4-1984

Ultrastructure of the Interface Between Stages of *Eimeria funduli/i> (Apicomplexa) and Hepatocytes of the Longnose Killifish, Fundulus similis*

William E. Hawkins
Gulf Coast Research Laboratory, William.Hawkins@usm.edu

John W. Fournie
United States Environmental Protection Agency, fournie.john@epa.gov

Robin M. Overstreet
Gulf Coast Research Laboratory, robin.overstreet@usm.edu

J. Parasit., 70(2), 1984, pp. 232–238

ULTRASTRUCTURE OF THE INTERFACE BETWEEN STAGES OF *EIMERIA FUNDULI* (APICOMPLEXA) AND HEPATOCYTES OF THE LONGNOSE KILLIFISH, *FUNDULUS SIMILIS*

William E. Hawkins, John W. Fournie, and Robin M. Overstreet
Gulf Coast Research Laboratory, Ocean Springs, Mississippi 39564

ABSTRACT: The interface between stages of *Eimeria funduli* and hepatocytes of the experimentally infected killifish *Fundulus similis* was studied ultrastructurally. Parasitophorous vacuoles (PV's) in which meronts, macrogamonts, and microgamonts developed were lined by an inner, smooth membrane and an outer, ribosome-studded membrane. The outer membrane bordered on the cytoplasm of the host cell, whereas the inner one limited the PV. The origins of these membranes have not been determined with certainty, but images were observed in which both membranes appeared to be continuous with the outer nuclear membrane of the host cell. Furthermore, the outer PV membrane was continuous with membranes of rough endoplasmic reticulum in the host cell. For stages which were rapidly growing or differentiating, the inner membrane blebbed into the PV. Blebbing ceased and ribosomes detached from the outer membrane after maturation of the meront or fertilization of the macrogamont. Blebbing appears to be a mechanism by which nutrients transfer from the host to the parasite. During sporogony, the inner PV membrane acquired a thin layer of electron dense material, but otherwise membranes lining the PV remained intact. The two PV membranes, probably together with dense material of parasitic origin lining the inner membrane, appear to serve as the oocyst wall enclosing the sporocysts until they are released in the intermediate host.

Parasitophorous vacuoles (PV's) of macrogamonts and microgamonts of *Eimeria funduli* Duszynski, Solangi and Overstreet, 1979, in experimentally infected specimens of the longnose killifish, *Fundulus similis* (Baird and Girard), were lined by two membranes that lay adjacent to the host cell (Hawkins et al., 1983a, b). An ultrastructural study of the oocyst of *E. funduli* in wild, naturally infected specimens of the Gulf killifish, *Fundulus grandis* Baird and Girard, failed to reveal an oocyst wall corresponding to that of coccidians from homeothermic hosts (Hawkins et al., 1983). The oocyst wall of *E. funduli* consisted of two membranes organized similarly to those of gamont PV's together with a thin layer of dense material, all of which were closely applied to the host cell. We therefore decided to examine other developmental stages of *E. funduli* to determine the origin of the PV membranes and the relationship between those membranes and the oocyst wall. Light microscopic observations on the intrahepatic stages of *E. funduli* and the timing of their development in experimentally infected longnose killifish have been reported (Hawkins et al., 1984). The present study examines some of those tissues ultrastructurally.

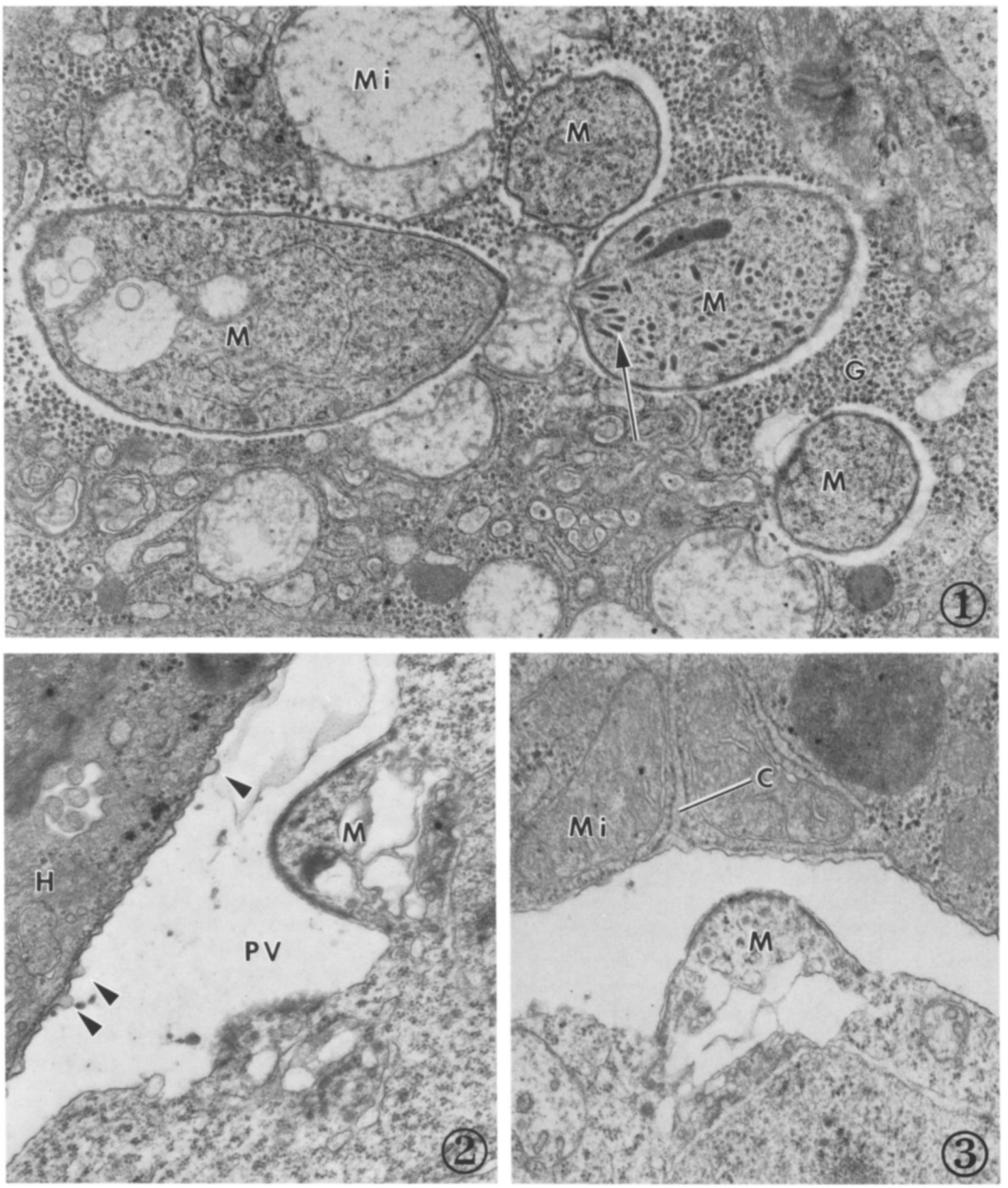
Received 29 March 1983; revised 9 November 1983; accepted 16 January 1984.

MATERIALS AND METHODS

Uninfected, wild specimens of *Fundulus similis* 56 to 83 mm in total length were collected by seining in a lagoon on Horn Island, about 20 km south of Ocean Springs, Mississippi. The fish were starved for 48 hr prior to being infected with *Eimeria funduli*. Infections were achieved by feeding each of the fish the cephalothoracic tissues from two specimens of the grass shrimp *Palaemonetes pugio* Holthuis. Grass shrimp had been fed minced liver containing sporulated oocysts from naturally infected specimens of *F. grandis*. Fish were maintained in a 75 liter glass aquarium with 23 ppt sea salt (Instant Ocean®) at about 17 C.

One or two fish were killed and livers were prepared for study at 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 21, 23, 25, 28, and 35 days post-infection (PI). Fish were killed by a blow to the head, and livers were quickly removed, minced, and fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (300 mOsm) for 3 hr. Tissues were rinsed in 0.1 M phosphate buffer, postfixed for 2 hr in 1% osmium tetroxide in 0.1 M phosphate buffer, rinsed in buffer, and dehydrated in ethanol. Following treatment with propylene oxide, tissues were embedded in epoxy resin.

Because stages that occur after zygote formation are difficult to fix and embed, we employed a modification of the double sectioning technique of Birch-Andersen et al. (1976) on tissues sampled after 13 days PI. Livers were mounted in Ames® O.C.T. embedding compound on a metal chuck, frozen with liquified carbon dioxide, and minced with a single-edged razor blade that had been cooled in a cryostat at −20 C. Minced tissues were allowed to thaw in 0.1 M phosphate-buffered 3.0% glutaraldehyde and then to fix for 2 hr. Tissues were subsequently processed as described above. To locate the intrahepatic stages of *E. funduli*, semi-thin (1–2 μm thick) sections were cut with an LKB ultramicrotome, mounted on glass slides, and stained with to-



FIGURES 1–3. **1.** Merozoites (M) in hepatocyte. Merozoites lie free in the cytoplasm and are not enclosed in parasitophorous vacuoles. Glycogen of hepatocyte (G); swollen mitochondrion (Mi); micronemes of merozoite (arrow). 9 days PI. × 15,100. **2.** Meront in parasitophorous vacuole (PV). Two membranes, an outer ribosome-studded one and an inner smooth one, line the parasitophorous vacuole. Note numerous relationships with blebs (arrowheads) on inner membrane. Developing merozoite (M); hepatocyte (H). 7 days PI. × 27,500. **3.** Meront in parasitophorous vacuole. Cisterna (C) of rough endoplasmic reticulum is continuous with cisterna between the two membranes that line the parasitophorous vacuole. Hepatocyte mitochondrion (Mi); developing merozoite (M). 7 days PI. ×27,000.

luidine blue. Thin sections were mounted on uncoated copper grids, stained with uranyl acetate and lead citrate, and examined with a Siemens 1A electron microscope.

RESULTS

The earliest developmental stage examined was the first generation merozoite. Sporozoites, which presumably no longer exist by day 4 post-infection (PI), were not observed in this study. Second generation merogony actively produced numerous merozoites leaving parasitophorous vacuoles (PV's) and entering host cells about nine days PI. Merozoites entered both hepatocytes and pancreatic acinar cells. However, we did not see merozoites in the act of penetration. Observations in this study are limited to hepatocyte infections because they were much more common than pancreatic ones. Sometimes as many as 10 merozoites infected an individual hepatocyte. Hepatocytes that contained recently penetrated merozoites had numerous vesicular, swollen mitochondria. Those merozoites typically lay free in the cytoplasm without a membrane-lined PV (Fig. 1).

Two membranes lined PV's of developing meronts (Figs. 2, 3), as well as microgamonts and macrogamonts as previously reported (Hawkins et al., 1983a, b). The outer PV membrane was ribosome-studded and limited the host cell's cytoplasm. The ribosomes lay on the cytoplasmic aspect of the outer membrane. The inner PV membrane faced the PV and had no ribosomes. That membrane of PV's containing those stages frequently blebbed into the PV's (Fig. 2). A space of about 15–30 nm separated the two membranes. The space between the two membranes sometimes continued into the host cell's cytoplasm where it was lined on both sides by membranes of rough endoplasmic reticulum (RER) (Fig. 3). These membranes coursed continuously with the outer PV membrane. Thus, the space, or cisterna, between the inner and outer PV membranes connected with RER cisternae of the host cell.

Parasitophorous vacuoles, especially those containing early developing stages, frequently had a close association with the host cell's nucleus (Fig. 4). We never observed a parasite penetrating the nuclear membrane nor a disrupted nuclear membrane. In Figure 4, the outer membrane of the hepatocyte nucleus coursed continuously as the outer PV membrane. No interruption in the ribosome lining existed at the transition between the outer nuclear membrane and the outer PV membrane. Likewise, the space between the two PV membranes was confluent with the perinuclear cisterna. The inner PV membrane also seemed to bear a close relationship with the host cell's nucleus. Where this membrane occurred adjacent to the hepatocyte nucleus in Figure 4, a typical, ribosome-studded, outer nuclear membrane did not occur. Instead, the inner PV membrane bordered the perinuclear cisterna. Figure 5 illustrates an hepatocyte nucleus-parasite interface in which the outer PV membrane coursed continuously with the outer nuclear membrane, but the inner PV membrane associated directly with the nucleus for only a short distance. Intravacuolar tubules did not occur nor did PV membranes form folds projecting into the PV.

Changes occurred in membranes lining PV's by the time they contained mature meronts and sporulating stages. In mature meronts and gamonts, the inner PV membrane had no blebs and few ribosomes attached to the outer membrane (Fig. 6) as they did in the developing forms. During sporulation, blebbing ceased completely, the width of the space between the two PV membranes diminished, and ribosomes no longer studded the outer PV membrane (Fig. 7). A layer of dense material of uncertain origin about 15 nm thick accumulated on the vacuolar side of the inner membrane. The dense material was separated by a clear space from the inner PV membrane. Occasionally, expansions of the space between the two PV membranes contained crystalline inclusions.

DISCUSSION

A single membrane lines the parasitophorous vacuoles (PV's) of most species of coccidians (Chobotar and Scholtyseck, 1982). Two membranes, however, line the PV's of all intrahepatic stages of *Eimeria funduli* and also some stages of species of *Isospora* Schneider, 1881 (see Ferguson et al., 1980; Pelster, 1973), *Toxoplasma gondii* (Nicolle and Manceaux, 1908) (see Pelster and Piekarski, 1971), *Sarcocystis fusiformis* (Railliet, 1897) (see Scholtyseck and Hilali, 1978), and *S. suihominis* Heydorn, 1977 (see Mehlhorn and Heydorn, 1979).

The host cell produces PV membranes of most coccidians (Chobotar and Scholtyseck, 1982). The intracellular origin of the membranes, however, varies. In *Plasmodium* spp., host cells phagocytose the coccidians, and the plasmalemma of the host cell in which the organism is phagocytosed forms the PV membrane (Ladda et al., 1969). Other eimerians, however, actively pen-

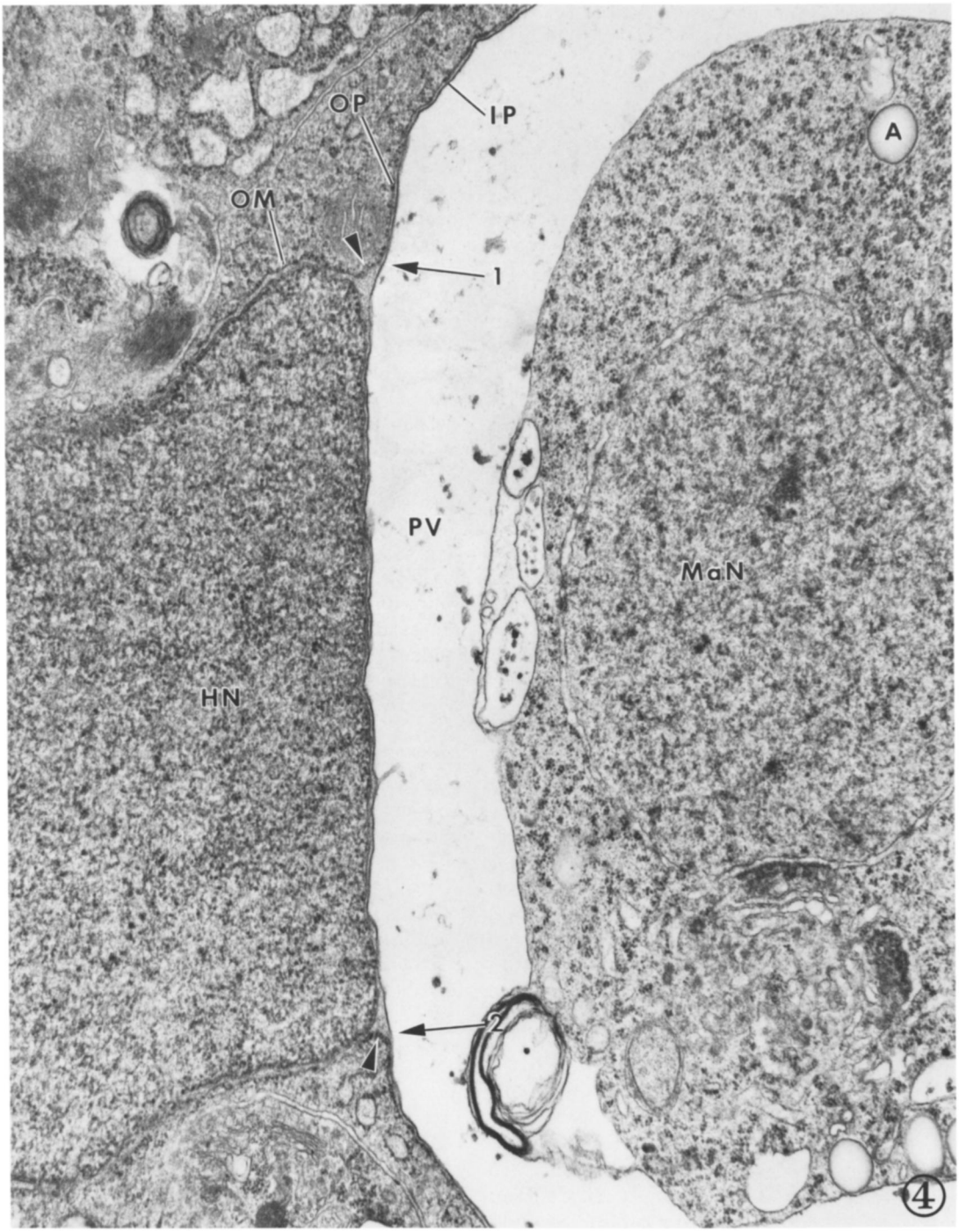
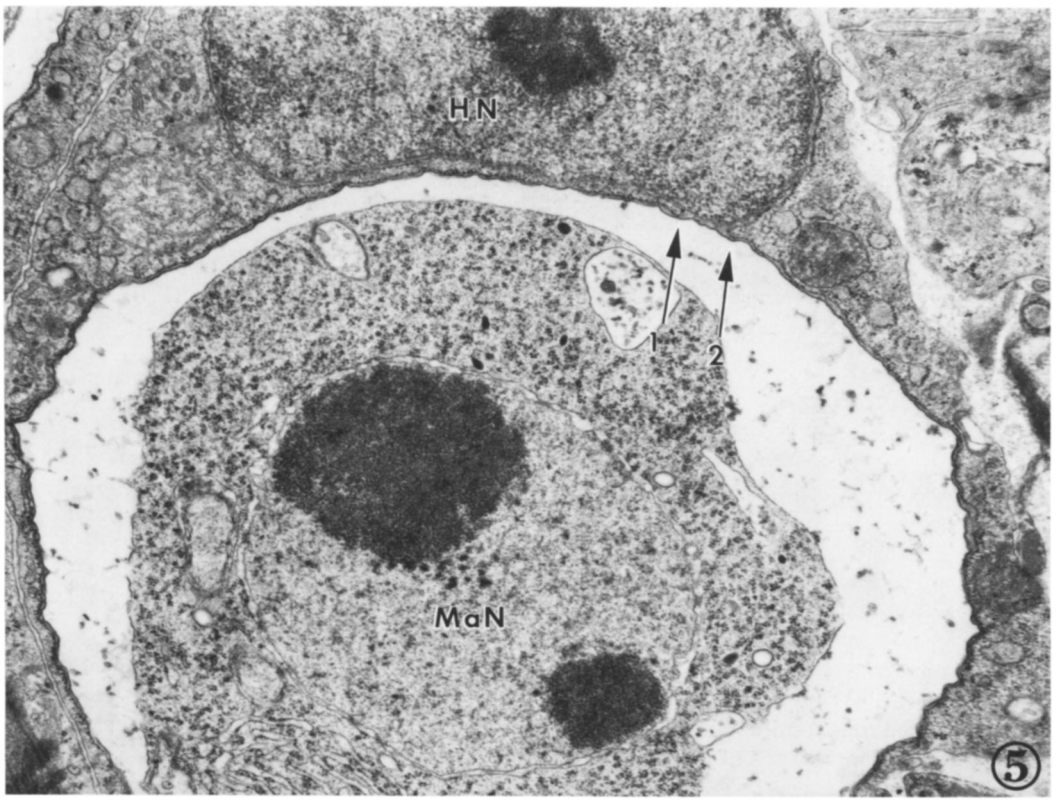
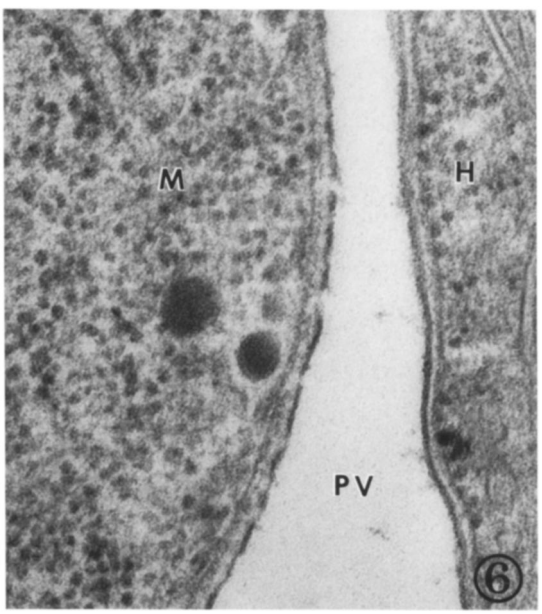
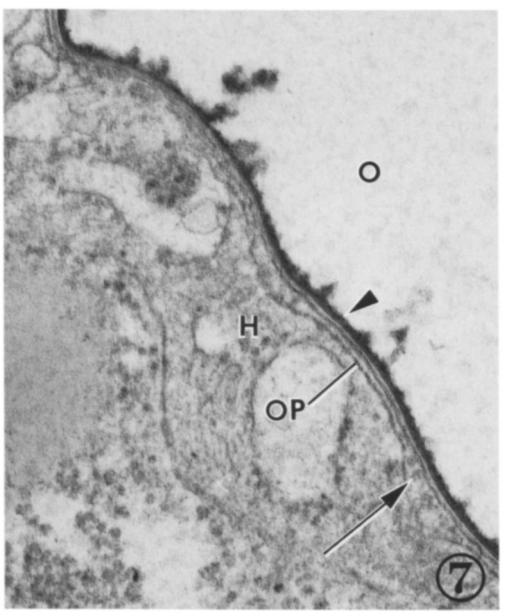


FIGURE 4. Developing macrogamont in parasitophorous vacuole (PV). The outer nuclear membrane (OM) is continuous with outer membrane (OP) of parasitophorous vacuole at the arrowheads. Between the arrows 1 and 2, the inner membrane (IP) of the parasitophorous vacuole appears to represent the outer nuclear membrane. Note that the perinuclear cisterna is continuous with the cisterna between the two membranes lining the parasitophorous vacuole. Also note that ribosomes line both the outer membrane of the parasitophorous vacuoles as well as the outer nuclear membrane. Macrogamont nucleus (MaN); amylopectin granule (A). 18 days PI. × 24,800.







FIGURES 5–7. **5.** Developing gamont in hepatocyte. The relationship between membranes of the parasitophorous vacuole and the outer nuclear membrane (between arrows 1 and 2) is not as extensive as in Figure 4. Hepatocyte nucleus (HN); macrogamont nucleus (MaN). 18 days PI. × 18,000. **6.** Mature merozoite in parasitophorous vacuole. Note lack of blebbing of inner membrane of parasitophorous vacuole (PV). Merozoite (M); hepatocyte (H). 8 days PI. × 83,200. **7.** Oocyst wall. Note outer membrane of parasitophorous vacuole (OP) is

etrate host cells, and the membranes appear to form *de novo* around the parasites after they penetrate into the host cell (Roberts et al., 1970). *Toxoplasma gondii* apparently utilizes both active invasion and, to a lesser degree, induced phagocytosis to enter host cells (Nichols and O'Conner, 1981). Toxoplasmas that entered by active invasion developed in PV's lined by several membranes, whereas those that were phagocytosed occurred in PV's lined by only a single membrane.

Mehlhorn and Heydorn (1979) suggested that the two membranes that lined PV's containing developmental stages of *S. suihominis* originated from the endoplasmic reticulum. Probably, a similar origin exists for *E. funduli*. Nevertheless, in the absence of intermediate stages, we can only speculate on the development of the PV membranes in *E. funduli*. Merozoites apparently penetrate hepatocytes because those stages occur free in the hepatocyte's cytoplasm without being enclosed in vacuoles. At least the outer, and possibly the inner, membrane originates from the host cell's RER. The ribosome-studded outer PV membrane often runs continuously with the outer membrane of the host cell's nuclear envelope and with RER membranes in the host cell's cytoplasm. Likewise, the space between the two PV membranes is confluent with the host cell's perinuclear cisterna and with RER cisternae in the host cell's cytoplasm. We do not know if the nuclear involvement is essential to the formation of the PV membranes or how long the nucleus-PV connections last.

The origin of the inner PV membrane is more questionable than the outer one. Perhaps the inner PV membrane, like the outer one, arises from RER. Evidence for this rests in observations of inner PV membranes lying adjacent to host cell nuclei in which the inner PV membrane appeared to serve as the outer membrane of the nuclear envelope. With such an origin, the merozoite might penetrate a hepatocyte, and the outer nuclear membrane of the hepatocyte's nuclear envelope might fold over the parasite. The relationship would resemble that between a developing mammalian organ such as the heart or lung and the fluid-filled pericardial or pleural sac into which they grow and where they gain two layers of membranes, visceral and parietal (e.g., Grant, 1962). Alternatively, the inner membrane might arise from the host plasmalemma or from membranes in the host's cytoplasm. If so, the outer nuclear membrane might fold over and envelop the inner PV membrane, thereby forming a second PV membrane. Relationships between coccidians and host cell nuclei have been noted for species of *Eimeria* and *Toxoplasma* (see Doran, 1982). Nichols et al. (1983) observed that toxoplasmas readily penetrated host plasmalemmas but not the nuclear envelope or other intracellular membranes.

We already suggested that the membranes lining PV's of gamonts constituted a modified cisterna of RER and that blebbing provided a mechanism by which nutrients were transferred from the host cell to the parasite (Hawkins et al., 1983a, b). We now know that blebbing occurs in PV's of all rapidly growing or differentiating stages of *E. funduli*. Our hypothesis is supported by the fact that PV membranes of sporulating stages and mature meronts appeared to atrophy, ribosomes detached from the outer PV membrane, and blebbing ceased. This would correspond with the end of the need for extensive transfer of nutrients from the host to the parasite. Meuller et al. (1981) proposed that "bleb-like protrusions" of the host cell PV membrane of early gamonts of *Eimeria contorta* Haberkorn, 1971, might play a role in nutrient transfer by increasing the host-parasite interface.

Oocyst walls of most piscine coccidians are very thin compared with the walls of coccidians in terrestrial hosts (Dyková and Lom, 1981; Overstreet, 1981). Thin oocyst walls closely applied to host cytoplasm have been reported for *E. variabilis* (Thélohan, 1893) by Davies (1978) and for *Goussia degiustii* (Molnár and Fernando, 1974) and *G. subepithelialis* (Moroff and Fiebiger, 1905) by Lom (1971). An electron micrograph in the report on *G. gadi* (Fiebiger, 1913) by Odense and Logan (1976) showed a thin membrane that could be a detached oocyst wall enclosing sporocysts. The oocyst wall of *E. fun-*

←

devoid of ribosomes in this stage. Electron dense material (arrowhead) lines the inner membrane. In places, the cisterna between the two membranes expands and contains inclusions (arrow). Freeze-processing. 30 days PI. × 64,000.

duli in naturally infected killifsh (Hawkins et al., 1983) was organized identically to the lining of the PV in sporogenous stages of experimental infections of *E. funduli* as shown in the present study. Duszynski et al. (1979) found that oocysts of *E. funduli* isolated by sugar flotation consisted of the thin layer of dense material plus the two membranes. Thus, it is likely that all three layers of the oocyst wall are associated with the sporocysts when they are ingested by the intermediate host. The layer of dense, presumed parasitic, material was applied to the host membrane facing the PV at sporoblast formation. We have not yet determined the origin of this dense material, but it probably is derived from wall-forming bodies (Hawkins et al., 1983a) and contributes to the resilience, impermeability, and longevity of the wall.

ACKNOWLEDGMENTS

We wish to thank Mrs. Debby Jennings, Mr. Robert Allen, and Mrs. Susan A. Fink for their technical assistance. This study was conducted in cooperation with the U.S. Department of Commerce, NOAA, National Marine Fisheries Service, under PL 88-309, Project 2-393-R.

LITERATURE CITED

BIRCH-ANDERSEN, A., D. J. P. FERGUSON, AND R. D. PONTEFRACT. 1976. A technique for obtaining thin sections of coccidian oocysts. Acta path. microbiol. scand. Sect. B **84:** 235–239.

CHOBOTAR, B., AND E. SCHOLTYSECK. 1982. Ultrastructure. *In* The biology of the Coccidia, P. L. Long (ed.). University Park Press, Baltimore, Maryland, pp. 101–166.

DAVIES, A. J. 1978. Coccidian parasites of intertidal fishes from Wales: systematics, development, and cytochemistry. J. Protozool. **25:** 15–21.

DORAN, D. J. 1982. Behavior of Coccidia *in vitro*. *In* The biology of the Coccidia, P. L. Long (ed.). University Park Press, Baltimore, Maryland, pp. 229–286.

DUSZYNSKI, D. W., M. A. SOLANGI, AND R. M. OVERSTREET. 1979. A new and unusual eimerian (Protozoa: Eimeriidae) from the liver of the Gulf killifish, *Fundulus grandis*. J. Wildl. Dis. **15:** 543–552.

DYKOVÁ, I., AND J. LOM. 1981. Fish coccidia: critical notes on life cycles, classification and pathogenicity. J. Fish Dis. **4:** 487–505.

FERGUSON, D. J. P., A. BIRCH-ANDERSEN, W. M. HUTCHINSON, AND C. H. SIIM. 1980. Ultrastructural observations on microgametogenesis and the structure of the microgamete of *Isospora felis*. Acta path. microbiol. scand. Sect. B **88:** 151–159.

GRANT, J. C. B. 1962. An atlas of anatomy. The Williams and Wilkins Company, Baltimore, Maryland, fig. 412.

HAWKINS, W. E., J. W. FOURNIE, AND R. M. OVERSTREET. 1984. Intra-hepatic stages of *Eimeria funduli* (Protista: Apicomplexa) in the longnose killifish, *Fundulus similis*. Trans. Am. microsc. Soc. **103:** 185–194.

———, ———, AND ———. 1983. Organization of sporulated oocysts of *Eimeria funduli* in the Gulf killifish, *Fundulus grandis*. J. Parasit. **69:** 496–503.

———, M. A. SOLANGI, AND R. M. OVERSTREET. 1983a. Ultrastructure of the macrogamont of *Eimeria funduli*, a coccidium parasitizing killifishes. J. Fish Dis. **6:** 33–43.

———, ———, AND ———. 1983b. Ultrastructure of the microgamont and microgamete of *Eimeria funduli*, a coccidium parasitizing killifishes. J. Fish Dis. **6:** 45–57.

LADDA, R., M. AIKAWA, AND H. SPRING. 1969. Penetration of erythrocytes by merozoites of mammalian and avian malarial parasites. J. Parasit. **55:** 633–644.

LOM, J. 1971. Remarks on the spore envelopes in fish coccidia. Folia Parasit. (Prague) **18:** 289–293.

MEHLHORN, H., AND A. O. HEYDORN. 1979. Electron microscopical study on gamogony of *Sarcocystis suihominis* in human tissue cultures. Z. ParasitKde **58:** 97–113.

MEULLER, B. E. G., S. S. DESSER, AND A. HABERKORN. 1981. Ultrastructure of developing gamonts of *Eimeria contorta* Haberkorn, 1971 (Protozoa, Sporozoa) with emphasis on the host-parasite interface. J. Parasit. **67:** 487–495.

NICHOLS, B. A., M. L. CHIAPPINO, AND G. R. O'CONNOR. 1983. Secretion from the rhoptries of *Toxoplasma gondii* during host cell invasion. J. Ultrastruct. Res. **83:** 85–98.

———, AND G. R. O'CONNOR. 1981. Penetration of mouse peritoneal macrophages by the protozoan *Toxoplasma gondii*. New evidence for active invasion and phagocytosis. Lab. Invest. **44:** 324–335.

ODENSE, P. H., AND V. H. LOGAN. 1976. Prevalence and morphology of *Eimeria gadi* (Fiebiger, 1913) in the haddock. J. Protozool. **23:** 564–571.

OVERSTREET, R. M. 1981. Species of *Eimeria* in non-epithelial sites. J. Protozool. **28:** 258–260.

PELSTER, B. 1973. Vergleichende elektronenmikroskopische Untersuchungen an den Makrogameten von *Isospora felis* und *I. rivolta*. Z. ParasitKde **41:** 29–46.

———, AND G. PIEKARSKI. 1971. Elektronenmikroskopische Analyse der Mikrogametenentwicklung bei *Toxoplasma gondii*. Z. ParasitKde **37:** 267–277.

ROBERTS, W. L., D. M. HAMMOND, AND C. A. SPEER. 1970. Ultrastructural study of the intra- and extracellular sporozoites of *Eimeria callospermophili*. J. Parasit. **56:** 907–917.

———, C. A. SPEER, AND D. M. HAMMOND. 1971. Penetration of *Eimeria larimerensis* sporozoites into cultured cells as observed with the light and electron microscopes. J. Parasit. **57:** 615–625.

SCHOLTYSECK, E., AND M. HILALI. 1978. Ultrastructural studies of the sexual stages of *Sarcocystis fusiformis* (Railliet, 1897) in domestic cats. Z. ParasitKde **56:** 205–209.