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Fine Structure of the Oocyst Walls of *Isospora serini* and *Isospora canaria* and Excystation of *Isospora serini* From the Canary, *Serinus canarius* L.

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SYNOPSIS. Oocysts of *Isospora serini* and *Isospora canaria*, from the canary *Serinus canarius*, were broken, added to a cell suspension, fixed in Karnovsky's fluid, and studied in the electron microscope. The oocyst wall of each species had an electron-lucent inner layer, a more osmiophilic middle layer and an outer layer of electron-lucent (*I. serini*) or electron-dense material interspersed with some electron-lucent material (*I. canaria*). A few, relatively large lipid-like bodies were present in the outer or middle layer of the oocyst wall of *I. canaria*. As many as 9 membranes were present in the oocyst wall of *I. canaria* and 3 in that of *I. serini*. When exposed to a trypsin-sodium taurocholate fluid, sporozoites of *I. serini* excysted from 5-month-old sporocysts *in vitro*, but not from sporocysts stored for more than 6 months. No excystation occurred in 15-month-old *I. canaria* sporocysts. Similarities and differences in excystation between *I. serini* and other *Isospora*, *Eimeria*, and *Sarcocystis* species are discussed.

Index Key Words: *Isospora serini*; *Isospora canaria*; oocyst wall; excystation; electron microscopy.

RECENTLY, Box (1) described the exogenous stages of *Isospora serini* (Aragão, 1933), which may be synonymous with *Atoxoplasma*, and *Isospora canaria* Box, 1975, from canaries, *Serinus canarius* Linnaeus. When examined in the light microscope the sporulated oocysts of these species are very similar structurally and differ only slightly in size. Our electron-microscopic findings with regard to the similarities and differences of the oocyst walls of *I. serini* and *I. canaria*, as well as light-microscopic observations on excystation of *I. serini*, are described herein.

MATERIALS AND METHODS

Sporulated oocysts of *I. canaria* and *I. serini* were sent to us by Dr. Edith D. Box, Department of Microbiology, The University of Texas Medical Branch, Galveston, in March, 1974. The *I. canaria* oocysts had been collected from canaries, *S. canarius*, in December, 1972; the oocysts of *I. serini* were collected from the same host species in September, 1973 and January-February, 1974. Both groups of oocysts were kept in 2.5% (w/v) aqueous $K_2Cr_2O_7$ solution at 4-5 C. For excystation studies, oocysts were concentrated by flotation in Sheather's sugar solution and then washed 3 × in Ringer's physiologic solution (RPS). Oocysts were concentrated by centrifugation (5 min at 115 *g*); the supernatant fluid was discarded and 1 drop of oocyst suspension in RPS was placed on a slide and to this drop was added a drop of 1.5% (w/v) sodium taurocholate (N.B. Co., Cleveland, Ohio) and 0.5% (w/v) trypsin (1:250, Difco) in RPS. To prevent drying, the above preparations were covered with coverglasses and sealed with Petrolatum. Oocysts were broken to release sporocysts using friction of the coverslip. The preparations were incubated at 37 C and then examined at various intervals at room temperature (~ 22 C) by bright-field microscopy to follow the excystation process. Photomicrographs were made using Panatomic-X 35-mm film within a Zeiss photomicroscope equipped with a 100 × Neofluar objective.

For electron-microscopic studies, a suspension of sporulated oocysts of *I. serini* or *I. canaria* was treated with NaOCl for 20 min or ground in a motor driven, Teflon-coated tissue grinder to break the walls and then added to a suspension of Detroit-6 (American Type Culture Collection, Rockville, Md.) cells obtained by treatment of monolayer cultures by a trypsin-versene solution. Use of the cells was necessary to form a pellet of oocyst walls. After the suspension was centrifuged at 850 *g* for 10 min, the pellet was fixed in Karnovsky's fluid (8), 3% or 15% (v/v) glutaraldehyde in 0.2 M cacodylate buffer for 4 to 8 hr at 22 C. The pellet was rinsed in 0.2 M cacodylate buffer for 12 hr at 5 C, placed in 1.5% (w/v) OsO_4 for 2 hr, dehydrated in ethanol and 2 changes of propylene oxide, and embedded in Epon 812. Sections were placed on 200-mesh grids, stained with uranyl acetate and lead citrate and examined with a Corinth 275 electron microscope.

RESULTS

Isospora serini (Figs. 3-12)

Sporulated oocysts of this species are spherical to subspherical and usually slightly smaller (cf. Figs. 1 & 3) than those of *I. canaria*, these findings being in agreement with those of Box (1). The sporocysts (Figs. 3, 4) of this species, however, have a distinct substiedal body which Box was unable to see. This substiedal body is ~ 3 μm deep and 5 μm wide, or almost 2 × larger than that found in *I. canaria*.

Excystation did not occur in intact oocysts exposed to the trypsin-sodium taurocholate solution for 3 hr or in free sporocysts exposed to RPS for an equivalent time. After treatment with the trypsin-sodium taurocholate medium, sporozoites excysted from only some of the sporocysts obtained from 5-month-old oocysts, but not from oocysts stored for more than 6 months. The following sequence of events was usually observed during

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† We are grateful to Dr. Edith Box for supplying oocysts of *Isospora canaria* and *Isospora serini* and for her cooperation and helpful suggestions during the course of this study.

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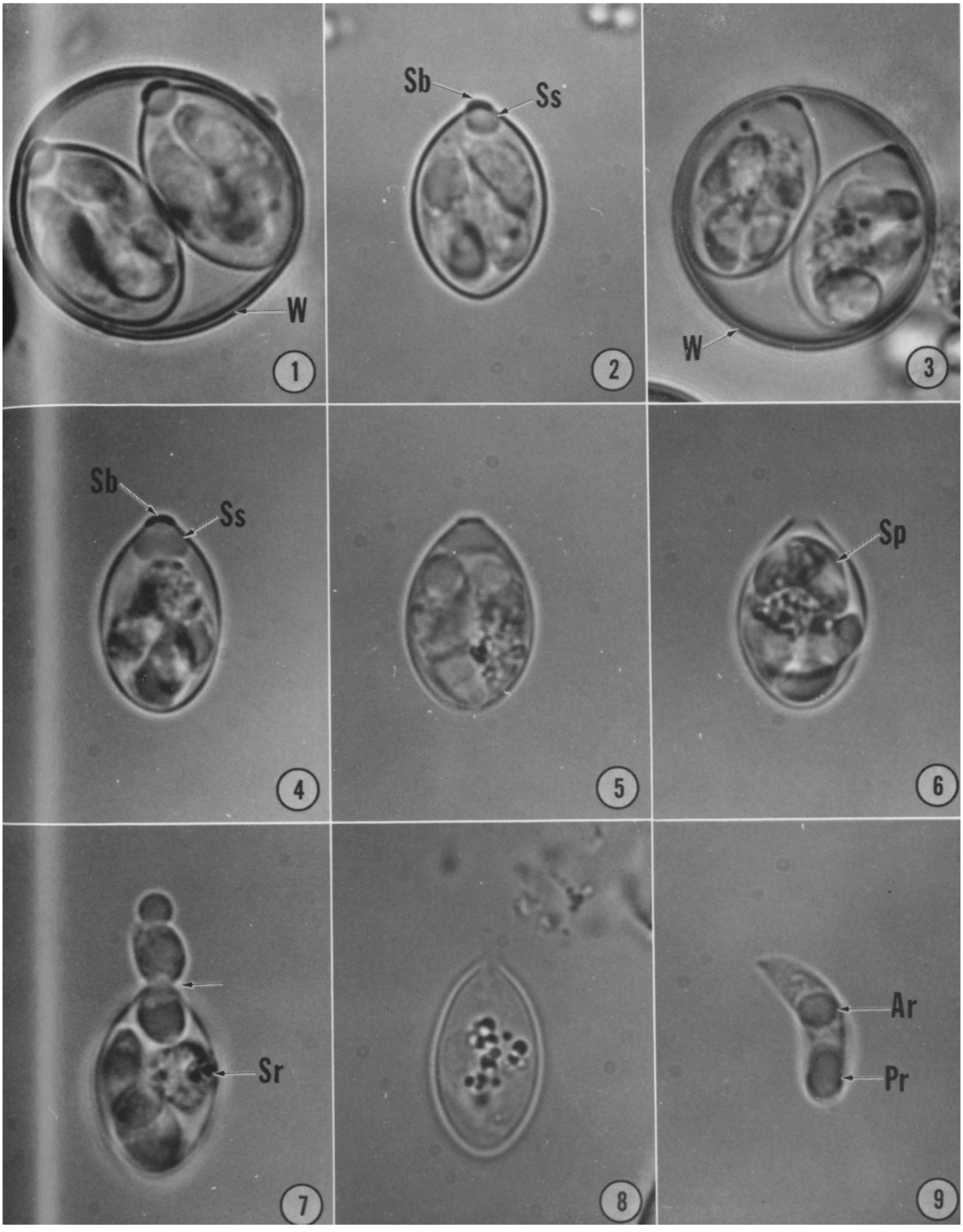
Figs. **1-9**. [Photomicrographs. Figs. 1, 2, *I. canaria*; Figs. 3-9, *I. serini*. All Figs. × 2120.] **1**. Oocyst in optical cross-section. **2**. Sporocyst; note distinct Stieda (Sb) and substiedal (Ss) bodies. **3**. Oocyst in optical cross-section. **4**. Sporocyst; note large substiedal body. **5**. Sporocyst after addition of trypsin-sodium taurocholate mixture; note that Stieda body has almost disappeared and substiedal body has become more transparent. **6**. Sporocyst after addition of excysting fluid; note disappearance of substiedal body. **7**. Sporozoite (Sp) excysting from sporocyst; note constriction (arrow) of sporozoite body at point of exit from sporocyst. **8**. Intact empty sporocyst after sprozoites have excysted. **9**. Free sporozoite; note the anterior (Ar) and posterior (Pr) refractile bodies.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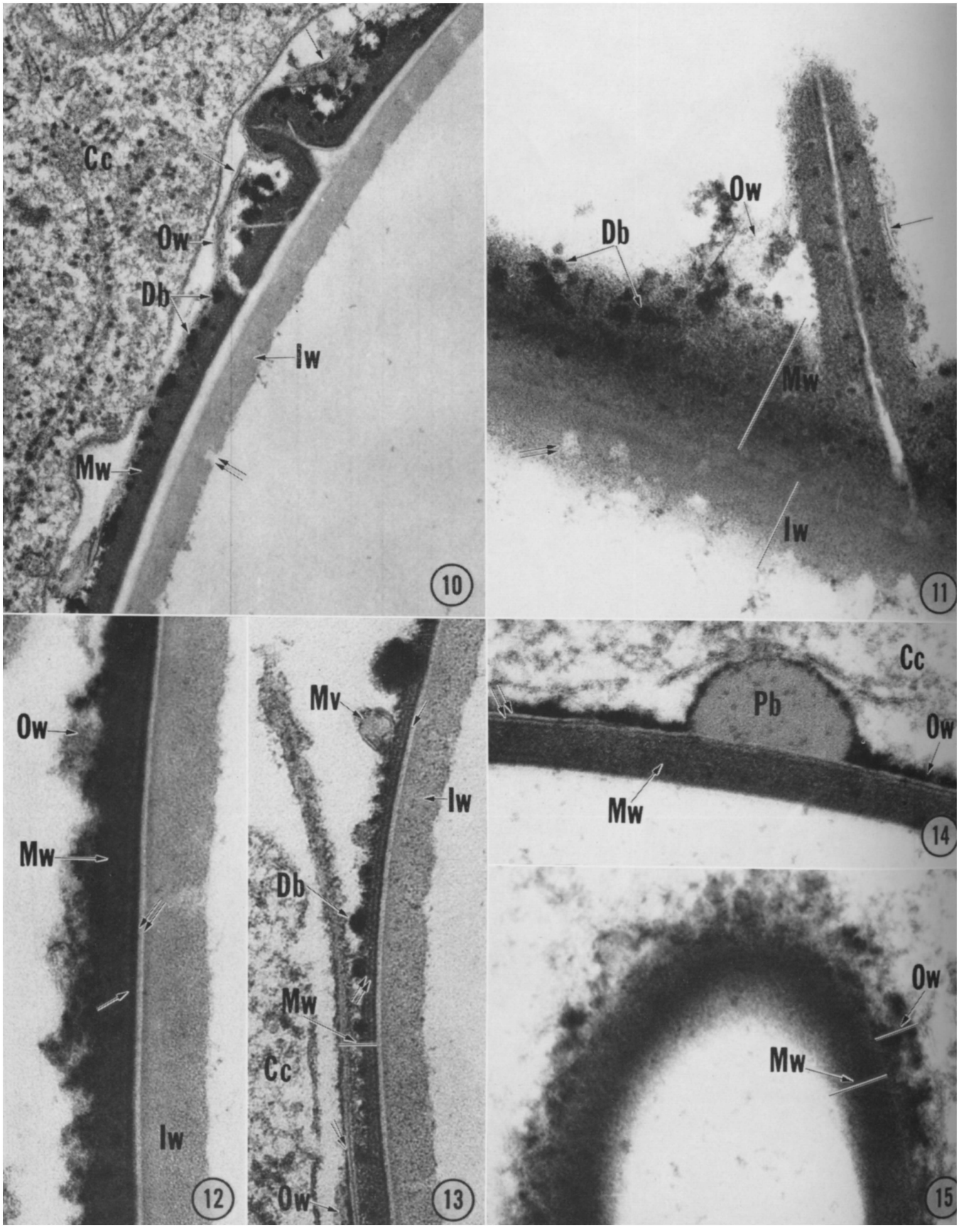
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excystation: (A) At 10-15 min after addition of the excysting fluid, the Stieda body changed in optical density and disappeared (Fig. 5). (B) One to 5 min later the substiedal body also disappeared *in situ* (Fig. 6). (C) Soon thereafter, sporozoites began to move about by slow gliding within the sporocyst. (D) Eventually a sporozoite would escape at one pole of the sporocyst with its body constricted as it passed through the gap in the sporocyst wall (Fig. 7). Sporozoites free of sporocysts (Fig. 9) were first seen at 35 min after exposure to the trypsin-sodium taurocholate mixture. Some sporozoites continued moving within sporocysts, which had no Stieda or substiedal bodies, for more than 60 min without excysting. After sporozoites had excysted, the sporocyst wall remained intact (Fig. 8) and did not collapse as reported for some *Isospora* species (6, 18). In those specimens in which excystation failed to occur, the Stieda and substiedal bodies, and later the contents of the sporocyst, all slowly disappeared.

The oocyst wall (Figs. 10-12) is composed of 3 layers: an electron-lucent inner, a more osmiophilic middle, and a relatively electron-lucent outer. These layers measure in thickness 100 (80-100), 110 (70-150), and 50 (25-90) nm, respectively. The inner layer consists of a homogeneous fine granular material and 2 closely applied unit membranes, which are present in the outermost portion (Fig. 12). Several indentations are present on the inner surface (Figs. 10, 11). The double-membrane complex of the inner layer is closely associated with an electron-dense line (Fig. 12) present in the innermost portion of the middle layer. The osmiophilic middle layer consists of a coarse granular matrix with numerous electron-dense spheroid bodies (10-65 nm) which are most numerous in the outer portion of the layer (Figs. 10, 11). The light-staining outer layer consists of a fine granular matrix (Figs. 11, 12) and a single unit membrane, usually adjacent to the middle layer (Fig. 11); occasionally, the membrane is more peripherally located (Fig. 10). In some preparations the middle and outer layers appear to slip upon the inner layer, throwing the 2 outer layers into numerous folds (Figs. 10, 11). After treatment with NaOCl, the inner layer usually separated from the middle layer and the 2 outer layers remained intact.

Isospora canaria
(Figs. 1, 2, 13-15)

Sporulated oocysts of this species were usually spherical and corresponded closely to the description given by Box (1). By the time we were able to do time-sequence excystation studies of these oocysts, they had been stored at 4-5 C in $K_2Cr_2O_7$ solution for 15 months. Although the oocysts and sporocysts appeared "normal" (Figs. 1, 2), sporozoites did not excyst from sporocysts treated with trypsin-sodium taurocholate at 37 C for 24 hr.

Three layers make up the oocyst wall (Figs. 13-15): an electron-lucent inner, an osmiophilic middle, and an outer layer, consisting of mostly osmiophilic material interspersed with some electron-lucent material. These layers measure in thickness 90 (80-100), 65 (50-110), and 25 (12-200) nm, respectively. The inner layer consists of a light-staining granular material (Fig. 13) which is somewhat coarser than that of *I. canaria*. Indentations of the inner layer are rarely seen. Two closely applied unit membranes of the inner layer are adjacent to the osmiophilic middle layer (Fig. 13). The inner portion of the middle layer consists of 4 or 5 closely applied unit membranes; the middle portion has unit membranes interspersed with electron-dense spheroid bodies; and the outer portion consists of 2 closely applied unit membranes (Fig. 13). The 2 membranes of the outermost portion often separate from the remainder of the middle layer (Fig. 13). Areas of the middle layer not covered with these 2 membranes contain membrane-bounded vesicles and numerous electron-dense bodies. The latter (65-200 nm) are larger in size than those (15-30 nm) in intact walls (Fig. 13). In many preparations the inner layer separates from the middle layer, whereas the 2 outer layers remain intact (Figs. 14, 15). Usually, the outer layer consists of a homogeneous electron-dense material of irregular thickness (Figs. 14, 15). Occasionally, lightly staining spheroid bodies (50-180 nm, Fig. 14), similar in appearance to lipid droplets, are present. It is difficult to determine if these bodies are part of the outer or middle layer, since the most peripherally located membrane of the middle layer appears continuous with these bodies (Fig. 14). The electron-dense material of the outer layer is continuous over the surface of the spheroid, lipid-like structures, which were not seen on the surface of NaOCl-treated oocyst walls. In such specimens, the inner layer usually was separated from the middle one, whereas the 2 outer layers remained intact.

DISCUSSION

Several investigators have described in detail the excystation of sporozoites of various species of *Eimeria* and *Isospora* (2, 4-7, 14, 18), but only a few mentioned the length of time the oocysts were stored before being used for excystation studies. Only Box (1) working with *I. canaria* and *I. serini* and Speer et al. (18), working with *Isospora canis* Nemeséri, 1959 from dogs stated the time that oocysts had been stored before their excystation experiments. In using relatively fresh oocysts (2-month-old), Speer et al. (18) experienced little difficulty in getting sporozoites to excyst. In experiments in this laboratory with *Isospora marquardti* Duszynski & Brunson, 1972 and 4 species of *Eimeria* from pikas (4, 5), the sporozoites of some species excysted while others did not when oocysts which had been stored for 12 months were used. In the present study, we found, as did Box (1), that sporozoites of *I. canaria* and *I. serini* would not excyst from oocysts stored for more than 6 months. For structural studies of *I. canaria* and *I. serini* sporozoites, Box (1) used sporocysts obtained from oocysts, which had been stored for less than 1 month and obtained numerous sporozoites. Most of the sporozoites appeared to have excysted after exposure to the excysting

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Fig. **10-15**. [Electronmicrographs of the oocyst walls. Figs. 10-12, *I. serini*; Figs. 13-15, *I. canaria*.] **10**. Oocyst wall with 3 layers. Note unit membrane (arrows) in outer layer, fold in middle (Mw) and outer layers (Ow) (upper right) and indentation (double arrow) of inner layer (Iw). Db, dense body. × 63,000. **11**. Tangential section of oocyst wall. Note fold of outer 2 layers at right side of micrograph, unit membrane (arrow) of outer layer (Ow) and indentations (double arrow) of inner layer (Iw). Db, dense body. × 105,000. **12**. Oocyst wall. Note 2 closely applied unit membranes (arrows) of inner layer (Iw) are seen adjacent to a dense line (arrow) of middle layer (Mw). Ow, outer layer of oocyst wall. × 168,000. **13**. Oocyst wall. The middle layer (Mw) has split in two. Unit membranes in inner (Iw) (arrow) and middle (Mw) (double arrow) layers are evident. Cc, cytoplasm of cultured cell; Db, dense body; Mv, membrane-bounded vesicle. × 105,000. **14**. Oocyst wall without inner layer. Unit membranes (double arrow) in middle layer (Mw) and adjacent to outer layer (Ow). A large spherical electron-lucent body (Pb) is seen in outer layer. Cc, cytoplasm of cultured cell. × 168,000. **15**. Tangential section of middle (Mw) and outer (Ow) layers of oocyst wall. × 168,000.

fluid for 15 or more min (Box, personal communication). In the present study, we found that sporozoites excysted from only some of the sporocysts from 5-month-old oocysts of *I. serini*, and not at all from sporocysts obtained from 15-month-old *I. canaria* oocysts. These findings suggest that the ability of sporozoites to excyst decreases rapidly in oocysts of these species stored at 4 C in $K_2Cr_2O_7$ solution, even though all structures within the oocysts appear normal.

The details of excystation have been studied in only a few species of *Isospora*. Sporocysts of some species have Stieda bodies, those of others do not. During excystation of *I. canis* (18), *Isospora endocallimici* Duszynski & File, 1974 (6) and *Isospora bigemina* (Stiles, 1891) Lühe, 1906 (large form) (unpublished data) sporocysts, which lack Stieda bodies, the sporocyst walls collapse and the sporozoites escape randomly. In an electron microscope study, Speer et al. (18) found that the sporocyst wall of *I. canis* consists of 4 curved plates. They suggested that the trypsin-sodium taurocholate acts upon the sites of apposition between 2 plates resulting in rupture of the sporocyst wall and collapse of the sporocyst. The sporocyst wall of *Sarcocystis tenella* Railliet, 1886 (11) is structurally similar to that of *I. canis*. Excystation in *S. tenella* may, therefore, be very similar to that in *I. canis*, *I. endocallimici* and *I. bigemina*. Excystation of *I. serini* (present study) and *I. marquardti* (4) sporocysts, which have Stieda bodies, is similar to that observed in *Eimeria* spp. (2, 5, 7, 14). In these species, the sporozoites exit through a gap at one pole of the sporocyst created by dissolution of the Stieda body, which occurs in the presence of the trypsin-sodium taurocholate. Thus, 2 considerably different means of excystation are found within the genus *Isospora*. Therefore, it seems likely that even further variation in the excystation process may exist in other species of *Isospora* as well as of *Eimeria*, *Sarcocystis*, and *Toxoplasma*.

The fine structure of the oocyst wall has been described for only a few species of coccidia (3, 9-19). In most of these, the wall has at least 2 prominent layers, usually an electron-lucent inner and a more osmiophilic outer layer. Soon after fertilization of a macrogamete by a microgamete, these 2 primary layers are formed at the surface of the zygote by disaggregation and then fusion of 2 types of wall-forming bodies (9, 17). Type I bodies form the outer, and type II bodies the inner layer. In some, *Eimeria tenella* (Railliet & Lucet, 1891) Fantham, 1909 (10), *Eimeria acervulina* Tyzzer, 1929 (9), *I. canis* (18) and *I. serini* and *I. canaria* (present study), a 3rd layer usually consisting of one or more membranes, is present on the outside. In *Eimeria perforans* (Leuckart, 1879) Sluiter & Swellengrebel, 1912 (17), *E. acervulina* (9), and *E. tenella* (10), this outermost layer apparently represents the original limiting membrane of the merozoite. In most species, the innermost layer appears to be relatively consistent in thickness and appearance, being composed of fine granular electron-lucent material, whereas more variation in thickness and composition occurs in the outer 1 or 2 layers. Roberts et al. (14) found hexagonal columnar projections in the outer layer of *Eimeria callospermophili* Henry, 1932 oocysts, and in some specimens round electron-dense granules were scattered along the surface of the outer layer. In *I. canaria* and *I. serini*, we found similar spheroid electron-dense bodies in the middle layer. Electron-dense knoblike projections occur on the outer and middle layers of the oocyst walls of *Eimeria larimerensis* Vetterling, 1964 (14) and *I. canis* (18), respectively. We found relatively large electron-lucent bodies in the outer or middle layer of *I. canaria* oocysts, but not in those of *I. serini*.

Although the wall-forming bodies probably account for the formation of the bulk of the oocyst wall, membranes which have been seen in the walls of some species may also play an important role. Lee & Millard (9) found 2 external membranes in the wall of *E. acervulina* oocysts, the most peripheral one being the original membrane of the merozoite. A single membrane was found in the outermost portion of the inner layer in the oocyst wall of *E. callospermophili*, and in *E. larimerensis* a single membrane was observed on the outside of the oocyst (14). In *E. perforans*, Scholtyseck et al. (17) found 5 unit membranes in the oocyst wall. Vetterling et al. (19) reported that the oocyst wall of *Sarcocystis* sp. consisted almost entirely of 4 fused membranes which formed a dense outer layer, 6 nm thick, and an electron-lucent, thin inner layer, 7 nm thick. We found as many as 9 membranes in the oocyst wall of *I. canaria* and 3 in that of *I. serini*. These membranes may arise in part from the membranes surrounding the wall-forming bodies, which are incorporated into the wall during its formation.

According to Mehlhorn & Scholtyseck (11), unsporulated *S. tenella* oocysts were surrounded by a single electron-lucent wall, 0.25 μm thick, which shrank during sporulation to 0.1 μm. Scholtyseck & Voight (15) observed the outer layer of the oocyst wall of *E. perforans* was lost soon after wall formation was complete. These findings suggest that there may be considerable change in structure of the oocyst wall after its formation, particularly after the oocyst has left the host.

REFERENCES

1. Box ED. 1975. Exogenous stages of *Isospora serini* (Aragão, 1933) and *Isospora canaria* sp. n. in the canary (*Serinus canarius*, L.). *J. Protozool.* **22**, 165-9.

2. Doran DJ, Farr MM. 1962. Excystation of the poultry coccidium *Eimeria acervulina*. *J. Protozool.* **9**, 154-61.

3. Dubremetz JF, Yvore P. 1971. Élaboration de la coque oocystique chez la coccidie *Eimeria necatrix* Johnson 1930 (Sporozoaires, Coccidiomorphes). Étude au microscope électronique. *C. R. Soc. Biol. Paris* **165**, 862.

4. Duszynski DW, Brunson JT. 1972. The structure of the oocyst and the excystation process of *Isospora marquardti* sp. n. from the Colorado pika, *Ochotona princeps*. *J. Protozool.* **19**, 257-9.

5. ———, ——— 1973. Structure of the oocysts and excystation processes of four *Eimeria* spp. (Protozoa: Eimeriidae) from the Colorado pika, *Ochotona princeps*. *J. Parasitol.* **59**, 28-34.

6. ———, File SK. 1974. Structure of the oocyst and excystation of sporozoites of *Isospora endocallimici* n. sp., from the marmoset *Callimico goeldii*. *Trans. Am. Microsc. Soc.* **93**, 403-8.

7. Hammond DM, Ernst JV, Chobotar B. 1970. Composition and function of the substiedal body in the sporocysts of *Eimeria utahensis*. *J. Parasitol.* **56**, 618-9.

8. Karnovsky MJ. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.* **27**, 137A-8A.

9. Lee DL, Millard BJ. 1971. The structure and development of the macrogamete and oocyst of *Eimeria acervulina*. *Parasitology* **62**, 31-4.

10. McLaren DJ. 1969. Observations on the fine structural changes associated with schizogony and gametogony in *Eimeria tenella*. *Parasitology* **59**, 563-74.

11. Mehlhorn H, Scholtyseck E. 1974. Licht und elektronenmikroskopishe Untersuchungen an Entwicklungsstadien von *Sarcocystis tenella* aus der Darmwand der Hauskatze. I. Die Oocysten und Sporocysten. *Z. Parasitenk.* **43**, 251-70.

12. Nyberg PA, Knapp SE. 1970. Scanning electron microscopy of *Eimeria tenella* oocysts. *Proc. Helm. Soc. Wash.* **37**, 29-32.

13. ———, ———. 1970. Effect of sodium hypochlorite on the oocyst wall of *Eimeria tenella* as shown by electron microscopy. *Proc. Helm. Soc. Wash.* **37**, 32-6.

14. Roberts WL, Speer CA, Hammond DM. 1970. Electron and light microscope studies of the oocyst walls, sporocysts, and excysting sporozoites of *Eimeria callospermophili* and *E. larimerensis*. *J. Parasitol.* **56**, 918-26.

15. Scholtyseck E, Voight WH. 1963. Die Bildung der Oocystenhülle bei *Eimeria perforans* (Sporozoa). *Z. Zellforsch. Mikrosk. Anat.* **62**, 279-92.

16. ———, Weissenfels N. 1956. Elektronenmikroskopische Untersuchungen von Sporozoen. I. Die Oocystenmembran des Hühnercoccids *Eimeria tenella*. *Arch. Protozool.* **101**, 215-22.
17. ———, Mehlhorn H, Hammond DM. 1971. Fine structure of macrogamonts and oocysts of coccidia and related organisms. *Z. Parasitenk.* **37**, 1-43.
18. Speer CA, Hammond DM, Mahrt JL, Roberts WL. 1973. Structure of the oocyst and sporocyst walls and excystation of sporozoites of *Isospora canis*. *J. Parasitol.* **59**, 35-40.
19. Vetterling JM, Pacheco ND, Fayer R. 1973. Fine structure of gametogony and oocyst formation in *Sarcocystis* sp. in cell culture. *J. Protozool.* **20**, 613-21.