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FIXATIVES, DECALCIFIERS AND ULTRASTRUCTURE OF THE ORGANIC REMNANTS FROM MURAL NACREOUS LAYERS OF FOSSIL CEPHALOPOD SHELLS

BY

Ch. GREGOIRE (*), G. GOFFINET (**) and M. F. VOSS-FOUCART (**)

(With 3 plates)

SUMMARY

The ultrastructure of the organic remnants has been compared in the TEM, after decalcification of the mural nacre of ammonites and fossil nautiloids by EDTA, which removes a soluble fraction, and after fixation and decalcification by formaldehyde-cetyl-pyridinium chloride-EDTA (CPC method) and chromium sulphate solutions, which are both considered to insure a better preservation of these organic remains.

The loose networks of altered trabeculae, frequently fused into membranes, which constitute the ultrastructure of the fossil organic remnants of nacre after decalcification by EDTA, are also found in the samples treated by the CPC method and by chromium sulphate. Continuous membranes, superimposed on the networks, are especially abundant in the material treated by chromium sulphate. It is concluded that the networks of altered trabeculae are not artifacts, but are the representative ultrastructures of the organic remnants of the nacreous layers in the fossils studied so far. It is suggested that disappearance of EDTA soluble substances does not distinctly alter the ultrastructure of the fossil organic residues.

(*) Formerly Laboratory of General and Comparative Biochemistry, University of Liège, Dieweg, 292, 1180 Brussels.

(**) Laboratory of Morphology, Systematics and Animal Ecology, University of Liège, Quai Van Beneden, 22, B-4020 Liège (Belgium).

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INTRODUCTION

Many remnants of the organic components of the nacre in ammonoids and fossil nautiloids of different ages (Pleistocene to Ordovician), decalcified in EDTA, appear in the TEM in the form of loose networks of differently altered trabeculae, of continuous membranes, and of small spheroidal or lenticular pebble-shaped particles, respectively produced by coalescence or fragmentation of these trabeculae (5-11, 4, 22)

As EDTA removes a soluble fraction from modern and fossil organic components of the nacre (15, 2, 13, 14, 18, 19, 23), these structures represent only the EDTA insoluble fraction of subsisting organic matrices, in which the amounts in biochemical components have been already considerably reduced during fossilization.

In order to appreciate the structural modifications caused by disappearance of the EDTA soluble fraction in the organic remnants of fossil nacre, fragments of nacreous layer of fossil cephalopods have been immersed in four fixatives and decalcifiers commonly used in the recent studies on mollusc shells, and their organic residues have been examined in the TEM.

MATERIAL AND METHODS

Ammonoids. — *Baculites claviformis* STEPHENSON (U. S. G. S. 25406) (998) (Cretaceous); *Leioceras opalinum* (Rein.) (405) (Jurassic); *Amaltheus spinatus* BRUGUIERE (408) (Lower Jurassic); *Stepheoceras* sp. (989) (Lower Jurassic); *Harpoceras mulgravium* SIMPSON, YOUNG and BIRD, 1937 (524) (Lower Jurassic); *Hildoceras* sp. (1008) (Lower Jurassic); *Carnites floridus* WULFEN (965) (Upper Triassic); *Anthracoceras wanlessi* PLUMMER and SCOTT 1937 (855) (Middle Pennsylvanian); *Agoniatites vanuxemi* (704) (Middle Devonian).

Nautiloids. — *Aturia luculuensis* (608) (Miocene); *Striacoceras typus* (Saemann) (703) (Middle Devonian); Large unidentified nautiloid, with a gyroconic conch (713) (Middle Devonian).

Fragments of nacreous layers, still composed of aragonite or diagenetically transformed into calcite, were fixed and decalcified as follows :

1. Decalcification in saturated or 0.5 M EDTA solutions (titriplex III Merck, Darmstadt) at pH 4.0 or 7.5.
2. Immersion of fragments of nacreous layers for seven days in solutions of 15 per cent glutaraldehyde, at pH 4.0 and 5.0, acting as fixative and decalcifier (989).
3. Immersion of fragments of nacreous layers in formaldehyde-cetylpyridinium-chloride (CPC) — EDTA, according to the method of WILLIAMS and JACKSON (21), in the modification applied to septal nacre by CRENSHAW and RISTEDT (3) (405, 408, 989, 524, 608, 855).
4. Immersion for various periods of time (48 hours to 25 days) of fragments and of powdered nacre in chromium III — sulphate solutions (pH 3.6), acting as fixatives and decalcifiers (20, 21, 17, 14) (998, 965, 524, 989, 703, 704, 713).

The organic remnants, placed on formvar coated screens and shadow-cast with platinum, were examined in a Siemens Elmiskop I, using a double condenser, a 200 μm condenser aperture, a 30 μm objective aperture and a cold stage.

OBSERVATIONS

E D T A . — As previously described on the basis of examination of several hundreds of fossil cephalopods (see Survey, 9), the biuret-positive organic remnants of decalcification by EDTA appear in shadow-cast preparations in the form of flat or cylindrical, widened or inflated, irregularly twisted and varicose trabeculae, arranged in loose networks (pl. 1, fig. 1; pl. 3, fig. 1), still frequently enclosed in polygonal fields delimited by intercrystalline cords identical to those shown in pl. 2, fig. 4. Membranes (pl. 2, fig. 3), rounded, scattered or clustered pebble-shaped particles and slabs (pl. 2, fig. 5), also observed among the remnants, are produced by coalescence or fragmentation of these trabeculae.

G l u t a r a l d e h y d e . — The organic material freed by glutaraldehyde contains the structures left by EDTA. However, fragmentation of the trabeculae and other structures into irregularly shaped small particles is more important.

C P C m e t h o d . — The organic structures (twisted trabeculae, membranes of coalescence, corpuscular fragments) left by the CPC-EDTA method are closely similar or identical to those recorded in the EDTA material (compare pl. 1, fig. 1 to fig. 2; pl. 2, fig. 3 to fig. 4, fig. 5 to fig. 6; pl. 3, fig. 1 to fig. 2).

Chromium sulphate. — The organic material freed by the chromium sulphate solutions consists of different structures : networks of trabeculae (pl. 1, figs. 3 and 4), membranes (pl. 1, figs. 3 and 4) and small particles (pl. 2, figs. 1 and 2). All these structures are also found in EDTA samples of organic remnants of many fossil nacres. However, in chromium sulphate preparations, the trabeculae are frequently more slender (pl. 2, fig. 1) and the spheroidal particles smaller (pl. 2, fig. 2), which suggests a general diffuse shrinkage of the structures. As shown in pl. 2, fig. 2, granular membranes have developed by tight agglutination of corpuscular debris of trabeculae. In other samples, clusters of particles seem to be coated with an additional, unidentified substance, which gives them an inflated appearance (pl. 3, fig. 3). Coarse networks of considerably flat ribbons of very large size and separated by an irregular fenestration (not shown) are out of proportion with the size and the shape of any shell organic matrix (see discussion).

DISCUSSION AND CONCLUSIONS

1. Consistent recording of networks of varicose trabeculae, occasionally fused into membranes, in samples decalcified in EDTA, fixed and decalcified in glutaraldehyde or by the CPC-EDTA method, suggests that these networks are not artifacts and can be considered as the representative ultrastructures of the altered organic matrices of the nacreous layers in the fossils selected in this study. A same profile of networks has been also observed in many other ammonoids and fossil nautiloids. These networks also characterize the ultrastructure of the organic matrices in the nacre of the modern *Nautilus* pyrolyzed experimentally without or under pressure (6, 8, 10).

2. BEHNCKE and ZELANDER (1), CRENSHAW and RISTEDT (3) consider glutaraldehyde as inadequate for preservation of acidic polysaccharides or of glycoproteins. The predominance, reported above, of fragments of trabeculae in samples decalcified in glutaraldehyde possibly indicates a fragility of the organic material greater than after treatment by EDTA.

3. The CPC method would preserve acidic polysaccharides (24, 3). Absence of additional structures in the present CPC material compared with the EDTA material may receive two explanations. The more probable is that acidic polysaccharides have disappeared from the samples during fossilization. In the other alternative, if still present, polysaccharidic substances could be diffusely incorporated within the trabeculae. Their elimination by EDTA would therefore not distinctly modify the ultrastructural appearance of these trabeculae.

4. SUNDSTRÖM and ZELANDER (21) recognized that decalcification of adult human enamel by aqueous solutions of chromium III sulphate is procedure-dependent. The effects of these solutions on the structures vary under the influence of several factors, such as pH, topography of the organic material in peripheral or central parts of the specimen and even nature of the support of the specimen in the solutions (for example, in specimens placed on gauge or on glass). As regards the problem of preservation of the original amount in organic material, SUNDSTRÖM and ZELANDER (21) consider the possibility of a definite loss of an acid-soluble fraction in enamel exposed to chromium sulphate. In molluscan shells, biochemical analyses of the organic remnants of decalcification of the *Nautilus* shell showed differences in amino acid composition between the proteins left after decalcification by EDTA and by chromium sulphate (15). The proteinic fraction left after decalcification in chromium sulphate solutions contained greater amounts of acidic amino acids. According to IWATA (15) these differences in amino acid composition presumably correspond to disappearance of a soluble protein during decalcification by EDTA. On the other hand, the perforations, which characterize the organic interlamellar membranes of nacre in the EDTA decalcified material, are absent in the remnants of decalcification by chromium sulphate. These membranes appear in the form of continuous sheets. For IWATA (14), this difference in structure is caused by dissolution by EDTA of intertrabecular substances filling the openings in the interlamellar membranes. IWATA considers these substances as the EDTA soluble fraction which would be fixed by chromium sulphate and then would remain in the intertrabecular areas. However, MUTVEI (16), in the interlamellar organic membranes of *Nautilus* nacre, GREGOIRE and MONTY (in GREGOIRE (11)) and GOFFINET, GREGOIRE and VOSS-FOUCART (12), in transverse sections of the same membranes, observed persistence of the intertrabecular membraneous bridges after treatment by EDTA. These divergent results suggest that the membraneous bridges cannot be safely identified to an EDTA soluble fraction.

In the present fossil samples treated with chromium sulphate, the organic remnants appear in the form of « networks » and of « membranes ». The representative trabeculae, which do not distinctly differ from those obtained after decalcification in EDTA, constitute the networks. Most of the membranes, which present some analogy with the « homogeneous material » of human enamel described by SUNDSTRÖM and ZELANDER (21) are produced by coalescence of trabeculae or by tight clustering of the pebble-shaped debris of trabeculae. These processes were also observed and formerly described in organic remnants of fossil nacre decalcified by EDTA (7, 9, 10). Other membranes seem to be precipitates produced by interactions between chromium and the calcium salts freed from the specimen. Finally, some salts also simulate arrangements into networks of small corpuscular or granular fragments. None of these miscellaneous structures, observed after use for decalcification of chro-

mium III sulphate, indicates preservation of specific structural elements which could have been lost during decalcification in EDTA. This suggestion needs biochemical confirmation.

A general shrinkage of the networks of trabeculae is also evident in certain samples. GOFFINET et al. (12) recently observed an identical shrinkage in ultrathin transverse sections of interlamellar networks of organic remnants from the nacre of the modern *Nautilus* decalcified in chromium III sulphate. In this material, a distinct increase in density was visible in the peripheral parts of the trabeculae and the intertrabecular spaces had disappeared or were considerably reduced by retraction or collapse of these trabeculae. As suggested by GOFFINET et al. (12), this general shrinkage might be caused by hardening (tanning) of the material. This suggestion is being checked on ultrathin sections of fossil conchiolin.

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EXPLANATION OF FIGURES

PLATE I

Figs, 1, 2, 3 and 4.

Stepheoceras sp. (Ammonite). Lower Jurassic, Lower Lias, Whitby, Yorkshire, England.

Fig. 1. The organic remnants after decalcification by EDTA of the nacreous layer of the shell wall consist of smooth, twisted, tortuous fragments of trabeculae, forming loose networks, mixed with spheroidal bodies produced by further dislocation of these trabeculae. Shadow-cast with platinum. × 48,000.

Fig. 2. The organic remnants of the nacreous layer of the shell wall, fixed and decalcified by the CPC method, do not differ in their ultrastructure from those shown in fig. 1. Shadow-cast with platinum. × 48,000.

Figs. 3 and 4. The organic remnants of the nacreous layer of the shell wall decalcified in chromium sulphate for 48 hours (fig. 3) and 25 days (fig. 4) consist, in this preparation, of thinly granular membranes, in which fragments of trabeculae identical to those shown in figs. 1 and 2 appear to be embedded, or are superposed (arrows) onto these membranes. Shadow-cast with platinum. Fig. 3 : × 60,000; fig. 4 : × 48,000.

PLATE II

Figs. 1 and 2. *Stepheoceras* sp. (see plate I). Organic remnants after decalcification by chromium sulphate (fig. 1 : 25 days; fig. 2 : 11 days) of the nacreous layer of the shell wall. In fig. 1, fragments of trabeculae appear identical to those shown in plate I, figs. 1 and 2, but they are considerably shrunk. In fig. 2, small corpuscles, produced by dislocation of the trabeculae, appear agglutinated into granular membranes. Figs. 1 and 2 : shadow-cast with platimum × 48,000.

Figs. 3 and 4. *Baculites claviformis* Stephenson (Ammonite) (393-14-998). Cretaceous, Senonian, Ripley formation, Mc Nairy Co, Tennessee, U. S. A. (U. S. G. S. 25406).

Figs. 3 and 4 show organic remnants of the nacreous layer of the shell wall after decalcification by EDTA (fig. 3) and chromium sulphate (fig. 4 : 6 days).

Fig. 3 shows a membrane of coalescence, in which fragments of trabeculae are embedded. Identical membranes enclosed in polygonal areas encircled by remnants of intercrystalline cords (arrows), appear shrunk (same magnification) (fig. 4). Shadow-cast with platinum. Figs. 3 and 4 : × 48,000.

Figs. 5 and 6. *Anthracoceras wanlessi* Plummer & Scott 1937 (Ammonite). Middle Pennsylvanian, Carbondale groups, Lewiston, Illinois, U. S. A.

Fig. 5. Organic remnants of mural nacre decalcified by EDTA. Fragmentation of trabeculae into small spheroidal bodies is one of the alterations consistently found among organic remnants of fossil shells of all ages. Shadow-cast with platinum. × 48,000.

Fig. 6. Organic remnants of mural nacre treated by the CPC method. Large rounded and lenticular particles and slabs, also frequently observed in material decalcified by EDTA alone, are mixed with fragments of trabeculae and particles of the same size as those shown in fig. 5. Shadow-cast with platinum. × 48,000.

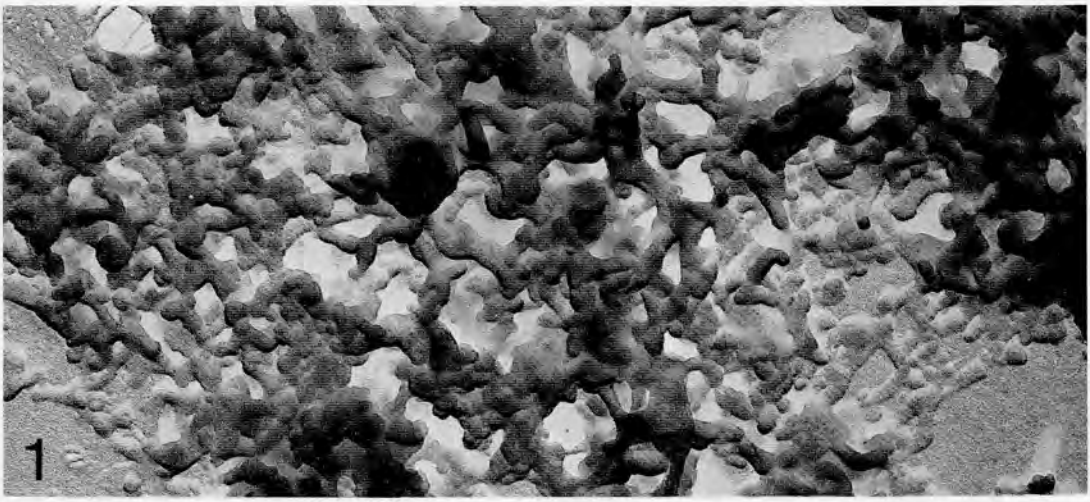
Plate III

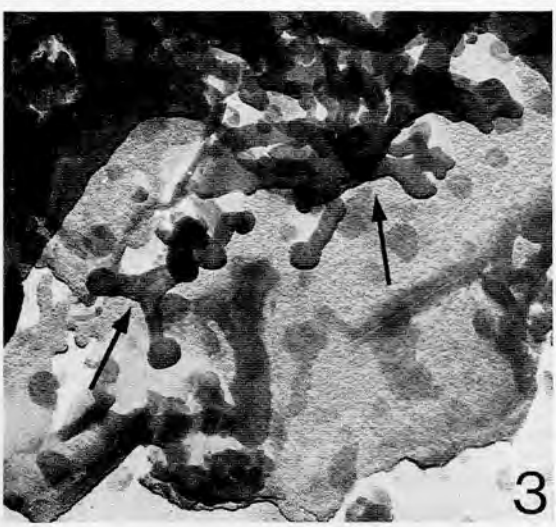
Harpoceras mulgravium Simpson, Young and Bird (Ammonite). Lower Jurassic, Lower Lias, Whitby, Yorkshire, England.

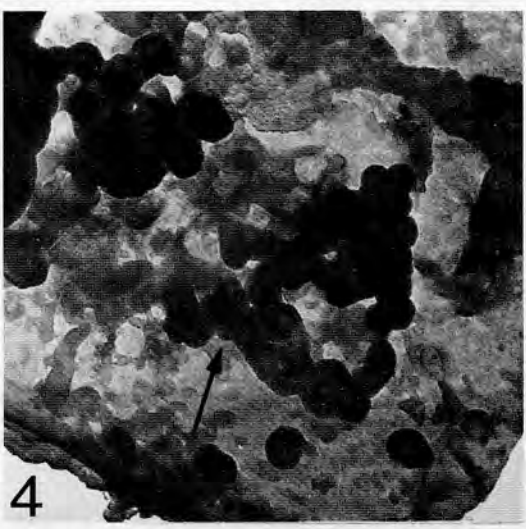
Fig. 1. Organic remnants of mural nacre decalcified by EDTA. Loose networks of trabeculae, some fragmented into rounded particles, are shown. Shadow-cast with platinum. × 48,000.

Fig. 2. Organic remnants of mural nacre after fixation and decalcification by the CPC method. The structure of the organic residues does not differ from that shown in fig. 1, especially in the left lower part of the figure. Shadow-cast with platinum. × 48,000.

Fig. 3. Organic remnants of mural nacre after decalcification for 5 days in chromium sulphate. The figure shows one of the heterogeneous aspects, in the form of coarse, clustered spheroidal structures, separated by an irregular fenestration of the organic residues left by this decalficier after 5 days (see the text). Shadow-cast with platinum. × 48,000.







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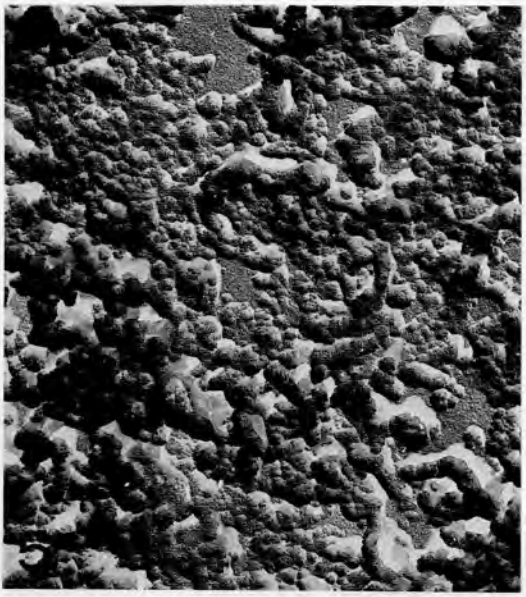

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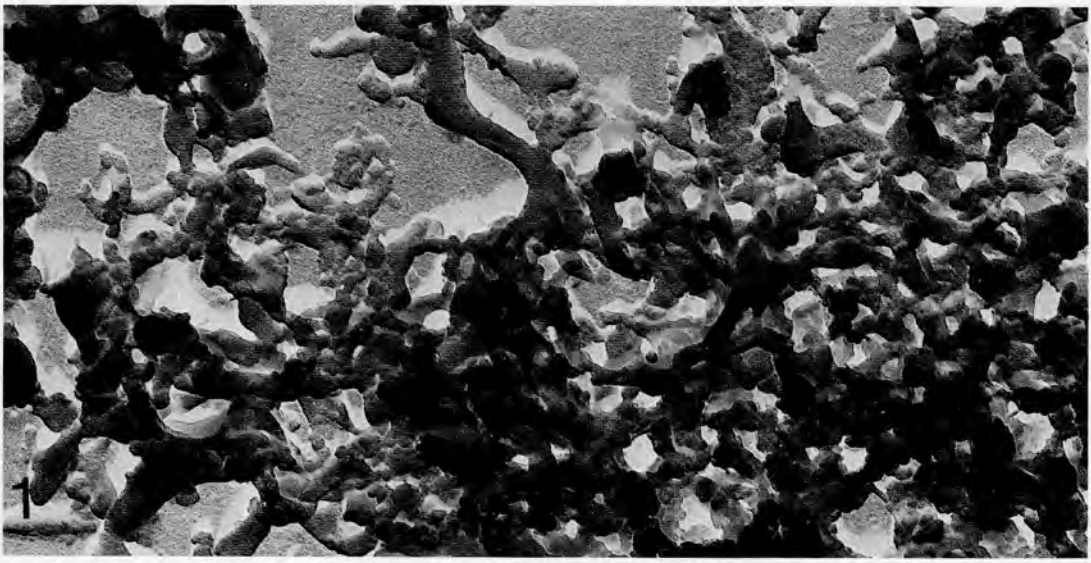

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