**References and Notes**

1. B. Angrist, *Stimulants: Neurochemical, Behavioral, and Clinical Perspectives*, I. Creese, Ed. (Raven, New York, 1983), p. 1; J. M. Davis, *Arch. Gen. Psychiatry* **33**, 858 (1976); ——— and R. Casper, *Drugs* **14**, 260 (1977).
2. P. A. Berger and K. Rexroth, *Schiz. Bull.* **6**, 102 (1980); G. M. Simpson, E. H. Pi, J. J. Sramek, *Drugs* **21**, 138 (1981); D. Tarsy, *Clin. Neuropharmacol.* **6** (Suppl. 1), S9 (1983).
3. S. O. Cole, *Neurosci. Biobehav. Rev.* **2**, 89 (1978); P. M. Groves and G. V. Rebec, *Annu. Rev. Psychol.* **27**, 91 (1976).
4. I. Creese, D. R. Sibley, M. W. Hamblin, S. E. Leff, *Annu. Rev. Neurosci.* **6**, 43 (1983); P. Seeman, *Pharmacol. Rev.* **32**, 229 (1980).
5. H. R. Burki, *Pharmacol. Ther.* **5**, 525 (1979); P. Jenner *et al.*, *Life Sci.* **23**, 545 (1978).
6. A. G. Ewing *et al.*, *Brain Res.* **261**, 101 (1983).
7. A. G. Ewing, R. M. Wightman, M. A. Dayton, *ibid.* **249**, 361 (1982); F. G. Gonon, M. J. Buda, M. Jouvet, J. F. Pujol, *ibid.* **223**, 69 (1981).
8. M. Hadjiconstantinou and N. H. Neff, *Neuropharmacology* **22**, 939 (1983).
9. S. Leff, D. R. Sibley, M. Hamblin, I. Creese, *Life Sci.* **29**, 2081 (1981).
10. G. V. Rebec, T. R. Bashore, K. S. Zimmerman, K. D. Alloway, *Neuropharmacology* **19**, 281 (1980).
11. Animals were housed in plexiglass chambers (35 by 35 by 45 cm) with a wire floor at least 48 hours before the first injection. Food and water were continuously available. Each chamber was contained in a sound-attentuating cubicle equipped with a one-way observation mirror for viewing the animals without disturbing them. The animals were maintained on a 12-hour bright-light (0800 to 2000), dim-light cycle; all injections were made between noon and 1400. Rats in each group were used only once to avoid residual drug effects.
12. The AA, freshly prepared each day and adjusted to *p*H 7.0 with 1*M* NaOH, was maintained under a steady stream of nitrogen until administration.
13. Amphetamine produces a wide variety of behavioral responses in the open field that are collectively called stereotyped because of their repetitive, invariant nature [A. Randrup and I. Munkvad, *J. Psychiatr. Res.* **11**, 1 (1974)]. Because each of these behaviors may be differentially affected by pharmacological and other manipulations, it is important to characterize each behavior individually over the entire time course of the amphetamine response to obtain an accurate behavioral description [G. V. Rebec and T. R. Bashore, *Neurosci. Biobehav. Rev.* **8**, 153 (1984)].
14. G. V. Rebec, E. E. Peirson, F. A. McPherson, K. Brugge, *Psychopharmacology* 77, 360 (1982); G. V. Rebec and D. S. Segal, *Pharmacol. Biochem. Behav.* **13**, 793 (1980).
15. As mean catalepsy time increased, so did the variance. To compensate for unequal within-group variances, statistical analysis was performed with a logarithmic transformation of the data.
16. R. E. Heikkila, F. S. Cabbat, L. Manzino, *Res. Commun. Chem. Pathol. Pharmacol.* **34**, 409 (1981); L. C. Tolbert, T. N. Thomas, L. D. Middaugh, J. W. Zemp, *Life Sci.* **25**, 2189 (1979).
17. L. Pauling, *Am. J. Psychiatry* **131**, 1251 (1974).


18. Supported by the National Institute on Drug Abuse (grant DA 02451 to G.V.R.). We also acknowledge the assistance of Glenn D. Anderson with our statistical analysis. Smith Kline & French Laboratories provided a generous supply of *d*-amphetamine sulfate.


* Present address: Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, Mo. 63110.



5 March 1984; accepted 4 October 1984


# Complete Development of Hepatic Stages of *Plasmodium falciparum* in Vitro


Abstract. *An in vitro model was developed to study the hepatic phase of* Plasmodium falciparum, *the only malaria parasite lethal to man. Primary cultures of human hepatocytes were inoculated with sporozoites of Brazilian and African strains of* P. falciparum. *On days 1 through 7 after inoculation examination of fluorescence-labeled and Giemsa-stained preparations demonstrated the presence of many intracellular parasites. In three separate sets of experiments all cultures were found to be infected with as many as 650 liver schizonts measuring up to 40 micrometers. After the addition of red blood cells, intraerythrocytic forms of* P. falciparum *were detected on days 12 and 13 by an immunofluorescence assay, indicating that the hepatic cycle had been completed in vitro.*


Among the developmental stages of plasmodia occurring in man, we know the least about the intrahepatic multiplication that follows inoculation of sporozoites by mosquitoes. This is not surprising, considering the inherent difficulty of studying these stages in humans and the scarcity of suitable hosts susceptible to the human parasites.

The alternative approach of reproducing the liver cycle in vitro is therefore an attractive one. Cultures can be easily manipulated and are essential in immunologic studies and studies of antiparasite drugs designed to act against the liver stage. This approach was recently successful in studies of *Plasmodium vivax* in which sporozoites introduced into cultures of functional human hepatocytes invaded the liver cells and developed to maturity, releasing merozoites infective for red blood cells (*1*, *2*). We report here a similar approach designed to produce the complete exoerythrocytic cycle of *Plasmodium falciparum*. in vitro.

Host cells were prepared by microperfusion with collagenase of a human liver fragment removed by biopsy (*2*). The perfused fragment was minced and the freed cells were suspended in medium and seeded in monoculture at a concentration of $5 \times 10^5$ cells per 35-mm petri dish. The cells were maintained for 24 to 48 hours before inoculation of sporozoites in supplemented minimal essential medium (MEM) (*3*).

The parasites used were an African isolate and a Brazilian clone of *P. falciparum*. Infective gametocytes were produced in vitro by static culture techniques slightly modified from those previously described (*4*). Eighteen-day cultures containing mature stage 5 gametocytes were fed to female *Anopheles stephensi* or *Anopheles freeborni* mosquitoes with a water-jacketed membrane feeder maintained at 37°C.

Sixteen days later, when sporozoites were present in the mosquito salivary glands, hepatocyte cultures were prepared for infection by removing spent medium and adding 0.5 ml of fresh medium. Five to ten pairs of aseptically dissected salivary glands were then introduced into each culture. Three hours later 1 ml of medium was added, after which the medium was changed daily. At various intervals after the infected salivary glands were introduced, the cultures were examined by the indirect fluorescent antibody test (IFAT) and by visible light microscopy before and after staining with Giemsa. The IFAT was performed on cultures that had been washed in phosphate-buffered saline (PBS) (*p*H 7.4) with gentle agitation for 30 minutes. Cultures were then fixed in methanol at 4°C, rinsed in PBS, and incubated with a diluted (1:50) hyperimmune serum from an African adult (titer was 1:50,000 against the erythrocytic stages). After three washes in PBS a fluorescein-conjugated antiserum to human immunoglobulins G, M, and A (Nordic) was used at a 1:40 dilution in 0.5 percent Evans blue.

In two sets of replicate experiments in which two groups of infected mosquitoes and hepatocytes from three different patients were used, liver schizonts of *P. falciparum* were observed in all cultures. Each culture contained dozens to hundreds of schizonts, with a maximum of approximately 650 schizonts per 35-mm petri dish (Fig. 1h). The schizonts were more numerous in proximity to the salivary glands and less numerous along the edge of the culture dish.

In cultures fixed at 20 hours, extracellular sporozoites were found attached to the surface of hepatocytes; a smaller number of rounded fluorescing bodies measuring 2 to 4 μm were observed inside the cells close to the nucleus (Fig. 1, a and b). These are interpreted as being early exoerythrocytic parasites, since corresponding forms were never observed in noninfected control cultures. At 64 hours after sporozoite inoculation, schizonts measuring 5 to 10 μm were often observed in juxtaposition to the hepatocyte nucleus, as shown in fluorescent and Giemsa-stained preparations (Figs. 1c and 2a). By day 5 schiz-

onts measured 10 to 20 μm (Fig. 2c). In cultures fixed on days 6 and 7 after inoculation, schizonts as large as 40 μm were found (Fig. 1, d to h, and Fig. 2d). Round forms were more in evidence than ovoid forms at this time, in contrast to schizonts observed in monkey liver sections on day 5 (*5*). The cytoplasm was poorly stained in preparations treated with Giemsa after IFAT (Fig. 2, a and b). However, in the cultures stained solely by Giemsa the late schizonts showed evidence of initiation of the aposchizogony described by Bray (*6*) and contained several hundred merozoites (Fig. 2d). In Giemsa-stained preparations at days 5 and 7 there was no clear area surrounding the schizont, as was found for these stages of *P. vivax* in culture (*2*).

Parasites varied in both size and staining pattern with IFAT. For the most part, cultured 5- and 6-day schizonts showed patterns of fluoresence resembling those seen previously in sections of infected liver, with the schizont periphery staining more brightly than the interior (Fig. 1d). However, the variety of fluorescence patterns was greater in vitro than in vivo. Some schizonts in vitro appeared as brightly fluorescing masses (Fig. 1d). Others contained several smaller fluorescing masses dispersed in the cytoplasm of the schizont (Fig. 1e) or on its periphery but without tracing a sharp outline of the parasite surface membrane (Fig. 1f); still others contained a well-defined nonfluorescing vacuole (Fig. 1g). When unstained specimens were viewed under white light after fluorescence, the vacuoles had a granular appearance that was refringent, and they did not stain with Giemsa. The vacuoles may have corresponded to degenerating parasites or they may simply have been artifacts of the fluorescence assay (vacuoles were not observed in cultures fixed and stained directly with Giemsa).

In a second series of experiments normal red blood cells (type O+) were added to the infected liver cultures on day 7 at a concentration of $10^9$ per petri dish. Cultures were kept thereafter under the standard conditions used for erythrocytic stage culture (*7*). When IFAT was used on thick-smear preparations to detect light blood infections (one parasite in $3 \times 10^5$ red blood cells is detectable with this technique), several blood cells infected with typical ring forms were seen in one of the three cultures studied on days 11 and 12.

It appears that hepatocytes may offer some advantage over hepatoma cells as hosts for the exoerythrocytic phase of this human parasite, since, despite abundant penetration, the parasite does not develop in hepatoma cells (*8*). Although the human hepatocytes in this study supported parasite differentiation and multiplication, the schizonts produced differed in certain aspects from those obtained in vivo. In general, they were smaller than those obtained on day 5 from man (30 to 50 μm) (*9*, *10*), *Cebus* monkeys (24 μm) (*11*), and chimpanzees (32 μm) (*6*) and those obtained on day 7 from *Saimiri* (100 μm) (*12*). Nonetheless, despite their smaller size, some schizonts in culture were nearly mature by day 7. In a recent experiment with *Saimiri sciureus* many schizonts were found on day 3, compared to a much smaller number on day 7 (*13*). These results

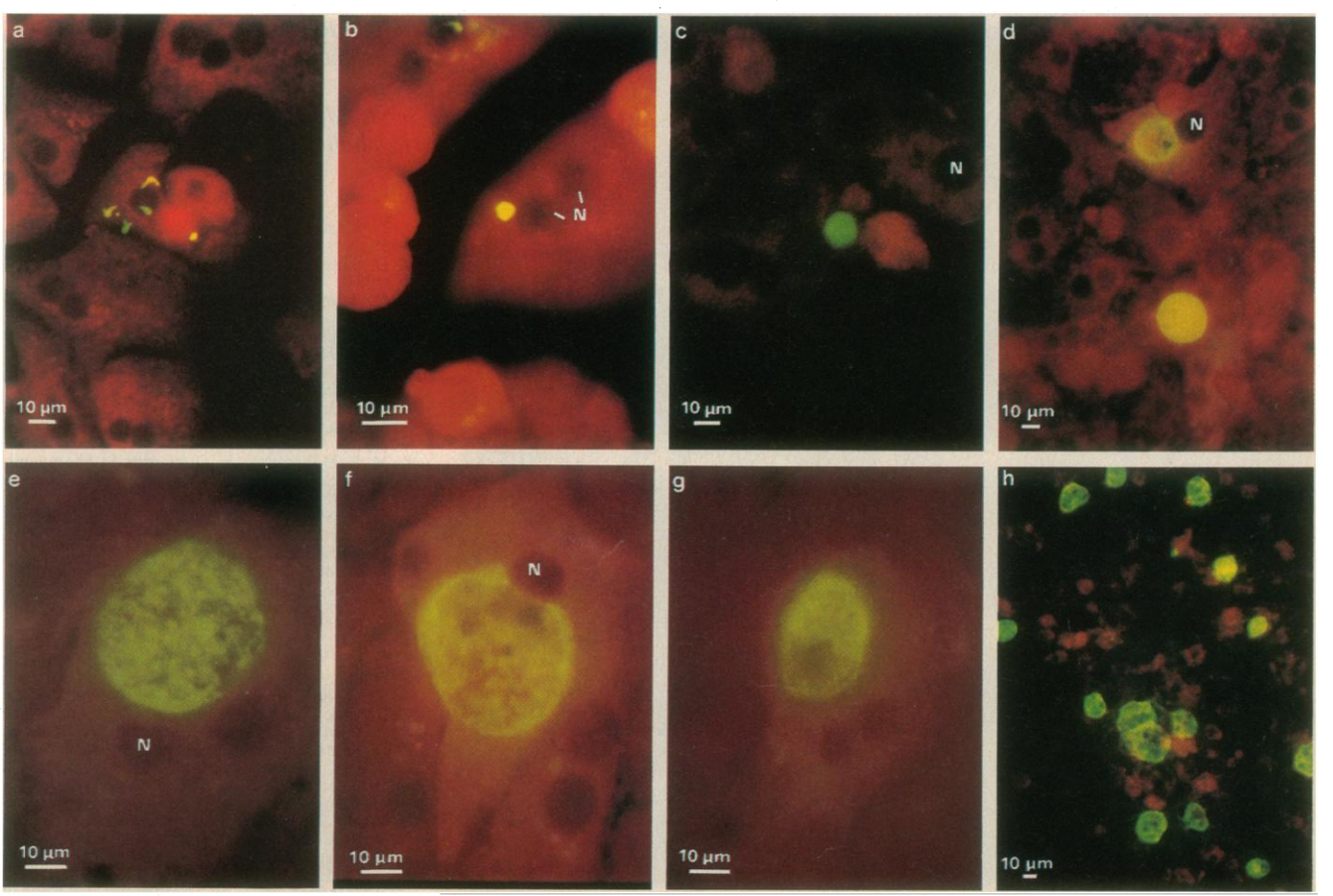


Fig. 1. Human hepatocytes infected with the liver stages of *P. falciparum* and stained by IFAT. (a and b) Cultures fixed 20 hours after sporozoite inoculation. (a) Sporozoites attached to the surface of a hepatocyte and a rounded, newly transformed trophozoite. (b) Enlargement of another newly transformed trophozoite. (c) Early schizont 64 hours after infection of the culture. (d to h) Cultures fixed 6 to 7 days after sporozoite inoculation. (f and g) Schizonts containing vacuoles devoid of antibody-binding material. Note the apparent weak fluorescence of the host cell.

suggest that, at least in *Saimiri*, many exoerythrocytic parasites never reach maturity and may be destroyed, whereas in culture the parasites persist even though they fail to achieve maximum growth. Considering the large number of sporozoites in each inoculum and the relatively modest number of exoerythrocytic forms found, it appears that many of the sporozoites do not penetrate the cultured hepatocytes. It has been proposed that, in vivo, sporozoites do not enter the hepatocyte directly but must first transit the Kupffer cells, where they may be processed in some way (*4*). Clearly, under the conditions used for these cultures, sporozoites enter at least certain hepatocytes directly and go on to develop into liver schizonts. At present it is impossible to say whether a small percentage of sporozoites in the inoculum are truly infectious, whether transit through a Kupffer cell would increase sporozoite infectivity, or whether only certain hepatocytes are susceptible to infection.

Although this in vitro system requires further refinement, it shows that the complete liver schizogony of *P. falciparum* can be obtained in vitro. The availability of this barely accessible stage in the laboratory, its reproducibility, and the number of recovered schizonts indicate that the system is already sufficient for use in some studies in immunology, biochemistry, and therapeutics.

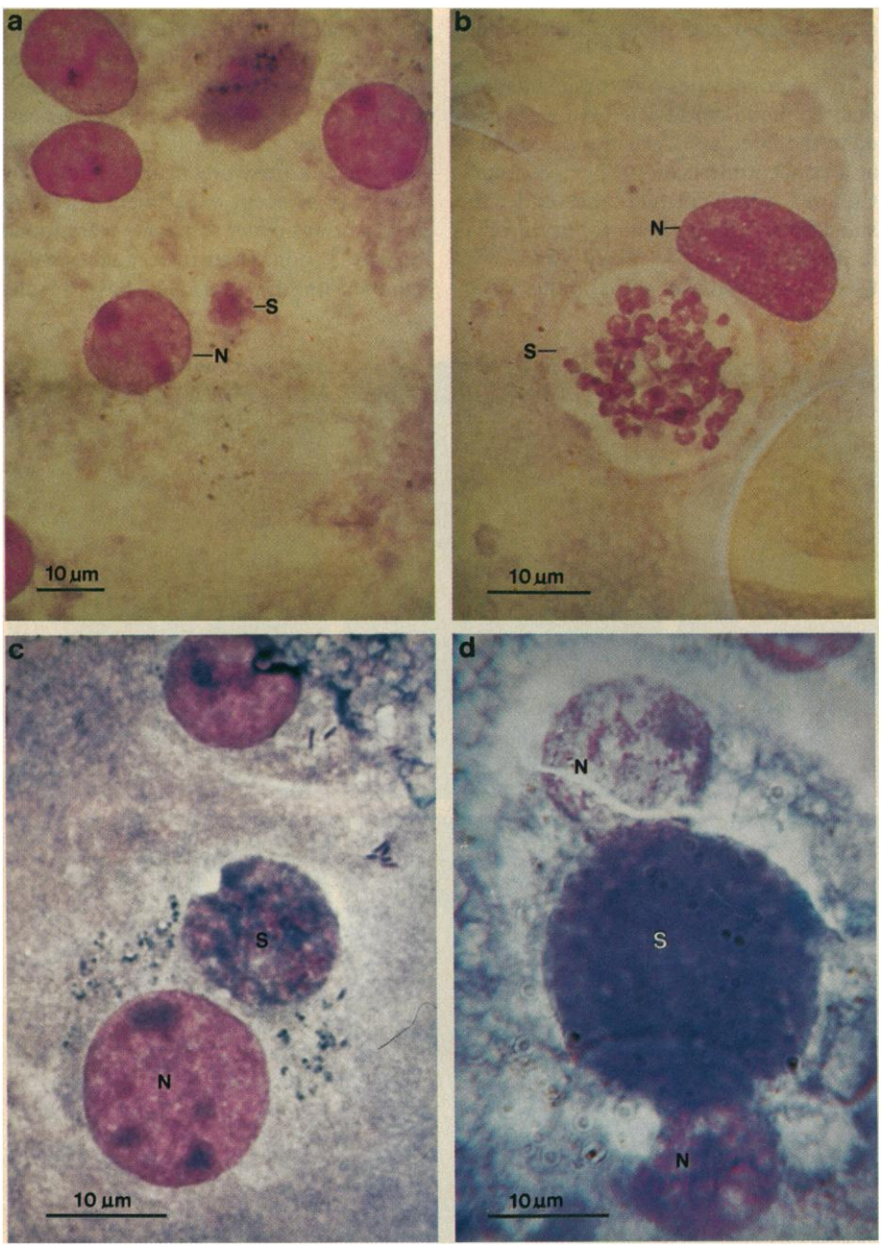


Fig. 2. Cultures stained with Giemsa. (a and b) Specimen stained with Giemsa after staining by IFAT (only the nuclei take up the stain). (a) A 64-hour schizont. (b) A 7-day schizont. (c and d) Specimens stained with Giemsa without previous testing by IFAT. (c) A 5-day schizont. (d) A 7-day schizont. Abbreviations: *N*, hepatocyte nucleus; *S*, schizont.


D. MAZIER
*Département de Parasitologie et de Médecine Tropicale, Groupe Hospitalier Pitié-Salpêtrière, 75013 Paris, France*
R. L. BEAUDOIN
*Naval Medical Research Institute, Bethesda, Maryland 20814*
S. MELLOUK, P. DRUILHE, B. TEXIER
*Département de Parasitologie et de Médecine Tropicale, Groupe Hospitalier Pitié-Salpêtrière*
J. TROSPER
*Naval Medical Research Institute*
F. MILTGEN, I. LANDAU
*Laboratoire de Zoologie-Vers, Associé au Centre National de la Recherche Scientifique, Museum National d'Histoire Naturelle, Paris Cédex 05, France*
C. PAUL
*Naval Medical Research Institute*
O. BRANDICOURT
*Département de Parasitologie et de Médecine Tropicale, Groupe Hospitalier Pitié-Salpêtrière*
C. GUGUEN-GUILLOUZO
*Unité de Recherches Hépatologiques, U 49, Institut National de la Santé et de la Recherche Médicale, Hôpital Pontchaillou, 35011 Rennes, France*
P. LANGLOIS
*Service de Chirurgie, Groupe Hospitalier Pitié-Salpêtrière*


**References and Notes**


1. D. Mazier *et al.*, *Ann. Parasitol. Hum. Comp.* **58** (No. 4), 405 (1983).
2. D. Mazier *et al.*, *Nature (London)* **307**, 367 (1984).
3. D. Mazier *et al.*, *C.R. Acad. Sci.* **294**, 963 (1982).
4. T. Ifediba and J. P. Vanderberg, *Nature (London)* **294**, 364 (1981).
5. P. Druilhe *et al.*, *Am. J. Trop. Med. Hyg.* **33**, 336 (1984).
6. R. S. Bray, *ibid.* **7**, 20 (1958).
7. W. Trager and J. B. Jensen, *Science* **193**, 673 (1976).
8. M. R. Hollingdale, E. H. Nardin, S. Tharavanij, A. L. Schwartz, R. S. Nussenzweig, *J. Immunol.* **132**, 909 (1984).
9. H. E. Shortt and P. C. C. Garnham, *Trans. R. Soc. Trop. Med. Hyg.* **41**, 785 (1948).
10. H. E. Shortt *et al.*, *ibid.* **44**, 405 (1951).
11. P. Druilhe, F. Miltgen, I. Landau, J. Rinjard, M. Gentilini, *C. R. Acad. Sci.* **294**, 511 (1982).
12. P. Druilhe *et al.*, unpublished results.
13. P. Druilhe *et al.*, unpublished results.
14. J. F. G. M. Meis, J. P. Verhave, P. H. K. Jap, J. H. E. T. Meuwissen, *Parasitology* **86**, 231 (1983).


15. This work was supported by Naval Medical Research and Development Command work unit 3M162770A870AF312, Institut National de la Santé et de la Recherche Médicale grant 841018, French Ministère de l'Industrie et de la Recherche contracts 83.L.0760 and 82.L.0787, and United Nations Development Programme/World Bank/World Health Organization Special Program on Training and Research in Tropical Diseases grants 840105 and 810108. We thank T. Ponnudurai for providing the NF54 isolate from West Africa and J. Williams for the 7G8 clone of a Brazilian strain of *P. falciparum*. We also thank G. Lecso, J. P. Chigot, B. Leckey, N. Pacheco, and E. McConnell for their cooperation.

17 September 1984; accepted 4 December 1984