The Morphology and Behavior of Living Exoerythrocytic Stages of *Plasmodium gallinaceum* and *P. fallax* and Their Host Cells*

By CLAY G. HUFF, Sc.D., ALAN C. PIPKIN, Ph.D., A. B. WEATHERSBY, Ph.D., and DINNIEMAUD V. JENSEN

(From the Division of Parasitology, Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland)

PLATES 30 TO 32

(Received for publication, May 1, 1959)

ABSTRACT

The morphology and behavior of living exoerythrocytic stages of *Plasmodium gallinaceum* and *P. fallax* were studied by the use of tissue cultures, phase contrast microscopy, and time-lapse cinephotomicrography. The morphology of exoerythrocytic stages of these two species was essentially that previously observed in fixed, stained material, with the following exceptions: (1) the presence of a filament on one end of the merozoite, (2) the absence of clefts in the cytoplasm of the large schizonts, and (3) the absence of a vacuole-like space around the parasite.

The following behavior was observed either directly or in time-lapse sequences: (1) emergence of merozoites from mature schizonts, (2) progressive motility of free merozoites, (3) entry of merozoites, both actively and passively, into host cells, (4) nuclear division in the parasite, (5) the various stages of schizogony, including final production of merozoites, (6) massive infection of host cells, and (7) phagocytosis of merozoites and attempted phagocytosis of mature schizonts by macrophages.

Exoerythrocytic stages of *P. fallax* differed from those of *P. gallinaceum* in that the merozoites of the former were (1) somewhat more curved in shape and (2) present in fewer numbers in mature schizonts.

The use of tissue culture, phase contrast microscopy, and time-lapse cinephotomicrography promises to solve many of the remaining problems concerning exoerythrocytic stages of malarial parasites and their interrelationships with host cells.

INTRODUCTION

Living exoerythrocytic stages of malarial parasites offer certain advantages for cytological study not offered by other cells. These stages are known to be obligate parasites of several different types of non-erythrocytic cells. The morphology of several species of these parasites has been previously studied by conventional histological methods. A few of them which are parasitic in birds are well suited for study in tissue culture by means of phase contrast microscopy, and time-lapse photomicrography. The cell-parasite complex offers the advantage that the interaction of two cells can be observed. One of these—the parasite—differs from the other—the host cell—in important characteristics such as its multinucleate nature, its method of nuclear division, and its inability to live extracellularly. Compared to many other intracellular parasites such as bacteria or rickettsiae the exoerythrocytic stages have the advantage of larger size.

The two species utilized in this study were *Plasmodium gallinaceum* and *P. fallax*, which are parasites of domestic fowl and which we have been able to adapt to continuous passage in chick embryos. The following account attempts (1) to

* The authors are grateful for the assistance given them by Miss Tsugiye Shiroishi, Mr. Richard N. Rossan, Dan G. Hartlieb, HM1, United States Navy, and Benjamin Raley, HM2, United States Navy.

describe the morphology of the living exoerythrocytic stages of these two species, (2) to compare these observations with published descriptions of fixed and stained material (see Huff and Coulston, 1944), (3) to describe their behavior based on visual observations and upon time-lapse moving pictures, and (4) to describe some of the interrelations of the exoerythrocytic stages and their host cells.

Materials and Methods

1. Strains.—The strains were continuously propagated in chick embryos by weekly serial passage of brain tissue from infected embryos onto the chorioallantoic membranes of normal 7 to 8 day old embryos by a modification of methods used by Zuckerman (1946), by Haas, Wilcox, and Ewing (1945), and Wilcox (personal communication, 1959). Eggs were candled, the position of the air sac in each marked, and an electric drill fitted with a dental carborundum wheel used to make a triangular cut in the shell in this region. To lessen the danger of contamination the shell membrane was left intact. A platinum tool heated to redness was then used to cut the membrane, and the opening into the air sac was completed by removing the triangular portion of the shell. Sterile forceps were introduced into this opening to make a small tear in the shell membrane above the embryo so as to expose a portion of the chorioallantois. Care was taken not to injure the chorioallantoic membrane and a piece of infected brain was then implanted upon the exposed membrane in the area having the greatest concentration of blood vessels. After 7 days' incubation at 37°C., the embryos were removed from the shell; brains and livers were removed, smeared, stained with Giemsa, and examined microscopically for exoerythrocytic stages. The most heavily infected brains were used to propagate the strain. One brain from a 14 to 15 day old embryo cut into 7 or 8 pieces provided sufficient inoculum for as many embryos. The most heavily infected livers were used to prepare monolayer tissue cultures as described below.

The *P. gallinaceum* strain was originated in this laboratory in 1954 by inoculating chick embryos intravenously with sporozoites following the method of Eichhorn (1940). The *P. fallax* strain was initiated in March 1957 by transferring portions of brain from a turkey that showed signs of brain involvement, onto the chorioallantoic membranes of chick embryos using the method of implantation described above. This bird had been inoculated intravenously 17 days previously with erythrocytic parasites; a brain smear indicated that numerous exoerythrocytic parasites were present.

2. Tissue Culture Methods.—The infected livers were washed 2 or 3 times in balanced salt solution to remove as many red blood cells and other debris as possible and were then trypsinized in a solution consisting of 0.25 per cent trypsin (Difco, 1:250) or "Tryptar" (Armour) in Hanks' balanced salt solution (Hanks and Wallace, 1949) using methods similar to that of Youngner (1954), Melnick *et al.* (1955). Trypsinization was continued for 10 to 15 minutes while spinning on a magnetic stirrer and resulted in a suspension for the most part of single cells and some small, cell aggregates. This cell suspension was centrifuged for 10 minutes at 1500 R.P.M. and a cell pack composed of 3 or sometimes 4 layers was formed. After aspirating the supernatant fluid, the mid-layer and particularly the cells just above the red cell pack were removed with a syringe and long needle and added to nutrient medium to give a 1 to 50 to 1 to 100 cell dilution. It was our experience that higher cell dilutions produced cultures which did not proliferate readily and which had too few parasites. One ml. portions of the resulting cell suspension were dispensed into each Porter flask which had two 11 x 11 mm. coverslips; or 0.5 ml. was dispensed into 16 x 150 mm. test tubes flattened on one side so as to accommodate one 11 x 22 mm. coverslip (Weiss and Dressler, 1958). The pH of the cultures was then adjusted to about 7.2 by gassing with a mixture of 5 per cent CO_2 and 95 per cent air according to Earle (personal communication, 1956). The cultures were incubated in a stationary position at 37°C. and were not disturbed for 18 to 42 hours.

The nutrient medium routinely employed was made up of 15 to 20 per cent chicken serum, 5 per cent chick embryo extract ultrafiltrate (EE-50), and 75 to 80 per cent Earle's balanced salt solution (Earle, 1943).[1] We recently substituted whole egg ultrafiltrate for the embryo extract and have found that it also stimulates growth effectively. Penicillin (50 units/ml.) and streptomycin (50 μg./ml.) were routinely added to the medium. Fluids on the cultures were usually replaced after about 42 hours incubation and on alternate days thereafter. The serum concentration was reduced to 10 per cent as soon as the cells had multiplied and migrated sufficiently to cover the floor of the flask or tube.

3. Microscopy and Photomicrography.—Phase contrast observations and photomicrographs were made with a Bausch & Lomb research microscope with positive, dark, phase contrast equipment and a Gamma cut-film photomicrographic camera (10 x 12.5 cm.). One valuable adjunct to satisfactory phase contrast microscopy consisted of an optical bench for supporting the microscope, the illuminator, and other equipment in rigid alignment. Illumination was obtained from a Bausch & Lomb ribbon filament lamp (108 watt, 6 volt) with condenser. A special condenser for concentrating more of the light from the band-shaped filament image was placed between the lamp and the mirror of the microscope (See Huff and Bronson, 1956). An interference filter (photomicrographic series) with peak wave length of 5500 A was used for observation.

[1] All of these biologicals were purchased from Microbiological Associates, Inc., Bethesda, Maryland.

The time-lapse cinephotomicrographic studies were made with a Model GF standard Zeiss-Winkel microscope with phase contrast condenser III Z/6 and illuminating system like that described above. Zeiss, × 100 and Wild, × 50, oil immersion phase contrast objectives and Zeiss, × 6, photo ocular were used. The time-lapse mechanism was built in our shops and consisted of a synchronous motor-driven cam activating electric microswitches controlling the light source. Length of exposure and interval between exposures were varied by changing the settings on the cams. The timer was connected mechanically to an Eastman cinespecial 16 mm. camera. Eastman plus-X, 16 mm. reversal and Shellburst linagraph negative stock films were used, the latter proving to be somewhat better for the purpose because of its sensitivity to yellow light.

Double-coverslip, temporary mounts were used for the most part in both still and time-lapse photographic studies. The 11 x 11 mm. coverslips on which the tissue had grown were removed from the culture chamber with a long platinum hook and mounted, culture side up, beneath a larger 22 x 22 mm. coverslip sealed to a standard microscope slide with petroleum jelly. A drop of the conditioned medium from the tissue culture flask was usually incorporated in this preparation to furnish sufficient fluid to fill the preparation completely. In such a double-coverslip preparation there is only the thickness of the No. 1 superimposed 22 x 22 mm. coverslip between the cells and the objective of the microscope. This resulted in excellent phase contrast images if care was taken to flatten the petroleum jelly seal equally on all four sides and to remove any thick bits of original inoculum so as to provide a level top surface next to the oil immersion objective.

Perfusion chambers were utilized in a few instances where longer observation and photographing of the parasites were desired. Some of the cultures photographed were grown in the removable-bottom type flask previously described (Pipkin and Mack, 1958) and mounted in a perfusion chamber essentially like that of Rose (1954). Other cultures were grown in the perfusion chamber from the start, employing in the later work a chamber designed by one of us (ABW) which consisted of a plexiglas body with attached No. 1 coverglass faces, and polyethylene perfusion tubes. This type of preparation has shown considerable promise and will be reported on separately.

OBSERVATIONS

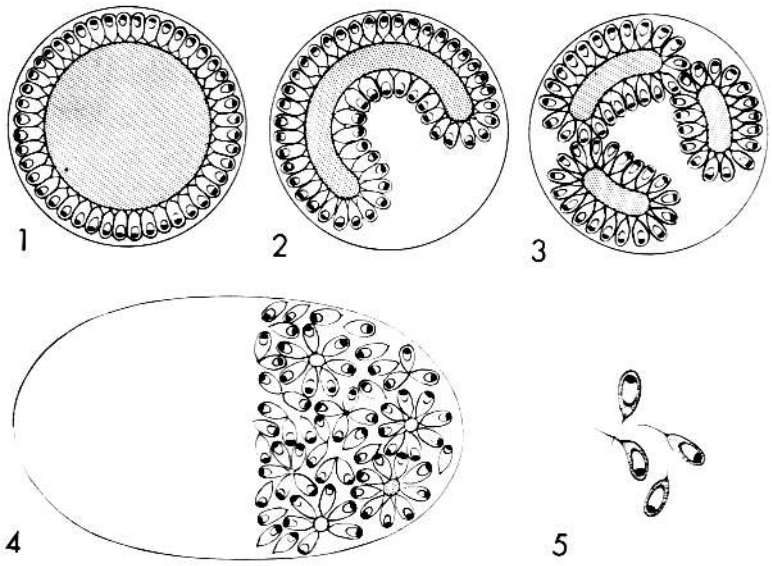
The exoerythrocytic stages of *P. fallax* were first described by Huff, Marchbank, Saroff, Scrimshaw, and Shiroishi (1950), but they were not described in detail. These authors found no differences between the stages they observed and those of *P. gallinaceum*, except the smaller number of merozoites in mature schizonts in *P. fallax*. They failed to find late exoerythrocytic stages (phanerozoites). However, these stages were subsequently reported in blood-induced infections in turkeys (Huff, 1954 *a*, *b*; 1957; and Rossan, 1957). Here we will describe the morphology and behavior of the exoerythrocytic stages, and make comparisons with findings in fixed and stained material, largely in terms of *P. gallinaceum*. Concerning *P. fallax*, it will then suffice to describe its deviations from *gallinaceum*.

PLASMODIUM GALLINACEUM

Merozoites.—When observed within the mature schizont the merozoites appeared to vary widely in size. Some of us had the impression that there were two distinct categories of schizonts in respect to the size of their merozoites. Although this impression had some support in the fact that one of our strains in chick embryos appeared to produce consistently larger merozoites than those in another, the matter was not subjected to a careful statistical analysis. An earlier study on the merozoite diameters of four species of avian *Plasmodium* (including *P. gallinaceum*) in fixed material failed to reveal any evidence of bimodality in the distribution of these measurements (Huff, 1954 *b*).

Emergence of merozoites from the mature schizont has been observed directly. The merozoites emerged either singly through a very small aperture in the wall of the mother schizont or in small numbers (Fig. 1). These observations were made upon tissue cultures in temporary preparations described above. On a few occasions when tissue cultures were being observed in Porter flasks under low magnification certain schizonts appeared to burst suddenly. This manner of emergence needs to be confirmed. In any event, it seems reasonable to assume that the manner and rapidity of emergence of the merozoites might depend upon the type of cell which is infected, the relative size of host cell and schizont, and the conditions in which the cells are living at the time of emergence.

The freed merozoites presented the elongate shape already known from stained material. They, however, differed from stained material in the presence of a filament at one pole which exceeded slightly the length of the body of the merozoite (approximately 1.2 times) (Text-fig. 5 and Fig. 2). The free merozoite had progressive motility in the direction of the filament and when attached by the filament, underwent rotation around the point of attachment. The rotation



TEXT-FIG. 1. First method of merozoite formation.

TEXT-FIG. 2. Second method of merozoite formation; invagination of peripheral layer of nuclei from which merozoites are formed.

TEXT-FIG. 3. Breaking up of double layer shown in Text-fig. 2 to form islands.

TEXT-FIG. 4. Third method of cytoplasmic schizogony without peripheral arrangement of nuclei or invagination.

TEXT-FIG. 5. Free merozoites with polar filaments.

was either clockwise or counterclockwise and it was frequently observed to change from one to the other. Under the conditions of observation (tissue cultures at 37°C.) the speed of rotation was about 2 to 5 times per second. The tip of the filament appeared slightly prehensile. Especially just prior to entrance into a host cell, the filament appeared to undergo a probing movement. Because of the refractivity of the merozoite wall it was not possible to study the internal morphology of the merozoite by phase contrast optics.

Penetration of chick embryo tissue culture cells by merozoites has been observed and still photographs have been made of various stages of this process. However, as yet, satisfactory time-lapse or normal speed moving pictures have not been made of this penetration.

On the basis of our observations so far, we believe that infection of the host cell may occur either passively or actively. Phagocytosis by macrophages was observed. In addition, what appeared to be passive entrance into cells resembling fibroblasts was observed. When a merozoite was touching the cell wall in the vicinity of the formation of a wave of membrane it was rapidly incorporated into the cell. In the more active type of penetration, after attaching itself to the cell wall of the host cell, the merozoite began penetration by inserting the filament into the cytoplasm of the host cell. The filament has been observed by phase contrast optics to lie within a small canal in the cytoplasm of the host cell. The entrance of the body of the merozoite into the host cell occurred rapidly; so rapidly that it has not yet been possible to observe the exact manner by which entrance occurred. On some occasions, after the filament was seen to penetrate the host cell it appeared to enlarge into a spatulate form. On the basis of time-lapse studies, the time required for the entrance of the body of the merozoite appears to be less than 3 minutes. It is interesting to note that Trager (1956) observed the penetration of an erythrocyte by a merozoite of *P. lophurae* in the matter of a few seconds.

After the merozoite had entered the cytoplasm of the host cell it rapidly lost its elongate form (Fig. 3), became rounded (Fig. 4), and the refractivity of its wall disappeared so that internal structures became clearly visible. By positive phase contrast the various structures appeared as follows: the nucleus in the young trophozoites was circular, paler than the cytoplasm, and about one-half the diameter of the trophozoite (Fig. 4, *N*). A dark, round organelle was associated

with the nucleus which corresponded in position and size with the organelle referred to as a nucleolus by Huff and Coulston (1944) (Fig. 4, *n*). We shall continue to refer to this structure as a nucleolus, admitting, however, that some other interpretation of its identity is possible. There appeared in the cytoplasm a bright vacuole, spherical in shape and approximately the same size as the nucleolus of the uninucleate trophozoite (Fig. 4, *V*). We interpret this as a food vacuole, again admitting that other interpretations are possible. Mitochondria were seen in all intracellular stages of the parasites. (An investigation of the fine structure of the exoerythrocytic stages by electron microscopy is in progress and we hope it will reveal more details of the mitochondria than can be seen or photographed by phase contrast microscopy.) Negative phase contrast optics reversed the light relationships between various structures in the parasite observed by positive phase contrast; *i.e.*, the nucleus and food vacuole were dark and the nucleolus and mitochondria were light (Figs. 11, 13, 16).

Nuclear Division.—We have photographed by time-lapse a few parasites undergoing nuclear division. We are not prepared at this writing to give with confidence a description of this process. Two points can be made at this time: namely, (1) that the process was very rapid, perhaps lasting no more than 3 or 4 minutes, and (2) that the nuclear membrane did not disappear during division of the nuclear material as it does in metazoan cells. The organelle, here called the nucleolus, underwent division subsequent to the division of the rest of the nucleus. A special study of mitosis is being made and will be reported when the details have been clarified.

Schizonts and Schizogony.—Schizogony in *Plasmodium* consists of (1) repeated nuclear divisions without concomitant cytoplasmic divisions, and (2) a final cytoplasmic division which provides separate portions of the parent cytoplasm for each nucleus. For convenience, the former will be referred to as *nuclear schizogony*, and the latter as *cytoplasmic schizogony*. For those stages between inception and completion of cytoplasmic schizogony we have used the term *presegmenter*. We have observed three processes by which cytoplasmic shizogony is effected. Since no differences were seen between *P. gallinaceum* and *P. fallax* in respect to these methods one description will suffice, and examples will be drawn from illustrations of each.

First, one peripheral layer of merozoites may be formed (Text-fig. 1). When this occurs, the nuclei come to the peripheral wall of the schizont and the nucleoli therein are outermost, sometimes actually protruding slightly into the limiting schizont wall (see Fig. 8). Splitting of the cytoplasm of the parent schizont then begins at the periphery (Fig. 9), and extends centrally until merozoites are completely formed (Fig. 18), the unused cytoplasm becoming the residual body (see Figs. 10, 15). The residual body in this type of schizogony is usually a spherical mass of varying size (Figs. 12, 15, 16), and the merozoites may vary from 12 or 15 to 100 or more.

The second method is one of invagination of the peripheral layer of nuclei from which the merozoites are formed. This process has long been known in Sporozoa and was well described by Wenyon (1926, **1,** see p. 67). The simpler type of invagination is shown in Text-fig. 2 and has been clearly observed in time-lapse sequences of moving pictures but is not shown in any of our still photomicrographs. As Wenyon pointed out, this invagination and vacuolation may be complex so that the cytoplasm appears as a highly vacuolated network. By observing many time-lapse sequences we have become convinced that the double layer of nuclei formed by invagination breaks up into two or more islands (see Text-fig. 3 and Fig. 15). In fact, this appears to be the most common process followed in the material we studied. Many photomicrographs of late stages in cytoplasmic schizogony could be explained in this manner (Figs. 14 to 16).

We have observed a third method of cytoplasmic schizogony in very large segmenting schizonts. These were followed in time-lapse sequences from beginning to completion. In this case no obvious peripheral arrangement of the nuclei preceded cytoplasmic schizogony and no invagination nor vacuolation occurred. Instead, the parent cytoplasm broke up directly into islands containing a few to several nuclei (see Text-fig. 4), and a continuation of the cytoplasmic division resulted in haphazard arrangement of large numbers of merozoites. Then the motion of the individual merozoites (largely Brownian, but in some degree vital motility) brought about a regrouping of merozoites into rosettes through attachments (perhaps by chance) of the filaments of the merozoites to small islands of residual cytoplasm. Hence, rosettes were formed primarily by the second process described above or, secondarily, by rearrangement

of merozoites. This fact would have been difficult to discover except through time-lapse cinephotomicrography.

Successive nuclear divisions produced schizonts closely resembling the uninucleate trophozoites just described, except for the presence of 2, 4, 8, or more nuclei and for their larger size (Fig. 5). A food vacuole was not always seen, but two or more were present in some of the larger schizonts. After the first 3 or 4 nuclear divisions it was impossible to count accurately the number of nuclei (Figs. 6 and 7). The schizonts continued to grow in size and the multiplication of the nuclei continued until they packed the space within the schizont wall (Fig. 7).

PLASMODIUM FALLAX

Merozoites.—The exoerythrocytic stages of this species were morphologically very like those of *P. gallinaceum.* The most obvious difference between them was that the merozoites of *P. fallax* were curved, while those of *P. gallinaceum* were usually straight (see Figs. 1 and 19). However, this was a matter of degree, so that it was not always possible to differentiate the two species on the basis of this characteristic. The motility of *P. gallinaceum* merozoites was also greater than that of the merozoites of *P. fallax.*

Uninucleate Trophozoites.—The differences between the intracellular stages of the two species are difficult to describe, but, in general, the internal structure of *P. fallax* was the more easily observed and more easily photographed (Figs. 6 and 7).

Schizonts.—During nuclear schizogony the schizonts of the two species were difficult to distinguish except on the basis of the same differences that distinguish uninucleate trophozoites. In agreement with the previous finding on fixed material we found that the number of merozoites in the mature schizont was smaller in *P. fallax* than in *P. gallinaceum.* In some instances in which their numbers were noticeably small, the merozoites were observed to be larger in size (Fig. 17).

Massive Infections of the Host Cell.—In tissue cultures of *P. gallinaceum* multiple infections of cells were often observed. More often than not, the parasites within a single cell were of varying size and degree of development. Although occasional examples of this difference in degree of development of the schizonts in the same cell were observed in *P. fallax* (Figs. 12 and 19), it was more frequently observed that a single cell might be infected with many parasites of this species, which were apparently of the same age (Figs. 20 to 22). Since the chances seem to be small that most of the merozoites from a segmenting schizont would invade the small cell, other explanations for these massive infections were sought. A clue was obtained in a few examples like Fig. 3. In one end of the infected cell of this figure are the remains of a schizont. The parasites still retain the shape of merozoites and an arrangement suggestive of the rosette structure so commonly observed in intact, mature schizonts. However, some of the other parasites have already begun to round up into the typical shape of uninucleate trophozoites. A reasonable explanation for these massive infections with parasites of similar size might be that a mature schizont ruptured inside the host cell and merozoites, instead of being expelled from the host cell, merely remained within its cytoplasm and began to grow into schizonts. It should be recalled that the merozoite of *P. fallax* has feeble motility and that the host cells in our tissue cultures were subjected to no extrinsic movement such as occurs in cells *in vivo.* These conditions would be favorable to this type of auto-infection of host cells. That such auto-infection does not happen *in vivo* is indicated by the failure to find examples of massive infection in fixed material from infected animals.

Evidence of cytopathic action of the parasites upon the host cells was observed only in the cells in which such massive infections occurred. In less advanced stages of development of this cytopathic action the cytoplasm surrounding the parasites became vacuolated. This vacuolation continued until the cytoplasm became a reticulum (Figs. 21, 22). As the process advanced the nucleus began to deteriorate and the cell very probably died. It should be emphasized that this evidence of harmful effect of the parasitism upon the host cell was seen only in these instances of massive infection of the cell and that in all other cases the relationship between the host cell and the exoerythrocytic stages appeared to be a harmonious one.

All intracellular stages exhibit varying degrees of motion. Because of the viscosity of both the cellular substance and the parasites, it is our belief that little, if any, of the motion seen visually or in time-lapse sequences is Brownian in character. Some of the motion is definitely due to vital

activity on the part of the host cell. In addition, there is some motion at nearly all times which must be considered as intrinsic to the parasites. In young trophozoites in which they are more clearly visible, structures such as the nucleus and mitochondria are seen to have activity similar in character to the corresponding structures in the host cell. In large multinucleate schizonts the nuclei undergo considerable movement and this results in motion of food vacuoles which one would assume incapable of intrinsic motion. During cytoplasmic schizogony the nearly formed, as well as the completed merozoites sometimes exhibited individual activity of greater degree. At the stage when several merozoites were attached to a residual body certain individual merozoites made rapid motions towards, and then away from the residual body. At times when the merozoites were arranged haphazardly they have been seen to undergo an accelerated gregarine- or worm-like motion which is visible microscopically. This period of accelerated action usually ended abruptly. On occasion in our time-lapse studies, we have observed several schizonts in the same host cell, and in some of these schizonts the internal activity was strikingly greater than that of adjacent schizonts.

Phagocytosis.— Active phagocytosis of free merozoites of both species of parasites by macrophages has been observed in time-lapse sequences of tissue cultures by cinephotomicrography. The pseudopods of the macrophages appeared to trap the merozoites by a wave-like action which can best be described by saying that the process resembled the submergence of a boat by an overtowering wave. With the passing of the wave the merozoite was seen to be within the cytoplasm of the macrophage.

Macrophages were attracted to mature schizonts and, in one time-lapse sequence one of them made repeated attempts to engulf the entire schizont (Fig. 23). A large portion of the cytoplasm of the macrophage was thrust against the schizont and smaller sheets of cytoplasm encircled as much of the schizont as was accessible. Traction was apparently exerted upon the schizont but, since the latter could not be detached from the host cell, the macrophage loosened its hold and retreated a short distance from the parasite—but returned soon after for another attack.

As yet we have been unable to film a macrophage sufficiently long after it has been seen to ingest a merozite to determine whether the parasite was able to grow within the macrophage. Normal growth of exoerythrocytic stages within macrophages has, however, been previously demonstrated in fixed and stained material (Huff and Coulston, 1945).

DISCUSSION

Comparison of Fixed and Stained Material with Living Parasites Seen with Phase Contrast Microscopy.—The appearance of exoerythrocytic stages by positive phase contrast microscopy resembled very closely their appearance in wet-fixed material stained with Maximow's hematoxylin-eosin-azur method, except for the colors in the latter. In other words, photomicrographs of the living exoerythrocytic stages taken by positive phase contrast resembled black and white photomicrographs of fixed and stained preparations. The appearance by negative phase contrast optics was like a photographic negative of the preparation under positive phase. Thus, under positive phase the cytoplasm of the parasites was dark gray, the nucleus was pale gray, the nucleolus was very dark, and the food vacuoles were very bright. No differences in morphology of the exoerythrocytic stages by the two methods were noted, except (1) the presence of a filament in the free merozoite, and (2) the absence of clefts in the cytoplasm of large schizonts when observed by phase contrast. Moreover, whereas in fixed and stained material there was nearly always a separation observed between the substance of the parasite and of the surrounding host cytoplasm, no such separation was observed by phase contrast. The most likely explanation for the absence of cytoplasmic clefts in and the vacuole-like space around the parasites is that these were artifacts due to fixation in the non-living material. However, we have not yet found an explanation for the absence of the filament on the merozoite in stained, fixed material. Attention should be called here to the work of Meyer and Oliviera (1947) who mentioned seeing these polar filaments on *P. gallinaceum* in tissue culture. The very close parallel between the appearance of living material in the phase contrast microscope and of material fixed in Zenker-formol solution and stained by the hematoxylin-eosin-azur method is further evidence of the advantages of the latter method over most other methods for preparing fixed, stained material. The great advantage of study of living material by phase contrast microscopy is, of course, that it permits

the study of the behavior as well as the morphology of both parasite and host cell. This advantage is so great as to make practically obsolete the study of stained, fixed material except under special conditions. Although this paper is devoted to the morphology and behavior of the exoerythrocytic stages as they appear in tissue culture under the most favorable known conditions, we are currently studying the effects of changing the physicochemical and biological environment of the tissue culture to determine the resulting effects on the parasites.

Unsolved Problems.—Although the question of susceptibility of the various types of host cells to invasion by the exoerythrocytic stages has been uppermost in our minds throughout the course of this work, we do not consider ourselves capable at present of stating unequivocally what cells may or may not be invaded in tissue culture. Because of the well known ability of cells to depart widely, when grown in tissue culture, from the morphology and behavior known *in vivo*, we believe that an intensive and many-sided study of the problem of identifying the cell types in tissue culture is required before we commit ourselves on this question. On the basis of fixed, stained cultures of chick embryo tissues infected with *P. gallinaceum*, Dubin (1952, 1954) reported that exoerythrocytic stages of this parasite could invade both pulmonary and hepatic epithelium. Since these tissues were not found to be invaded in the infected animal (Huff, 1957), we should be prepared to learn that the susceptibility of cells in tissue culture to malarial parasites may differ widely from those *in vivo*. For additional literature on the use of avian embryos and tissue culture in the cultivation of malarial parasites, see Pipkin and Jensen (1958).

Intracellular phagotrophy in erythrocytic stages of *P. lophurae* has been reported by Rudzinska and Trager (1957) on the basis of electron micrographic evidence. If exoerythrocytic stages are capable of phagotrophy, it should be possible to observe this process by means of time-lapse photography. Until now we have seen no evidence of it, but it deserves special study. In this respect, it is not definitely known whether the inclusions in the exoerythrocytic stages, which we called "food vacuoles," are analogous to the structures in *P. lophurae* referred to by the same term by Rudzinska and Trager.

As indicated in the section on observations, we have much to learn about the nature of the nuclear division that was observed in our time-lapse films. If this is not true mitosis, what light would this throw upon the systematic position of *Plasmodium* in the Sporozoa? Several of the latter are known to undergo mitosis similar to that in metazoa. Doflein and Reichenow (1929) discussed the deviations in protozoa from normal mitosis but none of the examples were close taxonomically to *Plasmodium*. We disagree with Wolcott (1957) in his interpretation of the nature of the organelle shown in phase contrast photographs of erythrocytic stages of five species of *Plasmodium*. We have tentatively called the corresponding structure in our material a nucleolus and we believe that it cannot be a chromosome because it is observable at all times and in all stages of development from merozoite to mature schizont. We have not yet seen chromosomes, even in the time-lapse sequences of nuclear division. Division of the structure which we have called the nucleolus occurred as the final step in nuclear division and did not resemble chromosome splitting in the least.

As yet, we have not succeeded in producing established cultures of either uninfected chicken cells or chicken cells infected with malarial parasites. Even in primary cultures the number of parasites usually decreased before the chick embryo cells ceased to grow. We are attempting to find ways of subculturing the infected chicken cells in cultures in fluid. Meyer and Musacchio (1958) have reported successful subculture of *P. gallinaceum* for 4 years uninterruptedly, but they alternated roller-tube culture with the hanging drop method. With the perfection of methods for more consistent and long-term cultivation, it will then be possible to make such quantitative measurements as the length of the schizogonic cycle, the time between nuclear divisions, and relation between type of host cell and size of the parasite.

Other items needing further research are (1) the more exact mechanism of penetration of merozoites, (2) the nature of the "food vacuole," (3) the nature of the organelle here called the nucleolus, (4) the structure of the filament on the merozoite, and (5) the identification of some of the unknown inclusions in the parasite, particularly the spindle-like figure outside the nucleus seen just prior to division of the latter.

BIBLIOGRAPHY

Doflein, F. and Reichenow, E., 1927–1929, Lehrbuch der Protozoenkunde, Jena, 5th edition.

Dubin, I. N., 1952, The cultivation of the exoerythrocytic forms of *Plasmodium gallinaceum* in tissue culture, *J. Infect. Dis.*, **91,** 33.

Dubin, I. N., 1954, Growth of exoerythrocytic forms of *Plasmodium gallinaceum* in epithelial cells in tissue culture, *Exp. Parasitol.*, **3,** 425.

Earle, W. R., 1943, Production of malignancy *in vitro*. IV. The mouse fibroblast cultures and changes seen in the living cells, *J. Nat. Cancer Inst.*, **4,** 165.

Earle, W. R., 1956, personal communication.

Eichhorn, E. A., 1940, Technique for intravenous inoculation of chick embryos, *Science*, **92,** 245.

Haas, V. H., Wilcox, A., and Ewing, F. M., 1945, Infection of chick embryos with non-pigmented forms of *Plasmodium gallinaceum*, *J. Nat. Malaria Soc.*, **4,** 279.

Hanks, J. H., and Wallace, R. E., 1949, Relation of oxygen and temperature in the preservation of tissues by refrigeration, *Proc. Soc. Exp. Biol. and Med.*, **71,** 196.

Huff, C. G., 1954 *a*, Changes in host-cell preferences in malarial parasites and their relation to splenic reticular cells, *J. Infect. Dis.*, **94,** 173.

Huff, C. G., 1954 *b*, Merozoite size in exoerythrocytic infections of *Plasmodium gallinaceum*, *P. fallax*, *P. lophurae*, and *P. cathemerium*, *Exp. Parasitol.*, **3,** 433.

Huff, C. G., 1957, Organ and tissue distribution of the exoerythrocytic stages of various avian malarial parasites, *Exp. Parasitol.*, **6,** 143.

Huff, C. G., and Bronson, J. F., 1956, Simple condensers for ribbon-filament and mercury vapor microscope lamps, *J. Biol. Photog. Assn.*, **24,** 121.

Huff, C. G., and Coulston, F., 1944, The development of *Plasmodium gallinaceum* from sporozoite to erythrocytic trophozoite, *J. Infect. Dis.*, **75,** 231.

Huff, C. G., Marchbank, D. F., Saroff, A. H., Scrimshaw, P. W., and Shiroishi, T., 1950, Experimental infections with *Plasmodium fallax* Schwetz isolated from the Uganda tufted guinea fowl *Numida meleagris major* Hartlaub, *J. Nat. Malaria Soc.*, **9,** 307.

Melnick, J. L., Rappaport, C., Banker, D., and Bhatt, P., 1955, Stabilized suspensions of monkey kidney cells suitable for intercontinental shipping, *Proc. Soc. Exp. Biol. and Med.*, **88,** 676.

Meyer, H., and Musacchio, M. O., 1958, *Plasmodium gallinaceum* in tissue cultures: results obtained during 4 years of uninterrupted cultivation of the parasite *in vitro*, *6th Internat. Cong. Trop. Med. and Malaria*, Lisbon, September 5–13, 1958, 293 (abstract).

Meyer, H., and de Oliviera, M. X., 1947, Estudio morfologico da forma exoeritrocitaria do "*Plasmodium gallinaceum*" em culturas de tecido. I., *Rev. brasil. biol.*, **7,** 327.

Pipkin, A. C., and Jensen, D. V., 1958, Avian embryos and tissue culture in the study of parasitic protozoa. I. Malarial parasites, *Exp. Parasitol.*, **7,** 491.

Pipkin, A. C., and Mack, A. D., 1958, A new tissue culture flask with demountable bottom, *Exp. Cell Research*, **14,** 219.

Rose, G., 1954, A separable and multipurpose culture chamber, *Texas Rep. Biol. and Med.*, **12,** 1074.

Rossan, R. N., 1957, The effect of antimalarial drugs on the exoerythrocytic and erythrocytic stages of blood-induced infections of *Plasmodium fallax* in the turkey, *Exp. Parasitol.*, **6,** 163.

Rudzinska, M. A., and Trager, W., 1957, Intracellular phagotrophy by malaria parasites: an electron microscope study of *Plasmodium lophurae*, *J. Protozool.*, **4,** 190.

Trager, W., 1956, The intracellular position of malarial parasites, *Tr. Roy. Soc. Trop. Med. and Hyg.*, **50,** 419.

Weiss, E., and Dressler, H. R., 1958, Growth of *Rickettsia prowazeki* in irradiated monolayer cultures of chick embryo entodermal cells, *J. Bact.*, **75,** 544.

Wenyon, C. M., 1926, Protozoology, London, Bailliere, Tindall and Cox, 2 vols.

Wilcox, A., 1959, personal communication.

Wolcott, G. B., 1957, Chromosome studies in the genus *Plasmodium*, *J. Protozool.*, **4,** 48.

Youngner, J. S., 1954, Monolayer tissue cultures. I. Preparation and standardization of suspensions of trypsin-dispersed monkey kidney cells, *Proc. Soc. Exp. Biol. and Med.*, **85,** 202.

Zuckerman, A., 1946, Infections with *Plasmodium gallinaceum* in chick embryos induced by exoerythrocytic and blood stages, *J. Infect. Dis.*, **79,** 1.

EXPLANATION OF PLATES

The photomicrographs in Figs. 1 to 23 were made from living specimens from tissue cultures of embryonic chick liver infected with *Plasmodium gallinaceum* and *P. fallax*; phase contrast optics. Fig. 2 was retouched to assure reproduction of the filament, *F*, on the merozoites; aside from the removal of a few scratches the other photomicrographs were not retouched.

PLATE 30

FIG. 1. A mature schizont of *P. fallax* releasing merozoites. Approx. × 1440.

FIG. 2. Free merozoites of *P. gallinaceum*. Note polar filaments, *F*. Approx. × 1440.

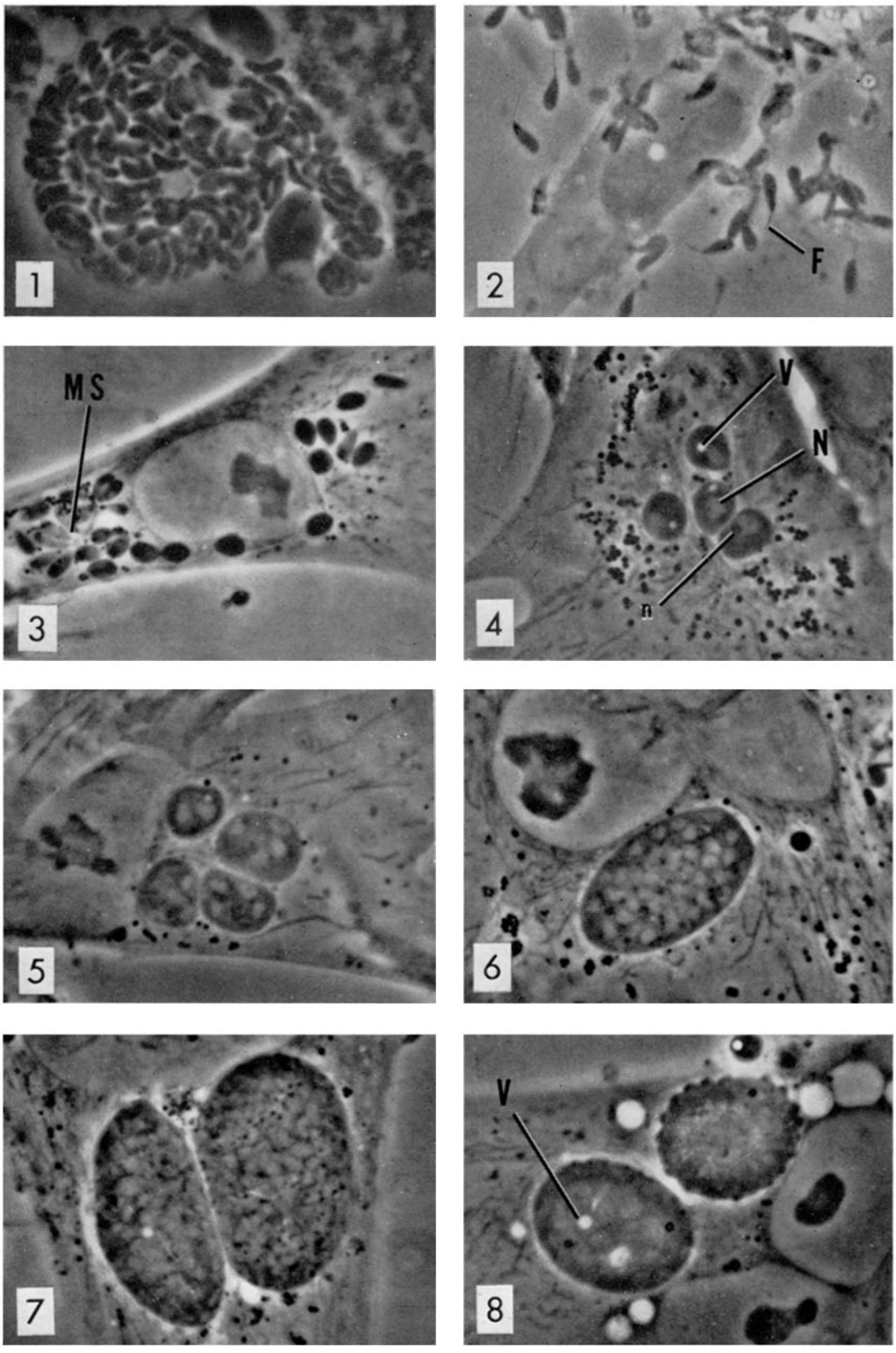
FIG. 3. Host cell containing remnants of a mature schizont, *MS*, of *P. fallax* and young trophozoites which may have developed from merozoites belonging to the schizont whose remnants are visible. Some merozoites have started to round up. × 747.

FIG. 4. Four uninucleate trophozoites of *P. gallinaceum* in a cell from embryonic chick liver. *N*, nucleus; *n*, nucleolus; *V*, food vacuole. × 1440.

FIG. 5. Four early schizonts of *P. gallinaceum*. Note the multiple nuclei. Approx. × 1440.

FIGS. 6 and 7. Schizonts of *P. fallax*. The numerous nuclei are beginning to fill the entire space inside the schizont wall; the two schizonts in Fig. 7 are in a more advanced state than the one in Fig. 6. × 867.

FIG. 8. Two large schizonts of *P. gallinaceum*. Note the protrusion of the nuclei into the schizont cell membrane; *V*, food vacuole. Approx. × 867.



(Huff *et al.*: Malarial exoerythrocytic stages)

PLATE 31

FIG. 9. Presegmenting schizont of *P. gallinaceum*. Note the beginning of cytoplasmic schizogony. Approx. × 867.

FIG. 10. Early stage of cytoplasmic schizogony in *P. gallinaceum*. Note peripheral arrangement of merozoites and the single large residual body, *RB*. × 560.

FIG. 11. Two schizonts of *P. fallax*. Negative phase contrast. The mature schizont is exceptionally small. × 480.

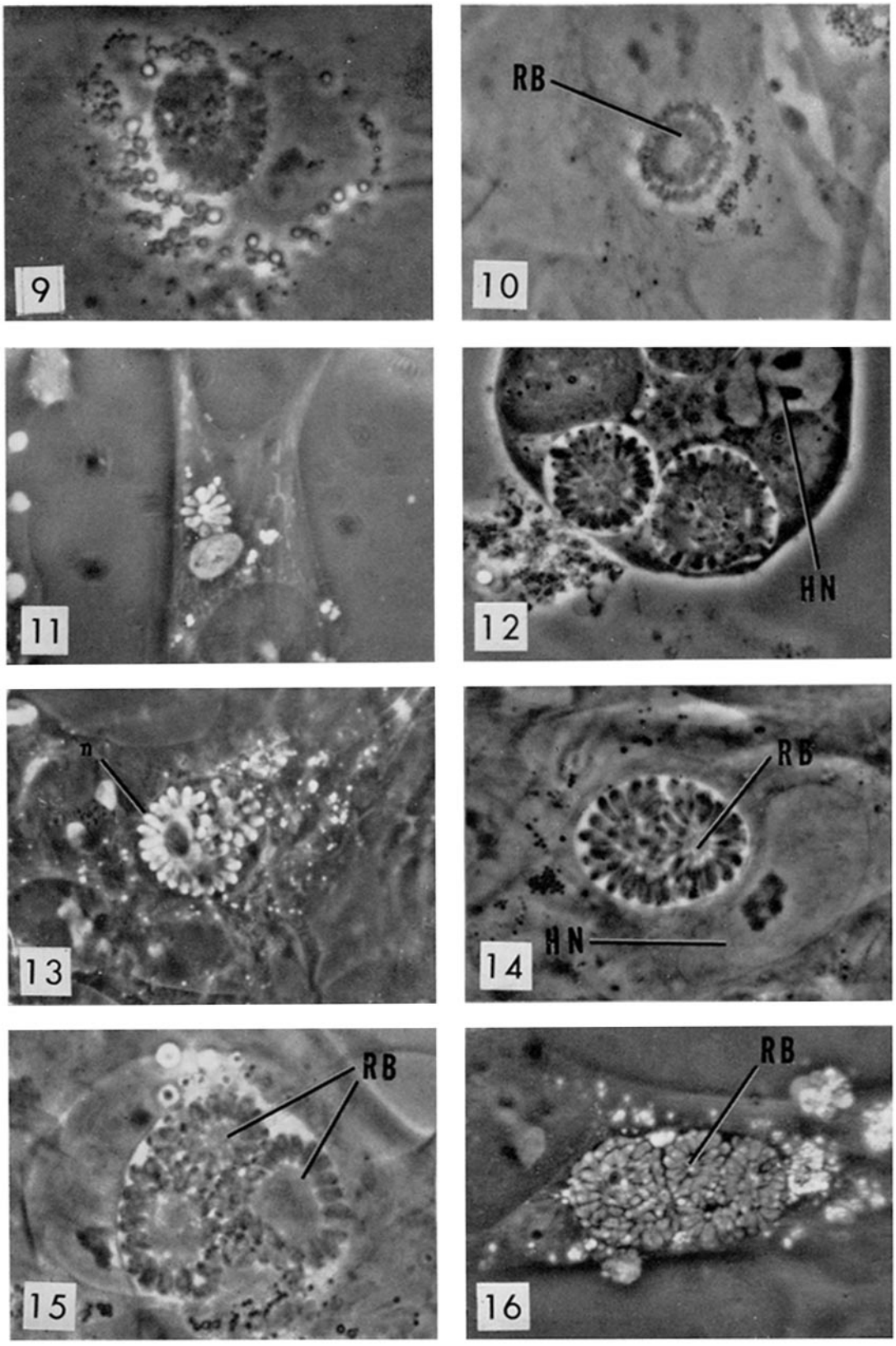
FIG. 12. Different stages in the maturation of schizonts of *P. fallax* in one cell. *HN*, host cell nucleus. Approx. × 867.

FIG. 13. Mature schizont of *P. fallax*; negative phase contrast. The nucleoli, *n*, are bright. Approx. × 872.

FIG. 14. Mature schizont of *P. fallax*. *HN*, host cell nucleus. *RB*, residual body. Approx. × 867.

FIG. 15. Mature schizont of *P. fallax* with multiple residual bodies, *RB*, and evidence of invagination as indicated in Text-fig. 2. × 680.

FIG. 16. A mature schizont of *P. fallax* photographed by negative phase contrast. Note multiple rosette formation around small residual bodies, *RB*. Approx. × 500.



(Huff *et al.*: Malarial exoerythrocytic stages)

PLATE 32

FIG. 17. Mature schizont of *P. fallax* with unusually large merozoites. Approx. × 867.

FIG. 18. Six parasites of *P. fallax* in different stages of schizogonic development and all within the same liver cell. × 560.

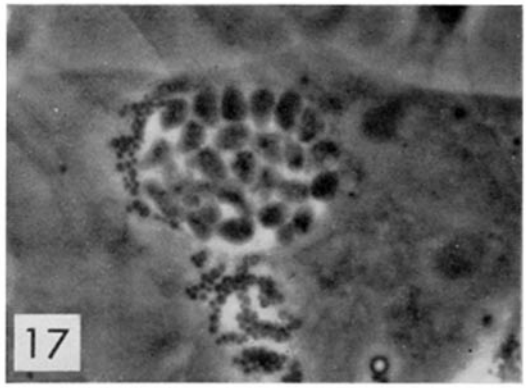
FIG. 19. One mature schizont and 16 young schizonts of *P. fallax* in the same host cell. × 560.

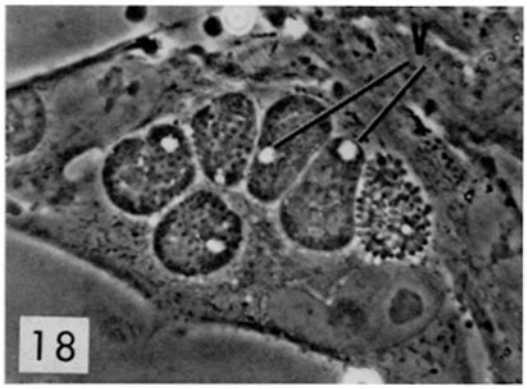
FIG. 20. More than 100 young trophozoites of *P. fallax* presumably coming from a mature schizont which developed in the same cell. × 1000.

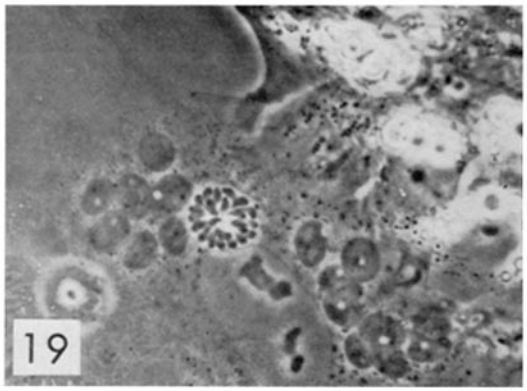
FIG. 21. Several half-developed schizonts of *P. fallax* in the same cell. Notice the network in the cytoplasm; cytopathic effect (*C*). × 560.

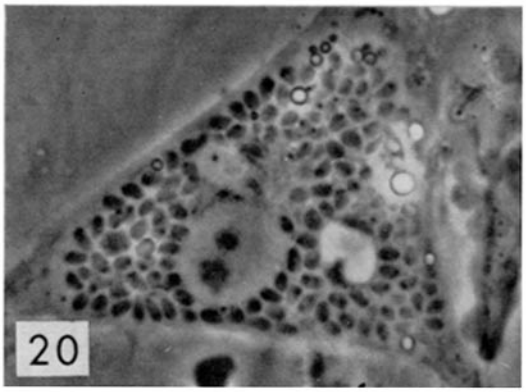
FIG. 22. Many large schizonts of *P. fallax* in one host cell. Note the cytopathic effect (*C*) on the cytoplasm of the host cell. × 560.

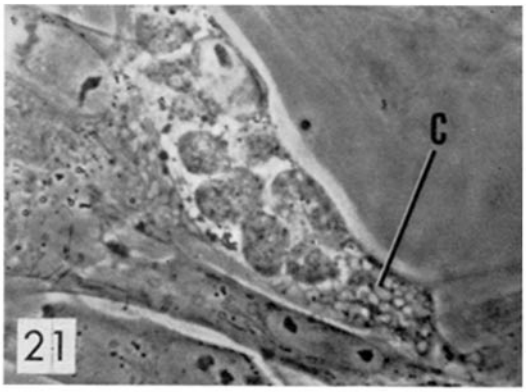
FIG. 23. Film strip from a time-lapse sequence showing attempts of a macrophage in tissue culture of embryonic chick liver to phagocytose a mature schizont of *P. fallax*. Arrows indicate protoplasmic processes of the macrophage attempting to encircle the schizont. Approx. × 628.

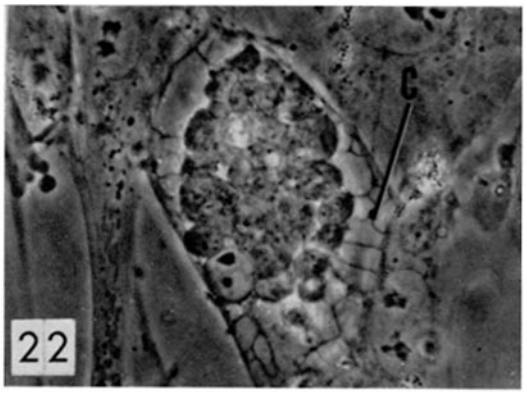


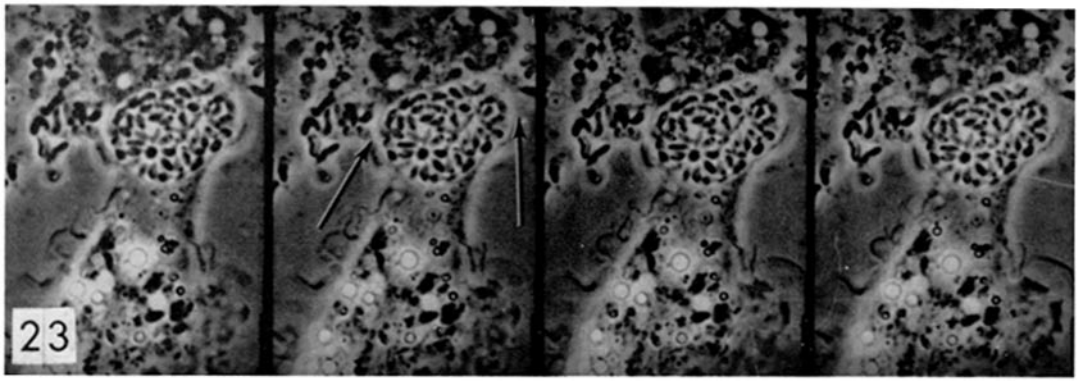












(Huff *et al.*: Malarial exoerythrocytic stages)