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Penetration of Calcium Carbonate Substrates by the Boring Sponge, *Cliona*

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SYNOPSIS. Clionid sponges are noted for their capacity to bore into calcareous substrates. During penetration the substrate is gradually destroyed as the sponge hollows out an extensive system of cavities and tunnels. Preliminary studies revealed that these excavations are produced as small fragments of calcareous material are removed by a special type of amoebocyte which exhibits an etching activity. Cellular penetration occurs along the interface where these cells contact the substrate and is characterized by a unique pattern of cell-substrate relationships. Each active cell releases a substance which dissolves the substrate around its edge, forming a linear etching which corresponds in size and shape to the contours of the cell. Deeper etching occurs as the cell edge, moving gradually downward through the initial etching, sinks into the substrate in a noose-like fashion. During this movement the cell border is drawn down through the slit-like crevice cut by the cell edge, while the nucleus remains in position on the surface of the substrate within the original etched outline. Eventually the undercutting action is completed and a small chip is freed from the substrate. Penetration is achieved by the precise cellular release of a chemical agent which dissolves the calcareous substrate along restricted zones of contact between cell and substrate.

Sponges of the genus *Cliona* inhabit cavities which they excavate in coral, the valves of living molluscs, dead shells, and calcareous rock. As the sponge bores, small fragments are detached from the substrate and expelled from the colony through the excurrent water canals. New cavities are established during the course of penetration and older ones are enlarged until the substrate becomes riddled with a network of interconnecting galleries and tunnels (Fig. 1).

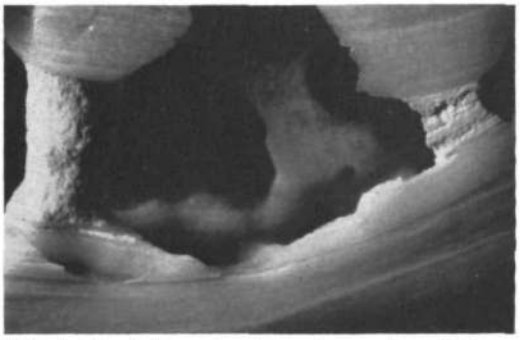

FIG. 1. Vertical section through a valve of *Crassostrea virginica*. The sponge tissue has been dissolved with chlorox to reveal the cavernous system of galleries which have been bored within the shell. ×15

This curious mode of existence has been examined by many investigators, and although no one has determined exactly how penetration of calcareous substrates is accomplished, several explanations have been offered. Mechanical fragmentation of the substrate was proposed by Topsent (1887) to account for the removal of the minute calcareous chips which typically accumulate on the surface of actively boring colonies. Revelle and Fairbridge (1957)

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and Ginsburg (1957) apparently overlooked the release of this particulate material and assumed that boring is achieved by the production of an acid which extensively dissolves calcium carbonate. Nassonow (1883) examined microscopically the boring activities of metamorphosed sponge larvae and saw sponge cells develop cytoplasmic extensions which insinuated themselves into the substrate, cutting out tiny calcareous particles in the process. Similar observations were reported by Cotte (1902) and Warburton (1958) on adult sponges. On the basis of these findings Nassonow (1883), Cotte (1902), and Warburton (1958) postulated that a localized dissolution of the calcareous substrate occurs along points of contact maintained between pseudopods or cytoplasmic processes and the substrate, the cytoplasmic extensions presumably releasing an acidic substance which dissolves the substrate enabling the sponge to cut out small particles. Of the many hypotheses which have been advanced to account for penetration, the last appears to be the most tenable. Penetration can undoubtedly be attributed to a highly complex pattern of cellular activities, but the specific functions which sponge cells perform are unknown. The primary objectives of this investigation were to define the spatial relationships which sponge cells establish with the substrate along sites of penetration and to describe the architectural nature of the substrate destruction.

EARLY PATTERNS OF SUBSTRATE DESTRUCTION

Sponge colonies were cultured in the laboratory in order to examine the mechanism of penetration. Large numbers of colonies could be maintained in a limited amount of space and a variety of experimental substrates could be employed. Cultures were set up by Warburton's (1958) technique, using colonies of *Cliona celata* growing in old oyster shell as the parental culture material. A piece of shell containing the living sponge was broken apart to expose the tissue lining the cavities. Small

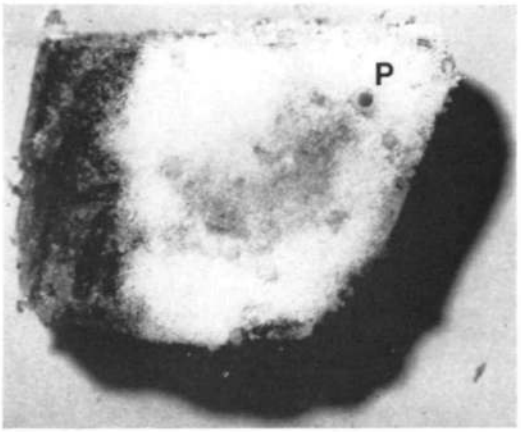

FIG. 2. This culture of *Cliona celata* was grown on Iceland Spar calcite for 46 days. The colony penetrated deep into the substrate and only the papillae (P) upon which the openings to the incurrent and excurrent water canals are situated are visible. The white granular debris is an accumulation of chips discharged during penetration. ×5

portions of the animal were teased out of the cavities and rinsed in filtered sea water to remove cellular debris which would foul the cultures. Next, clusters of sponge fragments approximately 0.5 ml in volume were placed upon blocks of substrate. Iceland Spar calcite was selected, as its transparency greatly facilitates microscopic observation of the destructive alterations produced by the sponge. The young cultures were kept in still water until colonial aggregates formed and attached to the surface of the substrate (about three days at 20°C) and were then transferred to running sea water. Within three to four weeks at 19-21°C, the colonies commence penetration (Fig. 2).

The first evidence of penetration is the appearance of closed elliptical to circular outlines on the surface of the substrate (Fig. 3). These lines are typically arranged into reticular patches. Incomplete outlines are frequently encountered which are semi-circular rather than elliptical or form a ring composed of several distinct crescents. Intermediate configurations of the latter type can be found in which the tips of the component arcs gradually approach one another and unite to form a

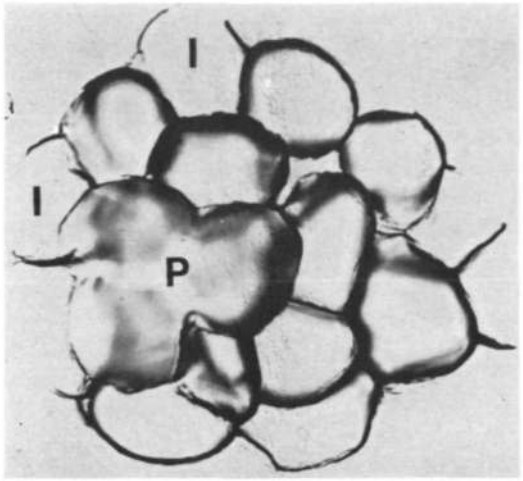

FIG. 3. A group of outlines (=etchings) on the surface of a prism of Iceland Spar calcite. This pattern was produced by a sheet of cells, each component cell forming a single etching around its edge. Outlines are elliptical or circular but exhibit departures from these general shapes, particularly along zones where adjacent etchings are in contact. Incomplete etchings (I) are conspicuous around the periphery of the pattern where more recently assembled cells have begun etching activity. The rims of three pits (P) are visible from which chips have been removed. ×1000

closed outline (Fig. 3). Each complete outline appears to develop by the extension and unification of several segments of curved line.

CELL-SUBSTRATE RELATIONSHIPS

Specimens of the substrate with cells attached to the surface in relatively undisturbed condition were obtained by dissecting the cultured sponge from the Iceland Spar calcite. The crystals with adherent cells were fixed in cold, phosphate-buffered osmium tetroxide, washed in buffer, stained with an aqueous solution of Azure II or Basic Fuchsin, dehydrated through a graded series of alcohols, cleared in xylene, and mounted in immersion oil.

Microscopic examination revealed that the outlines described above are actually linear etchings about 1μ in thickness. Only one cell type, which possesses a prominent nucleus and nucleolus and numerous basophilic granules, is typically associated with the etched outlines (Fig. 4). These cells lie enclosed within the small areas delineated by the contours of the etchings with the cell edge superimposed upon the line itself. In the case of the incomplete or semicircular type of outline the cell edge forms smooth connecting arcs across the gaps between the curved segments (crescents) of the unclosed etching. The spatial relationships which cells bear to etch lines can be accepted as evidence that etchings are formed along the cell edge. The configuration of any initial etching, therefore, conforms with the size and shape of the cell responsible for its production. Etching activity is localized within the cell edge but is probably not synchronized along the entire circumference. It may begin at a single or several points where the cell edge contacts the substrate and spread progressively around its periphery. Incomplete etchings reflect such a pattern of activity (Fig. 3).

As stated earlier, penetration is achieved by removing small chips from the substrate. This is a phenomenon in which many cells participate independently, each cell etching out a single chip. After an outline is etched, indicating the first dissolution of the substrate, deeper penetration ensues with the establishment of an additional set of cell-substrate relations. The entire cell edge next moves downward through the initial etching in a direction approximately normal to the original out-

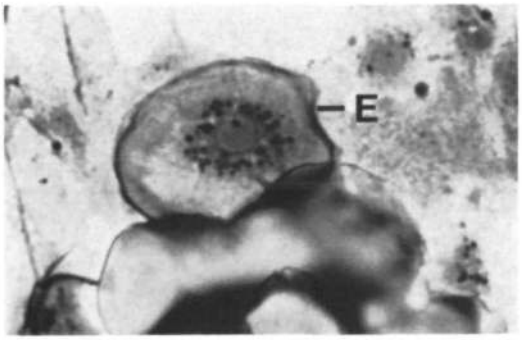

FIG. 4. The flat surface of a prism of Iceland Spar calcite with sponge cells attached. An etching-type cell containing many cytoplasmic granules lies in contact with the substrate and is surrounded by an etching (E) formed along its edge. Stained with Basic Fuchsin. ×1000

FIG. 5. Cross-sectional view of a cavity excavated by the sponge in oyster shell. A single chip (C) still occupies a pit within the walls of the cavity. Material prepared as in Figure 7. ×200.

line. As the cell edge insinuates itself deeper into the substrate, it dissolves a slit-like crevice into which the cell border is drawn. This band of cytoplasm, when stained, is clearly seen wedged within the crevice. During this stage the nucleus remains positioned in the center of the initial etching and the overall dimensions of the cell increase to accommodate the depth of penetration. The sinking movement of the penetrating rim of cytoplasm is analogous to a coring action. As the cell edge gradually etches deeper into the substrate, it constricts in a noose-like fashion so that the resulting crevice becomes conical or cup-shaped rather than tubular. Eventually a chip is etched free and rests within a shallow pit (Fig. 5). The manner in which the chips are dislodged from position and transported through the mesenchyme to the excurrent water canals remains purely speculative at present. Supposedly, the

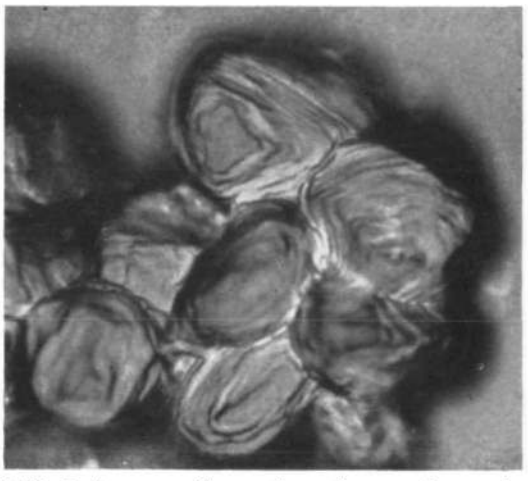

FIG. 6. A group of several contiguous pits on the surface of a prism of Iceland Spar calcite. ×800

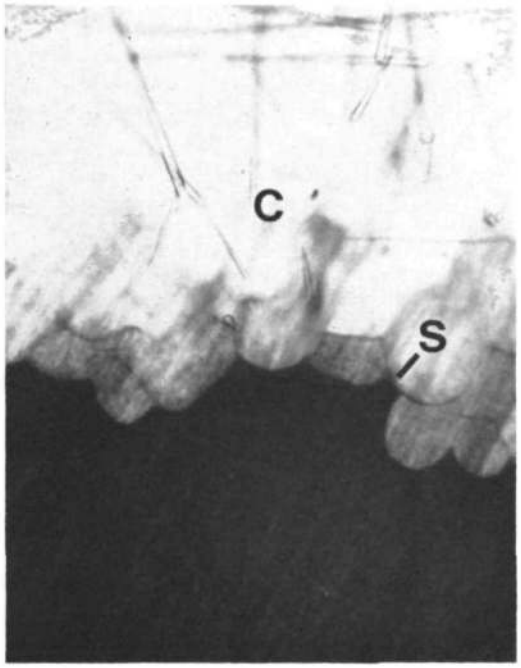

FIG. 7. Cross section through a cavity (C) which has been excavated by the sponge within oyster shell (S). Only a portion of the walls of the cavity is shown in this illustration. Pits lining the cavity have been cut from top to bottom and appear in this view as cup-shaped depressions. This section was cut at 80μ on a Gillings-Hamco Thin Sectioning Machine from a specimen embedded in araldite. ×250.

etching-type cells could exert sufficient pressure by amoeboid movement beneath the chips to initiate displacement. Once the chips have become completely detached from the substrate the pits are evident (Figs. 6, 7). Their walls characteristically exhibit a terrace-like sculpturing (Fig. 6). This pattern of relief is most likely produced by an intermittent dissolu-

tion of the substrate during which the cell edge makes limited vertical and lateral movements in an alternating sequence. It is not known whether the structural organization of a particular species of molluscan shell, or its crystalline form, is responsible for these displacements of the cell edge.

MORPHOLOGY OF THE CHIPS

Chips which have been etched out of the substrate are extremely angular and possess several flat, concave or convex facets which meet to form ridges and points (Fig. 8). Chips of calcitic (*Crassostrea virginica*) and aragonitic (*Mercenaria mercenaria*) shells are identical in shape to those removed from pure crystalline calcite. There is, however, a noticeable difference in average size among chips removed from each of these substrates. Sufficient data have not been obtained to determine whether this variability is significant. Neither the polymorphic form of the calcium carbonate nor the presence of organic material within the substrate alters the basic pattern of cellular penetration presented in the previous section.

Cells which engage in etching activity aggregate into flat sheets at many separate locations over the substrate. The disposition of adjacent cells within each group and the order in which the individual cells begin to etch determine the final shape of chips. The influence of these factors is illustrated in Figures 3, 4, and 9. Chips partially etched out already bear curved sides. Each of these facets represents the locus where an adjacent chip has been removed previously.

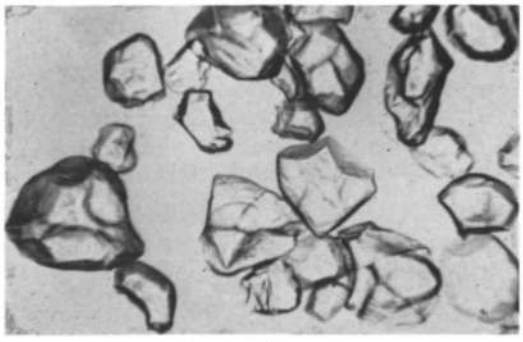

FIG. 8. Chips discharged by a sponge colony boring into Iceland Spar calcite. Although considerable variation in shape exists, their multi-sided form imparts a distinct angular appearance. Average dimensions for the greatest normal axes are $85\mu \times 62\mu$. ×1000

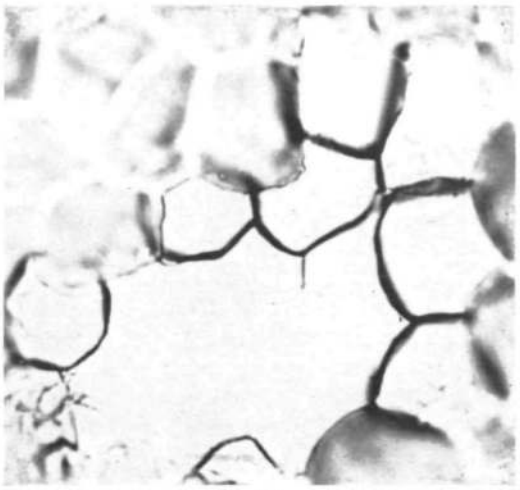

Fig. 9. A column of calcite protruding from a region of the substrate from which several layers of chips were removed by the sponge. Etchings are present along the top, and the sides of the column are scalloped at points where chips were completely removed. Subsequent chips removed from the edge of the column as it is destroyed will bear these curved facets. ×1000

PENETRATION OF NON-CALCIFIED SHELL LAYERS

Observations in the foregoing sections have dealt exclusively with the penetration of pure crystalline calcium carbonate (Iceland Spar calcite) and calcium carbonate crystals embedded within an organic matrix (oyster shell). The question arises as to whether a high proportion of organic material in the structural organization of the substrate could deter penetration. To test this possibility cultures were set up on pieces of periostracum, the proteinaceous outer layer of molluscan shell, obtained from the blue mussel, *Mytilus edulis*. The details of penetration have not been thoroughly analyzed, but initial observations indicate that the sponge is able to penetrate periostracum in much the

same manner as the calcareous substrates (Compare Figs. 3 and 10).

DISCUSSION

The results of this investigation are preliminary, and consequently many aspects of the mechanism of penetration must be left unexplored or only partially resolved. Significant advances have been made especially in clarifying cell-substrate relationships which are established during penetration. These associations have been defined in terms of the spatial relationships maintained between an actively etching cell and the attendant alterations in the substrate. In the course of penetration, a particular shape and orientation of the cell on or within the substrate is accompanied by a characteristic micropattern of substrate destruction. Initial etchings, for example, which are nearly circular, are produced only by those cells which have flattened against the substrate and assumed a circular shape. Similarly, deeper undercutting or etching occurs only as a cell gradually takes the shape of a hollow, lengthening cone, cutting into the substrate at its open apex.

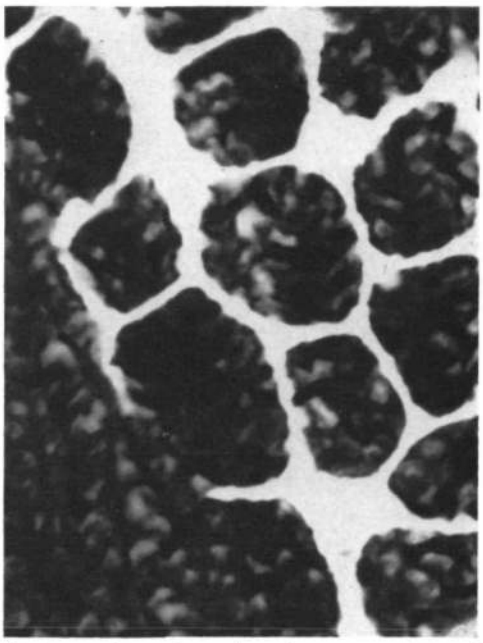

FIG. 10. Surface view of periostracum of *Mytilus edulis* showing a typical pattern of outlines etched by the sponge (compare with Fig. 3). The dark circular areas are chips which are still in position and the clear borders around them represent etchings. Intact sheets of periostracum utilized for cultures were obtained from whole shells decalcified in 3% HCl. ×1000

Etching activity is localized along the peripheral rim of cytoplasm which constitutes the cell edge and is not a general property of the entire cell surface. The release of the etching agent is precisely regulated, and dissolution is restricted to a narrow zone of contact maintained between the cell edge and the substrate. The most convincing evidence for this explanation of cellular penetration is the association which active cells bear to etched outlines during initial stages of penetration. In this situation the curvilinear cell edge coincides with the contour of the etching. I have never seen prominent pseudopodial extensions (Warburton, 1958) nor cytoplasmic processes (Nassonow, 1883; Cotte, 1902) directly participating in any phase of etching activity. However, it is conceivable that structural modifications of the cell edge may be present but will only be resolved at magnifications attainable with the electron microscope.

Although the developmental origin of the etching cells has not been determined, they have been regarded as mesenchymal cells (Cotte, 1902) or amoebocytes (Warburton, 1958). Actively etching amoebocytes possess a prominent nucleus and nucleolus and exhibit an intense cytoplasmic basophilia when stained with Azure II or Basic Fuchsin. These features suggest that the cells may be actively synthesizing protein. Etching cells also contain large basophilic granules concentrated around the nucleus but occasionally clustered in peripheral parts of the cell. It is tempting to speculate that these granules play a role in the elaboration or storage of a secretory product capable of dissolving calcareous substrates. However, confirmation of their chemical identity must await the results of additional histochemical tests.

Chips removed from molluscan shells of

calcite or aragonite or from either the prismatic or nacreous regions are remarkably similar in shape. Neither the calcium carbonate polymorph nor the structural organization of the substrate appears to exert any noticeable effect on the general configuration of chips. Their shape is entirely dependent upon the number and arrangement of adjacent cells in contact with the substrate and the order in which each cell begins to etch.

Mechanical aspects of the penetrative mechanism include the removal of chips out of pits, their subsequent passage through the mesenchyme, and transport *via* the excurrent water canals to the oscula through which they are discharged from the colony. Observations on all these phases are too fragmentary to warrant detailed discussion at this time. Presumably various amoeboid movements and manipulations of chips may be necessary to carry them across the mesenchyme (Warburton, 1958).

Little progress has been made in identifying the substance responsible for etching the substrate. Its chemical nature has been inferred from the observation that both mineralized and non-mineralized layers of molluscan shell are penetrated. Cotte (1902) and Warburton (1958) believed that an acid dissolves the calcified material but were unable to demonstrate an acidic pH along sites of penetration. The periostracum (Nassonow, 1924) and the occasional sheets of conchiolin (Old, 1942; Warburton, 1958) found within the calcified layers of *Crassostrea virginica* shell are also penetrated. To account for the independent dissolution of layers of conchiolin and calcified shell, Cotte (1902), Nassonow (1924), and Vosmaer (1933-35) proposed that in addition to an acid an enzyme may be produced which would break down the conchiolin. Although experimental evidence for such a dual system is lacking, it is possible that the etching agent is composed of a variety of substances, each exerting a specific chemical action on certain components of the substrate or modifying the chemistry of the cellular micro-environment to bring about a localized dissolution of the substrate.

The calcified layers of molluscan shell are composed of a highly structured organic matrix in which calcium carbonate crystals are embedded (Grégoire, Duchâteau, and Florkin, 1955; Travis, this symposium). It seems likely that both crystalline and organic components of these mineralized regions are dissolved during penetration. This is a reasonable supposition since it has been demonstrated that pure crystalline calcium carbonate (Iceland Spar calcite) as well as purely organic material is destroyed by the etching agent. In the penetration of shell, the cell edge, as it etches into the substrate, will inevitably encounter sheaths and lamellae of matrix enclosing crystals of calcium carbonate. Once these organic barriers are penetrated, the cell edge as it carves out a chip may either cut directly through prisms of the mineralized phase or continue penetration in a path delineated by the structural organization of the matrix. The organic matrix, therefore, constitutes the initial pathway for dissolution of the substrate, as the crystalline calcium carbonate will be exposed to the etching agent only after the integrity of the surrounding matrix has been destroyed (also see Travis, this symposium).

Sufficient evidence has been obtained through this study to suggest that the boring clionid sponges penetrate calcium carbonate substrates by a sequence of chemical and mechanical processes. Although each phase of the mechanism must be operative for effective penetration, the etching amoebocytes, since they initiate destruction of the substrate, perform the most essential role.

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