AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D. C. 20523

BIBLIOGRAPHIC INPUT SHEET

FOR AID USE ONLY

1. SUBJECT CLASSIFICATION
A. PRIMARY: Public Health
B. SECONDARY: General Public Health

2. TITLE AND SUBTITLE
In vitro ookinete development of the rodent malarial parasite Plasmodium berghei

3. AUTHOR(S)
Rosales-Ronquillo,M.C.; Silverman,P.H.

4. DOCUMENT DATE: 1974
5. NUMBER OF PAGES: 9p.
6. ARC NUMBER: ARC 614.4323-R773d

7. REFERENCE ORGANIZATION NAME AND ADDRESS
University of New Mexico
Department of Biology
Albuquerque, New Mexico 87131

8. SUPPLEMENTARY NOTES (Sponsoring Organization, Publishers, Availability)
(In J.of parasitology,v.60,no.5,p.819-824; and in v.60,no.6,p.1039-1040)

9. ABSTRACT
In vitro development of Plasmodium berghei ookinetes occurred in primary cell cultures as well as established cell lines from Anopheles stephensi Liston (Diptera: Culicidae). Two morphological forms of viable ookinetes were observed in cultures conditioned within a constant temperature ranging from 18±1 C to 21±1 C for 2 to 24 hr in fresh serum-free minimum essential medium (MEM) prior to inoculation of infected blood. Ookinetes exhibited either gliding, snakelike, or spiral forward movements. Some ookinetes apparently attempted to penetrate red blood cells (RBC), usually indenting the surface of the RBC. Factors which might influence ookinete formation such as temperature, pH, age of cell cultures employed, and length of time the cultures were maintained at constant temperatures (ranging from 18 ± 1 C to 21 ± 1 C) prior to inoculation of infected blood are discussed.

10. CONTROL NUMBER: PN-AAB-162
11. PRICE OF DOCUMENT:
12. DESCRIPTORS: Primary Cell Cultures, Innoculation, Ookinete Formation, Infected Blood, Temperature, PH, Age of Cell Culture
13. PROJECT NUMBER: 931-17-511-522
14. CONTRACT NUMBER: CSD-3689 Res.
15. TYPE OF DOCUMENT: Research Bulletin

AID 590-1 (4-74)

IN VITRO OOKINETE DEVELOPMENT OF THE RODENT MALARIAL PARASITE, *PLASMODIUM BERGHEI*

Maria C. Rosales-Ronquillo and Paul H. Silverman

Reprinted from the JOURNAL OF PARASITOLOGY
Vol. 60, No. 5, October 1974
p. 819–824
Made in United States of America
Copyright © 1974 by the American Society of Parasitologists

THE JOURNAL OF PARASITOLOGY
Vol. 60, No. 5, October 1974, p. 819–824

IN VITRO OOKINETE DEVELOPMENT OF THE RODENT MALARIAL PARASITE, *PLASMODIUM BERGHEI**

Maria C. Rosales-Ronquillo and Paul H. Silverman
Department of Biology, University of New Mexico, Albuquerque, New Mexico 87131

ABSTRACT: In vitro development of *Plasmodium berghei* ookinetes occurred in primary cell cultures as well as established cell lines from *Anopheles stephensi* Liston (Diptera: Culicidae). Two morphological forms of viable ookinetes were observed in cultures conditioned within a constant temperature ranging from 18 ± 1 C to 21 ± 1 C for 2 to 24 hr in fresh serum-free minimum essential medium (MEM) prior to inoculation of infected blood. Ookinetes exhibited either gliding, snakelike, or spiral forward movements. Some ookinetes apparently attempted to penetrate red blood cells (RBC), usually indenting the surface of the RBC. Factors which might influence ookinete formation such as temperature, pH, age of cell cultures employed, and length of time the cultures were maintained at constant temperatures (ranging from 18 ± 1 C to 21 ± 1 C) prior to inoculation of infected blood are discussed.

Many unsuccessful attempts have been made to develop an in vitro substrate that is physiologically and biochemically favorable to the cultivation of the complete mosquito cycle of the malarial parasite (Ball and Chao, 1957; Schneider, 1968; Walliker and Robertson, 1970). Recently, actively growing mosquito cells have been used to support in vitro development of the late stages of sporogonic forms of the malaria parasite (Ball and Chao, 1971, Schneider, 1972). However, the substrate appears to be inadequate to the cultivation of the early sporogonic forms

The successful in vitro induction of the exflagellation of microgametocytes, fertilization of macrogametocytes and formation of viable ookinetes of the rodent malarial parasite, *Plasmodium berghei*, using actively growing primary cell cultures derived from the immature stage of *Anopheles stephensi* (Rosales-Ronquillo, Simons, and Silverman, 1972) or an established *A. stephensi* cell line (Schneider, 1969) is reported herein.

MATERIALS AND METHODS

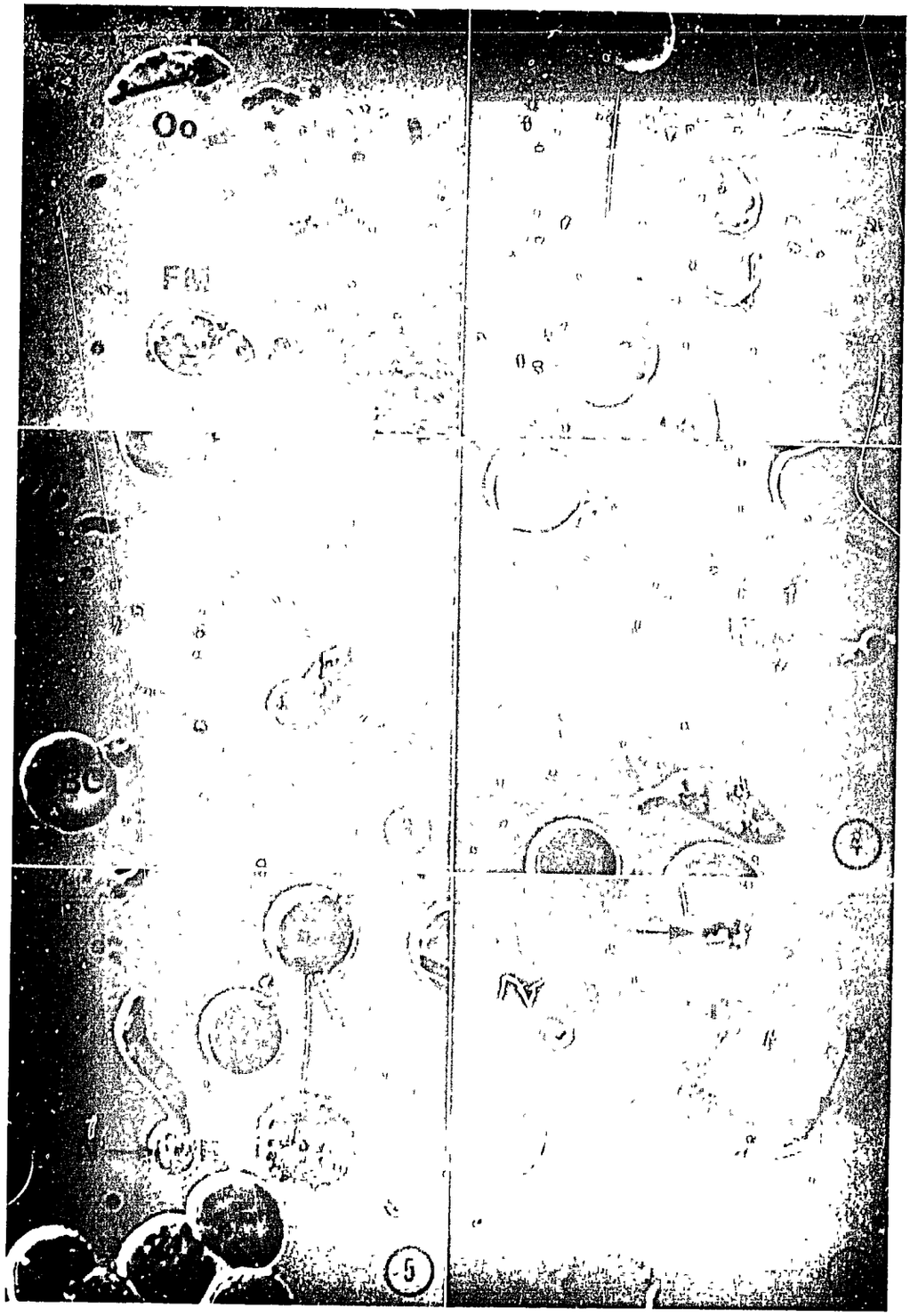
The NK65 strain of *P. berghei* was maintained routinely by blood passage in hamsters alternating with cyclic transmission through the mosquitoes

Primary cell cultures of *A. stephensi* (Rosales-Ronquillo, Simons, and Silverman, 1972) and monolayer cultures of *A. stephensi* cells (Schneider, 1969) were grown on 10- by 50-mm cover slips in 1 ml of their respective medium in Leighton tubes (16 mm by 125 mm) at 27 ± 1 C in the dark. Five- and 30-day-old primary cell cultures as well as monolayer cultures of anopheline cells, 5 to 7 days after subculture, were used. Cultures were conditioned prior to inoculation of injected blood at a constant temperature ranging from 15 ± 1 C to 24 ± 1 C in the dark. The length of conditioning time varied from 2 to 24 hr. Cultures were conditioned either in their old medium or in 1 ml fresh serum-free medium after removing the old medium. The serum-free medium consisted of 90% Eagle's minimum essential medium (MEM) with Eagle's salt solution but without sodium bicarbonate or glutamine. The pH of the medium was adjusted to 7.6 with 0.44% $NaHCO_3$. Cell-free media (MEM) with or without inactivated fetal bovine serum (FBS) were conditioned and used as the control cultures. Two-tenths ml of heparinized aseptically drawn infected hamster blood (0.35 ml heparin to 1 ml infected blood) was inoculated into each experimental and control tube using a sterilized 3-cc syringe equipped with an 18-gauge, 1.5-inch needle. Inoculated cultures were maintained at a constant temperature ranging from 15 ± 1 C to 24 ± 1 C in the dark. No antibiotics were employed throughout the experiments. The pH of the medium was measured in a Metrohm pH 102 model before and after the cultivation period.

Prior to inoculation into experimental and control tubes, blood was taken from the tail of gametocyte-carrying hamsters, placed on a slide, covered, and examined with a Zeiss–Nomarski interference-contrast microscope for high peak of macrogametocytes and exflagellating microgametocytes.

Appraisal of the cultivation experiments was done within a period of 50 hr after the initial inoculation of the infected blood Cultures were examined under a Zeiss–Nomarski interference-contrast microscope at 15 min after inoculation of infected blood, and at 5-hr intervals thereafter until the termination of the experiment. Prior to withdrawing a drop of the supernatant for appraisal of the culture, the experimental tube was agitated slightly. The culture drop was placed on slides, covered with a cover slip, and examined.

Received for publication 25 March 1974.
* Supported by the Agency for International Development, U. S. Department of State, Contract No. AID/csd-3689.


Oo
FBI
BG
4
5

At the termination of the experiment, the supernatant was drawn out, centrifuged at 500 rpm (RCF = 36.3) for 5 min. A few drops of the pellet were placed on slides and examined under a Zeiss–Nomarski interference-contrast microscope. Smears of the remaining pellet were made on slides, fixed and stained with either Giemsa or Oil red O, and examined under a bright-field microscope. The cover slip in each culture was removed, washed gently with fresh serum-free MEM, fixed and stained with either Giemsa or Oil red O, and examined with bright-field microscopy.

Photomicrographs were taken with a Zeiss C-35 camera. Motility of the ookinetes was recorded under Zeiss–Nomarski interference-contrast normal speed cinephotomicrography.

RESULTS

Development of fertilized macrogametes into well-developed ookinetes was observed only in both preconditioned cultures within the first 24 hr of cultivation. Exflagellating microgametocytes were first observed at about 7 to 10 min after the initial blood inoculation of these cultures. The process of fertilization was never observed. Early developing zygotes were similar in some structural characteristics to macrogametes (Fig. 1), being irregular in shape, with a nucleus located in the posterior one-half of the body. At 15 to 20 hr, ookinetes appeared to uncoil or elongate. During this time, a prominent bulbous structure, containing the dense mass of nuclear material, was present at the posterior end (Figs. 2, 3). As development proceeded, the nucleus moved anteriorly into the body of the ookinete, at which time the bulblike structure simultaneously decreased in size so that only a small protuberance remained (Fig. 4). In some ookinetes, the nucleus remained within the bulblike posterior end (Fig. 5). Mature ookinetes appeared commalike, blunt anteriorly and tapered posteriorly (Fig. 6). Each one measured approximately 12 μ long and 2.5 μ wide. An ovate nucleus enclosed by a nondescript nuclear membrane was usually located in the posterior half of the body. Pigment granules were observed in the anterior half of the body and circular patches were seen within the cytoplasm.

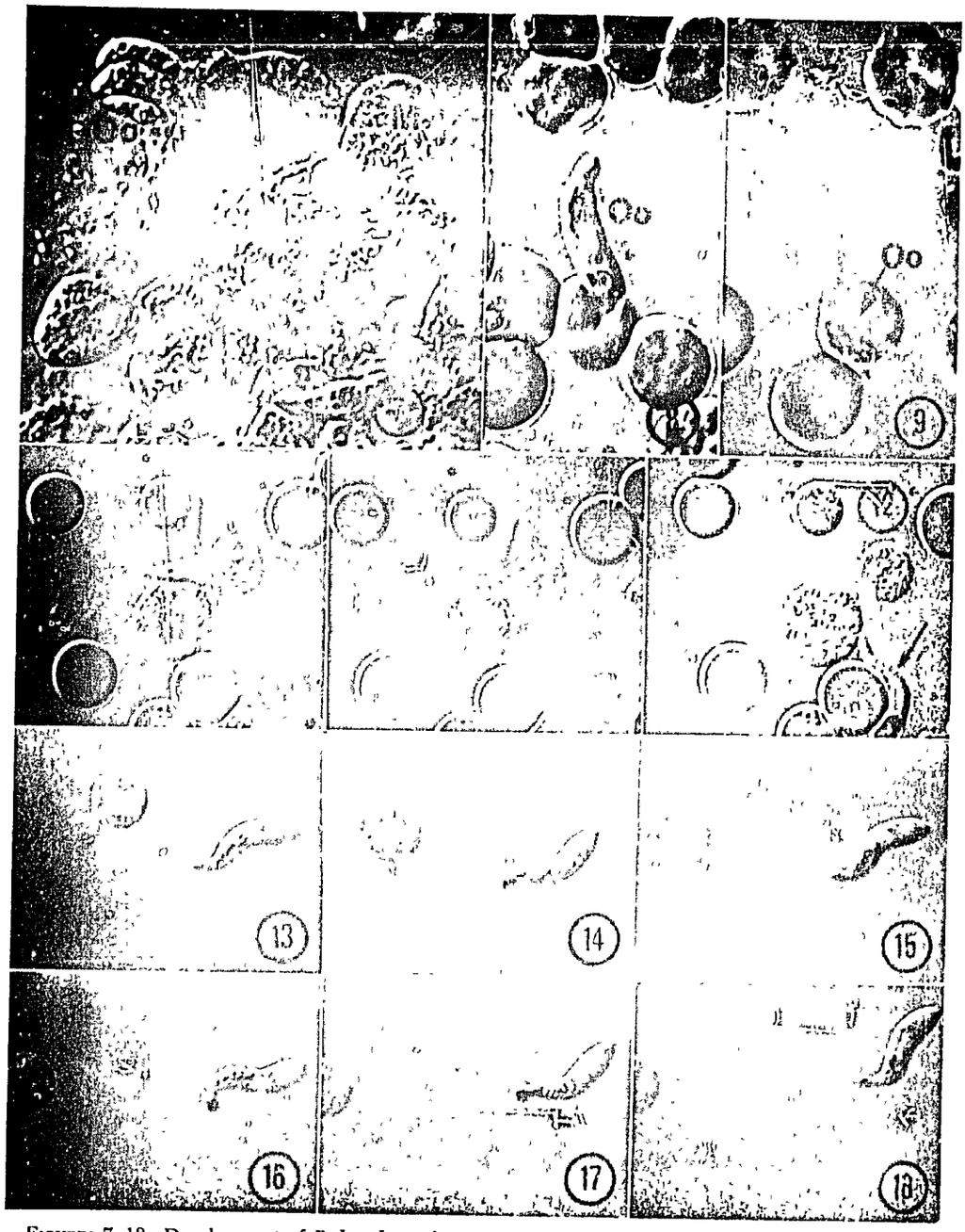
Occasionally, aggregation and clumping of macrogametocytes occurred in both cultures as early as 15 hr after blood inoculation (Fig. 7). At 20 hr, well-developed ookinetes, both commalike and elongated forms with a posterior bulbous end, were observed moving away singly from the aggregates or clusters.

Some mature ookinetes attempted to penetrate red blood cells (RBC) (Fig. 8). These ookinetes became motionless immediately after failing to penetrate red blood cells (RBC). This period of inactivity was then followed by degeneration of the ookinete.

The extent and mode of locomotion of the ookinete was best seen with Zeiss–Nomarski interference-contrast normal speed cinephotomicrography. Motile ookinetes were first seen at 18 hr into the cultivation period. Some exhibited a snakelike (Figs. 10–12) or a slight, wriggly, gliding movement (Figs. 13–18). Immature ookinetes which still had a posterior globular structure were also capable of similar movements. These activities lasted for as long as 15 hr in culture. At 40 hr, ookinetes progressively decreased in motility and ultimately remained stationary. Degeneration became evident about the 48th hr; ookinetes swelled to a rotund shape with indistinct nucleus and ill-defined cytoplasmic inclusions (Fig. 9).

←

FIGURES 1–9. Development of *Plasmodium berghei* ookinete in cell cultures. All are living specimens photographed with Zeiss–Nomarski interference-contrast microscopy Figs. 10–12. Ookinete movement taken at 30-sec intervals with Zeiss–Nomarski interference-contrast microscopy. Figs. 13–18. Ookinete movement taken at 1-min intervals. Hours after inoculation of infected blood into the culture substrate and magnification are listed in parentheses. Abbreviations: B, bulbous posterior end; CP, circular cytoplasmic patches, FM, fertilized macrogametocyte, M, macrogametocyte, N, nucleus; Oo, ookinete; P, posterior protuberance; G, pigment granules; RBC, red blood cells. **1.** Fertilized macrogametocyte and young developing ookinete (15, × 980). **2.** Early stage of zygote beginning to uncoil; note presence of a small elongating anterior end (arrow) (8; × 960). **3.** Elongating zygote, note presence of cytoplasmic inclusions at the anterior region (arrow) (8, × 1,100). **4.** Later stage of developing ookinetes; note presence of small protuberance at posterior end (arrow) (24, × 1,500). **5.** Well-developed ookinete with nuclear material within bulblike posterior end (24; × 1,400). **6.** Mature commalike ookinete with well-defined cytoplasmic patches, nucleus, pigment granule, and a small posterior protuberance; note lysed infected red blood cell (arrow) (24; × 1,500).



FIGURES 7–18. Development of *P. berghei* ookinete cont'd **7.** An aggregate of well-developed ookinetes and macrogametocytes (24; × 1,500). **8.** A mature ookinete attempting to penetrate a red blood cell (24; × 1,500). **9.** Degenerated ookinete (50; × 1,200). **10–12.** Snakelike movement of an ookinete (see arrow) (24; × 750). **13–18.** Mature ookinete exhibiting a slight, wriggly gliding movement (24; × 760).

DISCUSSION

Genesis of the early stages of the sporogonic forms of the malarial parasite, *P. berghei*, has been successfully demonstrated in primary cultures of cells of *A. stephensi* (Rosales-Ronquillo, Simons, and Silverman, 1972) and in an established cell line of *A. stephensi* (Schneider, 1969). After inoculation of these cultures with erythrocytic gametocytes obtained from plasmodium-infected hamsters, exflagellation of microgametocytes, fertilized macrogametes (zygotes), and formation of two structural forms of viable ookinetes were observed. Morphological characteristics and motility of the ookinetes do not differ from those found in vivo.

Culture experiments reported by earlier workers (Micks, de Caires, and Franco, 1948, Ball and Chao, 1964; Yoeli and Upmanis, 1968) indicated that the mosquito stomach probably possessed some substances necessary for maintaining the viability of the gametocytes, insuring exflagellation of microgametocytes, fertilization of macrogametes, and ookinete formation. Weatherbsy (1954, 1960) found that the mosquito stomach is not necessary to insure sporogonic development of the parasite by obtaining formation of oocysts in the hemocoel of mosquitoes. Alger (1968) demonstrated ookinete formation in heparinized and nonheparinized infected mouse blood sealed in capillary tubes. In the present study, we found that the mosquito gut is not essential for development of the early stages (ookinetes) of the sporogonic form of *P. berghei*. Since the cultures employed consist of cells and cellular outgrowths from mosquito larval fragments, the parasite is apparently not dependent upon specific host organs for development in vitro.

The early development of the sporogonic stages is apparently influenced by the temperature employed during cultivation, the particular age of both cultures used, the length of time the cultures were conditioned at 21 C in serum-free MEM, as well as the pH of the culture.

The temperature range which permits ookinete formation in vivo (18 to 21 C) in the natural host (Yoeli, 1965) also applies to our in vitro system. No ookinetes were observed in cultures maintained below 18 C or above 21 C.

Schneider (1972) and Ball and Chao (1971) suggested that primary mosquito cell cultures are probably more suitable for culturing the malaria parasite than the established anopheline cell line. Cells in primary culture probably retain more physiological and biochemical characteristics of the cells of their origin than cells in the established cell line which without doubt have already undergone extensive change and adaptation through a long period of subculturing. However, we found that more ookinetes developed in the anopheline cell line than in the 5-day-old and 30-day-old primary cultures. Presently, nothing conclusive can yet be stated, as to which will serve as a better culture system, until the capabilities of primary cultures of varying ages, e.g., 10-day-old, 15-day-old, 20-day-old, and 25-day-old, have been fully explored.

No development of the parasites was observed in either cell substrate which were conditioned in their old medium prior to inoculation with parasitized hamster blood. Failure of ookinetes to develop in these cultures may be due to toxicity caused by the accumulation of metabolic waste in the old medium which might have adverse effect on the gametocytes. No development was evident in medium alone (MEM) either with or without inactivated fetal bovine serum (FBS). It is likely that the MEM alone cannot fulfill the nutritional requirements of the developing parasite. On the other hand, MEM, which was fortified with inactivated FBS, also failed to support ookinete development.

The apparent conditioning period (2 to 24 hr) of the cell substrates at constant temperature (at either 18, 19, 20 or 21 ± 1 C) in fresh serum-free MEM prior to cultivation may have a direct and immediate bearing on the maturation of gametes, fertilization, and ookinete development. During this period the living cells may have enriched the media by release of substances that initiate exflagellation, insure fertilization, and support ookinete formation. Failure of the ookinete to develop further might be due to depletion or lack of some essential substances in the culture. However, prolonged conditioning for more than 24 hr did not elicit any sign of development beyond the ookinete stage.

Micks, de Caires, and Franco (1948) demon-

strated that the exflagellation of the male gametocytes of two malarial parasites, *Plasmodium cathemerium* and *Plasmodium relictum*, has no correlation to pH. Ball and Chao (1964) showed that early oocyst development of *Plasmodium relictum* in vitro occurred at a pH of about 7 and an alkaline medium of pH 8 is favorable to sporozoite production. We obtained successful ookinete formation of *P berghei* in both cultivation substrates within an initial pH range of 7.4 to 7.6 Structural integrity of the cell substrates and the formed ookinetes, as well as the motility of the latter, were still noted within a pH range of 7.6 to 8.0 40 hr into the cultivation period. This increase in alkalinity of the culture substrates as cultivation progresses may be due to the buffering capacity of the inoculated infected blood. Ball and Chao (1964) pointed out that a high pH is favorable to the development of the later stages of the sporogonic forms, therefore, this increase in alkalinity as cultivation progresses might be one of the essential factors for successful cultivation of the malarial parasite.

This investigation showed that a single culture of the entire sporogonic form of *P. berghei* cannot be obtained under the present cultural environment. It is apparent that our cultures can approximate the in vivo conditions for only a limited time. Ball and Chao (1964, 1971) and Schneider (1968, 1972) in their attempts to culture later stages obtained essentially similar results in that the oocysts developed for a short period of time, ranging from 4 to 5 days using primary cultures and established cell lines of mosquitoes. Their work as well as our investigation strongly indicate that the physiological as well as biochemical requirements of the different stages of the sporogonic forms are exacting. Efforts towards improving our cultivation substrate are presently in progress.

ACKNOWLEDGMENTS

The authors wish to acknowledge with appreciation the technical assistance of Gregory Nienaber, Miss Brenda Nowlin, and Thomas Stewart.

LITERATURE CITED

ALGER, NELDA. 1968. *In vitro* development of *Plasmodium berghei* ookinetes. Nature **218**: 774.

BALL, G. H, AND J. CHAO. 1957 Development *in vitro* of isolated oocysts of *Plasmodium relictum* J. Parasit **43**: 409–412.

———, AND ———. 1964. Cultivation of the insect cycle of plasmodia Am J. Trop. Med **13**: 181–192

———, AND ——— 1971 The cultivation of *Plasmodium relictum* in mosquito cell lines J. Parasit **57**: 391–395

MICKS, D W, P. F DE CAIRES, AND L B. FRANCO. 1948 The relationship of exflagellation in avian plasmodia to pH and immunity in the mosquito. Am J Hyg. **48**: 182–190.

ROSALES-RONQUILLO, MARIA C, R W. SIMONS, AND P H SILVERMAN. 1972 Long-term primary culture of cells of the mosquito *Anopheles stephensi* Ann. Entomol. Soc Am. **65**: 721–729

SCHNEIDER, I 1968. Cultivation *in vitro* of *Plasmodium gallinaceum* oocysts. Exp Parasit. **22**: 178–186.

——— 1969. Establishment of three diploid cell lines of *Anopheles stephensi* (Diptera: Culicidae) J Cell Biol. **42**: 603–606.

——— 1972 A comparative study of the development of the mosquito cycle of *Plasmodium cynomolgi* in primary cultures versus an established cell line of *Anopheles stephensi*. Proc. Helm Soc Wash **39**: 438–444.

WALLIKER, D, AND E ROBERTSON. 1970. Cultivation *in vitro* of sporozoites of *Plasmodium berghei* Tr Roy Soc. Trop Med. Hyg. **64**: 5.

WEATHERSBY, A B. 1954 The ectopic development of malarial oocysts. Exp Parasit. **3**: 538–543

———. 1960 Further studies on exogenous development of malaria in the hemocoels of mosquitoes Exp. Parasit **10**: 211–213.

YOELI, M. 1965 Studies on *Plasmodium berghei* in nature and under experimental conditions. Tr. Roy. Soc. Trop. Med. Hyg. **59**: 255–271.

———, AND R. S UPMANIS. 1968. *Plasmodium berghei* ookinete formation *in vitro*. Exp. Parasit. **22**: 122–128.

Reprinted from the JOURNAL OF PARASITOLOGY
Vol. 60, No. 6, December 1974
p. 1039–1040
Made in United States of America
Copyright © 1974 by the American Society of Parasitologists

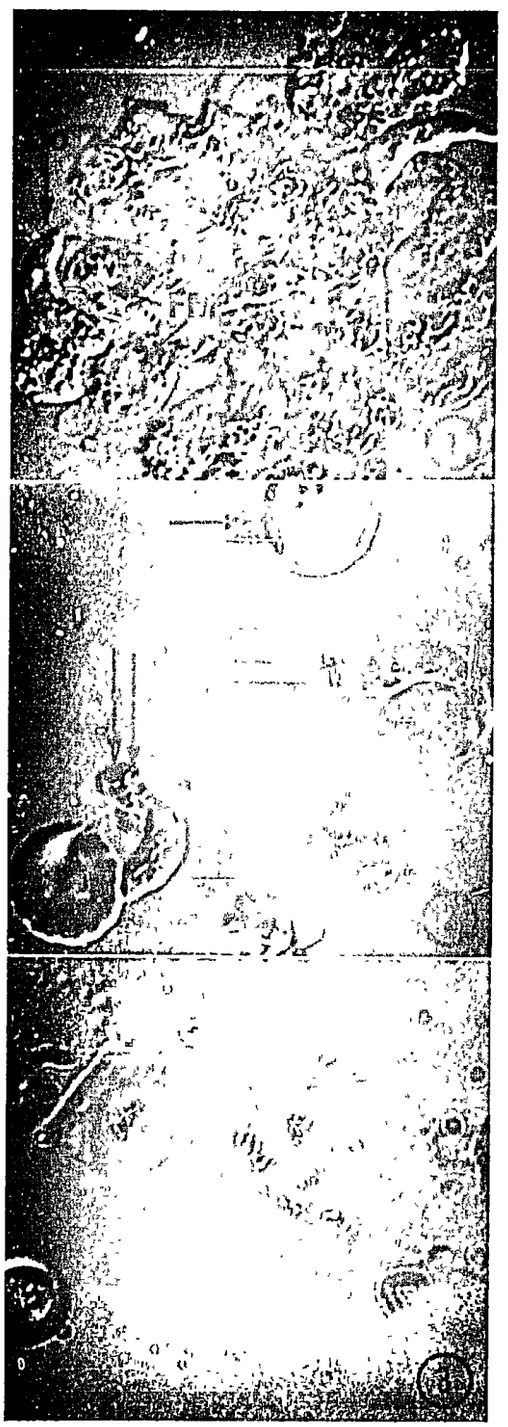
Plasmodium berghei Ookinete Formation in a Nonvector Cell Line

Rosales-Ronquillo and Silverman (1974, J. Parasit. **60:** 819–824) reported successful in vitro cultivation of ookinetes of *Plasmodium berghei* in primary cultures of mosquito cells (Rosales-Ronquillo et al., 1972, Ann. Entomol. Soc. Am. **65:** 721–729) and an established anopheline cell line (Schneider, 1969, J. Cell Biol. **42:** 603–606). However, both cultures were insufficient in supporting development beyond the ookinete stage. Dutky (1964, Am. J. Trop. Med. **13:** 193–194) suggested that nonvector cell lines might be more suitable for cultivating the entire mosquito cycle of the malarial parasite. Screening of various vector and nonvector host cell lines is currently being explored.

The ability of some fish cell lines to support growth of viruses (Officer, 1968, Proc. Soc. Exp. Bio. Med. **116:** 190–194; Wolf et al., 1968, J. Inf. Dis. **118:** 253–262) led us to investigate the feasibility of culturing the sporogonic forms of the malarial parasites in either the fathead minnow (FHM) line of epithelial cells (Gravell and Malsberger, 1965, Ann. N. Y. Acad. Sci. **126:** 555–565) or rainbow trout gonadal (RTG-2) cell line (Wolf and Quimby, 1962, Science **135:** 1065–1067).

Methods employed in this investigation were essentially the same as those described by Rosales-Ronquillo and Silverman (1974, loc. cit.).

Exflagellation of microgametocytes, fertilized macrogametocytes (zygotes), and ookinete development were seen in the fathead minnow cell line and proceeded on a schedule similar to that occurring in vivo (Garnham, 1965, Ann.



FIGURES 1–3. Photomicrographs of *Plasmodium* ookinete formation in fathead minnow (FHM) line of epithelial cells. All are living specimens photographed with Zeiss–Nomarski interference-contrast microscopy. Hours after inoculation of infected blood into cell culture and magnification in parentheses. Abbreviations: FM, fertilized macrogametocyte, FHM, fathead minnow cell, Oo, ookinete. **1.** An aggregate of fertilized macrogametocyte and ookinete (24, × 1,500). **2.** Two forms of mature ookinete, one has a bulbous posterior end (double arrows), the other ookinete is comma-shaped, note parasites (single arrow) inside red blood cell (30, × 1,500). **3.** An ookinete which appeared to be located within the intracytoplasmic vacuole (see arrow) of the culture cell (30, × 1,500).

Soc. Belge. Med. Trop. **45:** 259–266) as well as in the vector cultures in vitro (Rosales-Ronquillo and Silverman, 1974, loc. cit.). Aggregates of macrogametocytes (Fig. 1), the development of two forms of ookinetes at 18 to 24 hr (Fig. 2), and the mode and extent of locomotion of both forms of ookinetes were similar in vector as well as nonvector cultures. Fertilization of macrogametes by microgametes was not seen.

Although many ookinetes attempted to penetrate cells, entry of the ookinete into the cell was not observed. However, in some FHM cultures ookinetes appeared to be located within intracytoplasmic vacuoles of the host cells (Fig. 3). This was not observed in either of the mosquito culture systems. Light and electron microscopic studies are currently in progress in order to determine whether these ookinetes are intra- or extracellular. Ookinetes in intracytoplasmic vacuoles did not undergo any further development.

The results of the present investigation suggest that FHM cultures might have more potential capabilities than cultures of insect origin in supporting the development of ookinetes into oocysts. Further work along this line is currently in progress.

Development of the malarial organism in this nonvector culture indicates that the host-cell parasite specificity which occurs in vivo does not apply to cells and parasites in vitro. This is the first report of the development of ookinetes of *P. berghei* in an atypical pathogen–vector culture system.

Maria C. Rosales-Ronquillo, Gregory Nienaber, and Paul H. Silverman, Department of Biology, University of New Mexico, Albuquerque, New Mexico 87131 Supported by the Agency for International Development, U S Department of State, Contract No AID/csd-3689