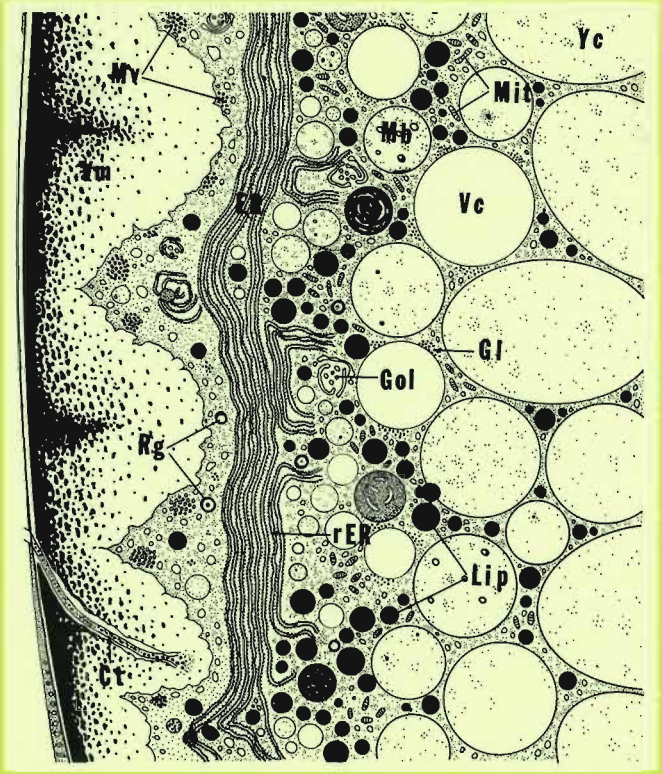
Keiichiro Miya
Edited by Toshinobu Yaginuma and Koichi Suzuki

The Early Embryonic Development of *Bombyx mori*

— An ultrastructural point of view —



GENDAITOSHO

Keiichiro Miya
Edited by Toshinobu Yaginuma and Koichi Suzuki

The Early Embryonic Development of *Bombyx mori*

— An ultrastructural point of view —

GENDAITOSHO

ISBN 4-906666-25-6

Published by GENDAITOSHO
Amenity-Tower 5F 11240 Tana, Sagamihara City
Kanagawa 229-1124, Japan

Editors' Preface

Insects are the most flourishing group of organisms on earth. The number of species has reached more than one million, possibly as much as ten millions, and it is estimated that the total insect population is a hundred million times higher than the size of mankind. This is the reason why the earth is called a planet of insects and why the insects are in the focus of many investigations. It is a significant task to learn how insects lead their lives and what makes them so successful in colonizing nearly every corner of the earth. If we understand insects, we can better grasp our own position in nature and handle environmental problems created by human expansion.

It goes without saying that some kinds of insects are regarded as nuisances worth extermination because they attack our food resources and transmit diseases. However, this is a view of a "master of nature", a role that we imposed on ourselves. Insects are an indispensable component of most ecosystems. Some became pests, from our point of view, when man began to grow some plants in unnaturally high densities and in monocultures and thereby established ideal conditions for these species to feed on such plants. Also, representative medical pest insects, such as mosquitoes, are considered to accidentally sting human beings instead of many other animal species in order to obtain the blood as their diets so that they themselves may survive.

Suppose that all the species of insects thoroughly disappeared from the earth. Most of the flowering plants would not be fertilized, thus failing to bear fruits and seeds. The decomposition of organic material, such as old leaves, would be hampered and soil fertility would be lost in a few years. Many microorganisms, such as bacteria and fungi, which are normally eaten by the insects, would proliferate and some would probably become pathogenic to man. On the other hand, thousands of animals including many fresh water fish, nearly all amphibians, most reptiles and birds, and most small mammals would lose their food. It is not difficult to imagine that the entire biosphere would collapse and man could not survive.

The contribution of most insects to our lives is indirect. The usefulness is obvious only in a few species, such as the honeybee or the silkworm. No one also doubts the contribution of *Drosophila melanogaster* towards understanding various aspects of life. *D. melanogaster* is one of the best examined organisms but is it possible to understand all insects on the basis of this knowledge? In the phylogenetic tree, a fly is as far from a silkworm as is man from a bird. We must obviously study other species, in addition to *D. melanogaster*, to comprehend insects. Fortunately, a great amount of knowledge on *Bombyx mori* has been piled up in our country, which is leading the field of Sericultural Sciences.

The present book entitled "The Early Embryonic Development of *Bombyx mori*" is concentrated on the ultrastructural morphology of early embryogenesis. It is published in English to facilitate the spreading of knowledge from Japan to other countries. Since the nucleotide sequencing of the genomes of several organisms has recently been fin-

ished, investigations are coming to the stage of clarifying the roles of individual genes. To understand the gene interplay in the developmental processes, the formation of cells and their constituting elements must first be tackled by ultrastructural studies. The process of early embryogenesis is a particularly suitable system for the developmental studies. Several novel approaches, which should complement the knowledge obtained in *D. melanogaster,* are suggested in this book. A combination of the information on the embryogenesis of the fruit fly and the silkworm will facilitate the understanding of early embryogenesis in all insects.

Dr. Keiichiro Miya (Iwate University Emeritus Professor) is the authority on Insect Embryology and Morphology. He plans to publish "A Pictorial Explanation of Silkworm Development" consisting of three volumes entitled Early Embryogenesis, Gametogenesis, and Organogenesis. He is still at work on this monumental undertaking. When meeting Dr. Miya on several occasions, we convinced him that it is desirable to publish a condensed version of his work as soon as possible. He agreed to prepare this book that covers Early Embryogenesis. We are honored by the invitation to take on the responsibility as the editors.

Acknowledgments

The publication of this book was supported by a year 2002 Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science (JSPS) (Grant-in-Aid for Publication of Scientific Research Results, no. 145316).

We wish to thank Mrs. Yasuyo Sakai and Dr. Toshiharu Tanaka for their help with the English edition, Miss. Kazuno Koide for her assistance with the word processor, and many colleagues for their suggestions and advice. We would particularly like to thank Dr. František Sehnal for his critical reading of the entire manuscript.

February 2003

Toshinobu Yaginuma
Graduate School of Bioagricultural Sciences, Nagoya University

Koichi Suzuki
School of Agricultural Sciences, Iwate University

Contents

Preface

A Pictorial Explanation of Silkworm Development

Early Embryogenesis

The motive of my studies on the embryogenesis of silkworms comes from a series of investigations about hereditary characters of the colors of silkworm eggs, which was led by Prof. Eisaku Kawaguchi in the Laboratory of Sericultural Sciences, Faculty of Agriculture, Hokkaido University in the 16th year of Showa (1941). Participating in the project as a student of the bachelor's course at that time, I took charge of making sections of eggs from the deposition to the hibernating stage to compare and examine the development of serosa pigment granules, one of the major factors determining the color of eggs in various silkworm races. After the discovery of large size cells in the diapausing embryos, the elucidation of their origin and the characterization of their features were regarded as a significant problem to be solved. This prompted me to focus my attention on this theme in my research for graduation.

In those days the major method in cytology and embryology was as follows: after a sample is fixed with a variety of fixatives, it is included in some embedding material, such as paraffin, to make sections that are stained with dyes and examined through a light microscope. The observation was limited to examining structural features of cells and comparing morphological differences of embryos of various developmental stages. The movements of a cell and the differentiation changes occurring with the lapse of time had to be presumed only from structural differences detected in the plural samples. Later, technical progress in phase-contrast microscopy made it possible to describe the structure of a living cell. It was an amazing step that revealed artifacts caused by the fixation procedures.

Because the silkworm egg is enclosed by a thick, tough chorion that prevents rapid penetration of fixatives, the fixation of cell organelles is often insufficient. Carnoy's fluid and hematoxylin-eosin staining were commonly used for the fixation and staining. Due to the elution of lipids, however, the cells often contracted and this prevented detailed investigations of structural processes during differentiation. Several kinds of other fixatives were tested and compared with each other. Finally, near satisfactory distinction of the big cells in question was obtained with heated Allen-Bouin's fluid. It was recognized that the big cells segregate from the germband in a particular region during an early stage of germband formation. Those cells were proved to be primordial germ cells by further examinations (Kawaguchi and Miya, 1943).

Since the 22nd year of Showa after the war (1947), investigations concerning germ cell differentiation have been resumed, and the following phenomena were examined: the movements of the primordial germ cells, the relationship between these movements and the formation of gonad anlagen originating in the mesoderm, the morphological

differentiation of the male and female gonads, and so on. A general description of the cascade of these processes was formulated (Miya, 1958, 1959a), however it proved necessary to go back to the study of oogenesis in order to understand embryogenesis, especially of the early stage. Consequently, we were obliged to extend our study to gametogenesis, including both the oogenesis and the spermatogenesis.

At that time, some amazing and effective devices, together with related cytological techniques, were developed for practical use. The significance of electron microscopy (scanning and transmission) and novel techniques of fixation, embedding, thin sectioning, and electron staining, etc. was immense. Also at Iwate University, advanced electron microscopes and some devices attached to them were made available to our investigations in the newly opened Laboratory of Electron Microscopy. But it was not easy to learn a developing technique and to make the best use of the functions of the new devices. We met similar difficulties as did our predecessors who learned and developed the classical fixation staining technique at the end of the 19th century. At the beginning, we sometimes reached wrong conclusions due to poor techniques, but over time the methods of electron microscopy were developed to near perfection. Just as the Carnoy's fluid fixation followed by the hematoxylin-eosin staining were reliably used for light microscopy, glutaraldehyde-osmium fixation, epoxy-resin embedding, and uranium-lead staining became trusty standards in the electron microscopy. We applied ultrastructural investigations to various developmental stages, ranging from gametogenesis to embryogenesis [the post-embryogenesis is left out because there is a splendid work by Akai (1976a,b)]. Occasional discrepancies in the results, reflecting the conditions of fixation and staining as well as the kinds of samples used in each experiment were inevitable, and they are commented on in the descriptions of the respective figures.

In any morphological investigation, the observation reveals only the state at the moment of fixation. It is predictable that a living cell will react to its environment and its morph will change all the time. Accordingly, in the case of ultrastructural morphology, it is difficult to decide whether two adjacent cells differing in their morphology perform different functions or if they are of the same kind and exhibit only changes that elapse with time. Notes are added to the figures in which this possibility may apply. More than twenty years have passed since I began to investigate ultrastructural morphology by electron microscopy and I have managed to touch the processes from gametogenesis to embryogenesis. There may be some research areas that have not been detailed satisfactorily. In other cases, I might have misunderstood a phenomenon because of a lack of appropriate research data. I am expressing my uncertainties with as many questions as possible, and it will be highly appreciated if you refer to them in your investigations.

My book consists of the following three volumes: I) Early Embryogenesis, II) Gametogenesis and III) Organogenesis. Volume I includes the ultrastructural morphology from the mature egg to the initiation of diapause; Volume II deals with the morphology of both male and female gonads and the formation of gametes. Finally, Volume III is about the morphological changes from diapause stage to larval hatching through the period of organ anlage formation.

It has been over fifty years since I started working on the embryology of insects, chiefly silkworms. I would like to express my gratitude to the late Prof. Eisaku Kawaguchi, my teacher who gave me a great chance to study and guided me courte-

ously until his last days. I also wish to thank the late Prof. Tetsuo Inukai for his valuable assistance and advice for my degree thesis, the late Prof. Toichi Uchida, the late Prof. Sajiro Makino and lastly Prof. Chihisa Watabane of Hokkaido University.

Needless to say, suggestions and words of encouragement from my superiors and colleagues are indispensable to continue and improve my investigations. I am very grateful to the late Seinosuke Omura (the Head of Sericultural Experiment Station), the late Prof. Hisao Ariga and the late Prof. Narumi Yoshitake (Tokyo University), the late Dr. Takeo Takami (Sericultural Experiment Station) and Dr. Hiromu Akai (Tokyo University of Agriculture), Dr. Yataro Tajima (Institute of Sericulture) and Emeritus Prof. Hiroshi Ando (Tsukuba University). Thanks must also be given to Emeritus Prof. Morihisa Kurihara and Prof. Koichi Suzuki (Iwate University) who assisted me as co-operative investigators during the project and the late Mr. Ichiro Tanimura who made a great contribution by operating electron microscopes and preparing a great number of samples (Laboratory of Electron Microscopy, Iwate University).

June, 2002

Keiichiro Miya
Kawagishi, Morioka, Japan

Introduction

The early embryogenesis of insects consists of the establishment of a body ground plan by maternal information that has been stored in the egg during oogenesis and by the spatial and temporal pattern of gene expression in the zygotic nuclei. The relative importance of these two factors and mode of their mutual interaction vary and depend on the species: in one case, the basal system is controlled by maternal information to a great extent, whereas in another case the maternal information is not so effective. The embryogenesis of silkworms is closer to the former model, and maternal determinants appear crucial for establishing the cephalocaudal embryonic axis, the dorsoventral polarity, the embryonic versus the extra-embryonic regions, as well as the areas of presumptive ectoderm and mesoderm. While the differentiation of primordial germ cells is triggered by the maternal determinants, the differentiation of gonads from the mesoderm is controlled by gene expression in the zygotic nuclei. This is why we must study oogenesis in order to understand the early embryogenesis. The origin of the constitutive elements of a mature egg has not been described sufficiently and further investigations on their physiological roles and their relation to the maternal information are also needed. The present book is limited to the ultrastructure of the mature egg and the changes occurring in early embryogenesis.

1 A brief history of embryological investigation on the silkworm

Thanks to the technical progress of light microscopy in the late 19th century, the eggs of insects came to be regarded as a subject of study in embryology. The first investigation on silkworm embryogenesis was carried out by Tichomiroff (1879). He made a report on the blastoderm formation, the differentiation of endoderm and the development of various organs such as the silk glands, tracheae, setae and so on. The cellularization of blastoderm was viewed as epigenesis within the egg according to Weismann (1863). On the other hand, Tichomiroff objected to Weismann's interpretation of the formation of the inner germband by ectoderm invagination. Further studies led him to the conclusion that mesoderm originates from cells separated from the primitive groove that appears at the middle line of an embryo and in the region of the tubular invagination that appears at the end of the blastopore.

In Japan, the first research on silkworm embryogenesis was conducted by Toyama (1896). While he highly esteemed Tichomiroff's investigation, Toyama noticed several unsatisfactory points. He especially focused his attention on the inaccuracies in the development staging, which were caused by the variability of the examined samples. He pointed out that the results were not practical. Then he carried out a series of investigations with the lapse of time. Eventually he succeeded in defining all major stages of embryogenesis from the egg architecture, the formation of blastoderm and

germband, the winter diapause, the period of embryogenesis resumption next spring, up to the formation of organs. This important work, which appeared in "*The Bulletin of the College of Agriculture, Tokyo Imperial University*" in 1902, is not only the first report covering the whole silkworm embryogenesis, but it is also evaluated as one of the major papers in lepidopterology. It is the first thorough description of insect embryogenesis worldwide. The formation of mesoderm and endoderm, a difficult question in those days, is described in detail. The formation of mesoderm proceeds in a variety of patterns according to the germband region. The mesoderm is formed by deep invagination at the front end of the blastopore, by an inward growth of the axial germband part in the gnathal region. In the ventral region the presumptive mesoderm is internalized by being overgrown by the ectoderm. The paper includes many other important and interesting views, for example: the endoderm (mid-intestine rudiment) of silkworms differentiates from cells at the bottom of the stomodeum and the proctodeum, histolysis and subsequent changes of an oral cell mass, the differentiation of salivary gland and subtracheal gland (prothoracic gland), the formation of an internal skeleton of a head, and so on. These investigations by Toyama were collected into one chapter in "*A Textbook of the Silkworm Egg*" in 1909, which was used as a manual by all who dealt with silkworm embryogenesis. Later, Ikeda (1910, 1912) examined embryogenesis from the blastoderm to diapause stage I. He reported these results together with his subsequent observations on the process of organ formation in the paper "*Embryonic Development*" published as a chapter in "*Experiments on Anatomy and Physiology of the Silkworm*" (1913).

When the basis of staging of silkworm embryogenesis became established, the attention of researches turned to (1) the development of practical technique useful in the incubation of silkworm eggs; for example, easy recognition of the developmental stages of silkworm embryos. Another task was (2) the reexamination of each phenomenon during embryogenesis and correction of the already described facts; for example, Iwasaki (1931, 1932) made observations of blastoderm cells, yolk cells and blood cells. His report shows that the membrane, which separates cleavage nuclei entering the periplasm, originates by inward invagination of the non-structural membrane that appeared on the surface of the periplasm. The yolk cell membrane produced by yolk cleavage is formed by further invaginations of the membrane produced during the blastoderm formation. He insisted that all blood cells are derived from the cells released from the oral cell mass at the mouth. Nakata (1935), who investigated the Malpighian tubules and the development of the posterior portion of the midgut, opposed the idea of Toyama that the midgut rudiment differentiates from the bottom of the proctodeum. He described that during midgut differentiation some of the ectodermal cells near the rear end of the caudal segment invaginate and begin to migrate into the embryo interior. Kawaguchi and Miya traced back to the period of germband formation to clarify the origin of primordial germ cells in another investigation (1943).

The study on embryogenesis around World War II was advanced enthusiastically by Takami. He successfully accomplished several research projects, such as the first analysis of silkworm embryogenesis by means of experimental embryology, the observation of living yolk cells, and the technical development of the *in vitro* culture of embryos. Wada (1955ab) made detailed examinations on the occurrence of the subesophageal body in relation to the blood cells, and followed Toyama's idea that the subesophageal body has its origin in the mesoderm and takes part chiefly in the differentiation of blood

cells. Miya tried to trace the fate of primordial germ cells and pursued problems of the gonad formation with the aid of cauterization (introduced by Takami) as well as with morphological observations. These works on silkworm embryogenesis were collected by Kuwana and Takami in the volume "*Embryology of Invertebrates*" edited by Kume and Dan (1957). They represent important contribution to insect embryology on a world scale.

The ultrastructural studies of silkworm started in the later half of the 20th century. Akai (1957, 1958) clarified the ultrastructure of chorion and Akutsu and Yoshitake (1974) subsequently examined the chorion of the "gray eggs". As to the inner features of eggs, Miya (1959b, 1960) observed the ultrastructure of serosa and yolk cells, and Takei and Nagashima (1975) compared the embryonic development of diapause and non-diapause eggs. Unfortunately, their immaturity in the microscopy technique to some extent disvalued their efforts.

Afterwards, with improved fixation procedures, the architecture of silkworm eggs and the ultrastructural features of embryogenesis could be elucidated. Okada (1970) made observations of the embryonic fine structures of the diapause and the non-diapause eggs. He found that several characteristic changes take place during the diapause, such as the vesicle-like mitochondria or the concentric arrangement of the rough endoplasmic reticulum (rER). Then Miya *et al.* (1972) made investigations on the serosa and yolk cells and discovered remarkable changes in the morphological structure of mitochondria. Takesue *et al.* (1976) compared the development and changes of yolk granules in the diapause eggs with the non-diapause eggs. The architecture of newly laid eggs, the changes precipitated by the sperm entry, the vitelline membrane, and the periplasm were emphasized in Miya's report (1978); Kobayashi and Miya (1987) assessed relationships between the embryonic and the extra-embryonic regions from the ultrastructural difference of rER attached to the periplasm of newly laid eggs. Concerning the blastoderm and the germband formation, Takesue *et al.* (1977, 1980) and Takesue and Keino (1980) examined the relation between migration of the cleavage nuclei and the microtubules, and insisted that the cytoplasmic membrane in the blastoderm cell formation is formed not by the invagination of oolemma, but by the protrusion of cleavage nuclei toward the surface of periplasm. As for the organogenesis, there are a couple of reports by Miya about gonads (1975), alimentary canal and Malpighian tubules (1976). Hashimoto and Miya (1987) compared the development of brain in the wild type and a lethal mutant (duplicate star-spots).

Among examinations based on scanning electron microscopy, I wish to mention reports on the gray eggs by Sakaguchi *et al.* (1973), on the chorion by Ohtsuki and his co-investigators (1974, 1977, 1982) and on the changes of the egg surface from the time of deposition to the stage of germband formation by Keino and Takesue (1982).

The above-mentioned "*Embryology of Invertebrates*" was later re-edited into "*Invertebrate Embryology* Vol. I & II" edited by Dan, Sekiguchi, Ando and Watanabe, and the investigations of silkworms after the descriptions by Kuwana and Takami (1957) are presented by Ando (1988) in the chapter "*Insects*" in Vol. II.

2 Considerations on the fixation and staining method

Since the silkworm egg is covered with a thick tough chorion like most other insect

eggs, which prevents rapid penetration of fixatives, it is necessary to search for the most appropriate kind of fixation. The penetration of osmium tetroxide is particularly poor and requires removal of the eggshell and cutting of the embryo. Before fertilization, the silkworm egg is extremely viscose between its chorion and the vitelline membrane, and removal of the eggshell is impossible. Once the egg is fertilized, however, the removal becomes possible because the change of the vitelline membrane into the "fertilization membrane" is associated with a reduction of viscosity. However, a lot of practice and time are required. Another obstacle is that the fixed yolk prevents penetration of other liquids, such as the embedding media. Therefore, mechanical separation of the region to be examined and the most appropriate arrangement of the size of sections and the angle against the vertical pole of the egg are required to obtain satisfactory micrographs. In the case of stabbing, penetration is unsatisfactory except around the stabbed spots.

With the progress of development and during the formation of cuticles on the serosa, the eggshell becomes easy to separate, and the fixation without chorion intervention is readily available. Moreover, it is possible to fix only the embryo separated from the yolk when necessary.

In regard to the obstacles described above, most examinations of the early embryonic development were done on specimens fixed with the osmium tetroxide at the first stage, and subsequently with the glutaraldehyde-osmium. The egg was typically cut and washed in ice-cold buffered 2.5% glutaraldehyde solution (veronal buffer, pH 7.4) and then fixed with ice-cold 1% osmium (veronal or phosphate buffer, pH 7.4) for 1.5 - 2 hr. In some cases, the egg was fixed in the former fixative for 1.5 - 2 hr and washed in the buffer for 30 min, and then fixed in osmium tetroxide solution for 2 hr. Subsequently, the egg was cleaned in the buffer again and dehydrated with ethanol series. The methacrylate resin and later the epoxy resin (Epon 812) were used for embedding. The method of uranyl-lead double stain was taken up. Uranyl acetate was followed by lead nitrate or lead citrate.

Although we paid much attention to the preparation of the samples, we have to admit a certain discordance among the results because we started with comparatively easy methods to cover a large range of embryogenesis. A note on the methodology was attached to the figure description whenever I deemed it important.

Chapter 1 Architecture of Mature Egg

A mature egg of the silkworm is normally a depressed spheroid, approximately 1.3 mm long, 1.0 mm wide and 0.6 mm thick. These features, however, vary according to races and breeding conditions. The micropylar channels open at the apical surface on one pole of the egg, where sperms enter into the egg. This pole is called the anterior, being distinguished it from the opposite pole that is called the posterior; the side with a somewhat convex shape is called the ventral and its opposite region is called the dorsal. The formation of embryonic anlage occurs in the ventral region. The egg cell is endowed by a normal cell membrane, enveloped by an extracellular vitelline membrane, and on the surface, protected by a thick tough chorion. The ooplasm can be divided into a superficial layer (the periplasm) and the central part with a reticular structure (the reticuloplasm). Most of the yolk is contained in the reticuloplasm, and the egg-nucleus is located at the dorsal side close to the micropylar region.

From an ultrastructural point of view, the definition of a "mature egg" has somewhat delicate aspects. In silkworm eggs, the sperms injected into the female copulatory pouch (bursa copulatrix) after mating migrate into the seminal receptacle (spermatheca) that serves as a sperm storage organ. During oviposition the sperm is released from the receptacle into the oviduct to fertilize the descending eggs before they are laid from the ovipositor. The moment of laying is commonly regarded as the beginning of embryogenesis and is taken as a time equal to zero in some situations when the egg laying is delayed, and thus the development may start in the female body. In exceptional cases, morphological changes can take place already during the egg detachment from the follicle epithelium, before it begins a descent through the oviduct (ovulation). This happens when parthenogenic development is precociously induced, for example by the treatment with hot water (Astaurov, 1967). Accordingly, the comparison of the egg ultrastructure before and after ovulation seems necessary. In this book, however, the morphology of eggs descending into the oviduct of pre-mating females is taken as a basis.

1.1 Eggshell

The eggshell consists of chorion and vitelline membrane, which are formed at the final stage of oogenesis. Their formation will be discussed in the chapter on oogenesis in another book on "Gametogenesis".

1.1.1 Chorion

The chorion of silkworms is the outermost egg envelope. It is formed by elastic and hard proteins and has a complicated structure that is compatible with physiological

functions such as the protection of an egg cell, the provision for sperm entry, gas change, and water retention. The boundaries of follicle cells are imprinted on the chorion surface in a characteristic pattern (egg patterns). Fig. 1A represents a typical surface structure of the chorion. The patterns vary according to races and regions: the boundaries sometimes swell to septa-like elevations as shown in the figure. In other chorions the boundaries may look like fine grooves. Aggregates of small callus-like piles of chorion may appear within the field defined by the imprints of cell boundaries. Several aeropyles open at the imprint encompassing more than three follicle cells in a specific egg region (Ohtsuki, 1979).

The chorion is composed of three layers, whose cross section is shown in Fig. 1B. The inner layer consists of two thin granular sublayers about 1 μm thick and a trabecular layer, with numerous spaces between the two (Fig. 2C). The middle layer consists of a pile of 30~35 fine-fibrous thin sublayers at about 10~12 μm thick (Fig. 2B). The outer layer is composed of 25~30 thin sublayers about 4 μm thick and very dense (Matsuzaki, 1968).

The surface of the micropylar region is considerably different from the remaining egg surface as demonstrated in Fig. 3A. It shows a petal-like pattern (micropylar rosette) with a micropylar opening (micropylar orifice) in the center. The number of micropylar channels is normally 3~4, but in some samples 6~8 channels are recognized (Ohtsuki, 1979; Kawaguchi *et al.*, 2002).

Three kinds of cells are involved in the formation of chorion in the micropylar region: micropylar channel-forming cells, micropylar orifice-forming cells and petal-like pattern (micropylar rosette)-forming cells (Yamauchi and Yoshitake, 1984) as represented in the drawing of Fig. 3B. Fig. 4A shows that the outer and inner layers are not detected in thin sections of the micropylar region (Akai, 1958; Miya, 1978, 1984a). Fig. 4B reveals that the lamellae of the middle layer, which is penetrated by micropylar channels made of the micropylar channel-forming cells, has an irregular arrangement (Akutsu and Yoshitake, 1974).

1.1.2 Vitelline membrane

The vitelline membrane used to be considered as a kind of simple noncellular membrane formed before the chorion formation. However, recent electron microscopic observations have revealed that the vitelline membrane structure is complex and differs in various insect species. The vitelline membrane of silkworms is composed of a thin layer adjoining the chorion and showing partly scarified piles, a subsequent electron dense layer about 0.25 μm thick, and a broad inner layer containing abundant electron dense irregularly-shaped granules scattered evenly among finer granules (Akutsu and Yoshitake, 1977 ; Miya, 1978, 1984a). In some observations, the outer thin layer is vague due to fixation conditions or specific features of the particular silkworm race (Fig. 5B). In the micropylar region, both the outer and the inner layers become thicker and in the immediate vicinity of the micropylar channel tubule, the outer layer reaches 2.0~2.5 μm in thickness. In this area, a wide space occurs in the outer layer, from the end of which a channel tubule (about 0.6 μm in diameter), crooked somewhat and enclosed by a thin membrane (about 6 nm thick), penetrates the inner layer and gets close to the oolemma but terminates in the inner layer without making any contact with the oolemma (Fig. 5A and Fig. 7A). The channel tubule contains many fine vesicles and

granules, which are presumably derived from the corn-like processes of its forming cells. These structural elements flow into the inner layer from the opening of the channel tubule (Fig. 7B).

1.2 Ooplasm and yolk system

As in most insect eggs, the ooplasm of silkworm eggs is divided into the periplasm, a superficial layer that is free from the yolk spheres, and the endoplasm or the reticuloplasm, which occupies the central part of the egg. The periplasm of silkworms is comparatively thick. Particularly, the anterior region specified for the sperm entry possesses a conspicuous ultrastructure different from the other regions. The determination of certain blastoderm cells to the primordial germ cells is thought to occur in the pole plasm as in Diptera. However, unlike in Diptera, the plasm in this silkworm egg does not exhibit any special structural features. The ribosomes and mitochondria are the most conspicuous ooplasmic organelles. Polysomes are normally not formed in unfertilized eggs. Instead, some part of the ribosomes are bound with an endoplasmic reticulum to form a cisterna-like or vesicular rough endoplasmic reticulum, which gives a particular arrangement to each region of the ooplasm.

1.2.1 Periplasm at the anterior region

The ultrastructure at the anterior region is represented diagrammatically in Fig. 6, and its electron micrograph is in Fig. 8. As discussed previously, the vitelline membrane increases the thickness at the anterior region, and the oolemma protrudes big cone-shaped processes into the inner layer, from which a great number of tiny processes extend. Around this area, the rough endoplasmic reticulum arranged in stacks parallel to the oolemma surface develops remarkably. A dense granule zone connects to the periphery of the stacked structure of the ER (the zone of endoplasmic reticulum in Fig. 9A). There are no other organelles but a small number of Golgi bodies in the rER zone. No mitochondria are distributed there. In the ooplasmic processes, there are medium-dense minute vesicles, 80 μm in diameter, and a minute vesicular zone of fine vacuoles (Fig. 9), which is also one of the most characteristic structures limited to the anterior region. Also, a few lipid droplets and round granules with thick cortex (they sometimes show concentric arrangements and includes calcium phosphate; Miya, 1984a) are detected in these processes.

A reticular structure connects to the inside of the ER zone, where yolk spherules (which are densely stained with toluidine blue and also have many needle-like structures), mitochondria, multivesicular bodies, lipid droplets, vacuoles of various size, and glycogen granules are recognized. The yolk spherules are obviously the structures that Takesue *et al.* (1976) described as Yg-d. Their ultrastructure varies as shown in Fig.13-B.

Between the rER zone of the anterior region and its adjacent periplasm, there is a zone that contains electron-dense granules, vesicles harboring these granules, and abundant mitochondria (the granule zone, Fig. 9B). These remarkable features of the granule zone can be presumed to have relation to the sperm entry. The significance of the anterior region for the sperm entry and its subsequent role in embryogenesis still re-

main to be solved in the future.

1.2.2 Periplasm outside the anterior region

It has been reported that silkworm egg is not completely ellipsoidal but bulges out on one side more than on the other; the chromophile properties of the ooplasm 1 hr after oviposition diversify according to the region, and the embryo develops at the more convex side of the egg (Takahashi and Yagi, 1926). Further investigations revealed that the chromophilic differences depend on the structure and the density of pyronine-positive granules, which attach to the periplasm in the presumptive embryonic region, and that they reflect the range of the embryonic and extra-embryonic blastoderm regions (Kobayashi and Miya, 1987, Fig. 10). From the ultrastructural point of view, the pyronine-positive granules correspond to the structures with concentric arrangement of the stacks of rough endoplasmic reticulum. As to the size and shape of the granules, there are regional differences as shown in Figs. 11 and 12. When embryogenesis starts, the pyronine-positive granules seen in the light microscope disappear in parallel with the disappearance of the stacked structures observed in the electron microscope. It remains to be clarified how much these structures participate in the determination of the embryonic region and the architecture of embryonic anlage.

In some Lepidoptera whose periplasm is comparatively thick, the periplasm sometimes consists of several layers that can be distinguished by the distribution of the organelles. For example, the periplasm of some Noctuidae and Tortricidae is divided into the outer layer, which includes bordered concavities, vesicles, multivesicular bodies, Golgi bodies, and cisternae or vesicles with dense granules. Further, the inner layer contains a great amount of mitochondria and stacks of rER arranged parallel to the egg surface (Ferenbach *et al.*, 1987). In some Acraeidae, an outer layer with multivesicular bodies and fine vesicles of ultrastructural granules, a middle layer which consists of yolk granules and lipid droplets, and an inner layer containing abundant mitochondria and rER are detected (Balinsky, 1986).

In the silkworm egg, the periplasm outside the anterior egg region is composed of a superficial layer, in which short rod-like mitochondria, lipid droplets, yolk spherules, vacuoles and glycogen granules are distributed in ooplasm rich in ribosomes, and an adjacent subcortical layer, which contains multivesicular bodies, a little larger vacuoles and C-yolk spheres within the reticular ooplasm. It is clearly distinguished from the inner yolk system. The oolemma protrudes with many microvilli into the inner layer of the vitelline membrane, and the rER zone with large concentric arrangements, which connects to the subcortical layer, is detected (Fig. 13A). Beside this structure, a remarkable difference in the distribution and morphological features of organelles and other inclusions between the presumptive embryonic region and the extra-embryonic region, is not discovered (Figs. 11 and 12). C-yolk spheres are the yolk spheres formed at the final stage of vitellogenesis. They are observed only in the subcortical layer and characterized by containing electron-dense spherules whose structures show many varieties; in some cases, vesicles and glycogen granules are observed (Takesue *et al.*, 1976; This spherule is described as Yg-2.). Also, there are many morphological varieties of yolk spherules (Fig. 13B).

1.2.3 Yolk system

The yolk system that occupies most of the egg is composed of the reticuloplasm with numerous yolk spheres, lipid droplets and glycogen granules. Free ribosomes, rough endoplasmic reticulum and mitochondria are scattered in the ooplasmic network, but no other structures are detectable. Two types of yolk spheres are distinguishable morphologically; the A-yolk spheres contain homogeneous fine particles (Fig. 14A) and the B-yolk spheres contain numerous electron-dense granules (Fig. 14B). The A-yolk spheres are abundant in the peripheral region.

1.3 Egg nucleus

The egg nucleus is situated on the dorsal side of the egg at a short distance from the micropylar region. An unfertilized egg stops its cleavage during the middle stage of the first maturation division. Fig. 15 represents the structure at this stage, where rows of electron-dense chromosomes in the equatorial plane are bound by some materials of middle electron density. These chromosomes come from the synaptonemal complex (Rasmussen and Holm, 1982) which make homologous chromosomes bind, and remain at the equatorial plane during the later stage. There are many stacks of rER with irregularly concentric arrangements, enclosing spindles that consist of microtubules adjacent to chromosomes. Small zones of dense fine particles (rER-associated rosettes) attach to the contractile regions of stacks that are scattered randomly in rER. The same situation occurs with stacks of rER at the micropylar region. Small sizes of Golgi bodies are also scattered, but no mitochondria can be observed. A lot of lipid droplets enclose the stacks (Fig. 16).

< Figure 1> Chorion

The superficial structure of chorion

The surface of chorion is imprinted by the boundaries of follicle cells that produced it and thereby shows a conspicuous pattern (the egg patterns). This figure is a scanning electron micrograph (SEM) of the upper central structure of an egg (Nichi 140). The imprints of the follicle cell boundaries are elevated, clearly demarcating small chorion fields beset with petit callus-like piles. At the imprints of the boundary of more than three cells, aeropyles open.

Arrow, aperopyle

(Modified from Ohtsuki, 1979)

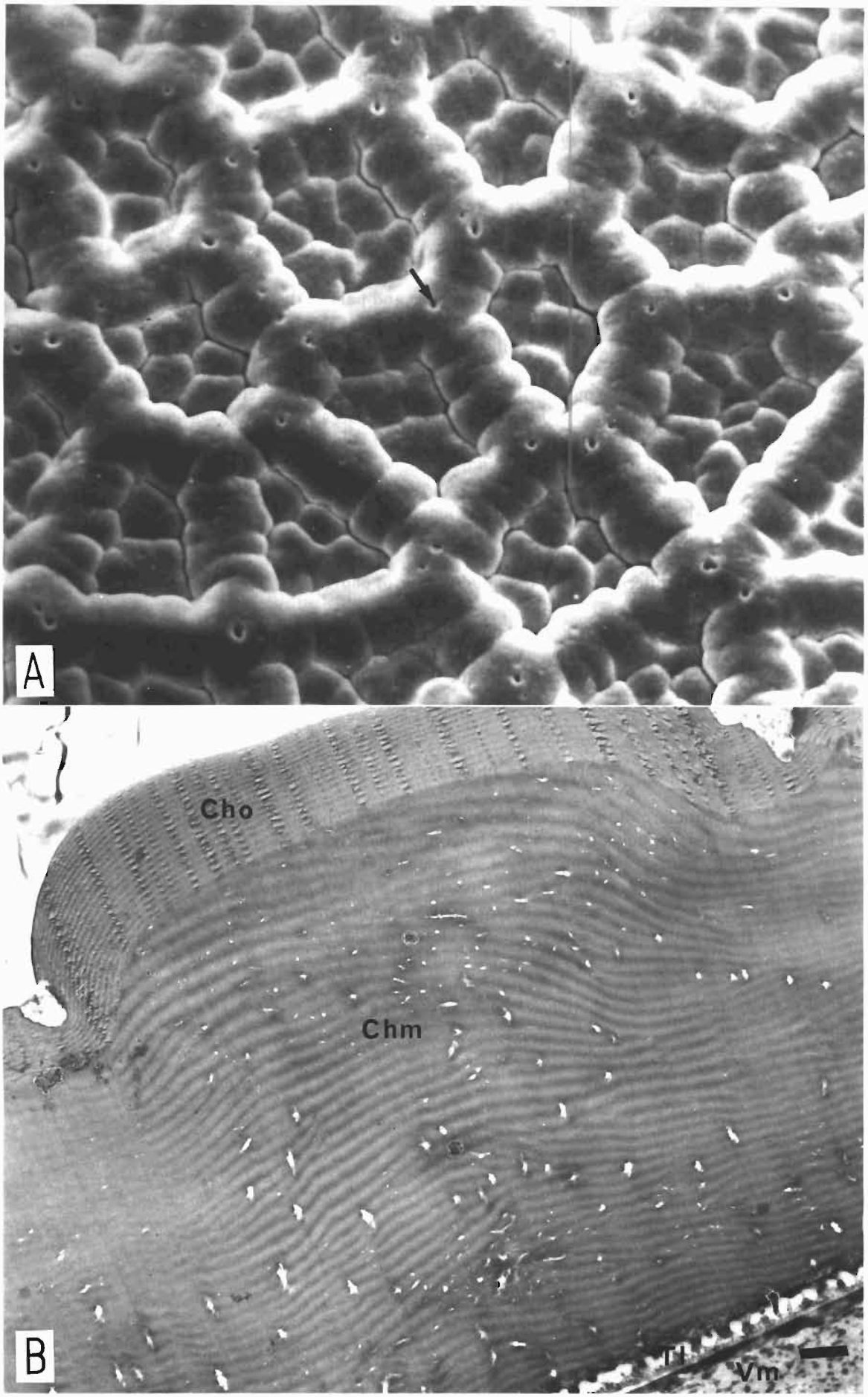
The section of chorion

This is a cross section of chorion in a lateral region of an egg of the commercial hybrid (Shunrei x Shogetsu). The chorion is composed of three layers; the inner layer which consists of two thin granular sublayers and a trabecular porous layer, the thick middle layer which displays a pile of abundant thin fine-fibrous sublayers with grain-like arrangement, and the outer layer (thinner than the middle layer) which shows a pile of thin layers with slightly high electron density. The thickness of chorion varies according to races. The European races have the thickest chorion, while the thickness is reduced in the Japanese, Chinese-univoltine, and maximally in the Chinese-bivoltine races. Even in the same egg, there are regional differences, for example its chorion becomes the thickest at the upper central region (Ohtsuki, 1979).

Chm, middle layer of chorion; **Cho**, outer layer of chorion; **T1**, inner layer of chorion; **Vm**, vitelline membrane

(Modified from Miya, 1978)

Note : The gultaraldehyde-osmium double fixation is adopted unless a note is added to a Figure. The scale indicates 1 μm unless a number is added to the bar.


A
Cho
Chm
B
Vm

< Figure 2> Chorion

Enlarged structure of chorion of the commercial hybrid (Shunrei x Shogetsu).

A. Enlarged micrograph of the outer layer and part of the middle layer

The outer layer consists of a pile of compact sublayers of a moderate electron density, about 0.2 μm thick. Sixteen thin layers are observed in this figure. Some dense material is outside the outer layer. This adhesive material is secreted from the female collaterial (mucous) gland during egg laying. The spaces between the outer sublayers, which are regarded as the imprints of follicle cell boundaries, and tubular structures between the thin sublayers of the middle layer, are the aeropyles.

Ap, aeropyles; **Chm**, middle layer of chorion; **Cho**, outer layer of chorion

B. Enlarged micrograph of the middle layer of chorion

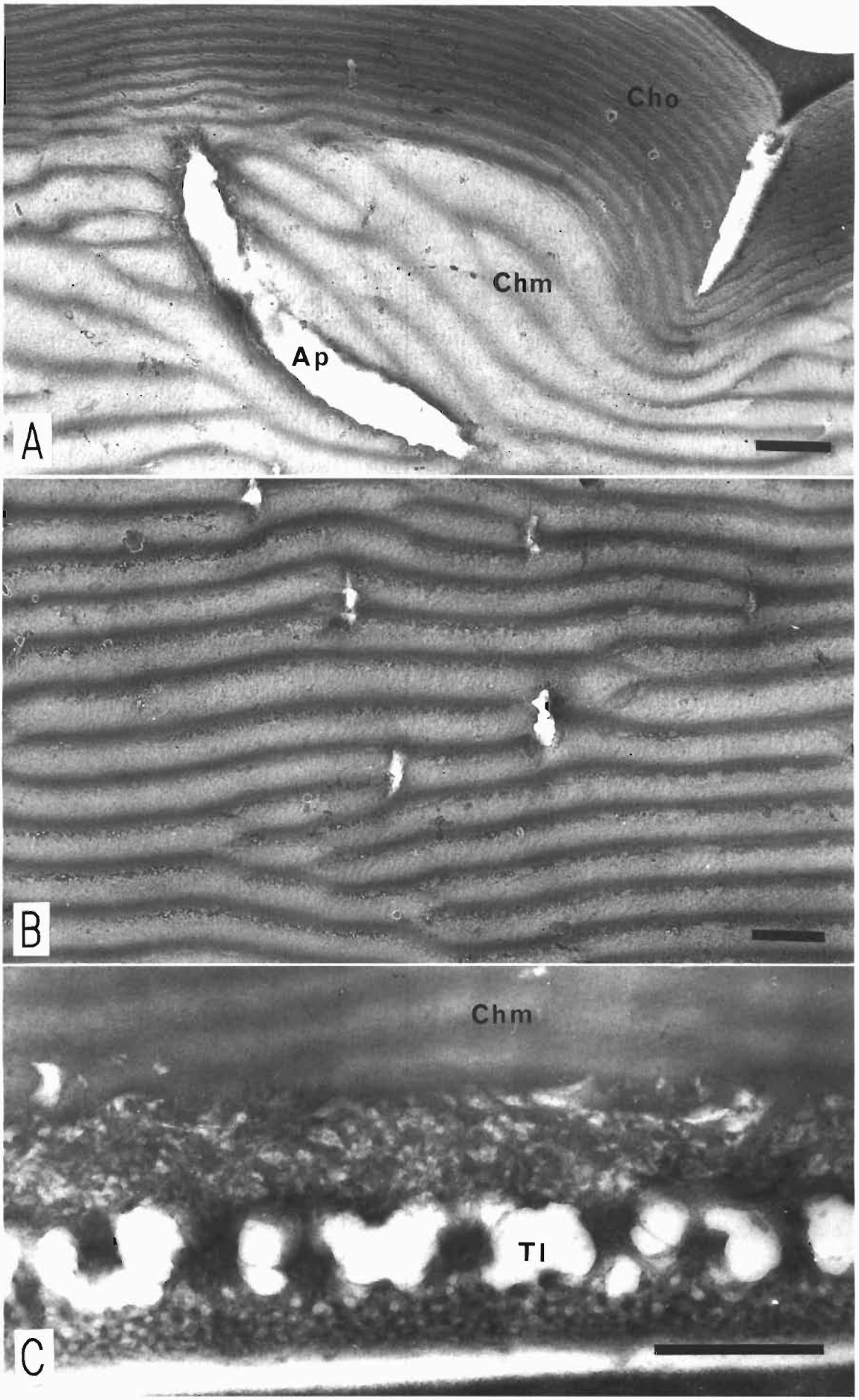
The middle layer displays a pile of thin sublayers about 0.4 μm thick with fine-fibrous grain-like arrangements. This sample displays 40 sublayers. Small irregular spaces are scattered among and within the sublayers that are less compact than the outer layer.

C. Enlarged micrograph of the inner layer and part of the middle layer

The structure of the inner layer is very complex. There are two thin layers and a trabecular layer between them. The innermost thin layer is about 0.2 μm in thickness and contains many unevenly distributed granular porosities. The middle trabecular layer is about 0.4 μm thick, and the outer layer is about 0.4 μm, containing a lot of sponge-like spaces. In some races, the outer layer is vague in certain chorion regions.

It seems that the inner layer has the closest relation with such physiological functions of the egg such as breathing (Hinton, 1969).

Chm, middle layer of chorion; **T1**, inner layer of chorion


Cho
Chm
Ap
A
B
Chm
TI
C

< Figure 3> Chorion

A. Superficial structure of chorion at the micropylar region

This figure is an SEM of the micropylar region of chorion of Nichi 140. The surface of chorion displays a completely different pattern, a petal-like pattern (rosette), and at its center the micropylar channels open. In this figure, 6 orifices of micropylar channels are observed.

(Modified from Ohtsuki, 1979)

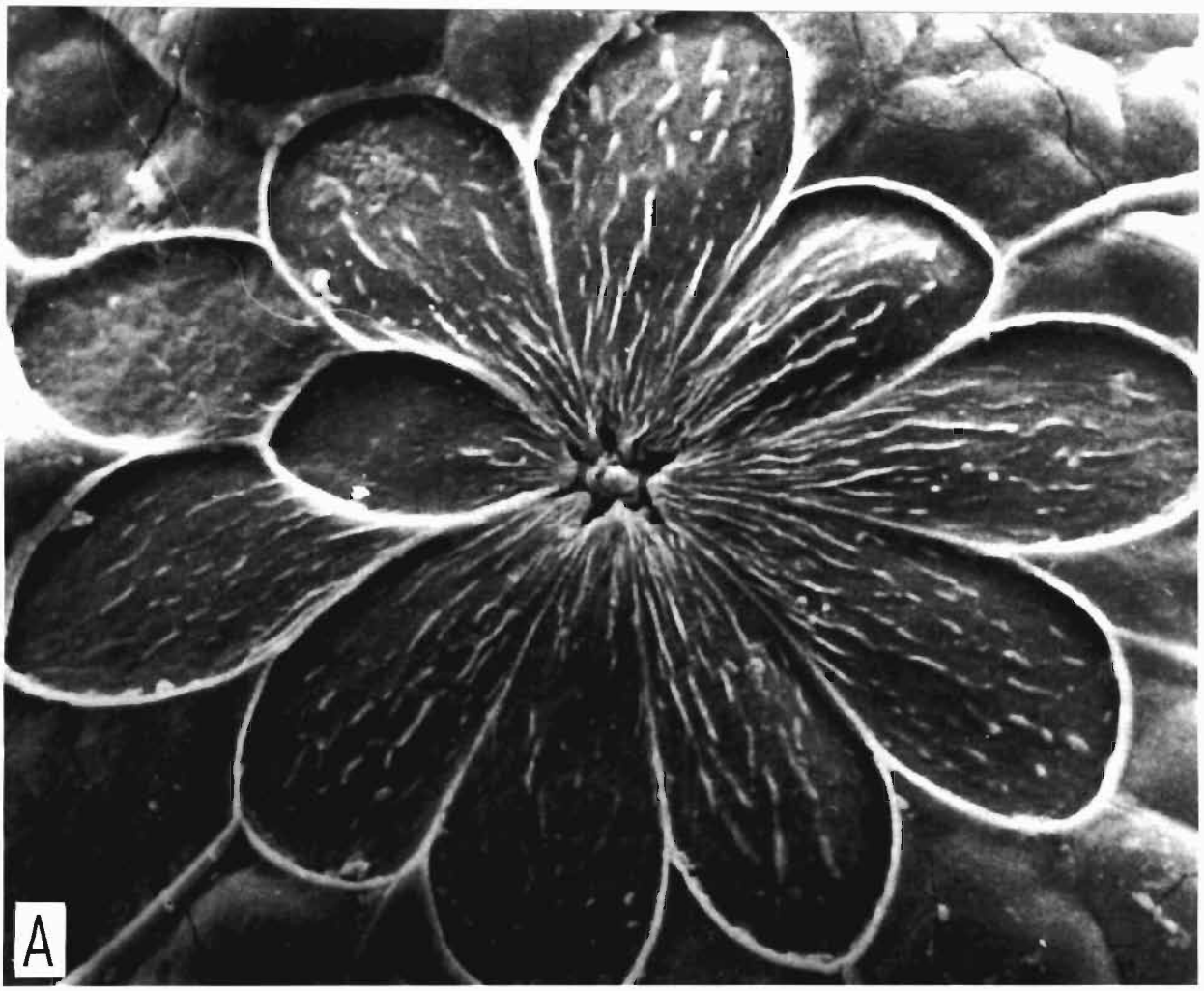
B. A diagrammatical drawing of the micropylar region of a mature egg

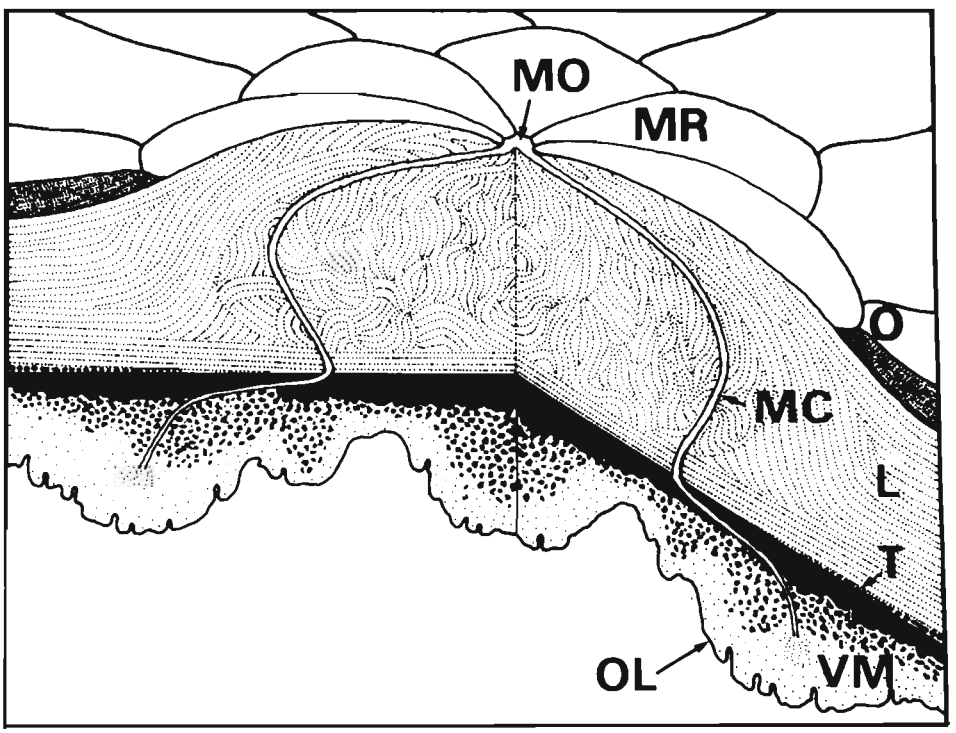
The three kinds of specialized follicle cells, i.e. the micropylar channel-forming cells, the micropylar orifice-forming cells and the petal-like pattern (rosette)-forming cells, are concerned with the formation of chorion at the micropylar region. The chorion at the micropylar region lacks the outer and inner layers. The sublayers of the middle layers show an irregular arrangement.

The micropylar channel tubules penetrate the middle layer of chorion with the help of some cytoplasmic processes of the micropylar channel-forming cells. The channel tubules penetrate the vitelline membrane too, but do not reach the oolemma. The end of the channel tubule opens within the vitelline membrane.

L, middle layer of chorion; **MC**, micropylar channel; **MO**, micropylar opening (orifice); **MR**, petal-like pattern (micropylar rosette); **O**, outer layer of chorion; **OL**, oolemma; **T**, inner layer of chorion; **VM** , vitelline membrane

(Modified from Yamauchi and Yoshitake, 1984)





B

< Figure 4 > Chorion

A. Structure of chorion at the micropylar and adjacent regions

The structure of chorion at the micropylar and adjacent regions is shown in an egg of the Daizo. As the outer layer comes up to the micropylar region, it becomes thinner and thinner, and it completely disappears at the micropylar region. At first the number of sublayers forming the outer layer is 9 in this figure. The outer layer becomes thinner and at the same time the number of thin sublayers reduces.

After the septum of the trabecular layer that occupies the central part of the inner layer disappears, a series of porosities are produced, and they become granular vesicles. They will, however, disappear completely at the micropylar region.

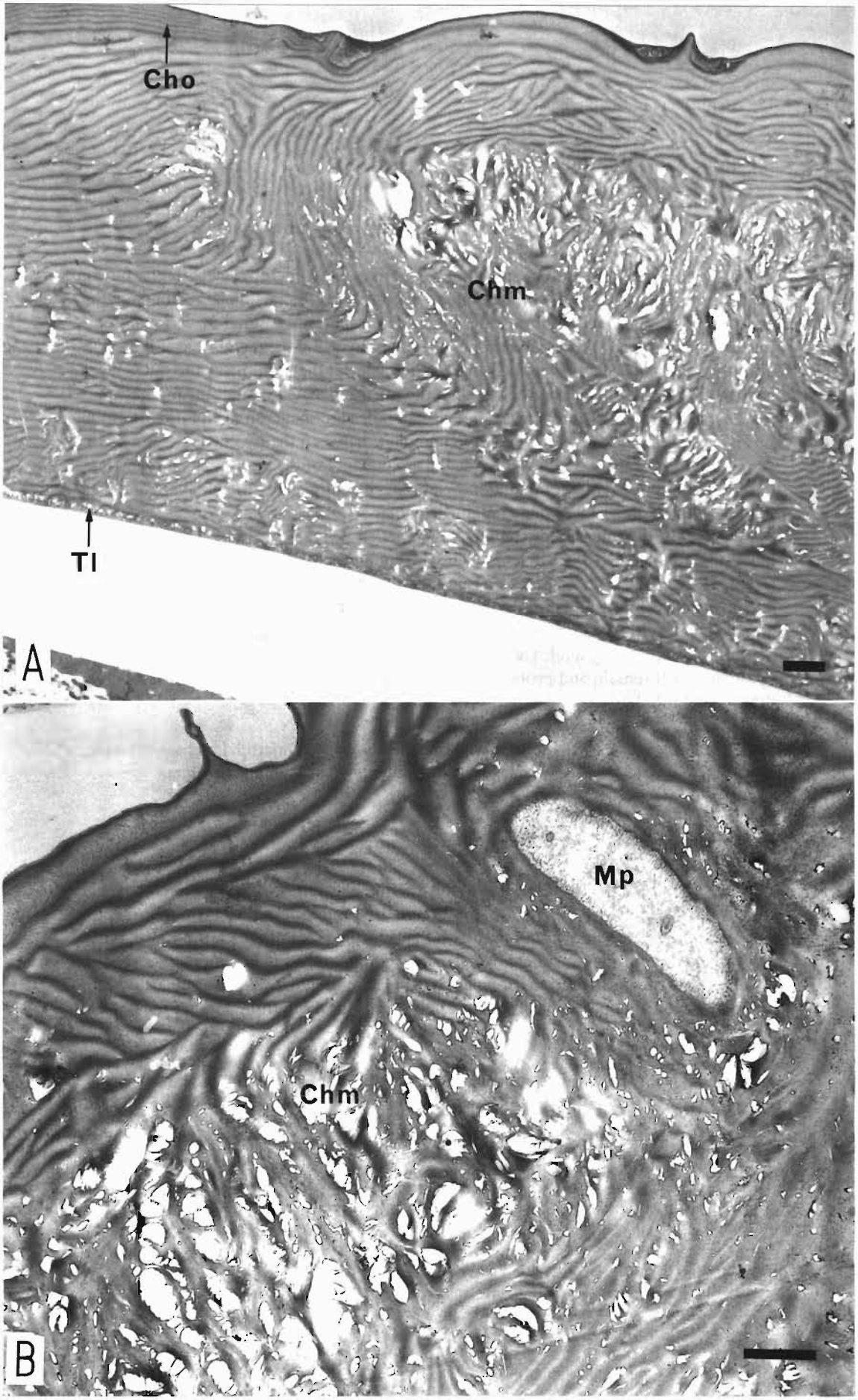
While 38 sublayers of the middle layer are arranged to make a stack parallel to the surface of the egg, the arrangement goes out of order at the micropylar region, and many spaces are scattered among the sublayers.

Chm, middle layer of chorion; **Cho**, outer layer of chorion; **Tl**, inner layer of chorion

B. Enlarged figure of chorion at the micropylar region

An enlarged micropylar region of chorion of a Daizo egg is shown. The stacks of sublayers of the middle layer are completely disturbed. There are various sizes of spaces at the central part. The right side of the figure shows a slant section of a micropylar channel, whose inner wall is penetrated with many microvilli, and a lot of fine particles are observed within the channel. Such structures will disappear after sperm goes through the channel.

Chm, middle layer of chorion; **Mp**, micropylar channel


Cho
Chm
TI
A
Mp
Chm
B

< Figure 5 > Vitelline membrane

A. Architecture of vitelline membrane at the micropylar region

The architecture of vitelline membrane of Daizo at the micropylar region is shown. In this sample, an extremely thin membrane about 3 nm thick is observed outside the outer layer of vitelline membrane, to which dense materials attach in various areas. Because some space is detected between the outer layer and the membrane at this position, the materials seem to be the same as the material that composes the outer layer (Arrow). The outer layer is rather thick at the micropylar region, and the thickest part reaches 2 μm. Many different sizes of dense granules are scattered from this part of the layer into the inner layer. However, the boundary between the two layers is not clear. Accordingly, it seems possible to divide the architecture of the vitelline membrane into the following three parts: a thin outer membrane, a central stack of sublayers of dense granules and an inner layer that contains irregular sizes of granules.

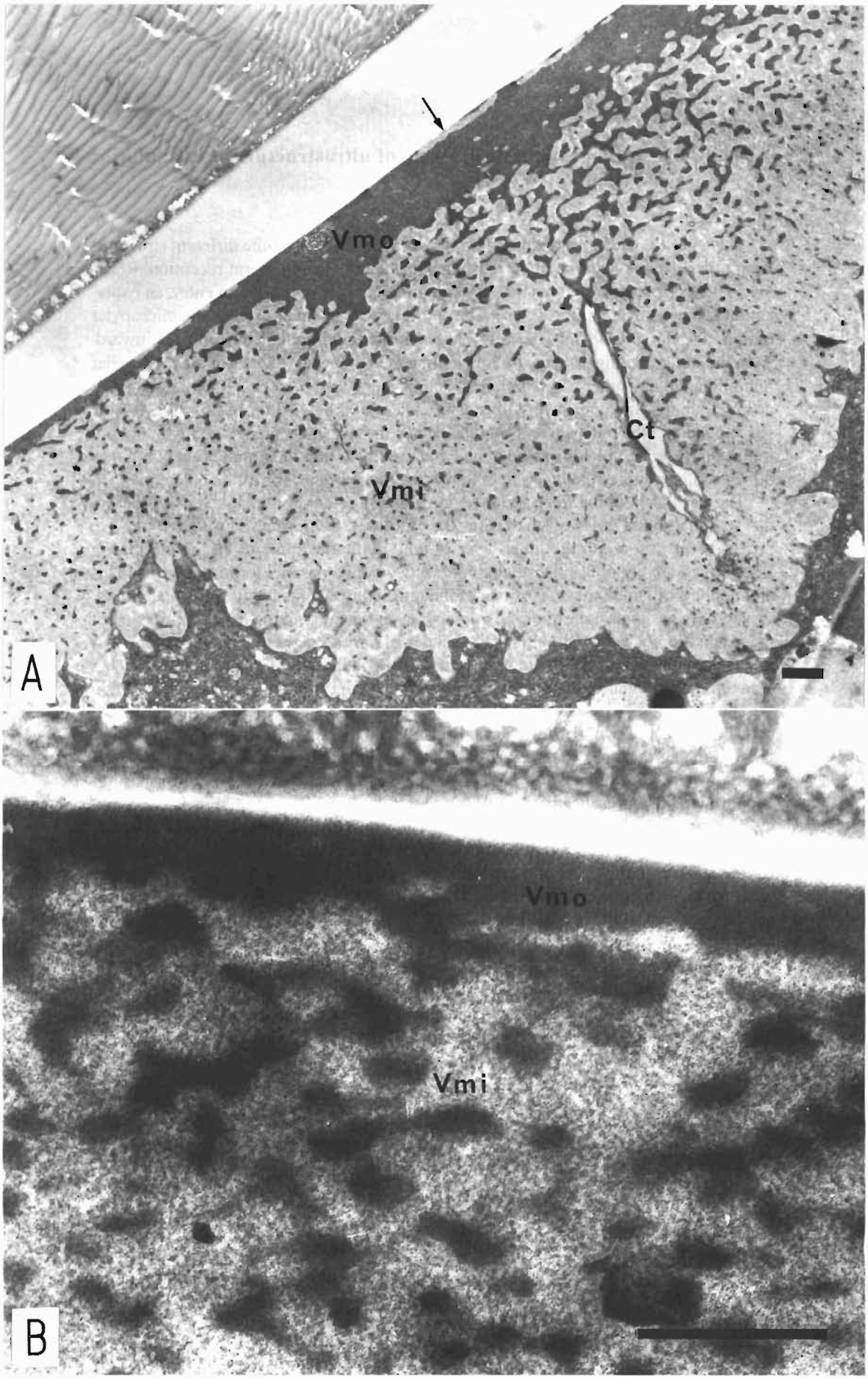
The end of the micropylar channel which penetrates the chorion is connected by a bugle-like tubule 16~18 μm in length, and this tubule stabs into the vitelline membrane (Akai, 1957; 1958). The tubule is a thin tube that consists of elastic chorion material and opens close to the oolemma, but is not in contact with it. This channel tubule is also composed of cytoplasmic processes of micropylar channel-forming cells. After its formation is completed, granules and vesicles that seem to be constitutive elements of the cytoplasm still remain and protrude into the inner layer of the vitelline membrane via the channel tubule.

Arrow, thin membrane at outermost vitelline membrane; **Ct**, micropylar channel tubule; **Vmi**, inner layer of vitelline membrane; **Vmo**, outer layer of vitelline membrane

B. Enlarged micrograph of the vitelline membrane in another region

The structure at the lateral region of the vitelline membrane of a commercial hybrid (Shunrei x Shogetsu) is shown. In this sample, the outermost thin membrane is not detected, maybe due to osmium tetroxide fixation. The lamellar electron-dense outer layer is about 0.4 μm in thickness, which is much thinner than in the micropylar region, and is easily distinguished from the inner layer with the irregular shaped granules. Within the inner layer, fine particles are distributed evenly, and intermingle with the above-mentioned granules.

Vmi, inner layer of vitelline membrane; **Vmo**, outer layer of vitelline membrane


Vmo
Ct
Vmi
A
Vmo
Vmi
B

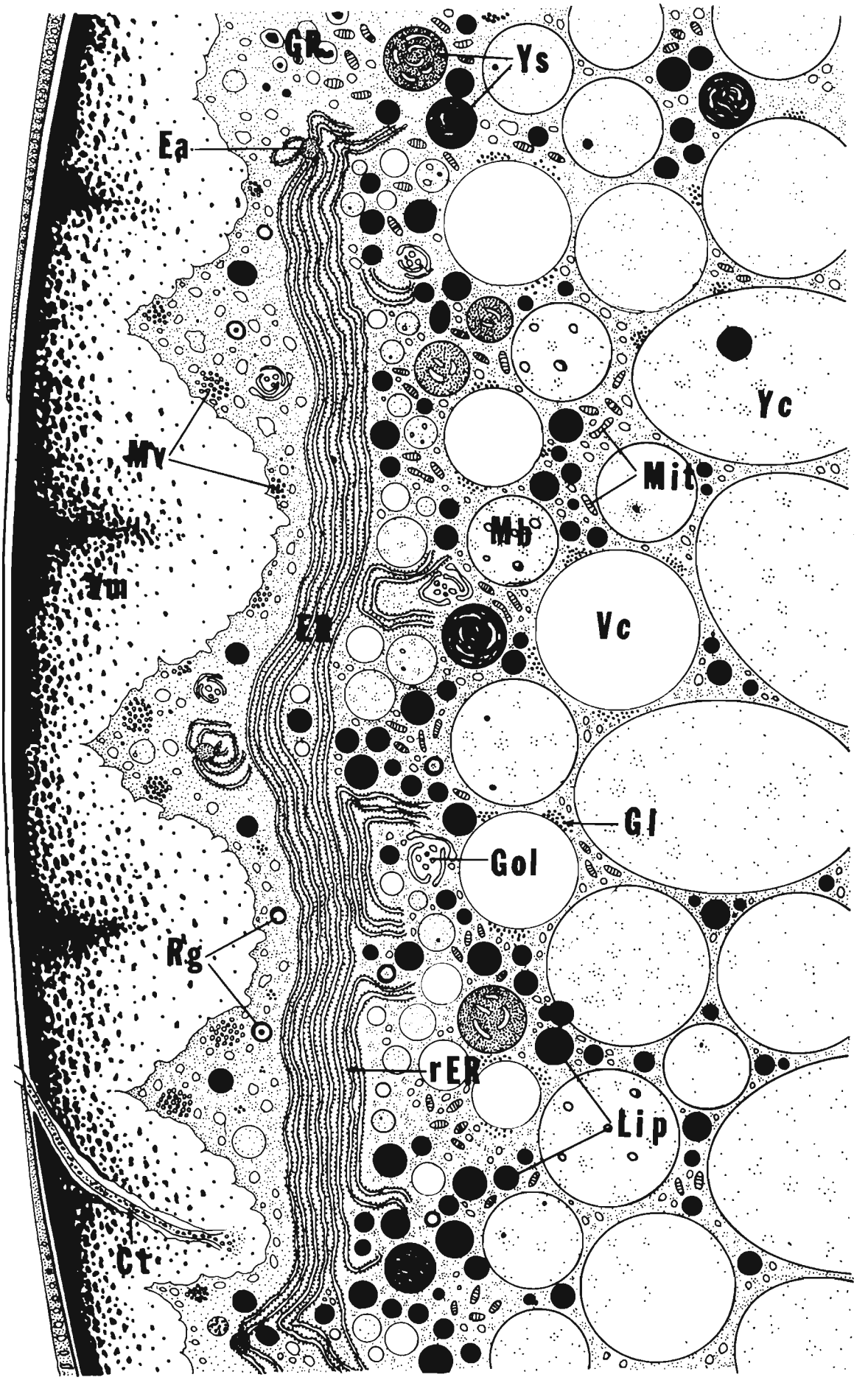
< Figure 6 > A diagrammatic drawing of ultrastructure at the anterior region

The anterior periplasm region of the silkworm egg exhibits quite different structures from other regions because of various phenomena concerning sperm reception. Relating to the periplasm structures that play a significant role in the sperm entry, an explanation has been made in this figure. The chorion lacks its inner layer in the micropylar region, and the micropylar channels that penetrate the middle layer protrude inward. Both the outer and inner layers of the vitelline membrane are thicker in the micropylar region than in any other egg region. Especially, at the spot pierced with the micropylar channel tubule, the outer layer gains its thickness of 2.0~2.5 μm. In this area, a wide space occurs in the outer layer. From the end of the space, a channel tubule (about 0.6 μm in diameter) crooked somewhat and enclosed by a thin membrane (about 6 nm in thickness), penetrates the inner layer to reach almost the surface of the oolemma.

The periplasm at the micropylar region shows a relatively thick layer of ooplasm that terminates in a series of small corn-like processes, from which short microvilli-like processes protrude into the inner layer of the vitelline membrane. In the periplasm, stacks of rough endoplasmic reticulum have developed parallel to the oolemma. This architecture of lamellar stacks is one of the most conspicuous features that can be observed only at the periplasm of the micropylar region. Within the ooplasm, outside of the stack, there are a great number of vesicles of various sizes and also aggregates of minute vesicles, about 80 nm in diameter, which contain less-dense materials. Such a structure is particular to the micropylar region called the "minute vesicle zone". Within the stacks of rough endoplasmic reticulum called the ER zone, there are no mitochondria, but some occur under the stacks. ER-associated rosettes made of electron-dense granules in a group stick to the end of the stacks. At the outside of the ER zone, there is an area where a lot of vesicles that contain dense granules and a lot of filiform mitochondria are also present (the granule zone).

The reticuloplasm lies adjacent to the periplasm. This ooplasmic network contains multivesicular bodies, a large size of vacuoles and a layer (subcortical layer) enclosing C-yolk spheres that consist of translucent materials and electron-dense spherules. The subcortical layer appears thicker at the anterior region and has less C-yolk spheres than any other region of the egg.

Ct, micropylar channel tubule; **Ea**, rER-associated rosette; **ER**, zone of endoplasmic reticulum; **Gl**, glycogen granule; **Gol**, Golgi body; **GR**, granule zone; **Lip**, lipid droplet; **Mb**, multivesicular body-like structure; **Mit**, mitochondrion; **Mv**, minute vesicle zone; **Rg**, thick-cortex round granule; **rER**, rough endoplasmic reticulum; **Vc**, vacuole; **Vm**, vitelline membrane; **Yc**, C-yolk sphere; **Ys**, yolk spherule


Gr
Ys
Ea
Yc
Mv
Mit
Mb
Vm
En
Vc
Gl
Gol
Rg
rER
Lip
Ct

< Figure 7 > Vitelline membrane at the micropylar region

A. Vitelline membrane at the micropylar region

The structure of the vitelline membrane of Daizo at the micropylar region is shown. The micropylar channel crooks and penetrates the middle layer of chorion and the outer layer of vitelline membrane. The outer layer is composed of the outermost thin layer and electron-dense sublayers. At the stabbing spot of micropylar channels, the outer layer remarkably increases the thickness and produces a wide space. Micropylar channel tubules run through this space. The inner layer is also thick, and it encloses scattered irregular-shaped dense granules. The micropylar channel tubule opens within the inner layer near the oolemma. Fine particles and minute vesicles are observed in the inner layer around the opening.

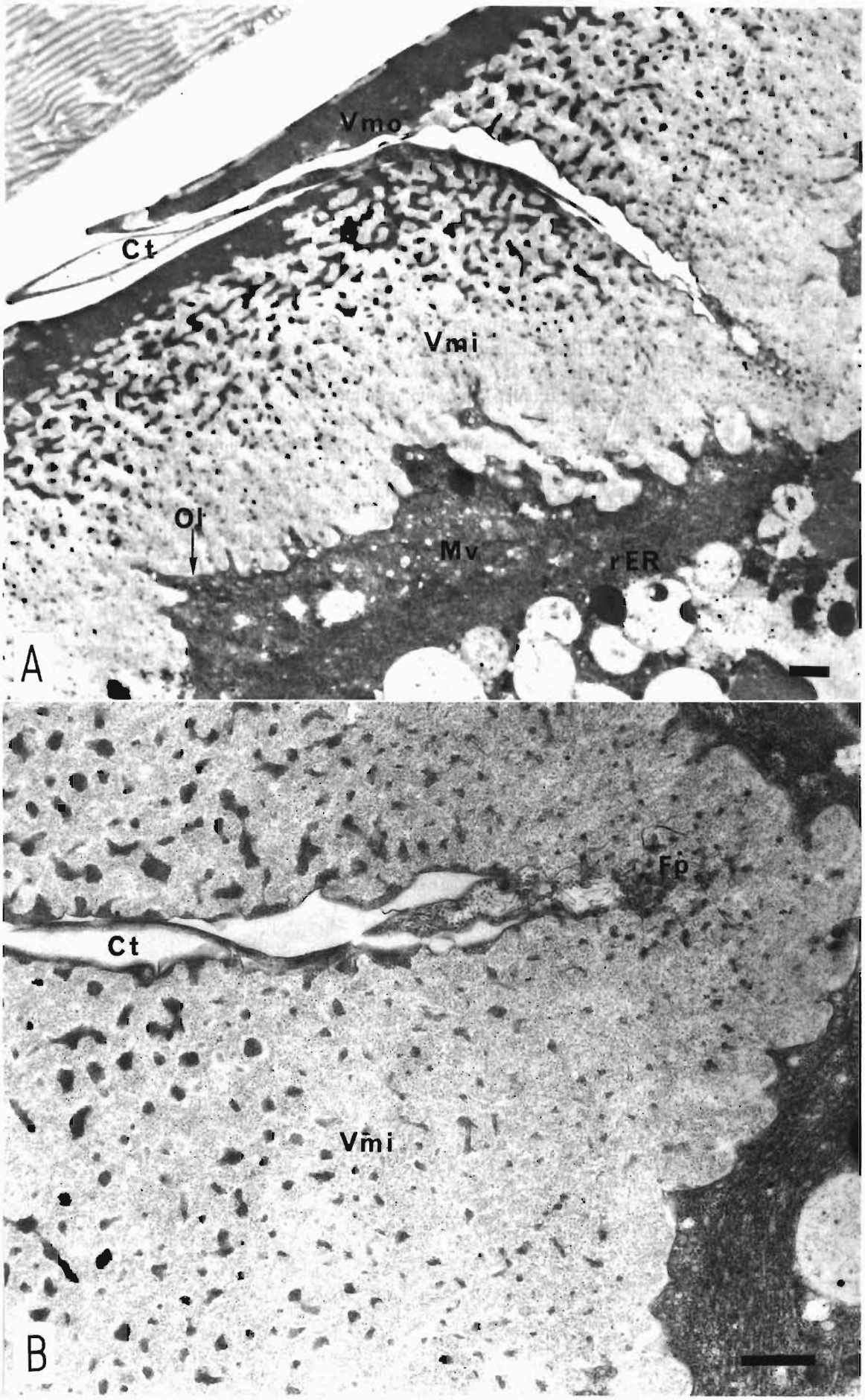
Ct, micropylar channel tubule; **Mv**, minute vesicle; **Ol**, oolemma; **rER**, rough endoplasmic reticulum; **Vmi**, inner layer of vitelline membrane; **Vmo**, outer layer of vitelline membrane

B. Enlarged micrograph of the micropylar channel tubule

An enlarged micrograph of the micropylar channel tubule of Daizo is shown. The inner layer of the vitelline membrane is tightly packed with large electron-dense granules at the area close to the outer layer. Both the size and the density gradually decrease toward the oolemma.

The micropylar channel tubule runs obliquely through the inner layer of the vitelline membrane but does not reach the oolemma. Instead it opens around the area. The channel tubule contains many minute vesicles and fine particles, which are presumed to be deposited by the corn-like processes of the forming cells. A part of these vesicles and particles flow through the channel tubule into the inner layer of the vitelline membrane.

Ct, micropylar channel tubule; **Fp**, group of fine particles and minute vesicles released from the channel tubule into the inner layer of Vmi; **Vmi**, inner layer of vitelline membrane


Vmo
Ct
Vmi
Ol
Mv
rER
A
Fp
Ct
Vmi
B

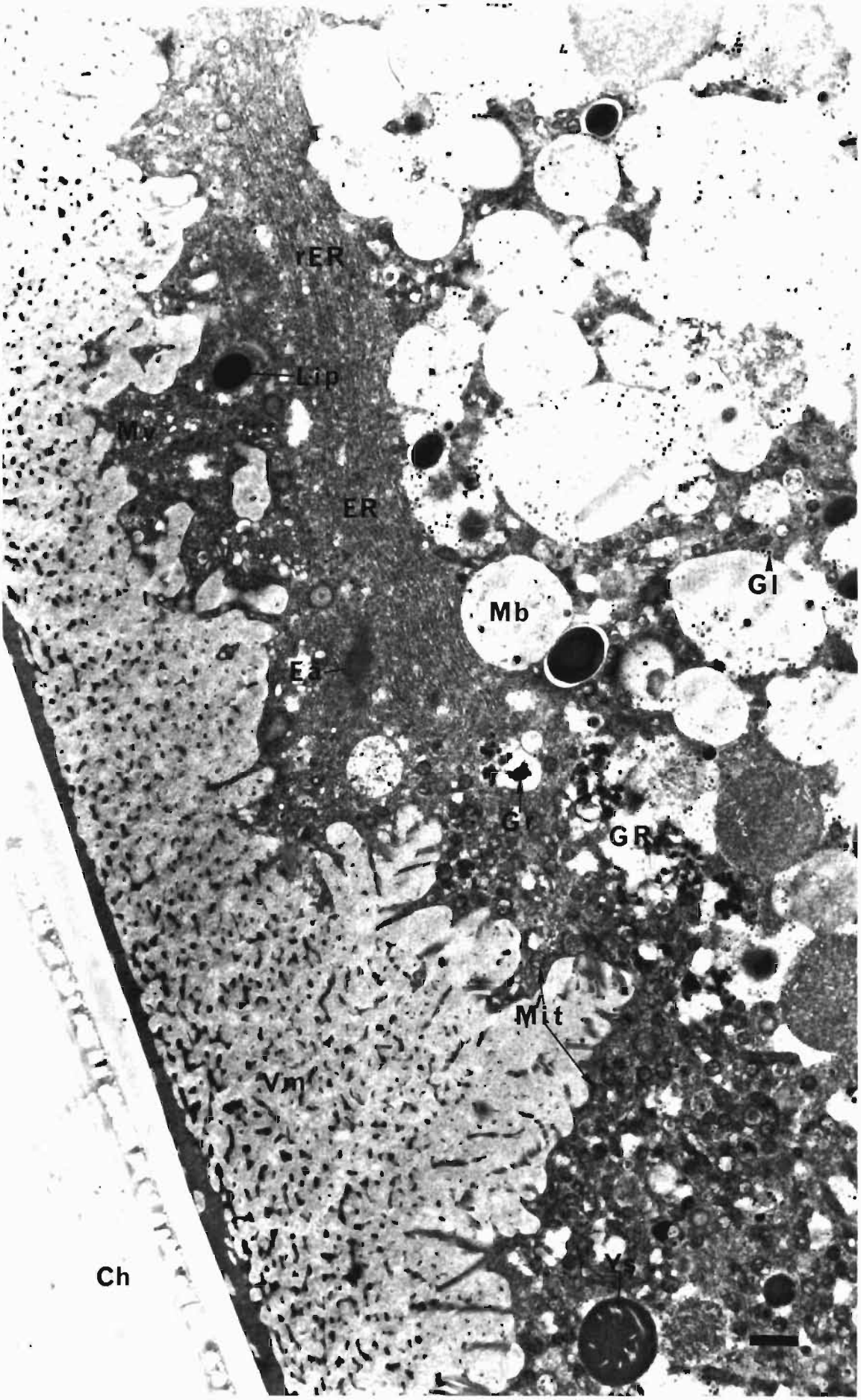
< Figure 8 > Ooplasm at the micropylar region

The figure shows the junction of the ER zone and the granule zone of the periplasm at the micropylar region of a Daizo egg. The periplasm at the micropylar region displays serial rows of cone-like ooplasmic processes, from which microvilli protrude into the inner layer of the vitelline membrane.

In the ER zone, stacks of rough endoplasmic reticulum parallel to the surface of the egg take priority, and ER-associated rosettes consisting of groups of electron-dense fine particles attach to the end of the stacks. Outside the stacks, there are numerous minute vesicles, a few lipid droplets, and round granules with a thick cortex. Inside the stacks, multivesicular body-like structures, various sizes of vacuoles, lipid droplets and glycogen granules are present. The ooplasm that fills the space contains a lot of short rod-like mitochondria.

The granule zone is characterized by a lack of rER and presence of many short rod-like mitochondria and small vesicles with electron-dense granules. In this area of the subcortical layer, there are also many yolk spherules, multivesicular body-like structures, vacuoles of diverse sizes, lipid droplets, C-yolk spheres, and glycogen granules. The granule zone occupies the boundary between the anterior region of the periplasm and the other regions of an egg.

Ch, chorion; **Ea**, rER-associated resette; **ER**, zone of endoplasmic reticulum; **Gl**, glycogen granule; **Gol**, Golgi body; **GR**, granule zone; **Gr**, electron-dense granule; **Lip**, lipid droplet; **Mb**, multivesicular body-like structure; **Mit**, mitochondrion; **Mv**, minute vesicle; **rER**, rough endoplasmic reticulum; **Vm**, vitelline membrane; **Ys**, yolk spherule


rER
Lip
Mv
ER
Gl
Mb
Ea
G
GR
Mit
Vm
Ch
Ys

< Figure 9 > Ooplasm at the micropylar region

A. Enlarged micrograph of the ER zone

An enlarged micrograph of the ER zone, which is the major area of the periplasm at the micropylar region of a Daizo egg, is shown. Its conspicuous feature is that over ten stacks of lamellar structure of rER are distributed on its superficial area. While this lamellar structure is usually arranged to make a stack parallel to the egg surface, sometimes it protrudes with a whorl line into cone-shaped cytoplasmic processes. ER-associated rosettes that are composed of electron-dense fine granules connect with the end of the stack structure and there exists a Golgi body. Within the ooplasmic cone-shaped processes, there are a lot of vesicles as well as a zone of minute vesicles which contain some material of less electron-density and are about 80 nm in diameter. The ER zone seems to be related with sperm reception and forms the star-like ooplasm that encloses the head area of the sperm segregated from the tail region.

There are various sizes of vacuoles, multivesicular body-like structures, lipid droplets and yolk spherules underneath the rER. Among these elements a lot of short rod-like mitochondria are observed. Some vacuoles include vesicles and possible glycogen granules.

Ea, rER-associated rosette; **Gl**, glycogen granule; **Gol**, Golgi body; **Lip**, lipid droplet; **Mb**, multivesicular body-like structure; **Mit**, mitochondrion; **Mv**, minute vesicle; **rER**, rough endoplasmic reticulum; **Vc**, vacuole; **Vm**, vitelline membrane; **Ys**, yolk spherule

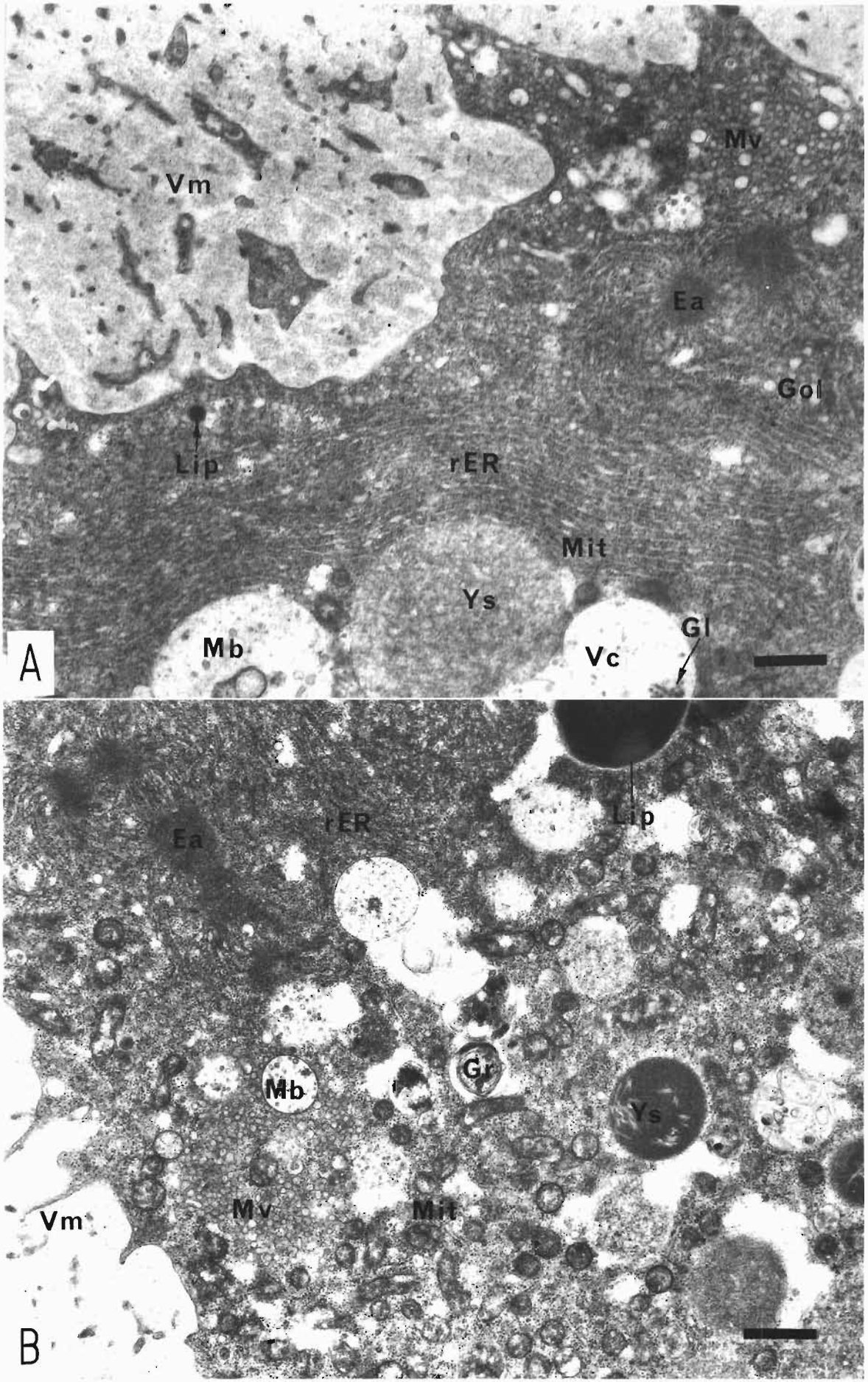
B. Enlarged granule zone

An enlarged micrograph of the granule zone adjacent to the ER zone of a Daizo egg is presented. This area is characterized by vesicles containing electron-dense granules and many rod-like mitochondria. The mitochondria possess electron-dense matrices, but their cisternae are indistinct. The other structural elements include multivesicular structures, lipid droplets and yolk spherules. The yolk spherules have many structural varieties; some have needle-like empty spaces within matrices, and others have vesicular or tubular spaces within less electron-dense matrices.

Neither the functions of the granule zone nor the natural features of the dense granules have not been investigated sufficiently yet.

In this figure, structural elements in both the granule zone and the ER zone are mixed with each other. The ER zone flows into the former and mitochondria into the latter.

Ea, rER-associated rosette; **Gr**, vesicles containing electron-dense granules; **Lip**, lipid droplet; **Mb**, multivesicular body-like structure; **Mit**, mitochondrion; **Mv**, minute vesicle; **rER**, rough endoplasmic reticulum; **Vm**, vitelline membrane; **Ys**, yolk spherule


Vm
Mv
Ea
Gol
Lip
rER
Mit
Ys
Mb
Vc
Gl
A
Ea
rER
Lip
Gr
Mb
Ys
Vm
Mv
Mit
B

< Figure 10 > Diagrammatical drawings showing the presumptive embryonic and extra-embryonic regions

A. Presumptive embryonic and extra-embryonic regions in the mature egg

These diagrams illustrate the distribution of pyronine-positive granules in a mature egg; Figure A (left and right) shows a frontal section and a cross section, respectively. In the frontal section, obvious pyronine-positive granules are observed only at the lateral sides of the egg, but not in its anterior and posterior regions. The following values as to the ranges without pyronine-positive granules were available: 20.3 ± 1.7% on the whole circumference of the egg at the anterior region; 16.8 ± 0.8% at the posterior region.

In the cross section, the above-mentioned pyronine-positive granules are seen in the lateral egg regions. The size of the granules is smaller in the ventral region and no granules can be observed in the dorsal region. The range without the granules at the dorsal region was measured and the gained value is 15.6 ± 1.6% on the whole circumference.

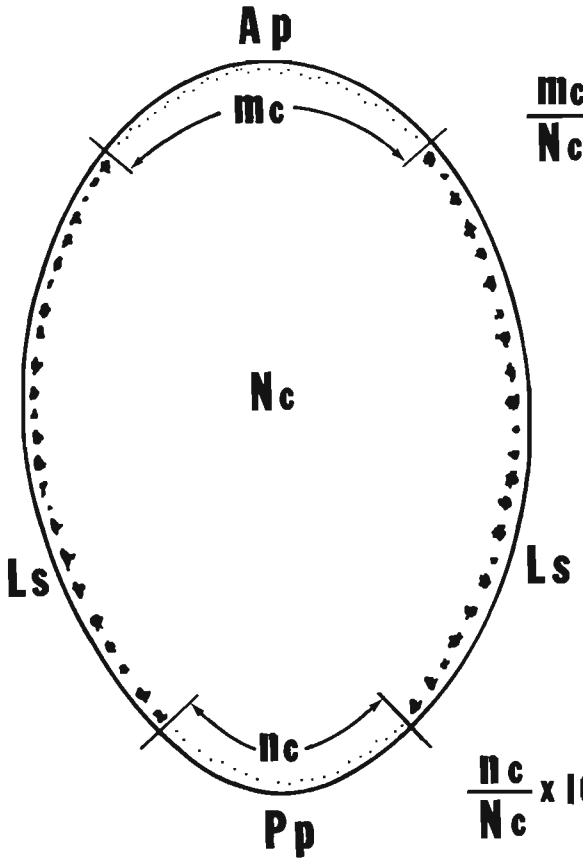
Ap, anterior pole; **Pp**, posterior pole; **Ls**, lateral side; **Vs**, ventral side; **Ds**, dorsal side; **Nc**, whole circumference of an egg in case of a frontal section; **mc**, range with no granules at the anterior region; **nc**, range with no granules at the posterior region; **Lc**, whole circumference of an egg in case of a cross section; **lc**, range with no granules at the dorsal region

B. Embryonic and extra-embryonic regions during the formation of embryonic anlage

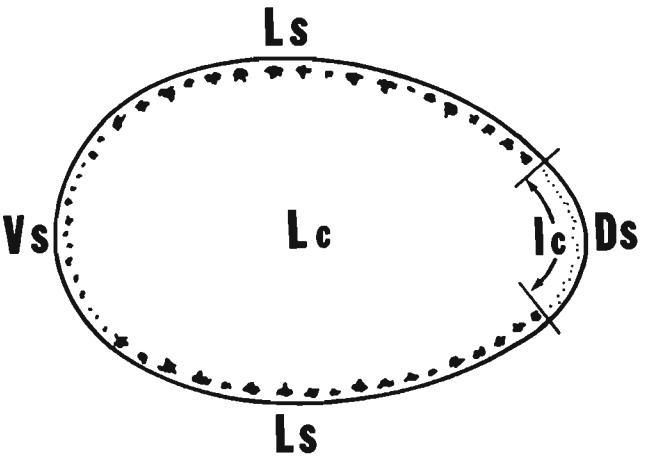
The embryonic and extra-embryonic regions are depicted during the formation of the embryonic anlage in the frontal (left in figure B) and cross sections (right in figure B), respectively. Embryonic anlage is well developed on both sides of the lateral region except on the anterior and posterior egg poles. The range of extra-embryonic region with cells destined to form the serosa encompasses 21.8 ± 5.7 % of the egg circumference in the anterior, and 14.4 ± 5.7 % in the posterior regions. In a cross section, the range of extra-embryonic blastoderm occupies 13.7 ± 5.2 % of the egg circumference. These values are consistent with the data on the distribution of the pyronine-positive granules (see above). The use of pyronine-positive granules as an indicator of the presumptive embryonic region can be suggested. As embryogenesis continues on, however, these granules gradually disappear. Therefore, we have not reached a conclusion that these granules are definitely the factor to determine the embryonic region. From an ultrastructual morphological point of view, pyronine-positive granules correspond to a large concentric arrangement of rER and its enclosing structural elements. But again, this structure collapses with the progress of development. Investigation on this point should be continued much further.

Ap, anterior pole; **Pp**, posterior pole; **Ls**, lateral side; **Vs**, ventral side; **Ds**, dorsal side; **Nb**, whole circumference of an egg in case of a frontal section; **mb**, extra-embryonic range at the anterior region; **nb**, extra-embryonic range at the posterior region; **Lb**, whole circumference of an egg in case of a cross section; **lb**, extra-embryonic range at the dorsal region

(Modified from Kobayashi and Miya, 1987)

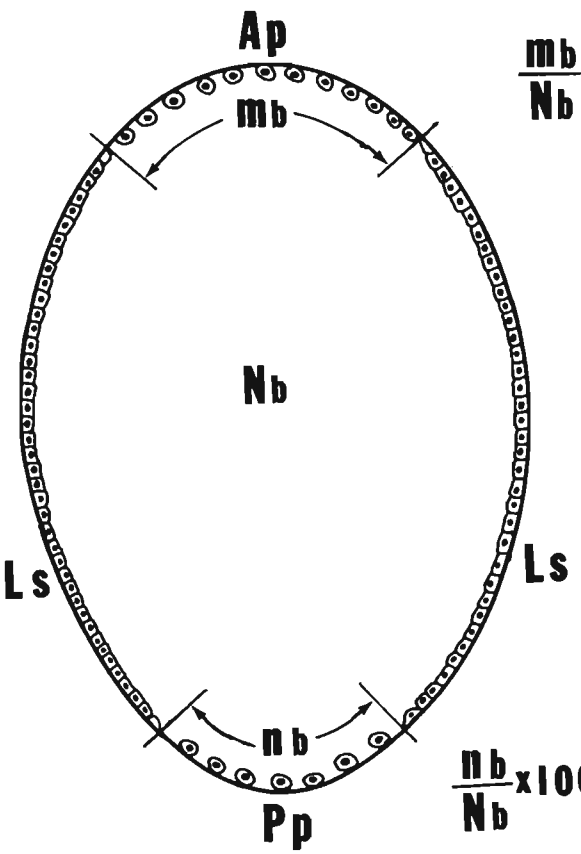


$\frac{mc}{Nc} \times 100 = 20.3 \pm 1.7\ (\%)$

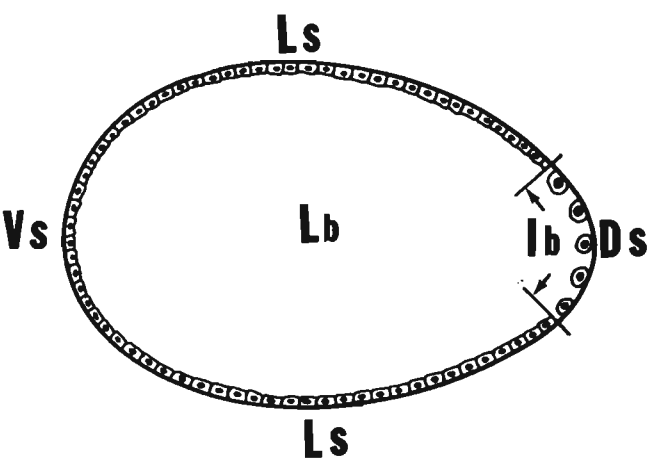


$\frac{lc}{Lc} \times 100 = 15.6 \pm 1.6\ (\%)$

$\frac{nc}{Nc} \times 100 = 16.8 \pm 0.8\ (\%)$



$\frac{mb}{Nb} \times 100 = 21.8 \pm 5.7\ (\%)$



$\frac{lb}{Lb} \times 100 = 13.7 \pm 5.2\ (\%)$

$\frac{nb}{Nb} \times 100 = 14.4 \pm 1.6\ (\%)$

< Figure 11 > Periplasm

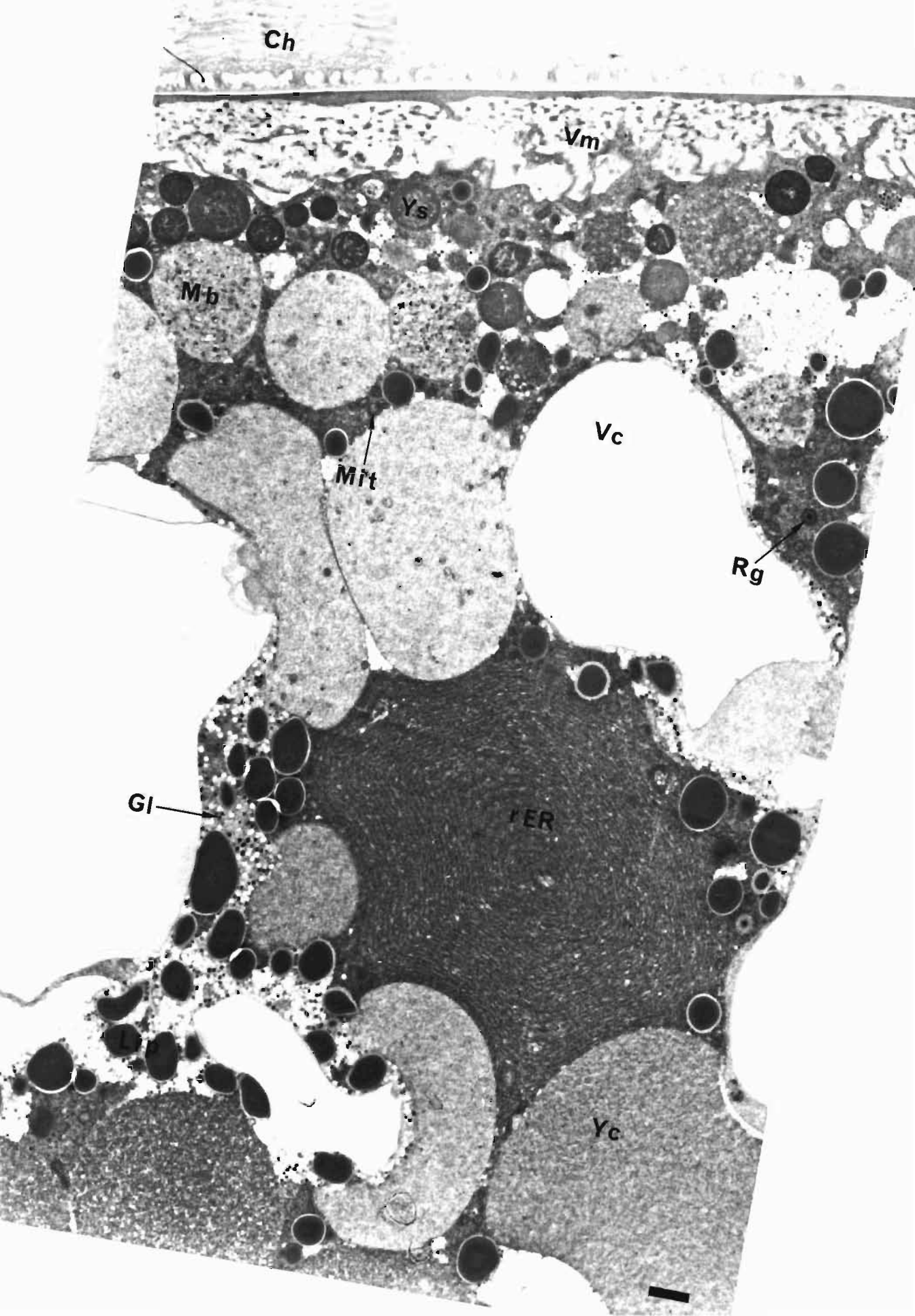
This is the structure of periplasm at the lateroventral region of a Daizo egg. The vitelline membrane is much thinner than in the micropylar region. In this figure, the outer layer of the vitelline membrane is 0.14~0.28 μm and the inner layer is about 2 μm thick. The oolemma protrudes microvilli into the inner layer.

On the surface of periplasm, a lot of yolk spherules, small lipid droplets, short rod-like mitochondria, multivesicular structures and glycogen granules are distributed. In yolk spherules some varieties in respect to electron-density or its inner structures are observed.

Large C-yolk spheres (which include electron-dense fine granules) and big vacuoles exist adjacent to the periplasm surface. In the space between them, there is a subcortical layer filled with the reticular plasm, in which rod-like mitochondria, thick-cortex round granules, lipid droplets and glycogen granules are scattered. The C-yolk sphere is the yolk that is formed at the final stage of vitellogenesis, and it exists only in a subcortical layer. It is characterized by a less electron-dense matrix containing high electron-dense fine granules. It also shows some variation; for example, it possesses vesicles and glycogen granules.

One of the remarkable features of the periplasm at the lateroventral region is the presence of pyronine-positive granules, as explained in the previous figure. From an ultrastructural point of view, these granules seem to correspond to a large concentric arrangement of rER and the structural element that surrounds the arrangement. This structure is distributed adjacent to the subcortical layer. A detailed explanation will be made in Fig. 13A.

Ch, chorion; **Gl**, glycogen granule; **Lip**, lipid droplet; **Mb**, multivesicular body-like structure; **Mit**, mitochondrion; **rER**, rough endoplasmic reticulum; **Rg**, thick-cortex round granule; **Vc**, vacuole; **Vm**, vitelline membrane; **Yc**, C-yolk sphere; **Ys**, yolk spherule


Ch
Vm
Ys
Mb
Mit
Vc
Rg
Gl
rER
Yc

< Figure 12 > Periplasm

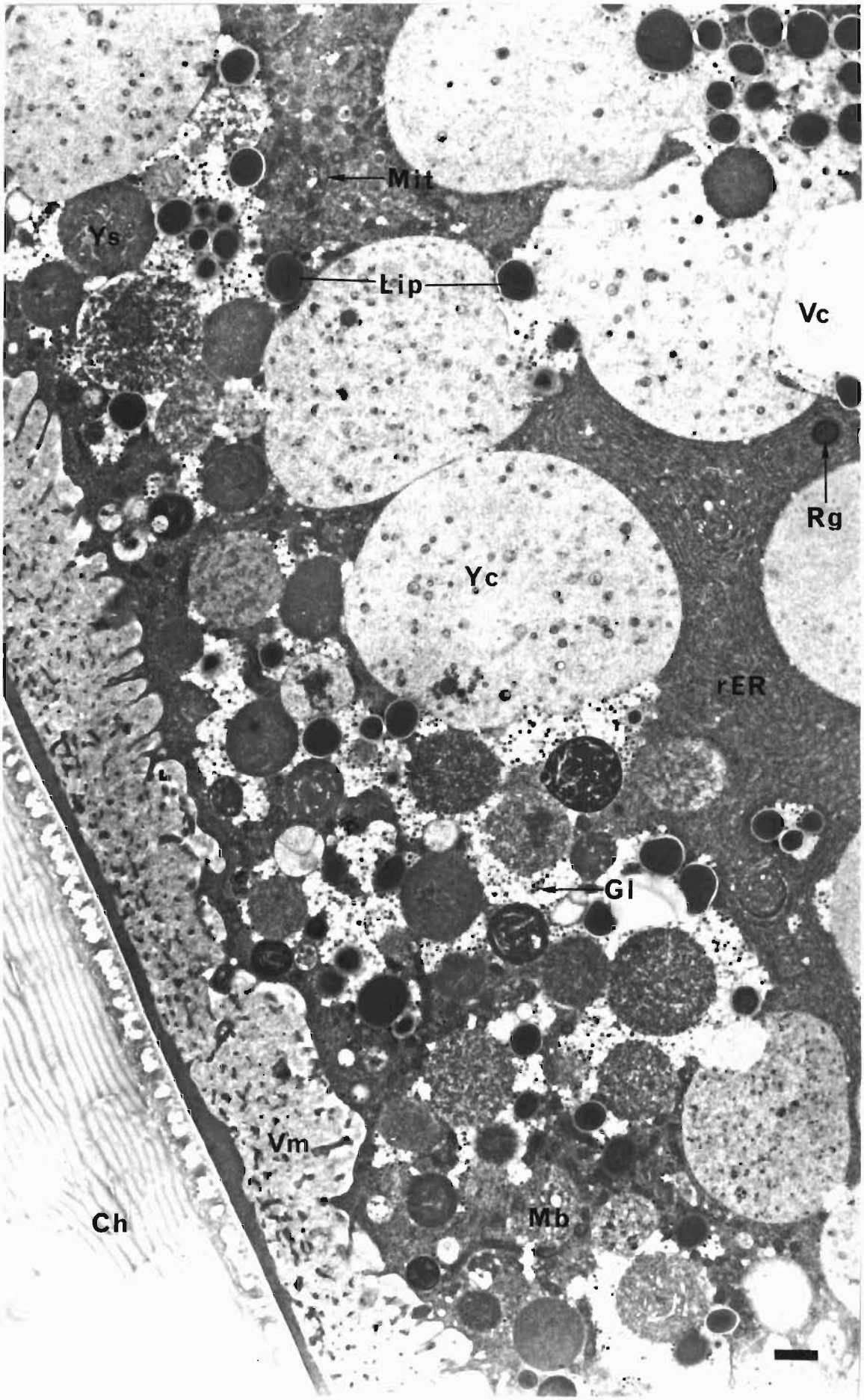
This micrograph shows the structure of periplasm in the dorsal region of a Daizo egg. The vitelline membrane is about the same as in the lateroventral region, being much thinner than in the micropylar region. The oolemma protrudes the microvilli into the inner layer of vitelline membrane.

In the apical area of the periplasm, there are a lot of yolk spherules, small lipid droplets, short rod-like mitochondria and multivesicular structures. Among these elements, there are a lot of spaces where glycogen granules are distributed. In yolk spherules many varieties are recognized just as in the cases at the lateroventral region.

The subcortical layer adjacent to the apical area of periplasm is occupied with large C-yolk spheres and vacuoles. The C-yolk in this figure appears full of ingredients, i.e., electron-dense granules, their enclosing vesicles, fine granules about 0.3μm in diameter, as well as small vacuoles are detected.

Although such a large concentric arrangement of rER as observed at the lateroventral region, can not be observed among the ooplasmic network structure, there is a wide territory that is occupied with stacks of rER. In other ooplasmic networks, rod-like mitochondria, lipid droplets and thick-cortex round granules are observed.

Ch, chorion; **Gl**, glycogen granule; **Lip**, lipid droplet; **Mb**, multivesicular body-like structure; **Mit**, mitochondrion; **rER**, rough endoplasmic reticulum; **Rg**, thick-cortex round granule; **Vc**, vacuole; **Vm**, vitelline membrane; **Yc**, C-yolk sphere; **Ys**, yolk spherule


Mit
Ys
Lip
Vc
Rg
Yc
rER
Gl
Vm
Ch
Mb

< Figure 13 > Periplasm

A. Enlarged micrograph of an concentric arrangement of rER

This is a micrograph of a large concentric arrangement of rER adjacent to the subcortical periplasm layer in the lateroventral region of a Daizo egg. The entire structure represents a shape of a star and contains stacks of rER with concentric arrangements every 0.07 μm approximately. There are no other organelles inside, but only rod-like mitochondria and lipid droplets are distributed around this structure.

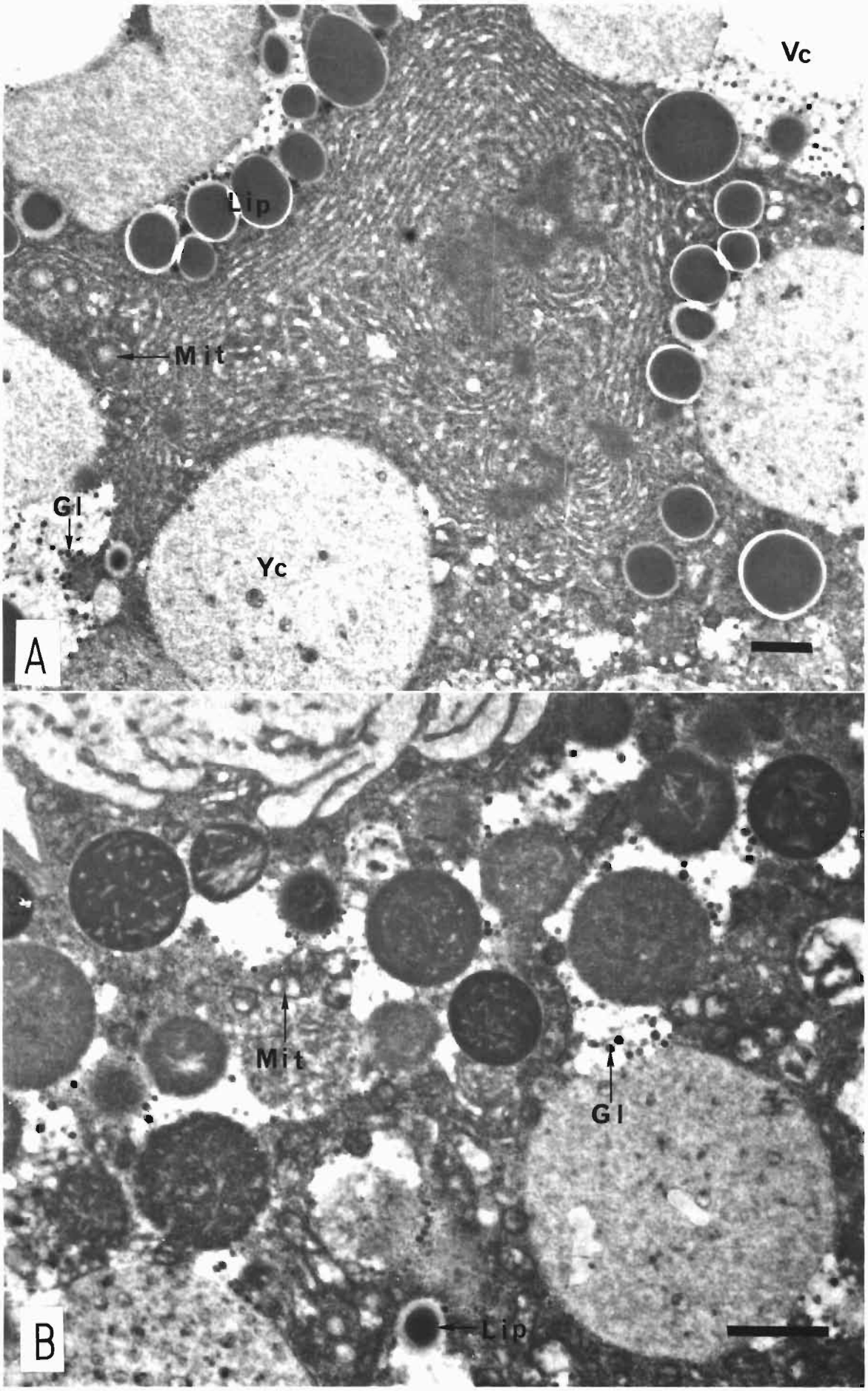
The star-like ooplasm is surrounded by large C-yolk spheres, vacuoles, lipid droplets and glycogen granules. What is observed as a large pyronine-positive granule through a light microscope in the Carnoy's fixation is considered to be a stacked group of these organelles.

Gl, glycogen granule; **Lip**, lipid droplet; **Mit**, mitochondria; **Vc**, vacuole; **Yc**, C-yolk sphere

B. Enlarged micrograph of yolk spherules

Structure of yolk spherules at the apical area of the periplasm in the dorsal region of a Daizo egg is shown. There are abundant and somewhat variable yolk spherules in this area. As a basic architecture, needle-like structures and light vesicular or small tubular structures are scattered in a high electron-dense matrix, and they display a variety of patterns. The density of the fine particles that build the matrix is low and some of them have less electron density as a whole. A thick section of the sample fixed with gultaraldehyde-osmium fixation is stained deep blue with toluidine blue.

Gl, glycogen granule; **Lip**, lipid droplet; **Mit**, mitochondrion


Vc
Lip
Mit
Gl
Yc
A
Mit
Gl
Lip
B

< Figure 14 > Yolk system

A. A-yolk sphere

The structure of an A-yolk sphere in the yolk system of a Daizo egg is represented. The yolk system that occupies most of the inner egg consists of the reticuloplasm that encloses proteid yolk spheres, lipid droplets and glycogen granules.

The structure can be morphologically classified in two types. In the A-yolk spheres, fine granules are evenly scattered, and its granule density determines the entire electron-density of the sphere. A-yolk spheres are present mainly around the yolk system.

Within this reticuloplasm, the ground substance including ribosomes and short rod-like mitochondria are observed.

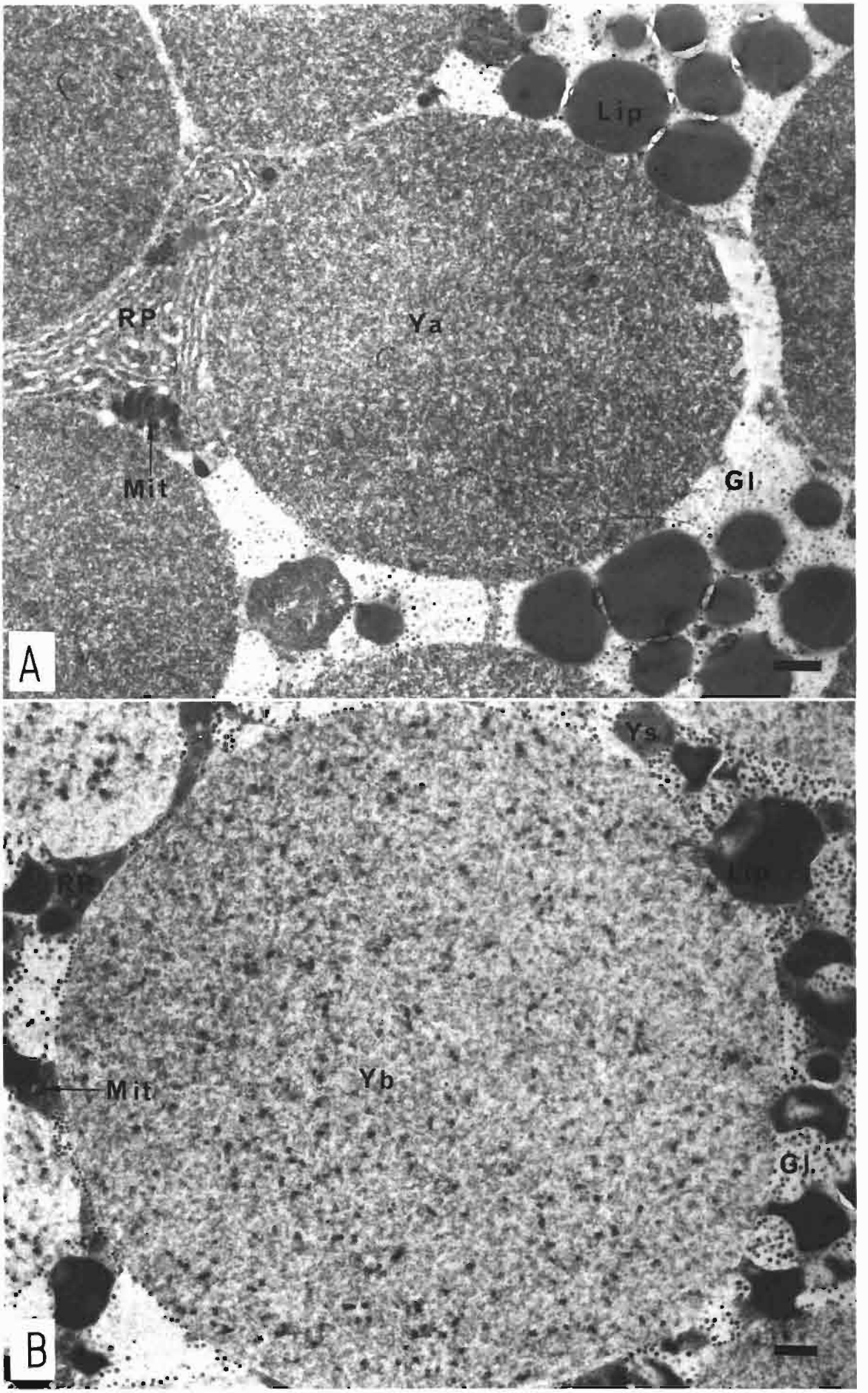
Gl, glycogen granule; **Lip**, lipid droplet; **Mit**, mitochondrion; **Rp**, reticuloplasm; **Ya**, A-yolk sphere

B. B-yolk sphere

The structure of a B-yolk sphere in the yolk system of a Daizo egg is shown. It is the same sample as Figure A above. B-yolk spheres mainly occupy the central part of the egg. A conspicuous feature of the B-yolk spheres is that they contain numerous electron-dense granules within the evenly distributed fine granulous ground substance.

In the reticuloplasm, the ground substance including ribosomes as well as short rod-like mitochondria are observed. Sometimes yolk spherules are distributed.

Gl, glycogen granule; **Lip**, lipid droplet; **Mit**, mitochondria; **Rp**, reticuloplasm; **Yb**, B-yolk sphere; **Ys**, yolk spherule

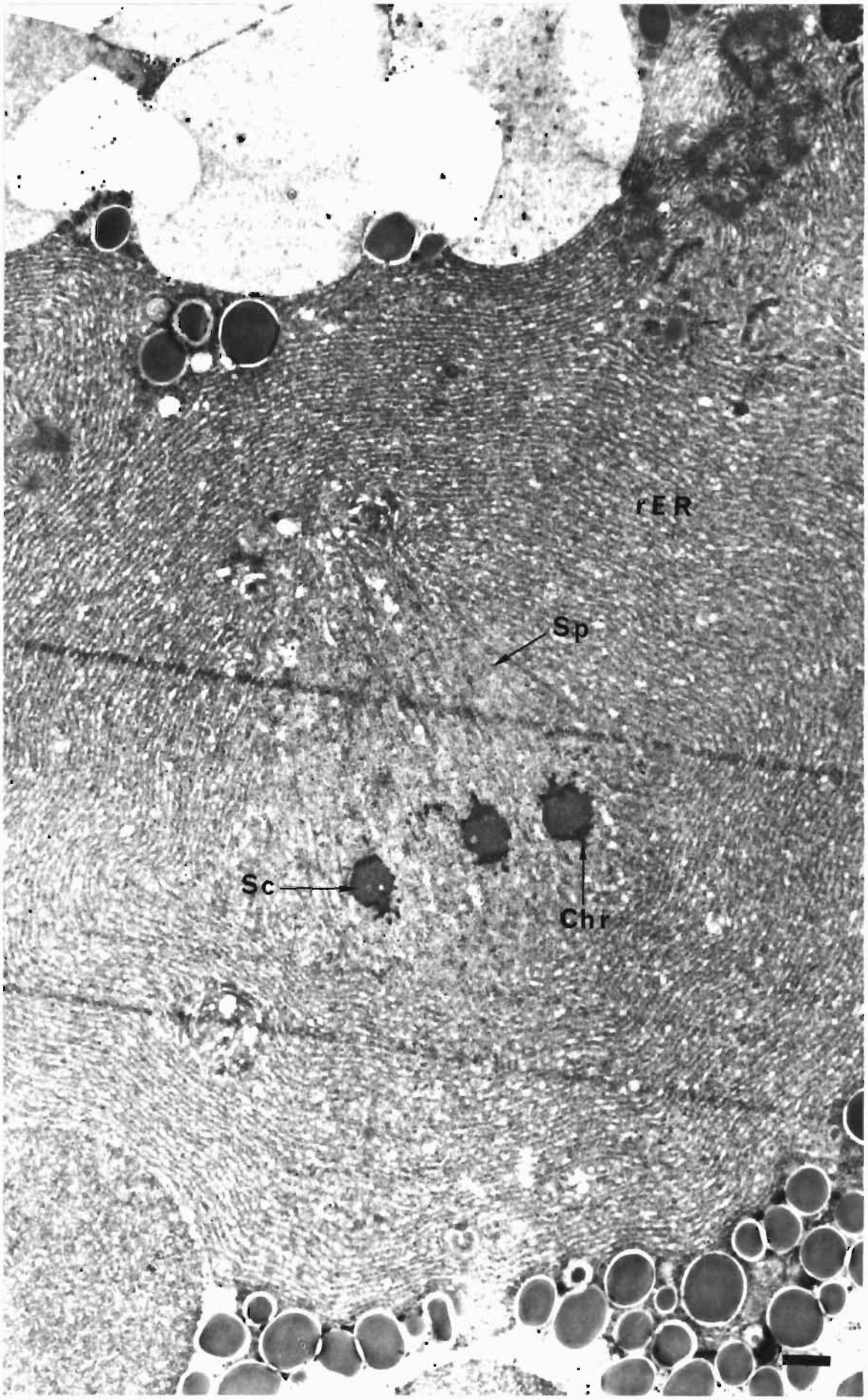

Lip
RP
Ya
Mit
Gl
A
Ys
Lip
RP
Mit
Yb
Gl
B

< Figure 15 > Egg nucleus

The nucleus of an unfertilized (no sperm-entry) silkworm egg stops dividing in the middle of the first maturation division and resumes meiosis after it is activated. This figure shows the structure of a nucleus of a Daizo egg dissected from the oviduct. During the zygotene stage of the meiotic prophase, homologous chromosomes are bound by the synaptonemal complex (Rasmussen and Holm, 1982) and are arranged in the equatorial plane as in the metaphase. The electron-dense structure in the figure represents each homologous chromosome and the less electron-dense material that connects the two structures represents the synaptonemal complex.

Each pair of chromosomes is attached to spindle fibers that are made up of fine tubular structures and abundant fine granules. The spindle is surrounded by the fine vesicular or tubular rough endoplasmic reticulum, part of which builds stacked structures. No other organelles or inclusions are present.

Chr, chromosome; **rER**, rough endoplasmic reticulum; **Sc**, derivative from the synaptonemal complex; **Sp**, spindle fiber

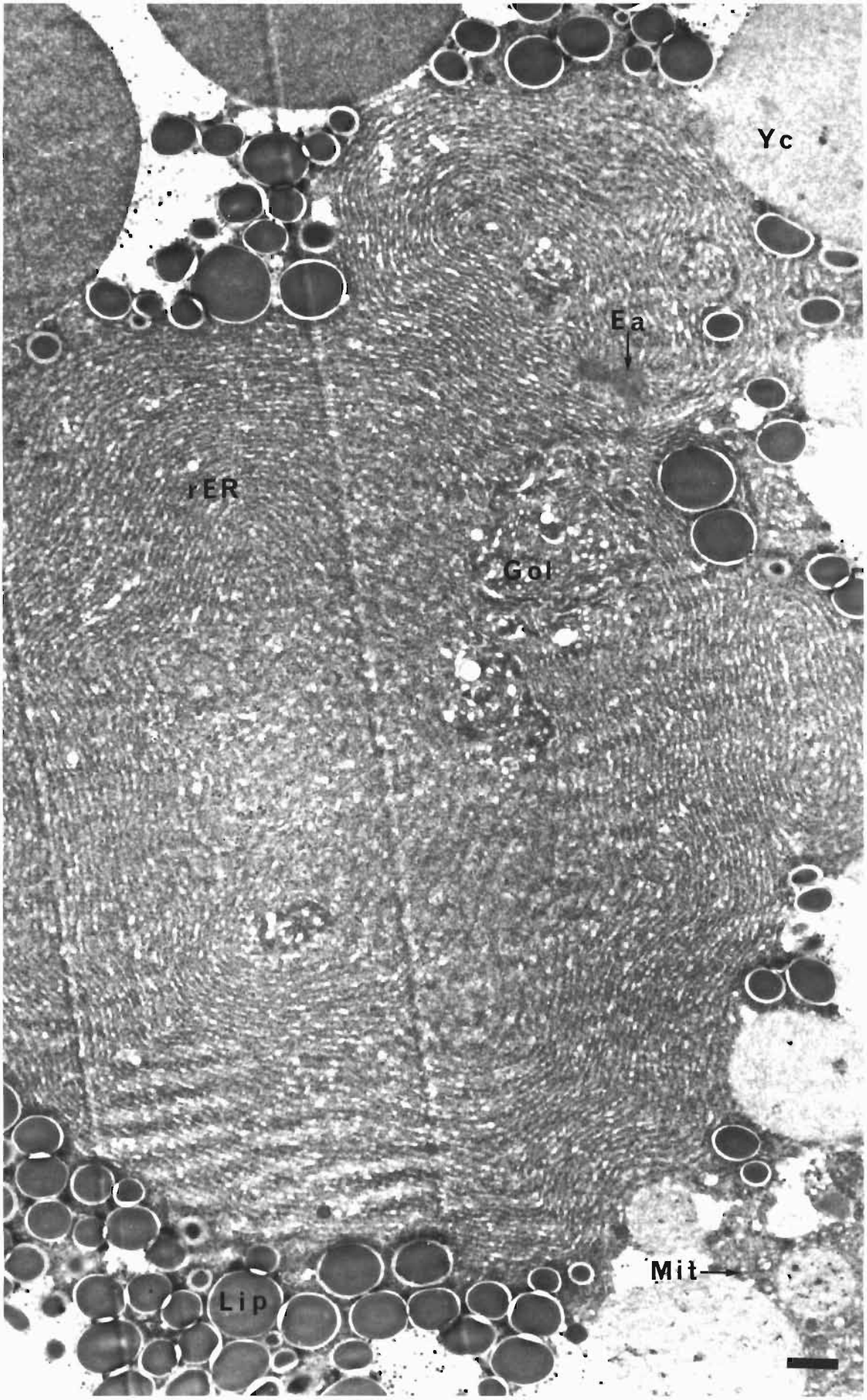

rER
Sp
Sc
Chr

< Figure 16 > Egg nucleus

This figure shows the structure of ooplasm around the nucleus in a Daizo egg. It is positioned dorsally in the micropylar region. As shown in the previous figure, an unfertilized (no sperm-entry) silkworm egg stops its division during the metaphase of the first maturation division, and stacks of rER accumulate within the ooplasm around the spindle. Only the rER-associated rosettes and the Golgi bodies can be detected, while no organelles like mitochondria were found. However, these are abundant within the reticuloplasm adjacent to the ooplasm that encloses the nucleus.

Also, as one of the most significant features of this structure, a lot of lipid droplets surround the large star-like ooplasm around the egg nucleus.

Ea, rER-associated rosette; **Gol**, Golgi body; **Lip**, lipid droplet; **Mit**, mitochondrion; **rER**, rough endoplasmic reticulum; **Yc**, C-yolk sphere


Yc
Ea
rER
Gol
Lip
Mit

Chapter 2 Stages of Embryogenesis

Zygote nucleus (synkaryon) formed by the fusion of the male and female pronuclei starts embryogenesis with continuously repeated mitotic divisions. Taking its morphological features into consideration, the whole process is classified as several developmental stages. In the case of silkworm, with the exception of its multivoltine and bivoltine races, the embryogenesis stops obligatorily in a comparatively early stage and the embryos enter diapause. Numerous investigations, important for practical sericulture, have been made on the handling of diapausing "eggs" and the methods of artificial diapause termination. Takami (1969) divided the development of silkworms into the following six stages: (1) pre-diapause, (2) diapause, (3) hibernation, (4) critical stage, (5) formation of organs, and (6) completion of larva. Subsequently, they were specified into 30 stages. The pre-diapause includes 7 stages: fertilization, cleavage, germanlage formation, yolk cleavage, pyriform-shaped stage, *Kokeshi* (China-spoon like)-shaped stage and Chemical spatula-shaped stage. The two stages of diapause are diapause I and diapause II. The hibernating period has 4 stages; the pre A, A, B-A and the B-B. The critical stage has the C-A and the C-B. The stages of organ formation are divided into 10; the D-A, D-B, appearance of labral appendages, shortening stage, cephalothoracic segmentation, blastokinesis, completion of blastokinesis, appearance of trichogen cells, appearance of setae and the appearance of tracheal taenidia. The completion of larva includes 5 stages; the head pigmentation I, head pigmentation II, body pigmentation I, body pigmentation II and the hatching.

Later, Ohtsuki (1979) replaced some expressions like A, B and C with others, and added some corrections to reclassify the development into 30 stages (Ref. Yamashita and Yaginuma, 1991). I follow his classification but a few corrections have been added to accentuate the ultrastructural point of view. The development after diapause will be described in Vol. III, "Organogenesis". Each stage of embryogenesis is characterized in Figs. 17 and 18.

2.1 Stage of fertilization

The zygote nucleus (synkaryon) is formed by fusion of the male and female pronuclei about two hours after perm entry.

2.2 Cleavage stage

Synchronous mitotic divisions produce a lot of cleavage nuclei that migrate toward the periphery and begin to penetrate the periplasm beginning about 10 hr after oviposition. From this stage to just before the diapause stage, some corrections are added,

based on ultrastructural observations.

2.3 Stage of blastoderm and germanlage formation

As will be mentioned later, cleavage nuclei of the silkworm do not reach and penetrate the periplasm simultaneously all over the egg but do so first only in its anterior-half. The cell membranes are formed soon after the nuclei have reached the periplasm, and a distinction between the syncytial and the cellular blastoderm is difficult. Furthermore, at the advanced stage when the cleavage nuclei occupy the whole surface of the egg, the germanlage can already be distinguished from the extra-embryonic region by higher cell density. Hence, in the silkworm there is no clear "blastoderm" as in some other insects. Takami named the described stage "germanlage formation", while Ohtsuki called it "blastoderm formation".

2.4 Stage of germband formation

The expressions of "germanlage" and "germband" in silkworm embryogenesis do not correspond to those used in recent general descriptions of insect embryology. For example, according to Sander *et al.* (1985), "germanlage" means a monocellular embryo after serosa segregates from the blastoderm and "germband" means the subsequent embryonic stage until gastrulation and segmentation. In my treatise, however, "germanlage" is defined as an embryo which is separated clearly from the extra-embryonic region and is still situated at the surface of an egg; 'germband' indicates an embryo from the stage of segregation from the surface and sinking into the egg, through gastrulation and segmentation, up to just before the diapause stage. Therefore, the stage of germband formation can be divided into a few more stages.

2.4.1 Completion of serosa

The cells in the extra-embryonic region gradually become flattened and migrate toward the lateral folds of germanlage that has segregated from the egg surface and begins to sink into the inner part of the egg. The extra-embryonic cells form a membrane (serosa) which encloses the whole egg. The germband at this stage is wide and extends symmetrically in a saddle-like arrangement on both sides of the ventral median line.

2.4.2 Initiation of yolk cleavage

The broad germband grows anteriorly and posteriorly, and at the same time its width begins to decrease. From this stage, the yolk constituents start to be rearranged. Lipid droplets gather around the nuclei, which were left within the yolk mass during the blastoderm formation. This stage precedes the phenomenon of yolk cleavage when yolk cells are formed around the yolk nuclei. A remarkable phenomenon of this stage is the exclusion of a part of cytoplasm at the anterior and posterior edges of the germband.

2.4.3 Differentiation of protocephalon and protocorm (pyriform-shaped stage)

The broad germband keeps growing anteriorly and posteriorly, and simultaneously becomes narrower in the center part. As a result, it assumes a pyriform shape and it becomes possible to distinguish the protocephalon from the protocorm. Amnion anlage cells differentiate at the periphery of the germband and become flattened as they extend over the ventral region to form the second embryonic envelope (amnion).

2.4.4 Spoon-shaped stage

Due to the growth of the germband, the protocephalon and protocorm become distinctly spoon-shaped, and the anterior and posterior ends of the germband curve into the inner part of the egg. The amnion is completed, and the yolk cleavage produces a yolk system consisting of numerous yolk cells. Gastrulation, which occurs by primitive groove invagination, starts from the center of the protocephalon.

2.4.5 Telson differentiation

The germband is long and is composed of ectoderm and mesoderm. The primitive segments, which originate from the mesoderm segmentation, become distinct. The posterior end is recognized as the telson, and also its inward invagination is advanced. Yolk cells are observed between the serosa and the amnion. In the diapause egg, it takes about 48 hr to reach this stage after oviposition.

The above-mentioned periods constitute the pre-diapause stage. I follow Ohtuski's classification and characterization of subsequent stages ranging from diapause to eclosion (the expressions in parentheses show classification by Takami).

2.5 Diapause stage I

The embryo migrates from the outermost egg surface to the inner part. Around the center, spaces without yolk cells can be observed.

2.6 Diapause stage II

The spaces that occupy the central part of the egg become larger. Free yolk cells are scarcely detected.

2.7 Hibernating stage I (stage pre-A)

If a diapausing egg is kept at a low-temperature (around 5℃) similar to natural winter conditions, it gradually terminates the diapause and starts its development when the appropriate temperature (about 25℃) is regained. The stage is called "the hibernating stage", which is divided into 4 stages. In this stage (stage pre-A by Takami), the egg has not completely terminated the diapause and a long time elapses to eclosion when it is

transferred to 25℃. The development of eggs within an egg batch is not simultaneous.

2.8 Hibernating stage II (stage A)

The egg almost completely terminates the diapause.

2.9 Hibernating stage III (stage B-A)

The aggregates of yolk cells begin to be separated.

2.10 Hibernating stage IV (stage B-B)

The mesoderm at the thoracic segment becomes considerably larger, and the aggregates of the yolk cells are mostly separated.

2.11 Critical stage I (stage C-A)

This indicates the period from the end of hibernation to the beginning of the organ formation. In an embryo during this stage, the mass of mesoderm cells in each segment conspicuously extends to both sides.

2.12 Critical stage II (stage C-B)

The protocephalon of the embryo sufficiently spreads. This stage occurs just before the appearance of the neural groove.

No remarkable changes in embryo morphology are recognized from the above-mentioned diapause, but ultrastructurally, morphological changes of the organelles are characteristic during these stages. There are a report on embryonic cells by Okada (1979) and also one on serosa cells and yolk cells by Miya *et al.* (1972). These reports will be described in another book, Vol. III, "Organogenesis".

2.13 Stage of appearance of neural groove (stage D-A)

The embryo begins to extend, and a neural groove appears along its ventral median line. The mass of mesoderm cells in each segment is divided into the right and left sides.

2.14 Stage of appearance of abdominal appendages (stage D-B)

Appendages appear at the gnathal and thoracic segments, and succeedingly at the

abdominal segments. The length of embryo in this stage is longest during all the stages up to the blastokinesis.

2.15 Stage of appearance of processes of labrum

A pair of callus-like processes of labrum appears at the front end of the protocephalon.

2.16 Shortening stage

The embryo shortens, and spiracular and silk gland invaginations occur.

2.17 Stage of cephalothoracic segmentation

The shortening of the embryo advances more, and gnathal segments unite, resulting in clear distinction of the future head from the thorax.

2.18 Blastokinesis (embryonic revolution)

The ventral part of embryo faces the ventral region of the egg. A sigmoid movement begins from the caudal end of the embryo that is gradually pushed with its dorsal side to the dorsal side of the egg. The blastokinesis is accomplished in a short time, but can be divided into three phases, because its positional changes are clearly detected.

2.19 Stage of completion of blastokinesis

When blastokinesis ends, the embryo starts growing again. The formation of the dorsal integument makes progress, and the alimentary canals are completed.

2.20 Stage of appearance of trichogen cells

On the surface of the embryo, small masses of trichogen cells are formed.

2.21 Stage of appearance of setae

Trichogen cells produce setae, which cover the surface of the embryo. Ocelli begin to be pigmented.

2.22 Stage of appearance of taenidia

Taenidia are formed in the trachea. During this stage, mandibles start to be pig-

mented, and the formation of larval organs is completed.

2.23 Stage of head pigmentation I

After the completion of larval organs, pigmentation of bodies makes progress to reach hatching. This pigmentation is divided into 4 stages. A head is pigmented with brown.

2.24 Stage of head pigmentation II

The serosa is swallowed, and the pigmented head looks blue from the outside. Except for the head, no other region has been pigmented yet.

2.25 Stage of body pigmentation I

Other regions start to be pigmented (in addition to the head).

2.26 Stage of body pigmentation II

The whole embryo is pigmented and looks blue.

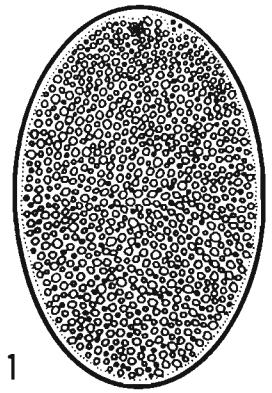
2.27 Stage of hatching

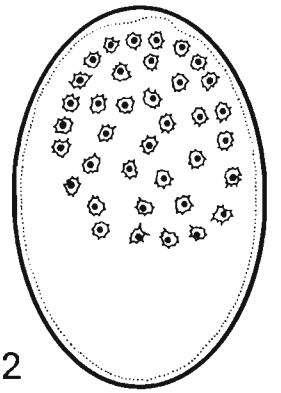
When the larval body is completed, the young larva eats and breaks the chorion around the micropylar region to hatch.

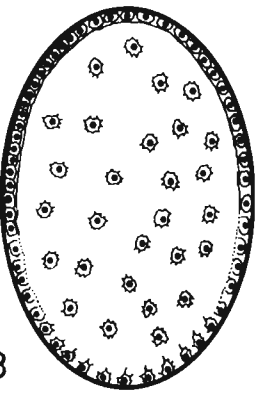
In non-diapause eggs, the diapause stage, hibernating stage and critical stage are absent.

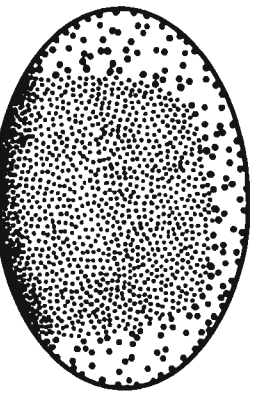
< Figure 17 > Developmental stages of embryos (Part 1)

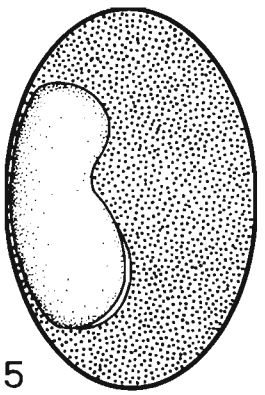
1. Fertilization (a longitudinal section): There are one zygote nucleus (synkaryon) and three polar body nuclei.
2. Cleavage (a longitudinal section): The number of cleavage nuclei increases by synchronous mitotic divisions, and the cleavage nuclei begin to migrate toward the periphery.
3. Blastoderm and germanlage formation (a longitudinal section): The cleavage nuclei gradually reach the egg surface beginning from the anterior region. Rapid formation of the cell membranes makes it difficult to distinguish the syncytial blastoderm from the cellular blastoderm. The cleavage nuclei which remain within the egg will differentiate to the yolk nuclei.
4. Germband formation I (completion of serosa) (a lateral view: all figures below are lateral views.): The germanlage separates from the egg surface and begins to sink into the inside. The blastoderm cells of the extra-embryonic region flatten and penetrate into the space where the germanlage had been present. The cellular membrane (serosa) which covers the whole egg is completed.
5. Germband formation II (differentiation of protocephalon and protocorm, pyriform-shaped stage): As a result of elongation and a decrease in width, the germband acquires a "pyriform-shape", and protocephalon and protocorm are recognized.
6. Germband formation III (spoon-shaped stage): By continuous extension of the germband, the protocephalon and the protocorm become obvious. The amnion and the yolk cleavage are completed.
7. Germband formation IV (telson differentiation): The germband elongates and the invagination of gastral groove (gastrulation) leads to the formation of separate ectoderm and mesoderm. Primitive segments become distinguishable due to mesoderm segmentation, and the posterior end of the embryo is recognized as the telson.
8. Diapause stage I: The embryo sinks into the inner part of the yolk mass. In the center of the egg, spaces without yolk cells can be observed.
9. Diapause stage II: The spaces in the central part reach their largest size.
10. Hibernating stage I: The embryo gradually terminates the diapause under low-temperature in the winter. It resumes its development in the appropriate temperature (around 25℃) only slowly because the diapause has not been completely terminated.
11. Hibernating stage II (stage A): The embryo almost completely terminates the diapause, but its development is suppressed under natural winter circumstances such as low temperatures.
12. Hibernating stage III (stage B-A): Yolk cells begin to be separated even at a low-temperature.
13. Hibernating stage IV (stage B-B): Most of the yolk cells are separated.
14. Critical stage I: A mass of mesoderm conspicuously extends to both the right and left sides.
15. Critical stage II: The protocephalon of the embryo sufficiently spreads.
16. Appearance of neural groove: The embryo begins to extend, and the mesoderm is divided into the right and left sides by the invagination of the neural groove.

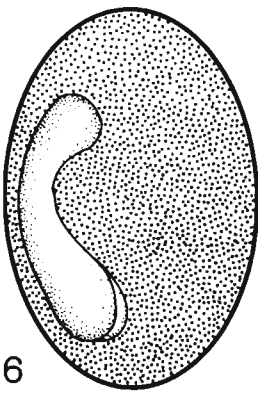

1

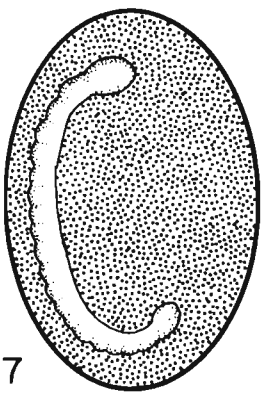

2

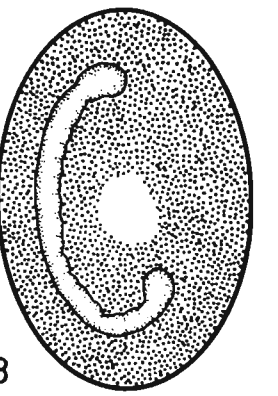

3

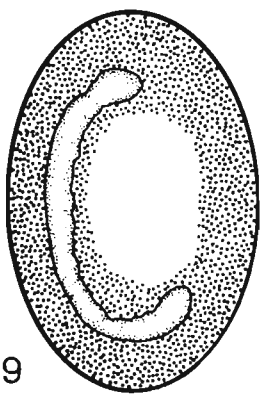

4

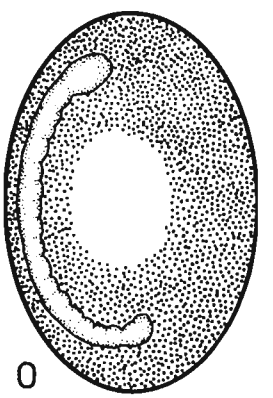

5

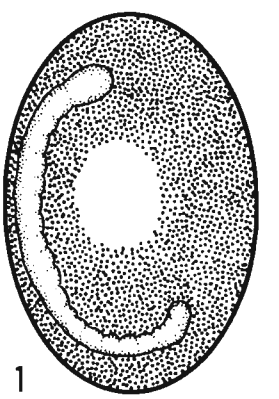

6

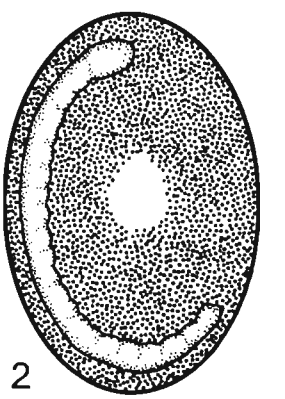

7

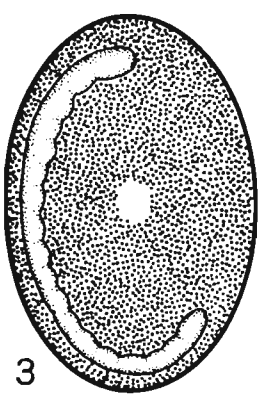

8

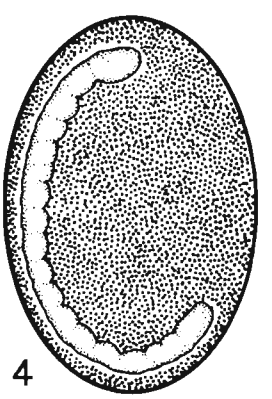

9

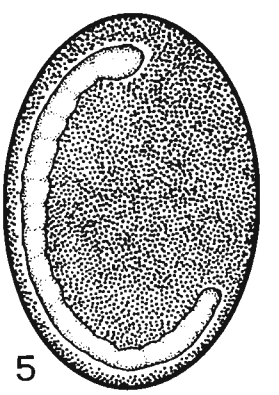

10

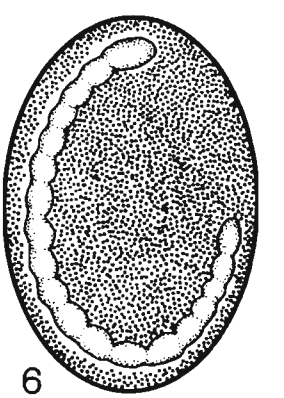

11


12


13


14

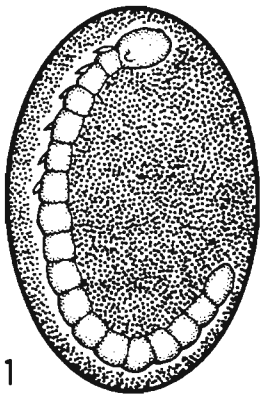

15

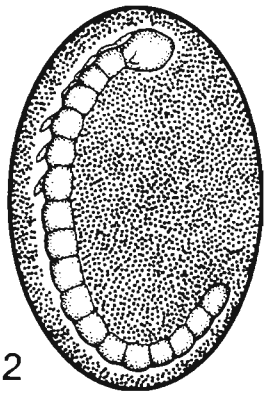

16

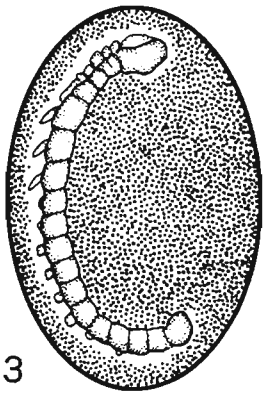
< Figure 18 > Developmental stages of embryos (Part 2)

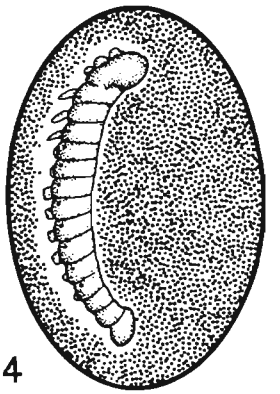
1. Appearance of abdominal appendages: Appendages appear on the gnathal and thoracic segments, and subsequently on the abdominal segments.
2. Appearance of processes of labrum: A pair of callus-like processes of labrum appears at the front end of the protocephalon.
3. Shortening stage: The shortening of embryo progresses, and spiracular invaginations develop.
4. Cephalothoracic segmentation: The shortening of the embryo advances. The gnathal segments unite, resulting in the distinction of the future head from the thorax.
5. Early stage of blastokinesis: The embryo is initially formed with its ventrum facing the ventral side of the egg. Later, with a sigmoid movement from the caudal end, the embryo changes its position and moves to the dorsal side. This movement is called blastokinesis (embryonic revolution) and it finishes in a short time. It is divided into the three stages.
6. Middle stage of blastokinesis: The embryo shows a sigmoid position.
7. Final stage of blastokinesis: The revolution of the tail part is completed.
8. Completion of blastokinesis: When blastokinesis ends, the embryo starts growing again. The dorsal integument formation is completed, except for part of the thorax.
9. Appearance of trichogen cells: Small masses of trichogen cells that are going to produce setae are formed on the embryo surface.
10. Appearance of setae: Bunches of setae are formed by the trichogen cells.
11. Appearance of taenidium: As taenidia are formed within the trachea, the layout of the tracheal system is visible from the outside. The formation of larval organs is completed.
12. Head pigmentation I: The head of the embryo is pigmented to a brown color.
13. Head pigmentation II: The pigmentation of the head is completed.
14. Body pigmentation I: Other body regions start to be pigmented.
15. Body pigmentation II: The whole embryo is pigmented and appears blue.
16. Hatching: When the larval body is completed, the larva eats and breaks the chorion in the micropylar region to hatch.

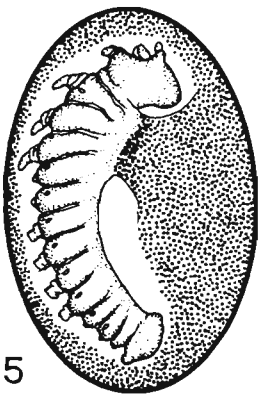
(Modified from Ohtsuki, 1979)

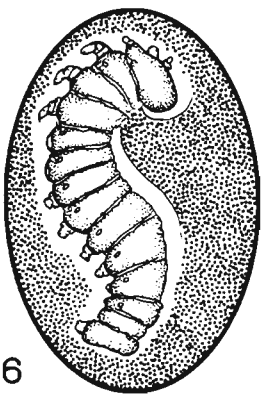

1

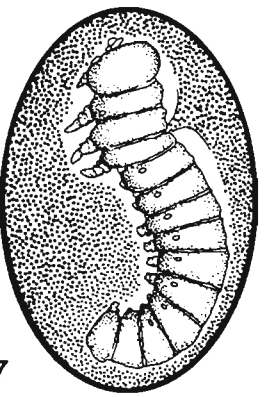

2

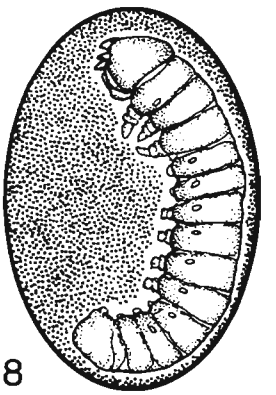

3

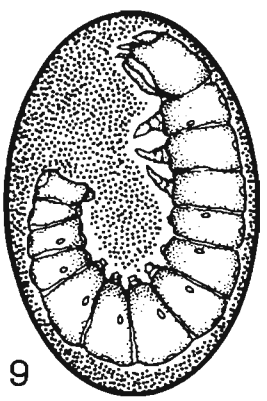

4

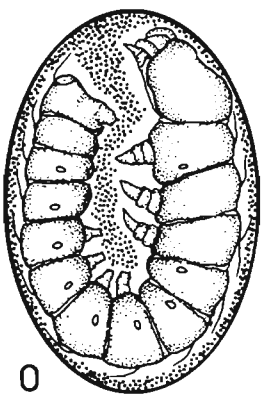

5

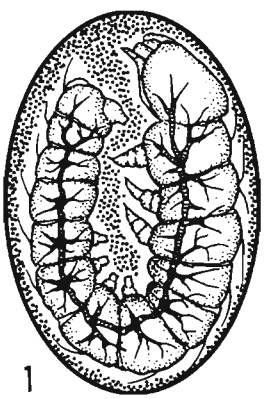

6

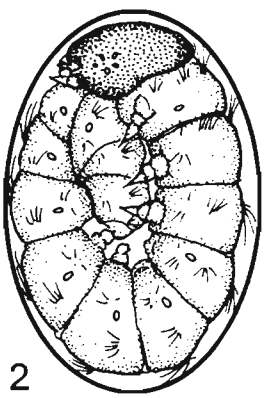

7

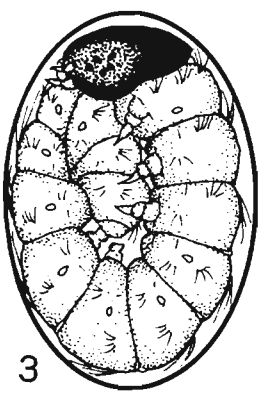

8

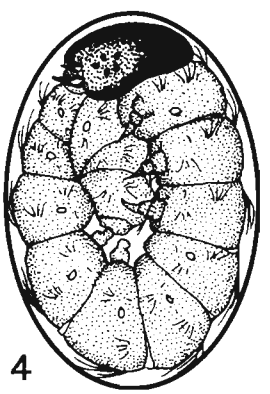

9

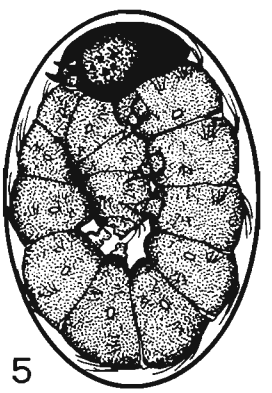

10

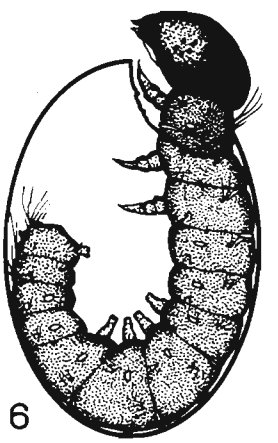

11


12


13


14


15


16

References

Akai, H. (1957) Studies on the electron microscopic structure of egg shell in *Bombyx mori* L. *J. Sericult. Sci. Jpn.*, **26**: 355-340 (Japanese).

Akai, H. (1958) Observations of the micropylar region of the chorion in the silkworm, *Bombyx mori* L. *Acta Sericol.*, **26**:19-21 (Japanese).

Akai, H. (1976a) *Ultrastructural Morphology of Insects*. 290p., Tokyo Univ. Press, Tokyo (Japanese).

Akai, H. (1976b) *Surface Structures of Insect*. 139p., Tokyo Univ. Press, Tokyo (Japanese).

Akutsu, S. and Yoshitake, N. (1974) Electron microscopic observations on the grey eggshell of the silkworm, *Bombyx mori* L. *J. Sericult. Sci., Jpn.* **43**: 461-466 (Japanese).

Akutsu, S. and Yoshitake, N. (1977) Electron microscopic observations on the vitelline membrane formation in the silkworm, *Bombyx mori* L., *J. Sericult. Sci. Jpn.*, **46**: 509-514 (Japanese).

Ando, H. (1988) Insects. In *Invertebrate Embryology* (Dan, K., Sekiguchi, K., Ando, H. and Watanabe, H., eds.), **Vol. II**, pp. 131-248. Baifuh-kan, Tokyo (Japanese).

Astaurov, B. L. (1967) Artificial parthenogenesis and exprimental polyploidy in silkworm. *J. Sericult. Sci. Jpn.*, **36**: 277-285.

Balinsky, B. I. (1986) Early differentiation in the egg of the butterfly, *Acraea horta* under normal condition and after ultravioloet irradiation. *Acta Embryol. Morphol. Experi. N. S.*, **16**: 103-141.

Fehrenbach, H., Dittrich, V. and Aissler, D. (1987) Eggshell fine structure of three lepidopteran pests: *Cydia pomonella* (L.) (Tortricidae), *Heliothis virescens* (Fabr.) and *Spodoptera littoralis* (Boisd.) (Noctuidae). *Int. J. Insect Morphol. Embryol.*, **16**: 201-141.

Frriedländer, M. and Gitay, H. (1972) The fate of the normal-ancleated spermatozoa in inseminated females of the silkworm, *Bombyx mori*. *J. Morphol.*, **138**: 121-129.

Gilbert, S. F. (2000) Developmental Biology (6th edition), Sinauer Associates Inc., Massachusetts.

Hashimoto, S. and Miya, K. (1987) Formation of brain of the normal and the embryonic lethal, "star-spot duplication" embryos in the silkworm, *Bombyx mori* (Bombycidae, Lipidoptera). In *Recent Advances in Embryology in Japan and Poland* (Ando, H. and Cz. Jura, eds.), pp. 237-254. Isebu Co. Ltd., Tsukuba.

Hinton, H. E. (1969) Respiratory systems of insect egg shells. Ann. Rev. Entomol., **14**: 343-368.

Ikeda, E. (1910) Embryonic development and morphological changes of *Bombyx mori*. Sangyoh-Shinpoh, **18**: 26-42 (Japanese).

Ikeda, E. (1912) Morphological change of silkworm eggs until 2 days from oviposition. Sangyoh-Shinpoh, **20**: 9-11; 43-47 (Japanese).

Ikeda, E. (1913) Embryonic Development. In *Experiments on Anatomy and Physiology of the Silkworm* (1513p.), pp. 1227-1337, Meibun-doh, Tokyo (Japanese).

Iwasaki, Y. (1931) Formation of blastoderm cell and yolk cell in the silkworm with some reference to the characteristics of yolk cell. *J. Appl. Zool. Jpn.*, **3**: 308-313 (Japanese).

Iwasaki, Y. (1932) The nature, formation and distribution of blood cells in the silkworm eggs. *J. Appl. Zool. Jpn.*, **4**: 4-7 (Japanese).

Kanda, T., Matsumura, H., and Ohtsuki, Y. (1974) Surface structure of silkworm (*Bombyx mori*) egg. I. Fine structure of micropylar apparatus. *J. Sericult. Sci. Jpn.*, **43**: 379-383 (Japanese with an English summary).

Kawaguchi, E. and Miya, K. (1943) Embryonale Entwicklung der Geschlechtszellen beim Seidenspinner, *Bombyx mori* L. *Jap. J. Genet.*, **19**: 133-134 (Japanese).

Kawaguchi, Y., Kusakabe, T. and Koga K. (2002) Morphological variation of micropylar apparatus in *Bombyx mori* egg. *J. Insect Biotechnol. Sericol.*, **71**: 49-54.

Kawamura, N. (1978) The early embryonic mitosis in normal and cooled eggs of the silkworm,

Bombyx mori. *J. Morphol.*, **158**: 57-72.

Keino, H. and Takesue, S. (1982) Scanning electron microscopic study on the early development of silkworm eggs (*Bombyx mori* L.). *Develop. Growth Differ.*, **24**: 287-294.

Kobayashi, Y. and Miya, K. (1987) Structure of egg cortex relating to presumptive embryonic and extraembryonic regions in the silkworm, *Bombyx mori* (Bombycidae, Lepidoptera). In *Recent Advances in Insect Embryology in Japan and Poland* (Ando, H. and Cz. Jura, eds.), pp.181-194, Isebu Co. Ltd., Tsukuba.

Kuwana, J. and Takami, T. (1957) Insecta. In *Embryology of Invertebrate* (Kume, M. and Dan, K., eds.). pp. 287-343, Baifukan, Tokyo (Japanese).

Matsuzaki, M. (1968) Electron microscopic observations on the chorion formation of the silkworm, *Bombyx mori* L. *J. Sericult. Sci. Jpn.* **37**: 483-490 (Japanese with an English summary).

Miya, K. (1958) Studies on the embryonic development of gonad in the silkworm, *Bombyx mori* L. Part I. Differentiation of germ cells. *J. Fac. Agric. Iwate Univ.*, **3**: 436-467.

Miya, K. (1959a) Studies on the embryonic development of the gonad in the silkworm, *Bombyx mori* L. Part II. Formation of the gonad. J. Fac. Agric. Iwate. Univ., 4: 126-151

Miya, K. (1959b) Electron microscopic studies on the embryonic development in the silkworm, *Bombyx mori*. I. Observations on the hibernating egg. *J. Seric. Sci. Jpn.*, **28**: 163-164 (Japanese).

Miya, K. (1960) Electron microscopic studies on the embryonic development in the silkworm, *Bombyx mori*. II. Fine structure of the serosa. *J. Seric. Sci. Jpn.*, **29**: 273 (Japanese).

Miya, K. (1975) Ultrastructural changes of embryonic cells during organogenesis in the silkworm, *Bombyx mori*. I. The gonad. *J. Fac. Agric. Iwate Univ.*, **12**: 329-363.

Miya, K. (1976) Ultrastructural changes of embryonic cells during organogenesis in the silkworm, *Bombyx mori*. II. The alimentary canal and the Malpighian tubules. *J. Fac. Agric. Iwate Univ.* **13**: 95-122.

Miya, K. (1978) Electron microscope studies on the early embryonic development of the silkworm, *Bombyx mori*. I Architecture of the newly laid egg and the changes by sperm entry. *J. Fac. Agric. Iwate Univ.* **14**: 11-35.

Miya, K. (1984a) Early embryogenesis of *Bombyx mori*. In *Insect Ultrastructure* (King, R. C. and Akai, H., eds.), **Vol. 2**, pp. 49-73, Plenum Press, New York.

Miya, K. (1984b) Early embryogenesis of "Kidney-shaped egg" in the silkmoth, *Bombxy mori* L. *J. Fac. Agric. Iwate Univ.*, **17**: 131-149.

Miya, K., Kurihara, M. and Tanimura, I. (1972) Changes of fine structures of the serosa cell and the yolk cell during diapause and post-diapause development in the silkworm, *Bombyx mori* L. *J. Fac. Agric. Iwate Univ.*, **11**: 51-87.

Nagy, L. M. (1995) A summary of lepidopteran embryogenesis and experimental embryology. In *Molecular Model Systems in the Lepidopte*ra (Goldsmith, M.R. and Wilkins, A.S., eds.), pp. 139-164, Cambridge Univ. Press, Cambridge.

Nagy, L., Riddiford, L. and Kiguchi, K. (1994) Morphogenesis in the early embryo of the lepidopteran *Bombyx mori*. *Dev. Biol.*, **165**: 137-151.

Nakamura, A., Amikura, R., Mukai, M., Kobayashi, S. and Lasko, P. F. (1996) Requirement for a noncoding RNA in *Drosophila* polar granules for germ cell establishment. *Science*, **274**: 2075-2079.

Nakao, H. (1999) Isolation and characterization of a *Bombyx vasa*-like gene. *Dev. Genes Evol.*, **209**, 312-316.

Nakata, T. (1935) Studies on the development of the Malpighian tube, and the early development of the posterior anlage of the mid-intestine in *Bombyx mori* L. *Rep. Inst. Seric. Fukuoka Pref. Jpn.*, 1-27 (Japanese).

Ohtsuki, Y. (1979) Silkworm eggs. In *A General Textbook of Sericulture* (The Japanese Society of Sericultural Science, ed.), pp. 156-173, Nihon Sanshi Shinbun-sha, Tokyo (Japanese).

Ohtsuki, Y. and Kanda, T. (1982) The fine structure of the egg shell of Bombycid and Satruniidmoth. In *The Ultrastructure and Functioning of Insect Cells* (Akai, H., King, R. C. and Morohoshi, S., eds.), pp.13-16, Soc. Insect Cells Jpn., Tokyo.

Ohtsuki, Y., Kanda, T., and Matsumura, H. (1977) Surface structure of silkworm (*Bombyx mori*) egg. II. Characteristics of different areas of egg surface. *J. Sericult. Sci. Jpn.* **46**: 45-50 (Japanese with an English summary).

Ohtsuki, Y., and Murakami, A. (1968) Nuclear division in the early embryonic development of the

silkworm, *Bombyx mori* L. *Zool. Mag.* 77: 383-387 (Japanese with an English summary).
Okada, M. (1970) Electron microscope studies on diapause embryos of the silkworm, *Bombxy mori* L. *Sci. Rep. Tokyo Kyoiku Daigaku, Ser. B*, **14**: 95-111.
Rasmussen, S. W. and Holm, P. B. (1982) The meiotic prophase in *Bombyx mori*. In *Insect Ultrastructure* (King, R. C. and Akai, H., eds.), **Vol. 1**, pp.61-85, Plenum Press, New York.
Sakaguchi, B., Chikushi, H. and Doira, H. (1973) Observation of eggshell structure controlled by gene action in *Bombyx mori*. *J. Fac. Agric. Kyushu Univ.*, **18**: 53-62.
Sander, K., Gutzeit, H. O. and Jackle, H. (1985) Insect embryogenesis: morphology, physiology, genetical and molecular aspects. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (Kerkut, G. A. and Gilbert, L. I., eds.), **Vol. 1**, pp. 319-386, Pergamon Press, Oxford, New York.
Suzuki, Y. (1995) Genes that are involved in morphogenesis and tissue differentiation of *Bombyx* embryos. In *Insect Biochemistry and Molecular Biology* (Ohnishi, E., Sonobe, H. and Takahashi, S. Y., eds.), pp. 301-315, Nagoya Univ. Press, Nagoya.
Takahashi, S. and Yagi, N. (1926) A defined region where an embryo develops in a *Bombyx* egg. Sangyoh-Shinpoh, (**393**): 266-268 (Japanese).
Takami, T. (1969) *A General Textbook of the Silkworm Eggs*. 371p., Zenkoku Sanshu Kyokai, Tokyo (Japanese).
Takei, R. and Nagashima, E. (1975) Electron microscope investigation on the early developmental stages of diapause and non-diapause eggs in the silkworm, *Bombyx mori* L. *J. Sericult. Sci. Jpn.* **44**: 118-124 (Japanese).
Takesue, S. and Keino, H. (1980) Mechanism of nuclear migration before blastoderm formation in the silkworm egg (*Bombyx mori* L). *Zool. Mag.*, **89**: 408.
Takesue, S., Keino, H. and Endo, K. (1976) Studies on the yolk granules of the silkworm, *Bombyx mori* L.: The morphology of diapause and non-diapause eggs during early developmental stages. *Wilhelm Roux's Archiv.*, **180**: 93-105.
Takesue, S., Keino, H. and Onitake, K. (1980) Blastoderm formation in the silkworm egg (*Bombyx mori* L.) *J. Embryol. Exp. Morphol.*, **60**: 117-124.
Tekesue, S., Onitake, K. and Keino, H. (1977) Studies on the blastoderm and germband formation during early embryogenesis in the silkworm, *Bombyx mori* L. *Zool. Mag.* **86**: 345 (Japanese).
Tichomiroff, A. (1879) Ueber die entwicklungsgeshichte des Seidenwurms. *Zool. Anz.*, **2**: 64-67.
Toyama, K. (1896) Structure of the eggs and development of the embryos in the silkworm, *Bombyx mori* L. Nogakukai-hoh, (**28**): 28-80 (Japanese).
Toyama, K. (1902) Contributions to the study of silkworms. I. On the embryology of the silkworm. *Bull. Coll. Agric. Tokyo Imp. Univ.*, **5**: 73-118.
Toyama, K. (1909) *A Textbook of the Silkworm Egg*. 785 p., Maruyama-sha, Tokyo (Japanese).
Wada, S. (1955a) Kenntnis der keimblatterheerkunft des Subosophagealkorpers am Embryo der Seidenraupe, *Bombyx mori* L. *J. Seric. Sci. Jpn.*, **24**: 114-117 (Japanese).
Wada, S. (1955b) Zur Frage des Subosophagealkorpers als hamopoetisches Gewebes am Embryo der Seodenraupe *Bombyx mori* (Vorlaufige Mittteilung). *J. Seric. Sci. Jpn.*, **24**: 311-313 (Japanese).
Weismann, A. (1863) Die Entwicklung der Dipteren im Ei. *Z. wiss. Zool.*, **13**:107-220.
Wolf, R. (1980) Migration and division of cleavage nuclei in the gall midge, *Wachtliella persicariae*. *Wilhelm Roux's Archiv.*, **188**: 65-73.
Yamashita, O. (1996) Diapause hormone of the silkworm, *Bombyx mori*: structure, gene expression and function. *J. Insect Physiol.*, **42**: 669-679.
Yamashita, O. and Yaginuma, T. (1991) Silkworm eggs at low temperatures: implications for sericulture. In *Insects at Low Temperature* (Lee, R.E.Jr., and Denlinger, D.L., eds.), pp. 424-445, Chapman and Hall, New York.
Yamauchi, H. and Yoshitake, N. (1984) Formation and ultrastructure of the micropylar apparatus in *Bombyx mori* ovarian follicles. *J. Morphol.*, **179**: 47-58.
Yamazaki, Y. and Okada, M. (1989) Differences in fine structures between normal and RNA-induced *Drosophila* pole cells. *Develop. Growth & Differ.*, **31**: 549-556.

Subject Index (except for Figure legends)

ISBN4-906666-25-6 C3045 ¥14000E