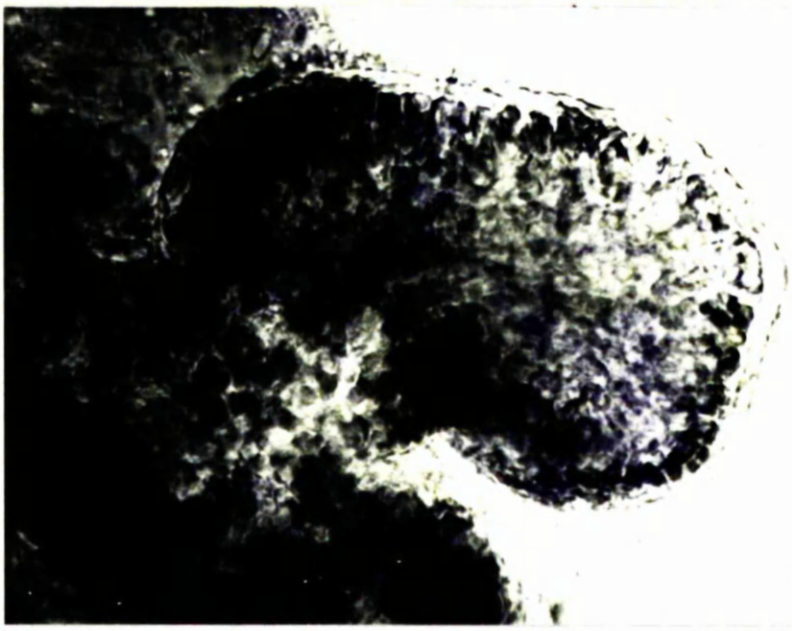

Fig. 28. Young isidium of Peltigera praetextata showing the single-layered cortex surrounding the phycobiont cells.

x 400 approx.

Fig. 29. Silhouette of a fully differentiated isidium with rhizinal hyphae growing out from the base.

x 40 approx.

that these are initiated primarily by an increase in the growth rate of the exposed phycobiontal groups. This was the view held by STRATO (1921) and by LINKOLA (1922). At the present stage of knowledge of the lichen symbiosis, no useful purpose can be served by dogmatism on this point, but experiments described in this work tend to show that the products of photosynthesis have a very limited translocation within the thallus. It is thus to be expected that any increase in growth of the mycobiont, apart from that of the medullary hyphae already mentioned, will be preceded by an increased supply of carbohydrate from the phycobiont cells in the near vicinity.

8. The relationship between regeneration growth* and isidia of species of Peltigera.

It is evident that the naturally occurring isidia on Peltigera praetextata and the prolific regeneration growth of the species in culture are closely associated characteristics. The latter type of growth is the direct result of a wound stimulus applied to the thallus when discs are cut; the former have been shown to arise from

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* In the preceding chapters the term "isidial growth" has been used when referring to this type of growth on Peltigera praetextata.

wounded parts of the thallus, although LINKOLA (1922) distinguished between wound isidia and true isidia. Close examination of field material of Peltigera praetextata and of material grown in the greenhouse shows that, prior to the formation of isidia along the margins of the lobes (isidia which are characterised by their vertical growth, and arrangement at right angles to the margin of the thallus), the margin near the apex of the lobe becomes finely nodular. This condition is considered to arise by increase in size of the metathallus by intercalary growth and the setting up of considerable strains in the marginal area behind the apices of the lobes. Inevitably there will occur slight cracking of the margin and thus the opportunity for localised outgrowth of the phycobiontal groups and associated hyphae.

A significant feature of isidia formation by Peltigera praetextata is their presence only on the older parts of the thallus, and in particular, along the margin at the junction between two lobes. There can be little doubt that these areas are subjected to greater strain than other parts of the thallus. Thus there is good reason to attribute the origin of true isidia (as defined by Linkola) as well as wound isidia, to a response to an interruption of the normal association between the two symbionts at these particular points on the thallus.

Whatever the morphological differences between the two types of isidia, there appears to be no sound premise on which to base differences in physiological function. It is not suggested however, that the taxonomic value of the isidia is, on this account, diminished.

The occurrence of "isidia" has been reported by THOMSON (1948) on Peltigera polydactyle, P. canina and also on P. horizontalis. During extensive observation of field material of these species, I have seen structures resembling isidia only on P. horizontalis. These consisted of a few minute dorsiventral lobules usually on the surface of the thallus. They had evidently arisen as the result of some mechanical injury to the thallus.

To further test the probability of the formation of isidia by these and other species (here the term "isidia" is used in the widest sense), discs of Peltigera canina, P. polydactyle, P. horizontalis and P. aphthosa were cultured on filter paper supplied with Bristol solution. These experiments showed that, with the exception of P. aphthosa, all the species produced regeneration nodules to a greater or lesser extent round the edges of the discs. Further growth of the nodules, particularly in the case of P. horizontalis, gave rise to a regeneration growth very similar to that of P. praetextata, although somewhat inferior in quantity (Figs. 30 and 31). In the very early

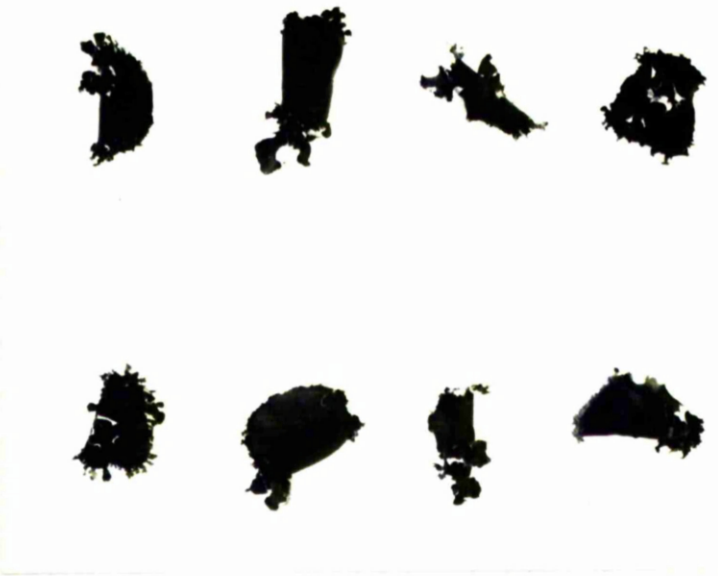

Fig. 30. Discs of Peltigera horizontalis showing the development of "isidia" round the margins and on the surface.

x 3/2

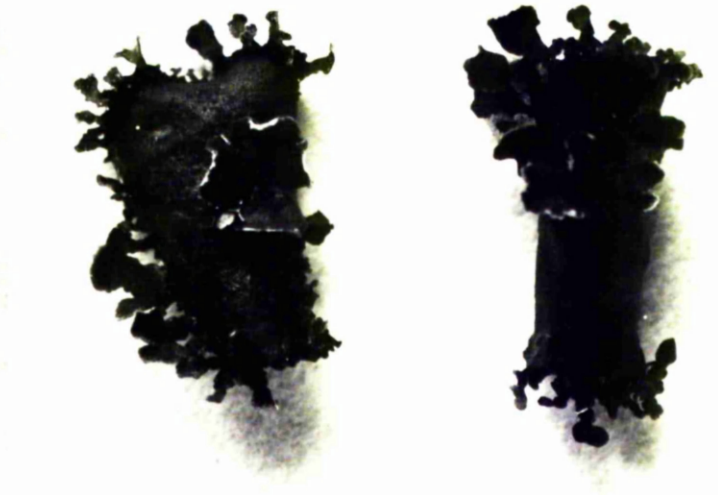

Fig. 31. Enlargement of two of the discs of Fig. 30, showing the outgrowths in greater detail.

x 9/2

stages of nodule formation it is difficult to distinguish between those of P. horizontalis and of P. praetextata. The cortical cells of both exhibit the convoluted outline described by DARBISHIRE (1926) as typical of isidia of P. praetextata. Figs. 32 and 33 show the nodule cortices of both species. The main point of differentiation however, is the early development of heteromery in the P. horizontalis structures, while those of P. praetextata may retain the near-homoiomerous condition indefinitely.

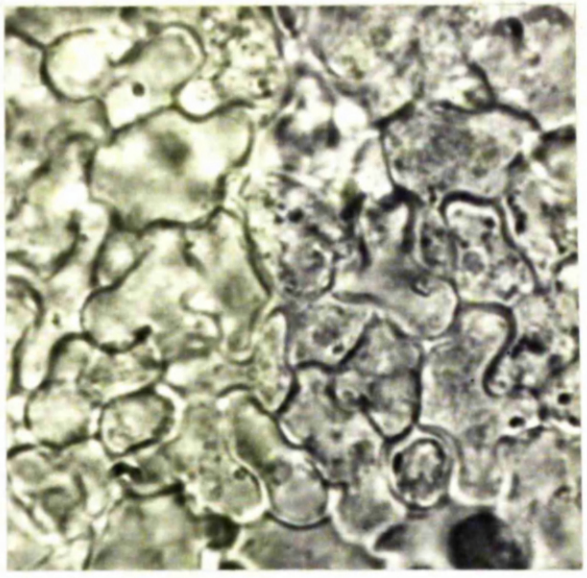

Fig. 32. Part of the cortex of an isidium of Peltigera praetextata showing the irregular cell-outline.

x 1000 approx.

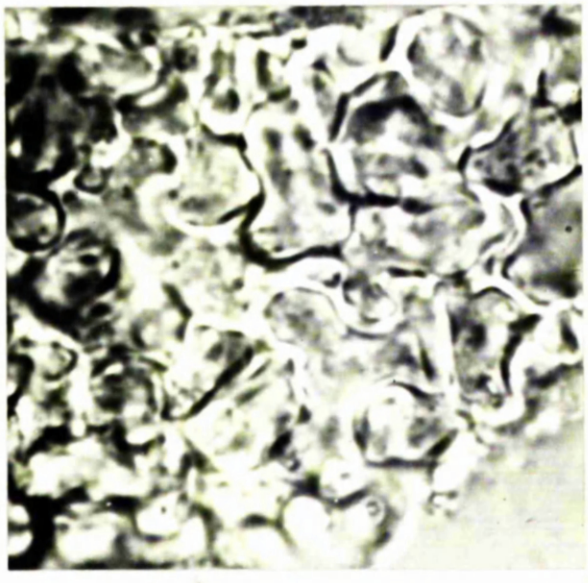

Fig. 33. Cortical cells of an "isidium" of Peltigera horizontalis.

x 1000 approx.

DISCUSSION OF DATA OBTAINED

The several disc experiments bring to light the importance of maintaining the correct degree of moisture in cultures, and illustrate the difficulties yet to be overcome in attempts to synthesise Peltigera from the two symbionts. In particular, the cultures on glass wool with controlled moisture conditions show that there is a close correlation between moisture content and balanced symbiotic growth. The failure of growth in the cultures with constant saturation must be partly due to the inability of CO_2 and O_2 to diffuse sufficiently rapidly through the waterlogged thallus. DARBISHIRE (1926) describes "chimneys" stretching from the hypothallus to the phycobiontal layer. It is through these that the major part of the gaseous exchange takes place, so that the growth of discs which are constantly saturated with culture solution must be limited to some extent. There is evidence however, that failure of these cultures is in large measure due to overgrowth of the phycobiont.

Although discs can be cultured in nitrogen-free Bristol solution, it has been shown that better growth is obtained with 0.25 gm. per litre of sodium nitrate. It was concluded from the disc experiments of Part I that some

of the nitrogen fixed by the phycobiont is taken up by the mycobiont in the formation of new symbiotic growth. The conditions under which these experiments were made were exactly similar to those described in the present section. In the latter experiments, the limiting factor in growth has evidently been the nitrogen supply to the discs. This may operate in either of two ways. In the nitrogen-free cultures, the growth of the phycobiont may be limited, or growth of the mycobiont may be limited, by the inadequate nitrogen supply. The available evidence points to a limitation in the growth of the phycobiont since otherwise, in nitrogen-free culture, there would have been a much greater than observed tendency for it to break away from the symbiotic state. In those cultures to which sodium nitrate was added, the increase in growth rate of the discs was probably due to the uptake of the nitrate by the mycobiont in preference to nitrogen fixed from the atmosphere.

It is of interest to compare the growth rate of discs of Peltigera praetextata in culture with that of the intact thallus under greenhouse conditions. The greatest mean dry weight of fourteen discs, after fourteen weeks in culture, was 107 mg. (Table 16, Expt. 2). This represented an increase to 2.6 times the original dry weight (41.5 mg.). During the thirteen-week period

from 20 April to 20 July, one lobe of the greenhouse plant increased in surface area from 0.25 sq. ins. to 0.81 sq. ins., i.e. to 3.24 times the original size. If it is assumed that the increase in surface area of the thallus is directly proportional to the increase in dry weight, i.e. that the thallus does not significantly increase in thickness, then one may take the increase in surface area as a measure of the dry weight increase. Thus the thallus may be stated to have increased to 3.24 times the original weight. But the dry weight of unit area of the thallus does increase with age due to growth in thickness, so that the value 3.24, corrected to allow for this increase, and also corrected to a fourteen-week period of growth, should be at least 3.6. It thus appears that the growth rate of the thallus under greenhouse conditions was about one and a half times that of discs, supplied with Bristol solution, under artificial light.

The overall aspect of discs in culture suggests that a better growth rate could be realised by using light of a higher intensity and by supplying nitrogen, for uptake by the mycobiont, in the organic form. But the possibility of doing so is questionable until it has proved possible to start off a new thallus from pure cultures of the two symbionts.

LINKOLA (1922) considers that naturally formed isidia are of limited growth. This is certainly the impression obtained when field material is examined, although instances can usually be found where some have superseded their neighbours to form miniature plants. The magnitude of the possible variation in size and form of isidial growth due to differences in the conditions of culture is illustrated by Figs. 22 and 24. Both represent discs which had been cultured for approximately similar periods but under widely differing moisture conditions.

Isidia have frequently been noted firmly attached by their rhizinae to stems of mosses growing up between two lobes of Peltigera thalli. That this is a common method of reproduction is revealed by an examination of the herbarium material of Peltigera praetextata in the Institute of Systematic Botany at Uppsala, Sweden. Of the forty six specimens available from the Nordic countries, twelve, which had been growing over various mosses when collected, were selected at random for critical examination. All but one of these showed examples of isidia having been removed from the parent thallus by growth of the moss shoots between the thallus lobes.

Although DARBISHIRE (1926) does not deny the possibility of isidia becoming detached, he tends to discount this because of their firm attachment to the thallus and, if

separated by natural means, a large part of the phycobiontal layer would be exposed, both on the parent thallus and at the base of the isidium. He considers this gross wounding to be inconsistent with natural dispersal. It has been shown however, that isidia, still attached to the parent thallus, may continue growth even though the latter has begun to decay (Fig. 24). This feature has been observed in plants grown in the greenhouse, and is also evident in a number of specimens in the Uppsala herbarium. Thus it is probable that isidia may serve as organs of reproduction by merely continuing their individual growth after the remainder of the thallus has died off.

LINDAHL (1953) does not agree with THOMSON (1948) that "isidia" may be produced on species such as Peltigera horizontalis. Fig. 31 however, illustrates a prolific growth of isidioid structures on discs of this species. Superficially they bear a strong resemblance to isidia of P. praetextata, but anatomically they show, from a very early stage, all the characteristics of the mature thallus. Specimens of P. rufescens and of P. polydactyla have been observed by Lindahl, "...with the margin of the thallus crispate and uneven, even broken up into small, quite irregular pieces." He distinguishes between these small lobelets and the isidia of P.praetextata by the horizontal growth of the former and the more or less

vertical arrangement of the latter. In all the few hundred discs which I have cultured, there has been no constant distinction between vertical and horizontal growth. This appears to be entirely a matter of chance, and is perhaps to be correlated with the question of available space. Discs with surface wounds produced, in some cases, simply lobed more or less horizontal structures, while vertically arranged, much dissected growths, were equally frequent under different moisture conditions.

Anatomically, the distinguishing feature between true isidia and wound isidia has been described by LINKOLA (1922) as being the continuity of the cortex of the former, with that of the parent thallus. Wound isidia are stated to arise by outgrowth of groups of the phycobiont where these have been exposed by injury to the thallus. Continuity of the cortex seems a slender criterion upon which to base the difference, and Linkola himself states, for wound isidia, " Dieses Rindenlager bildet im allgemeinen nicht eine direkte Fortsetzung der Kortikalschicht des Mutterthallus, doch sieht man auch Fälle, für welche dieses zutrifft. " The fact that in some cases, wound isidia show continuity of the cortex, suggests a correlation between this feature and the time of origin of the structures. Wound isidia are usually formed on

older parts of the thallus where the cortical cells have become fully differentiated, so that there is little likelihood of there being cortical continuity. On the other hand, if the near-apical margin of the thallus becomes injured and wound isidia are produced, the young incompletely differentiated cortical cells, or their progenitors, may invest the isidial primordia.

It is thus considered that no real distinction can be made between "true isidia" and "wound isidia" of the Peltigera praetextata type. It is suggested that from the experimental investigations of regeneration growth, wound isidia and true isidia are, in Nature, nearly the varied outcomes of differing chance combinations of certain physiological and environmental factors which it may (in future) prove possible to simulate in non-isidiose species.

There is however, a good case for differentiation, on taxonomic grounds, between the P. praetextata structures and the type of growth obtained on species such as P. horizontalis. As the great majority of isidia of the former arise from obvious centres of injury, this character, although not to be underestimated as a diagnostic feature, should perhaps be treated with some reserve when dealing with specimens bearing only a few scattered marginal isidia.

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SUMMARY OF PART II

1. Growth of discs of Peltigera praetextata in culture has been shown to be improved by the addition of combined nitrogen to the culture solution. It is considered that growth of the phycobiont of the discs is limited in nitrogen-free culture.

2. The optimum concentration of sodium nitrate and of potassium phosphate in Bristol solution, for culture of Peltigera discs at a light intensity of 150 foot candles, has been shown to be 0.25 gm. per litre.

3. The effect, on growth, of the moisture content of discs in culture was investigated and shown to have a strong influence on the type of isidia formed.

4. The growth rate of Peltigera praetextata under greenhouse conditions, as determined by the increase in surface area, was found to be greater than that of discs of the same species in culture. It is indicated however, that the conditions under which the discs have been cultured are close to the optimum.

5. The mechanisms associated with control of the amount of light reaching the phycobiontal layer of the lichen thallus are considered to be important in the regulation of the growth rate of the alga, not merely as protective

mechanisms against excessive insolation.

6. Observation of intact plants and of discs in culture indicates that isidia, which arise on the non-meristematic parts of the thallus, may continue growth after the parent thallus has died off.

7. Microscopic examination of the developmental stages of artificially produced isidia suggests that the "wound stimulus" is, in fact, a light stimulus perceived by the exposed phycobiont cells.

8. "Isidia" have been produced on cultured discs of Peltigera horizontalis, a non-isidiose species. These bear a strong resemblance to isidia of P. praetextata.

9. It is suggested on physiological evidence that no distinction can be made between true isidia, wound isidia and regeneration growth on species of Peltigera on which these may occur naturally, or on which they may be produced artificially. The value of isidia in the erection of P. praetextata as a separate species is not however, disputed.

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PART III

A short study of the phycobiont, isolated in bacteria-free culture, from *Peltigera praetextata*.

INTRODUCTION

The works of BARANETZKY (1867) and of ITZIGSOHN (1868) provide the first illustration that the "gonimia" (as the phycobionts of blue-green lichens were then termed) of Peltigera were identical with a free-living blue-green alga. The phycobiont was identified by Itzigsohn as Polycoccus punctiformis Kütz. This was later included in the genus Nostoc as Nostoc punctiforme (Kütz.) Hariot. Since the time of Baranetzky and Itzigsohn, the Peltigera phycobiont has been variously described as Nostoc lichenoides Vauch., N. peltigerae Let., N. aphaeriorum Vauch. and also as N. punctiforme (Kütz.) Hariot.

Comparatively few lichenologists have made a study of the phycobionts of the blue-green lichens. A good description of the Peltigera phycobiont in culture is given by LINKOLA (1920) who made isolations from seven species, including the cephalodia of P. aphthosa. All isolates were placed under Nostoc punctiforme, but it was recognised that there may be several physiological races or other small systematic units.

Further observations on the Peltigera phycobionts were made by DANILOV (1927). The formation of spores and of oblique divisions in the filament cells were figured.

Danilov accounted for the non-filamentous character of the phycobiont within the thallus as being a condition induced by the lack of space. He was able to reproduce this condition in culture.

The phycobionts of many species of Collema have recently been studied by DEGELIUS (1954) who concludes that the name Nostoc sphaericum Vauch., formerly applied to the phycobionts of the Collemas and Peltigeras, is no longer tenable for all species of Collema. The name Nostoc punctiforme is also rejected in view of the fact that the free-living form is probably only a development stage common to various other species of Nostoc. Thus with the elimination of N. punctiforme, the true identity of the Peltigera phycobiont remains uncertain. Nostoc sphaericum Vauch. is the epithet commonly applied, but it seems that an investigation of the Peltigeras, on lines similar to that by Degelius on the genus Collema, may reveal some differences in the several phycobionts which are sufficient to warrant their description as separate species.

The methods used by the early investigators in the isolation of the phycobionts of lichens have been criticised by many modern workers, and yet the results of their works have frequently been confirmed by the use of more refined techniques. The commonly employed method

of placing thin sections of a lichen thallus on a moist substrate is only at a disadvantage when a pure culture is required from a single cell of the phycobiont. Risk of contamination by epiphytic algae is great, even when individual cells are isolated from the thallus with a micromanipulator. This is especially so in the case of the green lichens.

In the short investigation described in the following pages, no attempt has been made to establish a clone of the phycobiont of Peltigera praetextata. Cultures were required only for investigations concerning germination of the lichen ascospores (Part IV), and thus no special precautions were taken to ensure isolation of a single cell of the phycobiont. The principal characteristics of Nostoc from Peltigera have been described and figured by LINKOLA (1920) and by DANILOV (1927). By way of confirmation however, the stages observed in the growth of the phycobiont in bacteria-free culture have also been outlined.

MATERIAL AND METHODS

Material of Peltigera praetextata for this investigation was obtained from the same sites as that used for experiments described in Part II. In certain instances, isolations were made from the actual specimens used in some of these experiments.

Green algal contaminants, should they appear in cultures of the phycobiont of Peltigera, can be easily distinguished and separated by sub-culturing. Thus there is no necessity to attempt their removal from the lichen thallus before the phycobiont is isolated. It is desirable however, to eliminate epiphytic blue-green algae from the thallus, as these are more difficult to distinguish from the phycobiont. Since the method of BOGUSCH (1944) has been found to be unreliable for the surface sterilisation of Peltigera (Part II), HENRIKSSON's (1951) method for the isolation of Nostoc from Collema tenax was used. Henriksson's lichen material was washed in sterile water, crushed on a slide in a drop of nutrient solution, and the free Nostoc filaments picked up with a micropipette for plating out.

Experience with disc cultures has shown that Bristol solution is adequate for symbiotic growth of the phycobiont of Peltigera praetextata. This medium, solidified with

1% agar, was therefore used for all isolations.

EXPERIMENTAL DETAIL

1. The isolation, in bacteria-free culture, of the phycobiont of Peltigera praetextata.

First experiments were carried out using material freshly gathered from the Dumfries-shire site. A small lobe of the thallus was thoroughly cleaned and all rhizinae removed, then vigorously shaken in several changes of sterile distilled water. The lobe was crushed with a ground-glass pestle in a drop of Bristol solution on a cavity slide, and the free Nostoc cells transferred with a micropipette to fresh Bristol solution. This procedure was repeated five times to ensure a high degree of freedom from contaminants. The final suspension was inoculated to 1% Bristol agar in Petri dishes. The cultures were illuminated with fluorescent lighting, at an intensity of approximately 150 f.c., for ten to twelve hours daily.

After some days in culture, the Nostoc cells began to divide with the production of short filaments of cells of smaller diameter than those in the thallus. Within

two to three weeks these filaments were seen to have migrated over the surface and also through the agar to the bottom of the plates. Sub-cultures were made to fresh Bristol agar from apparently clean parts of the plates, where it had been ascertained that only active hormogonia were present.

It has been shown in Part II that when discs of Peltigera are cultured under excessively moist conditions, or with a high level of nitrogen in the medium, the phycobiontal groups along the edges of the discs grow out of the thallus and spread over the substrate. In addition to overgrowth at the edges of the discs, the phycobiont colonies within the discs also showed a considerable increase in size (Fig. 34). In this condition the isolation of the phycobiont is facilitated since many of the colonies have already changed from the coccoid to the filamentous form.

For isolations from the proliferated colonies in the interior of the discs, the cortex was carefully stripped off with a sterile scalpel to expose the phycobiontal layer. A few of the colonies were lifted off with an inoculating needle to a drop of Bristol solution on a cavity slide, and crushed. Single short filaments were picked up with a micropipette and washed through several changes of Bristol solution before plating out on 1%

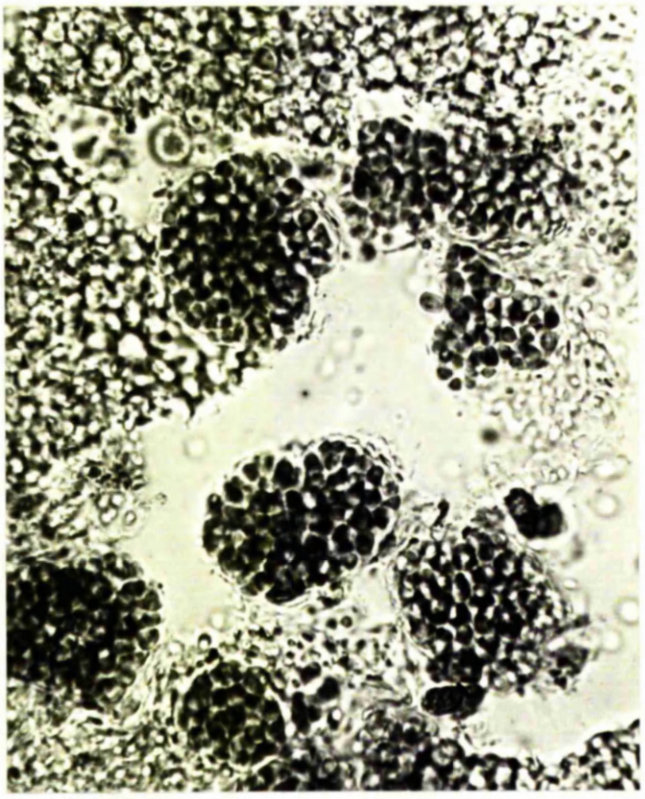

Fig. 34. Colonies of the phycobiont from the interior of a disc of Peltigera praetextata which had been cultured for five weeks in a mineral solution containing 1.7 gm. per litre of sodium nitrate. The colonies in this condition are many times the normal size.

x 400 approx.

1% Bristol agar. By this method of isolation, the probability of contamination of the cultures by epiphytic algae was greatly reduced. Further isolations were made from the phycobiont colonies which had grown out round the edges of the discs.

A total of eight successful isolations of the phycobiont of Peltigera praetextata were made by these three methods. In each case, the isolated alga displayed similar characteristics in culture thus indicating its identity with the phycobiont. Further sub-culturing ensured that any extraneous algae were eliminated.

From one of the cultures so obtained, a bacteria-free culture was achieved by irradiation with ultra violet light. Some of the surface growth of a young culture (in this stage consisting almost entirely of mobile filaments) was removed to Bristol solution in a test tube containing a few glass beads. The tube was shaken for five minutes at high speed on a flask shaker and the resulting fine suspension of Nostoc cells filtered through glass wool (FOGG, 1944) to a second test tube. Filtration was repeated until examination of a small drop of the suspension showed that only single cells of the alga, or filaments of no more than three or four cells, were present. The suspension was then diluted with Bristol solution so that a pipette-size drop contained approximately 100 cells

or filaments, and 3.5 ml. of this were transferred to a 25 ml. quartz flask. The flask was placed, on a shaker, at a distance of twenty inches from a "Hanovia" mercury vapour lamp. During irradiation, the suspension in the flask was gently shaken. At every half-minute of a six-minute irradiation period, a sample was withdrawn with a pipette and one drop inoculated to each of five Petri dishes containing 1% Bristol agar. The inoculum was distributed evenly over the surface with a glass spreader. All the cultures were placed under fluorescent lighting at a mean temperature of 21°C.

Examination of the cultures after three weeks showed that one plate of the 4.5 minute irradiation period appeared to be free of bacteria. This was sub-cultured to the following media to test for purity:

1. Bristol agar with 1% glucose.
2. Potato-dextrose-agar (200 gm. potatoes, 20 gm. dextrose, 20 gm. agar, 1000 ml. distilled water).
3. Peptone-dextrose-yeast extract-agar (bacteriological peptone 0.2%, dextrose 0.5%, yeast extract 0.2%, agar 2%).
4. Malt agar (malt extract 2%, agar 2%).
5. Lemco agar ("Lab" Lemco 10 gm., bacteriological peptone 10 gm., NaCl 5 gm., agar 20 gm., distilled water 1000 ml.).

6. Soluble starch agar (soluble starch 40 gm., Marmite 5 gm., agar 25 gm., distilled water 1000 ml.).
7. Ashby's mannitol-phosphate medium (SALLE, 1948).

No bacterial growth was evident in any of these media, either solidified with agar or in the liquid form. A stock culture for use in experiments to be described in Part IV was started on nitrogen-free Bristol agar slopes. This medium was chosen so that the growth of any chance contaminants arriving through the cotton wool plugs would be discoureged.

2. Characteristics of the phycobiont of Peltigera praetextata in bacteria-free culture.

In the symbiotic state, the phycobiont of Peltigera praetextata, in common with those of the other species of Peltigera, assumes the atypical "coccoid" form (DANILOV, 1927). Characteristic of this condition, which is said to result from the limitation of space within the thallus, is the aggregation of the phycobiont cells into small colonies, each surrounded by a firm gelatinous sheath. There may be up to about twenty round to oval cells in each colony. In no part of the mature thallus, not even in the marginal meristematic regions, is there any

evidence of the filamentous condition of the phycobiont.

It has not yet been established how new phycobiont colonies are formed within the thallus. If the assertion of DANILOV (1927), that the mycobiont draws nourishment from the mucilage secreted by these colonies, is correct, then it is probable that the continued dissolution of the non-living gelatinous sheaths of the marginal colonies by enzyme activity, enables the unrestricted division of the phycobiont cells. At any rate, microscopic examination of the meristematic regions reveals no evidence of the formation of hormogonia.

In contrast, the isolated phycobiont displays the filamentous condition typical of the free-living species of Nostoc. The comparatively high level of nutrients in the medium and the absence of competition for space, induce rapid transverse division of the coccoid Nostoc cells soon after inoculation to the culture medium. The long filaments thus formed (Fig. 35) give rise to either hormogonia or to chains of spores, depending on the conditions of culture. Fig. 36 shows a spore chain formed in an old culture on filter paper.

On media of a soft consistency (e.g. 1% Bristol agar) the first-formed filaments break up into short hormogonia which migrate over the surface of the plate and also completely permeate the agar. From the inoculation centre

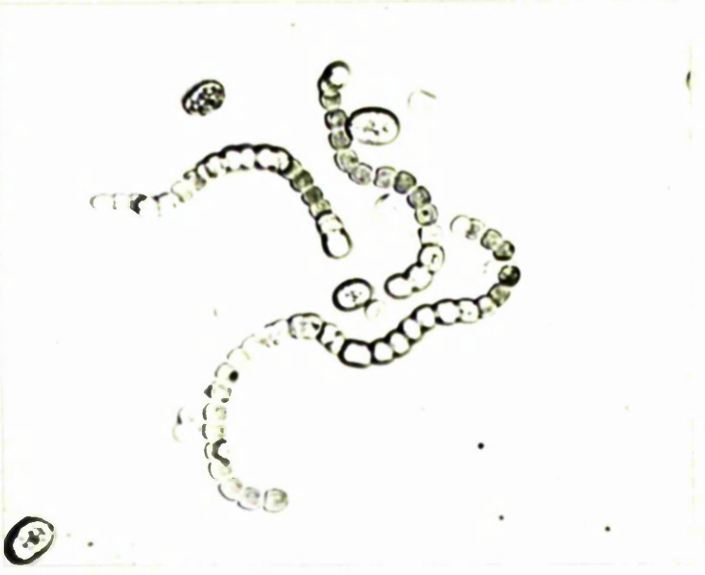

Fig. 35. Vegetative filaments of the phycobiont of Peltigera praetextata. Filaments such as these are formed directly from the coccoid cells isolated from the thallus.

x 600 approx.

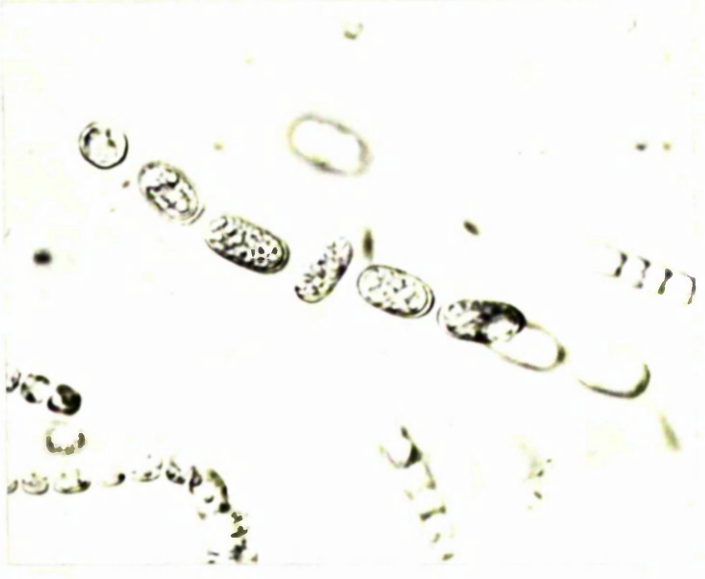

Fig. 36. Spore formation by the phycobiont of Peltigera praetextata. This was induced by establishing a culture of the phycobiont on filter paper supplied with a nutrient solution, then allowing the paper to dry out for a few weeks.

x 800 approx.

outwards, the hormogonia come to rest and the cells proceed to divide in both the transverse and longitudinal planes (Fig. 37). The filament, now incapable of movement, is thrown into folds and, with further new cell formation, colonies of the type illustrated by Fig. 38 are formed. They appear to consist of superimposed transverse rows of cells terminated at either end by a heterocyst. The latter are also frequently to be found in intercalary positions. In this condition there is a marked resemblance to the arrangement of the phycobiont cells in the young isidium (Fig. 27, Part II). The mature colony, reminiscent of the phycobiontal groups in the thallus, is invested by a well-defined gelatinous sheath inside which the Nostoc cells are massed together with no remaining semblance of the filamentous condition (Fig. 39).

Although no investigation was made of the optimum conditions for growth of the isolated phycobiont, it was established that it is capable of heterotrophic growth in the dark, with glucose as the source of energy. Chlorophyll formation is not inhibited but the rate of growth in the dark is not at all comparable with that in cultures on the same medium in the light. The problem to be overcome however, in future investigations of Peltigera praetextata, is not the creation of conditions

Fig. 37. An early stage in colony formation by the isolated phycobiont of Peltigera praetextata. The majority of the cells are in the process of division in the longitudinal plane.

x 800 approx.

Fig. 38. An intermediate stage in colony formation by the isolated phycobiont of Peltigera praetextata. The apparently polystichous condition is brought about by longitudinal divisions in the filament giving rise to transverse rows of cells.

x 800 approx.

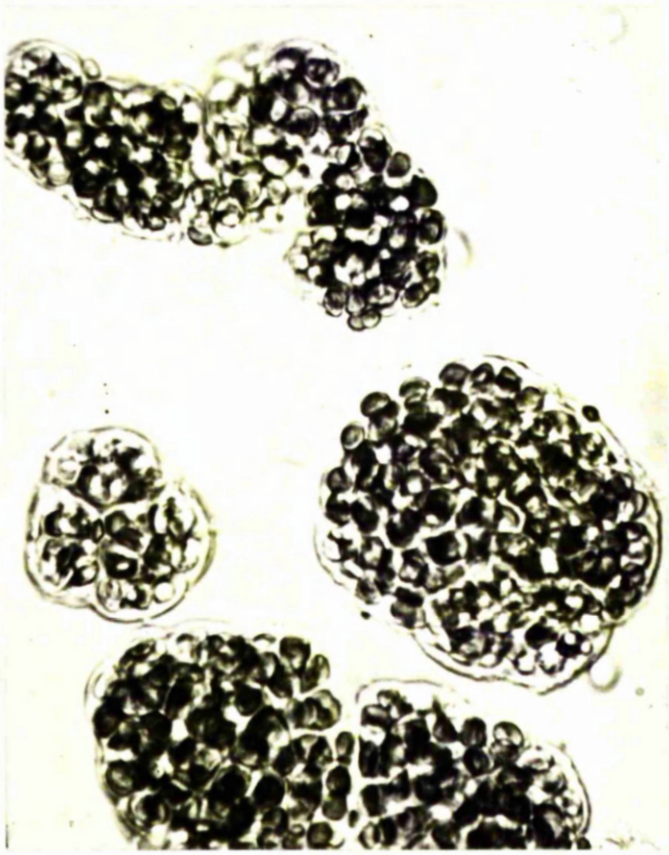

Fig. 39. Mature colonies of the isolated phycobiont of Peltigera praetextata. The filamentous condition is now totally obscured. Cell dimensions: 6.2-8.9 x 4.5-7.1 microns.

x 800 approx.

for optimal growth. Rather is it the realisation of conditions which will maintain the phycobiont at a rate of growth suitable for the re-establishment of the symbiosis from the isolated symbionts.

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SUMMARY OF PART III

1. The phycobiont (*Nostoc* sp.) of *Peltigera praetextata* has been isolated in pure culture, using three different methods.
2. A bacteria-free culture of the phycobiont was obtained by irradiation of a suspension of the alga with ultra violet light.
3. Stages in the development of the phycobiont in culture have been outlined. An apparently polystichous phase in colony formation is brought about by longitudinal division of the cells of the original filaments.
4. The phycobiont is capable of heterotrophic growth in the dark, with glucose as the source of energy. The formation of chlorophyll is not inhibited.

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PART IV

A study of the germination and further growth of ascospores of *Peltigera praetextata*.

INTRODUCTION

Throughout the numerous works on the isolation, in pure culture, of the mycobionts of lichens, few references have been made to the genus Peltigera. The reason for this is perhaps to be found in the statement by THOMAS (1939) which (translated) reads, " In spite of much research with different Peltigeromyces-species I had as little success as Werner in culturing a strain of this genus. The spores germinate in nutrient solution; on agar they perish."

The initial stages in germination of spores of Peltigera canina, P. horizontalis and P. polydactyla were described by TULASNE (1852). WERNER (1927), working with spores of three species of Peltigera, failed to obtain germ tubes longer than 110 microns. More recently THOMAS (l.c.) has made a study of spore ejection and germination in Peltigera. The conclusions of these authors may be summarised as follows:

1. Moist apothecia lose the ability to shed spores at temperatures of 30°C. and above.
2. Apothecia collected in December showed copious spore-shedding five hours after moistening. (No reference is made to viability of the spores.)

3. The optimum temperature for germination of spores of Peltigera horizontalis is 18°C. At temperatures of 24°C. and above, spores were destroyed.

4. In soil solution, spores of Peltigera produced germ tubes from the two terminal cells. In a proportion of the germ tubes, a terminal swelling was formed from which growth continued.

5. The maximum length of germ tube observed was 110 microns.

6. Viable spores were obtained from apothecia which had remained dry for several months.

It is of interest to note that the mycobionts of only three species of blue-green lichens have been successfully obtained in isolated culture. That of Collema pulposum was isolated by WERNER (1930), and DEGELIUS (1954) has cultured the mycobionts of Collema polycarpon and of C. subnigriscens in the presence of "special bacteria". In pure culture, spore germination and further growth were negligible. RYPÁČEK and TRTÍLKOVA-HELFERTOVÁ (1959) claim to have isolated two separate mycobionts from Peltigera aphthosa using Waren's solution. They also state that two were isolated from each of the species of the Cladonia coccifera group. These results are of little value in view of their statement that, " In the darkness and in the milieu rich in sugar, the Lichen

thallus produces already after 3 days a thick shaggy coating of the hyphes of the Lichen Fungus." There can be little doubt that one, at least, of the isolates was nothing more than a surface contaminant.

Considerable attention has been paid, on the other hand, to isolation of the mycobionts of the green lichens. In recent years this work has received a stimulus by the discovery that certain lichen substances exhibit antibiotic properties (BURKHOLDER et al., 1944).

It is unfortunate that the genus Peltigera, which has received much attention from systematist and physiologist, should have been practically by-passed by the many investigators who have turned their efforts to the isolation of the mycobionts of lichens. This is to be the more deplored in view of the ease with which Peltigera can be cultured under controlled conditions, and in view of its relatively fast growth rate and property of nitrogen fixation demonstrated in the foregoing chapters.

The following investigations were initiated in an attempt to repair this omission, and with the ultimate aim of synthesis of Peltigera from the two symbionts. It became apparent at an early stage however, that the statements of earlier workers in this field were amply justified. The investigations were continued in the hope that some information would be obtained about the complex

of factors influencing ascospore germination and further growth of the germ tubes.

MATERIAL AND METHODS

Apothecia of Peltigera praetextata and of Peltigera horizontalis were obtained from the following sites:

1. A disused limestone quarry on Springkell Estate in Dumfries-shire.
2. Fin Glen in the Campsie Hills, Stirlingshire.
3. Gonachan Glen near Fintry, Stirlingshire.
4. The Lake Bala district, North Wales.

In each case, entire plants were collected and kept until required for use, either planted out in glass-covered flower pots in the greenhouse, or dried in cardboard boxes. Some of the latter were stored at room temperature; the apothecia were removed from others and these stored at 3°C. in a refrigerator.

When required for use, an apothecium which appeared to be mature was removed from the thallus, with a small piece of the latter still attached, and immersed in distilled water for twenty to thirty minutes. For freshly gathered apothecia, this was found to be the

maximum permissible time of immersion as, in some cases, spore ejection started whilst the apothecium was still immersed in the distilled water. On the other hand, apothecia which had been stored dry for some time, frequently required a longer period of immersion.

Two principal methods were used for the inoculation of culture media with spores:

1. The moistened apothecium was placed on a pile of small filter paper discs (or other absorbent material) on a glass plate. The filter papers were saturated with distilled water to prevent the apothecium from drying out during the inoculation of the cultures. Spores were ejected upwards to agar media contained in Petri dishes inverted over the glass plate.

2. The apothecium was secured with vaseline to a glass plate and a drop of distilled water placed between the under surface of the apothecium and the glass. The plate was inverted over the media for downward ejection of the spores.

Both methods showed points of advantage and either was used according to the requirements of a particular experiment. A comparison of the attributes of the two methods is given below.

Method 1	Method 2
Can be used only for agar or silica-gel media.	Can be used for liquid media.
Only those spores which are ejected sufficiently high and in the correct direction are inoculated to the medium;	All spores ejected from the apothecium are inoculated to the medium.
There is less risk of contamination of the medium with bacteria or fungi.	The drop of distilled water placed beneath the apothecium frequently spreads to the upper (morphologically) surface thus trapping the spores.
The progress of spore ejection can be followed under the microscope.	

Diverse culture methods were used according to the nature of the media. Those which were in plentiful supply were contained in Petri dishes, but when only small quantities of medium were available, large drops were spread on glass plates supported by glass rod triangles in Petri dishes. The space below each plate was filled with distilled water to minimise drying out of the medium. For liquid cultures of the latter type, a ring of molten paraffin wax was pipetted to each plate

and the drop of medium placed inside the ring after it had solidified. Spreading of the medium over the glass plate was thus prevented.

Soil bacteria present in lichen apothecia are frequently ejected with the ascospores to the culture medium. No attempt was made in this investigation, to employ only spores which had been shown to be free from bacteria.

It was clearly impossible to predict the number of spores (if any) which would be ejected from an apothecium. Thus, for each experiment, a number of apothecia were moistened and that which showed the highest rate of ejection was selected for use. This criterion of selection however, was by no means infallible. In many instances a high initial rate of ejection was followed by a rapid decline before all cultures of a particular experiment had been inoculated. This entailed frequent repetition of many of the experiments in order to obtain reasonably satisfactory results.

Throughout this investigation, cultures were incubated for six days at 20°C. unless otherwise stated. These were examined under the low power of the microscope with a ruled coverslip inserted in the eyepiece to facilitate the counting of spores. Wherever possible, in the

assessment of per cent germination, or of other characteristic under investigation, counts were made of a minimum of 200 spores in a number of microscope fields selected at random. Spores were considered to have germinated even when only slight terminal swellings could be observed.

All media and distilled water were sterilised, except when otherwise stated, by autoclaving at 15 lbs. pressure for twenty minutes. Glassware was sterilised by dry heat at 160°C, for one to two hours.

EXPERIMENTAL DETAIL

1. An investigation of the suitability of various media for germination of ascospores of Peltigera praetextata.

As already indicated, THOMAS (1939) obtained germination of spores of Peltigera sp. in a soil solution; RYPÁČEK and TRTÍLKOVA-HELFERTOVÁ (1939) claim to have isolated two mycobionts from Peltigera aphthosa using Warén's solution (WARÉN, 1918-19), and WERNER (1927) used various synthetic media containing carbohydrates for germination of the spores of Peltigera canina. Preliminary

attempts to secure germination of the spores of Peltigera praetextata on the type of medium used by the above authors were beset with failure. Numerous other media (listed below) were also tried, in each case as a liquid medium and also solidified with 1% or 2% agar.

1. Malt extract (2%).
2. Malt extract-dextrose-yeast extract (malt extract 2%, dextrose 1%, yeast extract 0.2%).
3. Peptone-dextrose-yeast extract (bacteriological peptone 0.2%, dextrose 0.5%, yeast extract 0.2%).
4. Knop solution.
5. Czapek-Dox solution.
6. Beijerinck solution.
7. Bristol solution, modified (HENRIKSSON, 1951).
8. Aqueous extract of Peltigera praetextata (100 gm./l.).
9. Soil extract (500 gm./l.).
10. Tap water.

Germination, with the production of short unbranched germ tubes, was observed only in tap water.

A later observation showed that spores would germinate if inoculated to cultures of Nostoc (on Bristol agar) isolated from Peltigera praetextata. With this medium serving as a control for viability of the spores, all the media which had previously failed to support germination were re-tested. As before, none was found to

be suitable.

Further experiments using Nostoc cultures from which the algal growth had been removed, established the fact that germination took place on the residual agar (Fig. 40). In two instances, 90% and 93% germination was observed. Spores did not however, germinate on fresh Bristol agar control cultures. This seemed to indicate that some requirement for germination was excreted into the medium by the Nostoc. An aqueous extract of the Nostoc growth on Bristol agar was thus prepared and subsequently tested for ability to support germination. Several experiments were made with this extract in the liquid form and also solidified with 2% agar. In three of these, figures of 22%, 82% and 86% germination were recorded. Further tests were made in which some of the extract was sterilised by Seitz filtration and some by autoclaving at 15 lbs. pressure for twenty minutes. In the former case, the mean per cent germination (four cultures) was 55%, and in the latter, 49%. The requirement for germination was thus shown to be heat stable.

For further investigations, Nostoc extract was prepared according to a standard method, as follows:
A number of Petri dishes containing Bristol agar were inoculated with a heavy suspension of a bacteria-free culture of Nostoc isolated from Peltigera praetextata

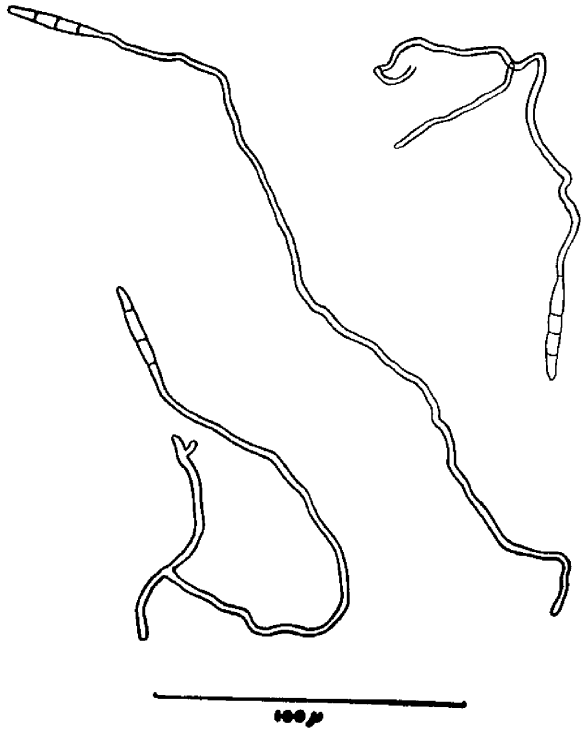

Fig. 40. Camera lucida drawing of spores of Peltigera praetextata cultured on the residual Bristol agar of a Nostoc culture.

as detailed in Part III. The suspension was prepared by adding about 0.1 gm. fresh weight of Nostoc to 10 ml. Bristol solution and shaking vigorously with a few sterile glass beads to break up the aggregates of filaments. A few drops of the suspension were pipetted to each plate and spread over the surface with a glass rod. The cultures were illuminated under fluorescent lighting for ten to twelve hours daily. After eight weeks under these conditions, the Nostoc growth, which had now thickly covered the surface of the agar, was scraped off with a large wire loop. Five gm. fresh weight of the Nostoc were suspended in 100 ml. distilled water for a few hours. The suspension was frozen, quickly thawed and Buchner filtered. The clear liquid was autoclaved at 15 lbs. pressure for twenty minutes, and stored at low temperature until required for use. Extracts prepared by this method were designated N.E.1.

2. Determination of the optimum concentration of Nostoc extract (N.E.1) for germination of spores of Peltigera praetextata.

A number of experiments, using N.E.1 solidified with 1% agar, indicated that higher proportions of spores germinated in diluted extract than in the undiluted extract.

Table 20 shows the combined results of three of these. A different apothecium was used to inoculate the cultures of each experiment. A more carefully controlled experiment was then set up to determine the most suitable strength of extract. In this case, ten dilutions ranging from x 1 to x 0.001 strength were used. Equal amounts of each dilution were transferred to Petri dishes and spore inoculations made from two apothecia. From one apothecium, dilutions of strengths 1 to 0.1 were inoculated, and from the other, dilutions of strengths 0.1 to 0.001. After six days the cultures were examined for spore germination, three counts of 100 spores being made for each. The data for mean per cent germination are shown in Table 21.

Table 20. Per cent germination of spores of Peltigera praetextata in three strengths of Nostoc extract (N.E.1).

Extract strength	Experiment					
	1		2		3	
1	0	0	40		1	0
0.5	0	0.5	69		0	0
0.25	7	2.5	68		5	4

Table 21. Mean per cent germination of spores of *Peltigera praetextata* in ten graded strengths of N.E.1.

Apo. No.	Extract strength									
	1	.5	.25	.1	.05	.02	.01	.005	.002	.001
1	0	6	32	53	-	-	-	-	-	-
2	-	-	-	75	48	28	9	6	2	3

From these data, the one-in-ten dilution appeared to be the optimum strength, i.e. 5 gm. fresh weight of *Nostoc* per litre. This strength was therefore taken as the most suitable for further investigations, and was designated N.E.2.

3. The effect of temperature on the germination of spores of *Peltigera praetextata* in *Nostoc* extract (N.E.2.).

THOMAS (1939) states that the optimum temperature for germination of spores of *Peltigera horizontalis* is 18°C., and that above 24°C. the spores fail to germinate. It seemed unlikely that there would be any significant difference in the case of spores of *P. praetextata*, but

two experiments were set up to establish comparable data for this species. Nostoc extract (N.E.2) was used in both experiments. The spores for each experiment were obtained from different apothecia. Cultures were incubated at temperatures of 6, 20, 26 and 30°C., and examined for germination after seven days. Table 22 shows the combined results of the two experiments.

Table 22. Mean per cent germination of spores of Peltigera proctexiata in N.E.2, at four temperatures.

Expt.	Temperature °C.			
	6	20	26	30
1	0	65	18	0
2	30	41	24	0

The optimum of the four temperatures is seen to be 20°C. Above this temperature there is a decrease in the per cent germination as noted by Thomas for Peltigera horizontalis. No facilities were available for temperatures within the range 6 to 20°C., so that it was not possible to establish the exact optimum. Such data

as have been obtained however, suggest a close agreement with the figure quoted by Thomas.

In these two experiments, as in all others, a spore was considered to have germinated even if only a small swelling was visible at either end. Thus, although at 20°C., germ tubes attained lengths of up to 70 microns, all germinated spores at 6°C. and at 26°C showed only very minute terminal swellings. The data of Table 22 also serve to show the variation in viability of spores from different apothecia.

4. A study of the factors causing rupture of the spores of Peltigera praetextata on various culture media.

During the investigation of the suitability of media for spore germination it was noticed that in several of the liquid media, and on all the agar media used, varying proportions of the spores showed rupture of one or both of the central cells. Many of the spores which had germinated were also seen to have ruptured. Fig. 41 shows stages in the disintegration of the spores on Bristol agar. Fig. 42 shows normal ungerminated spores. Progressive distension of the cells takes place, followed by breakage of the cell wall; the cell contents, rich in oil granules, become scattered over the surface of the

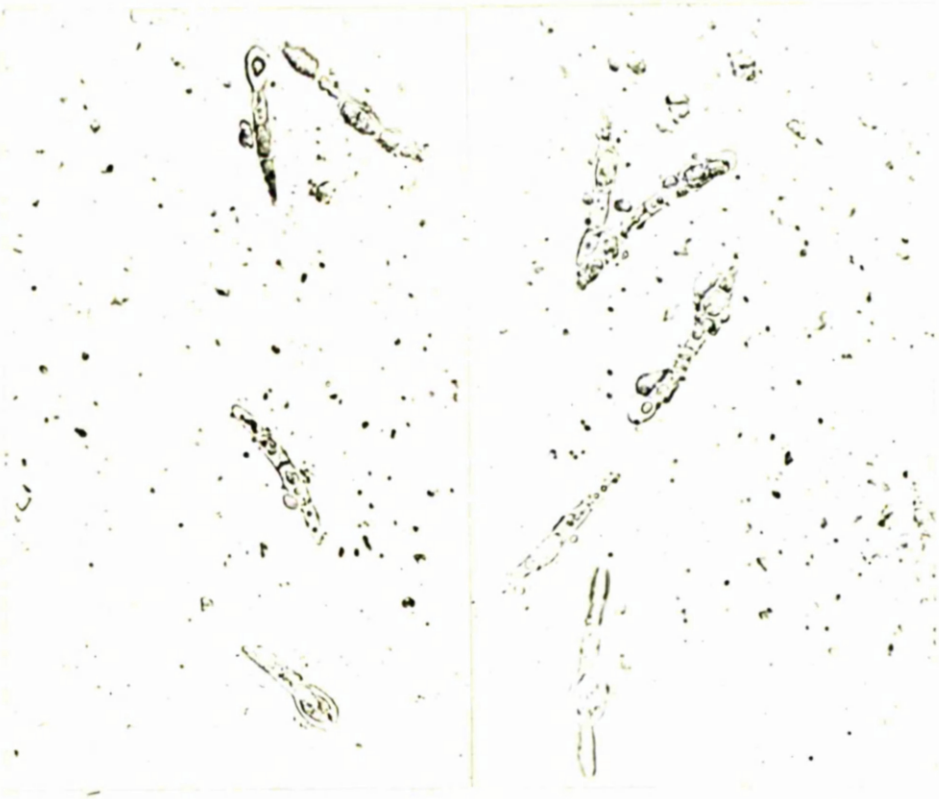

Fig. 41. Progressive stages in the rupture of spores of Peltigera praetextata on 1% Bristol agar. The spore contents can be seen scattered over the surface of the medium.

x 470

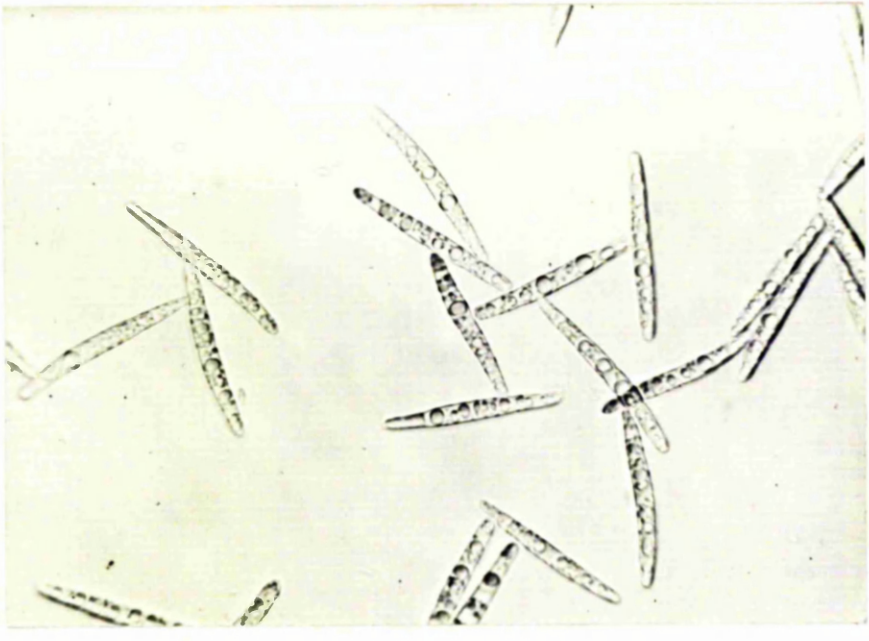

Fig. 42. Ungerminated spores of Peltigera praetextata, soon after discharge from the apothecium.

x 470

medium. THOMAS (1939) refers to this condition in the spores of Physcia stellaris on 4% malt agar as follows (translated), " Their contents are granular, the transverse walls distinct; they appear overnourished. Even the spore takes up nutrients, becomes thicker and bursts open." It is presumed that such a condition was also observed by Thomas in spores of Peltigera on agar media - " On agar they (the spores) perish."

Numerous authors (see QUISPEL, 1943-45 and DES ABBAYES, 1951) have isolated the mycobionts of green lichens, either from ascospores or from thallus fragments, on media solidified with agar. It is evident from these works and from my own observations that there is a marked difference between the spores of the green lichens and those of Peltigera regarding their tolerance of the conditions in culture media. Experiments previously described have shown that media containing agar are unsuitable for germination of Peltigera spores. It seemed however, that spore rupture was not occasioned solely by excessive richness of the medium, as was suggested by THOMAS (l.c.). An investigation into the causes of spore rupture in various media was thus undertaken.

Apothecia used were collected on 3 January 1955. At the time of collection and for some days previously, the ground was hard frozen. No rain or snow had fallen for

several days. There was thus a good chance that the apothecia contained a large number of mature asci. The apothecia, each with a small piece of attached thallus, were stored in darkness at 3°C. in a refrigerator until required for use. The first experiment was started on 6 February 1955, the second on 23 February, i.e. five and seven weeks after collection of the apothecia. Experiment 1 comprised 128 cultures, of which 83 were inoculated with spores from one apothecium, 23 from a second and 22 from a third. The following media were used:

Apothecium 1.

Aqueous solutions of glucose and sodium nitrate, in different proportions to give a series of seven C:N ratios (Table 31). Inoculations were also made to these media solidified with 2% agar.

N.E.1, 2% agar.

N.E.1, 2% agar, at dilutions of x1, 0.5, 0.25 and 0.125.

N.E.2, at dilutions as above.

2% malt extract.

2% malt extract agar.

Bristol solution.

2% Bristol agar.

Nostoc culture on Bristol agar.

Distilled water.

Apothecium 2.

N.E.1, 2% agar.

N.E.2, 2% agar, at dilutions of x1, 0.5, 0.25 and 0.125.

Tap water.

2% tap water agar.

2% Bristol agar.

Nostoc culture on Bristol agar.

Apothecium 3.

N.E.1, 2% agar.

N.E.1, 2% agar, at dilutions as above.

N.E.2, 2% agar, at dilutions as above.

2% Bristol agar.

Spores from the three apothecia were inoculated to the various media in a random sequence. Plates containing N.E.1 were inoculated at regular intervals throughout the ejection period of apothecium No.1. All the cultures, with the exception of those in distilled water, were incubated for seven days at 20°C. Two plates of each of the latter were incubated at 20, 26 and 30°C.

In the second experiment, 120 cultures were inoculated with spores from one apothecium. This experiment was planned as a repeat of the first but, owing to the failure of two of the apothecia to eject spores, some of the media were omitted. In addition, spores were ejected to the under surface of the agar of Nostoc cultures. The

latter, together with plates of N.E.1, were inoculated at regular intervals throughout the ejection period. All cultures were incubated at 20°C.

Data obtained regarding spore ejection.

Table 23 shows the respective times required for ejection to start, and the total periods during which ejection continued.

Table 23. Spore ejection from apothecia of Peltigera praetextata. Time required for ejection to commence and total period of continuous ejection.

Apo. No.	Time removed from water	Time spore ejection started	Time ceased	Total period of continuous ejection
1	9.54 a.m.	11.15 a.m.	3.03 p.m.	3h. 48m.
2	10.05 a.m.	11.25 a.m.	3.32 p.m.	4h. 7m.
3.	10.15 a.m.	12.10.p.m.	3.08 p.m.	2h. 58m.
4[*]	10.20 a.m.	12.10.p.m.	9.07 p.m.	8h. 57m.

[*] The apothecium used for the second experiment.

Since the four apothecia were collected from the same area at the same time, and kept under similar conditions, it is concluded that the differences in the ejection periods were due to variation in the state of maturity of the apothecia. That used for the second experiment was probably fully mature with a full spore complement. Considerable variation was also detected in the rate of spore ejection. Table 24 shows the number of cultures inoculated by each apothecium, and the range of time in minutes required for the inoculation of each with, as far as possible, a minimum of 200 spores.

Details of the number of spores ejected to each culture were recorded only for Experiment No. 2. Table 25 shows the approximate numbers of spores counted on various cultures throughout the period of ejection. No accurate estimate can be formed of the total number of spores ejected but, if the mean of the fourteen values in Table 25 is taken as the mean number ejected to each of the 120 cultures, it appears that some 30,000 spores have been ejected in about nine hours from one apothecium. It is thus indicated that there were some 3,500 to 4,000 mature asci in the apothecium.

Table 24. Rate of spore ejection from apothecia of *Peltigera praetextata*.

Apo. No.	Number of cultures inoculated	Range in time required for inoculation of 200 spores	Mean time required for inoculation of 200 spores
1	83	2- 8 min.	2.5 min.
2	23	5-16 min.	9.0 min.
3	22	5-14 min.	8.0 min.
4	120	2- 5 min.	2.5 min.

Table 25. Spore ejection rate at various intervals during the ejection period of an apothecium of *Peltigera praetextata*.

Ejection number	Approximate number of spores ejected	Time required for ejection min.
1st.	150	5
2nd.	200	5
3rd.	300	5
4th.	200	4
5th.	300	4
7th.	200	3
15th.	200	3
29th.	300	3
40th.	400	3
50th.	250	2
60th.	650	3
84th.	200	2
119th.	60	3
120th.	50	4

Data obtained regarding spore germination.

Spore germination was observed on the residual agar of the Nostoc cultures and on N.E.1. On the majority of the other media, less than 5% germination was recorded. Observations on spore rupture were thus unhampered by the occurrence of germination. It was established however, that the spores from the four apothecia were viable, the highest value being 93% germination.

Data obtained regarding spore rupture.

I Comparison of the degree of spore rupture in liquid media and on 2% agar media.

The fate of spores on Bristol solution, malt extract and on tap water agar serves to illustrate the effect of agar upon spore rupture. Data for per cent rupture of spores on these media, and in the same media without agar, are shown in Table 26. Although there is some variation according to the apothecium used, it is evident that the addition of 2% agar to Bristol solution and to tap water has caused a marked increase in disintegration of the spores. Since the liquid malt extract is also active in this respect, it is concluded that spore rupture is not produced by any purely physical characteristic of the agar, but rather by the presence of some metabolite.

Table 26. Per cent rupture of spores of Peltigera praetextata in media with and without agar.

Medium	Liquid				2% agar			
	Apothecium No.				Apothecium No.			
	1	2	3	4	1	2	3	4
Bristol solution	0	-	-	0	73	9	84	94
2% malt extract	84	-	-	90	97	-	-	91
Tap water	-	7	-	-	-	43	-	98

II Spore rupture related to the concentration of Nostoc extract (N.E.1).

The data for mean per cent spore rupture on the 54 Nostoc extract 2% agar cultures, together with the 16 liquid extract cultures, are set out in Table 27. Again there is seen to be considerable variation in the results for different apothecia, but a fairly constant relation is evident between extract strength and spore rupture. Comparison of the per cent rupture on the solid and in the liquid extract shows, as was deduced from the data

of Table 26, that the addition of agar has a marked effect on the degree of spore rupture.

Table 27. Mean per cent rupture of spores of Peltigera praetextata in four strengths of liquid Nostoc extract, and on the extract with 2% agar.

Extract strength	Liquid		2% agar			
	Apothecium No.					
	1	4	1	2	3	4
x 1	15	22	96	97	97	95[*]
x 0.5	5	27	94	75	97	95
x 0.25	0	7	79	78	86	83
x 0.125	0	4	51	38	37	60

[*] During inoculation of one of the four cultures of this strength, the apothecium was in contact with the medium. On examination of this culture, spore rupture was seen to be totally absent.

It has been noted that, during inoculation of one of the cultures with spores, the apothecium was in contact with the medium, and that none of the spores became ruptured. Examination of the culture after inoculation

showed that more than a thousand spores were present. Additional experiments have shown that when an apothecium becomes saturated, a thin water film appears on the hymenial surface. This has been observed to trap large numbers of spores ejected from the asci. It is thought that spores thus trapped would become fully turgid* and that, when released from the water film by physical contact with the medium, only a comparatively minor uptake of nutrients would take place, hence the absence of rupture.

III Spore rupture related to the time of ejection from the apothecium.

Spores were inoculated to N.E.1 from two of the four apothecia at various intervals during their periods of ejection to detect any physiological changes which might occur in the spores as the ejection periods continued. The data of the two experiments relating to this aspect of spore rupture are collected in Table 28. These data suggest that the spores of the two apothecia differed in

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* It has been observed, by examination of spores ejected to dry glass slides, that ejection from an apothecium commences some time before the spores are fully turgid.

some attribute which is reflected in the difference in susceptibility to rupture on the same medium.

Table 28. Variation in per cent rupture of spores of Peltigera praetextata, in N.E.1, with the time of ejection from the apothecium.

Ejection No.	Per cent rupture	
	Apothecium No.	
	1	4
4th.	40	98
11	29	-
12	-	99
18	-	-
28	32	-
29	-	100
40	24	100
49	25	-
50	-	99
59	32	-
60	-	100
74	-	99
81	2	-
82	1	-
84	-	100
97	-	64[M]
113	-	100
119	-	100

[M] The apothecium was in contact with the medium during this inoculation.

It is of interest to note the fall in per cent rupture of spores from the first apothecium while those of the second show, even at the end of the ejection period, complete rupture. At the 97th. ejection, the second apothecium was in contact with the medium. As in the previous case the smaller degree of rupture, here not so marked, is attributed to the higher turgidity of the spores which were inoculated to the medium from the water film over the apothecium.

These data suggest that, of the many spores ejected from apothecia in their natural habitats, a large proportion of those discharged soon after the apothecia become moistened by rain will possess the same capacity to rupture as found in these experiments. There will thus be small chance of these spores surviving.

IV The increase in per cent rupture of spores with time in culture.

It has been shown, for spores on Nostoc extract with 2% agar, that the degree of rupture is related to the concentration of the extract. The data of Table 27 were obtained from spore counts made after seven days' culture at 20°C. Some of these cultures were further examined after ten days. The two sets of data are brought together

for comparison in Table 29.

Table 29. Per cent rupture of spores of Peltigera praetextata after seven and ten days on four strengths of Nostoc extract 2% agar.

Extract strength	Seven days		Ten days	
	Apothecium No.			
	2	3	2	3
x 1	97	97	91	99
x 0.5	75	97	95	100
x 0.25	78	86	91	99
x 0.125	38	37	86	82

These data indicate that, in addition to the strength of the medium, the time of exposure to the medium also determines the amount of rupture of the spores. This suggests that the rupture-inducing substance in the agar enters the spore cells by a method other than by purely physical diffusion.

V Evidence for the diffusion through agar of the germination requirement of the spores.

Two Nostoc plates were inoculated with spores at the end of the ejection periods of the first and second apothecia. Three similar plates were inoculated by the fourth but in this case the spores were ejected to the under surface of the agar. Details of per cent germination and rupture of the spores on these media are collected in Table 30.

Table 30. Per cent germination and rupture of spores of Peltigera praetextata on the upper and lower surfaces of the agar of Nostoc cultures.

Agar Surface	Apothecium No.	Ejection No.	Per cent germination	Per cent rupture
Upper	1	83	93	0
"	2	23	90	0
Lower	4	11	25	Few
"	"	41	20	spores
"	"	106	10	ruptured

Germination of the spores on both surfaces indicates that the requirement for germination has diffused through the agar during the growth of the Nostoc. The low per cent germination of spores from the fourth apothecium is attributed to the lower concentration of this substance at the under surface than on the surface on which the alga was growing. Reference to page 117 shows that spores did not germinate on Bristol agar alone, and Table 26 shows that 94% of the spores ejected from apothecium No.4 disintegrated on the same medium. No exact record was made of the per cent rupture of the spores on the inverted Nostoc cultures, but it was evident that this was much less than 94% (less than 50%). It is suggested that this low figure for spore rupture on the under surface, and the absence of rupture on the upper surface of the cultures, is an indication of the interaction between the rupture inducing substance in the agar and the factor associated with germination excreted from the Nostoc growth.

VI The Carbon:Nitrogen ratio and spore rupture.

BROWN and HORNE (1926) state that the presence of swollen cells in the spores of Fusarium sp. is an indication of a high nitrogen content of the medium on

which the sporing mycelium is grown. It is explained that the C:N ratio is of importance in determining the form of the spores. It was thought that the C:N ratio may also be a factor concerned in the rupture of spores of Peltigera after ejection and, to test this hypothesis, spore cultures were set up using duplicate samples of the nine media listed in Table 31. Spores were also inoculated to these media solidified with 2% agar. The data of these tests are shown in Table 32.

Table 31. Composition of glucose-sodium nitrate media used to test the effect of the C:N ratio on rupture of spores of Peltigera praetextata.

Medium No.	Carbon (as glucose) (gm./l.)	Nitrogen (as $NaNO_3$) (gm./l.)	C : N ratio	Osmotic pressure (atmos.)
1	0.4	0.4	1	1.5
2	2.0	2.0	1	7.5
3	4.0	4.0	1	15.0
4	0.0	0.4	0	1.5
5	0.4	2.0	0.2	7.0
6	2.0	4.0	0.5	14.5
7	0.4	0.0	inf.	0.1
8	2.0	0.4	5	2.0
9	4.0	2.0	2	8.0

Table 32. Mean per cent rupture of spores of Peltigera praetextata on media of different C:N ratios.

Medium No.	C : N ratio	Liquid		2% agar	
		Apothecium No. 1	4	1	4
1	1	59	84	100	96
2	1	0	1	95	98
3	1	2	1	69	53
4	0	24	20	78	99
5	0.2	1	2	99	100
6	0.5	2	0	87	56
7	inf.	83	79	100	100
8	5	68	70	100	99
9	2	5	3	94	92

On liquid media 2, 3, 5, 6 and 9 there is seen to be less than 5% rupture of the spores, but examination of the respective C:N ratios reveals no obvious correlation between the two. It is to be noted however, that the nitrogen content of these five media is equal to, or greater than, 2 gm. per litre. This suggests that rupture of the spores is associated with endosmosis. If

it is assumed that the sodium nitrate was completely dissociated in solution, the osmotic pressures of the nine media will have the approximate values shown in Table 31. Spore rupture is thus seen to occur in media with an osmotic pressure of less than approximately seven atmospheres. This relationship does not however, explain the high per cent rupture obtained on the addition of 2% agar to the media. It is also evident that there is some other factor associated with uptake of one or other of the two constituents of these media. One would expect, if spore rupture were a feature caused solely by endosmosis, that complete disintegration would take place in distilled water. That this is not so however, is shown by the data obtained from spore cultures at three temperatures (see p. 126). Even after seven days at 30°C. these spores showed no signs of rupture. This suggests that the concentration of nutrients in the medium, and thus the osmotic pressure, are indirect causes of spore rupture. The primary cause is evidently some substance which increases the uptake of nutrients by the spores.

To confirm the observations made regarding spore rupture in the glucose-sodium nitrate solutions, a further test was carried out using a new method of inoculation. Despite the precautions taken in the previous experiments to ensure that the various solutions were inoculated with

a random sample of spores, it was felt that some possible differences in spore condition may have influenced the results for some of the media.

For this second test, an apothecium was moistened and fixed to the lid of a small Petri dish. Spores were collected for a number of hours in a small volume of distilled water. The spore collection was transferred to a 20 ml. centrifuge tube and centrifuged at 3,500 revs. per minute for one minute. The supernatant was drawn off and the remaining heavy spore suspension divided amongst nine centrifuge tubes so that each contained two drops of the suspension. These tubes were filled to near-capacity, each with one of the glucose-sodium nitrate solutions, and centrifuged as before. The supernatants were drawn off and each tube again filled with the appropriate solution and centrifuged. Part only of the final supernatants was drawn off so leaving sufficient in each tube for two drop cultures. By this method, the original distilled water suspension was replaced by suspensions of each of the nine glucose-sodium nitrate solutions. Any small trace of the two drops of distilled water left after double centrifugation was considered to have no effect on the concentration of the solutions. The data obtained from this test are shown in Table 33.

This experiment confirms the relation between osmotic pressure and spore rupture. Although the per cent rupture of spores in media 2, 3, 5, 6 and 2 is higher than in the previous experiment, the increase in spore rupture with decrease in the osmotic pressure is not obscured.

Table 33. Mean per cent rupture of spores of Peltigera praetextata in media of different C:N ratios.

Medium No.	C : N ratio	Osmotic pressure (atmos.)	Mean per cent rupture of spores
1	1	1.5	82
2	1	7.5	17
3	1	15.0	6
4	0	1.5	71
5	0.2	7.0	13
6	0.5	14.5	7
7	inf.	0.1	51
8	5	2.0	53
9	2	8.0	24

5. Further experiments relating to the effect of agar on spore rupture

Two experiments were set up to observe the variation, in per cent rupture and germination, between spores ejected to the surface of Nostoc extract (N.E.2) with 2% agar and spores suspended in the same medium. In each experiment, the suspensions in the agar were made by ejecting spores to known volumes of distilled water and then adding equal volumes of cooled Nostoc extract agar of double strength. The eight cultures of both experiments were incubated at room temperature for twelve days. The data for spore germination and rupture are shown in Table 34.

Table 34. Mean per cent germination and rupture of spores of Peltigera praetextata on the surface of Nostoc extract agar, and of spores suspended in the same medium.

Culture type	Per cent germination		Per cent rupture	
	Apothecium No. 1	2	1	2
Surface	0	0	100	100
Suspended	2	46	Few spores ruptured	

Spore germination, associated with a low per cent rupture, has taken place in the agar but not on the surface. Some interference has evidently occurred in the operation of the factor governing spore rupture. This may be due to lack of oxygen and suggests a possible link with the respiratory system of the spores.

In the investigations so far described, unpurified agar was used. It appeared probable however, that the substance causing spore rupture could be removed from the agar by chemical means. ROBBINS (1939) gives a method for the purification of agar by treatment with pyridine. This method was slightly modified for the present investigation in that the agar was treated with a 10% aqueous pyridine solution for 24 hours, in the proportions of 1 lb. agar to 5 1/2 litres of pyridine solution. The aqueous pyridine was drained off through muslin and the agar washed three times in distilled water, followed by two washings in 95% ethyl alcohol. The agar, now free from pyridine, was dried between layers of muslin. The purified agar was added to Nostoc extract (N.E.2) at 2% strength, and spores were ejected to this medium, to Nostoc extract with 2% unpurified agar and to the liquid extract, in which germination was known to take place (Table 21). Ejections were made at random to the various media and these examined for germination of the spores

after seven days at 20°C. The combined data of two such experiments are shown in Table 35.

Table 35. Per cent germination and rupture of spores of Peltigera praetextata on Nostoc extract with purified agar, and on the extract with unpurified agar.

Culture type	Per cent germination		Per cent rupture	
	Apothecium No.			
	1	2	1	2
Purified agar	72	72	12	35
	36	89	25	18
	54	69	47	12
	46	-	56	-
Unpurified agar	0	0	67	100
	0	0	100	99
	0	0	94	100
	0	-	100	-
Liquid extract	54	98	0	10
	70	89	0	7

Germination of spores on the extract with purified agar is seen to be almost as good as that in the control liquid medium, while no germination has taken place on the extract

with unpurified agar. There is still apparent however, a certain amount of spore rupture on the purified agar. This is thought to be due to incomplete extraction with the pyridine. The camera lucida drawings (Fig. 43) show the form of the spores and germ tubes on the purified agar. The long, branched tubes and the absence of terminal swellings are typical of germination in the liquid Nostoc extract (c.f. Fig. 45).

This experiment, while illustrating that pyridine treatment of agar caused a reduction in the tendency towards spore rupture, did not provide conclusive evidence that this was due to the removal of some specific substance or substances by the pyridine. In order to substantiate the data obtained and to determine whether spore rupture was produced by a constituent of the agar, the pyridine extractions were returned to the purified agar and this compared, in respect to rupture of inoculated spores, with unpurified agar.

In this experiment, 10 gm. of agar were treated three times in succession with 250 ml. of a 10% aqueous pyridine solution, allowing the agar to stand in each for 24 hours. After the final pyridine treatment, the agar was washed three times with distilled water and three times with 95% ethyl alcohol. These washings and the three pyridine

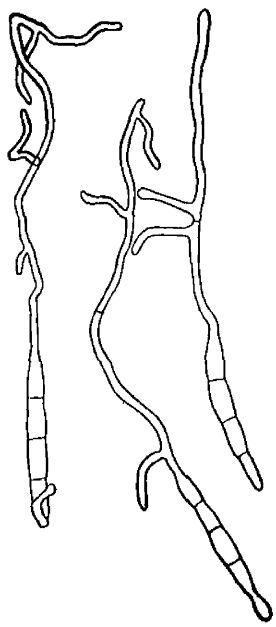

Fig. 43. Camera lucida drawing of spores of Peltigera praetextata cultured on Nostoc extract with 2% purified agar.

x 500 approx.

solutions were combined, and the pyridine removed by vacuum distillation. The residual aqueous solution was further evaporated to a small volume and this made up to 200 ml. with distilled water. This volume represented the extract of 10 gm. agar and contained 2.29 gm. solids.

The agar extract was added to N.E.2 in a concentration equivalent to 2% agar, and also in the same concentration to the extract with 2% purified agar. Spores were inoculated to these media and also to N.E.2, to N.E.2 with 2% unpurified agar and to N.E.2 with 2% purified agar. Four cultures of each were incubated at 20°C for seven days. The data of this experiment are shown in Table 36.

Consideration of these data reveals practically no difference between the per cent rupture of spores on purified agar to which the agar extract was added, and that on the unpurified agar. Also, the addition of agar extract to N.E.2 has caused a sharp increase in the per cent rupture of spores compared with that in the N.E.2 controls. It is thus indicated that some constituent of the agar is responsible for spore rupture and the masking of the capacity of the medium to support germination.

Table 36. Mean per cent germination and rupture of spores of Peltigera praetextata on Nostoc extract with reconstituted agar, compared with that on the extract with ordinary agar.

Medium	Mean per cent germination	Mean per cent rupture
N.E.2 with agar extract	0.5	76.5
N.E.2, 2% purified agar with agar extract	0	84
N.E.2, 2% agar	0	86
N.E.2, 2% purified agar	2	51
N.E.2 control	29	22

It is significant that no similar observation has been reported for lichen spores, other than that of THOMAS (1939) regarding the spores of Physcia stellaris on 4% malt agar. ROBBINS (1939) states that pyridine treatment of agar removes certain growth substances, and that Difco agar contains 0.041 gamma of biotin per gm. agar. Traces of thiamin or its derivatives are also stated to be present. The addition of vitamins to agar media

improves the growth of many free-living fungi, but the minute amounts already in agar seem surprisingly small to cause such complete disintegration of the spores of Peltigera.

To establish whether spore rupture is in some manner related to the vitamin content of agar, an experiment was carried out using media to which were added thiamin (in the form of aneurin hydrochloride) and biotin, in concentrations of 100 and 10 gamma per litre respectively. Nostoc extract (N.E.2), in which spore germination was known to take place, and No.3 of the glucose-sodium nitrate solutions were chosen as the most suitable media for this test. The latter medium has a C:N ratio of unity. It did not support germination and the degree of spore rupture was negligible (Table 32). In addition, Nostoc extract was treated with activated charcoal (LILLY and BARNETT, 1951) to remove growth substances, before addition of the above quantities of thiamin and biotin. Spores from one apothecium were inoculated in random sequence to the various media, two samples of each being inoculated. After seven days at 20°C. the cultures were examined for spore germination and rupture. These data are shown in Table 37.

Table 37. The effect of added thiamin (100 gamma/l.) and biotin (10 gamma/l.) on the germination and rupture of spores of Peltigera praetextata in Nostoc extract (N.E.2) and in the No.3 glucose-sodium nitrate solution.

Medium		Per cent germination		Per cent rupture		pH of medium	
		a	b	a	b	a	b
Nostoc extract	Thiamin	41	44	72	23	7.2	7.2
	Biotin	5	6	78	83	7.1	7.1
	Control	45	28	0	0	7.2	7.2
Charcoal treated Nostoc extract	Thiamin	11	0	62	85	7.2	7.2
	Biotin	1	0	82	48	7.2	7.1
	Control	23	2	1	16	7.2	7.0
Soln. 3	Thiamin	0	0	0	0	7.0	7.0
	Biotin	0	0	5-10	10	6.6	6.6
	Control	0	0	0	0	7.1	7.0

The addition of thiamin and biotin to N.E.2 has caused a sharp increase in the degree of spore rupture. The thiamin had little effect on spore germination, but this was much reduced by the addition of biotin. Somewhat similar results are evident for the charcoal treated extract, but it is of interest to note that the per cent germination in the control cultures was lower than that in the untreated extract controls. A vitamin requirement for germination is thus indicated.

Since the addition of thiamin and biotin to solution No.3 had scarcely any effect on the spores, it is deduced that spore rupture, caused by the addition of thiamin or biotin to Nostoc extract, is not the result of a purely chemical effect. Neither is the effect one of pH difference since the range in pH of the media was only 6.6 to 7.2. Rather is it to be considered as a secondary effect, probably acting through increased enzyme activity and hence increased uptake of nutrients from the medium. It is not suggested that the thiamin or biotin in agar are alone responsible for spore rupture. There are undoubtedly other growth substances present which may have a similar effect. For example, ROBBINS (1939) suggests the presence of a complex Z factor, one part of which (Z_1) is stated to be identical in biological action to guanine (ROBBINS and KAVANAGH, 1942).

The statement of THOMAS (1939), quoted on page 124 can now be qualified. Evidently it is not the richness of the medium as applied to the total nutrients present, but the richness in vitamins which has caused spores to "perish" on the agar media.

The data of Table 26 show that spores disintegrated in malt extract. The probability of an excessive vitamin content in this case was examined by culturing spores in 1% malt extract treated with activated charcoal. Spores were inoculated to the charcoal treated extract and to untreated extract controls, both solidified with 2% ordinary agar and with 2% purified agar. The cultures were examined for germination and rupture after 43 hours and after seven days at 20°C. The data obtained are shown in Table 38. Spores did not germinate on any of the media.

Comparison of the data obtained after 43 hours and after seven days further illustrates the increase in spore rupture with time (see Table 29). After seven days' incubation there was little difference between the per cent rupture of spores on the extract with ordinary agar and that on the extract with purified agar. This was also the case for the charcoal treated extract. Conversely, charcoal treatment of the malt extract has

caused a reduction in the degree of spore rupture in the presence of both ordinary agar and of purified agar. From this observation it is concluded that, as for agar itself, the vitamin content of malt extract is injurious to the spores.

Table 38. The effect of charcoal treatment of malt extract on the rupture of spores of Peltigera praetextata.

Medium	Per cent spore rupture					
	After 43 hours			After 7 days		
Malt extract 2% agar	43	26	8 (26)[H]	-	90	- (90)
Charcoal treated malt extract 2% agar	12	0	10 (7)	-	50	52 (51)
Malt extract 2% purified agar	14	14	2 (9)	80	90	80 (83)
Charcoal treated malt extract 2% purified agar	0	20	4 (8)	41	39	55 (45)

[H] The bracketed figures are the means of the several counts on each medium.

6. The effect of the addition of carbohydrate, on the germination and rupture of spores in Nostoc extract.

Previous experiments have shown that media with osmotic pressures of less than approximately seven atmospheres caused high degrees of spore rupture and that media with higher osmotic pressures were not so destructive to the spores. These observations were made from spore inocula in media containing glucose and sodium nitrate. All fungi, whether free-living or lichenized, require a source of carbon. In many cases glucose provides this adequately. Thus it was considered that the addition of glucose to Nostoc extract would improve germination and further growth of the spores.

An initial experiment was set up in which spores were inoculated to Nostoc extract with 1% glucose and 1% agar, also to the extract agar alone. The mean per cent germination of spores on the three control cultures after twelve days' incubation was 21%. Very few of the spores became ruptured. On the extract containing 1% glucose however, none of the spores germinated and all became severely ruptured.

A further experiment was carried out with graded amounts of glucose (0.01%, 0.025%, 0.05%) in Nostoc extract. The extract alone was used as a control medium. Cultures

were examined for germination after seven days at room temperature, counts of all the spores in each being made. These data are shown in Table 39.

Table 39. Per cent germination of spores of Peltigera praetextata in Nostoc extract with added glucose.

Per cent glucose	Per cent germination			
	Apothecium No. 1	2	3	4
0	80	98	93	91
0.01	53	78	78	45
0.025	-	-	-	7
0.05	3	-	-	-

The addition of glucose to Nostoc extract, even in micro-quantities, is seen to produce conditions which are not favourable to germination. Germinated spores in the extract with 0.01% glucose appeared similar in all respects to those in the control cultures, but in the extracts with the higher glucose concentrations, short unbranched germ tubes were produced. Many of the

spores in these cultures showed rupture of one or more cells. Thus, contrary to the findings for the glucose-sodium nitrate solutions, the addition of glucose to Nostoc extract causes a decrease in the per cent germination associated with rupture of the spore cells.

7. Germination of Peltigera spores in Nostoc extract treated with activated charcoal.

Rupture of spores in malt extract, and on Nostoc extract with 2% agar, has been shown to be related to the vitamin content of these media. Also, the data of Table 37 show that spore germination in Nostoc extract treated with activated charcoal was lower than in the complete extract. This latter observation indicated that germination was also related to the vitamin content of the medium independently of the relationship between the degree of rupture and of germination.

To further elucidate these findings, a number of experiments were carried out to confirm the effect of charcoal treatment of Nostoc extract on spore germination. For each experiment (five in number) the complete extract was used as a control medium. Cultures of four of the experiments were incubated for seven days at 20°C., the fifth at room temperature for ten days. The data for

mean per cent germination in the 29 cultures are shown in Table 40.

Table 40. Mean per cent germination of spores of *Peltigera praetextata* in *Nostoc* extract treated with activated charcoal.

Experiment No.	Per cent germination	
	Control extract	Treated extract
1	54	44
2	10	0
3	89	80
4	64	38
5	65	9

Germ tubes of spores in the treated extract were short and unbranched. Many showed the typical swellings indicating conditions unfavourable to germination. On the other hand, spores in the control extract developed much longer germ tubes which, in many instances, showed one or more branchings. The appearance of typical spores

in these media is shown by Figs. 44 and 45.

In experiment 3, the mean length was determined of the ten longest germ tubes in each of the twelve cultures. For the control extract this was 217 microns, and for the treated extract, 64 microns. Thus, although the differences in per cent germination in the two media were inconsistent, and in two of the tests, scarcely significant, there was in all cases a marked difference in the form and length of the germ tubes. The previous observation regarding removal of vitamins from Nostoc extract was thus confirmed.

To ascertain whether the substances adsorbed from Nostoc extract by the activated charcoal were, of themselves, capable of supporting germination of the spores, tests were made with the adsorbate added to No. 3 of the glucose-sodium nitrate solutions. The adsorbate was eluted from the charcoal with methylated ether and a volume of solution 3, equal to the volume of the original Nostoc extract, added to the ether. The latter was distilled off and the remaining solution autoclaved. Spores were inoculated to this medium, to charcoal treated extract and to controls of complete extract. Cultures were incubated for seven days at 20°C. The data of three such tests are collected in Table 41.

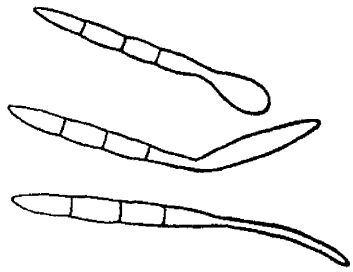

Fig. 44. Spores of Peltigera praetextata germinated in charcoal treated Nostoc extract.
Camera lucida drawing.
x 500 approx.

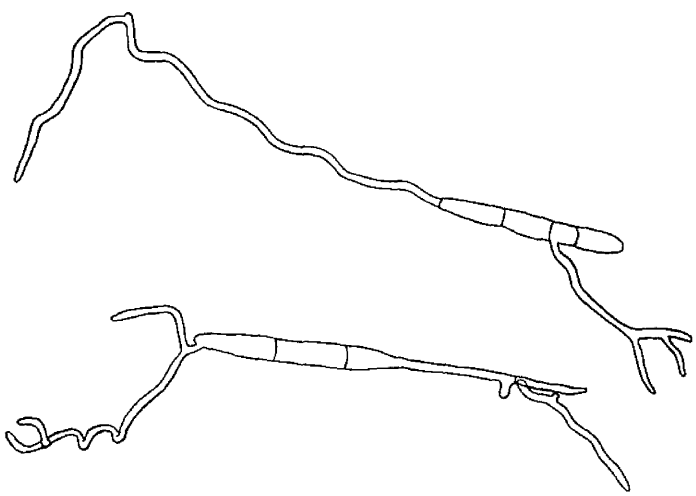

Fig. 45. Spores of Peltigera praetextata germinated in untreated Nostoc extract.
Camera lucida drawing.
x 500 approx.

Table 41. Mean per cent germination of spores of *Peltigera praetextata* in charcoal treated, and in untreated *Nostoc* extract, and in the charcoal-adsorbed fraction of *Nostoc* extract added to a glucose-sodium nitrate solution (No.3).

Medium	Per cent germination		
	Experiment No.		
	1	2	3
Nostoc extract	22	19	75
Charcoal treated extract	40	3	7
Ether eluate in solution No.3	0	0	0

The absence of germination in solution No.3 with added eluate indicates that the charcoal adsorbate is not the total requirement for germination. *Nostoc* extract thus contains some other substances, outwith the vitamin or growth substance class, which are essential for germination. The data for germination in the control extract and in the treated extract are again inconsistent, but, as before, there were marked differences in germ tube form. In particular, germ tubes in the treated extract of experiment 1 showed very typical starvation swellings.

There remained the possibility that the activity of the charcoal adsorbate was destroyed by elution with ether. Tests were thus made for spore germination in charcoal treated Nostoc extract to which the eluted adsorbate had been returned in concentration equivalent to that in the complete extract. Spores of Peltigera praetextata were used for two tests and for a third, spores of Peltigera horizontalis. The cultures were incubated for seven days at 20°C. The results of the three tests, comprising fifteen cultures, are shown in Table 42.

Table 42. Mean per cent germination of spores of Peltigera praetextata and of P. horizontalis in Nostoc extract, reconstituted after treatment with activated charcoal.

Medium	P. praetextata		P. horizontalis
	1	2	3
Nostoc extract	40	69	25
Charcoal treated extract	35	-	2
Charcoal treated extract with returned eluate	72	53	15

Germination in the reconstituted extract is seen to be comparable to that in the complete extract. Thus ether elution does not destroy the activity of the charcoal adsorbate, and the conclusions of the previous experiment remain valid.

These experiments suggest the presence of two distinct fractions of Nostoc extract necessary for spore germination, 1) a substance or substances of the vitamin class which can be removed by charcoal treatment of the extract, and 2) other substances, probably specific proportions of organic nutrients, which remain in the extract after charcoal treatment.

8. Silica-gel as a medium for spore culture.

Although agar can be rendered more suitable for spore culture by removing the excess of vitamins by pyridine treatment, this is a time-consuming task. Silica-gel, being a biologically inert substance, was thus considered as a possible substrate for further investigations. Before the advent of ion exchange resins, the preparation of a satisfactory gel left much to be desired and seldom could the gel be adequately sterilised. The ion exchange method (SMITH, 1951) has removed nearly all obstacles to the preparation of a gel, but the remaining disadvantages

are such that it has a limited application in this type of work.

The principal defect is the difficulty of obtaining a gel of a known and reproducible consistency. Differences can even be detected between individual plates of the same preparation of gel. A further disadvantage is the comparatively rapid drying out of the medium. Despite these drawbacks, unfailingly higher germination figures and better germ tube growth were obtained on gels impregnated with Nostoc extract than in the liquid extract or on the extract solidified with purified agar.

An initial test of silica-gel as a medium for spore culture showed that the per cent germination of ten cultures ranged from 12% on a very stiff gel, to 100% on a soft gel. Figs. 46 and 47 show the appearance of spores on a soft gel with Nostoc extract, after seven days at room temperature. Germ tubes were richly branched and many of the spores germinated from both terminal cells. The appearance of germ tubes from one or other of the central cells was a feature of silica-gel cultures (Fig. 47).

Perhaps the most striking effect of the addition of silica-gel to a culture medium is shown by the data of Table 43. The Bristol solution, in which Nostoc from Peltigera praetextata had been grown for some weeks, was

Fig. 46. Spores of Peltigera praetextata cultured on Nostoc extract with silica-gel.

x 195

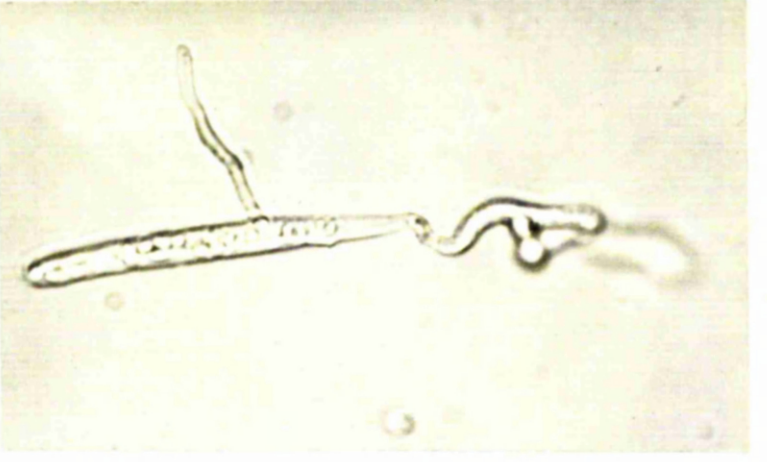

Fig. 47. An enlargement of a spore of Fig. 46 showing the germ tube arising from one of the central cells of the spore.

x 700

Table 43. Per cent germination of spores of Peltigera praetextata on a Nostoc culture solution gel.

Medium	Per cent germination		
	1	2	3
Nostoc culture solution with silica-gel	73	76	75
Nostoc culture solution	4	0	0

filtered off and part of it incorporated in a gel. The remainder, after suitable dilution, was used as a control medium. Counts of 100 spores on each culture after six days at room temperature, revealed that effectively no spores had germinated in the control solution, while those on the silica-gel showed germination consistent with that in Nostoc extract. It is unlikely that this effect was due to any property of the gel other than the creation of better conditions of aeration.

Further growth of germ tubes on silica-gel surpassed that obtained in Nostoc extract. The longest observed, in the several cultures previously mentioned, was 850 microns. Thus it is evident that the medium consistency, affecting the humidity and the availability of oxygen, is

a deciding factor in attempts to achieve further growth of the mycobiont of Peltigera praetextata.

9. Data regarding discharge and germination of spores from apothecia collected at different seasons.

Unlike the free-living discomycetous fungi, lichens produce apothecia which may persist for several years. They are, in most cases, perennial organs producing successive crops of ascospores throughout the year. It is generally accepted that spore production reaches a peak in the spring of the year. At this time, the viability of the spores is also said to be at a maximum, but, as DES ABBAYES (1951) says, "...les observations précises sur ce sujet sont rares."

WERNER (1927, 1930) states that spore ejection in Peltigera canina continues throughout the year, but only in March do spores emit germ tubes, whose growth is soon arrested. THOMAS (1939) found that apothecia of Peltigera horizontalis, collected in December, showed copious spore-shedding five hours after moistening. This same author also states that the ability of spores to germinate is not so closely bound to the seasons as was supposed by Werner.

Over the three years during which the numerous experiments previously described were carried out, a record was kept, for each apothecium, of the time of collection and of the conditions and length of storage. In addition, similar details were recorded for the apothecia used in other experiments which have not been described in this work. Such details were recorded for 83 apothecia of Peltigera praetextata and for four of Peltigera horizontalis. Table 44 shows the dates of collection of apothecia of Peltigera praetextata and the proportion of these with spores which were shown to be viable. In most cases, numerous apothecia were collected, of which selected individuals were used in the experimental work described.

Two of the 83 apothecia failed to eject spores after immersion in water. Those used soon after collection, e.g. the collections of July and September 1955, showed spore ejection within twenty to thirty minutes of moistening. On the other hand, spore ejection was delayed for four to five hours after moistening apothecia collected in January 1955 and used in June of that year. No relationship could be observed between the intensity of spore ejection and the season of collection. Likewise there was no obvious relationship between the per cent

Table 44. Dates of collection of apothecia of Peltigera praetextata, and the proportion with spores known to be viable.

Number of apothecia used from collection	Date of collection	Number with spores which germinated in Nostoc extract
6	Nov. 1953	1
14	July 1954	7
5	Sept. "	2
17	Jan. 1955	11
3	June "	3
13	July "	11
4	Sept. "	3
4	Oct. "	4
4	Dec. "	3
1	Feb. 1956	1
6	Mar. "	6
6	Apr. "	6

germination of spores from a particular apothecium and the time of collection.

For all seasons some, at least, of the apothecia discharged spores which germinated in Nostoc extract. Of the 83 apothecia used, spores from 23 failed to germinate. Spores from all but two of the latter were inoculated to media in which germination was not expected to occur. There is thus no evidence that these spores were non-viable. The two apothecia which shed non-viable spores were collected, one in July and the other in December.

Two of the apothecia of Peltigera horizontalis, collected in January, discharged a large number of spores but, since these were inoculated to media other than Nostoc extract, no indication of viability was obtained. The two remaining apothecia of this species were collected in April and in July 1955. Spores ejected from both, to Nostoc extract, showed good germination.

These data illustrate that, for the west of Scotland at least, viable spores are discharged from apothecia of Peltigera praetextata throughout the year. Viability is not confined to the month of March as was stated by WERNER (1930), for Peltigera canina. Although no evidence could be established that spores ejected at a particular season showed a greater capacity for germination than at other seasons, it is not disputed that such may be the case.

In the experiments described in this work, the spore complement of the apothecia used was that proportion of spores which happened to be mature at the time of ejection. Many of the later ejected spores would presumably come to full maturity where ejection continued for several hours. Thus no true indication of the manner of spore ejection has so far been obtained. However, in two additional experiments carried out in March and in April, an apothecium was affixed to a glass plate and suspended,

in daily succession, over some forty Petri dishes containing Nostoc extract. The small piece of thallus attached to the apothecium was so arranged that it dipped into the solution. The whole arrangement was placed under fluorescent lighting. Ejection of viable spores continued for ten days in one experiment, and for eleven days in the other. A noticeable feature of these experiments was the germination of spores within a few hours of ejection, after the apothecia had been continuously discharging spores for three to four days. Part of the apothecium used for one of the experiments was examined under the microscope for spores. Many of these were seen to have put out small germ tubes while still within the asci. This was presumably due to the prolonged immersion of the apothecium in the Nostoc extract.

Although there are disadvantages in this method of spore ejection, these two experiments illustrate that the pre-ejection condition of the spores is an important factor, and that this is closely dependent on the nutrition of the apothecium. Given suitable conditions and freedom from contamination by micro-organisms (both plant and animal), this method of obtaining spores would be most desirable. Unfortunately it is so nearly

impossible to remove contamination that one must hopefully await the advent of a pure culture of the lichen.

DISCUSSION OF DATA OBTAINED

Culture studies on Peltigera spores are considerably hampered by the difficulty experienced in obtaining uniform spore samples. A wide variation has been shown to exist between spores from apothecia collected from the same habitat and even between apothecia from the same plant. Few experiments can be planned to utilise spores from only one apothecium and those which have been possible are, rather unfortunately, the exception. The method described on page 142 overcame this difficulty to some extent and the results of the two experiments using the different methods of spore inoculation (Tables 32 and 33) are reasonably comparable. A large proportion of the discharged spores are however, lost in the process of centrifuging.

Contamination by bacteria and other micro-organisms is an unavoidable adjunct to spore culture 'en mass'. This has been present in nearly all the cultures of the experiments described. Fortunately, since the nutritional value of Nostoc extract is very low, in terms of bacterial growth, the experimental work has not been seriously affected by contaminants. But in media with a high organic nutrient content such as malt extract, and on all

media with unpurified agar, bacterial growth grossly interfered with the results of many experiments. Those which were obviously contaminated in part have been omitted from this work, but others have been included, such as the experiment on the effect of charcoal treatment of malt extract on spore rupture. Here, contamination was present in all of the 24 cultures and this has certainly influenced the results to some extent. Three of the cultures became so badly contaminated after seven days that spore counts were not made (Table 38). Despite this, the data obtained were true to expectation but, under different circumstances, the effect of contamination could not be overlooked.

It is possible however, to obtain clean spores by selection from an agar medium containing organic nutrients. THOMAS (1939) used this method with success for spores of green lichens but, if used for Peltigera spores, one has to contend with disintegration of the cells which has been shown to take place on media rich in nutrients, or which contain agar. The data of Tables 29 and 38 show that spore rupture increases with time in culture on an agar medium. Use may be made of this feature in the selection of bacteria-free spores before these begin to disintegrate. Observation of many spores in culture

certainly shows that a good proportion are free from bacteria.

The germination of spores in tap water (p.117) presented the peculiar feature of fusion between the germ tubes of separate spores. Various types are shown in Fig. 48. Such fusion is obviously a feature of malnutrition, as stated by THOMAS (1939) for spores of Physcia stellaris in distilled water. A very different form of germination was apparent in the presence of Nostoc cells, or in an extract of the latter. Here, the conditions were evidently ideal for germination as is shown by the long well-branched germ tubes (Figs. 40 and 45). It is doubtful however, whether the germ tubes in these media reached the stage where growth was entirely dependent upon nutrients withdrawn from the culture medium. The universal failure to obtain mycelium growth of a total length of more than 850 microns (p.164) points to the absence from the medium of some substance which is supplied to the developing germ tubes by the spore. This contention is supported by the appearance of spores after prolonged culture in Nostoc extract. All germinated spores became empty, leaving only the wall material, while spores which had not germinated, or which had put out only short tubes, still retained their granular cytoplasmic contents. Many of the cultures in Nostoc

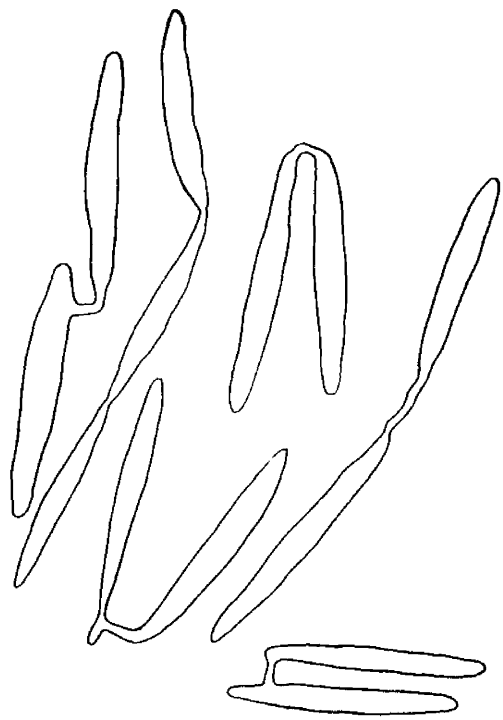

Fig. 48. Examples of germ tube fusion in spores of Peltigera praetextata, germinated in tap water. Freehand drawing.
x 600 approx.

extract, and on Nostoc plates, were left for some weeks in the hope that further growth of the germ tubes would develop. It is noteworthy that even in the presence of Nostoc cells, the partner of the mycobiont in symbiosis, growth of the germ tubes was arrested when the spore contents became exhausted.

The increase in spore germination in Nostoc extract with dilution to 5 gm. fresh weight per litre (Table 21) suggests a very low nutritional requirement. So low indeed that germination in soil solution was completely forestalled by disintegration of the spores. What then, is the probability of germination of spores ejected to the soil surrounding the parent plant ? It would appear that there is a hazard in the doubtful suitability of soil for germination, in addition to the mortality caused by failure to make contact with Nostoc filaments. This applies particularly to the large number of spores ejected in a short space of time soon after the apothecium has become moistened, and which exhibit a greater tendency towards rupture (p.135).

Spores in the various solutions of glucose and sodium nitrate showed a decrease in per cent rupture with increase in the concentration of the solution, and thus with increase in osmotic pressure. By extrapolation of the data for spore rupture to zero osmotic pressure,

one would expect to obtain a value approaching 100% rupture. But it has been shown (p.141) that spores did not disintegrate in distilled water even after seven days at 30°C. Further, there is evidence (p.155) that spore rupture in Nostoc extract is increased by the addition of glucose to a concentration of 1%. Tables 32 and 33 also show that most of the spores disintegrated in 0.1% glucose alone, with an osmotic pressure of 0.1 atmosphere, while in solutions of higher osmotic pressure, fewer spores became ruptured. These facts suggest that the basis of spore rupture is a progressive increase in the osmotic pressure of the cells followed by endosmosis and distention, to breaking point, of the cell walls. The spores shown in Fig. 41 show several of these stages, and in particular the very much swollen cells just prior to rupture.

Although spore rupture is caused by the abnormal uptake of osmotically active substances, it has been shown that there are diverse ways in which this condition may arise. Firstly this has been related to excessive vitamin content of the medium itself, e.g. of malt extract (Table 26), and also of the agar with which the media are solidified. Thiamin or biotin, added to Nostoc extract, reproduced a similar effect (Table 37). Secondly rupture is induced by the uptake of nutrients outwith the

control of any vitamin content of the medium. In such instances the degree of spore rupture has been shown to be an inverse function of the osmotic pressure of the culture medium (Tables 32 and 33).

The various experiments indicate that spore rupture is related to, 1) the vitamin content of the culture medium, or agar, 2) the concentration of the medium, 3) the osmotic pressure of the medium, 4) the availability of oxygen in the medium, 5) the length of time in culture, and 6) the time of ejection of spores from the apothecium. All these factors point toward spore rupture as being the result of a complex of processes each interacting with the other, but being finally controlled by the vitamin content of the culture medium, or of the spores themselves.

In Peltigera praetextata we thus have a lichen whose spores appear to be strictly dependent, for survival, upon nutrition from the living alga, and which present some remarkable differences from those of the green lichens.

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SUMMARY OF PART IV

1. Germination of the spores of Peltigera praetextata was obtained in an aqueous extract of Nostoc, the co-symbiont of this lichen.
2. The optimum temperature for spore germination was found to be in the region of 20°C.
3. Spore disintegration on culture media has been shown to be caused by the excessive vitamin content of the medium. The vitamin content of agar is also too high for normal germination.
4. The Carbon:Nitrogen ratio of the medium proved to have no effect on spore disintegration.
5. The addition of even micro-quantities of glucose to Nostoc extract had an adverse effect on spore germination. Higher concentrations of glucose caused complete spore rupture.
6. Spores did not germinate in soil solution, contrary to the finding of THOMAS (1939).
7. Silica-gel, but for the disadvantage of comparatively rapid drying out, is an ideal substitute for agar in spore culture work.
8. Spores are ejected from apothecia throughout the year. At no season are they incapable of germination.

9. There appears to be no obvious correlation between the per cent viability of spores and the season of the year in which spores are discharged from the apothecium.

10. A significant contribution is considered to be made to spore mortality in Nature by disintegration before germination has been able to take place.

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