TISSUE AND CELL, 1992 24 (5) 735–744

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FINE STRUCTURE OF THE CHORION OF *MANDUCA SEXTA* AND *SESAMIA NONAGRIOIDES* AS REVEALED BY SCANNING ELECTRON MICROSCOPY AND FREEZE-FRACTURING

Keywords: *Manduca sexta, Sesamia nonagrioides*, chorion, scanning electron microscopy, freeze-fracturing, helicoidal architecture, proteinaceous fibrils

ABSTRACT. The fine structure of *Manduca sexta* and *Sesamia nonagrioides* chorion was investigated by scanning electron microscopy and freeze-fracturing. In both species the mature chorion exhibits a complex ultrastructure on its outer surface, with a large number of aeropyles forming polygonal arrays. The micropyle is surrounded by a rosette of approximately 80 follicular cell imprints. Scanning electron microscopy of vertically ripped sections reveals that both chorions consist of two main layers: a trabecular layer closest to the oocyte and a lamellar layer. The technique of freeze-fracturing, utilizing single-sided and rotary shadowing, clearly shows that fibrils, approximately 3–4 nm in diameter, constitute chorionic lamellae in both species. The fibrils appear to have a 'beaded' structure, with a 2–3 nm axial periodicity. Freeze-fracturing also provides a direct visualization of the helicoidal arrangement of these fibrils for the formation of chorion supramolecular architecture.

Introduction

The silkmoth chorion, the major component of the eggshell, is a complex extracellular proteinaceous structure formed by the follicle cells which surround the oocyte. Its constituent proteins are predominantly organized as fibres embedded in a matrix (Smith *et al.*, 1971, Kafatos *et al.*, 1977; Papanicolaou *et al.*, 1986) which suggests analogies of chorion with vertebrate keratins and other fibre-matrix systems (Hamodrakas, 1984; Hamodrakas *et al.*, 1982a; 1982b; 1984; 1985; Regier *et al.*, 1983). The chorion consists of fibrous layers parallel to its surface. Between adjacent layers the direction of the fibres differs by a constant angle resulting in a helicoidal structure (Bouligand, 1972), a biological analogue of a cholesteric liquid crystal (Mazur *et al.*, 1982). The structure changes dramatically during morphogenesis and also varies locally, consistent with the biochemical complexity and the multiple physiological functions of the egg-shell (Kafatos *et al.*, 1977). Application of the technique of freeze-fracturing with single-sided and rotary shadowing and X-ray diffraction studies, revealed that silkmoth chorion consists of fibrils (also termed filaments hereafter), approximately 3 nm in diameter, and provided a direct visualization of the helicoidal arrangement of these filaments for the formation of chorion architecture (Hamodrakas *et al.*, 1986). The packing of the filaments as seen from freeze-fracturing is in good agreement with X-ray

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Received 9 December 1991.
Revised 25 June 1992.

data (Hamodrakas *et al.*, 1983; Hamodrakas *et al.*, 1986). These also suggest the prevalence of β-sheet structure for chorion proteins, in conjunction with evidence from laser-Raman and infrared spectroscopy studies (Hamodrakas *et al.*, 1982b; 1984; 1987) and analysis of chorion protein amino-acid sequences (Hamodrakas *et al.*, 1982a; 1985; 1988).

It has been proposed that the twisted anti-parallel β-pleated sheet is the molecular conformation which dictates the formation of the helicoidal architecture in proteinaceous eggshells (Hamodrakas, 1984; Hamodrakas *et al.*, 1988). This proposal was mainly based on data obtained from the silkmoth eggshell. However, there is still limited experimental evidence concerning this hypothesis (see, for example, Hamodrakas *et al.*, 1987).

In this report, we present data for scanning electron microscopy (SEM) and freeze-fracturing studies of proteinaceous chorions from two other Leipdopteran species, *Manduca sexta* and *Sesamia nonagrioides*, which: (a) confirm their lamellar organization, (b) provide a direct experimental visualization of chorion helicoidal architecture, and, (c) reveal that fibrils, approximately 3–4 nm in diameter, are the basic structural elements as in the case of silkmoth chorion (Hamodrakas *et al.*, 1986).

Materials and Methods

Preparation of purified chorions

Mature and ovulated follicles were dissected in Ringer's solution from female *Manduca sexta* and *Sesamia nonagrioides* adult insects. Follicles were cut in half with fine forceps and cleaned ultrasonically in 95% and 100% ethanol followed by distilled water, so that swollen epithelial cells and the vitelline membrane were peeled off the underlying and overlying chorion surface respectively. Chorions were selected under a dissecting microsope and air-dried.

Scanning electron microscopy

Samples of *Manduca sexta* and *Sesamia nonagrioides* chorions were prepared for scanning electron microscopy, utilizing preparative techniques described in detail by Margaritis *et al.*, 1980. A JEOL 840 scanning electron microscope, operating at 15 kV, was used.

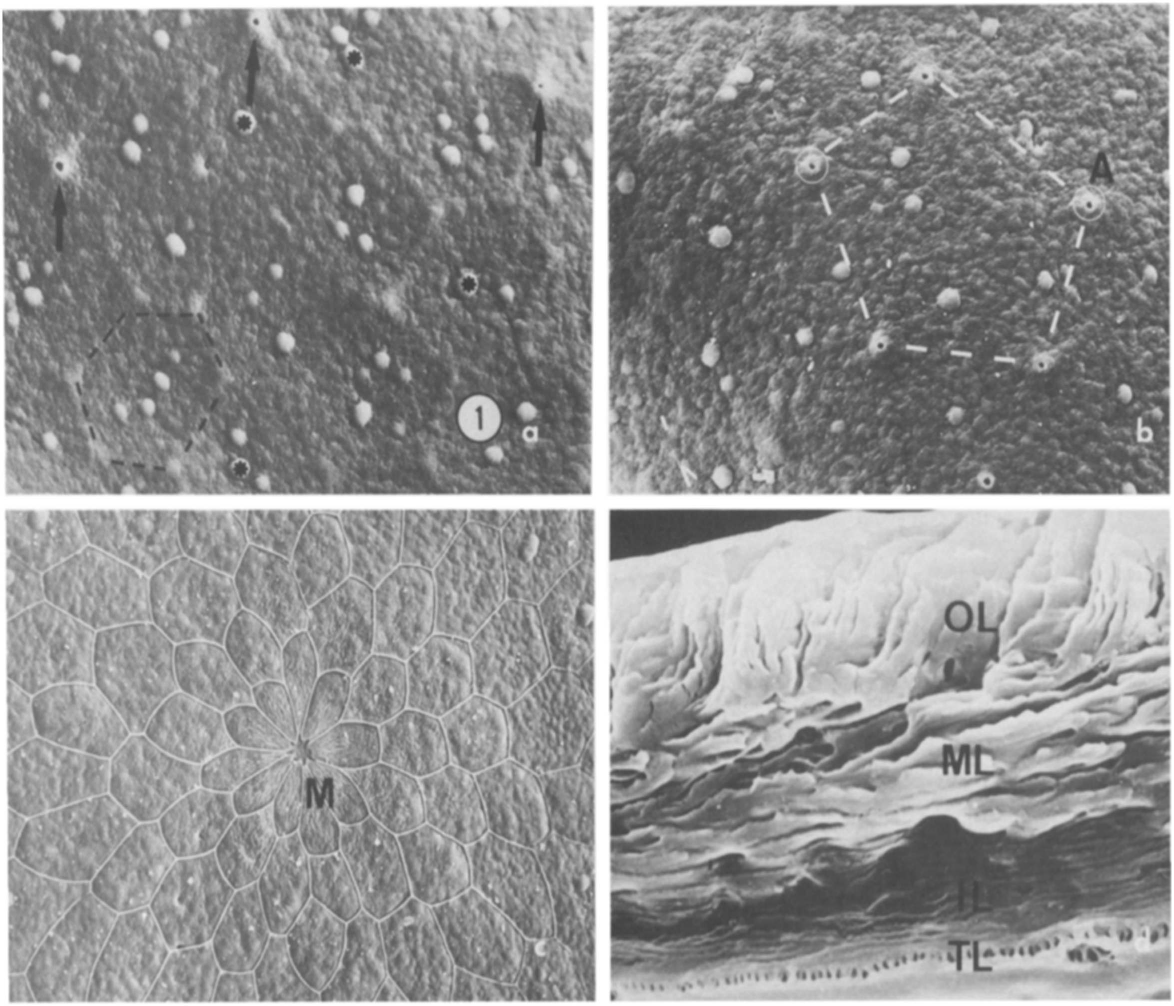
Freeze-fracture electron microscopy

Purified chorions were cut into small pieces in distilled water and deposited on thin copper holders which were then rapidly quenched in liquid propane. The samples were fractured at $-125°C$ with a liquid nitrogen cooled knife in vacuum greater than 10^{-6} Torr and replicated in a Balzers 301 freeze-etching unit. Freeze-etching was performed for *Sesamia nonagrioides* samples for 4 min. Half of the specimens were shadowed unidirectionally and the remainder were rotary shadowed (Margaritis *et al.*, 1977).

Metal evaporation was performed with electron bombardment guns using Pt-C electrodes. The Pt gun was set at a 35° angle to the specimen table surface, both for unidirectional and for rotary shadowing. The C gun was set directly above the specimen

Fig. 1. (a) View of *Manduca sexta* chorion surface. The follicle cell 'imprints' are marked by wide polygonal ridges (dotted lines) corresponding to the intercellular regions of the follicle cells. several knobs (*) are found within each 'imprint'. Aeropyles (arrows) are seen at the corners of polygons. (b) Aeropyles (A) are found at three-cell junctions. They are arranged in polygonal formations (dotted lines). (c) View of the micropyle (M), which is surrounded by approximately 80 follicle cell imprints. It is easily recognized by its fine ridges and the concentric organisation and elongated shape of the cell imprints. The micropyle area is devoid of aeropyles. (d) Section vertical to the surface of a *Manduca sexta* mature chorion, which shows lamellae lying above a thin trabecular layer (TL). The uniform in thickness lamellae of the inner zone (IL) are oriented parallel to the outer surfaces and to the oocyte. The middle zone (ML) consists of thicker and less orderly lamellae, whereas lamellae of the outer zone (OL) are oblique to the rest and to the chorion surface. Fig. 1a ×650; Fig. 1b ×700; Fig. 1c ×580; Fig. 1d × 5600.

Fig. 2. (a) Section vertical to the surface of a mature *Sesamia nonagrioides* chorion showing lamellae (L) lying above a rather thick trabecular layer (TL). The lamellae are oriented parallel to the outer surface of chorion. (b) Section vertical to the surface of a mature *Sesamia nonagrioides* chorion in the micropyle (M) area. The chorion consists of lamellae parallel to the outer surface and is devoid of a trabecular layer. Fig. 2a × 7600; Fig. 2b ×5100.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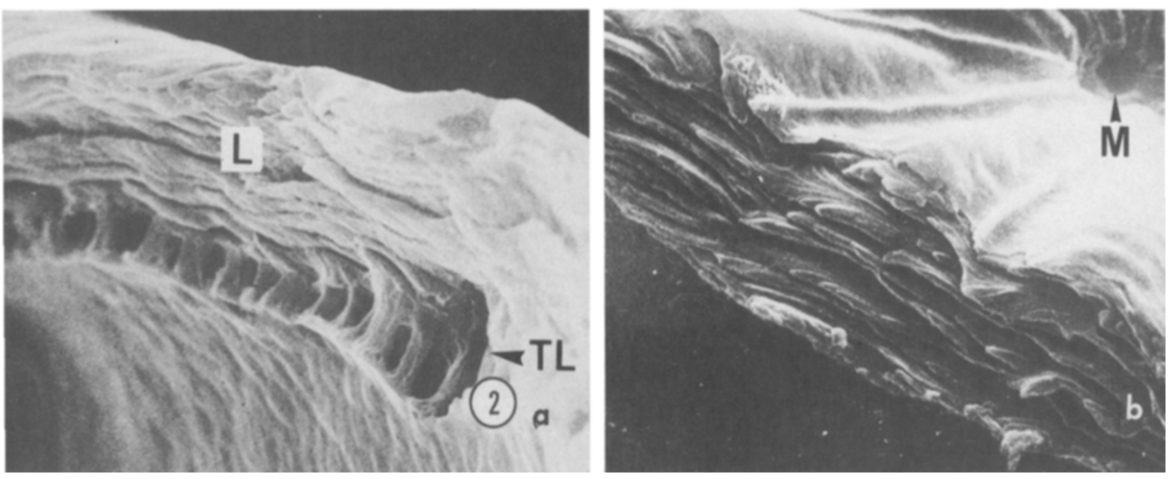
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table. The replicas were cleaned with sodium hypochlorite for 30 min, followed by chromic acid overnight for some specimens and, after distilled water washing, the were picked up on 400-mesh electron microscope grids.

Electron microassay was performed with a Philips EM301 microscope, operating at 60 or 80 kV.

All electron micrographs for freeze-fracturing are positive images, i.e. platinum deposits appear dark.

Results

Scanning electron microscopy

Manduca Sexta. A purified mature chorion has the shape of an ellipsoid of the 'flat' type (Fehrenbach *et al.*, 1987). The main axes of the ellipsoid are approximately 2·1, 1·6 and 1·6 mm in length (data not shown). A prominent feature of chorion's outer surface is a polygonal network of ridges (Fig. 1a). The ridges correspond to the edges of the follicular cells which secrete chorion. They are formed by overproduction of chorionic proteins in the intercellular spaces (Kafatos *et al.*, 1977). Each polygon corresponds to the overlying secretory cell—it is a follicular cell 'imprint'—and each ridge to a two-cell junction. 'Knobs', varying in number from one to four, are found in the center of each polygon. At ridge corners, aeropyles are found (Fig. 1b). These are round openings, 1·5 μm in diameter, leading to internal radial air-channels; they correspond to three-cell junctions and are arranged in polygonal groups at periodic distances of approximately 30 μm. The micropyle, through which sperm entry occurs, is discerned by its fine ridges and the concentric organization and elongated shape of the follicle cell imprints (Fig. 1c). Its external opening, 4·5 μm in diameter, is surrounded by a rosette of approximately 80 petal-shaped cell imprints. The micropylar rosette is continuous with the polygonal network of ridges and is devoid of aeropyles.

The first 'signs' of a helicoidal architecture are lamellae lying above a thin (0·3 μm) trabecular layer (TL), the first chorionic layer formed during choriogenesis (Fig. 1d). The lamellar layer can be divided into three zones: an inner zone (IL) (1·8 μm) in which thin lamellae are oriented parallel to the oocyte, a middle zone (ML) (2·2 μM), which consists of thicker and less orderly arranged lamellae and an outer zone (OL) (3·4 μm), which containes lamellae oblique to the rest and to the chorion surface. Transmission electron microscopy studies indicate that lamellae consist of protein fibres arranged helicoidally (Regier and Vlahos, 1988).

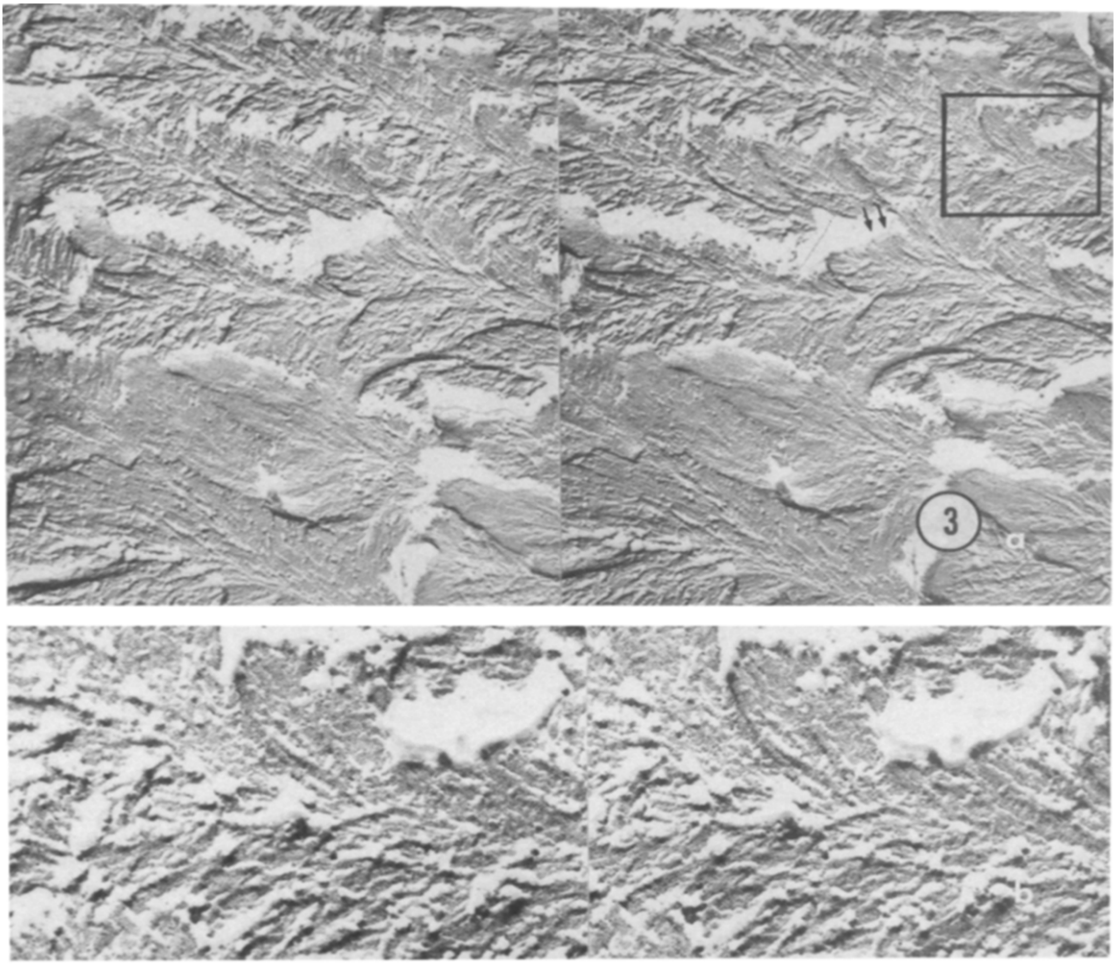
The thickness of chorion increases during choriogenesis up to 8·0 μm. However, near the end of choriogenesis it decreases slightly to 7·2 μm, in agreement with data obtained from transmission electron microscopy studies, due to a process termed compaction (Regier and Vlahos, 1988).

Sesamia nonagrioides. Details of the outer surface of *Sesamia nonagrioides* chorion have been described by Pucci and Forcina, 1984.

Scanning electron micrographs of vertically to the surface ripped sections of a mature chorion (Figs 2a, 2b) reveal that chorion consists of a few lamellae only, oriented parallel to its outer surface and to the oocyte, lying above a rather thick (1·5 μm) trabecular layer (Fig. 2a). The trabecular layer (TL) represents almost one-third of the overall

Fig. 3. (a) Stereoscopic view of a Pt/C replica of a freeze-fracture plane within the chorion of *Manduca sexta* (unidirectional shadowing). The fracture has advanced across successive lamellae producing a series of steps, revealing the helicoidal arrangement of its constituent proteinaceous fibrils, 3–4 nm in diameter (arrows). The apparent beading of the fibrils, probably suggests a helical fibrillar structure (see results and discussion sections). The angle between successive layers of fibrils, defining the helicoidal architecture is approximately 3°. (b) Higher magnification view of the boxed area of Figure 3a. Fig. 3a ×54,000; Fig. 3b ×135,000.

Fig. 4. (a) Stereoscopic view of a Pt/C replica of a freeze-fracture plane within the chorion of *Sesamia nonagrioides* (unidirectional shadowing) allows for direct visualization of the helicoidal pattern of architecture of its constituent fibrils (arrows). The main bulk of the chorion and also the trabecular layer (TL) can be seen. (b) At higher magnification, on apparent 2–3 nm beading of the 3–4 nm in diameter chorion fibrils is seen. Fig. 4a ×11,000; Fig. 4b × 77,000.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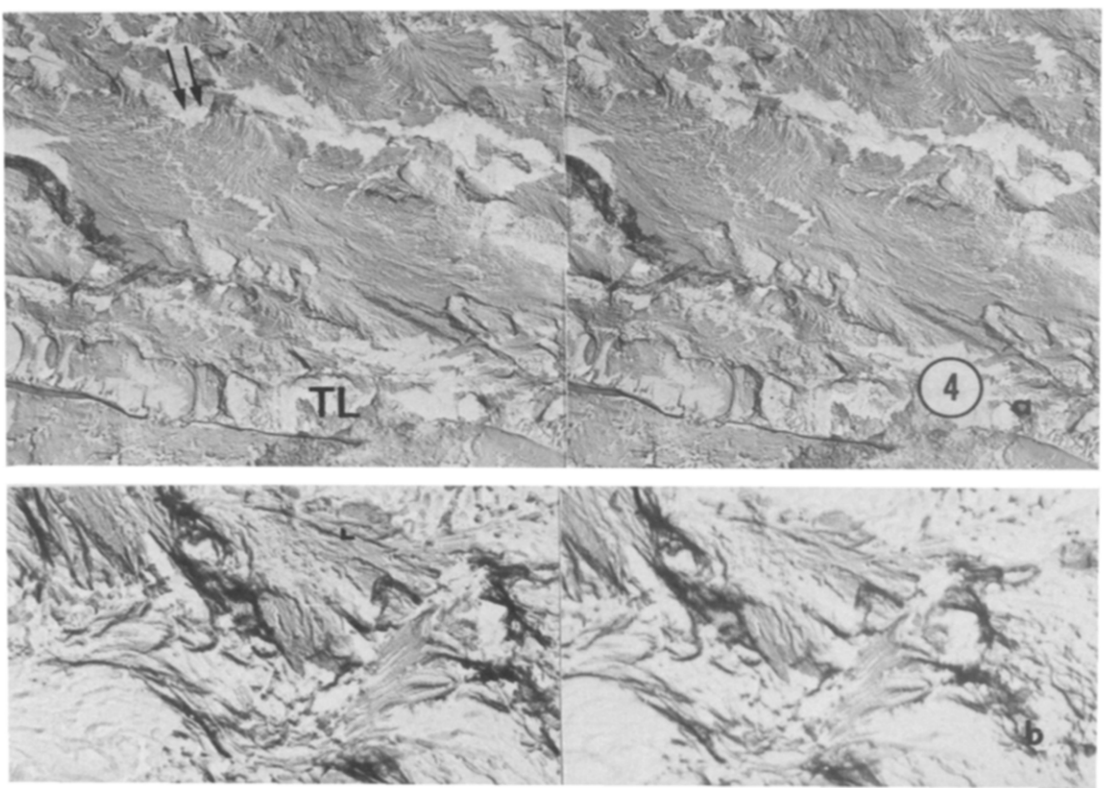
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thickness (5 μm) of the mature chorion. The micropylar area is devoid of a trabecular layer (Fig. 2b).

Freeze-Fracturing

Application of the freeze-fracturing technique both on *Manduca sexta* and *Sesamia nonagrioides* chorions reveals details of their fibrillar organization and their lamellar, helicoidal ultrastructure.

In unidirectionally shadowed replicas of *Manduca sexta* and *Sesamia nonagrioides* chorion, the helicoidal pattern of architecture of its constituent fibrils is directly visualized (Figs 3a, b, 4a, b, respectively). Fibrils are approximately 3–4 nm in diameter, as calculated from transverse (circles) and longitudinal (arrows) views (figs 3b, 4b). They appear to have a 'beaded' substructure along their long axis with an axial periodicity of approximately 2–3 nm. This substructure might imply a helical organization of their constituent protein molecule(s) (see, for example, Aebi *et al.*, 1983).

In rotary shadowed replicas of *Manduca sexta* and *Sesamia nonagrioides* chorions, the fracture has advanced across successive lamellae, producing a series of steps, which reveals the lamellar structure of chorion (Figs 5a, b, 6a, b, respectively). It can be seen that the fibrils are either straight or slightly curved. Transverse fracturing of the fibrils shows that they have diameters of approximately 3–4 nm (Figs 5b, 6b). Their packing arrangement results in periodicities of the same order of magnitude. Chorion fibrils observed longitudinally, show a fine (2–3 nm) periodical substructure (beading) along their long axis (Figs 5b, 6b). Details of the helicoidal architecture of chorion are also seen (Figs 5, 6).

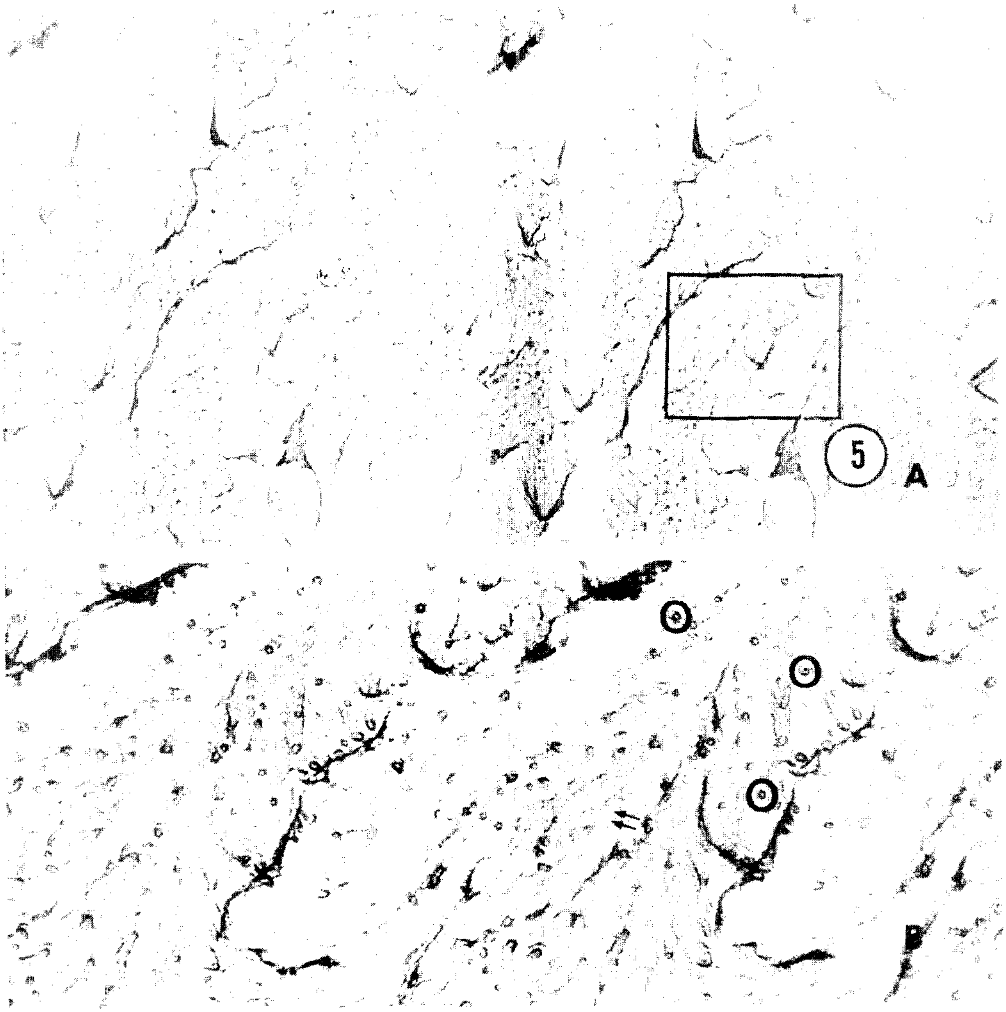
Discussion

Our work has been focused in attempts to identify the basic structural elements of the lepidopteran *Manduca sexta* and *Sesamia nonagrioides* proteinaceous chorions and their packing modes and, at the same time, to investigate their gross morphological features, making useful comparisons with the thoroughly studied system of silkmoth chorion (Kafatos *et al.*, 1977).

The outer surface of chorion in both species, shows structural elements and regional differentiation typical of lepidoptera (Kafatos *et al.*, 1977; Papanicolaou *et al.*, 1986; Fehrenbach *et al.*, 1987 and references therein). Apparently, the micropyle is similar in diameter in both species. For *Sesamia nonagrioides*, the network of ridges as well as the aeropyles appear to be absent in the micropyle area (Pucci *et al.*, 1984). Surprisingly, the aeropyles have the same diameter as those of *Manduca sexta* and are found only at the protrusions of the sculptured surface (Pucci and Forcina, 1984). Their arrangement indicates that they correspond to three-cell junctions (Sakaguchi *et al.*, 1973) and are formed by overproduction of chorionic proteins (Mazur *et al.*, 1980). Furthermore, the existence of aeropyles suggests that these openings connect the exterior of the egg with the trabecular layer (Barbier and Chauvin, 1974) and function like the respiratory plate of aquatic insect eggshells (Magaritis, 1985; Hinton, 1969; 1970; 1981; Wigglesworth and Beament, 1950). The

Fig. 5. (a) Stereoscopic view of a Pt/C replica of a freeze-fracture plane within the chorion of *Manduca sexta* (rotary shadowing). The fracture has advanced across successive lamellae producing a series of steps, revealing the lamellar structure of chorion and the helicoidal arrangement of its constituent fibrils. Fibrils are either straight or slightly curved and exhibit a beaded substructure. (b) At higher magnification, transverse fracturing of the fibrils (circles) reveals that they are approximately 3–4 nm in diameter. Fibrils observed longitudinally (arrows) exhibit a fine (2–3 nm) periodical substructure (beading) along their long axis. Fig. 5a ×44,000; Fig. 5b ×14,000.

Fig. 6. (a) Stereoscopic view of a Pt/C replica of a freeze-fracture plane within the chorion of *Sesamia nonagrioides* (rotary shadowing) showing clearly the helicoidal packing modes of its constituent beaded fibrils. (b) At higher magnification, 3–4 nm in diameter, chorion fibrils are either transversely (circles) or longitudinally (arrows) fractured. A periodic (2–3 nm) substructure (beading) can be discerned along the long axis of fibrils. Fig. 6a ×54,000; Fig. 6b ×100,000.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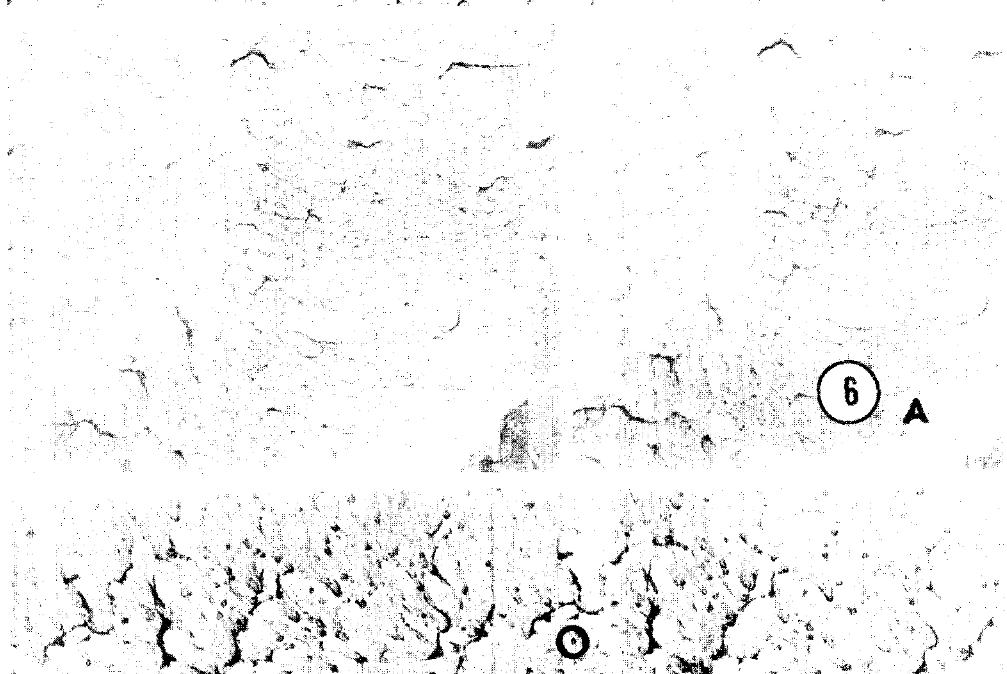
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absence of aeropyles in the micropyle area might be related to the absence of the trabecular layer in the same area. The latter is adapted for distributing air from the aeropyle channels over the entire surface of the underlying oocyte (Margaritis, 1985; Hinton, 1969).

In the Lepidoptera so far examined, the mechanisms operating during chorion morphogenesis, apparently share extensive similarities, as can be judged from the chorion surface and radial fine structure and their developmental changes (Kafatos *et al.*, 1977; Papanicolaou *et al.*, 1986; Fehrenbach *et al.*, 1987 and references therein; Regier and Vlahos, 1988; this work). Local variations presumably reflect inter-specific physiological needs and/or different local morphogenetic modes and protein chemistry. Typical examples of species-specific variations are the variable orientation of lamellae relative to the oocyte surface and their texture (for an exact definition of the term texture, see Bouligand, 1975) and the non-uniform thickness of the trabecular layer. The former, apparently related to the essential function of chorion of providing mechanical strength to external pressures, is exemplified by the variable orientation and texture of lamellae in *A. polyphemus* (Mazur *et al.*, 1980; 1982; 1989; Regier *et al.*, 1980; 1982), *B. mori* (Papanicolaou *et al.*, 1986) and *Manduca sexta* (Regier and Vlahos, 1988; this work), in contrast to the rather uniform orientation of lamellae in *Sesamia nonagrioides*. The unusually thick trabecular layer of *Sesamia nonagrioides* (1·5 μm), compared to the 0·4 μm and 0·6 μm thick layers of *A. polyphemus* (Mazur *et al.*, 1989) and *B. mori* respectively, possibly explains the sensitivity of *Sesamia nonagrioides* eggs under dry conditions (Pucci and Forcina, 1984 and references therein).

Direct visualization of the helicoidal architecture of *Manduca sexta* and *Sesamia nonagrioides* chorion provided by freeze-fracturing, confirms that the generalized model of Bouligand (1972) is valid in both species. Straight or curved fibrils are packed into sheets, sheets are stacked one on top of another, forming a helicoid. The same technique has also been used to directly visualize the helicoidal architecture in *A. polyphemus* chorion (Hamodrakas *et al.*, 1986), in the cuticle of a crayfish (Filshie and Smith, 1980) and also in studies of cholesteric liquid crystalline phases of polymer solutions (Livolant and Bouligand, 1989) and cholesteric liquid crystalline DNA (Rill *et al.*, 1989). Scanning electron microscopy provides also for the direct visualization of the helicoid in coelacanth scales (Giraud *et al.*, 1978), the cuticle in *Carcinus maenas* and the test of *Halocynthia papillosa* (Gubb, 1975).

The close analogy between the helicoidal structures of *Manduca sexta* and *Sesamia nonagrioides* chorions with the structure of true cholesteric liquid crystals (Friedel, 1922) suggests that these tissues are self-assembled by a mechanism very similar to the process allowing materials to form liquid crystals (see also Neville, 1975; Bouligand, 1978a; Mazure *et al.*, 1982): apparently, the helicoidal chorions should pass through a liquid crystalline phase before solidifying by disulphide bond formation at or near ovulation (Regier and Vlahos, 1988; Orfanidou and Hamodrakas, unpublished). Dislocations and defects are departures from the ideal model and disrupt the continuous formation of chorion (Mazur *et al.*, 1982).

Chorion fibrils in *Manduca sexta* and *Sesamia nonagrioides* have diameters of approximately 3–4 nm, similar in size to the fibrils of the silkmoth chorion (Hamodrakas *et al.*, 1986). This finding may be related to the observation that their constituent proteins have, apparently, similar molecular weights (Regier and Vlahos, 1988; Orfanidou and Hamodrakas, unpublished). It should be mentioned that systems in many respects analogous to silkmoth chorion, like the feather and scale keratins (Hamodrakas *et al.*, 1976) contain fibrous units, 3 nm in diameter (Fraser *et al.*, 1976, Stewart, 1977). Furthermore, in several systems of structural proteins composed also mainly of β-sheets, the basic units of structure have dimensions of the order of 3 nm (Geddes *et al.*, 1968, Dobb *et al.*, 1967, Burke *et al.*, 1972).

The beaded pattern of the fibrils, when viewed longitudinally, has a periodicity of 2–3 nm and might imply a helical substructure (see results). According to Rudall (1955) and more recently to Bouligand (1978b), a helicoidal structure can be generated from helical units. Recently, it has been proposed that, the twisted β-pleated sheet, a helical structure, is the molecular conformation which dictates the self-assembly of the helicoidal architecture of silkmoth chorion and of other proteinaceous eggshells (Hamodrakas, 1984). It remains to be demonstrated by more refined experimental and theoretical work (determination and analysis of primary structures of *Manduca sexta* and *Sesamia nonagrioides* chorion proteins, X-ray differentiation, laser-Raman and infrared spectroscopy studies of intact chorions) whether or not this proposal is correct in *Manduca sexta* and *Sesamia nonagrioides* chorions.

Acknowledgements

C.C.O. acknowledges the award of a BRIDGE short-term fellowship. We than Prof. I. Tsitsipis for providing *Sesamia nonagrioides* adult insects and Mr C. Grandchamp for technical assistance. S. J. H. and L. H. M. thank the University of Athens for financial support.

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