Fine Structure of the Exoerythrocytic Stage of Plasmodium cynomolgi

Abstract. *The light microscopy of primate exoerythrocytic stages (liver stage) of malaria is well described. This report demonstrates the ultrastructure of the 7-day exoerythrocytic stage of* Plasmodium cynomolgi *in the liver of a rhesus monkey.*

This is the first report describing the ultrastructure of a primate malaria exoerythrocyte (EE) body. The first report of this stage in primate malaria was in 1948, when Shortt and Garnham (*1*) recorded their observations of the EE body of *Plasmodium cynomolgi* in the liver of a rhesus monkey (*Macaca mulatta*). Since then the EE body in a number of nonhuman primate malarias and in all four human malarias has been described. Until the recent description of direct hepatic inoculation of sporozoites (*2*), concentrations of EE bodies in biopsy material were not sufficient to permit localization for electron microscopy. Even with this technique, only a few EE bodies are encountered in screening large quantities of tissue. It is appropriate that this first ultrastructural study deals with the same malaria species, *P. cynomolgi*, that Shortt and Garnham initially described.

Plasmodium cynomolgi (B strain) sporozoites were harvested in 10 percent monkey serum in saline from salivary glands of *Anopheles maculatus* mosquitoes after 15 days of extrinsic incubation. The sporozoites were inoculated directly into the liver of an uninfected rhesus monkey. A liver biopsy of the site of injection was taken on the 7th day after inoculation. The tissue was fixed for 6 hours in 3 percent glutaraldehyde containing 4 percent sucrose buffered with phosphate solution at *p*H 7.3, washed in phosphate buffer, and treated for 1 hour with phosphate-buffered osmium tetroxide. The tissue was embedded in Epon 812, and sections were cut with glass knives on an LKB ultramicrotome. The sections were stained with uranyl acetate and lead citrate (*3*) and examined with an RCA EMU-3H electron microscope.

Four EE bodies studied had an average size of 17.1 by 11.3 μm with a range of 14.0 to 21.0 by 8.5 to 15.5 μm. The liver cell border with its organelles is seen completely surrounding the EE body (Fig. 1). The liver cell is enlarged, and its nucleus is compressed to one side. The nucleus and organelles show no degenerative changes as a result of the parasitization by the malaria parasite.

The surface of the EE body is lobulated. Surrounding it is a membrane with an undulated outer surface and a smooth inner surface. A narrow space separates this outer membrane from a thin inner limiting membrane that closely approximates the cytoplasm of the EE body. Multiple irregularly shaped nuclei averaging 1.6 by 1.1 μm are seen throughout the EE body. There is no apparent pattern to their distribution. The nucleus has a homogeneous granular texture. A single nuclear membrane is only occasionally seen. Surrounding the nucleus is a clear space.

The cytoplasm of the EE body contains densely packed free ribosomes. In each section, there are five to six well-demarcated areas composed of ribosomes which are arranged in a linear fashion. Membranes of endoplasmic reticulum are absent. Mitochondria are present.

Two types of vacuoles are present. The first (type I), which varies in size from 0.3 to 1.5 μm, is round and has a distinct membrane. Most of these vacuoles appear empty, but a few contain membrane-like structures. The second type of vacuole (type II) is smaller, homogeneous, and more electron-dense. Type II vacuoles are round or occasionally crescent-shaped.

These initial ultrastructural findings on primate EE bodies confirm that they are within the hepatic cell. The electron micrographs clearly show the shape and distribution of nuclei in the EE body and demonstrate mitochondria and small vacuoles that are below the resolution of the light microscope. In routine histologic sections, the limiting membrane surrounding the EE body is difficult to see. The electron micrographs show it to be composed of two components. Further study is necessary to delineate the origin of the outer membrane and to determine whether the complicated feeding mechanisms described in avian malaria EE bodies (*4*) occur in the primate forms.

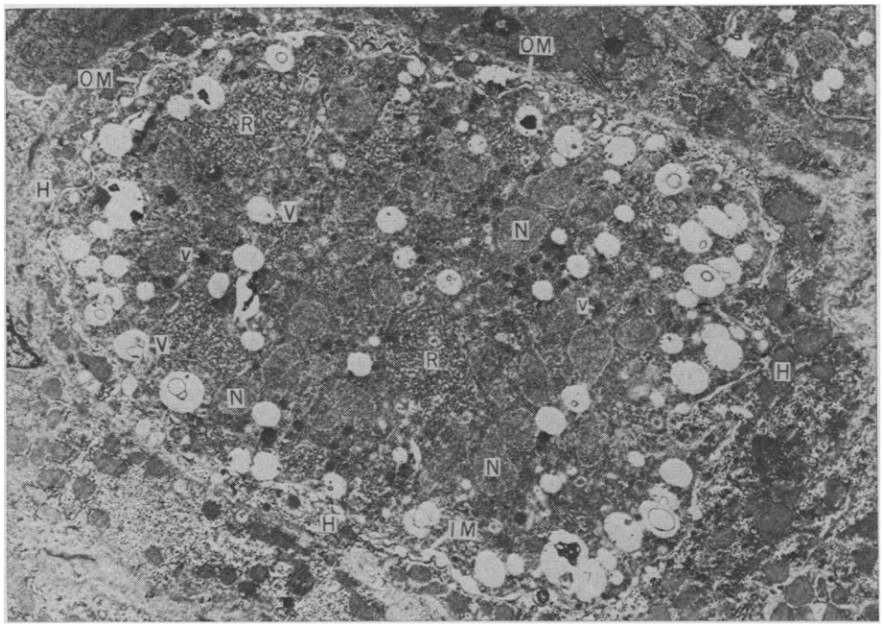

Fig. 1. Midsection of a 7-day-old exoerythrocytic body of *P. cynomolgi* in rhesus monkey liver. The parasitized hepatic cell (*H*) is seen surrounding the EE body. A wavy outer membrane (*OM*) and a thin inner membrane (*IM*) enclose the EE body. Several areas of linearly arranged ribosomes are seen (*R*). Nuclei (*N*) have a clear space surrounding them. Type I (*V*) and II (*v*) vacuoles are distributed throughout the EE body (×4600).

THOMAS SODEMAN
BERTRAM SCHNITZER
THOMAS DURKEE
Department of Pathology, University of Michigan, Ann Arbor 48104
PETER CONTACOS
Laboratory of Parasitic Diseases, Section on Primate Malaria, P.O. Box 80190, Chamblee, Georgia 30341

References and Notes

1. H. E. Shortt and P. C. C. Garnham, *Nature* **161**, 126 (1948).
2. J. R. Held, P. G. Contacos, J. R. Jumper, C. S. Smith, *J. Parasitol.* **53**, 656 (1967).
3. E. S. Reynolds, *J. Cell. Biol.* **17**, 208 (1963).
4. M. Aikawa, C. G. Huff, H. Sprinz, *Amer. J. Trop. Med. Hyg.* **17**, 156 (1968).

5. We thank William E. Collins and John R. Jumper for their assistance in obtaining this material for study.

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Acetylcholine Concentrations in Rat Brain: Diurnal Oscillation

Abstract. *A diurnal oscillation of acetylcholine concentrations in rat brain has been demonstrated by gas chromatography. Peak concentrations occur at 2 hours of light, and a trough is seen at 6 hours of darkness. This pattern is observed only in grouped rats, and emerges after at least 18 days of prior conditioning in an environment with controlled light, humidity, and temperature.*

In mammalian brain, the concentrations of some biogenic amines vary with a circadian periodicity (*1*). Also, light regulates the pineal activity of hydroxyindole-*O*-methyltransferase (*2*) and of 5-hydroxytryptophan decarboxylase (*3*), the enzymes responsible for synthesis of melatonin and serotonin, respectively. These results prompted us to study whether the brain concentrations of acetylcholine (ACh) exhibit a circadian rhythmicity. Such a periodicity is certainly important for interpreting experimental results of studies concerning long-term effects of drugs on brain ACh concentrations.

In confirmation of a report by Friedman and Walker (*4*), our results in rats show that brain ACh concentrations oscillate diurnally. However, whereas Friedman and Walker reported that the concentrations of ACh in the midbrain and caudate nucleus of rats peak at 4 hours of dark we found that the concentrations of ACh in the whole brain peak at 2 hours of light. These results were obtained with the use of a modification of an existing gas-chromatographic procedure for estimation of ACh in tissue extracts (*5*, *6*).

Sprague-Dawley, male, adult rats (Zivic Miller, Pittsburgh, Pennsylvania) (300 to 350 g) had unrestricted access to food and water, while housed either in groups of eight per cage or isolated (one animal per cage) in a soundproof room maintained at 23°C. The light cycle was controlled to provide 12 hours of light. The animals were decapitated at six evenly spaced times throughout a 24-hour cycle in groups of at least four rats each. Extreme care was taken to avoid causing undue stress or startling the animals. These experiments were all performed from October through February.

In our modified procedure we rapidly excised the brains (25 seconds) and homogenized them in 5 ml of ice-cold 0.4*N* perchloric acid with a Polytron ultraspeed homogenizer at full speed for 30 seconds. The brain weight was calculated by weighing the tubes before and after the brain was added. The final dilution of the homogenate was 4 ml of 0.4*N* perchloric acid per gram of wet brain. Brain homogenates were kept in ice for 30 minutes; they were then centrifuged in a Sorvall SS3 centrifuge in a cold room (2°C) at 9000*g* for 15 minutes.

A portion of the supernatant (3 ml) was transferred to another tube, and propionylcholine chloride (PrCh) (25 nmole; 25 μl in water) as an internal standard and 0.19 ml of potassium perchlorate (7.5*M*) in water were added. Propionylcholine chloride is a suitable internal standard because by a combination of gas chromatography and mass spectrometry it has been established that rat brain does not contain this compound (*7*). This mixture was centrifuged for 15 minutes at 9000*g* in the cold. Quaternary ammonium compounds in the supernatant (*p*H ranging between 4.20 and 4.45) were precipitated as the Reinecke salts in the presence of 50 m*M* tetraethylammonium chloride as coprecipitant. After the precipitates were freeze-dried, they were converted to their corresponding chlorides by batch treatment with Biorex 9 (Cl^-) (*5*) in methanol. The methanol was then transferred to another tube and dried prior to preparation for gas-chromatographic assay.

The esters were next demethylated with sodium benzenethiolate in methyl ethyl ketone and estimated by gas chromatography (*5*). Acetylcholine and PrCh are converted at this step to dimethylaminoethyl acetate and dimethylaminoethyl propionate, respectively, which, with the products of demethylation of other quaternary ammonium compounds, are extracted into chloroform (Fig. 1). Hexyldimethylamine was added as an external standard to each sample after demethylation with benzenethiolate was completed. This compound appears on the gas chromatogram with a retention time shorter than that of dimethylaminoethyl acetate (Fig. 1).

Acetylcholine concentrations reached a peak at 2 hours of light (L_2) and a trough at 6 hours of dark (D_6). In brains of rats kept in isolation (one animal per cage, 30 days or more) the ACh concentrations appeared statisti-

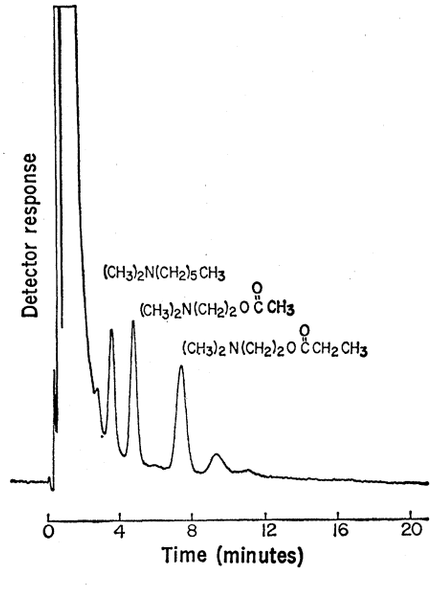

Fig. 1. Gas chromatogram of treated rat brain extract. Substances following solvent peak and identified by chemical formulas are, from left to right, hexyldimethylamine, external standard; dimethylaminoethyl acetate, demethylation product of endogenous acetylcholine; and dimethylaminoethyl propionate, demethylation product of propionylcholine, the internal standard. A silanized glass column (1.8 m; inside diameter, 2 mm) was packed with PAR 1 (80/120) coated with phenyldiethanolamine succinate (1 percent). The temperature of the column was 170°C; of the injection port, 240°C; and of the detector, 200°C. The flow rates were: carrier (N_2), 45 ml/min (80 psi); oxygen, 280 ml/min (20 psi); and hydrogen, 30 ml/min (26 psi). Dual flame ionization detectors were employed on a 5756 F&M gas chromatograph.