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Ultramicroscopic studies of the localization of adenosine triphosphate activity and H^3-glucose transport in *Toxoplasma gondii*

SHINKICHI AKAO

Department of Parasitology, School of Medicine, Keio University, Tokyo

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By the electronmicroscopic observations of *Toxoplasma gondii*, a number of organelles have been found by many investigators. It has nucleus, mitochondoria, Golgi complex and endoplasmic reticulum as is the case in other animal cells. Besides these, it has a polar ring at the acute end of the cell which is, according to Ogino & Yoneda (1966), composed of thickenings of outer and inner cell membranes and also of submembraneous fibrils. These fibrils, originating from the polar ring, diverge radially and extend longitudinally under the inner cell membrane. Just behind the polar ring there is an organelle, named conoid by Gustafson *et al.* (1954), in shape of a truncated cone which is, according to Ogino & Yoneda (1966), made of a compressed or tight spiral exhibiting an obliquely striped pattern. From inside the conoid, extend backwards a number of club-like organelles, the toxonemes. As the toxoneme or similar organelles have been found in *Sarcocystis*, *Besnoita*, *Lankesterella* and even in *Eimeria* and *Plasmodia*, Jacobs (1967) proposed a common name "microneme" in stead of toxoneme. In this paper, however, old and more popular name will be used. The purpose of the present study is to gain some clues to physiological functions of these peculiar organelles in connection with the metabolic activities of the parasite.

Materials and Methods

Electronmicroscopic studies on ATPase activity in animal cells have been reported by many investigators such as Nelson (1958), Tice & Barrnett (1962), Ashworth *et al.* (1963), Nagano (1965), Yasuzumi & Tsubo (1966) and Gillis & Page (1967). Methods applied in this study was essentially the same as those of previous authors. *Toxoplasma* cells were collected from 3 mice which had been infected with RH strain before 3 days. They were washed by centrifugation with acetate buffer (100 mM CH_3COONa, 4 mM $CaCl_2$, 4 mM $MgCl_2$) and prefixed with cold acetone, or glutaraldehyde (2% or 5%). Specimens without prefixation were also examined. They were then soaked into ATP medium (100 mM CH_3COONa, 4 mM $MgCl_2$, 4 mM $CaCl_2$: 1 mM Pb $(NO_3)_2$, 2 mM ATP) for 15 to 30 minutes at 36°C for sensitization and was washed two times by centrifugation with acetate buffer. Subsequently, in order to change invisible lead phosphate produced by the reaction to visible lead sulphate, the specimens were rinsed in sulfonic solution for 5 to 10 minutes. In the control experiment, the specimens were put into a solution of sodium fluoride (5%) or mersalyl (2.5 mM) in acetate buffer, the inhibitor of ATPase, before they were sensitized.

The sensitized materials were collected by

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centrifugation, fixed in 1% osmic acid solution, dehydrated by graded alcohol and embedded in stylene or epoxy resin. Ultrathinsections were made by Porter Blum Microtome, stained by saturated uranium solution and examined by Hitachi electron microscope Type HU-11 B, at 75 KV. Pictures were taken at 10,000–20,000 magnification and enlarged 2–4 times for publication.

Glucose transport in the *Toxoplasma* cell was studied by ultramicroautoradiography. D-glucose-6-H^3 solution having the specific radioactivity of 1.0 mC per 1.0 ml was diluted with distilled water before use and 0.5 ml amount having 100 μC was injected into the peritoneal cavity of mice which had been inoculated with RH strain 3 days ago.

The solution was pumped in and out several times to be well mixed with peritoneal fluid and kept in the peritoneal cavity for 30 minutes in one group and 60 minutes in another for sensitization. The peritoneal fluid was then drawn and centrifuged. The sediment was washed with phosphate buffer solution by centrifugation to remove free H^3-glucose. Ultrathinsections were made from these materials by the method stated above.

In vitro test, *Toxoplasma* cells were pooled from 5 mice which had been infected with RH strain 3 days before. They were washed with phosphate buffered saline pH 7.2 (P.B.S.) and suspended in the H^3-glucose medium stated above and kept at 37°C for 30 minutes or 60 minutes. They were then washed again with P.B.S. Methods of getting ultrathinsections from these materials were the same as those already described.

Ultrathinsections of in vivo and in vitro materials were mounted on sheet-mesh which had been carbon coated on both sides. They were then covered with nuclear track emulsion NR-H II (Konishi Roku Co. Ltd., Japan). In this procedure, the original emulsion was diluted ten times with distilled water, warmed in 40°C water bath and put on the ultrathinsections by platine wire loop method. After the emulsion was dried: ultrathinsections were put in a exposure box in which they were kept at 4°C for 3 weeks. All these procedures with the emulsion were carried out in the dark room under the safety red lamp. After the exposure, specimens were developed in either of the following two media.

1) Schwarz' formula

Monol	0.25 mg
Sodium sulphite	2.50
Hydroquinone	0.13
Sodium carbonate	1.50
Sodium thiosulphate	0.05
Aq. dest.	100.00 ml

2) Kikuchi's formula

A: Hydroquinone	1.0 gm
Citric acid	0.6
Aq. dest.	100.00 ml
B: 4.0% Silver nitrate	30.00 ml

A and B solutions are mixed before use.

The ultrathinsections developed in these media were kept in either of the following two media for 15–30 minutes to remove gelatin in the emulsion and also for the staining of specimens.

1) Mizuhira and Kurotaki's formula

Sodium hydroxide	2.0 g
Potasium sodium tartrate	1.6
Lead monoxide	saturate
Water	100 ml

The solution is diluted 20–100 times with water before use.

2) Akao's formula

Sodium hydroxide	2.0 g
Trypsin	1.0
Lead acetate	saturate
Water	100 ml

The solution is filtered through a filter paper and diluted 5–10 times with water.

The specimens were washed with water after the treatment and dried and were ready for examination.

In this kind of experiment, it is absolutely necessary to check the background fog in the nuclear track emulsion. For this purpose materials which were not sensitized with H^3-glucose were treated with exactly the same way as stated above. No background fog was recognized. The silver grains in the emulsion were also checked beforehand :

the emulsion membrane on the platine loop was mounted on the sheet-mesh, exposed to artificial light and developed to be examined by the electron microscope. The picture is shown in Fig. 1. The silver grains proved quite satisfactory, being uniform in size and distribution.

Results

1) Localization of ATPase activity

The localization of ATPase activity was indicated by the deposit of lead sulphate grains. ATPase activity was found most conspicuosly on cell membrane. Mitochondoria, toxoneme, Golgi complex, cytoplasmic vacuoles and so-called dense body were found to be positive for the reaction. Prefixation of the material with cold 5% glutaraldehyde gave the best results as compared with the treatment with 2% glutaraldehyde or cold acetone. Specimens without prefixation gave no positive reaction.

The surface of *Toxoplasma* body is covered with two membranes except at the polar ring where the inner membrane is lacking (Fig. 2). The lead grains were deposited in the layer between these two membranes throughout the whole body surface (Fig. 3). In places they were arranged in two rows adjacent to the two membranes (Fig. 4). Conoid, submembranous fibrils (arrow) were devoid of lead grains (Fig. 5). In mitochondoria, they were deposited on cristae which were finely branched and gave an alveolated appearance (Fig. 6). Golgi complex also exhibited positive reaction (Fig. 7).

Toxonemes have a distinct mesh-like configuration in the posterior enlarged portion while the anterior slender portion is almost homogeneous when prefixed with glutaraldehyde, as indicated by Ogino & Yoneda (1966) and Zypen & Piekarsky (1967). The lead grains were deposited only in the mesh-like configuration and not in the homogeneous portion (Figs. 3, 8, and 9). No grains were found on the surface of toxonemes.

Toxoplasma has osmophilic almost spherical bodies in its cytoplasm which may be called "dense body." Sometimes, a few lead grains were seen around these bodies (Fig. 4, arrow). In some cases, the reaction was positive on the nuclear membrane (Fig. 5). Vacuoles in the cytoplasm also had the grains along their limiting membrane (Fig. 9).

In control specimens in which the reaction was inhibited by fluoride or by mersalyl, the reaction was completely negative. In specimens which were not sensitized with ATP, the reaction was negative, too (Fig. 2).

2) The transport of H^3-glucose in *Toxoplasma*

The localization of H^3-glucose in *Toxoplasma* was indicated by the presence of reduced silver grains exposed to the radioactivity. In specimens taken from the mouse peritoneal cavity after 30 minutes sensitization, silver grains were found mostly on the cell membrane and in the peripheral cytoplasm (Fig. 10), and after 60 minutes of sensitization they were found in more central part of cytoplasm (Fig. 11), on the surface toxonemes (Fig. 12), membrane of Golgi complex (Fig. 13), and inside the cytoplasmic vacuoles (Fig. 14).

In specimens sensitized for 30 minutes in vitro, silver grains were found mostly in the peripheral cytoplasm and in specimens sensitized for 60 minutes, silver grains were accumulated in cytoplasmic vacuoles (Fig. 15). They were rarely found in Golgi complex or on toxonemes.

Whether or not *Toxoplasma* takes food by pinocytosis is a problem to be solved. In the present study, *Toxoplasma* cells were washed with P.B.S. and were kept in 1% solution of ferritin for 15 minutes at 36°C. Evidences of pinocytosis were not obtained by examinations of ultrathinsections of these materials. Control experiments carried out with *Trichomonas vaginalis* clearly demonstrated pictures of pinocytosis (Fig. 16, arrow indicates ferritin granules).

Discussion

Little is known about the physiological functions of organelles such as conoid, toxo-

nemes and submembraneous fibrils which are peculiar to *Toxoplasma* and its allied organisms. Gustafson *et al.* (1954) who were the first to describe these organelles, suggested that the conoid might be a device for penetration or intake of nutrition and toxonemes might imply secretory mechanisms associated with penetration or nutrition intake.

According to Ludvik (1963), the apex of the conoid is covered by a single layerd membrane which is sometimes invaginated inward where the narrow parts of toxonemes are situated. He suggested that the polar ring might have the function of sucker and conoid that of perforator and pinocytosis might be occuring in these parts. He also postulated that toxonemes might be secretory organelles capable of producing enzymatic substances to be discharged through the conoid.

Sheffield (1966) found a vesicle just posterior to the conoid of *Besnoitia jellisoni.* As its lumen was connected to the exterior through the conoid by a tubelike structure, he suggested that feeding could occur through this area. Micropyle was first found in *Toxoplasma* by Garnham & Bird (1962) and subsequently by Wildfuhr (1966) and Zypen & Piekarski (1967). Sheffield (1966) also found it in *Besnoitia.* Aikawa *et al.* (1966) described a similar structure in avian malarial parasites and demonstrated feeding mechanism of this structure. Sheffield (1966) also expressed the same opinion as to the function of the micropyle. Thus, *Toxoplasma* may probably take food through conoid and micropyle. To demonstrate these feeding mechanisms, the present author kept *Toxoplasma* in ferritin solution for 15 minutes at 36°C and examined their ultrathinsections by electronmicroscope. No ferritin particles were found in the cytoplasm of the parasite while *Trichomonas vaginalis* which were treated exactly in the same way exhibited pictures of active pinocytosis.

The presence of H^3-glucose and ATPase activity on and inside the cell membrane in this study suggests that the absorption of some nutrients is taking place actively through the body surface.

Capella & Kaufman (1964) studied the enzyme chemistry of *Toxoplasma* and found that glycolytic and Krebs' cycle enzymes were limited to the mitochondoria and no stain could be seen in cytoplasmic area by light microscope examinations. It would be very interesting problem to be solved whether these enzymes could be demostrated in toxonemes and other organelles by electron microscope observations.

By the autoradiography with H^3-glucose, it was suggested that this substance was absorbed from the body surface and diffused gradually into the cytoplasm. It was found in the unit membrane of Golgi complex and on the surface of toxonemes. These organelles probably utilize glucose as a material for energy source for their physiological activities.

The heavy radio-activity in the cytoplasmic vacuoles probably indicates the deposit of polysaccharides which contain H^3-glucose a sa constituent. These vacuoles probably represent PAS-positive granules which can be recognized in the cytoplasm by light microscope examination.

Summary

1) ATPase activity in *Toxoplasma* cell was most conspicuous on the cell wall which is composed of two membranes. This lead sulphate grains reaction products of ATPase activity were deposited irregularily or arranged in two rows in the layer between those two membranes.

2) In toxonemes, the ATPase activity was conspicuous in the posterior enlarged portion, the lead grains being deposited in its meshlike structure. The reaction was completely negative in the anterior slender portion which homogeneous in structure.

3) ATPase activity was recognized in Golgi complex, mitochondoria and cytoplasmic vacuoles.

4) H^3-glucose was detected by the autoradiography in the peripheral cytoplasm of *Toxoplasma* after the first 30 minutes of

sensitization. After 60 minutes it was found in Golgi complex, cytoplasmic vacuoles and on the surface of toxonemes.

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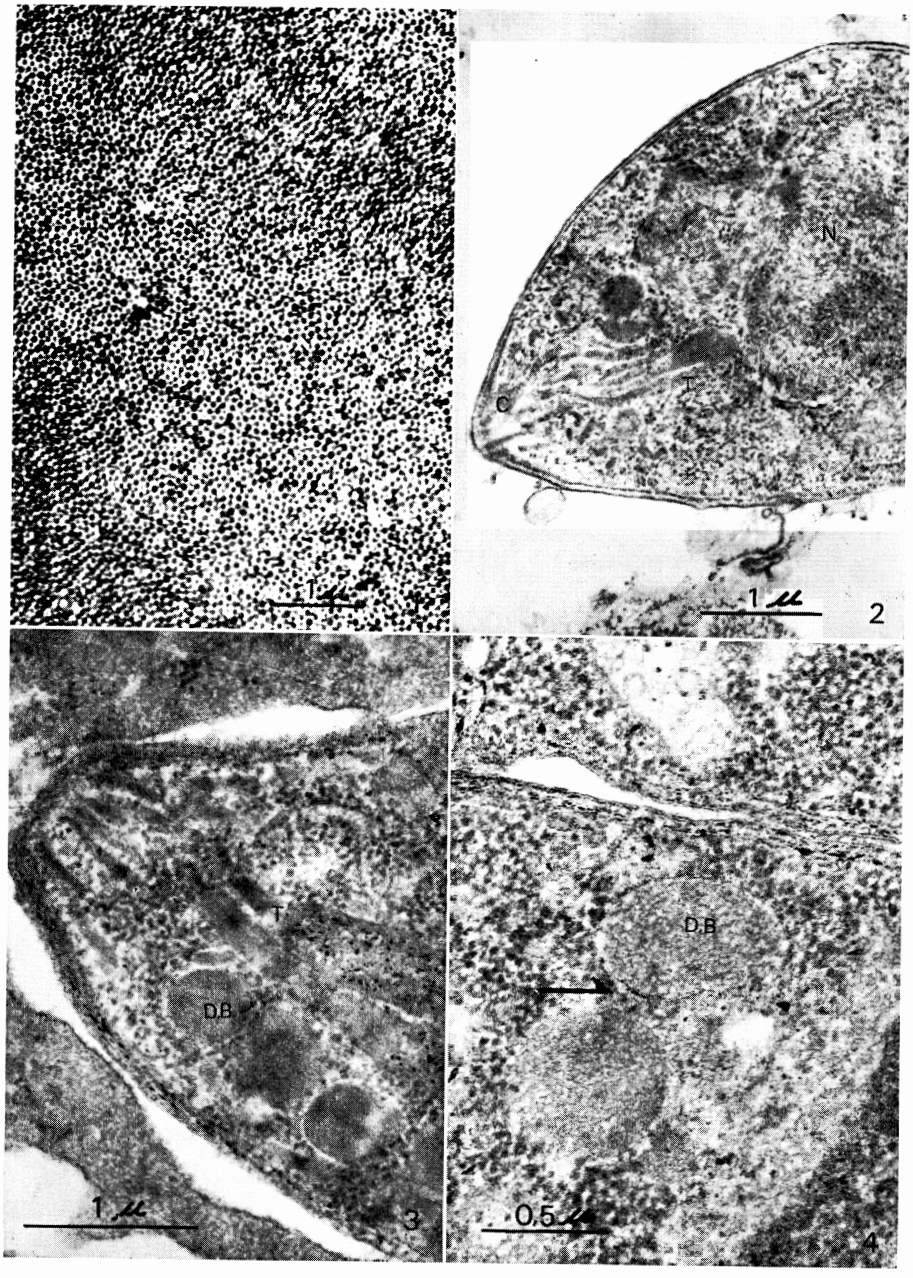
Explanation of Figures

Fig. 1. Reduced silver grains in the nuclear track emulsion which was extended on a platine wire loop and exposed to artificial light. 15,000×.

Fig. 2. A *Toxoplasma* cell of which the ATPase activity was inhibited by sodium fluoride. Lead sulphate grains are not seen. Notice that the plasma membrane at the acute end is single while the other part of the body is covered by double membranes. C: Conoid, N: Nucleus, T.: Toxoneme. 20,000×.

Fig. 3. The lead grains are deposited in the layer between two plasma membranes. They are distributedon the entire body surface. Their arrangement is not always regular. The lead grains can be seen in Toxonemes (T), too. Their distribution is limited to the mesh-like structure in the posterior enlarged portion. D.B.: Dense body. 30,000×.

Fig. 4. The lead grains are arranged in two rows in the layer between two plasma membranes. They are distributed on the outer surface of the inner membrane and inner surface of the outer membrane. Minute lead grains can be seen on the surface of so-called dense body (arrow). DB.: Dense body. 40,000×.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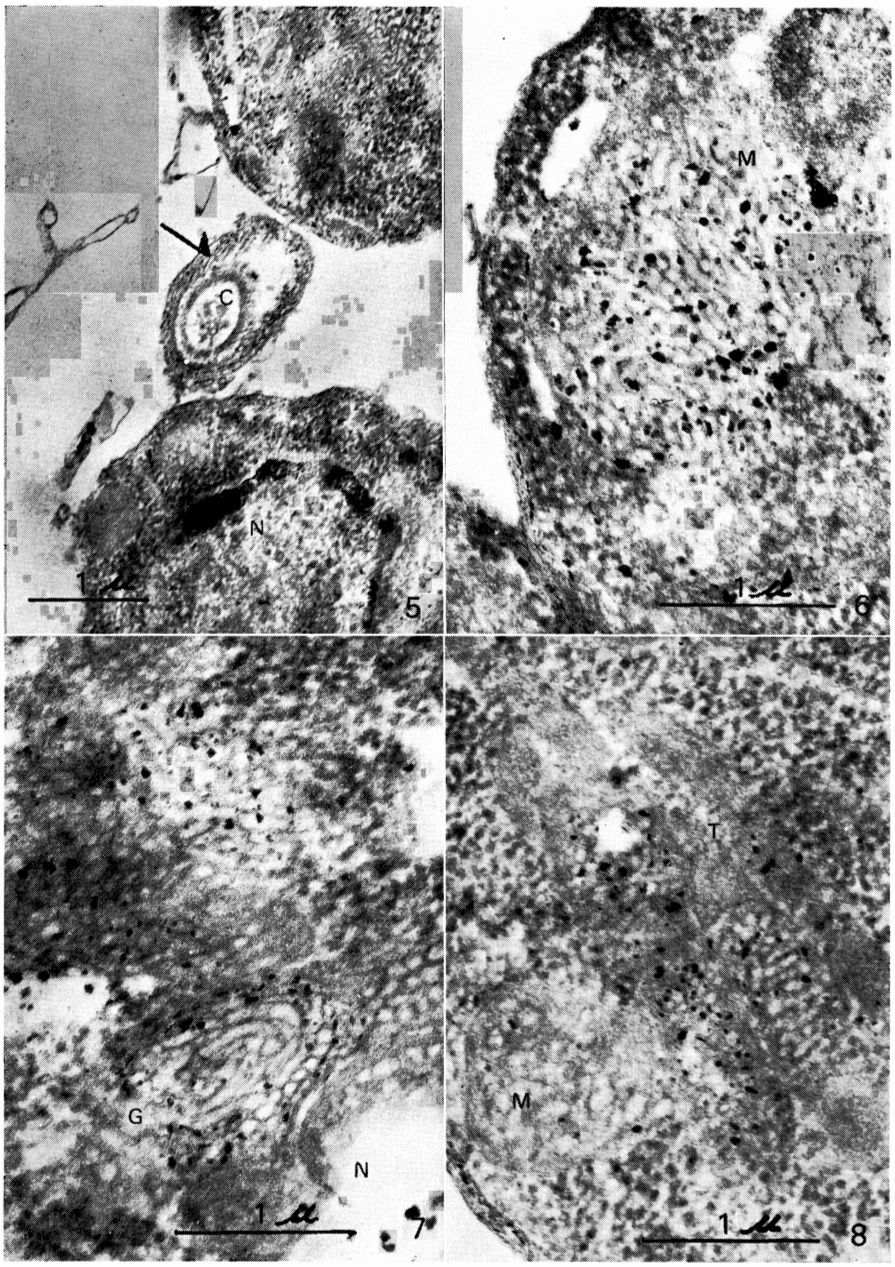

Fig. 5. Conoid (C) and submembraneous fibrils (arrow) are negative for ATPase reaction. The nuclear membrane shows dense deposite of lead in places. N: Nucleus. 20,000×

Fig. 6. A mitochondoria (M) showing finely alveolated structure of cristae. The lead grains seem to be distributed on the cristae and not in the matrix. 30,000×.

Fig. 7. A Golgi (G) complex having lead grains on its unit membranes. N: Nucleus. 30,000×.

Fig. 8. The enlarged posterior portion of the toxonemes. The lead grains are deposited in the mesh-like structure and not in the anterior homogeneous portion. M: Mitochondoria, T: Toxoneme. 30,000×.

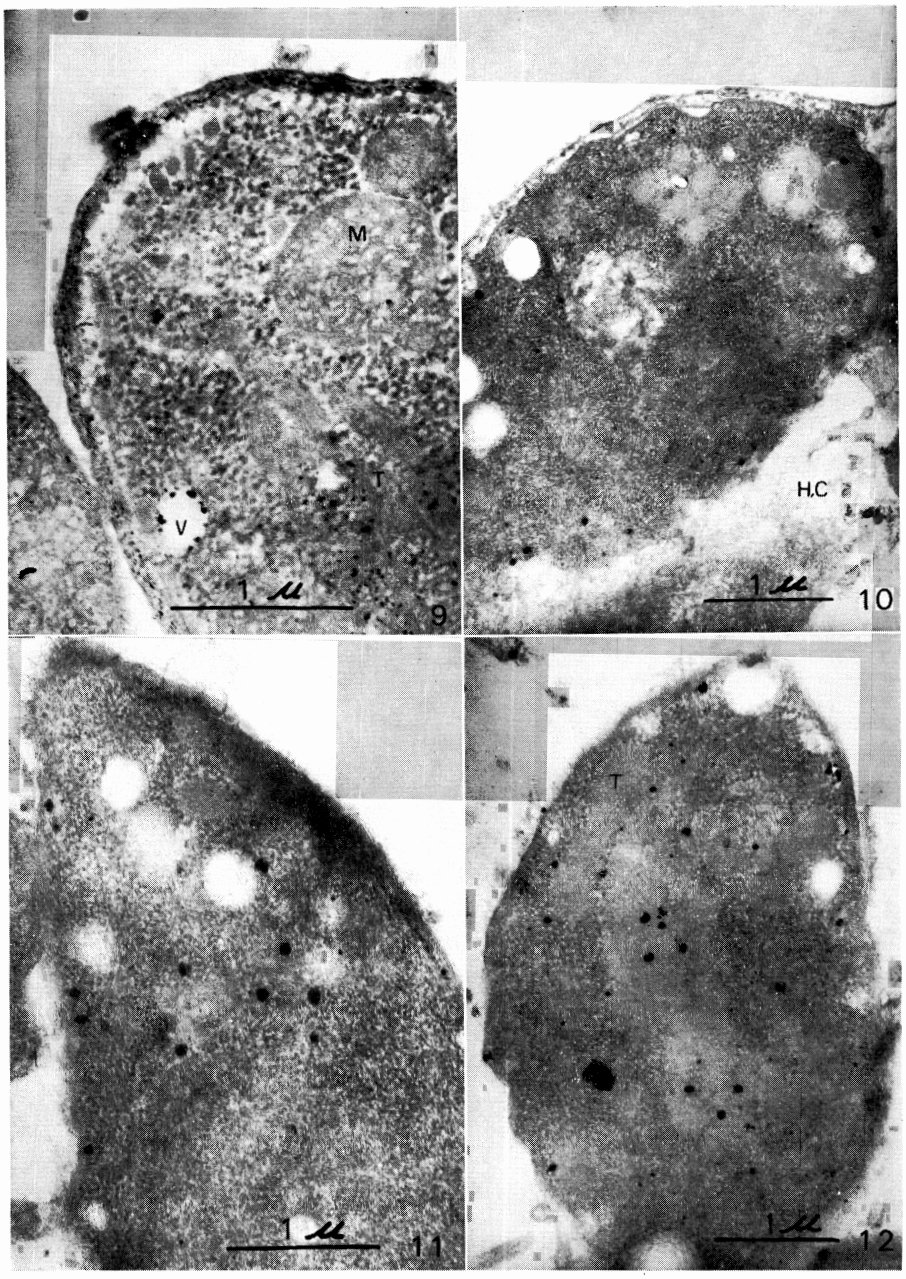

Fig. 9. A cytoplasmic vacuole (V) having the lead grains on its limiting membrane. The reaction in the cell membrane is conspicuos. The toxonemes (T) also show positive reaction in its mesh-like structure. M : Mitochondoria. 30,000×.

Fig. 10. Reduced silver grains (black dots) are distributed in the peripheral cytoplasm. (30 minutes' sensitization *in vivo*). HC : Host cell. 20,000×.

Fig. 11. Reduced silver grains are distributed in the peripheral as well as in central cytoplasm of the cell. (60 minutes' sensitization *in vivo*). 25,000×.

Fig. 12. Reduced silver grains are seen on the surface of toxonemes. (60 minutes' sensitization *in vivo*). 20,000×.

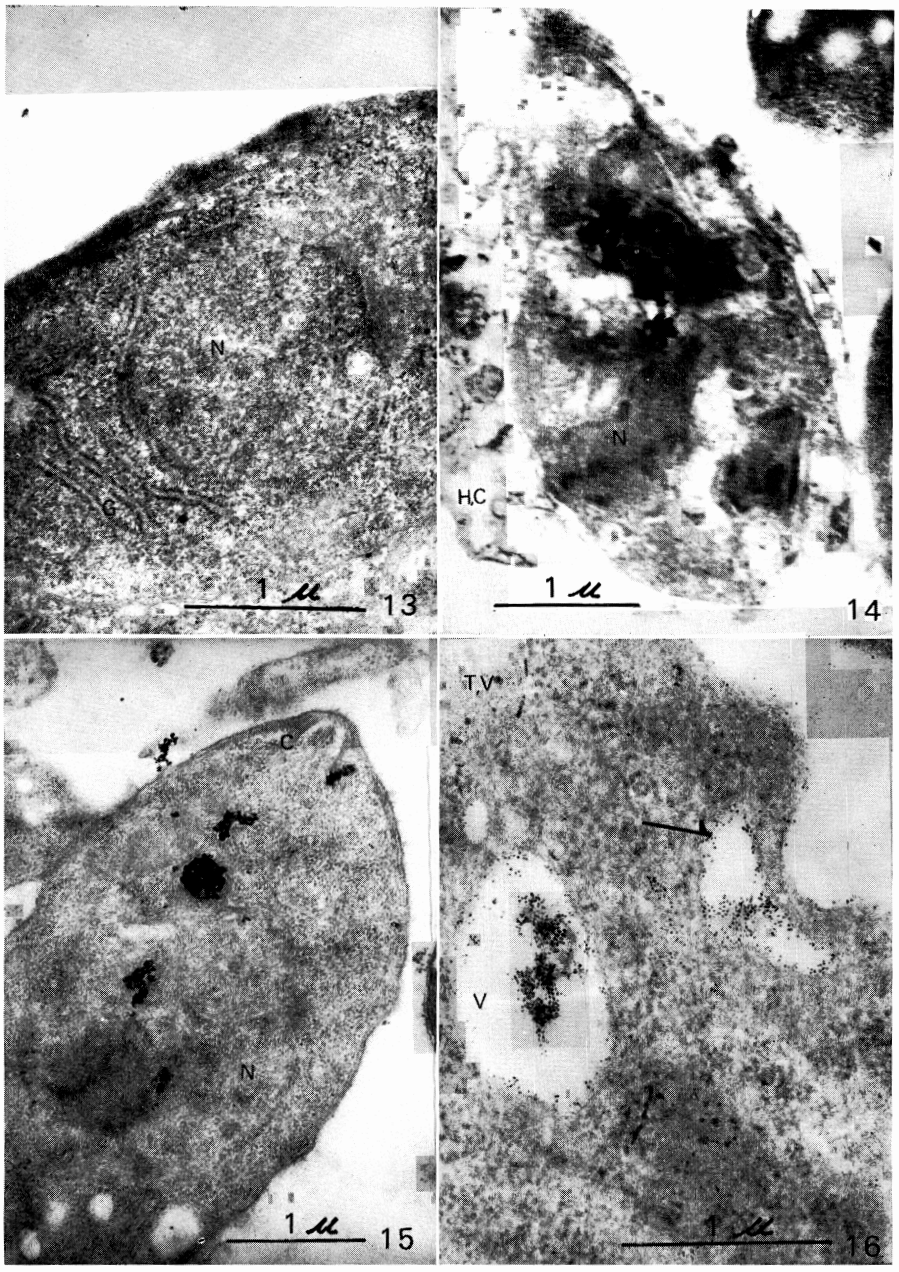

Fig. 13. Reduced silver grains on the unit membranes of Golgi complex (G). (60 minutes sensitization in vivo). N : Nucleus, 30,000×.

Fig. 14. A large cytoplasmic vacuole having a large amount of silver grains. This is probably a PAS-positive granule, often found by light microscope examination. (60 minutes sensitization in vivo). HC : Host cell, 25,000×.

Fig. 15. Silver grains are accumulated in cytoplasmic vacuoles. (60 minutes sensitization in vitro). C : Conoid, N : Nucleus

Fig. 16. Pinocytosis of *Trichomonas vaginaiis*. Ferritin particles (arrow) are being incorporated from body surface and doposited in cytoplasmic vacuoles. 35,000×.

Stamm) von *Toxoplasma gondii*. Zentbl. Barkt. I. Orig., 203, 495-517.
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Toxoplasma における ATP ase の局在および H^3-glucose とりこみに関する電顕的研究

赤 尾 信 吉
(慶応大学医学部寄生虫学教室)

近年電子顕徴鏡による *Toxoplasma* の研究が，数多く報告され虫体の徴細構造が詳細に知られるようになつた．然しながらその細胞内小器官における機能は大部分が未知である．Toxoplasma 体内に見出される徴細構造種々の原虫，即ち *Sarcocystis*, *Besnoitia*, *Lankesierella*, *Eimeria*, *Plasmodium*, 等にも見出されるもので，これら原虫が分類学上近似なものであることを示すと共に，これら細胞内小器官の機能を明らかにすることは，これら原虫類の生理及び病原性を明らかにする上に重要な問題である．本実験の結果は次の如くである．

1) *Toxoplasma* における ATP ase 活性は虫体の外皮の二層の膜構造部に著明に認められた．ATP ase による反応生成物は二層の間に規則的或いは不規則的に認められた．2) トキソネームにおいては，その末端部の網状構造部に特に著明に反応が認められた．しかしながら均質的な構造をもつ前端部では反応を認め得なかつた．3) ATP ase 活性はゴルジ体，ミトコンドリア．細胞内空胞等にも認められた．4) 電顕的オートラジオグラフィーによる H^3-glucose のとり込みは，最初の30分間感作で虫体細胞膜付近に証明され，60分間感作ではゴルジ体，細胞内空胞及びトキソネーム上に証明された．