Am. Zoologist, 9:881-885 (1969).

Decalcification at the Mantle-Shell Interface in Molluscs

Miles A. Crenshaw and Jerry M. Neff

Dental Research Center, University of North Carolina, Chapel Hill, North Carolina 27514

Synopsis. Decalcification at the mantle-shell interface in *Mercenaria mercenaria* was studied through the changes in the chemical composition of the extrapallial fluid, and by the measurement of Ca^{45}-deposition and solution. Measurements of O_2-tension demonstrated that the clam was anaerobic soon after the valves were closed. Measurements of calcium, carbon dioxide, and hydrogen ion concentration showed that all of these components of the extrapallial fluid increase with increasing time of closure. These measurements, and measurements of calcium and succinic acid in the tissues and fluids of the clam, indicated that succinic acid produced by the anaerobic metabolism of the clam was neutralized by the dissolution of previously deposited shell.

Evidence for the dissolution of molluscan shell during anaerobic periods is not new. Dugal (1939) showed that the shell of *Mercenaria mercenaria* was dissolved to buffer an organic acid produced by clams kept out of water for periods of days. He suggested this acid was lactic acid. More recent work by Awapara's group (Simpson and Awapara, 1966; Stokes and Awapara, 1968) indicated that this organic acid was probably succinic acid.

One of us (M.A.C.) has been determining the inorganic composition of the extrapallial fluids of marine bivalves. This study started from the suggestion by Wilbur (1964) that the extrapallial fluid, which is enclosed between the mantle and inner shell surface, is the medium from which shell is formed. The greatest variations in the inorganic composition of this fluid were in the concentrations of calcium, oxygen, hydrogen ions, and total carbon dioxide. The concentrations of these components depended upon whether the valves of the mollusc were open or closed at the time the sample was taken. When a mollusc closed its valves the concentrations of calcium, total carbon dioxide, and hydrogen ions increased. The increase in calcium was about three times that of carbon dioxide.

This work was supported by USPHS Grant DE-02668. The authors would like to acknowledge the technical assistance of Mrs. Dona B. King.

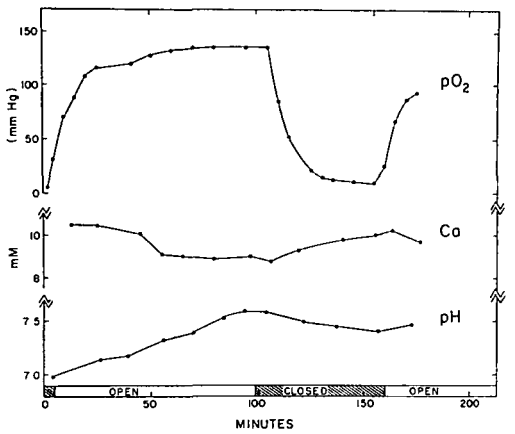

FIG. 1. The O_2-tension, Ca-concentration and pH of the extrapallial fluid of one clam with respect to the opening and closing of the valves. This cycle is indicated by the bar at the bottom of the graph.

Because the magnitude of the changes was greatest in *Mercenaria mercenaria,* we began a more thorough examination of the decalcification at the mantle-shell interface in this animal. Samples of the extrapallial fluid were obtained through a catheter cemented into a hole drilled through the shell between the adductor muscles and above the pallial line.

Figure 1 shows the concentrations of oxygen, calcium, and hydrogen ions in the extrapallial fluid of one clam when the valves were closed, opened, closed, and opened again. This cycle is shown at the bottom of the graph. This clam had been

closed for at least 4 hr when the first samples were taken. The clam was completely covered with sea water during this and the experimental periods.

The measurements of O_2-tension show that the clam was anaeorobic when the valves were closed. When the valves opened the O_2-tension increased, but the O_2-tension fell to less than 10 mmHg within 30 min after the valves closed again.

The pH of the extrapallial fluid was 6.91 before the valves opened. When the clam started ventilating the pH rose, and it fell again when the valves closed. The highest pH observed during ventilation was 7.59. When the first sample was allowed to equilibrate with room air for 30 min the pH rose from 6.91 to 7.48. This observation indicated that the low initial pH was not entirely due to an increased CO_2-tension.

The concentration of calcium in the extrapallial fluid decreased with ventilation from 11.0 to 8.8 mM. When the valves closed again the calcium increased. The lowest concentration of calcium observed was 0.2 mM above that of sea water. In other similar experiments the concentration of calcium in the extrapallial fluid decreased during ventilation but never reached that of sea water.

These results suggest that dissolution of the shell occurs in the normal ventilating cycle of *Mercenaria*.

Further evidence for decalcification at the mantle-shell interface is presented in Figure 2. Nine clams that had catheters inserted into their extrapallial cavities were painted with fingernail polish to prevent exchange of isotopic calcium at the outer shell surface. They were then placed in an aquarium containing 5 liters of sea water. A second catheter was inserted between the valves of each clam so the mantle cavity could be flushed with the external sea water during the course of the experiment.

At zero time, 0.1 mc Ca^{45} was added to the sea water, and the radioactivity in the sea water and extrapallial fluids was followed. These results are shown in the first

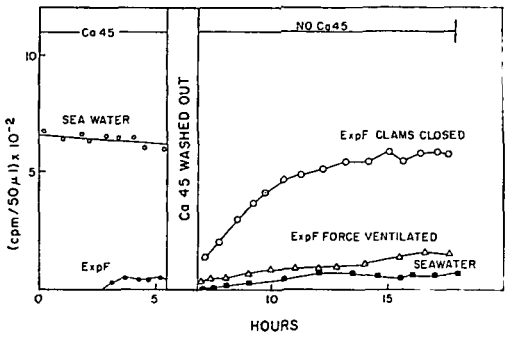

FIG. 2. The isotopic exchange of calcium between sea water and the extrapallial fluid (ExpF) and between the shell and extrapallial fluid.

5.5 hr on the graph. There was a slight decrease in the external Ca^{45} during this time. Radioactivity was detected in the extrapallial fluids of the clams about 2.75 hr after the isotope was added to sea water. The amount of Ca^{45} in the extrapallial fluid seems small, but isotopic dilution, by exchange at the inner shell surface, lowered the specific activity.

After 5.5 hr the sea water containing Ca^{45} was drained from the aquarium, and the aquarium was rinsed with four 5-liter volumes of fresh sea water. The irrigation of the mantle cavities of the clams was continued during this time. The procedure, which took 80 min, is indicated on the graph by the vertical column labelled "Ca^{45} washed out." Then 5 liters of fresh sea water were added to the aquarium.

At this time three of the clams were removed, the clams were shucked, and the shells were cleaned. The inner surface of each valve was dissolved in 10 ml of 0.01 N HCl. Samples of this dissolved shell were then assayed for radioactivity. The dissolved shell contained an average of 490 cpm/valve.

The fresh sea water in the aquarium and the extrapallial fluids of the six clams left in it were periodically assayed for radioactivity for an additional 11 hr after the Ca^{45} was washed out. The catheters were removed from the mantle cavities of three of the clams, and all three closed their valves.

There was a slight increase in the Ca^{45} in the extrapallial fluids of the three clams that were force-ventilated. An increased labelling was observed, to a lesser extent, in the sea water. These increases in radioactivity reflect the isotopic exchange at the inner shell surface and in the soft tissues and fluids of the clams.

The extrapallial fluids of the clams that were allowed to close showed a marked increase in Ca^{45} -concentration. Less than one-third of this increase can be accounted for by isotopic exchange, and the remainder represents dissolution of the shell.

After finding that the shell was dissolved during anaerobic periods we determined the organic acids responsible for the dissolution. We combined the mantle and extrapallial fluids of two clams that had been kept out of water for 24 hr, and prepared an acetone extract using the method of Frohman, Orten, and Smith (1951). We found the extract contained 17 mEq acid per liter of original fluid when we titrated it with 0.1N NaOH against phenolphthalein.

We then chromatographed a portion of this extract on Whatman No. 1 paper with ethanol:ammonia:water (80:5:15) (Hammen and Wilbur, 1959). Spots corresponding to succinic and lactic acids were the only ones revealed when the chromatogram was sprayed with 0.2% bromophenol blue in ethanol.

Duplicate assays with the method of Barker and Summerson (1941) showed that 2% of the acids in the extract was lactic acid. We found that 97% of the extracted acids was succinic acid. We used the method of Rodgers (1961) which is based on the reduction of 2,6-dichlorophenolindophenol by succinate in the presence of succinic dehydrogenase. The reduction of the indicator by the extract was completely inhibited by 1 mM malonate.

We then conducted a more extensive study of the relationship between production of succinic acid and dissolution of shell. Clams were removed from the aquarium after they had been siphoning for at least 30 min. Each clam was opened after being out of water for a time, and the mantle and extrapallial fluids drained into a beaker. A sample of the fluids was immediately diluted for determination of calcium by atomic absorption. A second sample of the fluids was extracted with acetone according to the procedure of Frohman, Orten, and Smith (1951).

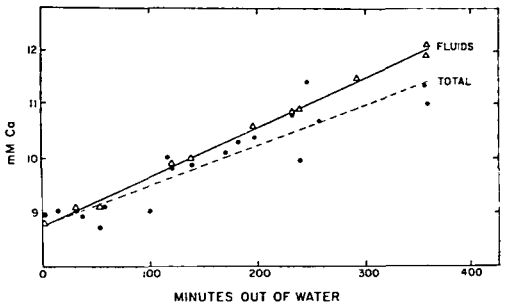

FIG. 3. The concentrations of calcium in the fluids and in the soft tissues and fluids (total) of clams with respect to time out of water.

The tissues were removed from the shell, combined with the unused portion of the fluids and brought to pH 2 with 2N HCl. This material was homogenized in an ice bath with a Sorvall Omnimixer. A sample of the homogenate was ashed at 500°C and diluted for Ca-determination. A second sample of the homogenate was extracted with acetone.

This procedure of combining the mantle and extrapallial fluids with the soft tissues was adopted because we could not completely drain the fluids from the tissues. This combination is shown in the graphs as the "total," and the concentrations are expressed as millimoles per kg of material. A rough estimate of the actual concentrations of tissue can be made by considering the fluids to have two times the weight of the soft tissues.

The concentration of calcium with respect to time out of water is illustrated in Figure 3. These data show that the shell was dissolved and that the increase in extrapallial fluid calcium observed earlier was not due to a redistribution of calcium between the soft tissues and the fluids. The rate of increase in the fluids was 0.5 mM Ca/hr, and in the total it was 0.4 mM/hr.

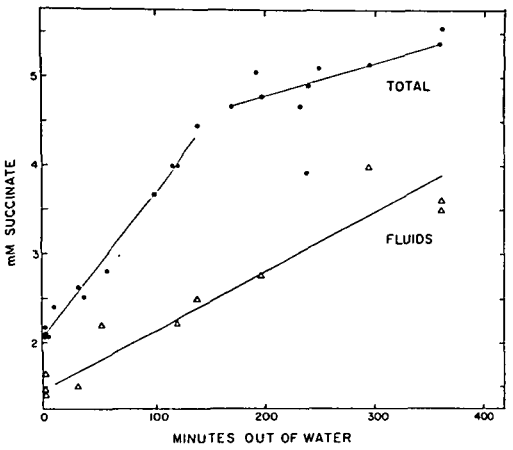

FIG. 4. The concentration of succinate in the fluids and in the soft tissues and fluids of clams with respect to time out of water.

This means that a 100-g clam lost about 2 mg of shell per hour.

Figure 4 shows the concentrations of succinic acid in the same clams. The clams which were opened immediately after being removed from sea water contained 1.5 to 2.0 mM succinate. The concentration of succinate in the fluid fraction increased steadily at 0.4 mM/hr. The total concentration of succinate increased rapidly for the first two hours, at about 1 mM/hr. After that time the rate of production of total succinate decreased to about that observed in the fluids. The reason for this sharp break in the curve is not known.

A comparison of the increases of succinate and calcium in the fluids shows that all but 20% of the calcium dissolved from the shell was used to neutralize succinic acid. Our earlier determinations indicated that the remaining 20% of the increase in calcium was neutralized by bicarbonate. This balance is difficult to calculate for the soft tissues because the rate of production of succinate was time-dependent.

Concentrations of lactic acid in the fluids and "total" were about the same and were constant at about 0.1 mM. Experiments to determine the recovery of lactic and succinic acids from sea water showed that our procedures accomplished complete recovery.

We then examined the changes in calcium and succinate in the extrapallial fluids of individual clams. The composition of this fluid would reflect, more clearly, the relationship between production of succinic acid and dissolution of shell. Two clams that had catheters in their extrapallial cavities were removed from an aquarium after they had been siphoning well-aerated sea water for 30 min. Samples of the extrapallial fluids were taken at intervals with calibrated microliter syringes and analyzed for calcium and succinate. The results are shown in Figure 5. There was evidence for individual variation, but the overall pattern was the same. The initial rates of production of succinate and dissolution of shell were high, and then the rates decreased. This pattern was very similar to the total succinate production shown in Figure 4. The succinic acid produced accounted for 80% of the calcium dissolved from the shell. These data demonstrate that the production of succinic acid is directly responsible for the dissolution of the shell.

Our results are summarized in Figure 6

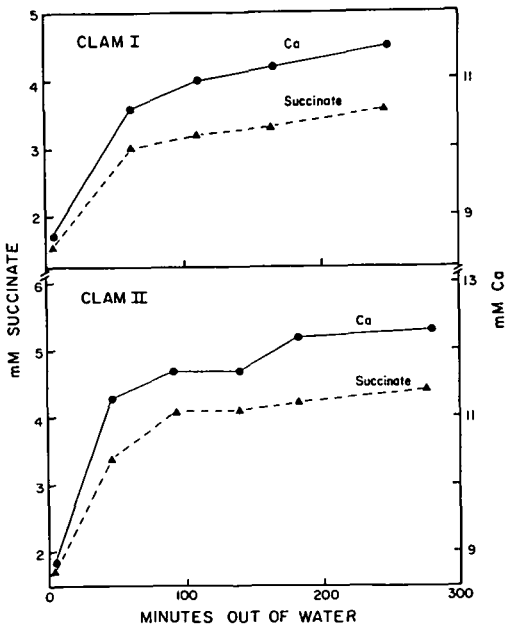

FIG. 5. The concentrations of calcium and succinate in the extrapallial fluids of two clams with respect to time out of water.

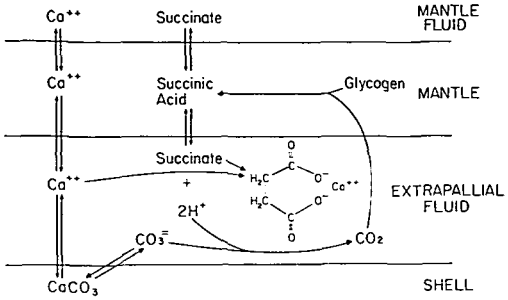

FIG. 6. Summary of the results presented in this paper.

and in the following statements. Under normal conditions *Mercenaria mercenaria* becomes anaerobic when the valves close. This period of anaerobiosis is accompanied by an accumulation of succinic acid. This succinic acid is produced by the fixation of CO_2 *via* the Wood-Werkman reaction (Hammen, 1966; Stokes and Awapara, 1966). Some of the CO_2 fixed into succinic acid originates from the shell carbonate when the shell is dissolved to neutralize the succinic acid. The evidence for this fixation was the disproportionate increase of calcium and total carbon dioxide during the anaerobic periods.

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