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Extracellular (axenic) development *in vitro* of the erythrocytic cycle of *Plasmodium falciparum*

(malaria/axenic cultivation)

WILLIAM TRAGER AND JONATHAN WILLIAMS

The Rockefeller University, New York, NY 10021-6399

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ABSTRACT **Merozoites of the erythrocytic stage of *Plasmodium falciparum* were suspended in erythrocyte sonicate medium with ATP and pyruvate and mixed with Matrigel to form a soft gel. The gel was overlaid with complete medium; this was replaced with fresh medium at 12, 24, and 36 hr. At these times and also at 45 hr rhodamine 123 was added to some cultures and gels were sampled. Viable extracellular forms showing rhodamine fluorescence were seen: rings at 12 hr, trophozoites and early schizonts with pigment at 36 hr, and late schizonts with developing merozoites at 45 hr. These merozoites were shown to be infective to erythrocytes added to the cultures at 45 hr. Electron micrographs of 36-hr trophozoites show the organisms to have only their single plasma membrane; no parasitophorous membrane is evident. We conclude that the complex process of entry, the intactness of the host erythrocyte, and the parasitophorous membrane are not essential to the development of a merozoite through its complete asexual cycle.**

The nature of the dependence of intracellular parasitic protozoa on their host cell is not understood. We have approached the problem by attempting to get extracellular cultivation of the intraerythrocytic stages of the human malaria parasite *Plasmodium falciparum*. Earlier work from this laboratory (1, 2) has shown that neither the complex process of entry into nor the intactness of the host cell is essential to the initial differentiation of a malarial merozoite into a trophic form. It is rather the milieu in which the merozoite finds itself. In an erythrocyte sonicate with ATP and pyruvate, up to 30% of merozoites developed into small trophic forms and about 5% developed into somewhat larger rings. Here we describe culture conditions that support extracellular development of a complete 2-day asexual cycle of reproduction. These conditions depend on embedding the merozoites in a gel overlaid with liquid medium that is replaced at appropriate intervals.

MATERIALS AND METHODS

Parasites. Stock cultures of *P. falciparum* were maintained in flow vessels (3). These were modified by making them twice as long and U-shaped. This doubled their capacity (to 2 ml of packed erythrocytes) without requiring the use of more medium or a faster rate of flow. We have used only clone HB-3 (4). Synchronization and handling of the cultures were done as described (1). A 3% (vol/vol) schizont suspension was incubated under culture conditions for 1.25 hr and then centrifuged 10 min at 200 × *g*. The supernatant containing merozoites and pigment clumps was then centrifuged 10 min at 1500 × *g*, all of the supernatant was removed, and the sedimented material was resuspended in an appropriate minimal volume of complete medium (see below). This suspension constituted the inoculum for the cultures; it had 100–400 × 10^6 merozoites per ml.

Culture Medium. An erythrocyte sonicate (see also ref. 1) was prepared in the following way. Whole human blood (type A^+) preserved in citrate/phosphate/dextrose (CPD) or CPD-adenine and not outdated was centrifuged at 500 × *g* for 10 min and the cells were washed three times in RP [complete culture medium of RPMI 1640 medium with hypoxanthine (50 mg/ml)/Hepes, pH 7.4 (5.94 g/liter)/sodium bicarbonate (2 g/liter) but without serum] and finally resuspended in RPS [complete medium with 10% (vol/vol) human serum] at 50% hematocrit. The suspension was usually kept overnight at 2°C. It was then centrifuged and the cells were washed once in KFS medium and finally resuspended in this medium at a 50% hematocrit. KFS medium was freshly mixed as follows: 40 ml of KF (see below)/1.6 ml of 5% (wt/vol) $NaHCO_3$/4.4 ml of human serum type A.

Medium KF was a high-potassium modification of RPMI 1640 medium prepared in the following way (using glass redistilled water throughout): 25 mg of hypoxanthine was dissolved in 10 ml of hot water and diluted with water to 400 ml. To this were then added 5.82 g of K-RPMI 1640 medium (this is a powder prepared by GIBCO to contain all the ingredients of RPMI 1640 medium without bicarbonate and with NaCl replaced by equimolar KCl)/2.97 g of Hepes (Sigma)/500 mg of bovine serum albumin (fraction V)/250 mg of fructose 1,6-diphosphate (trisodium salt, Sigma)/20 mg of β-nicotinamide dinucleotide (Sigma)/10 mg of calcium pantothenate/L-malic acid, sodium salt, prepared as a solution of 400 mg in 20 ml of water and neutralized to pH 6.9 with 0.5 M KOH. The preceding mixture was diluted with water to a final volume of 480 ml. It was filter-sterilized and stored frozen at −20°C.

The final suspension to be sonicated, usually 25–30 ml of a 50% erythrocyte suspension in KFS, was kept in ice water. It was sonicated in successive 5-ml portions in a 50-ml round-bottom glass centrifuge tube. The tube was held in ice water in the cup horn attachment of a Heat Systems ultrasonic processor model W-380. Sonication was for 2 min at a setting of 3 on the output control. The sonicates were pooled in a 50-ml plastic centrifuge tube kept on ice and then centrifuged at 5°C for 10 min at 1500 × *g* to sediment any possible remaining intact cells (very few were present). The supernatant was removed, except for the bottom 2 ml, and distributed in tubes in desired amounts, depending on the experiment to be done. Droplets of each portion were used for sterility tests and for microscopic examination. The sonicates were then frozen in a dry ice/ethanol bath and stored at −70°C until used (usually within 1–4 days).

The Gel. By embedding the merozoites in a gelatinous matrix, we hoped to achieve two ends. (*i*) We imagine it provides them with physical conditions more nearly like those in the interior of a cell. (*ii*) Having the organisms so embedded, with an overlay of liquid medium, makes possible provision of fresh medium with a minimum of disturbance, simply by changing the overlay.

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We have used the commercially available material Matrigel, from Collaborative Research. This is a solubilized tissue basement membrane containing laminin and collagen type IV as major constituents and also heparan sulfate, proteoglycan, and entactin. This remains liquid at temperatures of 4°C or lower but sets quickly to a gel at higher temperatures. We stored it at −20°C in vials, each containing 1 ml.

The Cultures. Culture media were completed a short time before the merozoite preparations would be ready.

An appropriate portion of sonicate was thawed. To 10 ml of sonicate was added 0.3 ml of a freshly thawed supplement to supply ATP at 2 mM and sodium pyruvate at 5 mM (final concentrations, see ref. 5).

Merozoites suspended in the complete medium were inoculated into wells of a 24-well Linbro plate in the following way. Two wells to be used for counts of the merozoites and for invasion controls received (per well) 0.05 ml of merozoite suspension plus 5 μl of a 50% erythrocyte suspension in RPS. These suspensions were mixed with a Pasteur pipet and a droplet was used to prepare a thin film. The experimental wells received (per well) 0.1 ml of merozoite suspension (containing 10–40 × 10^6 merozoites) followed by 50 μl of Matrigel. To do this, a vial of Matrigel thawed in ice water was held on ice. A beaker of sterile pipet tips that had been chilled overnight at −20°C was likewise held on ice. For each well, a fresh ice-cold pipet tip was used to deliver 50 μl and to mix it quickly but gently with the merozoite suspension. Completed plates were put in a prewarmed candlejar and incubated 20 min at 37°C (6). At this time the plate was removed. The invasion control wells then received (per well) 0.5 ml of RPS. The other wells, containing the gel, received (per well) an overlay of 0.5 ml of complete medium. Some wells received medium with a further supplement of CoA. The CoA was added as 0.3 ml of a 5 mM solution for each 10 ml of sonicate, to give a final concentration of 0.15 mM.

Separate plates were used for cultures to be terminated at different times. Blank wells not used for cultures received (per well) 0.5 ml of RPS. The plates were incubated in candlejars at 37°C. Aseptic technique was used throughout, and there was no need for antibiotics.

Because of the constraints of synchronization and the time needed to prepare the merozoite suspension, cultures were usually completed between the hours of 2000 and 2200. Accordingly, the first samples taken the following morning consisted of organisms after 12–14 hr of development. At this time, the invasion controls were centrifuged and the sedimented cells were used to prepare thin films that were stained with Giemsa stain. The gel cultures were treated in several ways. For some, the overlay was removed, and bits of gel were taken up with a Pasteur pipet, spread on a slide, and stained with Giemsa stain. More instructive results were obtained with the use of rhodamine 123. To the 0.5 ml of overlay was added 5 μl of a rhodamine solution at 1 mg/ml to give a rhodamine concentration of 10 μg/ml. This culture was incubated in the candlejar at 37°C for 1–2 hr. The overlay was then replaced with fresh complete medium that was left at room temperature for 30 min. This overlay was then removed. Bits of gel were prepared as wet mounts and these were examined for rhodamine fluorescence by epifluorescent microscopy at a magnification of ×1250 by using a BP 546 green interference combination excitor filter and an LP 590 red barrier filter with an FT 580 beam splitter (Zeiss).

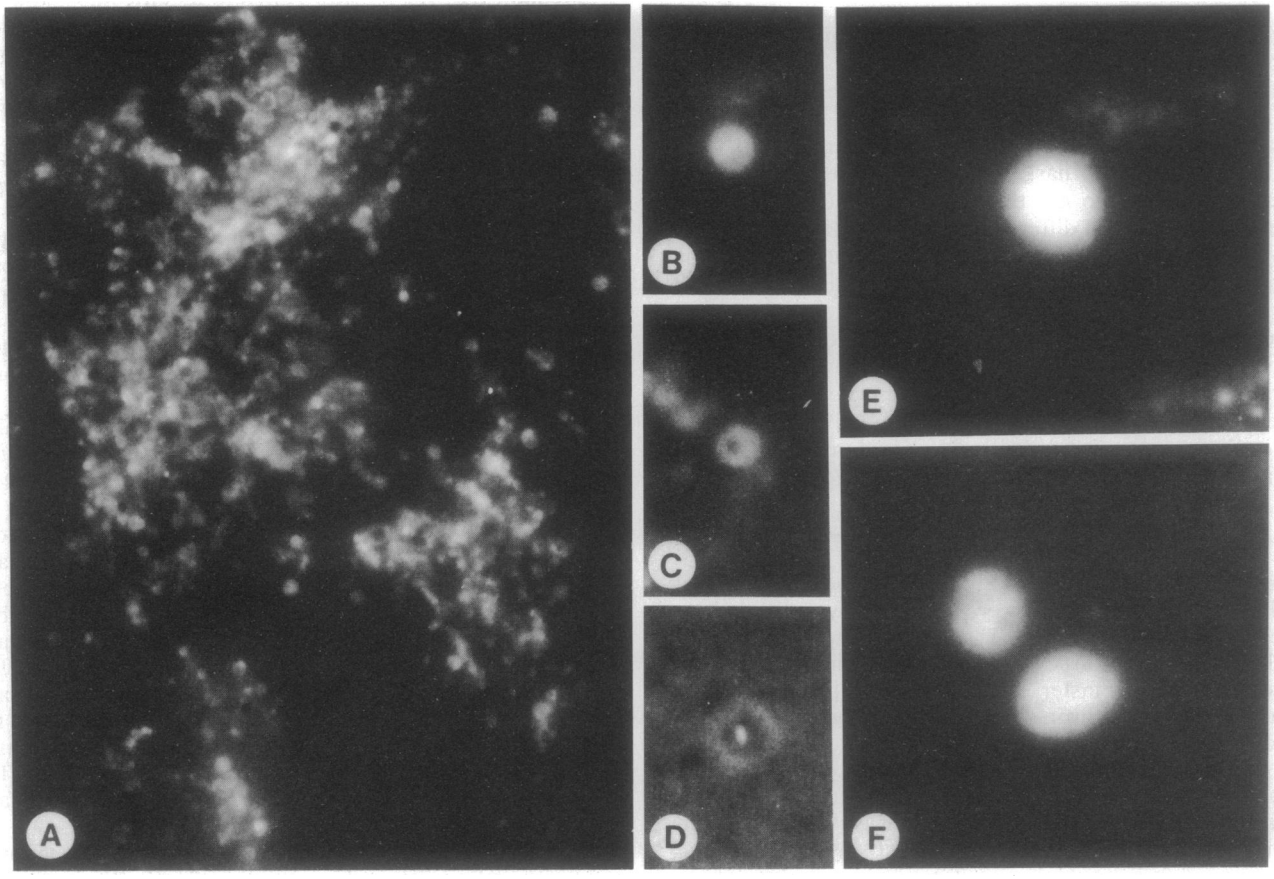

FIG. 1. Forms of *P. falciparum* developing extracellularly from merozoites in gel cultures after 12–14 hr (*A* and *B*), 36 hr (*C* and *D*), and 45 hr (*E* and *F*), as seen by rhodamine 123 fluorescence, except for *D* which is the phase-contrast image corresponding to *C*. The numerous brightly fluorescent rings, some in focus and others out of focus, embedded in a gel fragment are shown in *A*. (*B*) Large ring. Note the thinner portion corresponding to the "vacuole." (*C* and *D*) Trophozoite or early schizont with clumped pigment. (*E* and *F*) Late schizonts with forming merozoites. (*A*, ×1000; *B–F*, ×2000.)

To count the organisms, it was necessary to remove them from the gel. This was done with Dispase (Collaborative Research), an enzyme preparation from *Bacillus polymyxa* recommended for the digestion of Matrigel by the manufacturer. In preliminary experiments we determined that the minimal effective concentration of Dispase was supplied by the addition of 120 μl to the 0.5 ml of overlay, followed by incubation for 1 hr at 37°C. The contents of such a digested culture were then centrifuged, the sediment was mixed with 5 μl of the same 50% erythrocyte suspension used for the merozoite counts, and a Giemsa-stained film was prepared. Organisms could then be conveniently counted in relation to a fixed number of erythrocytes.

To maintain cultures beyond the first 12–14 hr, the overlay was replaced with fresh medium at 12 hr and again at 24 hr, and the cultures were sampled at 36 hr. Other cultures received fresh medium also at 36 hr and were sampled at 44–45 hr. For each change of medium, an appropriate portion of sonicate was thawed, provided with supplements (ATP and pyruvate, and CoA to some), and warmed in a water bath to 37°C. Usually, the overlay was removed from a pair of cultures and then immediately replaced with fresh medium (0.5 ml per well). Removed overlays were used to monitor pH.

For electron microscopy organisms were either fixed directly in fragments of gel or removed from the gel by digestion with Dispase (as described above) and then fixed. Fixation and subsequent procedures were as described (7).

RESULTS AND DISCUSSION

Morphology of the Axenic Parasites and Their Viability as Demonstrated by Uptake of Rhodamine 123. A favorable effect of the Matrigel was evident even in the first samples taken at 12–14 hr. Wet mounts of fragments of gel examined by phase-contrast microscopy showed more larger rings with motility than we had ever seen in earlier work with liquid medium. These, as well as the numerous small trophic forms, were made especially conspicuous if allowed to take up rhodamine 123 for 1–2 hr and then examined by epifluorescence. One could see many brightly fluorescent forms, demonstrating their viability (8) (Fig. 1*A*). The larger rings were especially interesting (Fig. 1*B*) because of their motility and because of the fine elongate pseudopodial extensions they showed. These may correspond to the membrane-lined extensions seen by electron microscopy in infected erythrocytes (see ref. 9). Stained slides prepared from the gel fragments showed the same small and larger ring forms noted from liquid culture. By digesting the gel with Dispase and then preparing stained slides of the organisms mixed with a fixed amount of normal erythrocytes, an estimate of the number of organisms could be made. In one experiment (E-166), for example, we found that from an initial inoculum of 7×10^6 merozoites per well, 23% developed to rings, of which 25% were large rings including some with pigment.

In cultures that received fresh overlay at 12 hr and at 24 hr and were examined at 36 hr, wet mounts of gel samples showed large trophozoites and early schizonts with clumped pigment. Again, these showed up especially well by rhodamine fluorescence (Fig. 1 *C* and *D*). Stained slides at this time had trophozoites and schizonts mostly with two nuclei. Counts from slides made from Dispase-treated material indicated that only ≈1% of the merozoites developed to these later stages. In one experiment (E-166), several stage II gametocytes developing extracellularly were noted in gel samples taken at 36 hr.

In cultures that also received fresh overlay at 36 hr and were examined at 44–46 hr, late schizonts were seen that fluoresced brightly with rhodamine (Fig. 1 *E* and *F*). In stained preparations schizonts with well-developed merozoites were present. Furthermore, merozoites showing rhodamine fluorescence and, hence, probably viable were noted in the wet mounts.

Infectivity of Merozoites Formed Axenically. Two experiments to test infectivity were carried out with similar positive results. A detailed description of one of these will at the same time provide for recapitulation of the kinds of observations made during the course of a typical experiment. For this experiment (E-167), the merozoite suspension in complete medium had 300×10^6 merozoites per ml with a contaminating initial population of only 3×10^4 schizonts per ml. Hence, each well had an inoculum of 30×10^6 merozoites. The small numbers of schizonts initially present were mostly extracellular and degenerated rapidly. They were not seen by rhodamine fluorescence in the 12- to 14-hr samples. Three Linbro plates were prepared. Plate 1 had two wells as invasion controls and two wells with a gel containing 0.1 ml of merozoite suspension plus 50 μl of Matrigel overlaid with 0.5 ml of complete medium, including the ATP/pyruvate supplement. For plates 2 and 3, each plate had three wells with the same gel and the same overlay and three wells with the same gel but with an overlay that also contained CoA at 0.15 mM.

Plate 1 was sampled at 12–14 hr. Slides prepared from the invasion controls showed an invasion rate (the number of intracellular rings as a percentage of the number of merozoites) of only 0.3%. Both of the experimental wells were exposed to rhodamine 123 and one of them was then digested with Dispase. Wet mounts from both showed many excellent brightly fluorescent rings. Duplicate stained slides from the Dispase-treated material mixed with 5 μl of 50% erythrocyte suspension gave an average count of 89 rings, including 37 larger ones, per 1000 erythrocytes. Since the merozoite count was 600 per 1000 erythrocytes, this indicated an overall initial development of 15%, with 6% becoming large (or 42% of the total of viable organisms). A count of 100 successive parasites seen on the slide prepared from the gel not treated with Dispase similarly showed 46% of the organisms to be large rings.

The six wells of plate 2 received fresh overlays of corresponding medium (with CoA for wells 4–6) at 12 hr and again at 24 hr. The overlays removed at these times had a pH value of 7.2–7.3. After 36 hr, all wells were exposed to rhodamine 123 for 1.5 hr. All then received fresh overlay. In addition, Dispase was added to wells 1, 2, 4, and 5. The digested contents of these wells were sedimented by centrifugation, each was mixed with 5 μl of 50% erythrocyte suspension, and slides were prepared. The gels in wells 3 and 6 were sampled directly for wet mounts and stained slides. The wet mounts

Table 1. Development of parasites at 36 hr: Counts from Dispase-digested material mixed with 5 μl of 50% erythrocyte suspension

Wells	Medium	Parasites, no. per 10,000 erythrocytes* R	T	S	T + S as % of initial merozoites†
1,2	Sonicate + ATP/pyruvate	7	38	13	0.4
4,5	Sonicate + ATP/pyruvate + CoA	10	63	21	0.7

*Whereas the rings and most of the trophozoites stained in a normal way, many of the schizonts stained weakly. R, rings; T, trophozoites; S, schizonts.

†Calculated by dividing the total trophozoites plus schizonts by 12,000 (the number of merozoites per 10,000 erythrocytes initially present). The same volume (5 μl) of the same 50% erythrocyte suspension was added to equivalent volumes of parasite suspension.

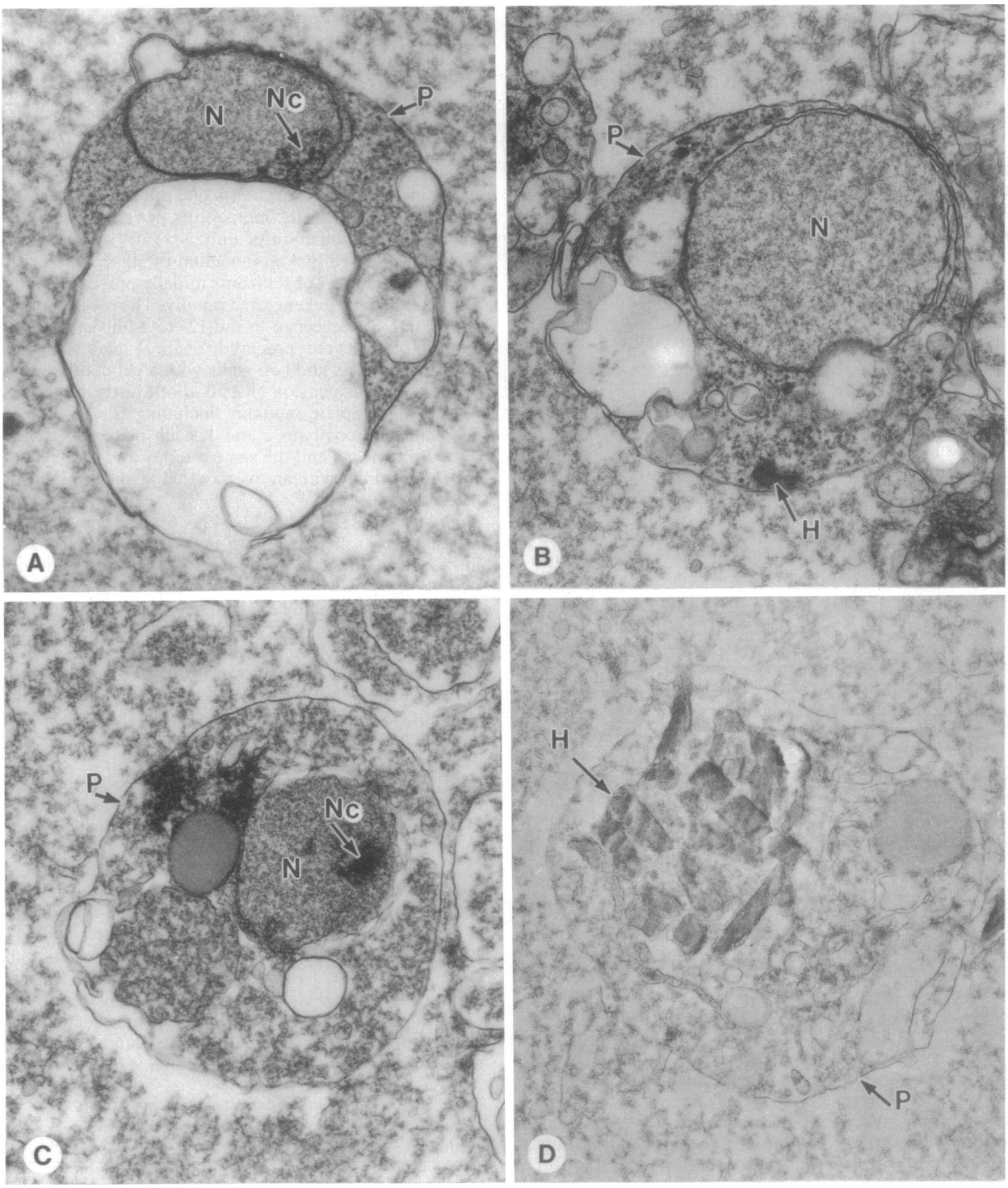

FIG. 2. Transmission electron micrographs of *P. falciparum* developing extracellularly in gel culture. (*A* and *B*) Rings at 12–14 hr fixed *in situ* in a gel fragment. (*C* and *D*) Late trophozoites from Dispase-treated material, showing nucleus in *C* and clumped pigment in *D*. Note the presence of a nucleolus, a feature of the Honduras-I strain and its clones to which this laboratory has previously called attention (10). P, plasma membrane; N, nucleus; Nc, nucleolus; H, hemozoin. (×30,000.)

both showed numerous fluorescent trophozoites and schizonts with clumped pigment (Fig. 1 *C* and *D*). The material from well 6 was particularly good. Wet mounts from the Dispase-treated wells showed similar fluorescent forms; these appeared more numerous and brighter in wells 4 and 5 than in wells 1 and 2. Counts of parasites in relation to the added erythrocytes on stained slides of this material gave the results summarized in Table 1. Although this result suggests a favorable effect of CoA, also seen in some earlier experiments (1), such an effect is not regularly reproducible.

The six wells of plate 3 were treated exactly like those of plate 2 at 12 and 24 hr and then again received fresh overlays at 36 hr. At 44 hr, wells 1, 2, 4, and 5 were treated in the following way. Each overlay was removed and replaced with 0.5 ml of a warm 1% suspension of type A^+ erythrocytes in RPS medium. The gel was then triturated with the tip of a Pasteur pipet. The plate was returned to the candlejar at 37°C. (Wells 3 and 6 were treated in other ways that gave no result and will not be further considered.) Wells 1, 2, 4, and 5 received fresh RPS 1 day later (day 3) and again on day 4. At

this time a 0.1-ml sample was removed and microcentrifuged, and the cells were used to prepare slides. The wells received fresh RPS on day 5, the well contents were centrifuged on day 6, and the sediment was used for stained slides. We thus had slides made at 2 days and at 4 days after addition of erythrocytes and breaking up of the gel. In each of the slides made at 2 days, a single intracellular trophozoite was found per 10,000 erythrocytes. In the slides made at 4 days, the counts were (per 10,000 erythrocytes) 5 and 2 rings for wells 1 and 2, respectively, and 4 rings and 7 rings plus 7 trophozoites for wells 4 and 5, respectively. All of these parasites were intracellular. They could only have been derived from infectious merozoites produced in the gel by the extracellular schizonts present after 44–46 hr of development.

Fine Structure of the Axenic Parasites. The organisms (Fig. 2) have much the same structure as corresponding stages developing intracellularly (7). They differ mainly in lacking a parasitophorous membrane, just as we had earlier found for the initial young rings developed in liquid medium (2, 5). This raises questions as to the role of the parasitophorous membrane. Whatever may be its role in nature, it has evidently been replaced or by-passed by the *in vitro* conditions we have provided.

Conclusion. It is clear that we now have conditions for the axenic development of at least one complete asexual erythrocytic cycle of *P. falciparum*. None of the following conditions is essential: an intact erythrocyte, the process of entry, or the parasitophorous membrane. It is equally clear, however, that there is much need for further improvement of the culture conditions. Although up to 30% of the merozoites show initial differentiation into trophic forms viable at 12 hr, with 5–6% appearing at this time as large rings, only ≈1% developed completely through the 2-day asexual cycle. The role of the Matrigel may be of special interest. Its striking effects on differentiation of tissue cultures (for example, see ref. 11) are not understood. For the parasites, it might have an essentially nonspecific role by providing a physical matrix or it is possible that certain of its protein constituents plays a part. It is also worth noting that the methods we have here described may be applicable in the axenic cultivation of other parasites that have been difficult to culture.

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