

Further Morphological Studies on the Formation and Structure of Hen's Eggshell by Scanning Electron Microscopy

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(Figs. 1-29)*

Morphological studies on the formation and structure of the avian egg shell have been made by a number of workers, including NATHUSIUS (1868,¹⁾ 1871²⁾), STEWART (1935),³⁾ ROMANOFF and ROMANOFF (1949),⁴⁾ SAJNER (1955),⁵⁾ SIMKISS and TYLER (1957),⁶⁾ SCHMIDT (1958,⁷⁾ 1960⁸⁾), MATHER and EPLING *et al.* (1962),⁹⁾ and SIMKISS (1968).¹⁰⁾ These workers studied the egg shell by light microscopy. SCHMIDT (1960,¹¹⁾ 1962¹²⁾), TEREPAKA (1963),¹³⁾¹⁴⁾ EL-BOUSHY and SIMONS *et al.* (1968)¹⁵⁾ studied it by polarized microscopy. MASSHOFF and STOLPMANN (1961),¹⁶⁾ HYEN (1963),¹⁷⁾ SIMONS and WIERTZ (1963),¹⁸⁾ MAYER and BAKER *et al.* (1973)¹⁹⁾ studied the egg shell by electron microscopy. Despite these researches, many problems still remain unsolved about the morphology of the egg shell. A major difficulty for the investigation of the shell consists always in the hardness of its structure. Therefore, it is necessary to approach the problem from a different angle.

The author tried to examine the egg shell by means of the scanning electron microscope. The apparatus was very efficient for the morphological investigation of the egg shell. The results obtained by this method have been reported in the previous papers (1969,²⁰⁾ 1970²¹⁾). The present paper deals with more detailed studies on the morphological aspects of the egg shell by scanning electron microscopy.

MATERIALS AND METHODS

Eggs of White Leghorn hens were used throughout these studies. Generally, the shell was treated in the following manner. It was fixed in a 10 % solution of formalin. Observation was made on its natural state, decalcified state, and the state of removal of organic matters from the shell. The shell was decalcified when

immersed in a 10 % solution of ethylenediaminetetraacetic acid (EDTA) buffered at pH 7. Organic matters were removed from the egg shell by two methods, intense and mild. In the former method, the shell was immersed in a 25 % solution of sodium hydroxide at room temperature for 48 hours. In the latter method, it was boiled in a 10 % solution of sodium sulfide for 5 minutes. In both methods, the treated shell was washed with water and dehydrated by passing through a series of increasing concentrations of acetone. Then it was dried at room temperature. For scanning electron microscopy, it was broken into small pieces, which were coated with gold and examined by a scanning electron microscope, JSM type U (Japan Electron Optics Laboratory, Ltd.), at an accelerating voltage of 15 KV.

1. Observation on Shell Formation

As is well known, the avian egg shell is composed of a shell membrane, a calcified layer or shell proper, and a cuticle from inside to outside. The shell membrane is subdivided into the inner and outer layer, and the calcified layer into the inner mammillary and the outer spongy layer. In his previous papers,²⁰⁾²¹⁾ the author has already described the general structure of the shell and the morphological aspects of shell formation. In the present chapter, closer observation was made on these subjects, particularly on the late stage of the shell formation.

Materials. Eggs completed after oviposition and eggs in various stages of shell formation were used in this study. Eggs in the process of shell formation were collected at random from the uterus of the oviduct, where the egg shell is formed, at a poultry eviscerating plant. They were classified grossly by the stage of shell formation, judging from the appearance and hardness of the shell. The egg shell was treated for observation by the method mentioned above.

Results. The findings in the early stage of shell formation have been described in the previous paper.²¹⁾ They are summarized here for reference. The first sign of shell formation was characterized by the deposition of minute sand-like granules on the fibers of the outer shell membrane. Then, the fibers became indistinct in their outline. After that, a large number of small concretions were produced not uniformly, but more or less dispersedly on the entire surface of the membrane. In a more advanced stage of growing, they developed into rough-surfaced pyramidal concretions. These concretions seemed to be composed of organic matter, since they were not decalcified with EDTA but dissolved in sodium sulfide or sodium hydroxide. They constituted the so-called "organic matrix core" which

early workers had observed by light microscopy. The organic matrix core played a significant role in the early stage of shell formation, because the subsequent development of mammillae occurred on the basis of it. It should be noted that

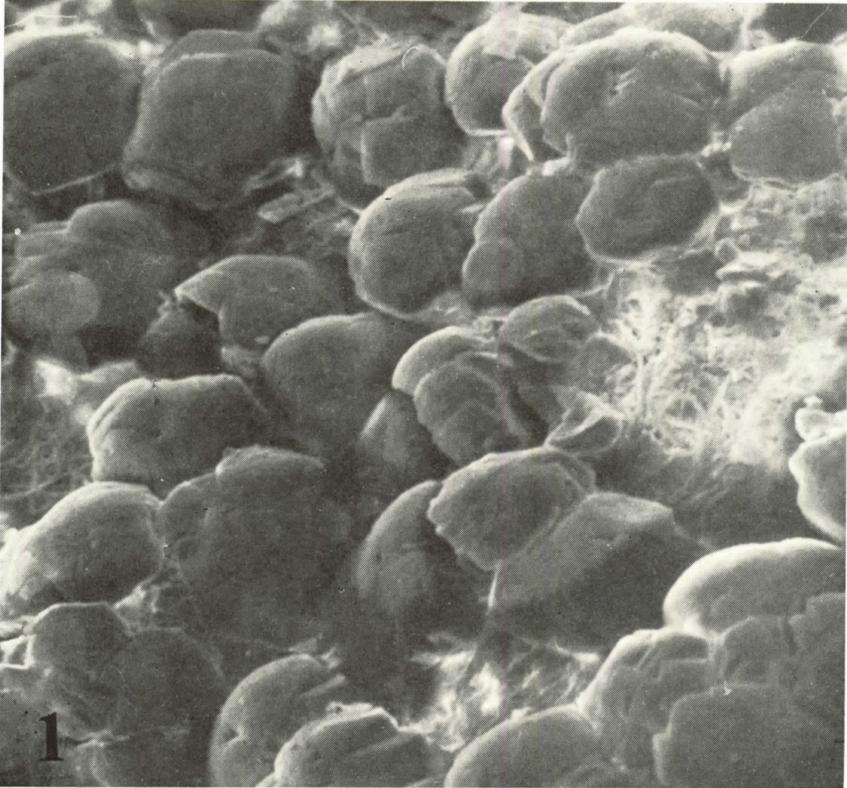


Fig. 1. Mammillae in the early stage of development. They are protruding like domes on the outer shell membrane and located irregularly. Most of them are composed of subunits. $\times 300$.

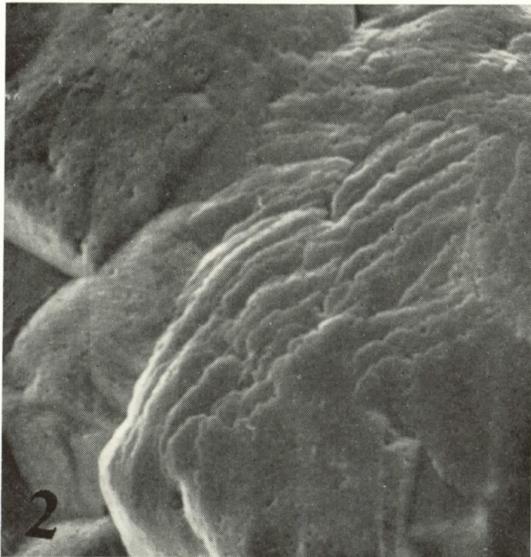


Fig. 2. A high-power magnification of mammillae shown in Fig. 1. Each mammilla has a streaky appearance with minute holes. $1,200$.

the first dispersed seeding of organic concretions on the shell membrane is particularly concerned with the formation of air pores, as mentioned below.

With the progress in the deposition of calcium around the organic matrix core, organic concretions developed gradually into an upward and lateral direction, while they were inhibited from growing inwards. The reason for this phenomenon will be proposed in the following chapter. As a result, the organic matrix core was embedded at the center and encrusted with calcite crystals. The mammillae in this early stage were conical in shape and rested solitarily on the membrane (Fig. 1). As shown in Fig. 1, the outer surface of each mammila appeared smooth at a low-power magnification, but it showed a highly streaky structure with minute holes at a high-power magnification (Fig. 2). This structure was not characterized as yet by any fibrous network within it.

With the advance of the process of shell formation, the mammillae enlarged gradually and the spaces among them became narrower and narrower. At last, they fused with one another to form a so-called mammillary layer (Fig. 3). The outer surface of the completed mammillary layer was uneven, due to the presence of mammillae protruding upward with a convex curvature. In addition, small spaces different in shape and dimension still remained at the site where the mammillae came together. Of them, the smallest spaces tended to close in the course of shell formation, while the relatively larger ones grew into air pores, which remained until the full accomplishment of the shell.

The spongy layer began to form itself on the mammillary layer after the completion of this layer. It was not formed, however, at the same time in an uniform process all over the mammillae. This evidence is shown in Fig. 4. In this figure, two types of mammillae are distinguished: rough-surfaced ones and smooth-surfaced ones. At a high-power magnification, mammillae of the former type were covered densely with large amounts of solid concretions showing an active deposition of calcium, while those of the latter type were not (Fig. 5). From this result, it seems likely that the formation of the spongy layer may be initiated to some extent by some factors, such as the maturity of mammillae.

The spongy layer in the early stage of shell formation was superimposed by a thin, board-like calcified layer which was newly formed (Fig. 7). This suggests that the formation of the spongy layer may have been promoted by the accumulation of the thin layer newly formed. These layers were irregular in form, but generally had a well-defined outline. They were formed first in spaces, including air pores, where mammillae came together. This process seemed to indicate that

the raw material of the spongy layer may have flowed out of the egg through spaces among the mammillae (Fig. 6). Materials originated from some other structures of the egg may probably be related to the formation of the spongy layer in the early stage of shell formation. In Fig. 7, at least three layers are differentiated from one another in the pattern of calcification. The most superficial

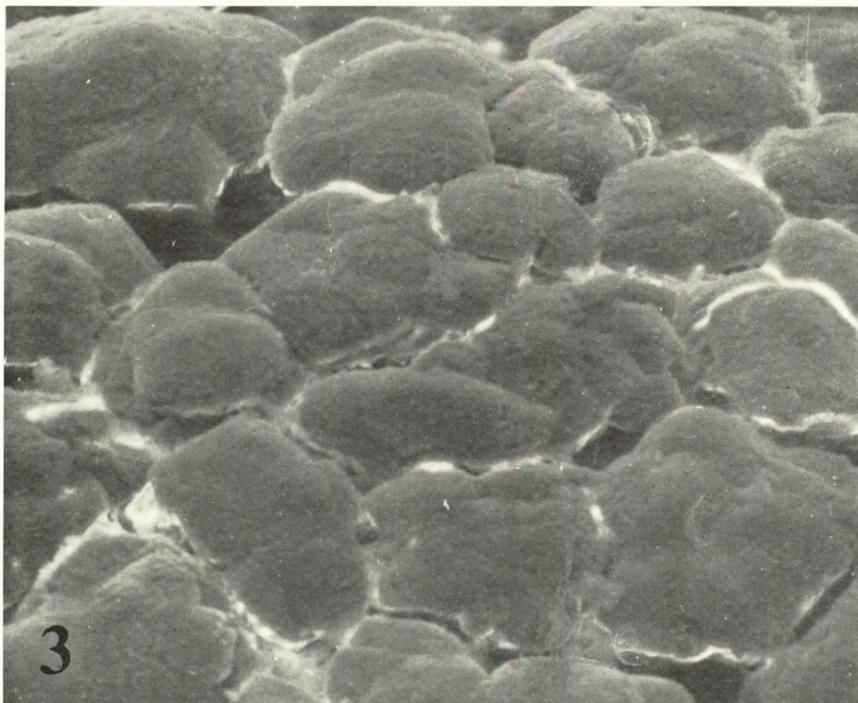


Fig. 3. An outer view of the almost completed mammillary layer. There are spaces varying in form and size in places where mammillae have come together. The outer surface is uneven. $\times 300$.

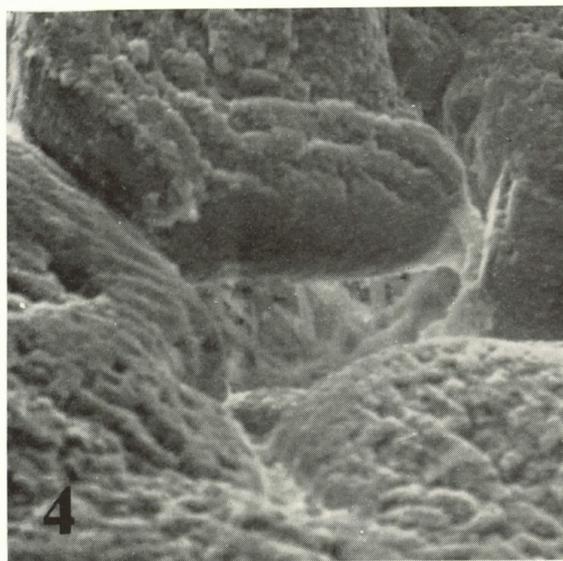


Fig. 4. Mammillae in the early stage of formation of the spongy layer. Mammillae at the bottom of the figure are more active in the deposition of calcium than those in the upper part of the figure. $\times 700$.

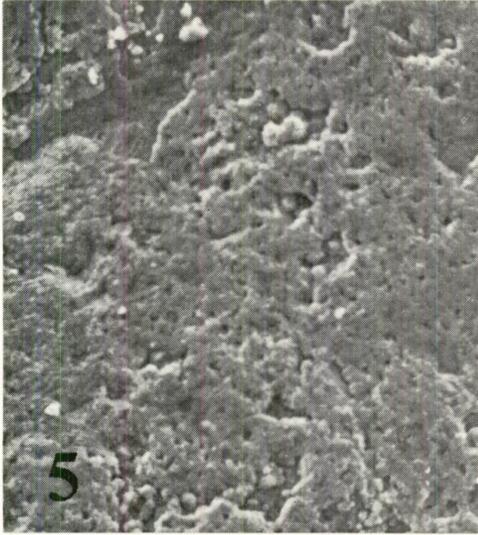


Fig. 5. A high-power magnification of mammillae active in the deposition of calcium. $\times 1,000$.



Fig. 6. Newly formed calcified layer of the spongy layer. It appears to have been squeezed out from the egg through the air canal. $\times 700$.



Fig. 7. Spongy layer in the early stage of formation. Thin, board-like calcified layers newly formed are superimposed layer upon layer. Superficial layers are coarser and more porous than deeper ones. $\times 200$.

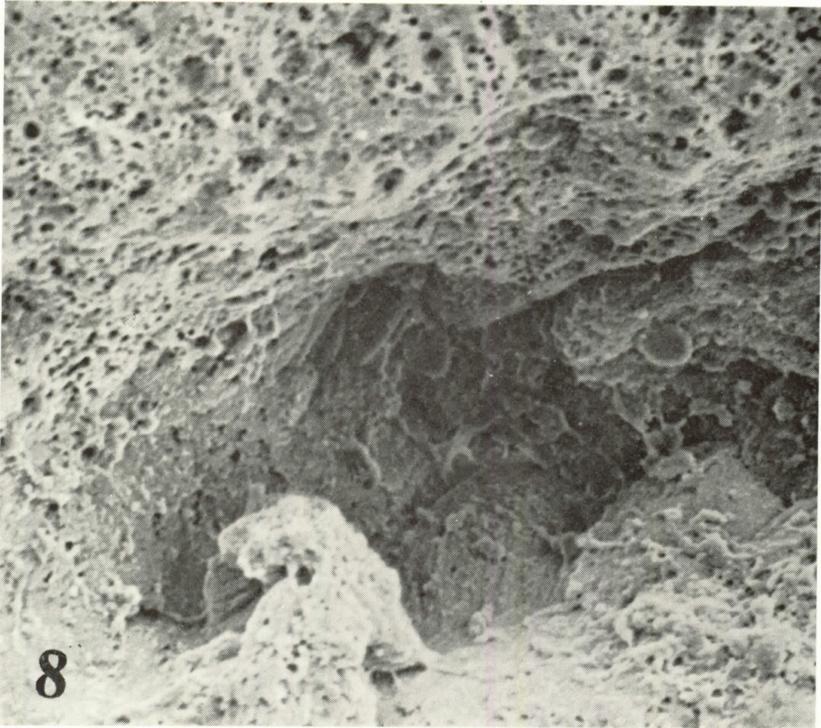


Fig. 8. A high-power magnification of the porous superficial layer shown in Fig. 7. This layer consists of a coarse network of matrix fibers and is cemented slightly with calcium. $\times 2,000$.

layer is highly porous, but the deeper layers are dense in texture. At a high-power magnification, the former layer appears to be made of a loose network of fibrous structure with meshes cemented with small amounts of calcium (Fig. 8). On the other hand, the latter layers are composed of more densely packed fibers, and the fibrous network has almost disappeared from them due to the cementing of meshes with calcium. This indicates clearly that the deeper layers are more calcified than the superficial layer. An intimate fashion showing the process of shell calcification is presented in Fig. 9. In this figure, two types, outer porous and inner dense, of newly formed layers are visible.

When observed at a high-power magnification, the outer porous layer had numerous fine rod-shaped processes and, at places, relatively large holes (Fig. 10). The processes stood vertically to the surface of the shell. They were about $0.1\text{--}0.3\ \mu$ in width. A question arose whether they were matrix fibers of the spongy layer or growing calcite rods. In their electron microscopic studies on the decalcified egg shell, SIMONS and WIERTZ¹⁸⁾ had found that the matrix fibers of the spongy layer were about $0.1\ \mu$ in diameter. The author (1969)²⁰⁾ reported from

his scanning electron microscopy of the decalcified shell that these fibers were composed of a network of main fibrils and collateral fibrils anastomosing with one another and that they were about $0.1\text{--}0.3\ \mu$ in diameter. Judging from these structures of matrix fibers, the rod-like processes may be growing matrix fibers. The inner dense layer was fully mineralized, with only a few minute canals remaining. Thus, growing matrix fibers were encrusted successively by the deposi-

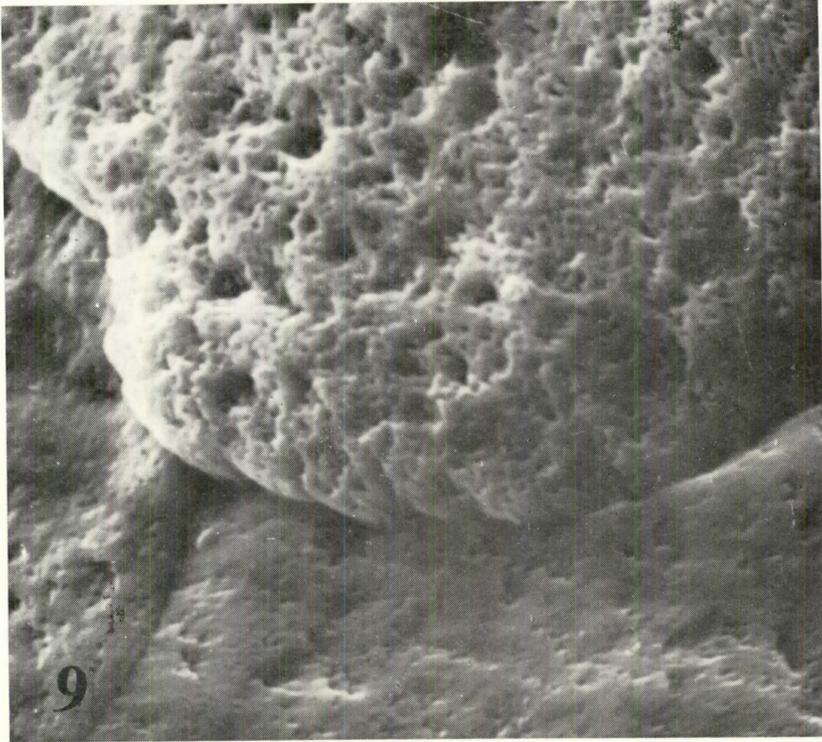


Fig. 9. Newly formed calcified layer of the spongy layer. The upper portion of the figure shows growing matrix fibers, the lower portion a highly mineralized layer cemented with calcium. $\times 2,000$.

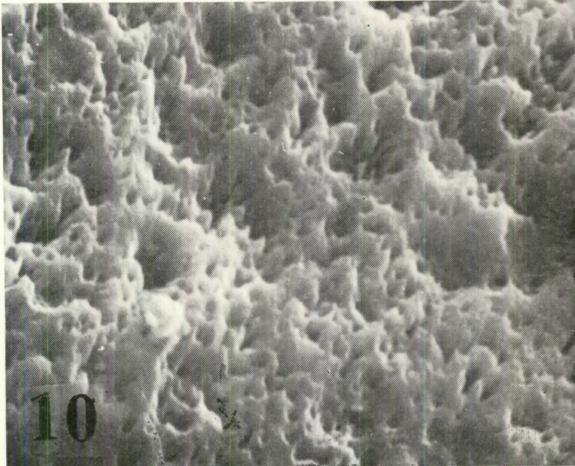


Fig. 10. A high-power magnification of growing matrix fibers shown in Fig. 9. Rod-like projections stand vertically on the shell surface. They are matrix fibers enveloped with calcium. $\times 3,500$.

tion of calcium to form a dense layer.

With the advance in the process of shell formation, the outer surface of the spongy layer became almost even and uniformly dense (Fig. 11). This change suggests that the accumulation of new calcite layers may have stopped, since it reached a certain portion of the entire thickness of the shell, and that only the deposition of calcium may have proceeded after that. The egg shell completed after oviposition became entirely dense and solid in the outer-surface view, as shown in Fig. 12.

From the above-mentioned morphological observation on shell formation, it may be concluded that there was a principal difference in the process of calcification of the shell between the mammillary and the spongy layers. In the pattern of calcification, the former exhibited an expanding growth around the organic matrix core of the mammilla, and the latter an additional growth upon layers newly formed. In addition, in the process of shell formation, the former was characterized by slow progress in mineralization without any definite pronounced formation of matrix fibers, whereas the latter showed rapid progress in growth and

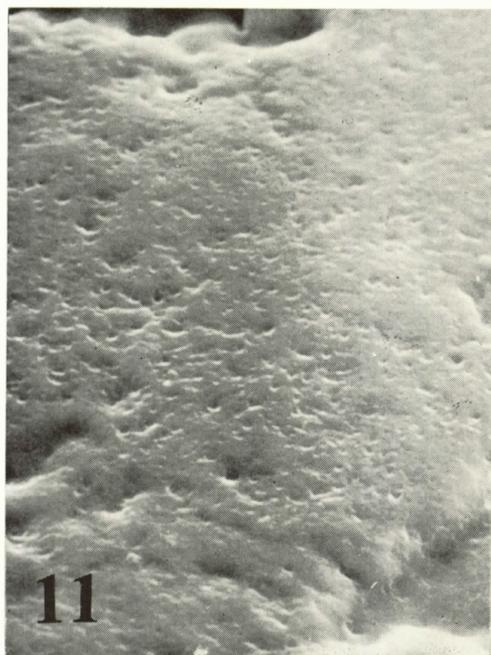


Fig. 11. An outer surface view of the almost completed spongy layer. The surface of the layer is nearly even and seems to be considerably dense and compact in texture. $\times 500$.



Fig. 12. An outer surface view of the shell after oviposition. The shell has a compact and solid appearance. Many air pores are open in shallow grooves on the shell surface. $\times 1,500$.

was always accompanied by newly produced matrix fibers. The process of formation of the spongy layer could be divided, though indistinctly, into two steps or stages. The primary or preliminary step was chiefly the formation of matrix fibers, due probably to raw materials originated from some structures of the egg. The primary step ended on the way of the formation of the spongy layer. The secondary step was the cementing with calcium salts of the network of the matrix fibers formed in the preliminary step. It continued until the end of full formation of the egg shell. COOKE and BALCH (1970)²²⁾ had reported that the matrix of the spongy layer was so abundant as to reach about one-third of the way into the shell. Their results may be interpreted by the morphological findings obtained throughout the course of shell formation as presented in this investigation.

II. Observation on Mammillae

In the observation on the shell formation mentioned above, it was particularly

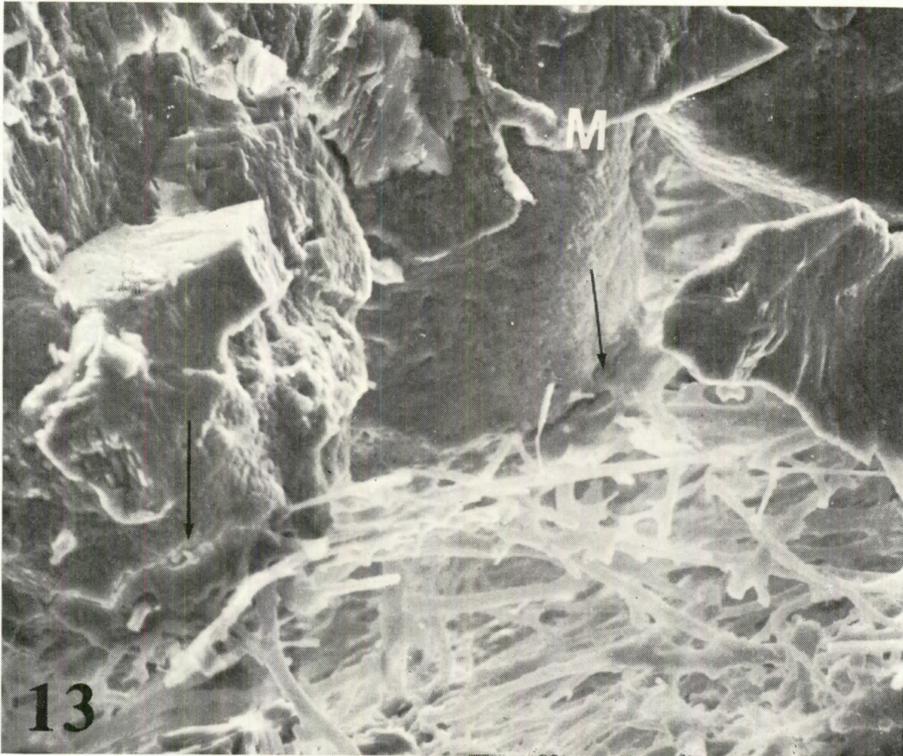


Fig. 13. Radial plane of a natural eggshell. Mammillae are situated on the outer shell membrane, with their tips embedded in the membrane. Notice the entrance of fibers from the membrane into the center of the tip. Arrows indicate the boundary between the tip and the rest of the mammilla. Letter M shows a torn envelope enclosing a mammilla. $\times 800$.

observed that the mammillae played an important role on the subsequent formation of the spongy layer. Moreover, the structure of the mammillae, especially the mammillary core, had long been a subject of discussion from the viewpoint of light microscopy. For this reason, the author tried to examine the structure of the mammillae in more detail. The present chapter deals with the structure of the mammillae as studied by scanning electron microscopy.

Materials. The materials used in this study have been collected from completed egg shells after oviposition. The egg shell was examined in its natural state and the state after removal of the organic matters.

Results. In the natural egg shell, the mammillae were hardly visible from the inside of the shell, since they were covered with an outer shell membrane. The radial plane of the shell, however, revealed the entire picture of the mammillae distinctly (Fig. 13). The mammillae were pendent from the eggshell proper with a wide base, and their tips rested on the outer shell membrane. Each mammilla



Fig. 14. An inner surface view of the shell from which organic matters have been removed. Mammillae are protruded rather regularly and different in shape from one another. Each mammilla has a cavernous structure varying in size and form at the tip. $\times 300$.

consisted of a narrow apex and the remaining wide base (hereafter referred to as the body of the mammilla). The apex is the mammillary core, as mentioned above. The mammillary core was embedded slightly in the outer shell membrane. At the apex of the mammillary core, some of the fibers derived from the outer shell membrane entered the center of the core clearly through canaliculi lying at the tip or on the side of each mammilla. This indicates that the mammillary core and the shell membrane are joined intimately with each other by means of a fibrous connection. The presence of the fibrous connection between the two structures has been shown by early workers by light microscopy. The present scanning electron microscopy, however, demonstrated the presence of such close fibrous connection as this more clearly and definitely.

When Fig. 13 is observed carefully, there is a slight crack just above the mammillary core. This crack shows the boundary between the mammillary core and the body of the mammilla. Even though the crack was induced by shrinkage of the mammillary core during the preparation of samples, it suggested that the junction between the core and the body of the mammilla may be loose. The two structures may probably be made of materials more or less heterogeneous to each other. On the other hand, the body of the mammilla appeared to be enclosed by a cuticle-like covering, which is indicated as a tearing membrane in the figure.

In the shell which had been treated slightly with sodium sulfide, the mammillae were visualized more clearly than in the untreated shell (Fig. 14). They protruded from the shell proper with fair regularity. They had all alike a cap-like structure on the tip, which was the mammillary core as observed above. Each mammilla had principally one core or sometimes two or more cores which had fused with one another. It was variable in size and form, depending upon the manner of fusion of the cores. A typical mammilla with one core was conical in shape. A large mammilla with some fused cores generally looked like a molar tooth. This type of mammilla is obvious to have been formed by such fusion of the bodies of some mammillae as corresponding to the fusion of cores. A clearer evidence for this change is given in Fig. 1, which presents mammillae in the early stage of formation. As is seen in this figure, most of the mammillae were formed by some subunits, though they might have been changed a little in the process of preparation of the samples. At any rate, the size and form of the mammilla seem to be determined by the first arrangement of organic cores on the shell membrane. As mentioned in the preceding chapter, matrix cores were formed disproportionately on the outer shell membrane. Consequently, it seems likely that mammillae may be

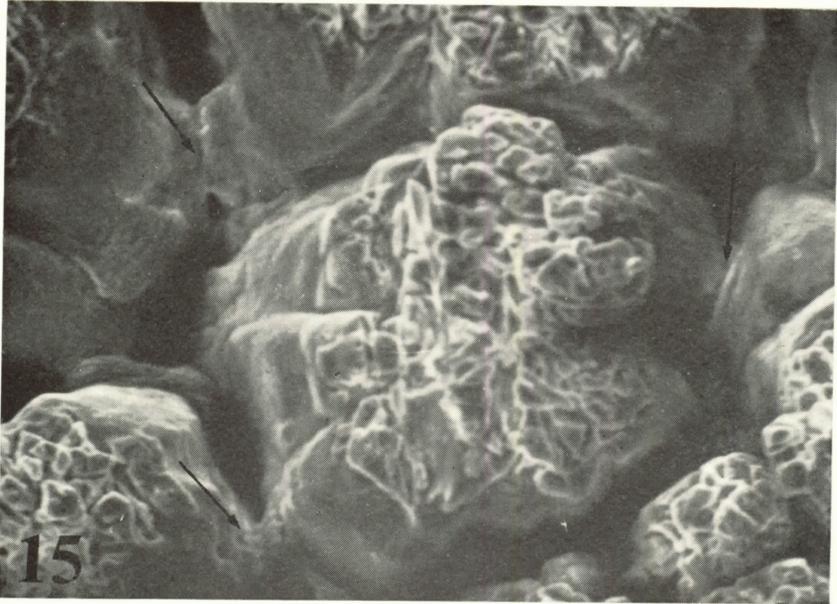


Fig. 15. A high-power magnification of the mammillae shown in Fig. 14. In the figure, molar-shaped mammillae are visible. Arrows indicate the connection between individual mammillae. $\times 800$.

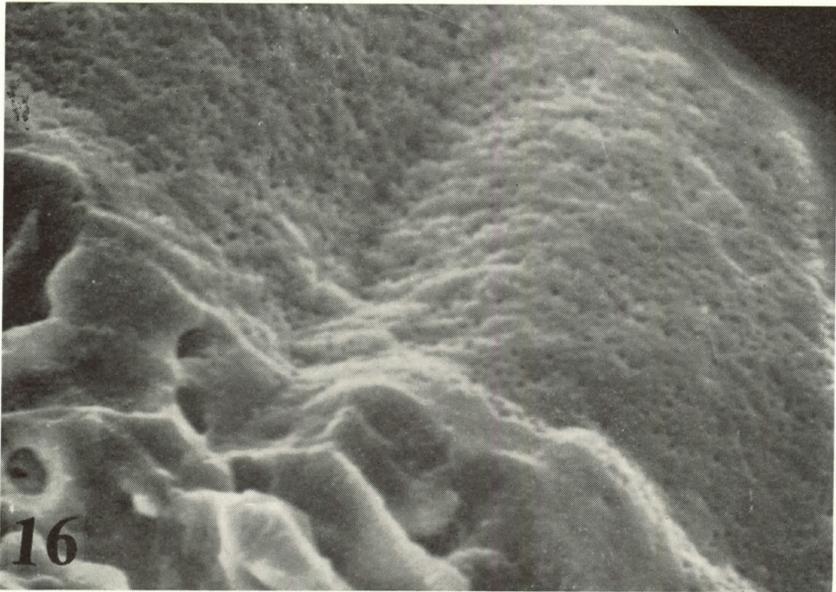


Fig. 16. A high-power magnification of a mammilla. The tip has canaliculi and appears to be more solid and dense than the wall. The wall consists of a network of fibrils and is cemented slightly with calcium. $\times 3,000$.

fused with one another to correct their irregular arrangement, and that this change may serve for the development of a well-arranged mammillary layer.

At a high-power magnification, the mammillary core showed a complicatedly incised structure with numerous canaliculi (Figs. 15 and 16). Judging from its compact and solid appearance, this canaliculated area seemed to have considerably been mineralized. The strange structure of the outer surface of the core is clear to have been brought about by the roots of fibers connecting the core and the outer shell membrane, as pointed out previously. The exact structure of the core could be represented by a mould preparation, as mentioned below in detail (Fig. 29). As is clear from this figure, the honeycomb structure, that is the replica of mammillae from which organic matters have been removed, has a pyramidal protrusion, *i. e.*, a mammillary core, and numerous fibrous roots of connecting fibers entering the core.

In a radial plane through the mammillae, the mammillary core is made of a superficial solid, canaliculated structure and an inner roughly cavernous structure. The former is a mineralized covering which encloses an organic core and the

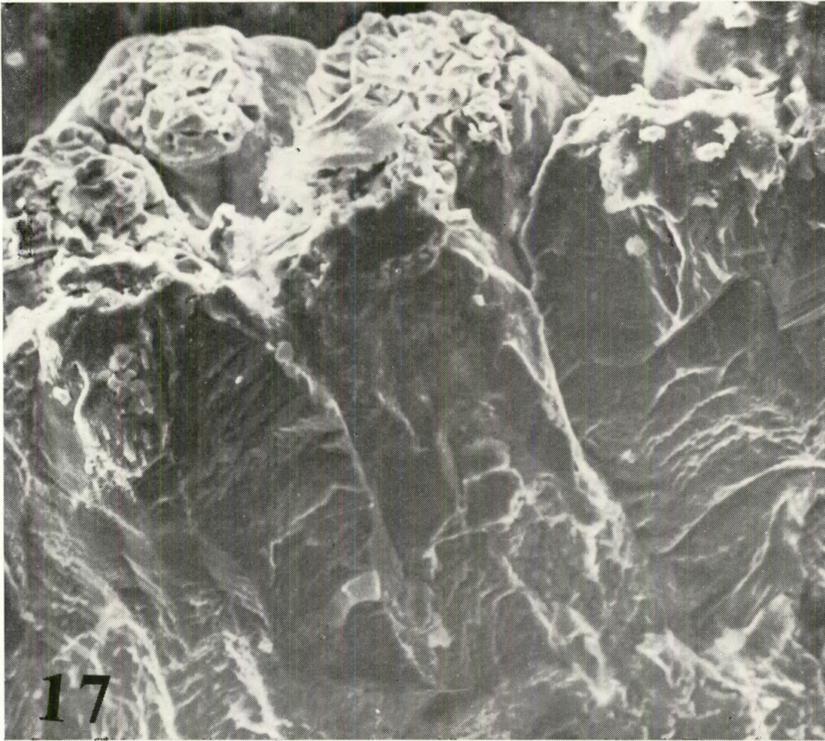


Fig. 17. Radial plane of the mammilla from which organic matters have been removed. Just below the cavernous structure are visible empty complex hollows in which organic matrix is situated. The body of the mammilla has striated structures extending radially from the central axis. $\times 500$.

latter a site where the organic core is present. From his light and polarized microscopic studies, TEREPA¹⁴) found that within the calcified tip of the mammilla there was an "organic matrix core" surrounded by a membranous ring encrusted with large calcite crystals. The results of the present investigation confirmed these findings of TEREPA.

The body of the mammillae was composed of a large central calcified part and such a peripheral cuticle-like covering as illustrated in Fig. 13 (Fig. 17). The central part of the mammilla is the site corresponding to the "dark area", name given to this part by early workers in their light microscopic studies. Under scanning electron microscopy, it shows itself as a striated structure when viewed from certain angles, but as a vague structure viewed from other angles (Fig. 17). The striations extend radially from the central axis. Judging from the diagrammatic representation of the fowl's egg shell, published by SIMKISS and TAYLER

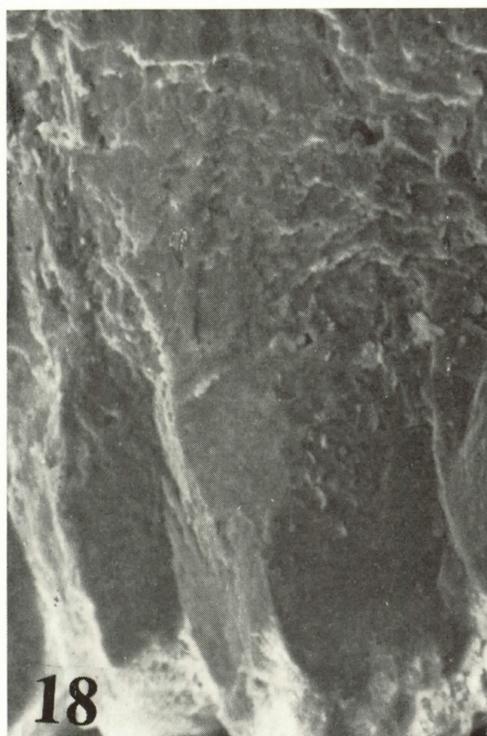


Fig. 18. Radial plane of the shell from which organic matters have been removed heavily. Notice large cavities in the central region of the mammilla from which organic matters have been removed by dissolution. $\times 200$.

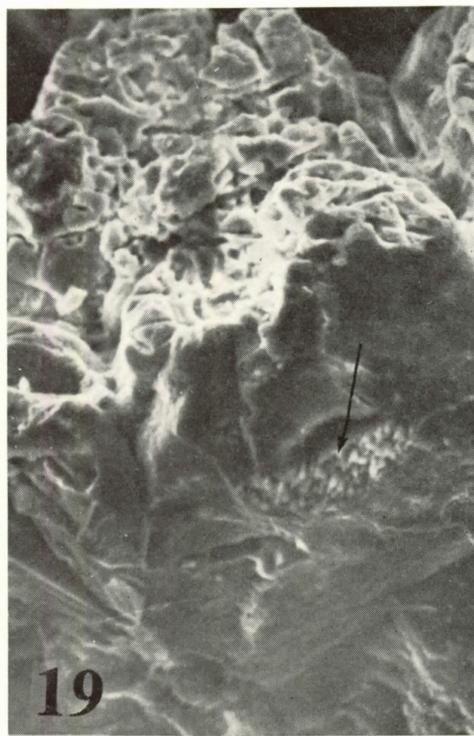


Fig. 19. Radial plane of the mammilla from which organic matters have been removed. Arrow indicates the end of an organic bridge which connects one mammilla with another. Notice that the bridge is composed of bundles of fibrils. $\times 500$.

(1971)²³), the striations seem to be calcite rods. In mammillae which had been treated vigorously with sodium hydroxide, the central portion became a large cavity (Fig. 18). TEREPAKA¹⁴) found that the "dark area" had been decalcified more readily than the remainder of the mammilla. This finding, as well as the results of the present studies, indicate that the central part of the mammilla is not mineralized so highly as the rest of the mammilla.

The cuticle-like covering appeared feeble and somewhat porous. At a high-power magnification, it seemed to be a coarse network of fibrils, cemented with small amounts of calcium (Fig. 16). Occasionally, a part of the covering joined with one of the adjacent mammillae at certain levels (Fig. 15). The resulting bridges were composed of rod-like processes, which might probably be fibrils forming bundles (Fig. 19).

A more definite evidence showing the morphological features of the mammilla



Fig. 20. An outer surface view of the mammilla in the early stage of development. The specimen has been decalcified. Notice a membranous structure and an aggregate mass remaining at the bottom of the figure. $\times 500$.

was observed in the decalcified mammilla in its almost completed stage (Fig. 20). As is seen in this figure, the mammilla remains unchanged only on the outer covering and small packed concretions containing fibrillar structures are present at the bottom, while the central portion is empty. The fibrillar structures of the concretions may be fibrils connecting the core and the body of the mammilla. This finding suggests clearly that the outer covering and the small residue, i. e. the organic core at the bottom, may have the same organic character, because both of them are resistant to decalcification.

In turn, the eggshell which had been treated with sodium hydroxide revealed obvious changes in the structure of the mammilla (Fig. 21). The most striking changes were the existence of a large depression at the tip and sharp etching of the sloping wall of the mammilla. They are obviously responsible for the resolution of the mammillary core and the outer covering, since these structures are of organic character. All these evidences lend decisive support to the opinion that the mammillary core as well as the outer covering which encloses the body of the mammilla may be highly organic. It is of interest to note that the mammilla is surrounded by an organic envelope, which allows the mammilla to extend in all the directions, except the inward direction, from the mammillary core. This core prevents the mammilla from growing inwards, as its internal surface is highly mineralized in the the early stage of formation, as mentioned above.

III. Observation on Hatched Eggshells

A number of investigators (SAJNER⁵), TYLER and SIMKISS²⁴), and TEREPA¹⁴) have pointed out that the shell membrane is detached from the shell on the 15th or 16th day of incubation, apart from such portion of the membrane as associated with the air cell. The membrane is separated at the core-mammillary boundary, accompanied by the mammillary core. It is not known as yet, however, how the liberation occurs to the hatching eggshell or how the membrane is liberated at this boundary. The manner of connection of the core-mammillary junction may probably be concerned with the liberation of the membrane. In order to solve this problem, the present investigation was performed on the core-mammillary junction in the hatched eggshell.

Materials. Eggs immediately after hatching were used. From the hatched shell, the outer shell membrane was stripped off with a pair of forceps. Separation of the membrane was very easy. Small pieces of the shell and the separated membrane were observed under the scanning electron microscope.

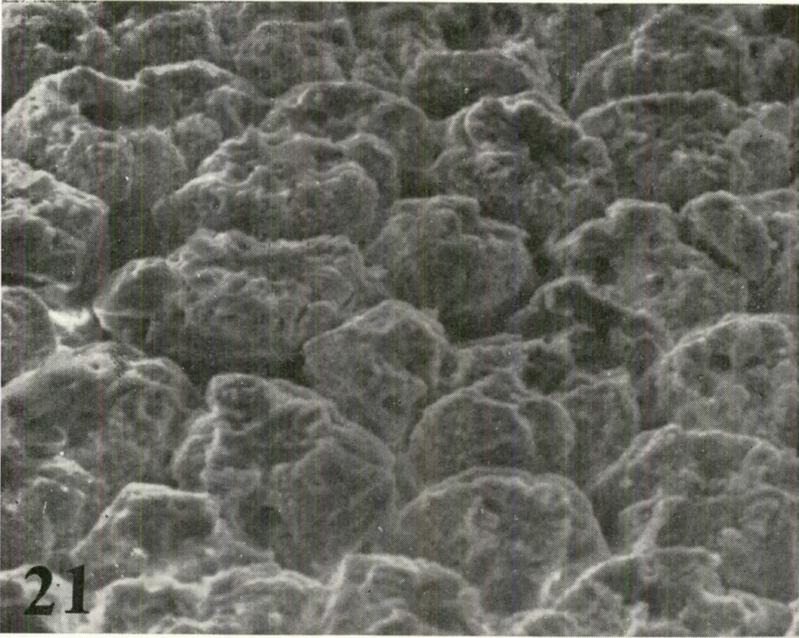


Fig. 21. An inner surface view of the shell from which organic matters have been removed heavily. Each mamilla has a large central depression at the tip and a sloping wall severely etched by removal of organic matters. $\times 300$.



Fig. 22. An outer surface view of the outer shell membrane liberated from the shell during the incubation period. The shell membrane has numerous concretions originated from the shell. $\times 300$.

Results. Fig. 22 shows the outer surface of the separated shell membrane. As is seen in this figure, the separated membrane has rough, angular concretions attached firmly to its surface. These concretions are the cores of the mammillae which have been carried by the shell membrane at the time of liberation. Whereas, the inside of the opposite shell has changed markedly in structure (Fig. 23). The most striking change is a large round depression at the top of the mammilla. The depression is clear to have been brought about by the removal of the mammillary core, because its shape is similar to that of the mammilla of the shell from which the mammillary core has been removed experimentally by the action of sodium hydroxide, as demonstrated in Fig. 21. From this result, it is confirmed that the liberation of the shell membrane occurred at the core-mammillary junction.

When the changed mammillae were observed at a high-power magnification, most of them had numerous rod-like projections on the concaved surface of the depression (Fig. 24). The projections seemed to be stretched radially from the center of depressions to the body of the mammillae. Particularly, they had irregular-shaped ends which were split mechanically. They were not calcite rods. Their split ends were composed of matrix fibers which were connected with the core-

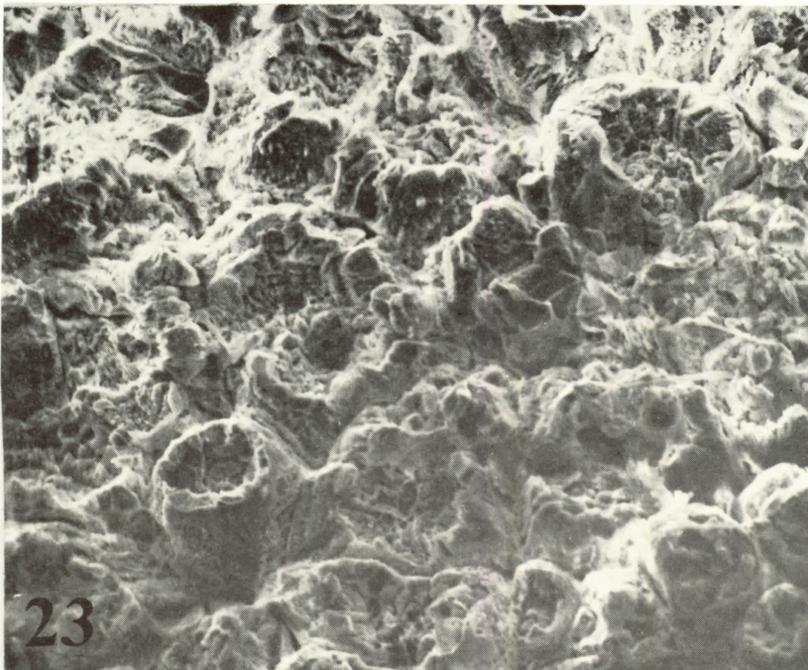


Fig. 23. An inner surface view of the hatched eggshell. Each mammilla has a large depression at the tip from which the matrix core has been liberated. $\times 300$.

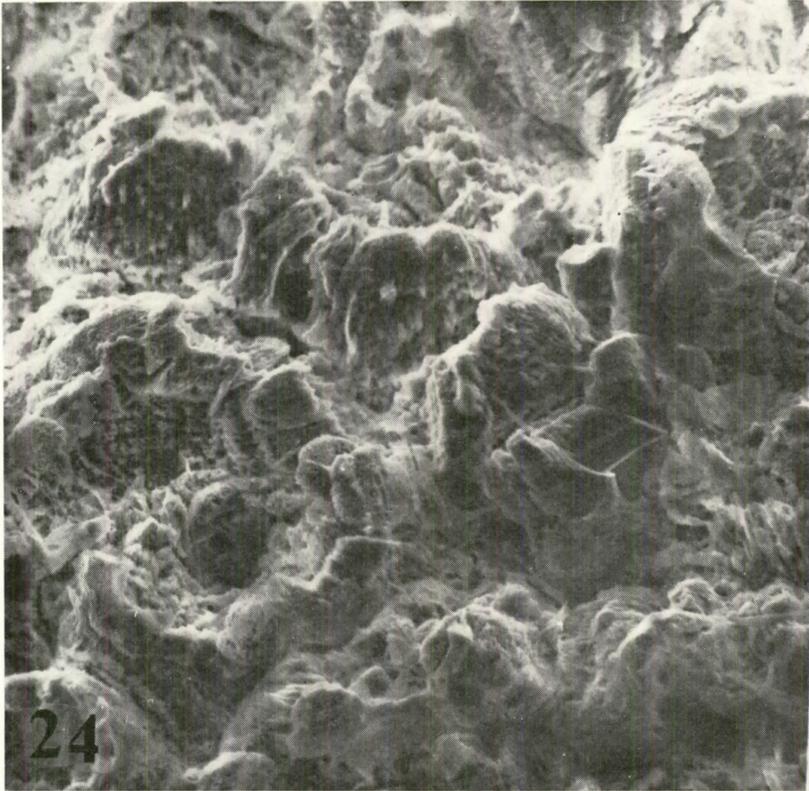


Fig. 24. A high-power magnification of Fig. 23. Large depressions have been produced at the tip of the mammillae by liberation of the mammillary cores. They have numerous rod-like projections. $\times 500$.

mammillary junction. From what is shown in Figs. 20 and 24, it was concluded that this core-mammillary junction was connected loosely with fibrils and had a weaker structure than the core-membrane juncture which was connected firmly with the outer shell membrane.

Concerning the liberation mechanism of the shell membrane during the incubation period, there are various opinions at present. BUCKNER and MARTIN *et al.* (1925)²⁵⁾ and JOHNSON and COMAR (1955)²⁶⁾ suggested that carbonic dioxide produced by the developing embryo might dissolve shell calcium and cause the liberation of the membrane. SAJNER⁵⁾ and TEREPAKA¹³⁾ speculated that the mammillae might be dissolved chemically by some enzymes or some actions within the mammillary core. TYLER and SIMKISS²⁴⁾, however, denied all these presumptions on the basis of their histochemical studies and observation on a plastic mould of the hatching eggshell.

From the above-mentioned morphological findings of the hatched eggshell, it

was possible to conclude that the liberation of the shell membrane was caused mainly by such mechanical attractive action as the constriction of the outer shell membrane, even though it took place chemically to some extent. When such attractive action acts on the shell membrane, separation will occur more easily in the core-mammillary boundary than in the core-membrane boundary, judging from the manner of connection of each boundary mentioned above.

As is well known, during the embryonic development, the inner shell membrane, which normally joins firmly with the outer shell membrane except around the air cell, is in intimate contact with the extra-allantochorionic membrane rich in blood vessels. By the formation of the embryonic membrane, it possibly attracts the outer shell membrane to its side, while its action does not work on the area around the air cell, because both membranes are naturally separated there from each other. For these reasons, the shell membrane is liberated during the incubation period, except the portion around the air cell.

IV. Observation on the Air Pore

The eggshell has a large number of minute canalicules, which are known as air pores. The morphological structure, size, and distribution of the air pores have been studied by early workers, including NATHUSIUS^{1,2)}, STEWART³⁾, ROMANOFF and ROMANOFF⁴⁾, and TYLER (1953²⁷⁾, 1955²⁹⁾, 1969³¹⁾). All these workers observed the air pores in cross sections of decalcified eggshells or stained specimens of eggshell. Exceptionally, TYLER (1953,²⁷⁾ 1956³⁰⁾) made an attempt to examine the air pore by preparing the mould of the air pore with plastic "Kaldock". As a result, he achieved an excellent figure showing the structure of the air pore. His mould, however, was presented in drawn pictures, since such mould could not be demonstrated easily in microphotographs. Using methods like that of Tyler, the three-dimensional structure of the air pore as presented by a methylmethacrylate cast was examined by scanning electron microscopy in this present investigation.

Preparation of samples. The eggshell completed after oviposition was broken into two pieces. Then the shell membrane adhering to the inside of the shell was grossly stripped off from each piece with a pair of forceps. It was treated with sodium hydroxide in the same manner as described in the chapter of materials and methods. The resultant material was washed thoroughly in running tap water for 24 hours and dried at room temperature. A half-sized eggshell was used. It was placed on a board with a hole adapted to its shape. A methylmethacrylate solution was poured in the inside of the shell. Then the specimen

was kept in an incubator at 80°C for 8 hours or more until the solution was solidified.

The resin of methylmethacrylate was prepared by the method of MURAKAMI (1971)³². Methylmethacrylate ester monomer, which is free of hydroquinone, was supplemented with about 1 % benzoyl peroxide and heated for 5 minutes until the temperature reached 80°C. After that this mixture was cooled off rapidly with water. Immediately before use, it was supplemented with 1.0–1.5 % dimethylaniline. An excellent sample was obtained when the viscosity of the resin and the passage of organic matter through the air pore had been adjusted. The viscosity of the resin was the most suitable when it was the same as that of glycerin. If the collodion is too low in the viscosity, it will be extruded through the air pore. If it is too high, it will not enter the air pore. The collodion cast thus prepared was immersed in a 5 % solution of nitric acid in order to dissolve the calcified shell. After the shell was dissolved completely, it was washed thoroughly in water. Small pieces of the collodion cast were examined under the scanning electron microscope.

Results. Fig. 12 shows the air pores of the completed egg shell from which organic matter has been removed. In it, the air pores are wide open on

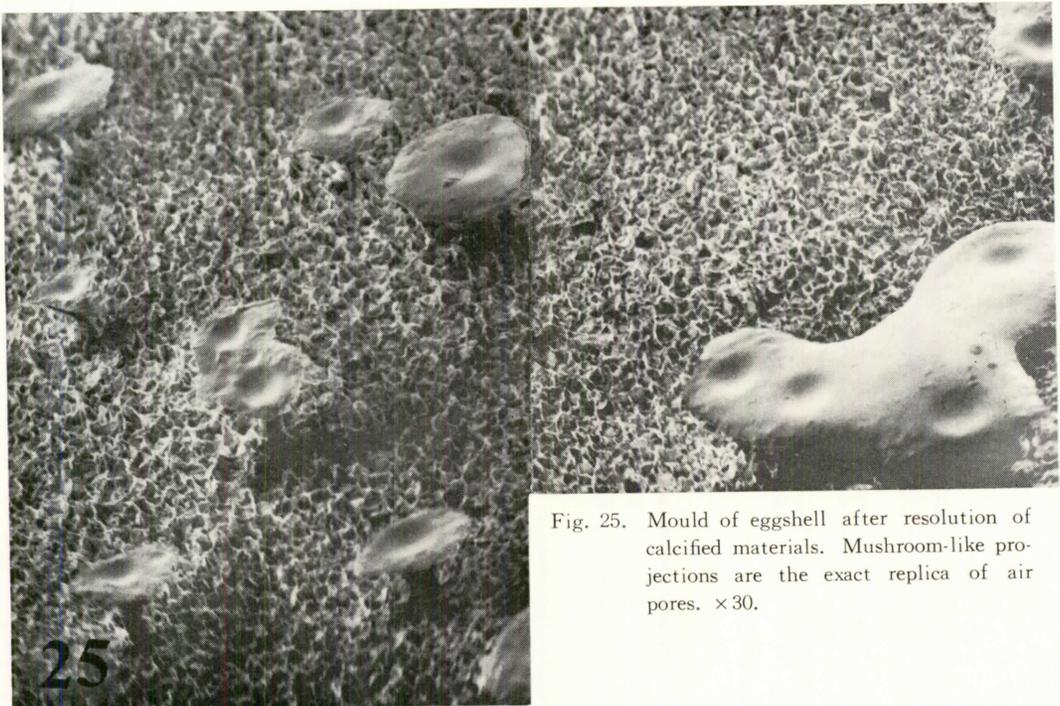


Fig. 25. Mould of eggshell after resolution of calcified materials. Mushroom-like projections are the exact replica of air pores. $\times 30$.

the shell surface. Their outer orifices are variable in shape and size. They are generally found at the bottom of shallow depressions on the shell surface separately or in groups.

The resin cast of the eggshell is presented in Fig. 25. As shown in this figure, a number of mushroom-like projections stand on the honeycomb-like structure. The projections exhibit the complete replica of the air canal. The umbrella-like enlarged portion at the tip of each projection corresponds to the shallow groove around the orifice mentioned above. The air canal has its origin from the mammillary layer as an inner orifice and runs straight through the entire thickness of the eggshell proper. It opens as an outer orifice. It is round in shape and the smallest in dimension at its origin. It is principally single and sometimes branches in two or three at its orifice (Figs. 26 and 27). These findings are different from those obtained by ROMANOFF and ROMANOFF⁴⁾ and TYLER³⁰⁾, who mentioned that the air canal gave off no branches in the hen's eggshell.

At a high-power magnification, a number of blind canals which do not stretch unto the outer surface are observed (Figs. 28 and 29). They have the appearance of triangular spikes varying in height. Occasionally, there are aborted canals which remain at higher levels of the shell thickness than the blind canals. The aborted canals are not so sharp at the tip as the blind ones. The presence of various types of canals within the shell has already been observed light microscop-



Fig. 26. An air pore having two canals. For explanation see Fig. 25, $\times 50$.



Fig. 27. An air pore having three canals. For explanation see Fig. 25, $\times 50$.

ically in cross sections of the shell by early workers. The present mould preparation of the shell made it possible to reveal the three-dimensional structure of the air pore.

It is of interest to note that various types of canals arise all alike from spaces where two or three mammillae come together. Generally, the true air pore tends to arise from a wide space among the mammillae, whereas abnormal pores usually arise from a narrow space. The abnormal canal is undoubtedly different from the true air pore, although it must have played the same role as the true air pore until they were obstructed by the deposition of shell materials. A suggested answer to this problem was mentioned in the preceding chapter.

TYLER³⁰⁾ raised the question of why some pores became closed by the succeeding deposition of shell materials, while others remained open. In connection to this question, ROMANOFF and ROMANOFF⁴⁾ described in their book titled "Avian Egg" that pores were formed in the places where the egg was in contact with the uterine epithelium. This description suggests that the uterine epithelium may be responsible for the formation of the air pores, although no definite evidence was given at all. TYLER³⁰⁾ speculated that three answer were possible to this problem. The one that he considered as the most possible was that the continual passage of

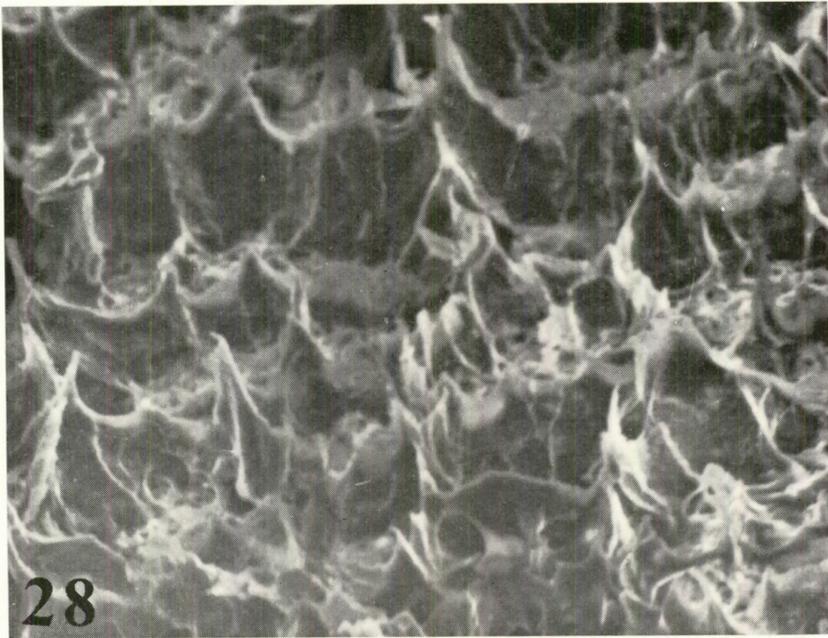


Fig. 28. Blind air canals in the mould preparation of eggshell. They look like triangular spicks. Notice that they arise from spaces where two or three mammillae have come together. For explanation see Fig. 25. $\times 300$.

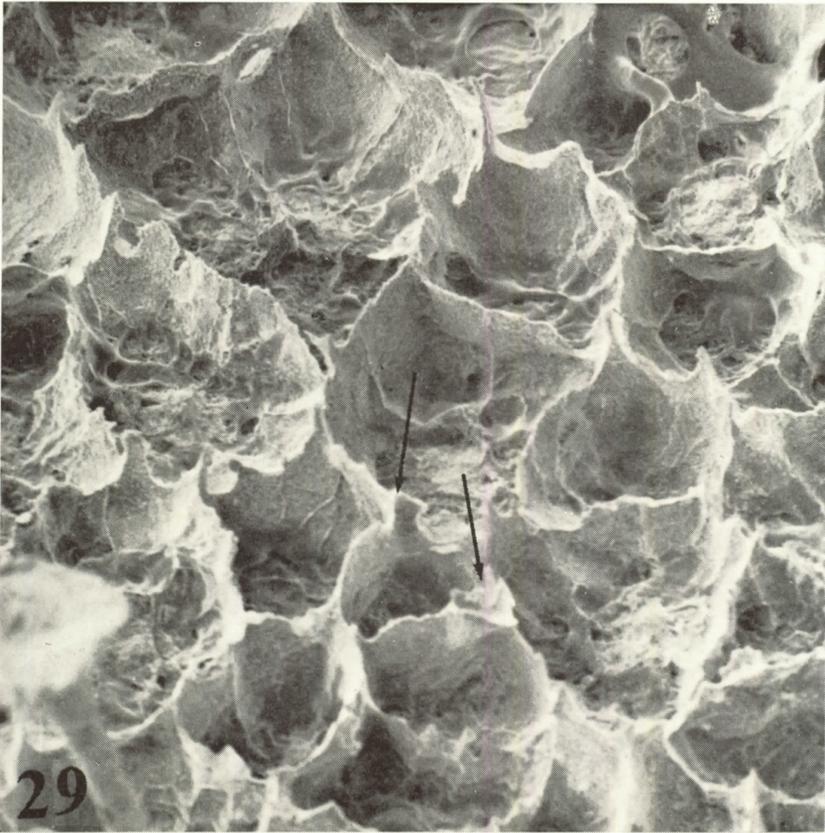


Fig. 29. Mould of mammillae after resolution of calcified materials from the shell. Honeycomb structures are the exact replica of mammillae. Notice that each cell has a pyramidal projection containing a mammillary core at the center. The cell is supplied with many fibrous structures, which are the roots of fibers connecting the mammillary core with the outer shell membrane. Numerous blind canals, aborted canals (arrows), and a true air canal (left corner) are visible. $\times 300$.

liquid and some gas from the uterus into the egg might give rise to the formation of the air pore. In fact, it is generally known that the inflow of uterine fluid into the egg causes the so-called "plumping" of the egg in the uterus.

In the author's opinion, it seems likely that the formation of the air pore may mainly be responsible for the dimension or form of the space where the mammillae come together and where an air canal is formed. Intermammillary spaces are surely made in the osculatory place of round mammillae. Moreover, they are variable in form and size. This situation is brought about by the irregular distribution and subsequent development of mammillae, as mentioned above. Ac-

cordingly, apart from the opinion of Tyler, it is possible that large intermammillary spaces may remain as air canals, despite the deposition of shell materials, whereas small ones may close in the process of shell formation to be blind or aborted canals.

SUMMARY

The three-dimensional structure of the hen's eggshell was studied by scanning electron microscopy. The morphological subjects of this study included the process of formation of the eggshell, the fine structure of the mammilla and air pore, and the morphological changes of the hatched eggshell.

The first step of shell formation began in the uterus of the oviduct with the formation of small organic concretions on the shell membrane. After these concretions had grown to some extent, they were encrusted with the deposition of calcium and became cone-shaped mammillae. They enlarged gradually upwards and in the lateral direction, while they were prevented from growing inwards, because the site of their presence had been mineralized early. The mammillae were enveloped by a cuticle-like covering. The enlargement of the mammillae proceeded by alternative formation of the covering and the subsequent mineralization of this covering. Finally, the mammillae were fused with one another to form a single layer in the mammillary stratum. The completed mammillae rested on the shell membrane, with their tips embedded in this stratum. They were connected firmly with the shell membrane by means of fibers derived from its outer layer. The mammillary core was connected loosely with the rest of the mammilla by a small amount of fibrils. The liberation of the shell membrane from the shell during the incubation period took place at the boundary between the core and the rest of the mammillae. It was considered to be induced due to the loose connection at this boundary.

After the completion of the mammillary layer, spaces varying in size and form were left behind in places where mammillae had come together. These intermammillary spaces seemed to play a certain role in the formation of a spongy layer. This layer was formed on the mammillary layer by the superimposition of new calcite layers. The process of calcification of the spongy layer consisted in two phases. The first step was the formation of a network of matrix fibers, and the second one the cementation of the meshes of the network with calcium.

The air pores of the eggshell showed themselves as mushroom-like projectins in the resin cast. They arose from the large intermammillary spaces and opened

in shallow grooves on the shell surface. In addition to true air pores, there existed a number of blind or aborted canals which had been closed on the way of the entire thickness of the shell. These canals arose from small intermammillary spaces. The formation mechanism of the air pore was discussed.

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走査電子顕微鏡下の鶏卵殻の構造と卵殻形成に関する知見補遺

藤 井 俊 策

前報に続いて鶏卵殻の構造と卵殻形成の形態的観察を、走査電子顕微鏡を用いて行なった。

鶏卵殻は周知のように、内側の乳頭層 (mamillary layer) と外側の海綿層 (spongy layer) からなっている。乳頭層形成の最初は、有機質性の微小な凝塊が外卵殻膜上に播種状に沈着することによって始まった。この凝塊は、乳頭層形成の中心である mamillary core となり、上方と側方に石灰沈着をともなってドーム状に発達し、最後にはお互いにゆ合して一層の乳頭層となった。乳頭は下方の卵殻膜側には生長しなかった。

完成した乳頭は、尖端の mamillary core をわずかに外卵殻膜内に埋没していた。各乳頭は形と大きさがかなり異なり、1乳頭に1mamillary core を有する典型的乳頭は円錐形を呈していた。大きな乳頭は、2～3のゆ合した mamillary core を持っており、一般に臼歯形であった。このように乳頭はほぼ円錐形を呈し、大小不同であるために、各乳頭がゆ合して乳頭層を作る際には、接触部に種々の形と広さの乳頭間隙が残る。この乳頭間隙から後に気孔が生じた。

乳頭はその周りを予備石灰沈着層ともいえる微細線維を含む有機質性の層で包まれていた。この層の形成とそれの石灰化が継続的に進行することによって、乳頭の生長発達が行われるものと考えられた。なお乳頭が卵殻膜側にのびないのは、この部が早く石灰化して予備石灰沈着層を持たないからのものであった。

乳頭は尖端の mamillary core 部に、外卵殻膜線維が進入することによって、卵殻膜と強固に結ばれていた。一方、mamillary core と乳頭固有部とは、微細な基質線維によって緩く結合していた。ふ卵中に卵殻膜と卵殻が分離するのは、この core と乳頭固有部の境界部であって、緩い結合様式のためと考えられた。

海綿層は乳頭部の完成後に引続いて形成された。海綿層の形成過程は大きく2段階に区別された。先ず基質線維からなる薄層が、乳頭層外表面の不平坦部を埋めるように積み重ねられた。この新生層の形成には、乳頭間隙を通して出る卵の内部の物質が関係しているように見えた。新生層は卵殻が一定の厚さに達すると、生長を止めた。その後はもっぱら、基質線維間隙に石灰沈着が進んだ。

卵殻の铸型標本では、気孔の立体的構造がよく観察された。真の気孔は、前記の比較的広い乳頭間隙からおこり、狭い間隙からおこるものは、途中で閉鎖されて盲管となる傾向があった。気孔の形成機構について論議した。