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Scanning Electron Microscopy of Shell Formation in Hen's Eggs

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(Figs. 1-19)

The shell of the avian egg is formed by a hard tissue consisting of about 95% of inorganic and 5% of organic substances. Structurally, it is composed of a shell membrane, the shell proper, and a cuticle layer from inside to outside. Papers have been published on the structural organization of the avian egg shell by a number of workers, including NATHUSIUS (1868)[1], STEWART (1935)[2], MORAN and HALE (1936)[3], ROMANOFF and ROMANOFF (1949)[4], SAJNER (1955)[5], SIMKISS and TYLER (1957)[6], MASSHOFF and STOLPMANN (1961)[7], MATHER and EPLING *et al.* (1962)[8], HEYN (1963)[9], SIMONS and WIERTZ (1963)[10], TEREPKA (1963[11], 1963[12]), and EL-BOUSHY and SIMONS *et al.* (1968)[13]. These workers used the optical microscope, polarization microscope, and electron microscope. It is already known that the shell proper of the egg shell is formed in the uterine portion of the oviduct while the egg stays in this portion for about 20 hours. The morphological mechanism of the shell formation, however, has been studied only by a few workers, including NATHUSIUS (1868)[1], and ROMANOFF and ROMANOFF (1949)[4], whose observations were naturally made by the conventional optical microscope.

In their previous study, the authors[14] examined the structure of the egg shell completed after oviposition by using the scanning electron microscope. This instrument was very usefull for the structural observation of such a hard tissue as the egg shell. In the present study, the morphological mechanism of the shell formation was examined in hen's eggs by scanning electron microscopy.

MATERIALS AND METHODS

Eggs in various stages of the shell formation of White Leghorn hens were used in this investigation. They had been removed at random from the uterus, where the egg is formed, in laying hens obtained from a poultry eviscerating plant. The eggs were classified grossly into six stages of developing shell, judging from the appearance and hardness of the shell. They were broken with a knife or a pair of scissors and their contents dripped off. The inside of the shell was washed shortly with physiological saline solution to remove adhering albumen. Then the shell was fixed in 10% formalin solution. Small pieces of the fixed shell were treated further in the following manners respectively, for observation: (1) No additional treatment, (2) immersion in 3% glacial acetic acid for 24 hours for decalcification, (3) dipping in 30–35% sodium hydroxide for 24 hours to remove the organic substances from the shell. They were washed with distilled water and dried at room temperature, or sometimes dehydrated through a series of graded acetone. The resulting specimens were coated with gold and examined under a scanning electron microscope, type JSM-2 (Japan Electron Optics Laboratory, Ltd.), at an accelerating voltage of 25 KV.

RESULTS

When eggs were examined by scanning electron microscopy just after having reached the uterine portion from the isthmus of the oviduct, they were enveloped only by a shell membrane. No deposition of calcium salts was observed as yet on the surface of the shell membrane. Fig. 1 shows the external appearance of the shell membrane of these eggs. As is clear from the figure, in which the outer shell membrane is illustrated, the shell membrane was found to have been formed completely at the isthmus, and has a sharply defined appearance. The outer shell membrane was formed by a relatively coarse meshwork of fibers running parallel to the shell surface. Each fiber took a thin tape-like form and was 3–4μ in width.

As soon as the shell formation started in eggs, minute sand-like granules first began to deposit on the surface of the shell membrane. The deposition of these granules was more remarkable with the advance in the process of shell formation. So that the outline of the shell membrane became more and more obscure (Fig. 2). Secondly, large numbers of small concretions arose scatteringly on the entire surface of the shell membrane, although they varied in distribution and size with the location on the shell surface. These concretions were slightly protruded on the shell surface, showing irregularity in shape (Figs. 3 and 4). At a glance, they were roughly constructed by what appeared to be calcific substance. They increased both in number and in size with the advance in the process of shell formation.

Then examination was made to determine whether these concretions were made of organic or inorganic material in nature. When the egg shell in this stage was decalcified with acid, it was not dissolved but remained almost the same in size. Smooth-surfaced projections kept their original positions even after decalcification (Fig. 5). On the other hand, when the egg shell was immersed in sodium hydroxide solution to remove organic substances, these concretions were all dissolved, as well as the shell membrane. From these results, it was reasonably suggested that the concretions deposited on the shell surface might be made mainly of organic material such as protein, although they seemed to be composed of calcific material. These concentrations of organic material acted as a whole to form the nucleus of calcification. Later, a mammillary knob developed from them.

An egg more advanced in the process of shell formation is illustrated in Fig. 6. As shown in the figure, organic concretions increased in size and height as a result of deposition of calcium salts. Eventually, they developed into sharp knobs. This is the early stage of formation of the mammillary knob. The early mammillary knob stood on the shell surface with a round base and a sharply pointed tip. With the advance in the process of shell formation, these knobs took the shape of a dome with a broad base and a somewhat flattened tip (Figs. 7 and 8). When decalcification occurred to the same egg as seen in Fig. 7, small organic cores remained in places corresponding to those of the preceding knobs (Fig. 9).

These dome-like protrusions were ultimately fused with one another to form a single calcium layer. This layer is the so-called mammillary layer. In general, eggs of these stages are called soft-shell eggs. Fig. 10 shows the outer surface of the well-formed mammillary layer. As shown in the figure, the mammillary knobs are arranged regularly, separated from one another with relatively deep sulci. Among the sulci there was a cannal of air pore (Fig. 11). When the same egg as seen in Fig. 10 was decalcified, small oval organic cores remained in the same state as observed before (Figs. 12 and 13). The remaining organic cores were more rounded and more compact in appearance than those in Figs. 5 and 9. This result indicates that the organic core located at the center of the mammillary knob was exposed by removal of the surrounding calcium. It is also suggested that the organic core may be a mass composed mainly of organic material and containing a little, inorganic material if any. Careful observation of Fig. 13 reveals that the organic core has been penetrated by some fibers stretching upwards from the outer shell membrane. This suggests a firm connection between the shell layer and the shell membrane.

The completed mammillary layer was examined for the mode of calcium deposition. In specimens from which organic matter had been removed, a calcite architecture was exhibited, as shown in Figs. 14–17. Figs. 14 and 15 illustrate the calcite architecture of the inner side of the mammillary layer, and Figs. 16 and 17 that of the outer side of it. As is clear from these figures, the mammillary layer at this stage was still roughly constructed by large crystals of calcite. When Figs. 16 and 17 are compared with Figs. 14 and 15, it is noted that the deposition of calcium in the outer side of the mammillary layer is denser and formed by smaller calcite crystals than that in the inner side of it.

After the formation of the mammillary layer in the shell, progress is made in the construction of a spongy layer. This is done by the additional deposition of calcium on the mammillary layer. Calcium salts were deposited progressively on the already formed mammillary layer in a radial direction. Fig. 18 shows the outer surface of the spongy layer almost completely formed. Fig. 19 shows the outer surface of the completed egg after oviposition. In these samples, organic matter had been removed from the shell. The deposition of calcium salts is poorer in the former figure than in the latter. This indicates that the formation of the spongy layer is progressive and continues until the oviposition of the egg. Calcium deposition of the shell seems to be caused additionally among calcite crystals already formed.

As the deposition of calcium salts proceeded, the shell layer increased gradually in thickness. Sulci among the mammillary knobs became indistinguishable by the thickening of the spongy layer. In the completed egg shell, as shown in Fig. 19, the shell has a very dense texture and a grossly plain surface. The interstices among the mammillary knobs disappeared entirely. The only indication for them was a slightly concave area with air pores open in it.

DISCUSSION

It is generally known that the shell proper of the avian egg is formed in the uterus by the secretions from the uterine glands, and that it is structurally composed of an inner mammillary layer and an outer spongy layer. From the present study on the developing egg in the uterus and the previous work on the completed egg after oviposition, some findings were obtained on the structural mechanism of the shell formation of the avian egg.

In the present study, the shell formation, which is a series of successive process of calcium deposition, was found to take place in two stages, an early and a late. In the early stage of shell formation, the mammillary layer was completed. In the late stage, the spongy layer was achieved. The early stage may be subdivided into two periods, one for the formation of an organic core and the other for the subsequent formation of mammillary knobs.

It has been generally accepted that the shell membrane, which consists of an inner and an outer shell membrane, is formed in the isthmus of the oviduct. In the present study, it was observed that as soon as eggs arrived at the uterus, they were enclosed by a fully formed shell membrane. The shell membrane at this stage had entirely the same appearance as that of the completed eggs which had been examined in the previous study. From these results, it is suggested that the shell membrane, once formed in the uterus, may undergo no morphological changes during the shell formation in the uterus.

The early stage of the shell formation began just after the egg had arrived at the uterus. The first indication of the shell formation was the deposition of fine granules on the surface of the shell membrane. These granules later developed into prominent protrusions. The protrusions on the shell surface had been formed not by the deposition of calcium, but by the accumulation of organic matter. This finding is supported by the fact that no protrusions were decalcified by acid, but that they disappeared entirely when treated with sodium hydroxide for removal of organic matter. Undoubtedly, these organic protrusions show the site of occurrence of initial calcification, since calcium salts have been deposited around them.

The formation of mammillary knobs followed the establishment of organic protrusions. It took place by the additional deposition of calcium salts around the organic protrusions. Consequently, the previous organic protrusions were embedded at the center of the mammillary knob and became an organic matrix. An organic protrusion which appeared in the early stage of the shell formation was the nucleus of calcification. In other words, it was the precursor of the mammillary knob. An early developing mammillary knob was very small in size at first. Later, it was enlarged by the progressive accumulation of calcium salts. At last, it was fused with one another and developed into a mammillary layer.

In the present study, the term organic core has been given collectively to masses of high concentration of organic matter which were located at the center of the mammillary knob. Many previous workers have already observed the presence of such core as this at the center of the mammillary knob. There has been, however, some disagreement on the location and the nature of the organic core. NATHUSIUS (1868)[1] was the first to observe an optically dark area at the center of the mammillary knob. STEWART (1935)[2], and ROMANOFF and ROMANOFF (1949)[4] have entertained the same opinion as NATHUSIUS. STEWART also stated that the core was primarily organic in nature, because it was insoluble in acid and readily disintegrated with alkali and by heat. On the other hand, SIMKISS and TYLER (1957)[6] demonstrated the presence of such organic core at the tip of the mammillary knob in their histochemical studies on the egg shell. TEREPKA (1963)[11] mentioned that if the term "core" meant the presence of high concentration of organic matter, the core would be present at the tip of the mammillary knob and considered organic in nature. He also asserted that it might be preferable to call the mammillary core observed by early workers the "mineral core", since this core was made of small-sized crystals of calcium which were a little different from crystals of calcium around them. The present authors observed in their previous investigation that the tip of the mammillary knob had a slight depression filled with organic matter. In addition, it was also observed that a large cavity was present at the center of the mammillary knob in the completed egg shell from which had been removed organic matter. This cavity was a space fit for the occupation by the organic core. In the present study, when the mammillary knobs were decalcified in varying stages of development, the organic core always remained at the site corresponding to the center of the mammillary knobs (Figs. 5, 9, and 12). The organic core remained almost the same in size and shape after decalcification, while the mammillary knobs varied greatly in size before decalcification. Judging from these results, it is clear that the core lying at the center of the mammillary knob is organic in nature. Therefore, it may be concluded that the core of the mammillary knob is located at the center of this knob and is organic in nature.

It is generally accepted that the shell membrane is attached firmly to the shell layer. ROMANOFF and ROMANOFF (1949)[4] described that the calcified tip of the mammillary knob was embedded in the shell membrane. TEREPKA (1963)[11] found that the calcified tip of the mammillary knob was buried in a membrane consisting of fibers which had come from the outer shell membrane and penetrated the calcified mammillary tip, bridging over gaps among the mammillae. The authors observed in their previous study that some of the fibers of the outer shell membrane entered the organic matrix through small canals running at the bottom of the calcified mammillary tip. It has been clearly recognized in the present study that the organic core of the mammillary knob is connected directly with fibers extending from the outer shell membrane (Figs. 12 and 13). This junction between the shell proper and the shell membrane may play some role in the shell formation.

The late stage of the shell formation begins by the formation of the spongy layer after the completion of the mammillary layer. In this stage, a gradual deposition of calcium salts proceeds on the mammillary layer. While the calcium deposition of the spongy layer extends in a radial direction from the already formed mammillary knob, calcite columns are formed in the entire shell layer. The findings of the calcite columns were given in a previous paper. The deposition of calcium salts in the spongy layer takes place simultaneously with the formation of plentiful matrix fibers. Calcium salts are deposited among matrix fibers. The matrix fibers of the shell layer were also described in a previous paper.

The shell-proper layer becomes thick and dense progressively in the texture of the shell, with the advance in the deposition of calcium. The late stage of the shell formation shows slower progress than the early stage. According to BURMESTER (1940)[15], shell material is deposited slowly during the first 3 hours after the egg reaches the uterus. As mentioned above, the early stage of the shell formation is represented by the formation of organic matter. So that shell material is small in amount.

SUMMARY

The morphological mechanism of the shell formation of hen's eggs was observed in developing eggs in the uterus by scanning electron microscopy. The shell formation was started in the egg just after the egg had arrived at the uterus. The first indication of the shell formation was the deposition of small sand-like granules on the shell surface. The subsequent indication of the shell formation was the appearance of small-sized organic protrusions on the shell surface. These protrusions increased in size as the shell formation advanced and formed nuclei of calcification. The deposition of calcium salts progressed around the organic protrusions, and mammillary knobs were formed. Previous organic concretions were encrusted by the deposition of calcium and became a central organic core, which was located at the center of the mammillary knob. Mammillary knobs were first small in size and later increased in size progressively. At last, they fused with one another to form a mammillary layer. After the formation of the mammillary layer, a spongy layer developed successively on the mammillary knob. It became thick with additional deposition of calcium salts in a radial direction.

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鶏卵の卵殻形成の走査電子鏡検

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鶏卵の卵殻形成の機構が，走査電子顕微鏡下で形態的に観察された．

卵管子宮部に下降した直後の卵は，卵殻膜のみに包まれた状態であった．卵殻の形成が始まると，まず卵殻膜表面に微細な粒子が沈着した．ついで卵殻膜の表面の所々に凝塊様隆起が現われた．この隆起は主として有機性物質より成り，石灰沈着の核のような作用をした．後にこの有機性基質の回りに石灰沈着が進み，乳頭状の突起となった．これが卵殻乳頭層の乳頭突起である．乳頭突起は次第に丘状に発達し，最後にはお互いにゆ合して一層の石灰層，すなわち乳頭層となった．

乳頭層の形成後は，石灰沈着が乳頭突起の上に，卵殻表面に放射状に進み，卵殻海綿層が形成された．卵殻海綿層の形成は徐々に行なわれた．

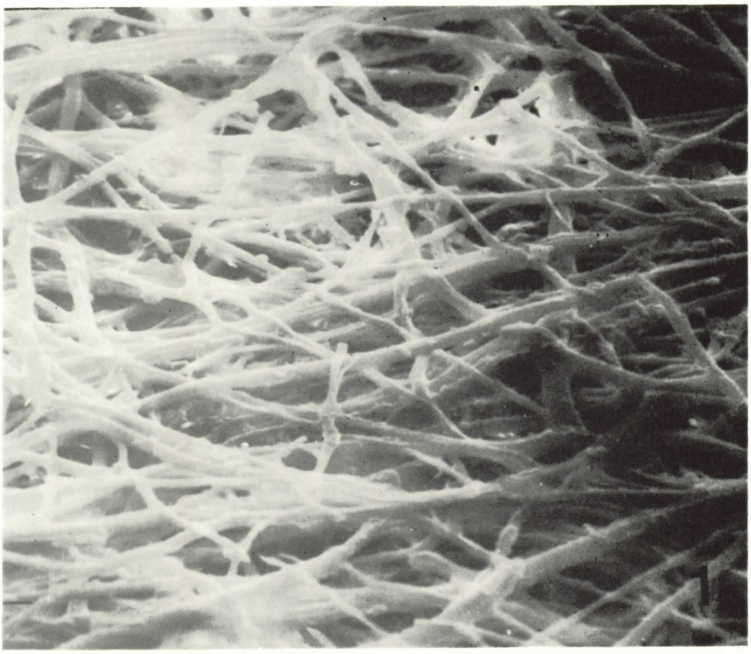

Fig. 1. The outer surface of an egg which has just arrived the uterus. The shell membrane has been fully formed and its demarcation is clear. ×1,000.

Fig. 2. The outer surface of an egg at the beginning of shell formation. Fine granules have been deposited on the shell membrane. No demarcation of the shell membrane is clear. ×1,000.

Fig. 3. The outer surface of an egg. Small protrusions have been formed on the shell surface. ×300.

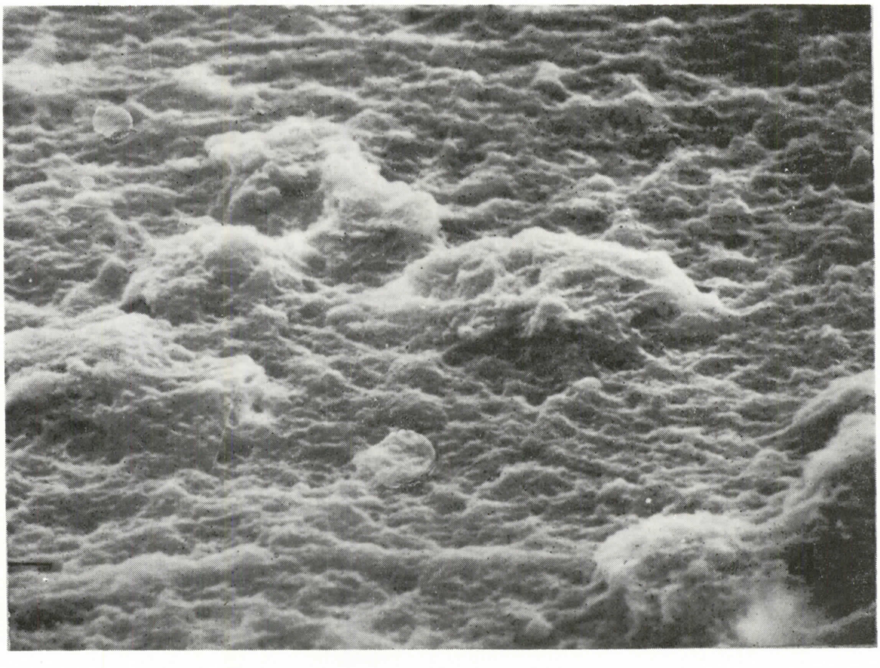

Fig. 4. A high-power magnification of Fig. 3. ×1,000.

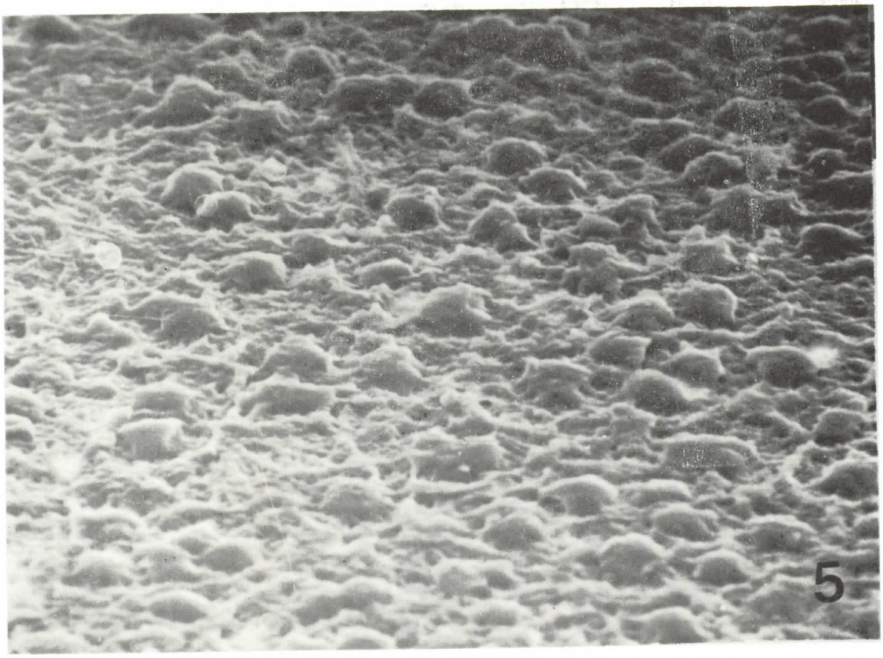

Fig. 5. Protrusions on the shell surface remaining after decalcification. ×300.

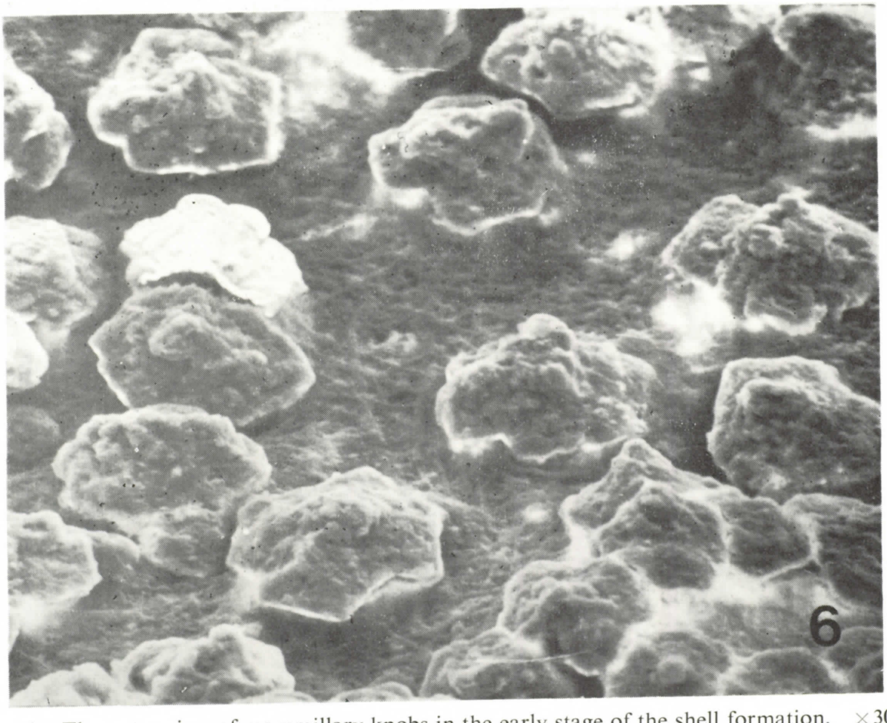

Fig. 6. The outer view of mammillary knobs in the early stage of the shell formation. ×300.

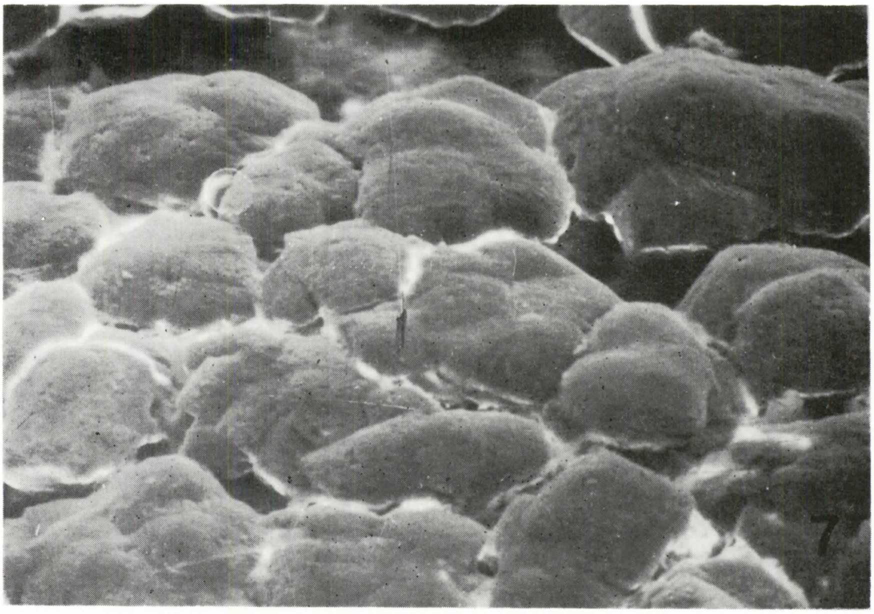

Fig. 7. The outer view of fairly developed mammillary knobs. ×300.

Fig. 8. A high-power magnification of Fig. 7. ×1,000.

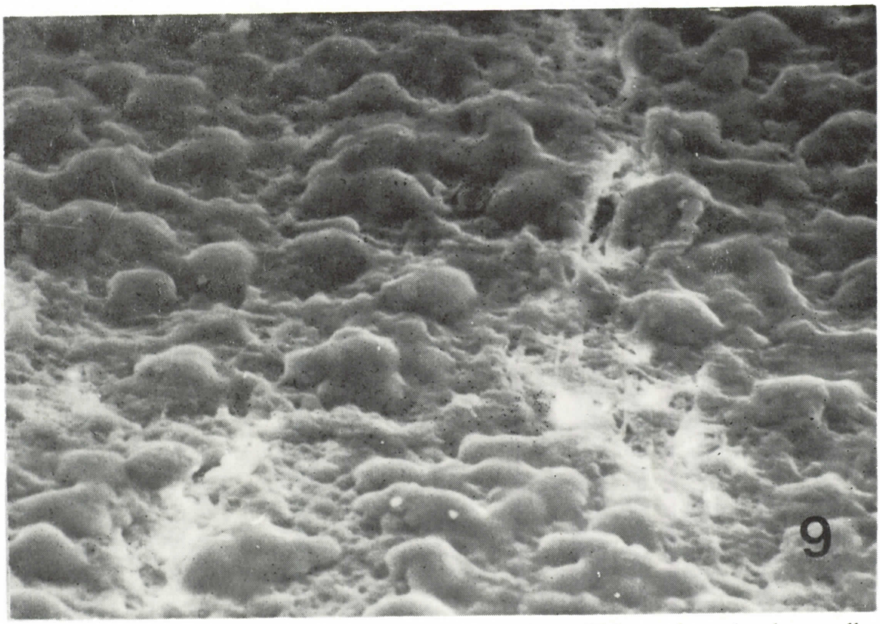

Fig. 9. Organic protrusions remaining after decalcification. This specimen has been collected from the same egg as used for Fig. 7. ×300.

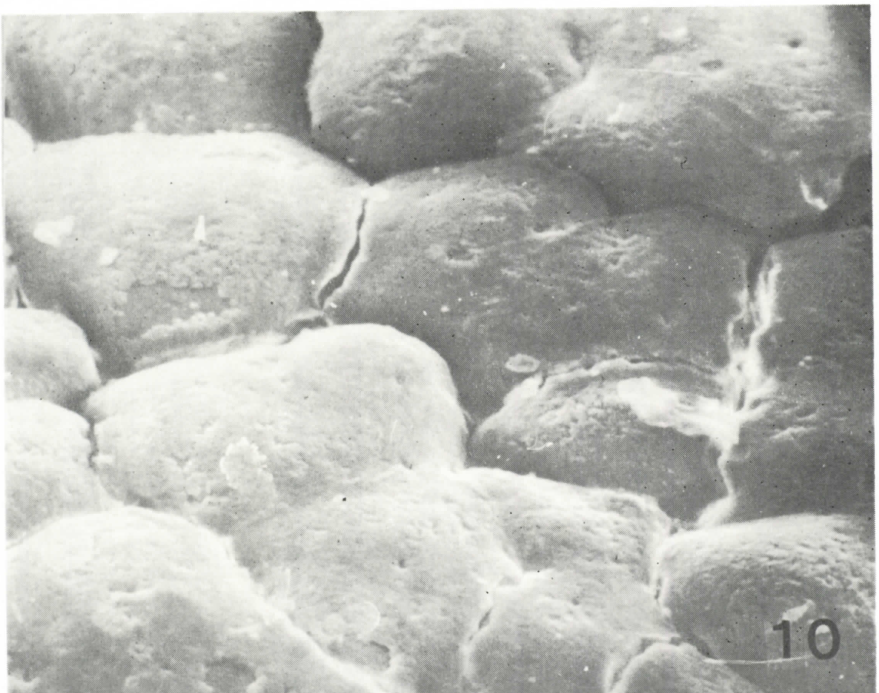

Fig. 10. The outer view of fully formed mammillary knobs. The knobs have been fused with one another to form a single layer which has constituted a mammillary layer. ×500.

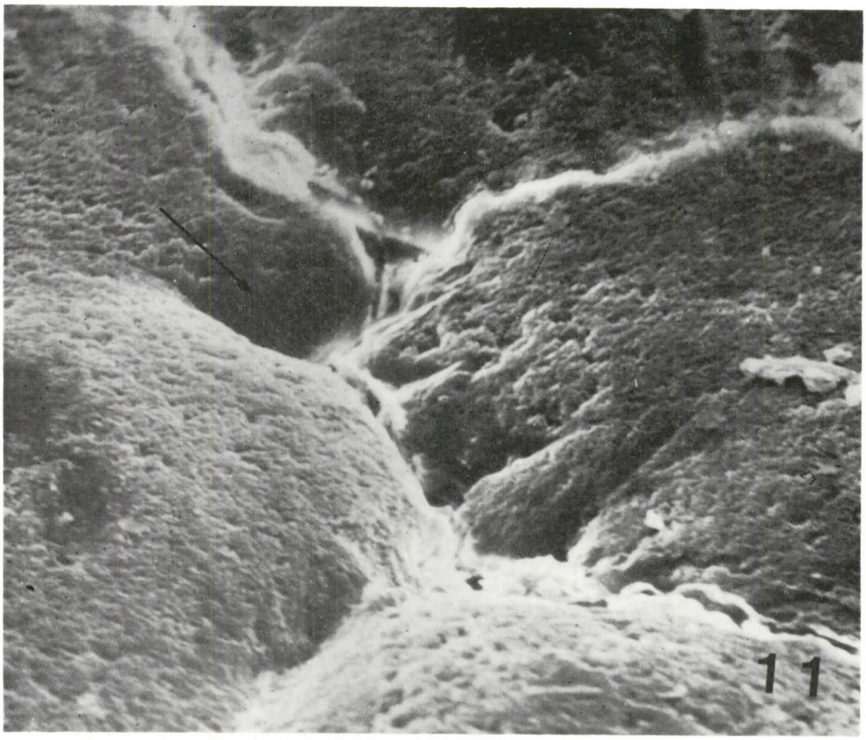

Fig. 11. The outer view of mammillary knobs which have been magnified. Arrow indicates the canal of an air pore. ×2,000.

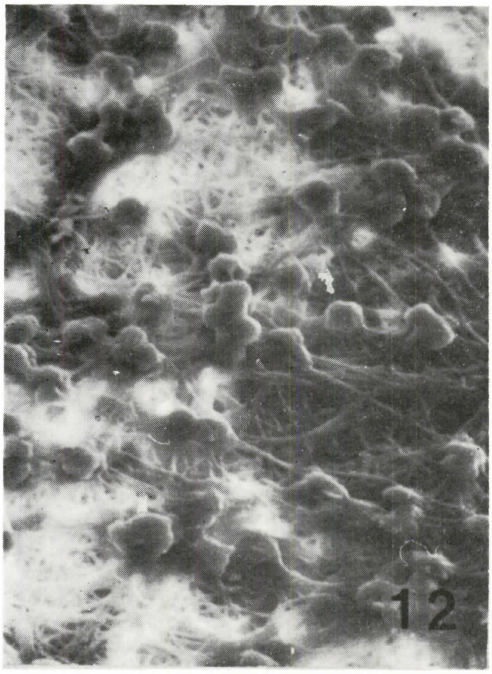

Fig. 12. Organic protrusions remaining after decalcification of the completed mammillary layer. ×300.

Fig. 13. A high-power magnification of Fig. 12. ×1,000.

Fig. 14. Calcite architecture of the inside of the mammillary layer from which organic matter has been removed. ×300.

Fig. 15. A high-power magnification of Fig. 14. ×1,000.

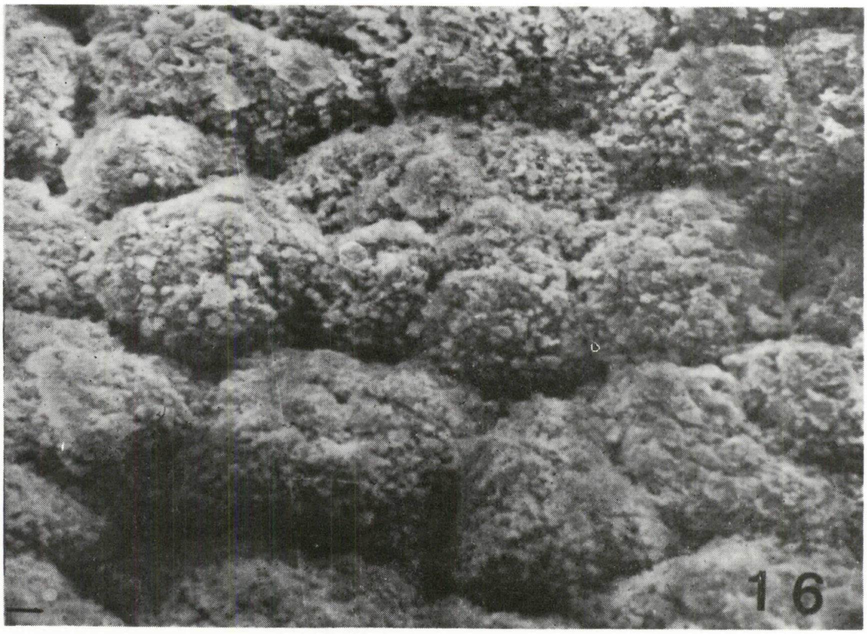

Fig. 16. Calcite architecture of the outside of the mammillary layer from which organic matter has been removed. ×300.

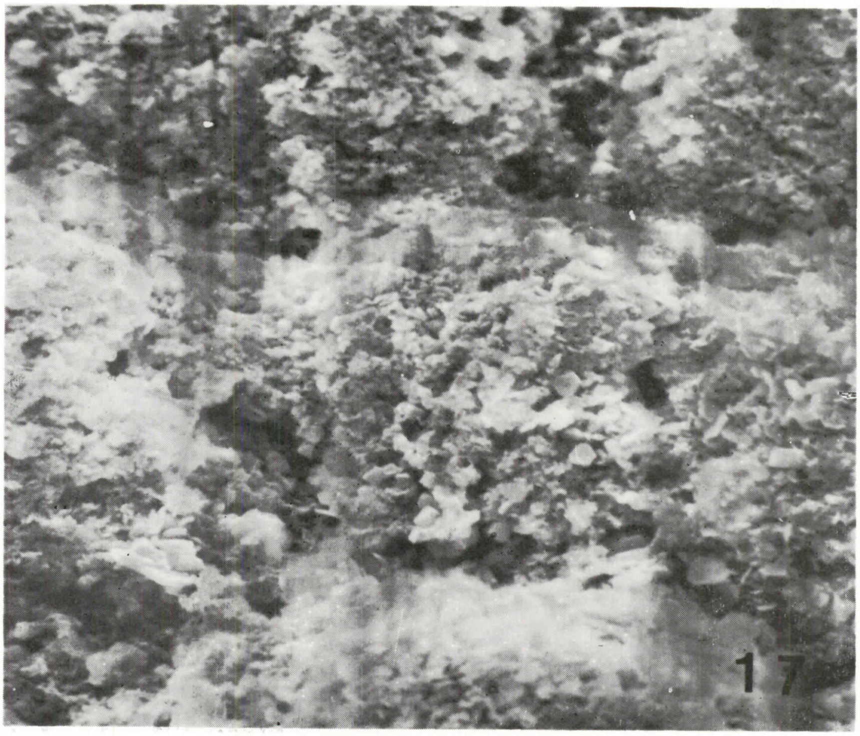

Fig. 17. A high-power magnification of Fig. 16. ×1,000.

Fig. 18. Calcite architecture of the outside of the almost completed spongy layer from which organic matter has been removed. ×300.

Fig. 19. The outer surface of the completed egg-shell after oviposition. Organic matter has been removed from the egg. ×1,000.