THE MUTUAL RELATIONS BETWEEN ALGAE AND FUNGI IN LICHENS

by

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(With Tab. V and VI).

CONTENTS

INTRODUCTION: THE DEFINITION OF SYMBIOSIS.

Symbiosis was defined by DE BARY (1878) as: „die Erscheinungen des Zusammenlebens ungleichnamiger Organismen". This definition covered the processes of mutualism, where both partners are benifited by the association, those of commensalism, where the profit is one-sided and those of parasitism, where one of the partners is harmed by the other one.

Many more recent investigators restricted the word symbiosis to the first-named case. The difference between mutualism and commensalism, however, even the difference between mutualism and parasitism is not as obvious as one was apt to think, as many symbioses consist of a labile equilibrium, which easily changes to the disadvantage to one of the partners. Moreover the criterium of mutual advantage can only be established after gathering a number of experimental facts, which are lacking for most symbioses. So it is advisable to use the word symbiosis in the sense of the definition of DE BARY. Then too, however, the limitation does not seem to be very pronounced. The well-known examples of micro-organisms, which, by supplying each other with accessory growth-requirements, can grow together in synthetic culture-solutions in which they cannot develop alone are generally described as "artificial symbiosis", particularly because it has been shown, that in many natural symbioses as well the exchange of accessory growth-factors (nutrilites) plays an important role. When, however, we enlarge the definition of symbiosis to these artificial symbioses we also have to include those cases, in which the two organisms do not live simultaneously in the culture-solution, but in which one of the "symbionts" makes the solution fit for the development of the second by the excretion of certain nutrilites, as the mechanism of this successive influence does not differ from the mechanism of the simultaneous one. But then we have to include under the definition of symbiosis all cases of successive influences as well, for instance cyclic cultures, hayburn, humus-formation, etc.

Does there exist a physiological, essential, difference between the mutual relations of symbionts and the mutual relations of cells or tissues in a higher developed organism, but for a greater complexity of the latter? Recent investigations show more and more that, on the contrary, there exist many points of similarity. I remind here of the

exchange of nutrilites in many cases of symbiosis and the exchange of hormones in higher organisms. Has the dependence of a cell in a tissue on the other parts of the organism not to be ascribed to the loss of certain potencies as well as has the dependence of one symbiont on the other? Many investigators have mentioned the functional analogy between certain structures of higher organisms with certain symbioses; enough to recall the ectotrophic mycorrhiza-fungi, which should perform the same function as the root-hairs of other plants, but especially the lichens, where from the association of an alga and a fungus a structure has arisen, which bears a so striking resemblance to a leaf of a higher plant, that more than one author has been seduced to phylogenetical speculations. From the association a new systematical unit should have developed, the plastids of green plants should be looked upon as modified green symbionts and so on. And, although speculations like these are difficult to justify morphologically, the supposition of a physiological similarity between those symbionts and certain parts of higher plants is at least a tempting and inspiring working-hypothesis.

So the recent physiological and biochemical investigations show more and more, that the mutual relations of the partners of a symbiosis show some resemblance to the mutual relations of other vital-units. BAAS-BECKING (1942) proposed in a meeting of the Netherlands academy of Sciences to use the term symbiosis in a wider sense as usual and to include: all mutual dependence of vital-units (cells, tissues, organs, organisms). These relations may have a parasitical, mutualistic or commensalistic character. This new definition of symbiosis is best illustrated by his survey of the symbiosis as given below:

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                                                     ⎧ obligate (irreversible
                                                     ⎪            potencies)
                                  ⎧ histosymbiosis  ⎨
                                  ⎪                  ⎪ facultative (reversible
               ⎧ homoiosymbiosis ⎨                   ⎩            potencies)
               ⎪                  ⎪
               ⎪                  ⎪                  ⎧ coenosymbiosis
               ⎪                  ⎩ autosymbiosis   ⎨
               ⎪                                     ⎩ gamosymbiosis
  symbiosis   ⎨
               ⎪                                     ⎧ endosymbiosis
               ⎪                  ⎧ simultaneous     ⎪    a: cyclic
               ⎪                  ⎪ (symbiosis s.s.) ⎨    b: repeating
               ⎩ heterosymbiosis ⎨                  ⎪ ectosymbiosis
                                  ⎪                  ⎪    a: natural
                                  ⎩ successive       ⎩    b: artificial
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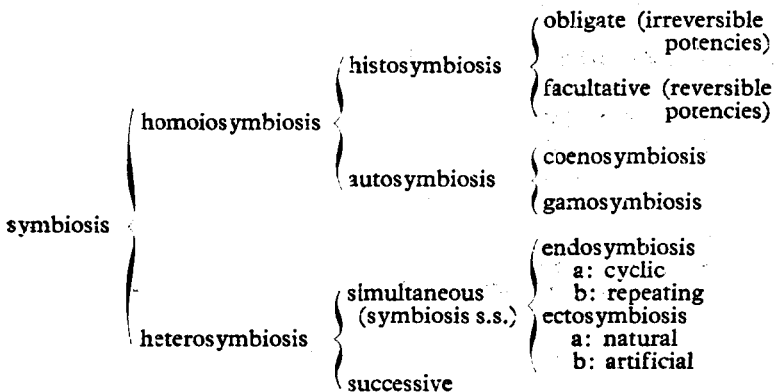
Here the simultaneous heterosymbiosis corresponds to the classical meaning of the word symbiosis. It has been divided into a cyclical endosymbiosis (Ardisia, Pavetta, Psychotria), a repeating endosymbiosis, with again and again a new infection (Gunnera, Legumes, endotrophic mycorrhiza) and an ectosymbiosis (ectotrophic mycorrhiza, lichens). The artificial symbiosis forms a special case of the ectosymbiosis. Closely related to the simultaneous heterosymbiosis is the successive heterosymbiosis (f.i. cyclic cultures, hayburn, humus formation).

The opposite of the heterosymbiosis — the mutual dependence of organisms belonging to different species — is formed by the homoiosymbiosis — the interdependence of vital-units belonging to the same species. The most simple case is formed by the coenosymbiosis — the mutual dependence of identical vital-units (social organisms, allelocatalysis); another example is formed by the gamosymbiosis — the mutual influence of the gametes (by means of crocitine in Chlamydomonas, by means of organic acids in mosses and ferns). The most complex case of homoiosymbiosis and of symbiosis in general is formed by the histosymbiosis — the mutual dependences of tissue-elements, who have lost their potentials in an irreversible (most animal tissues) or a reversible way (certain meristematic tissues of higher plants) and are highly dependent upon their "symbionts", although the cultures of tissues have shown that these vital-units too may live individually.

One could object to this definition of symbiosis, as the term symbiosis loses much of its original significance. On the other hand the cases of symbiosis s.s. become better defined by the comparison with physiologically related phenomena. As it stresses this relationship it may inspire to new working hypotheses.

Of course the limits between the different types of symbiosis are not very sharp. The scheme mentioned above must be considered as a survey, not as a dichotomic key.

In no symbiosis s.s. the resemblance to a histosymbiosis is so striking as in the lichen-symbiosis. It is easy to understand why the dual nature took long to be generally accepted. The similarity between lichens and green organisms like liverworts or even the leaves of phanerogams is so striking, that even recent lichenologists supposed, that a lichen is more than the sum of the two components: „die duale Natur ist heute nicht mehr das Wesen der Flechten — nein, ihr Wesen ist die neue Einheit" (TOBLER, 1934). The supposition of such a new unity is an inspiring hypothesis, yet our knowledge about the nature of this association is too small to justify conclusions like this. A better insight in the nature of this lichen-sym-

biosis will be important for the understanding of the symbiosis s.s. as well as for the symbiosis in its widest sense.

After the work of DE JONG (1938) upon the symbiosis of Ardisia investigations upon some other symbioses as well have been performed on the Botanical Institute of the Univerity of Leyden. I mention some work about the Nostoc-symbiosis of Gunnera by Mr. W. DIJKSHOORN, about the fungus-symbiosis of Lolium temulentum by Miss J. M. VAN ROON and Mr. J. V. D. DRIFT, while the investigation of the Ardisia-symbiosis was continued (BOK, 1941). On the following pages I shall give a survey of my work upon the lichen-symbiosis, which was carried out for the main part in the Botanical Institute mentioned above under the stimulating direction of Prof. Dr. L. G. M. BAAS BECKING, whom I thank for his great, enthusiastical and inspiring interest in my work. It was started at the Laboratory for Microbiology of the Technical College at Delft, whose director Prof. Dr. A. J. KLUYVER I want to thank for his continuous help and advice, while I want to thank Prof. Dr. W. H. ARISZ for the hospitality I received in the Botanical Institute of the University of Groningen, when I did not have the opportunity to finish my experiments in Leyden. Finally my thanks are due to Prof. Dr. G. v. ITERSON for his valuable help and Prof. Dr. F. KÖGL for his critical remarks on the chemical part of this work.

CHAPTER I

SURVEY OF THE LITERATURE.

Our modern conception about lichens thanks its origin to the Swiss botanists DE BARY and SCHWENDENER. Although many older investigators had occupied themselves with these remarkable organisms, their true nature had not been perceived. They had, however, generally reached the conclusion, that there existed a close relationship between lichens and fungi, as they found in lichens distinct hyphae and spore-formation in asci. The nature of the green cells however remained mysterious and with WALLROTH (1825—1827) they were generally regarded as reproductive organs or *gonidia*; a name still used to-day. Although this investigator observed the remarkable resemblance between gonidia and certain free-living algae, he concluded that apparently these free-living algae were nothing more than lichen-gonidia, which, in consequence of bad external

situations, had not been able to differentiate into lichens. The observation of TULASNE (1852), that in the mycelia formed after germination of the ascospores green cells developed, was very misleading. To-day we know, that these green cells developed because his cultures were not pure.

DE BARY (1864) was the first investigator who supposed that certain homoiomerous lichens like Collema resembled Nostocacae and Chroococcacae attacked by a fungus, a supposition, which did not evoke much criticism as this type of lichens was generally regarded as an isolated group of very primitive lichens. When, however, in 1867 his fellow-countryman SCHWENDENER enlarged this hypothesis to higher differentiated lichens and concluded, that all types of lichens had to be regarded as fungi parasitizing upon algae a grim and often very unkind criticism arose from two sides.

In the first place there were many systematical lichenologists, who refused to accept the idea of the dual nature of lichens. Their inconvincing arguments were soon repelled. The observations of SCHWENDENER himself (1869, 1872, 1873) and BORNET (1873) clearly showed the identity between lichen-gonidia and certain well-known algae, while after a study of the contact between algae and hyphae in the lichen-thallus it was obvious that this contact could not be explained by assuming an origin of the algae out of the hyphal-cells. The definite proof of the dual nature was delivered by synthesis-experiments, in which it was observed how the germinating spores formed hyphae, who enclosed the algal-cells, after which a thallus was formed. The first investigator who made experiments like this was REESS (1871), who experimented with Collema, soon followed by BORNET (1872) and TREUB (1873), who worked with higher differentiated lichens. Yet they observed only the beginning of a thallus-formation, a lichen was never formed. STAHL (1877) succeeded in this, because he made use of an object, where synthesis like this generally occur in nature, which with most other lichens is very doubtful. Endocarpon pusillum ejaculates together with its ascospores algal-cells: the so-called hymenial-gonidia and after sowing these hymenial-gonidia together with the ascospores on a suitable substrate he was able to observe the formation of a well developed lichen-thallus. The climax of all these experiments was given by BONNIER (1889), who was able to synthesize many well-known lichens like Parmelia and Xanthoria species even till apothecia were formed. Moreover he was the first investigator to use pure-culture methods. Yet the reliability of many of his observations has been doubted in recent times and indeed: his pure-culture methods did not appear to be very perfect and the pureness of his algal material in particular

was very doubtful, especially because he claims to have worked with an alga (Pleurococcus), which never occurs as gonidium in the lichens synthesized. Notwithstanding this his beautiful illustrations of the micro-synthesis clearly show that he has observed the first stages of a thallus-formation out of the two components. When finally Möller (1887) succeeded in isolating the fungal parts of many lichens without observing the formation of gonidia in the mycelia formed, the dual nature of lichens had to be generally accepted. The efforts of Elfving (1913 and even 1934) to defend in present days the old concept of the formation of the gonidia out of the hyphae must be regarded as a neglect of these older experiments.

In the second place the criticism against the ideas of Schwendener was pointed against his assertion that the fungus parasitised upon the alga. This seemed hardly in accordance with the high age reached by many lichens, without showing any degeneration of the algal part, which would have seemed to be the natural result of any parasitism. That Schwendener himself was struck by this difficulty appears from the fact, that in one of his later articles he proposes the term helotism (1869) for the nature of the association. In other words he supposed that the gonidia had to be regarded as "slaves" of the fungus. Soon, however, v. Tieghem (1874), de Bary (1879) and Reinke (1894 and already in 1872) proposed a new theory, which they called mutualistic symbiosis, or the consortium theory. In this theory the fungus was supposed to live from the assimilation products of the gonidia, while the gonidia used the inorganic salts absorbed by the fungus out of the substrate. So both partners did profit from the association and in fraternal coöperation they had formed a new systematic unity, which had already differentiated phylogenetically as such. In this form the hypothesis of the dual nature found much more approbation, especially among the systematical lichenologists, who now did not need to part their study-objects between the algae and the fungi and the consortium theory conquered the text-books of botany. Among the supporters Famintzin (1907) went as far as to consider symbiosis as a way in which new species could be formed. He even supposed, that the plastids of higher plants had to be regarded as modified green symbionts! When, however, we look for the experimental facts upon which the consortium theory was based it appears that these facts are lacking. The result was that a counter-criticism arose, which was pointed especially against the supposed advantage for the gonidia. For it is true, that the gonidia in a heteromerous thallus receive the inorganic salts from the fungus as they are in no direct contact with the substrate or the atmosphere, but it is doubtful whether, owing to this, the gonidia are in a better situation

than their free-living relatives, who can take the salts immediately out of the substrate or out of the moistening rain-water. The limited growth of the gonidia in the thallus and the often abundant development of the free-living aerial algae makes this very improbable. Moreover it is hardly conceivable that in slow growing organisms like this the provision with salts will be a limiting factor even though they are living on rather poor substrates. BEIJERINCK (1890) thought to have found a more important profit for the gonidia when he observed, that in pure culture they needed peptones. As in nature the nitrogen will be present in an inorganic form only, the symbiosis with a fungus, who can convert these inorganic nitrogenous salts into organic assimilatable compounds would be very important. But although this fact was confirmed by ARTARI (1902) who compared the gonidia of Xanthoria with the free-living Cystococcus infusionum and observed, that the first needed peptones and the second could make use of inorganic nitrogenous salts too, TREBOUX (1912) showed that all gonidia could live on inorganic nitrogen as well. Correctly he says, that we can only speak about a profit for the symbiosis, when the gonidia show a marked preference for organic nitrogen; this was not the case.

CHODAT (1913) called the attention to a third possibility. He stressed the fact that in pure culture Cystococcus gonidia not only grow very well on organic substrates in the absence of light, but that in the presence of light too the development was only appreciable, when a percentage of glucose was added to the medium. As it was by no means certain that the gonidia in a lichen-thallus could obtain enough light for their photosynthesis as they are hidden under a thick layer of hyphae, while many lichens are growing on rather dark places, he supposed that, in contrast to the usual conception, the lichen as a whole, and so the gonidia too, would live from the organic substances taken by the fungus from the substrate. And although recent investigations have shown that under optimal ecological conditions the lichens as a whole are autotrophic organisms, it has been shown by some of the same authors (STOCKER 1927, BOYSEN-JENSEN-MÜLLER 1929) that in lichens growing under suboptimal conditions a heterotrophic development of the lichen as a whole is indeed very probable.

In the fourth place we should mention those theories, which consider the relations between the water-household and the symbiosis. Already v. TIEGHEM (1874) supposed that the fungal hyphae protected the gonidia against desiccation. ZUKAL (1895) devoted a long publication to this subject, but it was only after the careful investigations of GOEBEL (1925), that the water relations of the lichen-thallus

were well understood. GOEBEL mentioned the occurrence of thick-walled hyphae, who were able to absorb water from a humid atmosphere. He supposed that during periods of drought they formed a water reserve, from which the gonidia could profit during some time. Yet it was hardly credible that this water-reserve would stand very long, so that it is very doubtful whether the gonidia are benifited by the presence of this water reserve. On page 519 we shall have occasion to say more upon this subject.

So we may conclude that up till now the physiological experiments did not make very clear whether, and if so how, the gonidia are favoured by the association. On the other side some histological arguments seemed to speak in favour of a parasitic behaviour of the fungus. In the first place many investigators observed how the fungus formed haustoria, who penetrated into the gonidia, resorbed their contents, after which these gonidia died (p.e. BORNET 1873, SCHNEIDER 1897, PEIRCE 1899, DANILOV 1910, SENFT 1916, NIENBURG 1917, FRY 1928, GEITLER 1933, 1934, 1937). In the second place it was observed that many lichens contained a number of dead algal cells, a fact inspiring ELENKIN (1902, 1904) to his theory of endosaprophytism. According to this theory the fungus secreted certain products, which killed the algae after which the fungus lived saprophytically from the dead algal cells. Most authors, however, think it more likely, that the killing of these gonidia is caused by bad ecological conditions. BACHMANN (1923) thought that these dead gonidia functioned as water-reservoirs and considered the dying of these gonidia as a "Stoffwechselpartialtod", that is a sacrifice for the benefit of other parts of the lichen-thallus. But the existence of haustoria was not considered either by many authors as a decisive proof for the parasitic character of the lichen-fungi as they remained limited to only a small percentage of gonidia, while most of the other gonidia were unharmed. In the recent articles of FRY and GEITLER, however, examples are given of lichens where all gonidia are attacked by haustoria. In these cases the gonidia are apparently unharmed (Lecidea spec.) while in Lempholemna they try to free themselves from the haustoria by remarkable acts of defense. The strongest opponent of the consortium-theory was the American lichenologist BRUCE FINK (1913), who considered lichens as fungi, who were living during a part of their life parasitically from an algal host and saprophytically from a dead substrate. Apart from his arguments referring to the occurrence of haustoria one of his most principal arguments was formed by the fact that lichens happen to occur on places where the gonidia are already found as free-living algae, so that the germinating ascospores may attack these algae like other parasitical fungi attack

their hosts. This argument is not correct, as Cystococcus (the goni-
dium of most lichens) is quite another alga than the free-living Pleuro-
coccus and allied species. Cystococcus only seldom occurs in a free-
living state.

Apart from the observations stated in which the algae seemed to
be harmed by their fungal partner other histological arguments seem
to speak again in favour of the reverse. A critical survey is to be
found in the book of TOBLER (1925). So BACHMANN (1923) observed,
that the formation of pyknidia by the fungus induced an increase of
the number of adjacent gonidia, a fact, which may be interpreted as
well by assuming the reverse, namely that a local increase of the
gonidia induces a formation of pyknidia by the fungus. Furthermore
we may recall the lively cell-multiplications of the gonidia in the
soredia, where they are closely surrounded by hyphae. But the
clearest histological observations of a promoting effect on the algae by
the fungus (although especially to the benefit¹of the fungus himself)
were made by NIENBURG (1917). In Pertusaria the growing rand-zone
only consists of hyphae. On the border of this rand-zone certain
gonidia are enclosed by hyphae, after which act they start vigorously
growing. After this they are pushed by these hyphae into the algal-
free randzone. In a more recent publication NIENBURG (1926) com-
pared the lichen fungus to ,,einem klugen Herrn, der seine Sklaven
— die Algen — gut füttert, damit er sie dann um so besser ausnützen
kann", a good definition of helotism.

A formative effect by the fungus upon the gonidia, without having
the right to speak about a promoting effect, was beautifully described
by GEITLER (1933—1938). P.e. in Endocarpon pusillum the algal
cells are larger in the thallus than in pure cultures, the hymenial
gonidia too are smaller but become larger after being enclosed by the
hyphae; the alga, on his part, attracts the hyphae although only over
a small distance; in Staurothele succedens the hymenial-gonidia are
formed like a Stichococcus, the gonidia in the thallus are round
(compare STAHL 1877); where blue-greens occur as gonidia the
ectoplasts are less abundant than in a free-living state; the "Nostoc"
gonidia of Placynthium nigrum in cultures turn out to be a Rivu-
laria! According to PEIRCE (1899) the gonidia of Ramalina reticulata
are not as beautiful as the free algal cells. BORNET (1873) already
described changes of the algae under the influence of the fungus.

The opposite fact, that the remarkable forms of the hyphae in the
thallus arise under the influence of the gonidia has been stressed by
MOREAU (1919—1921), who considered lichens as fungi, who are
suffering from an algal infection. By a strong resistance of the fungus
this "disease" has become chronic and seems to be a perfect sym-

biosis, but is, in reality, a reciprocal battle. He considered the lichen-thallus as an algo-cecidium.

From the above it will be clear that up till now nor the anatomical and histological, neither the few physiological investigations have been able to decide upon the old controversy between the mutualistic and parasitic theories. TOBLER (1925, 1934) is quite right when he stresses the uselessness of this controversy, while we are still ignorant about the physiology and the biology of the two components. He considers the lichen-symbiosis as a physiological equilibrium between the two components (compare ELENKIN 1906). This equilibrium is reached with the formation of a new morphological unit: the lichen-thallus and the production of new metabolic products: the lichenic acids. In many cases, however, bad ecological conditions will prevent the maintenance of this equilibrium, so that one of the partners is worsted. This physiological equilibrium is well illustrated by those cases where two algae take a part in the symbiosis like in the cepha-lodia or where two fungi compete like in the cases of parasymbiosis. In cultures it is very difficult to establish this equilibrium as either the alga or the fungus grows too fast but in nature as well failures like this may be found. So the synthesis of a lichen-thallus out of the two components only is possible on media poor in food substances (THOMAS 1939). This last investigator concluded that in the lichen-thallus the two components are living under suboptimal conditions.

The more important theories dealing with the physiology of the lichen-symbiosis are shown in the following summary:

1. The fungus thrives on the as-similation- or reserve pro-ducts of the alga (REINKE 1872, 1894, DE BARY 1879) in the following way:
   a) by diffusion through the contacts between hyphae and algal cells.
   b) saprophytically from dying algal cells (compare endo-saprophytism of ELENKIN 1902—1904).
   c. parasitically, from mate-rials obtained from algal cells by means of haustoria (BORNET 1873, SCHNEIDER 1897, PEIRCE 1899, DANI-

1. The alga thrives on inorganic salts, taken by the fungus from the substrate (REINKE 1872, 1894, V. TIEGHEM 1874, DE BARY 1879). Many other lichenologists disclaim the im-portance of this possibility.
2. The alga thrives on organic nitrogenous compounds pre-pared by the fungus from the available inorganic nitroge-nous salts (BEIJERINCK 1890. ARTARI 1902, denied by TRE-BOUX 1912).
3. The possibility of N-fixation by the fungus has never been considered.

LOV 1910, FINK 1913, 1914, SENFT 1916, NIENBURG 1917, FRY 1928, GEITLER 1933, 1934, 1937).

2. The fungus receives from the algae growth-promoting substances. Never been proved experimentally, only suggested by LEONIAN 1936, ZAKHAROWA 1938.

3. The fungus receives from the algae nitrogenous compounds as a consequence of eventual N-assimilation by the algae. This possibility has never been taken into account by any author. It has to be mentioned, however, in relation to the results obtained with other forms of symbiosis.

4. The alga uses organic substances, taken by the fungus from the substrate (CHODAT 1913, STOCKER 1927, BOYSEN-JENSEN-MÜLLER (1929).

5. The possibility of the fungus furnishing growth-promoting substances to the alga has never been suggested.

6. The fungus protects the alga against desiccation by
   a) its heavy cortex (ZUKAL 1895).
   b) its water-reserve in the „Quellhyphen" (GOEBEL 1926).

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STATEMENT OF THE PROBLEM.

The survey of the literature shows that our knowledge about the mutual relations between the two components is very scanty. There exists only one method to augment this knowledge: to cultivate both partners in pure culture and to investigate their physiological properties. Experiments like this, however, are very scanty! One of the most principal reasons for this failure has been the slow growth and the difficult isolation of the fungal components. I observed, however, that the fungi which live in symbiosis with proto-pleurococcoid algae (SCHMID 1933) in cultures resembled true lichen-fungi in many details, but that the growth-velocity of most of them was much better (QUISPEL 1942). It was evident, that these fungi formed an excellent object for the study of the symbiosis, although of course I had to be very cautious in applying the obtained results to true lichens as here the situations might be more complicated than in the more primitive symbiosis of the protopleurococcoid algae. Therefore the obtained results were always (if possible) tested on true lichen fungi. Apart from the investigation of these fungi I also examined the algal

symbionts. As the Cystococcus gonidia of most lichens are developing well in pure cultures, these lichen-components were investigated directly.

The following questions were considered:

1. the growth-requirements of the algae and the fungi. Especially the requirements of "nutrilites" [1]) were examined, as it has become known that the exchange of these substances is one of the most principal factors in a great many other symbioses, although in the lichen-symbiosis the function of these substances has never been considered (apart from some suggestions of LEONIAN 1936 and ZAKHAROVA 1938).

2. the origin of the lichenic acids. When TOBLER (1909) was right when he stated that these substances were characteristic for the symbiosis their origin had to be considered fundamentally important for the understanding of the mutual relations of the symbionts.

3. the importance of the symbiosis for the water-household of the lichen.

CHAPTER II.

THE ISOLATION AND THE DESCRIPTION OF THE SYMBIONTS.

A. THE ISOLATION AND DESCRIPTION OF THE FUNGI.

1. Literature:

While TULASNE observed and cultivated the isolated fungus partner, as already stated, in 1852, MÖLLER (1887) was the first investigator who succeeded in isolating lichen-fungi in pure culture. He made the asci ejaculate their spores into a drop of culture-solution (p.e. maltextract), while in some other cases he isolated the fungi from the spermatia. After three weeks the mycelia formed were visible with the naked eye and after a cultivation period of about three months the diameter of the mycelia varied between 1.5 and 8 mm (the latter diameter only was reached by the fungus of Arthonia vulgaris Schaer). With Buellia punctiformis Hffm. he made the remarkable observation that the spermatia gave a better growing mycelium than the asco-

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1) For the sake of uniformity I will always use the term nutrilites instead of the more usual words vitamines, hormones, growth factors, accessory substances etc.

spores, although after eight weeks the difference was no longer per-
ceptible. On transversal sections he was able to distinguish the follow-
ing layers: rhizoïds growing into the substrate, than a layer consisting
of a rather loose mycelium corresponding to the medulla of the
lichen-thallus, than a compact layer corresponding to the cortex,
while finally thin aerial hyphae rose into the air. Some hyphae con-
sisted of large, globular cells.

TOBLER (1909) described the fungus of Xanthoria parietina (L)Th.
Fr. and mentioned here too the stratified thallus and the similarity
with a lichen.

WAREN (1919—1920) described the fungi of Physcia ciliaris (L)
Th. Fr., Cladonia deformis (Ach.) Hoffm. and Lecidea fuliginea
Ach. The fungus of the Physcia was characterized by its very solid,
compact mycelia and the presence of large, globular cells, in which
he thought to observe the presence of chlorophyll (pupil of Elfving!).

The difficulties most investigators met, when they attempted to
isolate the fungal partners of lichens were so abundant, that the
publication of MÖLLER during a long time contained the only com-
plete descriptions of a number of lichen-fungi. So it could happen,
that CHODAT (1912) in apparent neglect of the work of MÖLLER con-
cluded that, in contrast to the gonidia the lichen-fungi had lost the
ability to live alone. In consequence the publications of WERNER
(1925, 1927) were fundamentally important. Partially together with
KILIAN (1924) he isolated a great number of lichen-fungi from the
ascospores, caught after ejaculation by the asci. It would go too far
to repeat all his descriptions for which I have to refer to the original
articles. I only want to mention the following points: the growth-
velocity of all fungi isolated was very low, the fungus of Baeomyces
roseus formed the only exception by forming mycelia with a diameter
of about 1.5 cm after a five month incubation; in all fungi, cultivated
on solid substrates, he observed the stratified thallus in which he
recognized the medulla, gonidial zone and cortex of the corresponding
lichen, in old mycelia cavities were formed. Furthermore he observed
rhizoïds penetrating into the substrate and aerial hyphae rising into
the air, whose terminal cells sometimes functioned as conidia. These
aerial hyphae sometimes were very long, sometimes they were lacking
in older mycelia. Some fungi formed here and there large, globular
cells; the cells rich in protoplasm were always filled with oil-drops.
The mycelia were mostly very compact and thick, the colour varied
with age and substrate, while in some cases the surrounding agar was
darkened. In liquid culture solutions they were badly developing.

The investigations of BARTUSCH (1932) and LANGE DE LA CAMP
(1933) brought little news. The latter hoped, that the fungus of

Collema glaucescens would grow better in culture than the fungi
of higher differentiated lichens. Her efforts to isolate this fungus
failed.

A great number of lichen-fungi was isolated by THOMAS (1939).
He isolated them by means of spore-ejaculations after which the
spores were brought directly upon the agar or only after germination.
Sometimes he inoculated his cultures with small hyphae, which had
been isolated with a micromanipulator. In order to distinguish the
fungi from the corresponding lichen he introduced a special termi-
nology: p.e. he called the fungus of Xanthoria parietina Xanthorio-
myces parietinae, the fungus of Physcia pulverulenta Physciomyces
pulverulentae etc., which prevented confusion between lichen and
fungus. The stratified mycelia observed by most authors were found
in his cultures too, but he was not convinced by the similarity with
a lichen-thallus as certain other fungi as well are composed of a more
compact outer layer and a less compact inner one. For the rest he
described the external habit of the mycelia on different substrates,
also grown in different temperature. All his mycelia showed an
elevated mode of growth, while, on some poor media, they were
penetrating deeply into the agar. The colour varied with the tempe-
rature and with the substrate, while often the surrounding agar was
darkened.

Moreover he described the isolation of the fungi which are living
in symbiosis with the algal-covers of Pleurococcus and allied species.
Although he did not say very much upon this subject he mentioned
that the growth of the dark coloured mycelia was better than with
the known lichen fungi and that he had been able to isolate eight
different forms.

2. **Isolation methods.**

I isolated the fungi out of the algal-covers in the following way:

a small amount of material of the algal cover was suspended in
water. As the adhering air prevents a good suspension of the mate-
rial, it was previously rubbed in a drop of water between two slides,
until all algae were moistened. The suspension was diluted to a
suitable concentration. Drops of this suspension were incubated as
hanging-drop cultures in the usual way; my intension was to obtain
hanging-drops containing only one small group of algae with ad-
hering hyphae. After a week incubation at 20° C. it appeared that
the hyphae had developed. Then the drops were examined micros-
copically to control that no other organisms had developed. When
this appeared not to be the case the drops were inoculated on a tube
containing malt-extract with 2 % agar.

After some weeks the fungi were visible with the naked eye.

Sometimes I made use of a micro-manipulator. With a small glass-capillary a group of algal-cells were taken out of a suspension of an algal-cover and brought into a drop of sterilized water. Out of this drop the group of algal cells (of course with adhering hyphae) was taken up for a second time and this manipulation was repeated five or six times, until it was likely that no other organisms were present in the drop. Then the drop was brought upon the agar. After both methods the identity of the fungi isolated as symbionts of the algae could not be doubted.

The lichen-fungi were isolated in the usual way. An apothecium was brought under a cover, which was fastened some mm above. After a day several ejaculated ascospores were visible upon this cover. They were suspended in a drop of sterilized water and with a glass-capillary one spore was isolated and transferred to a tube with malt-agar. In this way I isolated the fungus of Xanthoria parietina (L) Th.Fr.

3. Description of the fungi.

Without exception the fungi formed very hard, compact mycelia consisting of interlaced hyphae, so that they were very difficult to examine under the microscope. The structure of the mycelia could only be investigated by means of transversal sections, which were prepared according to LANGE DE LA CAMP (1933).

the mycelium was fixed with the following solution: water 100 cc, chromic acid 1 gram, acetic acid (concentrated) 1 cc, then it was embedded in paraffin and sections prepared with a microtome. As the hyphae were always distinctly coloured the sections remained unstained.

As the lack of typical fructifications did not enable me to make any classifications, I gave up attempts at classification. The descriptions refer to cultures upon malt-agar.

Fungus Pl 1; isolated from a cover of Pleurococcus vulgaris Boye Pet. on the bark of an oak in Delft (Tab.V, fig. 1, 2; text fig. 1, 2, 3, 4) solid, compact, very hard thallus, with a dark greyish-black colour in the hyphae, which colour diffuses into the surrounding agar. The young hyphae consist of thick-walled cells, 10—15 μ long, 4.5—6 μ thick, but soon oidia-like cells are formed, who are as long as they are thick. Old mycelia form a definitely stratified thallus consisting of a central layer formed by closely interlaced hyphae, in which the cells are longdrawn and a very compact exterior layer consisting of plectenchyma with isodiametrical cells in which no hyphae can be distinguished. Short aerial hyphae rise into the air, which are best
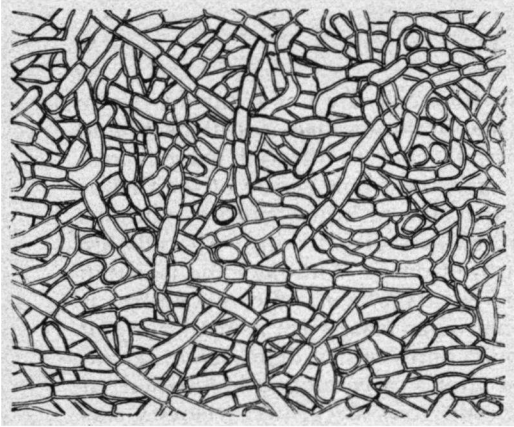
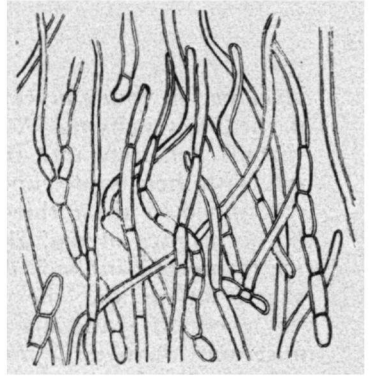Fig. 1. Interior plectenchyma transversal section.
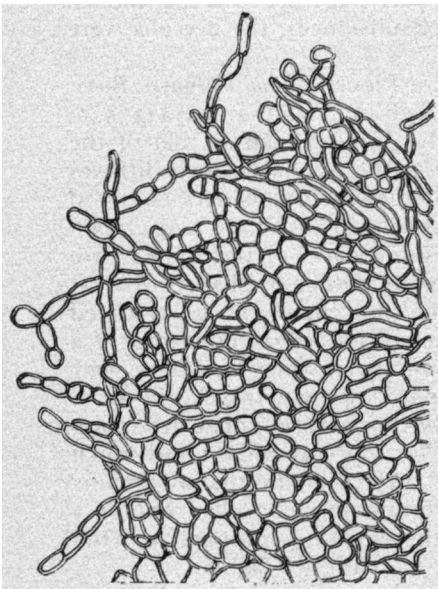650 ×

Fig. 3. Rhizoids.
650 ×

Fig. 2. Exterior plectenchyma and some
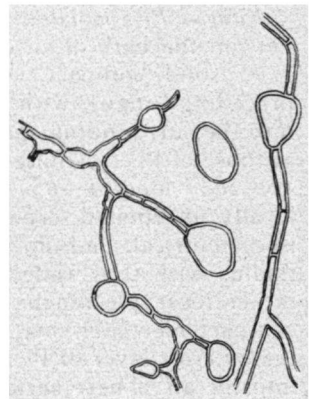aerial hyphae transversal section. 650 ×

Fig. 4. Swollen cells in liquid
culture solutions
650 ×

FUNGUS Pl I

developed in young mycelia and whose cells are not as thick as those of the plectenchyma. A great many thin-walled, locally septated rhizoids penetrate into the substrate and, as the old mycelia rest only with their border upon the agar, the whole centre of the thallus is occupied by these rhizoids. Especially in liquid culture-solutions the occurrence of big, globular cells is very striking. All cells are rich in protoplasm and filled with oil-drops. Although the isolation takes a lot of time, the growth-velocity, when once in culture, is very good. After inoculating a malt-agar plate with a small piece of mycelium (diameter 1 mm) a mycelium with a diameter of 3 cm. and a height of 1 cm. was formed after a three month incubation at 20° C. (tab. VI fig. 6). In liquids too, the growth-velocity was sufficient; in suitable liquid culture solutions in Erlenmeyer-flasks of 300 cc. containing 100 cc. liquid, mycelia were formed with a dry-weight of 1 gram after a two month incubation at 20° C. This heavy weight especially was caused by an abundant formation of an aerial mycelium upon the liquid (compare page 452). On agar media, containing only small amounts of foodsubstances, the mycelia were growing deeply into the agar.

Fungus Pl 2 isolated from a cover of Pleurococcus vulgaris Boye Pet. on the bark of an oak in Delft (Tab. V, fig. 3, 4. text fig. 5, 6, 7, 8). solid, compact, very hard thallus, not as high as that of the preceding fungus, with a dark colour in the hyphae, which diffuses into the surrounding agar. Yet the colonies are not as dark coloured as those of Pl. 1. Young hyphae consist of cells, who are long-drawn 10—20 μ long, 4—6 μ thick, while some parts of the hyphae are locally unseptated, especially at the ends. Older hyphae consist of isodiametrical, oidium-like cells. The mycelia form a stratified thallus with thin-walled rhizoids, an interior layer consisting of a rather loose plectenchyma, an exterior layer consisting of a very compact plectenchyma with not as many isodiametrical cells as in the exterior layer of Pl 1, while finally thin aerial hyphae are rising into the air. These aerial hyphae are far more abundant and much longer than those of Pl 1, so that the surface of the thallus obtains a rather wooly appearance. The protoplasm-rich cells contain a great number of oil-drops. The growth-velocity is comparable to the growth-velocity of Pl 1. (Tab. V fig. 4, Tab. VI fig. 6).

Fungus Ap 1 isolated from a cover of Apatococcus minor Edl on a stone wall in Delft. (text fig. 9) habitus as Pl 2, with the only difference, that the aerial hyphae have the peculiarity to aggregate, so that the surface shows a more prickly appearance. The hyphae are thin, only 1—3 μ thick, while the length of the cells varies from 4—14 μ, the septation is not always clearly perceptible. Here too I observed
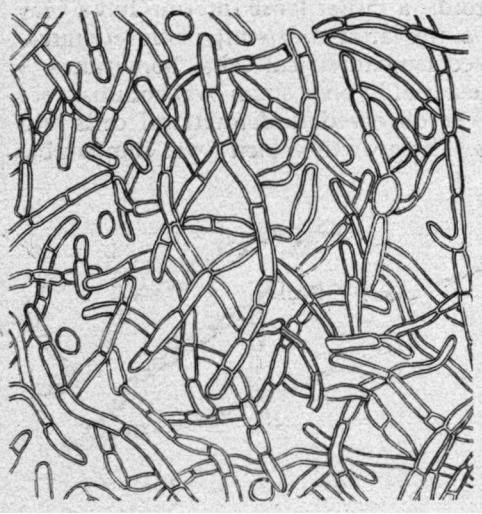
Fig. 5. Interior plectenchyma transversal
section. 650 ×

Fig. 7. Aerial hyphae.
650 ×
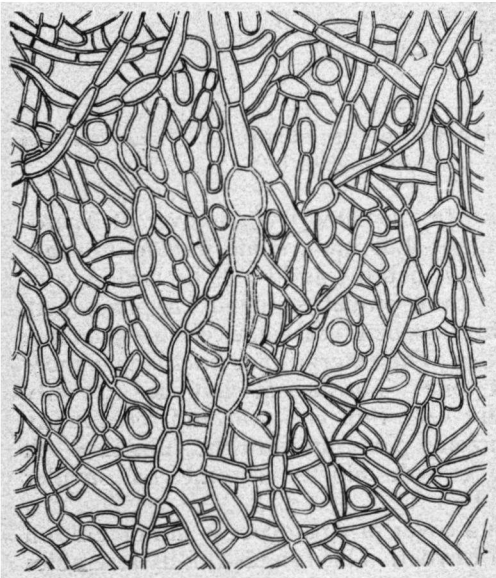
Fig. 6. Exterior plectenchyma transversal
section. 650 ×

Fig. 8. Rhizoids.
650 ×

FUNGUS Pl 2
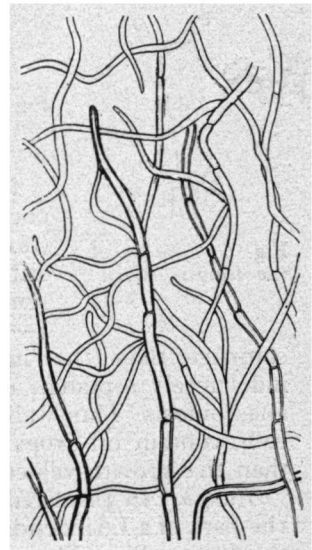
the stratified thallus with rhizoids, a rather loose interior and a very compact exterior plectenchyma, the latter consisting of closely interlaced hyphae. I never observed isodiametrical cells. Sometimes I observed, that at the end of the aerial hyphae small oval conidia were formed. In the hyphae I never observed isodiametrical cells. All cells were filled with oil-drops. The growth-velocity was comparable to the growth-velocity of Pl. 1.

Fig. 10. Hyphae of the fungus Ap 2, cultivated on malt-agar. 1000 ×.
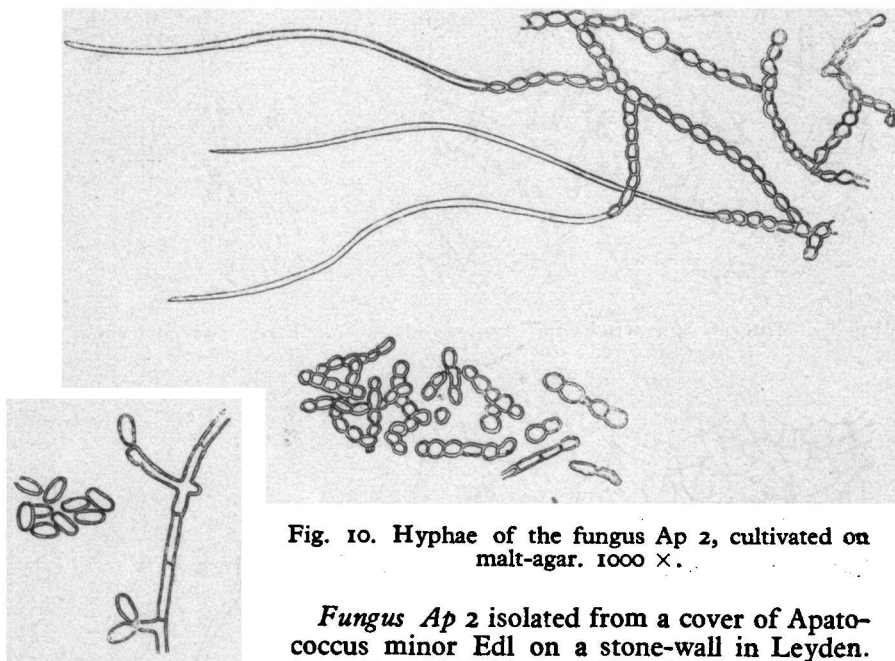
Fig. 9. Conidia of the fungus Ap. 1. 1000 ×.

Fungus Ap 2 isolated from a cover of Apatococcus minor Edl on a stone-wall in Leyden. (text fig. 10) habitus as Pl 2. The thallus consists of thin, much-septated hyphae with remarkably swollen yeast-like cells 4—9 μ long, 3—5 μ thick. The exterior plectenchyma is more compact than the interior one. The aerial hyphae are only indefinitely septated, they are very thin (1—3 μ) and form many anastomosis. Thin-walled rhizoids penetrate into the substrate. All cells contain oil-drops. The growth-velocity is somewhat smaller than the growth-velocity of Pl 1.

Fungus Ap 3 isolated from a cover of Apatococcus minor Edl. on the bark of a Liriodendron in Leyden. This fungus closely resembles the fungus Pl 1. The only difference is formed by the fact, that the

aerial hyphae are better developed, so that the surface of the thallus shows a more wooly appearance. Furthermore this fungus has the peculiarity that in very old thalli the exterior plectenchyma easily breaks down into small fragments.

Fungus C isolated from a heavy lichenized, soredium-like cover of Cystococcus spec. on the bark of an oak in Delft. As this cover must be regarded as an undeveloped lichen it is very probable, that this fungus too is a true lichen-fungus. The habit is identical with Pl 2. Microscopically the similarity with this fungus is very striking. The stratified thallus consists of a compact exterior plectenchyma with closely interlaced hyphae and short cells (4—10 μ long, 3—6 μ thick), an interior layer in which the hyphae are not as closely interlaced, rhizoids in the substrate and aerial hyphae rising into the air. The cells contain many oil-drops. The growth-velocity is not as good as the growth-velocity of the preceding species, although better than of most lichen-fungi. After inoculating a malt-agar-plate with a small piece of mycelium (diameter 1 mm.) a mycelium with a diameter of 1.5 cm and a height of 1 cm is formed after a three month incubation at 20° C. (compare Tab. VI fig. 6).

Finally I succeeded in isolating a fungus from a cover of Apatococcus minor Edl. on a stone-wall in Leyden, which was indistinguishable from the fungus Pl 1. This makes it probable, that these fungi are not specific to a certain algal species nor to certain substrates.

The only lichen-fungus I isolated from a well-developed lichen-thallus was Xanthoriomyces parietinae. Although this fungus has been described by several authors (TOBLER 1909, WERNER 1925, 1927, BARTUSCH 1932, LANGE DE LA CAMP 1933, THOMAS 1939) I will make some remarks upon my culture as there are some differences with the fungi described in literature. In agreement with WERNER (who gave the best description of this fungus) the hyphae consist of small, round cells, which only at the end grow out in the form of long, fringe-like threads (see text fig. 11). The stratified thallus already mentioned by most authors of course could be observed in my cultures too. Aerial hyphae only were visible in young mycelia as in old ones they are laying flatly upon the surface of the thallus. In old mycelia the surface of the thallus easily breaks down into small fragments. In all these respects my fungus was identical with the one described by WERNER. The most principal difference with the fungi described in literature was formed by the very dark colour of my fungus.

Although already I did mention the great similarity between true lichen-fungi and the symbionts of the aerial-algae and the better
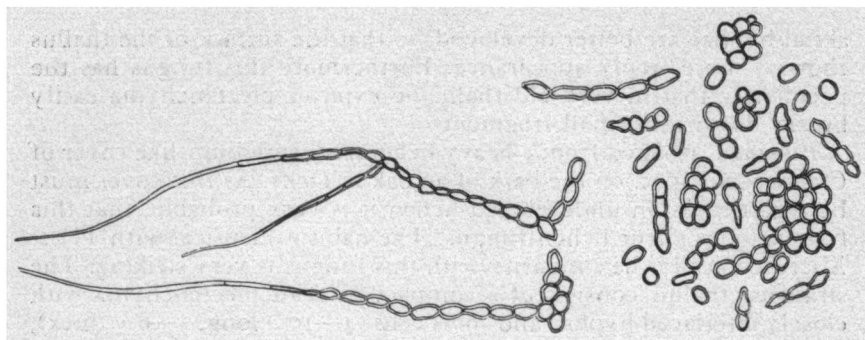
Fig. 11. Hyphae of Xanthoria parietina, cultivated on malt-agar.
1000 ×.

growth-velocity of the latter I again want to stress these facts as they
have to be considered as fundamentally important for the correct
interpretation of my experimental results. The better growth will
have been obvious from the facts mentioned. As an illustration I
refer to Tab. V, fig. 4, 5, Tab. VI, fig. 6.

As to the similarity between the symbionts of aerial-algae and
lichen-fungi: both are characterized by a solid, compact, very hard
thallus, by the thick-walled, protoplasm-rich cells, which are con-
taining many oil-drops, by the stratified thallus, while all the peculiar-
ities I described for my fungi have been described for some true
lichen fungi as well (e.g. the formation of conidia of fungus Ap 1
has been described for certain lichen-fungi by WERNER, the darkening
of the surrounding agar by WERNER and THOMAS etc.). The fungus
C, who most probably must be regarded as a true lichen-fungus can
hardly be distinguished from the symbionts of Pleurococcus- and
Apatococcus-covers and although Xanthoriomyces differed in details
the similarity with the other fungi is undeniable.

*So there are a lot of arguments to assume a close relationship between
the symbionts of aerial-algae and true lichen-fungi. The better growth-
velocity in cultures perhaps is caused by the fact that they are less
adapted to the symbiosis with an alga.*

B. THE ISOLATION, DESCRIPTION AND CLASSIFICA-
TION OF THE GONIDIA.

1. Literature.

As my experiments only were performed with Cystococcus-
gonidia (the Protococcus-gonidia of the old literature) I only will

mention those publications in which the isolation of these gonidia is described. Moreover other gonidia were seldom isolated.

The publications of FAMINTZIN and BARANETZKY (1867) and WORONIN (1872) who cultivated the gonidia of Physcia parietina and Cladonia species resp. of Physcia pulverulenta are only of a historical interest. The way in which they isolated these gonidia did not make it certain that the developing algae were the gonidia and no other algae, who only lived saprophytically upon the surface of the thallus. They classified these gonidia as Cystococcus.

The first pure culture of lichen-gonidia was performed by BEIJE-RINCK (1890). He isolated the gonidia of Xanthoria parietina by inoculating an agar-plate, containing inorganic salts only, with material taken from a section through the thallus. The infections, which developed notwithstanding the absence of organic substances were cut away, while the green colonies, which were visible after some weeks were transferred to an agar-plate containing glucose and peptones as here the development of the algae was much better.

ARTARI (1901) isolated the gonidia of Xanthoria parietina and Gasparina murorum. He classified them as Chlorococcum infusionum. According to TREBOUX (1912), however, they should be classified as Cystococcus humicola Naeg.

For the rest we observe in the older lichenological literature a remarkable neglect of the systematics of the green algae so that the Cystococcus-gonidia mostly were confused with free-living algae like Pleurococcus (Protococcus).

The first, who definitely put an end to this was CHODAT (1913). He warned for the existing danger that the isolations may be not the gonidia themselves, but closely resembling aerophilic algae, which only live epiphytically upon the surface of the thallus. Yet the method used by CHODAT did not avoid this danger. He proceeded as follows: the lichen was thouroughly washed with sterilized water, ground in a sterilized mortar and the resulting suspension diluted. With this dilution an agar-plate was inoculated. He continuously compared the developing algae with the gonidia in the thallus to be certain that they were identical. Together with KORNILOFF (1913) he isolated the gonidia of some Cladonia species. Although all these gonidia belonged to the genus Cystococcus, there were marked differences between the gonidia isolated from different Cladonia species. This investigation was extended by LETELLIER (1917), who observed important physiological differences between these gonidia.

The question arose whether every lichen-species contains its own Cystococcus species as gonidium, a problem investigated in a beautiful way by WARÈN (1920). His isolation method was much better

than that of CHODAT. He isolated the gonidia from a suspension of
the lichen with a small glass-capillary, with which they were trans-
ferred into a drop of sterilized water. From this drop they were again
transferred with the capillary into a second drop and so on until
he was certain, that no contaminating organisms were present. Then
he transferred the algal-cell to a tube with an agarized culture-
solution. Proceeding this way he was certain that the developing
algae indeed were identical with the gonidia. Most of the gonidia
isolated appeared to belong to Cystococcus, but as in the experiments
of CHODAT a great number of different species had to be distinguished.
So the gonidia of Cladonia were so different from the rest, that he
classified them in a subgenus Eleuterococcus. Generally every lichen-
species possessed its own specifical Cystococcus-species, but there
were some exceptions. For instance was the Cystococcus isolated
from a Dutch thallus of Xanthoria parietina quite different from the
Cystococcus isolated from Finnish thalli of this lichen. On the other
hand the same Cystococcus could be isolated from all Finnish thalli,
although they had been collected in quite different parts of Finland.
Of course he did not know whether the fungus of the Dutch Xan-
thoria was identical with the fungus of the Finnish specimens, so
that the problem of the specificity of the gonidia to certain lichen-
fungi could not yet be solved.

A new important contribution was given by JAAG (1929) in an in-
vestigation of the gonidia of Parmelia and Cladonia species. Here
it appeared, that every lichen-species possessed its own species of
Cystococcus. The mutual differences between the gonidia isolated
from a number of Cladonia-species were not as great as the differ-
ences between these Cladonia-gonidia and the gonidia isolated from
some Parmelia species. This specificity even was so marked, that the
same lichen-species appeared to possess another Cystococcus ac-
cording to the substrate from which it was collected. Yet this makes
it very doubtful whether the effect indeed is caused by a specificity
of the Cystococcus species to certain lichens. It seems more likely
that we only have to assume a great variability of the Cystococcus
species so that these different "species" are nothing more than
physiological and ecological races.

The publication of Miss RATHS (1938) made the supposed speci-
ficity yet more doubtful. Two of the three gonidia isolated from
thalli of Chaenotheca chrysocephala appeared to be identical with
the gonidium isolated by CHODAT from Cladonia endiviaepholia, the
third one was identical with the gonidium of Cladonia furcata!
Moreover she cultivated a free-living Cystococcus-species, which
had to be considered as a variety of Cystococcus endiviaepholiae.

Here for the first time the differences between the gonidia did not correspond to those of the lichens from which they had been isolated as was suggested by Warèn and Jaag.

Finally Thomas (1939) reached very important conclusions because he obtained pure cultures from many lichens, of the gonidia as well as of the fungi. So he was able to give the decisive proof that two thalli of Xanthoria parietina possessed the same fungus but two different Cystococcus species as gonidia. Moreover he succeeded in synthesizing a Cladonia pyxidata by bringing together the fungal component with a gonidium, which was not identical with the gonidium isolated from this lichen. So it was evident, that the much-praisen specificity of the gonidia was not as evident as had to be expected after the investigations of Warèn and Jaag. Perhaps we should assume that the genus Cystococcus consists of a great number of species and varieties. As most lichens multiplicate in a vegetative way by means of soredia and as it is by no means certain that in nature synthesis of lichens out of the two components often occur, we must not be surprised when specimens of a lichen in one country contain the same alga. Moreover it is probable, that most Cystococcus "species" are nothing more than physiological or ecological races.

The problem of the identity of certain free-living aerial-algae with certain gonidia is very important especially in relation to my experiments with the lichenized algal-covers. From the publication of Raths (1938) it is evident that indeed certain Cystococcus-gonidia may be found in a free-living state. In consequence there is no reason whatever to distinguish these free-living algae, which are described in literature as Cystococcus humicola Naeg., Cystococcus humicola Treboux, Cystococcus humicola Boye Pet., Chlorococcum sociabile Brand and Trebouxia arboricola Puymaly from the Cystococcus species occurring as gonidia in most lichens, especially because in our countries pure covers of these algae are never to be found as, in most cases, they are so heavy lichenized that they have to be considered as lichen-soredia, which in consequence of bad external situations could not differentiate into a lichen-thallus. When these covers are only lichenized in a small degree they are gradually connected with the soredial ones. In countries where, according to algo-logists, these algae are found as pure algal-covers, it would be very important to investigate whether these free-living Cystococcus consist of as many species and varieties as the gonidia.

In our countries, however, most algal-covers are not formed by Cystococcus species, but by the so-called proto-pleurococcoid algae (Brand 1925), that are all those algae, which formerly were described as Protococcus viridis or Pleurococcus vulgaris. Many authors

(CHODAT 1902, PRINTZ 1921) thought it probable that these covers were formed by more than one species, but it was BRAND (1925) who, after careful observations with the aid of vital-staining has much elucidated the classification of these forms. His observations were extended by BOYE PETERSEN (1928) and EDLICH (1936). According to the descriptions of these authors the algae found in the Dutch algal-covers should be classified under the following species:

Apatococcus Brand: cells mostly in groups of two or four cells, although there may be found many solitary cells too, the chromato-phore is parietal, clock-shaped and fills the whole cell. In some parts of the year, especially in early spring and in autumn this alga can be distinguished from the other proto-pleurococcoids by the forma-tion of aplanospores. The most servicable classification-characteristic is formed by the central ring of little globules, which is clearly visible after staining with neutral-red $\frac{1}{4}$ %. EDLICH distinguished two species: *perspicuus* with cells 7—15 μ and *minor* with cells 3—10 μ. Synonimes: Apatococcus lobatus (Chod.) Boye Pet., Pleurococcus lobatus Chodat, Pleurastrum lobatum Printz, Apatococcus vulgaris Brand.

Desmococcus vulgaris (Naeg.) Brand: cells mostly in groups of four cells, diameter 4—8 μ, while sometimes small threads are formed. The parietal chromatophore only fills a small part of the cell, a pyrenoid and a central ring are lacking, no aplanospores are formed. Synonyms: Pleurococcus vulgaris Naeg., Pleurococcus Naegelii Chodat, Protococcus viridus Wille. The principal difference with the genus Apatococcus is formed by the absence of spore-formation. (BOYE-PETERSEN). It is doubtful whether this never occurs in Desmo-coccus or has never been observed, as Apatococcus too does produce spores only very seldom and for instance never in cultures (see below).

Pleurococcus vulgaris Boye-Petersen. This alga too mostly forms groups of four cells, diameter 4—12 μ, it possesses a central chro-matophore with a marble-like surface and one pyrenoid, clearly visible after staining with JKJ. No spore-formation and no central-ring may be observed. This alga is usually called Pleurococcus vulgaris Meneghini (Pleurococcus calcarius Boye Pet.), which alga, however, forms great colonies, flat cell-plates and so on. As I did never observe them in the covers and neither BOYE PET. nor EDLICH did observe them, it is probable, that our alga is not identical with the one described by MENEGHINI. The latter alga most probably is related to the genus *Prasiola*.

Apatococcus and Pleurococcus are the most common aerial algae in the neighbourhood of Leyden and Delft. Apatococcus principally

occurred on stone walls, Pleurococcus on the bark of trees. After the dry months of the summer of 1941, all covers on the bark of the trees in the Leyden Hortus Botanicus mainly consisted of Apatococcus too. To-day no lichen is known where one of the proto-pleurococcoid algae has ever been found as gonidium. It is not impossible, however, that Endocarpon pallidum Ach. and perhaps other species of Endocarpon form an exception (GEITLER 1938). These lichens do not occur in the neighbourhood of Leyden and Delft.

2. Isolation-methods.

I isolated the gonidia by means of the method of JAAG (1929). The lichens were vigourously shaken with sterilized water to remove adhering impurities. Then the thallus was ground in a sterilized mortar with sterilized water. With a small glass-capillary having a diameter of about 50 μ and handled by means of a micromanipulator according to JANSE and PETERFI I isolated from the suspension formed an algal-cell with some adhering hyphae (this to be certain that the alga was a gonidium) and transferred this alga into a hanging-drop of sterilized water. This manipulation was repeated 5—6 times, till it was probable, that the algal-cell was not contaminated with other organisms. Herefore it was necessary to suck up and to blow out the algal-cell from the capillary with considerable force. In this way most of the adhering hyphae too were loosened from the alga, although mostly a small bit of fungus could not be removed neither after a small pressure with the glass-capillary as recommended by JAAG. Yet there were no objections to inoculate this hypha together with the algal-cell upon the agar as in most cases it did not develop. Only when I tried to isolate some proto-pleurococcoid algae in this way sometimes the fungus-partner developed instead of the alga. An objection to the presence of these hyphae was the greater danger that contaminating bacteria were present. Finally the algal-cell was transferred to a tube with a solution according to BEIJERINCK (water with 0.05 % NH_4NO_3, 0.02 % K_2HPO_4, 0.02 % $MgSO_4$ and 0.01 % $CaCl_2$) provided with 2 % glucose and 2 % agar. On media without glucose no development was observed. The tubes were incubated at room-temperature before a window at the north-side of the laboratory. The isolations were performed in autumn, as the summer-months are not fit for the cultivation of these algae, who are easily killed by high temperatures. Usually of the ten tubes inoculated one tube gave a good development, although only after a two months incubation the colonies were visible with the naked eye. The other tubes either remained sterile or they appeared to be contaminated with beautifully coloured bacteria. The developing algal colonies

were examined microscopically, while a plate of peptone-agar was inoculated with a suspension of the gonidia to controle, whether the culture was absolutely pure.

The isolation of proto-pleurococcoid algae was more difficult than the isolation of the gonidia, as had to be expected after the many failures described in literature (compare EDLICH 1936). Yet the method described above was fit for the isolation of these algae as well, although the colonies only became visible three or four months after inoculation.

3. Description of the cultures.

I isolated the gonidia from the following lichens: Xanthoria parietina (L)Th.Fr., recolted from a stone wall, Parmelia acetabulum (Neck.)Bub. and Physcia pulverulenta (Hoffm.)Nyl. both recolted from the bark of oaks [1]) and the aerial alga Apatococcus minor Edl. collected from a cover on a stone wall. Besides I made use of a culture of Apatococcus minor Edl. from the microbe-collection of the laboratory for microbiology at Delft, most probably the strain of "Pleurococcus vulgaris" originally isolated by BEIJERINCK (1898).

The three gonidia appeared to belong to the genus Cystococcus as had to be expected. So they were characterized by the following properties: round cells, with a central chromatophore with a lateral notch, a lobated outline and a central pyrenoid. Between the cell-wall and the chromatophore small grains were visible especially after staining with neutral-red. On agar-media without glucose in the light, the chromatophore was better developed and the pyrenoid better visible, than after development upon agar-media containing glucose. They reproduced in the following ways:

1. seldom by simple vegetative cell-multiplications.
2. mostly by the formation of autospores. We may distinguish two ways:
a) the cells divide into a small number of daughter cells (mostly eight).
b) the cells divide into a great number of spores.

According to WARÈN the first type of spore-formation is typical for the subgenus Eucystococcus, the second type for the subgenus Eleuterococcus. Nevertheless I observed both types in all gonidia isolated.
3. by the formation of zoospores. This way of reproduction can only be found in very young cultures. The mother-cell divides into a great many small cells, which when released have an oval form

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[1]) I am indebted many thanks to Mr R. A. Maas Geesteranus biol. drs., who kindly classified the lichens used.

with a pointed end on which the cilia are implanted. In old cultures no typical zoospores are formed, the cells already become round when yet being enclosed in the mother-cell so that at this moment this type of reproduction cannot be distinguished from the type mentioned as 2b.

4. by the formation of isogametes. These gametes are formed in an analogous way as the zoospores, but they copulate before developing. For the rest I refer to the descriptions and the illustrations given by CHODAT (1913), WARÈN (1920) and JAAG (1929).

The three gonidia mutually differed in the dimensions of the cells but especially in the form and the colour of the colonies on agar-media (Tab.VI, fig. 7 and text fig.12). The growth-velocity of the Xanthoria Cystococcus was somewhat smaller than the growth-velocity of the two other gonidia. I now give the description of the three gonidia:
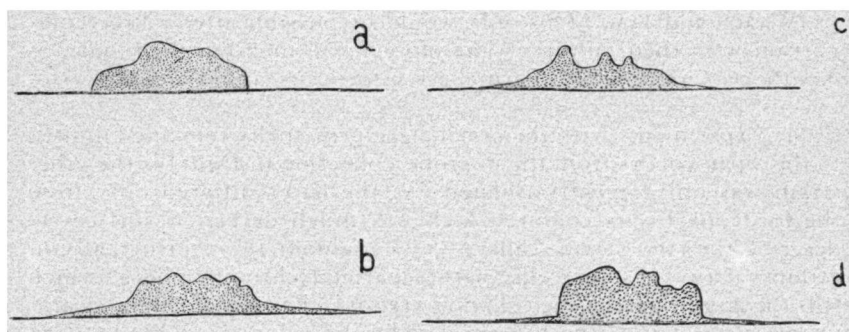
Fig. 12. Schematic transversal sections through the colonies of the three gonidia. *a*)Xanthoria parietina Beijerinck agar 2 % glucose in the light, *b*) Physica pulverulenta on Beijerinck agar 2 % glucose in the light, *c*) Physcia pulverulenta on malt-agar in the dark and *d*) Parmelia acetabulum on Beijerinck agar 2 % glucose in the light.

Cystococcus from *Xanthoria parietina* (L)Th.Fr.

In the light on BEIJERINCK-agar with 2 % glucose the colonies are shining-green with a soft, regulary wrinkled surface, in the dark on malt-agar the colour is dark-green and the surface is smooth. The colonies always have a considerable thickness, while the sides stay vertically upon the substrate. The diameters of the grown-up cell amount to 10—17 μ.

Cystococcus from *Parmelia acetabulum* (Neck.) Bub.

In the light on BEIJERINCK-agar with 2 % glucose the colonies protrude like small hills, the colour is vividly-green, the surface is

rather granular with a great many deep wrinkles. In young colonies the sides are steep, in older cultures they are spreading over the agar In the dark on malt-agar the colour of the colonies is somewhat darker. The dimensions of the grown-up cells amount to 14—18 μ, autosporeformations mainly as described at 2b.

Cystococcus from *Physcia pulverulenta* (Hoffm.) Nyl.

In the light on BEIJERINCK-agar with 2 % glucose the colour of the colonies is light-green, but in older colonies sometimes dark-green points are formed. The surface looks like small, rounded hills, while the sides of the colonie are spreading over the surface of the agar, showing a concentric structure. In the dark on malt-agar the colonies are dark-green, less high than in the light with deep wrink es over the surface. The dimensions of the grown-up cells amlount to 16—24 μ.

I gave up trying to identify my algae with the species described by WARÈN and JAAG. This only would be possible after a directcomparison with their cultures. Therefore I will mention them only as Xanthoria-Cystococcus, Parmelia-Cystococcus and Physcia-Cystococcus.

My experiments with the aerial algae principally remained limited to the Apatococcus from the microbe-collection in Delft, as the other strain was only recently isolated. As the latter differed only from the first one by its colour, which was much darker, it suffices to describe the first strain: cells 4—15 μ (seldom 18 μ), principally in groups of two or four cells, with a parietal chromatophore, which fills the largest part of the cell, no pyrenoid, the central ring is clearly visible after staining with neutral-red. The colonies on BEIJERINCK-agar with 2 % glucose in the light have a dull-green colour with an irregularly wrinkled surface, the sides of the colonie are steep. I never observed any spore-formation; BRAND too only observed spores in nature. I doubt whether these spores indeed belong to Apatococcus. Perhaps they are formed by another alga, which resembles Apatococcus, as many other algae may form sarcina-like groups of cells. When I tried to isolate the aplanospores with the micromanipulator the developing alga appeared to be: Chlorella ellipsoidea Gern. (Chlorococcum murorum Brand). When indeed the so-called spore-formation of Apatococcus is no reality the most principal difference with the other proto-pleurococcoids, especially with Desmococcus, would be invalid. This problem may be only solved by means of more extensive culture-experiments.

C. SYNTHESIS OF THE SYMBIOSIS.

Originally I intended to produce lichens or lichen-like stages by bringing together the two partners, especially for the experiments concerning the production of lichenic-acids. Furthermore the purposes of these synthesis were to establish the identity of my fungi as symbionts and to answer the question, whether the lichenized algal-covers could develop into true lichens, when the external situations were improved. The methodological difficulties, however, were so great, that this program could be realized only to a very small degree.

1. Literature.

In the survey of the general literature I already mentioned the old literature bearing upon this subject; I then showed, that only STAHL was able to synthesize a lichen-thallus, because he made use of a very good object and that the experiments of BONNIER (1877), who synthesized a great many lichens have been critisized in more recent publications (CHODAT 1913, THOMAS 1939). As, however, he was the only investigator who pretended to have synthesized these lichens in pure culture it is worth-while to mention the methods used. He brought together the alga and the fungus on a piece of sterilized bark or stone, which was hung over water in "Freudenreich flasks", while, when they were brought into a stream of sterilized air the results were far more successful. It is almost inconceivable how these cultures obtained the required salts as they were hung over the water and the air had passed some plugs of cotton-wool. However it cannot be doubted that in this way lichens could develop, although it is by no means certain that all these lichens were the results of synthesis as his algal-material certainly was very impure. All other investigators only could synthesize the beginning of a thallus, a lichen was never formed. TOBLER (1909) imputes this failure to the difficult establishment of the physiological equilibrium, so that either the alga or the fungus are growing too fast. His synthesis too did not pass the beginning of a thallus-formation. The illustrations given by his pupil BARTUSCH (1933) are very incredible. WERNER (1927) could not resynthesize the lichens from which he had isolated the fungi, either these fungi did not react upon the presence of the algae, or the algae were attacked. The experiments of THOMAS (1939) again are fundamentally important. He imputes the failure of many syntheses to the use of wrong substrates, whose contents of water or of food-substances are too high. When we know that after bringing soredia upon an agar-plate the two components develop individually we cannot expect, that a synthesis out of the components is to be

obtained on substrates like these. The following methods however were more successful: small pieces of pine-wood or elder-pith were partially immersed in 1.5 % Knop-agar and sterilized. The part not submersed in the agar was inoculated with the alga and the fungus. Although a lichen-thallus was never formed, the formation of soredial stages could be observed. When he made use of smaller flasks and decreased the concentration of the agar to 1 % the results were more successful. Elder-pith appeared to be the most suitable substrate. Although usually the production of small thalli was not surpassed, in one of the flasks a Cladonia developed with beautiful podetia. This was the first observation of the synthesis of a lichen out of the two components in an absolutely pure culture. Yet the method still was far from ideal, as in none of all other flasks (800!) such a beautiful result could be obtained, so that the success of the synthesis was entirely due to chance. Finally he made the following remarks about the methods to be used in future: a lichen only is possible under suboptimal conditions for both components. So the development of the two components may be favoured in no way whatever. The poorer the substrate, the better are the results. Furthermore he advised to change the humidity of the cultures within short intervals.

2. Own observations.

a) *Experiments with agar-media.*

A beginning synthesis only was possible on tap-water-agar. In agreement with the results of THOMAS other substrates as glucose-agar or malt-agar were not fit as the fungi grew individually along the gonidia and did not enclose them. On tap-water-agar I observed marked contacts between the fungus Pl 1 and Apatococcus minor, so that under the microscope no difference with the natural symbiosis was perceptible (text fig. 13). Afterwards the algae were enclosed in cavities of the mycelium (text fig. 14), but finally the algae were overgrown by the fungus. With the other fungi the results were not as beautiful. No synthesis with the Cystococcus species could be obtained as on these humid agar-media the formation of spores was too abundant.

b) *Experiments on plaster*:

On pieces of plaster soaked in sterilized tap-water the results were identical with those obtained on agar. This substrate too is too humid.

c) *Experiments according to* BONNIER.

The experiments of BONNIER with small stones hung over water in

447
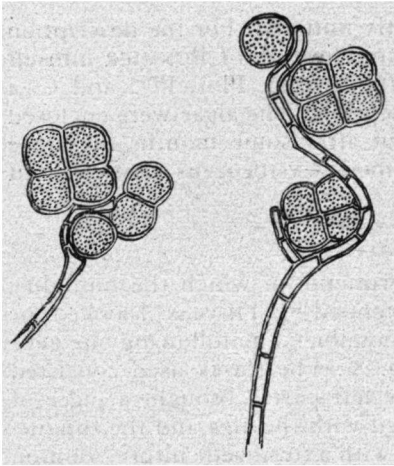
Fig. 13. Beginning synthesis of the fungus Pl 1 with Apatococcus minor on tap-water agar. 1000 ×.
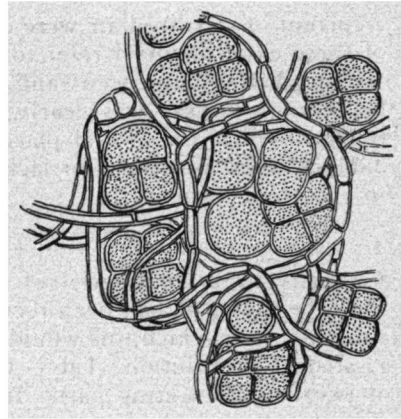
Fig. 14. Advanced stage of synthesis of the fungus Pl 1 with Apatococcus minor on tap-water agar. 1000 ×.
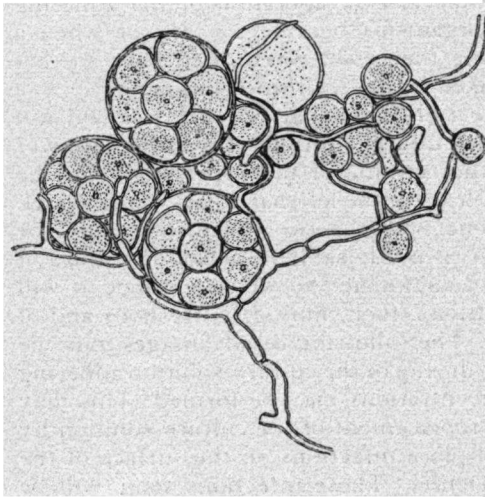
Fig. 15. Synthesis of the fungus C with the Cystococcus of Physcia pulverulenta on pieces of plaster in Beijerinck solution in the apparatus of table VI fig. 8. 1000 ×
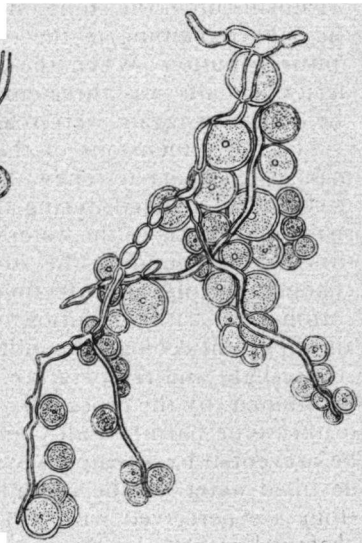
Fig. 16. Synthesis of Xanthoriomyces parietinae with the Cystococcus isolated from Xanthoria parietina on a piece of plaster in Beijerinck solution in the apparatus of table VI fig. 8 1000 ×.

a current of sterilized air were exactly imitated. For the description of these experiments I refer to the publication of BONNIER himself (1877). With Apatococcus and all fungi used (Pl 1, Pl 2 and C) a beginning synthesis was clearly perceptible. The algae were enclosed by the hyphae on several places but after some months the algae died off most probably for lack of food. Cystococcus was not used in these experiments.

d) *Experiments with varying humidity.*

Finally I started a series of experiments in which the humidity could be varied as had been recommended by THOMAS. I looked for a method with which this would be possible. The following one gave a satisfactory solution (Tab. VI, fig. 8). The flasks used consisted of two communicating parts. In the left part I brought a piece of stone or plaster, which was inoculated with the alga and the fungus. The other part of the flask was filled with a sterilized culture-solution (p.e. BEIJERINCK-solution or tap-water) till the base of the stone in the left vessel was immersed. The whole flask was mounted upon a little shelf, which could revolve and was fastened on a stand. By making capsize the flask the liquid in the left part fell, so that the organisms upon the stone only could live from the air-humidity and the salts remaining in the stone after evaporation of the adhering culture-solution. When the organisms became too dry or when a deficit of salts was threatening the vessel was turned back so that the organisms again were soaked by the culture-solution.

The big dimensions of the right-hand part of the flask did not make it necessary to renew the culture-solution, if possible however it could be changed during the running of the experiment. By the rising and falling of the water-level in the left-hand part of the vessel, the air, surrounding the stone with the two organisms, could be changed. To prevent infections it is advisable to poison the plugs of cotton-wool with a solution of sublimate. So we can change at will all important ecological conditions: food, humidity, fresh air and of course light and temperature. The following disadvantages must be mentioned: by the repeatedly drying of the culture-solution adhering to the stone harmful salt-concentrations may be formed. This may be prevented by a temporary replacement of the culture-solution by destilled water. In the second place infections on the surface of the stone are perceived with difficulty. These infections soon will be observed in the culture-solution too and when we control the sterility of this solution from time to time the sterility of the surface of the stone is sufficiently guaranteed. That the results (text fig. 15 and 16) are not as beautiful as had to be expected is caused for the main

part by my short practice with this method and by the town-atmos-
phere in which no lichens can develop. A striking success is only to
be expected, when the syntheses take place in free nature but as
they have to be regularly observed this was impossible. The first
stages of the symbiosis always were beautiful to observe; a synthesis
between the two components of Xanthoria parietina was developing
well, but became infected as I had not poisened the cotton-wool with
sublimate. Furthermore it appeared possible to synthesize the sym-
bionts of the algal-covers with the gonidia, from which sometimes a
soredial mass developed. Unfortunately these syntheses died off
during the summer-months.

The synthesized symbioses were too young to allow for experi-
ments. Yet they proved the identity of my fungi as symbionts, while
the last mentioned results made it probable that they are able to
produce higher developed stages of lichenisation. Whether this is
possible with Apatococcus and Pleurococcus is doubtfull, "soredia"
only were formed with Cystococcus, while in nature too soredia with
the proto-pleurococcoid algae have never been observed. Only more
syntheses may answer this problem.

D. CONCLUSIONS.

I have observed that the fungi which are living in symbiosis with
proto-pleurococcoid algae resemble true lichen-fungi in so many
details, that a close relationship has to be accepted. On the contrary
the proto-pleurococcoid algae themselves have never been found in
lichens, where mostly Cystococcus species occur as gonidia. From
artificial synthesis of the symbiosis it appeared that the symbionts of
these aerial-algae not only can live in symbiosis with the proto-
pleurococcoid algae, but too — and perhaps better — with Cysto-
coccus species. Further experiments must show, whether they can
produce true lichens. This is probably the case; firstly in consequence
of their observed relationship with true lichen-fungi, secondly be-
cause in nature a gradual transition is to be observed between algal-
covers and crustaceous lichens. I often found in Apatococcus covers
lichen-apothecia without the slightest indication of a thallus. A
microscopical examination did make it very probable, that these
apothecia had been formed by the symbionts of the aerial algae.
Trials to cultivate the fungi from the ascospores failed, so that the
definite proof could not be given.

With the necessary reserve I should like to interpret these liche-
nized algal-covers as follows: lichen-fungi who in consequence of
infavourable ecological conditions and the absence of their partner

Cystococcus cannot produce a lichen and do not get enough organic food to live saprophytically from the substrate entered into a symbiosis with proto-pleurococcoid algae. When, however, Cystococcus is present, the "surrounding" of this alga by the fungus will be far more enthusiastic and when the ecological conditions are favourable a lichen will be formed. Again I want to stress the hypothetical character of this interpretation.

The symbionts of the aerial-algae may be cultivated with more success than true lichen-fungi. This can be explained by assuming, that these symbionts are less adapted to the symbiosis than their lichen-forming relatives. By their better growth in cultures they are very well adapted for the investigation of the symbiosis. However, it is possible that in true lichens the relations between the two partners are more complicated; for this the difference in the growth-velocity is already an indirect indication.

The experiments described in the following pages were chiefly made with these symbionts of aerial algae, but if possible the principal results were tested upon Xanthoriomyces parietinae, the only true lichen-fungus I obtained in pure culture.

On the other hand I experimented with the algal-partners. These experiments, however, were performed with true lichen-gonidia as — for the lichen symbiosis — the proto-pleurococcoid algae are only of minor importance.

CHAPTER III.

THE GROWTH-REQUIREMENTS OF THE SYMBIONTS.

A. THE GROWTH-REQUIREMENTS OF THE FUNGI.

1. Literature.

There are only few publications referring to the growth-requirements of lichen-fungi in pure culture. From the many isolations of lichen-fungi we have to conclude, that apparently they behave like normal saprophytes, which are able to develop upon a great many organic substrates. Only in some recent publications some attention has been paid to the influence of the composition of the substrate upon the development of the lichen-fungi. So WERNER (1927) observed, that they prefer peptones to inorganic nitrogenous compounds, while they are able to liquify gelatine. THOMAS (1939) systematically compared the development of all isolated fungi upon four substrates to wit: malt-agar, peptone-agar, glucose-agar and

1/3 Knop-agar. Although he only intended to use the differences in the development upon these four media as characteristics for the classification, he too drew some conclusions as to the food-requirements of the fungi, which were, however, of little importance in consequence of the great complexity of his media. He divided his lichen-fungi into three groups: fungi belonging to the first group grew best upon malt-agar followed by peptone-agar, fungi belonging to the second group grew best on malt-agar followed by glucose-agar. The third group, only represented by one fungus Stereocaulomyces developed as well upon all substrates even upon the inorganic Knop-agar. It was evident, that this fungus could use organic substances of the agar, a fact already mentioned by WERNER for the fungus of Baeomyces roseus.

2. Own observations.

a) *methods*. As agar-media are not fit for quantitative experiments as firstly the agar is contaminated with many organic and inorganic substances and secondly upon solid substrates the cells are developing under quite inhomogenous conditions most experiments were performed in liquid culture-solutions. One could oppose that in this way the fungi are developing under very innatural conditions, but as I never found any differences, when I compared certain results with the results obtained in the same experiments performed upon agar-media or upon pieces of plaster soaked in culture-solution I do not think it necessary to make use of these more complicated and less reliable methods. As a culture-solution I used the solution according to CZAPEK-DOX, which has the following composition: 1000 cc H_2O, 50 gr. glucose, 2 gr. NH_4NO_3, 1 gr. KH_2PO_4, 0.5 gr. NaCl, 0.5 gr. $MgSO_4$. 7 H_2O, 0.02 gr. $FeCl_3$. All cultures were incubated at a room-temperature of about 15—20° C.

The homogenous inoculation of the experimental series was somewhat difficult as the fungi did not produce spores and the very hard mycelia could not be divided into equal parts. Usually I brought a small piece of mycelium in some cc. sterilized water and shook vigourously. So a suspension of loosened hyphae and small bits of mycelium was formed. After some minutes the latter had sedimentated, after which with a calibrated pipette equal amounts of the suspension were transferred to the flasks with the culture-solutions. With this method I obtained reliable results. The dry weight of the mycelia was measured by filtering the mycelium through a Buchner-funnel and transferring the wet mycelium to a watch-glass. Then the mycelium was dried in a vacuum-desiccator over $CaCl_2$ in a thermostat at 40° C. or in some cases to be mentioned by heating at

105° C. It appeared, that the dry weight was extremely dependent of the amount of aerial mycelium, which had developed upon the liquid, as the weight of the submersed hyphae was much smaller. This aerial mycelium only developed after a long incubation at optimal circumstances. As an abundant development of the aerial mycelium caused inreliable results it was advisable to examine the cultures after a one month incubation, when no aerial mycelium had been formed and the cultures were too old to make differences in the inoculation-strength influence the results.

- b) *the p_H of the cultures.* The p_H of the CZAPEK-DOX solution was sufficiently buffered; after sterilisation it amounted to 3.5 (measured with Lyphan indicator paper and an ionometer according to TRE-NELL). In some preliminary experiments the fungi Pl 1, Pl 2 and C were inoculated into solutions in which the KH_2PO_4 was replaced partially by H_3PO_4, K_2HPO_4 or K_3PO_4, so that the p_H of these solutions varied from 2.1—6.6. The development of the mycelia hardly was influenced by these changes of the p_H. As in all experiments to describe the p_H was situated between these limits and mostly was $\pm$ 3.5, the results certainly were not influenced by changes of the p_H of the culture-solutions. The fungi did not change the p_H of the solutions during their development with the exception of the fungi developing upon solutions with peptone as an only source of carbon. These solutions were made definitely more alcaline, most probably by a production of amines or other alcaline nitrogenous compounds out of the peptones (see page 460).

c) *the necessity of nutrilites.* Although all fungi developed well in solutions like malt-extract the development in the synthetic solution was very scanty. The replacement of the inorganic nitrogenous salts by asparagin did not better the results nor did the replacement of the glucose by other carbohydrates or poly-alcohols. The addition of small amounts of yeast-autolysate caused a good development. This yeast-autolysate was prepared in the following way:

    1 kg of bakers-yeast was ground in a mortar and suspended in 1 L. of water, the suspension was put into a thermostat of 55° C, where it was left for 24 hours, afterwards boiled and filtered. By addition of NaOH 30% the suspension was neutralized till the p_H was 6.4, boiled again and filtered and sterilized in an autoclave at 120° C, during 15 minutes.

The addition of peptones (Poulenc or Witte), which are always contaminated with nutrilites produced a similar effect. As I already mentioned in a previous communication (1942), with some fungi the addition of aneurin had a similar result, with other fungi however the effect of aneurin was either nil or remained far behind the effect

of the yeast-autolysate or the peptones. The behaviour of the fungi investigated is evident from table 1.

<div align="center">TABLE I.</div>

Dry weight of the mycelia of the fungi Pl 1, Pl 2, Ap 2, Ap 3, C and Xanthoriomyces in mg after a two month incubation at room-temperature in Erlenmeyer-flasks of 100 cc containing 25 cc Czapek-Dox solution. The fungus C (other experimental series) was cultivated in Erlenmeyers of 300 cc containing 100 cc liquid.

	control	10γ % aneurin	1 mg % peptones	1 cc% yeast-autolysate
fungus Pl 1	0.5	76.0	43.0	48.0
fungus Pl 2	0.1	1.5	28.0	47.0
fungus Ap 2	0.3	21.0	20.0	50.5
fungus Ap 3	0.0	0.0	22.0	44.0
fungus C	15.0	95.0	——	175.0
Xanth. par.	0.0	0.2	1.5	30.0

So it appears, that the fungus Pl 1 is the only one, where the effect of yeast-autolysate can be fully reproduced by aneurin, the effect of the yeast-autolysate even is somewhat smaller, which perhaps is caused by the presence of poisonous substances. The fungi Ap 2 and C are highly favoured by aneurin, but this effect is not as pronounced as the effect of the yeast-autolysate. The fungi Pl 2, Ap 3 and Xanthoriomyces hardly react upon the presence of aneurin, so that here other substances must account for the activity of the yeast-autolysate. In order to investigate the nature of these substances I modified the yeast-autolysate in the following ways.

1 : 25 cc of yeast-autolysate were boiled twice with norit, filtered, shaken with norit when cold, again filtered and filled up to 25 cc.

2 : 50 cc of yeast-autolysate were provided with a saturated solution of lead-acetate till no further precipitation was observed, the superfluous lead was removed with H_2S, the precipitate centrifuged, the H_2S removed with a current of air and the remaining liquid evaporated till the volume amounted to 50 cc.

3 : 25 cc of yeast-autolysate were provided with a saturated solution of $Ba(OH)_2$ till no further precipitation was observed, the superfluous $Ba(OH)_2$ removed with a current of CO_2, the precipitate centrifuged and the remaining liquid evaporated till the volume amounted to 25 cc.

4 : 25 cc of yeast-autolysate were provided with some drops of H_2O_2. After some hours the H_2O_2 was removed by boiling. The danger exists, that this solution still contained some H_2O_2.

5 : 50 cc of yeast-autolysate were made alcaline with some cc 30%

KOH, the solution filtered and sterilized at 120° C during 15 minutes. Then again the solution was filtered and if necessary filled up to 50 cc.

6 : 10 cc of yeast-autolysate were mixed with 90 cc alcohol 96 %. In consequence the solution obtained contained 86 % alcohol. The precipitate was removed by centrifuging, the alcohol distilled off and the remaining liquid brought to 10 cc.

7 : 25 cc of yeast-autolysate were shaken with 25 cc ether c.p. in an extraction-funnel. The precipitate formed between the two layers was filtered, the ether-layer again shaken with water and the water-layer again shaken with the ether, the ether- and water-layers were both brought to a volume of 25 cc.

All solutions described were neutralized till the p_H amounted to 6.4 and of each solution $\frac{1}{4}$ cc was added to 25 cc CZAPEK-DOX solution. The effect of these fractions upon the fungus Pl 2 is clearly visible from table II.

. TABLE II.

Dry weight of the fungus Pl 2 in mg. after a one month incubation in Erlen-meyer-flasks of 100 cc provided with 25 cc Czapek-Dox solution.

without yeast-autolysate		0.1 mg
yeast-autolysate		10.4 mg
—	shaken with norit	8.3 mg
—	precipitated with lead-acetate	9.3 mg
—	precipitated with Ba(OH)$_2$	0.7 mg
—	oxidized with H$_2$O$_2$	0.0 mg
—	heated with KOH	0.1 mg
—	precipitated with alcohol	11.3 mg
—	shaken with ether : water-fraction	11.5 mg
—	shaken with ether : ether-fraction	4.4 mg

The results need no commentary, the active principle cannot be removed with lead-acetate or alcohol, and is perhaps in a very small amount soluble in ether. Adsorption at norit only partially affects the activity, perhaps by the adsorption of aneurin. It is removed or destroyed by heating with KOH, oxidation with H$_2$O$_2$ and precipitation with Ba(OH)$_2$. The active substance resembles the factor MR in some details (SCHOPFER 1934, SCHOPFER and MOSER 1936, JANKE and SORGO 1939).

Thereupon I tried to identify the active substance with a number of nutrilites and amino-acids. Many well-known nutrilites were excluded by the results of the preceding experiments. I am indebted much thanks to prof. dr. F. KÖGL, who kindly procured the following solution of biocomponents : 1 cc water, 10 mg meso-inosite, 80 γ

aneurin, 80 γ adermin, 2 mg β alanin, 0.2 γ biotin and 12 γ pantho-
tenic acid. This solution partially was diluted 1 : 100 and this dilution
added to the cultures.

TABLE III.

Dry weight of the fungus Pl 2 in mg after a one month incubation at room-
temperature in Erlenmeyer-flasks of 100 cc provided with 25 cc Czapek-Dox
solution.

control	2.0 mg
1 mg % glycocoll	1.5 mg
10 mg % glycocoll	3.0 mg
1 mg % tyrosin	3.0 mg
10 mg % tyrosin	3.5 mg
1 mg % cystein	1.5 mg
10 mg % cystein	5.0 mg
1 mg % β alanin	17.5 mg
10 mg % β alanin	23.0 mg
10 mg % β alanin + 10 mg % glycocoll	
+10 mg % tyrosin + 10 mg % cystein	13.5 mg
0.25 cc % undiluted biossolution	13.5 mg
10 γ % hetero-auxin	6.0 mg
1 cc % yeast-autolysate	25.5 mg

So it appears, that the activity of the yeast-autolysate can be
accounted for by β alanin. As the effect of the yeast-autolysate is
only reached in high concentration the latter perhaps contains some
co-nutrilites or another related substance, which is physiologically
more active than β alanin. The addition of other amino-acids or other
bios-substances did not improve the result. Heteroauxin had a very
faint influence upon the development of this fungus.

When it was known, that one of the fungi was markedly favoured
by the presence of β alanin, this substance was added to cultures
of some other fungi as well.

TABLE IV.

Dry weight of the fungi Ap 2, Ap 3, C and Xanthoriomyces in mg after a one
month incubation in Erlenmeyer-flasks of 100 cc provided with 25 cc Czapek-
Dox solution with 10 γ % aneurin.

	Ap 2	Ap 3	C	Xanth.
control	4.5	0	19.8	0
1 mg % β alanin	5.4	7.8	10.3	0
1 cc % yeast-autolysate	10.1	12.7	24.0	2.5

While the fungus Ap 3 behaves identically with Pl 2, the fungi
Ap 3, C and Xanthoriomyces do not react to the presence of β alanin.

Especially for the latter fungus it is evident that the effect of the yeast-autolysate is caused by a factor yet unknown. As this is the only true lichen-fungus I possessed, it seemed worth-while to investigate whether the effect of the yeast-autolysate upon this fungus also is caused by a nutrilit.

<div align="center">TABLE V.</div>

Dry weight of the fungus Xanthoriomyces parietinae in mg after a three month incubation in Erlenmeyer-flasks of 100 cc provided with 25 cc Czapek-Dox solution.

control	2.3 mg
1 cc % concentrated bios-solution (see page 454)	33.5 mg
0.1 % peptone (Witte)	8.4 mg
0.1 % peptone + 10 γ % hetero-auxin	12.6 mg
0.1 % peptone + 1 cc % biossolution	35.1 mg

From table V it is evident, that the inability of the fungus of Xanthoria parietina to develop in synthetic culture-solutions is caused by the necessity of a nutrilit belonging to the bios-group. I have not further investigated, which bios-substance is responsible for this effect.

So we may conclude, that all fungi investigated cannot develop in synthetic culture-solutions as they have lost the power to synthesize some nutrilites. Partially these nutrilites could be identified with aneurin, β alanin and a yet unknown bios-substance.

Are these nutrilites absolutely necessary for the development or do they only augment the growth-velocity of the fungi? This question is essentially important in relation to the slow growth of lichens in nature. When these nutrilites would only augment the growth-velocity of the fungi and a small development without these nutrilites would be possible, there would be no reason to suppose, that in nature these fungi are provided with these substances. When, however, the presence of these nutrilites is absolutely indispensable, we must assume that in nature too they obtain them in some way. The very scanty development in synthetic culture-solutions already did make it probable that without nutrilites no growth at all is possible; the small development obtained could be wholly accounted for by the presence of nutrilites in the inoculation-material, taken from a culture upon malt-agar! The definite proof only could be given by the growth-curves on substrates with suboptimal concentrations of the nutrilites (FRIES 1938). When the nutrilites are absolutely necessary they must form a limiting factor when secured in suboptimal concentrations, so that the development of the mycelia soon will stop. When on the contrary the nutrilites only augment the

TAB. V.
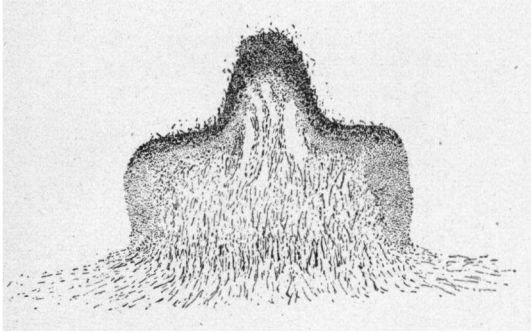
Fig. 1. Transversal section through a mycelium of the fungus Pl 1, showing the stratified thallus (somewhat schematized). 6 ×
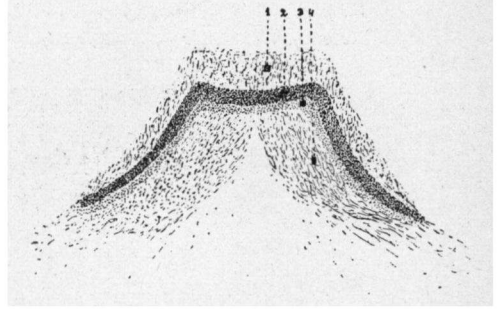
Fig. 3. Transversal section through the mycelium of the fungus Pl 2, showing the stratified mycelium, somewhat schematized 6 ×.

Fig. 2. Detail of the transversal section of fig. 1 showing rhizoids, the plektenchymous layers of which the exterior layer is the most compact, and some aerial hyphae. 60 ×

Fig. 4. Mycelium of the fungus Pl 2 after a three month incubation on malt-agar. The agar-plate had been inoculated with a small piece of mycelium (diameter 1 mm).The dark colouration of the surrounding agar and the lobated thallus are clearly visible.
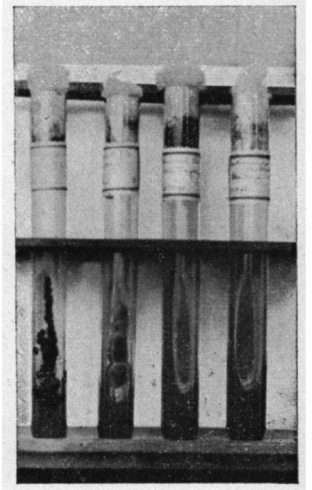
Fig. 5. Four tubes with malt-agar, simultaneously inoculated with ± equal amounts of the fungi (from left to right) Pl 1, Pl 2, C and Xanthoriomyces, after a one month incubation. Whilst the fungi Pl 1 and Pl 2 have well developed, in the cultures of the two other fungi hardly any development is perceptible.

TABLE VI.
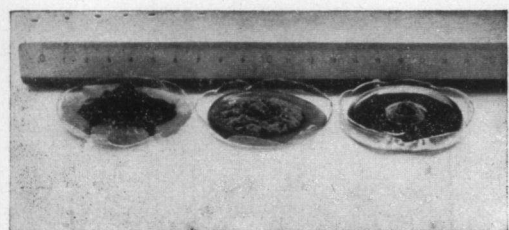
Fig. 6. Mycelia of the fungi (from left to right) Pl 1, Pl 2 and C after a three month incubation. The malt-agar plates had been inoculated with small pieces of mycelium (diameter 1 mm).
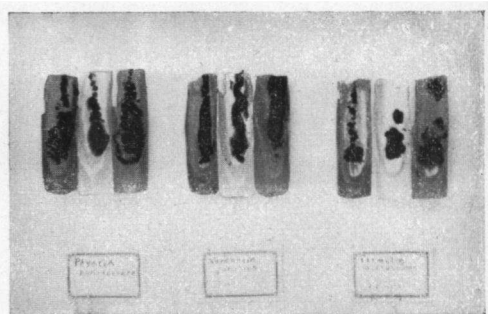
Fig. 7. The gonidia of Physcia pulverulenta, Xanthoria parietina and Parmelia acetabulum. Of each group of agar-tubes the left is malt-agar (incubated in the dark), the central Beyerinck agar 2 % glucose and the right pepton glucose agar, both incubated in the light.
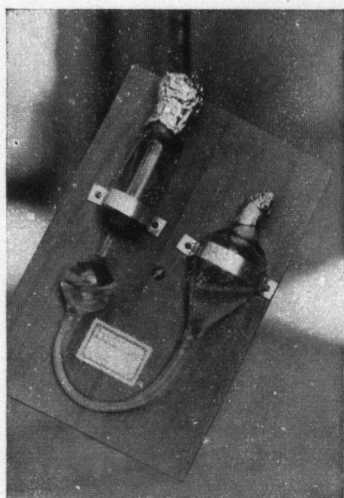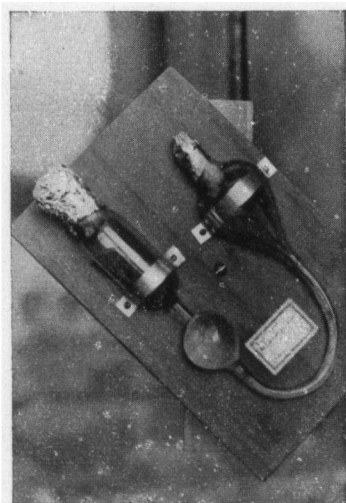
Fig. 8. Vessels for the synthesis of lichens out of the two components at varying humidity. a) the piece of plaster with the two components is watered with the culture solution b) the piece of plaster with the two components is left in an air-dry state.

growth-velocity the administration of suboptimal concentrations only will be perceived at the slope of the growth-curves, while the development only will stop, when some other factor has become limiting. Only in the first-mentioned case, there will be a relation between the final-crop and the quantity of nutrilites added to the culture-solution. Indeed I observed, that in the synthetic culture solutions without nutrilites the beginning development soon stopped at a moment when, in the solutions provided with nutrilites, the majority of the mycelium still had to be formed. This already did make it very probable that the nutrilites are absolutely necessary for the development of the fungi. To deliver the decisive proof for this assumption I constructed the growth-curves for the fungus with the most simple growth-requirements: Pl 1, when suboptimal con-
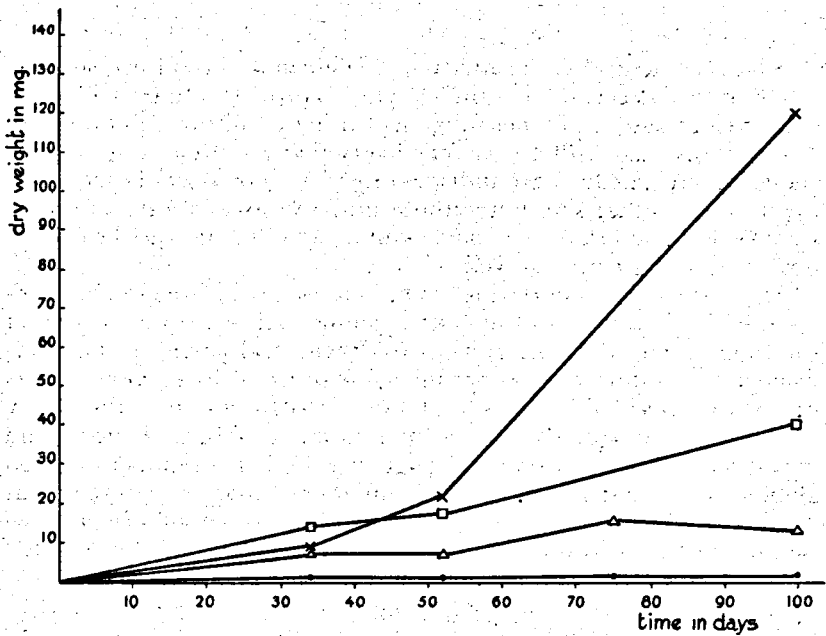
Fig. 17. Growth-curves of the fungus Pl 1 with suboptimal quantities o aneurin in the Czapek-Dox culture solution.
Abscis: time in days Ordinate: dry-weight in mg

●————● without aneurin
△————△ 0,001 γ % aneurin
□————□ 0,01 γ % aneurin
×————× 1,00 γ % aneurin

centrations of aneurin were added to the culture-solution. To this purpose a CZAPEK-DOX solution containing a certain amount of aneurin was divided over a number of Erlenmeyer-flasks of 100 cc, so that each flask contained 25 cc liquid and these flasks inoculated with equal amounts of a fungus-suspension. After some days in one of the flasks the dry weight of the mycelium was determined. The results are summarized in table VI and in text fig. 17.

TABLE VI.

Dry weight of the fungus Pl 1 in mg after incubation at roomtemperature in Erlenmeyerflasks of 100 cc provided with 25 cc Czapek-Dox solution inoculated 20.3.'42.

dates	0.000	0.001	0.01	1 γ % aneurin
21.4.'42	0.2	7.9	13.7	8.7 mg
9.5.'42	0.65	8.3	17.5	21.8 mg
2.6.'42	1.68	16.5	——	—— mg
26.6.'42	1.40	12.4	30.6	119.0 mg

So it is evident, that the addition of aneurin to the culture-solution is absolutely necessary for the development of the fungus Pl 1. As the development of all other fungi in synthetic culture solutions was as scanty as that of Pl 1 it is very likely that for these fungi as well the required nutrilites are indispensable. As moreover the development on any other solution without nutrilites is as scanty as on the CZAPEK-DOX solution, we may assume, that in nature they must obtain these nutrilites as well.

As the substrates upon which lichens are growing are not characterized by large amounts of nutrilites (stone-walls etc.) it is likely that the fungi obtain these nutrilites from their algal-partners. This was proved by the following experiments: a series of Erlenmeyer-flasks of 100 cc provided with 25 cc CZAPEK-DOX solution without any nutrilites was inoculated with a small number of algae, which could develop in these solutions (see page 470). When a number of green globules were visible along the bottom of the flasks they were inoculated with the fungi Pl 1 and Pl 2, which required aneurin resp. β alanin. From the results it is evident that the algae have secreted these nutrilites into the culture-solution.

TABLE VII.

Dry weight of the fungi Pl 1 and Pl 2 in mg after a two resp. one month incubation in Erlenmeyer flasks of 100 cc provided with 25 cc Czapek-Dox solution, which had been previously inoculated with algae.

	control	Apat. minor	Xanth. Cyst.	Parm. Cyst.
Pl 1	25	85	90	330
Pl 2	0.1	—	—	27

· Unfortunately I did not have the opportunity to finish an experiment in which I had inoculated Xanthoriomyces in a solution in which the Xanthoria Cystococcus had developed. As however this gonidium is able to develop in this synthetic solution and as bios-substances are of a general occurrence in living organisms, it is very likely, that the nutrilites, required by this fungus, may be provided for by its algal-partner.

d) *Carbon-requirements.* When the requirement of nutrilites was sufficiently analysed to make possible a development upon solutions of known composition I replaced the glucose in the CZAPEK-DOX solution by a number of other organic compounds in order to examine whether these compounds could be used as only sources of carbon. As it is not necessary to mention the dry weight of the mycelia formed I shall only indicate whether a definite development could be observed as compared with a control solution without carbon.

TABLE VIII.

Development of the fungi upon a Czapek-Dox solution provided with the required nutrilites in which the glucose has been replaced by other organic substances.

+ good development, — no development, blanko not investigated

source of carbon	concentration	Pl 1	Pl 2	C	Ap 2	Ap 3	Xanth
glucose	5 %	+	+	+	+	+	+
fructose	5 %	+	+	+			
galactose	5 %	+	+	+			
sucrose	5 %	+	+	+			
lactose	5 %	+	+	+			
arabinose	5 %	+	+				
glycerol	5 %	+	+				
erythritol	5 %	+	+	+			
mannitol	5 %	+	+	+			
amylum sol.	5 %	+	+	+			
cellulose	5 %	—	—	—			
pectin	5 %	—	—	—			
peptone (Witte)	5 %	—	+	—	+	+	+
Ca acetate	2 %	—	—	—			
Ca tartrate	2 %	—	—	—			
Ca oxalate	2 %	—	—	—			
Ca citrate	2 %	—	—	—			
tannin	2 %	—	—	—			
tannin	0.5 %	—	—	—			

From this table we may conclude, that the fungi can make use of a great number of organic substances. Many carbohydrates and poly-alcohols may be used, salts of organic acids were not fit as sources of carbon. Important are those substances for which it is likely that

they may act as sources of carbon in nature. Assimilation products of the algae like carbohydrates can be used as well as reserve-products like starch and polyalcohols (erythritol in Pleurococcus!). Cell-wall constituents like cellulose and pectin cannot be attacked, which explains the presence of many empty cell-walls in the necrophoral layers of many lichens. Most fungi can use peptones as only source of carbon, so that, as it was observed by WERNER that lichen fungi can liquify gelatine, it is not impossible that they can make use of the proteins of dead algal cells or of algal cells attacked by haustoria. It was observed, that when peptones had been used as source of carbon a rising of the pH was very striking, most probably by the production of alcaline nitrogenous substances from the peptones. Tannin could not be used and even appeared to be noxious in small concentrations.

e) *Nitrogen-requirements.* Although in contrast to most other symbioses the assimilation of atmospheric nitrogen by the lichen-fungi has never been supposed it was worth-while to look upon this possibility. Some of the fungi were inoculated into solutions in which the required nutrilites formed the only source of nitrogen. As the concentration of these nutrilites was only very small, they could not act as sources of nitrogen. The development upon these solutions was very scanty. The addition of 0.0001% sodium molybdate did not improve the results. When, however, I added a small amount of yeast-autolysate the development was abundant, the dry-weight of the formed mycelium was as great as I had ever observed before. Although it was probable that this development had only been caused by the presence of nitrogenous substances in the yeast-autolysate, I had to take into account that perhaps the yeast-autolysate contained some nutrilites, which had been necessary for the assimilation of the atmospheric nitrogen. A number of Erlenmeyer-flasks of 300 cc was filled with 100 cc CZAPEK-DOX solution containing 1 cc% yeast-autolysate. Of two flasks thus prepared one was inoculated with the fungus, the other served as control. In both flasks the nitrogen was determined by an ordinary Kjeldahl method after a two month incubation. As the pH of the culture-solution was 3.5 there existed no danger, that during the incubation-period volatile nitrogenous substances might have evaporated from the solution (compare LÖHNIS 1930). The mycelium was filtered from the culture-solution and dried at 105° C. The solution was acidified with some drops concentrated hydrochloric acid and evaporated in vacuum upon a water-bath. Then the mycelium was added to the dried culture solution and again dried at 105° C in a destruction flask, the dry material was destroyed with 20 cc H_2SO_4, 5 gr. K_2SO_4 and a trace

of CuSO$_4$ (all chemicals "pro analysis") until the solution was abso-
lutely colourless. Then the solution was diluted with 150 cc water,
60 cc NaOH 50 % was added and the escaping NH$_3$ destilled in a
Kjeldahl destillationapparatus. The NH$_3$ was bound by 25 cc 0.1 N
H$_2$SO$_4$ and this H$_2$SO$_4$ titrated with NaOH and methyl-red as in-
dicator. The results are shown in table IX.

TABLE IX.

Dry weight and nitrogen-content of mycelia and culture solutions of the
fungi Pl 1, Pl 2 and C after a two-three month incubation in Erlenmeyer
flasks of 300 cc provided with 100 cc of nitrogen-free Czapek-Dox solution
with 1 cc % yeast-autolysate.

	Pl 1	Pl 2	Pl 2	C
dry weight mycelium	860	1240	860	510 mg
nitrogen content of the mycelium + culture-sol.	7.39	12.25	12.42	11.95 mg
nitrogen content uninocu-lated control.	8.05	12.59	12.83	11.99 mg

So any assimilation of atmospheric nitrogen is altogether out of
the question. The small deficiency of nitrogen in all cultures as
compared to the controls may be easily explained by the evaporation
of some volatile nitrogenous compounds during the drying of the
mycelium.

When it was known that no atmospheric nitrogen was assimilated
the question arose, which nitrogenous compounds could then be used.
It appeared that all fungi could make use of amm. sulfate, potassium
nitrate, asparagin and peptones as sources of nitrogen, no specific
differences could be observed. Urea could never be used and was
in most cases definitely noxious.

B. THE GROWTH-REQUIREMENTS OF THE ALGAE.

1. Literature.

As all authors who have occupied themselves with the isolation
and the culture of the gonidia have made observations upon the
growth on different substrates the number of facts known about the
growth-requirements of the lichen-algae is much greater than those
about the growth-requirements of the fungal components.

Already BEIJERINCK (1890), the first investigator who isolated these
algae in pure culture, made an important observation. He observed
that the gonidia isolated developed much better upon substrates
containing glucose and peptones than upon inorganic media. As I

have mentioned in the general survey of the literature he concluded that the organic nitrogenous compounds necessary for the development of the gonidia should be contributed by the fungus.

ARTARI (1901, 1902) affirmed these observations. The gonidia of Xanthoria and Gasparinia not only grew much better upon organic substrates than upon inorganic ones, but they were able to grow upon organic media in the absence of CO_2 or of light. This did not appear as a peculiarity of lichen-gonidia as a great many other algae as well could live saprophytically on organic substrates. On the contrary ARTARI thought that the gonidia differed from all other algae by their preference for organic nitrogenous compounds as sources of nitrogen, which peculiarity he did not observe in the free living form of Cystococcus infusionum.

TREBOUX (1905) observed that many algae (among others lichen gonidia) were able to use salts of organic acids as sources of carbon. Especially acetates were beneficial in low concentrations. In relation to these observations I want to mention the observations of TOBLER (1911), that many lichen-fungi secrete crystals of calciumoxalate, which disappear when gonidia are present. The possibility exists, that this is caused by a resorption of this oxalate by the gonidia, although the observation of TOBLER is not at all convincing. Although TREBOUX confirmed the saprophytical mode of life of many gonidia he critisized the supposed necessity of organic nitrogenous compounds (1912). Firstly he showed, that ARTARI was wrong in considering C. infusionum as the free-living form of the gonidia of many lichens. It is quite another species, so that it is not surprising, that the growth-requirements differed. Secondly he observed, that the preference for peptones above inorganic nitrogenous salts was not at all as pronounced as had been suggested by BEIJERINCK and ARTARI. Ammonium-salts might be used as well. An important argument for a mutualistic symbiosis was rejected.

The observations of CHODAT (1913) and the conclusions based hereupon were very important. It appeared from his experiments and those of his pupil KORNILOFF (1913), that the gonidia of Cladonia species grew only slightly upon agar media without glucose (a diameter of 3 mm was reached after a five month incubation in the light) while on agar media containing glucose the development was considerable. Organic acids did not produce this effect, although in the dark they apparently could be used to a small degree as sources of carbon. Most gonidia preferred organic nitrogenous compounds above inorganic ones. On peptone-agar without glucose the development was scanty. Although in the dark the gonidia could develop well upon suitable substrates the development upon the same sub-

strates in the light always was somewhat better. Furthermore it appeared that they could liquify gelatin, which could be used as a source of nitrogen. In every respect these gonidia behaved like saprophytes. As I did already mention in the survey of the general literature CHODAT based upon these observations the hypothesis, that in the lichen-thallus the gonidia would profit from the organic substances taken by the fungus from the substrate. If, however, one would defend the old conceptions of the symbiosis it would be necessary to assume that the photosynthesis of the gonidia in the lichen-thallus was stimulated by an action of the fungus. In this respect it was interesting that the gonidia of Verrucaria species (belonging to the genus Coccobotrys) could not live saprophytically and were even harmed by the presence of glucose, the more so when we take into account, that these lichens live upon bare rocks.

LETELLIER (1917) compared the lichen-gonidia with certain free-living Cystococcus species and observed, that certain free-living species as well preferred organic nitrogenous compounds as sources of nitrogen.

From the above it will be clear that all authors agree that the Cystococcus gonidia develop much better upon organic substrates than upon inorganic media, but that there is no agreement as to the question whether organic nitrogenous compounds are preferred above inorganic nitrogenous salts. From the work of WAREN (1920) it became evident that this disagreement was caused mainly by specific differences between the gonidia of different lichens. In general growth was best in the presence of nitrogenous compounds like glycocol or alanin, but some gonidia grew as well upon ammonium-salts. Peptones and leucin were not as apt as the first-mentioned amino-acids. Acetamid only could be used by a small number of gonidia, while urea never was affected. As, however, most of his experiments were performed with the purpose to use the results for the classification of the different algae, they fall out of the scope of this survey. The same may be said of the work of JAAG (1929), from whose extensive experiments some points, however, should be mentioned. Upon inorganic substrates the growth of all gonidia (isolated from Cladonia and Parmelia species) was very scanty, although a faint development could be observed in the presence of continuous electric light. The concentration of glucose, which gave an optimal development sometimes amounted to ten percent, while even concentrations of 20 % still had a marked stimulating effect. On substrates, which were rich in glucose and salts strong light was necessary to give a maximal development while on poor substrates strong light was noxious. The gonidia of Cladonia preferred organic nitrogenous

compounds, the gonidia of Parmelia made use of inorganic salts as well. All gonidia could liquify gelatin, while the gonidia of Cladonia species appeared to be micronitrophilous as they could develop on agar media, to which no nitrogen had been added.

The investigations of RATHS (1938) and THOMAS (1939) in this respect brought only little news, they too observed the favourable effect of glucose and peptones and specific differences as to the preference of organic nitrogen. The last mentioned author stressed the great similarity between the food-requirements of the gonidia and of the fungi and remarked that a replenishment of each others food-requirements was out of the question, so that in a lichen-thallus the two components should live under suboptimal conditions.

So we may conclude that the principal result of all these investigations has been the universal preference for organic substrates of all Cystococcus gonidia. As the development upon inorganic media is very slight we have to suppose that the photosynthesis is not very pronounced. Direct measurements of the photosynthesis of lichen gonidia in pure-cultures never have been performed. There have been performed, however, a great many experiments upon the photosynthesis of lichens, which have proved that under favourable ecological conditions an assimilation-surplus is found (JUMELLE 1892, HENRICI 1918/19, STOCKER 1927, BOYSEN-JENSEN-MÜLLER 1929, SMYTH 1934, STALFELT 1939). Nevertheless some of these authors have concluded that these favourable conditions for certain lichens never will occur, so that in these lichens growth is only possible, when the fungus cooperates in providing organic substances (STOCKER 1927, BOYSEN-JENSEN-MÜLLER 1929). STALFELT (1939), however, claims that the possibilities of an autotrophism of lichens are more favourable than had been supposed by these authors, as the efficiency of photosynthesis is highly variable and for instance declines in the summer months and increases in winter, so that calculations about the photosynthesis during a year as had been performed by STOCKER are rather uncertain.

When, however, we take into account the small quantity of assimilating gonidia and the large amount of respiring fungus, we have to conclude from all these experiments that, like STOCKER expresses, the photosynthetic activity of the gonidia in the thallus is of a "erstaunlichen Leistungsfähigkeit". This high photosynthetic activity of the gonidia in the thallus is hardly in accordance with the poor development of these organisms when cultivated upon inorganic substrates. This discrepancy forms one of the most important problems in the lichen symbiosis.

2. Own observations.

a) *Preliminary experiments.* My first task was to control whether the gonidia, which I had isolated, preferred organic substrates to inorganic ones. This, indeed, was the case, as had to be expected. While all gonidia and even Apatococcus hardly developed on inorganic substrates, the addition of glucose had a marked influence. This influence already was perceivable at a concentration of o.1 % glucose, increased with the concentration to about 2 % glucose, was as marked when the concentration amounted to 10 % glucose, while the effect of 15 and 20 % glucose was not as obvious. That the glucose really was used as a source of carbon was evident from the fact that in the dark the algae developed upon these organic substrates nearly as well. The colour of the colonies in the light was (on glucose) not as dark as on inorganic media, although the colonies always were definitely green. On inorganic substrates the chromatophore was better developed than upon organic substrates, while the pyrenoid was clearly visible. This pyrenoid is a beautiful criterium to determine whether a Cystococcus cell has developed in an autotrophic or in a heterotrophic way. The beautiful pyrenoid and chromatophore of the gonidia in a lichen-thallus are an indirect indication that these gonidia live in an autotrophic way.

Besides glucose, fructose may be used as well, the effect of galactose is marked upon the gonidia of Physcia and Parmelia, while the Xanthoria Cystococcus prefers glucose, arabinose can only be used by the Physcia Cystococcus, sucrose had a definite effect upon all algae, while lactose and maltose cannot be used. As to the polyalcohols mannitol had a marked effect upon the gonidia, while erythritol could not be used. Amylum soluble had no effect, nor had glycerol, asparagin and peptones. A great number of salts of organic acids have been tested in different concentrations (Ca, Na and NH$_4$-salts of formic-, acetic-, lactic-, oxalic-, succinic-, tartaric-, and citric acid in concentrations varying form 0.5—2 %), but no stimulating effect could be observed. In relation to the experiment of TOBLER (1911) the question arose, whether these algae could not make use of these salts or only could use them to a so small degree, that the resulting development was even neglectible beside the autotrophic development. It appeared, however, that in the dark no development upon these organic salts could be observed, so that they cannot be used as a source of carbon. Only sodium acetate in small concentrations ($\frac{1}{4}$ %) produced a faint development.

I again want to stress the fact, that this stimulating effect of organic substances is not at all a peculiarity of the gonidia, but that it may be observed in a great many other algae as well. Nevertheless

the very poor development upon inorganic substrates is very re-markable as it has been shown that in the lichen-thallus the gonidia show an astounding photosynthetic activity. The bad development upon inorganic substrates as compared to organic media can it be explained in another way than by assuming that the photosynthetic activity in cultures is only very small? Certainly it is not caused by an infavourable composition of the BEIJERINCK solution, as firstly the glucose-solutions were provided with the same salts in the same concentration and secondly other culture solutions gave no better development (solutions according to DETMER, BRISTOL, MOLISCH and PRINGSHEIM as described in KUFFERATH 1930). Furthermore the phenomenon has been so often observed, that I do not need to say much more upon this subject.

As to the nitrogen-requirements I observed that on agar substrates ammonium salts could be used as well as asparagin, that peptone was not as suitable, nor was potassium nitrate, while potassium nitrite could never be used. On agar-media without nitrogen the development was so scanty, that there was no reason whatever to think of the assimilation of atmospheric nitrogen.

Before dealing with my further experiments I have to make some remarks upon the p_H of my cultures and the influence of the p_H upon the growth-velocity. The p_H of the BEIJERINCK solution (100 cc water, 0.05 gr NH_4NO_3, 0.02 gr. K_2HPO_4, 0.02 gr. $MgSO_4$ and 0.01 gr. $CaCl_2$) amounted to 6.0 and when provided with glucose 4.5 after sterilisation (determined with Lyphan indicator papers). As the solution was satisfactory buffered the p_H of the different cultures varied only within small degrees (4.5—4.8). In an orienting ex-periment I compared the development upon agar-media in which the p_H had been changed by replacing the K_2HPO_4 by KH_2PO_4 or K_3PO_4. From this experiment it appeared, that at p_H 4.0—7.4 the development was hardly dependant upon the p_H, at the neutral sides it was somewhat better than at the lower p_H. Yet the observed differ-ences were so small, that it was unlikely, that the small differences in the p_H of the culture-solutions in the experiments to be described could influence the results. While of course I always controled the p_H of the culture solutions I never had to correct the p_H.

b) *The influence of nutrilites upon the development.* After it had been shown, that the fungi could not develop without the presence of certain nutrilites in the culture solution, it seemed to be very important to investigate, whether the algae were also favoured by the presence of nutrilites. There is only little known about the in-fluence of theses substances upon green algae, I only mention some observations as to the effect of heteroauxin upon a number of uni-

cellular green algae by BRANNON (1937) and YIN (1937), which was denied by LEONIAN and LILLY (1937), but especially the recent experiments of ONDRATSCHEK (1940), who investigated the effect of a number of nutrilites upon some green algae (especially Haemato-coccus pluvialis). He observed that in heterotrophic cultures (acetate as source of carbon) aneurin and in autotrophic cultures ascorbic acid had a marked influence, although, however, the concentrations to be used were very high (ascorbic acid had an optimal effect in concen-trations of 1—10 mg %). Moreover the effect could only be observed in a small number of algae, most species being indifferent towards the addition of these substances.

The research after the effect of nutrilites upon green algae and especially upon these lichen-gonidia is made difficult by the fact that these organisms are highly favoured by the addition of assimi-lable organic substances in even very small concentrations. In con-sequence it seems unwarranted to conclude or even to suppose from the stimulating effect of a plant-extract, that this effect is due to a specific nutrilit. Extracts like these never can be used, so that we have to restrict our experiments to trials with a great many pure nutrilites, while then as well we have to take into account, that these nutrilites may only have served as sources of carbon. This difficulty is not as great, when we examine the algae under heterotrophic con-ditions, but as has been shown by ONDRATSCHEK (1940) the nutrilites, which are active upon heterotrophic algae need not be identical with the nutrilites, which stimulate these algae, when living autotrophi-cally. For the lichen-symbiosis it is more important to know, which nutrilites are necessary for the autotrophic — than for the hetero-trophic metabolism. Yet I began my experiments with an investi-gation on the behaviour of the gonidia towards some nutrilites, when growing under heterotrophic conditions in the dark as it cannot be doubted that, when the ecological conditions are suboptimal, the gonidia will live from the organic substances taken by the fungus from the substrate and, moreover, it is probable that nutrilites, which are absolutely necessary for the heterotrophic metabolism are in-dispensable as well, when no organic substances are present in the culture solution, although then the effect of these nutrilites may be masked by the absence of other substances, which are necessary for the photosynthesis (f.i. ascorbic acid). Finally we may obtain indi-cations, which may be used at the examination of the autotrophic cultures.

a) *Methods*: In all experiments I used the culture solution ac-cording to BEIJERINCK: water 100 cc, NH_4NO_3 0.05 gr., K_2HPO_4 0.02 gr., $MgSO_4$ 0.02 gr., $CaCl_2$ 0.01 gr., which for the heterotrophic

cultures was provided with 2 % glucose. The algae were cultivated in flasks of 50 cc containing 25 cc culture solution, excepted the autotrophic series, which were cultivated in Erlenmeyer flasks of 100 cc provided with 25 cc culture solution as a small layer of liquid was supposed to be more favourable for the diffusion of CO_2 into the liquid. The flasks containing glucose were incubated in a dark room at a room temperature of about 15—20° C., the flasks without glucose were placed before a window at the north-side of the laboratory. Care had to be taken, that in summer the cultures were not heated by the sun-rays, as a rising of the temperature causes an immediate dying of the algae. The flasks of one experimental series were inoculated with equal amounts of a suspension of algae in the culture solution. The algae were taken from young agar cultures, but in later experiments they were previously cultivated in liquid culture solutions without nutrilites (after heavy inoculation), and the other cultures inoculated with a suitable dilution of this culture, which warranted a greater uniformity of the inoculation.

The development of the gonidia was measured by counting of the number of algal-cells by means of a haemocytometer according to BÜRKER. During the running of my experiments this method appeared to be far from ideal, as the algae, especially the Cystococcus of the Parmelia and of the Xanthoria, had the peculiarity to aggregate after the cell-divisions, so that it was not possible to make an exact count. Only after a vigourous shaking of the culture reproducable results could be obtained. The ideal to construct the growth-curves in a number of experiments only could be realized within certain limits for the gonidia of Physcia pulverulenta. As, moreover, this alga showed the best growth-velocity, most of my experiments were performed with this gonidium. A second disadvantage of the counting-method was formed by the fact that we do not take into account that not only the number of algal-cells, but the dimensions as well are quite important for the estimation of the development. As it took a lot of time to estimate the average dimensions of the cells in a culture I only made some notes when the cell-dimensions were very deviating from those in the other cultures. That, in spite of these disadvantages, I always used this method is caused by the fact, that the other usual methods as well have their disadvantages. When we make use of a colorimetric or nephelometric method we do not take into account that the colour of the green cells varies highly with the composition of the organic substrate and is not at all an indication for the development of the algae (within certain limits), while a gravimetric determination of the algal material could not be used as the weight of the algae was too small compared to the inorganic

precipitations formed in most culture solutions after sterilisation.
The density of the inoculation and the incubation time will be
mentioned below.

β) The effect of nutrilites upon the heterotrophic development: As
I observed that the algae developed well in synthetic culture solutions
without any nutrilites when the inoculations were rather heavy I
started to investigate whether this was possible after a smaller ino-
culation as well. Simultaneously I inoculated some flasks containing
a BEIJERINCK solution with 2 % glucose, to which I had added ½ cc.
of an extract of the fungus Pl 1, which had been prepared as follows:

> a mycelium of about 500 mg, cultivated upon a CZAPEK-DOX
> solution with 10 γ % aneurin was filtered, ground in a mortar
> with some cc of water and diluted to 10 cc. Then this suspension
> was placed in a thermostat of 70° C during 12 hours, boiled,
> neutralized, boiled again and filtered.

The result is evident from table X.

TABLE X

number of algal cells per mm³ after a one-two month incubation.

alga isolated from:	inoculated with drops of suspension of algae					number of algal cells in one drop of the suspension used at the inoculation
	1 drop with	1 drop	2 drops	3 drops	10 drops	
		without fungus extract				
Xanthoria	388	27	74	—	104	47400
Parmelia	940	252	404	2850	2740	59800
Physcia	2640	34	122	122	7600	94800

From this experiment we have to conclude two important facts. In
the first place we observe that the strength of the development in-
creases with the strength of the inoculation. At the Physcia Cysto-
coccus this increase is not at all proportional to the increase of the
number of algal cells inoculated. In fact it is quite inconceivable that
differences in the density of the inoculation may be perceived after
a two-month incubation, so that it is more likely that the better
growth after a heavy inoculation is caused by the presence of some
favourable factors in the inoculation material. We observed further
that the fungus-extract contains substances which stimulate the
development of the algae.

Yet it was evident, that in all cultures a development of the algae
could be observed. As this still appeared to be the case when the
number of inoculated cells was very small (50 cells), although it took
very long before a development of the algae could be perceived in

that case, it might be doubted whether this fungus extract contained factors, which were absolutely indispensable for these algae. The growth-curves obtained of the Physcia Cystococcus on culture solutions with and without yeast-autolysate clearly showed that indeed the extract was not indispensable for the development. These growth-curves were obtained as follows; some flasks with and without yeast-autolysate (which as will be shown below has the same effect as the extract of fungus Pl 1) were inoculated with one drop of a suspension of the Physcia Cystococcus containing $\pm$ 10.000 cells per drop. From time to time a sample was taken by means of a sterilized pipette and examined in the haemocytometer. The results are evident from table XI and text fig. 18.

TABLE XI

number of cells per mm³ of the Physcia Cystococcus cultivated in Beijerinck solutions provided with 2 % glucose with or without yeast-autolysate inoculated with $\pm$ 10 000 cells 20-4-'42.

dates	without yeast-autolysate	with yeast-autolysate 1 cc %
15.5.42	104	1455
1.6.42	2866	2622
23.6.42	3430	4028

While in the solution containing yeast-autolysate the development started at once, the growth-curve in the synthetic culture solution showed a considerable lag-phase, but after some weeks a rapid increase of the number of algal cells could be observed, so that after some time, dependent upon the density of the inoculation, the number of algal cells was equal in all cultures. That the final number of algal cells is somewhat greater in the flasks containing yeast-autolysate may be easily explained by the greater amounts of foodsubstances. So the effect of the yeast- and the fungus extract consists only of a shortening of the lag-phase. The active substances of these extracts are not indispensable. In relation to the very slow growth of lichens in nature the importance of these substances may not be overrated. Nevertheless it seemed worth-while to investigate their nature.

When doing this it does not suffice to determine the final-crop as this appeared to be independent of the presence of the extracts, but I had to construct the growth-curves of every culture before I could draw any conclusions. The most illustrative observation was performed at the moment that, in the presence of the extract, the development already was pronounced, while in the controls no
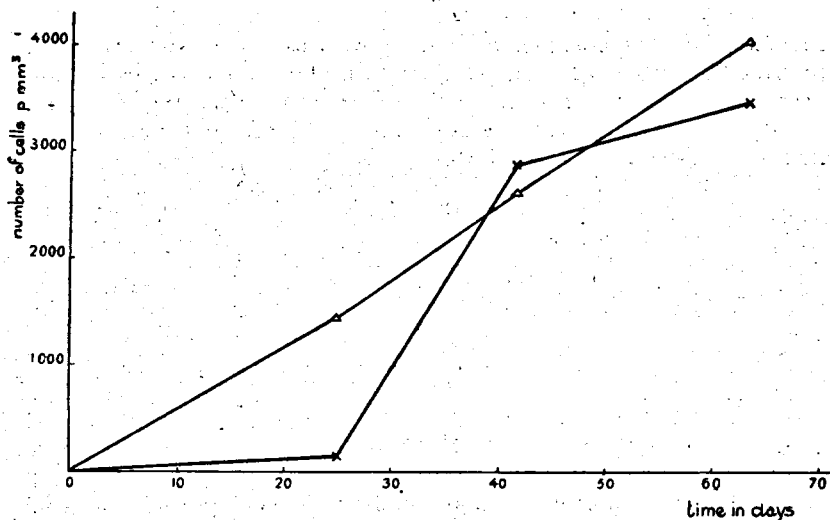
Fig. 18. Growth-curves of the Cystococcus from Physcia pulverulenta in a culture solution according to Beijerinck provided with 2 % glucose. △———△ with 1 cc % yeast-autolysate and ×———× without yeast-autolysate. Abscis: time in days. Ordinate: number of algal cells per mm³.

development was perceptible. With the Physcia Cystococcus this was the case after four weeks, with the two other algae after a one to two month incubation. When nothing else is mentioned, the results given below refer to the observations performed at this moment. As the development of the algae could be estimated by the green colour of the cultures this moment could be easily determined so that mostly it was not necessary to construct the whole growth-curve.

A second difficulty was formed by the dependence of the development on the density of the inoculation. Although it was advisable to choose this density as small as possible in order to make the effect of the extract more pronounced, on the other side the danger of an unequal inoculation was so great (in consequence of the difficult suspensability of the gonidia) that I choose the number of cells not too small. Mostly I inoculated the flasks with ± 10.000 cells.

Now to return to the experiment mentioned in table X. As the fungus from which the extract had been prepared had developed in a CZAPEK-DOX solution provided with 10 γ % aneurin and as ON-DRATSCHEK had observed that certain green algae were stimulated by the presence of aneurin I examined first whether the activity of the extract had to be ascribed to the activity of aneurin which, however,

seemed improbable as it had been shown that the algae were able to provide the fungus Pl 1 with this vitamin. To some cultures I added yeast-autolysate in order to find out whether this extract, which could be easily obtained in larger quantities than the fungus extract, had a similar effect as the latter.

TABLE XII

number of algal cells per mm^3

alga from	control	10 γ % aneurin	1 cc % yeast-auto-lysate
Xanthoria	22	32	317
Parmelia	0	0	500
Physcia	57	77	1548

So it appeared that aneurin had no effect, so that the stimulating substances indeed are synthesized by the fungus. Moreover it appeared that yeast-autolysate has a marked effect upon the development of the algae, so that I could make use of this extract in my further experiments.

Is this stimulating effect produced by some nutrilites or is it caused by the presence of a good source of carbon or nitrogen? I formerly observed that glucose is the best source of carbon for these algae so that it is not likely that the stimulating effect is caused by a better source of carbon as moreover the glucose was present in optimal concentrations. The presence of a better source of nitrogen was more likely as yeast-autolysate contains an abundance of nitrogenous substances and as it has been shown that certain amino-acids are preferred by many gonidia. Therefore I again examined the influence of yeast-autolysate in solutions in which the ammonium-nitrate was replaced by asparagin or peptones (Witte).

TABLE XIII

number of algal cells per mm^3

alga from	NH$_4$NO$_3$		asparagin		peptones		
	with	without	with	without	with	without	yeast-auto-lysate
Xanthoria	139	8	111	18	233	7	
Parmelia	72	2	46	21	200	9	
Physcia	182	1	333	20	222	0	

Asparagin appeared to give a much better growth than the ammonium salt, which is in contradiction with the results of page 466.

These results, however, had been obtained on agar-media which were heavily inoculated, so that it is obvious that here this preference for asparagin could not be observed. Yet this effect of the asparagin could not be compared to the effect of the yeast-autolysate, so that here certainly other factors are concerned. Furthermore it is not excluded that in using such high quantities of asparagin the stimulating effect had been produced by some contaminating substances.

. · The ashes of yeast-autolysate did not stimulate the growth of the algae so that we must assume that the active principles possess an organic character. In order to investigate the nature of these substances I provided a number of cultures with the fractions of yeast-autolysate described on page 453. I can only mention the results of the series inoculated with the Physcia and Parmelia Cystococcus as I did not have the opportunity to count the cultures inoculated with the Xanthoria Cystococcus. From the exterior appearance of these cultures, however, it was likely that this gonidium had reacted in the same way as the two other gonidia.

TABLE XIV
number of algal cells per mm³

extracts added to the culturesolutions	Physcia Cyst.	Parmelia Cyst.
control without yeast-autolysate	11	0
1 cc % yeast-autolysate	722	782
— shaken with norit	258	3
— precipitated with lead-acetate	89	11
— precipitated with Ba(OH)$_2$	2	0
— after oxidation with H$_2$O$_2$	10	0
— after heating with KOH	6	0
— after precipitation with alcohol	544	227
— shaken with ether: water layer	773	255
— shaken with ether: ether layer	7	0

We observe that the active principle cannot be dissolved in ether, cannot be precipitated with alcohol, while the activity is lost after treatment with KOH, Ba(OH)$_2$ and H$_2$O$_2$. After the action of norit and lead-acetate the effect is partially lost, which may indicate that more than one factor should be considered.

Furthermore I tried a number of nutrilites upon their stimulating activity, alone or in mutual combinations. Firstly I used the bios-solution obtained from Prof. Dr. F. KöGL in concentrations varying from 10 mg % m. inosite, 80 γ % aneurin, 80 γ % adermin, 2 mg % β alanin, 0.2 γ % biotin and 12 γ % panthotenic acid to 1/100 of this concentration. Furthermore I added these substances alone

(which could not be done with biotin and panthotenic acid as I did not possess these substances), heteroauxin 10 γ % and ascorbic acid in concentrations of 10 γ, 1 mg and 10 mg %. I need not reproduce these results as I never could observe any definite activity.

On the other hand in some experiments with the Physcia Cysto-coccus I observed a marked effect of nicotinic acid, although this effect could not be reproduced in later experiments. Whether this was caused by a great variability of the algae or by the use of other chemicals cannot be said; the experiments reproduced in table XV are so convincing that there is little doubt that at least in certain circumstances nicotinic acid has a marked effect upon these gonidia.

TABLE XV

added nutrilites	number of algal cells per mm³
control	12
10 γ % nicotinic acid	533
100 γ % nicotinic acid	1877
1000 γ % nicotinic acid	1988
10 γ % nicotinic acid +	
+ 1 cc % biossolution (strongest conc.)	1422
1 cc % yeast-autolysate	1666

The activity of the nicotinic acid alone was only comparable to the activity of the yeast-autolysate in high concentrations, the ac-tivity was definitely increased by the presence of bios-substances. While I could not observe the stimulating effect of nicotinic acid in later experiments, the effect of the nicotinic acid combined with the bios-substances was always evident, although not as marked as in the series of table XV. The effect then always remained far behind the effect of the yeast-autolysate, so that certainly in the autolysate still other factors are concerned.

I have mentioned that asparagin has a marked influence upon the development of the gonidia. I again have prepared a series of culture-solutions to which nicotinic acid and the bios-substances were added, but in which the NH_4NO_3 had been replaced by asparagin. Here again I did not have the opportunity to count the cultures of the Xanthoria Cystococcus, but again there were no reasons to suppose that this gonidium behaved at variance with the two other algae.

The activity of asparagin appeared to be markedly favoured by the addition of the bios-solution, which of the bios-substances produced this effect has not been analysed. The development then was in the Physcia Cystococcus even higher than with the yeast-autolysate, but

TABLE XVI

number of algal cells per mm^3

source of nitrogen	nutrilites	Physcia Cyst.	Parmelia Cyst.
NH$_4$NO$_3$		11	0
NH$_4$NO$_3$	1 cc % yeast-autolysate	722	782
asparagin		371	7
asparagin	1 cc % bios (conc. sol.)	1466	40
NH$_4$NO$_3$	100 γ % nicotinic acid.	22	0
NH$_4$NO$_3$	100 γ % nicotinic acid.		
	+ 1 cc % bios (conc. sol.)	302	7
asparagin	100 γ % nicotinic acid.	307	13

the Parmelia Cystococcus was much more favoured by the latter. Nicotinic acid did not augment the effect of asparagin, nor did it stimulate the growth with NH$_4$NO$_3$ in these experiments. Together with the biossubstances, however, it had a definite stimulating effect.

Although we cannot yet indicate the substances which cause the stimulating effect of the fungus extract and the yeast-autolysate, we can already remark that this effect is perhaps partially caused by the presence of organic sources of nitrogen, but that the main effect only is reached after the addition of certain nutrilites belonging to the bios group. Only in some experiments nicotinic acid had a definite influence. Again I want to stress the fact that these substances only stimulate the development but that they are not indispensable.

γ) *The effect of nutrilites upon the autotrophic development.* An investigation of the effect of nutrilites upon the autotrophic development of course is much more important for the problem of the symbiosis than the investigation of the effect of these substances upon the heterotrophic metabolism. When we observe the activity of nutrilites, which only stimulate the autotrophic development, we may conclude that apparently these nutrilites are favourable for the photosynthesis. As the growth-velocity of lichens and of the algae enclosed is only very small, we need not accept the necessity of a „growth-factor", when this factor is not absolutely indispensable. But, as we know that in the lichen-thallus the photosynthetic activity may be so great, that not only the algae themselves, but the in comparison enormous fungus-mycelium as well live from the assimilation products, while in cultures the gonidia hardly develop upon substrates devoid of organic food-substances, the demonstration of substances which could stimulate photosynthesis in these algae would be very important. Already CHODAT (1913) supposed that either the fungus would stimulate the photosynthesis of the algae or the algae would live from organic substances taken by the fungus from the substrate.

CHODAT himself and many other authors mainly considered the last mentioned hypothesis, the much more fascinating first possibility was neglected.

The first question which arose was, whether the growth in absolutely inorganic culture-solutions was impossible or only very slight. In literature it has been observed, that a slow growth in inorganic media indeed is possible (e.g. by JAAG 1929), but in these experiments the inoculation certainly was rather heavy. My algae could develop in inorganic BEIJERINCK solutions after heavy inoculations, which is hardly astonishing as we inoculate a great many reserve-substances (and nutrilites) as well, which might produce an initial development. When I inoculated with smaller quantities of cells no growth could be perceived. Even when I inoculated with ± 10 000 cells after three months no algae had developed. Of cource I do not know whether growth was visible after a longer period of incubation, but when we compare this result with the development in cultures containing glucose, we must assume that either photosynthesis is lacking or is an important limiting factor. This difference between the development in autotrophic and heterotrophic conditions was most evident for the Physcia Cystococcus, so that most experiments were performed with this gonidium.

We have to consider the possibility that the substances, which had a stimulating effect upon the heterotrophic metabolism could enable a development upon solutions without glucose as well. Neither the bios-solution nor nicotinic acid enabled an autotrophic development, asparagin, however, sometimes gave some development. A microscopical examination of the algal-cells, however, made doubtful if indeed these algae had developed autotrophically, as the chromatophores were not beautifully formed and no pyrenoid was visible. It therefore appeared more likely that the faint development had been caused by the presence of small amounts of assimilable substances (asparagin itself or contaminating substances). The influence of this substance upon the photosynthesis is doubtful.

Furthermore I added ascorbic acid to the culture solution, which had been observed to stimulate the autotrophic development of some green algae in the experiments of ONDRATSCHEK (1940). In table XVII the results are summarized of a series of experiments with the Physcia Cystococcus and the Parmelia Cystococcus. They were inoculated with ± 10 000 cells. Simultaneously I inoculated the same culture solutions with the Physcia Cystococcus and incubated them in the dark. As in none of these last-mentioned cultures any growth was perceptible the conclusion seems justified that ascorbic acid, in these concentrations, cannot enable a heterotrophic development. As

.glucose in the same concentrations already could enable a develop-
ment of the algae in the dark, we may assume that ascorbic acid
·cannot be used as a source of carbon for these algae. On the contrary,
.in the light, a definite development could be obtained by the presence
·of ascorbic acid.

TABLE XVII

:number of algal cells per mm³ after a three month incubation in the light.

percentage of ascorbic acid	Physcia Cyst.	Parmelia Cyst.
0	0	0
100 γ %	373	2
1 mg %	100	6
10 mg %	1200	59

Although the effect is very pronounced the development still is
not at all comparable with the development in glucose solutions.
Furthermore the optimal concentrations of ascorbic acid are very
high. We hardly have the right to speak about a nutrilit and it is
doubtful whether the fungus will secrete such high quantities of
ascorbic acid.

As ascorbic acid is oxidized by air, especially in more alcaline
:solutions, there is a good reason to believe that most of the ascorbic
.acid added to the culture solution is present in an oxidized, physiolo-
.gically inactive state, especially when we keep in mind the production
of oxygen by the assimilating algae. So the necessity of high per-
·centages of ascorbic acid does not prove that the reduced ascorbic
.acid is only active in as high a concentration.

To reduce the oxydation of the ascorbic acid during the incubation
I have inoculated the same series and cultivated them under bell-
glasses filled with the following gas-mixtures: Air, nitrogen (from
a commercial cylinder containing still some percentages of oxygen)
and hydrogen, each provided with 5 % CO_2. On account of the want
·of space in the bell-glasses this series only could be inoculated with
the Physcia Cystococcus, while only some cultures were inoculated
with the Xanthoria Cystococcus, as this alga had not been used in
the experiments of table XVII.

The results cannot be misinterpreted; the activity of ascorbic acid
in the atmosphere of nitrogen, but especially in the atmosphere of
hydrogen is already evident in the smallest concentrations used, so
that most probably still smaller concentrations may be effective.
*The resulting growth in the hydrogen atmosphere is as copious as in the
presence of glucose!* That the algae, however, were living autotrophi-

TABLE XVIII

number of algal cells per mm³ after a one month incubation in the light.

	air 5% CO₂	nitrogen 5% CO₂	hydrogen 5 % CO₂
Physcia control	o	o	o
100 γ % ascorbic acid	1	61	2700
1 mg % ascorbic acid	81	647	1267
10 mg % ascorbic acid	500	804	2007
Xanth. control	o		o
1 mg % ascorbic acid	4		100

cally is evident from the fact that in the dark ascorbic acid cannot be used as a source of carbon, while all cells in all cultures of table XVIII possessed a beautiful chromatophore and a distinct pyrenoid. So we may conclude that the small, or lacking, development in inorganic culture solutions is not caused by an inability to photo-synthesize, but only by the absence of a substance, which is indispensable for a good photosynthesis.

That this substance has the nature of a nutrilit is obvious as the active concentrations are very small, the effect of 100 γ % ascorbic acid already was so pronounced that certainly far smaller quantities will be active. As it was not active in heterotrophic cultures it is probable that its activity is directly linked with photosynthesis. One might object that in the heterotrophic cultures the effect of ascorbic acid could not be perceived, because here the glucoreducton, formed by the sterilisation of the glucose, already would have a similar effect (see below). In the experiments of ONDRATSCHEK (1940), who observed a similar effect of ascorbic acid upon the autotrophic development of some other algae, this effect could not be produced in heterotrophic cultures too, although he used sodium acetate as a source of carbon. Moreover it has become evident from many other observations, that ascorbic acid is in some way linked with the process of photosynthesis (compilation of these observations at GIROUD 1938 page 144).

My experiments do not answer the question whether ascorbic acid is a specific nutrilit. It might be possible, that its activity is only caused by its low reduction potential (E_0' at p_H 7 : —0.066 Volt. STERN 1935). Therefore I compared the effect of ascorbic acid with the effect of another reducing substance, which is widely distributed in nature: cystein (E_0' at p_H 7.0 : + 0.078 Volt. STERN 1935). It appeared that neither in air, nor in hydrogen cystein made possible an

autotrophic development. (concentrations 10 γ — 10 mg %). On the other hand it appeared from a preliminar experiment, that dioxy-maleic acid had a similar influence as ascorbic acid [1]). So the ascorbic acid is not strictly specific, although di-oxymaleic acid is a closely related substance. It is not unlikely that the promoting effect upon the photosynthesis of these algae is characteristic for all dienolic compounds. Perhaps even the stimulating effect of glucose in the light is not only due to its fitness as a food-substance, but also to the presence of small amounts of gluco-reductone formed at the sterili-sation of the glucose in the neutral or even weakly alkaline (before sterilisation) culture solutions. This would explain the phenomenon already observed by CHODAT (1913), that the development upon glucose in the light always is somewhat better than in the dark, a phenomenon which in my experiments always was very evident when I used very small amounts of glucose (10 mg %).

I do not know whether the ascorbic acid, or a physiologically equivalent substance, is absolutely indispensable for the photo-synthesis. To this purpose the growth-curves of the algae would have to be constructed in the presence of suboptimal amounts of ascorbic acid, but this has not yet been done. In any case the effect is very striking, when we think of the active assimilation in a lichen under optimal ecological conditions and the very slight or negligible development of the gonidia on purely inorganic media. So we are forced to assume that in a lichen-thallus as well the gonidia are stimulated to photosynthesis by the presence of ascorbic acid or related substances.

I have not yet been able to deliver the decisive proof. I could not demonstrate the production of substances reducing dichloro-phenol-indophenol by the fungus-symbionts in cultures, as has been ob-served in other moulds by BERNHAUER and GORLICH (1936). It is, however, possible that this will be the case, when we cultivate the fungi in other culture solutions than the CZAPEK-DOX solution used in these experiments. Furthermore the excretion of very small amounts will be active in the lichen-thallus, although the presence of these amounts could never be demonstrated when secreted in a culture solution, where it is very possible that they are oxidized during the long incubation. Finally it is possible that substances, which cannot be demonstrated by titration with dichloro-phenol-indophenol still possess a physiological influence upon photosyn-thesis, as the effect is not specific for ascorbic acid alone. In general it is unlikely that the fungi excrete ascorbic acid itself, as this sub-

stance has never been demonstrated in moulds. These organisms often produce considerable quantities of closely related substances like tetronic acid etc. (RAISTRICK 1940), furthermore the group

$$
\begin{array}{c}
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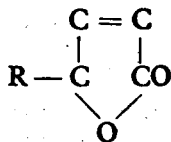
of ascorbic acid is found in many lichenic acids as well, a direct evidence that substances related to ascorbic acid play a role in the metabolism of lichens!

Although I thus could not deliver the decisive proof of the stimulation of the photosynthesis of the algae by the excretion of ascorbic acid or related substances by the lichen fungi, there is good evidence that this indeed will be the case.

C. CONCLUSIONS.

Before summarizing the facts mentioned in the preceding chapter I have to warn against generalisations. My experiments with the fungi mostly were performed with the symbionts of aerial algae, which are related to true lichen-fungi, but for which it is by no means proved that they are identical with certain true lichen-fungi, while I have only worked with one true lichen-fungus: Xanthoriomyces parietinae. As the most principal points in behaviour were met with all these fungi the possibility exists that these facts can be extended to other lichen-fungi as well. For the time being the obtained facts can only serve as lines of conduct for the investigation of other true lichen-fungi.

My experiments with the algae were all performed with true lichen-gonidia belonging to the genus Cystococcus. As in the most principal points they behaved identically, there is a good reason to extend the observed facts to other lichen-Cystococcus species as well. But it would be erroneous to extend these facts to gonidia belonging to other algal species. I recall here the observation of CHODAT (1913) that the gonidia of Verrucaria species belonging to Coccobotrys develop very well on purely inorganic substrates and are even harmed by the presence of glucose. When these green gonidia already behave essentially different from Cystococcus, it is obvious, that blue-green gonidia cannot be compared at all to the Cystococcus species.

Perhaps we may expect that the obtained facts may be extended to all heteromerous lichens with Cystococcus gonidia. They are only proved for one lichen; Xanthoria parietina (L) Th. Fr., of which I possessed both partners in pure cultures. The analogous behaviour of the other experimental objects is only an indicator that some generalisation is warranted.

Under this reservation I want to summarize the observations as follows:

a) Carbon supply. The fungi make use of the assimilation and reserve substances of the algae, some fungi use the proteins as well, cell-wall substances like cellulose and pectin cannot be used. The algae can live autotrophically, but also use organic substances taken by the fungus from the substrate, perhaps reserve substances of the fungi as well, but only simple carbohydrates are attacked, organic acids, like they are produced by many moulds, are very bad sources of carbon.

b) Nitrogen-supply. Both symbionts use inorganic as well as organic nitrogenous substances. The algae were only favoured by the presence of asparagin when the cultures were very young, as it shortened the lag-phase. In older cultures inorganic nitrogenous salts (ammonium salts) could be used as well. There was no reason to assume any assimilation of atmospheric nitrogen by one of the symbionts.

Here I want to add something. According to a number of Russian investigators (HENCKEL and YUZHAKOVA 1936, ZAKHAROVA 1938, and ISKINA 1938) Azotobacter should be present in all lichens, thus functioning as a third symbiont. Experiments performed by Miss J. K. H. WALENKAMP at the Botanical Institute in Leyden could not affirm these observations. She tried to isolate Azotobacter (or other nitrogen-fixing bacteria) from a great many lichens collected in the neighbourhood of Leyden, but the results were entirely negative. When we exclude the possibility of nitrogen-fixation in lichens, we have to make an exception for lichens with blue-green gonidia. As the possibility to fix atmospheric nitrogen has been proved for the free-living Nostoc commune (ALLISON 1935, BORTELS 1940) there is all reason to suppose that the Nostoc gonidia can perform the same feat.

c) The supplying with nutrilites. All fungi investigated could not develop on purely synthetic culture solutions without the addition of certain nutrilites to these solutions. These nutrilites appeared to be absolutely indispensable. Some of them already could be identified with aneurin and β alanin, while for the other substances it could be demonstrated that they belonged to the bios-group. It must be one of the first tasks of further lichenological investigations to

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find out whether this holds true for all lichen-fungi. It could be demonstrated that the algae can provide the fungi with these nutrilites.

When growing saprophytically the algae are favoured by certain nutrilites (some bios-substances, nicotinic acid), but these nutrilites were not absolutely indispensable for the development, they only shortened the lag-phase in cultures which were inoculated with small amounts of algae. So it is doubtful whether they will be very important for the problem of the symbiosis. For one of the fungi it could be demonstrated that it could synthesize these stimulating substances.

Far more important is the effect of ascorbic acid and related substances upon the autotrophic development, which is nil or very scanty without this substance. With reduced ascorbic acid (in an atmosphere of hydrogen with carbon dioxide) but as well with dioxymaleic acid and most probably with still other dienolic compounds a good autotrophic development was obtained. It is very probable that these substances act directly upon the photosynthesis as they are active only in autotrophic cultures. The definite proof that the fungi excrete such substances could not yet be delivered, but the "astounding" photosynthetic activity of the gonidia in a lichen-thallus makes it very probable. If this would be the case a definite benefit from the symbiosis for the alga would have been demonstrated.

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CHAPTER IV

THE PRODUCTION OF LICHENIC ACIDS.

A. INTRODUCTION.

On the preceding pages I described experiments with the two isolated compounds: alga and fungus. It may be asked whether such experiments suffice for a complete understanding of the economy of a lichen. Should a lichen-thallus only be regarded as the sum of the two components or is the lichen-thallus a new unity derived from the association of the two symbionts? Many authors defend the last-mentioned viewpoint. They assert that the lichen-thallus can be understood by adding the qualities of the alga to the qualities of the fungus no more than any other organism can be understood from the sum of the qualities of the separate cells. By the close contact between alga and fungus they should influence each others metabolism so

that a final metabolism should result, which differs from the metabolism of both components. This "Stoffwechsel eigner Art" (TOBLER 1925) resulted in the production of remarkable, specific substances: the lichenic acids. When this conception is the right one, a better insight into the origin of these substances will be very important for the better understanding of the lichen-symbiosis.

B. LITERATURE.

The first lichenic acid was discovered in 1826 by the chemist PFAFF, who isolated, from Cetraria islandica, a remarkable substance, which had not been found in any other organism before. This observation was soon followed by many others. Especially HESSE and ZOPF isolated, from many lichens, a great number of new substances. A survey of their work may be found in the well-known book of ZOPF (1907) "Die Flechtenstoffe". He showed that these substances were chemically far from related, so that they could only be defined by a biological definition: substances, which may be found in lichens in often considerable quantities and which have never been found in any other organism before. When the interest in the lichen-symbiosis decreased, these metabolic products were no longer investigated and only in recent times the constitution of a number of lichenic acids has been elucidated especially by the work of ROBERTSON and ASAHINA c.s. Before I refer to what is known about the biological origin of these products I will mention the chemical constitution of a number of lichenic acids making use of the classification of ASAHINA (1939):

A: Aliphatic substances

    1. fatty acids and lactones. Many important lichenic acids belong to this group e.g. proto-lichesterinic acid, nephrosterinic acid, caperatic acid etc. Their chemical constitution is best illustrated by the lichesterinic acid:

$$HOOC - CH - C = CH_2$$
$$| \quad |$$
$$CH \quad CO$$
$$CH_3 - (CH_2)_{12}O$$
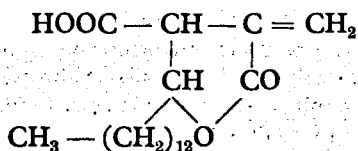
    2. neutral substances, which cannot be saponified by alkali. Their chemical constitution is rather unknown. Zeorin and leukotylin are tri-terpenic derivatives.

    3. polyalcohols, which may not be classified as lichenic acids,

as they are found in other organisms as well. Erythritol may be found esterified with certain lichenic acids f.i. with leca- noric acid in erythrin.

B: Aromatic substances.
1. derivatives of pulvinic acid e.g. pulvinic acid, vulpinic acid, calycin, stictaurin. Vulpinic acid has the following structure according to SPIEGEL:

$$C_6H_5 - C = C - C = C - C_6H_5$$

with COOCH$_3$, OH, CO —— O substituents
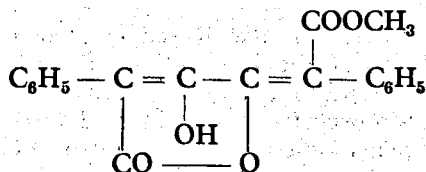
2. usnic acid, which structure has not yet been fully elucidated.
3. thiophanic acid.
4. depsids and depsidons of the phenol-carbonic acid orcin. The sceleton of their molecules has the following structure:

depsid   depsidon
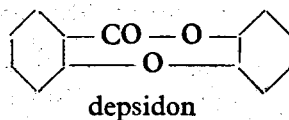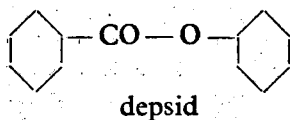
These substances are very important in consequence of their relationship with the tannins of higher plants. Many well- known lichenic acids belong to this group e.g. lecanoric acid, salazinic acid etc.
5. derivatives of anthraquinone, e.g. parietin, the well-known orange pigment of Xanthoria and Caloplaca species.

Which of the two symbionts produces these substances? All authors agree that they are finally excreted by the fungus. At a microscopical examination we always observe the crystals of the lichenic acids upon the hyphae, although they may be found especially upon those hyphae, which are in the neighbourhood of gonidia. The production by the fungus is very striking in Chiodecton sanguineum, a lichen investigated by TOBLER (1931). This lichen consists almost exclusively of hyphae which are heavily incrustated with the red Chiodectonic acid, but when the gonidia are present these crystals are lacking. TOBLER supposed that the metabolism of the fungus had been influenced by the algae, so that the excretion of the lichenic acids was suppressed. As TOBLER himself remarked, this case was

too exceptional to allow for generalisation. But in other cases I think the secretion of the lichenic acids by the hyphae to be not definitely proved, for firstly there are many lichenic acids which are present in so small amounts that the crystals cannot be observed in the thallus under the microscope and secondly a presence of crystals upon the hyphae does not demonstrate that these crystals are produced by these hyphae as it may be caused as well by a secondary incrustation.

Nevertheless we may assume that it is very likely that many lichenic acids are secreted by the hyphae. Then, however, the question arises, whether these substances are indeed formed by the fungus alone or only after the cooperation of the gonidia. It might be possible that they are only partially synthesized by the fungus, while the alga has produced some other part or that the alga had produced a substance, which has been changed by the fungus into the final lichenic acid. ZOPF (1909) held the opinion that the production of lichenic acids was caused by the cooperation of the two symbionts, as he was struck by the fact that these substances were highly specific to lichens and had never been found in any other organism, so that it was alluring to look after a connection between the symbiosis and the production of lichenic acids. His pupil TOBLER (1909) thought to have given the definite proof for this assumption; he cultivated the fungus of Xanthoria parietina in pure culture and did not observe any production of parietin, which may be easily demonstrated by the striking red colouration after addition of KOH. When, however, he had brought algae upon the mycelium and a beginning synthesis had been formed the presence of parietin could be demonstrated. His pupil BARTUSCH (1932) repeated this experiment in a rather inconvincing way. This experiment of TOBLER, however, does not prove the necessity of the symbiosis for the production of parietin, for we know that the metabolism of moulds is highly influenced by changes in the composition of the culture solution (MOLLIARD 1922, BUTKEWITCH and TIMOFEEWA 1935, PERQUIN 1938) and the effect of the synthesis with the algae might have been caused by a modification of the external conditions. The conclusion of TOBLER that the production of parietin was characteristic for the establishment of the physiological equilibrium of the symbiosis was premature.

Some doubt arose as to the supposed connection between the symbiosis and the production of lichenic acids, when it became known by the work of RAISTRICK c.s. (compilation of this work in 1932 and 1940), that many ordinary moulds synthesized a great number of remarkable metabolic products, which often showed some resemblance with certain lichenic acids, while finally he suc-

ceeded in isolating, from a strain of Aspergillus glaucus Link., an emodin mono-methyl ether, which was identical with parietin. This was the first observation that a lichenic acid had been synthesized by another organism than a lichen and the fact that this organism was a fungus did make it highly probable that the lichen-fungi as well might be able to synthesize such substances without the aid of an alga. The definite proof for this assumption was delivered by THOMAS (1936, 1937), who demonstrated the presence of parietin in pure cultures of Caloplacomyces and Xanthoriomyces (although only in a few strains of the latter fungus) and stictaurin in Candelariellomyces vitellinae. So these substances must be regarded as the result of the metabolism of the fungus alone. It may be doubted whether this is the case with all other lichenic acids as well. The lichenic-acids are chemically far from related, so that it is doubtful whether they should be biologically equivalent. Moreover THOMAS has only observed the production of lichenic acids in a small number of cultures; he does not mention a production of these substances in many other fungi, although they had been isolated from lichens, which in nature always contain remarkable quantities. Very interesting are the observations of THOMAS, that the production of lichenic-acids by isolated lichen-fungi is independent of the light, but is accelerated by the presence of many food substances as it has been found, that lichens growing in the light contain more lichenic acids than lichens developing in the shade (TOBLER 1925). It is evident, that the latter effect only is produced by a higher photosynthesis of the algae, so that there are a greater number of foodsubstances available for the fungus. The temperature did not influence the production of parietin in cultures, although stictaurin only was produced at temperatures higher than 9° C. In nature the production of lichenic acids may be influenced by the humidity (ZOPF 1907) and the oxygen of the air, as they are preferably secreted in parts of the thallus which are exposed to the air; most probably this effect is caused by a more active metabolism.

The biological importance of these lichenic acids is doubtful. ZOPF (1907) merely regarded them as excretion products. The opinion of previous authors (ZUKAL 1895), that the lichenic acids protected the thalli against damage by animals has been refuted by his experiments. Other authors thought that the acids lowered the transpiration, while GOEBEL (1926) observed that they protected the gonidia against water-infiltration. The latter possibility will be considered in chapter V, yet I may already remark that this function is not very important. So it is likely that the lichenic acids, which are

no more used in metabolism, are nothing more than excretion products.

After this survey of the literature I will describe my own experiments, which have already partially been published elsewhere (1942). As to more extensive treatises of this subject I refer to the book of ZOPF (1907), to the books of TOBLER (1925, 1933), to the survey of BRIEGER and THIES (1932), CZAPEK (1925) and to the more chemical compilations of KARRER (1926) and ASAHINA (1934, 1935, 1939).

C. ON THE PRESENCE OF LICHENIC ACIDS IN ALGAL-COVERS.

Before considering the question whether the symbionts of aerial algae, which had to be regarded as relatives to true lichen-fungi, could produce lichenic acids or similar substances in cultures I had to investigate whether the lichenized algal covers contained such substances. This question was the more important as lichenic acids had only been found in true lichens, while there was nothing known about specific metabolic products of "half-lichens". I began with the examination of the heavily lichenized soredial covers of Cystococcus, from which I had isolated the fungus C. As these covers had to be regarded as soredial or lepral forms of true lichens and as it was already known that soredia contain enormous quantities of lichenic acids, the presence of such substances in the Cystococcus covers had to be expected. Indeed it appeared, that all these covers, collected from the barks of a great many trees, contained a crystalline substance, which could be isolated as follows:

The algal-material was extracted in a Soxhlet-apparatus with acetone. When the extraction was complete the acetone was destilled off, after which a dark mass containing the "lichenic acid" and fats, chlorophyll etc. remained in the flask. The flask then was washed with chloroform and benzene to remove fats and chlorophyll. The lichenic acid could be obtained by filtration of these chloroformic and benzenic suspensions, after which the crystalline substance remaining in the filter was purified by crystallisation from alcohol and ether.

Characteristics: a colourless substance, crystallizing from alcohol in very small prismatic needles. It becomes brown at 240° and carbonizes at 260° C. It is soluble in hot alcohol and acetone, less soluble in hot ether, only slightly soluble in these solvents when cold. It is insoluble in benzene, petrol-ether and water. The substance shows the following characteristic colour reactions:

1. the alcoholic solution gives a red colouration with $FeCl_3$.
2. the solution in concentrated KOH has a yellow colour.
3. concentrated H_2SO_4 gives a brownish-red solution.
4. after boiling with NaOH and a drop of chloroform a red coloured solution is obtained, which fluoresces green after pouring into water (the so-called homo-fluorescein reaction characteristic for derivatives of orcin).
5. the crystals become yellowish-brown in a 1/50 solution of paraphenylenediamin, and yellow in a 1/50 solution of benzidin or alanin (reactions of ASAHINA 1934 given by depsids and depsidones with an aldehyde group).

All these reactions were as definite after ten times crystallisation from alcohol, so that they do not need to be described to an impurity (see below). Most probably this substance is related to salazinic acid although it is not identical with this depsidon.

Covers of *Pleurococcus vulgaris* Boye Pet. were extracted in the same way as the covers of Cystococcus with acetone, the acetone evaporated and the residu washed with cold chloroform and benzene. After filtration a crystalline substance remained in the filter, which showed a number of colour reactions. These colour reactions disappeared after recrystallisation so that they were produced by a contaminating substance. Whether this contaminating substance was a lichenic acid could not be decided, but as its quantity certainly was very small and the algal cover could have been mixed with lichensoredia it was useless to try to isolate this substance. The crystalline substance itself could be identified as erythritol. In no other ways the presence of a lichenic acid could be demonstrated.

On the other hand the covers of *Apatococcus minor* Edl. appeared to contain beside erythritol a remarkable substance, which certainly is identical with the "acide phycique" discovered in 1852 by LAMY. Instead of "acide phycique" I will use the provisional name apatococcin, as this substance is no free acid and the name "phycique" would cause confusions with substances like phycinic acid, physcion etc., which are far from related to apatococcin. LAMY already performed an elementary analysis and observed that after boiling with alkali a foamy solution ensued of the sodium salt. After the work of LAMY this substance went into oblivion and was only reinvestigated in 1927 by a pupil of Prof. Dr. G. VAN ITERSON: J. G. V. D. SANDE. (unpublished) [1]. From his observations the following facts may be mentioned. An elementary analysis showed the following values:

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[1] I owe much thanks to Prof. Dr. G. v. ITERSON for his permission to make use of these unpublished results.

C 69.90 %, H 11.93 % and N 3.00 %. The molecular-weight was determined according to RAST from the melting-point depression of campher and gave in two determinations as results 400 and 430, according to the method of BARGER 440—465 and 460—480 and from the boiling-point of the acetonic solution 470 and 480. From an extensive chemical investigation he drew the conclusions that the substance was neither a free base nor a free acid, that it was no alcaloid, no aldehyde, no derivative of pyrrol, phytosterol, pentose or anthracene and that it was no amine. The substance gave no biuret reaction. It was completely saturated. After boiling with acetic-anhydride the substance could be acetylated. By melting with solid NaOH a nitrogenous vapour escaped, in which microchemically a substance could be demonstrated which bears some resemblance to coniin. After the action of concentrated HNO_3 or H_2SO_4 an oily liquid was formed, which could not be crystallized.

As this substance, as will be shown below, had to be considered very important for the problem of the production of lichenic acids I have tried to elucidate the constitution of this substance in order to know whether it was related to certain lichenic acids. To this purpose I made use of the well-known books of MEYER (1938), ROSENTHALER (1914), GATTERMANN (1941) and BEHRENS-KLEY (1922).

P r e p a r a t i o n: the algal cover was extracted with acetone, the acetone destilled off and the residu washed with benzene and filtered through a glass-filter. In the filter remained the rough product, which could be purified by crystallisation from alcohol and from ether. Usually 1 kg of algal-material yielded 1 gram apatococcin.

C h a r a c t e r i s t i c s: It is a beautiful, white substance, con-sisting of very long threadlike crystals, melting at 139° C., soluble in most organic solvents, best in chloroform and pyridin, less in hot alcohol, acetone and ether, slightly in these solvents when cold, soluble in carbon-bisulfide, insoluble in water, by which it could not even be moistened. It sublimated at 1 cm pressure at 140—160° C. The crystals show a faint double-refraction. No colour reactions could be detected.

The empirical formula of the substance followed from elementary analysis performed by Mr. P. J. HUBERS, laboratory for organic chemistry, University of Amsterdam, which gave the following values in two determinations:

C 69.22 % H 11.33 % N 3.64 %
C 69.78 % H 11.73 % N 3.68 %

Although the tentative formula cannot be established with absolute

certainty she most probably is: $(C_{23}H_{45}O_4N)n$. This formula should yield

$$C\ 69.13\ \%\quad H\ 11.35\ \%\quad N\ 3.51\ \%$$

It is possible that the molecule of apatococcin contains one C atom or some H-atoms more or less.

The molecular weight was determined after the method of RAST by measuring the melting-point depression of campher (molecular freezing-point depression 39.7).

1.203 mg apatococcin were dissolved into 12.698 mg campher. The melting point depression amounted to 8.7° C. From this we calculate:

$$M = 39.7\ \frac{1000 \times 1.203}{12.698 \times 8.7} = 432.$$

With some reserve I propose the following elementary formula:

$$C_{23}H_{45}O_4N$$

In alcoholic solution the substance shows a neutral reaction. The chloroformic solution was shaken with 0.1 N NaOH, 1 N NaOH, 0.1 N HCl and 1 N HCl. The alkaline resp. acid fraction was acidified resp. made alkaline and shaken with chloroform. The five chloroformic fractions thus obtained were evaporated upon small watch-glasses. All apatococcin appeared to have remained in the original chloroformic solution. The substance could not be shaken out of its chloroformic solution neither by acids nor by bases, so that it is neither a free acid nor a free base.

By boiling with a dilute alcoholic solution of NaOH the substance is saponified: 10.41 mg apatococcin was dissolved into 1.983 cc NaOH 0.0456 N. It appeared, that 0.595 cc had been bound, which corresponds to 0.278 cc 0.1 N NaOH. So by saponification a one-basic acid has been formed. The alkaline solution had a foamy character. After acidification the free acid precipitated as a crystalline substance, consisting of very long, thread-like crystals, somewhat less flexible than the crystals of the original apatococcin. The acid melted at 160° C. The elementary-analysis performed by Mr. P. J. HUBER showed the following values:

$$C\ 68.28\ \%\quad H\ 11.26\ \%\quad N\ 3.94\ \%$$

Calculated for $C_{21}H_{41}O_4N$: C 67.89 % H 11.13 % N 3.77 %
for $C_{22}H_{43}O_4N$: C 68.53 % H 11.24 % N 3.63 %

Finally the original apatococcin again was sent to Mr. HUBERS for a methoxyl- and ethoxyl determination. The results were:

9.50 % OC_2H_5 or 6.55 % OCH_3
Calculated for one group OCH_3 7.77 % and for one group OC_2H_5
11.28 %. So we may conclude that most probably apatococcin con-
tains one group $COOCH_3$ (or $COOC_2H_5$).

Except by boiling with a dilute alcoholic solution of NaOH, the
substance is saponified by the prolonged action of cold concentrated
NaOH.

The action of hydrochloric acid did not change the substance:
20 mg apatococcin were boiled under a reflux-condensor with 1
N HCl in 70 % alcoholic solution, then the solution was diluted
with water and the quantitatively crystallizing substance filtered.
This substance could be identified with the unchanged apatococcin
(by mixed melting-point determination). After the action of con-
centrated HNO_3 or H_2SO_4 oily products were formed, which could
not be crystallized.

The substance seems saturated: when somewhat Na_2CO_3 and a
drop of a $KMnO_4$ solution was added to the cold alcoholic solution
of apatococcin, the red colour did not disappear, nor could I observe
a decolouration of bromine by the chloroformic solution.

The elementary formula makes it probable that apatococcin pos-
sesses a long paraffin chain, which also would account for the foamy
character of the sodium salt. In the esterified carboxylic group two
O atoms are present. My task was to determine the function of the
two other O and the N atom.

From the following experiments it is evident, that apatococcin is
neither an aldehyde nor a ketone:
the alcoholic solution gave no precipitation with phenylhydrazin
and sodium acetate.

20 mg apatococcin were boiled in an alcoholic solution with an
excess of phenylhydrazinic chloride dissolved in acetic acid. After
one hour of boiling the solution was diluted with water, after which
the unchanged apatococcin crystallized quantitatively.

According to V. D. SANDE the substance may be acetylated by
boiling during some days with acetic anhydride and a trace of sodium
acetate. Yet he did not determine the presence of the acetyl group
in the obtained substance, so that it is not quite certain that this
substance had been obtained by acetylation of the apatococcin. When
I repeated this experiment I indeed observed the very remarkable,
rhytmically crystallizing, crystals of the acetylation product, but
while after a short period of boiling (e.g. some hours) most of the
apatococcin had remained unchanged, after a long period of boiling,
as recommended by V. D. SANDE, most of the substance deteriorated
to an oily liquid. As acetylation usually is performed by boiling

with the acetic anhydride during some hours it is by no means certain that apatococcin may be acetylated as it is not impossible that the "acetylationproduct" had been formed by other reactions, e.g. dehydration. Yet we have to take into account that one or two groups OH are present in the molecule of apatococcin, although an eventual acetylation might have been performed at the nitrogen as well. It would be important to use other acetylation or benzoylation methods, as perhaps they will give more reliable results.

The function of the nitrogen is very important, as most lichenic acids contain no nitrogen, with the exception of picroroccelin, which surely is not related to apatococcin.

I have mentioned that apatococcin is no free base. This makes it improbable that it has the character of an amine or an alcaloid. I reacted upon the alcoholic solution with the following reagents: concentrated JKJ, potassium bismuth jodide, potassium mercuric jodide, phospho-tungstic acid, phospho-molybdenic acid and picric acid. So I did upon the solutions in chloroform and in carbonbisulfide and the solution of the sodium salt in water (STEPHENSON 1921, BEHRENS-KLEY 1922). All reactions had a negative result, so that an alcaloid character is highly improbable.

In different ways apatococcin was subjected to the action of HNO_2: some mg apatococcin were dissolved in concentrated acetic acid and small crystals of sodium nitrite added; when the gas-development had stopped the solution was diluted with water. The quantitatively precipitating crystals could be identified with un-changed apatococcin, which is in good accordance with the results of V. D. SANDE. Most probably apatococcin is no primary or secun-dary amine, no simple amide and no amino acid. The possibility, that apatococcin was a primary amine already had been rejected as the experiment of HOFFMANN with chloroform and sodium hydroxide gave a negative result, the odour of isonitrils could not be perceived. An amide group already was improbable, as the nitrogen could not be removed by saponification. As, however, many amids are difficult to saponify I tried to remove the nitrogen according to the method of BEAUVEAULT. This method had the disadvantage that apatococcin already is affected by concentrated sulphuric acid, which must be used as a solvent, so that it was difficult to show any affection by the nitrite. 20 mg apatococcin were dissolved in 1 cc concentrated sul-phuric acid and with a pipette I added 1 cc of a 3.6 % $NaNO_2$ solution under cooling with ice. Afterwards I heated to 60° C., while a heavy gas development could be observed. When this had finished the solution was poured into water and the partially oily precipitate was dried and sent to Mr HUBERS for a Nitrogen determination, which

yielded 3.06 %. So the nitrogen had not been removed by this action. Moreover I was able to separate the crystalline part of the precipitate from the oily rest by micro-vacuum sublimation after which it could be identified with the unchanged apatococcin.

That apatococcin should be an amino-acid already is improbable by the fact that, when dissolved in neutralized formol, even after boiling, no acid reaction could be observed (determined with Lyphan indicator papers). Furthermore ninhydrin gave no colour reaction.

It was possible, that apatococcin was a quaternary ammonium base as these bases often are not affected by NaOH, but react with moist silver oxyde. After mixing apatococcin with this reagent, even after heating, no change could be observed.

According to V. D. SANDE in the vapour, which escapes after melting with solid NaOH, a substance can be detected which resembles coniin. I repeated this experiment as follows: 100 mg apatococcin were melted with 1.5 gr. NaOH (p.a. Kahlbaum) and the developed vapour conducted through $^11_{10}$ N HCl. This HCl then possessed, especially when made alcaline, a remarkable odour, which reminded of the odour of coniin (but of course of acetamid as well). Yet the microchemical reactions made evident that most part of the nitrogen was present as ammonia: Nesslers reagent gave a brownish-orange coloured precipitate, platinic chloride and 2-4-dinitro a naphtol (KLEIN and STEINER 1928, STEINER and LÖFFLER 1929) gave crystals characteristic for ammonium. This result was contradictory to the results of V. D. SANDE. No alcaloid reactions could be observed. Then I tried to concentrate the substance responsible for the odour of coniin. The hydrochloric solution was evaporated and the residue dissolved in a small amount of water. This neutral concentrated solution indeed showed some reactions, which could not be ascribed to ammonium. With a concentrated solution of JKJ an oily precipitate was formed, while potassium bismuth jodide produced a crystalline precipitate, which highly resembled the precipitate obtained with coniin as illustrated by BEHRENS-KLEY. Without any doubt these reactions were caused by the substance which had been obtained by V. D. SANDE in much larger amounts. As in the experiments of V. D. SANDE the reaction with chloro-anil could not be obtained and after comparison with the reaction given by synthetic coniin (kindly put to my disposal by Prof. Dr. G. VAN ITERSON) it was evident that my substance was not identical with this preparation. However we have to be very cautious, as in my solution the reaction might have been prevented by the presence of contaminating substances. According to AMELINK (1928) the reaction with chloro-anil only is characteristic for synthetic coniin and cannot be obtained with

the natural occurring coniin as here contaminating companion-alcaloids interfere with the reaction. The reaction, which this author considered specific for the natural coniin, with $K_4Fe(CN)_6$, was not shown by my solution; I had no opportunity to perform this reaction with a sample of natural coniin. My solution did not show any reaction with Mayers reagent (potassium mercuric jodide) which is a strong argument against the presence of coniin or any other alcaloid in the solution. So there is no direct evidence that after melting with solid NaOH coniin can be formed, the odour of the destillate may be caused as well by acetamid, while we should not forget that the pure apatococcin already shows a very remarkable odour after heating, which was already mentioned by LAMY. Most of the nitrogen in the destillate was present as ammonia.

After heating with CaO or MgO or after zinc-destillation the nitrogen escaped, but in the destillates only ammonia could be detected. If the "coniin" had to be considered as an incomplete destruction-product of apatococcin obtained by melting with NaOH, it would have been expected in these less radical reactions as well. On the contrary I only observed the total destruction-product: ammonia. So I think it very likely, that this "coniin" is only a secondary product arisen from the melting with NaOH. There is no reason to suppose the presence of a preformed alcaloid structure in the molecule of apatococcin, as moreover the alcaloid character of the mysterious product in the destillate is far from evident.

C o n c l u s i o n s: We may conclude from the above experiments that apatococcin most probably possesses a long saturated paraffin chain and one esterified carboxylic group. The function of the nitrogen could not be detected. As apatococcin shows no alcaline properties it is not unlikely that the nitrogen in the molecule is already bound by a carboxylic group, for instance as a betain or as a complex acetamid or a lactam-ring.

When we consider the question whether apatococcin may be related to certain lichenic acids, it will be evident, that we have to look at the aliphatic lichenic acids like proto-lichesterinic acid. These too are substances with one or more carboxylic groups and a long paraffin chain. A chemical relationship between apatococcin and these lichenic acids is far from impossible. A principal difference is formed by the presence of nitrogen in apatococcin. Perhaps apatococcin has to be considered as a derivative of these lichenic acids or even as a proto-lichenic acid, which in the lichen is changed into the lichenic acid itself. In any case it will be important to elucidate the origin of this remarkable substance.

D. ON THE PRODUCTION OF LICHENIC ACIDS BY THE SYMBIONTS OF AERIAL ALGAE.

When I had shown, that the heavily lichenized Cystococcus covers contain lichenic acids and less heavily lichenized Apatococcus covers contain comparable substances like apatococcin it seemed worth-while to investigate whether the symbiontic fungi of these algal covers could produce these substances in pure cultures. Moreover, after the work of RAISTRICK and THOMAS, the assumption seemed war-ranted that these fungi, which had to be considered as relatives to true lichen-fungi, would be able to synthesize remarkable substances, which could be important for the problem of the production of lichenic acids. As the composition of the culture solution highly influences the metabolism of moulds it was necessary to cultivate my fungi on a great number of substrates. After some time, mostly two months, the mycelia were filtered and the culture solution tested with $FeCl_3$ upon phenolic compounds, extracted with ether in an extraction-funnel and the ether fraction evaporated upon a small watch-glass to detect ether-soluble crystalline substances. The my-celium was extracted with alcohol or acetone, this extract tested with $FeCl_3$ upon phenolic compounds and the rest of the extract evaporated on a watch-glass to detect crystalline substances, for which purpose it often was necessary to wash the watch-glass with water and petrol-ether in order to remove sugars and fats, which interfered with the observation of eventual lichenic acids. In some cases, as for instance in all cultures of the fungus Ap 1, a small part of the mycelium was sublimated in a micro-vacuum sublimation apparatus according to KLEIN-WERNER in order to detect sublimable lichenic acids or apatococcin, which can be easily recognized in sublimates by its very characteristic curved crystals (fig. 19). The experiments mainly were performed with the fungi Pl 1, Pl 2, Ap 1 and C. The growth-velocity of Xanthoriomyces was too small to make possible experiments like these, in cultures of this fungus on malt-agar I have not been able to observe parietin. All experiments were performed in Erlenmeyer flasks of 300 cc provided with 100 cc of a Czapek-Dox solution, when nothing else is mentioned. As, when performing these experiments, the growth-requirements of these fungi had not been analysed I always added 1 cc yeast-auto-lysate to the cultures.

Firstly I tried to demonstrate the production of lichenic acids in the culture solution of Czapek-Dox, (page 451) and the culture solution of RAULIN-THOM (1500 cc water, 75 gr. glucose, 4 gr. tartaric acid, 4 gr. ammoniumtartrate, 0,6 gr. amm. phosphate,

0.6 gr. K_2CO_3, 0.4 gr. $MgCO_3$, 0.25 gr. $(NH_4)_2SO_4$, 0.07 gr. $ZnSO_4$.7 H_2O and 0.07 gr. $FeSO_4$.7 H_2O), which had been mostly used in the experiments of RAISTRICK. In both solutions the results were negative. This might have been caused by a low intensity of the production, so that I had too little fungus material to detect the presence of a lichenic acid. With the fungi Pl 1 and Pl 2 I inoculated some mass-cultures in four Erlenmeyer flasks of 2 L., each filled with 750 cc Czapek-Dox solution. The extraction of the mycelia (12 gram Pl 1 and 10 gram Pl 2 dry-weight) yielded nothing. The solutions gave, even after concentration no colour reaction with $FeCl_3$.

Then I hoped to obtain better results by changing the culture-solution. As a standard medium I used the Czapek-Dox solution, which could be more easily modified than the unnecessarily complicated solution of Raulin-Thom. The variations performed consisted of the replacement of the ammonium nitrate by other sources of nitrogen, by changing the quantities of the salts, by adding other elements and so on. The solutions are described in table XX with the experimental numbers of the cultures inoculated. No results were obtained; neither a lichenic acid, nor a substance like those described by RAISTRICK, could be detected. A replacement of the glucose by peptones (at Pl 2) or erythritol did not better the results.

A great number of lichenic acids, namely the depsides and the depsidons show some similarity with the tannins. It is not excluded that certain lichen-gonidia are able to produce tannins, as they have been demonstrated in other algae (Spirogyra by v. WISSELINGH 1914), although microchemical reactions upon the presence of tannins in my algae only gave negative results (ferric-acetate, potassium bichromate, sodium molybdate). It might be possible, that the fungi would be able to attack these tannins, which would yield the simple crystalline depsids known as lichenic acids. As of course I did not know anything about the chemical nature of these eventual algal tannins, my experiments need remain limited to commercial tannin. I never observed that one of my fungi had been able to attack this substance, in only small concentrations (0.1 %) tannin was definitely toxic.

In a lichen-thallus the fungus will be provided with oxygen by the assimilating algae. It is a well-known fact that the lichenic acids are mainly secreted at parts of the thallus, which are exposed to the air. So it might be possible that a high percentage of oxygen might favour the production of lichenic acids. Cultures in a jar, filled with oxygen yielded a negative result, no lichenic acid nor a comparable substance could be demonstrated.

TABLE XX

fungi				concentration culture solutions										
Pl 1	Pl 2	Ap 1	C	glucose	KNO₃	NH₄NO₃	(NH₄)₂SO₄	pepton	KH₂PO₄	KCl	MgSO₄	FeCl₃	CaCl₂	MnSO₄
90	196	208	221	5		0.2			0.1	0.05	0.05	0.002		
92	218	216	222	5					0.1	0.05	0.05	0.002		
98	219	217	223	5					0.1	0.05	0.05	0.002		
100				5			0.2		0.1	0.05	0.05			
104			224	5	0.2	0.2			0.1	0.05	0.05	0.002	0.05	
106			225	5	0.2	0.2			0.1	0.05	0.05	0.002		
108				5		0.2			0.1	0.05	0.05	0.002	0.05	0.005
110			226	5		0.2			0.1	0.05	0.05	0.002		
112				5		0.05			0.05	0.05	0.05	0.002	0.05	0.005
167	197			5				1.0	0.1	0.05	0.05			
168	200			5				0.5	0.1	0.05	0.05			
169				1				0.1	0.1	0.05	0.05			
170				5				0.01	0.01	0.05	0.05	0.002		
171	201		227	5				0.1	0.1	0.05	0.05	0.002		
172				5				0.01	0.01	0.05	0.05	0.002		
173			228	5				0.1	0.01	0.05	0.05	0.002		
174	198			5		0.1			0.1	0.05	0.05	0.002		
176				5	0.01	0.01	0.01		0.1	0.05	0.05	0.002		
177				5					0.1	0.05	0.05	0.002		
178	199	210	229	5	0.01	0.01	0.01		0.01	0.05	0.05	0.002		
179	207	218	230	5	0.01				0.01	0.05	0.05	0.002		
180	206	219	231	5					0.01	0.05	0.05	0.002		
181				5					0.01	0.05	0.05	0.001		
182				5					0.01	0.01	0.01	0.001		
183	202	220	232	5		0.01	0.01		0.01	0.01	0.01	0.001		
175				5					0.01	0.01	0.01	0.001		
233	234	235	236	10		0.5			0.2	0.1	0.1	0.01		

numbers of the experiments

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Continuous illumination with electric light, low temperatures and high temperatures (0°—30° C) did not better the results.

One might oppose that cultures in liquid culture solutions are quite unnatural. Yet mycelia formed upon agar-media or pieces of plaster soaked in Czapek-Dox solution produced no lichenic acids. Finally I hoped that the addition of extracts of algae would stimulate my fungi to produce lichenic acids. As my cultures of algae yielded too small amounts of algal-material I had to use extracts of algal covers, which were prepared as follows:

> one part of algae was boiled during an hour with three — four parts of water, filtered and sterilized during 15 minutes at 120° Besides I used a suspension which had been prepared in the same way, but which had not been filtered.

As in these algal covers the fungi are only present in relatively small amounts, there will be only a small difference with extracts prepared from pure algae. Yet I had always to compare the results with an uninoculated control as this already showed a ferri-reaction, while in the suspensions of Apatococcus of course apatococcin was present. After a two month incubation of the fungi Pl 1 and Pl 2 in these extracts and suspensions no formations of a lichenic acid could be demonstrated.

Although of course many other variations in the culture solutions might be possible, it is unlikely that in this way these fungi can be stimulated to the production of lichenic acids or similar substances. The absence of the possibility to produce lichenic acids especially is remarkable for the fungus C, which had been isolated from the Cystococcus cover containing the described lichenic acid, while furthermore I want to stress the fact that the fungus Ap 1 did not produce apatococcin. In later experiments I was able to show that the fungi Ap 2 and Ap 3 developed upon malt-agar neither produced this substance, while the malt-agar cultures of Xanthoriomyces contained no parietin. Thomas too could only detect parietin in some strains of Xanthoriomyces.

E. ON THE PRODUCTION OF LICHENIC ACIDS BY THE ALGAE.

Most investigators were so convinced of the final production of lichenic acids by the fungi, that the production of such substances by the algae has never been considered. As the fungi, which I have investigated, produced no lichenic acids and no apatococcin in cultures it was worth-while to consider the possible production of these substances by the algae. As the quantity of algal material, which I

could obtain from my cultures was very small, this investigation had to be restricted to only few experiments.

Alcoholic extracts of cultures of Apatococcus and the three lichen-gonidia on BEIJERINCK-agar, BEIJERINCK-agar 2 % glucose, and 1 % glucose ½ % pepton agar yielded, even after concentration, no colour reactions with $FeCl_3$. After evaporating the extracts upon little watch-glasses I could, after washing with water and petrolether, observe no crystalline products. I only want to mention the presence of erythritol in the extracts of Apatococcus, which of course was removed by the washing with water.

Furthermore the investigation remained limited to trials to detect apatococcin in pure cultures of Apatococcus minor Edl. There were many reasons to suppose that apatococcin was produced by this alga:

1. apatococcin is present in considerable quantities in covers of Apatococcus, although the number of algal cells is much more important than the number of fungal hyphae.
2. when, nevertheless, the fungi would synthesize this substance, this would indicate, that the metabolism of the fungi yielded enormous amounts. This was hardly in accordance with the inability of the fungi to produce apatococcin in cultures. As, however, a great number of different fungi occur in the covers, it was not excluded that, by change, the fungus responsible for the production of apatococcin had not been isolated.
3. apatococcin is specific for covers of Apatococcus; in no way I could demonstrate this substance in covers of Pleurococcus. As I had observed that these covers possess a great number of fungi, which are not specific for certain algae (as they may be isolated as well from Apatococcus and Pleurococcus covers), the specificity of apatococcin to apatococcus-covers might be explained only by assuming that the alga is mainly responsible for the production.

When I tried to detect the presence of apatococcin in cultures of Apatococcus minor (strain of Delft) I made use of the micro-vacuum sublimation apparatus according to KLEIN-WERNER (described in KOFLER and KOFLER 1936). In natural covers apatococcin is easily recognizable in the sublimate by its very long, curved, threadlike crystals (text fig. 19). Although of course these crystals are contaminated it appeared to be possible to perform a micro-meltingpoint determination in an apparatus like that described in the book of KOFLER and KOFLER as well. On the average this melting-point was situated $\pm$ 10° below the melting point of pure apatococcin ($\pm$ 129° instead of 139° C). The bi-refraction was too small to be used as a characteristic.

From the algal cultures much more contaminating substances were

sublimated from the covers. Part of these substances were formed by erythritol, which could be removed by washing with water. As the danger was rather great that in spite of all precautions the crystals of apatococcin were purely mechanically washed away, this method could only be used when the quantity of apatococcin was abundant like in the sublimates from the covers. Otherwise it was advisable to drop a small amount of ether (p.a.) along the sublimate to a slide, upon which the ether evaporated. From this slide the crystals were, if necessary, again taken up by ether. When after evaporation of this ether extract no apatococcin was visible I concluded that the result was negative.

In the winter of 1940—1941 I examined a great number of cultures of the Delft strain of Apatococcus, which had been cultivated on the following sub- strates (all provided with 2 % agar).
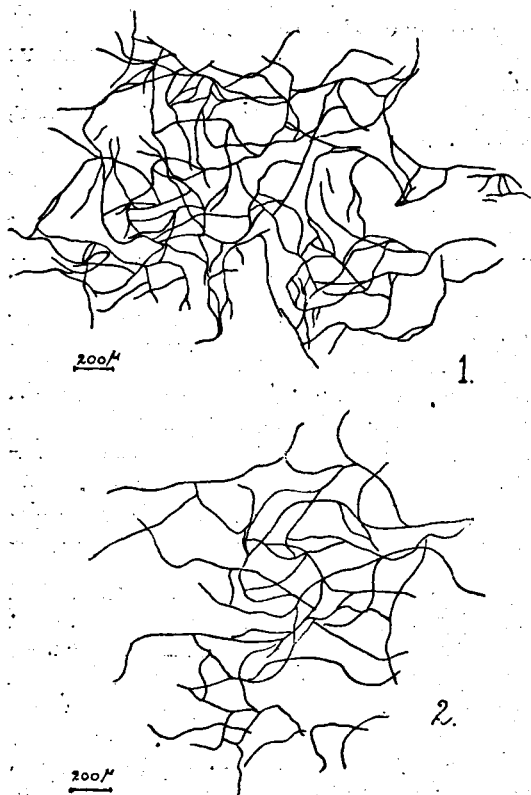
Fig. 19.

1) Sublimate from a cover of Apatococcus minor Edl. recolted from a stone wall.
2) sublimate from a pure culture of Apatococcus minor Edl. on Beijerinck agar with 2% glu- cose, which had died off during the hot days of June 1941.

BEIJERINCK solution
,,   ,,   2 % glucose
,,   ,,   2 % glucose and 0.5 % NH$_4$NO$_3$
,,   ,,   2 % glucose and 0.5 % pepton (Witte)
,,   ,,   2 % glucose and 0.5 % asparagin
,,   ,,   5 % glucose

BEIJERINCK solution 10 % glucose
    ,,   ,,   15 % glucose
    ,,   ,,   2 % glucose and the K_2HPO_4 replaced by
                    KH_2PO_4
malt agar
½ % peptone 2 % glucose.

In none of these cultures apatococcin could be demonstrated.

As it might be possible that apatococcin was present, bound to other cell-constituents, I tried to detect apatococcin after saponification with 30 % KOH during a day. The suspension in KOH then was acidified, filtered and the filtrate shaken with ether. The ether layer was evaporated on a small plate of copper, and the algae, which had remained in the filter, brought upon this plate. The plate was dried in a desiccator over $CaCl_2$, brought into the sublimation apparatus and subjected to a fractionated sublimation at 1 cm pressure. The fraction sublimating at 140—160° C. was examined. The sublimate was worked as I have described above. Sometimes I observed in these sublimates long, threadlike, crystals, which were too much contaminated to allow for an accurate melting-point determination; the melting-points observed did not exclude an identity with apatococcin. When I performed this experiment I did not yet know that apatococcin is saponified in this way to apatococcinic acid, which sublimates at higher temperatures. It is possible, however, that the impure apatococcinic acid already sublimated at lower temperatures; the crystals are too much alike to distinguish between these two substances.

When, however, I sublimated a number of Apatococcus cultures during the summer months of 1938 and 1941 I obtained far better results. In consequence of high temperatures a great many cells had died off, in 1941 even all cells had died and the cultures had become white. In the sublimates of these dead, colourless, cultures, in the strain from Delft as well as in the strain from Leyden, the characteristic crystals of apatococcin were already visible, without any washing with ether, directly in the sublimate, in the partially dead cultures of 1938 I observed them only when I had treated the sublimate with ether as described above (text fig. 19). With one of these crystals a melting-point determination could be performed, which yielded 129° C., so that there is little doubt that these crystals belong to apatococcin.

It might be asked whether apatococcin was produced at the dying of the algae or had been present in the living cells as well. The experiments with the saponified algae would be an argument for the

latter supposition, but they are by no means convincing. To our purpose it is more important to state that a substance, which shows a certain resemblance with some lichenic acids, is produced by the (living or dying) alga Apatococcus minor without the assistance of a fungal partner. Yet the quantity of apatococcin in the lichenized covers exceeds far the quantity in the cultures. This may be caused by the activity of the fungus, but there are other explications, which perhaps are more likely. When a cover develops, the old layers always are overgrown by the young cells. In consequence the old cells will be killed so that apatococcin is produced or set free. This apatococcin will accumulate, for it cannot be used in the metabolism of the vital cells and cannot be washed away by rain water, as these algal covers are not infiltrated by liquid water and moreover apatococcin is insoluble in water. So it need not surprise that the algal covers, which are several years old contain more apatococcin than the two months-old cultures. After the dry summer of 1941, in the covers on the bark of the trees in the Hortus Botanicus in Leyden, which usually consisted of Pleurococcus, Apatococcus had obtained the upper hand. Yet I could not detect apatococcin in the sublimates from these young apatococcus covers, although old covers of Apatococcus recolted from the bark of the same trees yielded considerable quantities. So there are reasons to account for the large quantities apatococcin in the lichenized algal covers by their great age. Moreover the greater quantities of erythritol in young Apatococcus cultures impedes the observation of the few crystals of apatococcin in the sublimates, while in the old covers only a small amount of erythritol is present.

We already have mentioned that covers of Pleurococcus vulgaris B.P.contain no apatococcin. This warrants the conclusion that apatococcin, like many lichenic acids, is a specific metabolic product. In cultures of the lichen-gonidia (living or dead, with or without previous saponification) cultivated on BEIJERINCK agar, BEIJERINCK agar 2 % glucose and 1 % glucose $\frac{1}{2}$ % peptone agar, no apatococcin could be detected. It is possible, that these algae synthesize other related substances, which cannot be demonstrated by sublimation (I remind for instance of apatococcinic acid, which only differs from apatococcin in some minor details and can be sublimated only with difficulty). As it was very difficult to demonstrate the known apatococcin in cultures of Apatococcus, a search after unknown related substances in cultures of the gonidia seemed a priori impossible. I only want to mention, that after sublimation of a culture of Cladonia gonidia from the collection of the laboratory for Microbiology in Delft small crystalline needles were present in the sublimate, which

could not be dissolved in water, but were soluble in most organic solvents. An identification of this substance was impossible. In the sublimates of my own cultures of gonidia no such crystals could be detected.

F. ON THE POSSIBLE CHANGE OF APATOCOCCIN BY THE FUNGI.

In the lichen-thallus lichenic acids are found mostly upon the hyphae. It might be possible that the lichen-fungi are able to attack substances like apatococcin and change them into lichenic acids e.g. by using the nitrogen and excreting the rest of the molecule. Yet the fungi Pl 1, Pl 2 and C could not act upon apatococcin nor upon the sodium salt of apatococcinic acid, which was used in some experiments in consequence of its solubility in water. I added these substances to culture solutions, which were poor in carbon or poor in nitrogen or to the normal CZAPEK-DOX solution. In all these experiments, however, the crystals of apatococcin remained unchanged. When bringing some crystals upon a mycelium the crystals were enclosed but were not affected. No influence of the presence of apatococcin upon the growth-velocity of these fungi could be observed.

G. CONCLUSIONS.

The most important facts, which might elucidate the origin of the lichenic acids may be summarized as follows:
From the literature it appeared that:
1. the fungus of Xanthoria parietina produces parietin only after synthesis with an alga (TOBLER 1909).
2. the fungus of other specimens of Xanthoria parietina and of Caloplaca species produces parietin in pure culture (THOMAS 1939).
3. the fungus of Candelariella vitellina produces stictaurin in pure culture (THOMAS 1939).
4. from the same investigation of THOMAS we may draw the conclusion that the fungi of many other lichens, which contain lichenic acids in nature, do not produce these substances in detectable amounts when cultivated.
5. ordinary moulds like Aspergillus and Penicillium species produce substances which are related to, or identical with, certain lichenic acids. (RAISTRICK c.s.).
From my observations it appeared:

6. the fungi which live in symbiosis with aerial algae and must be considered as relatives of true lichen fungi under no circumstances produced lichenic acids or similar substances when cultivated.

7. the alga Apatococcus minor Edl. produces a substance, called apatococcin, which bears some resemblance to certain lichenic acids.

In view of the great chemical diversity of the lichenic acids we may not be surprised that their origin is not identical. Certain lichenic acids like the derivatives of anthraquinone (parietin) or the derivatives of pulvinic acid (stictaurin) certainly are produced by the fungus alone, other lichenic acids f.i. the aliphatic fatty acids perhaps are mainly produced by the gonidia. As, however, the origin of most lichenic acids still is mysterious we have to take into account that they are produced after the cooperation of the two symbionts. ASAHINA (1937) suggested that the same lichen fungus might produce different lichenic acids, when being combined with different algae. The observation, that some algae are able to produce remarkable organic substances might be useful to explain this phenomenon.

It is a remarkable fact that the only substance for which a production after the cooperation of the two symbionts was thought to be experimentally established was the first substance for which a production by the fungus alone was definitely proved (parietin). This substance is found in all thalli of Xanthoria parietina, yet THOMAS could only observe the production of parietin in a small number of fungi isolated from these lichens. Yet there can be little doubt that the other fungi as well were able to produce parietin in the absence of algae. When TOBLER observed that the fungus of Xanthoria parietina produced parietin only after synthesis with an alga, he did not prove that by the symbiosis a new metabolic product had been formed. After the work of THOMAS it is evident that this product, which potentially can be produced by the fungus alone, after synthesis with the alga accumulated and in consequence could be demonstrated. The metabolism of the fungus was not changed in a qualitative, but only in a quantitative way, while in some strains of the fungus the latter type of metabolism already could be observed in pure cultures. We might compare the action of the algae to a kind of natural "Abfangverfahren". But in the same way we can suppose, that the many lichen-fungi, in which in cultures no lichenic acids could be detected, produce these substances after synthesis with the algae not because these lichenic acids are new metabolic products arisen from the association, but because they are substances which the fungus alone only produces in very small quantities, or as inter-

mediaries, and which now accumulate under the influence of the alga. Reversely the fungus might be able to accumulate certain metabolic products of the gonidia. The mechanism of this accumulation must remain indiscussed; it may be a simple change of the environment, an esterification with erythritol and so on.

The greater quantity of lichenic acids in the lichen-thallus as compared with the pure cultures of the components might be explained as well in a similar way as I have explained the great quantity of apatococcin in apatococcus covers (page 502). As lichen-thalli are mostly very old, excretionproducts, which cannot be washed away, will accumulate. As, however, young thalli already contain considerable quantities of lichenic acids this explanation seems to be not very important.

Finally I want to stress that for instance the Caloplacomyces cultures of Thomas contained parietin in quantities, which were comparable to the quantities of parietin in the lichen-thallus. Here the symbiosis was neither qualitatively nor quantitatively necessary for the production of this lichenic acid.

Most probably a great many other hypothesis might be formed upon the origin of the lichenic acids. A satisfactory explanation of the qualitative and quantitative production of these substances cannot be given. The fact, however, that certain fungi as well as certain algae produce lichenic acids or comparable substances in pure culture makes the problem of the origin of the lichenic acids lose much of its mysterious character. There is no important reason to consider these substances as the result of a specific metabolism of the symbiosis (Tobler 1925), nor to use them as an argument for a new unity of the lichen (Tobler 1934). This only could be justified, when these substances or the process in which these substances were formed had to be considered fundamentally important for the household of the lichen. As, however, it is very likely that most of these substances are nothing more than excretion-products, we must accept that, when these substances indeed are produced in more considerable quantities under the influence of the symbiosis, this symbiosis has made the metabolism of the two partners less efficient.

CHAPTER V

ON THE IMPORTANCE OF THE SYMBIOSIS FOR THE WATER-HOUSEHOLD.

A. LITERATURE.

The extreme ecological conditions to which many lichens are subjected were the reason that many investigators have considered the water-relations in the lichen-thallus. The most striking phenomenon was formed by the fact that lichens are able to live at ecological conditions which are not fit for the development of most other organisms. For instance SCHRÖTER remarks in his well-known book "Pflanzenleben der Alpen" (1926 page 759): "Man bedenke, Algen, die Kinder des Wassers und Pilze, die Geschöpfe der Feuchtigkeit und Dunkelheit treten zusammen zu einem Doppelwesen, das in der grellen Alpensonne an kahler Felswand der gewaltigen Verdunstung preisgegeben ist". When making remarks like these it was forgotten that the lichen-gonidia are not at all children of the water, but more or less extremely adapted aerophilic organisms, while it is known, that many solitary fungi as well may live upon bare rocks. (BACHMANN 1918).

The first who considered the water-relations to be important for the understanding of the nature of the symbiosis was V. TIEGHEM (1874). In a discussian after a lecture of WEDDELL for the Société Botanique de France he propagated not only the consortium theory (before DE BARY) but in a footnote he considered the possibility, that the fungus would protect the algae against desiccation.

In the publication of JUMELLE (1892) the first experiments were described concerning the water-relations of a lichen. The relation between the fresh-weight and the dry-weight of a lichen-thallus appeared to vary between 2.00—4.31 gr. related to 1 gr. dry-weight. The water-content varied with the water-content of the substrate. The resistence against desiccation was, in free-living algae, as high as in the gonidia belonging to the same species, so that he did not suppose that the fungus protected the algae against desiccation. The gas-exchange increased with the increase of the water-content, only when the water-content became very high a diminuation of the gas-exchange could be observed.

The considerations of ZUKAL (1895) have been much cited. He thought that the thick cortex functioned in hampering the evaporation of the water, which had been gathered in the algal-layers. Especially

the thick mucoid layer of blue-green gonidia would retain great quantities of water. This water should reach the algae after being absorbed by the cortex and its extremities and guided by capillary forces between the hyphae. He observed a relation between the thickness of the cortex and the desiccation to which the thallus was subjected; the greater the danger of desiccation, the thicker was the cortex. On very dry substrates the most exterior layers died off and formed something like a cuticula. The considerations of ZUKAL were less based upon experiments than upon analogies with higher plants.

SIEVERS (1908) and BACHMANN (1922, 1923), who performed a great many, although not very exact, experiments upon the water-household of lichens, confirmed the considerations of ZUKAL. The last-mentioned author introduced a new element in lichenology, the so-called necrophoral layers, layers of dead cells, whose empty membranes should function as water-reservoirs.

The theories of ZUKAL could not stand modern criticism. They were replaced by the much better founded concepts of GOEBEL (1926, 1927). GOEBEL considered the cortex not as a structure which, in analogy with the epiderm of higher plants, performed protecting functions, but as a structure which, by means of its thick easily imbibited cell-walls, would be very important for the absorption of water. In the centre of many lichens as well he observed such "Quell-hyphen", which could absorb important quantities of water. As most lichens are exposed to a strong evaporation he thought the water in these hyphae to be very important as water-reservoirs, although he admitted that this water-reserve would not stand very long. At the upper- and lower surface of a great many lichens he observed structures which were able to absorb water by capillary forces, but in the thallus the water was conducted by means of progressive imbibition. The gonidia should receive the water from the enveloping fungus, as they themselves are in no contact with the atmospheric water-vapour or with rain water. The gonidia are living in an air-chamber, which cannot be infiltrated with water, as the surrounding hyphae are so heavily incrustated with hydrophobic lichenic-acids, that they cannot be moistened with water. These air-chambers sometimes are in direct contact with the atmosphere by respiratory-openings, especially when the air-chambers are small. He illustrated the water-household of a great many lichens, so that the difference of their behaviour was very striking. Most important for the problem of the symbiosis was his following remark: "Es ist möglich, dasz die Herstellung der Luftkammern, in welchen die Algen leben, von diesen bedingt wird, dann, wenn sie die Pilzhyphen zum Ausscheiden der Flechtensäuren veranlassen. Wenn, wie das Bonnier annahm,

auch die starke Verdickung der Quellhyphenwände dem Einflusz der Algen zuzuschreiben sein sollte, so wurde die Auffassung, dasz es sich bei der Symbiose NUR um einen Helotismus handelte nicht mehr zutreffen. Denn die, das Funktionieren der Symbiose ermöglichenden Einrichtungen wären dann unter dem Einflusz der Alge auf den Pilz entstanden . . . . (1926 page 10).

This theory was supported by the independently performed experiments of STOCKER (1927), who stressed the fact that in a lichen-thallus no difference could be made between water-resorbing and water-excreting organs, as both functions were performed by the cortex. He further showed the importance of the imbibition of the walls of the hyphae and he performed some experiments upon the potency of this imbibition. He observed, that a lichen-thallus, when brought into a humid atmosphere at first increased in weight, but that this increase became less and less pronounced until finally the ultimate equilibrium was reached after a week! The uptake of liquid water was much more rapid, which he imputed to the fact, that the water had been conducted in the capillaries between the hyphae, so that these hyphae were moistened by the water over a far greater surface. The weight of a lichen appeared to vary with the relative humidity of the air. Furthermore he confirmed the observations of JUMELLE, that the gas-exchange increased with the water-content, but that, when the latter became very high, a decrease of the gas-exchange could be observed, most likely in consequence of an obstructed diffusion through the swollen cortex. An assimilation-surplus could only be observed after moistening with liquid water, a high relative humidity of the surrounding air was insufficient and resulted only a higher respiration. As the water is easily evaporated he supposed, that even on rainy days a photosynthetic inactivity has to be accepted. In nature dust would be the most effective way of moistening.

Less important are the publications of KOLUMBE (1927), who considered the absorption of water-vapour to be very important, as many lichens cannot be moistened by liquid water and often live in places, where they cannot be reached by liquid water (rain); of FRAYMOUTH (1928) who observed, in Parmelia physodes, the same relationship between respiration and water-content as had already been described by JUMELLE and STOCKER; and of SMYTH (1934), who described some observations on the water-absorption of Peltigera and mentioned that from humid, saturated, air less water was resorbed than from liquid water and that evaporation through the lower-surface was much more rapid than through the upper-surface. Finally I have to mention the experiments of RENNER (1934) upon the water-house-

hold of small epiphytes in Java, in which he experimented among
others with some lichens.

May these data form the basis for a theory of symbiosis? Certainly
not. For although there can be little doubt that the gonidia are
dependent upon the water taken up by the imbibition of the hyphae
and that the gonidia are protected against water-infiltration, it is not
proved that, in consequence, the algae in the thallus live in better
ecological conditions than their free-living relatives. So we firstly
have to inform ourselves about the water-household of the free-living
gonidia. About the water-household of free-living Cystococcus
species little is known, but the observations on the related proto-
pleurococcoid algae may be used as an orientation, the more as it
has been shown by FRITSCH and HAINES (1923), that these algae
react similarly to desiccation as Cystococcus. From their investigation
and the previous one of FRITSCH (1922) it appeared that Pleurococcus
(and as far as investigated the same holds true for Cystococcus) is
characterized by a highly viscous plasma, as this may be found in
other aerial-algae only after a high adaptation to drought. Vacuoles
are very small, so that imbibition will be far more important than
osmotic phenomena. Plasmolysis (perhaps it is better to talk about
plasma-contraction) is only obtained in very concentrated plas-
molytics. The increase of the resistance against plasmolysis at desic-
cation, which may be found in all aerophilic algae, can be observed
in Pleurococcus as well, but the results are less reproducible (FRAY-
MOUTH 1928 communicated, that this increase could be observed in
Cystococcus as well, but that the effect was much more pronounced
in the free-living forms than in the gonidia of Parmelia physodes).
The water-content is very low as compared to other aerophilic algae
and especially as compared to the water-content of water-algae.
From this water only a small part evaporates at desiccation, most of
the water is more firmly bound and can be removed only by heating
at 100° C. The percentage of this firmly bound water is greater than
in other aerial algae. By these peculiarities the proto-pleurococcoid
algae (and Cystococcus) are well-adapted to the life on the land and
they can endure desiccation. THOMAS (1933) observed that cul-
tivated lichen-gonidia could remain in an air-dry state without being
harmed.

ZEUCH (1934) observed, that Pleurococcus can multiply in an
atmosphere with a very low relative humidity. When the temperature
increased, the minimal relative humidity at which cell-multiplications
still were visible decreased, so that at 30° C the cells could yet divide
at a relative humidity of ± 50 %! In higher humidities, however,
this temperature was harmful, which was confirmed by EDLICH

(1936), while THOMAS (1939) confirmed this phenomenon for lichen-gonidia as well.

By these investigations, especially those of ZEUCH, the definite proof had been delivered, that proto-pleurococcoid algae can multiply in low relative humidities. More early SCHMID (1927) had thought to observe that liquid water was even dangerous to these algae, but that they were protected against the infiltration with liquid water by the adhering air-films, which prevented moistening with water. When after a very intensive moistening the cells were submersed, photosynthesis decreased and stopped, while the algae died off. Most probably, however, this had been caused by secondary circumstances, as these algae may be cultivated in liquid culture solutions!

Nevertheless the impossibility to moisten these algal-covers with rain water proved, that not only they could live from the atmospheric water-vapour, but that they could not use another source of water (except for the humidity of the substrate, which however on stone-walls may be neglected). The water-vapour is resorbed by the imbibition of the cell-walls (FRITSCH 1922).

OWN OBSERVATIONS:
B. THE WATER-HOUSEHOLD OF THE GONIDIA.

1. The resistance against desiccation.

That lichen-gonidia can endure a high degree of desiccation is obvious when we consider the dry substrates upon which a great many lichens occur. Experiments concerning the resistance against desiccation in cultures only have been performed by THOMAS (1939) who observed, that they could remain during eight days in an air-dry state without losing their vitality. Then, however, they died, perhaps by secondary causes. In experiments like these we must not forget that the gonidia have developed on humid agar media and thus will be less adapted to desiccation than the gonidia in a lichen-thallus.

I determined the strongest desiccation which could be endured by isolated gonidia as follows: the algae, taken from a two months old culture of the Xanthoria Cystococcus on BEIJERINCK agar 2 % glucose, were smeared upon a little watch-glass and brought into a desiccator over concentrated sulphuric acid. So here they were subjected to a degree of desiccation never to be found in nature. Every day a small amount of these algae was transferred to a cover with a hanging drop of maltagar, which was incubated in a moist chamber over water. After a day the greatest diameters were measured in two directions and the same diameters again measured after a week (the

first measurement was not performed the day of the inoculation to avoid an increase in volume by simply swelling of the dry material). The results are summarized in table XXI.

TABLE XXI

Diameters of the algal colonies 1 and 8 days after inoculation in a humid chamber on malt-agar, when being dried over conc. sulphuric acid. Diameters in μ.

	period of drying in days				
	0	1	3	5	7
after 1 day over water	110 × 116	90 × 128	98 × 98	102 × 108	139 × 153
after 8 days over water	220 × 204	122 × 179	122 × 130	143 × 163	187 × 214

It is evident that, even after a seven days desiccation over concentrated sulphuric acid, the algae have not lost their vitality. We may assume that in nature as well they will not die from desiccation, so that in this respect a protection by the fungus is not indispensable.

2. **The determination of the smallest relative humidity, which enables a development of the algae.**

From the investigations of ZEUCH (1934) it appeared, that Pleurococcus still may multiply in a relative humidity of 50 %, when the temperature is 30° C. He determined this minimal relative humidity by bringing a small amount of algae upon a cover, which was brought into a small desiccator over solutions of $CaCl_2$. Every day he counted the number of algal-cells. As these algal-cells could not obtain enough food on this bare cover each cell could only divide once or twice. The percentage of divided cells was a good criterium for the occurring development. Unfortunately this method could not be used for Cystococcus, as this alga multiplies seldom by simple cell-divisions, but mostly by the formation of spores. For this the reserve-substances did not suffice, even at a relative humidity of 100 % or in destilled water no multiplication could be observed. So the dependence of the multiplication upon the relative humidity had to be determined by the far less beautiful method of WALTER (1924). A glass-dish was partially filled with solutions of $CaCl_2$ and covered with a paraffined card-board, in which an opening was cut out. Upon a large cover a drop of agar with food-substances was brought and inoculated with a small group of algae. Then this cover was brought upon the opening in the card-board with the agar-drop at the

under-side and randed with paraffin. In consequence the algae could develop in an atmosphere with a known relative humidity, while the diameters of the group of algae could be measured under the microscope without transferring the algae from the moist-chamber. This method has the great disadvantage that we introduce a complicating factor: the agar. Firstly this agar will take water by imbibition, so that we have no certainty that the algae have taken the water directly from the atmosphere. Secondly the water-content of the agar and in consequence the concentration of the food-substances will vary with the relative humidity of the surrounding air. As a culture solution I used the solution according to BEIJERINCK provided with 2 % glucose and 2 % agar. As had been shown that much higher concentrations of glucose and salts are not harmful for the development of the algae, the increase of the concentrations after the evaporation of the water will not strongly interfere with the direct effect of the relative humidity upon the algae. Every group of algae was measured the day following the inoculation and after a week (that is 8 days after the inoculation). Here too the first determination was not performed the day of the inoculation in order to prevent the observations of changes in the volume of the algal-group by simply swelling or shrinking. Mostly I measured the largest diameter and the diameter transversally upon it, but when the group of algae had an irregular form this form was copied and two of the most characteristic dimensions measured. The concentrations of $CaCl_2$, required to give the asked relative humidities, were taken from the publication of ZEUCH (1934). The results are compiled in table XXII.

It appears that the lowest relative humidity, which enables a development of the algae is $\pm$ 91 %. At lower relative humidities no development could be observed, while even in most cases the algae still had somewhat shrunk. This minimal relative humidity is much higher than the one observed by ZEUCH. As, however, my Apatococcus too stopped its development at a relative humidity of 91 %, this difference with the results of ZEUCH is not caused by specific differences between the examined algae, but by the different methods used. In the method of ZEUCH we determine only the divisions of a small number of cells, in the method of WALTER we determine the increase in growth of a whole group of algae. It might be possible, that at a certain relative humidity some cells, which had just started dividing, performed this division, but that the metabolism was too much obstructed to enable any increase in volume of the divided cells or any further cell-divisions. These few cell-divisions will not be perceived in the method of WALTER; when with this method we state an increase of the volume of the group of algae it indicates a far

TABLE XXII

Diameters of the groups of algae in μ after one day and after eight days incubation on Beijerinck agar 2 % glucose (hanging drops of agar) in an atmosphere of known constant relative humidity. Temp. ± 15° C.

Rel. hum.	Parmelia Cyst.		Physcia Cyst.		Xanthoria Cyst.		Apatococcus minor	
	after 1 day	after 8 days	after 1 day	after 8 days	after 1 day	after 8 days	after 1 day	after 8 days
100 %	102.0	155.1	173.6	214.3	161.2	169.4	65.3	95.9
	85.7	126.5	126.5	147.0	130.6	159.2	55.1	79.6
98.9 %	44.9	81.6	175.5	204.1	112.3	122.4	85.7	110.2
	44.9	81.6	98.0	142.9	112.3	118.4	65.3	81.6
97.8 %	75.5	126.5	118.4	183.7	78.0	116.3	91.8	112.3
	57.1	93.9	118.4	177.6	114.3	126.5	75.5	100.0
96.4 %	75.5	98.0	171.4	214.3	120.4	122.4	71.4	83.7
	63.3	73.5	71.4	102.1	71.4	79.6	69.4	83.8
94.8 %	77.6	83.7	81.6	142.9	79.6	118.4	189.8	224.5
	57.1	83.7	79.6	101.2	65.3	100.0	126.5	147.0
91.1 %	65.3	65.3	106.1	116.3	117.0	157.2	79.6	79.6
	42.9	42.9	83.7	87.8	93.9	95.9	69.4	69.4
87.0 %	114.3	112.1	79.6	77.6	116.3	114.3	102.0	102.0
	69.4	61.2	85.7	81.4	118.4	116.3	93.9	93.9
81.0 %	85.7	85.7	138.9	138.9	87.8	85.7	118.4	118.4
	49.0	49.0	81.6	81.6	67.4	65.3	102.0	102.0

greater degree of activity. That indeed the relative humidity of 91 % is a cardinal point in the hydrature of the plasma of these gonidia is evident from the fact, that in higher relative humidities the cells look quite normal, but that in relative humidities beneath 91.0 % all cells are contracted.

3. Plasmolysis experiments.

This was confirmed by plasmolysis experiments. According to FRITSCH and HAINES (1923) plasmolysis of these algae only occurs in highly concentrated plasmolytics, much more concentrated than are used for the plasmolysis of other aerophilic algae. Furthermore those authors observed that there existed an enormous variation between the resistance against plasmolysis among the different cells. In my cultures this difference was not as pronounced, so that I could determine the concentration of sucrose in which plasmolysis (it is

better to use the term plasma-contraction) just occurred. The cells of the Xanthoria Cystococcus cultivated on BEIJERINCK agar 2 % glucose in the light all were contracted in solutions containing more than 2.0 mol. sucrose, in 2.0 mol. sucrose the percentage of contracted cells was 65 %, in 1.9 mol. only 15 %. In more diluted solutions of sucrose no contraction could be observed. The concentration of 1.9—2.0 mol.sucrose corresponds to a pressure of ± 110—120 atmospheres and a relative humidity of 91—92 %. This is in good agreement with the results obtained at desiccation.

FRITSCH and HAINES (1923) observed that in most aerial algae the resistance against plasmolysis increased with desiccation, but that in Pleurococcus and Cystococcus this phenomenon was far less evident in consequence of the very irregular and inreproducible results. Nevertheless FRAYMOUTH (1928) observed this phenomenon in Cystococcus (Trebouxia) as well, in free-living specimens, however, it was more marked than in the gonidia of Parmelia physodes. So it was likely that the gonidia in a thallus, which are subjected to desiccation, will contract at lower relative humidities than the gonidia cultivated on humid agar. I again have determined the percentages of contracted cells in sucrose solutions, now making use of transverse sections through a thallus of Xanthoria parietina collected from a stone-wall on a dry morning. In concentrations higher than 2.2 mol. sucrose all gonidia were contracted, at 2.2 mol some few cells retained their normal appearance, at 2.0 mol. 31 % of the cells was contracted, in 1.9 mol.only few cells were strongly and ± 10 % slightly plasmolysed. So a somewhat greater spreading of the results could be observed, but in general the cells tolerated somewhat higher concentrations of sucrose, some cells even remained uncontracted in a sucrose solution of 2.2 mol., which corresponds to a pressure of 140 atmospheres and a hydrature of 90%. Yet the difference between the gonidia from the thallus and the gonidia from the humid agar was not very pronounced.

4. Temperature and relative humidity.

According to ZEUCH (1934) the optimal and minimal relative humidity which enables cell-division in Pleurococcus decrease, when the temperature increases. My observations upon the influence of the relative humidity on the development were performed at room-temperature and as I have not repeated them at higher temperatures I cannot say whether the effect mentioned by ZEUCH can be observed in Cystococcus as well. In consequence of the great similarity between Pleurococcus and Cystococcus it is very probable that this will be the case. As in the summer months low relative humidities will

coincide with high temperatures the gonidia then will be able to perform their vital functions for a long time.

More important is the harmful effect of high temperatures when combined with high relative humidities, which has been described for proto-pleurococcoid algae by ZEUCH (1934) and EDLICH (1936). This harmful effect is so pronounced, that during the hot summer months of 1941 all my cultures of Apatococcus as well as those in the microbe collection of the laboratory for microbiology at Delft died off, only one culture upon malt-agar, which had been kept in a very cool place had survived. My Cystococcus cultures also soon died at relatively high temperatures, but fortunately they were not as sensitive as the cultures of Apatococcus. Yet in free nature these algae have to endure much higher temperatures than are harmful in cultures. This is possible because then they occur in a dry state. That indeed the lichen-gonidia as well only suffer from high temperatures, when they are moistened has already been shown in a preliminary experiment of THOMAS (1939). More accurate experiments (as have been performed with Apatococcus by EDLICH 1936) were missing. To obtain more data for the lichen-gonidia I have brought small amounts of algae upon pieces of plaster, which were watered with tap-water in a moist-chamber during 24 hours, so that all algal cells were saturated with water. Then they were brought into a desiccator over solutions of sulphuric acid of known concentration during 24 hours, after which these desiccators were placed in a thermostat during ten hours. Then they were brought back at room temperature and when they had been cooled the pieces of plaster were again watered with tap-water so that eventually shrunken cells could repair themselves. The number of dead algae then was determined by means of staining with a $1\,^0/_{00}$ solution of neutral-red. When only part of the cells had died off, the percentage of dead cells could only be determined with difficulty as many cells were only partially stained. For the rest the results were very obvious (table XXIII).

There is no doubt that indeed all these lichen-gonidia are more resistent to high temperatures at low- than at high relative humidities.

C. IMBIBITION EXPERIMENTS.

One of the most principal arguments for the theory of GOEBEL was formed by the high imbibition-capacity of certain hyphae, mentioned by GOEBEL (1925) as "Quellhyphen". Without any doubt it will be those hyphae which resorb the required water from the atmosphere and will provide the algae with this water, until the

TABLE XXIII

Percentage of dead cells (stained with $1\,^{o}/_{oo}$ neutral-red) after the influence of high temperatures during 10 hours in atmospheres with different relative humidities.

Rel. hum.	Xanthoria Cyst. 30°	Xanthoria Cyst. 40°	Parmelia Cyst. 40°	Physcia Cyst. 40°
100 %	36 %	84 %	90 %	38 %
96 %	7 %	59 %	76 %	28 %
93 %	6 %	53 %	15 %	—
82 %	7 %	25 %	15 %	13 %
69 %	6 %	17 %	9 %	16 %
60 %	7 %	22 %	7 %	6 %

hydrature of the algae will be equal to the hydrature of the fungus and the relative humidity of the surrounding atmosphere. The water-content of each partner, when this equilibrium is reached, can be determined by means of the imbibition-curves, which show the relation of the water-content to the relative humidity of the sur-rounding atmosphere. It seemed to be important to compare the imbibition-curves of the lichen-thallus with the imbibition-curves of the isolated components. Thus it might be observed whether the imbibition-capacity of the hyphae has been changed under the influence of the symbiosis, while a comparison between the im-bibition-curve of the thallus with the imbibition-curve of the isolated gonidia perhaps enables us to draw conclusions about eventual better water-relations for the gonidia in the thallus. As experimental object I used Xanthoria parietina (L) Th. Fr., the only lichen of which I possessed both partners in pure culture.

1. The imbibition-curve of the lichen.

A small thallus was brought into a small stoppered bottle over concentrated sulphuric acid and weighed at regular intervals. When the weight remained constant the thallus was quickly brought into a similar flask, which was partially filled with a solution of sulphuric acid of known concentration (determined with an areometer), so that the air above possessed a known relative humidity. From time to time the thallus was taken from the flask and weighed. To this purpose it was necessary to use a flask with a narrow opening, into which the thallus was brought in a small glass bucket, which hung from the stopper. When doing so it was possible to transfer the thallus out of and into the flasks without an undue mixing of the atmosphere in the flask with the air, which would have caused a temporary change of the relative humidity in the flask. I used flasks

of 25 cc filled with 10 cc H_2SO_4, the thalli used had a dry weight of ± 15—20 mg. They were weighed with a torsion-balance of the „Vereenigde Draadfabrieken Nijmegen", which enabled me to perform weighings with an accuracy of 0.1 mg in only a part of a minute, so that the loss of water by evaporation during the weighing could be neglected. When the weight of the thallus in the flask remained constant it again was brought over concentrated sulphuric acid and again the dry-weight determined in order to control whether the dry-weight had remained constant during the experiment. As the differences between the different thalli were only very small, it was not necessary to perform all weighings with the same thallus. The experiments were performed at room temperature of ± 15° C. As I could not keep this temperature constant, a small spreading of the observations was not to be avoided, at a relative humidity of 100 % the formation of condensation-water sometimes gave erroneous observations.

It appeared, that when bringing the dry thalli into an atmosphere with a relative humidity of 100 % it took 7 days before the equilibrium was reached, in atmospheres with smaller relative humidities this equilibrium was reached earlier. Inversily I observed that thalli, which were saturated with water, gave off their water within only some hours when brought over concentrated sulphuric acid, but that it took very long before an equilibrium in atmospheres with a high (although not saturated) relative humidity had been reached. So the equilibrium is rapidly reached (within some hours) when the relative humidity of the air is low, but it takes a long time, when this relative humidity is high. The imbibition-curve is constructed in text fig. 20, it shows the characteristic form of the imbibition-curve of a colloid (KATZ 1917).

2. The imbibition-curve of the fungus.

Xanthoriomyces parietinae was inoculated on a piece of plaster moistened with a Czapek-Dox solution provided with 1 cc % yeast-autolysate. This solution was not regularly renewed, but from time to time the mycelium was left in a dry state, so that the fungus could develop under circumstances which those in nature as far as possible equaled. I then constructed the imbibition-curve as described above (text fig. 20). The difference with the thallus was not very pronounced. This had to be expected, as in the thallus as well we only determine the imbibition of the fungus, as the algae form only a negligible part of the dry weight of the thallus. It is, however, remarkable that the maximal water uptake of the fungus is as great as the maximal water-uptake of the lichen in spite of the fact, that
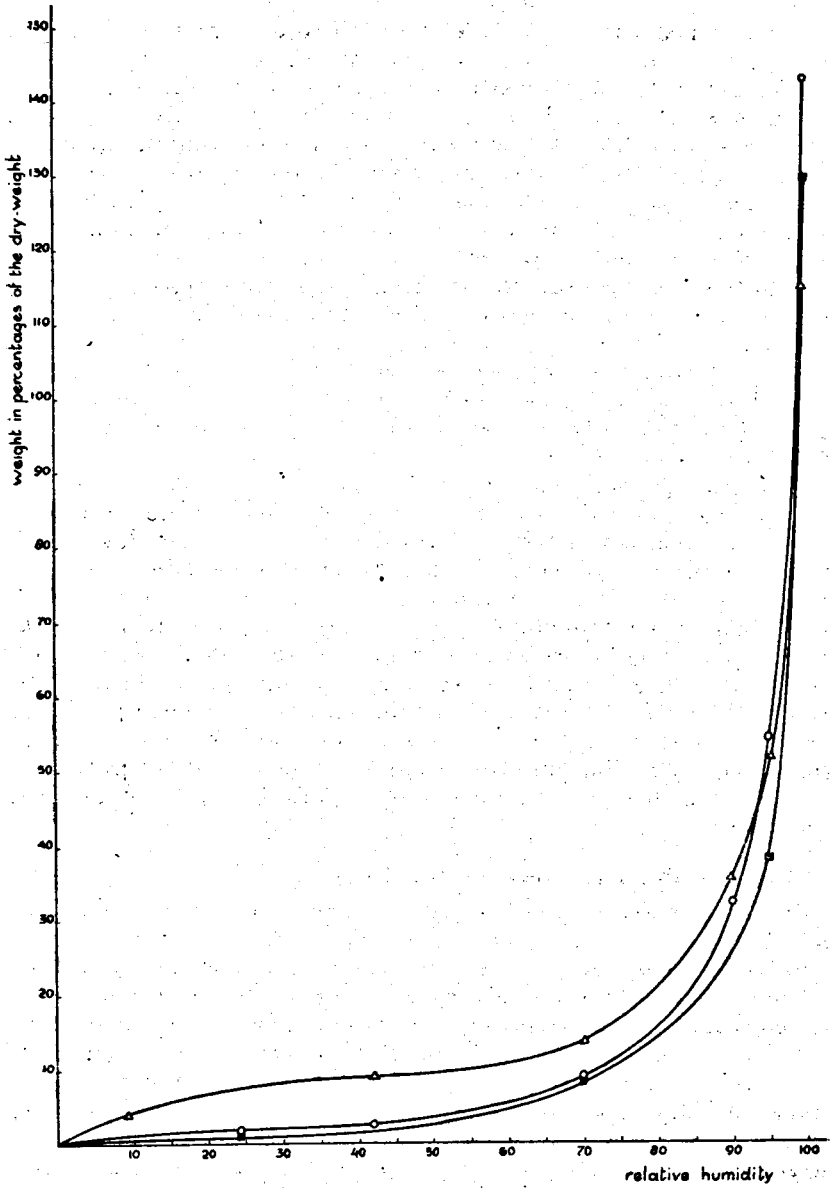
Fig. 20. Imbibition-curves of the thallus, the fungus and the alga of Xanthoria parietina (L) Th. Fr. Abscis: relative humidity. Ordinate: weight in percentages of the dry-weight.

△————△ imbibition-curve of the fungus
■————■ imbibition-curve of the thallus
O————O imbibition-curve of the alga.

in the thallus the fungus forms very thick-walled hyphae, as they cannot be found in pure cultures. It is obvious, that these thick-walled hyphae possess a greater quantity of wall-material, but that the imbibition of this wall-material is as great as the imbibition of the other cell-walls. Only in lower relative humidities the water-uptake of the fungus is somewhat more pronounced than the water-uptake of the thallus.

3. Imbibition-curve of the algae.

As the crumbly algal-material cannot be weighed apart, it was smeared on a small cover, upon which it dried as a flat cake and could be weighed together with the cover. For the rest the method used was quite identical with the one described above. As had to be expected the equilibrium of these small amounts of algae was reached much more earlier than of the thalli or the fungus. For the rest there is no essential difference with the imbibition-curves of these subjects (text fig. 20). The maximal water-uptake even was, percentually, somewhat greater than that of the thallus.

D. THE WATER-RESERVE IN THE HYPHAE.

In order to determine, in a direct way, whether the gonidia in the thallus are in some way protected against desiccation by the water-content of the hyphae I have brought small thalli, saturated with water, into small desiccators over solutions of $CaCl_2$ with a known concentration. After some time one of these thalli was ground in paraffin-oil and in the suspension obtained the number of contracted cells determined. This was repeated from time to time until this percentage did not change, and when it appeared, from simultaneously performed determinations of the dry-weight of one of the thalli, that equilibrium had been reached, the remaining thalli were brought back into the atmosphere with a relative humidity of 100 % and in the same way the decrease of the number of contracted cells and the increase of the number of repaired cells was observed. As the temperature of 15° C could not be kept constant, these latter experiments were not absolutely reproducible, as the condensation of water interfered with the results. So here only the best reproducible results are given with the restriction that the observed recovery of the algae may be somewhat exaggerated. My experimental object in these experiments was Ramalina fraxinea (L) Ach. This lichen is very suitable for experiments like this, as it is only fastened to the substrate with a small part of the thallus. In foliaceous lichens we must either leave a part of the substrate to the thallus,

so that we do not get a true image of the protecting influence of the water-content of the hyphae as the substrate will contain considerable amounts of water as well. Or we must isolate the substrate from the lichen which is practically impossible; for in this case incorrect results are also obtained as the evaporation through the lower-surface, which normally is never exposed to the air, will be much greater than the evaporation through the upper-surface. In Ramalina we can leave a small bit of the substrate to the thallus, which will not interfere with the results, while on the other hand it is not necessary to examine the lichen under unbiological conditions. I used small intact thalli and no parts of a thallus in order to experiment with lichens with an intact cortex over the whole surface. The results are evident from text fig. 21. Before interpreting this figure it is necessary to say a few words upon the velocity in which the isolated gonidia were influenced by the relative humidities used. For this purpose some thalli were ground in water and the
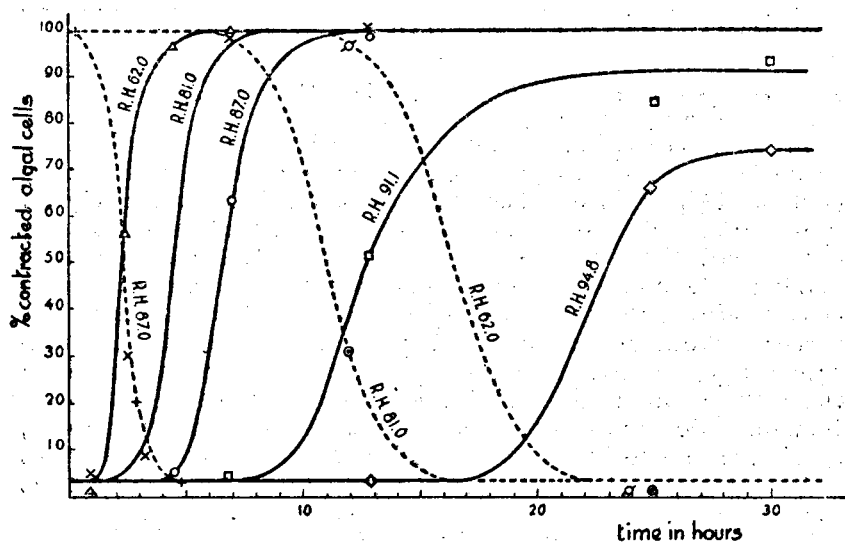

Fig. 21. ——— Curves showing the increase of the percentage of contracted algal cells of Ramalina fraxinea, when the moist thalli are transferred from an atmosphere with a relative humidity of 100 % to atmospheres with relative humidities lower than 100 %.
– – – – Curves showing the decrease of the percentage of contracted cells when the thalli are transferred from the atmospheres with low relative humidities to an atmosphere with a relative humidity of 100 %.
Abscis: time in hours.
Ordinate: percentage of the algal cells which are contracted.

obtained suspension brought upon a slide, dried and brought into an atmosphere saturated with water. Then they were transferred to atmospheres with different relative humidities and the increase of the number of contracted cells determined. When equilibrium had been reached they were brought back to the atmosphere with a relative humidity of 100 % and in the same way the increase of the number of repaired cells determined. It appeared that the gonidia upon this slide (which were the same gonidia as had been considered in the experiment of text fig. 21, but were unprotected by a fungus) had been influenced by the different relative humidities respectively after one — one and a half hour. When we compare this result to the result mentioned in fig. 21 with the gonidia protected by the water-reserve of the fungi it is evident that, at a strong desiccation, the algae in the thallus are hardly protected; but that the protective influence is present when the desiccation to which the lichen-thallus is subjected only is small. Then too, however, the protection is rather of a short duration. When, on the contrary, the desiccated lichens are transferred to a humid atmosphere, the recovery of the algae takes a long time dependent upon the degree of desiccation. So when a moist lichen is temporary brought into an atmosphere with a relative humidity of 62 % the loss of water is much more rapid than the water-uptake, so that the symbiosis seems to have a harmful effect on the water relations of the algae! Yet we must not forget that, in nature, however, lichens will be moistened with liquid water as well (rain, fog), which produces a much more rapid moistening. Furthermore, at high temperatures, which are often combined with the low relative humidities, it will be very important that the algae remain in a desiccated state. When a moist lichen is temporary brought into an atmosphere with a relatively high relative humidity (for instance 87 %) the water-uptake will be more rapid than the loss of the water, so that here the symbiosis certainly has a promoting effect upon the water-relations of the algae.

When we conclude that the water-content of the fungus may protect the algae against desiccation, it is obvious that this protecting influence is only very small and can only be observed with insignificant desiccations of the thallus. So the benefit for the algae only is very scanty and certainly is not sufficient as a base for a mutualistic theory of the symbiosis.

E. CONCLUSIONS.

As I have mentioned in the introduction of this chapter, GOEBEL concluded from his work upon the water-household of lichens (although under some preserve), that it was not justified to consider the association of the alga and the fungus in the lichen merely as helotism. Although GOEBEL did not mention the word mutualistic symbiosis, it is obvious that in this case the lichen-symbiosis should be classified as such. His main argument, that the structures which enable the functioning of the symbiosis have been formed by the fungus under the influence of the algae, may not be an argument for a mutualistic symbiosis. The only argument should be and may only be the experimental proof of a benefit from the association for the algae, when compared to its free-living relatives under the same external situations.

We may, however, draw the conclusion that it is highly probable that the gonidia are able to endure the external situations of drought to which a lichen is subjected, without any protection by a fungus. For we have seen that these algae survive a desiccation over concentrated sulphuric acid during more than a week, that these algae can develop in an atmosphere which is not saturated with water, because they can endure high osmotic pressures without contracting, while they can take up the water-vapour from the atmosphere in quantities, which are percentually comparable to those resorbed by the lichen-fungus. Finally I mentioned, that during the hot summer months it will be absolutely necessary that the algae are desiccated, as otherwise they are killed by the high temperature.

So the symbiosis certainly is not indispensable for the water-household of the algae, but it might be possible that, as GOEBEL suggested, the great quantities of water resorbed by the hyphae would form a water-reservoir, from which the algae could take the water, necessary for their vital functions. That, however, this water-reservoir would not function very efficiently already had been remarked by GOEBEL himself. It appeared from the experiment of text fig. 21, that this protection against desiccation could only be observed at a low degree of desiccation of the thallus. On the contrary it appeared that in consequence of the symbiosis the algae are much more dependent of liquid water than had been the case in a free-living state. The presence of this water-reservoir in the hyphae will be only of minor importance for the gonidia and certainly the benefit will be much too insignificant to base a mutualistic theory of the symbiosis upon.

Yet GOEBEL did not only refer to the existence of swelling hyphae,

but also to the existence of hyphae, which were so heavily incrustated with lichenic acids, that they protected the air-chamber, in which the gonidia live, against water-infiltration. That, however, this water-infiltration as such will have no harmful effect upon the algae already is evident from all my culture-experiments performed in liquid culture solutions. It might, however, be important, that no water surrounds the algae as the gas-diffusion would be hampered (which has indeed been stated by many authors when the water-content of the thallus was unusually high). Furthermore it might be possible that, when the temperature increases, this water could not escape, so that the algae were heated in moist conditions. But these factors are only important for algae which are enclosed in a lichen-thallus with a limited communication with the atmosphere. In free-living algae these factors are far less important. Moreover the proto-pleurococcoid algal-covers also cannot be infiltrated by liquid water, as they are covered with adhering air, so that it is highly probable that the same holds true for covers of the free-living Cystococcus. The covers of Cystococcus, which I observed in the neighbourhood of Leyden, indeed could not be infiltrated with water, but as they were heavily lichenized and contained large amounts of a lichenic acid, this observation is not convincing.

Certainly GOEBEL is right when he considers the thick-walled hyphae and the hyphae, covered with lichenic acids, as structures, which enable the functionating of the symbiosis, but they are no structures which play a role at the symbiosis itself.

Finally I want to remark that, of course, all these considerations only hold true for heteromerous lichens with Cystococcus gonidia. It is obvious that in homoiomerous lichens with Nostoc gonidia quite different water-relations will be found.

CHAPTER VI.

FINAL CONSIDERATIONS: THE LICHEN-SYMBIOSIS AS RELATED TO OTHER CASES OF SYMBIOSIS.

I will now bring together the most principal facts known about the mutual relations of the algae and the fungi in the lichen-thallus from the literature as well as from my own experiments. In the first place we will consider in which way the two partners can profit from the association.

It is evident that one of the most important profits for the fungus

lies in the provision with organic substances by the assimilation of the algae. It has been shown that the algae assimilate, when the external situations are favourable. The products formed by this assimilation and reserve-substances secondarily formed will be available to the fungus by means of the diffusion through the contacts between alga and fungus. Moreover the algae will provide the fungus with food-substances in a more indirect way as the fungi will live saprophytically from the dead algal cells or parasitically from living algal cells by means of haustoria. On the other side it is by no means certain that the ecological conditions always will allow a photosynthetic activity of the algae. Then it cannot be doubted that the fungus has to live saprophytically from the organic substances present in the substrate and even has to provide the algae with part of these substances.

A second profit from the symbiosis for the fungus is formed by its provision with nutrilites. It may be doubted whether all lichen-fungi cannot develop without the addition of certain indispensable nutrilites, but it is a fact that this will be so in a great many lichen-fungi. It has been shown that the algae can synthesize these nutrilites (aneurin, β alanin).

It might be possible that the algae can fix the nitrogen from the atmosphere, so that the fungus could live from the formed nitro-genous substances. This possibility, however, is very unlikely for the Cystococcus gonidia, yet we have to consider the possibility of a nitrogen-fixation in lichens with blue-green gonidia.

There is no reason to consider Azotobacter as a third symbiont, as the presence of this bacterium in dutch lichens could not be demonstrated.

It always was a great problem whether, and if so how, the algae would profit from the association. Initially it was assumed that the algae would require salts from the fungus. Although this certainly is the case, it should be doubted whether, in consequence, the algae are in a better situation than their free-living relatives, which can take these salts directly from the substrate.

Later it was assumed that the algae preferred organic nitrogenous substances as sources of nitrogen, which only could be provided by the fungus. Although indeed some gonidia prefer organic nitrogenous compounds as sources of nitrogen, this preference is not very marked, so that this profit for the algae from the association cannot be important.

The possibility that the fungi would be able to assimilate atmos-pheric nitrogen, so that the algae could profit from the produced nitrogenous substances is very unlikely.

When the ecological conditions are unfavourable for photo-synthesis, some gonidia will live from the reserve-substances of the fungus or from the organic substances taken by the latter from the substrate.

The protecting influence against desiccation by means of the water-reservoirs in the cell-walls of the hyphae has been overrated. This water-reserve does not function very efficiently, so that the protecting influence can only be perceived when the desiccation to which the thallus is subjected is only small. That the algae take the required water from the water resorbed by the hyphae and that certain hyphae, which are heavily incrustated with lichenic acids prevent a water-infiltration of the air-chamber in which the algae are living, certainly is important for the household of the lichen-thallus, but it is very doubtful whether this has to be regarded as a profit for the algae.

The nutrilites which stimulate the heterotrophic metabolism and which are synthesized by the fungi are not very important as the algae can synthesize these nutrilites themselves, be it in only small amounts.

On the other hand it is very important that ascorbic acid and phy-siologically related substances are able to stimulate the autotrophic development (most probably by stimulating the photosynthesis), as this autotrophic metabolism of course is much more important than the heterotrophic and, without nutrilites, is either nil or very scanty. Although the definite proof, that the lichen-fungi excrete such sub-stances, could not yet be delivered it is very probable that this will be the case as it has been shown that the photosynthetic activity of the gonidia in a lichen-thallus is very great.

It still is possible that the two symbionts are favoured by the association in other respects, which either I overlooked or still have to be discovered. Yet I suppose that to-day our knowledge of the lichen-symbiosis is best summarized as follows: (see textfig. 22)

the algae assimilate the atmospheric carbon-dioxide, which assimi-lation is stimulated by ascorbic acid (or) and related substances, which most probably are provided by the fungi; the fungi use the products formed by the assimilating algae, live saprophytically from dead algae or parasitically from cells attacked by haustoria. Moreover the algae provide the fungi with a number of indispensable nutrilites. When the external situations are unfavourable the algae will live hetero-trophically from the organic substances taken by the fungus from the substrate. Perhaps this heterotrophic metabolism of the gonidia is stimulated by certain nutrilites synthesized by the fungus. So we are certainly justified to consider this association as a mutualistic sym-
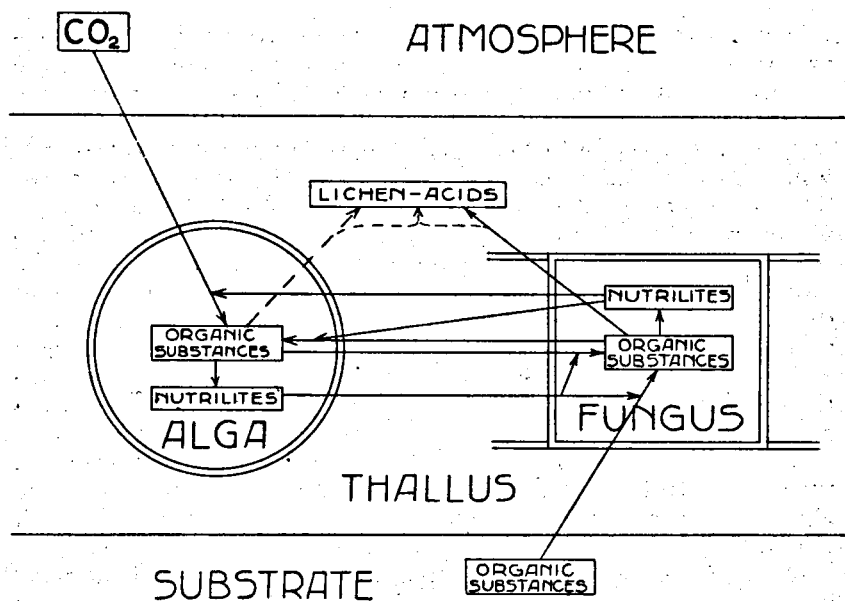
Fig. 22. Scheme, showing the mutual relations between alga and fungus in a lichen-thallus (explanation in the text).

biosis, which of course under unfavourable external situations may take a destructive character, while I want to stress the fact, that the exchange of nutrilites is one of the most principal factors in this association.

Again I want to warn against generalisations. It is not too speculative to extend this scheme to most heteromerous lichens with Cystococcus gonidia (the most important group of lichens!), although the decisive proof only has been given for Xanthoria parietina (L) Th. Fr. It is by no means certain (and even very improbable) that in other types of lichens the mutual relations between the two components always are covered by the scheme mentioned above:

In which way the lichen-symbiosis is related to other cases of symbiosis or should these cases be looked upon as fundamentally different?

According to TOBLER (1925) the lichen-symbiosis can be dis-tinguished from all other cases of symbiosis by the following facts: 1. an own specific metabolism characterized by the production of lichenic acids.

2. an own new morphological unity: the lichen-thallus.
3. an own new type of reproduction of both partners together in
   the soredia.

We have seen in Chapter IV, that there is no reason to regard the
lichenic acids as specific metabolic products; partially they are the
result of the metabolism of the fungus alone, partially certain algae
as well contain remarkable substances, which resemble lichenic-acids
in several details. When these substances are produced by the
association in larger amounts we only have the right to speak about
an accumulation of these substances. We have no right to consider
them as specific new metabolic substances. And even when these
substances would undergo some slight changes under the influence
of the association, it would not be warranted to consider them as
the result of a specific metabolism of the symbiosis, the less so as
most of these substances are merely excretion products, which are
of no importance for the household of the lichen (but for the pro-
tection of the gonidia against water infiltration in some cases).

As to the second point: the new morphological unity. During a
long time this point has drawn the attention of lichenologists and
formed one of the most important causes, that the dual nature of
the lichen took a long time to be accepted. When, however, we
consider the thallus as an algo-cecidium, a gall formed by the fungus
as a result of an algal-infection, as has been proposed by MOREAU
(1919-21) there are few reasons to consider this thallus as an essential
difference with other symbioses. The formation of galls in cases
of symbiosis may be universally observed; I only have to refer to
the root-nodules of Leguminous plants, the leaf-nodules of Ardisia
etc. In these cases, however, the galls can be recognized immediately
as such, because the dimensions of one of the symbionts are much
greater than the dimensions of the other one, so that the infection
and in consequence the gall only has a local character. In lichens,
however, both partners are micro-organisms, so that the gall has
changed the whole organism of the fungus. And that the gall shows
some remarkable adaptations to the infector, the alga, is again a
widely occurring phenomenon in nature. The problem of the for-
mation of the lichen-thallus is perhaps less a problem for the liche-
nologist, than a special and very interesting problem for the ceci-
dologist. This difference with other cases of symbiosis is perhaps
not as marked as one was apt to think. Moreover the lichen-fungi
in cultures already show the stratified layer, which is one of the
most remarkable peculiarities of the thallus.

And as to the third point: the reproduction of both partners
together can be observed in all cyclic symbioses. That in lichens this

simultaneous reproduction shows such a peculiar character again may be explained by the fact, that both partners are micro-organisms.

Finally TOBLER stressed the fact, that these points occur simultaneously. It is doubtful, whether this is a reason to distinguish the lichen-symbiosis from other cases of symbiosis in nature.

On the other hand it appeared from my experiments that there is one important and essential similarity between the lichen-symbiosis and a great many, if not all, other symbioses. For it has been shown that the exchange of nutrilites is one of the most important factors playing a role in most other symbioses as well.

This is best illustrated by the so-called artificial symbiosis: KöGL and FRIES (1937) observed that Nematospora Gossypii and Polyporus abietinus can develop together in synthetic culture-media, as Nematospora synthesizes the aneurin required by Polyporus, while Polyporus synthesizes the biotin required by Nematospora. A well-known example is the symbiosis between Rhodotorula rubra and Mucor Ramannianus described by MULLER and SCHOPFER (1937) and SCHOPFER (1938), which can develop together in solutions without aneurin as Rhodotorula synthesizes the thiazol- and Mucor Ramannianus the pyrimidin-part of the aneurin. Furthermore I want to mention the successive heterosymbiosis as described by ORR-EWING and READER (1928), who observed that Streptothrix corallines develops on media devoid of aneurin, when they were previously inoculated with Meningococcus.

As to the natural symbiosis: ASCHNER and RIES (1937) observed, that the influence of the symbionts of Pediculus vestimenti was effected by the excretion of vitamines, while KOCH (1933) demonstrated the same fact for the yeast-symbioses of Sitopedra panicea, as a small amount of yeast-extract could replace the living yeast-cells. It appeared that a great many active factors had to be considered (KOCH 1940).

One of the oldest observations, which gave evidence to the influence of nutrilites in botanical symbiosis was performed by MOCKERIDGE (1924), who observed that certain bacterial substances stimulate the growth of Lemna (most probably these substances belonged to the biosgroup).

In the symbiosis of Orchids BURGEFF (1934, 1936) observed that, although the seeds of many Orchids germinated on suitable organic substrates, the seeds of some species only could develop after the addition of an extract of Rhizoctonia or yeast. SCHAFFSTEIN (1938, 1941), who continued the investigation of the active principle (provisionally named vandophytin) showed that this substance most probably was a derivative of nicotinic acid. It must be remarked,

however, that the seeds of many Orchids synthesize this substance and that the specific activity of the symbiontic Rhizoctonia's is caused by other factors.

MELIN and LINDEBERG (1939) and MELIN and NYMAN (1940) observed that many mycorrhiza-fungi required aneurin and sometimes biotin. In this respect it is important to mention the observation of WEST (1939) that many seeds secrete aneurin and biotin into their medium.

Investigations of BAAS-BECKING and HULSMAN in Buitenzorg made probable that the Nostoc in Gunnera secretes heteroauxin, while DIJKSHOORN in Leyden (unpublished) showed that also the algae may receive substances from the Gunnera-plant.

In leguminous plants ALLISON (1933) showed that Rhizobium species required a nutrilit, which he called Co-enzyme R. From investigations of NILSSON, BJALFVE and BURSTRÖM (1938, 1939) it became evident that this co-enzyme R was identical with biotin, while, moreover, aneurin had some influence. The identity of Co-enzyme R with biotin was proved as well by the investigations of GYÖRGI, MELVILLE, BURK and DU VIGNEAUD (1940). MC BURNEY, BOLLE and WILLIAMS (1935) made the very important observation that bacteria-free species of Melilotus were markedly stimulated by panthotenic acid, although still no fixing of atmospheric nitrogen could be demonstrated. This panthotenic acid was produced by Rhizobium meliloti!

The root-nodules themselves should be formed by heteroauxin, synthesized by the bacteria from tryptophane (THIMANN 1936, LINK 1937). As, however, GEORGI and BEGUIN (1939) could not observe any difference between the production of heteroauxin by efficient and non-efficient strains of Rhizobium they thought this factor to be of minor importance for the symbiosis.

It is evident that even when we define symbiosis in its widest sense, as has been proposed by BAAS-BECKING (see introduction) the universal importance of the exchange of nutrilites still remains valid. I only want to remember to the work of MOEWUS, KUHN and WENDT (see for instance MOEWUS 1938), in which the importance of carotenoids (crocitin) upon the "gamosymbiosis" of Chlamydomonas species was discovered, but especially to the widespread effect of hormones in higher plants and animals ("histosymbiosis").

Of course it is possible that in many symbioses no exchange of nutrilites can be observed. Moreover the exchange of nutrilites certainly is not always the most important function of the symbiosis; for instance it will be as important for the lichen-fungi to be provided with organic food substances as to be provided with nutrilites. The

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main significance of mycorrhiza certainly is formed by the provision with salts by the fungi, while finally I only have to remind of the Orchids, where it has been shown that the specific influence of the symbionts is not connected with the availability of "vandophytin". Our provisional conclusion might be as follows;

In most — if not in all — cases, where vital units are dependent on each other (whether this dependence has a mutualistic or a parasitic character, whether it forms a homoio- or a heterosymbiosis) this dependence is caused by — or coupled with the exchange of nutrilites.

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SUMMARY.

1. The fungi which live in symbiosis with the aerial proto-pleuro-
   coccoid algae were isolated in pure culture. It appeared that
   there existed a great similarity between the morphological
   characters of these fungi and true lichen fungi. Yet the growth-
   velocity was much better. In consequence they formed an
   excellent object for the study of the symbiosis.
2. It appeared that these fungi could develop on media containing
   sugars, polyalcohols or starch, that some of them could use
   peptones as sources of carbon. Cellulose and pectin were not
   attacked, nor were salts of organic acids.
3. The fungi could not fix atmospheric nitrogen, there was no
   preference for organic sources of nitrogen over ammonium
   salts.
4. They could not develop in pure synthetic culture solutions, the
   addition of some nutrilites was absolutely indispensable. Partially
   these nutrilites could be identified with aneurin and β alanin.
5. The symbiontic algae (Apatococcus and a number of Cystococcus
   gonidia) can provide these nutrilites (or similar) to the fungi.
6. The fungus of Xanthoria parietina showed the same need of
   some nutrilites. It appeared that these nutrilites belonged to
   the bios group.
7. The Cystococcus gonidia of Xanthoria parietina, Physcia pul-
   verulenta and Parmelia acetabulum grew much better on organic
   substrates than on inorganic media. When the inoculation was
   not too heavy, after three months not a trace of development
   could be observed in culture solutions without organic sub-
   stances. Simple carbohydrates were the best sources of carbon;
   there was no marked preference for organic sources of nitrogen.
   There was no reason to suppose any fixing of atmospheric
   nitrogen.
8. The heterotrophic development of these gonidia was favoured
   by the addition of extracts from the symbiontic fungi but also
   by the addition of yeast-extract. These extracts were not ab-
   solutely necessary, their only influence consisted of a shortening
   of the lag-phase in growth. The active factors of these extracts
   partially belonged to the bios-group, in some cases nicotinic
   acid had a marked influence. Partially the effect might have

been caused by the presence of suitable sources of nitrogen.

9. The autotrophic development only was possible (or was highly stimulated) by the presence of ascorbic acid, dioxymaleic acid and (most probably still) by other dienolic compounds. (gluco-reducton). As these substances are easily oxidized by the air the effect was most pronounced, when the cultures were in-cubated in an atmosphere of hydrogen with 5 % carbon-dioxyde in the light. Then only 100 γ % ascorbic acid gave a development, which was comparable to the development on organic culture solutions.

10. Upon these facts a mutualistic theory of the symbiosis was based, in which apart from the provision with assimilation substances of the fungus by the algae the exchange of nutrilites played an important role.

11. A new method for the synthesis of the symbiosis is described, which makes it possible to change the humidity and the con-centration of the food-substances at will.

12. The fungi which live in symbiosis with the proto-pleurococcoid algae could produce no lichenic acids or similar substances, when cultivated under the most varying circumstances.

13. On the other hand it appeared that the alga Apatococcus minor synthesizes a remarkable metabolic product provisionally named apatococcin.

14. A chemical investigation of this substance showed that it posses-ses the following tentative formula $C_{23}H_{45}O_4N$, that it possesses one carboxylic group esterified with methanol or ethanol, that it most probably possesses a long paraffin chain. The function of the nitrogen could not be elucidated, the neutral character of the substance made probable, that the basic function of this nitrogen was anyhow bound by an acid group of the molecule. A relationship with certain aliphatic lichenic acids is probable.

15. The literature concerning the problem of the production of lichenic acids is reviewed and it was concluded that as certain fungi can produce certain lichenic acids, and certain algae can produce similar substances in pure culture the lichenic acids cannot be regarded as the result of a specific metabolism of the symbiosis. We have only the right to speak about an accumulation of these substances as a result of the symbiosis in some cases.

16. The water-household of the Cystococcus gonidia in pure culture was examined; it appeared that these algae most probably can endure the desiccation on the natural substrates of lichens without the protection of the fungus. After a week drying over concentrated sulphuric acid, they had not lost their vitality,

they could develop in a relative humidity of the atmosphere higher than 91 %, while high temperatures only could be endured, when the relative humidity was low.

17. There is only little difference between the imbibition-curves of the lichen-thallus, the lichen-fungus and the lichen-gonidia of the same lichen-species.

18. The protective influence of the fungus against desiccation of the algae only is very small and can only be perceived when the desiccation is not too intense.

19. The lichen-symbiosis is compared with other cases of symbiosis and it is concluded, that the differences are only apparent, while there is a great similarity as in all other symbiosis the exchange of nutrilites plays one of the most important roles.

20. This similarity still can be observed, when we extend the definition of symbiosis towards all dependences of vital units belonging to the same or to different species as has been proposed by BAAS-BECKING and for which definition some arguments are brought together in this paper.

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