

## THE FINE STRUCTURE OF CYSTS OF FRENKELIA (THE M-ORGANISM)

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**Abstract.** Electron microscope studies were carried out on cysts of *Frenkelia microti* and *F. glareoli* from brains of *Microtus agrestis* and *Clethrionomys glareolus* respectively. The relationship of the fine structure of *Frenkelia* to that of *Toxoplasma* and *Sarcocystis* is discussed.

All cysts of *Frenkelia* examined had corrugated walls made of a spongy inner layer and an outer electron-dense double membrane. The latter was more intricately folded in cysts of *F. glareoli*. The inner layer of the wall was continuous with septa which divided the interior of the cyst into compartments packed with mature and maturing zoites. The mature zoite was elongate with conoid, double polar rings, sub-pellicular fibrils, 2-4 paired organelles, numerous micronemes, a cytosome, a large posterior nucleus, a long mitochondrion and a distinctive thickening of the pellicle at the posterior end. Immature forms, rounded and slightly larger, were found principally along the periphery of the cyst wall and were more numerous in small or young cysts; they lacked the typical anterior organelles of Sporozoa and contained more abundant mitochondria than the elongate zoites. *Frenkelia* was shown to reproduce by endodyogeny.

In 1934, Findlay and Middleton described a protozoan parasite which formed large macroscopic cysts in the brain of *Microtus agrestis* (the European short-tailed vole), near Lake Vynwy in Wales. They believed the parasite to be the cause of the sudden periodic drop in the population of these rodents. The parasite was identified as *Toxoplasma* and named *Toxoplasma microti*. In 1953, Frenkel found the same or a very similar parasite in *Microtus modestus* in the New World. On the basis of differences in the morphology of the brain cysts and failure to transmit the parasite in the laboratory he reached the conclusion that Findlay and Middleton's parasite was not *Toxoplasma*, and temporarily named it the "M-organism". Since then this parasite has been recorded in the brains of several species of ericetid rodents belonging to the subfamily Microtinae in other temperate regions. Ludvík (1963) made comparative studies of the fine structure of "M-organism", *Toxoplasma* and *Sarcocystis*. In 1968, Biocca gave the "M-organism" the generic name *Frenkelia*.

The parasite is known only in its cerebral cystic form of which two types have been recognized. The first, a large, extensively lobulated cyst (Fig. 1\*\*) has been found in *Microtus agrestis* in Britain (Findlay and Middleton 1934; Tadros 1968) and Czechoslovakia (Šebek 1962, 1963); *Microtus modestus* in U.S.A. (Frenkel 1955, 1956, 1960); *M. arvalis* in Czechoslovakia (Šebek 1962); *Ondatra zibethica* in Canada

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\*\*) The Plates I-VIII will be found at the end of this issue.

(Karstad 1963); and *Lemmus lemmus* in Sweden (Enemar 1963). The second (Fig. 2), a smaller, round or oval cyst, was described from *Clethrionomys glareolus* (the bank vole) in Czechoslovakia (Erhardová 1955; Černá 1959; Šebek 1962), Sweden (Enemar 1963, Tadros 1968), Germany (Ludvík 1963), Britain (Tadros 1968), Poland (Doby et al. 1965); *C. rufocanus* and *C. rutilus* in U.S.S.R. (Zasuchin et al. 1958) and in *Arvicola sapidus* in France, (Doby et al. 1965). Biocca (1968) named the parasite which forms lobulated cysts in the brains of short-tailed voles, *Frenkelia microti* (Findlay and Middleton 1934), as the type species of the genus. The parasite with the rounded cyst in the brain of *C. glareolus* is now designated *F. glareoli* (Erhardová, 1955). The discovery in the future of a means of transmitting the parasite, followed by cross-infection experiments will prove or disprove the validity of the second species and establish whether or not the parasites seen in the brain of other species of rodents belong to these or other species of *Frenkelia*.

The present paper is a comparative study of the fine structure of the lobulated and rounded cysts of *Frenkelia* from the brains of *M. agrestis* and *C. glareolus* respectively.

## MATERIALS AND METHODS

*Clethrionomys glareolus* and *Microtus agrestis* were trapped alive at Lake Vyrnwy in Wales where Findlay and Middleton first saw the parasite in 1934 and where the infection is still enzootic. As yet the parasite can only be detected in the living vole by biopsy. Each animal was anaesthetized with ether and a small incision made in its scalp over the dome of the skull. The brain was examined through the transparent skull bones in diffuse daylight. When present in sufficient number moderately large cysts of *Frenkelia* were easily detected as white spots against the pale pink of the brain tissue (Bell et al. 1964). The infected animals, their scalps sutured, were isolated as a source of material for electron microscope studies. The voles were sacrificed over a period of eight months and cysts were removed to study different stages of development of the parasite. Examinations were made of both whole cysts and smears, the latter made by squashing individual cysts on slides under coverslips. Whole cysts free of brain tissue were conveniently obtained by gently shaking the infected brain in saline which caused a large number of cysts to drop out.

The free cysts were transferred to 3% glutaraldehyde in modified Rhodin and Zetterqvist buffer (see Garnham et al. 1967) and fixed at room temperature for two hours. After a wash in buffer overnight to remove excess glutaraldehyde, the cysts were transferred to 1% osmium tetroxide in buffer solution, for one hour. They were then washed again in buffer, dehydrated through graded ethanol solutions stained with 1% phosphotungstic acid in absolute ethanol for 30 minutes and embedded in epoxy resin (Araldite). Some cysts were fixed in 1% osmium tetroxide alone, others with 1.2% potassium permanganate in normal saline for one hour. Smears of zoites were fixed with same fixatives as the whole cysts, but only for 5–15 minutes.

## OBSEVATIONS

Similar features are seen in brain cysts of *Frenkelia* from both *C. glareolus* and *M. agrestis*. These features differ slightly according to the age of the cyst and the host. The cyst wall: In both species the wall is well defined and consists of two layers (Figs. 3, 4). The outer layer is composed of two unit membranes interrupted at intervals by numerous pores (Fig. 5b). This layer is more deeply folded, and has a more highly convoluted appearance in *C. glareolus* than in *M. agrestis* (Figs. 3, 4). The inner layer is a granular and mass of irregular thickness and electron density, which extends in places in the form of septa into the interior of the cyst, dividing it into compartments. The periphery of this mass contains membranes and organelles of differentiating zoites. The small cysts contain a greater thickness of this germinal material.

Septa: The inner layer of the cyst wall where continued as septa is not seen to possess any limiting membranes. In certain instances zoites appear to be forming

within some of the broader septa. The septa vary considerably in number and thickness and follow no constant pattern of arrangement.

**Cyst contents:** In section, cysts of *Frenkelia* are seen to be closely packed with a large number of organisms or zoites, arranged in clusters separated by septa. These organisms are of two distinct morphological types. The more numerous, probably the mature zoites, occupy most of the interior of the larger or more mature cysts. They are elongate and have the fine structural details of many sporozoan parasites. The other type is roughly spherical and mainly restricted in distribution to the peripheral areas of the mature cyst. Cells of this type constitute the bulk of organisms only in very small or young cyst. The mature zoites (Fig. 6): These elongate forms measure up to 10  $\mu$  in length and 3  $\mu$  in width. They are surrounded by a double unit membrane or pellicle which, at the conical anterior end, forms two polar rings by thickenings of the inner membrane (Fig. 7). Subpellicular fibrils run posteriorly from the region of these rings. A conoid lies at the anterior tip, and in longitudinal sections of this part is seen as a diagonally banded or spiral structure of 7 or 8 coils lying within the polar rings (Fig. 8). Associated with the conoid are the openings of the paired organelles; the latter extend posteriorly to swell out into club or flask shaped termination (Fig. 10). The most striking feature of the elongate zoite is the neatly packed rows of micronemes. These, interspersed with densely staining ribosomes, occupy an appreciable part of the anterior third of the zoite (Figs. 6, 10). The micronemes were too deeply stained in most preparations for details of their structure to be seen. However, in some sections of *Frenkelia microti*, they have the appearance of empty tubules (Fig. 9). A well defined cytostome (Fig. 11) is seen in the zone occupied by the micronemes. In cross section (Fig. 12) it appears as two concentric rings while in longitudinal section it is seen as a rimmed pit the walls of which are lined by both pellicular membranes. The bottom of the pit is composed of the outer membrane alone, the inner one being interrupted at this point. A large nucleus is situated in the posterior third of the body of the zoite. It is surrounded by the usual double membrane and has a large densely staining nucleolus (Figs. 6, 10). A number of vacuoles are arranged peripherally just within the pellicle, confined mainly to the posterior two-thirds of the zoite. These are one of the more striking features of the mature zoite, owing to their regularity of size and spacing (Fig. 13). Their possible origin as pinocytotic or phagotrophic vacuoles is indicated in Fig. 5. The mature zoites are in places well separated suggesting the presence of fluid within the cyst. A long mitochondrion is usually seen anterior to the nucleus. This exhibits a large number of tubular cristae (Figs. 10, 15). In one preparation we observed what looked like a slender neck connecting the mitochondrion to the exterior (Fig. 14). A cross section of the Golgi membrane between the nucleus and the mitochondrion is shown in Fig. 15. The posterior extremity of the zoite is pulled out into a nipple-like tail organelle, containing a thickened inner pellicular membrane (Fig. 16).

**The immature zoite:** Rounded zoites (Fig. 17) are seen in cysts of *F. microti* as well as *F. glareoli*, being particularly common in sections and smears of very young cysts fixed from the brain of a juvenile bank vole. They are larger than the elongate zoites measuring up to 5  $\mu$  in diameter. They do not possess conoid, polar rings, paired organelles or micronemes. Their nuclei occupy a larger portion of the cell volume. They have well developed Golgi zones and a large number of mitochondria (Figs. 18, 19). Ribosomes tend to be fewer in number and do not stain as densely as those of the mature zoites. A cytostome is present, in one instance two cytostomes were observed in the same cell. The cells contain a large number of inter-connecting vacuoles (Fig. 18).

**Dividing form:** Many of the zoites were found in the process of division. *Frenkelia* was seen to reproduce by endodyogeny, the type of division described for *Toxoplasma*

(Goldman et al. 1958) and *Besnoitia jellisoni* (Sheffield et al. 1966). Endodyogeny was the only mode of reproduction seen in our sections and was particularly noticeable in young cysts (Figs. 20, 21). Binary fission was not observed. Both round and elongate forms were seen to undergo endodyogeny, but in each instance, only elongate zoites with all the typical organelles were seen as the daughter cells.

## DISCUSSION

The numerous short blunt projections of the cyst wall of *Frenkelia* are similar to, but more pronounced than, the 'cogs' of the cyst wall of *Toxoplasma* (Garnham et al. 1962). They also resemble the 'villi' of the cyst wall in many species of *Sarcocystis*. It is interesting to note that the degree of development of these folds in the three parasites cited above, is proportional to the size of the cyst attained by each of them. The protuberances of the cyst wall increase its surface area affording a greater area of contact between the cyst and the surrounding brain tissue. This contact may facilitate the transfer of metabolites and the action of any enzymes produced by the parasite. In *F. microti*, where the surface area of the cyst is markedly increased by the deep lobulations, the cyst wall has fewer folds than *F. glareoli* which has a rounded or oval cyst. This again suggests a role related to metabolic activity.

It was not possible in this study to elucidate the stages of the formation of the cyst membrane of *Frenkelia*. Matsubayashi (1962) in electron-microscopic studies of *Toxoplasma* showed that the production of the cyst in the central nervous system in avirulent toxoplasmosis is accompanied by the deposition of granular precipitates on the limiting membrane of the vacuole surrounding the parasite in the invaded host cell. These granular particles fused together to give the solid cyst membrane. Light microscopic studies on the early stages of *Frenkelia* (Enemar 1963) indicate the intracellular position of the original infective forms in the brain. As the cyst increases in size a very thin membrane appears round the parasites, between them and the enlarged host cell nucleus. The wall proper probably develops from this membrane, possibly in the same way as that suggested by Matsubayashi for *Toxoplasma*. This is not however certain. The full sequence of the development of the cyst wall must await the successful transmission of the parasite in the laboratory or its cultivation *in vitro*.

The inner granular layer appears to be part of a spongy germinal matrix which originally filled the young cyst presumably derived from the cytoplasm and organelles of the original zoite. This undifferentiated matrix gradually decreases in volume as it is reorganized into the developing zoites. Eventually all that is left of it is a thin layer of unequal thickness around the periphery of the cyst and long fine branching remnant strands between aggregations of zoites. These strands are the septa. In other work in preparation (W. A. Tadros) on the ultrastructure of *Sarcocystis* in voles this has been found to be the method of formation of septa in the cysts of this Sarcosporidian. We suggest that soluble materials diffusing into the granular layer of the cyst wall may also diffuse into the deeper regions of the cyst along these septa and thus reach the zoites in most regions of the cyst.

Under the light microscope, mature cysts of *F. glareoli* often exhibit a number of empty compartments bound by septa in the centre of the cyst. Enemar (1963) has suggested that these compartments are used for storing waste products which may prove harmful to the zoites. However, it seems unlikely that these fine strands, shown here to be unlimited by any membrane, are capable of selective permeability, or of retaining soluble materials against a concentration gradient. An alternative explanation for these empty compartments is that the zoites in the centre of the cyst degenerate,

being too far away from the cyst surface to receive sufficient nutrition, or to dispose of their waste products. On the other hand it may be that the original matrix becomes used up before any central zoites are formed thus leaving a central fluid reservoir.

Like *Sarcocystis* the zoites of *Frenkelia* are of two morphological types. Levine (1961) referred to the elongate and rounded forms of *Sarcocystis* as trophozoites and trophoblasts respectively. The term trophozoite has been defined by the W. H. O. in a recent report on *Toxoplasma* (1969) as: "The individual which develops inside the pseudocyst as a result of endodyogeny". The term is therefore not valid for describing zoites within the true cysts of *Frenkelia*. Senaud (1967) referred to the elongate zoite of *Sarcocystis* as "Endodyocyte" and to the rounded form as "Heterometrocyte". These terms are slightly cumbersome but are adequate until the revision of the terminology of zoites in the light of the coccidian affinity of the Toxoplasmea. The fine structure of the elongate forms of *Frenkelia* is almost indistinguishable from *Sarcocystis* and strongly reminiscent of that of *Toxoplasma*. The possession of a conoid, polar rings, cytostome and paired organelles or rhoptries is strong evidence for placing *Frenkelia*, as Ludvík proposed in 1963, amongst the Sporozoa, even though the life cycle is not yet known. The conoid and paired organelles are generally regarded as organelles of penetration. The possession of these by zoites of *Frenkelia* suggests that at some stage in the life cycle, the elongate forms actively penetrate cell membranes. The parasite perhaps has a coccidia-like developmental cycle in the alimentary canal of a carnivore similar to that recently described for *Toxoplasma* in the gut of the domestic cat (Work and Hutchison 1969; Hutchison et al. 1970; Overdulve 1970). The spiral structure of the conoid is similar to that described by Sheffield (1966) for *Besnoitia jellisoni*, by Sheffield and Melton (1968) for *Toxoplasma gondii* and by Ryley (1969) for the sporozoite of *Eimeria tenella*. Sheffield points out that the conoid may change its shape rather like a spring. Our own observations on *Frenkelia* support this view and indicate that the spirally wound element is tubular in nature and may assist the penetration of membranes by acting like an engineer's twist drill.

Garnham et al. 1960 suggest that the subpellicular fibrils of the sporozoite of malarial parasites are contractile and capable of producing feeble motility. The wave in the pellicle seen in some of the zoites (Fig. 7) suggests that these fibrils may play a similar role in *Frenkelia*. Further, their co-ordinated contraction might depress the level of the polar rings, to which they are attached, allowing the conoid to protrude, thus assisting its suggested function of boring through membranes.

The tightly packed parallel structures found in the anterior part of the zoite are remarkably similar to the sarconemes of *Sarcocystis* (Ludvík 1958a, 1960, 1963), the toxonemes in *Toxoplasma* (Garnham, Baker and Bird 1962a), the lankesterellonemes in *Lankesterella* (Garnham et al. 1963) and the "cytoplasmastrange" of the first generation merozoite of *E. perforans* and *E. steidae* (Scholtyseck and Pierkarski 1965). We refer to these structures in *Frenkelia* as micronemes following Senaud (1967) and Levine (1961). Ryley (1969) has suggested that the micronemes of the sporozoite of *E. tenella* are bulbous terminations of fine tubules, rather than long convoluted tubules cut at different levels, to give this regular pattern. Some of our sections clearly demonstrate the hollow tubular nature of the micronemes of *Frenkelia*. This may have been due to their being empty when they were fixed. It is suggested that the large number of apparently empty vacuoles found regularly arranged just beneath the pellicle in the posterior two-thirds of the mature zoite may represent the location of metabolites which have passed through or are awaiting passage out of the pellicle. Very similar vacuoles are seen in *Sarcocystis* zoites.

Although little is known of the life cycle of *Frenkelia*, we envisage the reproduction in the brain of the rodent to be as follows: Differentiated and specialized

organelles assemble within the germinal matrix; these are best seen at the periphery of the larger cysts in the inner cyst wall layer. Further development occurs and the cells, finally enveloped by a double membrane take up an independent existence as elongate or rounded cells. These rounded cells either undergo endodyogeny to produce mature parasites or develop into elongated mature zoites. These latter also undergo endodyogeny to produce more mature elongated zoites.

The present studies confirm the close relationship of *Frenkelia* to *Toxoplasma* and more specially to *Sarcocystis*. This striking morphological similarity leads us to predict that *Frenkelia* will be shown to have a coccidian life cycle similar to that recently described for *Toxoplasma*.

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## ТОНКАЯ СТРУКТУРА ЦИСТ *FRENKELIA* (М-ОРГАНИЗМ)

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**Резюме.** Цисты *Frenkelia microti* и *F. glareoli* из мозга полевок *Microtus agrestis* и *Clethrionomys glareolus* изучались с применением электронного микроскопа. В работе излагается связь между тонкими структурами *Frenkelia* на одной и *Toxoplasma* и *Sarcocystis* на другой стороне.

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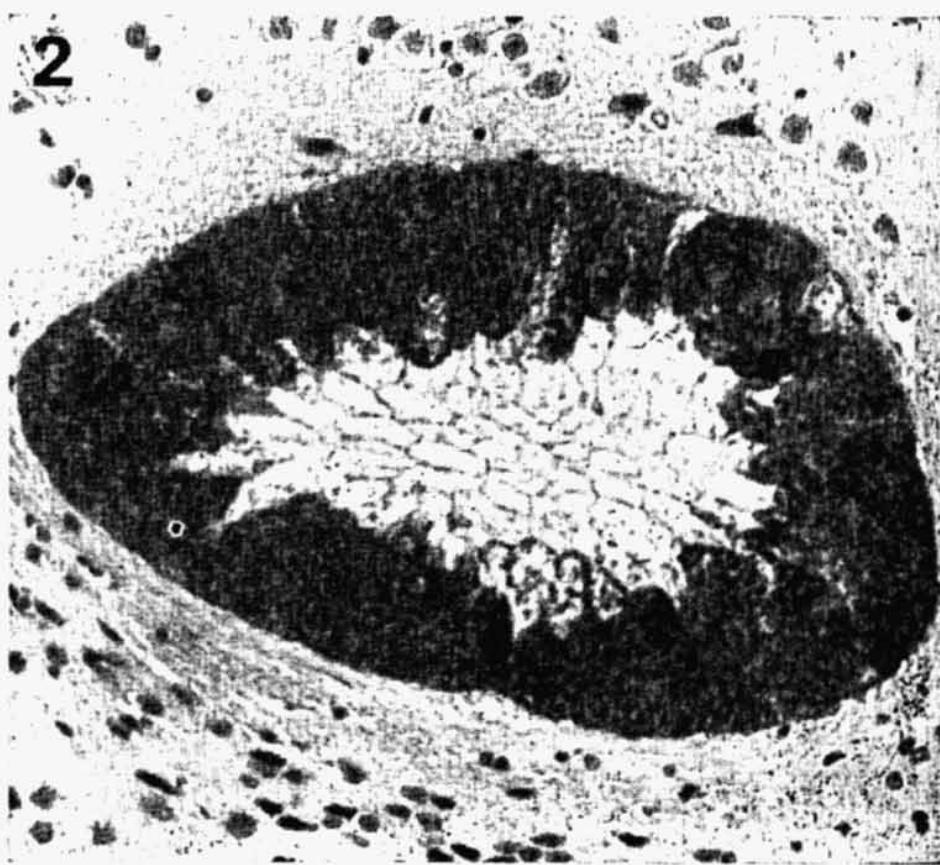
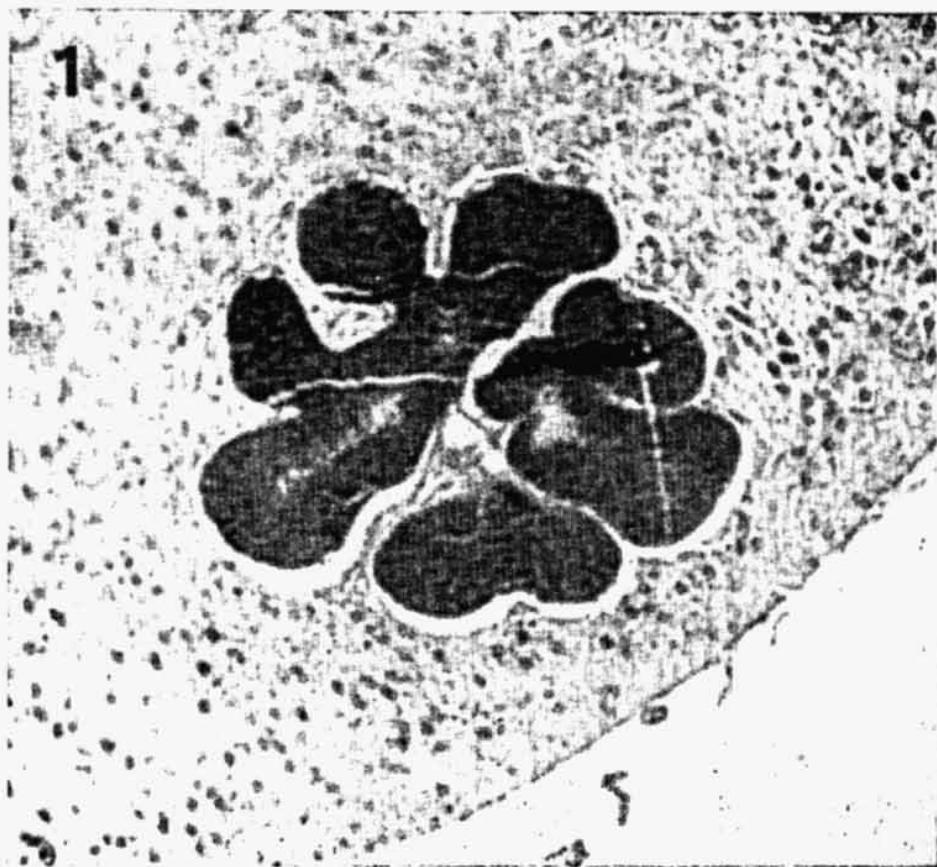
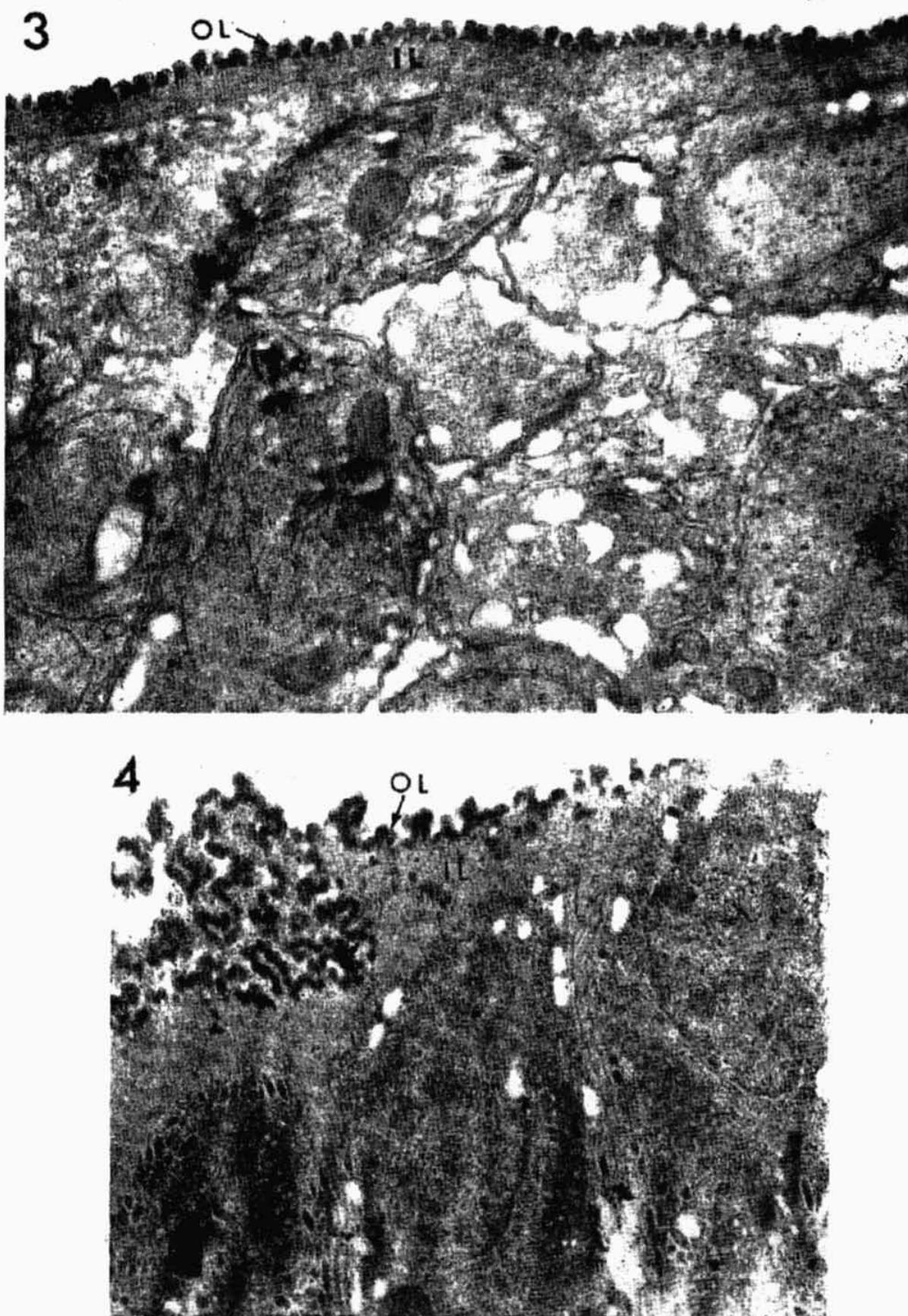


Plate I

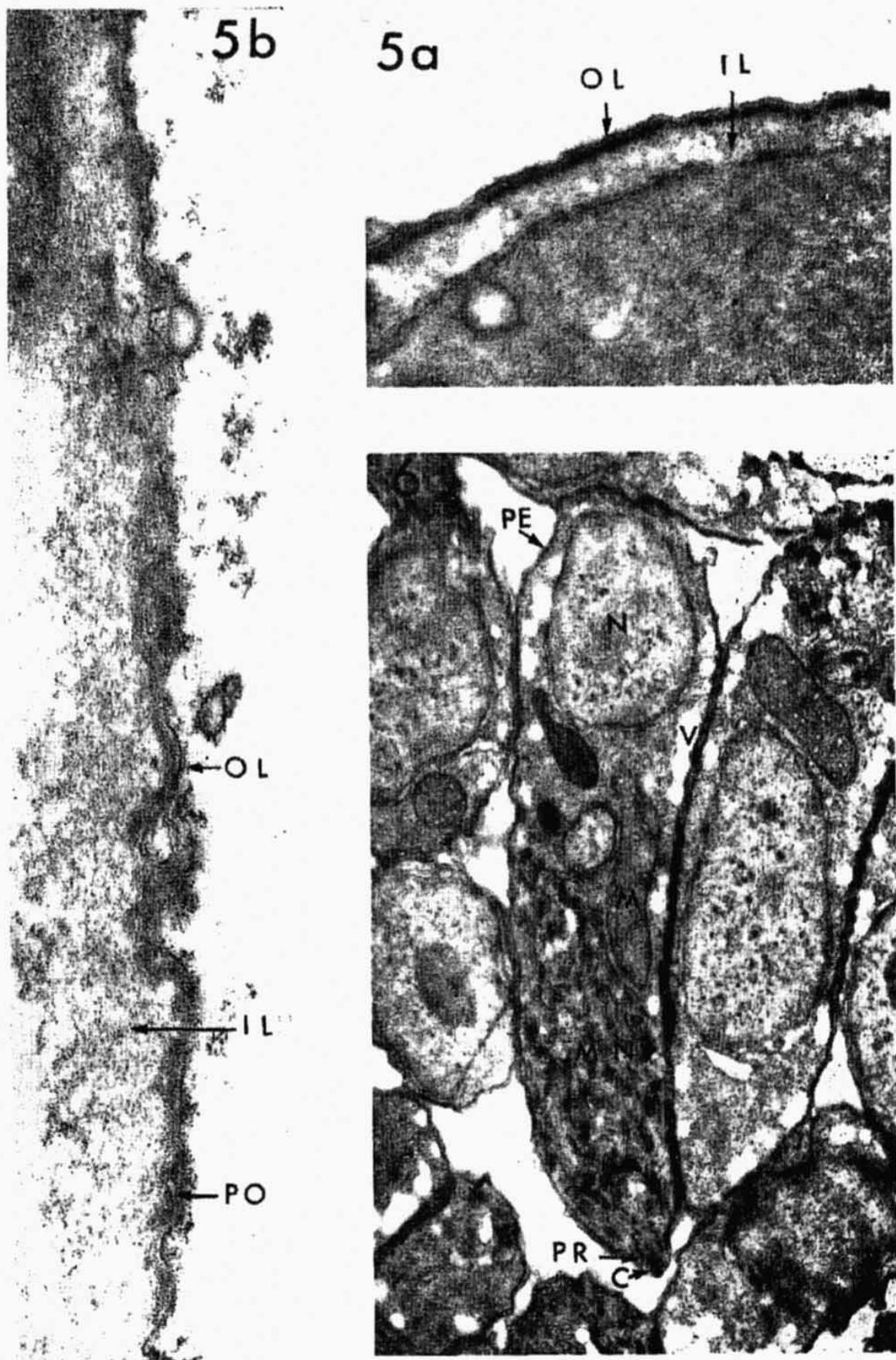
Fig. 1. Diagram of lobulated cyst of *Frenkelia microti*.

Fig. 2. Diagram of the rounded cyst of *Frenkelia glareoli*.

**Plate II**

**Fig. 3.** Cyst wall of *Frenkelia* from brain of *Microtus agrestis*. OL — outer layer, IL — inner layer. Fixed with osmium tetroxide ( $\times 30,000$ ).

**Fig. 4.** Cyst wall of *Frenkelia* from brain of *Clethrionomys glareolus* showing marked convolutions in the outer layer. OL — outer layer, IL — inner layer. Fixed with glutaraldehyde and osmium tetroxide ( $\times 30,000$ ).

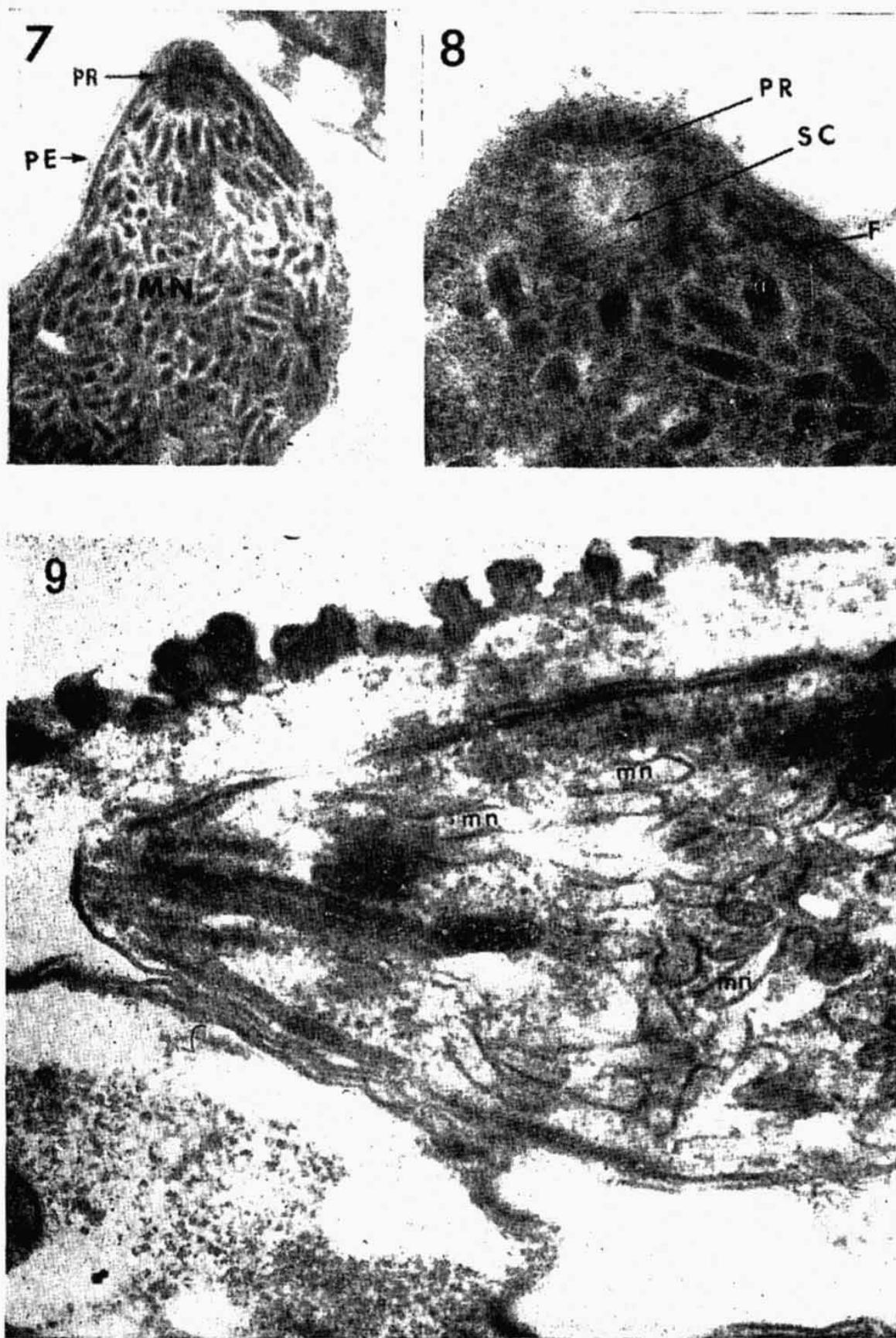


## Plate III

Fig. 5a. Higher magnification of the outer layer of the cyst wall of *Frenkelia glareoli* showing that it consists of two unit membranes. OL — outer layer, IL — inner layer. Fixed with potassium permanganate ( $\times 50,000$ ).

Fig. 5b. Cyst wall of *Frenkelia glareoli* showing possible pores PO in the outer wall. Fixed with potassium permanganate ( $\times 52,000$ ).

Fig. 6. Mature zoite of *Frenkelia glareoli*. PE — pellicle, C — conoid; PR — polar ring, MN — micro-nemes, M — mitochondrion, N — nucleus, V — regularly arranged vacuoles under the pellicle. Fixed with osmium tetroxide ( $\times 24,000$ ).

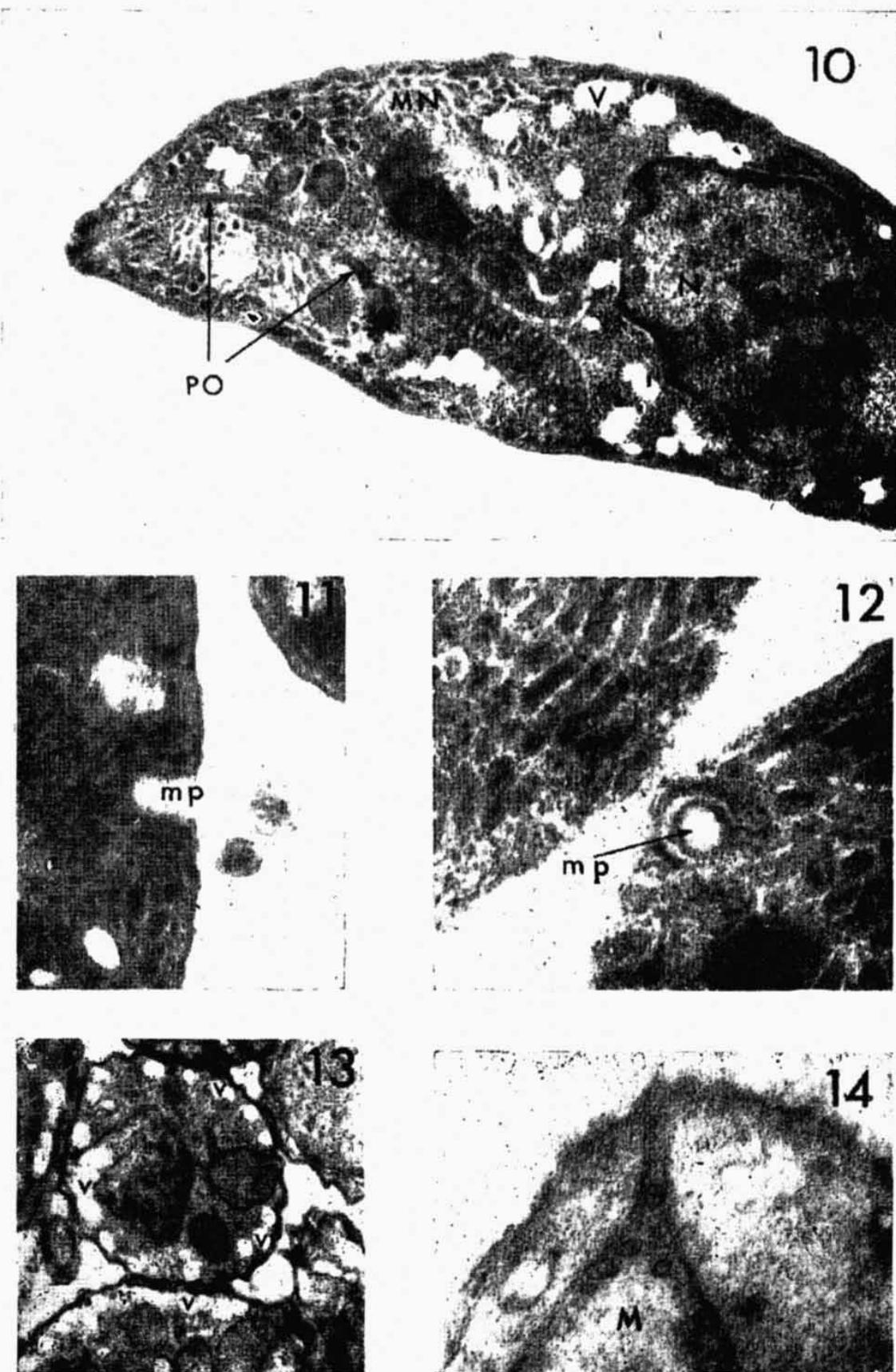


## Plate IV

Fig. 7. Elongate zoite of *Frenkelia glareoli* showing conical anterior end. PR — polar rings, PE — pellicle, MN — micronemes.

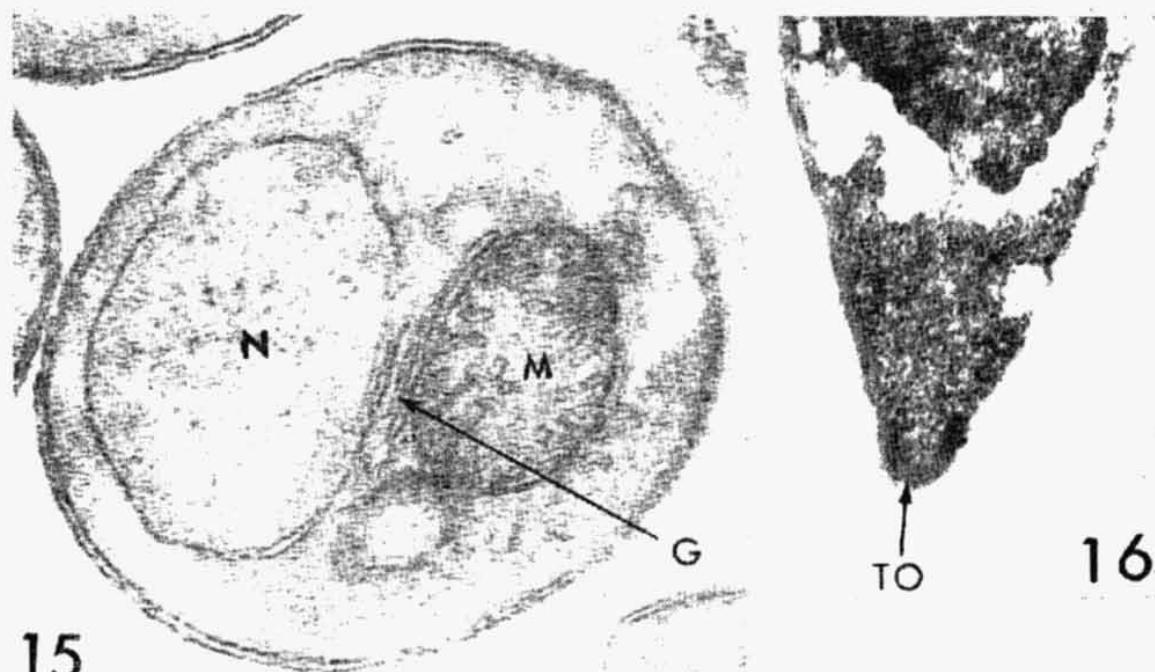
Fig. 8. Anterior end of elongate zoite of *Frenkelia* showing SC — spiral coil of the conoid, PR — polar rings, F — subpellicular fibrils. Fixed with glutaraldehyde and osmium tetroxide ( $\times 50,000$ ).

Fig. 9. Longitudinal section of the anterior end of an elongate zoite of *Frenkelia*. mn — micronemes. Fixed in glutaraldehyde and osmium tetroxide ( $\times 40,000$ ).



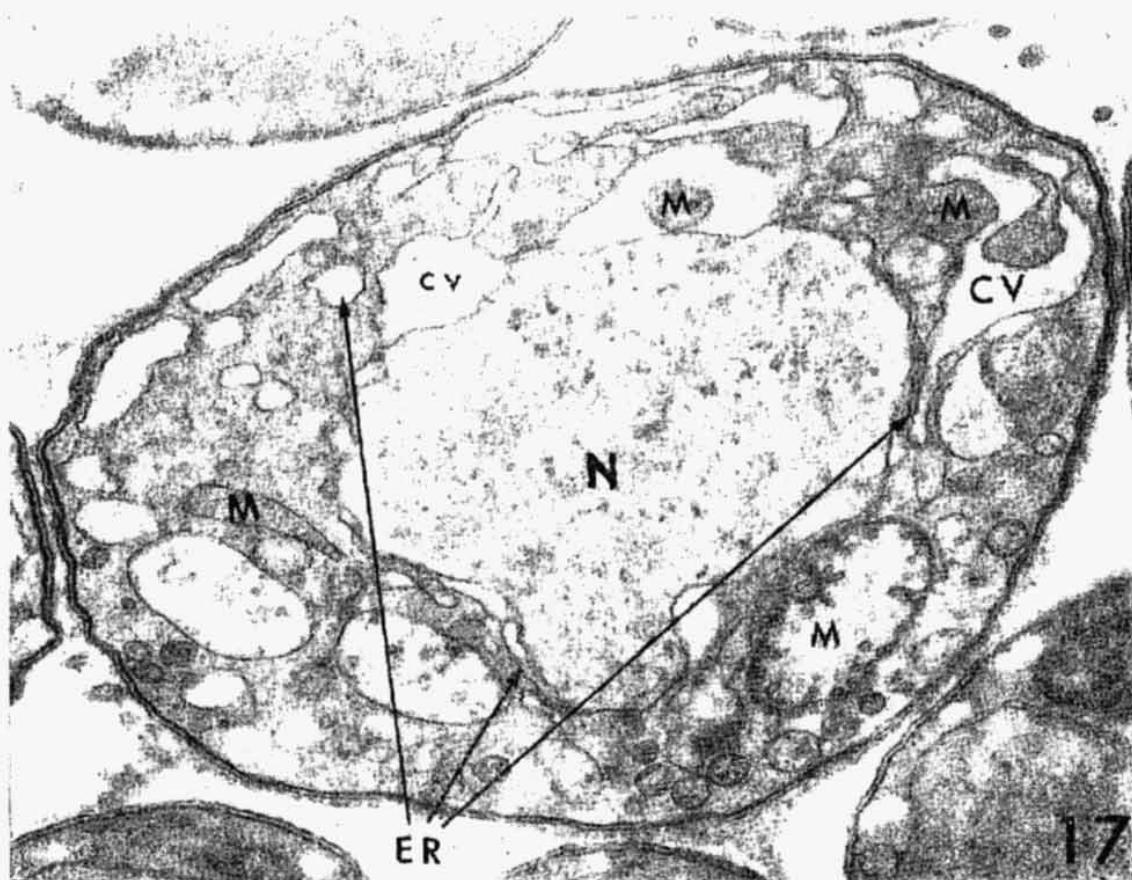
## Plate V

**Fig. 10.** Longitudinal section of anterior end of mature zoite. MN — mieronemes, appearing as empty tubules with limiting unit membranes. V — vacuole, PO — paired organelles, N — nucleus. Fixed with osmium tetroxide ( $\times 100,000$ ). **Fig. 11.** Longitudinal section through the cytostome, mp. Fixed with glutaraldehyde and osmium tetroxide ( $\times 72,000$ ). **Fig. 12.** Cross section through the cytostome mp which appears as two concentric rings. Fixed with glutaraldehyde and osmium tetroxide ( $\times 72,000$ ). **Fig. 13.** Cross section through a mature zoite showing the regular arrangement of the subpellicular vacuoles V. Fixed with osmium tetroxide ( $\times 24,000$ ). **Fig. 14.** Curious apparent connection between mitochondrion, M and pelicle of mature zoite. Fixed in potassium permanganate ( $\times 54,000$ ).



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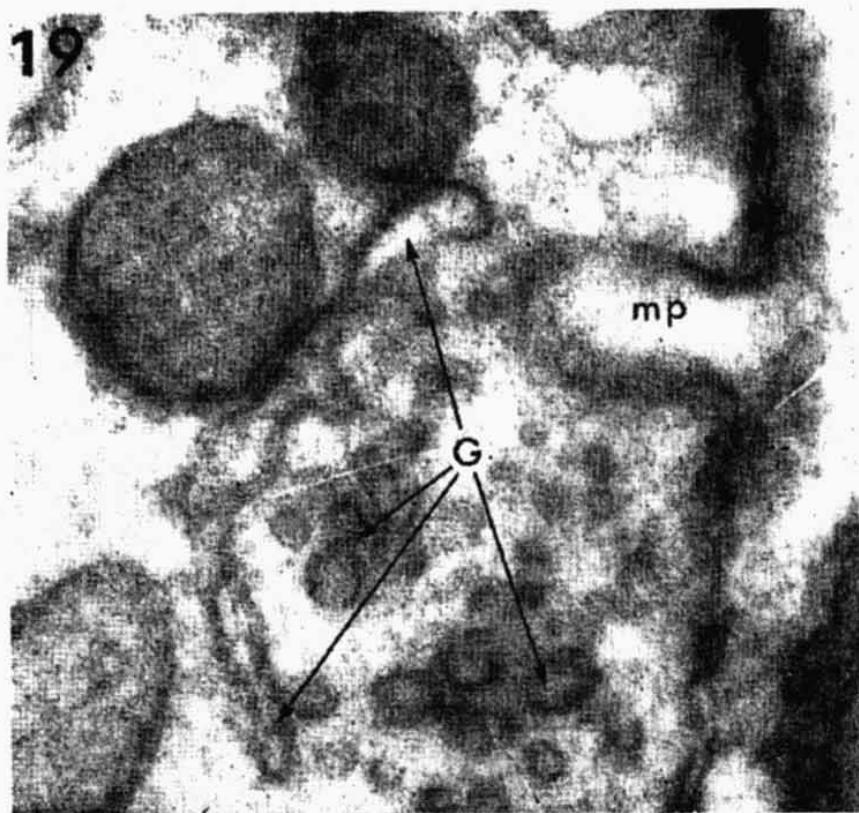
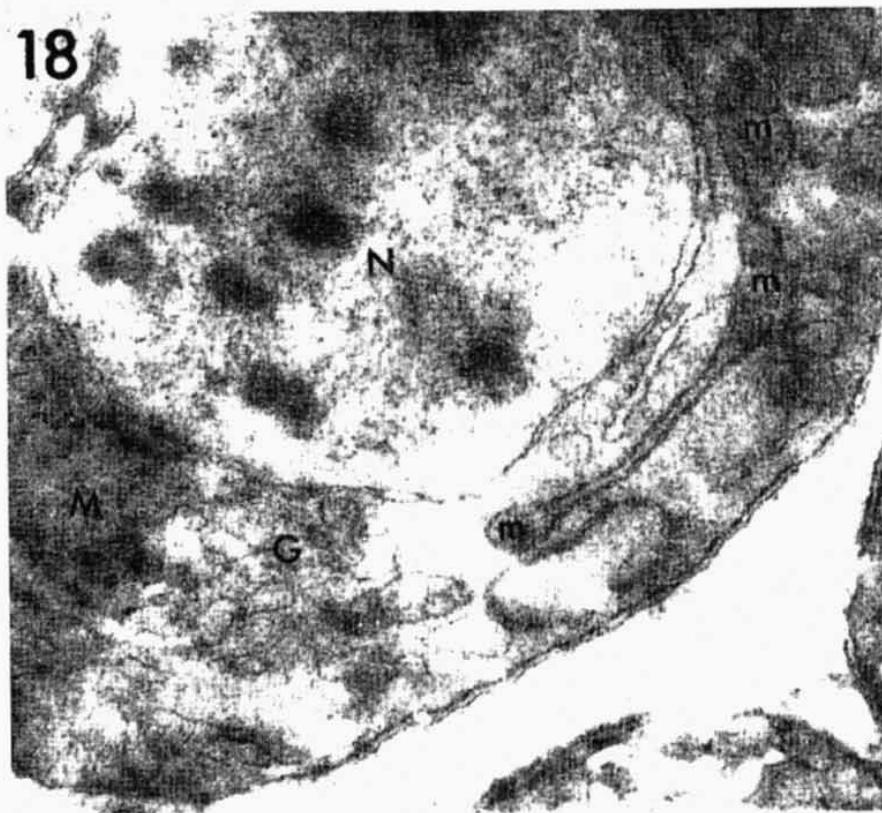
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**Plate VI**

**Fig. 15.** Mature zoite showing the Golgi membranes G. N — nucleus, M — mitochondria. Fixed with potassium permanganate ( $\times 48,000$ ).

**Fig. 16.** Longitudinal section of the posterior end of a mature zoite showing a nipple-like pellicular thickening forming a "tail" organelle — TO. Fixed with glutaraldehyde and osmium tetroxide ( $\times 60,000$ ).

**Fig. 17.** Section of immature rounded zoite. M — mitochondria, ER — endoplasmic reticulum, N — nucleus, CV — large communicating vacuoles. Fixed with potassium permanganate ( $\times 26,000$ ).



## Plate VII

**Fig. 18.** Immature zoite of *Frenkelia*. M — extensive mitochondrial membranes, G — Golgi membranes, N — nucleus. Fixed in potassium permanganate ( $\times 40,000$ ).

**Fig. 19.** Portion of pellicle of rounded zoite. G — Golgi apparatus. Fixed in glutaraldehyde and osmium tetroxide ( $\times 80,000$ ).

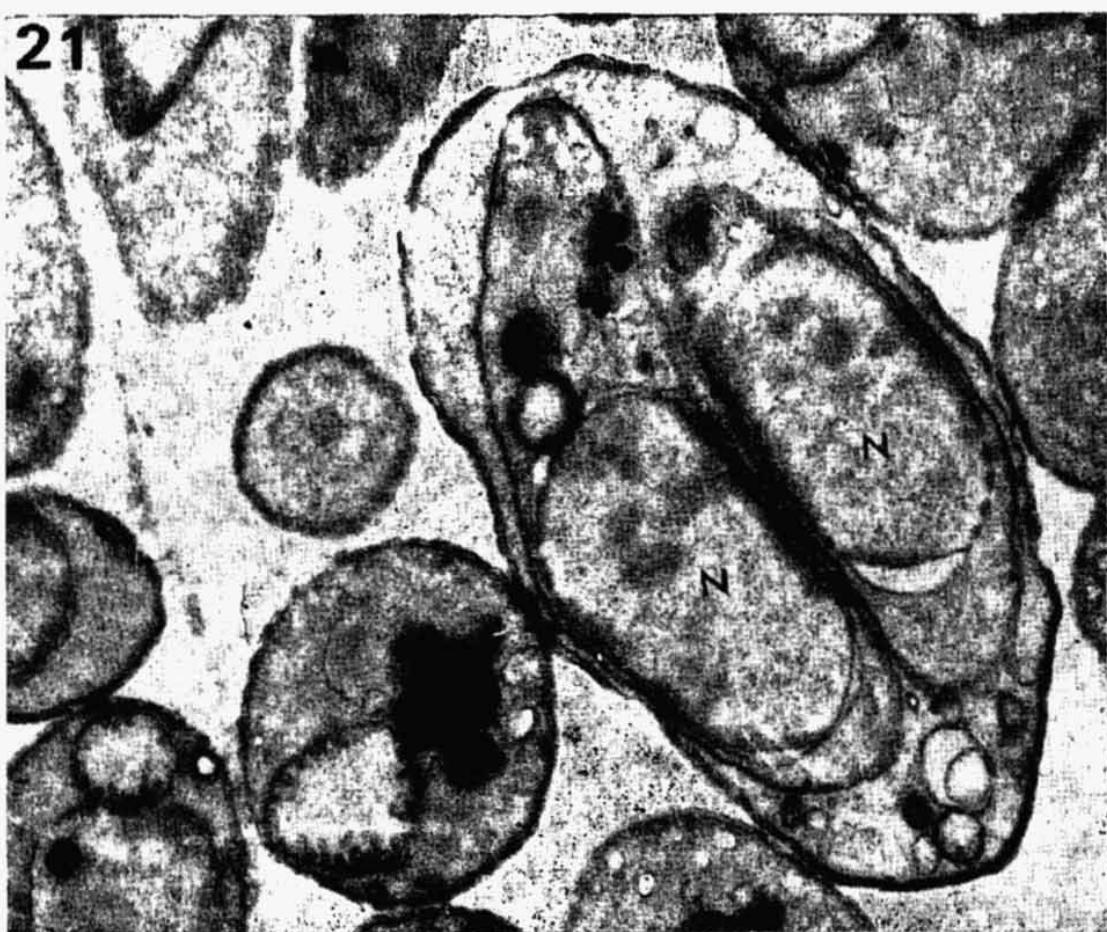
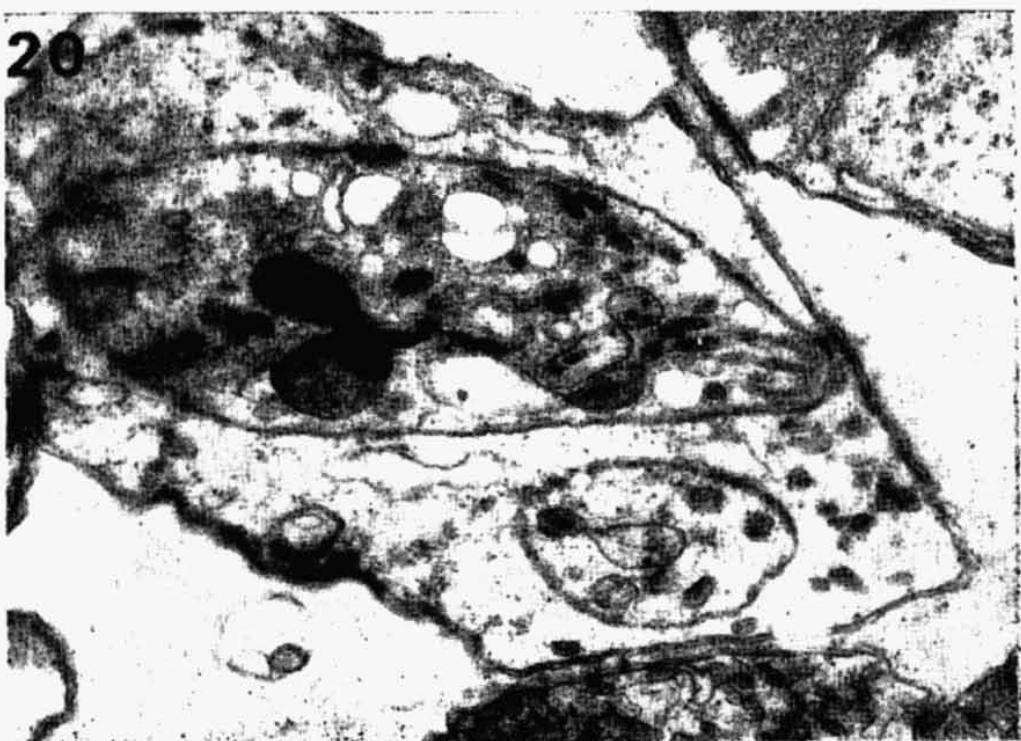


Plate VIII

Fig. 20. Reproduction of *Frenkelia* by endodyogeny. Fixed with osmium tetroxide. ( $\times 40,000$ ).  
Fig. 21. *Frenkelia* zoite undergoing endodyogeny. N — nucleus. Fixed with glutaraldehyde and osmium tetroxide ( $\times 22,000$ ).