X-RAY DIFFRACTION STUDY OF THE INSOLUBLE ORGANIC MATRIX OF MOLLUSK SHELLS

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1. Introduction

Mollusk shells are generally composed of calcium carbonate in an organic matrix. The organic matrix is observed to form prior to mineralization [1,2], and it has been suggested that some of the matrix protein components may serve as a template for mineral deposition [3–6]. The calcium carbonate of the shell can be dissolved in EDTA leaving an insoluble fraction which comprises the bulk of the organic matrix [5,7,8] and consists of a heterogeneous mixture of proteins and carbohydrate [9–12]. X-ray diffraction patterns reported for various organic matrices [13–17] are quite poor in detail and, apart from suggestions that they may contain α- and β-keratin-type features [16,17], have not led to structural interpretations. We describe here a new X-ray fiber diffraction study of insoluble organic matrices of shells representing the three major classes of mollusks. This enabled us to establish that the ordered protein components in the insoluble fraction adopt an antiparallel β-sheet conformation and that, where recognizable, the polysaccharide phase is chitin. We have determined the structural parameters of these phases, and have also investigated the relative orientations of the protein, mineral crystals and, in some cases, the chitin for several shell layers.

2. Experimental

Shells of *Nautilus repertus* (Palau), *Mytilus californianus* and *Crassostrea irredescens* (Baja California, Mexico), *Crassostrea gigas* (Morro Bay, California), *Tectus dentatus* and *Pinctada radiata* (Gulf of Elat, Red Sea) were collected alive, air-dried and stored. Individual shell layers were separated, cleaned and decalcified in 10% EDTA in phosphate buffer (pH 7) containing 0.1% sodium azide. In some cases fixatives were added to the decalcifying solution, but this was found not to affect the X-ray results. The two fixatives used were 0.25% glutaraldehyde and 4% formaldehyde, 0.5% cetylpyridinium chloride [18]. These were added with the aim of stabilizing the soluble components of the organic matrix and investigating whether these had an ordered structure distinct from that of the insoluble components. After decalcification, the salts were removed by exhaustive washing with distilled water. The organic matrix samples were carefully air-dried on glass slides so as to preserve as well as possible the geometric configuration of the microstructural units.

A few specimens were prepared from *N. repertus* nacreous layer for which the protein was removed from the chitin phase by boiling in 5% aqueous KOH for 24 h, followed by dipping in 3 N HCl and thorough washing with distilled water [19]. X-ray photographs of the organic matrices were taken at room humidity with Cu Kα radiation on a Norelco Chesley microcamera or on a Searle Franks double-mirror camera. X-ray photographs of the mineral phase were taken on the microcamera and on a precession camera with either Cu Kα or Mo Kα radiation.

3. Results

X-ray diffraction patterns were obtained from samples of 10 organic matrices, which differed taxonomically in shell micro-architecture and mineralogy. Although, they varied greatly in quality, the patterns showed similar features and can be conveniently

divided into two categories. The first (type 1) is exemplified by the matrix of the nacreous layer from the septum of *N. repertus* and the second (type 2) by the prismatic layer of *M. californianus*. The *N. repertus* matrix is exceptionally well oriented and gave 3 distinct X-ray patterns when photographed with the X-ray beam in 3 orthogonal directions, one perpendicular to, and two in the plane of the film-like specimen. One of these views is shown in fig.1(a) and the main X-ray spacings are listed in table 1. These include a subset of reflections which corresponds to the β-chitin pattern obtained from the pen of the squid *Loligo* [20], which has been interpreted as a hydrated form [19]. This was confirmed by the observation of only the β-chitin pattern in samples of matrix from which the protein had been extracted. The chitin fiber axis (*b*-axis in table 1(a)) lies along a well defined direction within the plane of the specimen, that is parallel to the interlamellar matrices. It also lies along the direction of bilateral symmetry of the animal suggesting that the molecular components X-rayed are still in their in vivo positions. There is however essentially random orientation of the chitin structure about this axis, as in photographs taken with the X-ray beam along the direction of the *b*-axis (orientation (3) in table 1) the equatorial reflections in fig.1(a) do not break up into *a*-axis and *c*-axis components, but appear as rings.

The *N. repertus* reflections, which do not belong to the β-chitin pattern, have spacings and orientations consistent with an orthogonal unit cell with $a = 9.5$ Å, $b = 6.9$ Å and $c = 15.0$ Å (cf. table 1(b)). These cell dimensions and the indices of the most intense reflections, including 002, 201 and 211, are strongly suggestive of the type of β-pleated-sheet structure that occurs in many silk-fibroins and related polypeptides [21,23] that is with antiparallel polypeptide chains and staggering of the β-sheets along the *a* direction so that rows of side-chains in adjacent sheets interweave each other. The orientation of the protein reflections (table 1(b)) indicate that this structure has a unique orientation in 3 dimensions with the polypeptide-chain direction (*b*-axis) lying in the plane of the specimen, but perpendicular to the chitin fiber axis, and the side chain direction (*c*-axis) perpendicular to the plane of the specimen and hence to the interlamellar matrices (fig.2).

The X-ray pattern obtained from the *M. californianus* prismatic layer (fig.1(b)) is also consistent with a silk-fibroin-like β-pleated-sheet structure with the same *a* and *b* axial length as in *N. repertus* but a shorter *c* axis (table 1(c)). We have not observed any

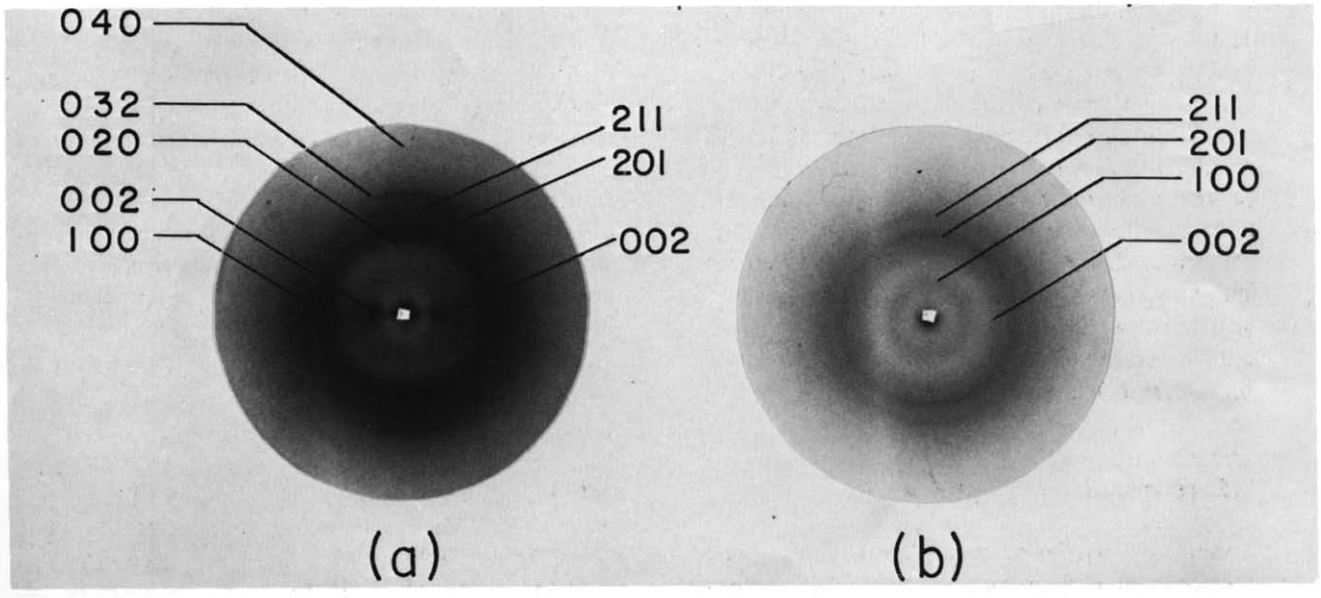

Fig.1. X-ray diffraction photographs of insoluble organic matrix from (a) *N. repertus* nacreous layer, (b) *M. californianus* prismatic layer. Both specimens were in orientation (2) and indices of the strongest refections are shown; chitin by arrows from the left and protein by arrows from the right (cf. table 1).

Table 1
Prominent reflections in X-ray patterns of organic matrices from *N. repertus* nacreous and *M. californianus* prismatic layers: (a) *N. repertus*, chitin; (b) *N. repertus*, protein; (c) *M. californianus*, protein

	I_o	Orientation			d_o(Å)	*hkl*	d_c(Å)
		1	2	3			
(a)	vs	E	E	–	11.5 (broad)	002	
	m	M	M	–	5.14	020	
	vs	E	E	–	4.6 (broad)	100	
						102	
	s	M	M	–	3.33	032	
	mw	M	M	–	2.58	040	
(b)	mw	–	E	E	7.53	002	7.50
	s	M	M	–	4.53	201	4.53
	m	M	M	M	3.79	211	3.79
	w	E	M	M	2.24	130	2.24
	w	–	M	M	2.05	230	2.07
(c)	mw	–	–	–	9.5 (broad)	100	9.50
	mw	–	E	E	5.50	002	5.50
	vs	–	M	M	4.39	201	4.36
	ms	–	M	M	3.70	211	3.68
	w	–	E	E	3.04	113	3.06
	w	–	M	M	2.25	130	2.24
	w	–	–	–	2.05	230	2.07

Observed intensities (I_o) were estimated as very strong (vs), strong (s), moderately strong (ms), medium (m), moderately weak (mw) or weak (w) and are listed for orientation (2). Observed orientations are given for the X-ray beam in three orthogonal directions (1) perpendicular to and (2) and (3) in the plane of the specimen. They are listed as equatorial (E), meridional (M), or (–) where for various reasons they were not clearly observed. Observed spacings (d_o) can be compared with those calculated (d_c) for the protein structures for orthogonal unit cells with $a = 9.5$ Å, $b = 6.9$ Å, $c = 15.0$ Å for *N. repertus* and $a = 9.5$ Å, $b = 6.9$ Å, $c = 11.0$ Å for *M. californianus*. Indices (*hkl*) are assigned to the protein reflections on the basis of these cells and for the chitin reflections in accordance with those proposed for β-chitin B hydrates [19]

chitin features in this pattern, nor even from the small amount of residue obtained from the matrix after removal of the protein. The *M. californianus* prismatic layer matrix showed only partial orientation indicating that the β-pleated sheets are randomly oriented in the plane of the specimen with the side chains (*c*-axis) perpendicular to this plane and therefore perpendicular to the long axis of the prisms.

We have obtained only a few preliminary X-ray photographs from organic matrices of other mollusk shells, but these generally resemble the two described above and presumably imply similar structures. *M. californianus* and *P. radiata* nacreous layers and *C. irredescens* foliated layer gave patterns very similar in spacing and intensity distribution to that of *M. californianus* prismatic layer (type 2), with no evidence of chitin. By contrast, *T. dentatus* prismatic layer shows a pattern dominated by chitin features with little or no contribution from the protein phase, whereas *T. dentatus* nacreous layer shows both protein and chitin features and resembles a poorly oriented pattern of *N. repertus* nacreous layer (type 1). A somewhat similar radical intensity distribution was observed in photographs of *N. repertus* and *P. radiata* prismatic and *C. gigas* foliated layers, but these 3 patterns, consisting essentially of two broad rings, are

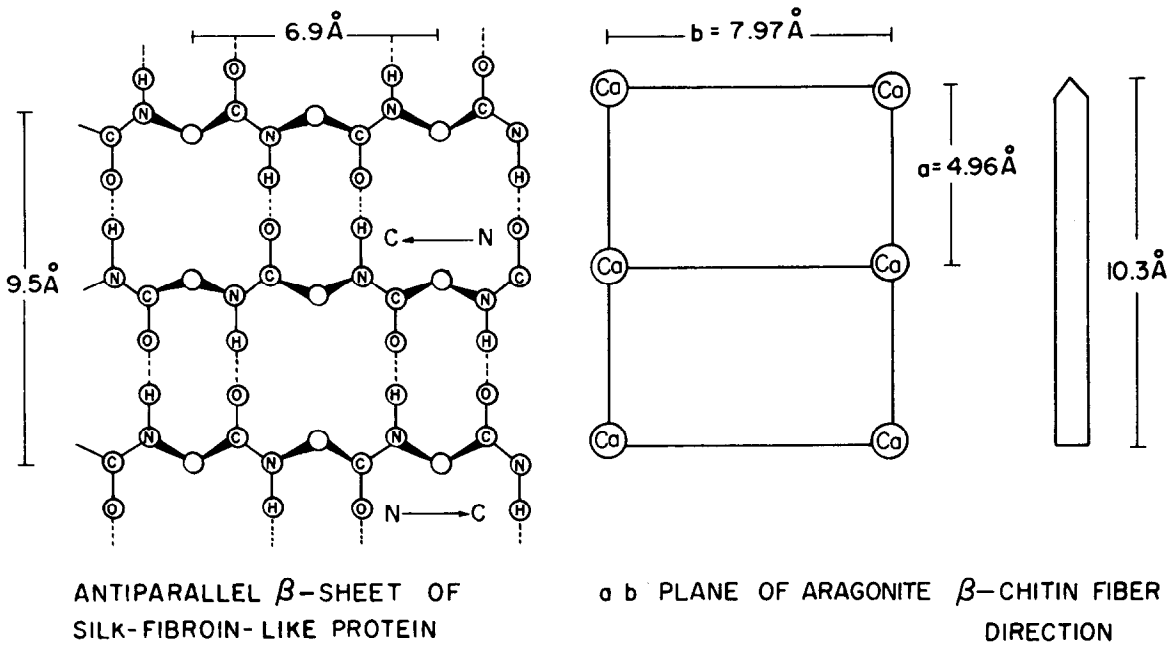

Fig.2. Schematic representation of the spatial relationships between protein sheets aragonite crystals and chitin fibers in the nacreous layer of *N. repertus*.

too diffuse and unoriented for clear identification. Table 2 summarises the results we obtained from the various specimens.

By studying incompletely decalcified *N. repertus* nacreous layer we have been able to observe the orientation of the aragonite crystals in relation to the structure of the organic matrix. The 5 Å *a*-axis of the aragonite orthorhombic cell is well lined up with the chitin *b*-axis (protein *a*-axis) and the *b*- and *c*-axes of aragonite lie along the *b*- and *c*-axes of the protein, respectively (fig.2). Thus there is a well-defined spatial relationship between the orientations of the protein, chitin and aragonite. The orientations of the crystallographic axes of the other nacreous-layer aragonites were also compared to their associated insoluble organic matrices (table 2). In all 3 cases the *c*-axes of both the protein β-sheet structure and of the aragonite are oriented perpendicular to the nacreous interlamellar matrices. In *T. dentatus* and *M. californianus* the *a*- and *b*-axes of the aragonite and also of associated matrix proteins are randomly or very poorly oriented. However, in *P. radiata* the aragonite *a*- and *b*-axes are well oriented, but we have observed no preferred in plane orientations for the corresponding axes of the protein structure.

4. Discussion

Our X-ray diffraction results are consistent with those of studies using other techniques. Infrared spectra of insoluble matrices indicate β-sheet structures, probably in an antiparallel conformation [24], and the amino acid compositions of insoluble shell matrices resemble those of silk fibroins [25] in their content of glycine, alanine and serine, which together typically constitute >50% of the total [26]. The axial orientations we have observed in nacreous layer aragonite crystals are essentially in agreement with the results of previous diffraction studies [27,28].

Chitin is present in some mollusk shells, but absent in others, as determined by the chitosan test [29] and confirmed with chitinases [30], and there is evidence suggestive of covalent binding between chitin and protein in several types of tissues [20,31]. Such binding or some strong non-covalent interactions between chitin and protein could explain the fixed relative orientations of these two components in the nacreous layer of *N. repertus* (fig.2). However, we have not observed any of the long periodicities, suggestive of an intimate structural complex of chitin and protein, that have been reported for several

Table 2
Comparison of shell layer ultrastructures, mineralogies and protein conformations of the species studied

Species	Shell layer ultrastructure	Mineralogy	Protein conformation
Nautilus repertus (Cephalopoda)	Nacreous (septum)	Aragonite (a,b moderately oriented, c well oriented)	β-Sheet type 1[a] (a,b,c oriented)
	Prismatic	Aragonite	Unoriented diffuse pattern
Tectus dentatus (Gastropoda)	Nacreous	Aragonite (a,b random in plane, c well oriented)	β-Sheet type 1[a] (a,b random in plane, c oriented)
	Prismatic	Aragonite	?[a] (Protein produced no detectable reflections)
Mytilus californianus (Bivalvia)	Nacreous	Aragonite (a,b poorly oriented, c well oriented)	β-Sheet type 2 (a,b random in plane, c oriented)
	Prismatic	Calcite	β-Sheet type 2 (a,b random in plane, c oriented)
Pinctada radiata (Bivalvia)	Nacreous	Aragonite (a,b,c well oriented)	β-Sheet type 2 (a,b random in plane, c oriented)
	Prismatic	Calcite	Unoriented diffuse pattern
Crassostrea irredescens (Bivalvia)	Foliated	Calcite	β-Sheet type 2
Crassostrea gigas (Bivalvia)	Foliated	Calcite	Unoriented diffuse pattern

[a] Oriented chitin structure observed

other systems [20]. On the contrary, the different kinds of orientation which we have observed for chitin and protein definitely preclude any cocrystallization in a mixed lattice. Our results, in fact, fit very well with a picture of chitin fibrils lined up in a definite direction on a protein surface, as has been visualised in an electron micrograph study of alkali- and chitinase-susceptible components of the nacreous layer of *Nautilus macromphalus* [30].

The silk-fibroin like protein, in some cases associated with chitin, may provide the structural basis for the elaboration of the organic matrix framework around which the mineral is deposited. After shell formation is complete, it probably contributes to the mechanical properties of the shell itself [32]. The definite spatial relationship on a molecular level between organic and mineral components, as observed in *N. repertus* nacreous layer is consistent with the notion that the ordered 'structural framework' within this insoluble fraction in some way directs mineral crystal formation. Further work is required to determine whether the spatial relationships observed here extend to the organic matrix–mineral interfaces before more definite conclusions can be drawn about the role of the insoluble organic matrix in mineral formation.

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