NADH. The result of such assays after 10, 32, and 42 hours of centrifugation is illustrated in Fig. 1, *a–c*, respectively. The best separation of myoinositol kinase and adenosine triphosphatase was achieved after 42 hours.

We were not successful in coupling the purified myoinositol-kinase–catalyzed reaction with the oxidation of NADH. The assay solutions inhibited myoinositol kinase.

Identification of the particular isomer of myoinositol phosphate formed by the enzyme-catalyzed reaction was achieved by descending chromatography in a mixture of isopropyl alcohol, ammonia, and water (70:10:20) at 40°C for 32 hours (*5*). Radioactive myoinositol phosphate was eluted from electrophoretograms and treated with activated charcoal to remove nucleotides. The product was then mixed with each of the possible isomers of myoinositol phosphate (the 1-, 2-, 4- and 5-) and spotted on Whatman No. 1 chromatography paper. The 1- and 3-, and 4- and 6- isomers are D,L pairs and are indistinguishable by our techniques. The chromatograms were analyzed with a radiochromatogram scanner to locate the radioactive product which had been mixed with an authentic unlabeled isomer. Each chromatogram was then sprayed with Hanes-Isherwood reagent to locate the phosphate portion of the unlabeled isomer (*6*). The product of myoinositol kinase was found to migrate as myoinositol-1-phosphate and was separated from the other isomers (see Fig. 2).

Verification of the identity of the enzymic reaction product was carried out by the following procedure: Myoinositol-2-phosphate (Cal Biochem) was submitted to weak acid hydrolysis (1*N* HCl at 80° to 85°C for 30 minutes) to yield an equilibrium mixture of the 1- and 2- phosphates (*7*). The hydrolysis solution was neutralized with 1*N* NH_4OH, mixed with radioactive product and chromatographed as described. Myoinositol-1- and -2-phosphates were run as standards. The location of the radioactive product again corresponded to that of the 1-phosphate and was separated completely from the 2-phosphate.

Tentative identification of the product as myoinositol-1-phosphate opens the question of whether this enzymic reaction is the initial step in polysaccharide synthesis. It seems unlikely that the 1-phosphate (which would result in formation of a sugar-6-phosphate) would be in this pathway. This reaction may be the initial step in synthesis of phytic acid, the hexaphosphate ester of myoinositol, or, perhaps it has some other as yet unknown function.

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4 October 1965

Artificial Reestablishment of the Lichen Cladonia cristatella

Abstract. *Slow drying stimulated the artificial development of fruiting structures by* Cladonia cristatella *Tuck. Fructification of the fungal partner occurred in the absence of its algal symbiont. Only newly isolated mycobionts produced the reproductive structures. Soredia and squamules were formed in response to the joint effect of slow drying and nutrient-poor conditions.*

Perhaps the most puzzling problem in lichenology has been the inability to reconstitute a lichen association in laboratory culture. Although considerable progress has been made in this direction (*1*), the final step in the lichen development sequence (*2*), that is, fruiting of the lichen fungus, has never been attained. The persistent record of failure in attempts to achieve this stage has hampered progress in both experimental and descriptive lichenology and has raised doubts as to whether the separate symbionts still retain the capacity to reestablish well-defined lichenized associations.

The experimental procedure for the first part of my investigation was as follows: Spores from the lichen's fruiting bodies (apothecia) were allowed to discharge into 250-ml erlenmeyer flasks which contained either soil-extract or malt-yeast-extract agar (*3*). One set of flasks contained only the agar medium. In another set each flask contained, in addition to the agar medium, a fragment of rotted wood, apple or maple, which was taken from the original substrate on which some of the test specimens were growing. The wood fragments were moistened with distilled water and placed either horizontally on the bottom of the flask or in a slanted position. The flasks with the wood were autoclaved and kept for 24 hours at 37°C to allow for germination of any viable contaminant spores on the wood. Medium was then added to the flasks to a level which would not fully cover the surface of the wood, and the flasks were autoclaved again. Each of the two sets of flasks was divided into two groups. One group received a suspension of the alga *Trebouxia erici* Ahm. (*4*), a phycobiont of *Cladonia cristatella* which has been maintained in culture for 7 years, and the other group received only the suspensory medium (*5*) and not the alga. Spore discharge into the flasks was accomplished by placing washed apothecia from the lichen onto petri dish covers, by means of vaseline, and placing these covers over the mouths of the flasks. The covers were kept in this position for as long as 2 days, or until a sufficient spore discharge had been obtained. One of the covers with apothecia was placed over the bottom of a petri dish filled with agar. By examining the dish at periodic intervals, I could determine the relative amount of spore discharge from the population of lichen which was being used. The covers then were replaced with sterile cotton plugs and the flasks were incubated at 20°C. After germination of the spores, which again could be determined by examining the "control" petri dish, a suspension of the alga from a young, liquid culture was added to one set of the flasks. All flasks were kept under constant illumination at a light intensity of about 1100 lu/m².

After 2 weeks, the alga formed a heavy, continuous growth over the agar surface and parts of the wood fragments. Scattered colonies of the

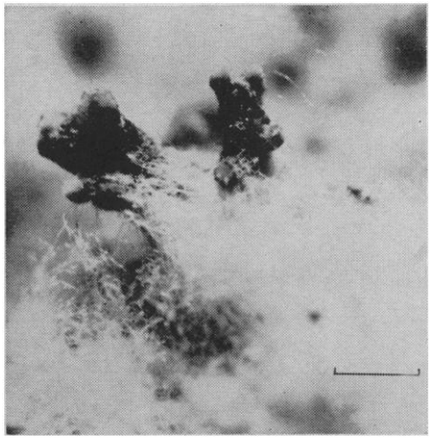

Fig. 1. Apothecium formed by the lichen fungus, *Cladonia cristatella*, in culture. Scale, 0.5 mm. [A. A. Blaker]

fungus became visible about a month after spore discharge and after 2½ months they measured 3 to 5 mm in diameter. Initial development of the fungus occurred more rapidly in the presence of the alga. The symbionts were loosely associated. The nutrient media supported independent growth of both organisms and thus precluded the establishment of a lichenized condition (*1*). In the algal flasks and on the wood fragments, the fungal colonies were loosely mycelial at first and later developed a compact, mounded morphology which is typical of lichen fungi on nutrient agar. As the cultures began to dry, 3 to 4 months after spore discharge, reproductive structures appeared on the fungal colonies. Colonies which were growing on the wood fragments generally were first to form these structures; those near the margin of the agar medium where shrinkage from the flask or wood fragment had occurred, were next, and those colonies in the central part of the agar medium were last.

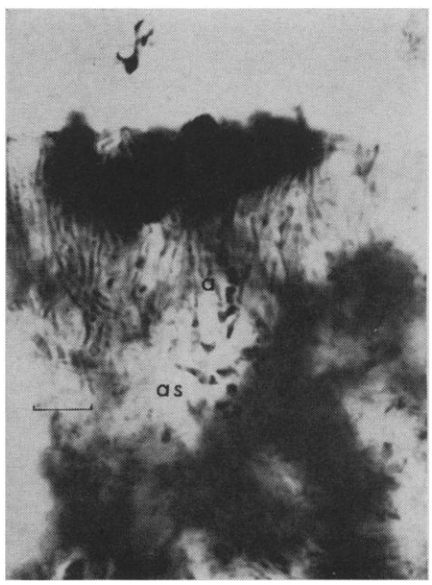

Fig. 2. Hematoxylin-eosin stained section of a young apothecium. *a*, Ascogonial filament; *as*, ascogenous hyphae. Scale, 10 μ. [A. A. Blaker]

Pycnidia appeared first. These structures were formed by most of the colonies in a culture. They were of different types: stalked and sessile, brown, red, or orange in color. Some pycnidia were flask-shaped with a dark-brown exterior and a reddish interior. This type of pycnidium is formed commonly by the naturally occurring lichen. The pycnidia exuded their spores in gelatinous strings. Pycniospores were identical with those formed under natural conditions, both in type and failure to germinate after 4 weeks on soil-extract agar. Apothecia were not as common as pycnidia, and they appeared later. However, no specific pattern in the sequence of development of the two types of reproductive structures could be determined. The young apothecia were either sessile on a colony, usually in clusters of a dozen or more, or they were borne singly on small podetia (about 1 mm high), sometimes branched, which emerged from the colony (Fig. 1). Sections of the apothecia revealed typical reproductive tissues, that is, a palisade layer of hyphae, corresponding to the paraphyses, capped by a red pigmented epithecium which often was encrusted in portions by red crystals. Fusions between hyphae of the palisade layer were common.

Ascogonial filaments (carpogonia) and ascogenous hyphae were seen clearly in sections of 3-month-old apothecia stained with hematoxylin-eosin (Fig. 2), but asci were not evident. Ascogonial filaments originated in tissue below the paraphyses and extended to the epithecium. The filaments were as long as 50 μ and consisted of as many as 11 cells. The basal cells of the filaments were about 5 to 7 μ in diameter while the cells toward the tip were narrower and longer. In several instances, with crushed preparations of young apothecia, I saw a pycniospore attached to the tip of a hypha which extended beyond the epithecium (Fig. 3). The spores could not be dislodged either by pressure on the coverslip or by alternate drying and wetting of the preparation. In one case, a connection between the spore and hyphal tip was seen. A thick gelatinous matrix was evident around the tips of some of these hyphae. Because of the thickness of the sections, I could not trace these hyphae back into the apothecium to see whether they were the trichogynes of ascogonial branches. These extended hyphae, however, are similar to what have been described as trichogynes in other species of *Cladonia* (*6*).

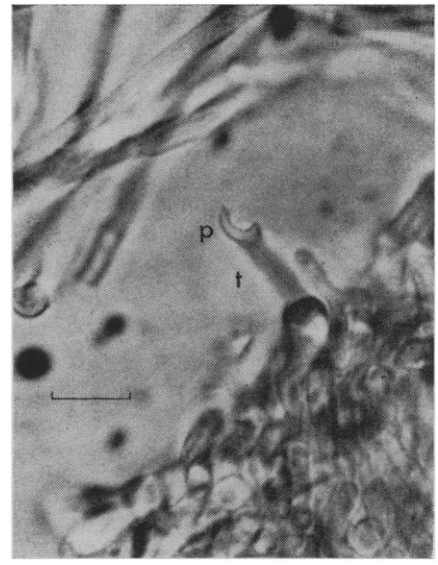

Fig. 3. Crescent-shaped pycniospore (*p*) attached to the tip of a trichogyne (*t*) which extends out from a young apothecium. Scale, 10 μ.

Formation of apothecia and pycnidia was independent of the alga, the wood, and the media used in this study. Apothecia were seen on colonies in a flask containing malt-yeast-extract agar, when the alga, but not the wood, was present; they were also seen on colonies on the same agar medium when a wood fragment, but not the alga, was present. The number of reproductive structures, however, was greatest in flasks which contained both the alga and the wood. There was no substrate specificity. Colonies derived from specimens collected on soil readily formed fruiting structures on the rotted wood.

In all flasks, the stimulus for the formation of the reproductive structures appeared to be a slow drying of the culture. Another factor, that is, concentration of nutrient material, which would vary concomitantly with drying, also was considered. Further experiments provided additional information in this respect.

The second part of my investigation was designed to verify the pro-

duction of fruiting structures by the fungus in the absence of an alga. Colonies of newly isolated (2½ months) mycobionts were taken from slants of the malt-yeast-extract agar and placed directly onto wood fragments, including sections of pith, which were embedded partially in the agar. Because of the slow growth of lichen fungi, the colonies underwent considerable drying before they established a functional contact with the substrate. The drying was evidenced in the discoloration and shrinkage of parts of the colony surface. Numerous pycnidia appeared on the colonies about 2 months after inoculation on areas where drying had occurred. Apothecia developed in the same areas shortly after the pycnidia but they were less common. Both types of reproductive structures appeared on the colonies on pith as well as the other types of wood. As each colony made full contact with the substrate its growth rate increased, and a loose mycelial fuzz of new growth covered the old colony surface.

Mycobionts which had been in culture for several years did not form any reproductive structures after 4 months under similar experimental conditions. A suspension of the alga was washed over the actively growing fungal colonies to observe whether this would stimulate their production of fruiting structures. No reproductive structures developed after an additional 2½ months incubation. During this period, small groups of the alga had developed on the fungal colonies. As these cultures dried, patches of well-defined soredia as well as a few squamules appeared on the upper surface of the fungal colonies. The squamules had a structural organization similar to that found in the naturally occurring lichen. The algal cells of the soredia were penetrated by intracellular haustoria. There was a progressive development of these lichenized structures, which accompanied the progressive drying of the symbionts on the wood. A similar drying of the symbionts on the nutrient agar did not cause them to form these lichenized structures.

The following conclusions and assumptions have been reached on the basis of this investigation. (i) The algal partner is not a necessary prerequisite to the fructification of the lichen fungus. Under natural conditions, however, because of the nutritional needs of the fungus, the alga may well be necessary and the thallus which is formed by the fungus in order to house its algal symbiont may be a reflection of that need. (ii) The fungal partner in culture loses its ability to form fruiting structures but is still capable of forming the lichenized structures. (iii) It would be reasonable to assume that the spores of this lichen are functional in the natural reestablishment of the lichenized state.

The discovery that drying is an important stimulus to the full development of a lichen seems a simple solution to a long-lasting problem. If, however, we recognize and understand the environment in which these associations operate, then the solution becomes an obvious as well as a simple one.

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1 October 1965

Calcite Deposition during Shell Repair by the Aragonitic Gastropod Murex fulvescens

Abstract. *Shell repair was induced by coating the inner surfaces of gastropod shells with nail polish. In aragonitic gastropods initial deposition on the nail-polish membrane was of aragonite spherulites and, in one species, polygonal calcite crystals; later the normal crossed-lamellar structure of the shell was restored.*

Various materials placed within the extrapalleal cavity in contact with the mantle tissues of molluscs evoke shell repair and become encrusted with deposits of $CaCO_3$ (*1*). Deposition of $CaCO_3$ polymorphs other than those characteristic of the normal shell of the animal (*2*), and several crystal types (*3*), have been observed under various experimental conditions. Unfortunately the experimental results may not permit strict comparison because of the different methods and materials used to evoke shell repair. The Hirata mantle-cover-slips-shell preparation is of limited use in some bivalves; it is mainly for morphological studies (*4*). An easy method, probably feasible for all molluscs, has been devised and applied to some gastropods. Coating the internal shell surface with nail polish provides a sufficient stimulus for shell repair, with minimal damage to the shell. X-ray and morphological studies of early stages of shell repair are possible because of the relatively low x-ray-absorption coefficient of the coating material and of its satisfactory transmission of light.

Two species of marine gastropods will be discussed. Young and adult *Busycon (Fulgur) caricum* (Gmelin) and adult *Murex fulvescens* Sowerby were collected near the Duke University Marine Laboratory at Beaufort, North Carolina. The feet were forced inside the shells by C-clamps applied against the opercula. Then the exposed inner surface of each shell was dried, wiped with acetone, and painted with commercial red nail polish (*5*) to form a series of thin membranes near the peristomal edge of the shell and at some distance from it (Fig. 1*a*). When the nail polish had dried, the animals were returned to aerated tanks of sea water at 21°C. In a few hours all animals were moving around. Their mantles were extended to the edges of the shells and apparently covered all the coated areas. After 15 or 30 days the animals were killed by immersion in 10-percent formalin in sea water; the membranes of polish and the material covering them were easily peeled off, dehydrated in ethanol, and dried.

White deposits were found on the membranes of both *Busycon* and *Murex* killed after 15 days (Fig. 1). In *Busycon* these deposits consisted of dense granules, 5 to 10 μ in diameter, packed