Histol Histopath (1992) 7: 339-345

Histology and Histopathology

A scanning and transmission electron microscopic study of the membranes of chicken egg

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Summary. Questions regarding the structure of the inner and outer shell membranes of the chicken egg were addressed in this study by correlating observations from light microscopy and scanning and transmission electron microscopy. The egg membrane had a limiting membrane, which measured .9 to .15 μm in thickness and appeared to be a continuous and an impervious layer, but the shell membrane did not. Under the SEM, each membrane was seen to be made up of several fibre layers. In the tear preparations viewed under the SEM two layers were observed in the egg membranes and three to five layers in the shell membrane, with an apparent plane of cleavage between each layer. Each fibre was made up of a central core and an outer mantle layers. The central core was perforated by channels which measured .08 to 1.11 μm in diameter and ran longitudinally along the length of the fibre. Between the mantle layer and the fibre core was a gap or cleft measuring between .03 to .07μm. The diameter of the fibres of the inner layer of the egg membrane ranged between .08 to .64μm, whereas those of the outer layer of the same membrane ranged from .05 to 1.11 μm. Fibres in the shell membrane ranged from .11 to 4.14 μm diameter.

Key words: Chicken, Egg membrane

Introduction

The structure of the avian egg membranes has been extensively studied with the light microscope since the early part of this century. The findings reviewed by Romanoff and Romanoff (1949), Hodges (1974) and Gilbert (1974) showed that the avian egg is enclosed by two layers of membranes - an inner and an outer. The inner membrane, also called the *egg membrane (membrana putaminis)* encloses the albumen while the outer membrane, also called the *shell membrana (membrana testae)* is interposed between the egg membrane and the inner surface of the shell. At the blunt end of the egg, the two membranes are separated by an air-sac (Romijn and Roos, 1938; Romanoff and Romanoff, 1949; Hodges, 1974); elsewhere they loosely contact and form a single layer (Simons and Wiertz, 1963). The membrane fibres are formed from oviducal glands secretions (Hodges, 1974) as the egg spirals back and forth during its passage down the isthmus. These fibres form an intricate, interlacing network (Hodges, 1974).

The membranes vary in tickness; for example, the shell membrane of Leghorn eggs is about three times thicker than the egg membrane (Romanoff and Romanoff, 1949). There is also a relationship between the thickness of the membrane and the size of the egg, and the diameters of the fibres and mesh size of the membranes (Romanoff and Romanoff, 1949; Bellairs and Boyde, 1969; Candlish, 1970). Scanning (SEM) and transmission electron (TEM) microscopic studies (Tyler, 1969; Tung and Richards, 1972; Becking, 1975) have provided better estimates of fibre size than those which were determined from early light microscopic studies. The present study utilises SEM and TEM to determine egg membrane structure and fibre size in the region of the air-sac.

Materials and methods

Three brown Leghorn chicken eggs from the same flock of hens which were in their 6th month of production were used. The eggs were broken at the equator and the yolk was drained off. The egg membrane at the blunt end of the egg was carefully removed with iris scissors and transferred to a Petri dish. It was then fixed in a solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in .1M

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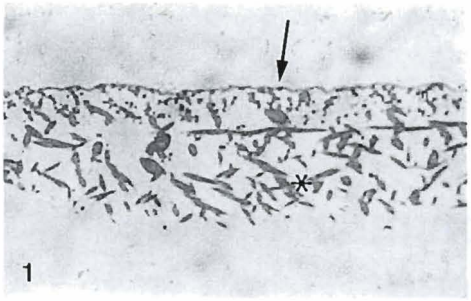
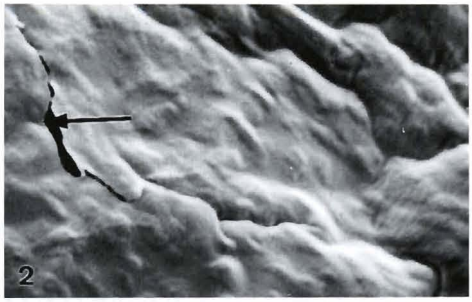
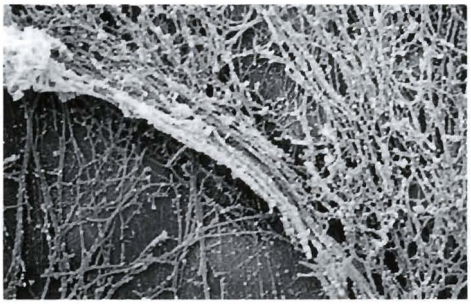
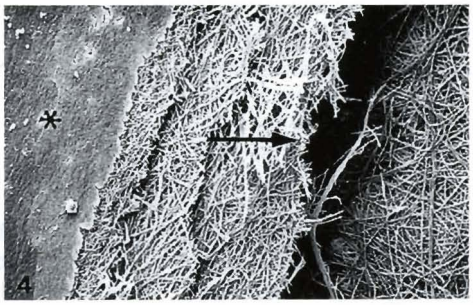
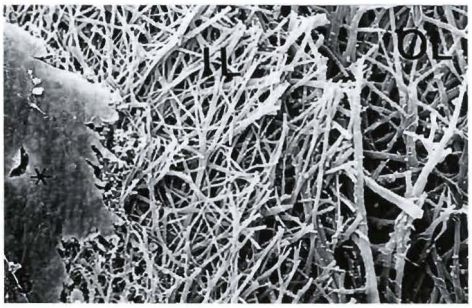
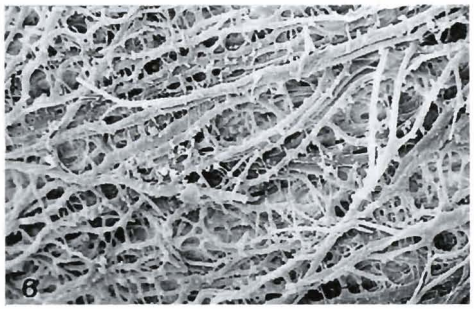


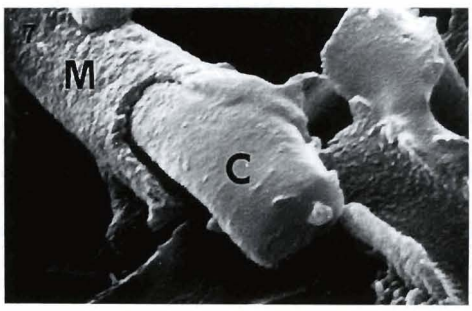












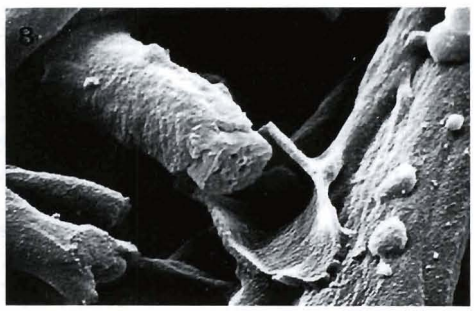


Fig. 1. A 1 μm thick section of the egg membrane showing the limiting membrane (arrow), the outer layer of large diameter fibres (asterisk) and, in between, the inner layer of small diameter fibres. x 130

Fig. 2. SEM micrographs of the inner surface of the limiting membrane showing the surface undulations and humps. The crack (arrow) is an artifact. x 2,975

Fig. 3. SEM micrograph showing strands of albumen adhering to the inner surface of the limiting membrane. x 400

Fig. 4. SEM micrograph of a tear preparation of the egg membrane showing a terraced appearance of the fibres. (Asterisk = limiting membrane. Arrow = inner and outer layer cleavage plane). x 65

Fig. 5. SEM micrograph of the egg membrane. Asterisk = limiting membrane. (IL = inner layer of fibres. OL = outer layer of fibres). x 270

Fig. 6. SEM micrograph showing the fibres of the egg membrane branching and criss-crossing. x 270

Fig. 7. SEM micrograph of the fibre core (C) surrounded by the manthle layer (M). x 8,500

Fig. 8. SEM micrograph of the fibre core whose cut surface shows several tube-like channels. x 5,000

phosphate buffer (pH 7.2-7.4), kept at 4°C. After 3 days, the samples were cut into small pieces, some of which were torn into two pieces each using watchmaker's forceps so as to produce "tear preparations". Small pieces of the shell and shell membranes above the air-sac were also removed and fixed for 3 days before they were decalcified in glacial acetic acid for 1 week. After that, all the tissues were osmicated in 1% osmium tetroxide in .01 M phosphate buffer, pH 7.3, for 2 hours.

For SEM, the tissues were dehydrated with graded ethanol solution and critical point dried with liquid carbon dioxide in a Polaron E 3100 Series II critical point apparatus (Polaron Equipment Ltd., U.K.). Then they were mounted on stubs with silver paint and gold-coated in a Polaron E 5100 Series II Cool sputter coater (Polaron Equipment Ltd., U.K.) and viewed in a Philips SEM 505 scanning electron microscope.

For TEM study, the tissues were embedded in Araldite after dehydration. Semithin sections (1 μm thick) were cut on a Reichert OmU_3 ultra-microtome and stained with toluidine blue, while ultrathin sections of gold interference colour were stained with uranyl acetate and lead citrate and viewed under a Philips 400T EM. All measurements of fibre diameter were made with a Zeiss Morphomat 10 semi-automatic image analyser. Since most of the fibres were cut obliquely, only their short diameters were measured. For this

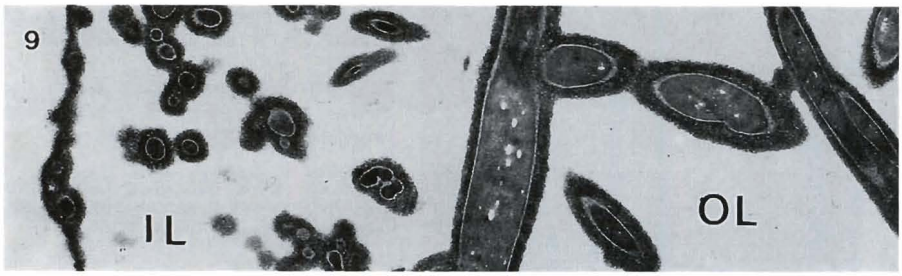


Fig. 9. TEM micrograph showing the limiting membrane and the inner (IL) and outer (OL) layers of the egg membrane. x 10,500

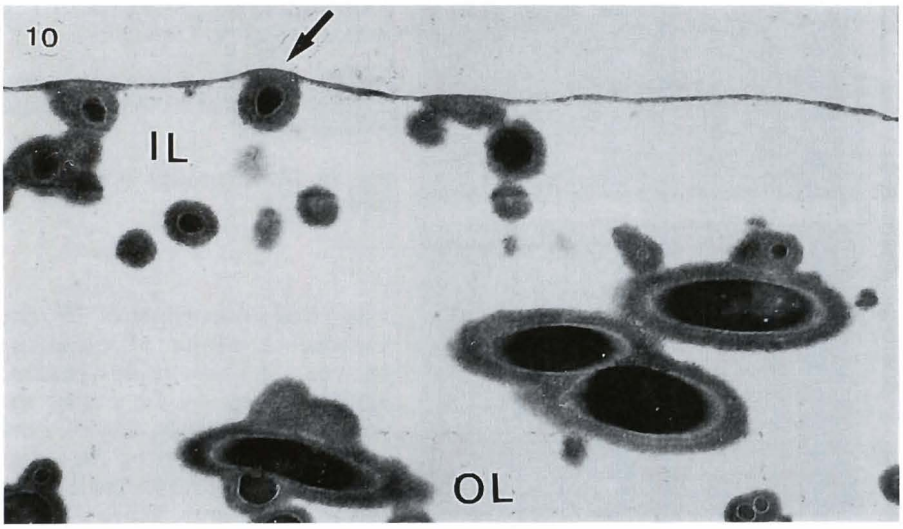


Fig. 10. TEM micrograph showing the inner layer of fibres of the egg membrane adhering to the limiting membrane (arrow). Some fibres share the same mantle layer. (IL = inner layer, OL = outer layer) x 6,300

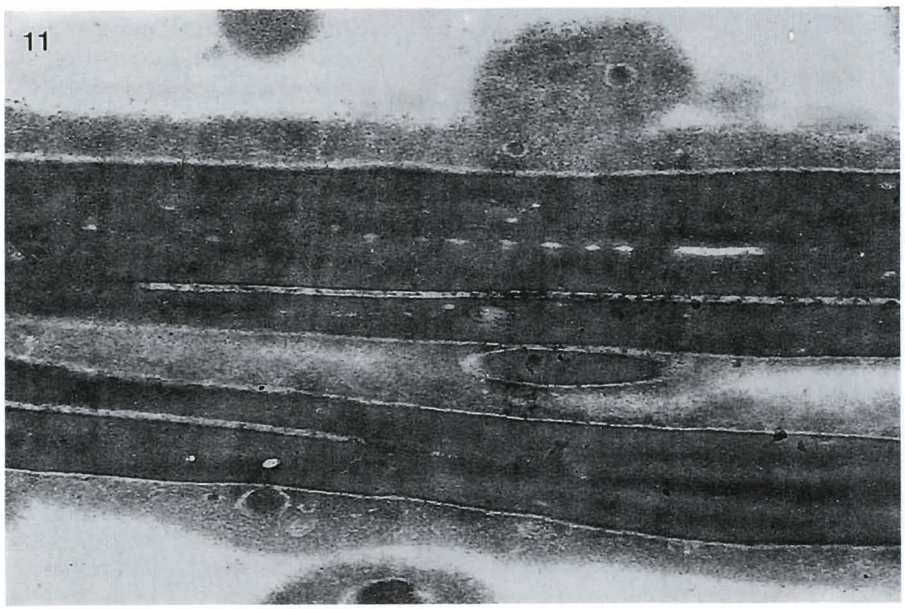


Fig. 11. TEM micrograph showing the longitudinal section through two fibres of the egg membrane and the longitudinal channels running in the fibre core. x 14,000

purpose, measurements were made on electron micrographs with a print magnification of between x 6,300 and x 15,000. For measuring smaller structures such as the limiting membrane and the cleft between the mantle layer and the fibre core, micrographs of print magnification of between x 30000 and x 63000 were used. The means and standard deviations (SD) of the fibre diameters were calculated and significant differences were determined by the t-test.

Results

Egg membrane

Semithin sections. Light microscopy revealed that the egg membrane was made up of an inner and outer layer (Fig. 1). On its inner surface, the inner layer of fibres was lined by a limiting membrane (Fig. 1, arrow). The limiting membrane appeared as a continuous, impervious layer separating the fibres from the albumen. Some of the innermost fibres appeared to adhere to it whereas the outer layer of fibres confronting the air-sac was not covered by a membrane.

The thickness of each layer of fibres was variable although the outer layer was generally thicker than the inner (Fig. 1). In addition, the fibres of the outer layer generally appeared to be larger in diameter than those of the inner layer (Fig. 1). SEM micrograph showed the limiting membrane as a continuous sheet of tissue with an undulating, featureless surface. The undulations were due to the presence of numerous humps which were more obvious when the specimen was viewed at a tilt angle of 60º (Fig. 2). Strands of albumen were frequently observed on the inner surface of the limiting membrane (Fig. 3).

Under the lower power SEM, fibres of egg

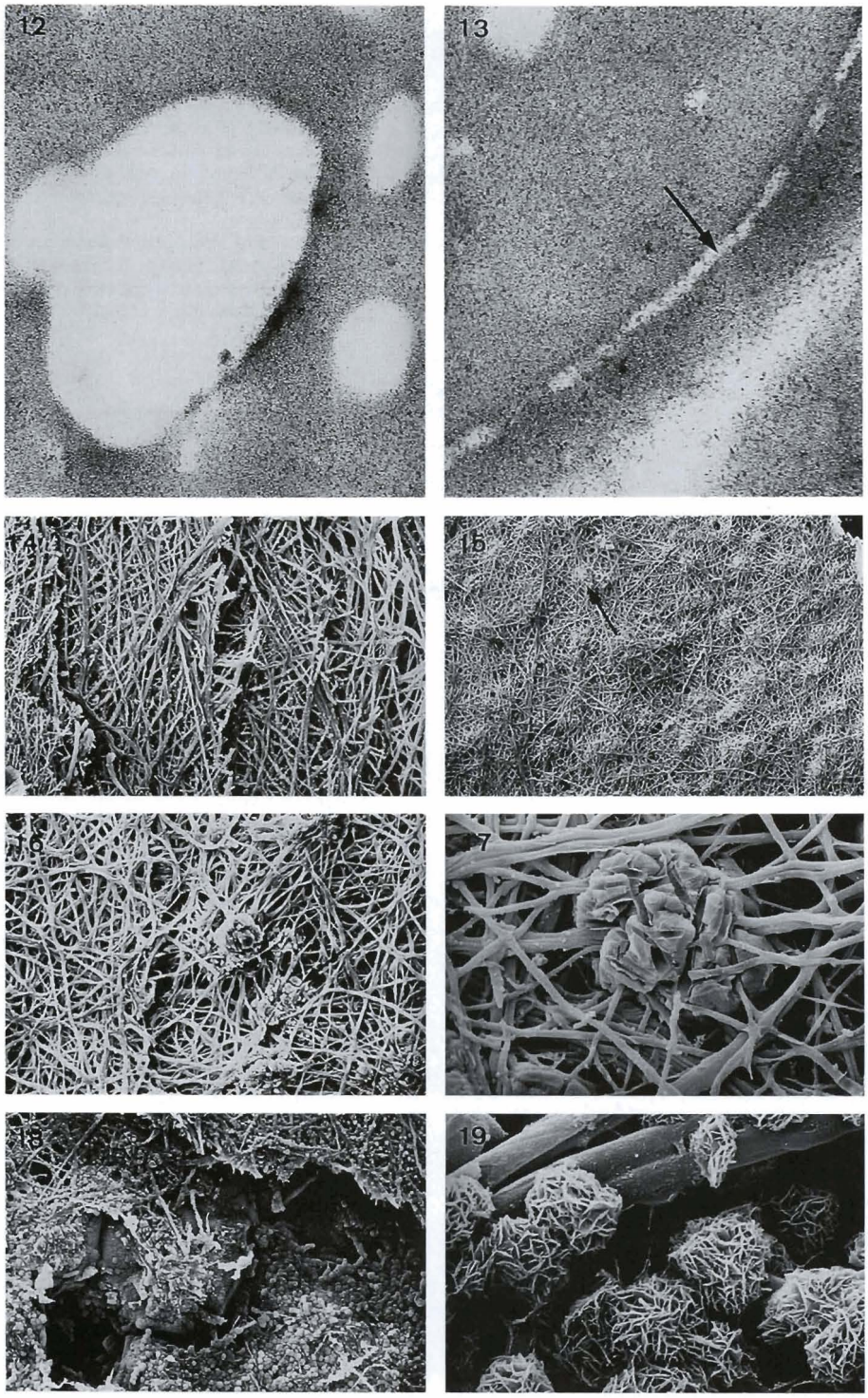


Fig. 12. TEM micrograph of a cross-section of the channels in the fibre core. X 63000

Fig. 13. TEM micrograph of the gap between the fibre core and the mantle layer of egg membrane fibre. x 80,500

Fig. 14. SEM micrograph of a tear preparation of the shell membrane showing terracing of the fibres. x 135

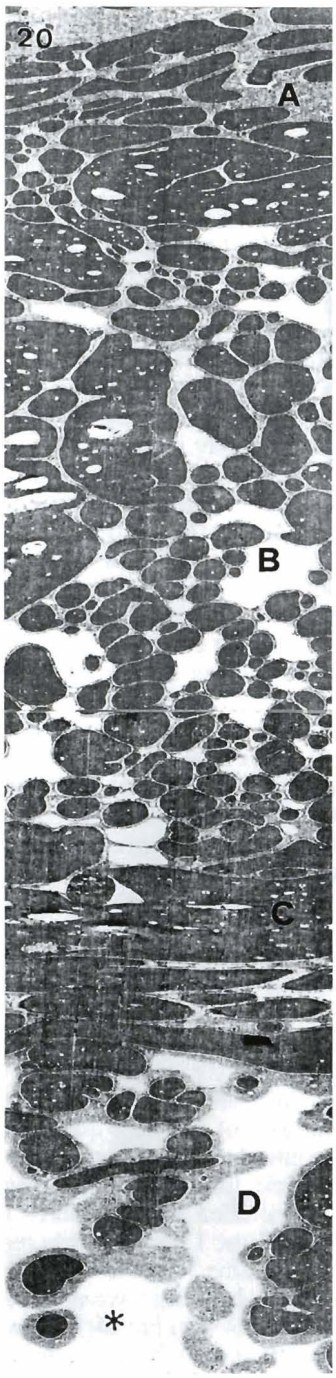
Figs. 15 - 17. SEM micrographs at different magnifications. (x 65, x 390 and x 550) showing attachment of the mamillary cores to fibres of the shell membrane (arrow).

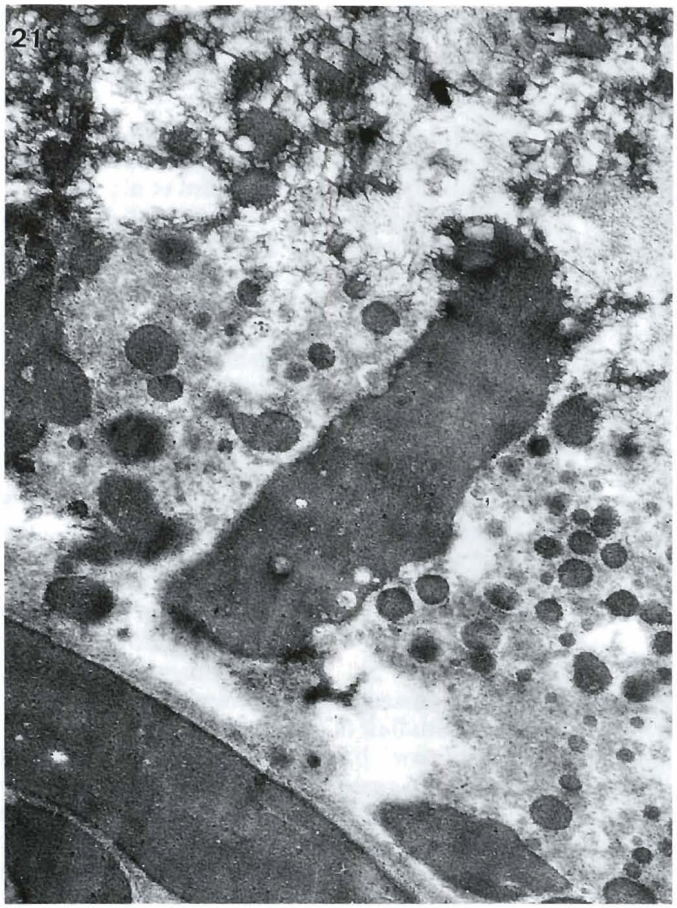
Fig. 18. SEM micrograph of the mamillary core after removal of the fibres of the shell membrane. x 125

Fig. 19. SEM micrograph of the globules seen in Fig. 18.

membrane appeared as an intricately-woven network (Fig. 4) which presented a stratified appearance. The fibres ran in all directions and criss-crossed each other; some appeared to anastomose while others did not (Figs. 5, 6). At higher magnification, two distinct layers of fibres could be distinguished. When the specimen was tilted at an angle of 15º and viewed, a plane of cleavage between the two layers became apparent (Fig. 4). Generally, the fibres of the inner layer were smaller than those of the outer layer (Fig. 5). When the broken ends of some fibres were examined, they were observed to be made up of a central core which was surrounded by a sheath-like structure called the *mantle* (Fig. 7). On the surface of the central core were small tubercle-like structures (Fig. 7). Examination of the cut surface of a fibre core showed that it was perforated by several "holes" (Fig. 8). In addition, a small cleft separated the mantle layer from the fibre core (Fig. 7).

TEM study. The limiting membrane appeared to be a featureless, amorphous membrane which was not uniformly thick throughout the length of the fibres which were examined (Figs. 9, 10). A total of 50 measurements were made from five pieces of egg membrane removed from the blunt end of the three eggs; the mean thickness of the membrane varied from .09 to .15 μm. At irregular intervals along the length of the membrane, some inner-layer fibres were observed to be adherent to its inner surface (Fig. 10). At such sites, the mantle layer of the fibre appeared to fuse with the limiting membrane,





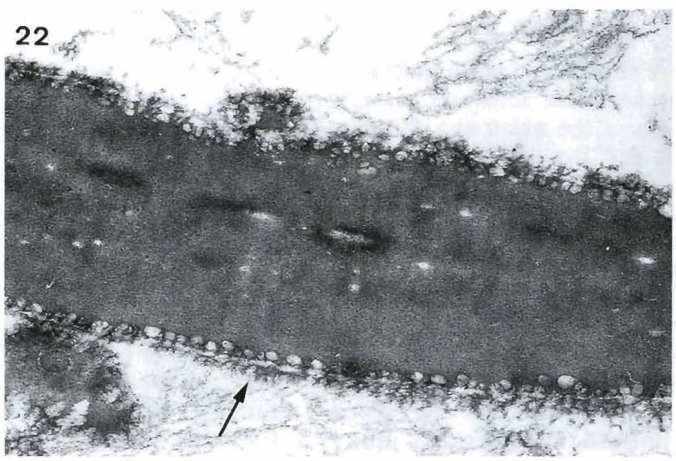


Fig. 20. TEM micrograph of a cross-section of the egg membrane. Four layers can be distinguished (A - D). Asterisk = surface facing the air-sac. x 3,600

Fig. 21. TEM micrograph of a decalcified egg shell showing the site of attachment of a mamillary core to the shell membrane. x 17,500

Fig. 22. TEM micrograph of a fibre near the attachment of the mamillary core. (Arrow = fibrillary material and subsurface vesicles). x 22,750

thereby producing a slight elevation of the latter; these could be the sites where the surface humps were observed under the SEM (Fig. 2).

The short diameters of 300 fibres of the outer layer and 200 fibres of the outer layer were measured. For the inner layer, these diameters ranged from .08 to 0.64 µm (mean = .30 µm; SD = .12 µm) and for the outer layer, they ranged from 0.5 to 1.11 µm (mean = .60 m; SD = .21). Differences between these diameter means were statistically significant ($P < .01$).

The TEM of fibres which were cut longitudinally confirmed the SEM observation of the fibre core being separated from the mantle layer by a gap or cleft (Figs. 9, 11). A high power TEM showed that these channels, measuring between .02 and .13 µm in diameter, were not membrane-bound and that they were devoid of any content (Fig. 12). The mantle layer, which varied from .13 to .35 µm in thickness, was separated from the fibre core by a cleft (Fig. 13) which measured between .03 to .09 µm.

Shell membrane

SEM study. In tear preparations of the shell and the attached shell membrane, stratification of the fibres into three to five layers was observed (Fig. 14). The outer layers of the membrane was characterized by prominent accumulations of material which occupied the interstices between the fibres (Figs. 15 - 17); these sites were the attachment points of the mamillary cores to the fibres of the shell membrane (Fig. 18). Numerous globules of about 3 - 4 µm diameter, whose surfaces showed creases or folds, were also observed in the vicinity of the mamillary core and in the interstices between the fibres (Fig. 19).

TEM study. At lower power TEM, the fibres were seen to be organized into groups of bundles. In each bundle, the fibres were all oriented similarly; frequently, a bundle of fibres sectioned longitudinally or obliquely alternated with another in which the fibres were sections transversely (Fig. 20). The short diameter of 300 fibres ranged from .11 to 4.14 (mean = 1.37 μm; SD = .76 μm). The larger fibres tended to be located more superficially (i.e. nearer the shell). The mean thickness of the mantle layer was .39 μm (SD = .20 μm). The diameters of the longitudinal channels in the fibre core ranged from .08 to 1.11 μm (mean = .27 μm; SD = .20 μm). The gaps between the fibre core and the mantle layer varied from .03 to .07 μm.

At the attachment sites of the mamillary cores, many small profiles measuring about 3 μm in diameter were present (Fig. 21). These structures probably corresponded to the globules observed with the SEM (see above and Fig. 18). Fibrillar material was also observed on the surface of and in the intervals between the fibres (Fig. 22).

Discussion

Terminology. Since the inititation of light microscopic studies until the more recent ultrastructural studies of the structure of the avian egg, the term "membrane" has been traditionally used to denote that part of the tissue which separates the albumen from the shell. It should be stressed, however, that these "membranes" are not true biological membranes but are merely investments or layers of material laid down as the egg moves down the isthmus of the oviduct.

The limiting membrane. A thin limiting membrane, which is continuous and impervious, separates the egg membrane from the albumen of the avian egg (Simons and Wiertz, 1963; Bellairs and Boyde, 1969). Such a membrane has also been described in the reptilian egg, for example in the chelonid (Solomon and Baird, 1976), trionyx (Packard and Packard, 1979) and kinosterid turtles (Packard et al., 1984a).

Simons and Wiertz (1963) estimated the limiting membrane to be about 2.7 μm thick, but the present TEM study shows that it is much thinner and that it is not of uniform thickness but varies from .09 to .15 μm. The inner surface (i.e. the one which faces the albumen) is smooth but presents many undulations. The present TEM observations suggest that these undulations are, in part, produced by the fibres which run beneath it. No fenestrations have been observed. This observation has a functional significance since it has been suggested that the limiting membrane not only separates the egg membrane from the extraembryonic membrane but also may provide defense against bacterial invasion (Tung and Richard, 1972; Tung et al., 1979).

The egg and shell membranes. The existence of two major layers of membrane in the avian egg has been known from early light microscopic studies. A double layer of membrane, however, has not been observed in reptilian eggs, for example, the kinosterid (Packard et al., 1984a,b) and chelonid turtles (Packard, 1980) and tuatara, an evolutionarily ancient squamatic reptile (Packard et al., 1982).

Moran and Hale (1936) reported that it was possible to dissect the egg membrane into two layers and the shell membrane into three layers. This was supported by Simons and Wiertz (1936) who reported that the egg membrane was made up of three layers and the shell membrane of six layers. But Simkiss (1958) could not distinguish separate layers in the two membranes in histological sections. On the basis of SEM observations of tear preparation, the present study suggests that the egg membrane may be composed of at least two distinct layers of fibres and the shell membrane of three or more layers. However, the possibility that the stratification of the fibres seen in the SEM of tear preparation could have been artifactual cannot be ruled out. But if the stratification is not an artifact, then it would suggest that the laying down of fibres is not one continuous process but interrupted at intervals as the egg spirals down the isthmus.

Fibres. A great variability in the size of the fibres in both the egg and shell membranes has long been known. The fibres in the shell membrane show greater variability than those in the egg membrane. In the present study, the smallest fibres are found to be located nearest the egg albumen (mean = .3 μm; SD = .12 μm) and the largest ones near the shell (mean = 1.37 μm; SD = .76). The present results concur with those of Simons and Wiertz (1963), Candlish (1970), Draper et al. (1972) and Wong et al. (1984) and confirm that the early figures reported by Romankewitsch (1932) and Moran and Hale (1936) were grossly overestimated. It also supports the idea that the fibres get larger during the later stages of the passage of the egg down the oviduct (Romanoff and Romanoff, 1949; Draper et al., 1972; Hodges, 1974). In their immunofluorescence study, Wong et al. (1984) concluded that the fibres in the shell membrane contained Type I collagen whereas those of the egg membrane contained Type 5 collagen, although the characteristic 67 nm banding have not been observed under TEM. Stevenson (1980) has also shown that these fibres could be digested by a bacterial protease, Pronase P.

Mashoff and Stolpmann (1961) described each as being made up of a core surrounded by a mantle with Bellairs and Boyde (1969) called the "medulla" and "cortex", respectively. These two components of the fibre have been observed in the present and other studies (Simons and Wiertz, 1963; Candlish, 1970; Draper et al., 1972). The two components are separated by a gap which Draper et al. (1972) called "halo". Under high power TEM in the present study, some fuzzy material bridging the gap between the mantle layer and the fibre

core was observed in the present study which Bellairs and Boyde (1968) and Candlish (1970) described as "delicate strands". Draper et al. (1972) observed that these width of the gap was fairly constant and suggested that the gap might be a shirnkage artifact. Fibrillar material was also observed on the surface of the mantle layer of the fibres. Wong et al. (1984) have shown that these structures contain glycosaminoglycans.

The presence of "holes" in the fibre core has been noted by Simons (1971). There has been some suggestion that these profiles represented "small holes" or "hard inclusions" (Simons, 1971; Draper et al., 1972). It is apparent from the present study that these profiles, which appear as holes in transverse sections of the fibre core, appeared as long channels in longitudinal sections of the fibre core. The functional significance of these channels is not known.

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Accepted January 22, 1992