ULTRASTRUCTURE OF THE PELLICULAR COMPLEX OF *PLASMODIUM FALLAX*

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ABSTRACT

The exoerythrocytic merozoites of *Plasmodium fallax* grown in a tissue-culture system have been investigated by negative staining and thin-sectioning techniques, and the respective results have been compared. Negative staining provided additional information, corroborated findings obtained with thin sectioning, and contributed particularly to the study of the pellicular complex of the merozoites which has been demonstrated as being composed of three layers: a thin outer membrane, a thick interrupted inner membrane, and a partial layer of microtubules. Observations made of negatively stained parasites revealed that the thick, interrupted inner membrane in thin sections is actually a labyrinthine structure and covers the entire surface of the merozoite, except at the regions of the conoid and the cytostome. The microtubules which radiate from the conoid to the posterior end demonstrated a transverse periodicity and filamental subunits parallel to the axis of the microtubule. The detailed structure of the conoid and the cytostome is also described.

INTRODUCTION

In previous papers (1, 2), we described the fine structure of the erythrocytic stages of certain avian malarial parasites, *Plasmodium fallax*, *Plasmodium lophurae*, and *Plasmodium cathemerium*, using the thin-sectioning technique. More recently we have studied both the erythrocytic and exoerythrocytic stages of *P. elongatum* with the same technique (3). These studies revealed that both the erythrocytic and exoerythrocytic merozoites of all of these avian malarial paraties are almost identical and possess the same organelles. Hepler and his colleagues (11) studied the fine structure of the exoerythrocytic stages of *P. fallax* grown in a tissue-culture system and likewise reported the identical organelles which had previously been found in the merozoites of other parasites. There seemed still to exist, however, several unanswered problems, pertaining particularly to features of structure of the pellicular complex of these merozoites which were not revealed by thin sectioning alone. The pellicular complex of the merozoites is demonstrated as being composed of three layers: a thin outer membrane, a thick interrupted inner membrane, and a layer of microtubules. Since the parasite is rather large in size, 3.5 μ in length and 2 μ in width, a thin section of these merozoites does not afford a three-dimensional view. Also, the extent to which this pellicular complex covers the surface of the parasite is not clear.

In order to investigate these problems, we have attempted to study the whole merozoite, without sectioning, by means of negative staining; particular emphasis has been given to the correlation of findings on the pellicular complex obtained with negative staining and thin-sectioning techniques. For this purpose, the exoerythrocytic stages of *P. fallax* grown in a tissue-culture system (6) appear

to be an ideal model, since the merozoites are found free in the culture medium, and we can apply the negative staining technique directly to them without any interference from the host cell.

MATERIALS AND METHODS

The exoerythrocytic stages of *Plasmodium fallax* used in this study were grown in a tissue-culture system (6) at the Naval Medical Research Institute, Bethesda, Md. 14-day-old turkey embryos were inoculated with embryonic turkey brain previously infected with *P. fallax*. The heavily infected brain tissue of these embryos was collected 6 days after inoculation and was injected into a tissue culture system in culture T-flasks. The culture media in the T flasks contained 50% diploid growth medium, and 50% of a mixture composed of 90% mixture 199 (Microbiological Associates, Inc., Bethesda, Md.), 10% fetal calf serum, and 1×10^{-4} M folinic acid.

Culture media which contained numerous, free merozoites were collected and centrifuged for 10 min at 300 *g*. The host cells infected with *P. fallax* were usually adherent to the culture T-flask, and the culture media itself usually contained mainly the free merozoites of *P. fallax*.

After centrifugation the supernatant was discarded. For negative staining, distilled water was added to the resulting pellets. They were sonicated in an ultrasonic dissonicator for 10 min to be ruptured and were again centrifuged for 10 min at 300 *g*. A small portion of the pellets was placed into a drop of 2% PTA solution, and 300-mesh copper grids coated with Formvar were touched to the PTA drop containing parasites.

After the excess solution was removed from the grid with filter paper, the specimens were examined with a Siemens Elmiskop 1A.

For the thin-sectioning technique, the pellets obtained from the initial centrifugation were fixed, dehydrated, and embedded. Pellets were fixed in 1.25% glutaraldehyde solution (19) buffered with 0.05 M phosphate solution (pH 7.3) containing 4% sucrose for 1 hr at room temperature. Following fixation, the material was placed in phosphate buffer solution, with several changes, for 1 hr. After having been washed in the buffer solution, the material was postfixed in 1% OsO_4 in a phosphate buffer solution for 1 hr. The material was dehydrated in an ascending series of alcohol and in propylene oxide, and finally was embedded in Epon 812.

Thin sections were cut on a Porter-Blum MT-2 ultramicrotome with a Dupont diamond knife. The sections were mounted on copper grids and were stained with 1% uranyl acetate and lead citrate (18). These sections were also examined with a Siemens Elmiskop 1A.

OBSERVATIONS

Various shapes of the exoerythrocytic merozoites of *P. fallax* have been observed by thin-sectioning technique. These varied shapes are apparently due to the different planes of sectioning of the parasites. In a true transverse section the merozoite is ovoid in shape (Fig. 2), and in a longitudinal section it appears elliptical-to-elongated (Fig. 1). The widest diameter is in the anterior portion, at about one-third the distance between the anterior and posterior ends (Fig. 1). The results obtained with negative staining agree with those obtained with thin sections, in that most of the exoerythrocytic merozoites of *P. fallax* are elliptical-to-elongated in shape (Fig. 4) and measure 3–4 μ in length and 1.5 μ in greatest width. Occasionally, the merozoites studied by negative staining have an apparently oval configuration. This may result from the failure of these merozoites to flatten completely on the Formvar film coating the copper grids.

Observations made on transverse sections of the exoerythrocytic merozoites of *P. fallax* indicate that the pellicle of these merozoites is composed of three layers: a thin outer membrane, a thick interrupted inner membrane, and a layer of microtubules (Figs. 1, 2). In any given section of the merozoite, the thin outer membrane seems to cover the entire surface of the merozoite and to limit the parasite from its outer environment. Negative staining confirms the presence of a continuous outermost membrane covering the surface of the merozoite.

The thick, inner membrane is about 150 *A* wide in thin sections and is therefore about twice as thick as the outer membrane. It is composed of interrupted segments which vary in length (Figs. 1, 2). The distance between interrruptions also varies from place to place. In some sections these interruptions are rarely observed, whereas in others they are distinctly noticeable. These findings on the thick interrupted inner membrane are also consistent with the findings on the erythrocytic stages of avian malarial parasites, including *P. fallax*, *P. lophurae*, *P. cathemerium* (1), and *P. elongatum* (3), and on the exoerythrocytic stages of *P. elongatum* (3) which we have previously studied. When the sections were cut tangentially to the surface of the erythrocytic merozoites of these parasites, we occasionally observed a small portion of a meshwork in an arrangement similar to that of chicken wire (1).

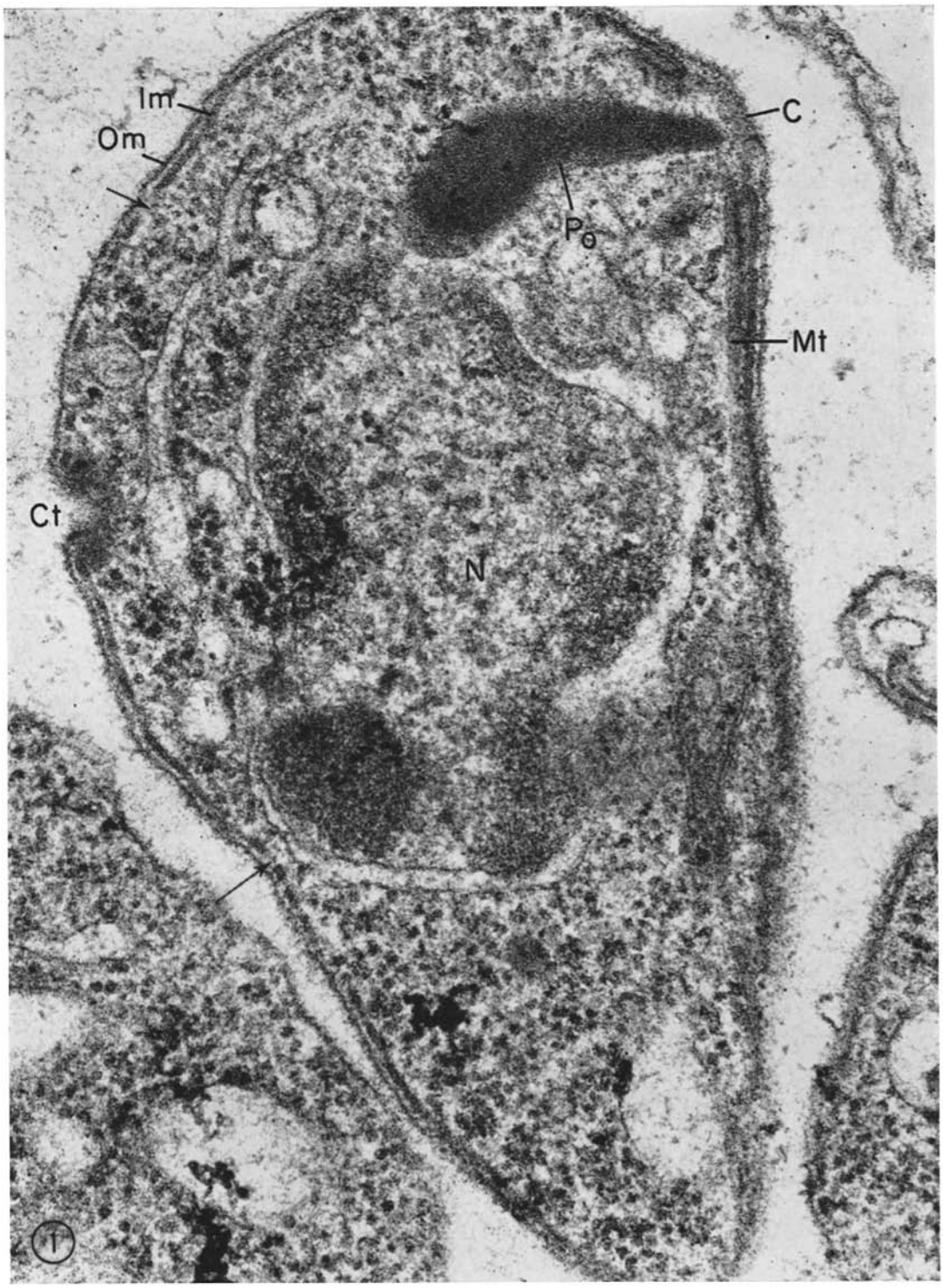


FIGURE 1 A longitudinal section of an elliptically shaped merozoite of *P. fallax*. Conoid (*C*) is located at the slightly lateral portion of the anterior end and is closely associated with paired organelle (*Po*). (Our use of the term conoid differs slightly from the original use by Gustafson et al. See footnote in the text.) Nucleus (*N*) is situated in the midsection of the merozoite. A longitudinal section of the cytostome (*Ct*) at the lateral portion of the parasite shows the thick lateral wall and a thin membrane of the base. Pellicle is composed of the thin outer membrane (*Om*), a thick, interrupted inner membrane (*Im*), and a partial layer of microtubules (*Mt*). In some areas, the interruptions (arrows) of the inner membrane are prominent. × 89,000.

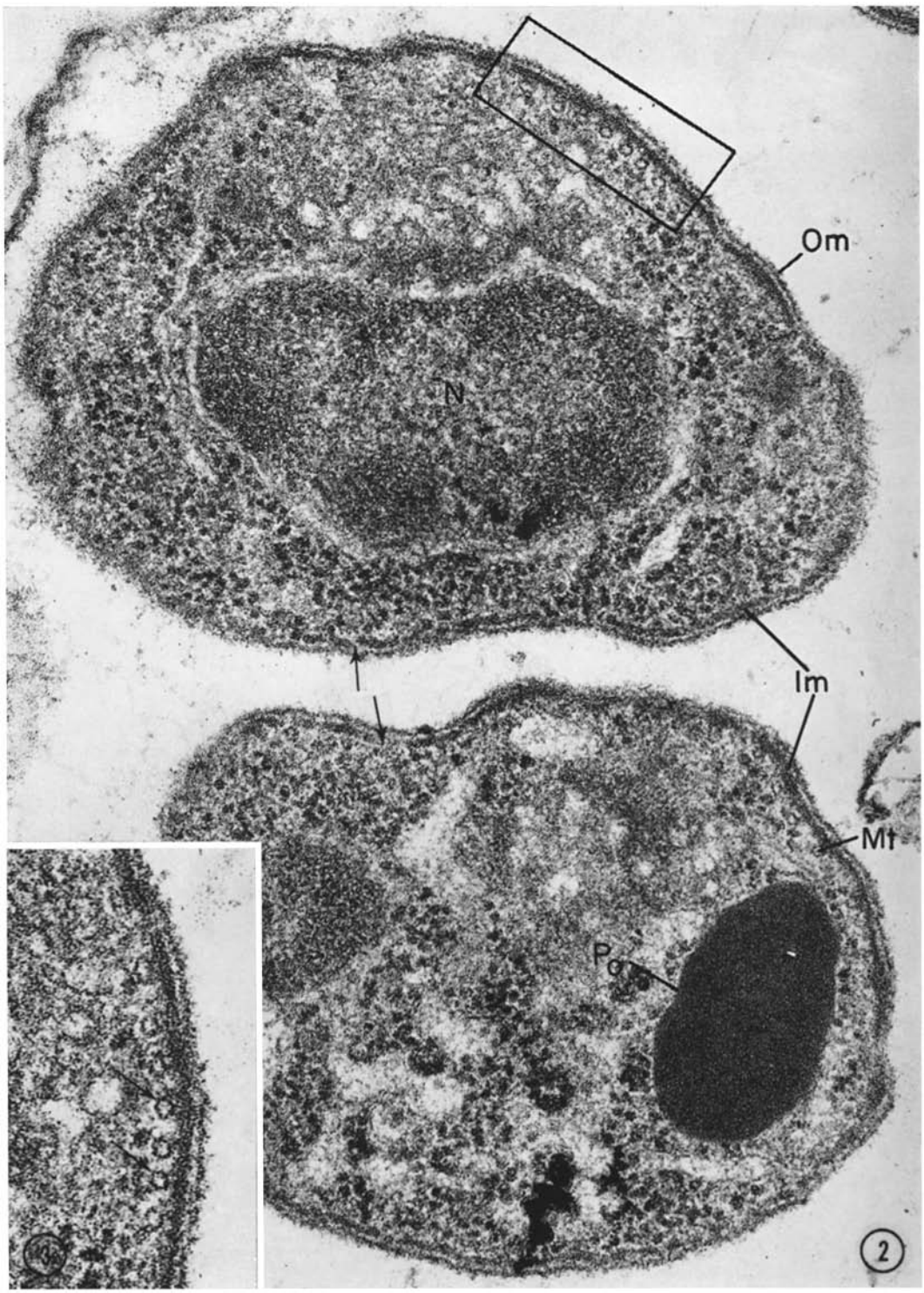


FIGURE 2 A transverse section of two merozoites. These parasites are covered with a thin outer membrane (*Om*), a thick, interrupted inner membrane (*Im*), and a partial layer of microtubules (*Mt*). The interruptions (arrows) of the inner membrane occur irregularly, and in some areas they are hardly observed. The lower merozoite shows a cross-section of one unit of the osmiophilic paired organelle (*Po*). *N*, nucleus. × 89,000.

FIGURE 3 A higher magnification of the pellicular complex shown in the rectangular area in Fig. 2. Each microtubule (arrows) is composed of a densely stained cortex and a slightly stained core. × 106,000.

The exoerythrocytic merozoites of *P. fallax* subjected to osmotic shock apparently are partially ruptured, and their cytoplasm may escape from the parasite. Thus, the structure of the remaining pellicle can be demonstrated more clearly and extensively by negative staining. Negative staining of an osmotically shocked merozoite reveals that the thick, interrupted inner membrane seen in thin sections is actually a labyrinthine structure (Figs. 4, 5) without a consistent pattern, rather than a structure resembling chicken wire. This labyrinthine structure appears to cover the entire surface of the merozoite, except at the regions of the conoid and the cytostome. Each septum of the labyrinthine structure is negatively stained and its thickness measures approximately 190–280 A. The space between these septa is electron opaque and shows no definite structural details (Fig. 5).

A layer of pellicular microtubules is always located just beneath the thick, interrupted inner membrane in thin sections (Figs. 2, 3). However, we have never observed in any given section that the layer of microtubules encircles the entire surface of the merozoite. Each microtubule measures approximately 220 A in over-all diameter and is composed of a lightly stained central core and a densely stained cortex (Fig. 3). The microtubules appear to radiate from the base of the anterior structure, that is the polar rings of the conoid,[1] to the posterior end (Fig. 9). In thin sections of the merozoites, however, we were not able to determine the ending of these microtubules. By negative staining, we have demonstrated that these microtubules radiate from the outermost polar ring of the conoid and terminate at the posterior end of the merozoite (Figs. 4, 8). We also observed that the outermost polar ring of the conoid appears to be directly connected with the microtubules (Figs. 6, 8).

Each microtubule observed by negative staining measures 190–230 A in the over-all diameter and is composed of an electron-opaque core and a negatively stained cortex. The diameter of the core is rather difficult to determine in these micrographs because the boundaries are not distinctly delineated, but it appears to be approximately 160 A. Close observation reveals that the microtubule possesses fine, filamental subunits which run parallel along it (Figs. 8, 10). These fine filaments appear to be beaded and to demonstrate transverse periodicity (Figs. 8, 10).

Although negative staining of the exoerythrocytic merozoites of *P. fallax* provides more knowledge of the microtubular structure, it does not clarify the still obscure distribution of the microtubules along the surface of the parasite. In some instances, we have counted 24–26 microtubules radiating from the outer polar ring of the conoid in an even distribution (Fig. 8). Some of these microtubules do reach the posterior end of the merozoite (Fig. 4), although we could not determine whether all of them terminate at the posterior end.

The conoid of the exoerythrocytic merozoite of *P. fallax* is usually located at a slightly lateral portion of the anterior end and appears, by both thin sectioning (Fig. 1) and negative-staining techniques, to be an inverted funnel-shaped structure. In sections, the base of the conoid is encircled by three osmiophilic polar rings. Corresponding to this observation, the conoid as observed also by negative staining is encircled by negatively stained polar rings (Figs. 6, 8), but it is rather difficult to determine their number. We have observed a regular, periodic arrangement of fine lines on these negatively stained rings (Figs. 6, 8).

Near the conoid, osmiophilic, paired organelles of tear-drop shape are observed in longitudinal sections (Fig. 1). A narrow ductule appears to extend from each unit of the paired organelle to the tip of the conoid. In transverse sections of the anterior end of the merozoites, the paired organelles are seen as two oval osmiophilic structures. Negative staining also shows similar structures, such as one or two dark, osmiophilic, oval structures near the conoid. The matrix of these oval structures is positively stained and is homogeneously osmiophilic.

In longitudinal sections, the cytostome, or mouth of the parasite, is a pellicular depression whose lateral walls are darkly stained, thick, linear segments and whose base is a thin membrane (Fig. 1). In face-view sections, the cytostome is encircled by a darkly stained, concentric, double ring sur-

[1] Our use of the term conoid differs slightly from its original use by Gustafson et al. (1954. *Am. J. Trop. Med. Hyg.* 3:1008). We use the term to refer to the truncated anterior end, demarcated by polar rings, since there seems to be no better term to identify the cone-shaped anterior end. Gustafson et al. used the term conoid to refer to the dense, cone-shaped structure inside the anterior end which we have not found in the merozoites of avian malarial parasites.

rounding an electron-transparent central area. The inner diameter of the cytostome measures 80 mμ and the outer diameter measures 170 mμ. In the negatively stained whole merozoite, a face view of the cytostome shows it to be a circular structure composed of an electron-opaque central area surrounded by a negatively stained double ring (Figs. 4, 7). The rings show a fine, linear periodicity similar to what was seen in the polar rings of the conoid. The inner and outer diameters of the cytostome in negatively stained merozoites are similar to those of the cytostome in thin sections. Occasionally, there are two cytostomes in a single merozoite.

Thin sections reveal many other organelles, such as the nucleus, mitochondrion, and spherical body, as well as endoplasmic reticulum and ribosomes, as seen in the erythrocytic and exoerythrocytic merozoites of other malarial parasites. Details of these organelles are not clearly differentiated by means of negative staining, although the outlines of the organelles can sometimes be seen.

DISCUSSION

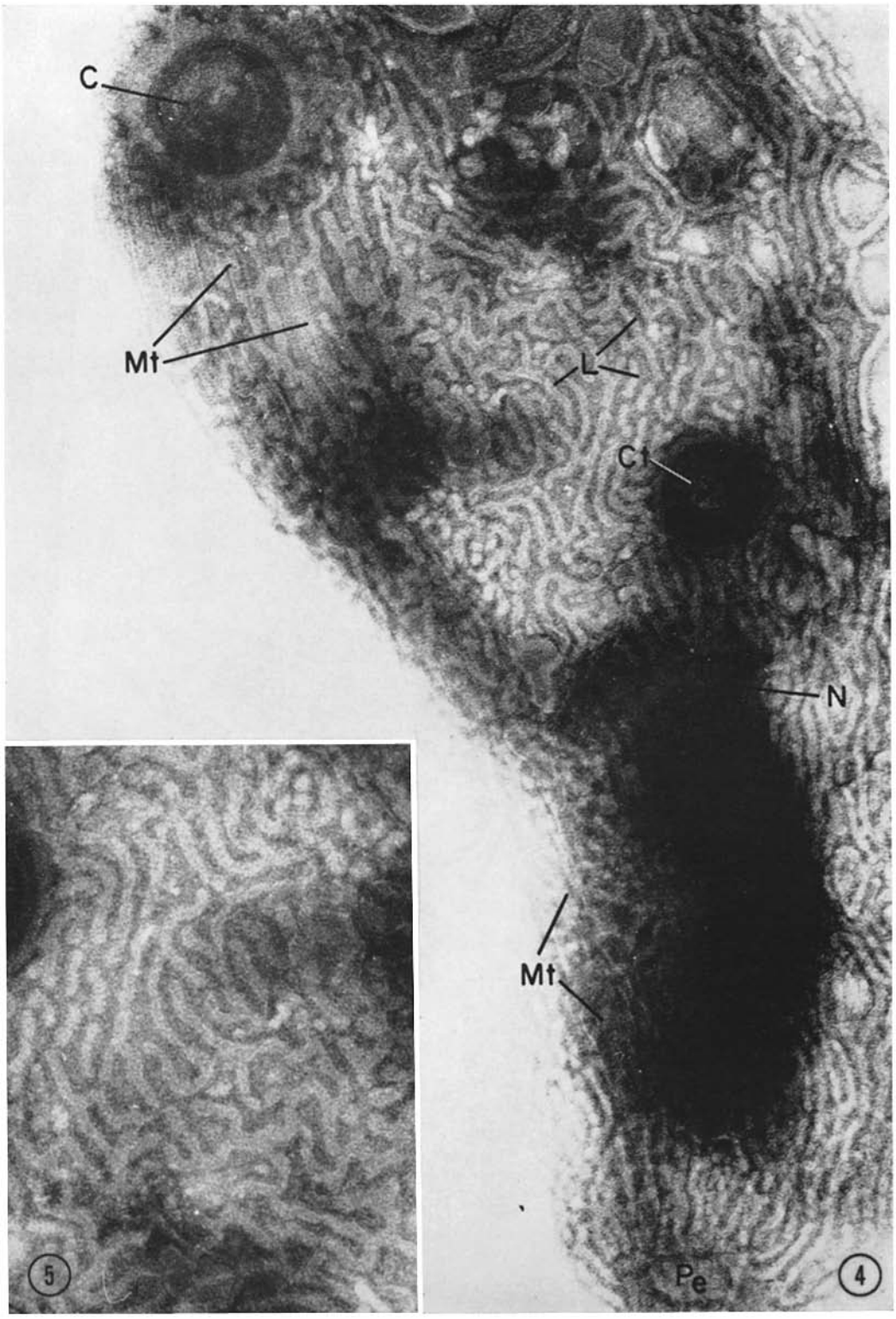
In the present study the negative-staining technique applied to an osmotically shocked merozoite of the exoerythrocytic stages of *P. fallax* has provided additional information and corroborated findings already obtained with the thin-sectioning technique. However, it is certain that negative staining alone could not provide adequate knowledge for the understanding of merozoite fine structure. The combination of negative-staining and thin-sectioning techniques is, therefore, an ideal method for the morphological study of the parasites by electron microscopy since one technique complements and confirms the other.

Negative staining contributed particularly to the study of the pellicular complex of the avian malarial parasite. Although a great deal of knowledge of the pellicular complex of the merozoites of various avian malarial parasites has been obtained by thin sectioning (1, 3), several aspects have remained inadequately understood. From observations of thin sections of the merozoites of both erythrocytic and exoerythrocytic stages of avian malarial parasites, the pellicular complex has been documented as having three layers: a thin outer membrane, a thick interrupted inner membrane, and a layer of microtubules. However, in thin sections we were able to observe only small areas of the pellicular complex, and we were not certain as to what extent these three layers of the pellicle cover the merozoite. The structure which in particular was not well revealed by thin sectioning was the arrangement of the thick, interrupted inner membrane observed in limited areas of the pellicle. Although we were reasonably certain that the thick, interrupted inner membrane observed in sections tangential to the parasite surface has an arrangement similar to that of chicken wire (1), the evidence was still not established. The negative-staining technique has added to our knowledge and has clarified our previous assumption on the pellicular complex of the merozoites of avian malarial parasites. The reason for failure to reach a definite conclusion with thin sectioning was apparently the large size of the parasites and the fact that tangential sections of small areas of the merozoite surface were inadequate for demonstrating the entire arrangement of the pellicular complex.

From our investigations on the asexual stages of avian malarial parasites, it is now reasonable to state that all merozoites of these parasites are covered with a three-layered pellicular complex. These three layers are a thin continuous outer membrane, a labyrinthine meshwork, and a layer of microtubules. The avian malarial parasites studied include the erythrocytic merozoites of *P.*

FIGURE 4 Negatively stained exoerythrocytic merozite of *P. fallax* demonstrating a conoid (*C*), a cytostome (*Ct*), and a pellicular complex. The merozoite is almost entirely covered with the complex labyrinthine structure (*L*). From the base of the conoid, several microtubules (*Mt*) radiate to the posterior end (*Pe*). The nucleus (*N*) is observed as a darkened area. × 101,000.

FIGURE 5 A higher magnification of a negatively stained merozoite showing the details of the labyrinthine structure of the pellicular complex. × 150,000.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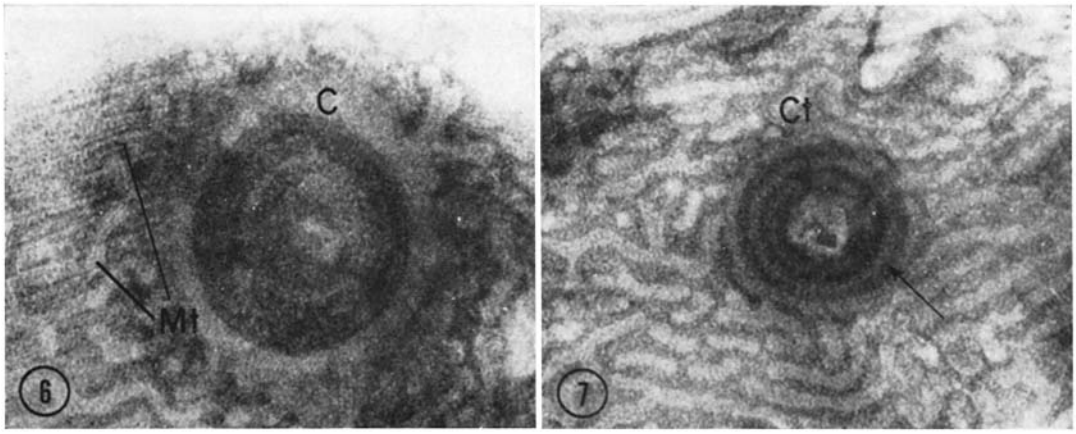

FIGURE 6 Negatively stained exoerythrocytic merozoite demonstrating a face view of the conoid (*C*) at a higher magnification. The polar rings reveal a fine linear periodicity. Several microtubules (*Mt*) radiate from the conoid. × 114,000.

FIGURE 7 A higher magnification of a negatively stained face view of the cytostome (*Ct*). The cytostome is surrounded by a double electron-translucent ring with fine linear periodicity (arrow). Notice the relationship between the labyrinthine structure and the cytostome. × 114,000.

fallax, *P. lophurae*, *P. cathemerium* (1), and *P. elongatum* (3) as well as the exoerythrocytic merozoites of *P. fallax* and *P. elongatum* (3).

A similar pellicular complex has been reported in sections of sporozoites of various malarial parasites (8, 22) as well as other protozoa such as trypanosomes (8), *Lankesterella* (8), *Toxoplasma* (9), *Sarcocystis* (14), *Besnoitia* (20), and *Eimeria* (21). All these protozoa have been described as having a double membrane and a layer of microtubules.

The outer membrane of these protozoa may be the membrane which limits the protozoa from the external environment and may be analogous to the plasma membrane of other living cells. Although thin sectioning reveals that in all these protozoa the inner membrane is closely aligned to the outer membrane, there appears to be no report that this inner membrane is arranged like a labyrinth, and shows irregular interruptions. This labyrinthine structure seen in the erythrocytic and exoerythrocytic merozoites of avian malarial parasites may probably be unique to these particular parasites.

The function of this labyrinthine structure is not clear, although we have assumed that it may serve as a cytoskeleton and provide rigidity for the merozoite. This supposition is supported by the fact that a merozoite has to pass through a free, living stage during which period it is outside the host cell and is entirely unprotected. Other evidence for the rigidity of the labyrinthine structure derives from the fact that this structure breaks down as the parasite starts to expand its size in the trophozoite stage (1, 11). This appears to indicate that the labyrinthine structure may interfere with the expansion and growth of the malarial parasites within the host cell.

The pellicular microtubules of the exoerythrocytic merozoites of *P. fallax* observed with negative staining are similar in appearance to microtubules reported in various animal (5, 7, 10) and plant cells (13). Grimstone and Klug (10), who studied the fibers in flagella of *Trichonympha* and other flagellates, found that each subfiber of the outer fibers in these flagella clearly revealed a longitudinal striation owing to the presence of fine filaments as seen with negative staining. They also described a transverse periodicity in the subfibers. These observations are extremely similar to our findings for the microtubules presented in the present study. Gall (7), studying a group of negatively stained microtubules from the marginal bands of a newt erythrocyte, also reported that the microtubular wall is composed of longitudinal subunits which are beaded fibrils running parallel to the long axis of the microtubules. Pease (17)

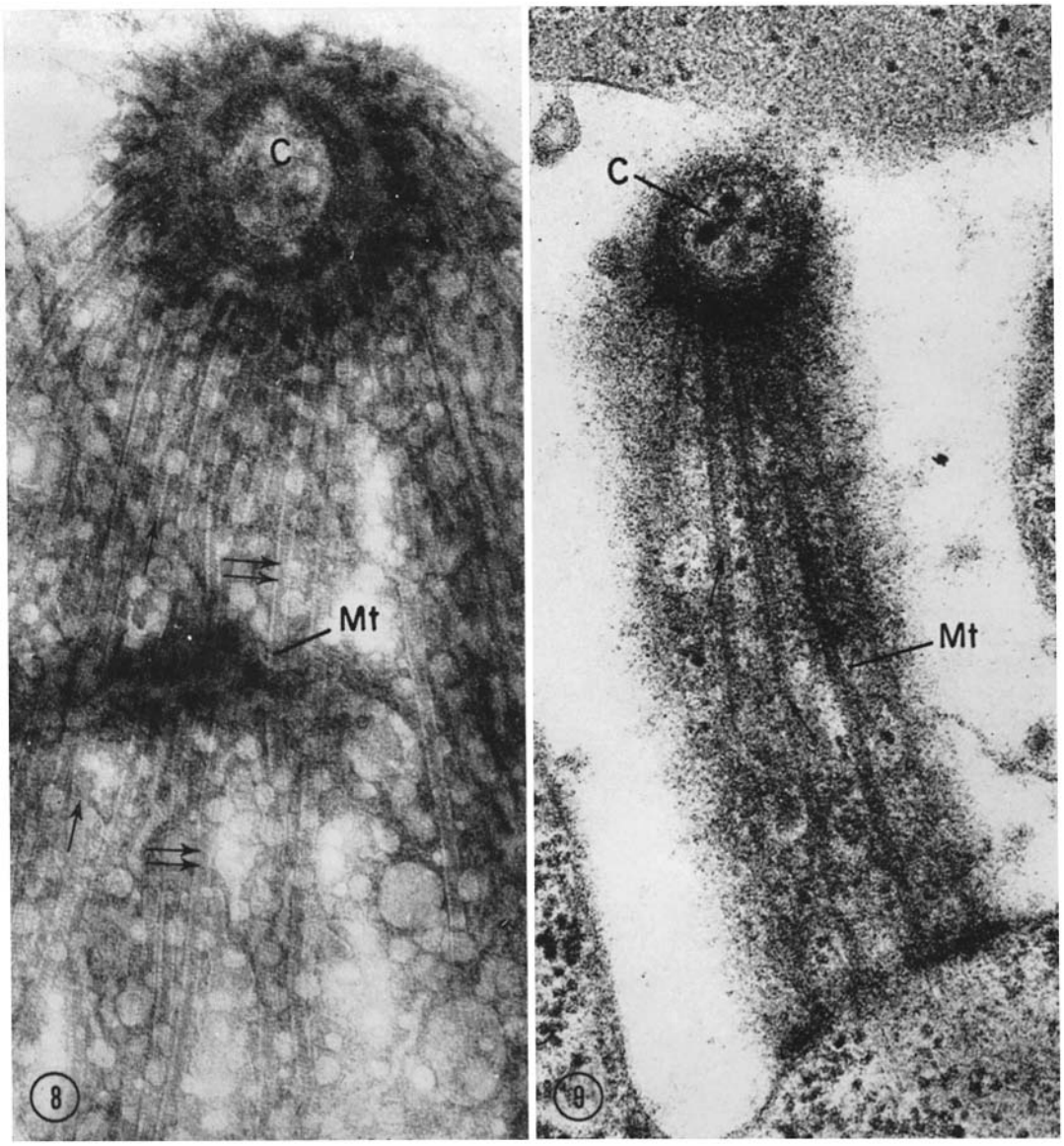

FIGURE 8 Negatively stained exoerythrocytic merozoite showing microtubules (*Mt*) which radiate posteriorly from the polar rings of the conoid (*C*). Each microtubule possesses filamental subunits (double arrows) with a transverse periodicity (single arrows). Notice the connection between the microtubules and the outer polar ring. Negatively stained globules on and around the microtubules may probably be an artifact. × 102,000.

FIGURE 9 A longitudinal section of a newly forming erythrocytic merozoite of *P. fallax* reveals the microtubules (*Mt*) radiating posteriorly from the conoid (*C*). The microtubules show faintly transverse periodicity (arrows). × 87,000.

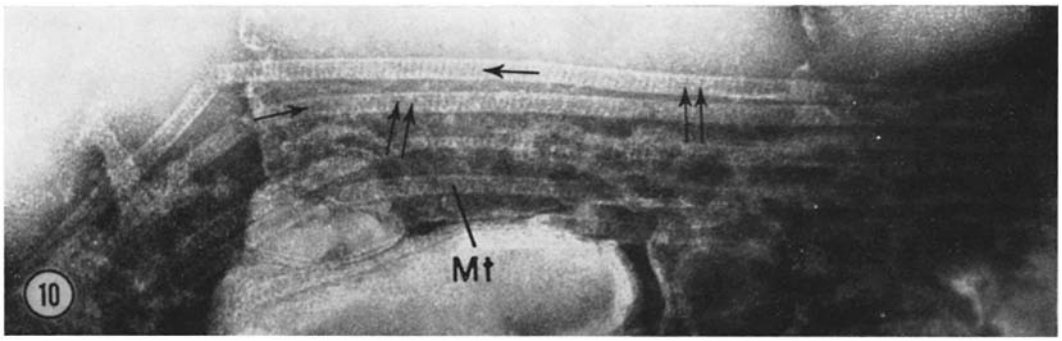

FIGURE 10 Another example of the negatively stained microtubules showing transverse periodicity (single arrows) and filaments (double arrows) parallel to the axis of the microtubule (*Mt*). × 135,000.

and André and Thiéry (4) also reported similar subunits in ciliary and flagellar fibers revealed by negative staining.

The function of the pellicular microtubules or fibers is still not clear. Meyer and Porter (16) observed a layer of the pellicular microtubules beneath the plasma membrane of *Trypanosoma cruzi* and suggested that they may be composed of contractile protein which may function in the motility of the organism; these authors cite the disappearance of these organelles after treatment with trypsin or after prolonged fixation with osmium tetroxide. Judge and Anderson (12) have also reported fibrils (microtubules) in trypanosomes and suggested, as another possibility, that these fibrils may be fine channels that secrete or conduct the secretion out of the organism via the conoid. Burton (5) has suggested that the microtubules of fluke spermatozoa may provide stability, as do those of tapeworm spermatozoa described by Lumsden (15), because they are resistant to sonication and bending. The function of the microtubules in the merozoites of avian malarial parasites may be motility, since the merozoites are a motile form, albeit for a very short period. Further, the microtubules disappear in subsequent stages when the parasites become nonmotile. However, we cannot overlook the possibility that the microtubules may also provide stability to the merozoites.

The presence of two cytostomes in the exoerythrocytic merozoites of *P. fallax* as demonstrated by negative staining is an interesting new finding. So far, we have not observed two cytostomes in a single merozoite which we have studied by the thin-sectioning technique (2, 3). The fact that the merozoite is large in size may readily account for this new finding.

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