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An Electron-Microscopical Study

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ISOLATED ERYTHROCYTIC FORMS OF *PLASMODIUM BERGHEI*
AN ELECTRON-MICROSCOPICAL STUDY*

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ABSTRACT: The fine structure of *Plasmodium berghei* was studied after its release from rat erythrocytes by one of four methods—cell disintegration by French press, or lysis by saponin, antiserum, or distilled water. Cell disintegration by French press released the largest proportion of structurally intact parasites generally lacking closely associated arrays of erythrocyte membranes. However, the total number of parasites harvested by this method was comparatively few. On the other hand, the saponin-freed parasite material contained a large number of parasites, most of which appeared intact, but several parallel arrays of host membranes were also present. Lysis by antiserum freed some apparently intact parasites, but there were also several damaged forms and unbroken cells. Lysis by distilled water yielded very few healthy parasites; most freed forms contained vacuolar inclusions or were broken into smaller pieces. A double-unit membrane limiting the parasite cytoplasm, at least at intervals, was evident in several of the electron micrographs of isolated *P. berghei* and of *P. berghei* found outside the host cell in infected whole blood. Alterations in the nucleoplasm and varied forms of a double-membraned vacuole were frequently observed in the freed parasites. Food vacuoles and cytostomes, when present, appeared normal in the isolated parasites.

A variety of approaches in malaria research—metabolic,[1-4] physiological,[5,6] antigenic,[7-12] immunologic,[13,14] chemotherapeutic,[1,15] biochemical,[16-23] and cultivation[24-27]—have all included some attempts to use organisms freed from the host cells. Plasmodia have been released from their host erythrocytes by several means, the most popular of which include hypotonic lysis with distilled water,[7,9,17,18,20,21] saponin lysis,[1,5,8,10-12,14,15,17,19,22,23,28-30] and lysis by means of hemolytic antiserum.[2-4,6,13,24-27,31] In addition, investigators have applied mechanical treatment such as ultrasonic disintegration[16] and the French-press method.[7] The employment of enzymes to improve the separation of the parasites from host material, especially from the nuclei of avian erythrocytes, has also been described.[11,23,29,32] The choice of a particular method of lysis varies with the specific needs of the researcher. Workers such as Zuckerman,[33] and Sherman,[11] for example, interested in an antigenic examination of plasmodia, preferred the use of saponin, which yields a large quantity of free parasites whose structure or antigens appear relatively intact. The presence of exogenous protein and the effort of preparing large amounts of specific antiserum are also avoided when saponin lysis is selected in preference to lysis with antiserum. On the other hand, Trager,[25-27] in his effort to obtain viable extracellular parasites for *in vitro* studies, employed the hemolytic antiserum and complement method introduced by Speck, Moulder, and Evans,[6] a lytic system thought to be less injurious to the parasites, and resulting in preparations retaining a considerable degree of metabolic activity.

Two major characteristics of the lysates that are frequently described, especially in the original reports of lysis, are the presence of host remnants and the intactness of the parasite, the latter sometimes verified by *in vivo* tests of viability. To date, these two characteristics have been examined mainly at the light-microscope level. Exceptions include the electron micrographs of Bahr in 1966 showing *Plasmodium berghei* released by means of pepsin at pH 1.6 or by sonication[16] and Theakston, Fletcher, and Maegraith's reference to (though not published) electron

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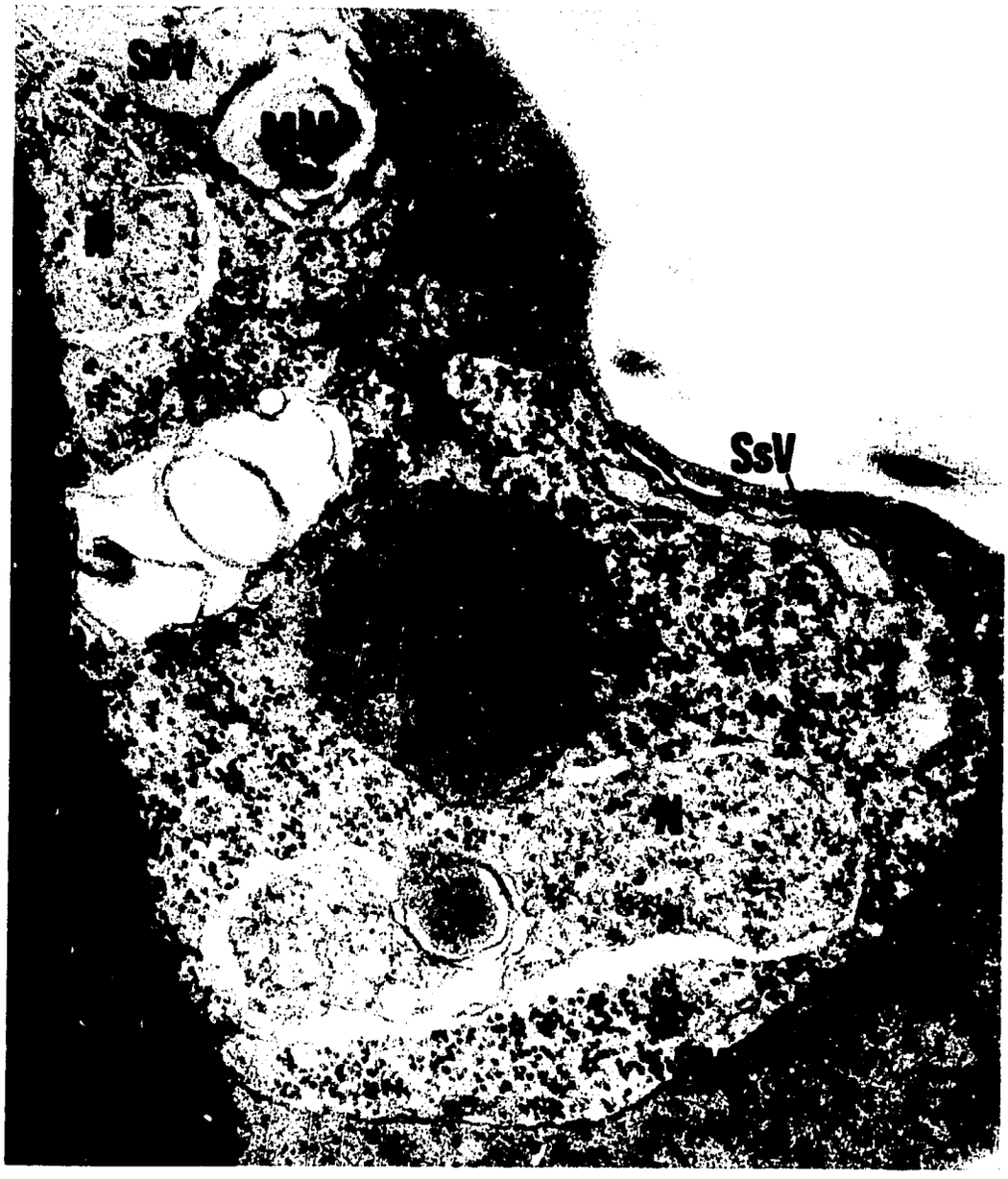


FIGURE 1. Electron micrograph showing portions of two trophozoites of *P. berghei* infecting a rat reticulocyte. Note especially the cytostome (Ct), large food vacuole (FV), cup-shaped nucleus (N) of more or less uniform density, plasma membrane (PM), sausage-shaped vacuoles (SsV), and multilaminated-membraned body (MM). The multilaminated-membraned body appears in close association with the sausage-shaped vacuole and the plasma membrane (× 51,000).

micrographs of *Plasmodium vinckei* freed by hemolytic antiserum.[34] In addition, Kudzinska and Trager reported fine-structural studies with *Plasmodium lophurae* cultured *in vitro* outside the host cell.[35]

We now report ultrastructural observations with *P. berghei* freed from rat erythrocytes by one of four different methods—cell disintegration by French press, or lysis by saponin, distilled water, or antiserum.

FIGURE 2. The multilaminated-membraned body (MM) is apparently contained within the sausage-shaped vacuole (SsV), which has assumed a doughnut-like appearance in this trophozoite of *P. berghei*. There is also a possible connection (*arrow*) between the multilaminated-membraned body (MM) and the double-unit membrane (PM) at the parasite's surface (× 56,000).

MATERIALS AND METHODS

Groups of at least five young Sprague-Dawley rats with near maximum parasitemia (30 to 40%) of the NK65 strain of *P. berghei* (supplied by Dr. Meir Yoeli, New York University School of Medicine, New York, N. Y.) were bled by cardiac puncture in the presence of neutralized Alsever's solution or sodium citrate. Erythrocytes were obtained from the pooled blood by centrifugation and were washed three times with phosphate-buffered saline solution, pH 7.0 to 7.2, care being taken to remove any buffy coat after each spin.

Washed cells lysed by Eastman saponin according to the method of Spira and Zuckerman[32] yielded parasite pellets at 12,000 × G, which were washed free of visible contaminants.

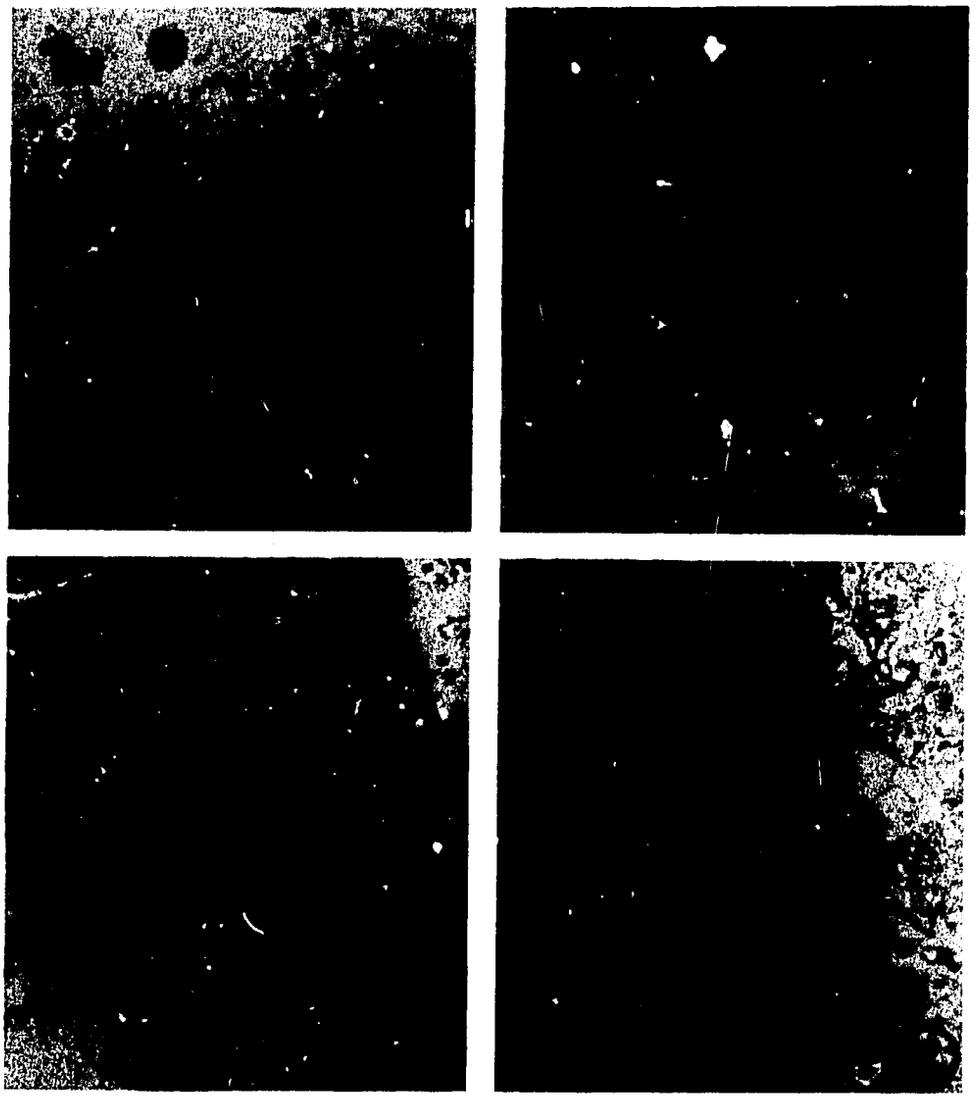
Cells designated for French-press disintegration were handled by the method of D'Antonio and co-workers[7] at a pressure of less than 1,000 psi. An initial centrifugation at 50 × G was followed by a collection and washing of the freed parasites at about 12,000 × G.

The preparation of rabbit anti-rat erythrocyte serum samples and its use with complement in the technique of lysis with antiserum were carried out following the method of Speck, Moulder, and Evans.[6] The solid material was separated and washed by centrifugation at 900 to 1,000 × G.

Hypotonic lysis was performed on washed erythrocytes by subjecting them to at least 10 volumes of distilled water. The resulting lysate was centrifuged and washed four times in phosphate-buffered saline solution, pH 7.0 to 7.2.

The pellets of parasites freed by each of these four methods were handled as identically as possible during the preparation for electron-microscope studies. They were minced finely and fixed in a solution of 6% glutaraldehyde in 0.2 M PO_4 buffer, pH 7.2 for 1 hour at 4°C. After a wash in sucrose-PO_4 buffer, at least overnight, the material was fixed again for 1 hour in 1% osmic acid, in 0.2 M PO_4 buffer, pH 7.2. Dehydration was carried out in an ascending ethanol series to propylene oxide; finally, the pellets were em-

FIGURE 3. A segmenter of *P. berghei* showing budding merozoites. The thickened inner membrane of the merozoites is evident (*arrows*). The individual nuclei (N) exhibit dense granular masses, especially at the periphery. A cytostome (Ct) is also evident. Pigment granules (PG) appear to be concentrated in the mother schizont (× 37,000).

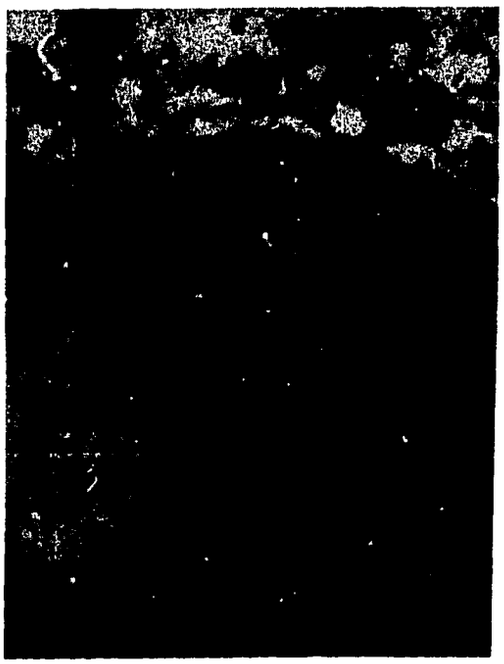


FIGURES 4–7. **4.** A thin section through a pellet of *P. berghei* parasites isolated by saponin lysis. Numerous intact parasites and one damaged form (De) along with parallel arrays of host membrane (HM) are evident (× 5,500). **5.** A thin section through an isolate of *P. berghei* parasites freed by cell disintegration by French press. Only two intact parasites (P) are visible among the pieces of cellular debris (× 6,300). **6.** A thin section through a pellet of *P. berghei* parasites isolated by antiserum lysis. Part of an intact erythrocyte (1), a form which may be a freed parasite but likely is a lysed reticulocyte (2), a parasite surrounded by host components (3), a parasite in a more intact blood cell (4), and a portion of a white blood cell (5) are all visible (× 5,500). **7.** A thin section through an isolate of *P. berghei* parasites freed by distilled-water lysis. A damaged parasite containing many vacuoles (1) and a portion of a rat blood cell (2) are evident (× 5,500).

bedded in Epon 812. Thin sections were cut on a Reichert OMU-2 ultramicrotome with a diamond knife and were mounted on naked 400-mesh copper grids. The sections were stained with saturated uranyl acetate for 1 hour followed by Reynolds' lead citrate for about 5 minutes. The grids were examined in a Hitachi HU 11C microscope.

After cell disintegration by French press and lysis by distilled water, the number of whole,

FIGURE 8. A preparation (× 21,000) of *P. berghei* parasites after saponin lysis. Double-membraned vacuoles containing vesicles (Dv) or internal membranes (Di), or both, are observed. A small circular body, possibly a multilaminated-membraned body, is seen in a double-membraned vacuole (*arrow*). Other possible multilaminated-membraned (MM) structures and a sausage-shaped vacuole (SsV) are present. Alterations in the nuclei of the parasites include a nucleolar-like patch (Nc) and a clumping or aggregation of larger dense particles (Cp). The endoplasmic reticulum (ER) occurs as wide vesicles with a light matrix. A food vacuole (FV) of low density is apparent in one of the parasites.



FIGURES 9 and 10. **9.** A portion of a *P. berghei* trophozoite in a saponin-prepared isolate shows a double-membraned vacuole with vesicular elements (Dv). Remnants of host-membrane material (HM) lie outside the double unit membrane at the surface (PM) of the parasite (× 63,000). **10.** A double-membraned vacuole having an inner-membrane structure (Di) is prominent in a *P. berghei* trophozoite after saponin lysis of the host erythrocyte (× 55,000).

freed parasites was relatively few. The technique of first sampling a block by thick section (1 μ) on the ultramicrotome, then examining this section in the light microscope after staining with toluidine blue, proved helpful in locating the freed parasites in thin sections.

RESULTS

Intracellular Forms

For comparing intracellular forms with isolated forms, a representative electron micrograph of two trophozoites infecting a rat reticulocyte (Fig. 1) illustrates several characteristics of these intracellular asexual forms. Note in particular the cytosome and also large food vacuole, the nucleus of more or less uniform density, the double-unit membrane limiting the parasite cytoplasm, the sausage-shaped vacuole, and the multilaminated-membraned body. The latter structure, also termed a concentric double-membraned body,[36] was frequently found in association with the plasma membrane or the sausage-shaped vacuole of the parasite (Figs. 1 and 2). A typical segmenter of *P. berghei* (Fig. 3) shows especially a thickened inner membrane, large nucleus of irregular density, and a cytostome in the individual merozoites as well as a concentration of pigment granules in the mother schizont.

FIGURE 11. A parallel array of host membranes (HM) is illustrated in a *P. berghei* parasite freed by saponin lysis. A conspicuous nucleolar-like body (Nc) is accompanied by aggregations of dense particles (Cp) in the compact nucleoplasm. In the cytoplasm of the parasite, there are suggestions of a typical sausage-shaped vacuole (SsV) and multilaminated-membraned body (MM), and vesicles containing pigment granules (Vp) are visible. Numerous ribosomes (RNP) and endoplasmic reticulum (ER) exhibiting wide channels of low density are also evident (× 68,000).

HM
HM
11

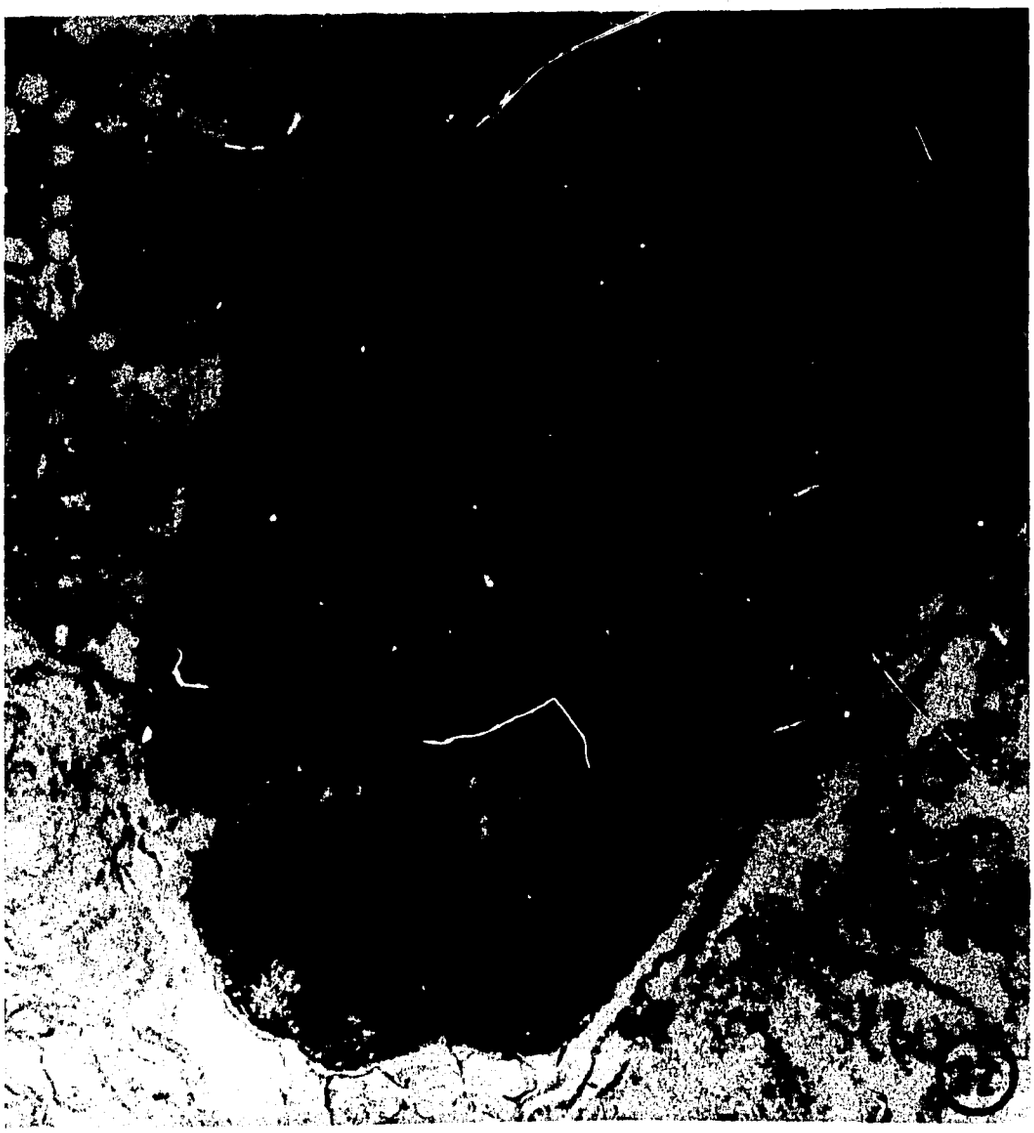


FIGURE 12. An early schizont of *P. berghei* from an isolate prepared by saponin lysis exhibits a characteristic thickening in its outer membrane (*arrows*) and the presence of more than one nuclei (N). A parallel array of host membranes (HM) is observed in close association with the parasite. A double-membraned vacuole (Dv) is evident and appears to contain swollen tubules or vesicles. In addition, single membrane-bound vesicles with pigment granules (Vp) are seen. Note the apparent break (B) in the plasma membrane of the parasite (× 35,000).

Isolated Forms

General Characteristics of Isolates

Figure 4, made at relatively low magnification, illustrates a pellet of *P. berghei* parasites freed from the erythrocytes by saponin lysis. The harvest after lysis by saponin contained numerous freed parasites, apparently intact and often grouped so closely together that it was impossible to photograph one entire parasite without including the borders of a neighboring one (Fig. 4). Only a few damaged parasites and no intact erythrocytes were observed after lysis by saponin;

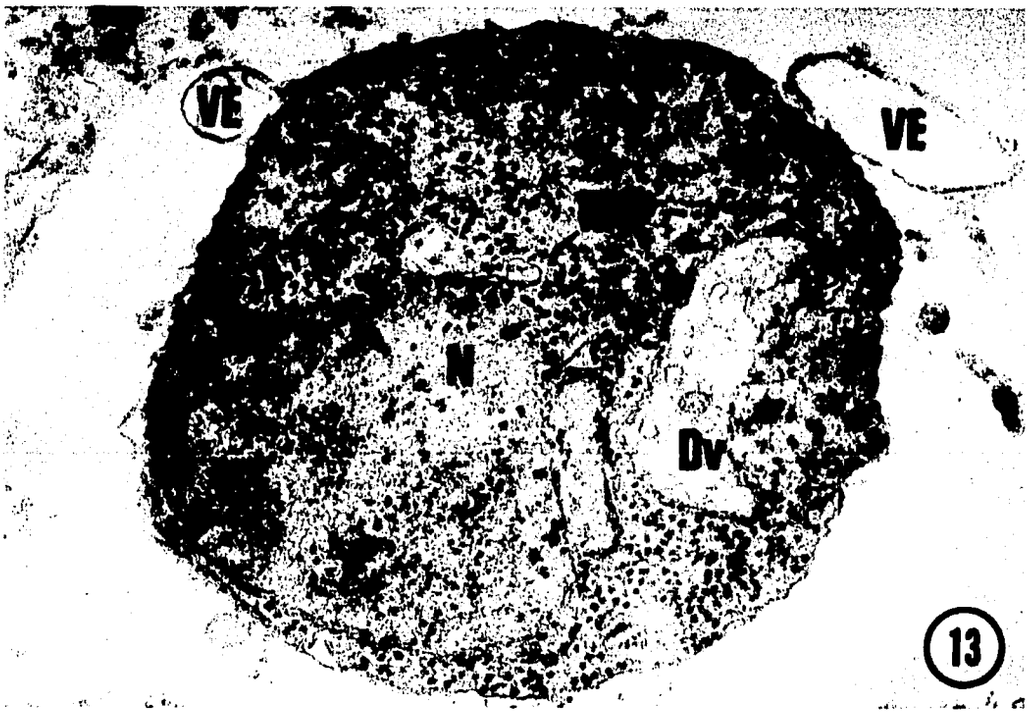


FIGURE 13. *P. berghei* freed from a rat erythrocyte by cell disintegration by French press. Density alterations are obvious in the compact nucleus (N), and numerous ribosomes (RNP) are found in the cytoplasm. A double-membraned vacuole (Dv) appears to enclose a light matrix with a few vesicular components. The double-unit membrane limiting the parasite cytoplasm is occasionally thrown into vacuolar extensions (VE). The parasite appears relatively free of host contamination, although small remnants may be present (× 61,000).

the finding of a parasite in a partially lysed host cell was rare.

As illustrated in Figure 5, a low-power field of a thin section of French-press isolated parasites usually displayed only a small number of parasites among the pieces of cellular debris. These parasites generally appeared healthy. Damaged forms and intact erythrocytes or parasitized red cells were infrequent.

The preparations lysed by antiserum contained a wide range of material consisting of many intact red cells, infected cells, parasites in partially lysed cells, and freed parasites, often relatively intact (Fig. 6). Sometimes after lysis by antiserum, the parasites appeared damaged or altered to a varying degree (Figs. 21 and 22). The cyto- and nucleoplasms looked empty or light in density, and vacuolation and membrane elaboration was evident. In apparently more advanced stages of destruction, aggregation of cytoplasmic components was observed (Fig. 22).

Only a few whole parasites were usually encountered in a low-power field of a thin-sectioned, distilled-water isolate (Fig. 7). Under the conditions of this study, lysis by distilled water of infected cells yielded parasites all injured to a greater or lesser degree. Many damaged parasites exhibited an altered internal organization similar to that just described for some parasites freed by lysis with antiserum (Fig. 21). Several others occurred as a very dense form, with the cytoplasm appearing to be condensed or concentrated around a number of vacuolar spaces. These vacuoles were present peripherally (Fig. 23) or seemed to join and form large holes in the parasite (Fig. 24). They also existed as long extensions out of the parasite (Fig. 25). The parasite may eventually be broken in this way, for small pieces of parasite cytoplasm were prevalent in many sections of these preparations (Fig. 25).

Stages

Parasites observed after lysis by any one of the four methods that we used were commonly

FIGURE 14. A *P. berghei* trophozoite freed by cell disintegration by French press exhibits a cup-shaped nucleus (N) and prominent, intact food vacuoles (FV). A double-unit membrane (PM) is visible at intervals along the parasite's surface. Occasionally the two membranes appear to be pulled away from the parasite cytoplasm or from each other (*arrows*). A few host-membrane remnants (HM) are evident (× 55,000).

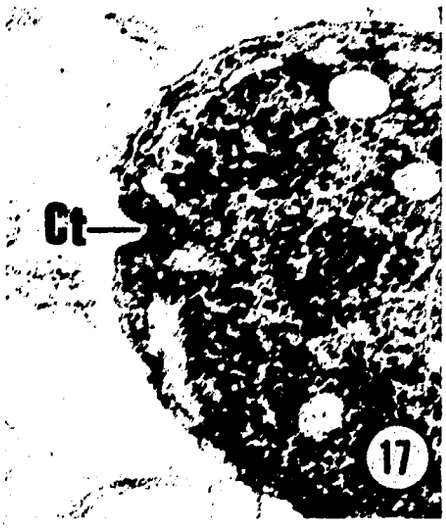
of the trophozoite stage. Schizonts (Fig. 12) were seldom encountered. Some small parasites resembled the merozoites that are seen forming in the segmenters of intracellular *P. berghei*. For example, Figure 15 demonstrates a parasite that was freed by French-press disintegration and appears to be a merozoite.

Limiting Membranes

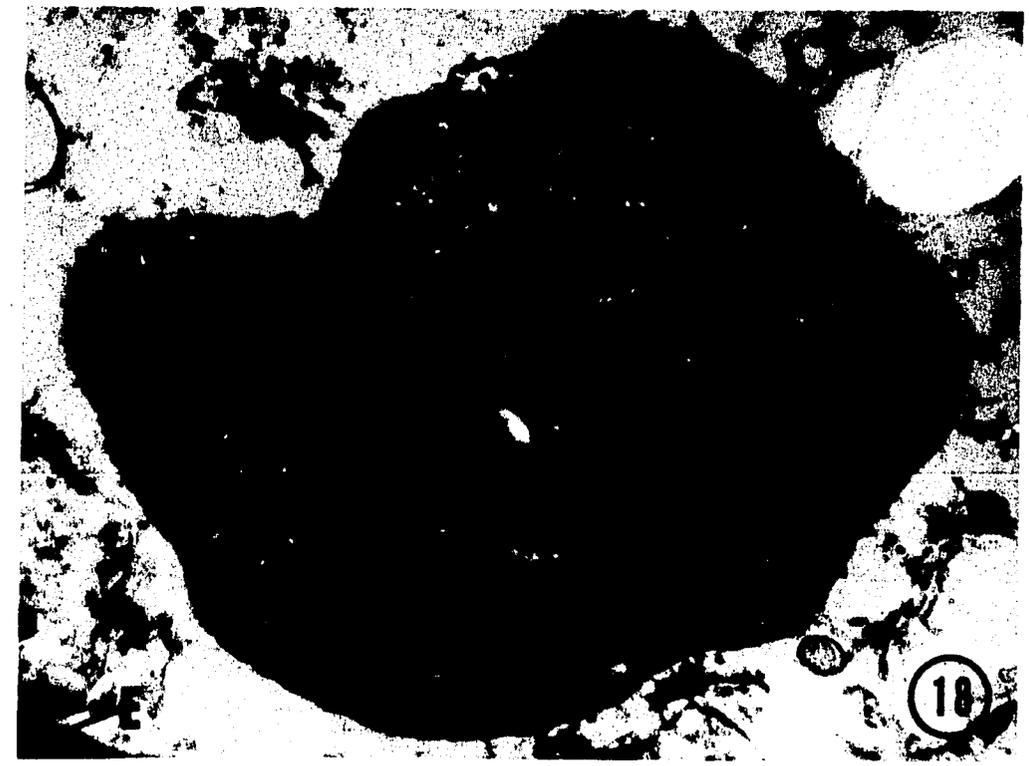
Host

In this study *host membrane* denotes the membrane material found close to the parasite but external to the double-unit membrane often observed limiting the cytoplasm of the freed parasites.

The close association of host-membrane material was common in saponin-prepared isolates. In several of these preparations, the host membranes occurred as a parallel array of membrane layers, many applied to or located close to the limiting membranes of the parasite (Figs. 4, 11, and 12). No parallel arrays of host-membrane material were evident in isolates prepared by French-press disintegration (Figs. 5, 13, 14, and 15). Extraneous membrane material, when present, usually occurred as small individual pieces that appeared



FIGURES 15–17. **15.** A merozoite of *P. berghei* prepared by cell disintegration by French press shows evidence of a three-layered limiting membrane (*arrows*). A large nuclear region (N) is visible. Dense bodies (D) also appear in the cytoplasm (× 72,000). **16.** An oblique section of a cytostome (Ct) of *P. berghei* freed by cell disintegration by French press appearing as a dense crescent-shaped depression in the outer surface of the parasite (× 56,000). **17.** A cross-section of the cytostome (Ct) of a French press-freed *P. berghei* merozoite. Dark areas bounding the invagination are evident (× 50,000).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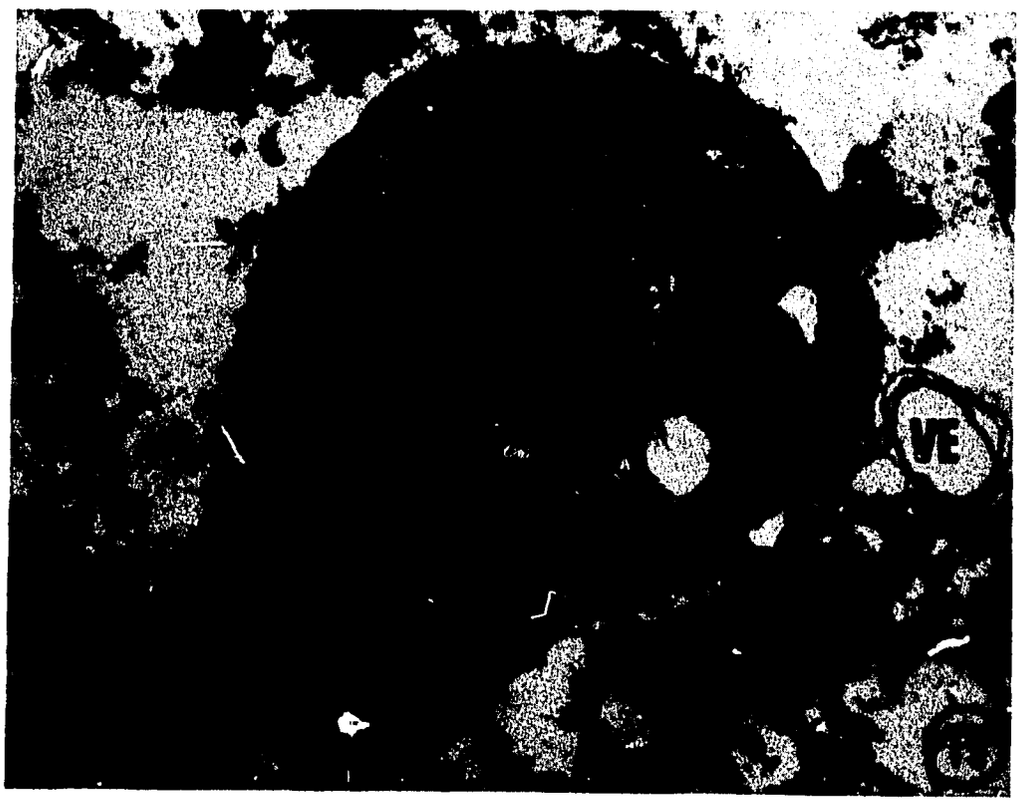
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FIGURE 20. A large and swollen double-membraned vacuole (Dv) with a few small inclusions is illustrated in a portion of a *P. berghei* trophozoite freed by antiserum lysis. A double-unit membrane (PM) limiting the parasite is also evident (× 55,000).

to approach the parasite surface only in a few sites. In *P. berghei* prepared by antiserum lysis, host membrane was occasionally encountered around part of the parasite. However, extensive parallel arrays of host membranes were not seen. Finally, host-membrane material was often observed not as parallel arrays but in close association with parasites isolated from their host cells by distilled-water lysis (Fig. 23).

Parasite

In many sections, freed *P. berghei* trophozoites exhibited a double-unit membrane limiting the cytoplasm and visible at least at intervals along the parasite's surface (Figs. 9, 14, and 20). A triple-layered outer membrane was distinguishable in some sections of isolated merozoites (Fig. 15), and the thick inner membrane characteristic of schizonts was also detected (Fig. 12).

The plasma membrane sometimes appeared relatively intact. However, evaginations and large vacuolar extensions (Figs. 13, 19, and 25) were not uncommon. Occasionally, breaks were evident in the plasma membrane (Fig. 21), or the two unit membranes were pulled away from the parasite cytoplasm or from each other (Fig. 14).

Nucleus

The nuclei of isolated parasites generally appeared compact even in the trophozoite stage (Figs. 11 and 13). In addition, the nuclei of both trophozoites and small dense forms, probably merozoites, often exhibited prominent inclusions. These inclusions included nucleolar-like patches (Figs. 8, 11, and 19) and clumping or aggregation of dense particles (Figs. 8, 11, 19, and 21).

Structures associated with food intake

Food vacuoles (Figs. 14 and 22) and vesicles containing hemozoin particles (Figs. 11, 12, and 23) often appeared essentially unaltered in the freed parasites, although large food vacuoles were

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FIGURES 18 and 19. **18.** *P. berghei* freed from its host erythrocyte by antiserum lysis. The membranes limiting the parasite appear reasonably intact, as does the internal cytoplasmic structure. Parallel membranes of the endoplasmic reticulum (ER) are visible in the cytoplasm. Note the presence of a portion of an intact erythrocyte (E) in the preparation (× 66,000). **19.** A nucleolar-like patch (Nc) and considerable clumping (Cp) are observed in an antiserum lysis-freed *P. berghei*. A double-membraned vacuole with a few inclusions (Dv) is evident, along with a vacuolar extension (VE) apparently related to the parasite (× 45,000).

sometimes observed to be lighter in density than the parasite cytoplasm (Fig. 8). Of special interest was the apparent resistance of food vacuoles to some of the destructive action of antiserum lysis, where damaged parasites contained what appeared to be relatively intact inclusions of host cytoplasm (Figs. 21 and 22). Cytostomes were observed infrequently and were seen particularly in French-press preparations; they appeared normal when present (Figs. 16 and 17).

Membraned organelles

Sausage-shaped vacuoles and multilaminated-membraned bodies (Figs. 8, 11, and 25) characteristic of intracellular *P. berghei* were evident in parasites freed by all four methods of release considered here.

In saponin-prepared isolates particularly, and to a lesser extent in the rest of the isolates, double-membraned organelles with atypical features were also found. For example, double-membraned vacuoles varying in size and containing a number of distinct vesicular elements were common (Figs. 8, 9, 12, and 13). On the other hand, an internal double membrane often in a scalloped pattern was present in some of the double-membraned vacuoles; the matrix around this membrane system was usually more or less uniform in density and without discrete vesicular elements (Figs. 8 and 10). A small concentric-membraned structure suggestive of an early multilaminated-membraned body was occasionally observed along with the vesicular elements within a few double-membraned vacuoles (Fig. 8). Finally, large and swollen double-membraned vacuoles with a few small inclusions (Fig. 20) were infrequently noted, especially in damaged parasites.

Other cytoplasmic structures

In sections of freed *P. berghei* where the parasites looked reasonably intact, the ribosomes appeared normal and plentiful in the cytoplasm (Figs. 11 and 13). As indicated earlier, in badly injured parasites, clumping was prominent in the cytoplasm, and no typical ribosomes were visible (Fig. 22).

The endoplasmic reticulum was sometimes poorly developed in the isolated parasites. In many saponin-prepared parasites, however, the endoplasmic reticulum appeared as relatively wide vesicles with a light matrix (Figs. 8 and 11). Parallel membranes of the endoplasmic reticulum were evident in Figure 18, which shows a parasite freed by antiserum lysis.

Dense bodies, possibly toxonemes, are suggested in Figure 15 and appear similar to these structures found in intracellular merozoites.

DISCUSSION

General Characteristics of Isolates

The total number of intact, freed parasites released by French-press disintegration was significantly less than that resulting from saponin or antiserum lysis of an equivalent amount of parasitized red blood cells. It appears that the French press effectively disintegrated not only the red blood cells, but also a proportion of the parasites as well. However, of those parasites that survived French-press isolation, most were structurally intact and comparatively free of the closely associated host membrane arrays frequently seen in the saponin-freed material. Lysis by antiserum freed some apparently intact parasites, but both damaged parasites and unbroken host cells were also observed. Lysis by distilled water yielded very few healthy parasites, and most freed forms contained vacuolar inclusions or were broken into smaller pieces.

These results are somewhat specific to the conditions of the four methods of lysis selected for study here. Bovarnick and co-workers, for example, using saponin, found that the extent of hemolysis seemed to vary with the concentration

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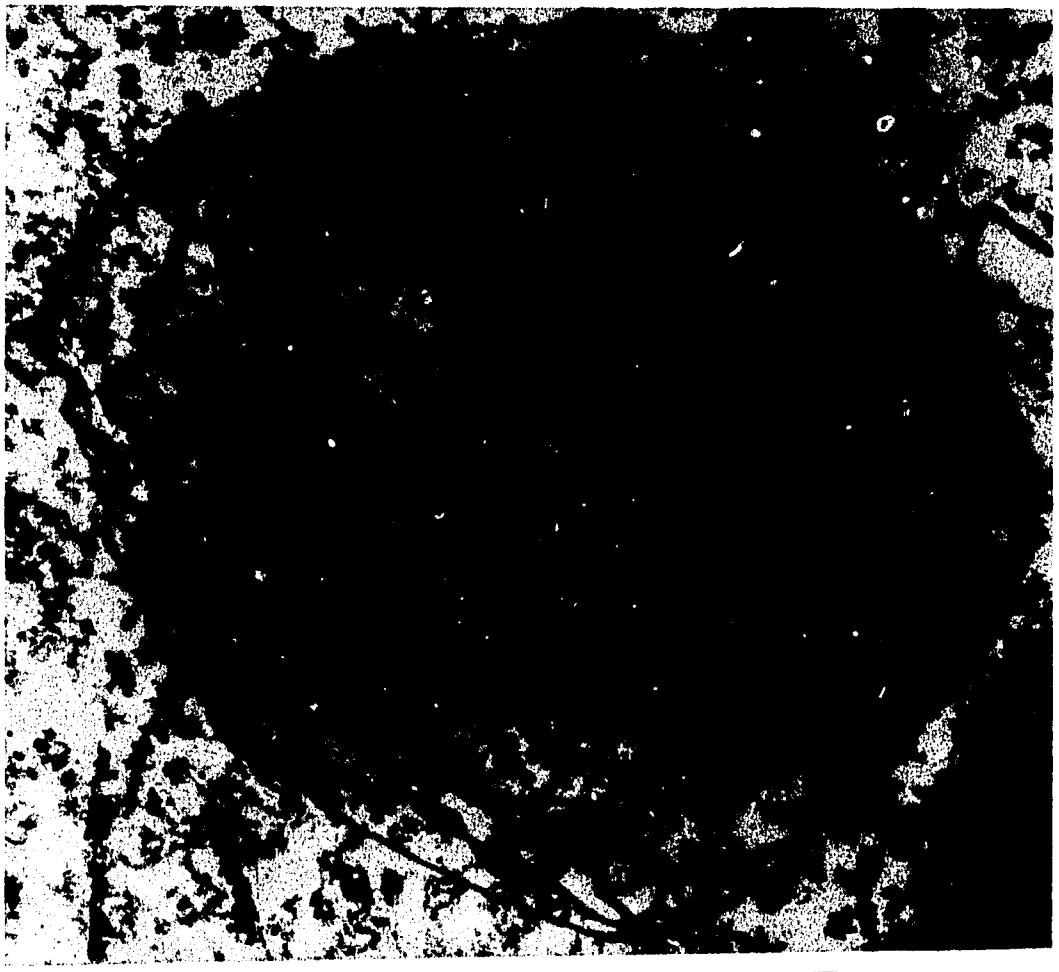
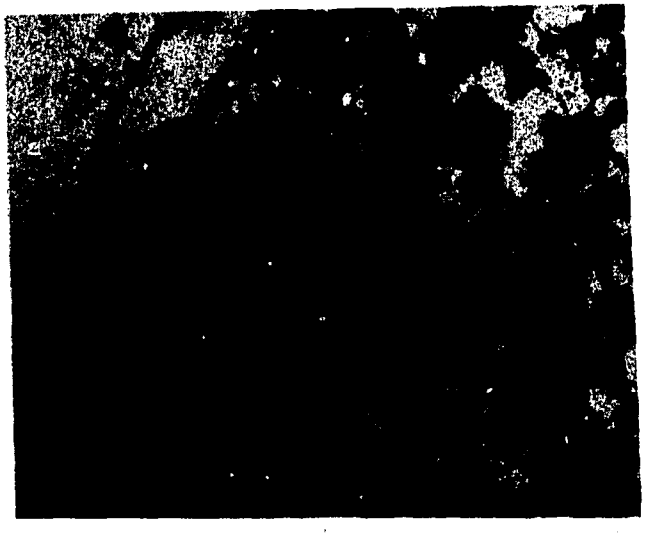
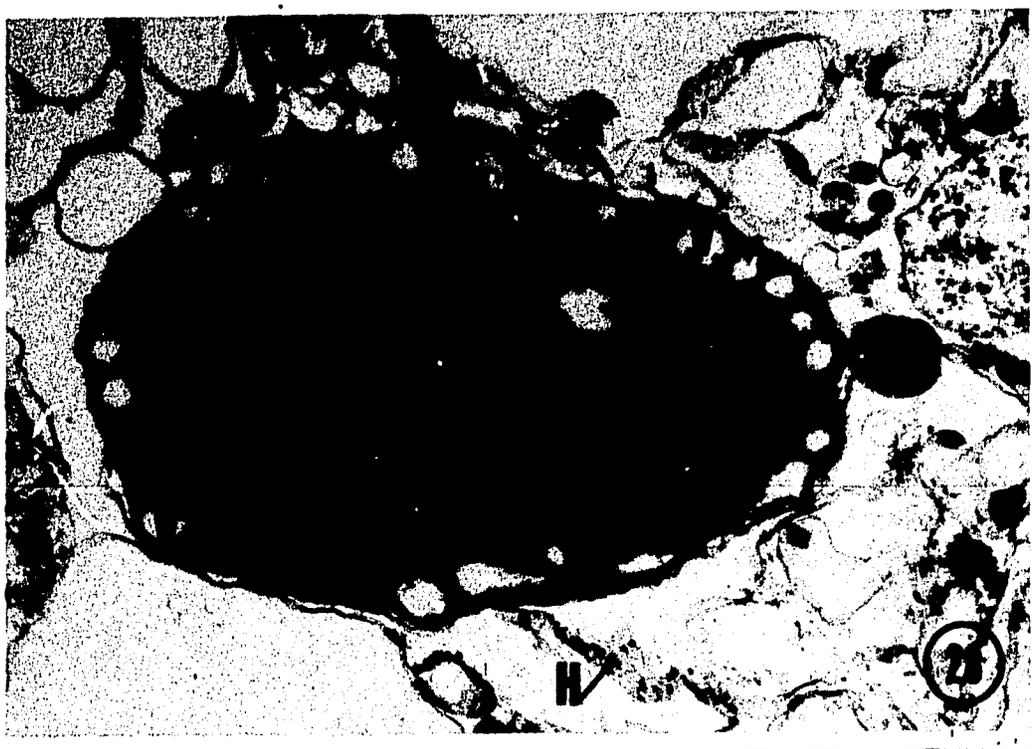
FIGURES 21 and 22. **21.** A damaged *P. berghei* after lysis by antiserum of infected cells displays a clumping (Cp) in the nucleus (N), a cytoplasm that is light in density, except for an area resembling host cytoplasm (HC), some membrane alterations (Ma), and a break (B) in the double-unit membrane at the parasite's surface. An intact erythrocyte (E) is visible in the section (× 50,000). **22.** Extensive clumping in the cytoplasm (Cc) is observed in a damaged antiserum lysis-freed *P. berghei*. An apparent inclusion of host cytoplasm (HC) looks normal (×66,000).



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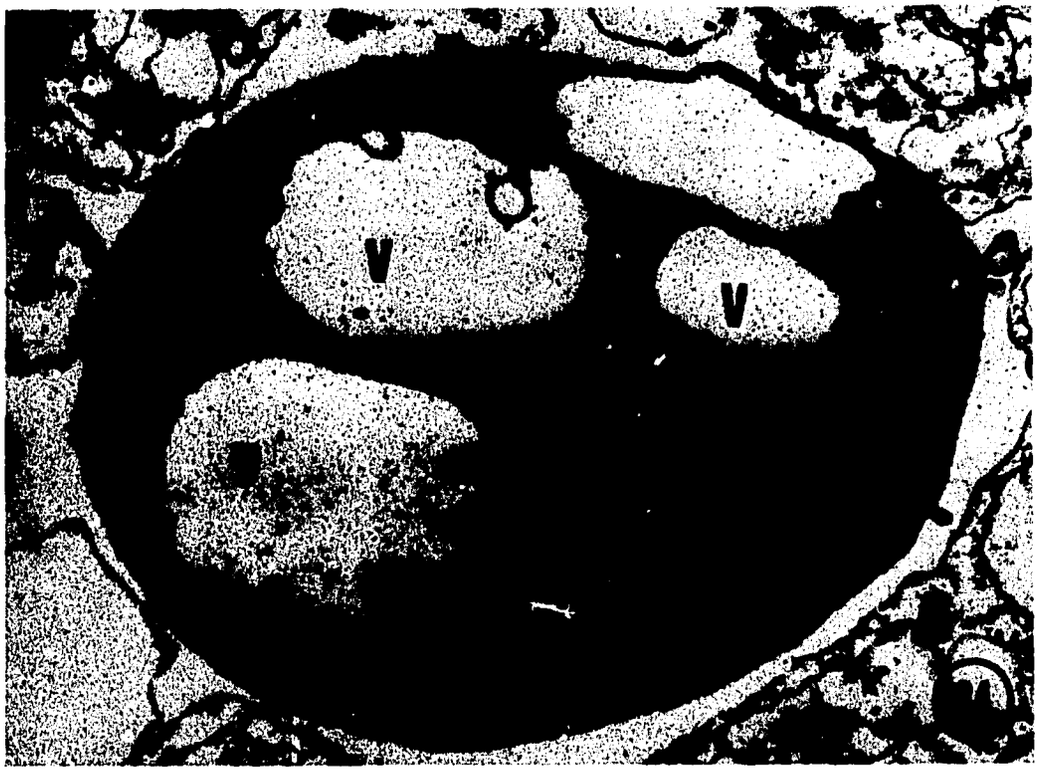
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FIGURE 25. A vacuolar extension (VE) is conspicuous in a *P. berghei* parasite after distilled-water lysis of infected blood. Note a small multilaminated-membraned body (MM) in a vacuole within the parasite. Odd pieces of parasite cytoplasm (P) are indicated in the vicinity of the parasite (× 40,000).

of saponin and the temperature at which the cells were in contact with the saponin.[1] Bowman and his colleagues reported a very small percentage of intact erythrocytes after their antiserum treatment,[2] unlike the relatively large number of intact or partially lysed erythrocytes observed here. In the antiserum technique, agitation during incubation with the serum appears important in reducing the development of clumps of agglutinated red blood cells. Differences in agitation methods and in subsequent procedures for collecting the freed parasites by centrifugation may have contributed to the differences in our preparations with regard to the contamination with host cells. Deegan and Maegraith exposed infected cells to only two times their volume (as opposed to at least 10 volumes used here) of distilled water and described the collection of swollen but intact parasites.[17] Such a swollen state is reminiscent of some of the earlier forms of damage with vacuole formation observed in our distilled-water preparations.

Variation may also result from the use of different host cells or parasites, or both. Investigators interested in obtaining freed avian malaria parasites, for example, must also deal with the host cell nucleus as a source of contamination. On the other hand, the parasite itself may react differently to the methods of release. D'Antonio, for instance, has observed that *P. berghei* appears to

FIGURES 23 and 24. **23.** A *P. berghei* parasite prepared by distilled-water lysis of rat erythrocytes is observed to feature a series of peripheral vacuoles (V) and to exhibit a cytoplasm that is very dense and apparently lacking in endoplasmic reticulum. Vesicles containing pigment granules (Vp) are obvious in the cytoplasm. Host material (H) appears to be closely associated with the parasite (× 43,000). **24.** Large vacuoles or holes (V) dominate a distilled water-prepared *P. berghei,* which also displays a condensed cytoplasm (× 47,000).

FIGURE 26. Two *P. berghei* trophozoites found outside the host cell in a preparation of infected blood. A double-unit membrane limiting the cytoplasm of the parasites (*arrows*) is evident, as is the presence of host membrane (HM) and cytoplasmic (HC) materials (× 32,000).

be more susceptible to mechanical pressure treatment than *Plasmodium knowlesi*, and a lower pressure with the French pressure cell must be used in order to obtain intact parasites.*

Stages

That large parasites may be broken or fragmented during release from the host cells is suggested in the ultrastructural studies of isolates prepared by all four methods. Larger parasites, especially segmenters, were absent from most of the thin sections examined.

Rudzinska and Trager reported that *P. lophurae* merozoites that had formed extracellularly *in vitro* differed from those formed within a schizont in a host cell in being spherical in shape.[83] The merozoites of *P. lophurae* are more ovoid in the

* Dr. L. E. D'Antonio, Department of Zoology, University of Illinois, Urbana. Personal communication.

intracellular schizont. In this study, small forms, which appeared to be extracellular merozoites of *P. berghei,* do not vary much in size or shape from those observed within the schizonts located in host erythrocytes.

Limiting Membranes

Host material

As indicated earlier, *host membranes* are considered here to be the membrane material close to the parasites but outside the double-unit membrane usually found at the surface of the isolated parasites. There is another possible interpretation, however. The double-unit membrane has also been observed to border intracellular plasmodia,[30] and its origin is currently in question. Aikawa[40] and Hepler *et al.*[41] have proposed that the outer of the double-unit membrane is not of parasite origin but is formed by the host cell in response to the infection. If their theory proves to be correct, the second membrane limiting the parasite, as seen at least at intervals in many of our freed specimens, would also represent host-membrane material.

It is generally believed that after lysis by saponin,[37] antiserum,[38] or exposure to a hypotonic medium,[37] holes or defects are produced in the membrane of the red blood cell with the resulting escape of hemoglobin. In the instance of erythrocytes infected with plasmodia, it seems reasonable to assume that after such a lysis, the red-cell membrane, still relatively intact or partially broken, will collapse and surround the parasite, forming a range of associations with it. Reports concerned with the release of parasites by the three methods mentioned above—saponin, antiserum, and hypotonic lysis—note the presence of stromal material close to or attached to the freed parasites.[2,9,17,39] In the preparations studied here after saponin lysis, host membranes were also evident, often as a parallel array appearing sometimes to envelop the parasite. Host membranes were also visible in the antiserum and distilled-water lysates, but in a less elaborate way, often only pieces of the membrane appearing close to the parasite. Likewise, only a few host-membrane components were found associated with most of the parasites freed by French press. According to D'Antonio and colleagues, mechanical disintegration employing the French press tends to destroy or disintegrate the red-cell membrane, many pieces of which can be removed by centrifugation of the intact parasites.[7] Our results tend to support this interpretation.

Parasite

As discussed above, there are many reports of a double-unit membrane surrounding plasmodia located within the erythrocytes.[30] We likewise saw two-unit membranes, at least at intervals, limiting several of the isolated parasites. Parasites accidentally found outside the host cell in infected whole-blood preparations also exhibited an intact double membrane (Fig. 26); Rudzinska and Trager reported a similar occurrence in *Plasmodium coatneyi.*[12] Theakston *et al.* also described a double-unit membrane in *P. vinckei* after hemolytic antiserum treatment.[31] Bahr, on the other hand, claimed that only a single-unit membrane limited the cytoplasm of freed *P. berghei* in his preparations, and he described the existence of erythrocyte membranes wrapped tightly around the parasites.[10] The question of the identity of the outer of the double-unit membrane that limits intracellular malaria parasites is again of significance. Some of the extraneous host-membrane material observed by Bahr may be interpreted instead as the second unit membrane of the parasite that has been pulled periodically from the surface of the parasite during the lytic treatment.

Nucleus

Peters reported the presence of a nucleolus in what he described as a penetrating merozoite of *P. berghei.*[43] We have not observed any nucleoli in the nuclei of intracellular *P. berghei* parasites. However, we have seen a dense chromatin-like distribution in the merozoites of a segmenting *P. berghei* (Fig. 3), which closely resembles that described in the merozoites of avian plasmodia.[10]

The discovery in isolated parasites of nucleolar-like patches and additional dense nuclear inclusions that resemble the smaller particulates and the clumping of ribosome particles reported by Ladda[41] after exposure of *P. berghei*-infected cells to chloroquine *in vitro* is of special interest. Some of this nuclear alteration (particularly the clumping) may represent a form of nonspecific damage paralleling the effect of certain drugs. The almost complete absence of nuclear inclusions

in *P. berghei* parasites that we accidentally found outside the host cells (Fig. 26) and not exposed to extensive lytic procedures, tends to favor this proposal, although only a few of these parasites were available for examination. The well-defined nucleolar-like body, as well as some of the other dense material present in many of the freed preparations may have a physiological significance as the rest of the parasite often appeared intact.

Structures associated with food intake

The apparent resistance of food vacuoles to some of the destructive action evident in the antiserum-lysis preparations is puzzling. The technique involves the exposure of infected cells to a rat-erythrocyte antiserum and complement. One might expect the hemolytic action to extend to the components of the erythrocyte cytoplasm that occur as inclusions in the parasite, especially when the parasite itself is often damaged to the point that permeability is enhanced. Nevertheless, the presence of the relatively intact food vacuoles in the absence of the surrounding erythrocyte, both here and in several other sections of isolates prepared by the various methods, strengthens Rudzinska's and Trager's[30] and Trager's[45] stand that the large host inclusions observed in malaria parasites are not simply the result of the irregular contour of the parasite.

Membraned organelles

The true significance of two membranous organelles—the sausage-shaped vacuole and the multilaminated-membraned body (also termed a concentric double-membraned body)[31]—that occur repeatedly in the erythrocytic stages of mammalian malaria parasites[30] has not been determined. A mitochondrial function has been proposed for both organelles.[34,36,30] In the instance of the former structure in *P. vinckei*,[34] this hypothesis has been based in part on the occasional finding of crista-like inclusions. We have only rarely observed distinct inclusions in the sausage-shaped vacuoles of intracellular *P. berghei*, and these few examples involved just one or two inclusions per vacuole. The sausage-shaped vacuole has also been described as a fluid-filled reservoir that supports or maintains osmotic stability between parasite and host cell.[34,36] The multilaminated-membraned body has been given additional significance as a possible membrane-proliferating apparatus or as a type of Golgi structure.[34]

Although the presence of these two organelles is usually observed at the periphery of the parasite in close association with the plasma membrane, they also appear away from the outer surface; the multilaminated-membraned body, in particular, is often seen in close proximity to the nuclear membranes or to the membranes surrounding a large food vacuole. In addition, the multilaminated-membraned body may appear to be contained within or possibly derived from the sausage-shaped vacuole (Figs. 1 and 2). It may also appear as a vesicular structure if cut in a different plane.[34] There appears then to be an elaboration of membrane structures, presumably for a variety of functions—all structures perhaps originating at the outer plasma membrane and retaining many membranous associations after leaving the surface of the parasite.

It is interesting that in freed parasites, this elaboration of membrane structures is carried even further to forms that are similar to sections of the sausage-shaped vacuole but exhibit a well-defined internal organization. In saponin-prepared parasites especially, double-membraned vacuoles containing vesicles were common (Fig. 9) and bear resemblance to the typical protozoan mitochondria described in avian malaria.[40] Their frequent appearance in the freed parasites was accompanied by a reduction in the number of sausage-shaped vacuoles and multilaminated-membraned bodies. Like the sausage-shaped vacuoles, they may contain a multilaminated-membraned body that appears along with the vesicular elements. These observations suggest that at least some of the double-membraned vacuoles seen in freed parasites may be structural expressions similar in nature or function to that of the sausage-shaped vacuoles and multilaminated-membraned bodies of intracellular *P. berghei*. Possibly some of these double-membraned vacuoles represent forms with mitochondrial function in parasites subjected to abnormal conditions. Mitochondria are flexible structures known to respond readily to environmental changes. Rudzinska and Vickerman reviewed the alterations in mitochondrial structure with variation in factors such as the state of aerobiosis, the presence of adequate food, and the age of the organism.[30] Metabolic differences in isolated *P. berghei* para-

sites as, for example, the decreased ability to oxidize lactate,[2] may possibly be reflected in a change in mitochondrial structure and, in turn, explain, in part, the double-membraned vacuoles described here in the freed parasites.

There is also the possibility that the double-membraned vacuoles are simply manifestations of damage; certainly some of the forms do suggest this. For example, the large double-membraned vacuoles with a few inclusions observed in the antiserum-lysis preparations (Fig. 20) imitate the swollen mitochondria reported in *Plasmodium fallax* exposed to primaquine in tissue culture.[40] Also, the double-membraned vacuoles with internal membrane systems, often of a scalloped nature, resemble structures described in *P. berghei* after treatment with chloroquine *in vitro*.[41]

Other cytoplasmic structures

The cytoplasmic clumping observed in badly damaged freed parasites closely resembled that described by Ladda[44] after he exposed *P. berghei*-infected cells to certain drugs—for example, to a high concentration of chloroquine. The presence of a poorly developed endoplasmic reticulum in some of the isolated parasites is not unusual. Rudzinska and Vickerman, in their 1968 review, noted that the endoplasmic reticulum of malaria parasites is not too well developed.[30] Furthermore, it varies considerably during the growth of the organism and may appear sometimes to be absent from the parasite, but at other times to fill the cytoplasm.

CONCLUSION

It appears impossible to prepare freed malaria parasites completely devoid of host contamination. Parasites may be cleaned of surrounding host membranes and cytoplasmic remnants. However, food vacuoles, degraded hemoglobin, and other host products are incorporated in the cytoplasm of the parasite and may not be removed without destruction of the parasite itself. This is the nature of an obligate intracellular parasitic relation. Nevertheless, simplification of the host-parasite system by isolation of the parasites, however incomplete the isolation, appears useful for a variety of experimental studies. The electron microscope, it appears, may play a role in defining this simplified system in terms of the amount and nature of host remnants and the apparent intactness of the parasite.

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