AN APPLICATION OF CRITICAL POINT DRYING TO THE COMPARISON OF MODERN AND FOSSILIZED SOFT TISSUES OF FISHES

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ABSTRACT. Critical-point-dried samples of recent biological soft tissues can be used to make accurate comparisons with exceptionally well-preserved fossil material. This technique has distinct advantages over thin sections of biological tissues, as palaeontologists are often more familiar with observing three-dimensionally preserved material. This technique offers important opportunities for comparative taphonomic and anatomical studies, especially for palaeontologists working with exceptionally well-preserved soft tissues such as may be found in early diagenetic concretions.

CRITICAL point drying (CPD), the drying of biological tissues at the critical temperature and pressure of carbon dioxide, is a technique which allows the examination in three dimensions of soft tissues under a vacuum, and hence is ideal for scanning electron microscopy (SEM). The technique produces a minimum of artefacts during the drying process compared with freeze drying and drying in air, and the CP-dried tissues may be coated with a variety of electrically conductive materials.

Biological specimens dried in air are greatly distorted by surface tensional processes, unless they are composed of particularly rigid biopolymers such as chitin. CPD involves taking the liquid in which a specimen is immersed to its critical point; that is the temperature (T_c) and pressure (P_c) at which the liquid changes imperceptibly from a liquid to a gas (or vice versa). At this point, surface tension is zero, and fluid may be released from the tissue causing a minimal amount of morphological change. The procedure is based on Anderson (1951), who also describes in more detail the principles behind the method. An alternative to CPD is freeze drying (Boyde 1974). Freeze drying offers excellent opportunities for examining fractured surfaces, but it also has a number of serious drawbacks, including severe tissue damage due to ice crystal formation (Hayat 1978), and we do not recommend its use here.

We demonstrate an application of CPD to a palaeontological problem, an attempt to resolve an early diagenetic event preserved in pre-compaction concretions. Martill (1988) suggested that the preservation of phosphatized soft tissues in Cretaceous fishes from the Santana Formation of Brazil took place very rapidly, and prior to burial. Since burial rates in the deposit are unknown, however, the time interval in which phosphatization took place is also unknown. The soft tissues discovered by Martill show a number of features related to the decomposition and collapse of the tissues. Thus it was believed that phosphatization accompanied active decay rather than post-dating a physico-chemically induced interruption of the decomposition process, but this supposition remained to be tested.

On this basis, it should be possible to mimic decomposition processes taking place on the sea floor under laboratory conditions, to sample the decaying tissue at various intervals, and observe changes in tissue morphology with time. CP-dried samples of partly decomposed tissue could then be examined using the SEM, and direct comparisons made between recent and fossil material.

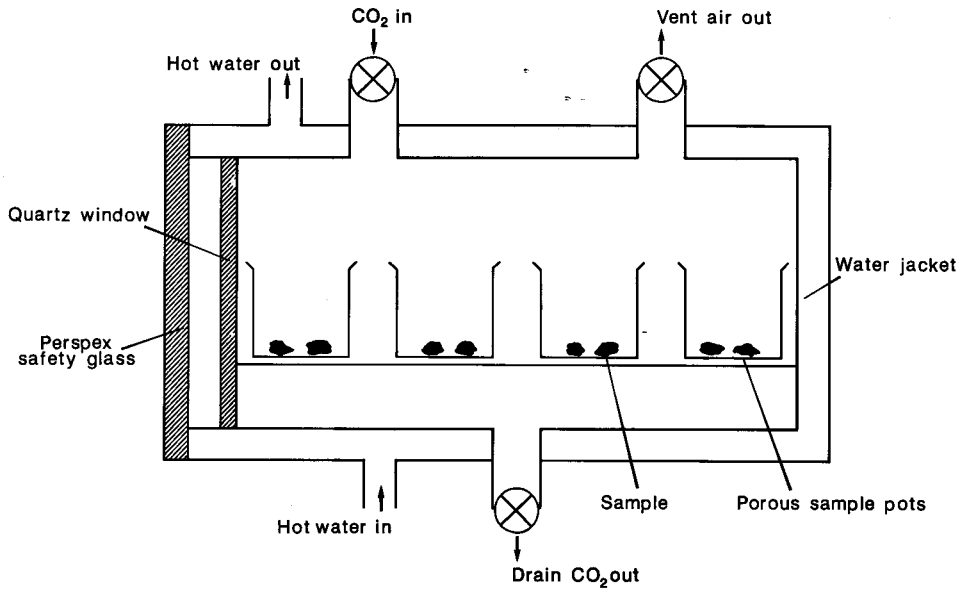
METHOD

A rainbow trout (*Salmo gairdneri* Richardson), 20 cm in length, was killed and placed in a bath of fresh sea water with a specific gravity of 1·02 (normal salinity). Samples of striated muscle, gill filament with secondary lamellae, and ovaries were removed at death. Further samples were removed at hourly intervals up to 4 hours,

[Palaeontology, Vol. 33, Part 2, 1990, pp. 423–428, 1 pl.]

and then at 6 hours, 24 hours, 48 hours, and 7 days. The experiment was carried out at room temperature in a fume cupboard.

The samples of tissue were immediately fixed for 1·5 hours in 1% osmium tetroxide buffered in phosphate at pH 7·2 to prevent further post-mortem degradation. After two washes in the buffer the tissue was dehydrated by immersion in a series of acetones (30%, 50% – 10 minutes each, then 70%, 85%, 90%, 95%, and 100% for 15 minutes each). The 100% acetone wash was repeated. At this stage all the water in the tissue had been replaced by acetone (alternative methods of dehydration use ethanol or freons, as described by Cohen 1979). The samples were then placed in porous vessels flooded with acetone, and loaded into the CP-drying bomb (text-fig. 1). This was then filled with liquid carbon dioxide and flushed through three or four times over a 3·5 hour period to expel all the acetone from the samples, replacing it with CO_2. The half-filled bomb was gently heated to bring the CO_2 to its critical point and then vented (T_c = 31 °C, P_c = 72·9 atmospheres). Biological tissues cannot be CP-dried using water as the ambient fluid as the higher temperature and pressure required is not practical (water T_c = 374 °C, P_c = 217·7 atmospheres). The CP-dried samples were sputter-coated with gold and examined using a Hitachi S-520 SEM at 20 kV.

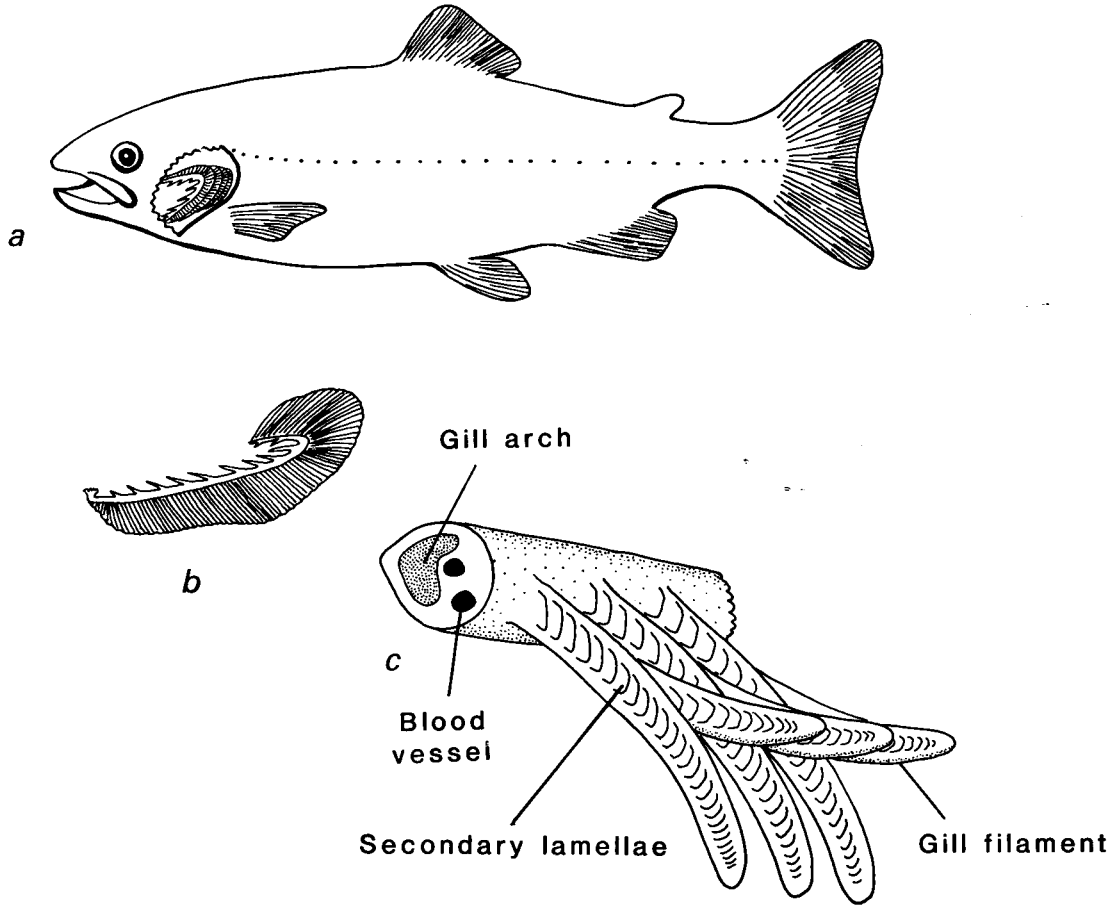


TEXT-FIG. 1. Schematic diagram of the critical point-drying bomb. Samples are placed in porous beakers supported on a boat. The apparatus is heated by a hot water circulatory system. CO_2 enters through a regulatory valve. The operation can be observed through a clear Perspex safety shield in front of a silica window.

Fossilized soft tissues were obtained from teleost fish in pre-compaction concretions from the Santana Formation (Lower Cretaceous, Aptian/Albian), of the Chapada do Araripe, Ceara, north-east Brazil. They were prepared by partial digestion in 10% acetic acid so that the fish skeleton remained partly within the concretion. This allows the soft tissues to be more readily identified since they remain *in situ* within the fossil. The fossil material was also sputter-coated with gold and examined on the same SEM, but at 15 kV to minimize beam damage (*note*: high kV at high magnifications have been found to damage some phosphatized soft tissues).

RESULTS

The morphology of all the recent CPD soft tissues examined changed with time after death. Here we discuss only the gill filaments and associated secondary lamellae (text-fig. 2). In the recent samples, secondary lamellae of the gills at time zero are erect and well spaced (plate 1, fig. 1). At high magnification, detail on the surface of individual cells is seen at time zero, but this begins to break down after only one hour (plate 1, fig. 8). As time of decay progresses, individual secondary



TEXT-FIG. 2. Diagram showing position of secondary lamellae and gill filaments in the rainbow trout (*Salmo gairdneri*). *a*, Trout with operculum removed to reveal gill arches. *b*, Single gill arch with gill filaments. *c*, Detail of gill arch with three filaments showing position of secondary lamellae. Based loosely on Hughes and Morgan (1973).

lamellae collapse, possibly due to lack of blood pressure and also because of gravity (plate 1, fig. 3). This collapse produces a prominent kink at the base of each lamella (plate 1, fig. 9). After two hours, the epithelial cells of the recent secondary lamellae begin to detach, leaving only a connective tissue lining (plate 1, fig. 6). Epithelial tissue covering the gill ray begins to detach from the gill ray after four hours. A similar detachment is also seen in the fossil material (plate 1, fig. 4). Gills sampled after seven days show very little identifiable soft tissue remaining. However, bone surfaces show numerous colonies of microorganisms, including spherical and rod-shaped bacteria (plate 1, fig. 10).

A large number of the fossil gills show kinks at the base of each lamella (plate 1, fig. 7a, b), although many of the fossil secondary lamellae are preserved fully erect and well spaced (plate 1, fig. 2a, b). Individual epithelial cells are only rarely preserved in the fossil material, but they can sometimes be seen on the surface of gill rays. They do not show details of cell wall ultrastructure, but this may be because of an inability of this preservational style to resolve these features.

DISCUSSION

Timing of phosphatization

Results of the experimental decomposition of the trout show that very rapid morphological change occurs in delicate soft tissues such as gill filaments and secondary lamellae. The exceptional preservation of these tissues in fossil fish from the Santana Formation, together with the preservation of artefacts brought about by decomposition, show that phosphatization was clearly an extremely early and rapid diagenetic event. Temperature and salinity are likely to have been major influences controlling the rate of reactions and hence rate of decay of the fossil fish. The temperature of the Santana sea floor has yet to be detemined isotopically, but its position within the palaeo-tropics, and its generally shallow aspect suggest warm, rather than cold, bottom-water conditions. Present-day salinity has been assumed for the Santana Formation for the purpose of this experiment, although a number of authors have considered the salinity to range from fresh to hypersaline (see Martill 1988 for a review).

Comparisons between Recent and fossil material at normal salinity and room temperature suggest that phosphatization must have taken place within the first five hours of death of the fish, although preservation of epithelial cells in place suggests a possibly earlier event, *c.* 1–2 hours. This indicates that very high concentrations of dissolved phosphate were available for rapid precipitation on to nucleating sites. Although the oxygen level of the Santana sea floor is not known precisely, the presence of arthropods and rare molluscs shows that anoxia had not been reached. However, Allison (1988) has shown that most carcasses in marine environments undergo anoxic decomposition even in well-oxygenated water. We therefore assume that oxygen levels are not as important as hitherto believed.

Use of CPD by palaeontologists

This example shows how critical point drying can be used to help solve a specific palaeontological problem, in this case, one of taphonomic and diagenetic importance. However, there are numerous other taphonomic, taxonomic, and palaeobiological applications for the technique. Palaeobotanists

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EXPLANATION OF PLATE 1

Comparison between fossil soft tissues and recent trout gills after various times of decomposition. (Fossil material prefixed DM is currently held by the first author at the Open University, but will be transferred to the University of Leicester, Department of Geology.)

Fig. 1. Section of CPD fresh trout gill filament with erect secondary lamellae, × 150.

Fig. 2. DM 50. *a*, Section of fossil gill filament from Santana Formation, × 150. *b*, Detail of four erect fossil secondary lamellae showing preservation fabric of phosphatic microspheres, × 700.

Fig. 3. Three CPD trout gill filaments after one hour of decomposition. Two of the filaments show detached portions of epithelial cells revealing connective tissue lining of blood vessels, × 70.

Fig. 4. DM 63. Fossil gill filaments showing same features observed in fig. 3, × 70.

Fig. 5. CPD trout gill filament after one hour of decomposition, with post-mortem break-up following boundary between epithelial cells, × 300.

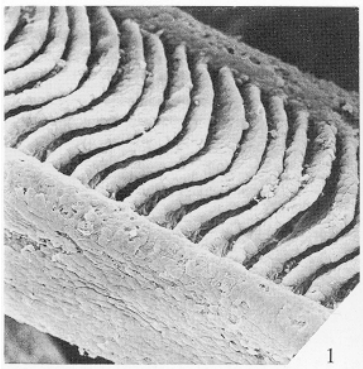
Fig. 6. CPD trout gill filament with secondary lamellae after four hours of decomposition. The epithelial cells have detached from the lamellae leaving only a connective tissue lining. Collapse has produced kinking of the lamellae at their bases. Some shrivelling of the connective tissue lining of the blood vessel has taken place, × 200.

Fig. 7. DM 101. *a*, Fossil gill filament showing similar features to those seen in figure 6, × 400. *b*, Detail of secondary lamellae/blood vessel junction, × 1000.

Fig. 8. CPD trout gill after one hour of decomposition, showing collapsed epithelial cells, × 2000.

Fig. 9. CPD trout gill filament showing detail of secondary lamellae/blood vessel junction, after four hours decomposition, × 400.

Fig. 10. CPD trout gill after seven days of decomposition. All gill tissue has decomposed, only isolated colonies of bacteria remaining on exposed bone surfaces.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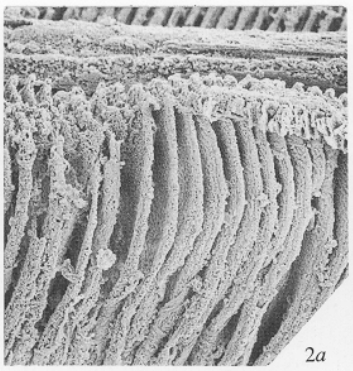
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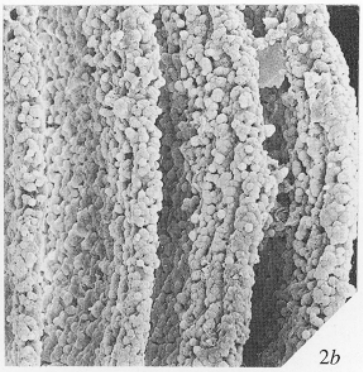
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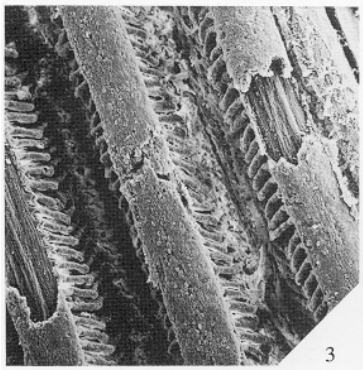
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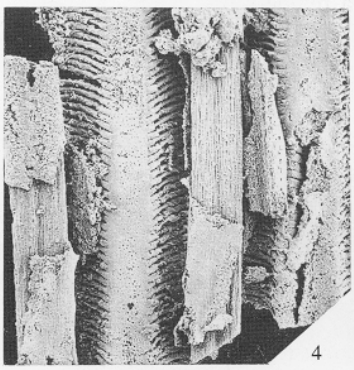
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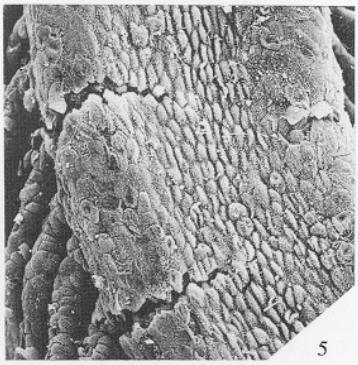
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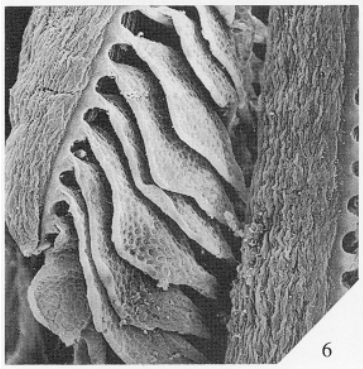
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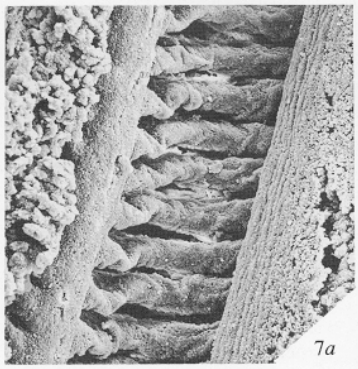
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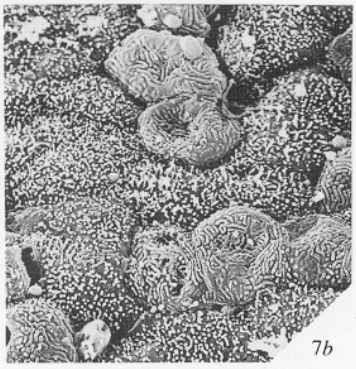
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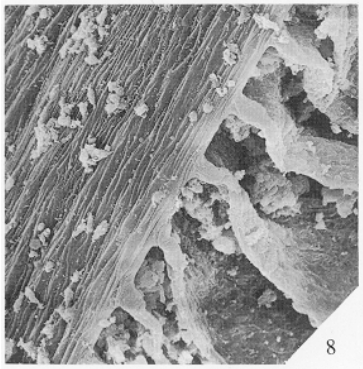
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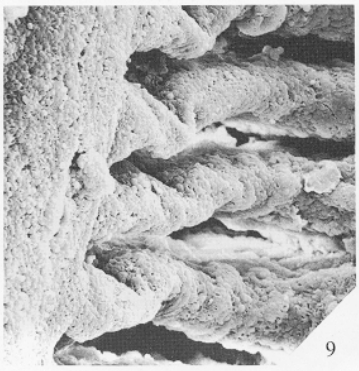
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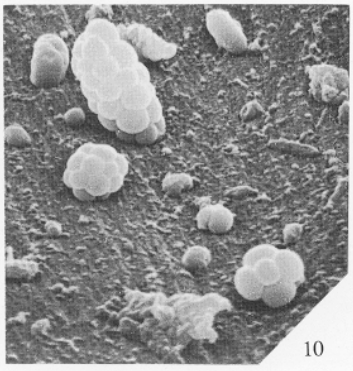
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MARTILL and HARPER, *Critical point drying*

familiar with CPD (e.g. Hill 1987) have used it successfully for comparative anatomical studies. Wherever fossil material requires comparison with recent material, especially at high magnifications, this technique allows direct comparisons of three-dimensional material to be made. Very few artefacts are introduced during preparation, although it should be pointed out that there may be some shrinkage of the tissue, and at very high magnifications it is sometimes possible to see osmium tetroxide crystallized on some surfaces from over-osmication.

CP-dried material stored in anhydrous conditions has a long shelf life. Bivalve mollusc material prepared by Harper has persisted for at least 12 months without discernible deterioration.

Acknowledgements. We thank the Biology Department of the Open University for use of their CPD facility, and Tony King for helpful instruction. Use of the SEM at the Department of Geology, University of Leicester is gratefully acknowledged, with special thanks to Rod Branson. Roy Clements commented on the manuscript. This work was funded by an Open University Research Grant awarded to D.M. L.H. is funded by NERC.

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Typescript received 1 March 1989
Revised typescript received 27 May 1989