THE FEEDING MECHANISM OF AVIAN MALARIAL PARASITES

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ABSTRACT

Electron microscope studies of the erythrocytic forms, including gametocytes and asexual schizonts, of the protozoa *Plasmodium fallax*, *P. lophurae*, and *P. cathemerium*, have revealed a "cytostome," a specialized organelle of the pellicular membrane which is active in the ingestion of host cell cytoplasm. In material fixed in glutaraldehyde and postfixed in OsO_4, the cytostome appears in face view as a pore limited by two dense circular membranes and having an inside diameter of approximately 190 mμ. In cross-section, the cytostome is a cavity bounded on each side by two dense segments corresponding to the two dense circles observed in face view; its base consists of a single unit membrane. In the process of feeding, the cytostome cavity enlarges by expansion of its membrane, permitting a large quantity of red cell cytoplasm to come into contact with the cytostome wall. Subsequent digestion of erythrocyte cytoplasm occurs exclusively in food vacuoles which emanate from the cytostome invagination. As digestion progresses, the food vacuoles initially stain more densely and there is a marked build-up of hemozoin granules. In the final stage of digestion, a single membrane surrounds a cluster of residual pigment particles and very little of the original host cell cytoplasm remains. The cytostome in exoerythrocytic stages of *P. fallax* has been observed only in merozoites and does not seem to play the same role in the feeding mechanism.

INTRODUCTION

The mechanism of feeding in malarial parasites has been the subject of a number of papers, most notably those from the laboratory of Rudzinska and Trager (17–20). Their studies on the fine structure of actively growing erythrocytic stages of *Plasmodium lophurae* and *P. berghei* demonstrated the presence of food vacuoles which they believed to be formed by random invagination of the parasite membrane around red cell cytoplasm. They interpreted this process as intracellular phagotrophy and have suggested that this is the mechanism by which malarial parasites engulf red cell cytoplasm. Similar findings, also interpreted as intracellular phagotrophy, have been made by Duncan et al. (2) in the study of the fine structure of gametocytes of *P. cathemerium*, and more recently by Ristic and Kreier (15) in the study of the asexual erythrocytic stages of *P. gallinaceum*.

In the course of our study of the fine structure of both exoerythrocytic and erythrocytic stages of *P. fallax* and of the erythrocytic stages of *P. lophurae* and *P. cathemerium*, using newer and improved techniques of tissue preservation, we have attempted to further clarify the mechanism of feeding. A specialized pore on the surface of the organism, not previously described in either

erythrocytic or exoerythrocytic stages of *Plasmodium*, appears in the erythrocytic forms to be the place through which the parasite ingests red cell cytoplasm. Because of its involvement in feeding we are calling this surface pore a "cytostome."

This paper will be particularly concerned with a detailed description of the cytostome and its ultrastructural relationship with the engulfment of red cell cytoplasm. The process of food ingestion in the erythrocytic forms of *Plasmodium* will be analyzed and compared with examples of cytostomal feeding in other protozoa. Finally, special attention will be devoted to a discussion of the apparent differences in the mechanism of feeding between the actively growing erythrocytic and exoerythrocytic forms. A more extensive study of the fine structure of other aspects of developing exoerythrocytic and erythrocytic malarial parasites will be presented in a forthcoming paper.

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 (1) derived from embryonic turkey brain cells, served as part of the material for this study. The initial culture material was prepared by inoculating 14-day-old turkey embryos with material previously isolated from an infected turkey brain. After 6 days of incubation, the 20-day-old embryos were sacrificed and the brain tissue collected. Portions of heavily infected tissue were trypsinized and then inoculated in culture flasks (T flasks) in a medium containing 50% Diploid Growth Medium, and 50% of a mixture composed of 90% mix 199 (Microbiological Associates, Inc., Bethesda), 10% fetal calf serum, and 1×10^{-7} M folinic 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 spun down 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 erythrocytic stages of *P. fallax* and *P. lophurae* (obtained from Dr. R. B. McGhee, University of Georgia, Athens, Georgia) were maintained in turkeys, and of *P. cathemerium* (obtained from Dr. R. D. Manwell, Syracuse University, Syracuse, New York) in canaries through successive blood passages. To obtain material for examination in the electron microscope, about 1 ml of blood was drawn from birds showing 40 to 60% parasitemia.

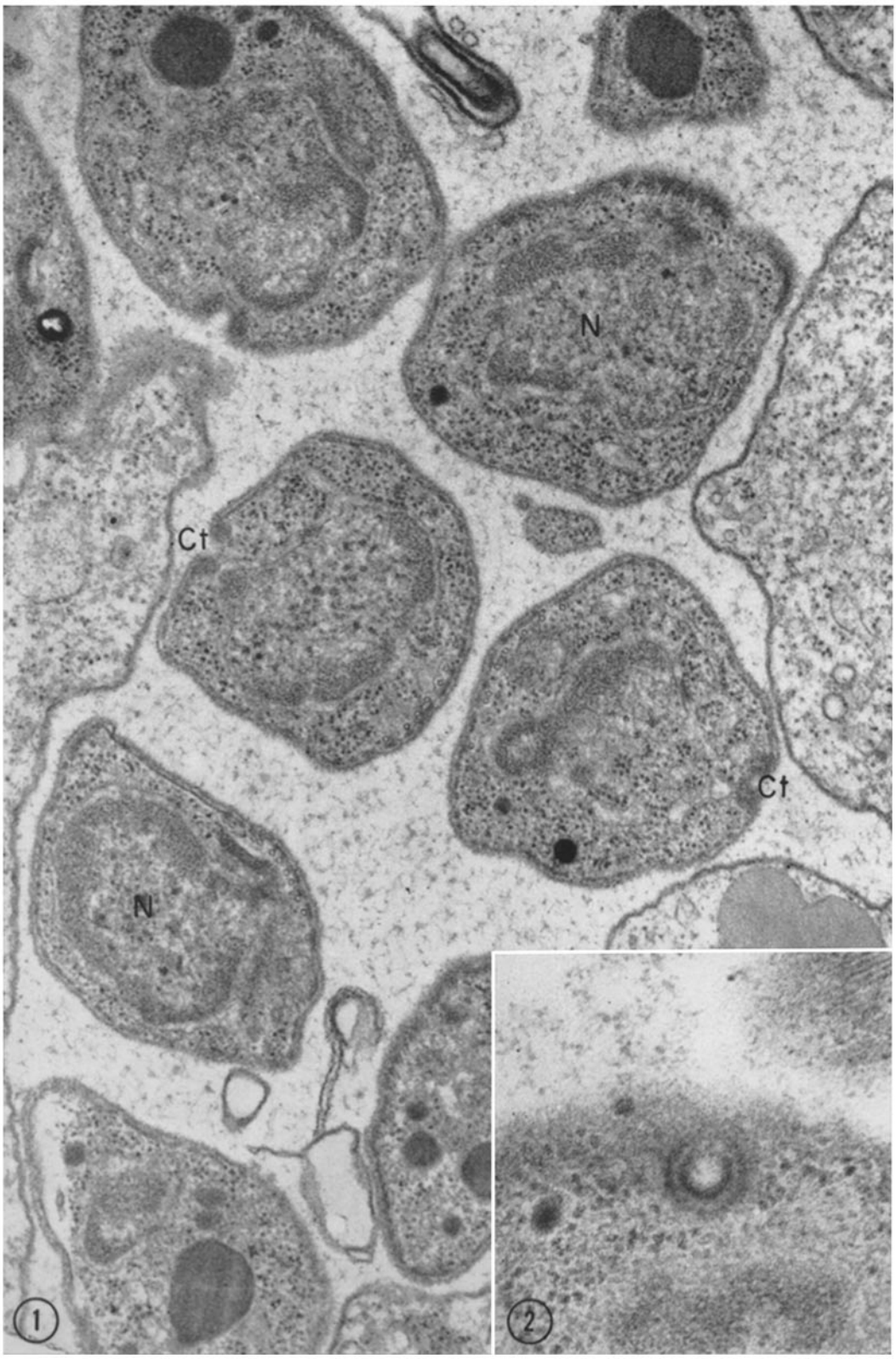
The subsequent stages for preparing tissue for analysis in the electron microscope were very similar for both the exoerythrocytic and erythrocytic forms and, therefore, will be considered together. The tissue was fixed for 1 hr in 1.25% glutaraldehyde solution, buffered with 0.05 M PO_4 at pH 7.3 and containing 4% sucrose. In the case of the erythrocytic forms, the blood was centrifuged after fixation for 10 min, at 200 *g*. The blood or brain tissue culture cells were washed in a buffered PO_4-sucrose solution with 3 changes. The material was left in the last wash for at least 1 hr, and frequently it was left overnight at 4°C.

Abbreviations

Ct, cytostome
F, food vacuoles
M, mitochondrion
Mb, marginal bands
Mp, malarial pigment granules
N, nucleus
Po, paired organelles
Sb, spherical body

FIGURE 1 A group of mature exoerythrocytic merozoites of *P. fallax* still enclosed by the cytoplasm of the turkey culture host cell. Three examples of cytostomes (*Ct*) are evident. Each merozoite possesses a thin outer membrane and a thicker inner membrane. Beneath the inner membrane, cross-sections of a few microtubules can be seen. The cytoplasm of the merozoite contains a large nucleus (*N*), elements of endoplasmic reticulum, and ribosomes. × 40,000.

FIGURE 2 A tangential section along the outer surface of an exoerythrocytic merozoite of *P. fallax* shows the two prominent concentric circles constituting the cytostome. × 68,000.


N
Ct
Ct
N
1
2

Following the wash, 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 finally embedded in Epon 812 according to the method of Luft (11). In some cases, the tissue was stained in 5 to 10% uranyl acetate in absolute ethanol prior to embedding.

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 (if they had not already been stained in the dehydration) and with lead citrate (14). The sections were examined in either an RCA EMU-3G or a Siemens Elmiskop 1A.

OBSERVATIONS

Studies on the ultrastructure of the exoerythrocytic forms of *Plasmodium fallax* and on the erythrocytic forms of *P. fallax*, *P. lophurae*, and *P. cathemerium* have revealed the presence of a specialized structure, or cytostome, on the surface of the parasite. In the exoerythrocytic stages of *P. fallax*, the cytostome has been observed only in the mature merozoite, whereas in the erythrocytic stages of the three parasites studied the cytostome has been observed in all phases of development including gametocytes, and appears in each case to be functioning in the uptake of erythrocyte cytoplasm.

1. *The Cytostome in Erythrocytic and Exoerythrocytic Merozoites*

In exoerythrocytic and erythrocytic merozoites, the cytostome occupies a position about midway between the anterior and posterior ends of the merozoite and appears as a depression in its pellicle. While cytostomes are not observed in every section, no more than one has been observed in any given plasmodial cell. Fig. 1 clearly shows the cytostomal depression in cross-sections of exoerythrocytic merozoites of *P. fallax*. The cytostomal pore of the exoerythrocytic merozoite usually measures 80 to 100 mμ in the inside diameter and approximately 250 mμ in the outside diameter. The cytostome on the erythrocytic merozoite resembles that in the exoerythrocytic merozoite in every respect except that it is much larger (Figs. 3 to 6). The inner diameter measures 170 to 200 mμ, or about twice that for the cytostome of the exoerythrocytic forms, while the outer diameter measures 280 to 340 mμ. The inner cross-sectional area, therefore, would be nearly four times greater in the erythrocytic cytostome, and this may have some significant relationship to feeding.

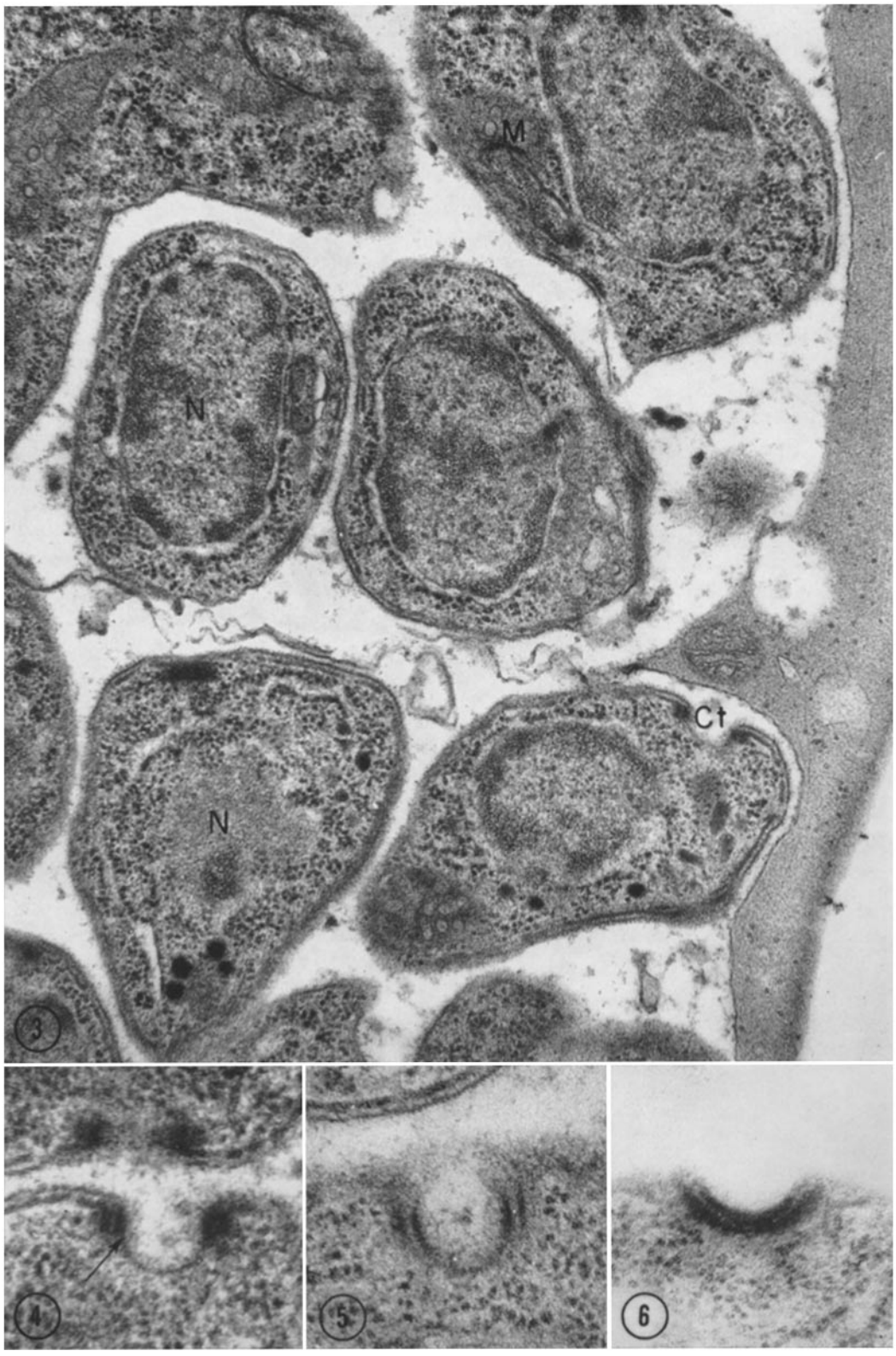
The wall of the cytostome, either exoerythrocytic or erythrocytic, when viewed in cross-section consists of two very short, densely staining lines (Figs. 1, 3, and 4), so prominent that they facilitate the finding of the cytostome in the examination of the micrographs. In some micrographs (Figs. 3 and 4) the inner limiting thick segment of the cytostome appears to be continuous with the thin single membrane which forms the base of the depression. The outer dark segment attaches to the inner pellicular membrane which runs over the entire surface of the merozoite (Figs. 1, 3, and 4).

FIGURE 3 A cluster of mature erythrocytic merozoites of *P. cathemerium* within a red blood cell. On one merozoite a prominent cytostomal depression (*Ct*) is visible. The sides of the cytostome are bounded by two short dark segments and the base is formed by a single membrane. Erythrocytic merozoites possess a double-layered pellicle similar to that of the exoerythrocytic merozoite. Nuclei (*N*), mitochondria (*M*), endoplasmic reticulum, and ribosomes are evident. × 40,000.

FIGURE 4 A high magnification micrograph reveals in closer detail a cross-section of the cytostome of an erythrocytic merozoite of *P. lophurae*. The dark segments bounding the cytostome are particularly prominent. The inner segment is continuous with the basal membrane of the cytostome (arrow), while the outer segment is continuous with the thick inner membrane of the merozoite pellicle. × 68,000.

FIGURE 5 Face view of the cytostome of the erythrocytic merozoite of *P. fallax*, showing the two concentric circles which correspond to the short segments observed in cross-section. Compare this face view micrograph with the face view of Fig. 2, and note the much larger size of the erythrocytic cytostome. × 68,000.

FIGURE 6 An oblique section of a cytostome of *P. fallax*, depicting the crescent-shaped depression. × 68,000.


M
N
N
Ct
3
4
5
6

Micrographs from sections cut tangential to the surface of the merozoite reveal the circular profile of the cytostome. The two concentric circles observed in Figs. 2 and 5 correspond to the short, dark segments seen earlier in the cross-sections. In the erythrocytic merozoite which is not yet actively feeding, the lumen of the cytostome is electron transparent (Figs. 3 to 5). Slightly more oblique sections of the cytostome (Fig. 6) show a crescent-shaped depression. The two densely staining circles seen in face view have been superimposed upon each other in the oblique section and yield a single wide, but rather diffuse border in which very little fine structure can be discerned.

2. *The Cytostome in Actively Feeding Erythrocytic Stages*

An erythrocytic merozoite, after invading a new red blood cell, "rounds up" and begins ingesting host cell cytoplasm which is largely composed of hemoglobin and, therefore, stains uniformly with osmium tetroxide. Since these are avian red blood cells they have a nucleus and, in addition, the cytoplasm contains a few organelles such as mitochondria, endoplasmic reticulum, and marginal bands (Fig. 7).

In an early stage of feeding in *P. cathemerium* (Fig. 7), a small amount of host cell cytoplasm containing hemoglobin bulges into the orifice of the cytostome. Additional examples of initial steps in the feeding mechanism, also in *P. cathemerium* (Figs. 8 and 9), show young actively growing trophozoites with slightly larger cytostome cavities containing red cell cytoplasm. In these early stages, two distinct membranes surround the invagination. The membrane next to the parasite cytoplasm, the basal membrane of the cytostome, expands inward creating a cavity which precedes the ingestion of red cell cytoplasm (Fig. 7). The second membrane, immediately adjacent to the red cell cytoplasm, is the membrane originally produced by the red cell in response to infection and functions in separating the parasite from the host cell cytoplasm.

When an actively feeding cytostome is observed in face view, its lumen is no longer electron transparent, as it appeared earlier in merozoites, but now possesses a staining characteristic of the red cell cytoplasm (Figs. 10 to 13). This similarity in staining leaves little doubt that the material observed in the expanded cytostome cavity is red cell cytoplasm. The presence of host cytoplasm in the lumen of the cytostome of a presegmenter (a schizont undergoing cytoplasmic division) (Fig. 19) suggests that the parasite is still actively feeding at a late stage of development.

In the process of feeding, the cytostome cavity grows to a rather large size; some invaginations have been observed which penetrate almost 1 μ (850 mμ) into the parasite cytoplasm. Even though large amounts of host cell cytoplasm bulge into the cytostome cavity, the diameter of the orifice does not change.

When the invagination reaches a certain—though undetermined—size, the food vacuoles pinch off from it. The newly formed food vacuoles appear to have only a single membrane envelope in contrast to the invaginations containing host cell cytoplasm within the cytostome which are bounded by the above-mentioned double membrane (Figs. 15 to 18).

Digestion of host cell cytoplasm by the parasite occurs within these food vacuoles. As digestion progresses, the engulfed red cell cytoplasm initially becomes more densely stained and there is a marked build-up of residual pigment particles (Figs. 14, 16 to 18). In the final stages of digestion, a single membrane surrounds a cluster of residual pigment particles and some red cell cytoplasm of markedly reduced density (Fig. 20).

Occasionally found within trophozoites are aggregates of host cell cytoplasm which are limited by a double membrane and are lacking any connection with the cytostome (Fig. 14) or with the host cytoplasm. This observation can be interpreted in one of two ways: either the cytostome is in a plane different from the one in which the section is made, or the seemingly pinched-off host cell cytoplasm may have resulted from an invagination of the trophozoite plasma membrane at a place on the parasite other than the cytostome. The latter has been found to occur to a slight degree in *P. lophurae* (Fig. 14). Within a single parasite of *P. lophurae*, examples showing several invaginations which are continuous with the host cytoplasm have been observed. In other parasites, *P. fallax* and *P. cathemerium*, this phenomenon is rare. In any event, these double membrane-bounded aggregates have always the same electron opacity as host cell cytoplasm and do not contain malarial pigment granules. They are probably related to the ameboid motion of the parasite and should be

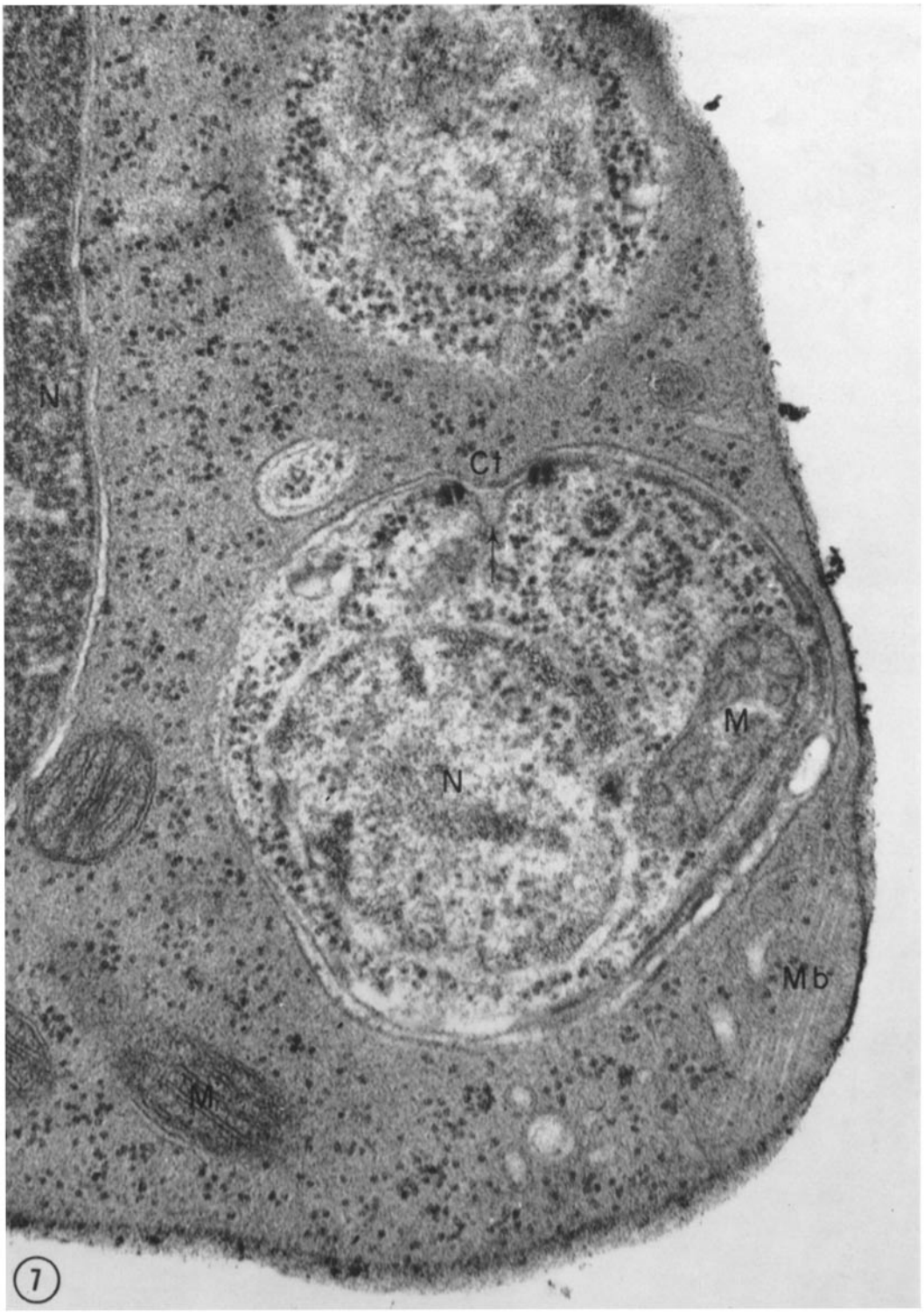
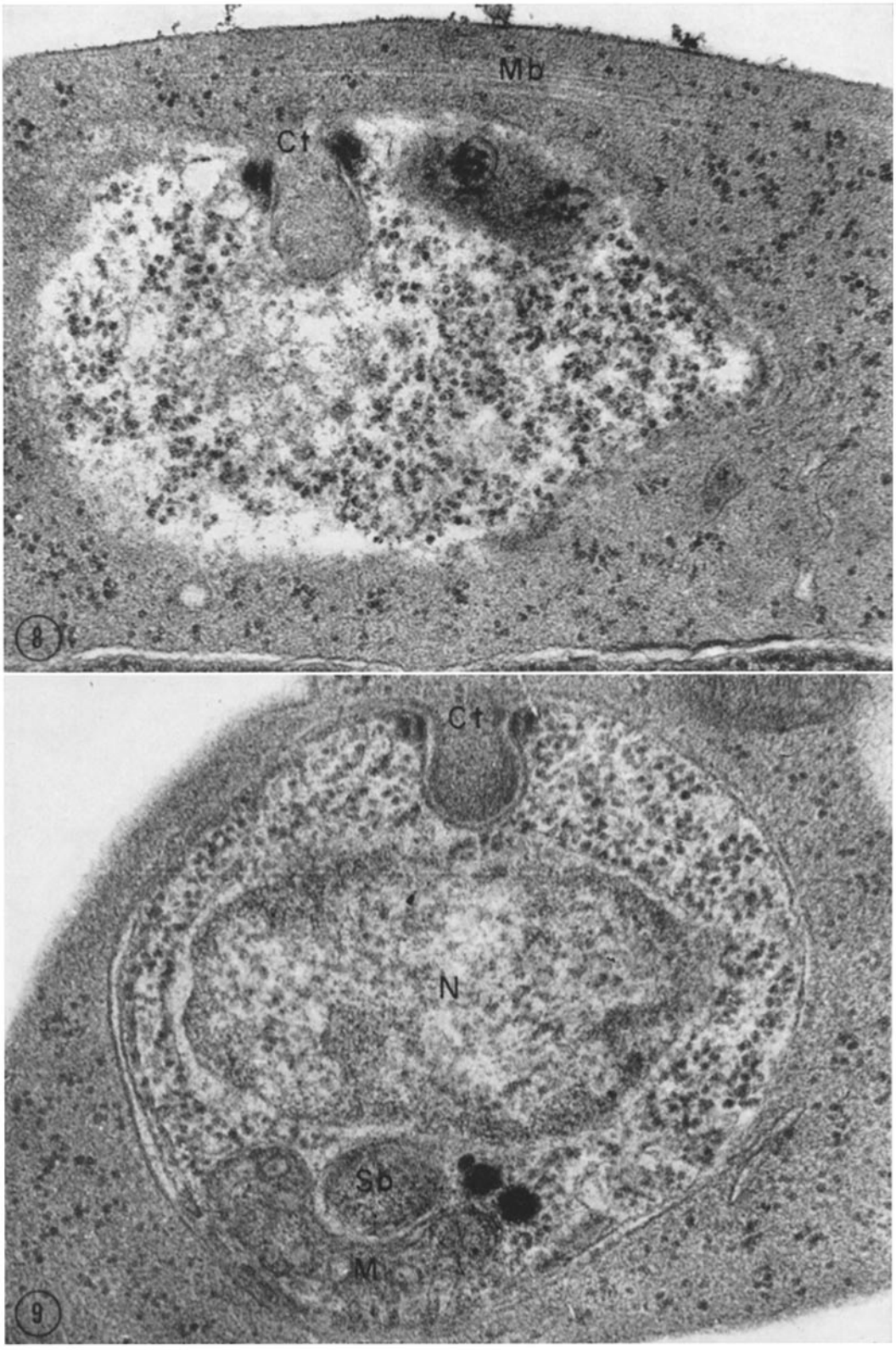
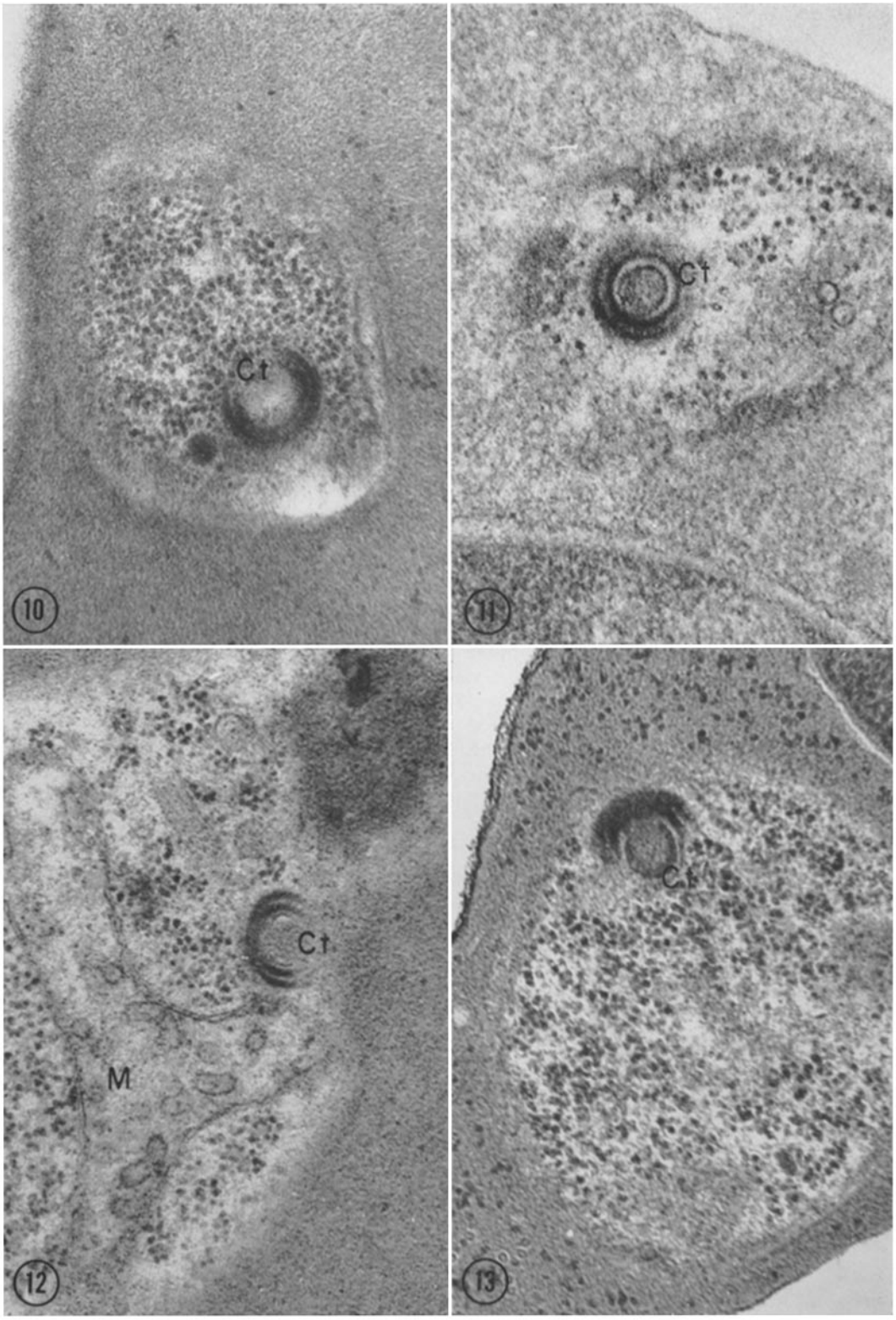


FIGURE 7 An early stage of feeding in an erythrocytic trophozoite of *P. cathemerium*. The basal membrane of the cytostome (*Ct*) extends inward (arrow) into the parasite cytoplasm and precedes the entrance of red cell cytoplasm. The dark linear segments of the cytostome are prominent. Nucleus (*N*), mitochondrion (*M*), and ribosomes are observed in both the parasite and host cell. Some microtubular structures or "marginal bands" (*Mb*) can be seen in the outer periphery of the host cell. × 68,000.



FIGURES 8 and 9 Two examples of trophozoites of *P. cathemerium* in relatively early stages of feeding. The red cell cytoplasm bulges into the cytostome (*Ct*) and is limited by two distinct membranes, the inner membrane originating from the host cell and the outer membrane arising from the basal membrane of the cytostome. Nucleus (*N*), mitochondrion (*M*), and spherical body (*Sb*) are also observed. × 74,000.



FIGURES 10 to 13 Face views of cytostomes (*Ct*) ingesting red cell cytoplasm. Figs. 10 and 11 are examples of *P. cathemerium*, and Figs. 12 and 13 are of *P. fallax*. Fig. 11 clearly shows three concentric circles characteristic of feeding cytostomes. The inner circle is the host cell membrane; the second and third circles correspond to the two dark segments of the parasite cytostome. The diameter of the cytostome has not changed during feeding. × 68,000.

clearly distinguished from the true food vacuoles as well as from host cell cytoplasm within the cytostome cavity.

Continued efforts to disclose cytostomal feeding in the developing exoerythrocytic parasites have been unsuccessful. Furthermore, in large, actively growing exoerythrocytic schizonts, we have not been able to identify food vacuoles or any process at the surface of the parasite which might indicate feeding by phagotrophy.

3. *Cytostomes in Gametocytes*

The cytostome has also been observed in gametocytes of *P. fallax*, *P. lophurae*, and *P. cathemerium*, and here, too, it appears to be involved in feeding. Gametocytes are characterized by a single large nucleus, and numerous oval osmiophilic bodies, probably composed of lipid, which are randomly scattered throughout the cytoplasm, and, in addition, the gametocyte is surrounded by a triple outer membrane system as formerly shown by Duncan et al. (2). By these structural features the gametocytes are easily distinguished from the asexual stages undergoing schizogony. Fig. 21 shows a gametocyte of *P. cathemerium* in the process of ingesting host cell cytoplasm through its cytostomal pore. The diameter of the pore on the inside measures 170 to 200 mμ, and on the outside 300 mμ. Just as in the asexual forms, the thick inner boundary of the cytostome appears to be continuous with the membrane forming the base of the cytostome. Morphologically, the process of feeding seems to be identical with that in asexual stages.

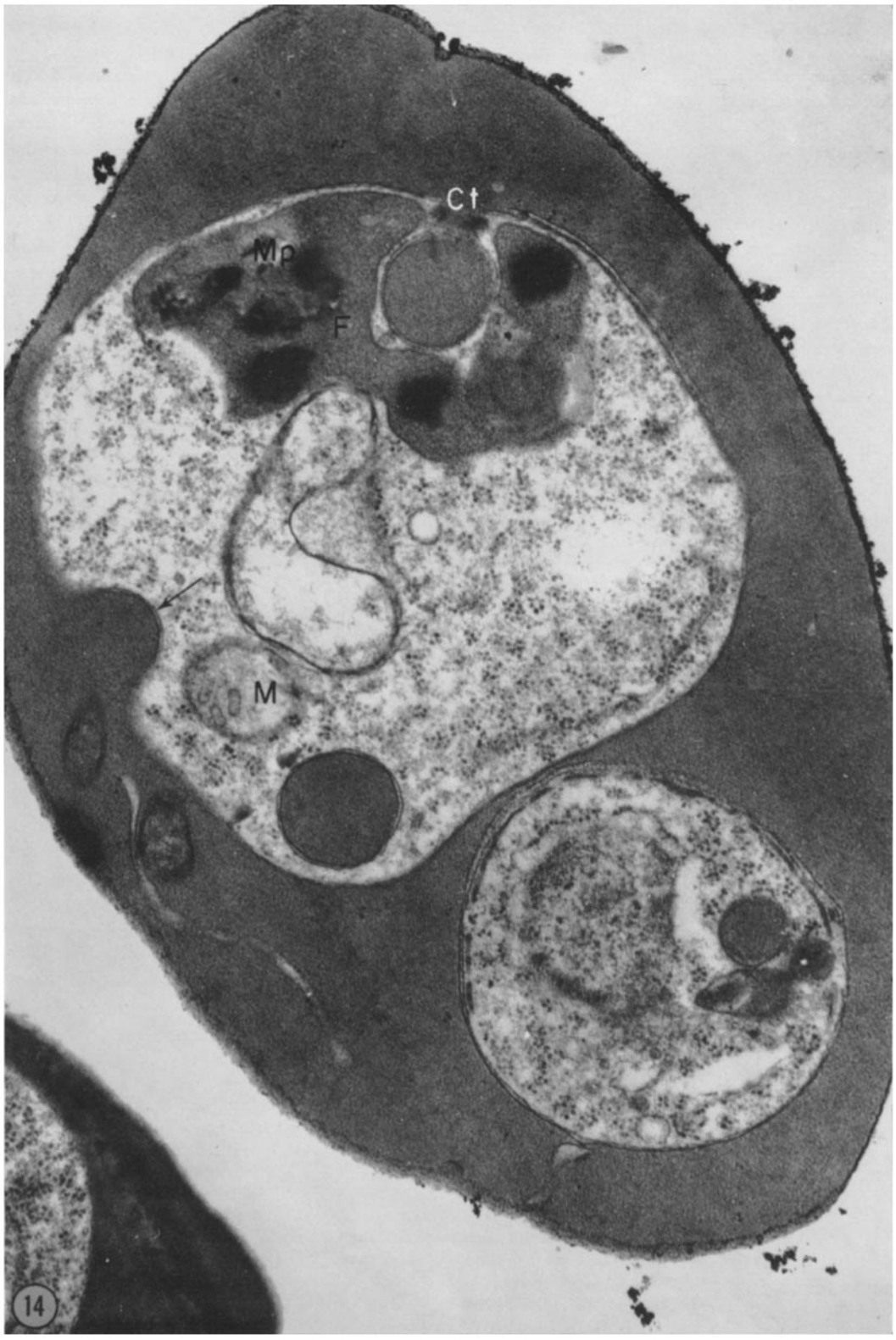
DISCUSSION

In 1961 Garnham et al. (6), in a fine structure study of sporozoites of *P. falciparum*, described an organelle morphologically identical with the cytostome which we have reported in this paper. This organelle, named by them "micropyle" and considered by them to be the place through which the sporoplasm emerged, appeared in cross-section as a depression in the pellicle of the sporozoite, and to be bounded on each side by two densely stained segments, whereas tangential sections showed that it was composed of two dense concentric circles. According to Garnham et al., this pellicular depression has an inner diameter of 80 to 90 mμ, or nearly identical with the dimension which we have reported for the inner diameter of the cytostome of the exoerythrocytic merozoites of *P. fallax*.

Since the time of this early report, additional work by Garnham and coworkers on sporozoites of *P. vivax* (3, 7), *P. brasilianum* (3, 7), *P. bastianelli* (7), *P. ovale* (7), and *P. cynomolgi* (3), on sporozoites of *Lankasterella* (3, 4), and on cystic and proliferative forms of *Toxoplasma* (3, 5) have revealed the presence of a similar pellicular pore structure. Studies by Ludvik (10) have demonstrated the presence of a similar structure in *P. cathemerium*, organism M, *Sarcosporidia*, and *Toxoplasma*. In each case in which measurements have been reported, the structure had an inner diameter identical with that of the cytostome of the exoerythrocytic merozoite which we have observed. However, in no case has any evidence been presented which would shed light on its role, although Garnham (3) and Garnham et al. (7) have reaffirmed their view that the pore functions as the place of sporoplasm emergence.

In the course of our studies, considerable evidence has accumulated which casts doubt on the function of the pellicular pore as the place of sporoplasm emergence and on the possibility that a sporoplasm emerges at all. The fact that the pore is found on merozoites, the proliferative form of *Plasmodium*, presents a question of the possibility of its function as a "micropyle." In addition, we

FIGURE 14 An example of a more advanced stage of feeding in *P. lophurae*. A large complex food vacuole (*F*) surrounds the cytostome, the cavity of which is filled with host cell cytoplasm. Malarial pigment particles (*Mp*) are accumulating in the food vacuole. Another bulge of host cell cytoplasm can be observed in the lower left of the same parasite (arrow). However, there is no indication of a cytostome at its base. Near by and opposite from the cytostome, there is a mass of host cell cytoplasm surrounded by a double membrane. Such seeming inclusions, which occasionally occur in *P. lophurae*, are probably due to tangential cuts from the uneven parasite surface. A young trophozoite is observed in the lower right of the same erythrocyte. × 36,000.


Ct
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14

know from the cinematographic studies of Huff et al. (9), that once an exoerythrocytic merozoite enters a new cell it rounds up and initiates the exoerythrocytic cycle, and that no example of extrusion of the cell contents has been observed. Furthermore, in an extension of the light microscopic studies of Huff et al., we have been able with the aid of the electron microscope to confirm their findings with examples of merozoites in various stages of rounding up. Again, there has been no indication of emergence of cell contents.

The most important finding from our work has been the consistent structural relationship of the pellicular pore with the intake of host cytoplasm. We have observed this phenomenon in the erythrocytic stages of all the parasites so far studied and, therefore, recommend changing the name of the pellicular pore from "micropyle" to "cytostome," because the latter term indicates its role in feeding.

The term cytostome dates back to the time of the early light microscopic studies on protozoa, in which it was used to designate the pore through which an organism engulfed food. Commonly, cytostomes were restricted to ciliates and flagellates. In recent years, with the aid of the electron microscope, a more extensive analysis of cytostomes has been possible (13). Reports from different laboratories show that the structures as well as the mechanism of feeding of cytostomes may vary widely, depending on the particular species of protozoon.

For example, the cytostome of *Trypanosoma mega* (22) appears as a long funnel-shaped gullet which extends rather deeply into the cell. By culturing *T. mega* with ferritin, Steinert and Novikoff (22) were able to demonstrate an aggregation of electron-opaque particles in the region of the cytostome and to follow their subsequent incorporation into the cell, presumably by pinocytosis. Pitelka (12) has described a structure in *Bodo saltans* morphologically similar to the cytostome of *T. mega* and which possibly functions in the uptake of food.

The cytostome of *Paranema trichophorum* (16) appears funnel-shaped and similar to that in *T. mega*, but differs in that it is structurally associated with two large intracytoplasmic pharyngeal rods which assist the organism in feeding, by attaching to large particles of food. In contrast to *T. mega*, feeding in *Paranema* involves the intake of relatively large particles of food, probably through a process of phagocytosis.

Quite recently Vivier and Schrevel (23), in fine structure studies on the intestinal parasite, *Sabellaria aveolata*, demonstrated the presence of four distinct surface organelles, one of which closely resembles the cytostome of *Plasmodium* reported in this paper. This organelle appears as a depression in the pellicle, and is bounded on its side by two dense membranes and at its base by a single membrane. Although this structure has not been designated a "cytostome," it, nevertheless, seems to be active in the uptake and subsequent digestion of food as evidenced by the diffuse appearance of its basal membrane and the apparent migration of a granular material from its basal perimeter into the cytoplasm of the parasite. While there is considerable similarity in structure between the feed-

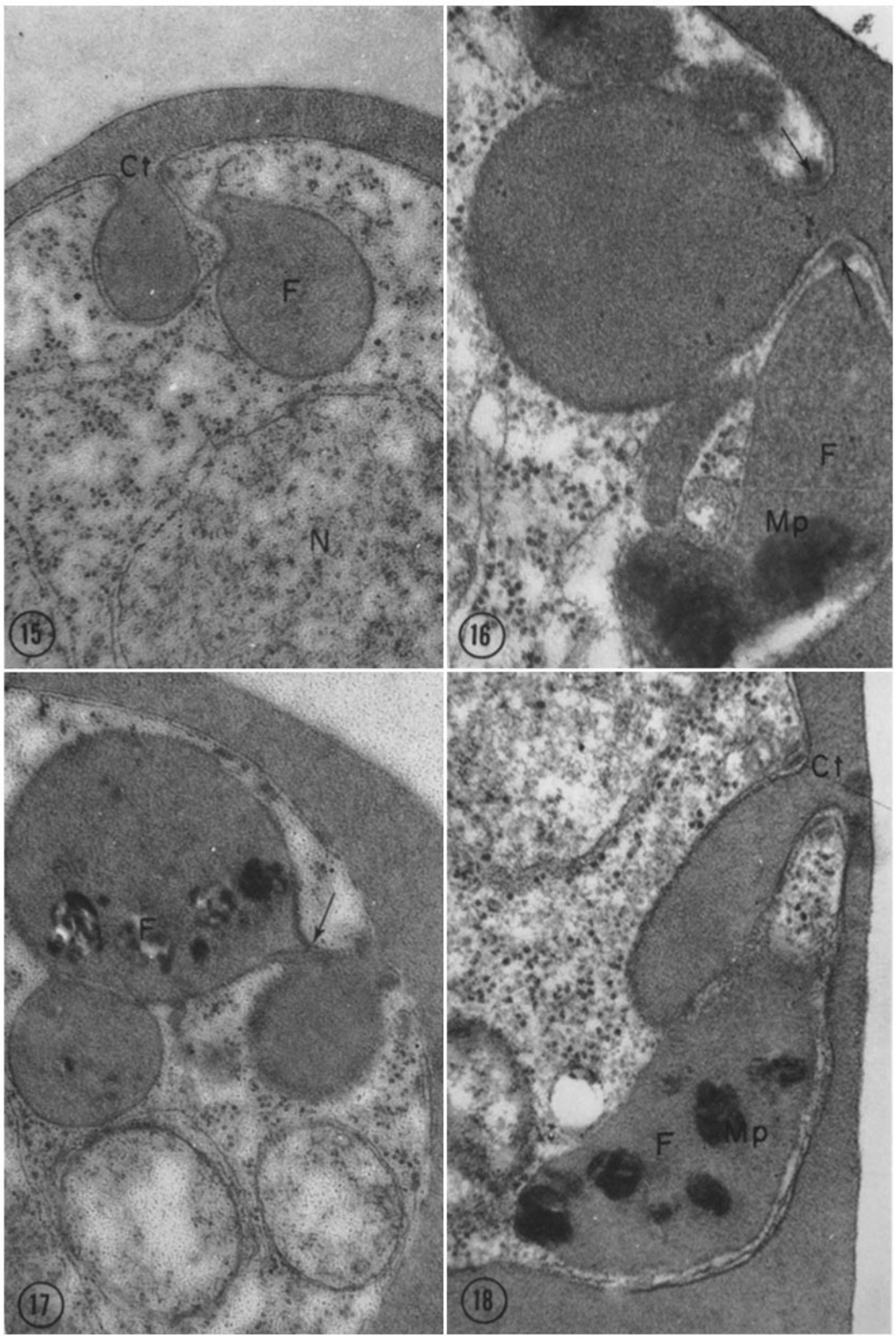
FIGURES 15 to 18 Four examples showing the formation of food vacuoles in actively growing trophozoites of *P. lophurae*. All, × 65,000.

FIGURE 15 A food vacuole (*F*) surrounded by a single membrane is pinching off from the wall of the cytostome (*Ct*). Note the double membrane delimiting the red cell cytoplasm which bulges through the cytostomal orifice.

FIGURE 16 Food vacuoles containing malarial pigment (*Mp*) are clustered in the vicinity of a large cytostomal invagination containing red cell cytoplasm. The two dark segments bounding the cytostome are quite prominent (arrow).

FIGURE 17 An example of food vacuoles (*F*) blebbing off from the bulge of host cell cytoplasm. The food vacuoles are still connected by a thin strand to the bulge (arrow).

FIGURE 18 A large food vacuole (*F*) containing malarial pigment (*Mp*) is surrounded by a single membrane while the adjacent bulge of host cell is limited by two membranes.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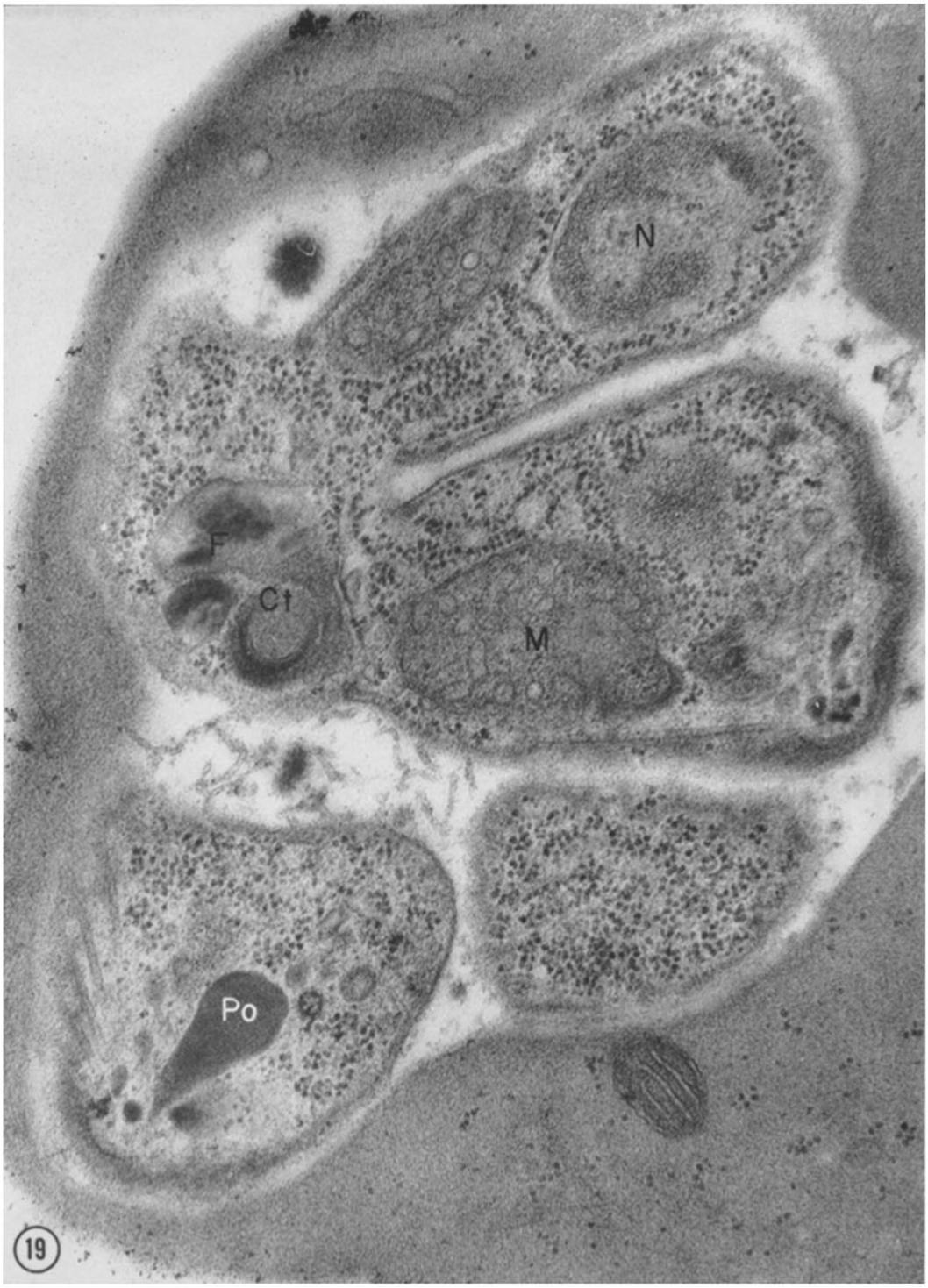

FIGURE 19 A late developmental stage in *P. fallax*; the merozoites have nearly completed their budding from the central cytoplasmic mass. The cytostome (*Ct*) in face view contains red cell cytoplasm, indicating that it is still ingesting red cell cytoplasm. The food vacuole (*F*) adjacent to the cytostome contains particles of malarial pigment. In the lower left a budding merozoite has been sectioned near its anterior tip, revealing a few radiating microtubules and a large sinuous dense body or "paired organelle" (*Po*) (*See* Garnham et al.). × 60,000.

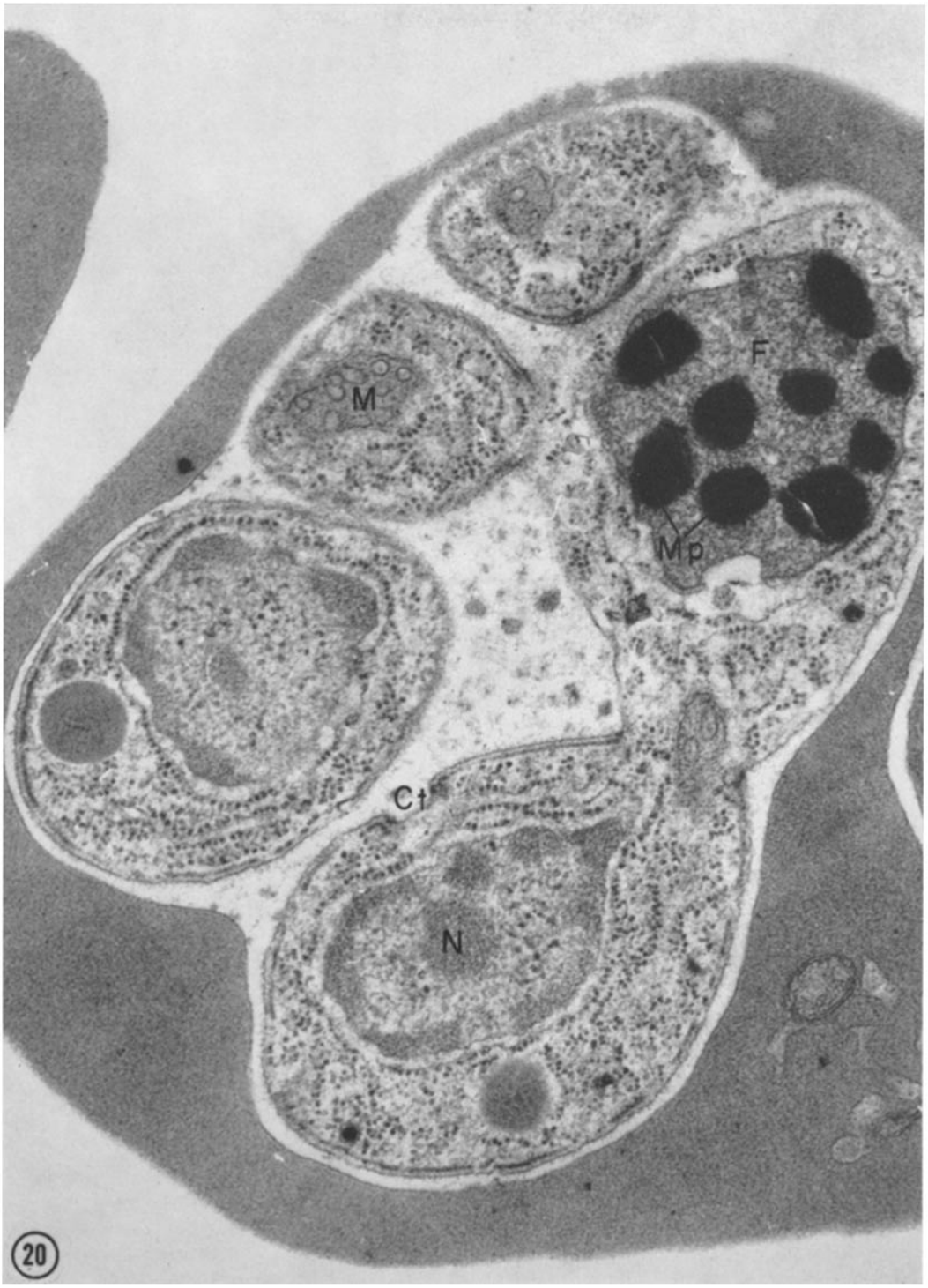

FIGURE 20 A late stage in segmentation in *P. fallax*. Two merozoites are still connected to the central body by a thin neck of cytoplasm. A large food vacuole (*F*) within the residual cytoplasmic mass contains numerous granules of malarial pigment (*Mp*). The red cell cytoplasm of the food vacuole is lightly stained, a further indication that extensive digestion has taken place. A cytostome (*Ct*) is evident in one of the budding merozoites. × 36,500.

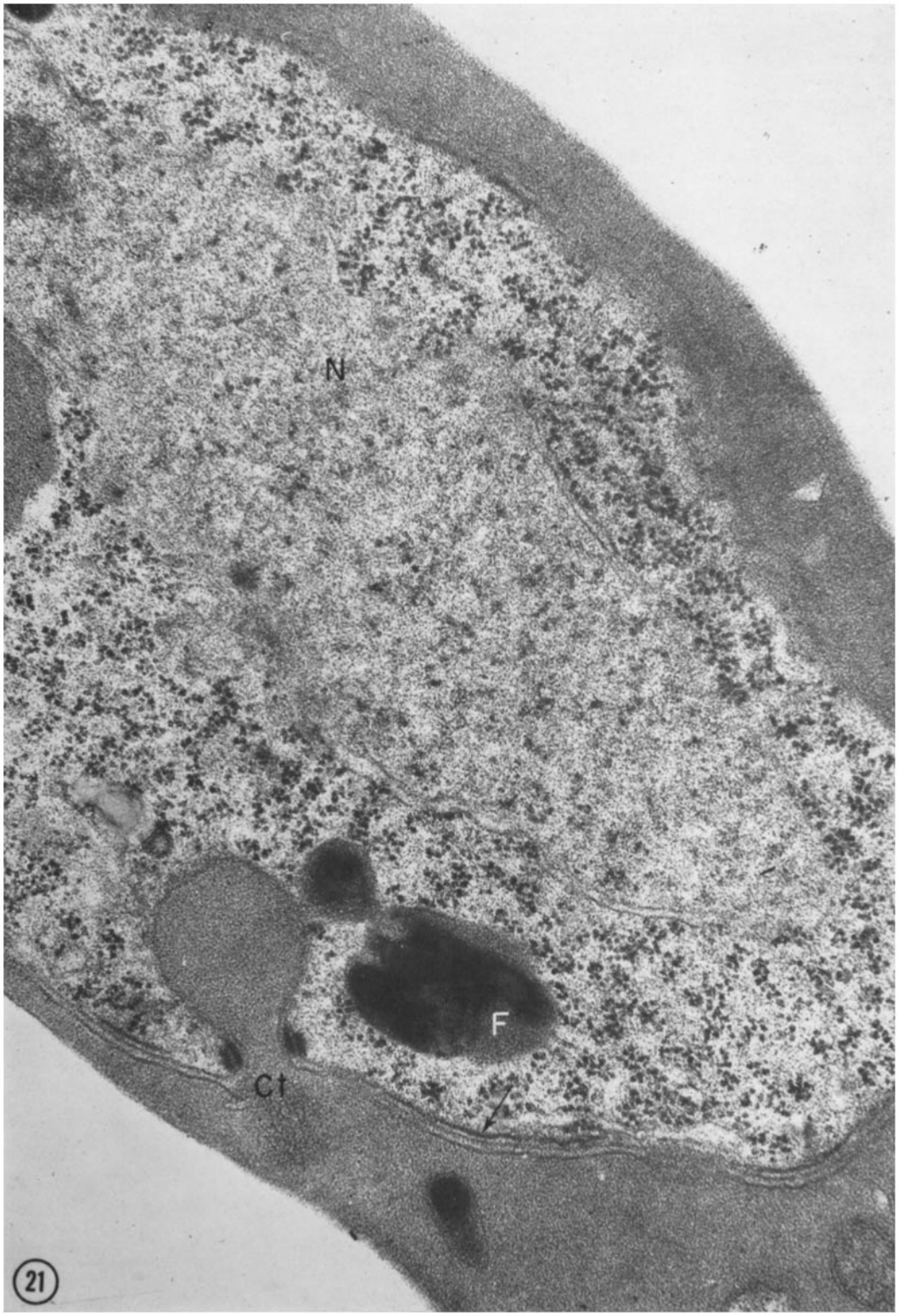

FIGURE 21 A gametocyte of *P. cathemerium* actively ingesting red cell cytoplasm through its cytostome (*Ct*). The food vacuole (*F*) adjacent to the invagination contains particles of malarial pigment. Gametocytes are characterized by a triple-layered outer membrane (arrow) and a single large irregularly shaped nucleus (*N*). × 54,000.

ing pores of *Sabellaria* and *Plasmodium*, there is, however, considerable difference in the process by which food is ingested: for example, the uptake of relatively large discrete quantities of food within food vacuoles in *Plasmodium* as opposed to the diffusion or migration of a small, granular material in *Sabellaria*.

Feeding in malarial parasites has already been described in a series of classic papers by Rudzinska and Trager (17 to 20). In studies on *P. berghei* and *P. lophurae*, they observed large invaginations of red cell cytoplasm which they interpreted as intracellular phagotrophic food vacuoles. While they did not observe the cytostome, the food vacuole complex shown by Rudzinska and Trager (which we believe originates from the cytostome) is morphologically similar to what we have described. Of particular note in their description is the thin neck of the food vacuole which compares closely to the cytostomal region in our work. However, close examination of their micrographs does not reveal the dense segments characteristic of the cytostome.

The factors which account for the discrepancies between our work and that of Rudzinska and Trager are probably the different procedures used in preparing tissue for examination in the electron microscope. Since the time of the original work of Rudzinska and Trager, many advances have been made in the technique of preparing tissue, particularly in embedding and fixation. The Epoxy resins, for example, have virtually replaced the methacrylates as an embedding medium. Perhaps even more important has been the introduction of glutaraldehyde fixation and OsO_4 postfixation by Sabatini et al. (21) in 1963. Due to these changes it is now possible to retain much more cytoplasmic detail than before. In our opinion, these improvements in handling tissues are responsible for the preservation of the cytostome.

We have noticed that one of the parasites, *P. lophurae*, also studied by Rudzinska and Trager, is somewhat more complicated, in that additional invaginations occasionally occur along with the one through the cytostome. This we attribute to the amoeboid motion of the parasite within the erythrocyte.

Additional evidence supporting cytostomal feeding comes from our observations on developing gametocytes. Here, again, the cytostome is actively involved in the uptake of red cell cytoplasm. It seems unlikely that the triple-layered pellicle, characteristic of gametocytes throughout their entire development, would be flexible enough to allow invaginations and subsequent pinching off of vacuoles of red cell cytoplasm. The cytostomal depression, on the other hand, is a region on the surface of the parasite in which only one parasite membrane separates the parasite cytoplasm from the erythrocyte and, therefore, would most likely be the place of hemoglobin ingestion.

Engulfment of host cytoplasm by gametocytes has also been noted by Duncan et al. (2) who point out that, even though gametocytes and asexual forms of *Plasmodium* show striking differences in their plasma membrane, their food vacuoles appear similar, and those authors conclude that their mechanism of feeding must be the same.

We conclude that feeding in erythrocytic stages of *Plasmodium*, either in gametocytes or in asexual trophozoites and schizonts, is more than simple, random invagination of the parasite plasma membrane and engulfment of red cell cytoplasm by phagotrophy; it involves a specific organelle, the cytostome and a two-step process. The red cell cytoplasm first accumulates within the cytostome cavity and is secondarily incorporated into food vacuoles emanating from the cytostome wall. We do not know whether the intake of red cell cytoplasm into the cytostome is due to an active process established in the parasite or whether it results from expansion of the growing parasite in the erythrocyte which creates a pressure forcing red cell cytoplasm through the pore of the cytostome. The process of red cell cytoplasm uptake and the subsequent pinching off of food vacuoles would seem to fit the current concepts of pinocytosis (8), although it must be argued that it is a more specialized case since feeding occurs through a specific organelle.

It is interesting to note that, while we have repeatedly observed the cytostome cavity filled with host cell cytoplasm in asexual forms and gametocytes of the erythrocytic cycle, we have never observed an analogous phenomenon in the developmental stages of the exoerythrocytic cycle. Nor have we ever witnessed any particulate engulfment or phagotrophy of host cell cytoplasm by growing exoerythrocytic stages.

Because the growth rate of the exoerythrocytic forms far exceeds that of the erythrocytic forms (in a few days 200 or more new merozoites may emerge from a single exoerythrocytic infection as opposed to 10 to 20 from an erythrocytic infection), great quantities of food must be needed. Because

of the lack of any evidence for cytostomal feeding or for either phagocytosis or pinocytosis, we suggest that the growing exoerythrocytic stages obtain food by a process of diffusion.

It should be pointed out that the merozoite in the exoerythrocytic phase of development enters into a cell which has a much more heterogeneous and probably a nutritionally more complete composition than the erythrocyte. The cytoplasm of the brain tissue culture cell, for example, is richly endowed with mitochondria, Golgi bodies, endoplasmic reticulum, and ribosomes and, therefore, appears to have all the apparatus necessary for the synthesis of the nutrients and metabolites required by the developing parasite. Simple diffusion of sugars, amino acids, ATP, vitamins, etc. from the host cell cytoplasm into the region of the parasite would supply a trophozoite with all the precursors necessary for active growth.

The growing erythrocytic parasite, on the other hand, resides in a rather homogeneous environment, composed largely of hemoglobin. For this reason, the available food may be low in some of the required nutrients. The cytostome may have evolved as a specialized organelle for feeding, enabling the developing parasite to take in large amounts of red cell cytoplasm from which a sufficient quantity of the necessary metabolites could be extracted.

The differences in size of the cytostome between the exoerythrocytic merozoites and the erythrocytic developmental stages may have some relation to its function in feeding. As mentioned under Observations, the cytostome in the erythrocytic stages is twice the diameter of, and hence is four times larger in cross-sectional area than the cytostome in the exoerythrocytic merozoites. The greater size would enable the developing erythrocytic stages to take in large amounts of food. Furthermore, to the best of our knowledge, only the erythrocytic forms actively ingest food through the cytostome. It is interesting to note that the structure, similar to the cytostome, reported by Garnham and coworkers (3, 6, 7) in sporozoites of different species of *Plasmodium*, has the same dimensions as the cytostome in the exoerythrocytic merozoites. If our idea of the dormancy of the cytostome in the exoerythrocytic merozoite is correct, then we would assume that the cytostome-like structure, or "micropyle," is nonfunctional in sporozoites. This assumption is tentative, pending the study of early stages of development of the cryptozoite by electron microscopy.

From our studies of different stages of *P. fallax*, we conclude that a dramatic change occurs in the size of the cytostome when the exoerythrocytic merozoite differentiates into a merozoite capable of invading a red blood cell. Such infections of new host cells would be unsuccessful if the merozoite could not adapt to its new environment by enlarging its cytostome. The fact that gametocytes, which also ingest hemoglobin, have a cytostome similar in size to the one in the asexual erythrocytic forms, supports our view that enlargement of the cytostome is a prerequisite for ingestion of erythrocyte cytoplasm, and, therefore, for survival of the parasite in the red blood cell.

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REFERENCES

1. Davis, A. G., Huff, C. G., and Palmer, T. T., Procedures for maximum production of exoerythrocytic stages of *Plasmodium fallax* in tissue culture, *Exp. parasitol.*, in press.
2. Duncan, D., Street, J., Julian, S. R., and Michs, D. O., Electron microscopic observations of the gametocytes of a malarial parasite (*Plasmodium cathemerium*), *Texas Rep. Biol. Med.*, 1959, **17**, 314.
3. Garnham, P. C. C., The ultrastructure of Haemosporidia and allied protozoa with special reference to the motile stages, Proceedings of the First International Congress of Protozoology, Prague, 1961, (J. Ludvik, J. Lom, and J. Vavra, editors), New York, Academic Press, Inc., 1963, 427.
4. Garnham, P. C. C., Baker, J. R., and Bird, R. G., The fine structure of *Lankesterella garnhami*, *J. Protozool.*, 1962, **9**, 107.
5. Garnham, P. C. C., Baker, J. R., and Bird, R. G., Fine structure of the cystic form of *Toxoplasma gondii*, *Brit. Med. J.*, 1962, **5271**, 83.
6. Garnham, P. C. C., Bird, R. G., Baker, J. R.,

and BRAY, R. S., Electron microscope studies of motile stages of malaria parasites. II. The fine structure of the sporozoites of *Laverania* (Plasmodium) *falcipara*, *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1961, **55**, 98.

7. GARNHAM, P. C. C., BIRD, R. G., and BAKER, J. R., Electron microscope studies of motile stages of malaria parasites. IV. The fine structure of the sporozoites of four species of *Plasmodium*, *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1963, **57**, 27.
8. HOLTER, H., Pinocytosis, *in* International Review of Cytology, Vol. VIII. (G. H. Bowne and J. F. Daniel, editors), New York, Academic Press, Inc., 1959, 481.
9. HUFF, C. G., PIPKIN, A. C., WEATHERSBY, A. B., and JENSEN, D. V., The morphology and behavior of living exoerythrocytic stages of *Plasmodium gallinaceum* and *P. fallax* and their host cells, *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 93.
10. LUDVIK, J., Electron microscopic study of some parasitic protozoa, Proceedings of the First International Congress of Protozoology, Prague, 1961, (J. Ludvik, J. Lom, and J. Vavra, editors), New York, Academic Press, Inc., 1963, 387.
11. LUFT, J. H., Improvements in Epoxy resin embedding methods, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 409.
12. PITELKA, D. R., Observations on the kinetoplast-mitochondrion and the cytostome of *Bodo*, *Exp. Cell Research*, 1961, **25**, 87.
13. PITELKA, D. R., Electron-Microscopic Structure of Protozoa, New York, The Macmillan Co., 1963, 140.
14. REYNOLDS, E. S., The use of lead citrate at high pH as an electron-opaque stain in electron microscopy, *J. Cell Biol.*, 1963, **17**, 208.
15. RISTIC, M. and KREIER, J. P., The fine structure of the erythrocytic forms of *Plasmodium gallinaceum* as revealed by electron microscopy, *J. Trop. Med. and Hyg.*, 1964, **67**, 509.
16. ROTH, L. E., An electron microscope study of the cytology of the protozoan *Peranema trichophorum*, *J. Protozool.*, 1959, **6**, 107.
17. RUDZINSKA, M. A., and TRAGER, W., Intracellular phagotrophy by malaria parasites: an electron microscope study of *Plasmodium lophurae*, *J. Protozool.*, 1957, **4**, 190.
18. RUDZINSKA, M. A., and TRAGER, W., Electron-microscope studies on malarial parasites, *Triangle*, 1959, **4**, 97.
19. RUDZINSKA, M. A., and TRAGER, W., Phagotrophy and two new structures in the malaria parasite, *Plasmodium berghei*, *J. Biophysic. and Biochem. Cytol.*, 1959, **6**, 103.
20. RUDZINSKA, M. A., and TRAGER, W., The role of the cytoplasm during reproduction in a malarial parasite (*Plasmodium lophurae*) as revealed by electron microscopy, *J. Protozool.*, 1961, **8**, 307.
21. SABATINI, D. D., BENSCH, K., and BARRNETT, R. J., Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation, *J. Cell Biol.*, 1963, **17**, 19.
22. STEINERT, M., and NOVIKOFF, A. B., The existence of a cytostome and the occurrence of pinocytosis in the trypanosome, *Trypanosoma mega*, *J. Biophysic. and Biochem. Cytol.*, 1960, **8**, 563.
23. VIVIER, E., and SCHREVEL, J., Étude au microscope électronique, d'une grégarine du gerne, *Selenidium*, parasite de *Sabellaria alveolata*, L., *J. Micr.*, 1964, **3**, 651.