Mem. Fac. Agr. Kagoshima Univ., **17**, 165–174, 1981

Electron Microscopical Observations on the Egg of *Echinococcus multilocularis*

Tsukasa SAKAMOTO
(*Laboratory of Veterinary Pathology*)
Received for Publication September 10, 1980

Introduction

Light microscopical observations on the hexacanth embryos of cyclophyllidian cestodes have been reported by a number of workers[10]. Electron microscopy on the cyclophyllidian eggs by several investigators has revealed some ultrastructural features. Particularly, Morseth[6] studied on the structures of embryos of *Echinococcus granulosus* together with other taeniid cestodes using transmission electron microscope. However, the observation on the egg of *E. multilocularis* was not yet reported. The present study was undertaken to describe the fine structures of the egg of *E. multilocularis*.

Materials and Methods

Adults of *Echinococcus multilocularis* Leuckart, 1863 were obtained from the dogs 33 and 34 days after the experimental infection. The entire worms were rinsed with in ice-cold phosphate-buffered solution to pH 7.4, and fixed for 2 hours at 4°C in 5.28% glutaraldehyde buffered to pH 7.4. After the first fixation, the worms were washed briefly in ice-cold phosphate-buffered solution, and postfixed for one hour in 2.0% osmium tetroxide at 4°C. The worms were washed again in cold buffer, passed to propylene oxide and a 1: 1 mixture of propylene oxide and Epon 812. The materials were then embedded in Epon 812, and sectioned by the ultramicrotome. Polymerization was carried out at 55°C for 18 hours. The sections were mounted on nickel grids and stained with saturated uranyl acetate and lead citrate.

The thicker sections obtained from the same blocks were stained with toluidine blue and PAS, and examined under a light microscope for identification of the area of the electron microscopical preparations.

A part of adults were used for freeze-fructuring electron microscopy. The samples were prefixed for 12 hours with ice-cold 2% glutaraldehyde buffered, using phosphate or cacodylate buffer, to pH 7.4, and transferred to 40% glycerol in 0.85% saline solution. The samples on a brass holder were rapidly frozen in Freon 12 at liquid N_2 temperature. The freeze-fructuring was carried out using JEOL–EF–FED freeze-etching apparatus. Replication was performed with platinum-carbon. The replicas were coated with carbon, made to be floated on distilled water, cleaned by adding a hypochlorite solution, rinsed three times with distilled water, and placed on 400-mesh grids. The

This work was presented at the 77th Meeting of the Japanese Society of Veterinary Science, on 6th–9th April, 1974 in Tokyo, Japan.

specimens were observed, using JEM-100 electron microscope.

Results

1. Light microscopical findings of the eggs

The outer surface of egg was covered with egg shell (capsule) which was lined with vitelline layer consisting of outer and inner envelopes. The oncosphere is covered with embryophore. An oncospheral membrane is situated between the inner envelope and embryophore. The oncosphere is a bilaterally symmetrical, spherical embryo armed with three pairs of hooks arranged in a hemisphere. Each hook consists of a curved blade, a short collar (guard) and a long handle (shank). The cuticle (tegument) is lined with an irregular network of contractile muscle fibers. The muscle fibers attaching to each of the hooks were composed of two kinds of muscle bands, the both ends of one of those muscle bands connect the collar of hooks with the inner surface of cuticle near other hooks, and the both ends of another band connect the base of handle with the opposite pole of the oncosphere. The somatic cells of oncosphere are composed of the cells of two types as follows: The cells of one type which are situated beneath the cuticle (tegument) of hook region and the cells of another type which are the undifferentiated cells located in the opposite hemisphere of oncosphere. Two large gland cells showing to be positive in PAS reaction, are located symmetrically below the lateral hook. They are considered to be penetration gland cells. Besides, the flame cells are found to be moving among parenchyma under phase contrast microscope.

2. Electron microscopical findings of the eggs

The envelopes surrounding oncosphere consist of eight layers or membranes as follows: From the outermost, the egg shell (capsule) surrounds the surface of egg, vitelline layer comes second, next are arranged in order outer embryophoric membrane, embryophore, granular layer, basal layer and oncospheral membrane. The egg capsule is cytoplasmic membrane (unit membrane) which the two dense laminae are separated by an opaque interspace. The vitelline layer in electron microscopy is equivalent for the outer envelope in light microscopy. The layer contains some nuclei of vitelline cell, abundant mitochondria, glycogen-granules, crystalline substances and vacuoles. The nuclei and mitochondria are degenerated in mature eggs. The inner surface of the vitelline layer is lined with unit membrane (outer embryophoric membrane).

In the observation of the replicas prepared by freeze-etching of the eggs, two kinds of crater-shaped pores consisting of large pores and small ones, and the groove of reticulated joints among the polygonal embryophoric blocks are seen on the surface of outer embryophoric membrane. The small pores which have one to three granules in them, are about 0.06 μm in diameter, and are distributed densely all over the surface of the membrane. The large pores having many granules in them are noted sporadically among the small ones, located on the intersecting points of the groove of reticulated joint among the embryophoric blocks.

The electron dense embryophoric blocks are arranged beneath the outer embryophoric membrane (the plasma membrane of embryopholic cell). The embryopholic blocks appear to be irregularly polygonal in cross section, and are longer in length than in width. The blocks contain many lacumae and vermicular channels. The lacuna contain the circular bodies which are various in density. The granular layer is packed with numerous ribosomes, and contains nuclei having a prominent nucleolus in each, mitochondria, and electron dense circular bodies. The inner surface of the granular layer was covered with cytoplasmic membrane (basal membrane of granular layer).

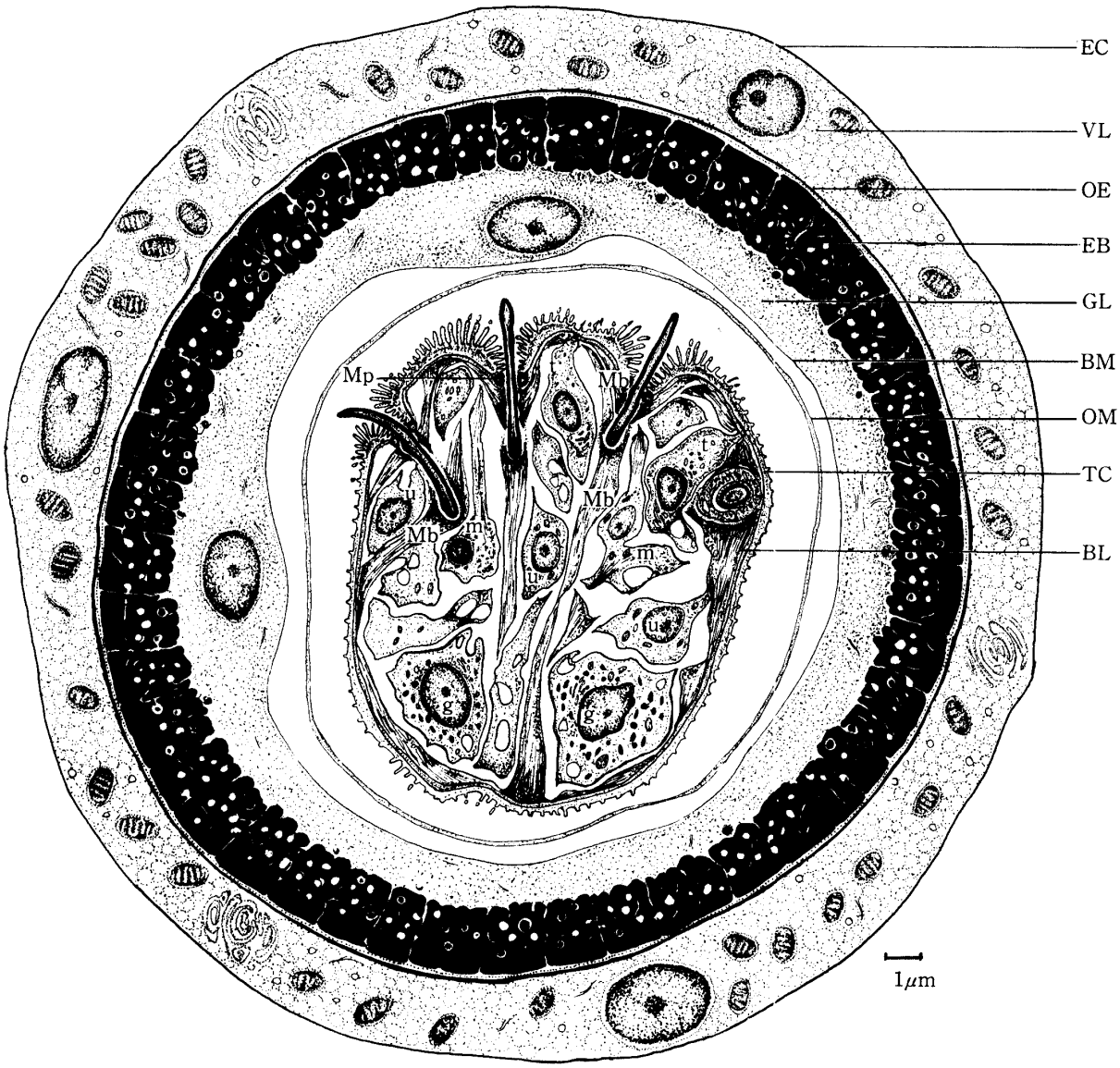


Fig. 1. Schematic drawing of egg of *Echinococcus multilocularis*.
EC, egg capsule; VL, vitelline layer; OE, outer embryophoric membrane; EB, embryophoric block; GL, granular layer; BM, basal membrane of granular layer; OM, oncospheral membrane; TC, tegumental cytoplasm of oncosphere; BL, basal lamina lining tegumental cytoplasm; Mb, muscle band; Mp, midpiece; t, tegumental cell; m, muscle cell; u, undifferentiated cell; g, gland cell

The oncospheral membrane is seen attached to the basal membrane or separated from it. The oncopheral membrane is composed of two pairs of laminae, lies between the basal membrane of the granular layer and tegumental cytoplasm (limiting membrane) of the oncosphere itself.

The surface of oncosphere was covered with superficial, syncytial cytoplasm (distal cytoplasm: tegumental cytoplasm) which is continuous with tegumental cells (perikarya: perinuclear cytoplasm) embedded in parenchyma. The outer surface of the tegumental cytoplasm has numerous microvilli. The microvilli are covered with a unit membrane which is continuous over the outer surface of tegumental cytoplasm of oncosphere. These microvilli grow dense and long in the region surrounding hooks. On the contrary, the microvilli are sparse and short in the opposite side. Numerous vesicles which contain electron dense materials were seen in the tegumental cytoplasm. The

tegumental cytoplasm is lined with basal lamina. The muscle consists of somatic musclature and hook musclature. The somatic muscle almost entirely encircles itself beneath the tegumental cytoplasm. The handle (: shaft: shank) of hook is surrounded by sheath of tegumental cytoplasm which is encircled by the basal lamina. The base of hook is embedded in the cytoplasm consisting of midpiece and oncoblast (hook-forming cell). The desmosome connects itself between the sheath of tegumental cytoplasm and cytoplasm of the oncoblast. The hook muscles attach to the basal lamina surrounding the cytoplasm arround hook. The wide band of distal part of hook muscle attaches to the basal lamina lining tegumental cytoplasm of oncosphere. A number of α glycogen particles are contained in the cytoplasm of muscle cells. The large secretory cells (penetrating gland cells) containing many electron dense bodies, and undifferentiated cells containing numerous free ribosomes and tubular smooth endoplasmic reticula, were recognized among the cells of parenchyma of oncospheres.

Discussion

Rybicka[10] has pointed out that much confusion has arisen in the terminology used to describe light microscopical findings of the embryonic envelopes of cyclophyllidean cestodes and summarized the various terms used in many papers into four terms: (1) capsule, (2) outer envelope, (3) inner envelope and (4) oncospheral membrane. Swiderski[13] described that electron microscopy showed five envelopes around the developing embryo of *Catenotaenia pusilla*: (1) capsule, (2) outer envelope, (3) inner envelope, (4) embryophore and (5) oncospheral membrane. Morseth[6] reported that the embryophoric envelopes of taeniid eggs are made up of eight distinct layers and membranes: (1) egg capsule, (2) vitelline layer, (3) outer embryophoric membrane, (4) embryophore, (5) granular layer, (6) basal membrane of granular layer, (7) oncospheral membrane and (8) limiting membrane. The present electron microscopy revealed that the envelopes of egg of *Echinococcus multilocularis* are essentially similar to those of taeniid eggs reported by Morseth[6]. The oncosphere of *E. multilocularis* is covered by tegumental cytoplasm having microvilli. The tegumental cytoplasm is corresponding to outer (cytoplasmic) coat used by Collin[1,2], limiting membrane by Morseth[6] and oncospheral membrane by Swiderski[13]. The cellular structures of echinococcal oncosphere observed in this experiment are essentially analogous to those of various species of cyclophyllidean cestodes reported by many workers[7].

Stvortsvo[12] reported that eggs of *E. granulosus* and *Taenia saginata* immersed in 10% formalin solution for two hours remained infective. Nosik[8] also recognized the remaining infectiousness of hydatid eggs immersed in alcohol and formalin for several hours. Meymarian and Schwabe[5] described that a high percentage of echinococcal oncospheres also survived after immersion for 24 hours in 1 to 2% tide detergent, after 24 hours in 5 to 20% formalin, Roccal, Lysol and 70 to 95% ethyl alcohol. Parnell[9] tested the ovicidal action of 50 kinds of non-organic compounds on eggs of *Taenia ovis* and *T. hydatigena,* and Mackie and Parnell[3] tested more than 50 classes of organic and non-organic compounds including germicides. They reported that all of the drugs tested were ineffective. Sakamoto et al.[11] stated that the eggs of *E. multilocularis* soaked in each of the solutions or suspensions of 5% arecoline hydrobromide, 5% bithinol sulfoxide, 5% bunamidine hydrochloride, 5% niclosamide, 2% bis(2-hydroxy-3-nitro-5-chlorophenyl)sulfide and 0.2% dithiazanine iodide were infective. The high resistance of the taeniid eggs against ovicidal agent is considered to be depending for their impermeability upon the thick embryophore blocks held together by a cement substance. On the other hand, Maymarian[4] observed that the embryophore

of *E. granulosus* was broken down for a little while by the digestion of the cement like substance uniting the embryophoral blocks through the use of pancreatin solution. The present electron microscopy exhibited that there are numerous lacunae and tubules containing various electron dense substance distributed at frequent intervals within the embryophoral block. The similar findings were recognized also in the eggs of *E. granulosus* by Morseth[6]. In the present observation the replicas of eggs by freeze-etching, two kinds of crateriform pores packed with granules were found distributed all over the surface of outer embryophoric membrane. Particularly, the large-sized pores located on the intersecting points of reticular joints among the blocks. Accordingly, it can be conjectured that the impermeable outer embryophoric membrane may be changed into porous membrane by digestion of granules packed in the pores with digestive juice. It is considered that the ultrastructural findings obtained in the present observations is full of suggestion for the investigation of ovicidal agents against echinococcal eggs.

Summary

The ultrastructure of egg of *Echinococcus multilocularis* Leuckart, 1863 was observed using routine and freeze-fracturing method. The envelopes surrounding oncosphere are made up of six layers and membranes: in order from the outermost toward inside, (1) egg capsule, (2) vitelline layer, (3) outer embryophoric membrane, (4) embryophore, (5) granular layer, and (6) oncospheral membrane. The oncosphere is covered with syncytial cytoplasm (tegumental cytoplasm) having microvilli. A basal lamina is lined on the inner surface of tegumental cytoplasm. The network of transverse somatic muscle-fibers lines the inside of the basal lamina. Oncospheral hook is surrounded by the sheath of cytoplasm. The basal part of hook is inserted in the midpiece and the cytoplasm of oncoblast. The cytoplasm surrounding the hook is covered with the basal lamina. The end of the hook muscle fiber attaches to electron opaque patch on its basal lamina encircling the tegumental and sheath cytoplasm. Two gland cells and several undifferentiated cells were found among the muscle cells and perikaria of the tegumental cells.

Acknowledgements

The author wishes to express his cordial thanks to Prof. I. Kono and Mr. N. Yasuda of this laboratory for their kind advice and help in this study.

References

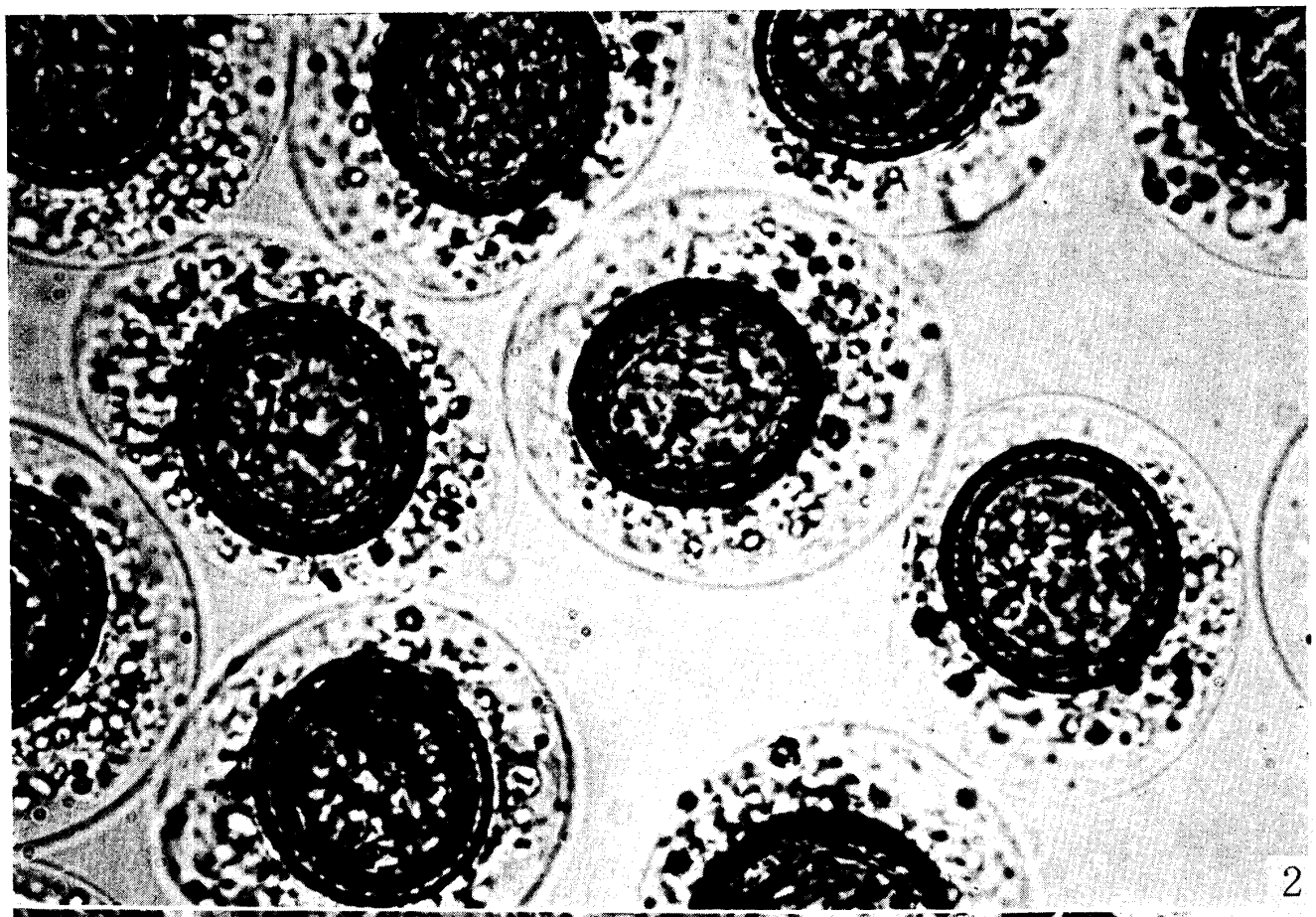
1) Collin, W. K.: The cellular organization of hatched oncospheres of *Hymenolepis citelli* (Cestoda, Cyclophyllidea). *J. Parasit.*, **55**, 149–166 (1969)
2) Collin, W. K.: Electron microscope studies of the muscle and hook systems of hatched oncospheres of *Hymenolepis citelli* McLeod, 1933 (Cestoda: Cyclophyllidea). *Ibid.*, **54**, 74–88 (1968)
3) Mackie, I. and Parnell, I. W.: Some observation taeniid ovicides: The effects of some organic compounds and pesticides on activity and hatching. *J. Helminth.*, **41**, 167–210 (1967)
4) Meymerian, E.: Host-parasite relationships in *Echinococcus*. VI. Hatching and activation of *Echinococcus granulosus* ova *in vitro*. *Am. J. trop. Med. Hyg.*, **10**, 719–726 (1961)
5) Meymerian, E. and Schwabe, C. W.: Host-parasite relationships in echinococcosis. VII. Resistance of the ova of *Echinococcus granulosus* to germicides. *Ibid.*, **11**, 360–364 (1962)
6) Morseth, D. J.: Ultrastructure of developing taeniid embryophores and associated structures. *Expl Parasit.*, **16**, 207–216 (1963)

7) Nieland, M. L.: Electron microscope observations on the egg of *Taenia taeniaeformis*. *J. Parasit.*, **54**, 957–969 (1968)
8) Nosik, A. F.: Resistance of the oncospheres of *Echinococcus granulosus* to some physical and chemical factors. *Trud. khar'kov. vet. Inst.*, **21**, 304–311 (1952) [*Helminth. Abstr.*, **21**, 382 (1952)]
9) Rarnell, I. W.: Some observations on taeniid ovicides: Screening techniques, and the effects of some inorganic compounds. *J. Helminth.*, **39**, 257–272 (1965)
10) Rybicka, K.: The embryonic envelops in cyclophyllidean cestodes. *Acta parasit. pol.*, **13**, 25–34 (1965)
11) Sakamoto, T. et al.: Studies on pharmaco-therapy against larval and adult multilocular echinococcosis. I. Anthelmintic and ovicidal effects of drugs against adult *Echinococcus*. *Jap. J. Parasit.*, **20**, 120–131 (1971)
12) Skvortsov, A. A.: Egg structure of *Taeniarhynchus saginatus* and its control. *Zoologicheskii Zhurnal*, **21**, 10–18 (1942)
13) Swiderski, Z.: Electron microscopy of embryonic envelope formation by the cestodes *Catenotaenia pusilla*. *Expl Parasit.*, **23**, 104–113 (1968)

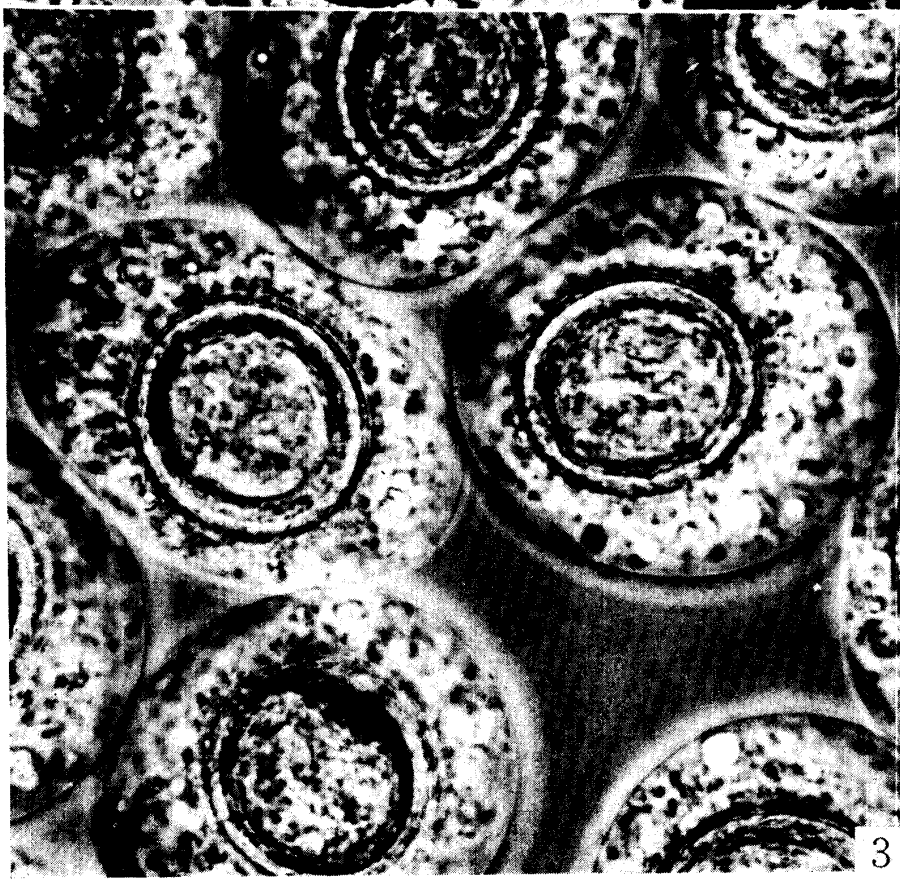
Explanation of figures

All figures are photomicrographs of eggs of *Echinococcus multilocularis*.

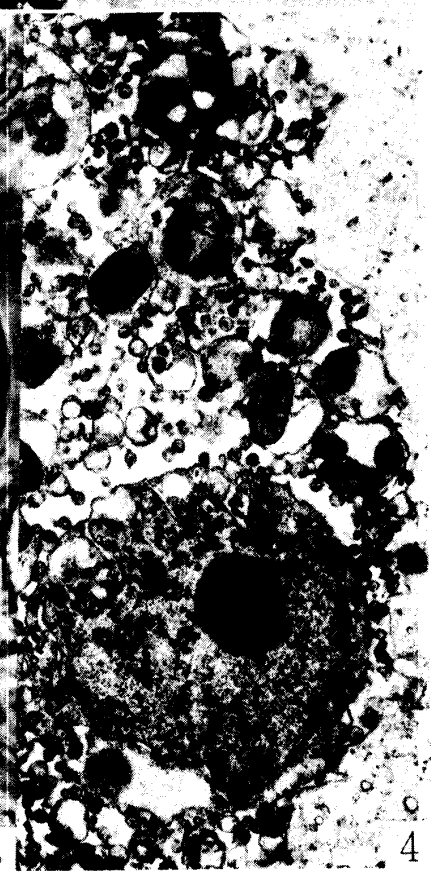
Fig. 2. Photomicrograph of echinococcal eggs. ×780
Fig. 3. Phase contrast photomicrograph of the eggs. ×780
Fig. 4. Vitelline layer containing nucleus, mitochondria and vesicles. ×6,000
Fig. 5. Vitelline layer and embryophore. ×6,000
Fig. 6. Vitelline layer and embryophore. ×4,000
Fig. 7. Vitelline layer and embryophore. ×12,000
Fig. 8. Whole egg. ×3,000
Figs. 9 and 10. Electron photomicrograph of replica of egg prepared by freeze-etching method. ×10,000
Fig. 11. Cross section of embryophore. Embryophoric blocks having microtubules and lacuna containing circular bodies. ×12,000
Fig. 12. Embryophore and oncosphere. Nucleus is seen in granular layer. ×6,000
Fig. 13. Oncosphere covered with oncospheral membrane. ×5,500
Fig. 14. Hooks, tegument and muscle cells in oncosphere. ×12,000



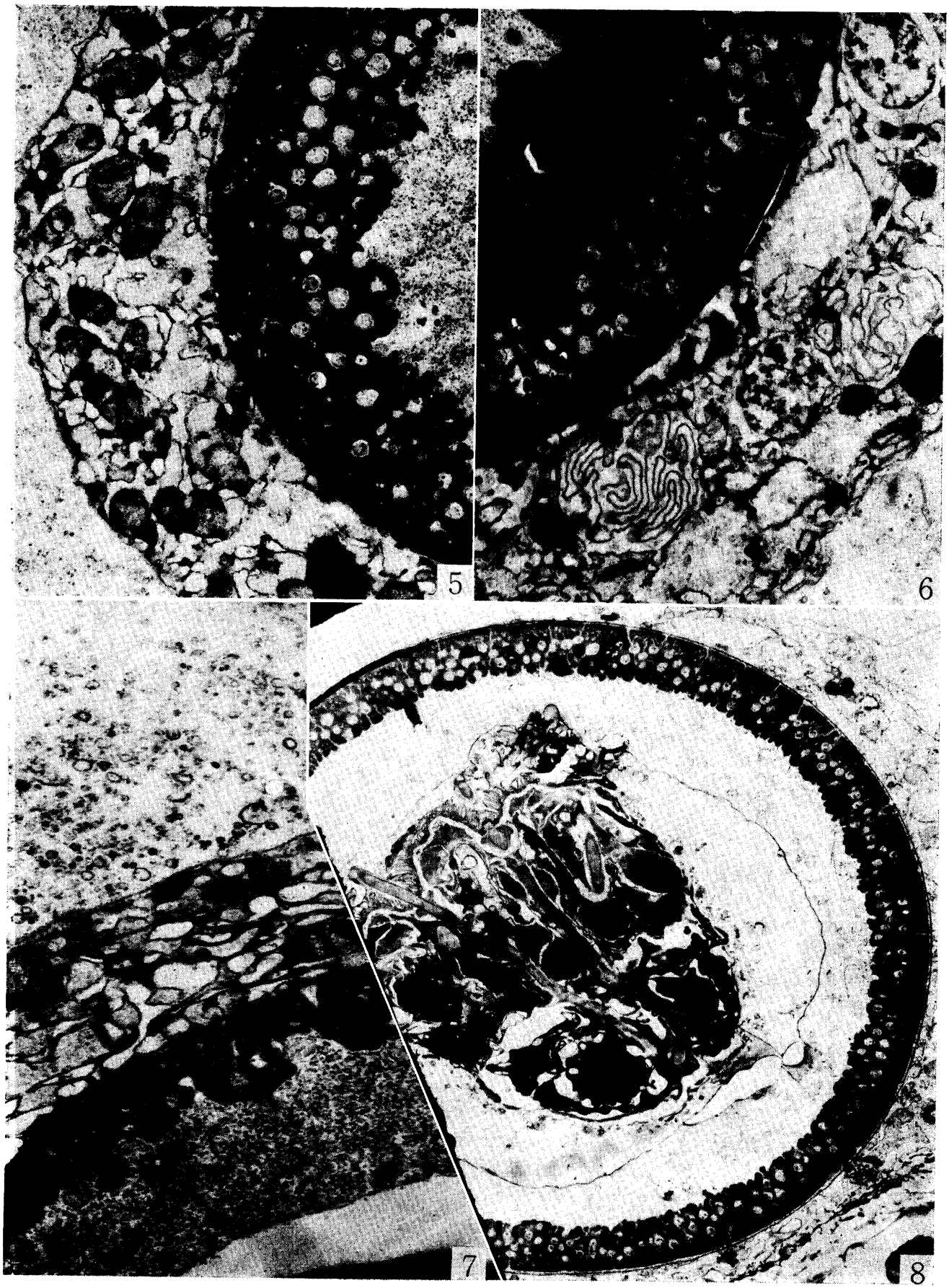
2

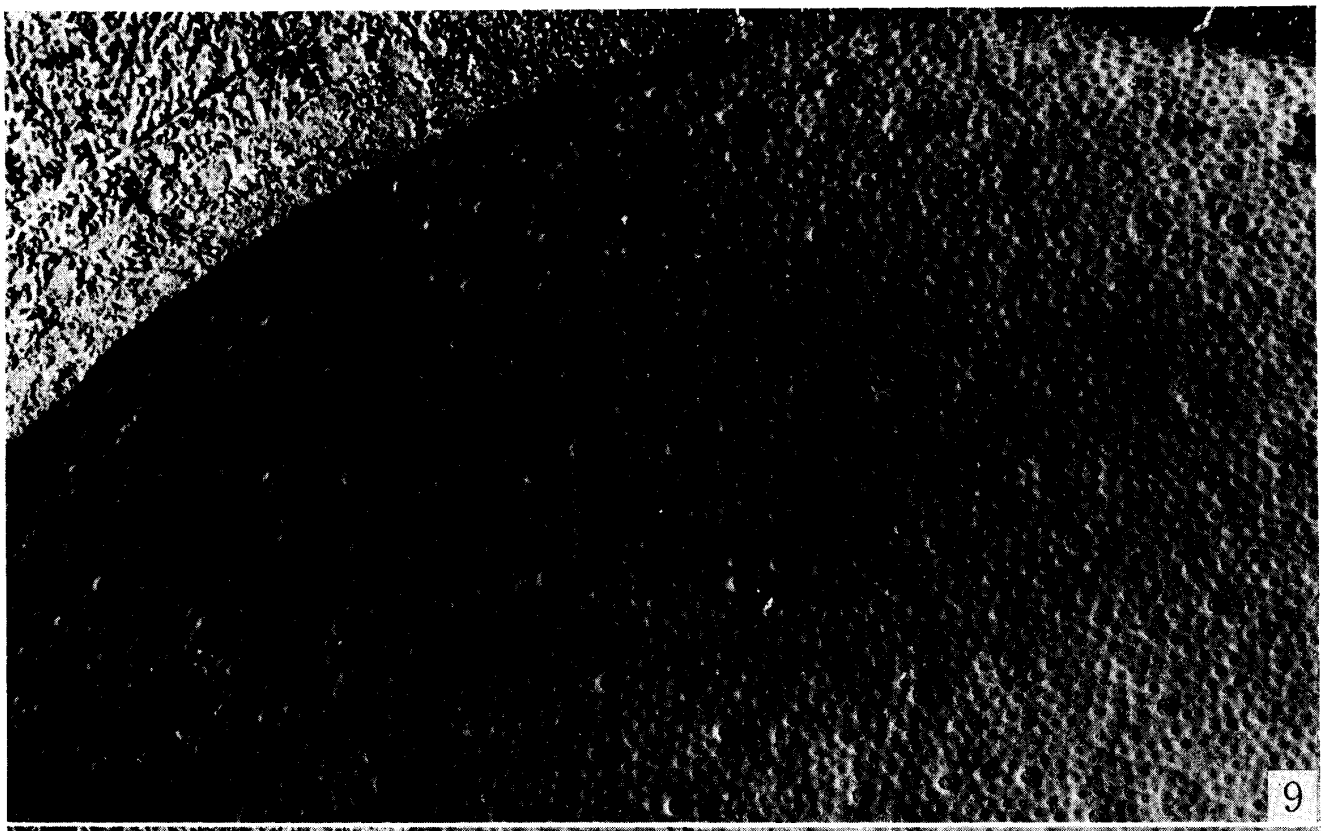


3

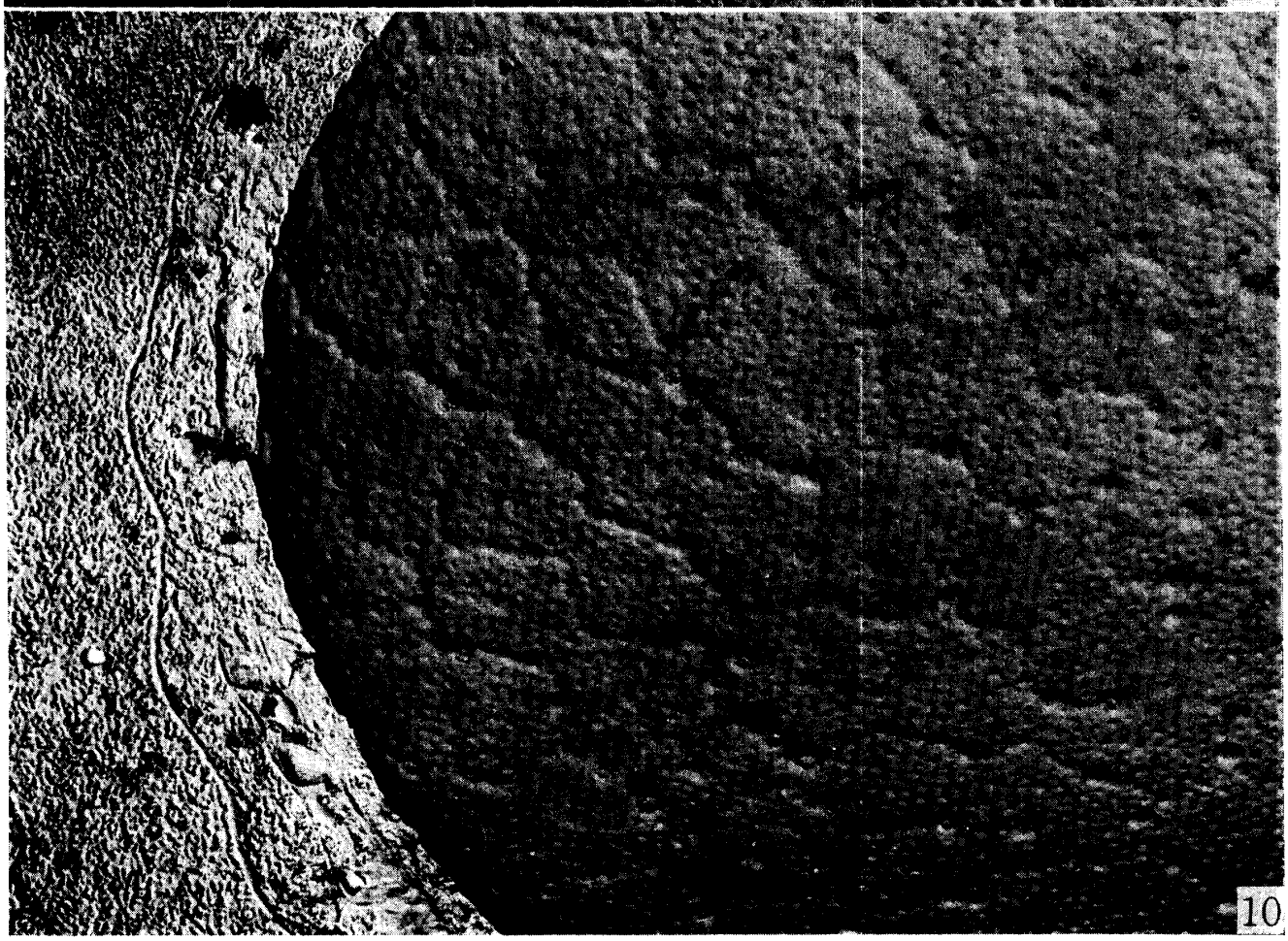


4

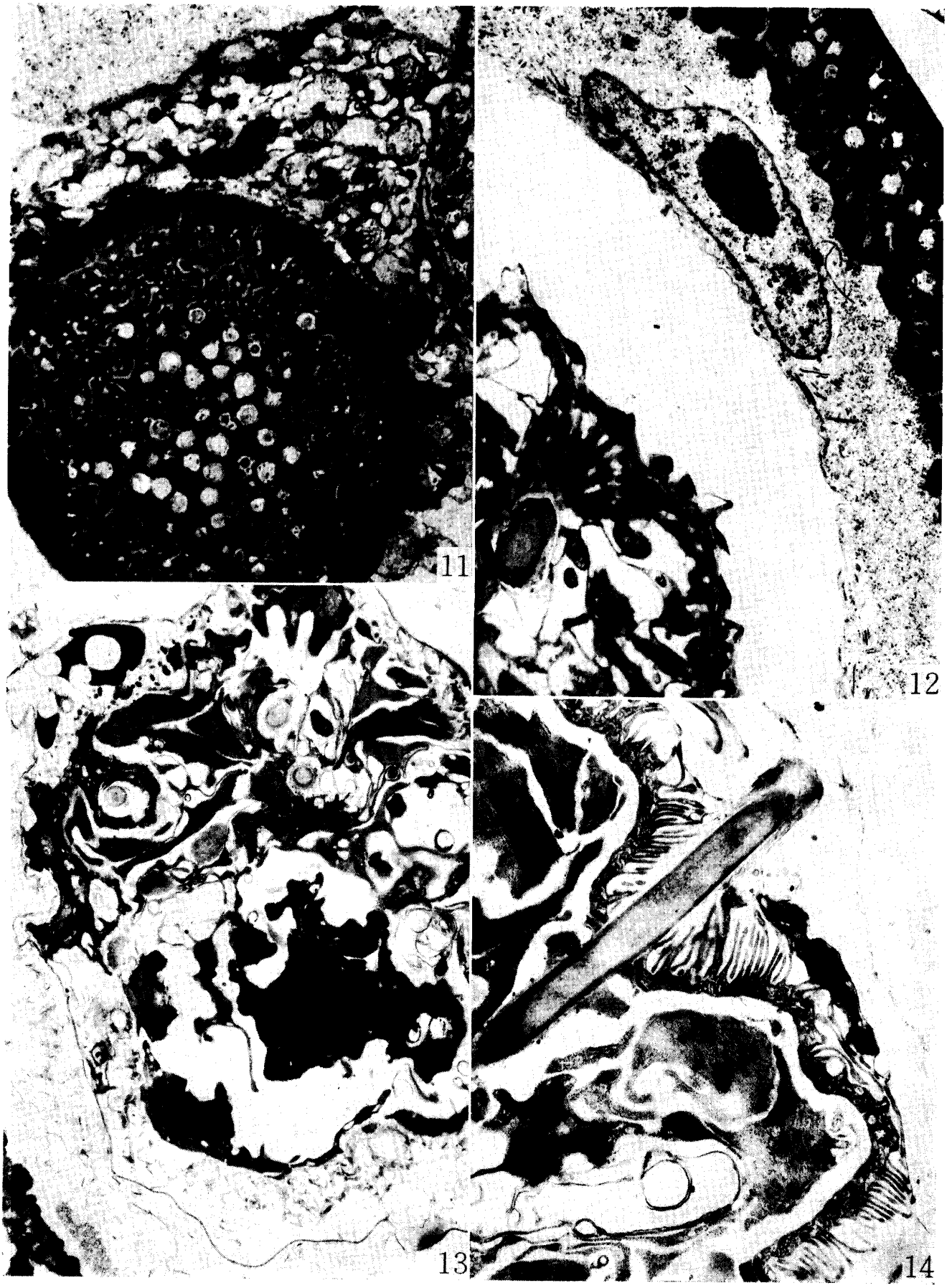

5
6
7
8



9



10


11
12
13
14