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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3. Lichen specimens were collected from rotted apple and maple woods, and from the soil of an open roadside bank and an open grassy field, all in the area of Worcester, Mass.
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5. Either *Trebouxia* organic nutrient medium [R. C. Starr, *Am. J. Botany* **47**, 67 (1960)] without the soil extract; Bold's mineral medium [T. R. Deason and H. C. Bold, *Phycological Studies I. Exploratory Studies of Texas Soil Algae*, Univ. of Texas Publ. No. 6022 (1960)]; or a phosphate buffer (50 ml of 1*M* KH_2PO_4, 29 ml of 1*N* NaOH, 421 ml H_2O).
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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

together to form masses 30 to 100 μ in diameter. In *Murex* two crystalline morphologies on the membranes were noted: (i) rather flat spherulites about 30 to 150 μ in diameter, similar to those of *Busycon*, and (ii) polygons, apparently homogeneous in structure, irregular in shape, about 10 to 120 μ long, and 15 μ thick.

The spherulites displayed higher x-ray absorption at periphery than at center, similar to that observed by Abolins-Krogis (*3*), and a very definite radial arrangement of the mineral crystals (Fig. 1*c*). An irregular pattern was formed by the boundaries of adjacent spherulites. In polarized light the aggregates showed high birefringence and, on rotation of the microscope stage, the typical sweeping isogyres of radial crystals. Single polygons were scattered among the spherulites (Fig. 1*d*); sometimes the polygons formed mosaic patterns, with boundaries remarkable for their straight edges (Fig. 1*e*). Individual polygons displayed high birefringence and sharp extinction on rotation of the stage, behaving as single crystals (Fig. 1*f*). Microradiographic techniques revealed no internal structure, but the boundaries between adjacent polygons were easily recognized because of their low x-ray absorption (Fig. 1*e*). The aggregates, as well as the normal shell material of both species, were shown by x-ray diffraction to be aragonitic, but the polygons in *Murex* were large crystals of calcite.

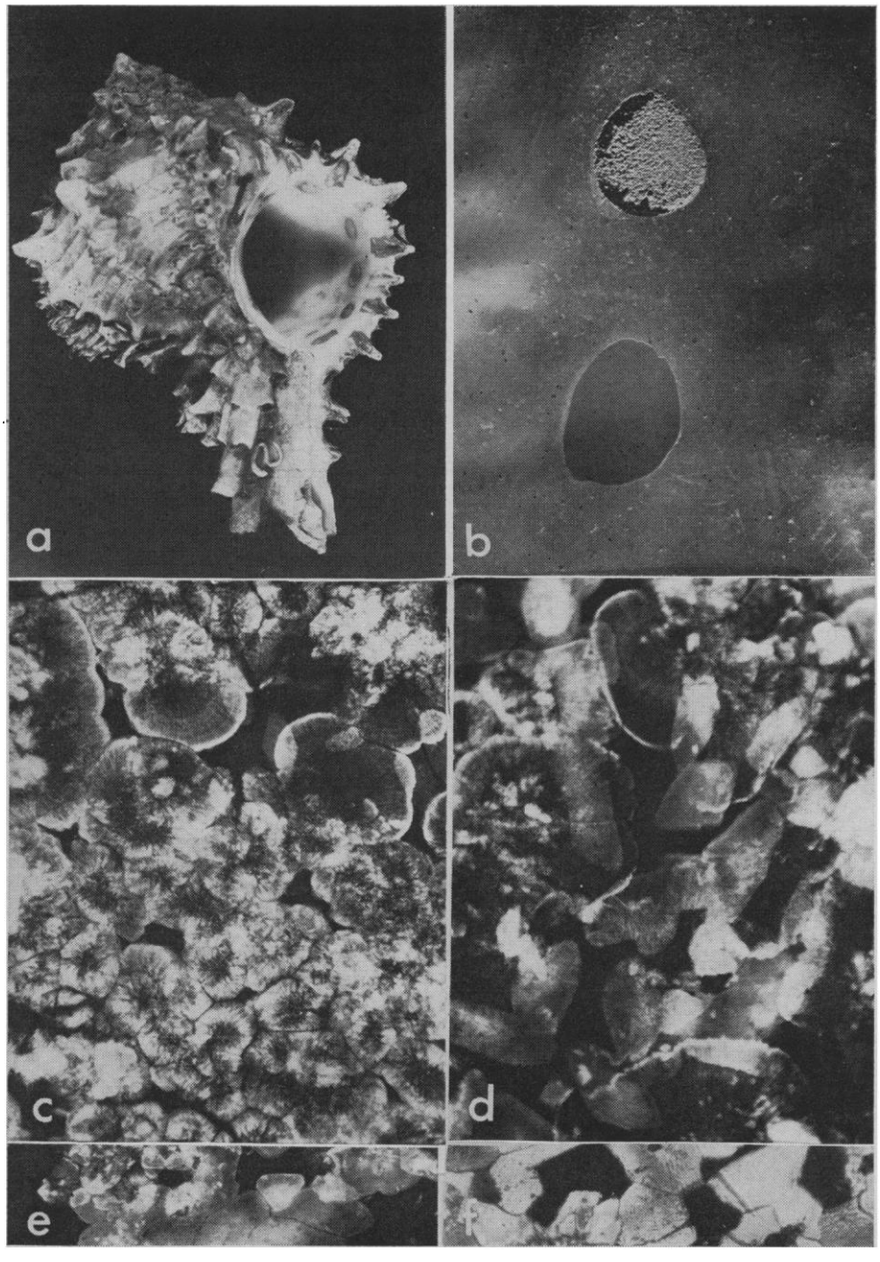

Fig. 1. *Murex fulvescens* Sowerby. (*a*) Shell, showing nail-polish membranes after 30 days; ⅙ natural size. (*b*) Spherulitic deposits after 15 days (× 5). (*c*) Spherulitic deposits (× 90). (*d*) Spherulites and calcite polygons (× 180). (*e*) Calcite polygon mosaic (× 120). (*f*) Another portion of calcite polygon mosaic under crossed nicols (× 120). (Parts *c*–*e* are contact microradiographs.)

Although the spherulites disappeared after decalcification in ethylenediaminetetraacetic acid (*6*), the outlines of the polygonal patterns persisted. The outlines may be sheets of dense, nonmineralized, organic material. This suggestion was supported by electron-microscope observations of thin undecalcified cross sections: the boundaries between polygonal crystals of calcite were about 1 μ in width and appeared to be free of mineral (see *1*, fig. 11, p. 262).

In both *Busycon* and *Murex* after 15 days most of the surface of the shell around the nail-polish membranes appeared to be finely granular and made opaque by an easily removable chalky deposit; this altered surface showed only vague elements of the crossed-lamellar surface pattern observed in normal shells. X-ray diffraction showed that the chalky deposit was aragonite in both species. Beneath the membranes the normal crossed-lamellar surface pattern was completely preserved. Animals killed after 30 days showed significant differences: some membranes in *Murex* and *Busycon* were covered by thick deposits of normal shell material having crossed-lamellar patterns; there were almost no chalky deposits on the shell surfaces, and only on those membranes located at the edges of the shells were there spherulitic deposits similar to those found after 15 days.

Shell repair after nail-polish coating has been also surveyed in the gastropods *Thais floridana, Fasciolaria hunteria, Busycon perversum, Polinices duplicatus, Nassarius obsoletus*, and *N. vibex*; in all, there was similar spherulitic deposition on the membranes, but with difference in quantity and rate between the various species.

The fact that the shell surface adjacent to nail-polish membranes appears to be covered at first with a chalky aragonitic deposit and later with a regular crossed-lamellar structure, as in the normally grown shell, suggests that in this region the chalky deposits may represent dissolution of the shell to obtain Ca^{++}, an early event in the whole process of shell repair (*7*). The

large single crystals of calcite, with intercrystalline sheets of apparently nonmineralized organic material, found in *Murex*, strongly resemble the prismatic structure in bivalves (*8*). Atypical $CaCO_3$ polymorphs are found during shell repair (*2*), but the presence of the well-ordered framework, as well as calcite, in a normally aragonitic shell of gastropods has not been previously reported. Both the spherulites and the abnormal calcite structures are probably responses of the shell to pathological conditions. It is not yet known whether they persist as inclusions or are resorbed during restoration of the normal crossed-lamellar surface pattern.

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Proteins and Disulfide Groups in the Aggregation of Dissociated Cells of Sea Sponges

Abstract. *A sponge extract that produced specific aggregation of dissociated cells was treated with various enzyme preparations to determine which enzymes would destroy its aggregating properties. The results indicate that proteins play a key role in the aggregating effect of the extract on dissociated, glutaraldehyde-fixed sponge cells. Further studies confirm the necessity of calcium for the aggregation and indicate the necessity of intact disulfide groups.*

Moscona (*1*) and Humphreys (*2*) have shown that extracts of certain sponges are capable of aggregating the dissociated cells of the same species of sponge. To determine the chemical nature of the responsible material in the extract, we studied a Chilian marine sponge (*Haliclona variabilis*), using a crude extract of this sponge and its chemically dissociated cells, according to Humphreys' techniques (*2*). Our object was to make advances in this field which could contribute to the understanding of several biological phenomena related to the cell surface in higher organisms, such as cell recognition and selective mutual attachment of cells (*3*); changes in cellular adhesiveness preceding cell movement in normal (*4*) and malignant tissues (*5*), which are particularly important in embryonic morphogenesis and tumor invasiveness; and possibly adhesion of circulating tumor cells to the vascular endothelium (*6*).

Preliminary studies by Moscona (*1*) indicate that the binding properties of the intercellular material in sea sponges are resistant to ribonuclease, deoxyribonuclease, and collagenase but susceptible to treatment with periodate and thus suggest that carbohydrates might play a role in aggregation of dissociated sea-sponge cells. However, because periodate is a very powerful oxidizing agent which may act upon other molecules, including proteins with hydroxyl groups (*7*), we decided to use milder agents to establish the chemical nature of the cell-binding material.

For this purpose, the crude extract, before being used in the aggregating experiments, was treated with a variety of enzymes. Chemically dissociated cells were fixed with 25-percent glutaraldehyde (7:1), washed, and suspended in normal, cold sea water. The crude extract was dialyzed at 2°C against 2 m*M* $CaCl_2$ for 72 hours and stored at 2°C. The cell aggregation test was performed at 2° to 4°C with a rotatory shaker (*8*). Under these conditions the fixed cells did not aggregate unless crude extract was added, as reported by Moscona (*1*). Calcium is necessary both for stabilization of the specific cell-binding substance present in the crude extract and for cell aggregation in the presence of this material (*1*, *2*).

Chemical analysis of our crude extract revealed protein and sugar to be the two main components (Table 1). These results confirm those of Margoliash *et al.* (*9*), who performed chemical studies on similar but more highly purified material. To determine whether one or both components were involved in cell aggregation, the crude extract was treated with different hydrolases acting on peptide bonds (12 enzymes), or glycoside compounds (seven enzymes). In addition, ribonuclease and four hydrolases which split different kinds of ester bonds were also used. Most of the enzymes were dissolved in 2 m*M* $CaCl_2$, except for eight prepared with appropriate buffers. Usually they were used at the *p*H and concentration recommended (*10*). K-zyme, which is a filtrate from *Streptomyces fradiae* cultures (*11*), and a purified preparation of keratinase from this filtrate (*12*) were dissolved in 0.1*M* carbonate-bicarbonate buffer at *p*H 9.

Before the enzymatic incubation, 1 volume of the enzyme solution was mixed with 9 volumes of the calcium-stabilized crude extract. In controls, 1 volume of the enzyme solution was replaced by the same amount of 2 m*M* $CaCl_2$, or by 1 volume of a solution in which the enzyme was inactivated by different procedures. In each case the activity of the enzyme in 2 m*M* $CaCl_2$, with or without buffer, was tested with a suitable known biochemical or cytochemical system. Temperature and length of incubation were those usually recommended, and we began with the shortest incubation. If the crude extract proved to be resistant, incubation was prolonged at a lower temperature (30°C or 25°C), because of its thermosensitivity. After incubation, the mixture was chilled, and, if it contained buffer, it was dialyzed for 12 hours at 2°C against 2 m*M* $CaCl_2$.

For the cell aggregation test, 1.5 ml of the incubated mixture as just described was mixed with 1.5 ml of the sponge-cell suspension in a 25-ml erlenmeyer flask, and the flask was placed in a special rotatory shaker (New Brunswick Scientific Co., model S35) and rotated in a cold room (2° to