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Observations of the Egg-shell Structures Controlled by Gene Action in *Bombyx mori*

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The surface patterns and the structures of transverse sections of the egg-shells of mutant *Grey egg* alleles in the silkworm, *Bombyx mori*, have been examined with the scanning electron microscope.

The principal surface pattern of the egg-shell is a network of hexagons or pentagons. The shapes and the sizes of the polygons are different among the strains of *Grey egg* alleles.

The micropyle on the anterior end is surrounded with a pattern like a single-petaled flower. These patterns do not so much differ among strains of Grey egg alleles.

Small pores, aeropyles, open at each corner of the polygons, on the knobs which rest in every polygon, or at the furrow between the knobs. The location of the aeropyles differs among different strains.

The structures of transverse sections of the egg-shell are composed of the outer, middle, and *inner layers. Grey egg alleles* affect mainly the formation of the middle layer in the egg-shells and cause an abnormal lamellar structure in the layer. The gene interactions between Grey egg alleles are also noted in the formation of the middle layer.

INTRODUCTION

Many aspects of replication, protein synthesis, and mechanisms of gene regulation have been solved by modern molecular biology through the studies of viruses and bacteria. But the fundamental problem of morphogenesis followed by cell differentiation has been left behind, because of the complexity of eukaryote cells as compared with prokaryote cells. The surface and inner structure of the egg-shell in the insect are affected by the arrangement and secretory functions of the ovarian follicular cells. To make clear the structure of genetically abnormal egg-shell seems to provide a clue for solving the problem of the cellular function and morphogenesis by gene action.

The patterns of the egg-shell surface in *Bombyx mori* had been studied by Omura and Kataoka (1943) and it was demonstrated that the sizes and the forms of surface patterns were different among various races. Genetical and morphological stuides on the egg-shell of the *Grey egg* mutants in *Bombyx mori* were made by Tanaka (1932), Chikushi (1950, 1954), Takasaki (1955), and Sado and Chikushi (1958), and it has been demonstrated that the surface patterns and the inner structures are controlled by *Grey egg* alleles, although the morphological studies were made chiefly with the light microscope.

Here we describe the results of observation of the surface patterns and inner structures, which have been unable to resolve by the light microscope, of egg-shells of *Grey* egg mutants. We also discuss some of the gene action of *Grey* egg alleles.

MATERIALS AND METHODS

Most of the silkworm strains of various mutants of the egg-shell used have been maintained in the laboratory. Detailed descriptions of mutants used are given in the monograph of silkworm stocks by Chikushi (1972).

Because the egg-shell characters are determind by maternal genotype, genotypes which will be described here are shown as female genetic constitutions.

Egg **of standard type (p22):** This strain has originated from the Japanese race "Yamatonishiki" and has been maintained since 1924. The egg-shell is untinged and translucent. The shape of the egg is elliptic in circumference, slightly narrow on the anterior end, and dorso-ventrally flat.

Egg heteiozygous for Grey egg and normal ($Gr/+$): The ***Grey egg*** was found as a spontaneous mutant and introduced by Toyama in 1912. The gene is dominant to the normal (standard type) and located at 6.9 on the second chromosome. The features of the egg-shell are opaque and milky white. The shape of the egg is slightly ellipsoidal.

Egg homozygous for Grey egg (Gr/Gr): The egg-shell is untinged and translucent such that as normal (Sado and Chikushi, 1958). The shape of the egg is slightly ellipsoidal.

Egg homozygous for Bird-eyed egg (Gr^B/Gr^B): It is characteristics of the living egg that a circular dark brownish area is seen in the center of the egg and the other circumference shows opaqueness with milky white. Occurrence of the colored area in the center is due to optical combination of the translucent character of the egg-shell with the deep brownish serosal pigment. The egg looks like a bird's eye. This mutant has been found spontaneously by Takasaki and Ichimaru (1958), and it is dominant. This gene is an allele of *Gr*.

Egg homozygous for collapsing egg (Gr^{col}/Gr^{col}): The feature of the egg is spindle shape immediately after oviposition, but completely depressed to the bottom for half an hour after oviposition owing to excessive evaporation of water from the egg. This gene mutation has been found spontaneously in the second chromosome derived from p22 **strain in the progeny of the cross between** Gr/Gr and normal (p22) **by Sado and Chikushi (1958).** The gene Gr^{col} is an allele of ***Gr*** and recessive to the normal.

Egg heterozygous for Grey egg and collapsing egg (Gr/Gr^{col}): The shape of the egg is like a corn and about one-third of the tip area of egg-shell is translucent. The remaining part is opaque.

Egg heterozygous for X-ray induced Grey egg X-l and normal ($Gr^{X_1}/+$):

The egg-shell shows opaqueness and this character segregates unstably. Some batches show all normal, all grey or mixture with the both eggs, at the case may be. This mutant has been induced by X-ray irradiation (Takasaki, 1958) and may involve a deficiency in the vicinity of *Gr* locus on the second chromosome.

On the observation of egg-shell surface, the eggs were washed with distilled water. The specimens were then doubly coated with carbon and gold by the shadowing apparatus of the JEE 4C type. In the case of observation of the cross section the egg-shells were gently torn by needles. The other procedures were the same as that of the egg-shell surface. The observations were carried out by the JSM 2 type scanning electron microscope.

RESULTS OF OBSERVATIONS

I. Surface structures of the egg-shells

Principal surface structure as seen in the standard type egg consists of network pattern. A unit of the network is enclosed with projecting boundary and takes the shape of a regular or irregular polygon. Several small knobs or tubercles formed rest in the polygon (Fig. 1). Small pores, aeropyles, are seen at the furrow between the knobs (Fig. 10). The micropyles are at the anterior end of the egg-shell. The micropyle is surrounded with the surface pattern like a single-petaled flower (Fig. 2). The number of the petals is eight to ten. Two micropyles can be seen in the center of the flower pattern and their diameter is about 0.5 μ. Since the number and the form of the petals do not differ much among strains used, we do not give details.

Standard type (Normal) egg

As was already shown by Sado and Chikushi (1958), the surface structure of the egg-shell in the standard type (p 22) is covered by network arrangement (Fig. 3). A unit of the network takes the shape of a hexagon or pentagon by projecting boundaries about 7 μ wide. Six to eight small knobs rest in every polygon. The diameter of the knobs is 4 to 8 μ. Small pores are in the furrows between adjacent knobs (Fig. 10). The pores occasionally open on the knobs.

Gr/+ egg

The pattern of surface of the egg-shell is similar in appearance to the standard type, but the boundaries of the polygon are rather flat. The width of a boundary is 10 μ. The number of the knobs is four to five, rather small in number as compared with the standard type (Fig. 4). The knobs are 7 to 10 μ wide. Small pores are seen at the corners of the polygon (Fig. 11).

Gr/Gr *egg*

The polygons on the surface of the egg-shell are rather irregular and take the shape of quadrilateral or pentagon (Fig. 5). The width of the boundaries of these polygons is 7 to 10 μ. One to five small knobs rest in every polygon and their forms are irregular. The knobs are usually 5 to 10 μ wide. Small pores are seen on the knobs (Fig. 12), but not at the corners of the polygon.

Gr^B/Gr^B *egg*

The boundaries of network and the number or the shape of knobs in every polygon of the egg-shell are similar to that of *Gr/+ egg* described previously (Fig. 6). Since this living egg has a dark brownish colored spot which is seen in the center of the egg as already described, it is suspected that surface patterns of the dark brownish spot may be different from the other area of the egg. However, a clear difference in the pattern cannot be found anywhere on the surface. Small pores are seen at the corners of the polygon (Fig. 13).

Gr^{col}/Gr^{col} egg

The patterns of the polygon forming network of this egg-shell are somewhat irregular, showing triangular, quadrilateral or pentagonal shape (Fig. 7). The knobs in every polygon are very irregular and flat (Fig. 14). The small pores open at the corners of the polygon.

Gr/G^{col} egg

The boundaries of network are flattish and irregular (Fig. 8). The polygons show irregularly pentagonal or hexagonal shape. The knobs surrounded by the polygon are also flattish. The shapes of the knobs are variable and the outline of each knob is indistinct. One to three knobs rest in every polygon. As mentioned already, the egg shell has translucent and opaque areas, but the surface pattern of the former do not differ much from the latter. The small pores open at the corners of polygon as seen in the homozygote for collapsing egg and in some other allelic constitutions.

$Gr^{X_1}/+$ egg

The features of boundaries of the network and the knobs are very similar to that of *Gr/+ egg.* But the boundary which is formed into sequential arrangement of several tubercles is more prominent as compared with the other mutant types (Fig. 9). Small pores open at the corners of polygon (Fig. 15).

Size of the polygon as a unit of network on the egg-shell

The sizes of polygon among various races of the silkworm as well as the wild silkworm, ***Bombyx mandarina,*** had been measured by Omura and Kataoka (1943). They were able to show that the sizes of polygon were different among various races, According to their results, the actual size measured by the net-micrometer of a light microscope were from ***600*** to 1,200 square microns. Our measurments are shown in Table 1. The sizes of poygon are small as compared with those measured by Omura and Kataoka.

Since the sizes of polygons differed slightly in different places on the surface, the measurement of the sizes was made on a flat surface in the center area about half-way between the poles of the egg. An area of 10 to 15 polygons in each micrograph was measured by use of a section paper. The sizes of polygon in ***Gr*** mutants are mostly larger than that of the normal (p 22), except for Gr^B/Gr^B and Gr^{col}/Gr^{col}.

II. Structures revealed by sections of the egg-shells

Standard type egg

The egg-shell of this type consists of three very distinct layers as already

Table 1. Sizes of polygon as a unit of network of the egg-shell in various genotypic strains of Gr alleles.

Strains	Square micron	Standard deviation
p22 (+/+)	132	± 25.09
$Gr/+$	217	± 42.14
Gr/Gr	178	± 25.36
Gr^{B}/Gr^{B}	95	± 29.53
Gr^{col}/Gr^{col}	139	± 42.77
Gr/Gr^{col}	175	± 21.09
$Gr^{X1}/+$	153	± 22.79

reported by Chikushi (1954) and Sado and Chikushi (1958). The outer, middle and inner layers have often been refered to as exochorion, midchorion and endochorion, respectively. According to Hinton's suggestion (Hinton, 1968, 1969), these three layers are here simply called the outer, middle and inner layer.

The outer layer of the shell can be divided further into three sublayers ; the outer, middle and inner sublayers, and they are 0.2 to 0.4 μ, 0.9 to 1.5μ and 0.3 to 0.6 μ thick, respectively (Fig. 16). The outer and the middle sublayers may consist of solid and compact sheet of chorionin, while the inner sublayer consists of rather loose chorionin which may be similar to that of the middle layer. Vertical ditches in the outer layer are seen sporadically. The ditch passes through from the surface to the borderline between the outer and middle layer. The ditch, which may be a part of structural respiratory system of the egg, is about 0.1 μ wide.

The middle layer is about 4 μ thick and is relatively uniform in the most parts of the shell. The scanning microscopic observation at a magnification of 3,000 times clearly revealed that the middle layer is composed of more than 20 thin layers which are unresolved with a light microscope (Fig. 16). The thin layers are piled up in parallel with each other and form many subunits which are sequentially connected. The subunits are usually 0.7 to 1.5 μ long, but some are much longer, and about 0.15 μ wide. The many lines which may be formed of attached points of the subunit are seen in the vertical direction.

The inner layer is about 0.3 μ thick and the thickness is very uniform. Small square holes are set in a line in the layer and this structure may be meshwork constructed in the inner layer as described by Hinton (1967).

Gr/+ egg

The egg-shell structure of this type consists of major three layers as shown in the standard type (Fig. 17).

The outer layer, about 1.2 μ thick, may be made of solid and compact sheet of chorionin. The layer is much thinner than that of the normal egg-shell and sequentially connected with irregular arc-like subunits. The narrow ditches in the vertical direction are seen in places.

The middle layer is about 8 μ thick and thicker than that of the normal egg-shell. The many thin lamellae as the subunit in the middle layer are seen, but the middle layer shows strikingly chinky, for the parallel arrangement of the thin layer is very discomposed. In some parts the layers adhere to each other and make lumps. As a whole of view, the layer has an appearance of entangled waves.

The inner layer is about 0.3 μ thick. Small holes are arranged linearly. This feature is the same as that of the normal egg-shell.

Gr/Gr egg

The egg-shell structure of this type consists of major three layers ; the outer, middle and inner layers (Fig. 18).

The outer layer consists of two distinct sublayers, namely, the outer and inner sublayers. The outer sublayer is about 0.3 μ thick and may be compactly packed by chorionin. The inner sublayer is 0.5 to 0.7 μ thick and may be formed of more or less solid chorionin.

The middle layer is about 5 μ thick. In this layer many V-shaped lamellar structures join to side by side and they are arranged regularly in horizontal way. The unit of the lamellae is 0.15 to 0.3 μ wide and about 10 μ long, being not uniform. It is of interest that these features are not shown in the egg-shell of *Gr/+* mentioned previously.

The inner layer is about 0.4 μ thick and small holes set in array linearly. This feature is similar to that of the normal egg-shell.

Gr^{B}/Gr^{B} egg

As previously described, the living egg shows a dark brownish area owing to translucent egg-shell in the center of the egg surface, and the other area is opaque. Thus the observations were carried out on both the center and the outer area.

The structure of the translucent center area consists of major three layers; the outer, middle and inner layers (Fig. 19). The borderline between the outer and middle layer is not so clear. The thickness, put together with the outer and middle layers, is about 10 μ. The structure of the two-thirds outer part of the layer shows that relatively smooth lamellae are piled up in parallel. However, in the one-third inner part of the layer, arrangement of the lamellae shows the irregularity like the high and low waves. The inner layer is clearly seen and the thickness is about 0.5 μ. Many small holes are arranged linearly.

In the opaque area of the egg, the structure is very different from the translucent center area. This area consists of the outer, middle and inner layers (Fig. 20). The outer layer is about 2 μ thick. The surface line of the layer appears to be markedly uneven. In the layer vertical ditches are seen sporadically. The structure of the middle layer is also different from the center area, namely, many lamellae are layered with horizontally or vertically inclined directions and the lamellae adhere in places. Some cracks or holes can be seen in places of the layer. This layer is 13 to 15 μ thick. The inner layer is formed of one sheet in which small holes are arranged linearly. This layer is about 0.5 μ thick.

Gr^{col}/Gr^{col} egg

The structure of the egg-shell consists of major three layers ; the outer, middle and inner layers (Fig. 21).

The thickness of the outer layer is varied, 0.4 to 0.8 μ. The borderline between the outer and middle layer cannot be clearly distinguished in some places.

The middle layer is about 6 μ thick. Many thin lamellae are irregularly arranged in the layer and show strikingly chinky features, being more excessive than that of the middle layer in ***Gr/Gr^{col}* egg.** This features may be closely related with the fact that the egg-shell of this egg is completely depressed to the bottom for half an hour after oviposition owing to the loss of water and the rapid evaporation from the egg.

The inner layer of the egg-shell is about 0.3 to 0.6 μ thick and has many small holes which are linearly arranged. But the holes cannot distinctly be observed in some places.

***Gr/Gr^{col}* egg**

The feature of the egg-shell shows translucent and opaque area as already pointed out. Thus the observations were carried out on both of them.

The structure of the opaque area of the egg-shell consists of the outer, middle and inner layers (Fig. 22). The outer layer is about 0.6 μ thick and the surface of the layer shows irregularly wavy line. The thickness of the middle layer is extremely varied, 6.3 to 10.5 μ. This layer is constructed of many irregular lamellar plates like ragged wave and of many irregular clumps, with the consequence that the layer is very porous. In addition of these features, the irregular cracks can be seen in the vertical direction in places. The inner layer is about 0.4 μ thick and many small holes are arranged linearly in the layer.

The structure of the translucent area also consists of the outer, middle and inner layers (Fig. 23). The thickness of the outer layer is varied, 0.5 to 0.9 μ and the surface line of the layer shows irregularly wavy. The thickness of the middle layer is also extremely varied, 3 to 6 μ. The structure of the middle layer is markedly different from that of the opaque area, namely, many lamellae are piled up in horizontally parallel with each other. The horizontal ditches lie scatteringly in the layer. The parallel arrangement of the lamellae appears somewhat irregularly wavy.

***Gr^{X_1}/+* egg**

The structure of the egg-shell consists of major three layers: the outer, middle and inner layers (Fig. 24).

The outer layer is 1.5 to 3.0 μ thick and the linearity of this layer is relatively irregular.

The thickness of the middle layer is slightly varied, 6.8 to 8.4 μ. This layer is constructed of irregularly arranged and clumped lamellae in the vertical direction. The layer is remarkably porous and irregular crevices appear in the vertical direction.

The inner layer is about 0.8 μ thick and many small holes are arranged linearly.

DISCUSSION

It has been shown from the observation that the patterns and the sizes of polygon as a unit of network on the egg surface differ among different mutant strains of the *Grey egg* alleles. The $Gr/+$ strain used has been maintained by the cross between p22 and Gr/Gr strain. On the other hand, Gr^{col}/Gr^{col} strain has been established as a spontaneous mutant from the progeny of the cross between p22 and Gr/Gr as previously mentioned. So that, pedigree of $Gr/+$, Gr/Gr^{col}, Gr^{col}/Gr^{col}, Gr/Gr and p22 strains will have colse relationship. These strains have also been shown diffeffrent in surface patterns of the egg-shells (Figs. 3, 4, 5, 7 and 8). It may be said from these facts that *Gr* alleles control surface patterns of the egg-shells. However, as the differences of the surface patterns of the egg-shells had been found among different races (Omura and Kataoka, 1943), it will be necessary for more systematically genetic analysis.

It has been clearly shown from the experiments that actions of the *Gr* alleles are mainly associated with the peculiar structure in the transverse sections of the egg-shell. Because, there is a very close relation between gene action and opaqueness in the grey eggs on account of the status being found only in the egg-shell of *Gr* mutants.

Gr alleles may mainly control the formation of the middle layer of egg-shell. As already pointed out by Sado and Chikushi (1958), the thickness and the structure of the egg-shells of $Gr/+$ and Gr/Gr are very different. The thickness of the $Gr/+$ egg-shell is about 10.5 μ and the lamellar structure of their middle layer is very irregular and remarkably porous. On the other hand, the thickness of the shell of Gr/Gr is about 6.5 μ showing the same order of the standard type, while the structure of their middle layer is more regular and compact than that of $Gr/+$. It can be said from these facts that the actions of $Gr/+$ concerned with the structure of the middle layer might be more effective than that of Gr/Gr. In general, the action of a dominant gene is more predominant in homozygous condition than in heterozygous as to expression of the character, but the case of *Gr* alleles is paradoxy. One explanation for this phenomenon will be made that when *Gr* and + genes may act as antagonistic and the both genes may be codominant, distortion of structure in the middle layer of $Gr/+$ may more remarkably appear than that of Gr/Gr.

According to Machida (1940), endochorion (inner and middle layers) and exochorion (outer layer) of the egg-shell of the silkworm are secreted by the follicular cells and they consist of accumulations of peculiar masses that appear in cytoplasm of the cells. But he has been unable to observe detailed process of the egg-shell formation. Since *Gr* is dominant, it is reasonable that the action of *Gr* appears on the heterozygote for *Gr* and normal. The *Gr* may act on the orientation of lamellae at the time of secretion in accordance with the quantitative or qualitative difference of peculiar chorionin masses formed in the follicular cells. However, it is not yet known actually why the structures of the egg-shell between $Gr/+$ and Gr/Gr are remarkably different as shown in Figures 17 and 18.

It is of interest that the action of Gr^{B} appears differently on the structure of the middle layer between the center and the other remaining area in an egg. It may be due to the different cellular function of chorionin secretion in the follicular cells responsible for the formation of the two area and this may suggest that the gene action may appear at the cellular level. The similar situation can be found in the case of the heterozygote for *Gr* and Gr^{col} as shown in

Figures *22* and *23*.

In comparison with the structures of the middle layer between $Gr/Gr, Gr^{col}/Gr^{col}$ and Gr/Gr^{col}, the features are very different in each case. It can be said from the facts that the mechanisms of interactions between the *Grey egg* alleles may be involved in the formation of the egg-shell structure.

Thus one might expect that the actions of the *Grey egg* alleles may have changed the orientation or even the structure of associated proteins in the middle layer of the egg-shell and that the change may be caused by the process and function of secretion from the follicular cells.

Concerning with the structure of the egg-shells, Hinton (1969, 1970) has very extensively and beautifully reviewed from view point of respiratory system in the eggs of many insect species. According to his articles, many terrestrial eggs of butterflies and the other insects have not a plastron which is the respiratory system of the egg-shell, but nevertheless have a layer of gas in the inner part of the egg-shell. It would be said from our observations that the respiratory system of the egg-shell in the silkworm may be similar to the case of the butterflies. It was observed in the experiment that the small pores, aeropyles, open on the coigns of the polygon pattern on the surface of the $Gr/+, Gr^{B}/Gr^{B}, Gr/Gr^{col}, Gr^{col}/Gr^{col}$ and $Gr^{X_1}/+$ egg-shell. On the other hand, the aeropyles were unable to be observed at the corners of the polygon in the standard type (p22) and the Gr/Gr egg-shell, but they were observed on surface of the knob or at the furrow between the knobs. These openings of the aeropyles may be somewhat different among strains or races in the silkworm. For instance, in the Chinese race many aeropyles opened everywhere on the surface of knobs and the borderlines of polygon of the network, though the photograph is not presented here. However, in the Japanese race, for example, the standard type used in this experiment, the aeropyles mainly open at the furrow between the knobs in every polygon. It is not yet known from the facts that whether these differences in opening the aeropyles are due to the action of Gr alleles or not. As for the aeropyles in various strains and races of the silkworm, it would be essential to study more extensively for an understanding in respiratory systems. Most of the batches belonging to the *Grey egg* strains show low hatchability. These facts may be due to poor respiration or aberrant water evaporation owing to the abnormal structure of the egg-shell. In order to clarify the mechanisms of formation of the egg-shell controlled by the *Gr* alleles, analyses of proteins and amino acid of the egg-shell are now underway.

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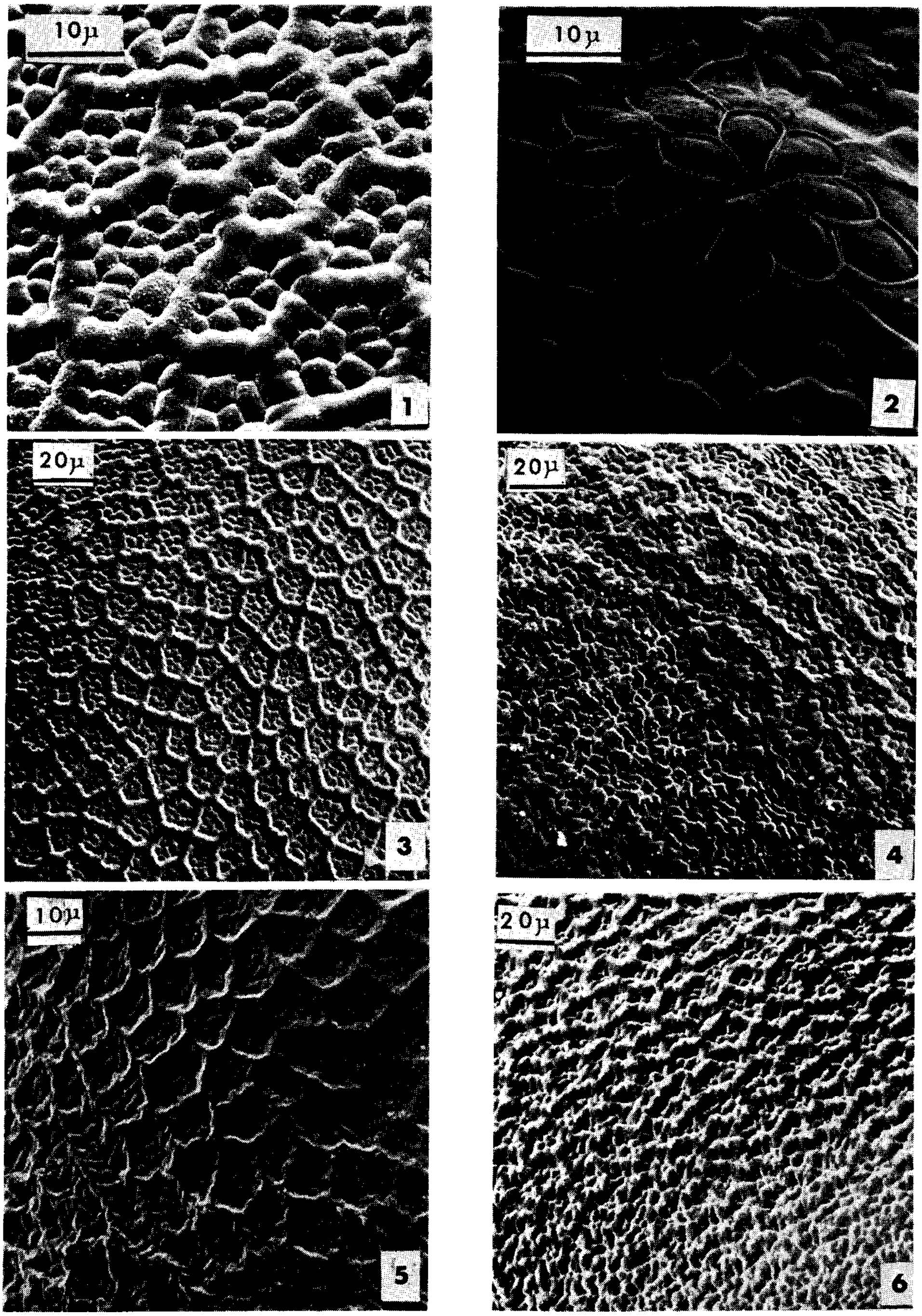
Explanation of Plate I

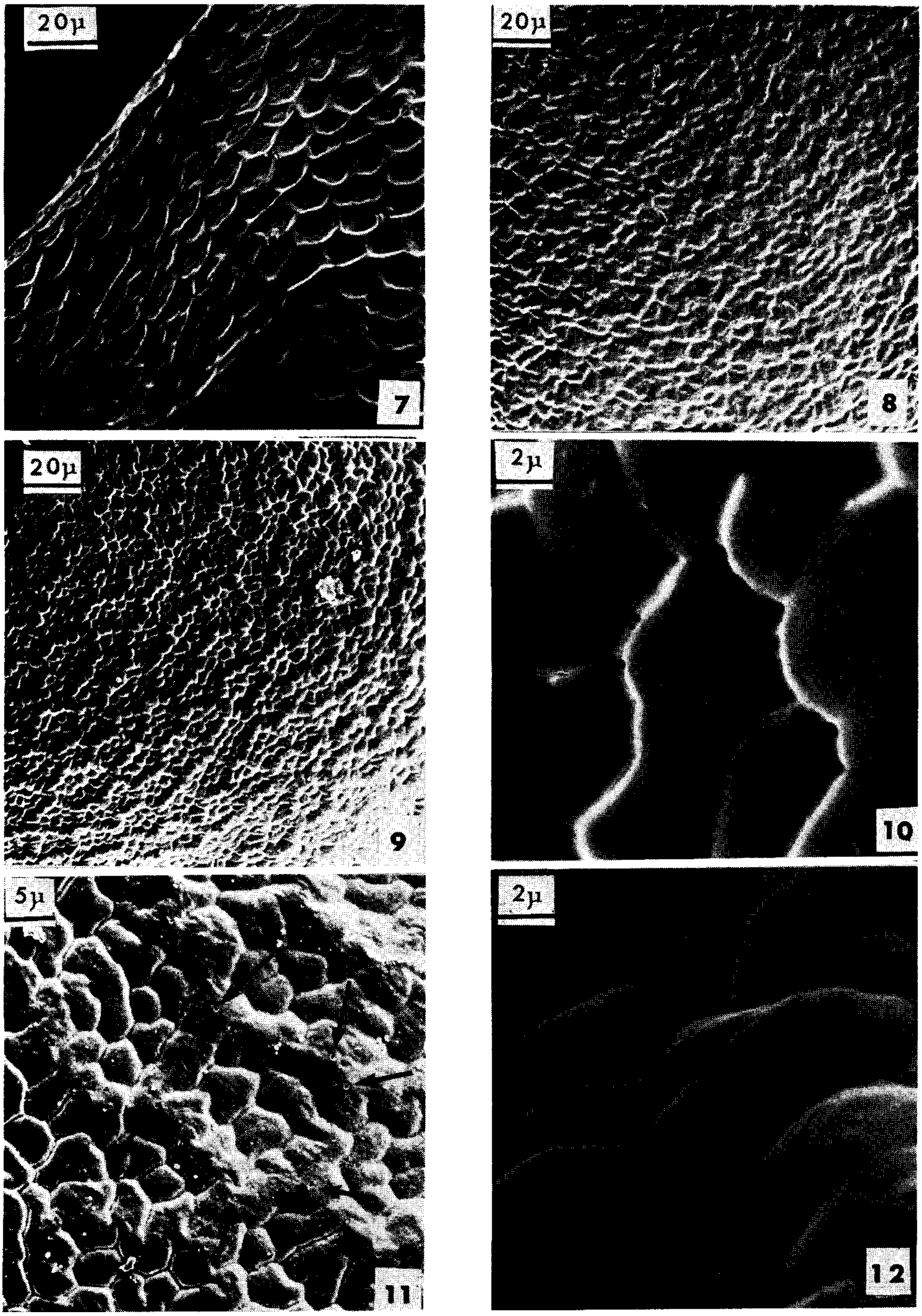
Fig. 1. Surface about half-way between the poles of egg of the standard type (p22). This photograph shows a typical surface pattern of the standard type (Japanese race) in the silkworm. **Fig. 2.** Surface of the anterior pole of the standard type. In the center of the pattern which is like a single-petaled flower, micropyle, can be seen. **Fig. 3.** Surface of the standard type (p22). **Fig. 4.** Surface of $Gr/+$ *egg*. **Fig. 5.** Surface of Gr/Gr *egg.* **Fig. 6.** Surface of Gr^B/Gr^B egg.



Stereoscan electron micrographs of egg-shells of ***Bombyx mori***

Explanation of Plate II

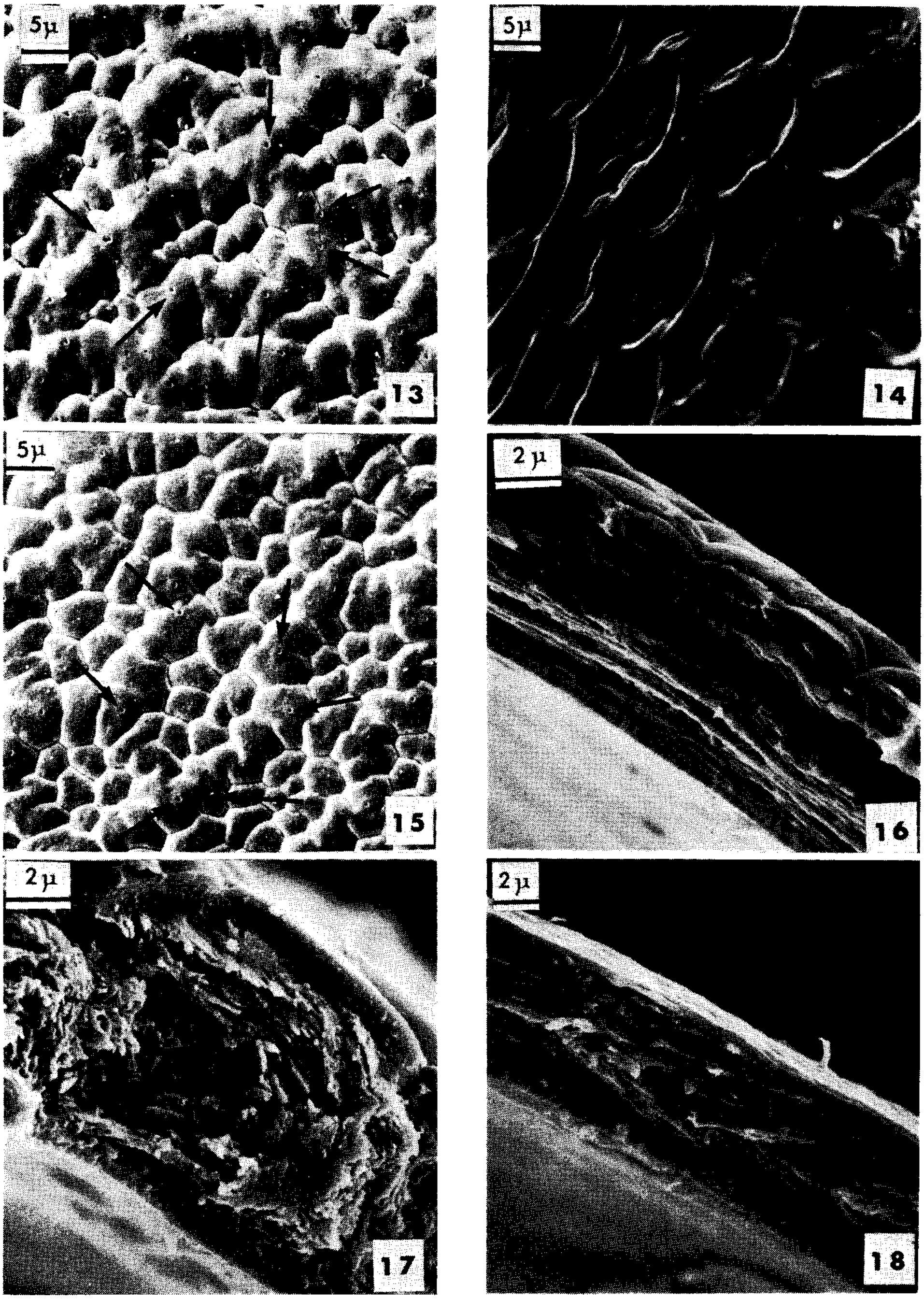
Fig. 7. Surface of Gr^{col}/Gr^{col} egg. **Fig. 8.** Surface of Gr/Gr^{col} egg. **Fig. 9.** Surface of $Gr^{X1}/+$ egg. **Fig. 10.** Surface of the standard type revealed at high magnification. Small pores, aeropyles, can be seen at the furrow between the knobs. **Fig. 11.** Surface of $Gr/+$ egg revealed at high magnification. Arrows show the small pores, aeropyles. **Fig. 12.** Surface of Gr/Gr egg revealed at high magnification. The small pores, aeropyles, can be seen on the knobs.



Stereoscan electron micrographs of egg-shells of ***Bombyx mori***

Explanation of Plate III

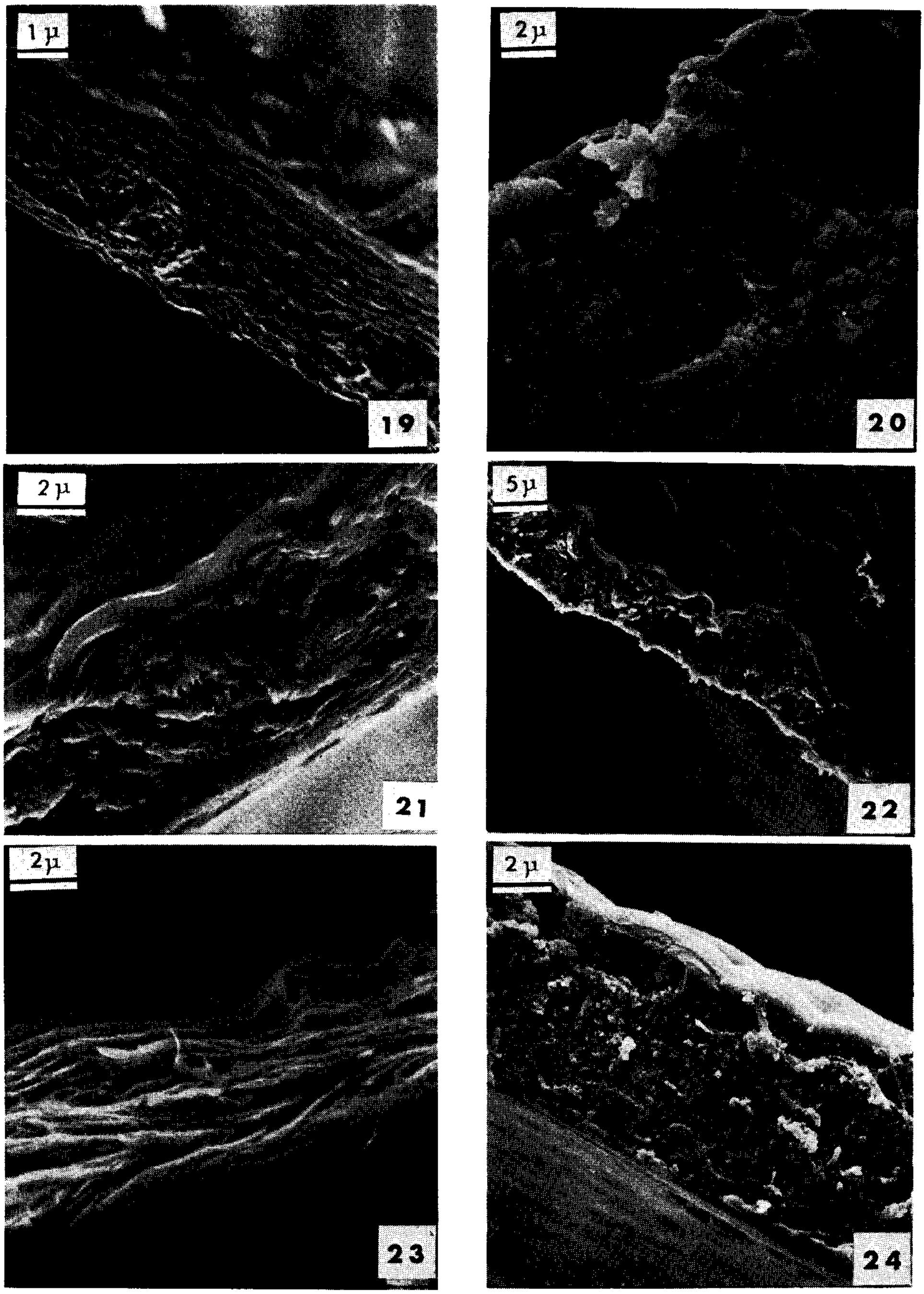
Fig. **13.** Surface of Gr^{B}/Gr^{B} egg revealed at high magnification. Arrows show the small pores, aeropyles. Fig. 14. Surface of Gr^{col}/Gr^{col} egg revealed at high magnification. The small pores, aeropyles, can be seen at the corners of the polygon pattern. Fig. 15. Surface of $Gr^{X1}/+$ *egg* revealed at high magnification. Arrows show the small pores, aeropyles. Fig. 16. Section of the standard type (p 22). Fig. 17. Section of egg-shell of $Gr/+$. **Fig. 18.** Section of egg-shell of Gr/Gr.



Stereoscan electron micrographs of egg-shells of *Bombyx mori*

Explanation of Plate IV

Fig. 19. Section of translucent area half-way between the poles of egg-shell of Gr^{B}/Gr^{B} *egg.* **Fig. 20.** Section of egg-shell in opaque area of Gr^{B}/Gr^{B} *egg.* **Fig. 21.** Section of egg-shell of Gr^{col}/Gr^{col}. **Fig. 22.** Section of egg-shell in opaque area of Gr/Gr^{col} *egg.* **Fig. 23.** Section of egg-shell in translucent area of Gr/Gr^{col} egg. **Fig. 24.** Section of egg-shell of $Gr^{X1}/+$.



Stereoscan electron micrographs of egg-shells of *Bombyx mori*