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# Early generation merogonies of *Sarcocystis muriviperae* in liver and muscles of white mice

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**Abstract.** Early development of the coccidium *Sarcocystis muriviperae* Matuschka, Heydorn, Mehlhorn, Abd-Al-Aal, Diesing et Bichler, 1987 is described from experimentally infected white mice fed sporocysts from naturally infected *Vipera palaestinae* and *Coluber jugularis*. Although the course of infection was similar, mice infected with the sporocysts from the first host survived an inoculum of up to 200,000 sporocysts, while others infected with the second, succumbed to inocula exceeding 40,000 sporocysts in 7–10 days post infection (p.i.). Histological and ultrastructural studies revealed merogony in the hepatocytes during days 7–10 p.i. and onset of sarcocyst development by days 19–21 p.i. The livers of infected mice are grossly enlarged and of a mottled whitish colour due to severe neutrophil inflammatory infiltration, apparently stimulated by host cell residues or from defunct disaggregating meronts at the end of the merogony cycle. Early sarcocysts undergo a further division by endopolygeny before proceeding to division by endodyogeny.

There are several accounts on the development of coccidia of the genus *Sarcocystis* in the intermediate hosts prior to the formation of sarcocysts (Dubey et al. 1989), including fine structural data on the initial merogony stages. Precystic stages of *Sarcocystis* spp. of large mammals and of birds develop mainly in the vascular endothelium of diverse organs (Pacheco and Fayer 1977, Heydorn and Mehlhorn 1978, Dubey et al. 1980, Speer and Dubey 1981, Smith et al. 1987). Species found in rodents usually develop in the liver in hepatocytes (Sénaud and Černá, 1978, Cawthorn and Brooks 1985), whereas species developing in rats undergo merogonous development in both endothelial and in other connective tissue cells in lungs, spleen and kidneys (Zaman and Colley 1975, Brehm and Frank 1980). The only available accounts of the fine structure of the initial merogony stages of the snake-rodent *Sarcocystis* species are those of Mehlhorn and Matuschka (1986) and Matuschka et al. (1987) for *S. clethrionomyelaphis* and *S. muriviperae,* respectively. There is some documentation on the ultrastructure of the early sarcocysts and development of the sarcocyst wall for species developing in avian and mammalian definitive hosts (Pacheco et al. 1978, R. J. Cawthorn's figures 19 and 24 in Dubey et al. 1989), but none for snake-rodent species. The present study provides a detailed description of the developmental process of *Sarcocystis muriviperae* in livers of laboratory mice (*Mus musculus*) fed sporocysts obtained from *Vipera palaestinae* ("viper"), and *Coluber jugularis* (black snake) and the early development of the sarcocysts in the muscles of infected mice.

## MATERIALS AND METHODS

*Sarcocystis muriviperae* infections from naturally infected vipers and black snakes from the coastal plain of Israel were maintained in laboratory mice and captive snakes (Finkelman and Paperna, unpublished). Ultrastructural studies on the early stages of infection in the liver were made using suckling and weaned mice fed with 50,000–150,000 sporocysts extracted from feces of vipers and sacrificed 7–11 days p.i. Fine structure of the early stages in muscle was studied in mice fed an uncounted number of sporocysts obtained from black snakes and sacrificed on day 21 p.i.

For light microscopy (LM) histology tissues were fixed in 10 % neutral buffered formalin and, following dehydration in ascending alcohols, were embedded in glycol methacrylate (GMA of Agar, UK). Sections, 3–4 μm thick, were cut with a glass knife on a Sorval JB4 microtome and stained with Meyer's haemalum, using eosin or eosin and phloxin, as counterstains.

For transmission electron microscopy (TEM) studies, tissues were fixed in Karnovski or in 2.5 % glutaraldehyde in cacodylate buffer (0.1m, pH 7.4) for 24 h at 40°C, and after repeated rinsing in the same cacodylate buffer, they were post-fixed in 1 % osmium tetroxide, in the same buffer, for 1 h. After rinsing in the same buffer, the material was dehydrated in graded alcohols and embedded in either Epon 812 or Agar 100 (Agar Co., UK). Thin sections, cut on a Reichert Ultracut with a diamond knife, were stained on-grid with uranil acetate and lead citrate and examined with a Jeol 100CX TEM.

## RESULTS

### Infection in the liver

LM histological examination of mouse liver on days 7 to 11 p.i. revealed undivided meronts of approximately 20 × 15 μm in size (Fig. 1), dividing meronts and free merozoites. Meronts before division reach 32 × 17–20 μm in size and their nucleus undergoes extensive lobulation before becoming fragmented into daughter nuclei (Fig. 2). Merozoites measured 5 × 1.2 μm. They differentiated from the daughter nuclei and ultimately form a characteristic rosette, 22–27 × 18–22 μm in size (Fig. 3), before becoming scattered (Figs. 4, 5) and dispersed into the tissue (Figs. 6,7). No parasites could be traced in the liver after day 11 p.i. and no parasites of any stage could be detected in the spleen, the kidneys or the lungs, at any time p.i.

Mice inoculated with heavy doses of sporocysts from either of the two snakes developed grossly enlarged, mottled whitish livers. Young, suckling mice, were particularly susceptible to infection with sporocysts from black snakes and all mice receiving inocula exceeding 40,000 sporocysts succumbed by days 8–10 p.i. Mice of same age, however, survived inocula of up to 200,000 sporocysts from vipers. Only 2 out of 5 young mice survived inoculations with over 200,000–300,000 viper sporocysts, and all with doses above 300,000 died by day 11 p.i.

The histological picture of mice inoculated with oocysts from both the vipers and the black snakes was similar, revealing numerous foci or extensive diffuse areas of inflamation and necrosis. The tissue, infiltrated by heterophilic granulocytes, and in some areas also by eosinophils, was comprised of hepatocytes undergoing hydropic degeneration in a matrix of hyaline and necrotic cellular residue (Figs. 8–10). The developing parasites, e.g. meronts in various stages of division, occurred only in the relatively undamaged tissue, outside the foci of inflammation, and only exceptionally at the periphery of the lesion (Figs. 8, 9). Infiltration initially developed at the site of the defunct meront. Neighbouring hepatocytes containing some released merozoites, demonstrated clear signs of damage (Fig. 7). Inflammatory conditions persisted in mice beyond 11 days p.i. but were resolved in those surviving to the stage of muscle infection (21 days p.i.).

### Electron microscopy

Meronts within the hepatocytes were bounded by a fine two-layered wall and were not enclosed in a parasitophorous vacuole. The several large nuclei of the developing meronts were highly lobulated, each with several nucleoli (Fig. 11). Following further nuclear division merozoite anlagen develop by endopolygeny in the meronts' cytoplasm (Fig. 12): these are filled with large electron lucent vesicles apparently containing amylopectin (Fig. 13). Emerging merozoites (Fig. 14), characteristically lacked rhoptries, and their anterior portion contained a few micronemes. Only residues of the large vesicles seen in earlier stages remain in the differentiated merozoites (Fig. 15). The meront residuum contained residual nuclei, food vacuoles and large mitochondria (Fig. 15). Alongside the differentiated meronts there also occurred others which yielded heavily vacuolated and probably defunct merozoites. Released merozoites were located within a matrix of cellular debris and the residue of defunct premature meronts (Fig. 16). Merozoites were also seen free in the cytoplasm of polymorphonuclear leucocytes and monocytes (Figs. 17, 18). The surface of their pellicle was densely covered with distinct electron dense droplets (Fig. 19).

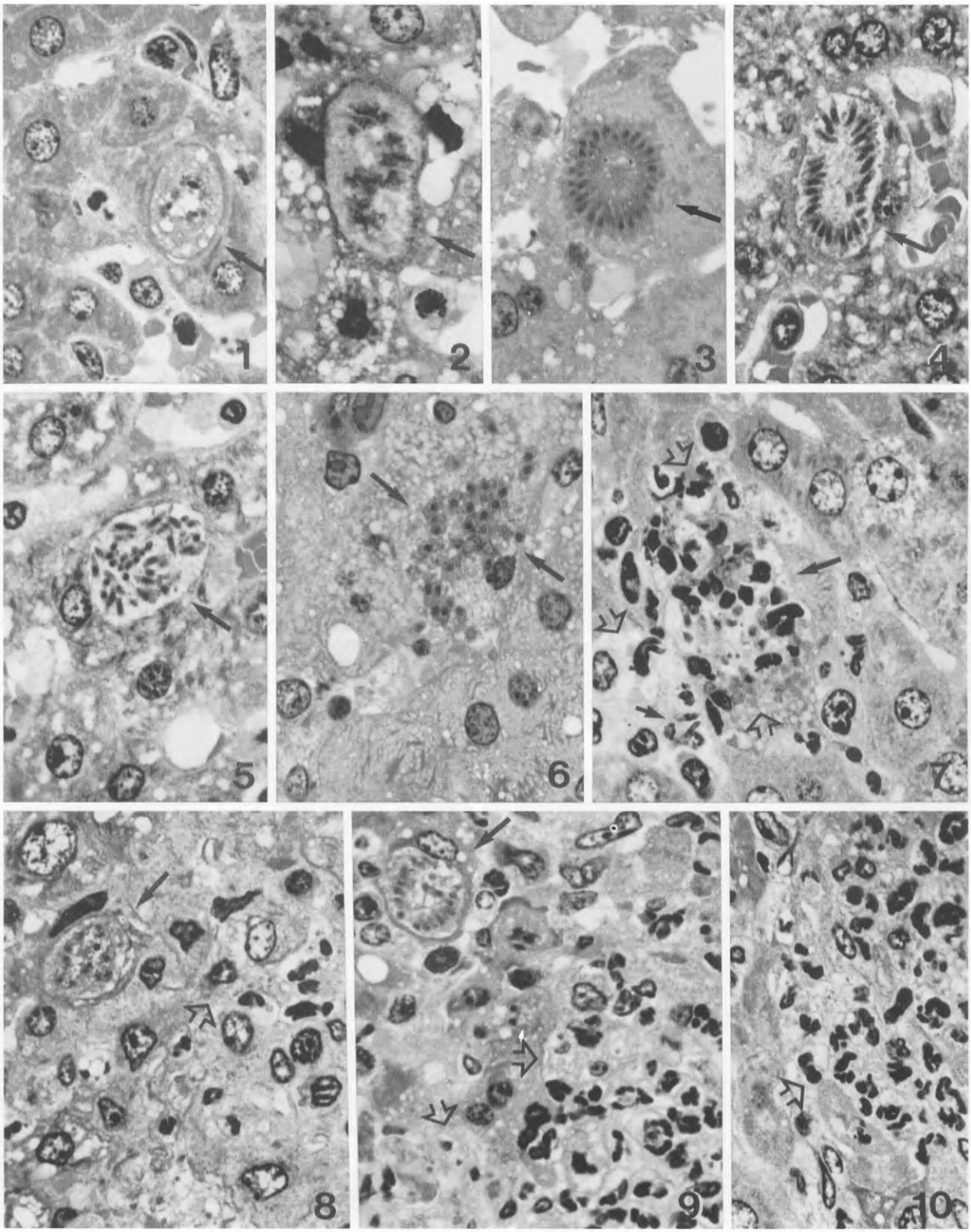
### Early stages in the muscles

Sarcocysts were first detected in the muscles of mice fed both viper and black snake sporocysts on days 19 –21 p.i. In a mouse fed on black snake sporocysts and sacrificed by day 21 p.i. for electron microscopy, much of the muscle contained sarcocysts, but many were already degenerated (Fig. 20). Histology revealed both early stage sarcocysts and older ones. The first, measuring about 13 × 9 – 27 × 16 μm, contained zoites with large lobulated nuclei characteristic for endopolygeny, as well as metrocytes with a single small nucleus. Older sarcocysts measuring 31 × 18 μm, 45 × 18 μm, and 58 × 29 μm, contained 20, 50 and 61 metrocytes, respectively (Fig. 21).

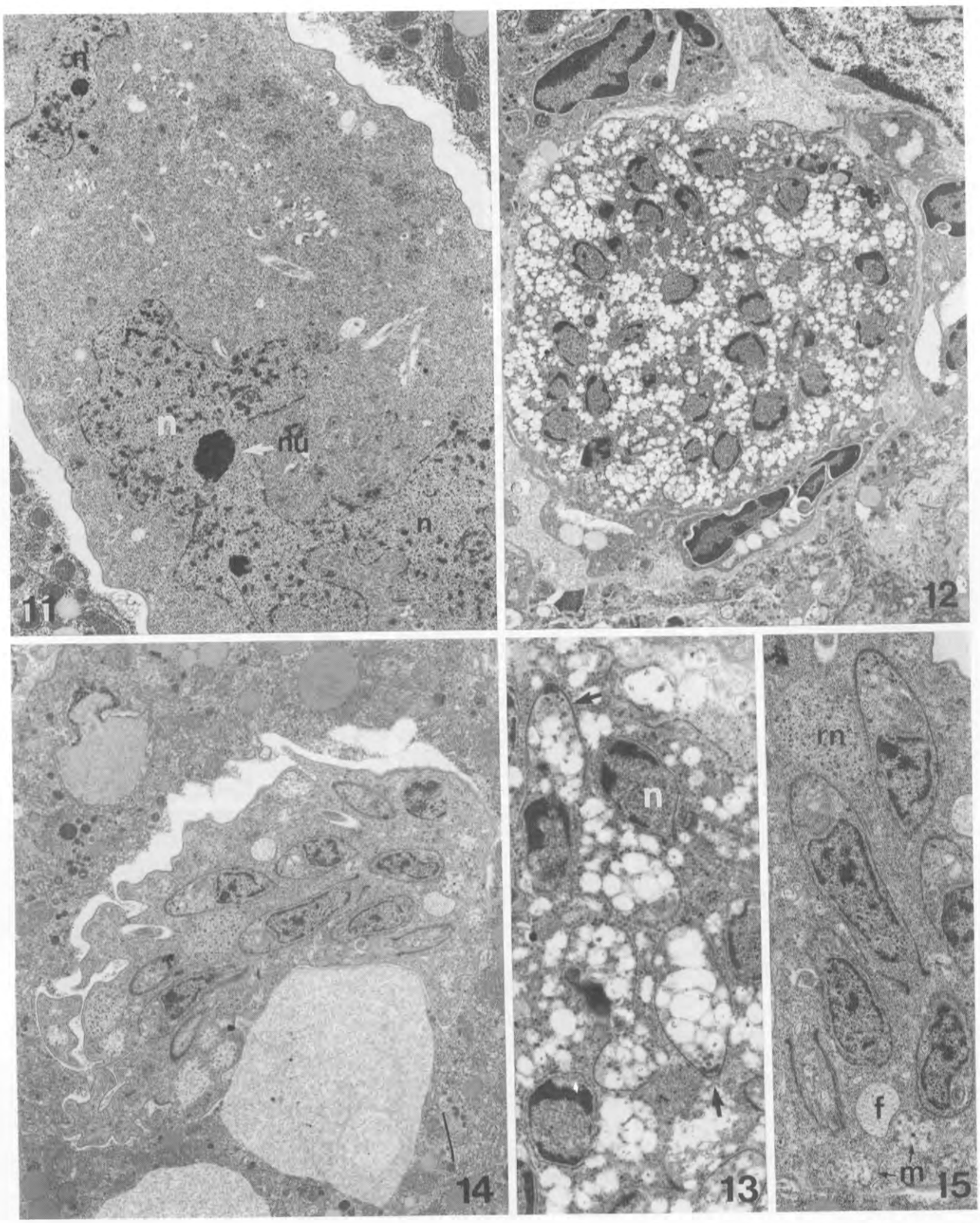
### Electron microscopy

Unizoite sarcocysts (Fig. 22) had a primary wall thrown into numerous anastomosing and branching electron dense protrusions alternating with micropyle-like "windows" of a single unit membrane (Fig. 23). The zoite occupied about half of the volume of the sarcocyst the rest being comprised of a cytoplasmic layer (granular layer of Dubey et al. 1989, or cyst's ground substance of Scholtyseck et al. 1974). This contained many granules and vesicles, either lucent or filled with electron dense residues. This zoite still retains the remains of an apical complex, accompanied by subpellicular microtubules, and it is bound by an incomplete membrane. Its cytoplasm contains large mitochondria, rough and smooth endoplasmic reticulum and a variety of empty vesicles or others filled with a fragmented substance.

Other sarcocysts contained a single large zoite with a dividing nucleus (Figs. 24, 25) together with several smaller metrocytes dividing by endodyogeny. Some cross sections of sarcocysts already contained only metrocytes (Figs. 26, 27). Both zoites and metrocytes were bound by two unit membranes, accompanied with large




**Figs. 1–10.** Light micrographs of histological sections of livers from white mice, 9–10 days p.i. with 100000–150000 sporocysts of *Sarcocystis muriviperae* from the viper, H&E, × 1000. **Fig. 1.** Young meront. **Fig. 2.** Meront with lobulated daughter nuclei. **Fig. 3.** A rosette. **Figs. 4, 5.** Formation of merozoites. **Fig. 6.** Scattered merozoites. **Fig. 7.** Hepatocyte necrosis and neutrophil infiltration (open arrow) in a site of past merogony. Merozoites may be seen at site (bold arrow). **Fig. 8, 9.** Neutrophil infiltration (open arrow) together with a dividing meront (bold arrow). **Fig. 10.** Focus of inflammatory infiltration (open arrow).




**Figs. 11–19.** Transmission electron micrographs of meronts of *Sarcocystis miruviperae* before and after division, in the liver of a mouse 10 days p.i. with 100000 sporocysts from a viper. **Fig. 11.** Meront prior to division showing lobulated nuclei (n) with prominent nucleoli (nu), × 5050. **Figs. 12, 13** (Fig. 13 – enlarged view of Fig. 12). Meront with divided nuclei (n) with primordia of merozoites (arrow), × 4000 and × 10000. **Figs. 14, 15** (Fig. 15 – enlarged view of Fig. 14). Meronts in advanced stage of division into merozoites, meront's cytoplasm contains residual nuclei (rn), food vacuoles (f) and mitochondria (m), × 4400 and × 7400.

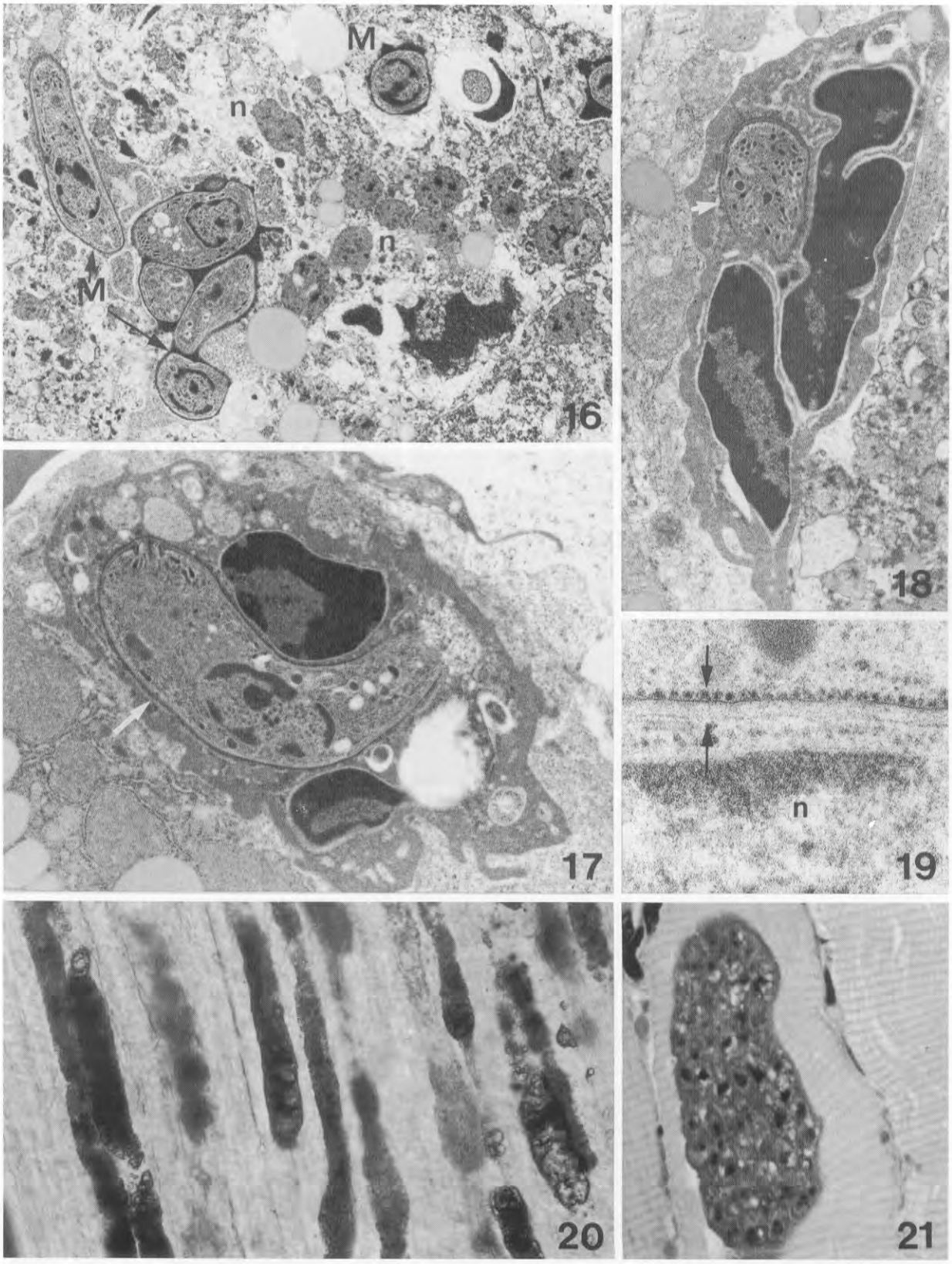
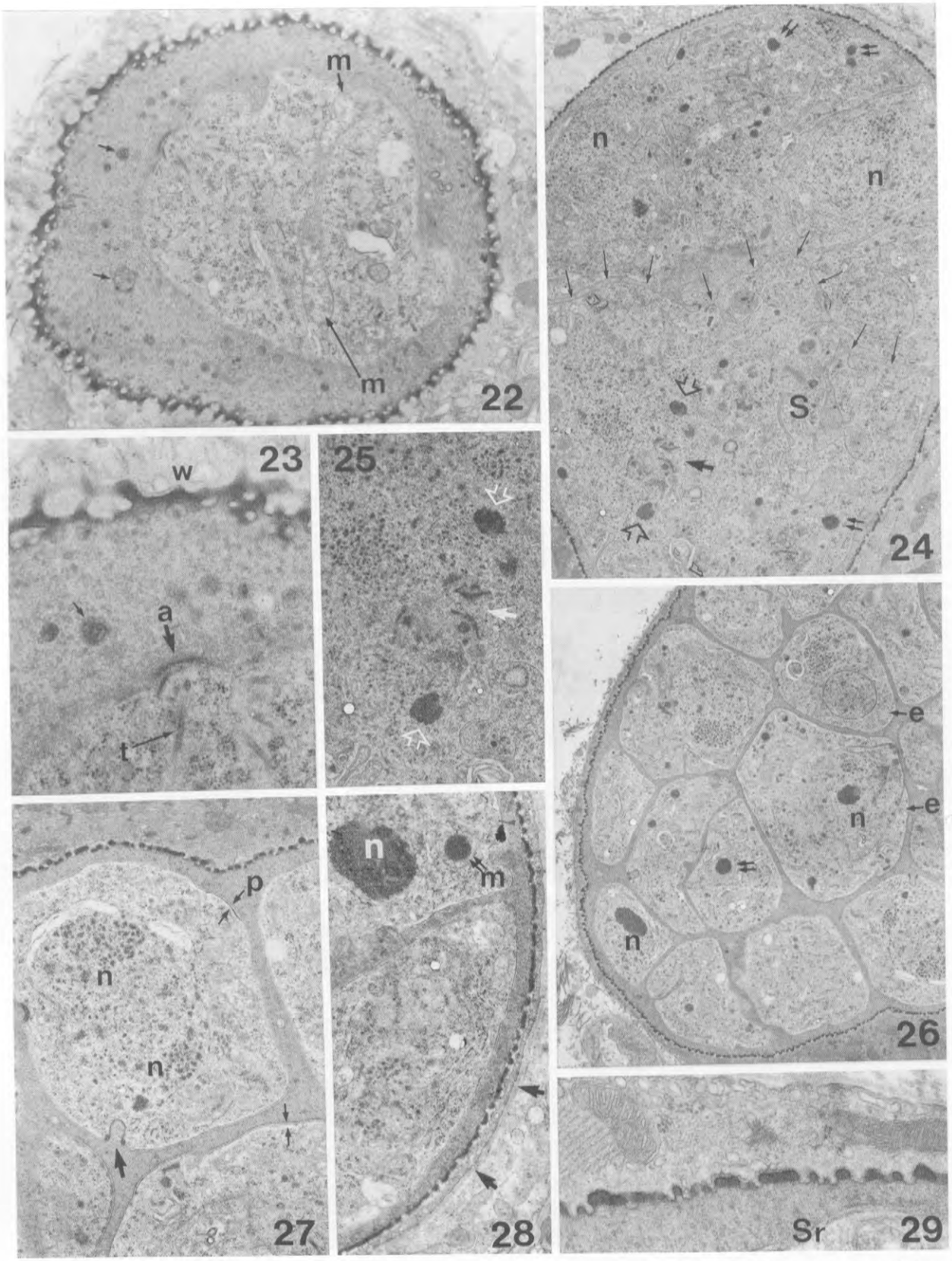



**Fig. 16.** Free merozoites (M) of *Sarcocystis miruviperae* and the residue of a defunct meront showing scattered nuclei (n) within a matrix of cellular residue and debris, × 6450. **Figs. 17, 18.** Merozoites in polymorphonuclear leucocytes, × 12200 and × 11900. **Fig. 19.** High magnification of the merozoite pellicle overlaid with electron-dense droplets, × 104000. **Fig. 20.** Light micrograph of a compressed unfixed piece of muscle with a heavy load of intact and degenerating sarcocyst from a mouse 21 days p.i. with sporocysts from a black snake, × 150. **Fig. 21.** Light micrograph of a sarcocyst in histological section of mouse muscle, 21 days p.i., with sporocysts from a black snake, × 1000.




**Figs. 22–29.** Transmission electron micrographs of young sarcocysts in the muscle of a white mouse, 21 days p.i., with sporocysts from a black snake. **Figs. 22, 23** (Fig. 23 – enlarged views of Fig. 22). Unizoite sarcocysts with apical complex (a), microtubules (t), very large mitochondrion (m) and primary wall (w) with numerous protrusions. The superficial cytoplasmic layer contains numerous granules (small arrows), × 14800 and × 29600. **Fig. 24.** Young sarcocyst with a large meront (S, outlined with

micropores. The zoites and the metrocytes filled most of the sarcocyst leaving only a marginal "granular" layer (Fig. 28). The cytoplasm of the zoite and the metrocytes contained mitochondria with a heavy electron-dense aggregate (Figs. 24, 26, 28). The primary wall of all these sarcocysts was comprised of a single membrane juxtaposed with irregularly interrupted thick electron-dense thick layer (Figs. 28, 29). The primary wall was sometimes adhered to a vesiculated superficial layer, seemingly of host origin (Fig. 28).

## DISCUSSION

If infection is heavy enough, early merogony in the liver of the white mouse appears to induce a severe pathology. The merogonous stage of infection is particulary damaging to small-sized intermediate hosts, when located in the hepatocytes (snake-mouse species of *Sarcocystis* – Bledsoe 1980, Mehlhorn and Matuschka 1986; bird of prey-rodent species – Tadros 1981, Cawthorn and Brooks 1985) or in vascular endothelial cells, particularly of the lungs (python-rat *S. singaporensis*– Zaman and Colley 1975, or *S. falcatula* in the budgerigar – Smith et al. 1987, 1989). It seems that the damage induced by inflammatory response in the liver is far more extensive than that one would expect from direct response to the presence of parasites in the liver. Inflammatory changes never occurred around meront-infected hepatocytes. Infiltration of neutrophils (sometime together with eosinophils) in the mice liver coincided with the disintegration of the hepatocytes following completion of the merogony process. Such disintegrating liver cells could be the source of substances causing the inflammatory response. Observed disaggregating defunct, incompletely developed meronts may be another, or contributory source for substances eliciting inflammatory responses. The inflammatory process subsided later on, while merozoites were still remaining in the tissues. Wenyon (1926) in his discussion on the pathology of *Sarcocystis* mentions early workers' findings that some *Sarcocystis* species contain a substance referred to as "sarcocystin", which is highly toxic to a variety of animals. In *S. cruzi* infections, inflammatory response was attributed to the release of antigens during the rupture of second generation meronts (Dubey et al. 1982).

Species of *Sarcocystis* vary in their pathogenicity to their intermediate host, and there is also interspecific variation in the timing of the crisis, which appears to coincide with the final stages of merogony: this may differ among the various *Sarcocystis* species. *S. clethrionomyelaphis* caused illness and death by days 8–9 p.i. in voles receiving as few as 1000 sporocysts (Mehlhorn and Matuschka 1986), and *S. idahoensis* was fatal to deer mice exposed to 15,000 sporocysts 5–6 days p.i. (Bledsoe 1980). On the other hand, according to Matuschka et al. (1987) white mice became ill (by days 9–11 p.i.) only when fed doses of 600,000 sporocysts of *S. muriviperae* from *V. palaestina*. In our experiments the mice succumbed after feeding 231,000 *S. muriviperae* sporocytes obtained from vipers, and as few as 43,000 sporocysts of seemingly the same species obtained from black snakes. Arguments for conspecificity are presented elsewhere (Finkelman and Paperna, unpublished): natural infections studied in vipers and black snakes showed oocysts to be of similar sizes; the parasites from both snake species have the same restriction of the intermediate host to *Mus musculus* and they have the same fine structure of the primary sarcocyst wall. There is also a complete conformity in the structural and fine structural affinities of the gamogonous stages (Paperna and Finkelman 1996). The fine structure and the division process by endopolygeny seen in *S. muriviperae* liver meronts appears to be the same as that of other *Sarcocystis* species, whether they develop in the hepatocytes or in endothelial cells, and in both small (rodents) and large (pigs, cattle) intermediate hosts (Pacheco and Fayer 1977, Heydorn and Mehlhorn 1978, Sénaud and Černá 1978, Speer and Dubey 1981). Only those of *S. falcatula* from the budgerigar (Smith et al. 1989) seem to be different. In *S. tenella*, two merozoite anlagen emerge from a spindle formed at each lobe of the nucleus (Speer and Dubey 1981). This was not seen in our material. The rosette arrangement of merozoites of divided meronts described by Dubey et al. (1989) for *S. rauschorum* was seen only by LM in our material. Rosette formation is also implied from the peripherally arranged merozoites in TEM image of mature *S. clethrionomyelaphis* meronts presented by Mehlhorn and Matuschka (1986).*

It has been suggested that *Sarcocystis* species developing in small intermediate hosts such as rodents

← small arrows) containing dividing nuclei (open arrows), with what appears to be a spindle (bold arrow), and a few metrocytes each with one nucleus (n). Mitochondria with an electron dense aggregate (see Fig. 28) are marked with double fine arrows, × 5610. **Fig. 25.** Enlarged view of the dividing nuclei in the meront of Fig. 24, × 10300. **Fig. 26.** Sarcocyst with metrocytes, some dividing by endodyogeny (e). Inactive cells show a condensed nucleus (n); the other electron dense bodies are the condensed deposits in the mitochondria (double fine arrows). **Fig. 27.** Metrocyte prior to division (n – nuclei) with double membrane wall (p) and a micropore (bold arrow), × 10300. **Fig. 28.** Metrocyte with condensed nucleus (n), and mitochondia with heavy deposit (double arrows). The primary wall is juxtaposed to a superficial layer of presumably host origin (bold arrows), × 10400. **Fig. 29.** Enlarged view of the primary wall of a young sarcocyst (Sr), × 40900.

* The mature *S. muriviperae* meront shown by Matuschka et al. (1987, Fig. 10) seems to be part of a *S. clethrionomyelaphis* meront shown in an electron micrograph by Mehlhorn and Matuschka (1986, Fig. 3).

(mostly in hepatocytes) have only a single precystic stage, while in larger hosts there are several merogonous divisions (in endothelial cells) before sarcocysts are formed in the muscles (Dubey et al. 1989). In *S. singaporensis*-infected rats, additional meront generations occur in vascular endothelial cells of various organs in the span of days 11–22 p.i. (Brehm and Frank 1980, Beaver and Maleckar 1981). The span of 11 to 21 days p.i. prior to the first detection of young *S. muriviperae* in the muscles may suggest additional extramuscular merogonous generations, but none has yet been found in the infected white mice. The zoites in the early muscle sarcocysts in our infected white mice seem to precede the appearance of metrocytes and apparently divide by endopolygeny. In other species the earliest observed sarcocysts already contained metrocytes dividing by endodyogeny (Dubey et al. 1989).

We were unable to detect the stage preceeding the formation of the primary cyst wall, i.e. within a parasitophorous vacuole, as reported by Mehlhorn et al. (1975). The primary wall of the early unizoite cyst we observed appears to be the same as of the unizoite and young *S. rauschorum* sarcocysts (R. J. Cawthorn's figures in Dubey et al. 1989), and are structurally reminiscent of the primary wall of mature *S. muris* sarcocysts described by Sheffield et al. (1977).

It is suggested that the variable structures of primary walls, even the complex ones, seem to derive from a simple primary wall, enclosing young sarcocysts. In *S. rauschorum*, the the primary wall undulating character seen in the early sarcocysts is retained in mature cysts (Dubey et al. 1989).

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