

THE FINE STRUCTURE OF THE EXOERYTHROCYTIC STAGES OF *PLASMODIUM FALLAX*

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ABSTRACT

The fine structure of the exoerythrocytic cycle of an avian malarial parasite, *Plasmodium fallax*, has been analyzed using preparations grown in a tissue culture system derived from embryonic turkey brain cells which were fixed in glutaraldehyde-OsO₄. The mature merozoite, an elongated cell 3- to 4- μ long and 1- to 2- μ wide, is ensheathed in a complex double-layered pellicle. The anterior end consists of a conoid, from which emanate two lobed paired organelles and several closely associated dense bodies. A nucleus is situated in the mid portion of the cell, while a single mitochondrion wrapped around a spherical body is found in the posterior end. On the pellicle of the merozoite near the nucleus a cytostomal cavity, 80 to 100 m μ in diameter, is located. Based on changes in fine structure, the subsequent sequence of development is divided into three phases: first, the dedifferentiation phase, in which the merozoite loses many complex structures, i.e. the conoid, paired organelles, dense bodies, spherical body, and the thick inner layers of the pellicle, and transforms into a trophozoite; second, the growth phase, which consists of many nuclear divisions as well as parallel increases in mitochondria, endoplasmic reticulum, and ribosomes; and third, the redifferentiation and cytoplasmic schizogony phase, in which the specialized organelles reappear as the new merozoites bud off from the mother schizont.

INTRODUCTION

The recognition of an exoerythrocytic cycle of malarial parasites in their vertebrate host represents a major advance in our understanding of malarial infections (3). As an intermediate between sporozoites from the mosquito and the erythrocytic phase, the exoerythrocytic stage constitutes an essential link in the complete cyclical development of a malarial parasite. Observations on the morphology of exoerythrocytic forms, however, have been largely confined to the light microscope, and, to date, only two brief studies on their fine structure have been made (27, 28). The relative sparsity of host cells invaded by this stage of the developing parasite has created a major difficulty in locating

examples and has been one of the main factors limiting their study by electron microscopy.

An improved tissue culture technique has recently been developed (4) which greatly facilitates study by electron microscopy. With this technique, it has been possible to obtain cultures infected heavily enough for us to observe examples of developing parasites in each low magnification field on the electron microscope. With a background in parasite structure and development provided by light microscopy (15) and phase micrography (16) and the use of the favorable tissue culture system, we undertook a more penetrating analysis of the exoerythrocytic stage of *P. fallax* at the fine struc-

ture level. In our study, we have given special attention to the ultrastructure of the motile forms, the mature merozoites, and we have attempted to assess the significance and function of their numerous specialized organelles and structures. We have also followed the changes which occur in these structures as the parasite undergoes its development in the tissue culture cells.

MATERIALS AND METHODS

Exoerythrocytic stages of *Plasmodium fallax* (maintained in turkeys at the Naval Medical Research Institute, Bethesda, Maryland), which were grown in a tissue culture system derived from embryonic turkey brain cells, served as the material for this study. The initial culture was prepared by inoculating 14-day-old turkey embryos with material previously isolated from an infected embryonic turkey brain. Six days after challenge, the 20-day-old embryos were sacrificed and the brain tissue collected.

Portions of heavily infected tissue were trypsinized and then inoculated into culture flasks (T flasks) in a medium containing 50% Diploid Growth Medium, and 50% of a mixture composed of 90% mixture 199 (Microbiological Associates, Inc., Bethesda), 10% fetal calf serum, and 1×10^{-4} M folic acid. Both brain cells and parasites flourished in this medium.

Cultures which exhibited a confluent sheet of tissue cells with a considerable number of parasitized cells were selected for examination in the electron microscope. To prepare the tissue for analysis, the cells were first incubated with trypsin at 37°C for 5 to 10 min or until most of them had become detached from the surface of the culture flask. The freed cells were transferred to a centrifuge tube and centrifuged at 300 *g* for 10 min. The supernatant was discarded and the remaining pellet carried through the subsequent stages of fixation, dehydration, and embedding.

The tissue was fixed for 1 hr in 1.25% glutaraldehyde solution, buffered with 0.05 M PO_4 at pH 7.3 and containing 4% sucrose (40). Following fixation, the material was washed in 3 changes of buffer. The material was left in the last wash for at least 1 hr and frequently was left overnight at 4°C. Finally the material was postfixed in 1% OsO_4 in a buffered PO_4 -sucrose solution for 1 hr.

Dehydration was carried out in a graded ethanol series, and the tissue was embedded in Epon 812 according to the method of Luft (25).

Thin sections were cut on a Porter-Blum MT-2 ultramicrotome with a DuPont diamond knife. The sections were mounted on 300- or 400-Cu mesh grids and counterstained with uranyl acetate and lead citrate (33). The sections were examined in either an RCA EMU 3G or a Siemens Elmiskop 1A.

OBSERVATIONS

Through our observations of many electron micrographs and comparison with the studies previously made by light microscopy (15) and phase cinematography (16-18, 42), we have been able to establish a pattern of change in fine structure which the parasite undergoes during its growth. We have arranged the micrographs in an order corresponding to what we believe is the developmental sequence.

Normal Tissue Culture Cells

An uninfected turkey brain culture cell (Fig. 1), shown as a point of reference, exhibits an ultrastructure common to many cells. A large nucleus with a prominent nucleolus dominates the central region of the cell, while the surrounding cytoplasm contains numerous organelles, including mitochondria, Golgi bodies, endoplasmic reticulum, and ribosomes. In some tissue cells, both infected and uninfected, we occasionally observe droplets 0.5 to 1 μ in diameter which stain evenly and are thought to be lipid. It is difficult to identify what particular type of brain cell these are since they have been maintained in tissue culture, by periodic transfer, for a considerable length of time and have probably dedifferentiated.

Mature Merozoites

The form of the parasite which invades the tissue culture cell and initiates the developmental cycle is the merozoite. These motile organisms are elongated, usually 3 to 4 μ long and 1 to 2 μ wide (Fig. 2). They have an anterior end composed of a conoid, a conically shaped structure with three encircling polar rings (Fig. 6), from which emanate two evenly staining bodies comparable to the structures Garnham et al. (9) named "paired organelles" (Fig. 8). Since the plane of the section varies considerably, it is not unusual to observe only one of the pair (Figs. 2, and 6). Detailed examination has not revealed any internal structure within the paired organelles. In the anterior end of the merozoite a number of dense bodies are located, measuring approximately 0.1 μ in diameter and having a more granular texture than their neighboring paired organelles (Fig. 2). Similar structures in sporozoites have been called convoluted tubules by Garnham et al. (12), but, since they appear to us neither convoluted nor tubular,

we prefer to use the more general term dense bodies.

A spherical nucleus bounded by a double membrane resides in the central portion of the cell (Figs. 2, 3, and 8). The densely staining granular substance observed distributed around its edge is presumably chromatin. Ribosomes line the outer membrane surrounding the nucleus (Fig. 3).

A single mitochondrion is situated in the posterior end of the merozoite (Figs. 2 and 5). Its tubular cristae are similar to those of many protozoan mitochondria (32) and differ markedly from the lamellar cristae of the host tissue mitochondria (Fig. 8). Frequently, a mitochondrion is found conspicuously wrapped around a spherical body of unknown identity (Figs. 2, 5, and 8). This rather striking and consistent association is observed only in the mature merozoite. The spherical body can be distinguished from the mitochondrion by the presence of a homogeneous granular internal matrix, and by the presence of an indeterminate number of membranes enclosing it (Fig. 5).

The matrix of the merozoite cytoplasm contains a few elements of the endoplasmic reticulum and numerous ribosomes (Fig. 2).

The merozoite possesses a complex pellicle composed of two distinct membrane layers (Figs. 2, and 3). The outer layer is a single membrane and is the limiting structure of the merozoite. Interior to this is a second and thicker membrane layer, which, in cross-section, contains numerous evenly spaced interruptions indicating the presence of many pores (Fig. 3). Longitudinal sections only occasionally show these interruptions. Beneath the thick inner membrane of the pellicle, groups of microtubules are frequently observed (Fig. 3). They are circular in cross-section, measuring approximately 24 μ in diameter, and have a densely stained cortex and a lightly stained core. The microtubules radiate from the base of the conoid and extend posteriorly, covering the entire surface of the merozoite.

On the surface of the merozoite, approximately midway between the anterior and posterior ends, a depression or cavity is observed which is 80 to 100 μ wide on its inside and is bounded by two short dense segments (Fig. 4). Since erythrocytic forms of *P. fallax*, *P. lophurae*, and *P. cathemerium* have been shown to ingest red blood cell cytoplasm through this cavity, it has been named "cytostome." A detailed account of its structure in both the erythrocytic and exoerythrocytic forms has been presented in an earlier publication (2).

A summary of our observations on the fine structure of the mature merozoite is presented in a diagram in Fig. 7.

Early Stages of Infection: Merozoite to Trophozoite

Merozoites which have just invaded the host cell cytoplasm are shown in Fig. 8. Multiple infections, similar to the one shown here, occur with great frequency in the tissue culture system and probably result from the rupturing of a segmenter within the host so that the newly released merozoites reinfect the same cell. This phenomenon has been pointed out by Huff et al. (16) as an anomaly of the tissue culture system, since multiple infections have not been observed in vivo.

At this early stage of infection, the merozoites still possess their characteristic elongated shape and numerous complex organelles. They have not yet become rounded or started their transformation into trophozoites. Attempts to obtain micrographs of still earlier phases in the invasion and examples of the process of entry itself have proved unsuccessful. We know, however, from the many light microscope studies, that the actual entry is a rapid and elusive process, observed only a few times (18).

The host cell cytoplasm surrounding these newly entered merozoites in Fig. 8 contains a large accumulation of vesicles which suggest that damage has taken place. It is difficult, however, to assess the significance of host cell changes at this stage since numerous examples of the cytoplasm of cells containing parasites at much later stages of development display a normal structure with the presence of organized elements of the endoplasmic reticulum, ribosomes, Golgi bodies, and mitochondria (Figs. 11 and 12). An impression gained from viewing numerous different cultures is that there is a significant variation in host cell cytoplasm between cultures, irrespective of the degree of infection, i.e. some show many more lipid droplets and vesicles than others. The vesicular nature of the host cytoplasm of Fig. 8, therefore, may not be due to the effect of the parasite.

Very shortly after entry, the merozoite becomes rounded and undergoes a series of transformations (Figs. 9 and 10). The conoid becomes widened and diffuse in appearance and the closely associated paired organelles and small dense bodies become considerably smaller and less conspicuous (Fig. 9), suggesting to us that they are in the process of breaking down. The mitochondrion remains but

its associated spherical body becomes swollen, loses its electron opacity, and eventually disappears (Fig. 15).

By tracing the thick inner membrane of the merozoite pellicle (Fig. 9), we find that many portions have disappeared, and they are also presumed to be in the process of breaking down. Occasionally, the outer limiting membrane pulls away from the thick inner membrane (Fig. 10) which subsequently lyses. Concomitant with the loss of the thick inner membrane is the disappearance of the layer of pellicular microtubules. The resulting degradation of the thick inner membrane and pellicular microtubules seems to destroy the rigidity and shape of the merozoite and permits rounding (Fig. 10).

The combined effects of the degeneration of the numerous specialized organelles leaves the trophozoite in a dedifferentiated state in which it retains only a few essential structures, a nucleus, mitochondrion, elements of the endoplasmic reticulum, and ribosomes, all bounded by the limiting membrane of the parasite.

External to the parasite, an outer membrane is formed which completely encloses the rounding trophozoite (Figs. 9 and 10). This is quite clearly not one of the parasite membranes since in many places, where the merozoite pellicle has not broken down, the triple-layered structure of the pellicle can be observed in addition to this outermost membrane. Because the latter is completely out-

side of the parasite, even in such regions as the conoid, and because there appears to be no connection between it and the limiting membrane of the parasite, we suggest that this outermost structure, characteristic of trophozoites and other growing forms, is produced by the host cell cytoplasm.

In early stages of infection, when the host membrane is first formed, small projections on its surface are evident which appear similar to those observed on coated vesicles (35). These projections, however, soon disappear and subsequently the host membrane becomes thick and darkly stained compared with the limiting membrane of the parasites (Figs. 11 and 12).

Growth of the Parasite: The Schizont

In its dedifferentiated state the trophozoite undergoes remarkable growth (Figs. 11 and 12). Marked synthesis takes place within the expanding parasite, and in 2 or 3 days as many as 200 new nuclei and mitochondria are formed from the original ones supplied by the invading merozoite. The 200 or so merozoites which will bud off from the fully grown parasite will then receive one each of these nuclei and mitochondria.

The arrangement of microtubular spindle fibers (Fig. 13) conforms closely to that in mitotic divisions noted by Huff et al. (16, 17). The fibers together form a fan-shaped cluster, while individual

Abbreviations

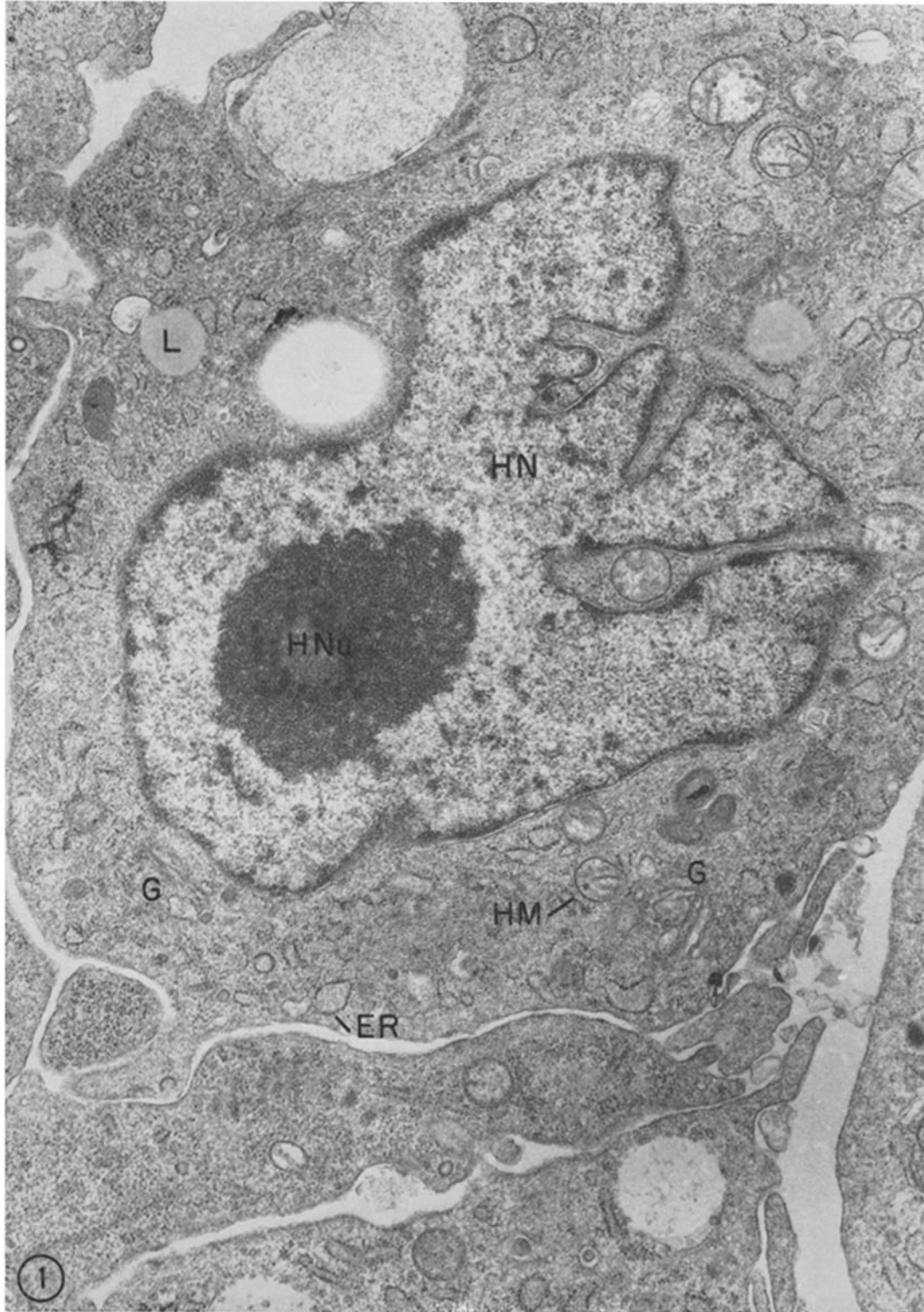
Parasite

C, conoid
Ct, cytostome
D, dense bodies
ER, endoplasmic reticulum
Im, thick inner membrane
Om, outer membrane of pellicle
M, mitochondrion
Mt, pellicular microtubules
N, nucleus
Nu, nucleolus
Po, paired organelles

R, ribosomes

Sb, spherical body
Sf, spindle fibers
Host cell
ER, endoplasmic reticulum
G, Golgi bodies
HM, Host mitochondrion
HN, Host nucleus
HNu, host nucleolus
Hm, host membrane
L, droplet

FIGURE 1 An electron micrograph of a typical uninfected turkey brain cell which has been grown in tissue culture. A large nucleus (*HN*) and prominent nucleolus (*HNu*) are observed in the center of the cell. The cytoplasm contains elements of the endoplasmic reticulum (*ER*), ribosomes, mitochondria (*HM*), Golgi bodies (*G*), and occasional droplets (*L*), presumably lipid. $\times 29,000$.



members have a dense cortex and a light core and measure 20 to 22 μ in diameter. The nuclear membrane does not break down during division, except at the point at which the spindle fibers are attached. Careful examination of several dividing nuclei failed to reveal chromosomes or any structures associated with the spindle fibers. Within the nuclei we frequently observe prominent nucleoli, which appear to be composed of densely packed clusters of ribosomes (Fig. 12). While we have not determined what ultrastructural changes they undergo during division, it is already known from cinemicrographic studies that nucleoli do not break down, but appear to split in the final stages of daughter nuclei formation (16).

Mitochondria presumably increase in number by fission. Examples have been observed where constriction, suggestive of cleavage points, occurs along elongated mitochondria (Fig. 14).

Around actively growing parasites extensions of the thick outermost membrane are frequently observed coursing into the host cell cytoplasm where

they occasionally end in a bleb (Figs. 11 and 12). The limiting membrane of the parasite has never been observed extending into these folds. Such phenomena may be examples of host cell processes which are involved in synthesizing this membrane, and are further indication that the outermost membrane is produced by the host cell cytoplasm.

The cytostomal cavity has been observed in young actively growing forms of the parasite (Figs. 16 to 19). As the parasite grows in size, the likelihood of finding the cytostome decreases. At present, we are uncertain of its ultimate fate, as to whether the cytostome persists or eventually disappears and at what stage this may occur. Close examination of several examples, however, has not revealed structures which we feel are related to host cytoplasm ingestion, such as expansion of the cytostome cavity, extensions of the basal membrane of the cytostome into the parasite cytoplasm, formation of food vacuoles, or projections of the outer, host-produced membrane into the cytostome cavity. As observed in the mature merozoite,

FIGURE 2 to 6 Micrographs from sections made at different angles, showing the characteristic fine structure of the mature exoerythrocytic merozoite of *P. fallax*.

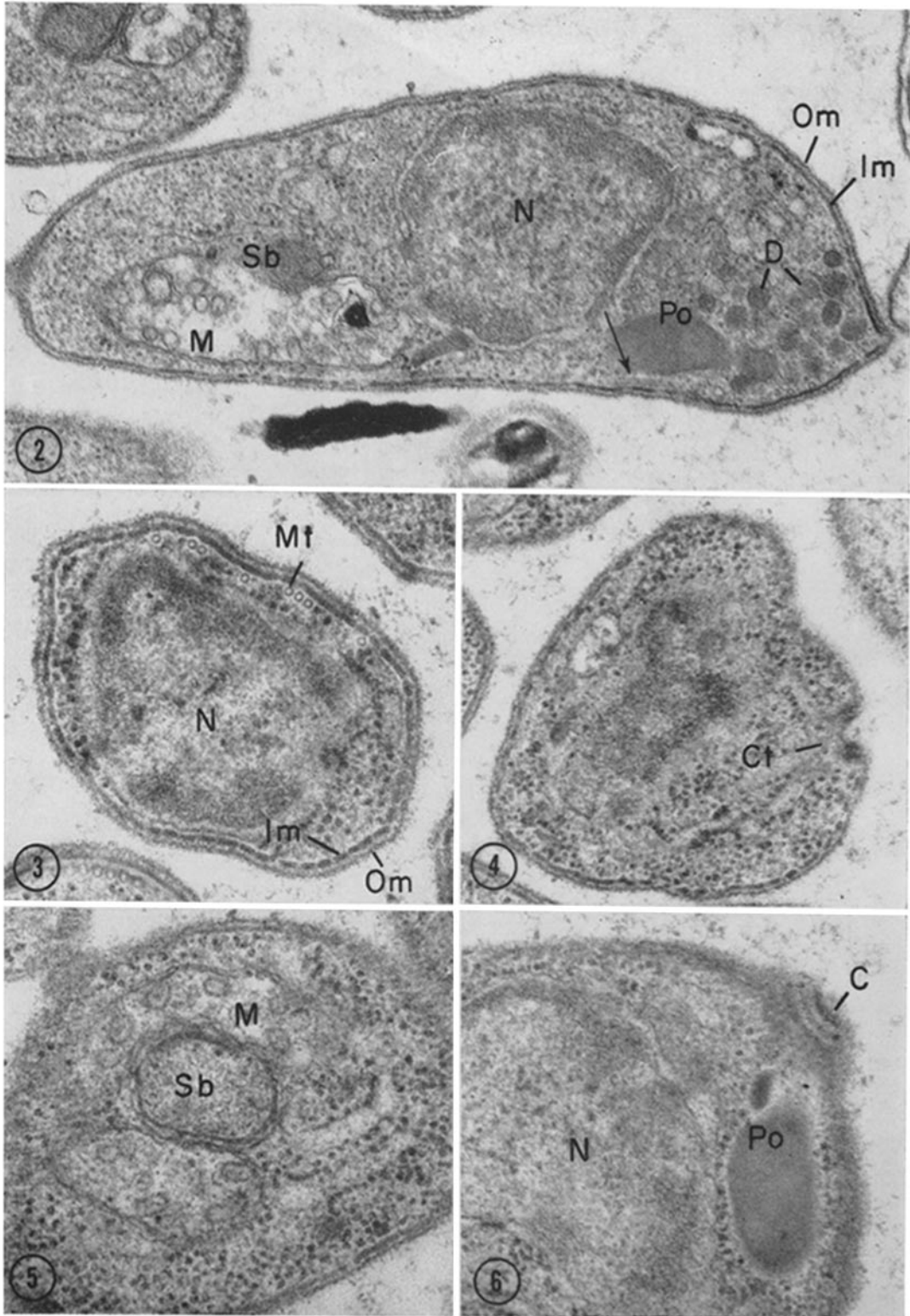
FIGURE 2 A longitudinal section reveals the elongated form of the merozoite. The anterior end on the right contains numerous dense bodies (*D*) and a portion of one of the paired organelles (*Po*). A nucleus (*N*) is situated in the mid section of the cell, while a single mitochondrion (*M*) and its associated spherical body (*Sb*) occupy the posterior part of the cell. Ribosomes and a few elements of the endoplasmic reticulum form the matrix of the cytoplasm. The pellicle is composed of the thin outer membrane (*Om*) and a thick inner membrane (*Im*). A faint trace of a microtubule sectioned longitudinally is visible (arrow). $\times 41,000$.

FIGURE 3 A cross-section of a merozoite, showing the complex pellicle. Note the evenly spaced interruptions in the thick inner membrane (*Im*). The circular profile of a pellicular microtubule (*Mt*) can be observed. Individual tubules are approximately 24 μ in diameter. $\times 58,000$.

FIGURE 4 The cytostome (*Ct*) on mature merozoites forms a depression in the pellicle. It is 80 to 100 μ wide on the inside and is bounded on each side by two short dense segments. When observed in a longitudinal section of a merozoite, the cytostome is located near the nucleus and approximately midway between the anterior and posterior ends of the merozoite. $\times 43,000$.

FIGURE 5 A section through the posterior end of a merozoite reveals a mitochondrion (*M*) wrapped around a spherical body (*Sb*). The mitochondrion has tubular cristae and is bounded by a double membrane. The spherical body has a granular matrix and is bounded by a series of membranes. $\times 51,000$.

FIGURE 6 An oblique section through the anterior end of a merozoite shows the region of the conoid (*C*). Its cone-shape and three encircling polar rings are evident. One of the paired organelles and a nucleus are also visible. $\times 48,000$.



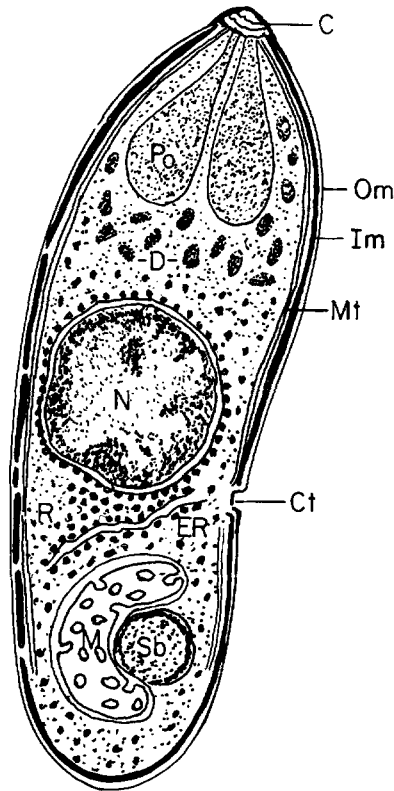


FIGURE 7 A diagrammatic sketch of the detailed fine structure of a mature merozoite as observed in a longitudinal section. Approximately $\times 50,000$.

the cytostome is bounded by two short dense segments on each side of a cavity. It is interesting to note that the cytostome cavity, on the growing parasites we have seen, measures only 40 to 50 $m\mu$ in inside diameter, or approximately one-half the diameter observed (80 to 100 $m\mu$) in mature merozoites. (Compare cytostomal size in Fig. 4 with those in Figs. 17 and 18.) While reduction in the size of the cytostomal opening appears to have taken place, intermediate forms with a cytostomal opening between 45 and 90 $m\mu$ have not been observed.

Cytoplasmic Schizogony: The Formation of New Merozoites

After considerable growth and many nuclear divisions, the process of cytoplasmic schizogony which produces new merozoites begins. The first indication is the appearance on the surface of the schizont of areas in which the limiting membrane

becomes considerably thicker (Fig. 20). Adjacent to these regions are observed large, oval dense bodies; since two such bodies are occasionally apparent at a single locus, they are probably the paired organelles characteristic of merozoites (Fig. 23). Within the cytoplasm of the schizont, swirls of organized elements of the endoplasmic reticulum become conspicuous (Fig. 20).

In subsequent stages of cytoplasmic schizogony the thickened regions of membrane grow outward, assuming a cone shape (Fig. 21). The previously thick membrane can now be distinguished as two separate membranes, a thin outer one, which corresponds to the limiting structure of the mature merozoite, and a thick inner membrane which will become the inner membrane of the merozoite. Close examination of the two membranes shows that the outer one is a single unit structure 6 to 7 $m\mu$ wide whereas the inner one is composed of two closely packed unit structures and has a total width of 15 $m\mu$ (Figs. 22 and 23). Areas on the surface of the schizont which are not differentiating into merozoites are bounded by the single limiting membrane only (Figs. 21, 22, 24, and 25). It is important to note that the thin outer membrane is the one that has been the limiting membrane of the merozoite. All changes in structure, such as the elaboration of the thick inner membrane, occur internal to the outer membrane of the parasite.

During the process of cytoplasmic schizogony the outer limiting membrane of the parasite separates from the host membrane, creating a large clear space into which the budding merozoites grow.

As buds grow outward, the cytoplasmic elements which had been formed in the growing schizont flow into them (Figs. 22 to 25). First, the anterior end forms and includes the conoid on the outside, and the closely associated paired organelles and the dense bodies (Fig. 24). The nucleus enters next, followed by the single mitochondrion with its associated spherical body (Fig. 25). The matrix of the budding merozoite is filled with elements of the endoplasmic reticulum and ribosomes. In the final stage the bud, which now has the characteristic shape of a mature merozoite, pinches off from the central cytoplasmic mass (Fig. 25).

A few cytoplasmic elements, including some endoplasmic reticulum and ribosomes, appear not to enter into budding merozoites and are presumably left behind. It is known from the light micro-

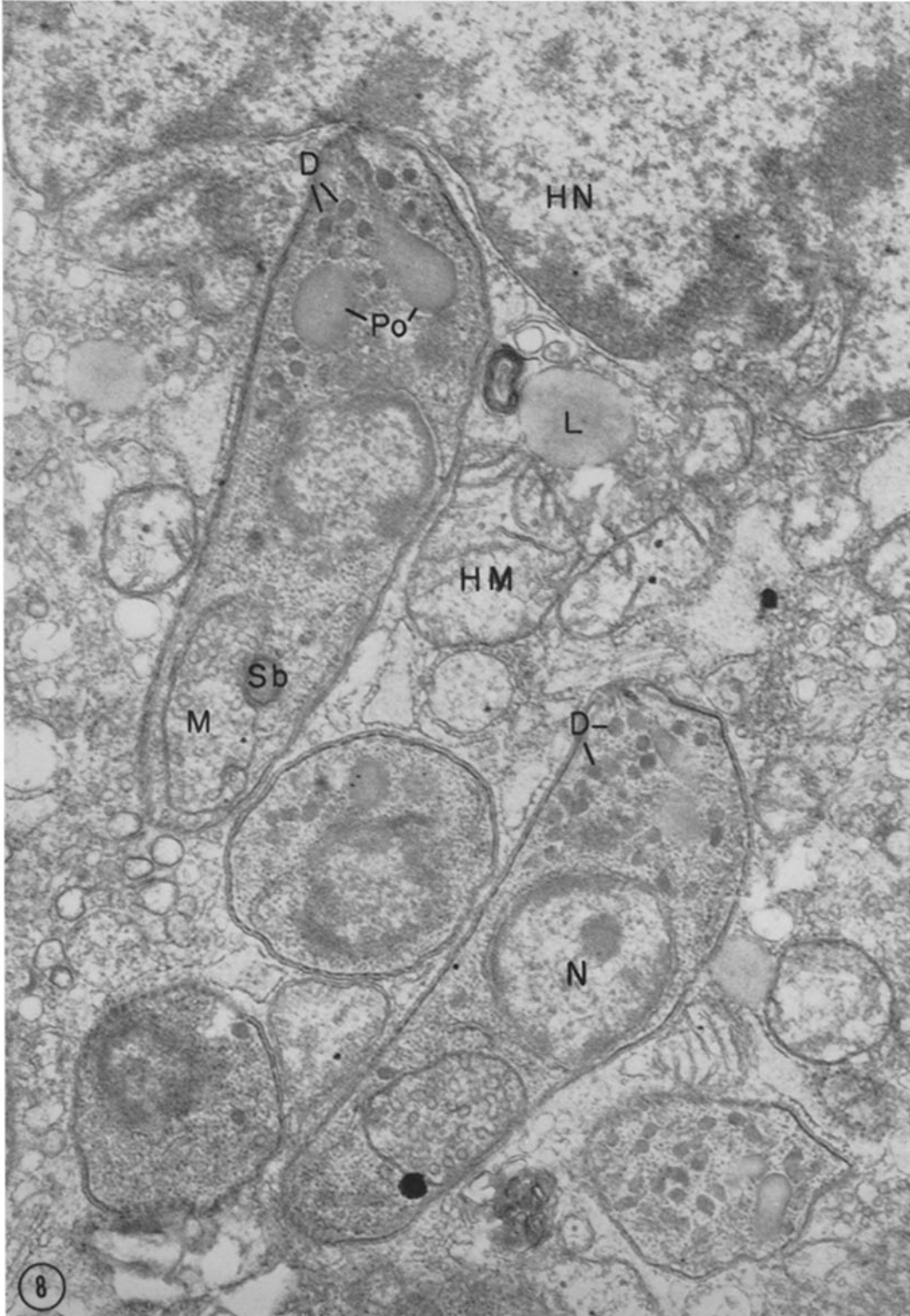


FIGURE 8 An early stage of infection, showing five merozoites embedded in the cytoplasm of a host cell. Longitudinal sections of two of the merozoites reveal the presence of the specialized organelles and structures characteristic of merozoites. The host cell mitochondria (*HM*) have lamellar cristae, in contrast to the tubular cristae of the parasite mitochondria (*M*). The host cell cytoplasm contains numerous vesicles, but their significance and possible relation to the invading parasites is unknown. $\times 28,000$.

scope studies of Huff et al. (16) that more than one crop of merozoites can be produced from a single large schizont. Therefore, the cytoplasmic elements which remain in the schizont at this point may be incorporated into merozoites which bud off at a later time. The entire process of cytoplasmic schizogony is well coordinated, and each new merozoite buds off in perfect synchrony with the ones adjacent to it.

DISCUSSION

Our observations on the exoerythrocytic cycle of *P. fallax* reveal a distinct pattern in the sequence of development. On the basis of the intricate changes in fine structure, we would distinguish the following three phases: first, the dedifferentiation phase, in which the merozoite loses its complex structure and transforms into a trophozoite; second, the growth phase, which consists of many nuclear divisions as well as parallel increases in mitochondria, endoplasmic reticulum, and ribosomes; and third, the redifferentiation and cytoplasmic schizogony phase, in which the specialized organelles and structures reappear and finally regroup in merozoites which eventually bud off from the mother schizont.

Within the parasite, the developmental processes and metabolic reactions must be well coordinated with one another, as the extensive organization and unity in development would seem to indicate. The growth phase and especially the later stages of redifferentiation and cytoplasmic schizogony do not occur randomly, but are characterized by a high degree of precision. During schizogony, for instance, there is a simultaneous appearance of complex organelles at many loci over the entire

surface of the schizont. Subsequently, when these numerous loci grow outward, their development is synchronous and the organelles which become part of the merozoite aggregate and flow into the growing buds in a definite sequence. The highly controlled and coordinated processes of the developing parasite result in the formation of many merozoites, each of which is identical and possesses great complexity.

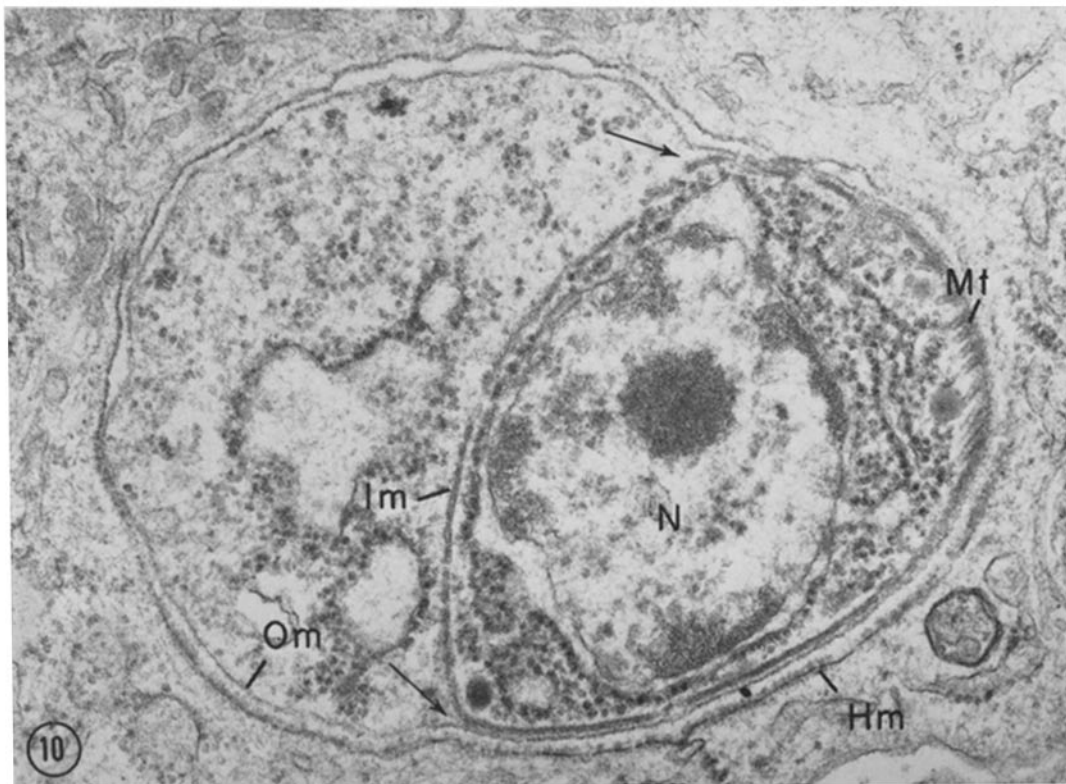
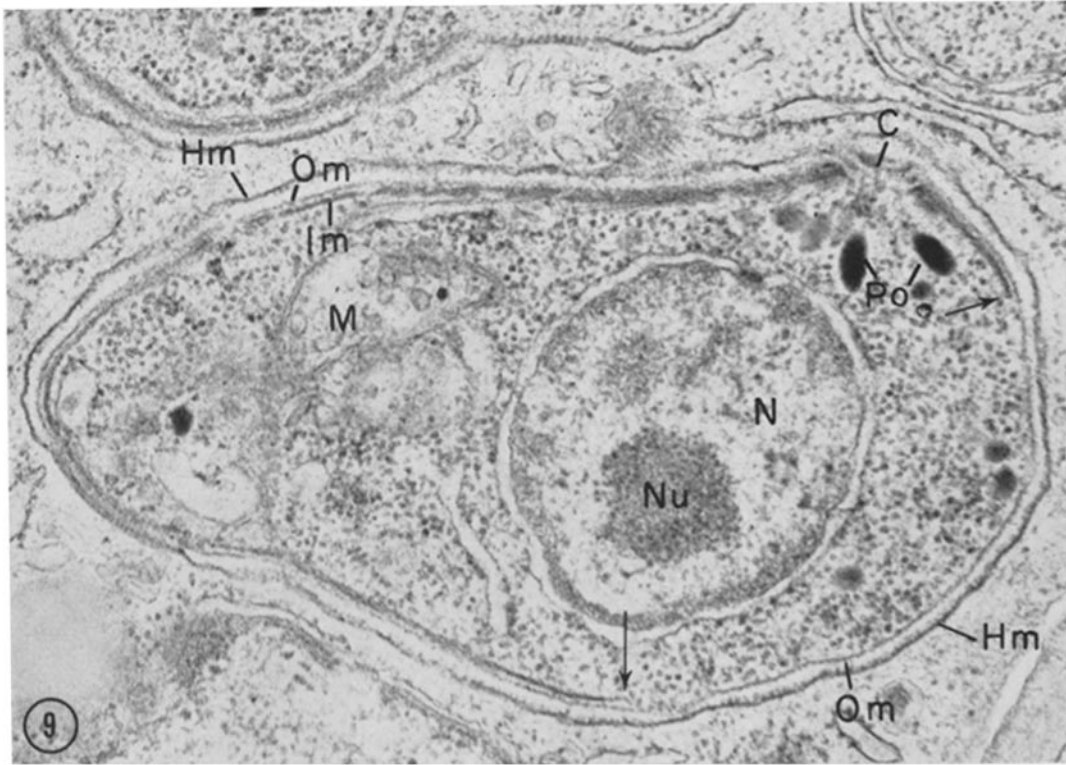
The developmental cycle we have observed with the electron microscope closely resembles that in the earlier findings of Huff et al. (16, 17) who studied living exoerythrocytic forms of *P. fallax* and *P. gallinaceum* with phase microscopy and cinemicrography. Those authors noted that in the first stage of infection the merozoites which were rounding into trophozoites lost much of their refractivity and thus their contents were easier to view. We have found that the specialized organelles and structures deteriorate in this first stage, and we feel that the loss in refractivity is a consequence of the deterioration.

Huff et al. (16) divide the subsequent stage of development into two parts: (1) nuclear schizogony, which includes many nuclear divisions without any cytoplasmic division, and (2) cytoplasmic schizogony, or the process of budding which yields the new merozoites. These two stages, we feel, correspond to the stages of growth and of redifferentiation and cytoplasmic schizogony as depicted in the electron microscope. Our studies show, however, that nuclear division is accompanied by a proliferation of all cellular components, namely the mitochondria, endoplasmic reticulum, and ribosomes.

We have also been able to observe the more de-

FIGURE 9 An early stage of infection, showing a merozoite in the process of rounding into a trophozoite. The changes in fine structure in the parasite which are evident at this point include: a widening of the conoid (*C*), the reduction in size of the paired organelles (*Po*) and of dense bodies, and the dissolution of large portions of the thick inner membrane (arrows). An additional membrane (*Hm*) which completely encloses the parasite is now visible. It lies outside of the parasite and shows no connection with it and is believed to be produced by the host cell. $\times 41,000$.

FIGURE 10 An example of an early stage of infection. The thick inner membrane (*Im*) of the parasite along much of its surface has pulled away from the outer membrane (arrows) of the parasite. Again, as in Fig. 9, the inner membrane (*Im*) appears to be in the process of breaking down. A few remnants of microtubules sectioned obliquely are evident. The host-produced membrane (*Hm*) completely surrounds the parasite and at this stage has numerous projections on its surface (lower right). $\times 57,000$.



tailed structural changes involved in nuclear division itself which light microscopy failed to show. Of particular interest are the large pores in the nuclear membrane at the place at which the spindle fibers converge. That these pores are the only places on the nuclear membrane which have been observed to break down, and that they are structurally close to the spindle fibers suggest that they may be active in spindle fiber formation. Analogous findings by Roth et al. (34) from a fine structure study of amoebae have revealed that pores over the entire surface of the nuclear envelope enlarge at a time when the spindle fibers are being synthesized. It has already been established in studies on nuclear division in sea urchin eggs that a major portion of the protein used in the construction of the spindle apparatus is derived from the cytoplasm (26). It would appear that the pores observed in dividing nuclei of *Plasmodium fallax* might be direct channels permitting the flow of large quantities of protein from the cytoplasm to the nucleus where it aggregates into spindle fibers.

The late stages in development of cytoplasmic schizogony have been documented in the time-lapse movies of Huff et al. (17) and Weathersby et al. (42). The first indication of schizogony is the appearance of slight protrusions or bulges over the entire surface of the parasite. Within each protrusion, a dark granule frequently resolved into two parts was observed. Huff et al. (16) tentatively interpreted these paired dark granules as nucleoli, although it was unclear to them why there were two. Wolcott (43) observed these same dark granules in earlier studies on the structure of several species of *Plasmodium*, and thought they were chromosomes. It is clear from the present study that the paired dark granules observed in the light microscope are neither nucleoli nor chromosomes but the paired organelles which appear in the tips of young buds as they grow out from the mother schizont.

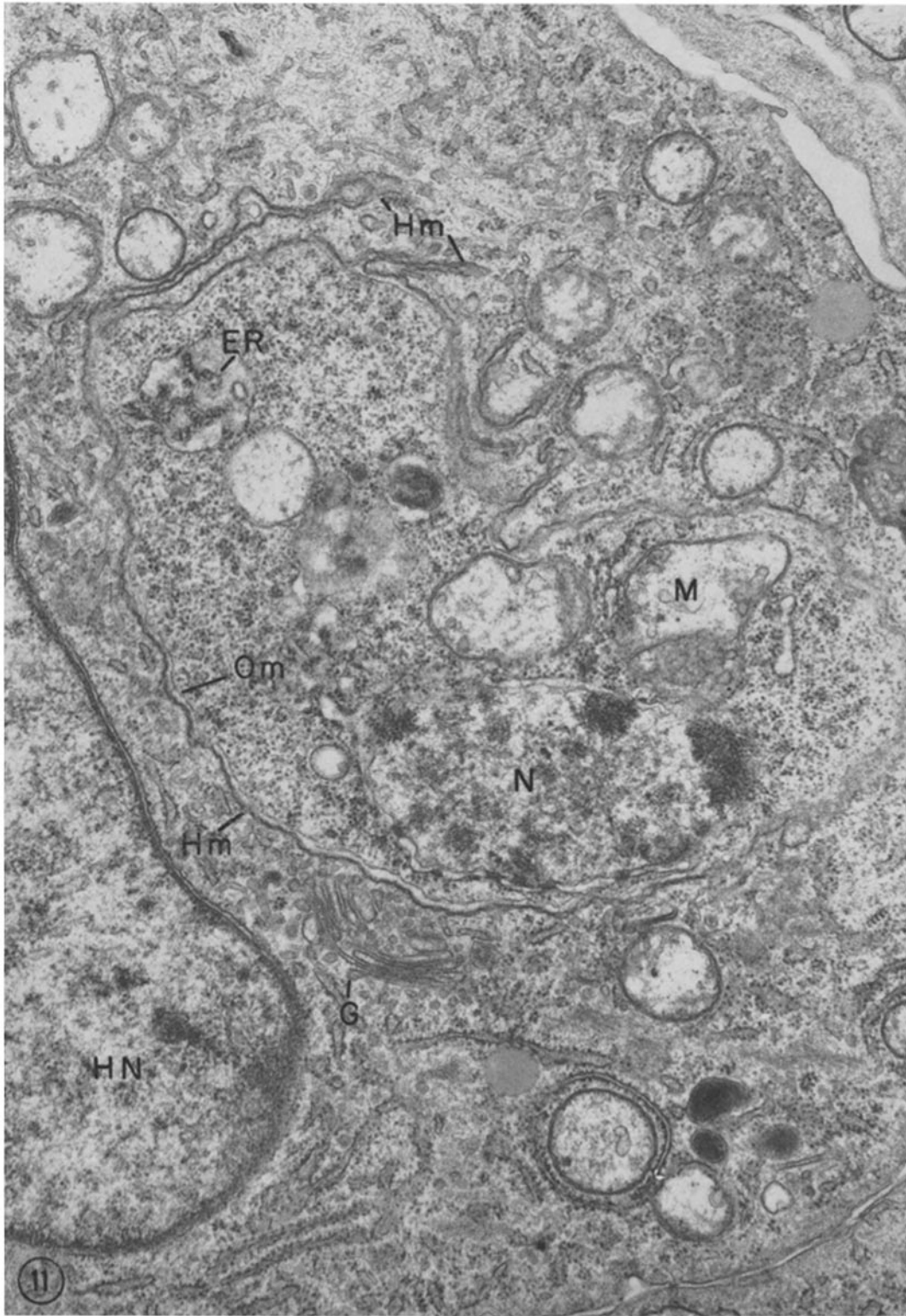
Only a few studies on the fine structure of exo-erythrocytic forms have been reported and these have concentrated mainly on the early and late stages in the developmental sequence (27, 28). In a study of *P. gallinaceum* grown in tissue culture cells, Meyer and Oliveira de Musacchio (27) drew attention to segmenting forms and to structures inside the budding merozoite. After a merozoite infected a cell and started to develop, these authors noted that its cytoplasm became less dense and that this density reappeared later during cytoplasmic schizogony. They did not observe, however, the intricate process of dedifferentiation and subsequent redifferentiation that we have reported.

Other reproduction cycles in the genus *Plasmodium*, namely sporozoite formation and erythrocytic merozoite formation, have been analyzed in the electron microscope. Duncan et al. (5) noted that when sporozoites bud from an oocyst, there is a thickening of the surface membrane at those regions where schizogony actually occurs, while areas on the surface of the oocyst which were not segmenting were covered by a single membrane. This finding corresponds with our observations on the elaboration of internal membranes around budding merozoites.

Of particular importance to our study is the report of the fine structure of the development of *P. lophurae* in its erythrocytic cycle made by Rudzinska and Trager (38). They found a similar concentration and subsequent flow of organelles into segmenting merozoites which we have reported. They did not observe many of the organelles which we find in mature merozoites, such as the conoid, small dense bodies, pellicular microtubules, or the spherical body associated with the mitochondrion.

Significant differences in interpretation, particularly of the role of the parasite membranes in the growing schizont, exist between our work and that

FIGURE 11 A parasite in its fully dedifferentiated state, containing a nucleus (*N*), mitochondrion (*M*), elements of the endoplasmic reticulum, and ribosomes. The original outer membrane (*Om*) of the merozoite is still the limiting membrane of the parasite, although it has expanded greatly as a consequence of parasite growth. The host membrane (*Hm*) is conspicuously denser than the outer membrane of the parasite. Foldings of the host membrane, exclusive of parasite membranes, can be observed extending deeply into the host cell cytoplasm. The surrounding host cytoplasm appears to be undamaged and contains Golgi bodies (*G*), mitochondria, elements of the endoplasmic reticulum, and ribosomes. $\times 30,000$.



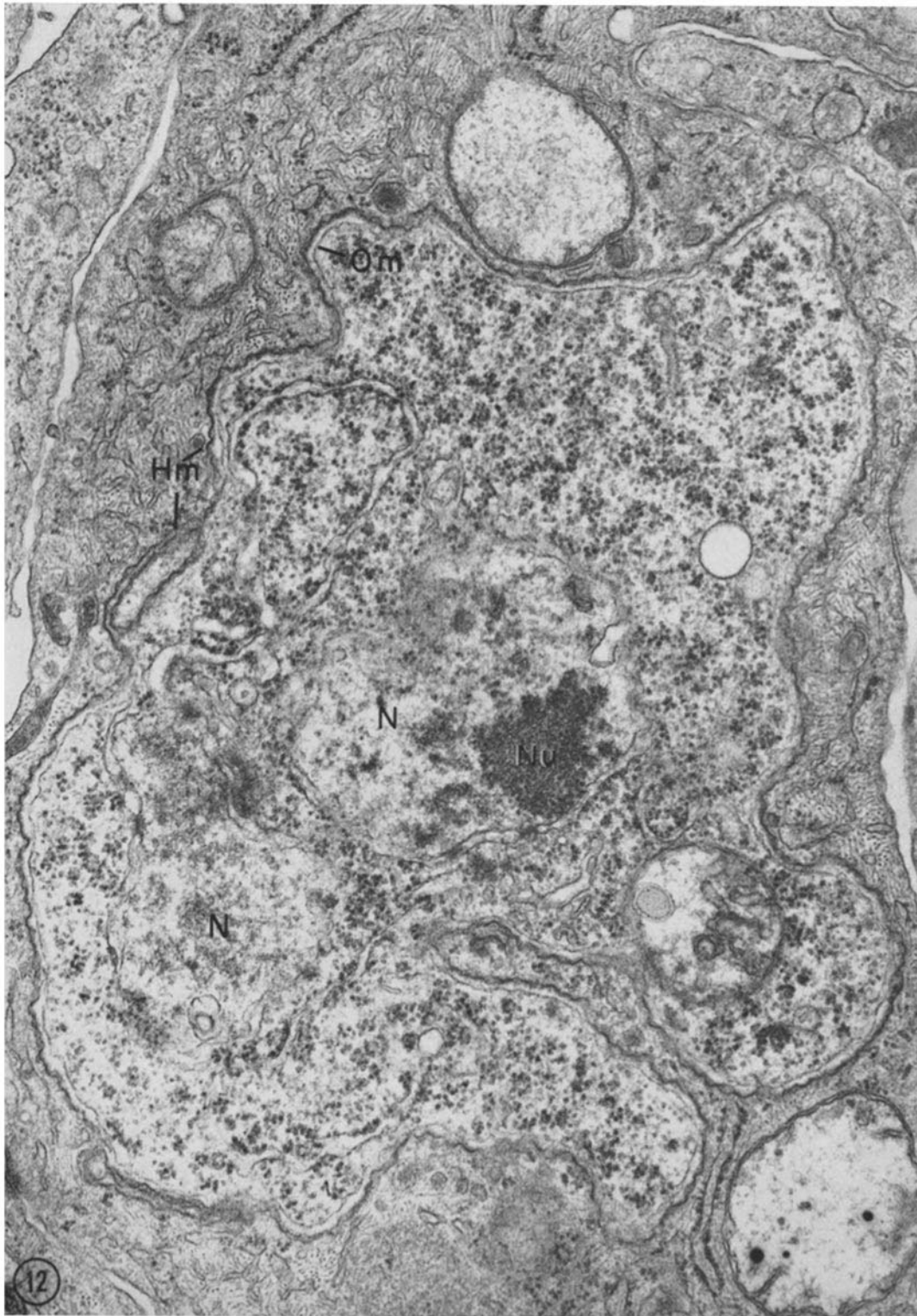


FIGURE 12 A more advanced stage in the growing form of the parasite. Cross-sections through two nuclear areas (*N*) are observed. One nucleus contains a prominent nucleolus (*Nu*) which appears to be composed of densely packed clusters of ribosomes. The parasite has an irregular shape. Foldings of the host membrane (*Hm*) can be observed extending into the host cytoplasm. $\times 42,000$.

of Rudzinska and Trager (38). While they state that the limiting membrane originally bounding the merozoite does not participate in the formation of new budding merozoites, we have found that the outer membrane of the invading merozoites remains the limiting membrane throughout all subsequent changes and developmental stages. This outer membrane undergoes extensive growth as the parasite increases in volume, and in the final stages of development becomes the limiting membrane of the new merozoites as they bud. All changes in parasite structure occur internal to this membrane, and except for its growth there appears to be no change in either its structure or function.

We should like to reiterate that the parasite in its exoerythrocytic developmental stages is also separated from the host cell by another and more prominent membrane, one which we believe is produced by the host cytoplasm.

Our findings on the sequential development of the parasite in its exoerythrocytic form are convincingly supported by the recent work of Aikawa (1) on the fine structure of the erythrocytic cycle of three avian parasites, *P. lophurae*, *P. cathemerium*, and *P. fallax*. From his work, it is apparent that development in these three parasites is fundamentally the same, and resembles strikingly the development of the parasite in the exoerythrocytic cycle. The differences between the erythrocytic and exoerythrocytic forms already known from light microscope studies, such as the smaller number and rounder shape of the merozoites produced from an erythrocytic schizont, are amply reaffirmed by Aikawa (1). However, of greater relevance to this work is the presence in the merozoite of the same organelles which we have reported, i.e. the conoid, paired organelles, dense bodies, mitochondrion and its associated spherical body, cytostome, and the complex triple-layered pellicle. Aikawa (1) has also found developmental sequences and changes in fine structure identical to the ones described in this paper.

Since our study deals with a model tissue culture system, in which parasite development might differ significantly from that in the living host, it is reassuring to find that the development of normal in vivo erythrocytic forms as demonstrated by Aikawa (1) is virtually identical to that which we have reported for in vitro grown exoerythrocytic forms. The striking similarity of the two kinds of asexual development is reason for our confidence that the development of the exoerythrocytic forms in the

tissue culture cells is analogous to that in a living avian host.

The exceedingly intricate structure of the merozoite has led us to speculate on the possible function of its many presumably specialized organelles. It should be pointed out that these specialized structures are not unique to erythrocytic and exoerythrocytic merozoites, since most of them (exclusive of the spherical body associated with the mitochondrion) have been observed in sporozoites (6, 9, 10, 12) and ookinetes (11) of *Plasmodium*. Electron microscope studies also show similar organelles in *Toxoplasma* (7, 13, 14, 20, 21, 24), *Sarcocystis* (21-23), and *Lankesterella* (8), organisms which are taxonomically related to *Plasmodium*. In each case, the motile form of the parasite is the one possessing certain specialized organelles.

The conoid, as first described and named by Gustafson et al. (14) in *Toxoplasma*, appears on the anterior end of the merozoite and is generally thought to be concerned with the attachment and subsequent penetration of the parasite into the host cell. We find this an attractive hypothesis, though a final proof of such an activity remains incomplete.

The paired organelles and dense bodies, observed in malarial parasites, which appear similar to the toxonemes of *Toxoplasma* and the sarconemes of *Sarcocystis*, have also been implicated in the processes of penetration of the parasite into the host cell (6, 9). Garnham et al. (9) have suggested that the paired organelles observed in sporozoites might contain proteolytic enzymes which would assist in lysing the plasmalemma of the host cell and thus allow the parasite to enter. Again, this theory has not been proven but seems plausible. Our own work shows that the paired organelles and neighboring dense bodies are among the first organelles to disappear in newly penetrated merozoites. Their early disappearance implies that the organelles are used in the initial stage of infection, and possibly in the actual entry process itself. Their close structural association with the conoid, and the presence of channels to the outside through the region of the conoid (Fig. 8), further suggest that the paired organelles are active in penetration.

The spherical body associated with the mitochondrion appears to be, at present, unique to *Plasmodium*, and as yet has been observed only in the exoerythrocytic and erythrocytic merozoites. Its close structural relationship with the mitochondrion suggests that it may have a functional relationship also. In mature merozoites capable of

moving in a free state from one cell to another, there must be great requirement for energy, and it seems reasonable that the spherical body could be a rich deposit of a substrate, such as lipid, to which the mitochondrion would have immediate access and which it could quickly convert into the necessary ATP. Palade (30) has observed, in pancreatic cells of the fasted guinea pig, similar configurations in which mitochondria are wrapped around lipid droplets. He suggests that the lipid droplet might be composed of fatty acids which the fatty acid oxidases of the mitochondrion would be able to completely metabolize. Another example is found in fertilized sea urchin eggs in which groups of mitochondria have been observed conspicuously associated with large lipid droplets (31).

The complex pellicle serves a number of related functions. The outermost membrane appears to be the limiting structure of the merozoite. The thick inner membrane probably functions as a cytoskeleton to give the merozoite its shape. The pellicular microtubules could serve as a cytoskeleton, but more likely are the structures of motility. Complex pellicles have been observed in the motile forms of all malaria parasites so far studied, and they appear to be common features of many different protozoa as reviewed by Pitelka (32). Microtubules are frequently associated with the pellicle of protozoa and have been particularly well described in trypanosomes (19, 41). Meyer and Porter (29) were the first to suggest that the pellicular fibrils observed in electron micrographs of trypanosomes are composed of contractile proteins which might function in the motility of the organism.

The cytostome does not appear to function in

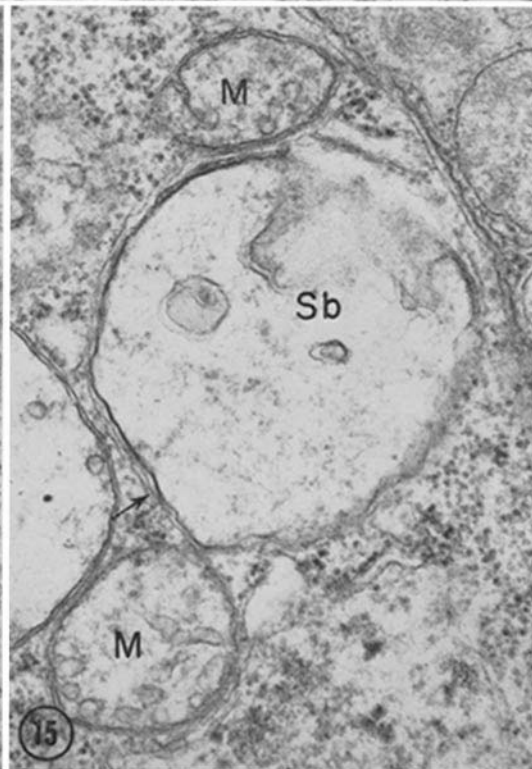
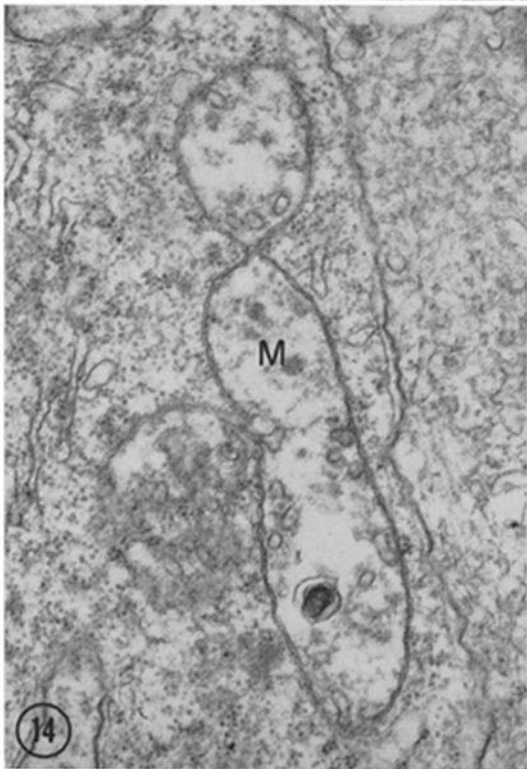
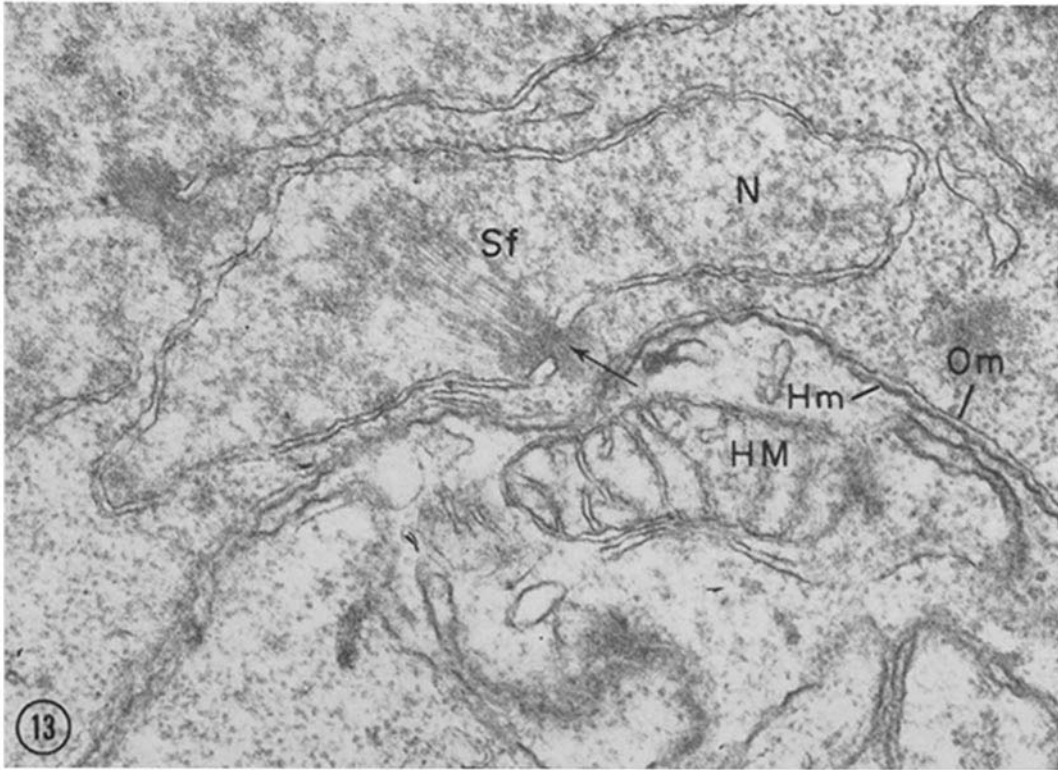
food intake in the exoerythrocytic forms of the parasite, though it is established that it is the cavity through which the growing erythrocytic forms take in red blood cell cytoplasm (2). We see no examples of host cytoplasm invagination and ingestion, and the decreased diameter of the opening of the cytostome in growing exoerythrocytic forms, only half as wide as the same structure in mature merozoites, is compatible with its inactivity.

It seems clear, too, that the cytostome does not function as a "micropyle" or the place of protoplasm emergence as suggested by Garnham and co-workers (6, 10). Searching for a possible explanation for this cavity on the surface of sporozoites, Garnham et al. (10) theorized that upon infection sporoplasm would emerge through this pellicular pore and initiate the preerythrocytic cycle, hence the name "micropyle." They argued that the triple-layered pellicle, because of its rigidity, would not allow the sporozoite to become rounded and start its developmental cycle, and that it would, therefore, be necessary for sporoplasm to be extruded. Two lines of evidence, however, shed doubt on the pellicular cavity as a place of sporoplasm emergence; firstly, it is involved in red blood cell cytoplasm uptake, the reason for changing its name from "micropyle" to "cytostome" (2); and secondly, as we have shown in this paper, in the merozoite the inner layers of the thick pellicle, which closely resembles that of the sporozoite, break down shortly after entry, thus destroying any rigidity, and permit the rounding of the organism. We would presume that a similar phenomenon occurs in the development of sporozoites.

FIGURE 13 A parasite nucleus (*N*) in the process of dividing. A fan-shaped group of spindle fibers (*Sf*) can be observed extending part way across the nucleus. The individual fibers are microtubular in structure, having a dense cortex and a light core, and measure approximately 20 $m\mu$ in diameter. The nuclear membrane does not break down except at the point where the spindle fibers converge (arrow). At this region a homogeneous material is evident, bridging the gap between nucleus and cytoplasm. $\times 45,000$.

FIGURE 14 An elongated mitochondrion of the parasite, which is greatly constricted and presumably in the process of dividing. $\times 31,000$.

FIGURE 15 The spherical body (*Sb*) in growing forms of the parasite becomes swollen, and its contents appear diluted and stained lightly. It can easily be distinguished from the mitochondrion by the presence of a triple-enclosing membrane and the absence of tubular cristae. It eventually disappears and is not observed in advanced stages of development. $\times 50,000$.



The possibility exists that the cytostome is totally inactive in both the sporozoite and exoerythrocytic forms, and that it is carried through these stages only to function in the erythrocytic cycle.

The apparent inactivity of the cytostome poses a question concerning the mechanism of feeding in the growing exoerythrocytic forms. While we continually observe irregularities in the surface of growing parasites (Fig. 12), we have never discovered processes which could be interpreted as host cytoplasm engulfment or phagotrophy as described by Rudzinska and Trager (36, 37) and Rudzinska et al. (39) in growing erythrocytic forms. Nor have we been able to identify pinocytosis at the surface of the limiting membrane of the parasite. In addition, we have not observed, within

the parasite, vesicles containing what we could identify as host cell cytoplasm. Our findings suggest that the exoerythrocytic stage feeds by a process involving diffusion of necessary metabolites and precursors, in the form of small molecules, from the host cytoplasm into the parasite. The presence in the parasite of mitochondria and of an extensive matrix of endoplasmic reticulum and ribosomes indicates that it is capable of synthesizing its own ATP and its own structural proteins and enzymes required for metabolic reactions.

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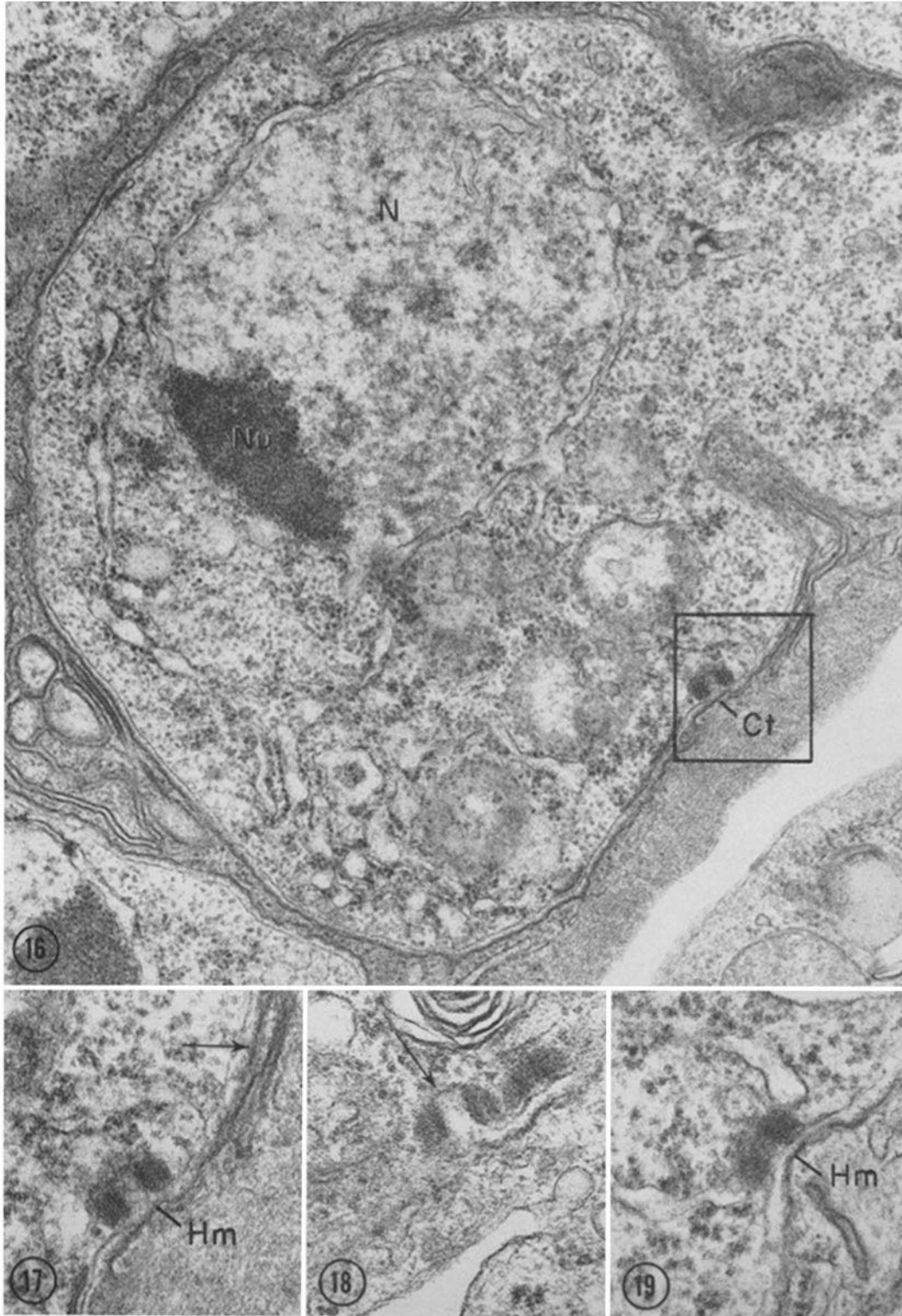
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FIGURE 16 A cross-section through a cytostome (*Ct*) observed on the surface of an actively growing parasite. $\times 42,000$.

FIGURE 17 A higher magnification view of the cytostome in Fig. 16. It is clearly evident that the host membrane (*Hm*) extends across the opening of the cytostome cavity and does not enter into it. Note the unit structure of the host membrane (arrow). $\times 84,000$.

FIGURE 18 A second example of a cytostome on a growing parasite. The two short segments characteristic of cytostomes are particularly prominent. In both Figs. 17 and 18, measurements show that the cytostome has an inner diameter of 40 to 50 μ or about one-half its diameter in the merozoite (compare with Fig. 4). The lumen of the cytostome cavity is clear, thus presumably empty, and the basal membrane (arrow) has not expanded. $\times 84,000$.

FIGURE 19 A third example of a cytostome again shows that the host membrane does not extend into its cavity. Observations on all three cytostomes shown here support our view that they do not function in feeding in the exoerythrocytic forms of the parasite. (See text for a discussion). $\times 84,000$.



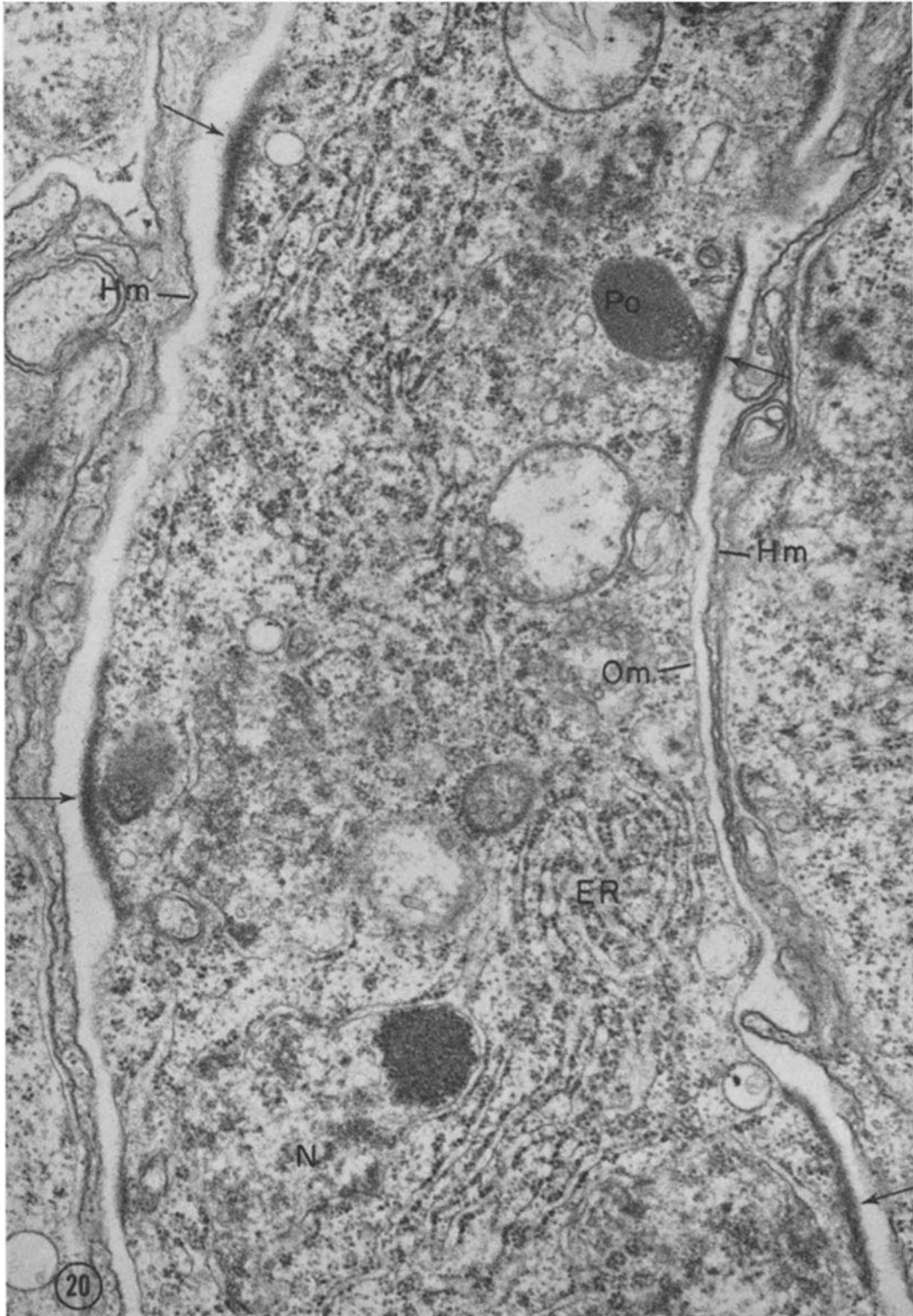


FIGURE 20 A very early stage in cytoplasmic schizogony in the parasite. Distinct areas on the surface of the parasite become thickened (arrows). Adjacent to these areas large oval bodies, presumably the paired organelles, are observed. Within the parasite cytoplasm, compact swirls of the granular endoplasmic reticulum (*ER*) are evident. The outer membrane of the schizont has separated from the host membrane, leaving a wide clear area. In subsequent stages, this clear area becomes more pronounced. $\times 36,000$.

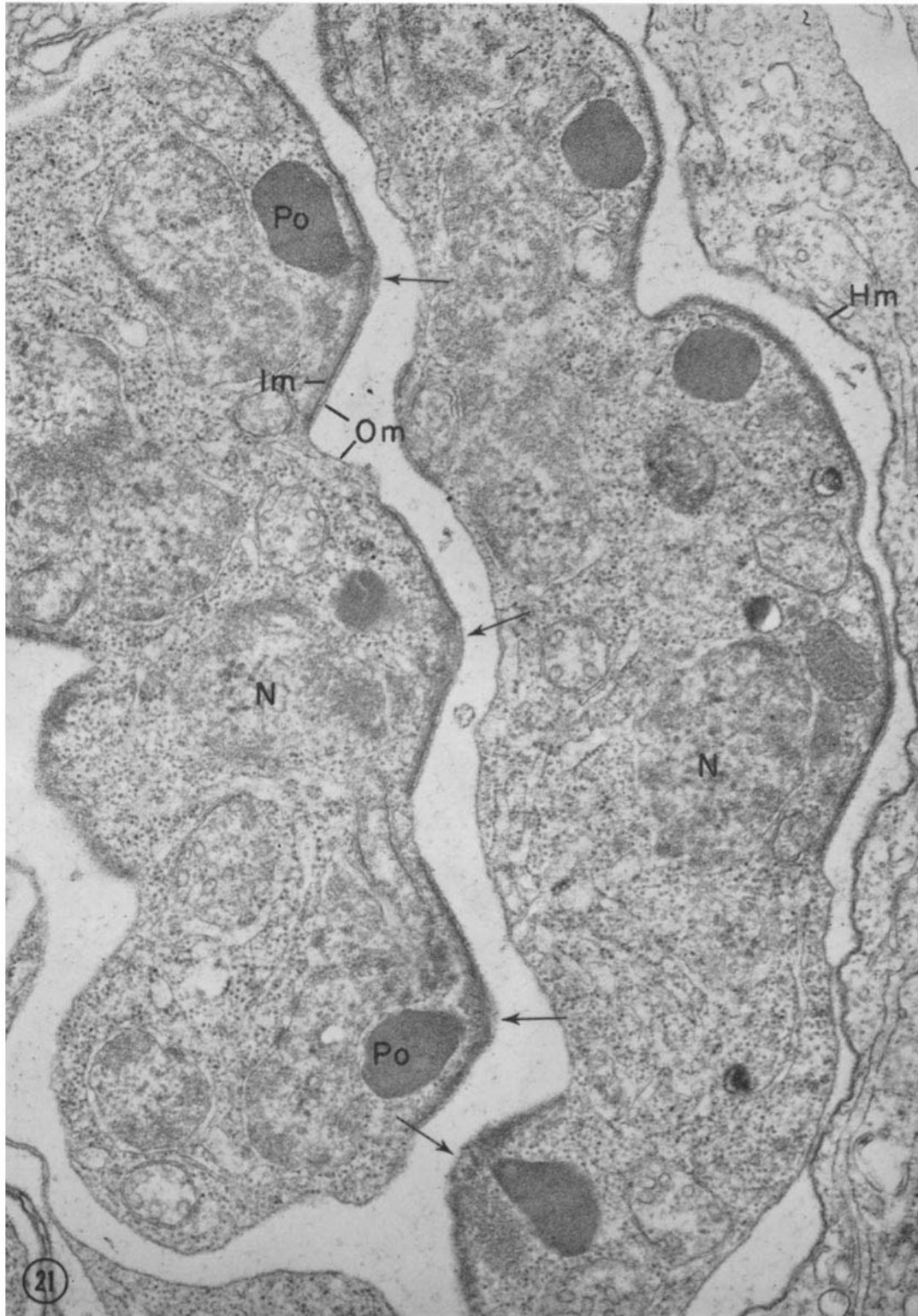
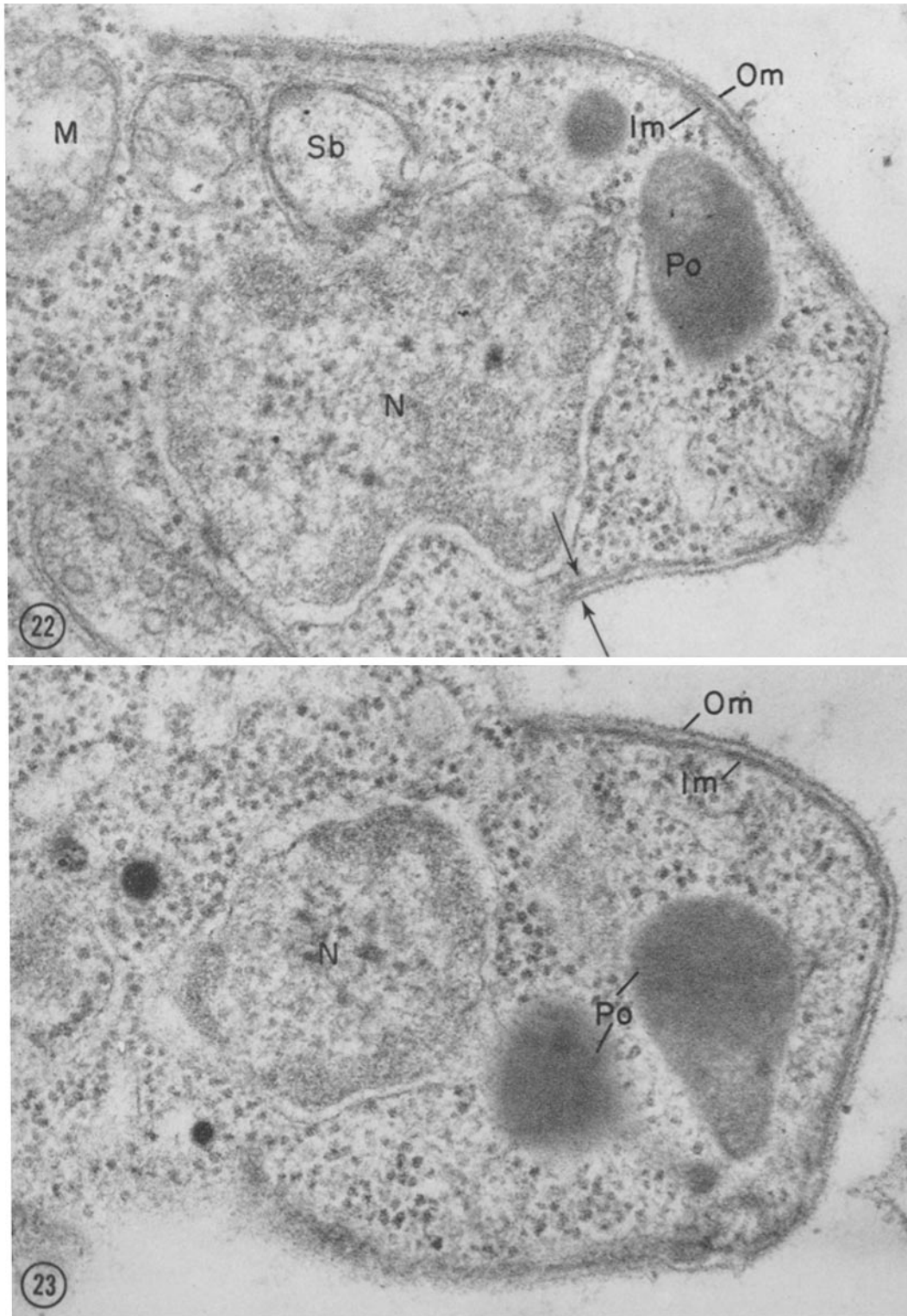


FIGURE 21 A section through the surface of a schizont, showing the growing buds (arrows). The thickened areas grow out, becoming pyramid shaped. Two membranes can now be distinguished, a thin outer and a thick inner, corresponding to the double layer of the merozoite pellicle. Areas on the schizont between the growing buds are covered by a single membrane only. Large oval bodies are most likely a single member of the paired organelles (*Po*). Numerous nuclei and mitochondria are visible. $\times 36,000$.



FIGURES 22 AND 23 Two examples, at higher magnification, of growing buds in early stages of development. Paired organelles (*Po*), nuclei (*N*), mitochondria (*M*), and associated spherical body (*Sb*) are observed in the process of flowing into the expanding bud. Note the fine structure of the pellicle in Fig. 22 (arrows). The outer membrane is a unit structure 6 to 7 μ wide, whereas the inner membrane is composed of two closely packed unit structures with a total width of 15 μ . $\times 80,000$.

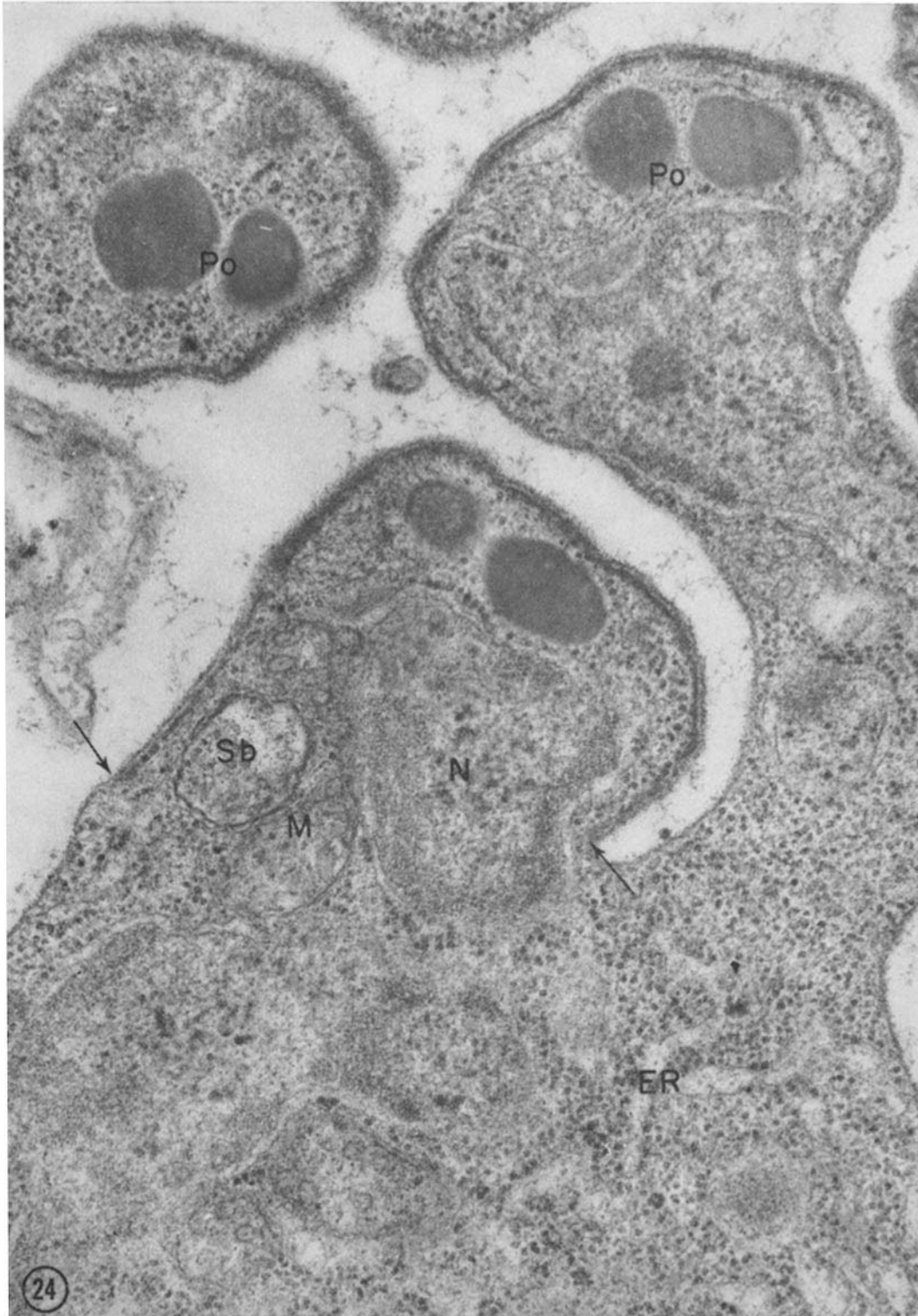


FIGURE 24 A more advanced stage of cytoplasmic schizogony. A double-layered pellicle covers the areas which are budding, whereas a single membrane bounds the adjacent regions (arrows show the transition). Paired organelles are observed in the anterior ends of the bud. $\times 48,000$.



FIGURE 25 Two merozoites which have virtually completed their budding from the mother schizont. The paired organelles and dense bodies have formed in the anterior tips of the merozoites. In sequence, the nucleus, the mitochondrion with its associated spherical body, the elements of the endoplasmic reticulum and the ribosomes flow in. Only the final pinching-off is incomplete. A sharp transition is evident at the zone in which the thick inner membrane terminates (arrows). Elements of the endoplasmic reticulum and ribosomes are still present in the schizont. $\times 58,000$.

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