LIGHT AND ELECTRON MICROSCOPIC OBSERVATIONS OF *IN VITRO* EXCYSTATION AND ASEXUAL DEVELOPMENT OF TWO BOVINE *SARCOCYSTIS* SPECIES (APICOMPLEXA, SARCOCYSTIDAE).

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of Master of Science

in the Department of Pathology and Microbiology

Faculty of Veterinary Medicine

University of Prince Edward Island

Janice M. Gillis

Charlottetown, P.E.I.

November 22, 1993

ISBN 0-315-88455-X

CONDITIONS OF USE OF THESIS

The author has agreed that the Library, University of Prince Edward Island, may make this thesis freely available for inspection. Moreover, the author has agreed that permission for extensive copying of this thesis for scholarly purposes may be granted by the professor or professors who supervised the thesis work recorded herein or, in their absence, by the Chairperson of the Department or the Dean of the Faculty in which the thesis work was done. It is understood that due recognition will be given to the author of this thesis and to the University of Prince Edward Island in any use of the material in this thesis. Copying or publication or any other use of the thesis for financial gain without approval by the University of Prince Edward Island and the author's written permission is prohibited.

Requests for permission to copy or to make any other use of material in this thesis in whole or in part should be addressed to:

Chairman of the Department of Pathology and Microbiology,

Faculty of Veterinary Medicine

University of Prince Edward Island

Charlottetown, P.E.I.

Canada, C1A 4P3

SIGNATURE PAGES

iii-iv

REMOVED

ABSTRACT

Acute infection with species of *Sarcocystis* (Phylum Apicomplexa) can cause severe, often fatal disease in bovine intermediate hosts. Decreased growth, production, and reproductive success can also occur with chronic sarcocystosis. Elucidation of the mechanism of excystation (i.e. release of infective sporozoites from sporocysts) and host-parasite interactions involved in *Sarcocystis* spp. infections are necessary to develop preventive control measures. Asexual developmental stages of *Sarcocystis cruzi* and *S. hirsuta* were utilized in *in vitro* light and electron microscopic studies. Specifically, excystation of sporozoites and their subsequent invasion of and development in host cells were examined. Various excystation protocols were evaluated. Deletion of trypsinization and addition of an elutriation procedure produced highly variable excystation rates, with viable sporozoites. Addition of proteinase K gave higher, more consistent rates and better reduced debris in preparations of *S. cruzi* sporozoites. The process and ultrastructure of excystation of *S. cruzi* and *S. hirsuta* were similar. Neither sporocysts nor sporozoites were sufficiently morphologically distinct for rapid, reliable species differentiation. However, significant differences ($P=0.000$, Student's T-test) in length, area, and perimeter exist. Both sporocysts and sporozoites of *S. cruzi* are larger than *S. hirsuta*. The tissue culture and processing technique of Kingsley and Cole (1988) was modified for host cell invasion experiments. The time of maximum host cell penetration post inoculation with *S. cruzi* sporozoites was 15 min and 1 h for bovine pulmonary artery endothelial cells and rat heart myoblasts, respectively. Scanning and transmission electron microscopic evidence supported active invasion by sporozoites as one component of cell invasion. Asexual development of *S. cruzi* occurred in each of four tissue culture lines tested (Bovine pulmonary artery endothelium, Rat heart myoblasts, Rat skeletal muscle myoblasts, and African green monkey kidney cells). Schizogony was visualized in all cell types; sarcocysts did not develop. Suitability of host cell type was assessed by quantification of merozoite production. Although endothelial monolayers were most productive *in vitro*, reduced efficiency of the merozoite harvesting technique used herein did not allow extrapolation to *in vivo* development. That schizogony and merozoite release occurred in various host species and cell types indicates that, *in vitro*, the range of host cell specificity is broad.

DEDICATION

To my parents, Louis and Mary McKenna, with much appreciation.

ACKNOWLEDGEMENTS

I am beholden to my faculty supervisor, Dr. Richard J. Cawthorn, and the Department of Pathology and Microbiology, for the opportunity to conduct graduate research and study. Dr. Cawthorn's support and guidance is gratefully acknowledged. Also, direction provided by MSc. Supervisory Committee members: Doctors R.J. Long, A. Lopez, F. Markham, and G. Wright was sincerely appreciated.

Special thanks are extended to Dr. James Amend, Department of Anatomy and Physiology, for his assistance particularly, in the initial phase of this project. Technical and moral support provided by Dana Coles, Robert Maloney, Dianne Friesen, Susan Plourde was vital. Additionally, I thank Eileen Kinch and Sharon Martin for their patience and help throughout preparation of this manuscript.

Research was facilitated through awards provided by:

- Natural Sciences and Engineering Research Council of Canada Postgraduate Scholarship
- Norden Graduate Research Award
- Saint Dunstan's University Graduate Scholarship
- Smith-Kline Beecham Research Award to J.M. Gillis

Also supported, in part, by operating grant from the Natural Sciences and Engineering Research Council of Canada to R.J. Cawthorn.

Last but foremost, I thank my husband, Edwin, for being an inspiration. Moreover, his nurturing of our two daughters, Jaimee and Emma, has ultimately permitted completion of this thesis.

TABLE OF CONTENTS

LIST OF TABLES PAGE

LIST OF FIGURES

LIST OF FIGURE ABBREVIATIONS

Ag	-	Amylopectin granule
Ac	-	Apical complex
Ar	-	Apical rings
Cm	-	Central microtubules
Co	-	Conoid
Cb	-	Crystalloid body
Cs	-	Conoid subunits (=microtubules)
Db	-	Dense body/granule
Dr	-	Duct of rhoptry
Er	-	Endoplasmic reticulum
Gc	-	Golgi complex
Hc	-	Host cell cytoplasm
Hn	-	Host cell nucleus
Hp	-	Host cell plasmalemma
Is	-	Immature schizont (= meront)
Iw	-	Inner layer of sporocyst wall
Im	-	Inner membrane complex
Ip	-	Interposed strip of sporocyst wall
Lb	-	Lipid body
Ms	-	Mature schizont/meront
Mz	-	Merozoite
Mn	-	Microneme
Mi	-	Mitochondrion
Mt	-	Microtubule
Mv	-	Microvilli
Mw	-	Membranous whorls

Mb	-	Multivesicular body
No	-	Nucleolus
Nu	-	Nucleus
Ow	-	Outer layer of sporocyst wall
Pp	-	Parasite plasmalemma
Pv	-	Parasitophorous vacuole
Pe	-	Pellicle
Pr	-	Polar rings
Rh	-	Rhoptry
Sc	-	Sporocyst
Sp	-	Sporocyst plate
Sr	-	Sporocyst residuum = Residual granules
Ss	-	Sporocyst suture
Sw	-	Sporocyst wall
Sz	-	Sporozoite
Sbr	-	Sporozoite body ridges
Sm	-	Subpellicular microtubule
Vn	-	Venation of sporocyst

CHAPTER I - INTRODUCTION

Sarcocystis species are protozoan parasites of the phylum Apicomplexa (Levine, 1970) having indirect life cycles (Frenkel, 1977). These cyst-forming coccidia (see section 2.1.1 for complete classification) have worldwide distribution in reptiles and all higher vertebrates including human beings (Fayer and Dubey, 1986). Over 120 named species exist (Levine, 1988). Information vital to clarification and understanding of systematics, developmental cycles, and effects on host populations is only recently available (Cawthorn and Speer, 1990). Species of *Sarcocystis* which are pathogenic cause a disease (i.e. sarcocystosis = sarcosporidiosis = sarcosporidiasis) which is of greater importance in the intermediate (prey/herbivore) host(s) than in the definitive (predator/carnivore) host(s) (Dubey *et al.*, 1989). Both fatal, acute infections and chronic illness result in substantial economic losses to livestock producers (Dubey and Fayer, 1983). Non-fatal cases can cause poor growth, reproductive failure, and reduced productivity including decreased milk production (Stalheim *et al.*, 1980; Dubey and Fayer, 1983; Herbert and Smith, 1987). For literature reviews of sarcocystosis and *Sarcocystis* spp. see Smith (1981), Dubey and Fayer (1983), Herbert and Smith (1987), Dubey *et al.* (1989), and Cawthorn and Speer (1990).

Although *Sarcocystis* spp. have been intensively studied since Heydorn and Rommel's (1972) discovery of the complete heteroxenous life cycle, several basic questions remain unanswered. Details of ultrastructure and life cycles of many *Sarcocystis* spp. are lacking (Levine, 1988). Host and cell specificity for each

developmental stage and their pathogenic mechanisms are undetermined. Mechanisms of host cell invasion and evasion of host cell defences are also unknown. Elucidation of the host-parasite interactions involved in *Sarcocystis* spp. infections should facilitate the development of preventive or protective control measures such as accurate, early diagnosis; anti-*Sarcocystis* vaccines; and effective chemotherapy.

The following chapter provides background information on *Sarcocystis* spp. and highlights recent studies of sarcocystosis. *Sarcocystis cruzi* and *S. hirsuta,* which have canid and felid definitive hosts, respectively, are common parasitic infections of cattle. These two species are particularly detailed in the following literature review since *S. cruzi* and *S. hirsuta* were used *in vitro* studies reported herein. The prospectus for the present study (section 2.4) provides the rationale for, and an outline of, subsequent experiments. The specifics of *Sarcocystis* spp. excystment and *in vitro* development including host cell invasion are reviewed in pertinent experimental chapters.

CHAPTER II - LITERATURE REVIEW

2.1 Species of *Sarcocystis* (general)

2.1.1 History and classification

Parasitic cysts found in the skeletal muscle of a house mouse *Mus musculus* (Miescher, 1843) were the first recognized stage of *Sarcocystis* spp., hence the generic name (i.e. sarco = muscle). This protozoan parasite is classified as:

Phylum	-	Apicomplexa; Levine, 1970
Class	-	Sporozoasida; Leuckart, 1879
Order	-	Euccidiorida; Leger and Duboscq, 1910
Family	-	Sarcocystidae; Poche, 1913
Genus	-	*Sarcocystis*; Lankester, 1882
Species	-	About 125 named species (56 species of known definitive and intermediate host(s))

Type specimen	-	*S. miescheriana* (Kuhn, 1865) Labbe, 1899
Related genera	-	*Toxoplasma*, *Isospora*, *Besnoitia*, *Hammondia*, *Frenkelia*, *Caryospora*, and *Neospora*

The historical taxonomy of *Sarcocystis* species highlights problems of ambiguous nomenclature and misclassification. The subject was recently reviewed (Dubey *et al.*, 1989; Cawthorn and Speer, 1990). To avoid taxonomic discrepancies, proper identification of organisms should precede classification (Bandoni and Duszynski, 1988). Previously, *Sarcocystis* species designation was by host occurrence and appearance of sarcocysts in host species (Wenyon, 1926). Confusion arose from improper or inadequate description of type specimens and demonstration of structurally distinct sarcocysts within a given host species (Dubey

et al., 1989). Following discovery of heteroxenous life cycles, further species designations were based on intermediate and definitive host specificity (Levine and Tadros, 1980).

Earlier documentation did not allow verification of previously described species (e.g. *S. cruzi*, *S. hirsuta*, *S. tenella*) with those species of known life cycles; therefore, a new nomenclature system combining names of intermediate and definitive hosts (e.g. *S. bovicanis* = *S. cruzi*, *S. bovifelis* = *S. hirsuta*, *S. ovifelis* = *S. tenella*) was proposed (Heydorn *et al.*, 1975). Levine (1986) contested replacement of old names and acrimonious debate ensued (Frenkel *et al.*, 1987; Levine and Baker, 1987). Frenkel *et al.* (1979, 1984) petitioned the International Commission of Zoological Nomenclature to rename *Sarcocystis* spp.; however, original names were upheld (Melville, 1980, 1984). Ultimately, the Commission-sanctioned names may be less confusing since cases of relatively broad host specificity occur within *Sarcocystis* spp. (Crum *et al.*, 1981; Fayer *et al.*, 1982). Additionally, two or more species of *Sarcocystis* can simultaneously develop in an intermediate or a definitive host (reviewed in Cawthorn and Speer, 1990).

Confirmation of heteroxeneity among apicomplexans also prompted reevaluation of Hoare's (1933) classification scheme which was based on number of sporozoites per sporocyst and sporocysts per oocyst and assumed all coccidia to be monoxenous (Levine, 1987). Subsequent classifications defined *Sarcocystis* spp. as either facultatively heteroxenous or obligatorily heteroxenous (Levine, 1982). With discovery of monoxenous *Sarcocystis* spp., (Pak, 1979; Matuschka

1987), taxonomic reevaluation of the *Sarcocystis* genus or redefinition of taxa levels are required. History (i.e. genealogy of a species) should provide the most stable (i.e. maximum information with minimal overlap) means to catalog organisms; however, the most recent, widely accepted classification of coccidia (Levine, 1988) is partially one of convenience. Thus, conflicts arise when evolutionary relationships are overridden.

The taxonomy of *Sarcocystis* spp. currently depends on differences in parasite morphology and host specificity (Levine and Tadros, 1980). Variability of host specificity, mentioned above, limits usefulness of this criterion as the discriminating factor. Barta (1989) cautions against use of either host or tissue specificity or heteroxenous versus monoxenous life cycles for classification at higher taxa levels. The use of morphologic data as a means of classifying organisms has been critiqued and the limitations of its use discussed (Long and Joyner, 1984). Cawthorn and Speer (1990) noted that sarcocyst walls are species-specific, although they are not species unique. Moreover, heterogeneity of sarcocysts previously assumed to be a single species requires further investigation (Ford *et al.*, 1987). Recent biochemical techniques (including isoenzyme electrophoresis) have, however, provided genetic evidence supporting classification of *Sarcocystis* spp. by morphological characters (O'Donoghue *et al.*, 1986; Ford *et al.*, 1987). Isoelectric focusing has also permitted unequivocal differentiation of three *Sarcocystis* spp. in European sheep (*Ovis* spp. from local breeders in Lower Saxony, West Germany) (Tenter *et al.*, 1989).

Ideally, the maximum number of obtainable characters or traits should be examined to determine relationships in phylogenetic classifications (Fink, 1986). Thus any classification using a single criterion (e.g. sarcocyst structure, length of pre-patent period, method of excystation, site or host specificity) or criteria in limited combination proves less valuable. Overall, improved presentation of type specimens of *Sarcocystis* spp. (Bandoni and Duszynski, 1988) and identification of additional features of a given species (eg. physiological and behavioral characteristics) should facilitate species designations (O'Donoghue *et al.*, 1986).

Future use of antibody/antigen detection techniques, protein and DNA sequencing and DNA/RNA hybridization to differentiate and characterize parasitic species should result in further rearranging and refining of *Sarcocystis* taxonomy. Ribosomal RNA sequencing has already raised serious questions concerning validity of the present classification of apicomplexans (Johnson *et al.*, 1987); surprising genetic diversity may lead to expansion of higher taxa (Johnson and Baverstock, 1989). Additionally, the hapantotype concept (reviewed in Williams, 1986) should facilitate classification of species of the genus *Sarcocystis* with relatively complex life cycles. This concept replaces a single 'type specimen' with a group of specimens representing several stages in the life cycle of a given species.

2.1.2 Life cycle

Discussion of classification (section 2.1.1) has alluded to developmental variations which exist among species of *Sarcocystis*. The life cycle of *S. cruzi* is

an example of an obligatory heteroxenous life cycle with sexual (gametogony and sporogony) and asexual (schizogony=merogony) development occurring in definitive and intermediate hosts, respectively. Figures 2.1 and 2.2 illustrate the basic developmental cycle of *Sarcocystis* spp. and particulars of the *S. cruzi* life cycle, respectively. Details of parasite development differ among *Sarcocystis* species, e.g. two generations of meronts occurring in *S. cruzi* have not been observed in most other species; sarcocysts are usually the only asexual stage found (Levine, 1982). Additionally, McKenna and Charleston (1991) suggest that location of excystation (i.e. the small intestine of intermediate hosts) may be variable. The life cycles of many *Sarcocystis* spp. are still unknown; both definitive and intermediate hosts have not been identified for all named species. *Sarcocystis cruzi* demonstrates transmission via predator - prey or scavenger - carrion relationships. Until recently *Sarcocystis* asexual stages in canine or feline definitive hosts were assumed to be aberrant infections in debilitated or immune compromised hosts (Hill *et al.*, 1988). Dubey and Speer's (1991) characterization of *S. canis* n. sp. represents the first report of a *Sarcocystis* species that forms schizonts in dog tissue. With the discovery of the life cycle and mode of transmission of this new species, the term "Predator-Prey Coccidia" may no longer be synonymous with "The Sarcocystidae". Alternate mechanisms for transmission of *Sarcocystis* spp. are discussed in Epidemiology of infection and disease (section 2.1.3).

Definitive hosts of *S. cruzi* (i.e. Canidae) are infected by ingesting either

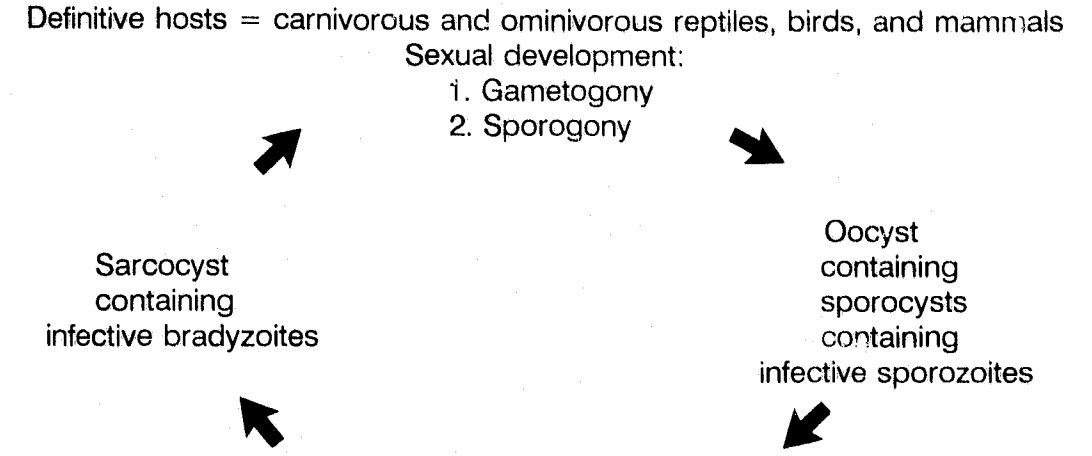


Fig. 2.1 - Developmental Cycle of Species of *Sarcocystis*

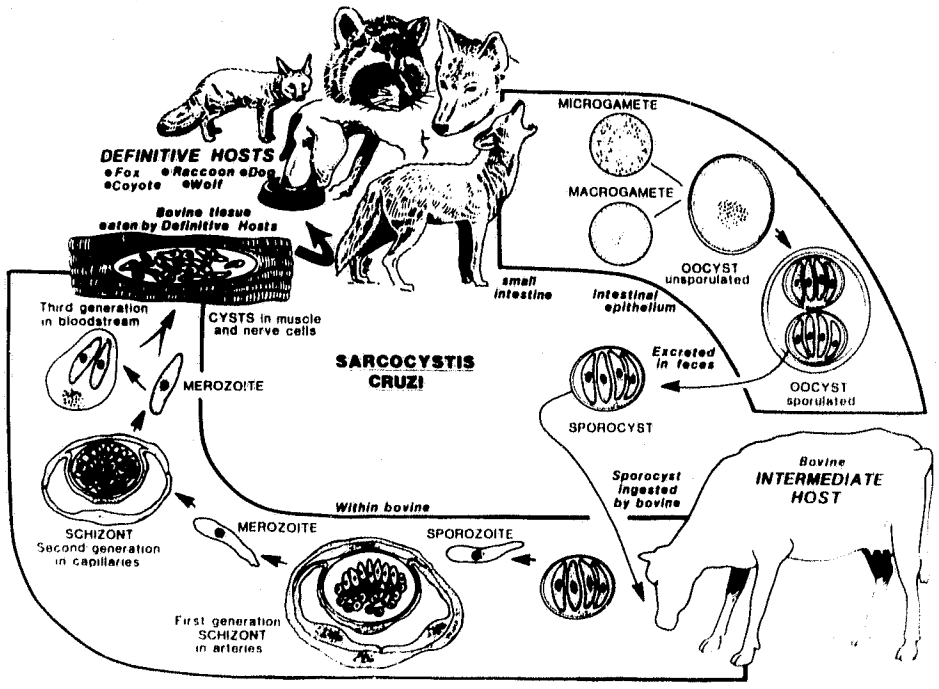


Fig. 2.2 - Life Cycle of *Sarcocystis cruzi* (From Dubey, JP, Speer, CA, and Fayer, R. *Sarcocystosis of Animals and Man*, 1989. With permission.)

muscle or nerve tissue of intermediate hosts (Bovidae) containing infective sarcocysts (Fayer *et al.*, 1976). Banana-shaped bradyzoites, released upon digestion of sarcocysts, invade epithelial cells of the small intestine and transform into male (micro-) and female (macro-) gametes (Fayer, 1974). Fertilization takes place in enterocytes or goblet cells and the resulting zygotes develop into oocysts in the lamina propria (Sheffield and Fayer, 1980; Dubey, 1982). An oocyst undergoes sporulation producing two sporocysts each containing four sporozoites (see Fig. 2.2). Rupture of the fragile oocyst wall may result from intestinal contractions which force sporulated oocysts from the lamina propria through the villar tips into the intestinal lumen (Box and Duszynski, 1980). Rarely, both oocysts and sporocysts are found in faeces (Fayer and Dubey, 1986).

Intermediate hosts are infected by ingesting oocyst- or sporocyst-contaminated feed or water. Digestive enzymes and bile induce excystation of sporozoites in the small intestine. The path of *S. cruzi* sporozoites from point of excystation until initial development in the arteries of mesenteric lymph nodes is not known; however, sporozoite transport via portal circulation is postulated (Dubey, 1982). Apparently two generations of schizogony (i.e. multiple fission) / merogony (i.e. fission producing merozoites) precede formation of intramuscular cysts (Dubey, 1981). First-generation schizonts (= meronts) develop to merozoites by the process of endopolygeny (see section 2.2.2 for detail of methods of asexual division). Development occurs in close association with endothelium of arteries, particularly those of the intestine and mesenteric lymph

nodes (Fayer, 1977a; Dubey *et al.*, 1980). The second schizogonous generation occurs in capillaries throughout the body (Fayer and Johnson, 1973). Host cell type parasitised by 1st- and 2nd-generation schizonts is unclear (see Pacheco and Fayer, 1977; Dubey, 1982; Dubey *et al.*, 1982; Dubey and Fayer, 1983; Speer and Dubey, 1986). Endothelial cells are generally accepted as the host cell; however, schizonts are frequently observed below the endothelial layer in unidentified cells. Additional division of merozoites by endodyogeny occurs in mononuclear cells (Fayer, 1979; Dubey, 1982); the extent of this type of reproduction in *Sarcocystis* species is not known. Terminal-generation merozoites released in the blood stream eventually penetrate muscle (rarely nerve) cells and form sarcocysts by endodyogeny (Fayer and Johnson, 1974). Immature sarcocysts contain metrocytes which are not infective to the definitive host (Mehlhorn and Frenkel, 1980). Infective bradyzoites develop approximately 10 weeks after ingestion of sporocysts (Dubey, 1976). Completion of the life cycle occurs when the definitive host ingests tissue harbouring infective cysts.

2.1.3 Epidemiology of infection and disease

Sarcocystis species can have cosmopolitan distribution with relatively high prevalence (Cawthorn and Speer, 1990). It is only an assumption that occurrence of a particular species does not vary in different host populations throughout the world (Ford *et al.*, 1987). Dubey *et al.* (1989) reported that some *Sarcocystis* spp. are found less frequently than others, e.g. felids appear to be less suitable definitive hosts. The geographical locale of a given species of *Sarcocystis* is

limited by distribution of intermediate and definitive hosts; however, a single species may use several of either type of host. Moreover, either host type can be infected with several *Sarcocystis* species (Cawthorn and Speer, 1990). That some lizard species practising autotomy and cannibalism can serve as intermediate and/or definitive host for *Sarcocystis* (Matuschka and Bannert, 1989) has raised the question whether cannibalism or scavenging has a major role in transmission of *Sarcocystis* spp. among other carnivorous or omnivorous hosts. Invertebrates can act as transport hosts for *Sarcocystis* spp. (Smith and Frenkel, 1978; Markus, 1980;) and they have contributed to serious outbreaks of *S. falcatula* in psittacines (Clubb and Frenkel, 1992). Whether these hosts significantly enhance transmission of disease in all species of *Sarcocystis* has not been adequately determined in field studies. Experimental designs should ensure contamination via this route does not occur (Smith and Frenkel, 1978).

Dubey *et al.* (1989) highlight factors contributing to high prevalence of infection observed in *Sarcocystis* spp., notably: a) use of multiple hosts by a single species, b) harbouring of several species in a single host, and c) prolonged shedding of large numbers (Ford, 1974; Dubey, 1980) of sporulated (i.e. immediately infectious) oocysts by definitive hosts after ingestion of relatively small amounts of infected intermediate host tissue (Fayer, 1977; Dubey, 1980). Significant immunity to reshedding of sporocysts is not observed (Heydorn and Rommel, 1972; Srivastava *et al.*, 1987). Ability of sporulated oocysts and

sporocysts to withstand freezing temperatures, and thus overwinter, also facilitates infections (Dubey *et al.*, 1978; Cawthorn *et al.*, 1984; Leek, 1986).

Overall, the genus *Sarcocystis* contains highly successful parasites. There are isolated cases wherein hosts are rarely infected with *Sarcocystis* spp. (Dubey *et al.*, 1989). Contrasting and comparing these hosts with those which are commonly infected may provide insight into the factors which affect host susceptibility and resistance. Ultimately, the mechanisms of host-parasite interactions influential in infection and disease must be determined.

2.2 Description of *Sarcocystis* spp. of cattle

2.2.1 Host-parasite relationships

Three species of *Sarcocystis* parasitize cattle. *Sarcocystis cruzi* (Hasselmann, 1926; Wenyon, 1926); *S. hirsuta* (Moule, 1888); and *S. hominis* (Railliet and Lucet, 1891; Dubey, 1976) have Canidae (dog, coyote, fox, wolf and raccoon), Felidae (cat), and primates (human, monkey, and baboon) as definitive hosts, respectively (Dubey *et al.*, 1989). *Sarcocystis* spp. are generally more specific for intermediate hosts than for definitive hosts (Dubey and Fayer, 1983); however, cattle and domestic cats are the only hosts of *S. hirsuta* (Dubey, 1982; Dubey, 1983; Bottner *et al.*, 1987).

Infections of *S. hominis* in humans are commonly reported in Europe but not in the United States (Fayer and Dubey, 1986); the extent of infection resulting in clinical illness is not known. Levine (1985) suggests *S. hominis* is more

common in humans than is generally believed. Rommel (1985) notes 7%, 5%, and 15% of randomly collected faecal samples from humans, cats, and dogs, respectively, contain sporocysts; however, this represents all *Sarcocystis* spp., including those of cattle. Bovine *Sarcocystis* spp. are considered nonpathogenic in the above definitive hosts. Human hosts with natural infections of *S. hominis* have, however, reported nausea, abdominal pain, diarrhoea, and anorexia (Dubey, 1976; Dubey and Fayer, 1983). Diarrhoea also occurs in experimental dogs and other carnivores fed *S. cruzi*-infected beef; however, change in diet could influence this nonspecific clinical sign (Dubey and Fayer, 1983).

Disease (sarcocystosis) is of greater economic concern in intermediate hosts. Sarcocysts found in muscle are usually described as innocuous, eliciting neither host response nor clinical signs. Filho and Miraglia (1977) have reported that sarcocysts produce a toxin, sarcocystin, but its composition and effect(s) are not adequately detailed in the literature. Maturation and rupture of schizogonous phases preceding cyst formation is the primary cause of severe acute disease (see section 2.3.5 for details of pathogenesis). Of the three species of *Sarcocystis* which infect cattle, *S. cruzi* is the most common and most pathogenic (Fayer and Dubey, 1986). Prevalence of *Sarcocystis* spp. infection in cattle in the United States is extremely high. Virtually 100% of cattle show past or current infection using various techniques to detect anti-sarcocystosis antibodies or parasitic sarcocysts in muscle tissue (Fayer and Dubey, 1986; Dubey *et al.*, 1989). Prevalence in Canadian cattle seems to be comparable (Cawthorn, personal

communication) (see Table I for prevalence of *Sarcocystis* spp. of cattle elsewhere). As previously noted, simultaneous development of *S. cruzi*, *S. hirsuta*, or *S. hominis* can occur. However, the majority of observed sarcocysts are produced via the canine cycle (see Table II for comparison of the developmental stages of bovine *Sarcocystis* spp.). The pathogenicity of bovine *Sarcocystis* spp. is dose dependent. Mean lethal dose of sporocysts of *S. cruzi* is 200,000 (Fayer and Dubey, 1986). In the less pathogenic *S. hirsuta*-infections, cattle that ingest 100,000 or more sporocysts develop only fever, diarrhoea, and mild anaemia (Dubey, 1983). *Sarcocystis hominis* is least pathogenic with 1,000,000 sporocysts producing only mild anaemia in the bovine host (Dubey *et al*., 1988).

2.2.2 Ultrastructure of *Sarcocystis* spp.

The scope of this study limits the subsequent discussion of *Sarcocystis* spp. ultrastructure to the *asexual* life cycle stages found in intermediate hosts. Illustrations of ultrastructural features described below are presented in Figs. 2.3-2.9. For descriptions of the ultrastructure of the *sexual* life cycle stages in definitive hosts consult Dubey *et al*. (1989) and Cawthorn and Speer (1990). Additional details of merogony, gametogony and sporogony in coccidia are given by Chobotar and Scholtyseck (1982) and Ball and Pittilo (1990). Discussion of *S. hominis* is also limited in this and subsequent sections since a) this species was not utilized in experiments reported herein and b) few ultrastructural studies have been conducted on this mildly pathogenic species.

Table I - Prevalence of bovine *Sarcocystis* species in cattle with natural mixed infections (as determined by presence of tissue sarcocysts at slaughter).[a]

Country of origin	# of Animals examined	% positive for Sarcocystis spp.	% positive with		
			S. cruzi	*S. hirsuta*	*S. hominis*
Austria	1657	90.5	63.0	43.0	49.5
Brazil	168	96.0	69.0	8.0	4.0
Germany	1066	91.3	48.0	37.5	33.5
New Zealand	500	100.0	98.0	80.0	unknown

[a]Data from Gut (1982) and Dubey, Speer, and Fayer (1989).

Table II - Comparison of bovine developmental stages of *Sarcocystis cruzi* and *S. hirsuta*

	S. cruzi[a]	*S. hirsuta*[b]
1st-generation meronts		
Location	arterioles (several organs)	arterioles (mesentery and intestine)
Duration (DPI*)	7-26	7-23
Peak development (DPI)	15	15
Size of meronts (μm)	41.0 x 17.5	37.2 x 22.3
Size of merozoites (μm)	6.3 x 1.5	5.1 x 1.2
2nd-generation meronts		
Location	capillaries (several organs)	capillaries (striated muscle and heart)
Duration (DPI)	19-46	15-23
Peak development (DPI)	24-28	16
Size of meronts (μm)	19.6 x 11.0	13.9 x 6.5
Size of merozoites (μm)	7.9 x 1.5	4.0 x 1.5
Parasitemia Duration (DPI)	24-46	11
Intraleukocytic multiplication	Yes	No
Sarcocysts Location	skeletal muscle, heart, and CNS	skeletal muscle only
Maturation time (DPI)	86	75
Wall thickness (μm)	<1.0	3-6

[a]Data from Dubey (1982a)
[b]Data from Dubey (1982b) and Fayer and Dubey (1986)
*DPI = Days post infection

The motile, penetrating stages of *Sarcocystis* i.e. sporozoites, merozoites, and bradyzoites, share common ultrastructural features which typify apicomplexans. All features are illustrated in sporozoites of *S. cruzi*, the infective stage for cattle. This elongated, fusiform stage is bound by a tri-membrane pellicle consisting of an outer plasmalemma and an inner double membrane complex (IMC) (Chobotar and Scholtyseck, 1982) (Fig. 2.3). Freeze-fracture technique demonstrates that the latter is comprised of closely apposed flattened vesicles with a series of regularly arranged intramembranous particles (D'Haese *et al.*, 1977). The functional significance of this organelle is unknown.

Sporozoites of *Sarcocystis* spp. also possess an "apical complex" composed of a conoid, polar and apical rings, rhoptries, micronemes, subpellicular and central (=internal) microtubules and a micropore (Figs. 2.4-2.6). The most anterior structures are the electron-dense apical (= preconoidal) rings, of unknown function (Fig. 2.5). Two rings are the norm for *Sarcocystis* spp. zoites; however, a third is suspected in *S. tenella* bradyzoites (Porchet and Torpier, 1977). *Sarcocystis cruzi* sporozoites appear to have two apical and two polar rings. The latter are positioned and described as for other coccidia, i.e. polar ring one (PR1) at the terminal point of the IMC with anterior and posterior electron-dense, medial projections and polar ring two (PR2) located posteriorly, serving as an attachment site for subpellicular microtubules (SM).

Zoites of *Sarcocystis* spp. have 22 SM (Scholtyseck *et al.*, 1970) radiating at regular intervals from PR2 and extending 1/2 to 2/3 the length of the parasite

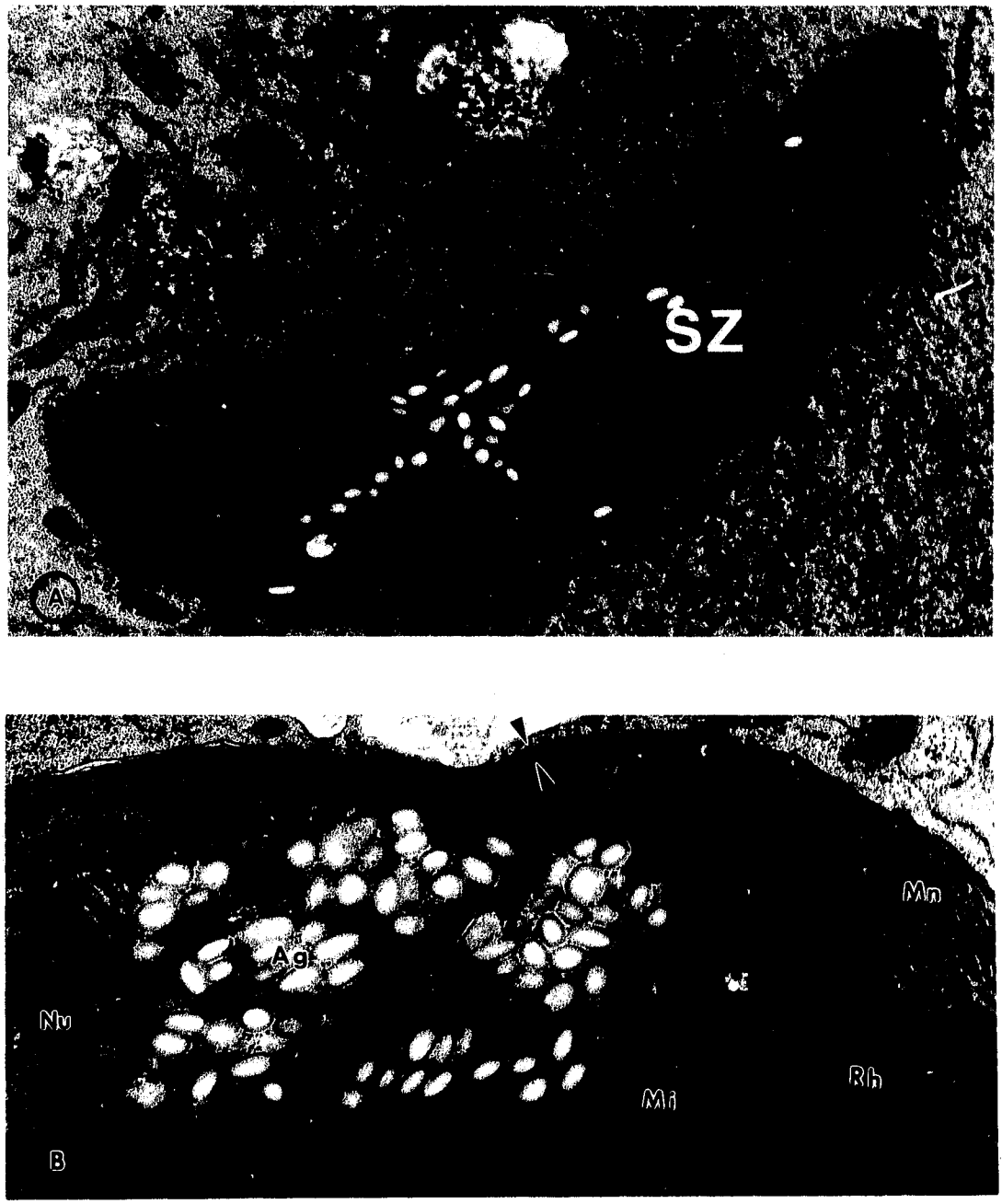


Fig. 2.3 A) Longitudinal section of *S. cruzi* sporozoite in tissue culture (x15,400).
B) Higher magnification of characteristic organelles and tri-membrane pellicle (arrowheads) of *S. cruzi* sporozoite (x25,000).

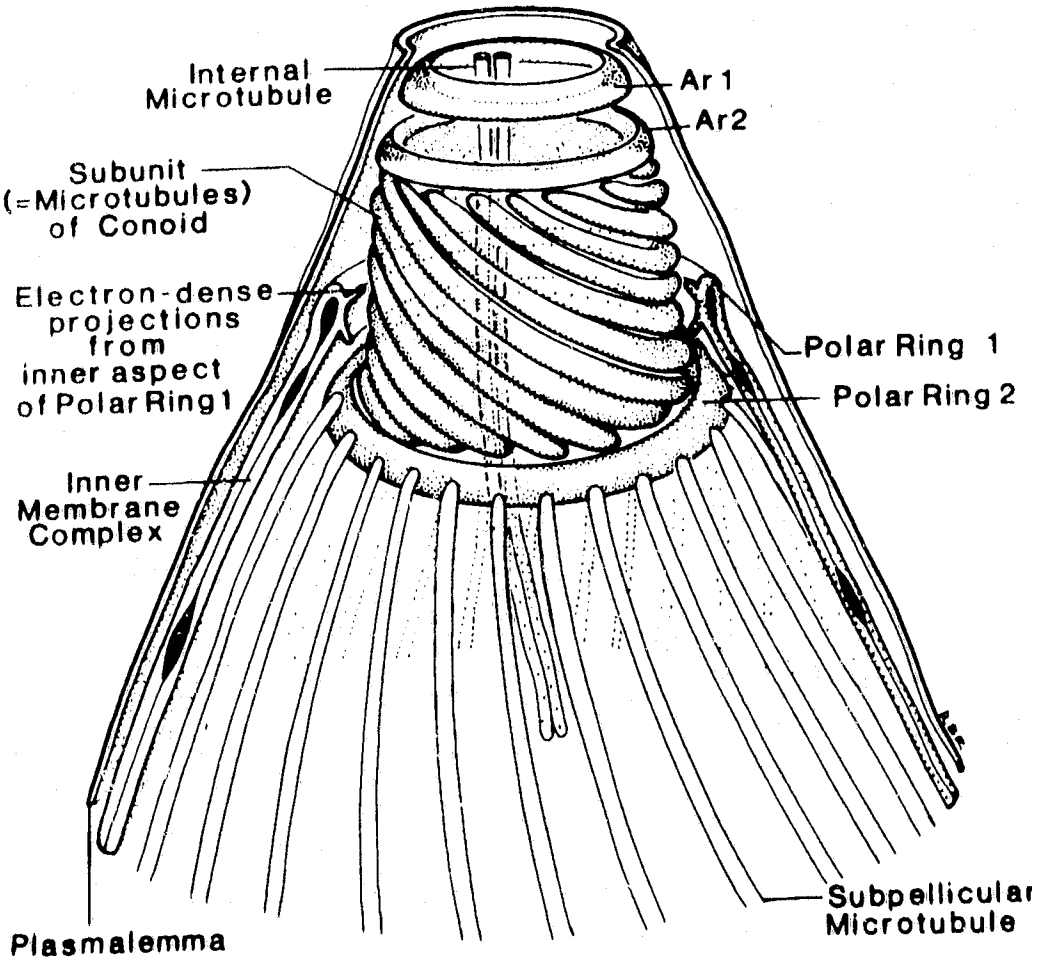


Fig. 2.4 Schematic representation of apical complex of *Sarcocystis* species. (From Dubey, JP, Speer, CA, and Fayer, R. *Sarcocystosis of Animals and Man*, 1989. With permission.)

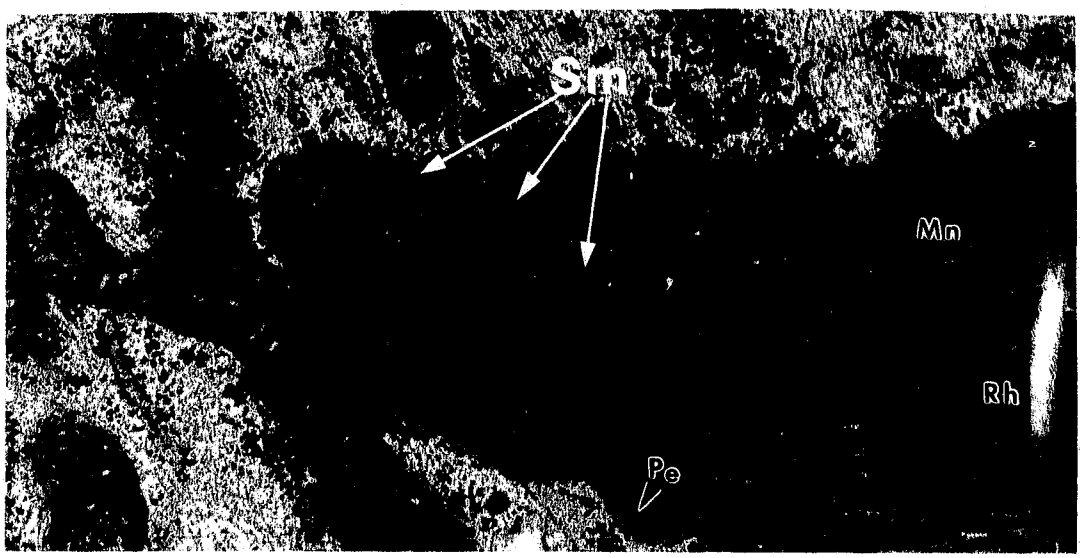


Fig. 2.5 Apical complex of *S. cruzi* sporozoite. Note apical and polar rings and subpellicular microtubules (x44,500).

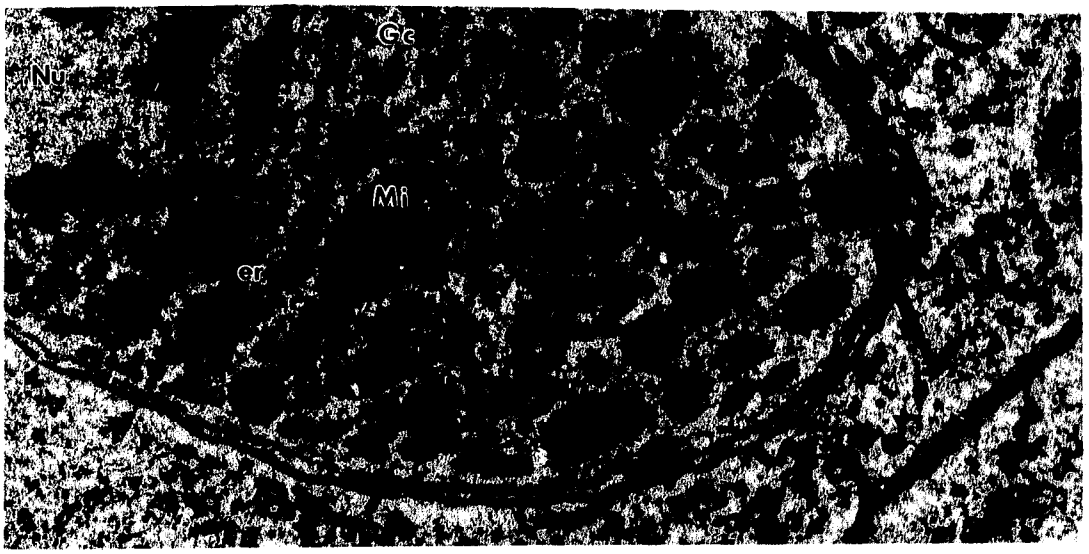


Fig. 2.6 Apical end of *S. cruzi* sporozoite. Note spirally arranged subunit (=microtubules) of conoid (arrow) (x33,000).

(Dubey *et al.*, 1989). Subpellicular microtubules likely provide stability and maintain structural integrity; however, their role in zoite motility is controversial. For example, antimicrotubular agents such as colchicine did not affect the motility of sporozoites of *Eimeria* sp. (Jensen and Edgar, 1976) but, they inhibited motility of *Sarcocystis* sp. merozoites (D'Haese *et al.*, 1977). A role in the transport of cytoplasmic elements has also been suggested for SM. Lastly, number and arrangement of SM is corresponds with the linear pattern observed in the IMC, suggesting a functional interaction (Porchet and Torpier, 1977; Dubremetz and Torpier, 1978).

The conoid, a prominent feature of the apical complex, is also of microtubular origin. This hollow, conical, truncated organelle is composed of spirally arranged subunits, closely associated with apical and polar rings (Scholtyseck *et al.*, 1970). Number of microtubule subunits varies by genus and species; 20 have been reported in *Sarcocystis* sp. (Porchet-Hennere, 1975). An additional two microtubules are longitudinally and eccentrically located in the cone's interior. The conoid and the two central (=internal) microtubules probably function in cell penetration and conoid extension and retraction, respectively (Chobotar and Scholtyseck, 1982).

All asexual stages of species of *Sarcocystis* have micropores (Dubey *et al.*, 1989). Both active and inactive micropores appear as invaginations of the plasmalemma which interrupt the inner membrane complex (Scholtyseck, 1973). Pores resemble pinocytotic vesicles except the neck of the invaginated membrane

is thickened to form an electron-dense collar. In cross section, thickenings of the plasmalemma and IMC appear as two concentric rings (Scholtyseck, 1979). Serial sectioning to determine the number of micropores in each stage has not been conducted; however, in other coccidia, numbers increase with size of the developmental stage, e.g. more micropores characterize schizont versus zoite stages (Ferguson *et al.*, 1977; Porchet-Hennere, 1977). Such a phenomenon supports the proposed role of micropores to be ingestion of nutrients for parasite growth and maintenance (Senaud *et al.*, 1976).

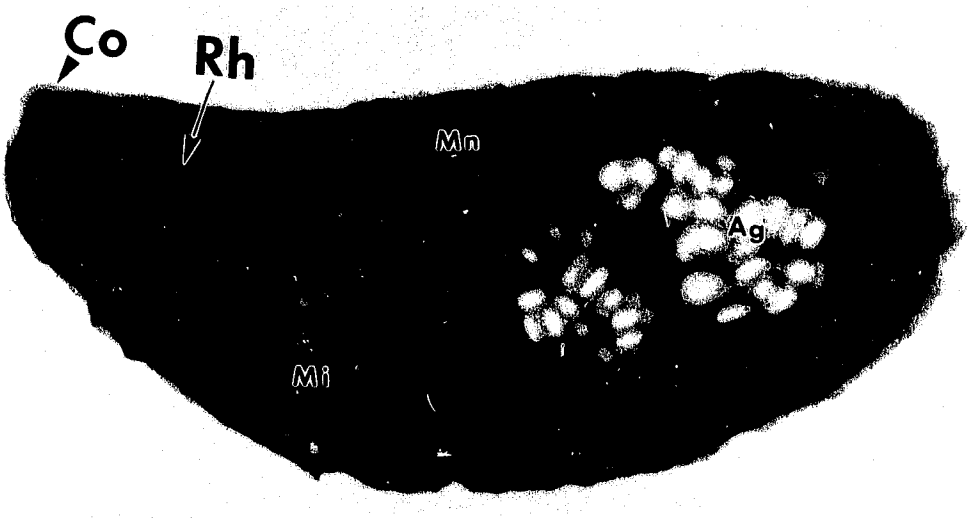
Rhoptries and micronemes, additional ultrastructural features of apicomplexans, are membrane-bound, electron-dense, elongate organelles concentrated in the anterior portion of zoites. The former are typically larger and club-shaped (Fig. 2.7) with their tapered duct portion extending through the conoid to the apex of zoites (Fig. 2.8). Micronemes are more numerous, bacilliform structures frequently observed in tightly packed parallel rows (Fig. 2.9). The tortuous nature of rhoptries makes interpretation of their numbers more difficult in tissue sections. Two to several rhoptries are reported for *Sarcocystis* species (Porchet-Hennere and Nicolas, 1983); *S. cruzi* sporozoites have at least 5 distinct rhoptry duct systems. Interconnection of rhoptries and micronemes has been reported in *Sarcocystis* spp. (Entzeroth *et al.*, 1991) but its significance and extent are controversial.

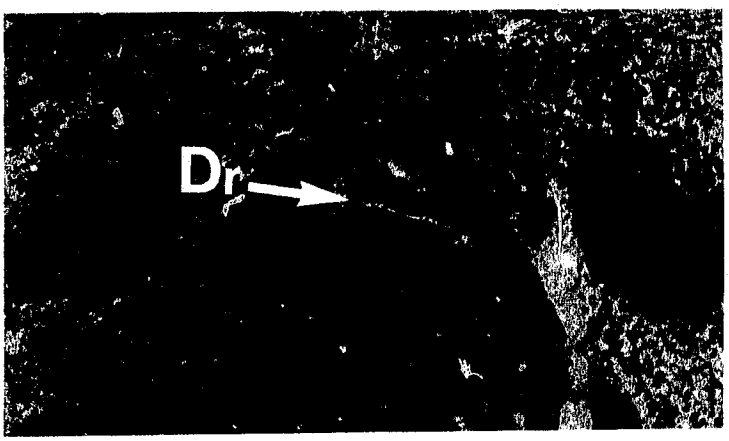
A role in host cell invasion was first proposed for rhoptries and micronemes solely based on their location. This function was supported by isolation of a penetration-enhancing factor from rhoptries (Lycke *et al.*, 1975) and an apparent

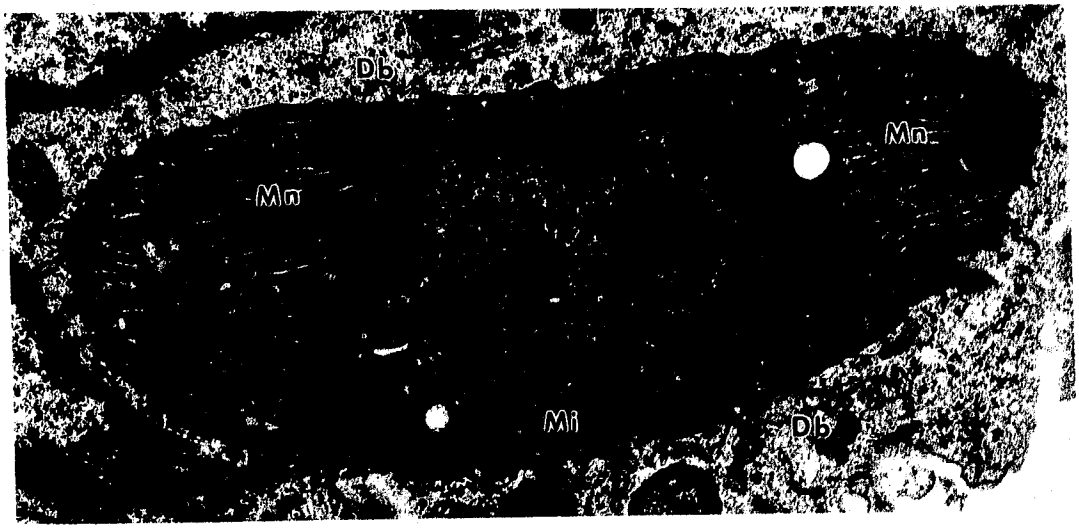
Fig. 2.7 *Sarcocystis* spp. sporozoite with rhoptries in longitudinal section. Note conoid at apex of sporozoite and posterior organelles (x22,250).

Fig. 2.8 Cross section of anterior end of *Sarcocystis* spp. sporozoite. Note ducts of rhoptries (arrow) passing through rings of the apical complex (x52,000).

Fig. 2.9 *Sarcocystis* spp. merozoite with tightly packed, parallel micronemes. Note also circular appearance of mitochondrial cristae and peripherally located dense bodies (x25,200).


Co
Rh
Mn
Ag
Mi


Dr


Db
Mn
Mn
Mi
Db

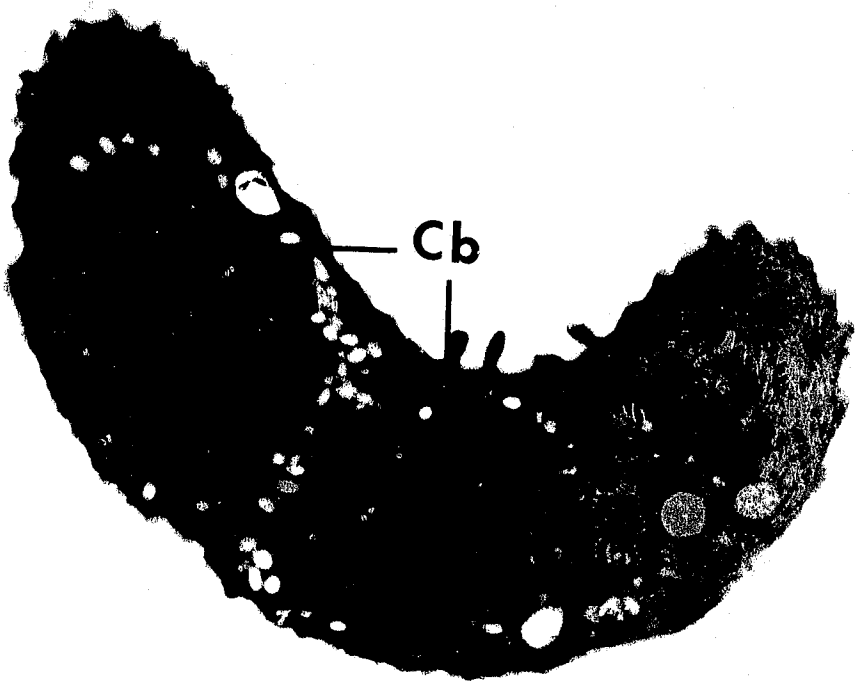
"exocytosis" of organelle contents after host cell invasion (Jensen and Edgar, 1976). Currently, efforts concentrate on a) characterization of rhoptry and microneme products and b) elucidation of their structural and functional relationships (Perkins, 1992). The majority of rhoptry research has been conducted on *Toxoplasma gondii* and *Eimeria* spp.; however, microneme proteins have been characterized in *Sarcocystis* spp. (Dubremetz and Dissous, 1980; Pohl *et al.*, 1989). No identical protein has been isolated from both rhoptries and micronemes indicating they are distinct with separate origins (Perkins, 1992). This also supports separate functions of these organelles; however, overlap cannot be ruled out, particularly if connections unite these organelles.

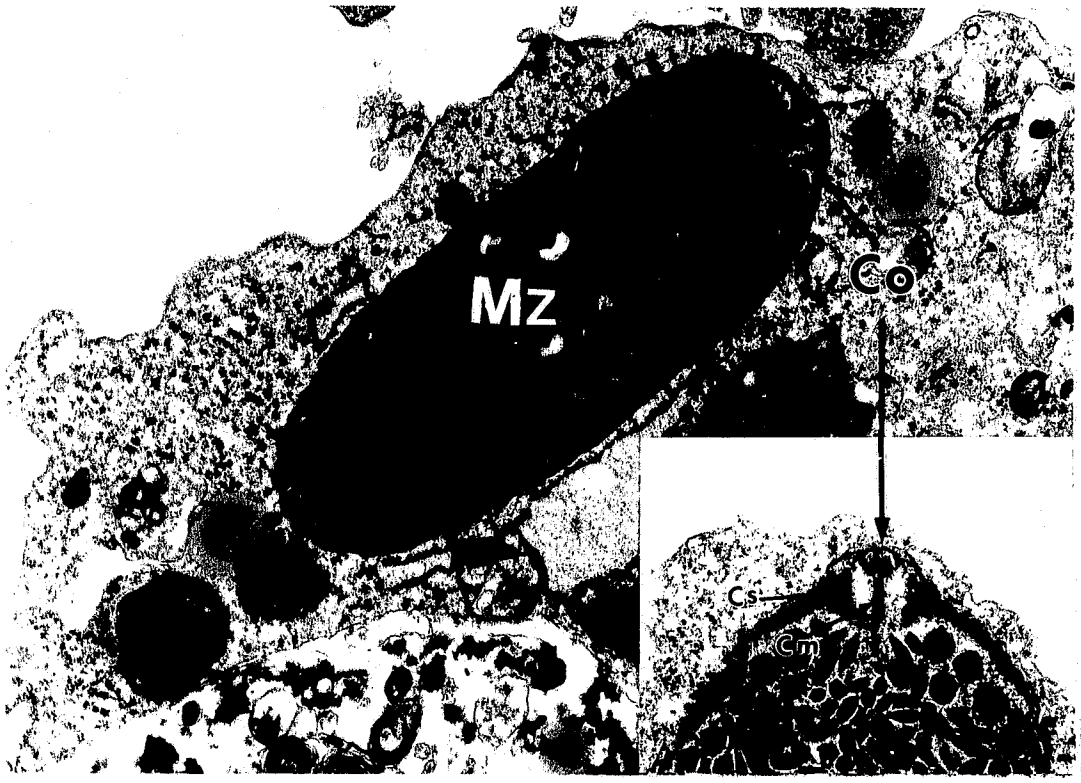
Refractile bodies, usually the most prominent and distinctive structure of sporozoites (Chobotar and Scholtyseck, 1982), are absent in *Sarcocystis* species. Crystalloid bodies, a unique feature of some apicomplexan sporozoites, may be an analogous organelle. The latter are irregularly shaped, nonmembrane-bound structures consisting of regularly arranged electron-dense and -lucent granules (Fig. 2.10). Both refractile and crystalloid bodies are speculated to function as an energy source for the parasite.

Merozoites and bradyzoites differ from sporozoites of *Sarcocystis* spp. by the absence of rhoptries and crystalloid bodies, respectively (Fig. 2.11). Size can also be used to differentiate and speciate sporozoites and merozoites of *S. cruzi* and *S. hirsuta* (see section 4.3.1 - Table IV for sporozoite sizes). Bradyzoite size

Fig. 2.10 *Sarcocystis* spp. sporozoite. Note regularly arranged spherical subunits of large, nonmembrane-bound crystalloid bodies (x20,400).

Fig. 2.11 *Sarcocystis cruzi* merozoite. Note retracted conoid at apex of zoite and lack of rhoptries (x18,000). Inset: higher magnification of an extended merozoite conoid. Note microtubular subunits of conoid and central microtubules (x34,000).


Cb


Mz
Co
Cs
Cm

is not useful for identification since dimensions can be quite variable in live preparations and difficult to measure in tissue sections (Dubey *et al.*, 1989).

Other organelles found in asexual stages include nucleus, Golgi complex, mitochondria, smooth and rough endoplasmic reticulum, ribosomes, and multivesicular bodies. Additionally, fibrillar elements (eg. cytoplasmic microtubules and microfilaments) and inclusion bodies (eg. dense granules, amylopectin, and lipid) occur. Consult Ball and Pittilo (1990) and Dubey *et al.* (1989) for descriptions of their function(s) in coccidia and *Sarcocystis* species, respectively.

Sporozoite transformation to ovoid schizonts/meronts occurs free in the host cell cytoplasm, i.e. not within a parasitophorous vacuole (Dubey *et al.*, 1980). Transformation begins shortly after host cell penetration and is characterized by: a) gradual disappearance of apical complex organelles, inner membrane complex, and subpellicular microtubules; b) increases in nuclear size and number of nucleoli; c) proliferation of endoplasmic reticulum, ribosomes and mitochondria; and d) reduction in amylopectin granules (Pacheco and Fayer, 1977). Autophagic vacuoles, multivesicular bodies and micropores are also prominent at this stage of development. The formation of merozoites within meronts is by "synchronous multiple endopolygenesis" (=endopolygeny) wherein: a) merozoite development begins centrally in association with nuclei, centrocones, and centrioles; b) numerous daughter merozoite anlagen appear before completion of nuclear division; and c) ultimately, mature merozoites are simultaneously released at the meront surface via a budding process (Cerna and Senaud, 1977).

In this method of merogony, relative age of meronts is easily determined by nuclear appearance (particularly, size and degree of lobation), extent of separation of daughter merozoites from nuclear material, and location of merozoite anlagen relative to the limiting membrane of the parasite. Examples of young, intermediate, and mature meronts and details of their distinguishing features are provided in chapter VI. At maturity, host cell rupture accompanies merozoite release. Sarcocyst formation begins when terminal-generation merozoites of *S. cruzi* invade muscle cells (primarily cardiac or skeletal) or, less frequently, nerve cells. Frenkel (1990) questions whether cysts reported in brain tissue are actually intravascular precystic schizonts or merozoites; ultrastructural confirmation is necessary. *Mature S. hirsuta* and *S. hominis* sarcocysts have been demonstrated in only skeletal muscle (Fayer and Dubey, 1986; Dubey *et al.*, 1988). An immature cyst in myocardium is reported for *S. hominis* (Dubey *et al.*, 1988).

Ultrastructural development of sarcocysts has not been completely described in any *Sarcocystis* species; some detail of cyst formation in cattle is given in Mehlhorn *et al.* (1975) and Pacheco *et al.* (1978). The terminal-stage merozoite and its surrounding parasitophorous vacuolar membrane give rise to metrocytes and the rudimentary sarcocyst wall, respectively (Chobotar and Scholtyseck, 1982). Dubey *et al.* (1989) and Cawthorn and Speer (1990) provide brief descriptions of metrocyte and cyst wall structures, respectively. Metrocytes form by endodyogeny, a process similar to endopolygeny except only two new metrocytes are produced as each of the original merozoites transforms. Once

formed, some metrocytes may divide to produce new metrocytes. Eventually, most metrocytes within sarcocysts develop into bradyzoites, the stage infective to definitive hosts.

Sarcocystis cruzi sarcocysts are microscopic (<500 μm long) whereas *S. hirsuta* cysts may be as large as 8 mm long by 1 mm wide (Bottner *et al.*, 1987). Whether *S. hominis* produces macroscopic sarcocysts remains undetermined (Dubey *et al.*, 1988). *Sarcocystis cruzi* and *S. hirsuta* can be distinguished histologically by thickness of the cyst wall, i.e. <1 μm and 3-6 μm, respectively (Briggs and Foreyt, 1985). Differentiation of *S. hirsuta* and *S. hominis* is more difficult requiring examination of sarcocyst ultrastructure (Mehlhorn *et al.*, 1976; Dubey *et al.*, 1988). The sarcocyst wall has taxonomic importance and is well described for these and other *Sarcocystis* species (see Dubey *et al.*, 1989); however, changes in cyst wall ultrastructure over time (i.e. with state of maturity) have been noted (Bottner *et al.*, 1987; Dubey *et al.*, 1988; Friesen *et al.* 1989). Temporal examination of sarcocysts would complete descriptions and facilitate identification of some *Sarcocystis* species.

2.3 Sarcocystosis in cattle

2.3.1 Disease outbreaks

Dalmeny, Saskatchewan was the location of the first reported natural outbreak of bovine sarcocystosis in Canada (Corner *et al.*, 1963). Since the etiologic agent was not immediately determined, the term 'Dalmeny disease' often

appears in the early literature. Subsequently, other field cases of sarcocystosis were identified in both dairy and beef breeds, worldwide (Meads, 1976; Frelier *et al.*, 1977; Clegg *et al.*, 1978; Landsverk, 1979; Giles *et al.*, 1980; Collery and Weavers, 1981; Carrigan, 1986). These cases represented acute infections which were frequently fatal; however, subclinical and chronic forms of sarcocystosis also occur. Clinical and pathologic findings associated with each form of the disease are given below.

2.3.2 Clinical signs

Sarcocystosis is a systemic disease which results in a wide range of clinical signs. The clinical presentation of acute sarcocystosis may include: anorexia or inappetence, fever, shivering, decreased milk production, diarrhoea, anaemia, peripheral lymph node enlargement, hypersalivation, muscle tremors, dyspnea, jaundice, hyperexcitability or lethargy, retained fetus, abortion, and stillbirths (Fayer and Johnson, 1973, 1974; Johnson *et al.*, 1975; Fayer *et al.*, 1976; Stalheim *et al.* 1980; Frelier, 1980; Dubey *et al.*, 1982; Foreyt *et al.*, 1986). Neurologic signs including opisthotonos, extension of the limbs, and ataxia have also been observed in infected animals (Johnson *et al.*, 1975). Clinical signs can progress rapidly resulting in prostration and death two or three days after the onset of illness (Dubey and Fayer, 1983; Foreyt *et al.*, 1986). Central nervous system (CNS) signs are the "end-stage" indicators which signal impending death (Dubey *et al.*, 1989).

Death can also result after a more prolonged illness characterized by weight loss, alopecia, and cachexia, in addition to some or all of the above clinical signs. Although more chronic processes are evident, this is not the "chronic form" of sarcocystosis *per se*. These cases were exposed to the same source of infection (and at the same time) as the more acute cases. However, the animals that did not die acutely likely received a smaller dose of infective sporocysts or had a higher natural resistance to disease.

Recovery from acute disease is possible (Dubey and Fayer, 1983). Alternatively, some animals may lapse into the true chronic form of disease. In either case, growth rates do not return to normal and "stunting" is apparent in surviving cattle (Fayer and Elsasser, 1991). Factors which determine whether an infected animal dies acutely, recovers, or develops chronic disease are unknown.

Chronic infections manifest as low grade illnesses which may be characterized by emaciation, pale or icteric mucous membranes, mandibular edema, exophthalmia, lameness, decreased milk yield or agalactia, muscle atrophy, alopecia (particularly of tail, neck, rump and around eyes and ears), hyperexcitability, hypersalivation, nasal discharge, postparturient cachexia, abortion, and death (Fayer and Dubey, 1986). Central nervous system signs including nystagmus, recumbency with a walking gait, and opisthotonos have also been observed (Fayer *et al.*, 1983). Usually, reduced rate and efficiency of weight gain are the only indicators of chronic infection (Briggs and Foreyt, 1985). In some animals

disease is silent with no outward sign of infection and normal clinical laboratory findings (Cawthorn and Speer, 1990).

2.3.3 Pathology of acute and subacute disease

Animals severely affected with sarcocystosis that have died naturally or were euthanized may show only slight gross lesions at necropsy (Dubey *et al.*, 1989). However, pallor of mucous membranes and visceral organs, serous atrophy of fat, generalized lymphadenopathy, icterus, visceral edema, ascites, hydrothorax, hydropericardium, and petechiae and ecchymoses of heart, brain, skeletal muscle, and serosal surfaces have been noted (Fayer and Johnson, 1973, 1974; Johnson *et al.*, 1975; Fayer *et al.*, 1976; Dubey *et al.*, 1982; Foreyt *et al.*, 1986). Dark red mottling on pale skeletal muscles may manifest as characteristic stripes (depending on plane of section) (Fayer *et al.*, 1976; Dubey *et al.*, 1982). Haemorrhage may also appear in the sclera of the eyes (Dubey and Fayer, 1983).

Histologically, haemorrhage, edema and mononuclear cell infiltrates are visible throughout the body particularly in the heart, brain, liver, lung, kidney, striated muscles (Johnson *et al.*, 1975; Dubey *et al.*, 1982; Nakamura *et al.*, 1982). Multifocal degeneration or necrosis is prominent in heart, skeletal muscle and kidney and is found to a lesser extent in other internal organs (Fayer and Dubey, 1986). Depending on stage of infection, schizonts may or may not be evidently associated with the endothelium of affected tissues; regardless, vasculitis is a significant feature of infection (Dubey *et al.*, 1982). Inconsistent findings in sarcocystosis include calcium deposition in affected muscle, fibrinous thrombi in

capillaries, nonsuppurative meningitis, focal gliosis, and thymic atrophy due to lymphocyte depletion (Johnson *et al.*, 1974; Johnson *et al.*, 1975; Frelier *et al.*, 1979; Frelier, 1980; Dubey *et al.*, 1982; Dubey and Fayer, 1983).

Clinical pathology of acute sarcocystosis shows elevation in creatinine phosphokinase, lactate dehydrogenase, alanine aminotransferase, and sorbitol dehydrogenase enzyme activities, particularly in the acute phase of the disease (Fayer and Prasse, 1981; Prasse and Fayer, 1981; Frelier and Lewis, 1984; Mahaffey *et al.*, 1986). Hyperbilirubinemia and increases in blood urea nitrogen are consistently observed in infected animals (Mahrt and Fayer, 1975; Dubey *et al.*, 1982; Dubey and Fayer, 1983). There can be as much as a 75% decrease in packed cell volumes with corresponding decreases in erythrocyte counts and haemoglobin levels (Dubey and Fayer, 1983; Frelier and Lewis, 1984). Platelet dysfunction, increased prothrombin time, and increased fibrin degradation product concentration have also been noted (Dubey and Fayer, 1983; Fayer and Dubey, 1986). Lastly, lower serum values of glucose, calcium, sodium, chloride and proteins are reported in acute sarcocystosis (Dubey and Fayer, 1983; Fayer and Dubey, 1986).

2.3.4 Pathology of chronic disease

Chronically affected hosts frequently have no remarkable gross lesions (Fayer and Dubey, 1986) except for serous atrophy of fat (Dubey *et al.*, 1989). Briggs and Foreyt (1985) contend that sarcocysts in muscle can be detected by careful visual inspection. Such macroscopic cysts likely result from *S. hirsuta*

infection (Dubey *et al.*, 1989). In *Sarcocystis* spp. infections, the histologic changes associated with parasitic muscle cysts are usually unremarkable. Mild myositis and myocyte necrosis, confined to parasitized muscle cells, are frequently reported. The association of sarcocysts and myositis is not clear, moreover, histology of sarcocystosis cases does not necessarily correlate with clinical signs (Cawthorn *et al.*, 1990; Cawthorn and Speer, 1990).

2.3.5 Pathogenesis

Severity of disease directly relates to number of sporocysts ingested, e.g. a minimum of 50,000 are required to produce clinical signs of *S. cruzi* infection. Host age *per se* does not influence pathogenicity; however, disease may be more severe in previously unexposed or lightly exposed animals i.e. animals which have never consumed sporocysts or have ingested only small numbers of sporocysts. In *Sarcocystis* spp. acute and chronic disease result from schizogonous activity and sarcocyst formation, respectively (Cawthorn and Speer, 1990).

Pathogenesis of chronic sarcocystosis is not clearly known. Sarcotoxins and lectins associated with bradyzoites of sarcocysts may be important (Dubey *et al.*, 1989). Tumor necrosis factor (TNF) depresses activities associated with growth and is associated with wasting disease (Dubey *et al.*, 1989; Fayer and Elsasser, 1991). An *in vitro* assay for production and detection of TNF has shown that lysates of *S. cruzi* bradyzoites stimulate murine macrophages to produce this cytokine (Fayer et al. (1988). Alteration of growth-regulating hormones (Elsasser

et al., 1986, 1988) may contribute to the poor weight gains and low feed efficiency of chronic disease.

In acute disease, bimodal pyrexia (39.5-42 °C) 15-18 days post infection (DPI) and 28-30 DPI correlates with maturation and rupture of schizonts (Fayer and Johnson, 1973; Johnson *et al.*, 1975; Stalheim *et al.*, 1980; Dubey *et al.*, 1982). However, not all animals show the first fever peak (Dubey *et al.*, 1982). Pyrogen or prostaglandin release is likely associated with parasitemia (Dubey *et al.*, 1989). Daugschies' *et al.* (1989) swine research has produced the first report of elevated prostaglandin levels in acute sarcocystosis (discussed below).

Cattle clinically normal during 1st-generation merogony, become sick coincident with maturation and rupture of 2nd-generation meronts (Dubey and Fayer, 1983). The inflammatory response which accompanies acute disease may result from antigens or metabolic products from zoites, immature schizonts, or disrupted host cells (Fayer and Dubey, 1986; Dubey *et al.*, 1989). Why some schizonts elicit no apparent tissue response (Johnson *et al.*, 1975) is unexplained. The mechanism of haemorrhage in sarcocystosis is also unexplained.

Myositis reflects myocyte penetration by merozoites (Cawthorn and Speer, 1990); again, products liberated from the host cell or merozoites may be the stimuli for the observed inflammation (Dubey *et al.*, 1982). Alternatively, Dubey (1983) suggests that myositis in *S. hirsuta* infection is due to capillary necrosis resulting from the presence of 2nd-generation meronts within these vessels. The myositis (and inflammation in other organs) generally subsides as sarcocyst

formation begins (Dubey and Fayer, 1983). Degenerative changes in muscle tissue and severe clinical signs do not always accompany cyst formation (Fayer and Johnson, 1974). Dubey *et al.* (1982) observed as much as 1/3 of muscle tissue to be composed of sarcocysts, but did not observe a significant inflammatory response in affected muscles. When present, lesions are not necessarily limited to the vicinity of sarcocysts and the number of cysts is not related to severity of disease (Frelier *et al.*, 1979). Granulomatous myositis may follow sarcocyst rupture; the rupture stimulus is unknown (Dubey and Fayer, 1983).

The principal pathophysiologic effect of acute sarcocystosis is a regenerative, normocytic, normochromic anaemia (Frelier *et al.*, 1977; Fayer and Prasse, 1981). Haemorrhage and splenic removal of erythrocytes cannot account for the severity of anaemia. Fayer and Prasse (1981) and Frelier and Lewis (1984) suggest that anaemia associated with bovine sarcocystosis is an extravascular haemolytic event. Whether haemolysis is immune-mediated is unclear (Frelier and Lewis, 1984; Mahaffey *et al.*, 1986). Corticosteroids are commonly used in the treatment of immune-mediated anaemia in humans and animals because these drugs have an ability to interfere with the phagocytic activity of macrophages (Petz and Garratty, 1980; Switzer and Jain, 1981). That prednisolone acetate administration failed to suppress red cell clearance in acutely affected calves, supports a non-immune-mediated anaemia (Collery, 1989). Capillary extravasation resulting from intravascular coagulopathy has also been suggested as a

mechanism of anaemia in sarcocystosis (Dubey *et al.*, 1989). Whether parasite toxins or host cell metabolites influence the anaemia is unknown.

Poor weight gains seen with sarcocystosis are not caused by decreased feed intake alone. Fayer and Lynch (1979) reported evidence of physiological effects beyond those induced by nutritional status eg. renal malfunction and muscle catabolism. Hormone disturbances further compromise feed efficiency and growth (Elsasser *et al.*, 1986; Elsasser *et al.*, 1988). The factors modulating growth, including immune response to *S. cruzi* via cytokine interactions with the endocrine system, are reviewed in Fayer and Elasser (1991).

Abortion in sarcocystosis is not due to cachexia (Stalheim, *et al.*, 1980). The mechanism of abortion and why *Sarcocystis* spp. have been infrequently isolated from aborted fetal tissues are not known. Toxins are an unlikely cause but, hormonal imbalance and pyrexia may be significant factors (Stalheim *et al.*, 1980). Whether the profound anaemia in acute sarcocystosis directly induces or influences abortion has not been determined. Elevated plasma levels of prostanoids (specifically prostaglandins E_2 and $F_{2\alpha}$) have been demonstrated in acute sarcocystosis in pigs (Strohlein, 1986; Daugschies *et al.*, 1989). Some properties of PGE_2 (i.e. increased permeability of blood vessels, pyretic activity, and stimulation of myometrial contraction) can be linked to clinical sarcocystosis (e.g. extravasation of fluid, fever, and abortion). *Sarcocystis miescheriana*-infected sows had increases in $PGF_{2\alpha}$ immediately prior to aborting (i.e. 11-12 DPI) (Strohlein, 1986). Increased $PGF_{2\alpha}$ also occurs in plasma of *S. cruzi*-infected,

aborting cows (Baetz *et al.*, 1981); however, unlike PGE_2, elevated levels of $PGF_{2\alpha}$ are assumed to reflect *Sarcocystis*-induced abortion, not trigger it (Daugschies *et al.*, 1989). Since prostanoids have major roles in the inflammatory process and regulation of haemodynamics, alteration of their metabolism could account for additional pathogenic effects associated with sarcocystosis (e.g. the above mentioned inflammation and haemorrhage).

The nervous signs associated with sarcocystosis cannot be explained by severity of anatomic lesions in the central nervous system (CNS). Stackhouse *et al.* (1987) and Dubey *et al.* (1989) suggest toxin release from one or more life cycle stages of the parasite could exacerbate CNS signs. Hypoproteinemia and vasculitis cause edema and excess fluid in body cavities (Cawthorn and Speer, 1990; Dubey *et al.*, 1989). Since the heart is often severely affected, its decreased function and congestion may contribute to ascites, hydrothorax, and hydropericardium (Briggs and Foreyt, 1985). Anorexia may account for the decreases in glucose, calcium, sodium, and chloride (Briggs and Foreyt, 1985). Fayer and Dubey (1986) and Dubey *et al.* (1989) suggest poor nutrition and microthrombi could contribute to the alopecia observed in sarcocystosis.

Lastly, pathogenesis of natural and experimental sarcocystosis may vary e.g. pedal and buccal ulcerations and eosinophilic myositis are occasionally described in cattle naturally infected with *Sarcocystis* spp. but have never been observed in an experimentally infected animal (Dubey *et al.*, 1989). The differences in clinical signs and types of lesions could reflect repeated infection

with *Sarcocystis* spp., the specific isolate/strain of parasite, or the age or breed of host animal (Dubey *et al.*, 1982). Restricted access of secondary pathogens to experimental animals could also be a factor. Immunosuppression and increased susceptibility to other diseases (i.e. bacterial, viral, or parasitic) have been reported in infections with *Sarcocystis* spp. (Frelier, 1980; Fayer, 1982; Dubey, 1983). Further research is necessary to explain the observed variability in clinical signs and lesions between experimental and natural infections of sarcocystosis.

2.3.6 Management strategies for control

In sarcocystosis outbreaks, effective control measures can only be implemented once a proper diagnosis has been reached. History, clinical signs, and necropsy findings (especially muscle haemorrhage without inflammation) will provide a presumptive diagnosis of sarcocystosis, but the disease is difficult to confirm without histologic examination of tissues and serology because of the nonspecific nature of clinical signs (Foreyt *et al.*, 1986). Serum analysis for antibody to *Sarcocystis* antigen by indirect hemagglutination, immunofluorescence, and ELISA has proven useful in studies of natural outbreaks and in laboratory studies (Dubey, 1976; Lunde and Fayer, 1977; Hong *et al.*, 1982). However, the sensitivities of these tests require improvement and none are commercially available. Antibody levels in acute disease may not be adequate to confirm sarcocystosis, although testing is reliable 3 months postinfection (Fayer and Prasse, 1981). Titers begin to rise 30 DPI and may reach a peak of 1:39,000 by 90 DPI (Lunde and Fayer, 1977; Stalheim *et al.*, 1980).

The causative agent in acute sarcocystosis is not easily found in host tissues. By the time illness is apparent in the bovine host, most meronts have ruptured and are not obvious. Merozoites associated with parasitemia are often too few to be routinely detectable on blood smears. Lymph node or muscle biopsy at necropsy may help detect parasite stages, particularly vascular schizonts (Fayer and Dubey, 1986). Gut (1982) found trypsinization of muscle a reliable method for detection of sarcocysts, but the method is time consuming and may require large amounts of tissue. Stereoscopy is a more rapid, equally effective technique for visualizing parasitic muscle cysts and it does not require elaborate equipment (Gut, 1982). The finding of mature sarcocysts does not indicate sarcocystosis, only previous exposure. It may be necessary to rule out other protozoal agents, such as *Neospora caninum* or, to a lesser extent, *Toxoplasma gondii*.

Inspection of the affected animals' feeding, housing, and pasture areas is essential to determine the origin of infection. Carnivore interaction with cattle must be assessed since the main mode of transmission of sarcocystosis is the faecal-oral route i.e. contamination of cattle feed or water with infected canine faeces (Dubey and Fayer, 1983). In acute outbreaks, the farm dog is usually the source of the infective sporocysts (Frelier *et al.*, 1977; Giles *et al.*, 1980; Foreyt *et al.*, 1986). Only rarely have merozoites or schizonts been isolated from fetal tissue (Munday and Black, 1976; Dubey and Bergeron, 1982; Hong *et al.*, 1982); thus,

intrauterine transmission is unlikely. Colostrum is neither infective nor protective for offspring (Fayer *et al.*, 1982; Fayer *et al.*, 1976).

Interruption of the *Sarcocystis* spp. life cycle is the only effective means to control sarcocystosis. Infected livestock carcasses are often scavenged by carnivores; therefore, deadstock should be properly disposed of by burial or incineration. Raw meat (including offal) remains infective during refrigeration (2-4 °C) for up to 20 days; however, freezing (-18 to -20 °C) or cooking (60-65 °C) kills infective bradyzoites (Fayer, 1975; Stalheim *et al.*, 1980; Koudela and Steinhauser, 1984). Carnivores should be excluded from feeding, watering, bedding and feed storage areas for cattle. These measures reduce: a) reinfection of carnivores and b) sporocyst contamination; however, they are difficult to implement practically (Cawthorn and Speer, 1990). Elimination of reinfections when pasturing animals is likely impossible due to the presence of wild carnivores (Rommel, 1985). Farmer education is vital to prevent initial or recurring disease.

Currently, no prophylactic or therapeutic agents prevent oocyst development in definitive hosts (Fayer and Dubey, 1986). Some drugs are effective in prevention and treatment of sarcocystosis in the intermediate host (reviewed by Cawthorn and Speer, 1990); however, their efficacy has not been determined in field trials. Experimental immunization with live or irradiated sporocysts has produced limited protective immunity (Fayer and Dubey, 1984). Repeated natural infections do not significantly decrease susceptibility to infection (Srivastava *et al.*, 1987; Dubey *et al.*, 1989). Cattle cannot be cross protected by inoculation with

less pathogenic species. For example *S. hirsuta* does not protect against *S. cruzi* infection (Fayer and Dubey, 1984). Research continues on immunogenicity of different life cycle stages of this parasite and the mechanisms involved in persistence of immunity. Presently, veterinary assistance consists of symptomatic treatment and supportive therapy, both of which are often ineffectual. Prognosis and economics of sarcocystosis now dictate culling over treatment.

2.3.7 Economic Significance

Acute outbreaks of sarcocystosis can be economically devastating to individual farmers. The associated mortality rate is difficult to evaluate because significant epidemiologic data are often missing from outbreak descriptions. For example, the number of animals at risk (and for what given time period) has not been consistently reported. Alternatively, the case fatality rate (CFR), which best describes severity of acute disease (Martin *et al.*, 1987), can be calculated. Averaging data from Corner *et al.* (1963), Frelier *et al.* (1977), Giles *et al.* (1980), and Carrigan (1986), the CFR (= number of deaths/total number acquiring disease) observed in sarcocystosis is 60 percent. Although CFR in acute sarcocystosis is high, the chronic form of disease is responsible for a greater overall financial loss to producers. The major effect of sarcocystosis is likely reduction in the animals' weight gain (Herbert and Smith, 1987). In dairy herds, the reduced milk yields also have an immediate, significant economic impact (Fayer *et al.*, 1982). Additional losses result from reproductive failure (Stalheim et al., 1980). The effect of sarcocystosis on subsequent pregnancies is unknown.

Immunosuppression may result in increased susceptibility to other pathogens and contribute substantially to production losses (Frelier, 1980). Deaths and veterinary costs further decrease profits.

Carcass condemnation (in whole or in part) and meat downgrading result in large annual losses in the beef industry. There are both direct and indirect effects of *Sarcocystis* spp. infection which influence grading of meat at inspection e.g. presence of macroscopic sarcocysts and poor body conditioning, respectively. Additionally, eosinophilic myositis (a common cause of condemnation) has also been attributed to sarcocystosis. Evidence supporting this claim is conflicting (Jensen *et al.*, 1986; Granstrom *et al.*, 1989); however, Ely and Fox (1989) recently demonstrated that cattle condemned for eosinophilic myositis have higher antibody levels to *S. cruzi* than control animals. Unfortunately, the percentage of animals and meat culled at slaughter due to overt or underlying sarcocystosis cannot be readily determined from abattoir records. Financial losses based on poor feed conversion, failure to grow, lower milk production, reproductive problems, and obvious clinical disease are even more difficult to determine. Fayer and Dubey (1986) have estimated the ante- and postmortem losses in the cattle industry resulting from infections with *Sarcocystis* spp. at nearly $100 million (U.S.) annually.

Assessment of sarcocystosis impact is further complicated by the widespread, subtle nature of the chronic disease. *Sarcocystis* spp. infect up to 98% of slaughtered cattle (Fayer, 1982). Serologically, 100% of cattle tested had

antibody to *S. cruzi* antigens in their sera (Fayer and Dubey, 1986); thus, most infections are asymptomatic or misdiagnosed. The true prevalence of disease (versus infection) is unknown. Performance cannot be accurately compared if infected and uninfected animals are not readily differentiated. Dubey *et al.* (1989) assert that wide prevalence of pathogenic *Sarcocystis* spp. will ensure increased recognition of clinical disease when better diagnostic tests are available. Significant control efforts are not likely to occur until the financial losses which accrue can be tangibly demonstrated to producers. Currently, education and research are the keys to changing sarcocystosis from an accepted environmental risk to an unnecessary one.

2.4 Prospectus for present study

The remainder of this thesis examines three different *in vitro* events in the life cycle of *Sarcocystis* spp. occurring in the bovine intermediate host, specifically: a) excystation (release of sporozoites, infective for intermediate hosts, from oocysts and sporocysts), b) host cell invasion by sporozoites, and c) subsequent schizogonous development (=asexual development) from sporozoite to merozoite.

Firstly, in chapter IV, *S. cruzi* and *S. hirsuta* are used to compare and contrast the process of excystation by scanning and transmission electron microscopy. Without sporocyst breakdown no infection can occur. Detailed knowledge of this phenomenon could result in intervention which interrupts the life cycle at the level of transmission between definitive and intermediate host. Conversely, efficient excystation is required for *in vitro* experimentation with asexual

stages. Again, mechanisms must be revealed before the *in vivo* environment can be reliably duplicated. Additionally, the diagnostic potential of morphologic features of *S. cruzi* and *S. hirsuta* sporocysts are explored.

In chapter V, host-parasite interactions of cell invasion by *S. cruzi* sporozoites are examined in an electron microscopic study. Understanding basic biology of this process facilitates elucidation of underlying mechanisms which ultimately provide new areas and avenues for prevention or control of sarcocystosis.

Lastly, in chapter VI, development of the more pathogenic *S. cruzi* is followed for an extended period in various cell lines by light and transmission electron microscopy. In *Sarcocystis* spp. the number and location of schizogonous phases are indeterminate. The determination of host cell specificity and suitability is important for both *in vivo* control (eg. prophylactic and therapeutic approaches) as well as *in vitro* research (eg. completing the entire *Sarcocystis* life cycle in tissue culture).

Purpose and specific objective(s) of each of the experimental chapters are given in introductory sections i.e. sections 4.1, 5.1, and 6.1.

CHAPTER III - MATERIALS AND METHODS (GENERAL)

This section contains general methods employed in all experiments. Variations for specific experiments are detailed under 'Materials and methods' in individual chapters.

Where possible tissue culture techniques replaced *in vivo* experimentation using cattle. When the experimental design necessitated infection of cats and dogs all pertinent guidelines of the "Guide to the Care and Use of Experimental Animals" Canadian Council on Animal Care (1980-1984) were followed.

3.1 Collection of sporocysts

Dogs (Bioresearch Inc., Senneville, PQ) (n=4 or 5 per collection period) and cats (Handibear Services Co., Breadalbane, PE) (n= 4 or 5 per collection period) were fed naturally infected bovine hearts and diaphragms from a local abattoir. Their faeces were collected daily and examined for *Sarcocystis* spp. sporocysts using sucrose floatation and phase contrast microscopy. When samples were positive for sporocysts (usually 7-14 DPI), faeces were collected and held at 4 °C for subsequent sporocyst recovery (see section 3.3). After a 2-3 week period of faeces collection, animals were euthanized by barbiturate overdose i.e. 105.6 mg/kg Euthanyl® (barbiturate content = 240 mg/ml pentobarbitol) (MTC Pharmaceuticals, Cambridge, ON). Sporocysts in the intestinal tract were recovered as described in section 3.2.

Collection and purification techniques described below were adapted from Dubey (1980) and Speer (1983). Haemocytometer counts estimated numbers of sporocysts recovered. Sporocyst storage conditions were as described by Leek and Fayer (1979).

3.2 Sporocyst isolation from intestine

Once peristalsis ceased, the entire intestinal tract was cut into 0.3 m sections and digesta was squeezed from the lumen. The pieces of intestine were longitudinally split and the mucosa and submucosa were scraped from underlying musculature with a glass slide. Scrapings were placed in 20 volumes of phosphate buffered saline (PBS) containing 4-6 drops of detergent (Tween- 80) (Can Lab, Moncton, NB). The mixture was then blended 1 min in a Waring commercial blender at high speed, shaken gently 1 h on an orbital shaker, and centrifuged 10 min at 1200 xg. Sediments obtained were rehomogenized in 20 volumes of PBS. This procedure was repeated three times and final sediments resuspended in 5-10 volumes of Hank's balanced salt solution with 10,000 U penicillin/ml, 0.01 g streptomycin/ml, 0.05 g fungizone/ml, and 500 U mycostatin/ml (HBSS-PSFM, pH 7.4). Samples were stored at 4 °C for up to 24 months.

3.3 Sporocyst isolation from faeces

Faeces pooled from daily collections were diluted in 20 ml of tap water per 10 g of faeces and blended 1 min in a commercial blender at high speed. To remove debris, the resulting mixture was poured through larger to smaller sieves

(mesh sizes 2.0 mm, 850 μm, and 425 μm) into 250 ml centrifuge bottles. After centrifuging for 10 min at 500 xg, supernates were discarded and sufficient 2 M Sheather's sugar (3420 g sugar, 2070 ml double distilled H_2O, 36.36 ml 40% formaldehyde) added to each bottle to transfer remaining solids to one large container. Five times the volume of Sheather's medium used in transfering the solids was then added to the container and the contents thoroughly mixed.

Sporocysts were isolated using gradient centrifugation. Cold tap water was carefully layered onto 35 ml of the sugar/faeces mixture in a 50 ml tube and centrifuged at 500 xg for 20 min. The top layer and the interface which contained the sporocysts were removed, diluted with 1.5-2.0 liters of tap water, and centrifuged in 250 ml bottles at 500 xg. The sporocyst suspension was further concentrated by: a) decanting the supernates, b) resuspending in 50 ml PBS (number of 50 ml tubes depending on amount of mat erial recovered) and c) centrifuging for 10 min at 500 xg. Steps a), b), and c) were repeated twice more and final pellet(s) stored in 20 volumes of HBSS-PSFM at 4 °C.

3.4 Excystation of sporocysts

Sarcocystis spp. were excysted according to Cawthorn *et al.* (1986) with modifications. The sporocyst suspensions that were prepared in sections 3.2 and 3.3 were vacuum filtered through two layers of cheesecloth, pelleted by centrifugation 500 xg and pretreated with 2.6% aqueous sodium hypochlorite solution (NaOCl) for 30 min at 4 °C. Sporocysts were washed by centrifugation with cold HBSS until the odour and color of NaOCl were not apparent.

An additional step of centrifugal elutriation proven useful for purification of *Eimeria* sporocysts and sporozoites (Stotish *et al.*, 1977; Russman, 1982) was incorporated to further clean sporocyst suspensions. Apparatus, techn ique and method to determine flow rates and rotor speed were described by Bachere *et al.* (1988). Briefly, sporocysts suspended in HBSS medium were loaded into the JE-6B Elutriation System and Rotor (Beckman Instruments Inc., Mississauga, ON) in a Beckman J-6M/E refrigerated centrifuge. Fractions were collected at flow rates of 5, 20, 30, 40, and 45 ml/min at 500 xg at 4 °C and examined for the presence of sporocysts with a phase contrast Zeiss compound microscope (Carl Zeiss Canada Ltd., Don Mills, ON). Elutriated sporocysts were further treated with 2.6% NaOCl, washed with sterile HBSS, concentrated by centrifugation 500 xg, resuspended in 5 ml of filter sterilized excysting fluid (10% bovine bile in HBSS, pH 7.4), and incubated at 38 °C in 5% CO_2, 95% air atmosphere for 4 h.

Tubes were inverted several times after 2 h of incubation to mix sporocysts and excysting fluid (EF). To remove EF from excysted sporozoites, 5-10 ml of RPMI-1640 culture medium (GIBCO, Grand Island, NY) were added and the resulting suspension pelleted by centrifugation at 500 xg. After resuspending in fresh medium, excystation rates were determined by haemocytometer using the formula of Cawthorn *et al.* (1986) i.e. [number of free sporozoites/ (number of free sporozoites + number of sporozoites in sporocysts)] x 100.

Prior to inoculation of cell cultures, excysted sporozoites were washed twice by centrifugation in RPMI-1640. Pelleted sporozoites were then resuspended in

fresh medium containing 5% fetal bovine serum (FBS), 50 ug/ml dihydrostreptomycin and 50 U/ml penicillin G and their numbers estimated by haemocytometer.

Protocols varied for host cell invasion and *in vitro* development experiments; cell culture technique and EM processing differed as detailed in respective chapters. Incubation conditions of 38°C in 5% CO_2-95% air atmosphere in a NAPCO 6300 continuous flow CO_2 incubator were constant for all cultures.

CHAPTER IV - COMPARATIVE EXCYSTATION OF *SARCOCYSTIS CRUZI* AND *S. HIRSUTA*

4.1 Introduction

Excystation of sporozoites has been examined in species of *Eimeria*, *Isospora*, *Toxoplasma*, *Sarcocystis*, *Cryptosporidium* and *Caryospora*, with *Eimeria* spp. being the most intensively studied. Earlier studies on the stimuli for *in vitro* excystation are reviewed in Ryley (1973) and Wang (1982). Both the process of excystation and the factors which induce it differ among genera of Apicomplexa and even among species within a genus. Moreover, the ultrastructure of the process of excystation is also variable, related to oocyst and sporocyst structure.

Apparently, excystation is a two "phase" process wherein reducing agents (e.g. cysteine, sodium dithionite) or reducing conditions (e.g. anaerobic, CO_2 atmosphere) prepare oocysts or sporocysts to respond to digestive enzymes (e.g. trypsin, chymotrypsin) and/or bile or bile salts (e.g. deoxycholate, glycotaurocholate). Presumably, the first (primary/pretreatment) and the second (secondary/treatment) "phases" mimic the environment of the stomach/rumen and small intestine, respectively (Long and Speer, 1977; McKenna and Charleston, 1982).

Appropriate stimuli result in one of two distinct "patterns" of excystation linked to sporocyst structure (Duszynski and Speer, 1976). Sporozoites are randomly released by collapse of the sporocyst wall at the site of apposition of sporocyst plates delineated by "suture lines". Alternatively, sporozoites are

released through a gap at one pole of the sporocyst created by dissolution of the Stieda body.

Although techniques which induce excystation have been elucidated for several species of coccidia (reviewed by Long and Speer, 1977), their precise mechanism of action is unknown. Careful examination of the ultrastructure of excystation is necessary to correlate effects of excysting fluids with sporocyst breakdown.

Since high numbers of contaminant-free sporozoites are necessary for *in vitro* experiments, excystation research has previously concentrated on improvement of excystation rate (ER) (Fayer and Leek, 1973; Leek and Fayer, 1979; Stockdale *et al.*, 1985; Cawthorn *et al.*, 1986). Ultrastructure of sporocysts is described for only a few coccidia (Speer *et al.*, 1973; Speer *et al.*, 1976; Christie *et al.*, 1978; Overstreet *et al.*, 1984). Within the Sarcocystidae, ultrastructural details are limited to *S. tenella* (Melhorn and Scholtzsech, 1974), *S. suihominis* (Becker *et al.*, 1979), *S. debonei* (Box *et al.*, 1980), *S. suicanis* (Strohlein and Prestwood, 1986) and *S. cruzi* (Dubey *et al.*, 1989; Cawthorn and Speer, 1990).

Mode of excystation and structure of sporocysts have been linked to Frenkel's (1977) life cycle dependent classification (Box *et al.*, 1980). More recent reclassifications, particularly among the less studied aquatic coccidia, are based, in part, on sporocyst wall and associated structures such as sporocyst veils and sporopodia (Dykova and Lom, 1981; Overstreet *et al*, 1984). However, the

taxonomic value of the ultrastructure and morphology of oocysts and sporocysts requires further exploration.

The purposes of experimentation detailed in this chapter are threefold. First, to determine, through systematic *in vitro* comparisons, whether the process of excystation is similar for *S. cruzi* and *S. hirsuta*. Second, to ascertain whether the ultrastructure of sporocysts or sporozoites can be used to identify and differentiate between these two species. Third, to gain insight into *in vitro* mechanisms of excystation. The present study also provides evaluation of different excystation protocols; specifically, comparison of excystation rates and viability and ultrastructure of zoites released.

4.2 Materials and methods

To compare excystation processes, sporocysts of *S. cruzi* and *S. hirsuta* were elutriated and excysted as described in Chapter III. Viability and infectivity of sporozoites released from sporocysts were tested in bovine pulmonary artery endothelial cells (CPAs) (#CCL 207) (American Type Culture Collection (ATCC), Rockville, MD). See Appendix A for details of cell lines and their respective tissue culture medium.

Separate excystation trials with *S. cruzi* compared the efficiency of modifications herein (i.e. modification 1 - addition of an elutriation process to clean unexcysted sporocysts; and modification 2 - alteration of the excysting fluid composition, specifically deletion of trypsin) with: a) the proteinase K technique of Ndiritu *et al.* (1993) for excysting *S. cruzi* and b) the control excystation protocol

(Cawthorn *et al.*, 1986). Sporocysts collected from experimental infections conducted up to 17 months and up to 24 months earlier were used in excystation trials with the proteinase K and control protocols, respectively. A Bioquant (BQ System IV) Hi Pad Digitizer (R&M Biometrics Inc., Nashville, TN) system facilitated sporocyst and sporozoite measurement.

4.2.1 Processing for scanning electron microscopy

Samples of sporocyst suspensions were taken at 15 min intervals during the 4 h incubation period and processed for scanning electron microscopy (SEM) as follows (NB: excystation to assess effects of proteinase K was sampled at 4 h only):

Excysting fluid was removed from sporocysts (and free sporozoites) by centrifugation and the pellet resuspended in 1 ml of Millonig's phosphate buffered saline (MPBS) (Appendix C for formulation). The suspension was passed through a 0.6 μm nuclepore filter (Nuclepore Canada Inc., Toronto, ON) which had been previously flushed with 6 drops of 5% fetal bovine serum (FBS) in the same buffer. Sporocysts were then fixed by passing 5 ml of 2.5% glutaraldehyde in MPBS through the filter. Filters were then removed and placed in fixative overnight at 4 °C. Subsequently, processing followed our routine SEM protocol (see Appendix B). Specimens were examined and photographed using a Hitachi H-7110 Scanning System electron microscope operating at 25 kV. Micrographs were taken on Polaroid 55 Positive/Negative instant sheet film (Polaroid Corporation, Cambridge, MA) and developed and fixed in Kodak developer D-19 (Eastman

Kodak Company, Rochester, NY) and Kodak general purpose hardening fixer (Cat. no. 1238146) (Kodak Canada Inc., Toronto, ON), respectively. Final prints were produced on Kodak Polycontrast III RC glossy paper (Eastman Kodak Company, Toronto, ON) using a Kodak ektamatic automatic print processor (Model 214-K) (Eastman Kodak Company, Toronto, ON).

4.2.2 Processing for transmission electron microscopy

Preliminary experiments indicated that pelleted samples of sporocyst/EF suspensions which were fixed and treated as tissue blocks resulted in fragile sporocyst pellets. Moreover, sporocyst sections cut from these pellets showed instability in the electron beam during electron microscopic examination. Consequently, samples of sporocysts/EF suspensions were first inoculated on to tissue culture cells and then processed for transmission electron microscopy (TEM) using Kingsley and Coles (1988) modified method as described in Chapter V sections 2.1 and 2.2. To prevent unstable sporocyst sections, three different embedding media (detailed below) were used.

Prior to the cell culture inoculations, samples of the sporocyst/EF suspension were washed by centrifugation in RPMI-1640 culture medium. Pelleted sporocysts were then resuspended in a small volume of RPMI-1640 medium and inoculated onto monolayers of bovine pulmonary artery endothelial (CPA) (ATCC #CCL 207) cells and rat heart myoblast (H9C2) (ATCC #CRL 1446). The inoculated monolayers were incubated for 15 min to allow sporocysts to settle. Post incubation, monolayers were fixed in 2.5% glutaraldehyde in 0.1 M phosphate

buffer (pH 7.4, 230 mosm). Subsequent TEM processing was identical to section 5.2.2; representative samples of *S. cruzi* and *S. hirsuta* were embedded: a) directly from alcohol to epon/araldite, b) from propylene oxide to epon and c) from alcohol to Spurr's resin (see Appendices B and C for formulations and EM processing schedules). Polymerized blocks were sectioned on a Reichert-Jung Ultracut E microtome (Cambridge Instruments Co., Austria) stained with uranyl acetate (Appendix C) and lead citrate (Appendix C). Sections were examined and photographed in a Hitachi H-7000 or H-600 electron microscope operating at 75 kV. Micrographs were photographed on Kodak electron microscope film Estar Thick Base 4489 (Eastman Kodak Company, Rochester, NY) and developed, fixed, and printed as in section 4.2.1.

4.3 Results

Of the total number of sporocysts loaded into the elutration system, each elutriation run/trial (n=30) returned, on average, 36.6% "cleaned" sporocysts i.e. more than 36.6% of the starting number of sporocysts were recovered; however, only 36.6% were sufficiently free of debris to procede with excystation. Excystation rates (ER) for *S. cruzi* and *S. hirsuta* ranged from 1.2 to 49.0%, averaging 23.7% (no. of trials = 28), and from 2.9 to 82.1%, averaging 28.8% (no. of trials = 24), respectively. *Sarcocystis cruzi* trials using the modified and control protocols showed no significant difference in ER. In both species, excysted sporozoites were active and infective for CPA cultures, regardless of protocol.

The trypsin-deleted protocol provided extremely variable yields of clean sporocysts; debris often obscured structures such that preparations were useless for SEM. Composition and consistency of proteinase K preparations was significantly improved i.e. cleaner sporozoites, free of debris and mucus, were obtained (Ndiritu *et al.*, 1993). Neither technique resulted in detectable damage to sporocysts or sporozoites. Excysted sporozoites were infective for CPA cultures.

4.3.1 SEM results

Intact sporocysts of *S. cruzi* and *S. hirsuta* exhibited a four-plate structure delineated by suture lines joined at oblique angles (Fig. 4.1). Sporocyst rotation (Fig. 4.2) demonstrates plate orientation and shape i.e. similar, equidistant, ovoid plates whose raised margins meet at angles greater than 90 degrees. Surface venations were also present in both species, but more prominent in *S. hirsuta* (Figs. 4.1 and 4.2). Comparative sporocyst and sporozoite parameters measured are listed in Tables III and IV, respectively, *S. cruzi* being larger than *S. hirsuta*.

Excystation of *S. cruzi* and *S. hirsuta* proceeded similarly (Fig. 4.3A-H). Excystation of the intact sporocyst (Fig. 4.3A) began with indentation at the junction of longitudinal and transverse sutures (Fig. 4.3B). Invagination (and subsequent splitting of sutures) proceeded along suture lines, particularly toward the poles (Fig. 4.3C,D). Continued inward curling of plate margins (Fig. 4.3E,F) resulted in sporocyst collapse (Fig. 4.3G) and freeing of sporozoites (Fig. 4.3H); however, complete breakdown was not necessary for zoite release (Fig. 4.3D).

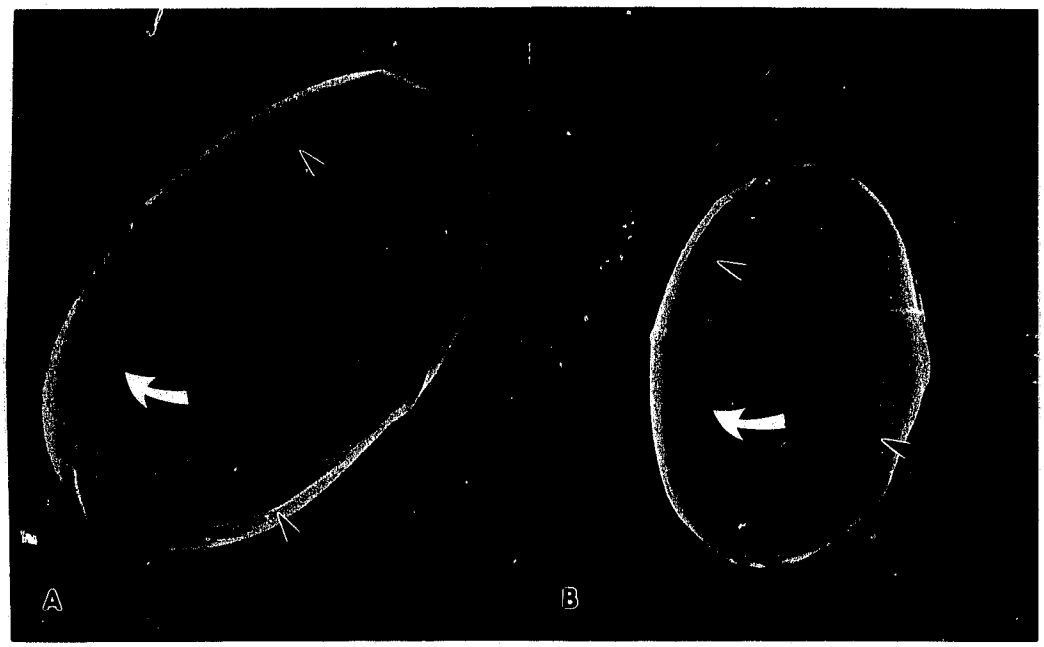
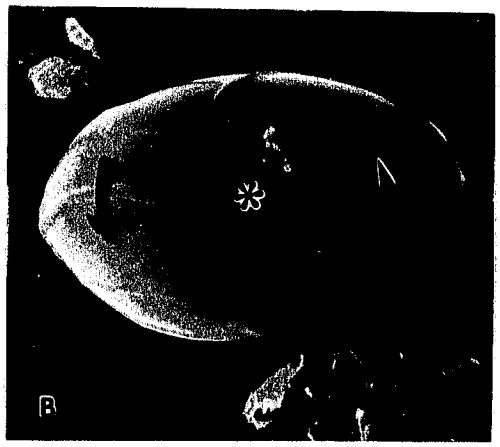


Fig. 4.1 Structure and comparative size of:
A) *Sarcocystis cruzi* sporocyst (x5000) and
B) *Sarcocystis hirsuta* sporocyst (x5000)
Note raised sporocyst sutures (curved arrow) which divide sporocysts into four "plates" and sporocyst surface venation (arrowheads).

Fig. 4.2 *Sarcocystis hirsuta* sporocysts at different stages of rotation (A-C). Curved arrow indicates direction of sporocyst rotation, double arrow indicates "suture lines" delineating plate margins, and arrowhead indicates sporocyst venations. Note shape and structure of one of four sporocyst plates ("*"). (x5000).


A


B


C

Table III - Comparative Sporocyst Parameters for *Sarcocystis cruzi* (n=30) and *S. hirsuta* (n=30), *in vitro*.

	S. cruzi	*S. hirsuta*
	$\bar{x} \pm$ S.E.M*	$\bar{x} \pm$ S.E.M
Length (μm)**	18.25 ± 0.21	15.49 ± 0.22
Width (μm)	11.80 ± 0.16	11.43 ± 0.23
Area (μm)**	107.92 ± 1.16	76.91 ± 1.11
Perimeter (μm)**	54.00 ± 0.95	45.48 ± 0.88
Shape factor++	0.472	0.478

* Mean ($\bar{x}$) values ± standard error of the mean
** Indicates significant difference (P=0.000) by Student's T-test
++ Calculated as: $p^2/4\pi a$ where p=perimeter and a=area

Table IV - Comparative Sporozoite Parameters for *Sarcocystis cruzi* (n=15) and *S. hirsuta* (n=15), *in vitro*.

	S. cruzi	*S. hirsuta*
	$\bar{x} \pm$ S.E.M*	$\bar{x} \pm$ S.E.M
Length (μm)**	16.41 ± 0.67	11.30 ± 0.33
Width (μm)	4.34 ± 0.20	4.09 ± 0.19
Area (μm)**	13.74 ± 0.41	8.78 ± 0.26
Perimeter (μm)**	35.30 ± 1.19	28.93 ± 1.29
Shape factor++	0.143	0.142

* Mean ($\bar{x}$) values ± standard error of the mean
** Indicates significant difference (P=0.000) by Student's T-test
++ Calculated as: $p^2/4\pi a$ where p=perimeter and a=area

Fig. 4.3 *Sarcocystis cruzi* demonstrating successive stages of sporocyst breakdown and sporozoite release in the excystation process of *Sarcocystis* species. All figures (x5000) except F) and H) at (x4000) and (x7000), respectively.

A) Fusiform sporozoites (four) visible through the sporocyst wall of an intact sporocyst.

B) Initiation of sporocyst breakdown. Note invagination of sporocyst wall at junction of transverse (single arrow) and longitudinal (double arrow) sporocyst sutures.

C) Continued invagination at suture lines creates an opening/split in the sporocyst wall. Note residual granules in sporocyst interior.

D) Progressive widening of opening of sporocyst wall at dehiscent sutures (arrows) releases interior sporozoites.

E) Interior view of sporocyst showing inward curling of lip-like margins of sporocyst plates (arrow) along suture lines. No changes in exterior surface structure or venation (arrowhead) are detectable.

F) and G) Continued inward curling of plate margins (arrow) results in progressive separation of sporocyst plates. Surface venation (arrowhead) remains visible.

H) Sporocyst plate remnants and one of four recently excysted sporozoites.

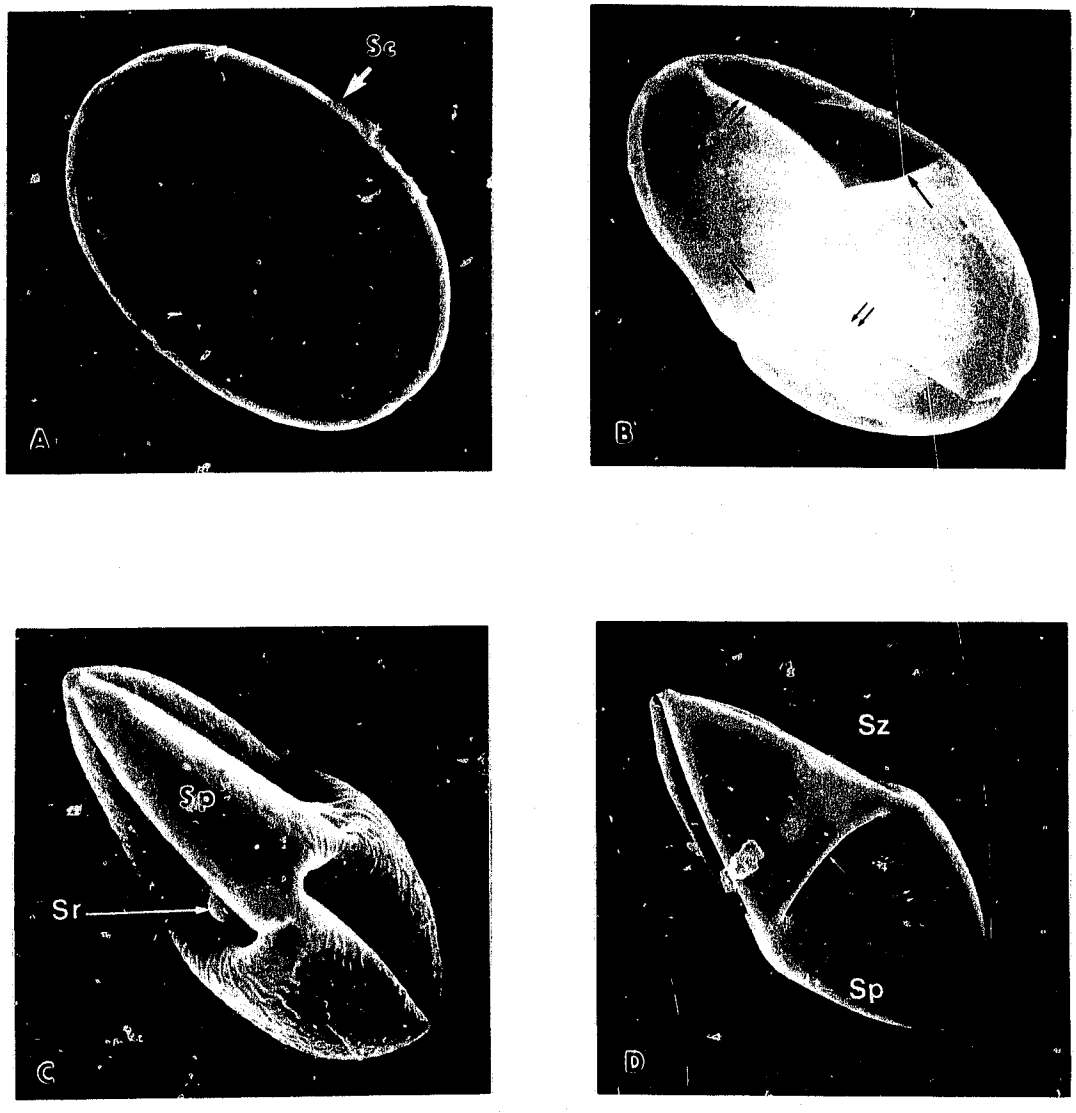
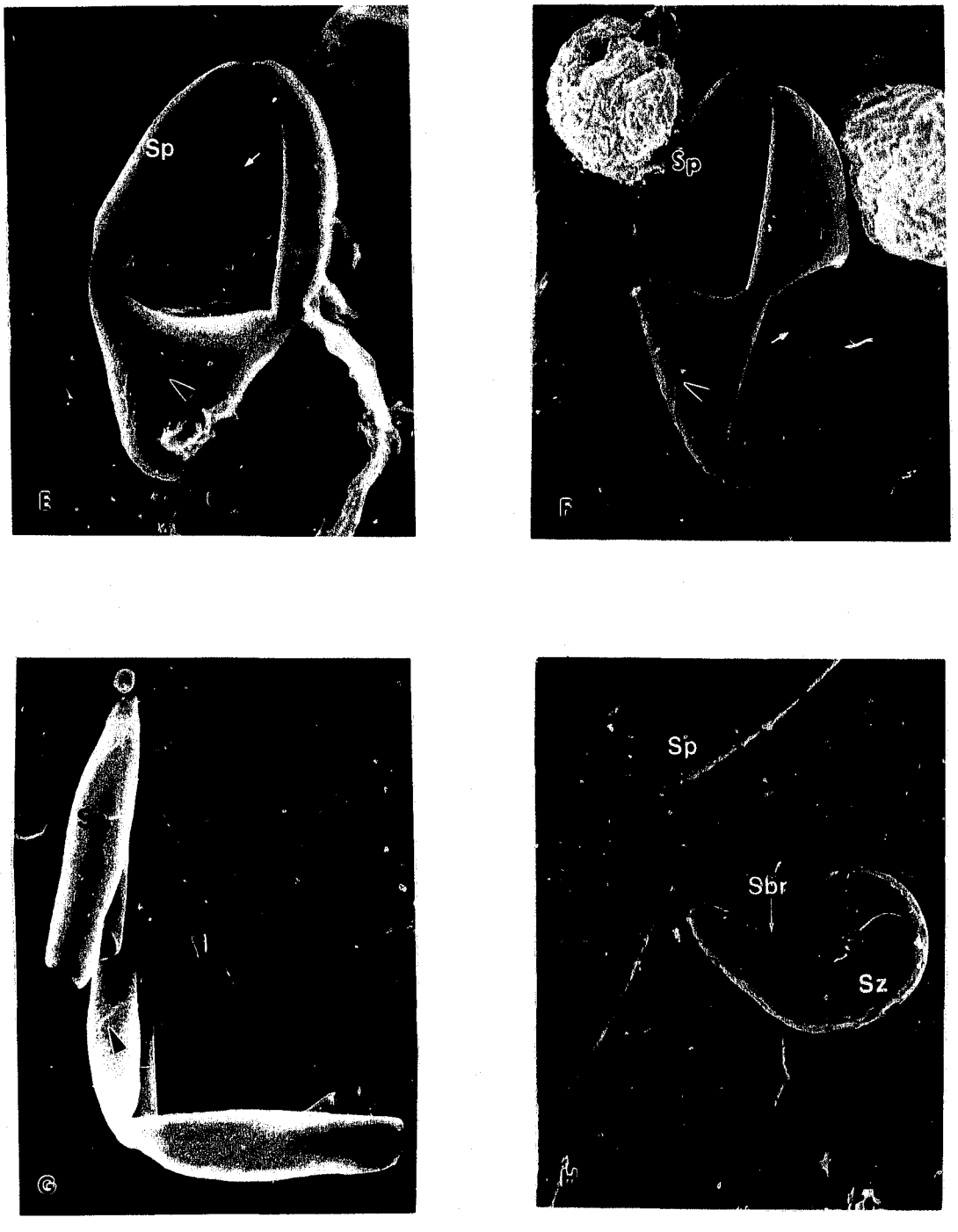

Sc
A
B
Sp
Sr
C
Sz
Sp
D

Fig. 4.3 continued...



Orientation of plate residues ranged from all 4 plates remaining attached to all 4 completely separated from, yet associated with, each other. Intact oocysts (or oocyst wall remnants) were rarely seen (Fig. 4.4).

4.3.2 TEM results

None of the three embedding protocols stabilized sporocysts in the electron beam; however, Spurr's formulation reduced the number of sporocysts that ripped out of tissue sections during ultrathin sectioning.

Sporocyst walls of *S. cruzi* and *S. hirsuta* had analogous ultrastructure which was conserved post-excystation (Fig. 4.5). A distinct 145 nm thick inner wall (IW) (93 nm *S. hirsuta*) with transverse striations of a 14 nm periodicity (19 nm *S. hirsuta*) expanded at sporocyst plate margins (Fig. 4.6). The thinner 55 nm thick outer wall (OW) (38 nm *S. hirsuta*) was comprised of at least two 10 nm thick "unit membranes" (Fig. 4.7A). Alternating light and dark layers were a reflection of areas of electron dense material interposed between these membranes (Fig. 4.7B). The inner and outer limits of individual unit membranes were themselves slightly electron dense, contributing to the electron density of the intervening "strip". Beneath the outer layers, a less clearly defined region appeared to contain 1 or 2 additional layers much more closely applied to the IW (Fig. 4.8).

Layers of the OW were usually more distinguishable at the region overlying dehiscent sutures (Fig. 4.9). Intact, continuous unit membranes were frequently

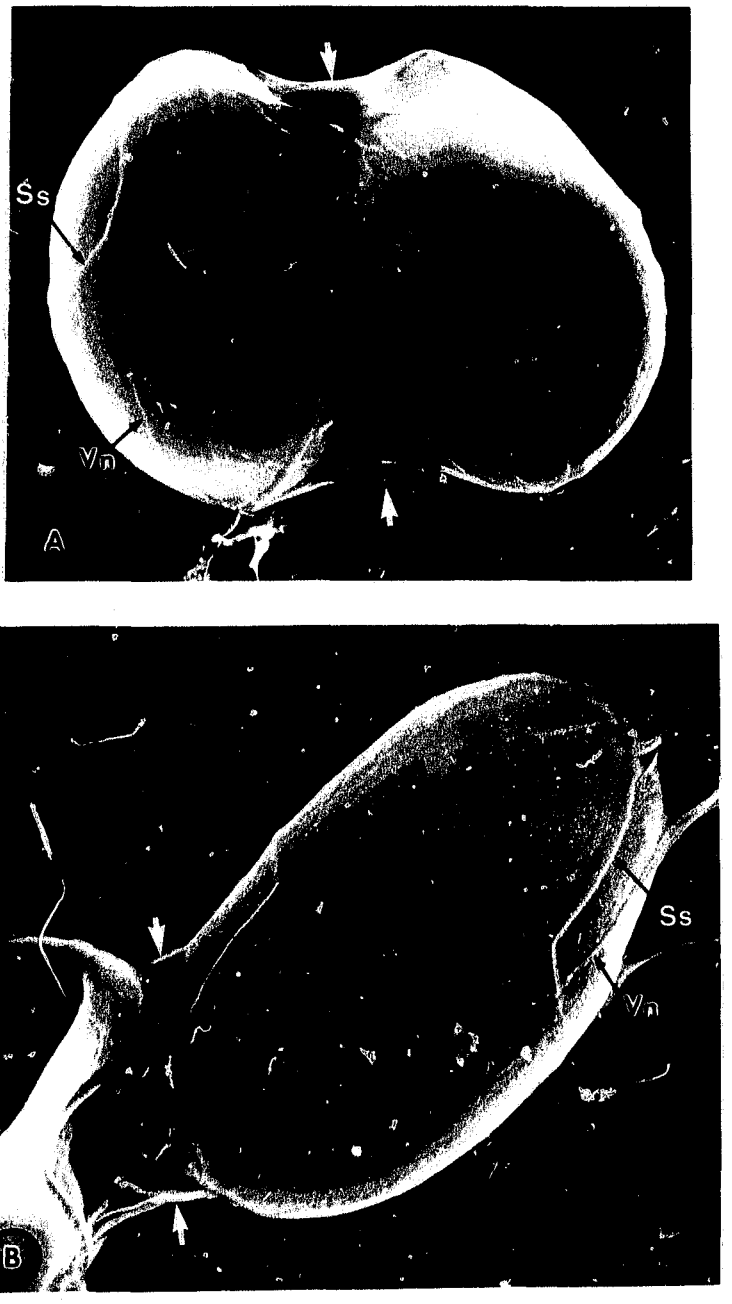
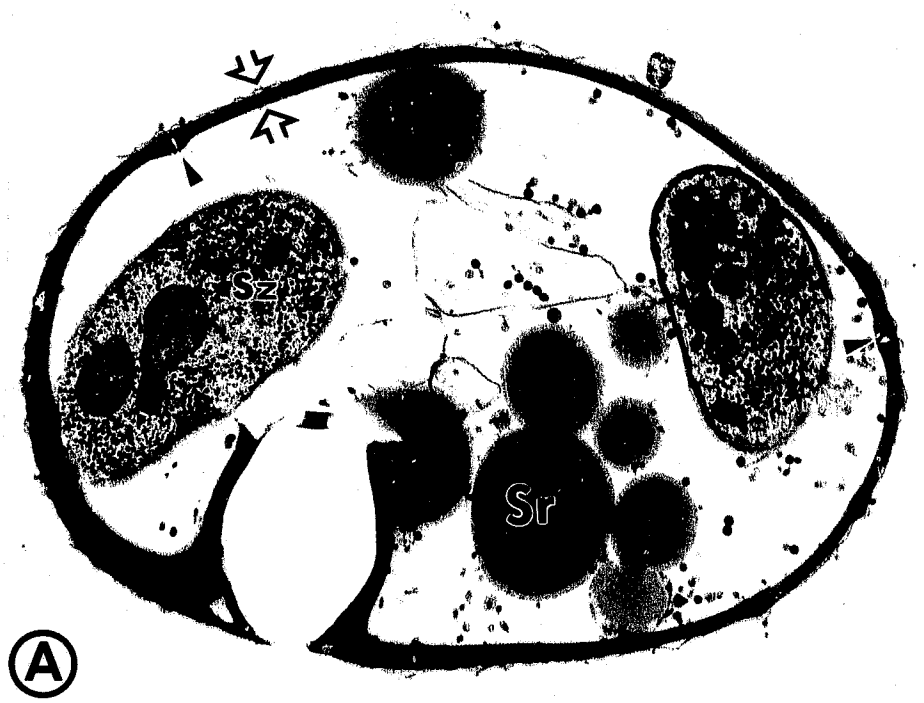


Fig. 4.4 A) *Sarcocystis hirsuta* oocyst with sporocyst sutures and surface venation visible through the thin membrane-like oocyst wall (arrows) (x6000).

B) *Sarcocystis cruzi* sporocysts with oocyst wall remnant visible at arrows (x5000).

Fig. 4.5 Comparative ultrastructure of:
A) *Sarcocystis hirsuta* sporocyst (x16,000) and
B) *Sarcocystis cruzi* sporocyst (x10,000)
Note interior sporozoites (Sz) and similarities in sporocyst residual material (Sr), wall structure (open arrows) and plate margins (arrowheads).


Sz
Sr
A

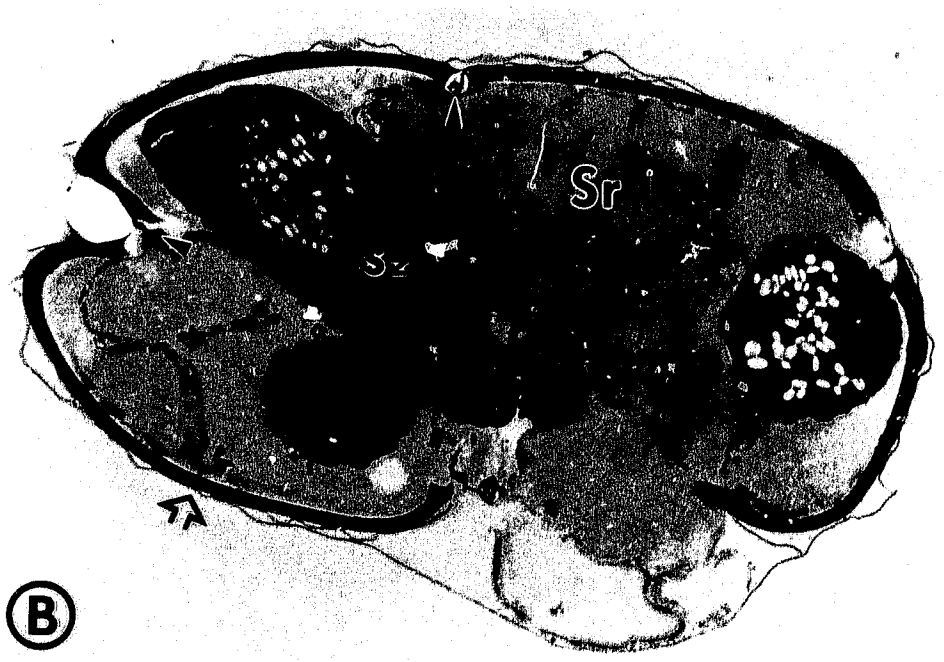
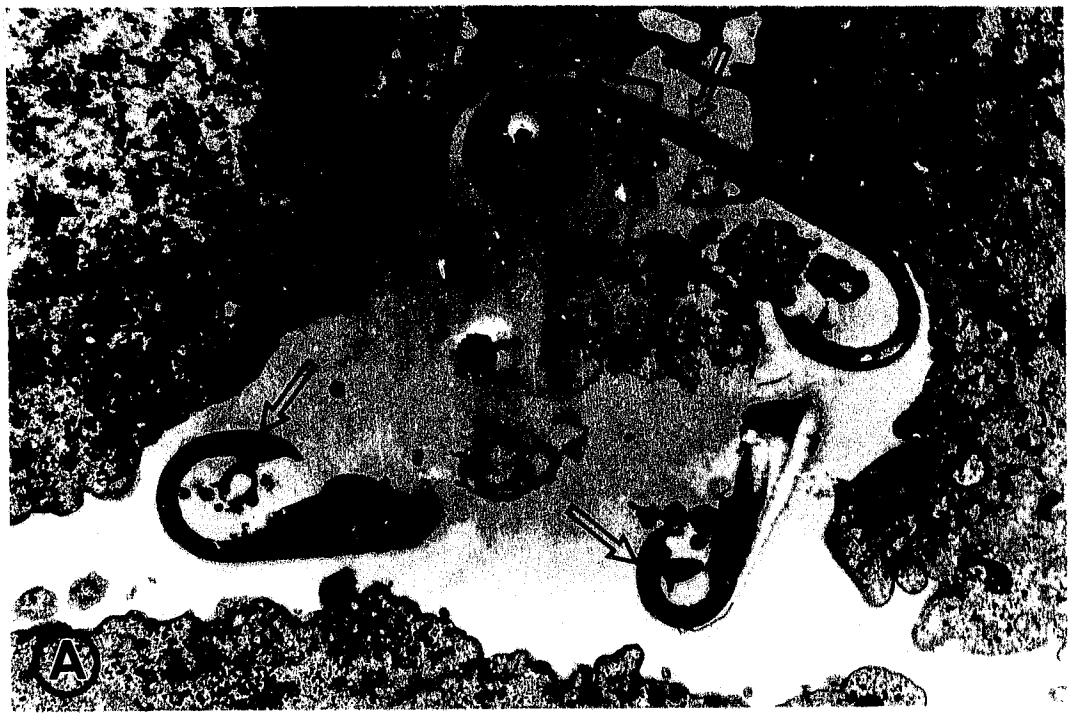
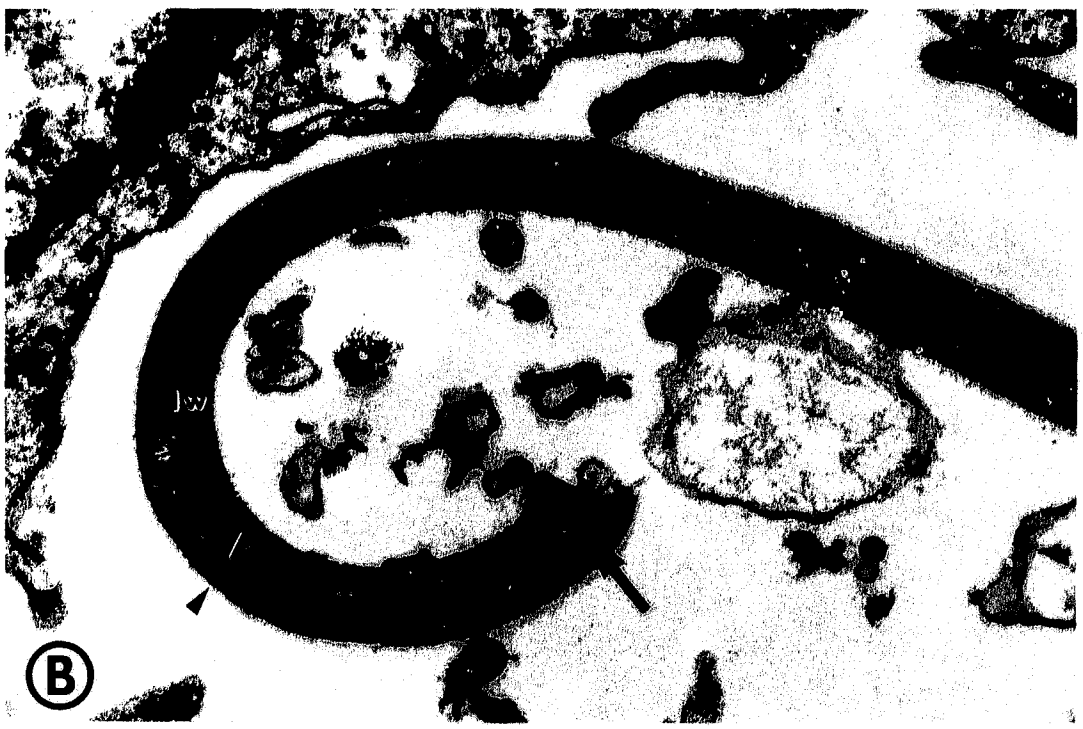

Sr
Sz
B

Fig. 4.6 A) *Sarcocystis* spp. sporocyst wall remnants, post excystation (open arrows) (x13,300).

B) Higher magnification of boxed area in A) showing transversely striated inner wall (lw) and its expanded margin at the site of sporocyst plate apposition (arrow). Note electron-lucent and electron-dense layering of the outer wall (between arrowheads) (x52,000).


A


Jaw
B

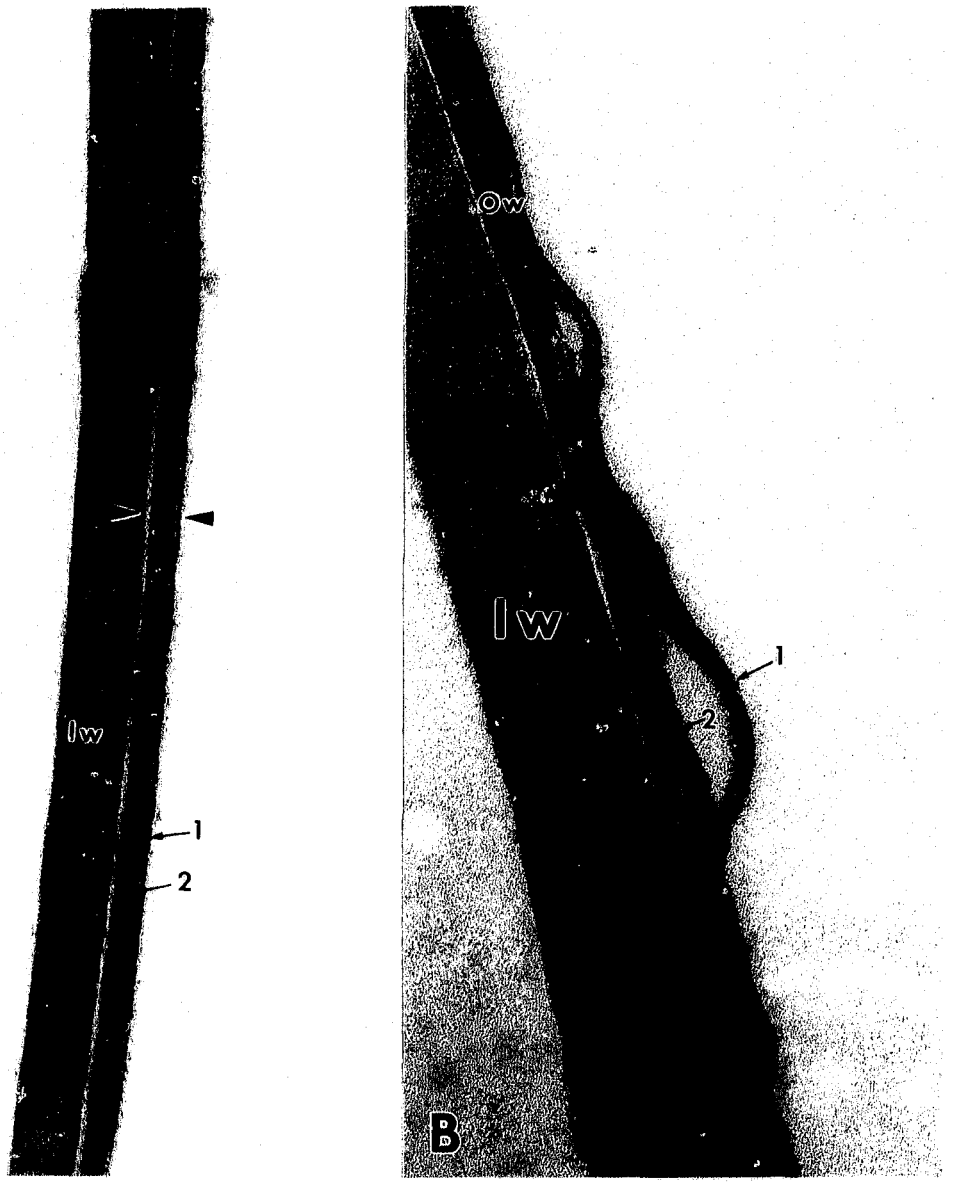
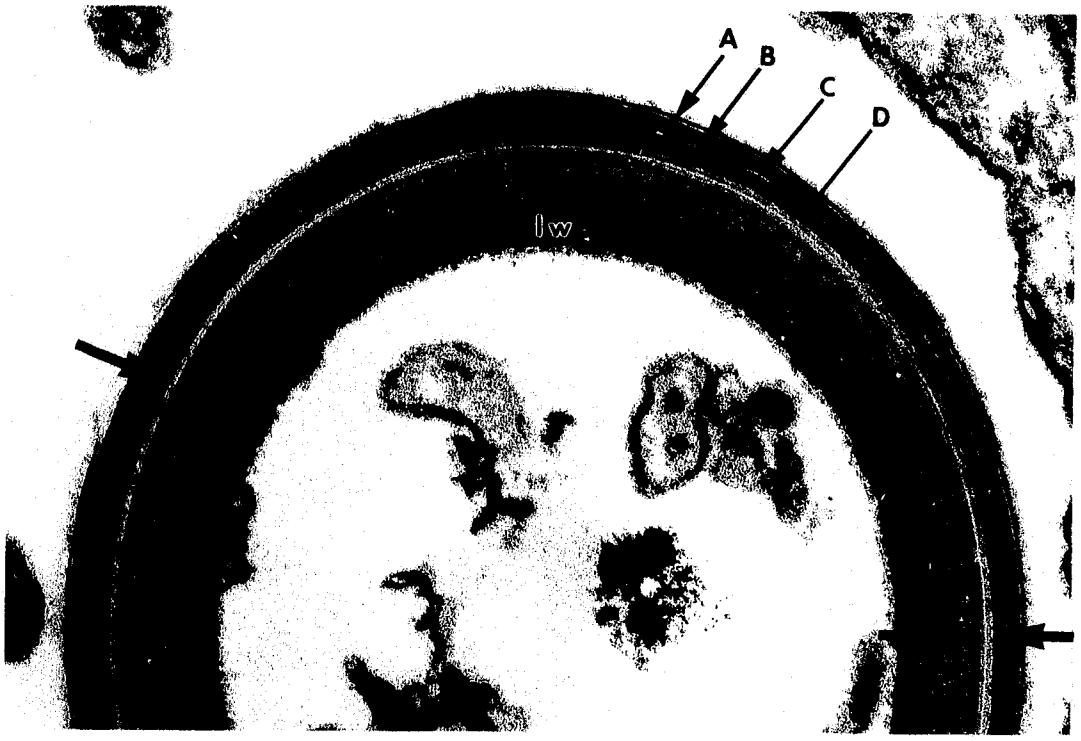


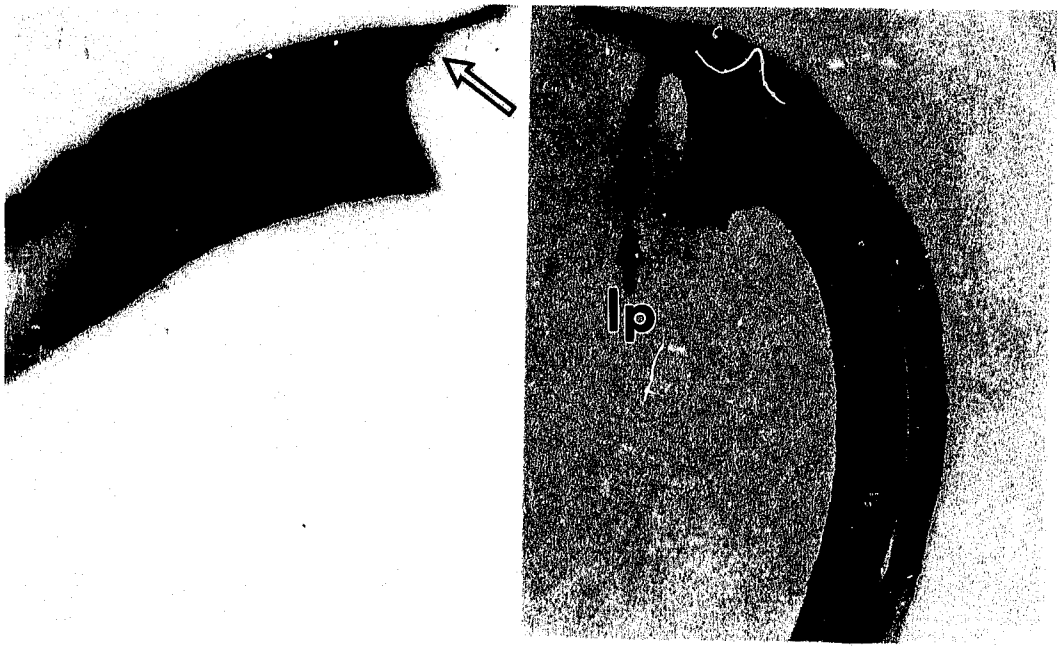
Fig. 4.7 A) *Sarcocystis* spp. sporocyst wall. Note outer wall (between arrowheads) showing electron lucent unit membranes (two) separated by electron dense material (x77,000).

B) Higher magnification of inner and outer sporocyst wall. Unit membranes (1 and 2) have focal separation from each other and from underlying layers. Note relative electron density of membranes and interposed material (x150,000).

Fig. 4.8 Sporocyst wall of *S. cruzi*. The four prominent layers of the outer wall are denoted by A-D. Less easily distinguished layering is present at the interface of the inner (IW) and outer (OW) walls (i.e. between arrows) (x103,000).

Fig. 4.9 Cross section of dehiscent suture of *S. cruzi* sporocyst (each micrograph depicts one half of the split suture). Note prominence of OW layers overlying sporocyst plate margin (open arrow) and the presence of an interposing strip (lp) between plate margins (x71,250).


A
B
C
D
lw


lp

observed over the interposing strip (IP) of plate margins even after excystation had occurred. The IP itself consisted of at least two discrete subunits (Fig. 4.10). Surface venation was reflected in both the IW and OW as an outward bulge (Fig. 4.11). This bulge was not always detectable on the lumenal side of the wall, although after excystation, it became more noticeable in curled plates. Artifactual separation of IW and OW was observed (Fig. 4.12). Some sporocysts had either a finely or a coarsely granular appearance which was usually restricted to the outer wall (Fig. 4.13). Intact oocysts (or oocyst wall remnants) were not seen.

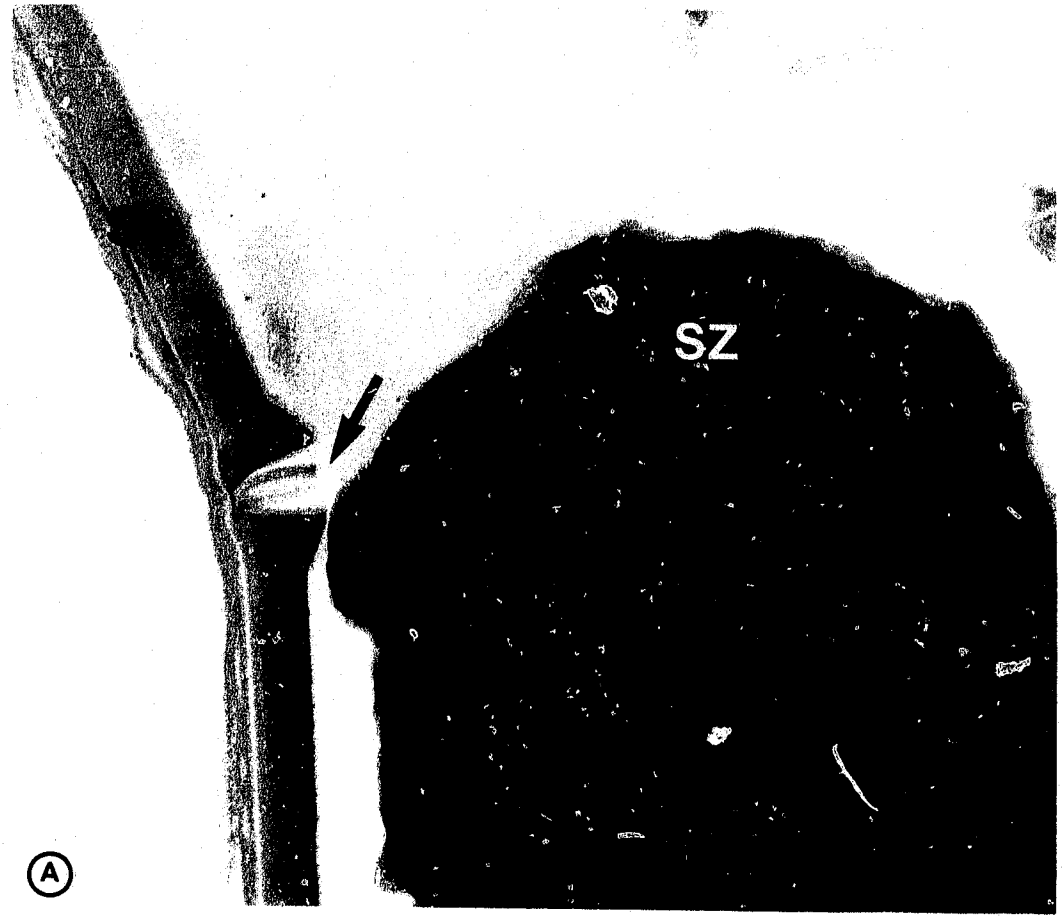
4.4 Discussion

Low excystation rates observed with deletion of trypsin were somewhat unexpected since *S. cruzi* and *S. hirsuta* excyst without trypsin (Fayer and Leek, 1973; Cawthorn and Speer, 1990). Elutriation was not a factor in rate of excystation. Percent return of sporocysts was the fraction of sporocysts eluted which were adequately cleaned for excystation. With an average return of only 36.6%, elutriation was deemed unsatisfactory for purification of *S. cruzi* or *S. hirsuta* sporocysts collected from either faeces or intestine.

Incubation media, time in storage, pretreatment conditions, EF, time in EF, pH and temperature at storage and excystation all could influence *in vitro* ER (Fayer and Leek, 1984). Unlike most coccidia, hosts of *Sarcocystis* pass single sporocysts and few sporulated oocysts in faeces (Levine, 1982). The fragile oocyst wall usually ruptures when oocysts move to the intestinal lumen (Box and Duszynski, 1980). Lack of protection from an oocyst wall appears to make

Fig. 4.10 A) High magnification of *Sarcocystis* spp. sporocyst and interior sporozoite (Sz). Note interposed strip (arrow) of sporocyst wall (Sw) plate margin (x50,000).

B) Higher magnification of plate margin in A). The interposed strip (Ip) of the inner sporocyst wall is composed of two distinct electron dense subunits (x117,000).


SZ
A

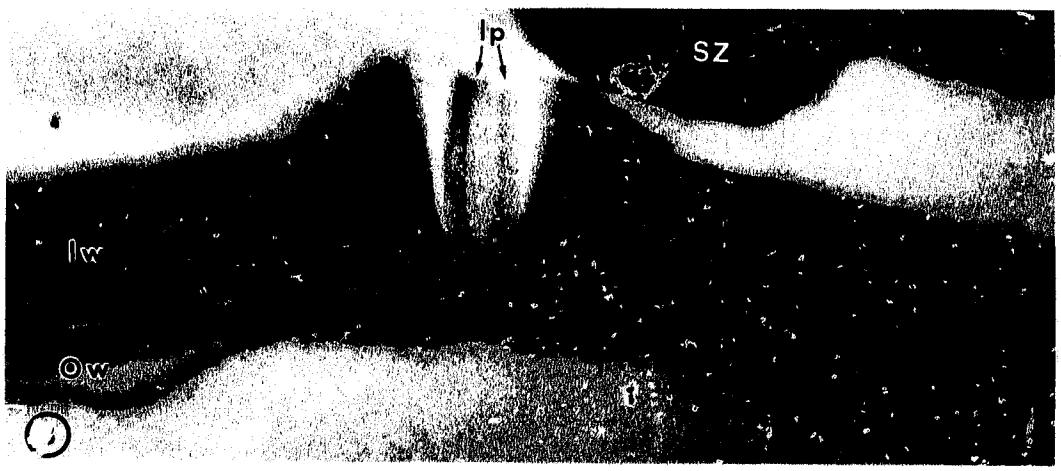
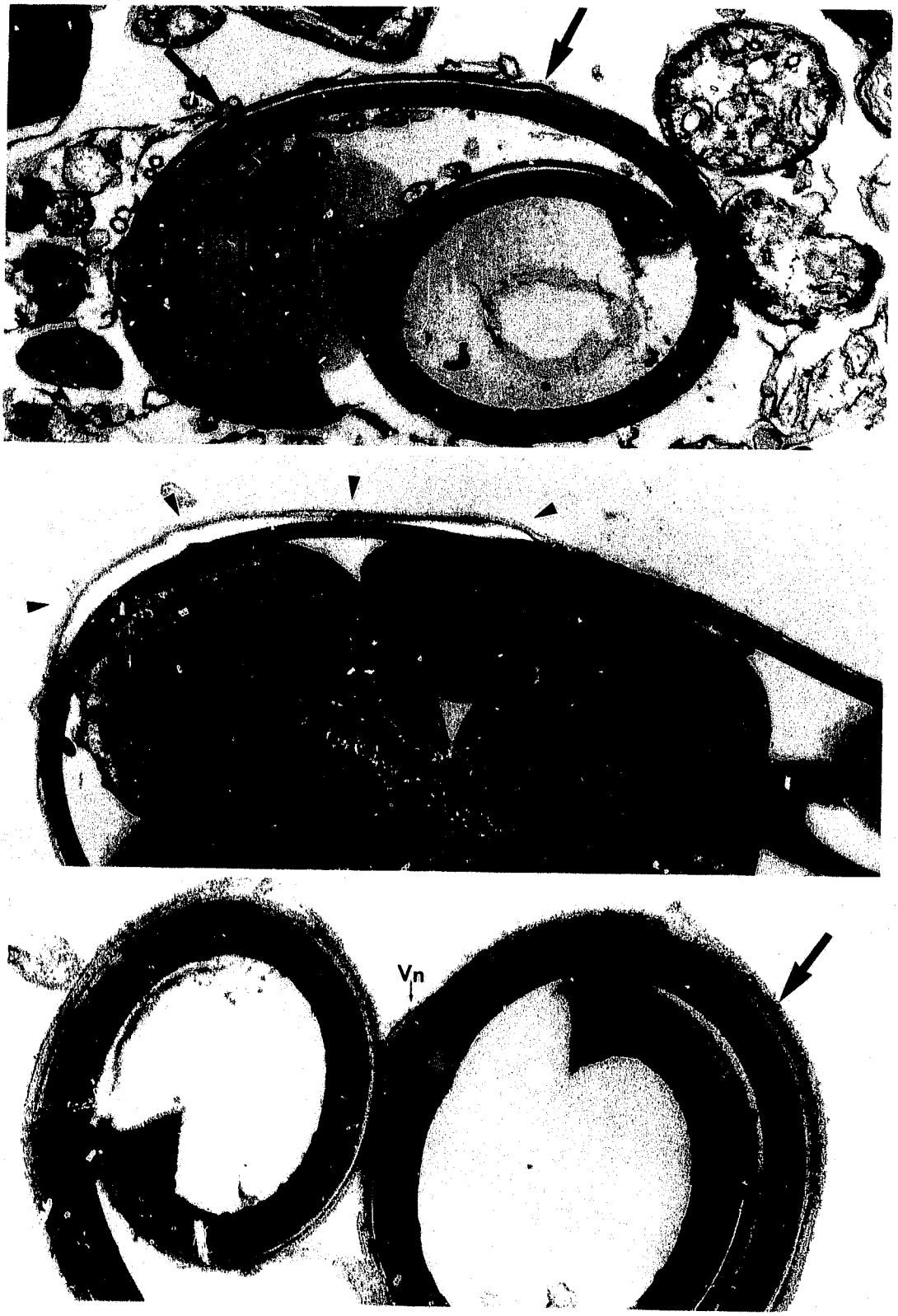

lp
SZ
lw
ow

Fig. 4.11 Venation (arrows) of the inner and outer wall of *Sarcocystis* sp. sporocysts (x39,100).

Fig. 4.12 *Sarcocystis hirsuta* sporocyst with artifactual separation of outer and inner sporocyst wall (arrowheads) (x28,500).

Fig. 4.13 Cross section of sporocyst plate of *S. hirsuta*. Note the inward curling of plate margins post excystation and granular appearance of the outer sporocyst wall (arrow) (x74,000).


Vn

Sarcocystis sporocysts more vulnerable to chemical disinfectants (Leek and Fayer, 1979) and perhaps renders them more susceptible to loss of viability during storage.

Cawthorn *et al.* (1986) noted no reduction in ER between 1 - 2.5 month-old and 38 month-old *S. capracanis* sporocysts; Strohlein and Prestwood (1986) obtained satisfactory excystation rates from *S. suicanis* preparations up to 24 months old. However, species variability in duration of viability cannot be discounted. Sporocyst age may have contributed to inconsistent, decreased ER. Since other above mentioned factors remained consistent between modified and control protocols, inability to duplicate the high excystation rates achieved by Cawthorn *et al.* (1986) remains unexplained.

The process and ultrastructure of excystation of *S. cruzi* and *S. hirsuta* were similar. However, there were significant differences ($P=0.000$, Student's T-test) in length, area, and perimeter of sporocyst and sporozoite stages (Tables III and IV, respectively) with *S. cruzi* being larger in dimension than *S. hirsuta*. Smaller size of *S. hirsuta* was also reflected in sporocyst wall measurements, but not in sporocyst or sporozoite width.

In both species of *Sarcocystis* examined, excystation begins with breakdown of the IW at the junction point of longitudinal and transverse sutures. Although equatorial (transverse) sutures are reported to dissolve first (Cawthorn and Speer, 1990), they appeared relatively stable, frequently remaining intact until the splitting process had extended the entire length of the sporocyst. Since plates

curled inwards obscuring the lumenal side of the sporocyst wall, SEM preparations were not useful for studying fine detail of this process.

Plate number (four) and configuration were consistent with other species of *Sarcocystis* (Tombes, 1979; Box *et al*., 1980; Strohlein and Prestwood, 1986). Distinct sporocyst venations, first reported in *S. suicanis* (Strohlein and Prestwood, 1986), were apparent on the surface of *S. cruzi* and *S. hirsuta* sporocysts. Closer scrutiny might reveal unique patterns of venation; however, this is impractical for diagnostic purposes. Similarly, although *S. hirsuta* venation was more prominent, this is a relative observation with little diagnostic value. Number of layers in the sporocyst wall has also been suggested as a simple means of species differentiation. While the IW is easily visualized and characterized, description of the OW can be subjective when OW layers are indistinct or closely apposed. The OW in this study showed only two prominent, membrane-like, electron lucent layers which were interpreted to be the "unit membranes" depicted by Box *et al*. (1980). These unit membranes were separated from each other and underlying structures by relatively electron-dense material. Two membranes with darker interposed material manifest as four discrete layers, yet the structure of the OW could not be reconciled with Box's *et al* (1980) description of four layers in OW of *S. debonei*.

Although Strohlein and Prestwood (1986) described the OW of *S. suicanis* with 3 dense and 3 lucent bands, the OW appears to have the same wall structure as *S. cruzi* and *S. hirsuta*. The OW of *S. tenella* is also characterized by two prominent layers (Mehlhorn and Scholtyseck, 1974). Overall, there is disparity

concerning the ultrastructure of the wall of sporocysts of *Sarcocystis* species. Long and Joyner (1984) note that processing techniques account for discrepancies in *Eimeria* oocyst structure. Similar problems may be occurring with *Sarcocystis* sporocysts. Treatment with NaOCl did not remove outer wall layers as reported by Dubey *et al.*, (1989) and Cawthorn and Speer, (1990). Additionally, there was apparently artifactual separation of inner and outer walls. The granular appearance of some sporocyst walls was also interpreted as artifact because it was not consistently observed; moreover, no counterpart was observed with SEM.

In species of *Isospora*, a thickening in the OW above sutures or a thin strip above the IP may indicate sites where sporocyst breakdown is initiated (Speer *et al.*, 1973; Speer *et al.*, 1976). Although species of *Sarcocystis* undergo a similar pattern of excystation, neither of these structures are reported in this or other *Sarcocystis* studies. The impetus for collapse appears internal with splitting of IW's always progressing from interior to exterior.

Definitive answers concerning mechanisms of excystation remain elusive. Various hypotheses have been proposed for the involvement of first and second phase stimuli (reviewed by Sommerville and Rogers, 1987) yet these stimuli are not universally required. Even among *Sarcocystis* species excystation requirements are different (McKenna and Charleston, 1991). Moreover, conditions that produce *in vitro* excystation do not necessarily reflect *in vivo* conditions. Overall, experimental protocols mimic a much more complex series of biochemical reactions occurring in the digestive tract. Our inconsistent success implies that the

host environment has not been adequately duplicated. The specific host or parasite products involved in excystation must first be identified before mechanisms of action can be fully understood.

CHAPTER V - HOST CELL INVASION BY SPOROZOITES OF *SARCOCYSTIS CRUZI*

5.1 Introduction

Research involving "invading stages" of Apicomplexa (and other parasites) is currently spearheaded by a search for potent surface epitopes for vaccine production. This has diverted attention from research on the cell biology of host-parasite interactions including invasion, nutrition, and survival i.e. resistance to host defence mechanisms (Tait and Sacks, 1988). The following chapter concentrates on invasion which is more accurately described as: recognition/attachment, reorientation, and finally, penetration (Russell, 1983).

An understanding of the mechanism of invasion is vital; invasion initiates the developmental cycle that results in host damage. Furthermore, there can be no reinvasion without release from infected cells. Once excysted, sporozoites must "seek" and invade (and perhaps exit and reinvade) host cells for continuation of the life cycle; similarly, merozoites and microgametes must be capable of leaving host cells and entering new ones (Dubey *et al*, 1989).

Concentration on parasites with medical or veterinary significance has restricted the view of invasion: species of *Eimeria*, *Toxoplasma*, and particularly, *Plasmodium* (see Speer, 1983; Werk, 1985; Breuer, 1985 for respective reviews) have been intensively studied yet major questions (reviewed in Sinden, 1985) remain unanswered. Identical molecular mechanisms are unlikely to exist, even

among closely related species; thus, expansion of study in other genera of Apicomplexa is warranted.

Host cells are apparently accessed by: a) forceful rupture of or b) gradual invagination of plasma membranes, resulting in an intracellular parasite free in the cytoplasm or enclosed in a parasitophorous vacuole (PV), respectively. However, exceptions and complicating factors exist, eg. PV formation may be absent or delayed (Chobotar and Scholtyseck, 1982). Furthermore, whether phagocytosis by host cells, or active penetration by zoites or some combination occur is controversial, eg. *Toxoplasma gondii* shows equally strong evidence for parasite and host participation in invasion (Nichols and O'Connor, 1981; Werk, 1985).

Several invasion models have been proposed: Aikawa *et al.* (1978) described a "moving junction" wherein a zone of continuous attachment and detachment of parasite and host membranes occurs, likely receptor dependent. Russell (1983) presented a "capping model" which assumes adherence and displacement of membranes by means of anionic sites and actin-based contractions, respectively. Other models include movement by membrane flow phenomena and induction of receptor-mediated phagocytosis (see Michel *et al.*, 1980 and Russell, 1983 for details).

Proposed models should incorporate the apical complex and associated organelles implicated in invasion. Similarly, involvement of parasite and host cytoskeletal elements require further investigation. Specific roles for rhoptries, micronemes, and dense bodies are emerging (see Bannister *et al.*, 1986; Adams

et al., 1990; Torii *et al.*, 1989, respectively) although details of achievement of particular functions are lacking.

The highly conserved morphology of invasive stages supports functional similarity among various apicomplexan genera (Dubremetz, 1990) but, does not mandate it. Examples of diversity and specialization include formation of a secondary PV in *S. muris* (Entzeroth, 1985) and formation of unique dense bands near the conoid in *Cryptosporidium* spp. (Lumb *et al.*, 1988). Additionally, there are discrepancies, e.g. invasion by apicomplexans usually proceeds when the zoite stage reorientates such that its apex is aligned with the host membrane (Perkins, 1992). However, Weathersby (1987) observed *Plasmodium* spp. merozoites enter tissue culture cells posterior end first.

Membrane surfaces (both parasite and host) are perhaps the most obvious in their diversification. Comparing specificity of *Plasmodium*-merozoites for erythrocytes with that of *Toxoplasma*-tachyzoites which enter almost all mammalian cells (and assume various intracellular locations) supports Sinden's (1985) view that specific receptors cannot mediate penetration of all membranes. Moreover, membrane alterations occur preinvasion (e.g. capping and shedding of immune complexes) (Speer *et al.*, 1985), during invasion (e.g. insertion of membranous rhoptry secretions) (Bannister and Mitchell, 1989) and postinvasion (e.g. channel formation in membranes upon dense granule exocytosis) (Torii *et al.*, 1989).

The significance of membrane changes in the invasion process are becoming better defined. However, Joiner (1991) notes most information on

rhoptry function and parasitophorous vacuole formation is derived from only *Plasmodium species*. Basic mechanism(s) of host cell invasion must be examined on a broad comparative basis to validate specific observations, elucidate underlying mechanisms and optimize protocols for prevention and control of sarcocystosis.

The purpose of experimentation detailed in this chapter was to document the ultrastructure of host cell invasion by *S. cruzi* sporozoites and to obtain insight into the mechanism(s) of attachment and penetration (and possibly exiting and reentry of cells) preceding *in vitro* development. The present study also provides critical evaluation of a rapid tissue culture technique for TEM and SEM of invasion experiments.

5.2 Materials and methods

5.2.1 Testing of cell culture technique

A simple, rapid method of culturing and processing *Sarcocystis*-infected cell lines was necessary. Aclar® (Pro-Plastics, Morristown, NJ), a flexible thermoplastic film composed of fluorinated-chlorinated resins, is extensively used to culture cells. Its properties make the product stable and amenable to subsequent light and electron microscopy (Masurovsky and Bunge, 1989). Furthermore, the film is useful for correlating SEM and TEM observations, particularly surface features (Kingsley and Cole, 1988).

The technique of Kingsley and Cole (1988) was assessed in preliminary experiments testing suitability of Aclar as a cell culture substrate for eight ATCC cell lines (consult Appendix A for description of cell lines and their recommended culture media). Incorporation of a staining procedure could facilitate subsequent EM processing. P-phenylenediamine (Fisher Scientific Co., Fair Lawn, NJ) rapidly enhances detail of coccidia without producing artifact (Douglass and Speer, 1983). Further preliminary experiments comparing staining capabilities and ultrastructural effects of p-phenylenediamine and toluidine blue (our routine EM protocol as control - Appendix B) were conducted with the former proving superior. Subsequently, the methods of Kingsley and Cole (1988) were used with the addition of p-phenylenediamine staining and other significant modifications detailed in section 5.3.1 of this chapter.

Lastly, inadequate fixation observed in preliminary work prompted experimentation altering both pH and osmolarity of fixatives to better correspond with various tissue culture media employed. Concentration of fixatives and buffers reported herein gave the best results.

5.2.2 Invasion of cell cultures

5.2.2.1 Preparation and inoculation of tissue cultures

Sporocyst collection and excystation were as detailed in Chapter III. Approximately 5 x 10^4 *S. cruzi* sporozoites were inoculated into each well of six Multiwell® 24-well culture plates (Becton Dickinson, Lincoln Park, NJ) containing

gas sterilized [Sterilization conditions: Oxyfume® (88% Freon and 12% Ethylene oxide) for 1 3/4 hr at 49.4 °C] 127 μm thick Aclar-33C discs which had been previously seeded with 5 x 10^4 cells of either bovine pulmonary artery endothelial cells (CPAs) or rat heart myoblasts (H9C2's) and incubated at 38 °C in 5% CO_2-95% air atmosphere until monolayers formed (approx. 24 h).

Intervals in the sampling regime (30 sec, 5 min, 15 min, 30 min, 1 h, and 2 h) were spread to pinpoint the invasion window for *S. cruzi* sporozoites. At each sample time, discs of CPA and H9C2 monolayers were removed and fixed in 2.5% glutaraldehyde in 0.15 M phosphate buffer (pH 7.36, 315 mosm) for 30 min. Samples were then rinsed in phosphate buffer and postfixed in 1% osmium tetroxide in the same buffer for 30 min at 4 °C. After a final buffer rinse, Aclar discs were cut in half for separate SEM and TEM processing. Storage was in phosphate buffer (pH 7.4, 315 mmol/kg) at 4 °C for a maximum of 5 days prior to further EM processing.

5.2.3 SEM preparation

Fixed monolayers were dehydrated by placing Aclar discs in glass petri dishes containing increasing ethanol concentrations (50%, 70%, 95% and 100%). Our routine SEM processing schedule (Appendix B) was followed except times were reduced to 30 sec. Throughout dehydration and following procedures, the cell side of discs faced upward. While ensuring cells remained submerged in precooled (4 °C) 100% ethanol, specimens were put in Histo Prep biopsy bags (Allied Fisher Scientific, USA). Bags were weighted with paper clips and loaded

into a Balzers Union CPD 020 critical point dryer (Balzers High Vacuum Products, Mississauga, ON). Discs were attached to SEM stubs with silver paint (JB EM, Montreal, PQ) or conductive carbon paint (Structure Probe, Inc., Westchester, PA) and gold coated for 6 min (at 10 mA, 10^{-1} mbar vacuum) in a Polaron PS3 SEM Coating Unit (Polaron Equipment Ltd., Watford, England). Stubs were stored in a vacuum desiccator until examined and photographed in a Hitachi H-7110 Scanning System electron microscope operating at 25 kV. Photography supplies and production of final prints was as in section 4.2.1.

5.2.4 TEM preparation

Fixed monolayers were partially dehydrated in 50% ethanol (10 sec) and 70% ethanol (10 sec) before staining with 1% (w/v) p-phenylenediamine in 70% ethanol (30 sec or until staining visible). Dehydration continued in 95% ethanol (10 sec) and 100% ethanol (10 sec X2). Discs were submerged in 50:50, 100% ethanol:epon/araldite (see Appendix C for formulation) and cut to fit No. 4 gelatin capsules (TUB Enterprises, North Augusta, ON) (approx. 1.5 mm^2). Disc pieces were positioned, cell side up, on a supported clean sheet of Aclar and a drop of pure epon/araldite placed on each one. Finally, labelled, epon/araldite-filled gelatin capsule were inverted over the pieces (see Fig. 5.1). The Aclar sheet was carefully placed in a polymerizing oven overnight at 70 °C. Polymerized blocks were sectioned on a Reichert-Jung Ultracut E microtome, stained with uranyl acetate and lead citrate, and examined and photographed in a Hitachi H-7000 or H-600

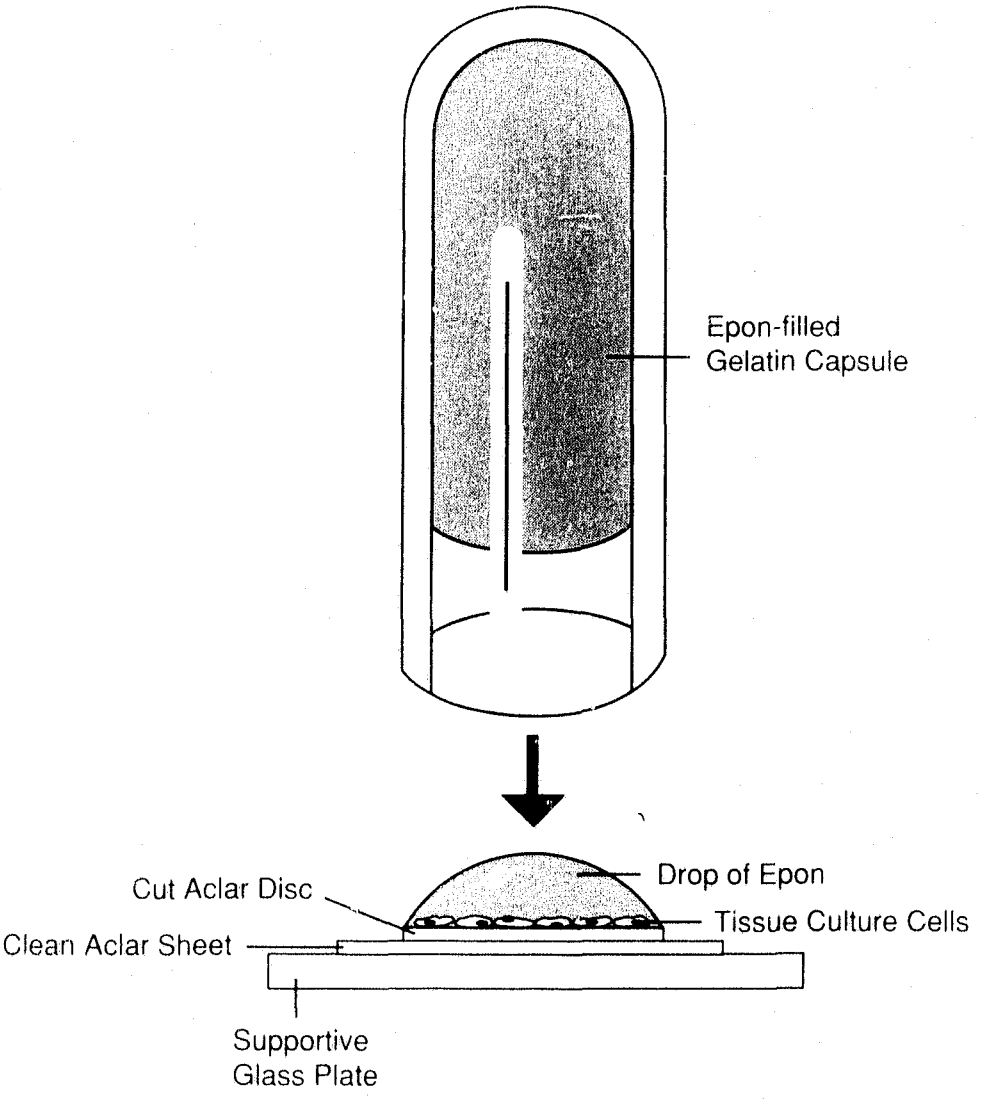


Fig. 5.1 Schematic representation of direct embedding technique used for transmission electron microscopy of *Sarcocystis cruzi* infected tissue culture monolayers grown on Aclar film.

electron microscope operating at 75 kV. Photography supplies and production of final prints was as in section 4.2.2.

Material was also preserved for light microscopy by using similarly processed discs which had *not* been cut to fit capsules. Intact discs were placed on the Aclar sheet, covered with a few drops of epon, polymerized without gelatin capsules and then taped to standard glass slides for microscopic viewing.

5.3 Results

5.3.1 Testing of cell culture technique

Preliminary testing confirmed the suitability of Aclar film as a cell culture substrate. All eight cell lines tested (CPA, M617, VERO, H9C2, L-6, G-8, Nor-10 and BALB/c C1.7) gave satisfactory results; however, the material did not perform as previously described by Kingsley and Cole (1988). Aclar discs were noticeably warped after steam sterilization and they became less rigid when exposed to propylene oxide during TEM processing, necessitating: a) a switch from steam autoclaving to gas sterilization of discs and b) discontinuation of the use of propylene oxide as the transfer agent from alcohol to epon. The latter modification facilitated direct embedding (Fig. 5.1) which replaced gluing polymerized specimens to blank blocks.

Aclar was readily separated from the embedding medium when done shortly after polymerization. Delaying the removal of discs resulted in Aclar residues that remained on the block face and required sectioning for removal.

This resulted in more difficulty aligning the block face to the microtome knife. Sections retrieved either split or wrinkled where Aclar-epon interface remained. Additional problems included: discs floating to the surface of culture wells, cell growth on the underside of discs, and bacterial contamination. Lastly, "bubbling" at the surface of SEM preparations frequently occurred when scanning in TV mode at relatively low magnification (2000x) and accelerating voltage (25 kV). When the stage position was stationary for more than 1-2 min, expanding bubbles either obscured specimens or produced direct specimen damage.

5.3.2 Invasion of cell cultures

5.3.2.1 SEM results

Sporozoite (SZ) and cell surfaces were well preserved. Both CPA and H9C2 preparations were relatively free of debris. Sporozoites exhibited counter-clockwise spiralling of body ridges which was not restricted to invading SZs (Fig. 5.2). Most did not have conoids fully extended and a few free SZs (i.e. unattached to host cell) had small "blebs" of membrane-like material intimately associated with the apex of the anterior end (Fig. 5.3).

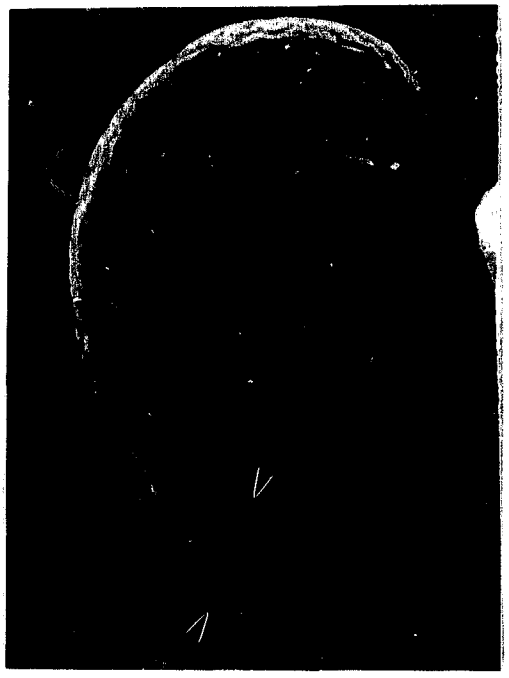
In CPA cultures, no parasites were observed inside cells at 30 sec. Only one SZ was found on the surface of the preparation and it was not attached to a cell surface. At 5 min PI SZs appeared more numerous on cell surfaces (with no particular orientation) and a few had entered cells (Fig. 5.4). The majority of SZs could not be positively identified as either entering or exiting cells (Fig. 5.5). Both

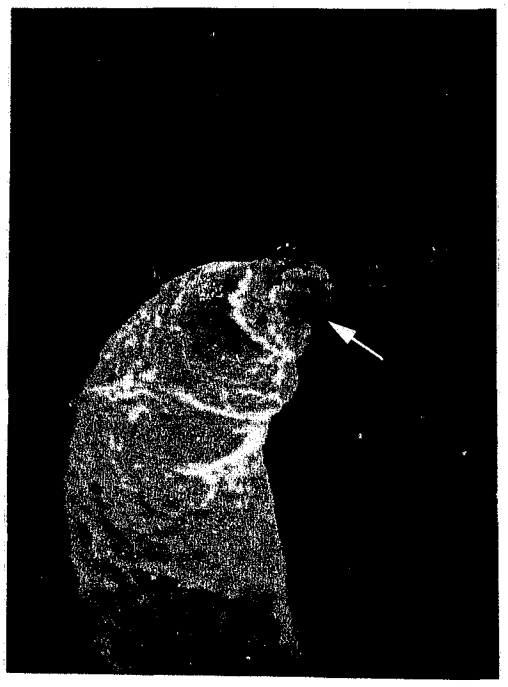
Fig. 5.2 *Sarcocystis* spp. sporozoite. Note body ridge spiralling (b veen arrowheads) (x13,300).

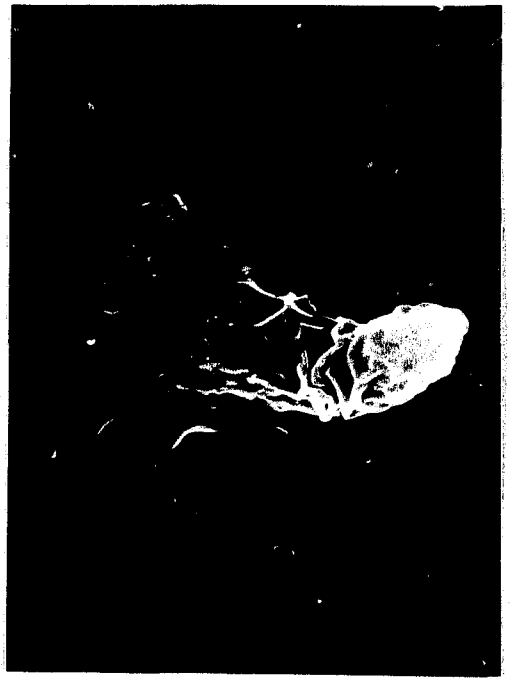
Fig. 5.3 Apex of *S. cruzi* sporozoite. Note membranous material (arrow) which is intimately associated with some preinvasion i.e. extracellular sporozoites (x20,000).

Fig. 5.4 Sporozoite of *S. cruzi* in bovine pulmonary artery endothelial cell 2 h post sporozoite inoculation of tissue cultures (x4500).

Fig. 5.5 *Sarcocystis cruzi* sporozoite entering (or exiting) bovine pulmonary artery endothelium 1 h post sporozoite inoculation of tissue cultures (x7000)







ends of zoites had a conoid-like protrusion at their apices (Fig. 5.6). Additionally, the conoid and apical complex was not always at the more tapered end of SZs. Marked conoid extension (Fig. 5.7) facilitated anterior and posterior orientation of parasites. By 15 min, the number and activity of SZs seemed to peak: attachment to cells was usually at the more tapered end; the region overlying the anterior microtubule system became more prominent (Figs. 5.8); and marked SZ flexion was noted (Fig. 5.9). Examination of subsequent samples (i.e. 30 min, 1h and 2h) demonstrated a steady increase in number of parasitized cells and a steady decline in extracellular SZs. By 2h PI SZs were rarely seen outside cells.

There were no surface projections from the cell toward SZs; plasma membranes of parasite and host merged smoothly into one continuous unit without demarcation (Figs. 5.10 and 5.11). Some penetrating SZs were constricted (approximately half their original diameter) as they passed through the cell wall (Fig. 5.12). A shallow depression in the cell surface accompanied some invaders and not others.

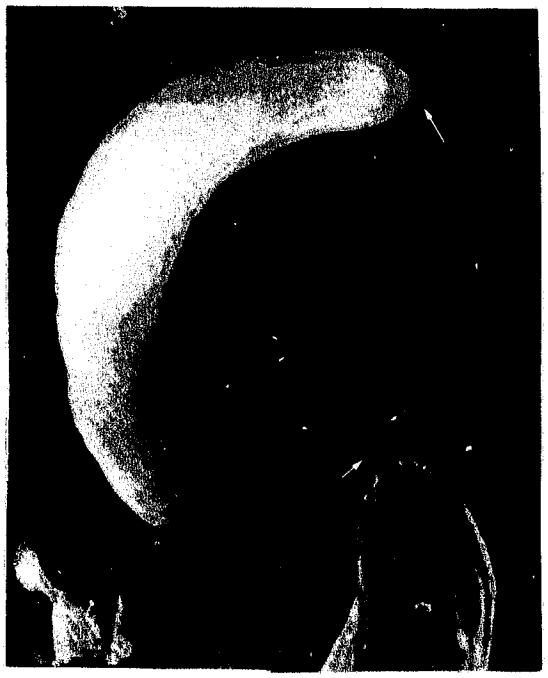
Sporozoites which had penetrated cells were most frequently seen lying immediately beneath the plasma membrane; the entire SZ outline projecting from the host cell surface (Fig. 5.13). Deeper penetrating SZs were recognized by their characteristic banana shape which contrasted against the darker surrounding host cell (Fig. 5.14). Most SZs maintained their preinvasion conformation but shortening and thickening (i.e. a fatter appearance) sometimes occurred postinvasion. Parasitized cells were usually infected with more than one SZ and up to 5 per cell

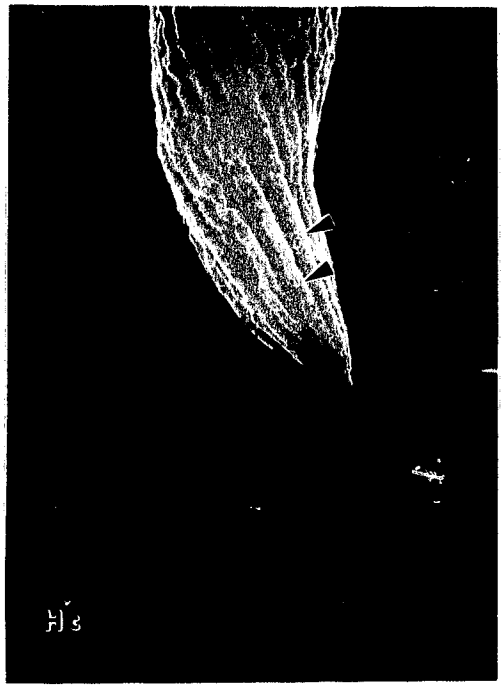
Fig. 5.6 Conoid-like projections (arrowheads) at tapered ends of *Sarcocystis* spp. sporozoite (x 14,000).

Fig. 5.7 Extended conoid (arrow) of *Sarcocystis* spp. sporozoite (x10,500). Inset: Note lancet-like projection (small arrow) at apex of *Sarcocystis* spp. conoid (arrow) (x15,000).

Fig. 5.8 *Sarcocystis cruzi* sporozoite attached to host tissue culture cell. Note prominent ridges (arrowheads) in the sporozoite plasmalemma overlying the region of subpellicular microtubules (x13,000).

Fig. 5.9 *Sarcocystis cruzi* sporozoites 15 min post sporozoite inoculation of bovine pulmonary artery endothelial cells. Note flexion (arrow) typical of peak invasive activity (x8050).





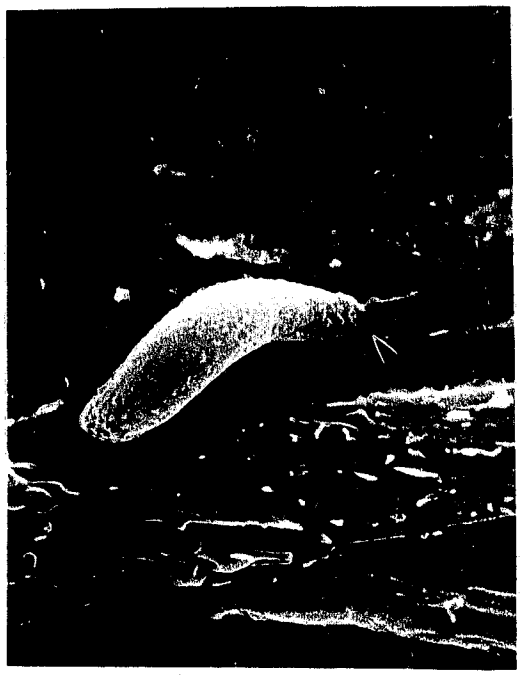

Hc

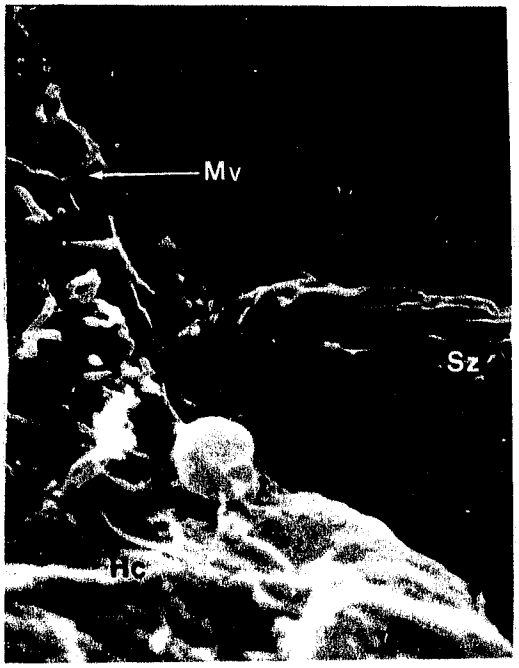
Fig. 5.10 *Sarcocystis* spp. sporozoite entering rat heart myoblast 1 h post sporozoite inoculation. Note continuity of parasite and host plasmalemmas (arrowhead) and lack of microvillar projections at tne point of host parasite contact (x5800).

Fig. 5.11 High magnification of sporozoite attachment to rat heart myoblast 2 h post sporozoite inoculation. Extension of host cell microvillar projections over the zoite surface, typical of a phagocytic process, is not evident (x20,000).

Fig. 5.12 Sporozoite penetrating tissue cultured endothelial cell 15 min post sporozoite inoculation. Note depression in host cell membrane (arrowheads) and constriction of sporozoite (arrow) at point of penetration (x11,500).

Fig. 5.13 *Sarcocystis cruzi* sporozoite 30 min post sporozoite inoculation of bovine pulmonary artery endothelial cells. Note location of the sporozoite just internal to the host cell plasma membrana (x8100).




Mv
Sz
Hc


Sz
Hs

(4 in H9C2s) were noted. Additionally, SZs were often seen "paired" inside cells i.e. two in close proximity to one another. Tears in the host cell plasma membrane were common at: a) sites where SZ penetration occurred (Fig. 5.15); and b) overlying intracellular SZs (Fig. 5.16). Sporocysts were also visible inside cells (Fig. 5.17).

The H9C2 cultures paralleled CPA results except no SZs had successfully penetrated until 15 min PI (versus 5 min with CPAs). Overall, fewer sporozoites invaded this cell line; consequently relatively more extracellular SZs were visible at the 2 h point.

5.3.2.2 TEM results

Transmission EM confirmed SEM observations of anterior and posterior SZ's protrusions (Fig. 5.18), membranous secretions from apical end of zoites (Fig. 5.19), and phagocytosis of sporocysts (Fig. 5.20). Ultrastructure of the conoid and interior organelles permitted rapid orientation of parasites. Maximum sporozoite penetration of both CPA and H9C2 cultures (Fig. 5.21) corresponded with the SEM intervals; however, extensive sectioning could not reveal sporozoites penetrating host cells at right angles. Tangential sections which were obtained (Fig. 5.22) did not provide adequate detail of the penetration process; specifically, ultrastructure of parasite and host membrane interaction.

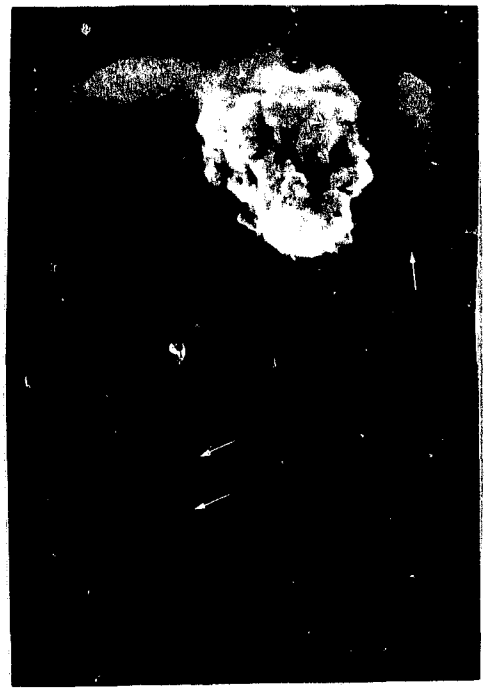
A loss of density of rhoptry contents after host cell penetration resulted in a "ghost-like" or mottled appearance of this organelle. Intracellular and

Fig. 5.14 Sporozoites (arrows) of *S. cruzi* in bovine pulmonary artery endothelial cell 2 h post sporozoite inoculation. Note: The deeper penetration of zoites into the host cell cytoplasm relative to Fig. 5.13 (x3300).

Fig. 5.15 High magnification of rat heart myoblast 15 min post inoculation with *S. cruzi* sporozoites. Note point of host cell penetration (arrow) by sporozoite and host membrane remnant (arrowhead) covering apex of zoite. Open arrow indicates cell surface microvilli (x14,950).

Fig. 5.16 Rat heart myoblast 2 h post inoculation with *S. cruzi* sporozoites. Note artifactual tear in host cell membrane (arrowheads) overlying the intracellular sporozoite (arrow) (x9500).

Fig. 5.17 Phagocytized *S. cruzi* sporocyst in rat heart myoblast 1 h post sporozoite inoculation. Sporocyst sutures (white arrow) and venation (black arrow) are visible through the host cell plasmalemma (x3200).



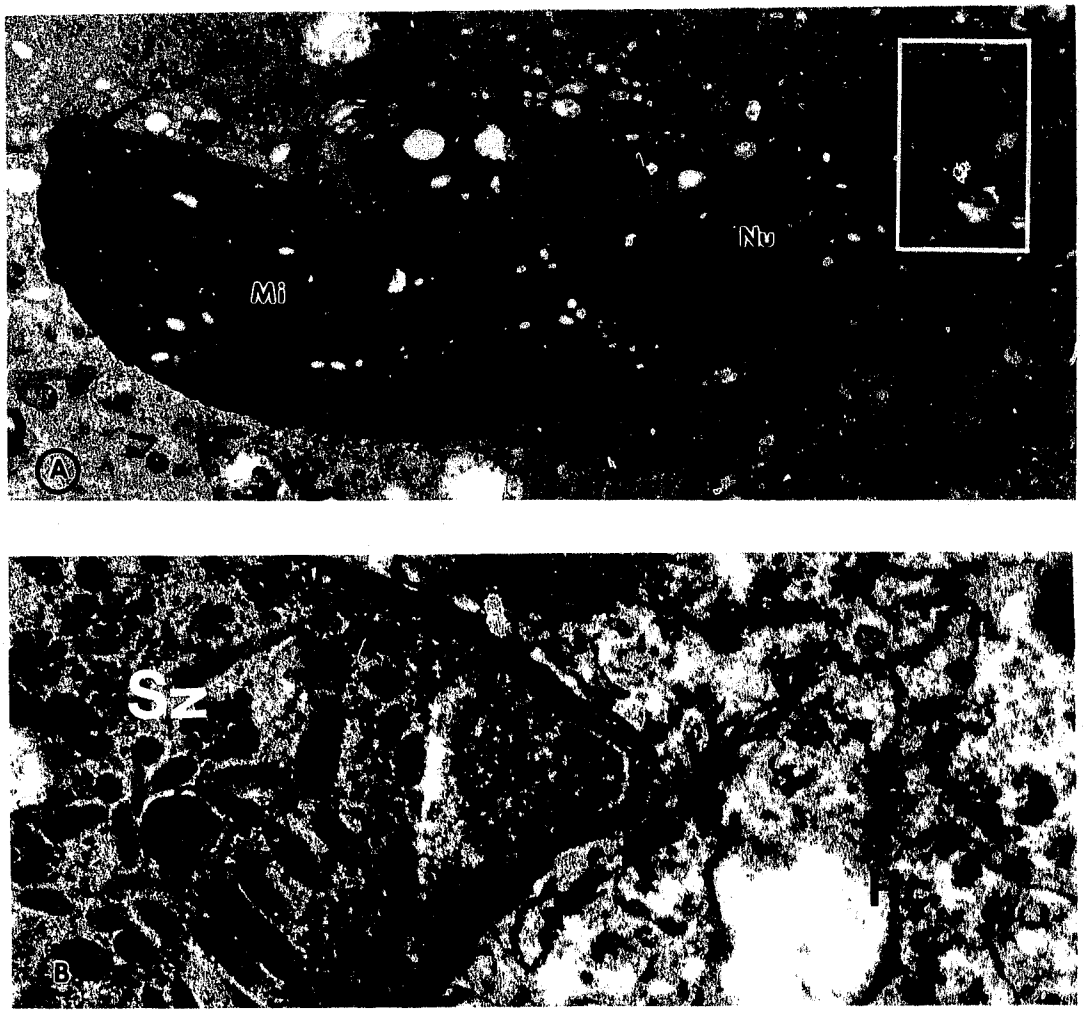


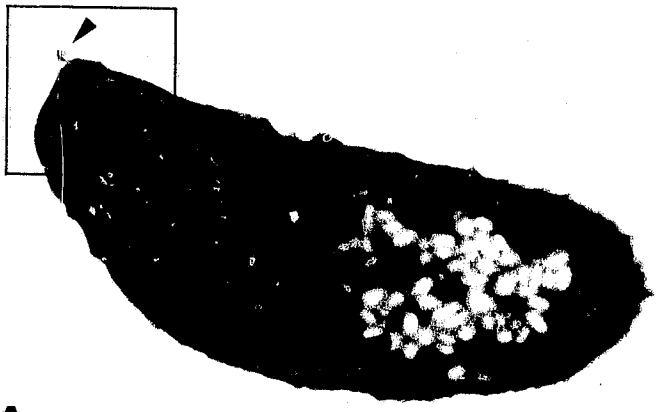
Fig. 5.18 A) Longitudinal section of *S. cruzi* sporozoite in rat heart myoblast 2 h post sporozoite inoculation. Note conoid (Co) and conoid-like projection (boxed in) at anterior and posterior zoite, respectively (x12,875).

B) Higher magnification of a *S. cruzi* sporozoite showing ultrastructure of the posterior projection (arrow) (x77,250).

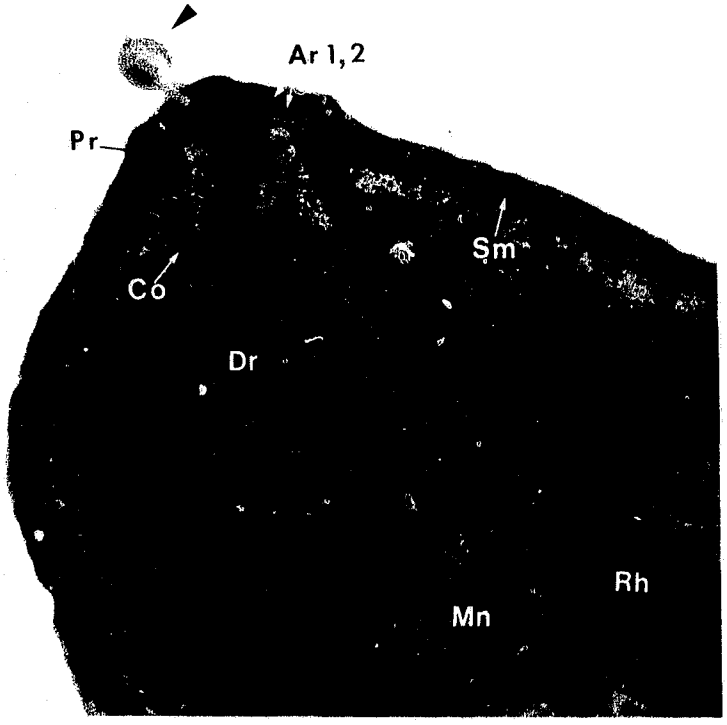
Fig. 5.19

A) Extracellular (i.e. preinvasion) *S. cruzi* sporozoite with small "bleb" (arrowhead) of membrane-bound material at zoite apex (x 15,000).

B) Higher magnification of boxed area in A). Note that the membrane surrounding material at arrowhead is continuous with that of the outer sporozoite pellicle. Although this apical "bleb" appears to have originated from within the conoid region, the point of origin of this material is not visible (x75,000).



A



B

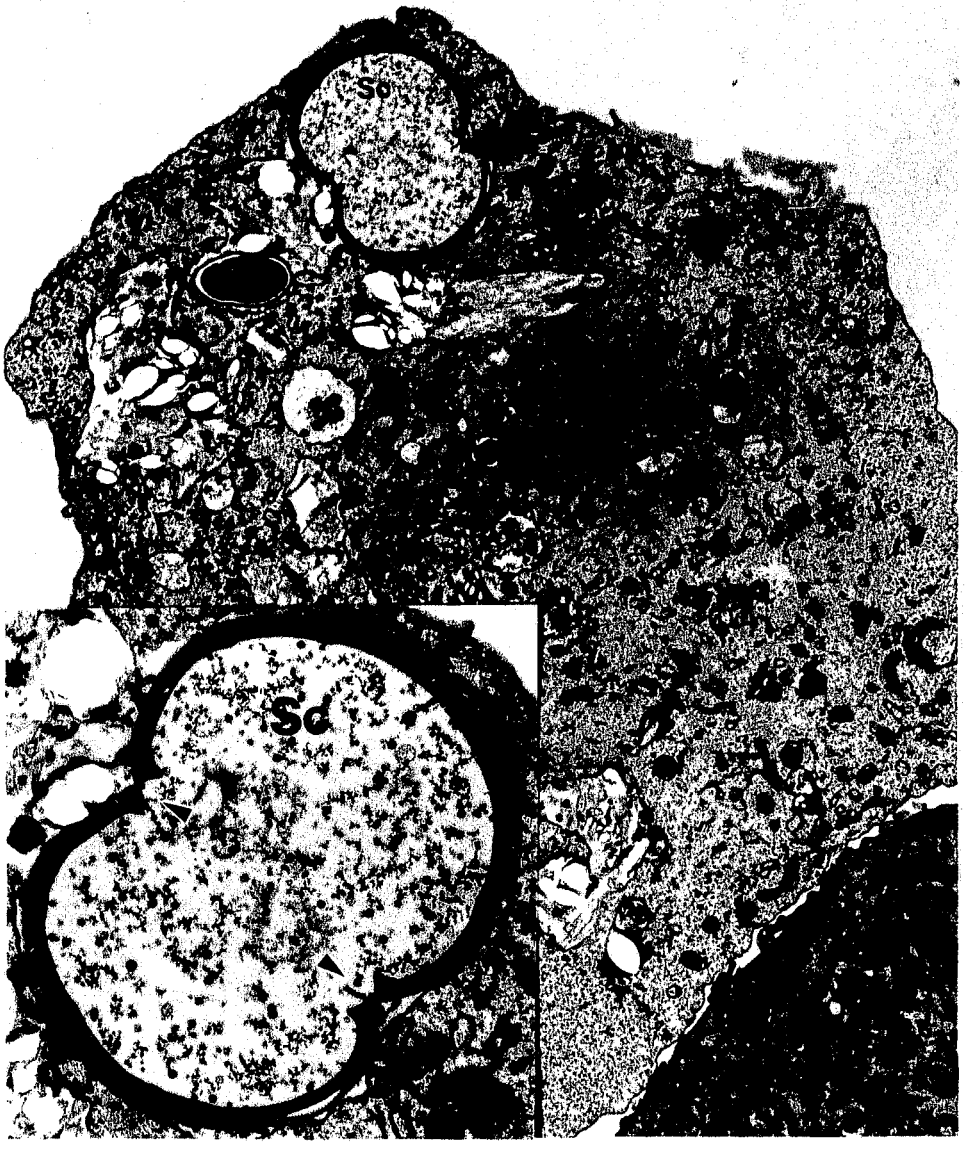


Fig. 5.20 Bovine pulmonary artery endothelial cell with phagocytized *S. cruzi* sporocyst 15 min post sporozoite inoculation (x6250). Inset: higher magnification of cross sectioned sporocyst. Note interposed strip (arrowheads) at sporocyst plate margins (x13,600).

Fig. 5.21 *Sarcocystis cruzi* sporozoites (arrows) in: A) bovine pulmonary artery endothelial cell (x6250) and in B) multinucleate myotube of rat heart myoblast 2 h post sporozoite inoculation (x3860).

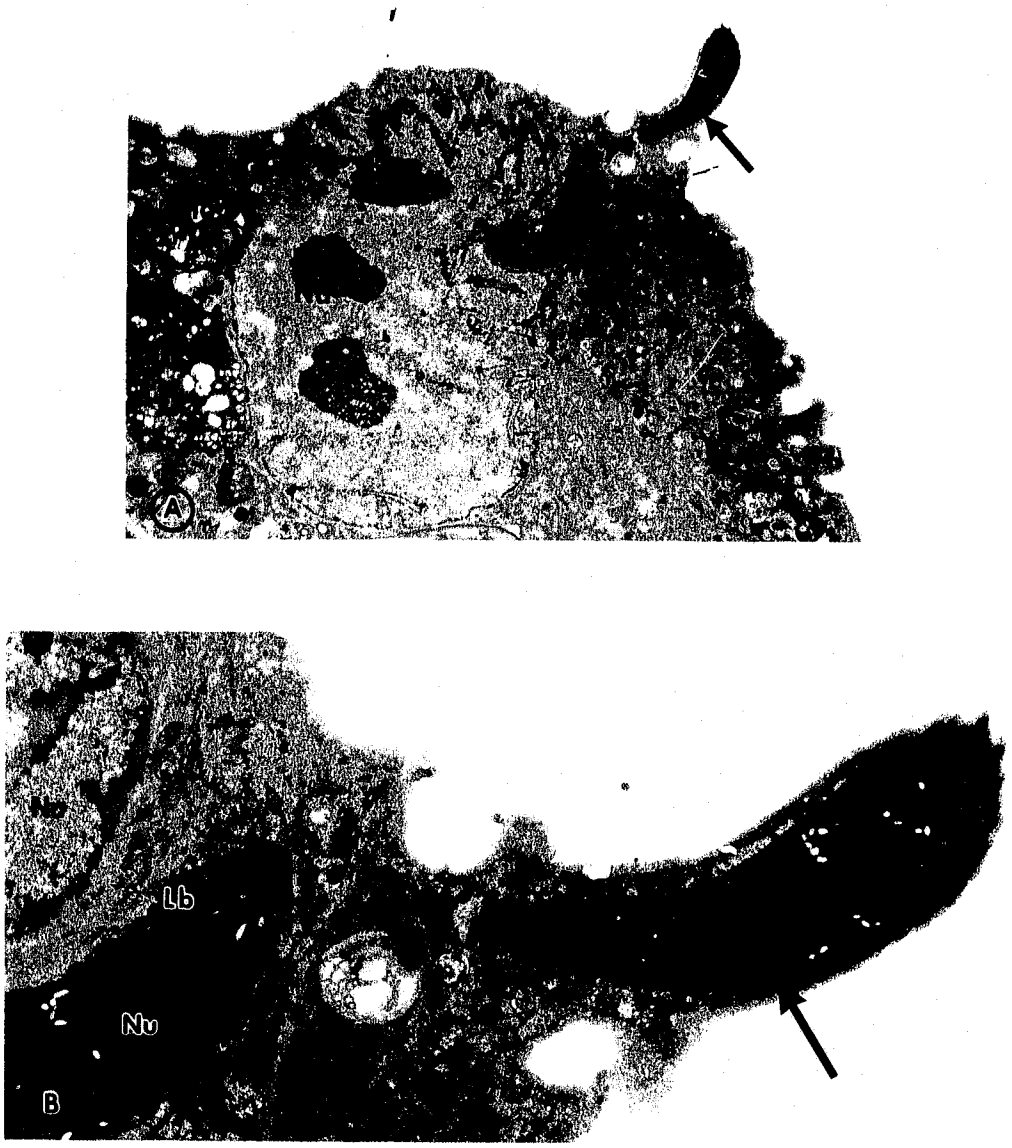


Fig. 5.22 A) Longitudinal section of *S. cruzi* sporozoite post penetration (open arrow) and tangential section of zoite (arrow) entering or exiting host cell, 1 h post sporozoite inoculation of bovine pulmonary artery endothelial cells (x2500).
B) Higher magnification of the sporozoites in A) (x10,000).

extracellular SZs are depicted in Figures 5.23 and 5.24 for comparison of rhoptries (see also Fig. 5.18B to observe this phenomenon). Alternatively, relative proportion of rhoptries:micronemes appeared to decrease. The changes in rhoptry density or number were more obvious at later sample times i.e. 1-2 hr post sporozoite inoculation.

Membranous material was frequently observed around intercellular SZs (Fig. 5.25). A space of variable width separated the parasite plasmalemma from the surrounding membrane(s). Although ultrastructure was reminiscent of a PV, SZs were never observed entirely enclosed within a membrane-bound vacuole. Artifactual separation of SZs and host cell cytoplasm did create nonmembrane-bound spaces around some zoites (Fig. 5.26). Lastly, several SZs had peripherally located granules of intermediate density which opened to the host cell cytoplasm via pore-like structures (Fig. 5.27). The electron dense collar typical of micropores was not associated with these 'pore' structures. Whether an exocytotic or endocytotic process was occurring could not be determined. Nor was the origin or fate of granule contents obvious.

5.4 Discussion

5.4.1 Culture technique

The tissue culture technique of Kingsley and Cole (1988) was successfully used in this invasion study; however, significant modifications were made. Moreover, several discrepancies with their results were apparent.

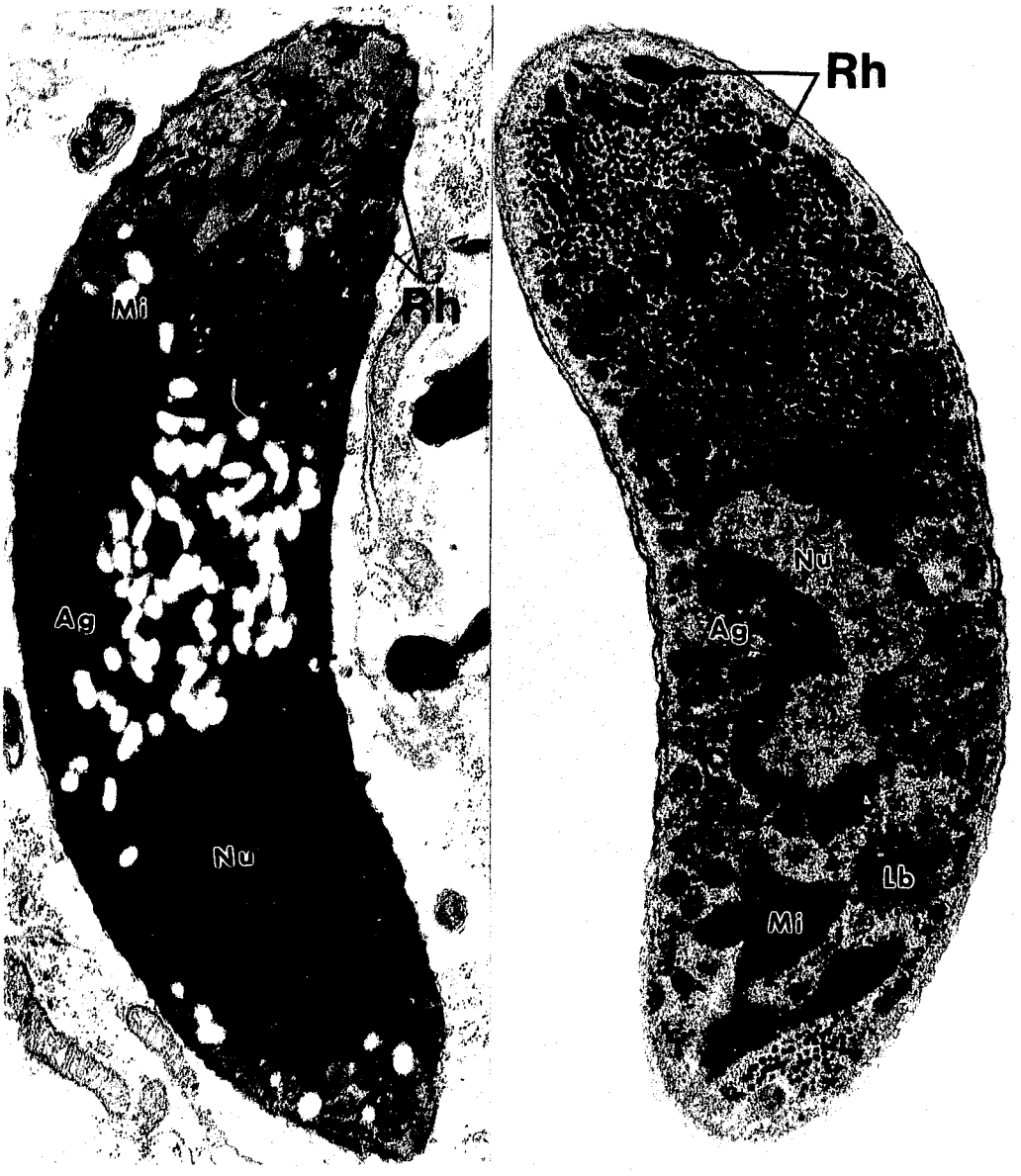
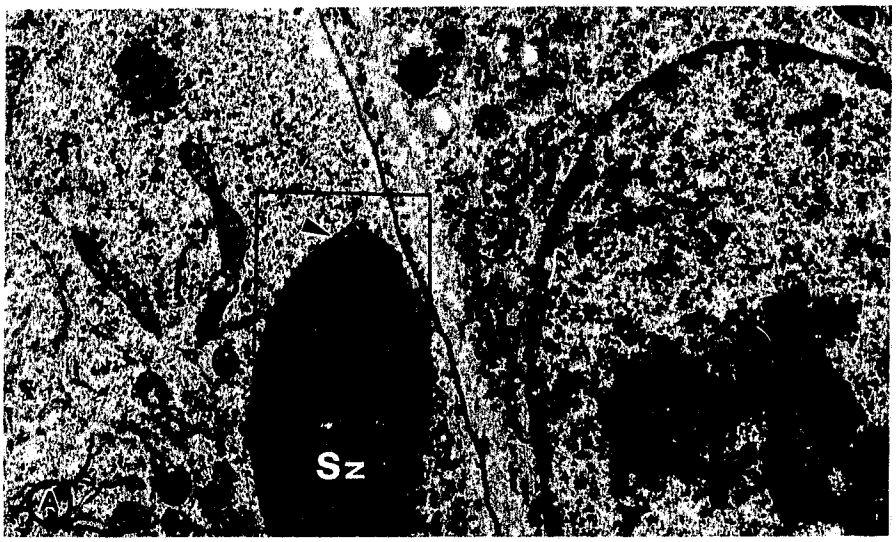


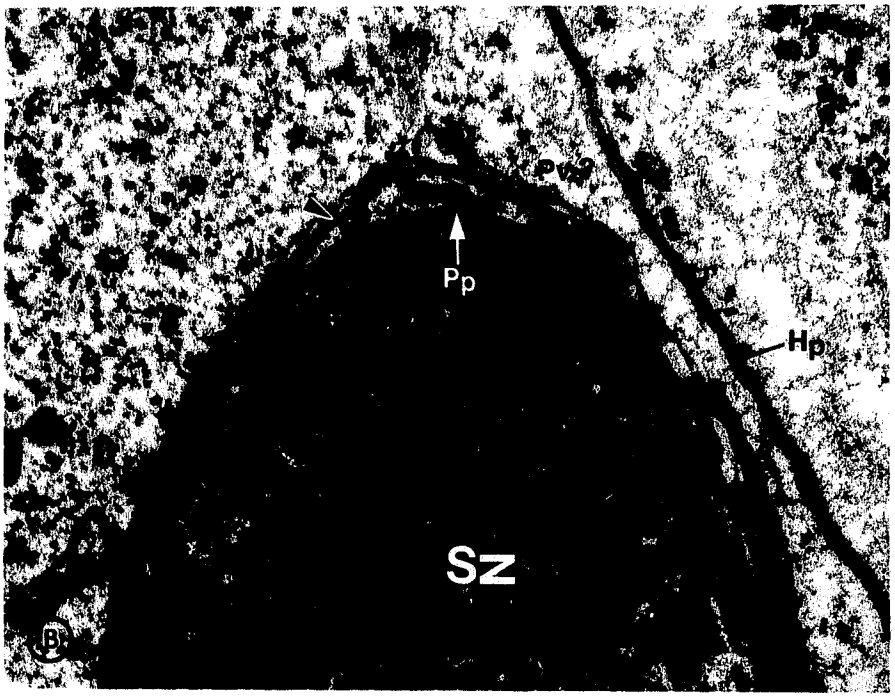
Fig. 5.23 *Sarcocystis cruzi* sporozoite in tissue cultured bovine endothelial cell 2 h post sporozoite inoculation. Note decreased electron density of rhoptries (Rh) at apex of zoite giving a "ghost-like" appearance to this organelle (x19,200).

Fig. 5.24 Excysted, extracellular *S. cruzi* sporozoite 4 h post exposure to excystation stimuli. Note electron density of rhoptries (Rh) in the anterior portion of the zoite (x21,000).

Fig. 5.25 A) Membranes (arrowhead), reminiscent of parasitophorous vacuolar membranes, surrounding *S. cruzi* sporozoite in bovine endothelial cells 15 min post sporozoite inoculation (x11,500).

B) Higher magnification of boxed area in A). A space (= parasitophorous vacuole?) of variable width separates membranous material (arrowhead) from the parasite plasmalemma (Pp). Note that neither the outermost membrane nor membranes within the space are continuous around the entire zoite and that their origin is unclear (x50,000).


Sz
A


Pp
Hp
Sz
B

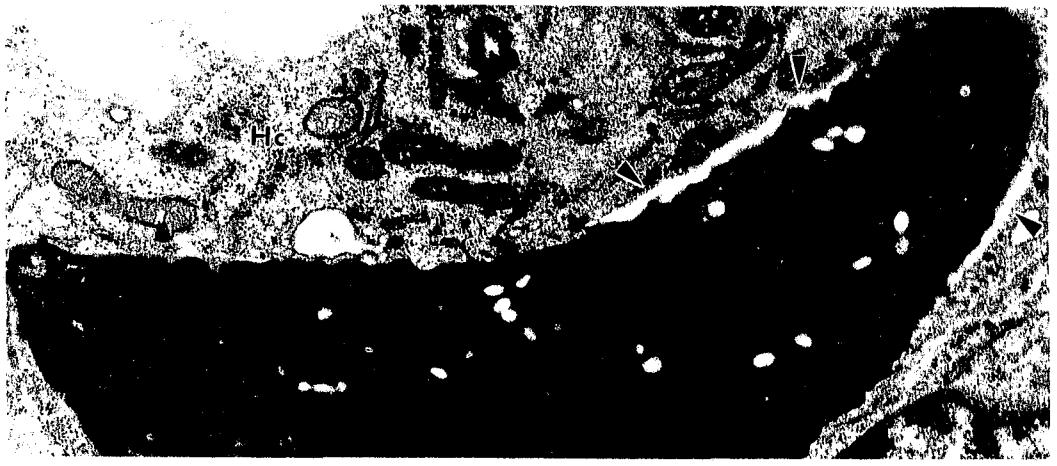


Fig. 5.26 Artifactual separation (arrowheads) of *S. cruzi* sporozoite and host endothelial cell cytoplasm 2 h post sporozoite inoculation (x20,000).

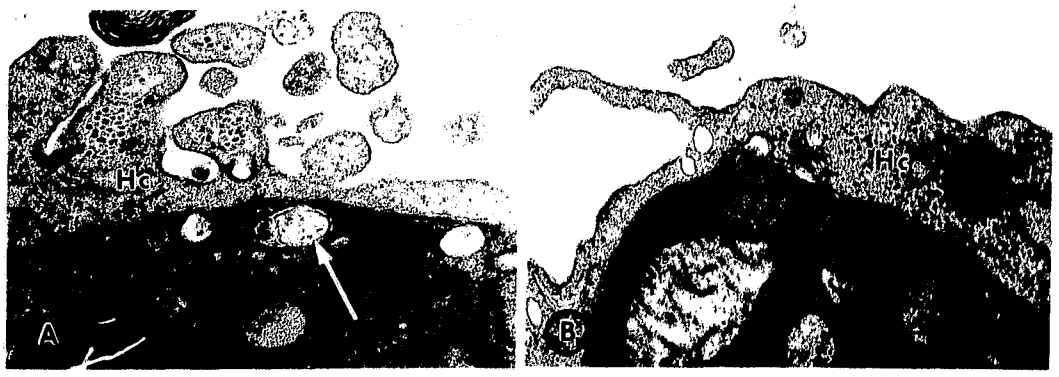


Fig. 5.27 Sections of *S. cruzi* sporozoite in rat heart myoblast 2 h post sporozoite inoculation (x33,900). A) Note peripherally located granules/vesicles (arrow) of intermediate electron density. B) An opening from one granule/vesicle to the host cell cytoplasm is visible at arrow.

The warping of Aclar discs was unexplained. Although Aclar film melting point (202 °C) is relatively low, conditions in the steam autoclave (121 °C for 15 min) should have been compatible; others routinely use steam. Subsequently, gas sterilization was determined to be compatible with Aclar.

Direct embedding eliminated the tedious and time consuming step of gluing polymerized specimens to blank blocks. Tissue infiltration by embedding media was complete; however, only a maximum two cell thickness was used. This technique may not be useful for long term experiments wherein monolayers frequently overgrow.

Kingsley and Cole (1988) recommended leaving Aclar film in place (i.e. attached to the polymerized medium) as long as possible to support and protect embedded cells. If all Aclar is stripped away from the TEM invasion specimens, cells and parasite are located right at the surface of the block face (see Fig. 5.1). Since the film provided a barrier to physical damage, it was left *in situ* until individual blocks were sectioned. However, the nonstick property of Aclar was not permanent (at least when interfaced with epon); thus polymerized blocks/gelatin capsules should be trimmed and Aclar film removed before sectioning or storage.

The "bubbling" observed at SEM represents expanding of the Aclar film; melting point is the likely factor. Scanning conditions under which this occurs (TV mode, low magnifications) are essential to locate small specimen(s) which are not uniformly distributed or are few in number. This frequently applies in study of cell

invasion by apicomplexans; thus, caution is recommended before proceeding with Aclar for SEM of parasites.

Overall, the Kingsley and Cole (1988) technique was useful; most problems encountered were minor and rectifiable. There are, however, major points to note: 1) Osmification alone did not provide adequate contrast to culture cells or sporozoites; incorporation of p-phenylenediamine staining significantly increased efficiency by facilitating orientation and subsequent processing of Aclar discs. 2) For uniform monolayer specimens direct embedding into blocks proved more efficient, decreasing manipulation of Aclar discs and eliminating the time-consuming gluing procedure.

Contamination problems experienced (i.e. some culture wells were rapidly overgrown with bacteria postinoculation) were not related to culture technique. Difficulty obtaining clean sporocyst (hence sporozoite) preparations (discussed in chapter IV) was likely the cause. The sporozoites inoculated into the wells of tissue culture plates had been washed post excystation but significant debris remained. Penicillin and streptomycin levels in culture media were not always suppressive. Excystation protocols were further modified to correct this problem; however, based on previous success and that direct embedding was untested in long term experiments, cell culture in polystyrene flasks was pursued for subsequent *in vitro* development studies (Chapter VI).

5.4.2 Invasion of cell cultures

Host cell penetration by coccidian zoites is usually very rapid requiring only 1-2 sec for some species (Russell, 1983). Thus, surprisingly the sampling regime reported herein (first sample 30 sec PI) demonstrated the peak invasive activity at 15 min and 1 h PI of CPAs and H9C2s, respectively. Postexcystation, SZ washes remove bile and its components which increase SZ motility (Speer, 1983). Woodmansee *et al.* (1987) noted morphologic changes in *Cryptosporidium* SZs and decreased motility after removing excess excystation fluids. Whether this delays invasion is unknown. Viscosity of inoculum also affects SZ movement or settling; thus, debris remaining after washes could have further delayed attachment. Invasion times are highly specific to protocol i.e. variation in sporozoite preparation (eg. purification or excystation procedures) preinoculation could significantly delay or enhance penetration. Additionally, cell culture itself has many variables (eg. media composition, culture temperature, condition of cells) which might alter invasion times. Variations based on cell type are also reported (Doran, 1982); therefore, the failure of SZs to penetrate into H9C2s as rapidly or as readily as into CPAs was not unexpected.

The "drilling" action of SZs suggests a role in penetration (Werk, 1985). Direction of SZ body ridge spiralling can be confusing and descriptions vary depending on whether viewing from anterior to posterior or vice versa. *Sarcocystis cruzi* SZs had the same twisting pattern and direction as *Toxoplasma gondii* tachyzoites; therefore, to be consistent with Chiappino *et al.* (1984), a counter-clockwise "designation" was given. Their description of a counter-

clockwise "torsion" (i.e. the force itself, not the direction of spiralling) requires that the leading, conoid end is generating force in a counter-clockwise drilling-type motion. Nichols and Chiappino (1987) postulated movement of the polar rings diagonally along the conoid subunits during conoid extension and retraction causes twisting that results in body torsion. Alternatively, structures of the apical complex may be relatively stationary and the force driving penetration is generated by the zoite's posterior end. In the latter case, a "clockwise" torsion would be necessary to create the observed body ridge spiralling.

In SEM preparations the SZs on cell surfaces at 5 min were assumed attached because they had not been washed away as occurred at the previous sample interval. However, there was no indication of the mechanism by which SZs maintained their position. Moreover, it was difficult to confirm whether zoites were entering or exiting cells because anterior and posterior of SZs appeared very similar. Most graphical depictions of SZs show the apical complex at the more tapered end of the zoite. This was variable depending on SZ flexion, twisting, and length i.e. the conoid and apical complex frequently appeared at the blunt (less tapered) end depending on SZ orientation.

Sporozoite shape change (shorter, fatter) after penetration was infrequent; how and why it occurs is not known. Roberts *et al.* (1971) noted that the anterior of *Eimeria* SZs appeared to swell and decrease in length during invasion. Vetterling *et al.* (1973) also reported *Sarcocystis* sp. zoites to be shorter and wider postinvasion. Interestingly, this is also the SZ shape Speer *et al.*, (1983) produced

by exposing coccidia SZ to monoclonal antibodies. Similarly, significance of host membrane depression at SZ entry sites is unknown. This feature is not consistent among coccidian genera and was not consistent within this study.

Individual host cells were heavily parasitized and sporozoites were frequently seen in close proximity to each other within cells. This may reflect increased susceptibility to subsequent invasion once a host cell has been penetrated by a SZ. While such a phenomenon would facilitate infection, long term benefit to parasites is questionable i.e. any one cell would have an optimum SZ carrying capacity since only a limited number of developing meronts could be sustained.

Cell membranes were often torn at penetration sites and overlying intracellular SZs. However, this likely resulted from artifactual mechanical damage during SEM processing rather than SZ action. Sporozoites that protruded prominently at the cell surfaces would be subjected to more shear force in processing. These SZs typically had associated tears whereas deeper penetrating ones did not. Additionally, the continuity of host-parasite membrane argues against parasite-induced ruptures.

In *T. gondii*, host cell membrane projections such as filopodia or lamellipodia accompanied attached tachyzoites (Chiappino *et al.*, 1984) and in *S. muris* pseudopodia-like extensions apparently derived from host surface microvilli attached to the extracellular part of invading merozoites (Entzeroth, 1985). These features, characteristic of a phagocytic process, were not observed with *S. cruzi*

sporozoites; nevertheless, CPA and H9C2 cells did have this capability as evidenced by engulfed sporocysts. Adams and Bushell (1989) also showed that *E. vermiformis* SZs were not internalized in this manner. Lack of surface projections supports active penetration by SZs but does not rule out host cell participation. Sporozoite entry may differ from other "zoite" stages regardless of the conserved morphology.

It is difficult to demonstrate sporozoites during host cell penetration (i.e. at precisely the moment the host plasma membrane is contacted and "breached" by the SZ) (Long and Speer, 1977). In this study, no sporozoites were visualized by TEM during invasion. Lack of information on the mechanism of internalization influences ability to confirm or discount parasitophorous vacuole formation i.e. any vacuole formed may undergo rapid change or dissipation post penetration if its function in invasion of the host cell is complete. Dubey *et al.* (1980) also suggest escape from PVs is possible in *Sarcocystis* species. Although *S. cruzi* is not believed to have a PV associated with asexual life cycle stages, host cell penetration has not been observed in any studies which dismiss its presence (Chobotar and Scholtyseck, 1982). Parasitophorous vacuoles have been identified with bradyzoites of several *Sarcocystis* spp. (Vetterling, 1973; Entzeroth, 1984, 1985; Jantzen and Entzeroth, 1987). The suggestion of a PV around sporozoites (and merozoites) requires confirmation.

Changes in rhoptry density, particularly at later sample times, likely indicate involvement in invasion and postinvasion processes egs. PV formation, evasion of

host defenses, or other aspects of intracellular development. Granules observed at the periphery of zoites could not be identified as dense granules. Their reduced electron density could reflect a loss of granule content by exocytosis. This phenomenon is reported in *T. gondii* (Leriche and Dubremetz, 1990) and *P. knowlesi* (Torii *et al.*, 1989) and some *Sarcocystis* spp. (Entzeroth *et al.*, 1986; Jantzen and Entzeroth, 1987). Mechanism and function are currently speculative; however, the role of dense granules appear to be different from that of rhoptries and micronemes (Torii *et al.*, 1989). One hypothesis is that dense granules play a part in avoiding fusion of host lysosomes with the PV (Leriche and Dubremetz (1990). Entzeroth *et al.* (1991) have recently identified a new population of granules in *S. muris* which have an intermediate density similar to those observed herein. Further study is necessary to discern whether *S. cruzi* also has two populations of granules and to rule out endocytosis at their pore-like openings.

Since a) the invasion window has been identified for the protocol reported herein and b) improved excystation rates have been recorded for *Sarcocystis* sp. (Ndiritu et al., 1993), higher sporozoite inoculations at 15 min and 1 h for CPA and H9C2 cell, respectively, should facilitate visualization of host-parasite interaction at invasion and complete the limited information on PV formation and dense granule exocytosis. Immunoelectron microscopy of *S. cruzi* is essential to label organelles implicated in invasion and accurately trace dissemination of their product(s) within parasite stages and host cells.

CHAPTER VI - IN VITRO DEVELOPMENT OF MEROGONOUS STAGES OF *SARCOCYSTIS CRUZI*

6.1 Introduction

Discovery of the two host life cycle of *Sarcocystis cruzi* (Heydorn and Rommel, 1972) was followed by experimental induction of bovine sarcocystosis to determine infectivity and pathogenicity (detailed in section 3.1) of this organism. Subsequent *S. cruzi* research concentrated on *in vivo* studies to elucidate fate (i.e. location and developmental stages) of infective sarcocysts in definitive hosts (Fayer, 1974; Fayer and Johnson, 1975; Dubey *et al.*, 1980; Dubey, 1982a) and infective sporocysts in intermediate hosts (Fayer, 1977, 1979; Dubey *et al.*, 1980, 1982; Dubey, 1981, 1982a). Additionally, cross transmission studies (Fayer *et al.*, 1976; Dubey, 1980) were conducted to ascertain degree of host species specificity.

In vitro cultivation of *Sarcocystis* spp. sexual stages had been previously demonstrated by Fayer (1970). However, the significance of his findings were not fully understood until gametogony was observed in cell cultures inoculated with bradyzoites from muscle cysts of *S. falcatula* (Fayer, 1972); resulting oocysts confirmed *Sarcocystis* spp. were coccidia. Sporogony also begins *in vitro* (Vetterling, *et al.*, 1973; Becker, *et al.*, 1979) but has not been completed in culture systems. *Sarcocystis cruzi* was the first *Sarcocystis* spp. to have asexual stages successfully cultured *in vitro* (Speer and Dubey, 1986; Speer *et al.*, 1986b). This was particularly noteworthy since the asexual phase of the life cycle is pathogenic,

causing serious disease in intermediate hosts (Speer and Burgess, 1987); thus, a means to obtain large numbers of merozoites for physiological, immunological, and biochemical research was established. Vascular (asexual) development of *S. capracanis*, *S. tenella*, and *S. hirsuta* has since been adapted to cell culture (Speer *et al.* 1986a; Cawthorn *et al.*, 1989) with sporozoites giving rise to numerous schizonts and merozoites; sarcocyst formation, however, has not been induced *in vitro*.

In vitro cultivation of *Sarcocystis* spp. aids diagnostic, prophylactic, and therapeutic advances while furthering knowledge of physiology, immunology, and pathology of sarcocystosis (Cawthorn and Speer, 1990). *In vitro* systems have been extensively used to document ultrastructure of various life cycle stages of *Sarcocystis* spp. since *in vivo* development appears to proceed similarly. Generation of monoclonal antibodies for detection and, perhaps, ultimately, for prevention of disease have been facilitated by *in vitro* cultures (Speer and Burgess, 1988; Burgess *et al.*, 1988). Additionally, merozoites produced *in vitro* may be useful in development of diagnostic genomic probes for *Sarcocystis* spp. as has been conducted by Kibenge *et al.* (1991) using *S. cruzi* sporozoite DNA.

Host-parasite relationships *in vivo* and *in vitro* have been relatively unexplored. In definitive hosts of *Sarcocystis* spp., various cell types are parasitized (Dubey *et al.*, 1982; Dubey, 1983). However, in intermediate hosts, the location and number of schizogonous generations is not conclusive (Levine, 1986). Neither the type of cell parasitized nor subsequent cellular pathogenesis have

been determined (Speer and Dubey, 1986). Elucidation of factors which dictate whether sporozoites will enter and develop in a cell could lead to prevention of infection. Details of host-parasite interactions after completion of invasion are also limited and unclear. Ultrastructure varies at the point of host cell-parasite contact. Entzeroth (1983) found a unit membrane surrounding meronts in intermediate host cells after infection with *Sarcocystis* sporocysts recovered from dogs. Others consider *S. cruzi* meronts to be free in the host cytoplasm (Pacheco and Fayer, 1977; Dubey *et al.*, 1980). Variations may be species specific or they may vary depending on type of cell parasitized. Understanding the role of host-parasite associations and interactions in both sexual and asexual development may allow interventions which will interrupt *Sarcocystis* life cycles.

The purpose of experimentation detailed in this chapter was to determine whether the vascular phase of *S. cruzi* will undergo similar development in different host cell populations *in vitro*. Transmission EM was used to compare the ultrastructure of development in various cell lines and provide insight into host-parasite interactions of asexual stages of *S. cruzi*.

6.2 Materials and methods

6.2.1 Preparation and inoculation of tissue cultures

Cultivation of *S. cruzi* sporozoites and merozoites followed methods of Speer and Dubey (1986) and Speer *et al.* (1986a, 1986b). Approximately 1×10^5 or 2×10^5 sporozoites were inoculated into sterile 25 cm^2 polystyrene culture flasks

(Corning Glass Works, Corning, NY) containing monolayers of bovine pulmonary artery endothelial cells (CPA) (ATCC - CCL 207), rat heart myoblasts (H9C2) (ATCC - CRL 1446), rat skeletal muscle myoblast (L-6) (ATCC - CRL 1458), mouse muscle (Nor 10), African green monkey kidney (VERO) (ATCC - CCL 81) culture lines. For each infected cell line, an equal number of matched, uninfected culture flasks served as controls. The latter cells were treated identically to the sporozoite cultures and fixed and processed for EM following the same sampling regime.

Flasks were incubated in 5% CO_2 - 95% air atmosphere at 38 °C for 10 weeks, changing culture media on alternate days. The recommended medium for each cell line (see Appendix A) was used with the exception of CPAs. A medium problem was suspected in preliminary experiments which revealed an unidentified crystal-like material in this cell line. A switch from RPMI 1640 to M-199 (Gibco Laboratories, Grand Island, NY) with 10% FBS was made, and use of M-199 became routine. Varying media FBS concentration (range 2-20%) maintained monolayers for the duration of the experiment. Cultures were examined daily and photographed with a Nikon phase-contrast inverted microscope.

To assess relative productivity of cell lines, merozoites were counted at two-day intervals when schizonts were rupturing. Flasks were gently rocked 20x and the medium decanted and centrifuged at 500 xg for 10 min. After resuspension in fresh medium, merozoite numbers were estimated by haemocytometer with a phase-contrast Zeiss compound microscope.

6.2.2 TEM preparation

Control and sample flasks of infected CPAs and H9C2s were processed weekly for TEM whereas, infected VERO and L-6 lines were only processed at termination of the 10 week experimental period.

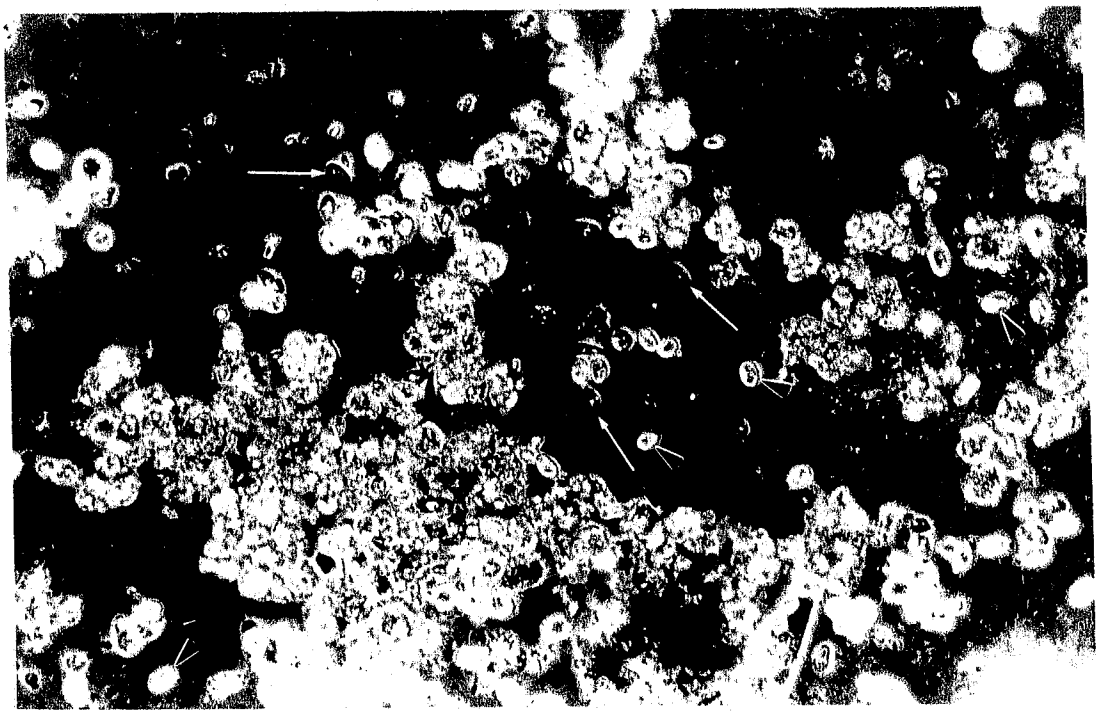
A 0.25% trypsin-versene solution removed monolayers (and parasites) from flasks for concentration by centrifugation at 500 xg for 10 minutes. Pelleted monolayers were then fixed in 2.5% glutaraldehyde in standard phosphate buffer (0.15 M, pH 7.3) or in a modified Millonig's phosphate buffer (see Appendix C for buffer formulation). Further processing of monolayers was as outlined in Appendix B. Specimens were sectioned on a Reichert Ultracut E microtome, stained with uranyl acetate and lead citrate and examined and photographed using a Hitachi H-7000 or H-600 electron microscope operated at 75 kV. Photomicrographs were photographed, developed, and printed as described in section 4.2.2.

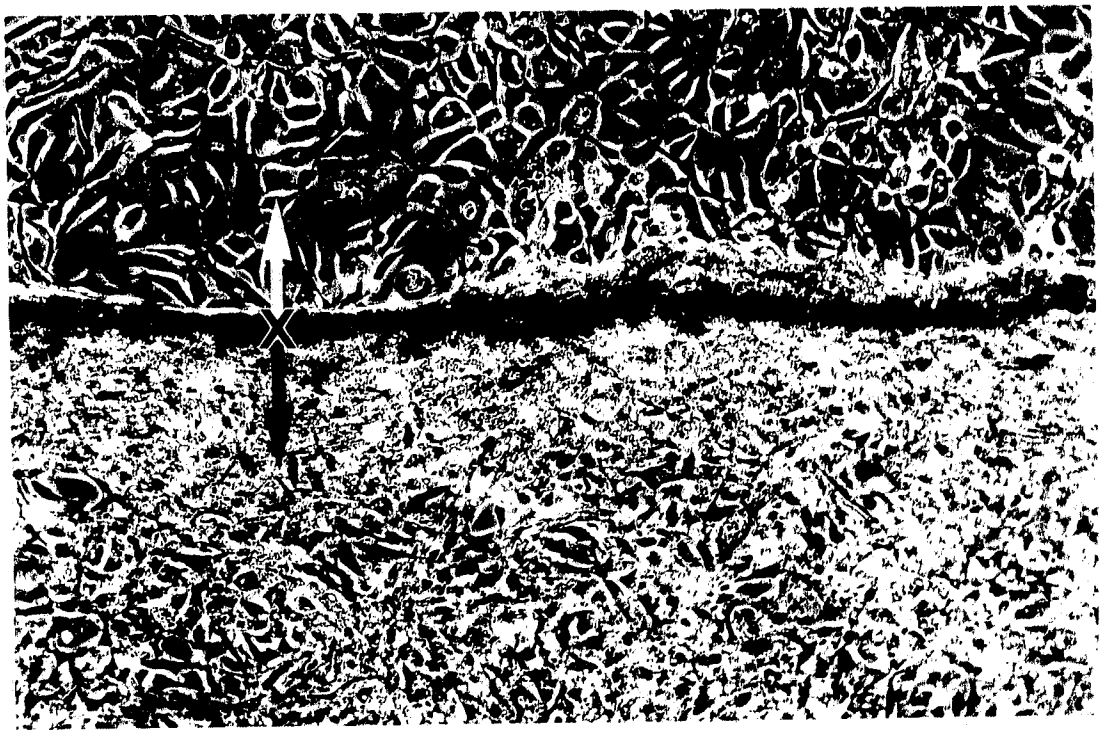
6.3 Results

Monolayers were generally healthy over the 10 wk period although sporocyst remnants and other excystation debris settled and could not be adequately washed from culture vessels 24 hr post SZ inoculation (Fig. 6.1). Some cell vacuolization and detachment with subsequent monolayer degeneration was noted. Regeneration of monolayers occurred after FBS concentration was augmented to 20% (Fig 6.2). The CPAs were most susceptible to alterations in culture conditions which stressed cells. Additionally, CPA's organized to form tubular structures reminiscent of capillaries (Fig. 6.3); similar cell aggregation was

Fig. 6.1 Light micrograph of tissue culture vessel containing post excystation debris prior to the 24 h wash of monolayers (i.e. <24 h post sporozoite inoculation). Note unexcysted (arrowheads) and excysted (arrows) *S. cruzi* sporocysts. Motile sporozoites and cultured cells not visible in the plane of focus (x90).

Fig. 6.2 Typical bovine pulmonary artery endothelial tissue culture 5 wk post sporozoite inoculation. Older cells (black arrow) are covered with excystation debris and the edge of the monolayer is detached at "X". Cell regeneration (white arrow) has occurred below (x44).





not observed in any of the other cell lines. A few CPA and H9C2 flasks had yeast contamination (Fig. 6.4) and were treated with 500 ug/ml Fungizone® (Flow Laboratories, Mississauga, Ontario). When number of yeast particles became reduced (usually after 2 media changes), a maintenance dosage of 250 μg of Fungizone® was used for one week and then discontinued. No further contamination was observed.

Sarcocystis cruzi sporozoites penetrated all cell lines and produced merozoites in all except Nor 10s. The latter underwent extensive contraction activity (3 DPI) such that monolayers floated from the culture substrate and sufficient medium coverage of cells could not be maintained; therefore, the Nor 10 cell line was removed from the experiment. The H9C2s and L-6s cells became syncytial (Fig. 6.5) and coordinated contractions of myofibers also raised and clumped monolayers (Fig. 6.6). Both lines remained attached to flask bottoms and could be covered by adding more medium. Overtime, centres of clumped cells appeared to degenerate (Fig. 6.6).

Meronts and merozoites were first visualized in CPA and H9C2 cultures at 24 and 33 DPI and 30 and 39 DPI, respectively (see Figs. 6.7-6.9). Transmission electron microscopy was required for positive confirmation of development in L-6s and VEROs. A total of 4.87×10^5 merozoites was recovered from 4 CPA culture flasks from 34-50 DPI. During the same period only 0.078×10^5 merozoites were harvested from 4 H9C2 flasks (see Table V). Counts were not continued on L-6 and VERO lines because merozoites were not observed by LM. Released

Fig. 6.3 Capillary-like tubes formed in endothelial cell cultures (8 wk post sporozoite inoculation); a larger and smaller tube are visible between arrows (x43).

Fig. 6.4 Yeast infected culture of bovine pulmonary artery endothelial cells 5 wk post sporozoite inoculation. Note yeast particles (arrows) and merozoites (arrowheads) free in culture medium. A few merozoites have invaded host endothelial cells (open arrow) (x98).

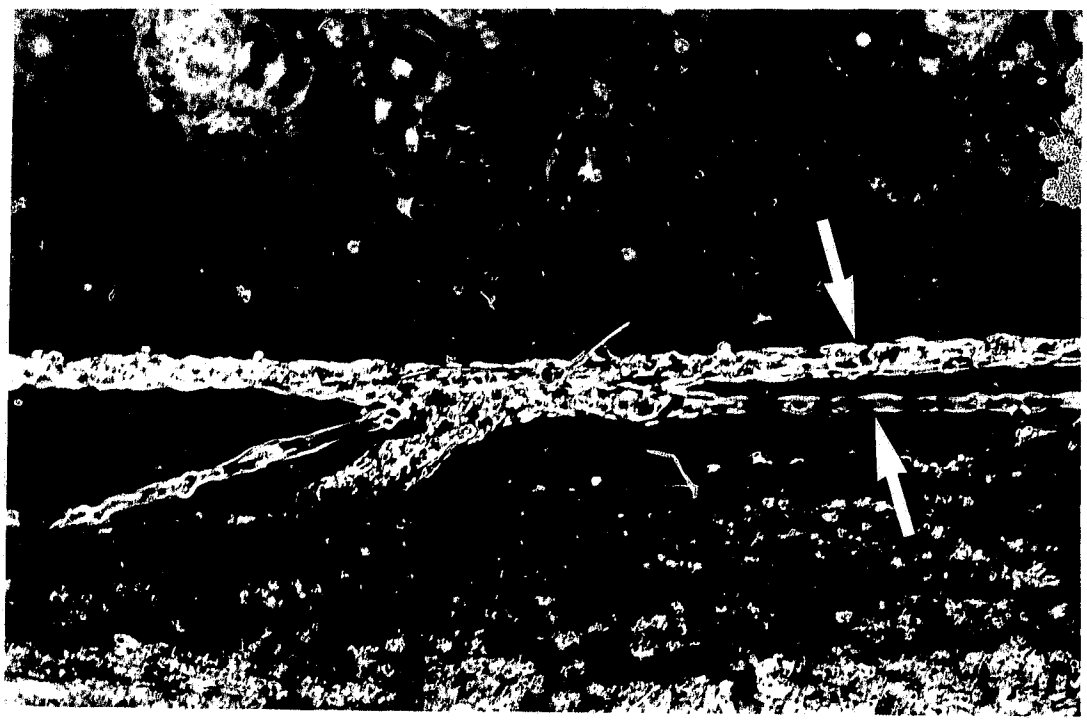
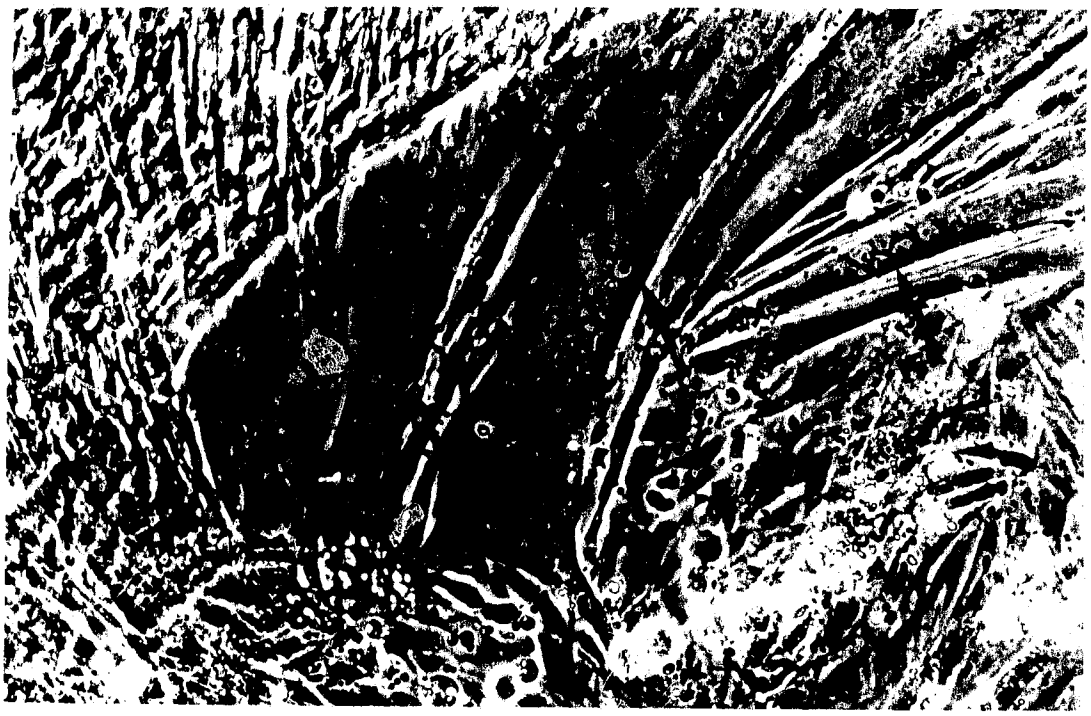


Fig. 6.5 Syncytial rat heart myoblasts in tissue culture 6 wk post sporozoite inoculation. Note formation of multinucleate myotubes (arrows) (x106).

Fig. 6.6 Rat heart myoblasts in tissue culture 5 wk post sporozoite inoculation. Culture is raised and clumped (at arrows) from coordinated contraction of myofibers. Note cells in the centre of clumps have lost definition and appear to be degenerating (compare with peripheral myoblasts) (x43).



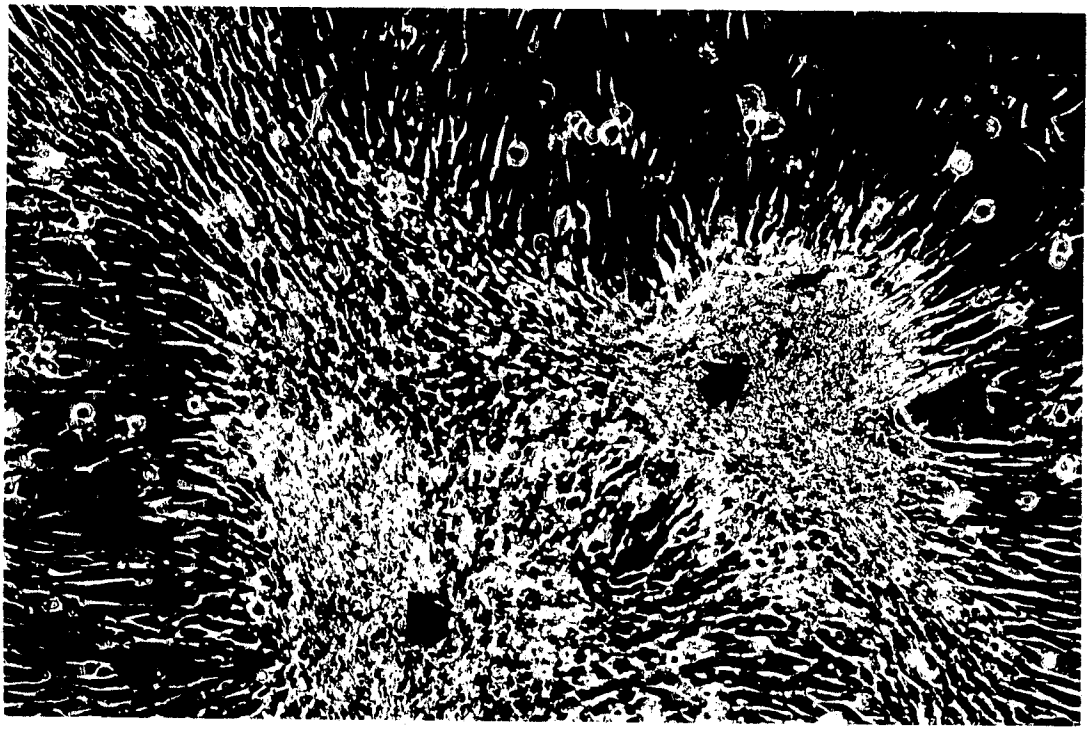


Fig. 6.7

A) Numerous merozoites in tissue cultured rat heart myoblasts, 7 wk post sporozoite inoculation. Note several mature meronts at center of boxed area (x94).

B) Higher magnification of boxed area in A). Note merozoites (arrowheads) and rosette-like meronts (arrow) with individual merozoites budding at the periphery of the host cell (x200).


A

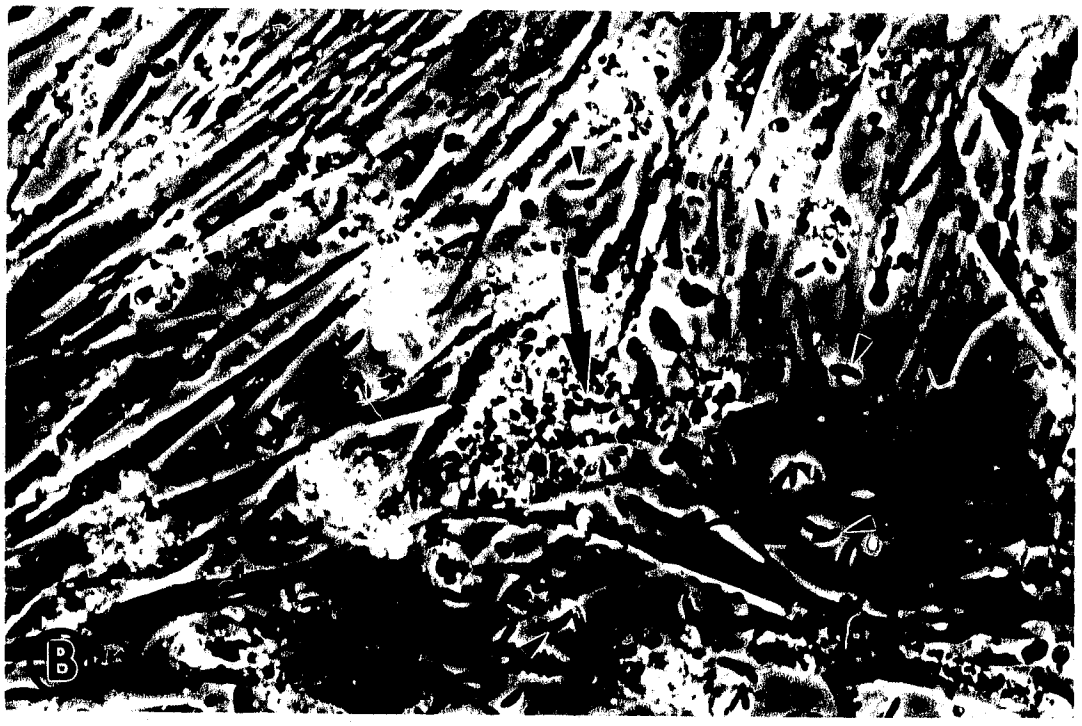
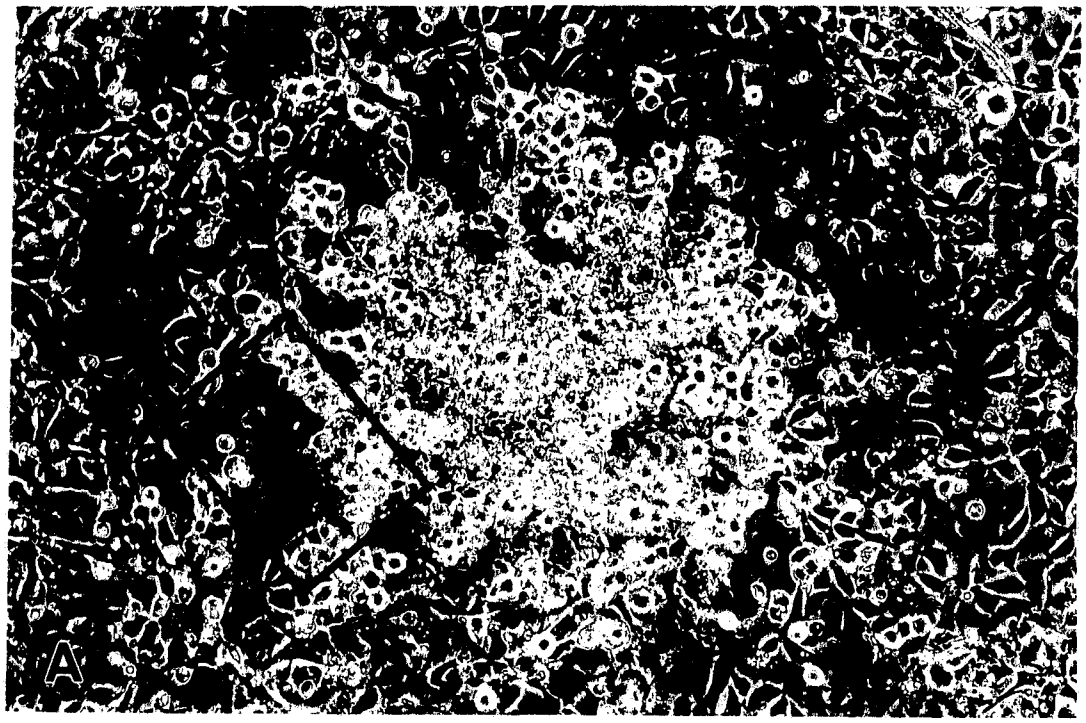
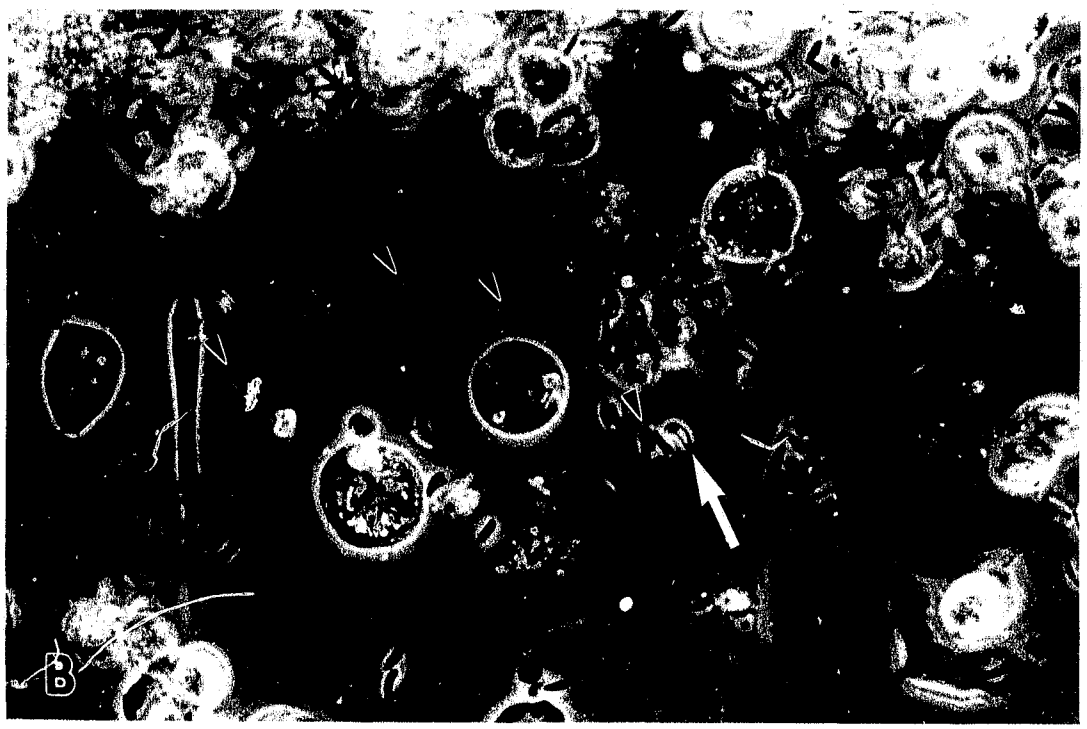

B

Fig. 6.8

A) Numerous rupturing meronts in monolayer of bovine pulmonary artery endothelial cells 5 wk post sporozoite inoculation. The centrally located cluster of refractile meronts is releasing merozoites which are barely visible between circular meronts and at the periphery of the meront cluster (x44).

B) Higher magnification of area similar to box in A) shows released merozoites (arrowheads). Note different size and refractility of the similarly shaped merozoite and sporozoite (arrow) (x197).


A


B

merozoites exhibited characteristic gliding and pivoting movements and most actively invaded adjacent cells (Fig. 6.10). Membranous material similar to that associated with invading sporozoites (see Fig. 5.25, chapter V) surrounded some intracellular merozoites (Fig. 6.11) but typical PV formation was not evident. In H9C2s, merozoite penetration of the host cell nucleus also occurred (Fig. 6.12). Schizogony was obvious in CPAs and H9C2s until termination of the experiments at 70 DPI. However, the number of schizogonous generations could not be determined due to asynchronous development of meronts. Sporozoites in cultures were last seen by LM at 36 DPI (Fig. 6.8B); however, TEM results confirmed they were present for the entire experiment. Sporozoites seen by TEM at 70 DPI were all intracellular.

Table V - Numbers of *Sarcocystis cruzi* merozoites harvested from four 25-cm^2 culture flasks of bovine pulmonary artery endothelial cells (CPA) and of rat heart myoblasts (H9C2) from 34-50 days post sporozoite inoculation.

DPI[a]	# CPA MZs ($x10^5$)	# H9C2 MZs ($x10^5$)
34	0.23	-
36	0.28	-
41	0.61	0.008
45	1.37	0.023
48	1.44	0.031
50	0.94	0.016
TOTAL	4.87	0.078

[a]DPI = days post inoculation of each flash with 2 x 10^5 sporozoites

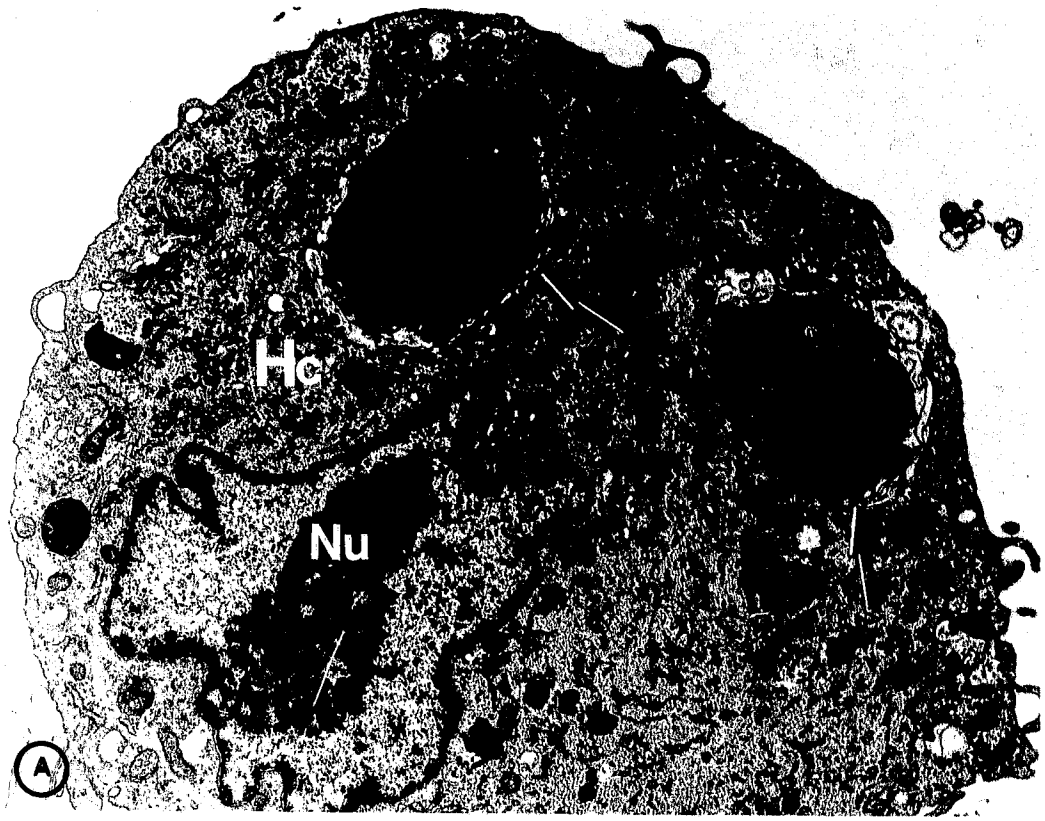
Fig. 6.9 High magnification of bovine endothelial cell (arrow) containing a mature meront. Note large number of free merozoites in surrounding tissue culture medium (x240).

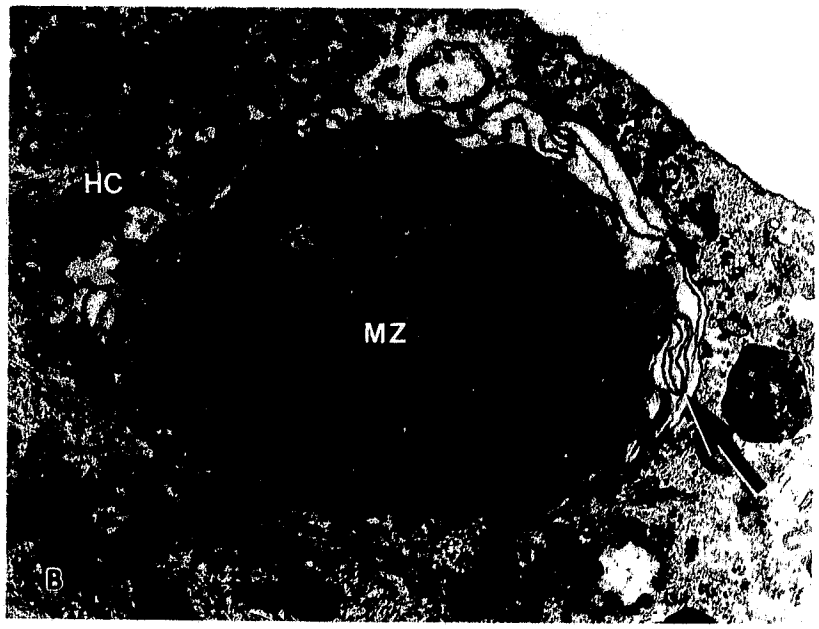
Fig. 6.10 Merozoite-infected bovine endothelial cells (arrows) in tissue culture 4 wk post sporozoite inoculation. Note excystation debris (i.e. sporocysts (double arrows)) remaining from initial sporozoite inoculation (x175).

Fig. 6.11

A) *Sarcocystis cruzi* merozoites in tissue cultured bovine pulmonary artery endothelial cell 10 wk post sporozoite inoculation. Note membranous material surrounding merozoites (arrows) (x13,100).

B) Higher magnification of merozoite in a). Note layering of membranes around zoite (arrow) (x32,400).


Hc
Nu
A


HC
MZ
B

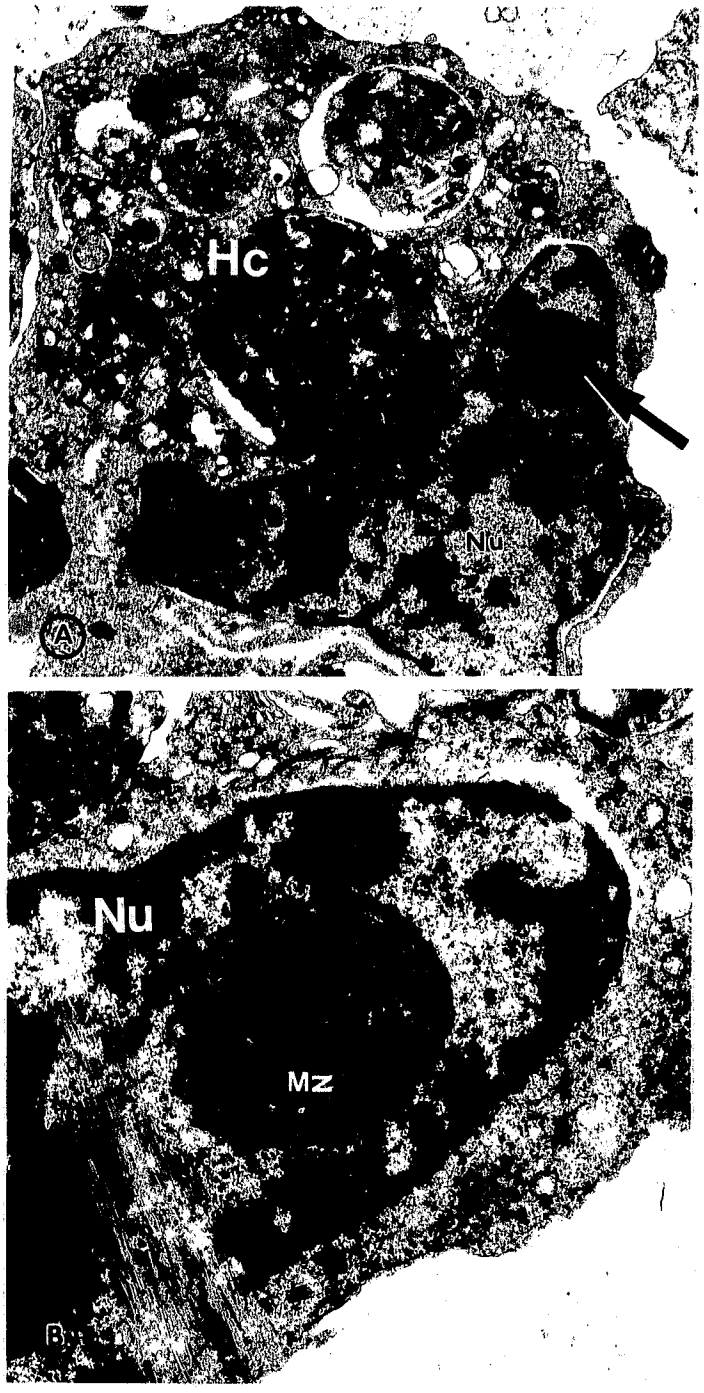


Fig. 6.12

A) *Sarcocystis cruzi* merozoite (arrow) in nucleus of tissue cultured rat heart myoblast, 3 wk post sporozoite inoculation (x6750).

B) Higher magnification of merozoite in a) (x18,900).

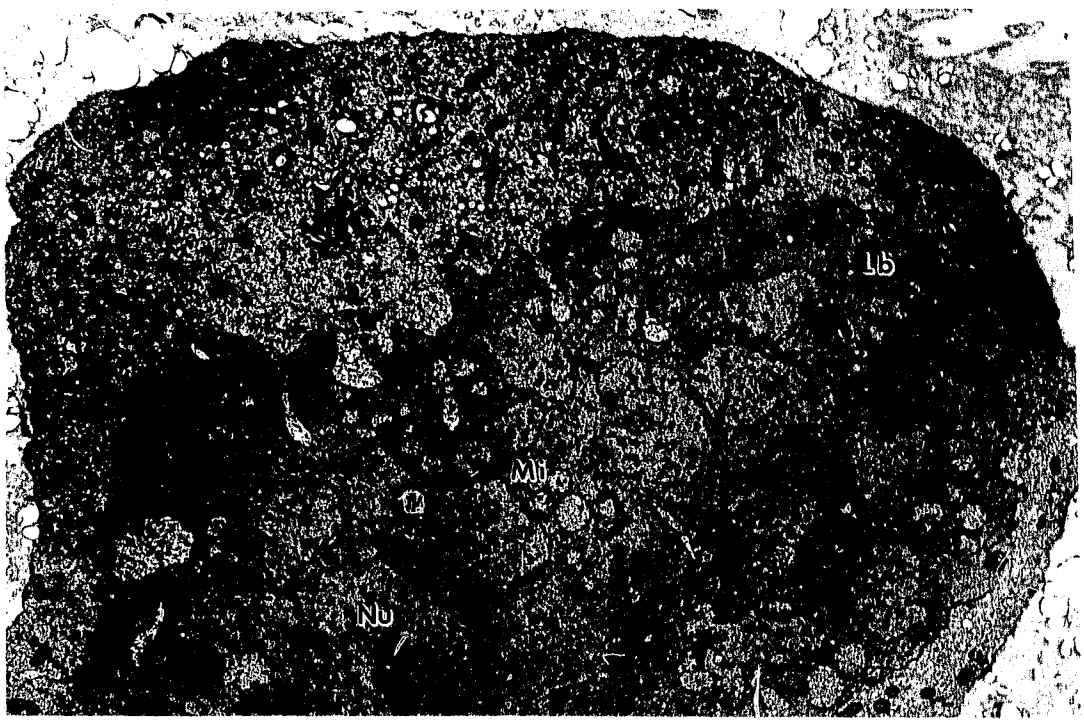
Transmission EM demonstrated sporozoites and merozoites in all cultures from 7-70 DPI and 28-70 DPI, respectively. Various stages of endopolygeny and mature meronts were evident in CPAs from 28-70 DPI and in H9C2s at 19 DPI (Figs. 6.13-6.16 and 6.17-6.19, respectively). Cultured cells harbouring late stage or mature meronts had disrupted membranes, swollen or fragmented mitochondria, poor ultrastructural definition, and vacuolated cytoplasm which lacked the homogeneity of that observed in neighbouring uninfected or control cells (Fig. 6.20). Overall, there was decreased electron density of nuclear structures and host cytoplasm. Cells recently invaded by merozoites did not exhibit the above changes even in heavily parasitized cells (Fig. 6.21). All intracellular parasite stages were well preserved and appeared healthy. Early- or late-stage meronts were not found in L-6 or VERO cell lines. Sarcocyst formation was not observed.

6.4 Discussion

Periods when cells detached and monolayers became sparse were not episodic, rather they were directly linked to an introduced stressor; for examples, initial sporozoite inoculum, addition of antifungal agent, and merozoite release. Uninfected cells had clear ultrastructural definition. In contrast, host cells with maturing meronts had the appearance of improperly or poorly fixed tissue indicating schizogony taxes the infected cell. Degeneration in centres of clumped (contracted) cell lines likely resulted because media nutrients and air or CO_2 could not efficiently or sufficiently reach inner cells. This may partially explain reduced

Fig. 6.13 Immature *S. cruzi* schizont in tissue cultured bovine pulmonary artery endothelial cell, 4 wk post sporozoite inoculation. Note the enlargement and division of the schizont nucleus characteristic of early development (x4900).

Fig. 6.14 Higher magnification of an immature *S. cruzi* schizont. Note invagination of the parasite plasmalemma (arrows) around nuclear lobes (x6125).


Lb
Mi
Nu

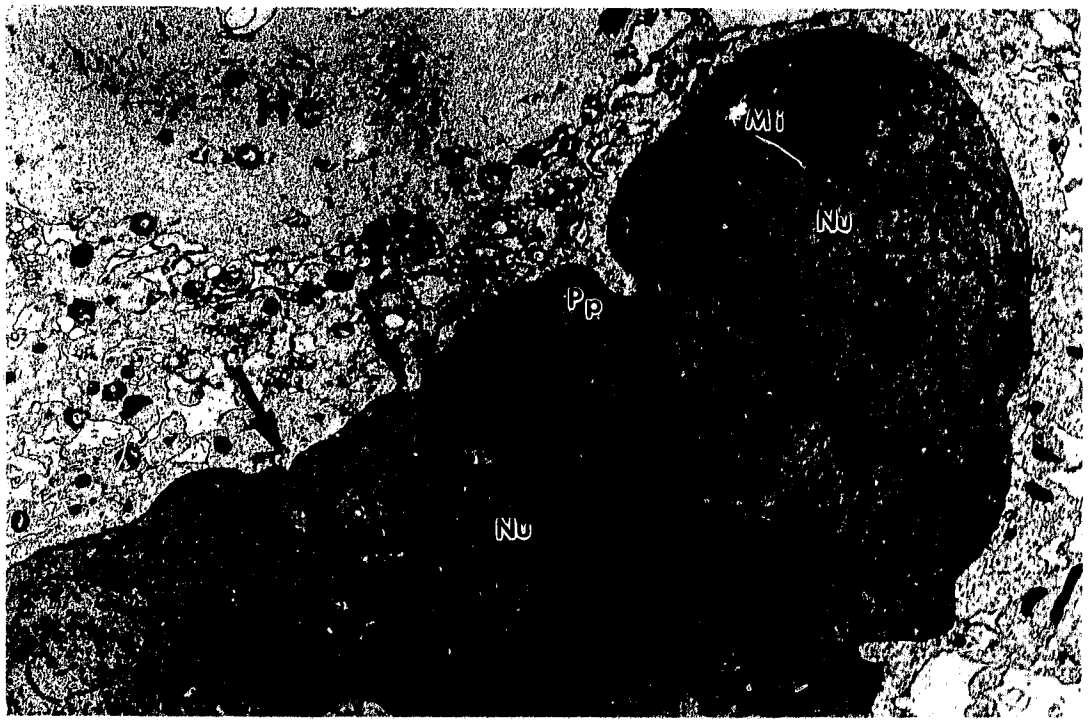
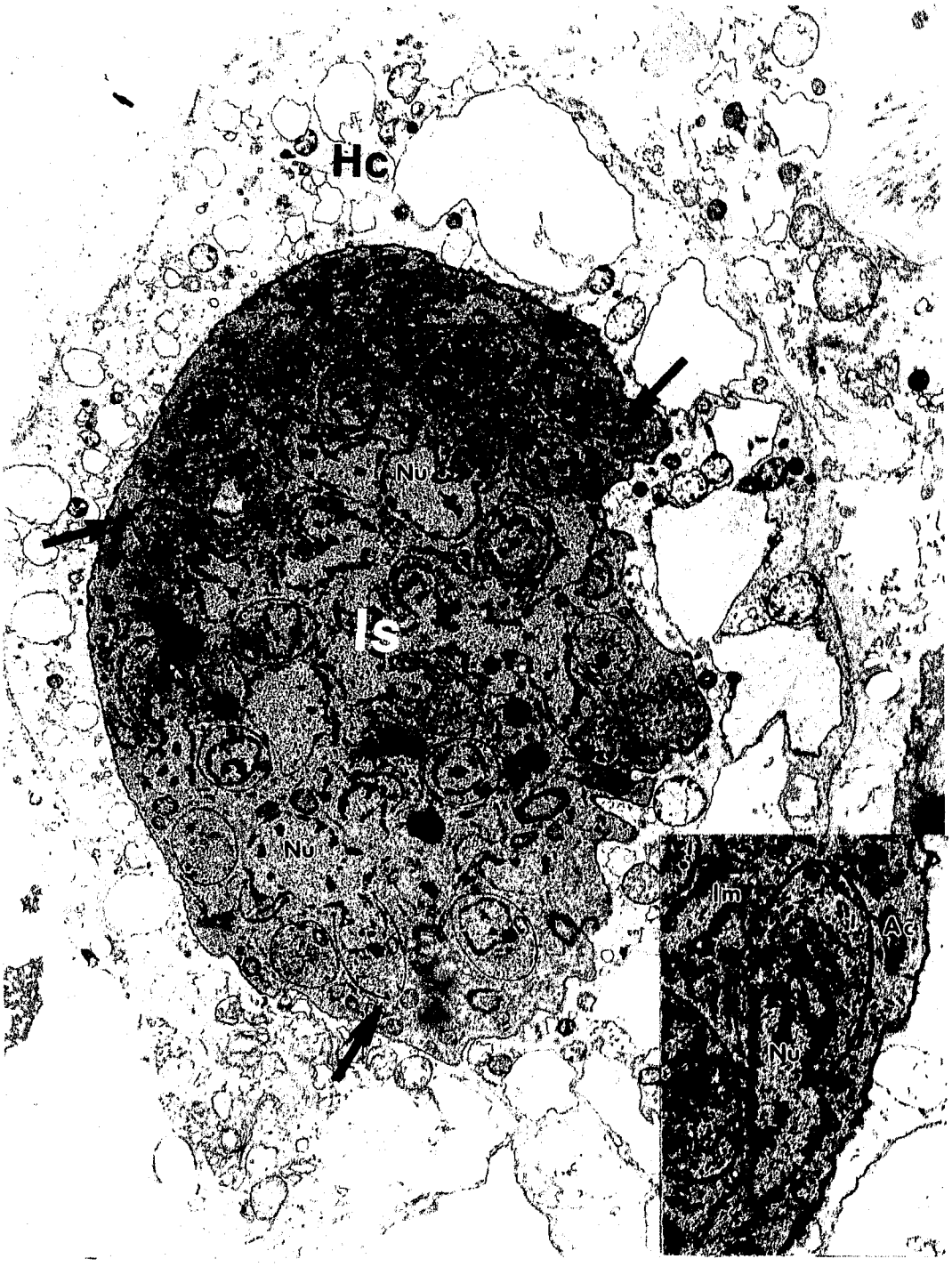

Mi
Nu
Pp
Nu

Fig. 6.15 *Sarcocystis cruzi* schizont in tissue cultured bovine pulmonary artery endothelial cell, 4 wk post sporozoite inoculation. Note schizont has developed to an intermediate stage wherein apical complex (arrows) and characteristic organelles of developing daughter merozoites appear (x6000). Inset: Higher magnification of a developing merozoite. Note inner membrane complex (Im) grows posteriorly around each lobe of the dividing nucleus (x12,495).


Hc
Nu
Is
Nu
Im
Ac
Nu

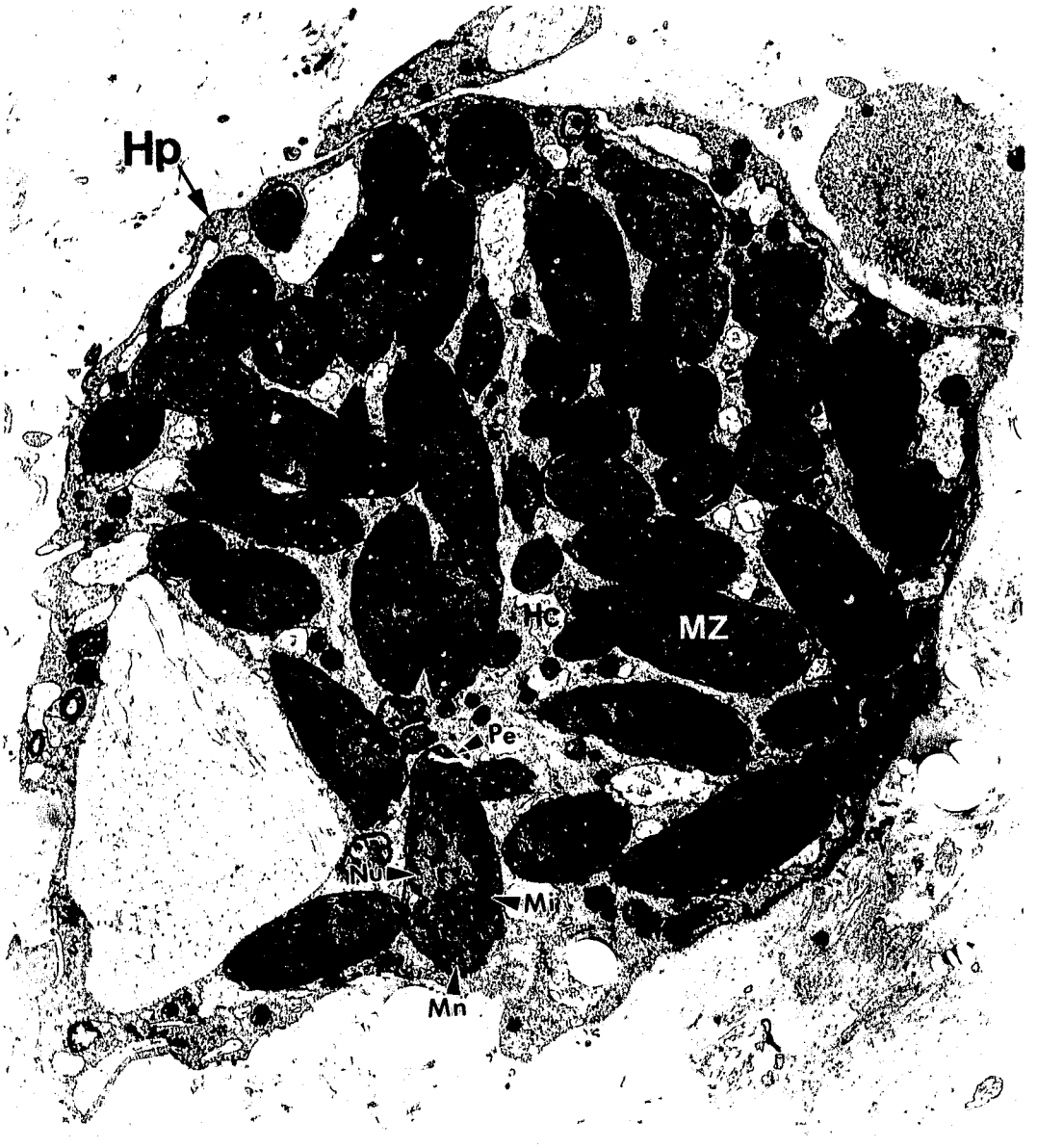


Fig. 6.16 Mature *S. cruzi* schizont in tissue cultured bovine pulmonary artery endothelial cell, 4 wk post sporozoite inoculation. The inner membrane complex of each developing merozoite (see figure 6.15) has fused with the limiting membrane of the schizont to complete merozoite pellicle (Pe) formation. Remnants of the original "mother cell" (i.e. early schizont) are indistinguishable from the host cell (x6000).

Fig. 6.17 Early *S. cruzi* schizont in rat heart myoblast culture, 3 wk post sporozoite inoculation. The parasite nucleus has enlarged and begun to divide. Initial infoldings of the schizont limiting membrane are visible at arrowheads (x7500).

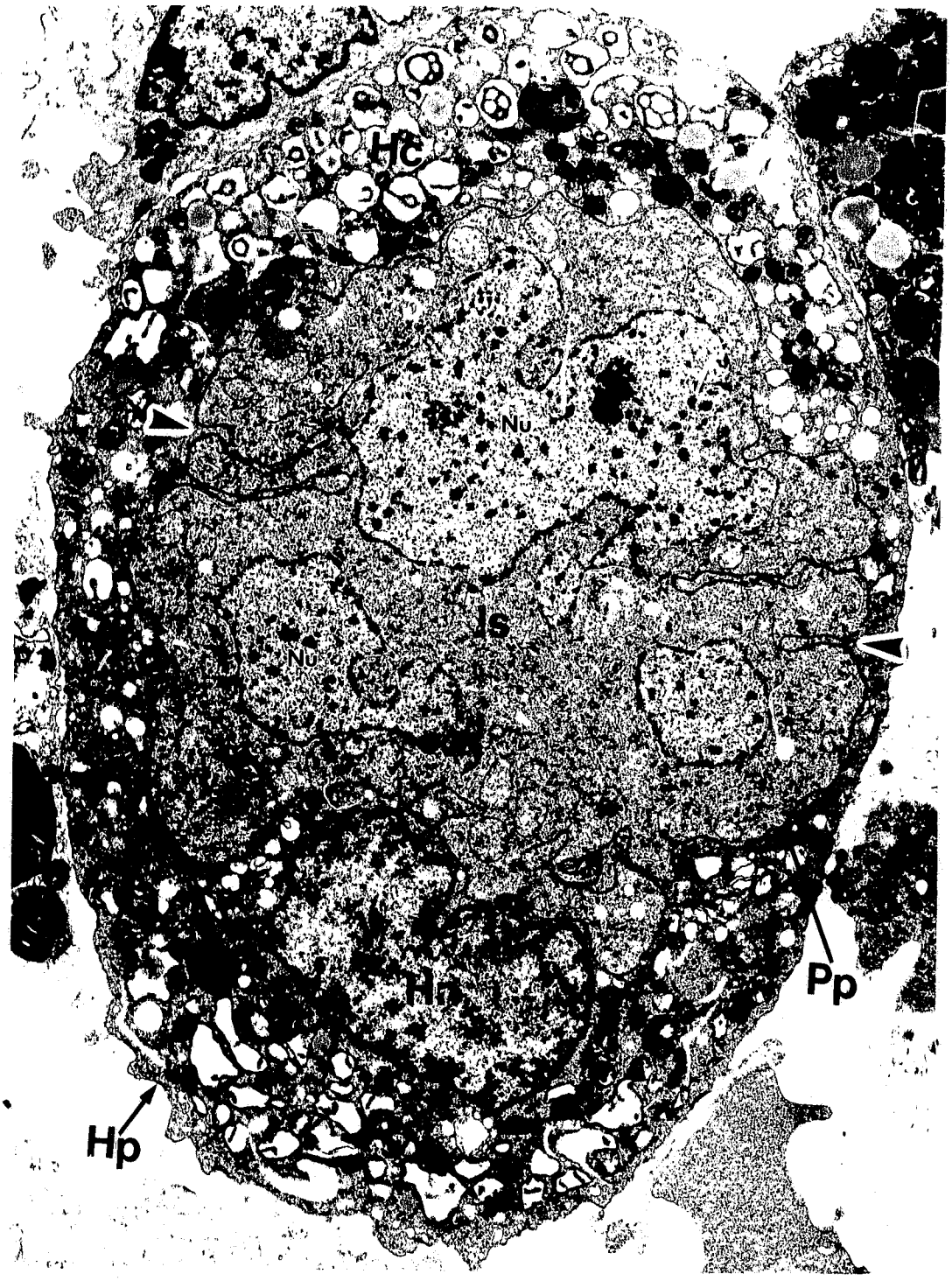

Hc
Nu
Nu
Is
Hp
Pp
Hp

Fig. 6.18 Intermediate-stage *S. cruzi* schizont in cultured rat heart myoblast, 3 wk post sporozoite inoculation. Note developing daughter merozoites (arrows) and infoldings (arrowheads) of the schizont limiting membrane which ultimately surround individual merozoites (x6750).

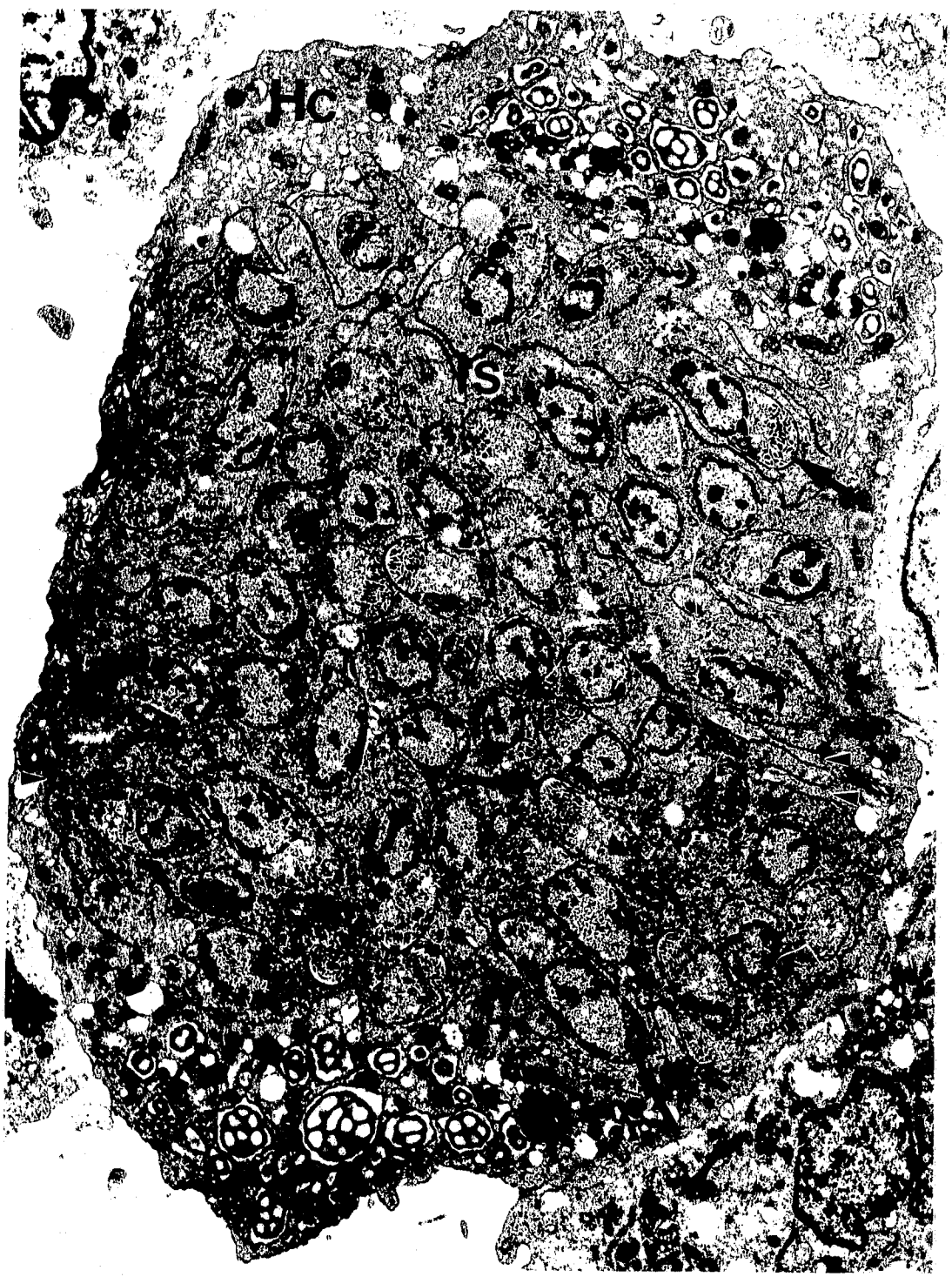

Hc
S

Fig. 6.19 Mature *S. cruzi* merozoites in cultured rat heart myoblast, 3 wk post sporozoite inoculation. Remnants of the original "mother cell" (i.e. early schizont) are indistinguishable from the host cell (x5400).

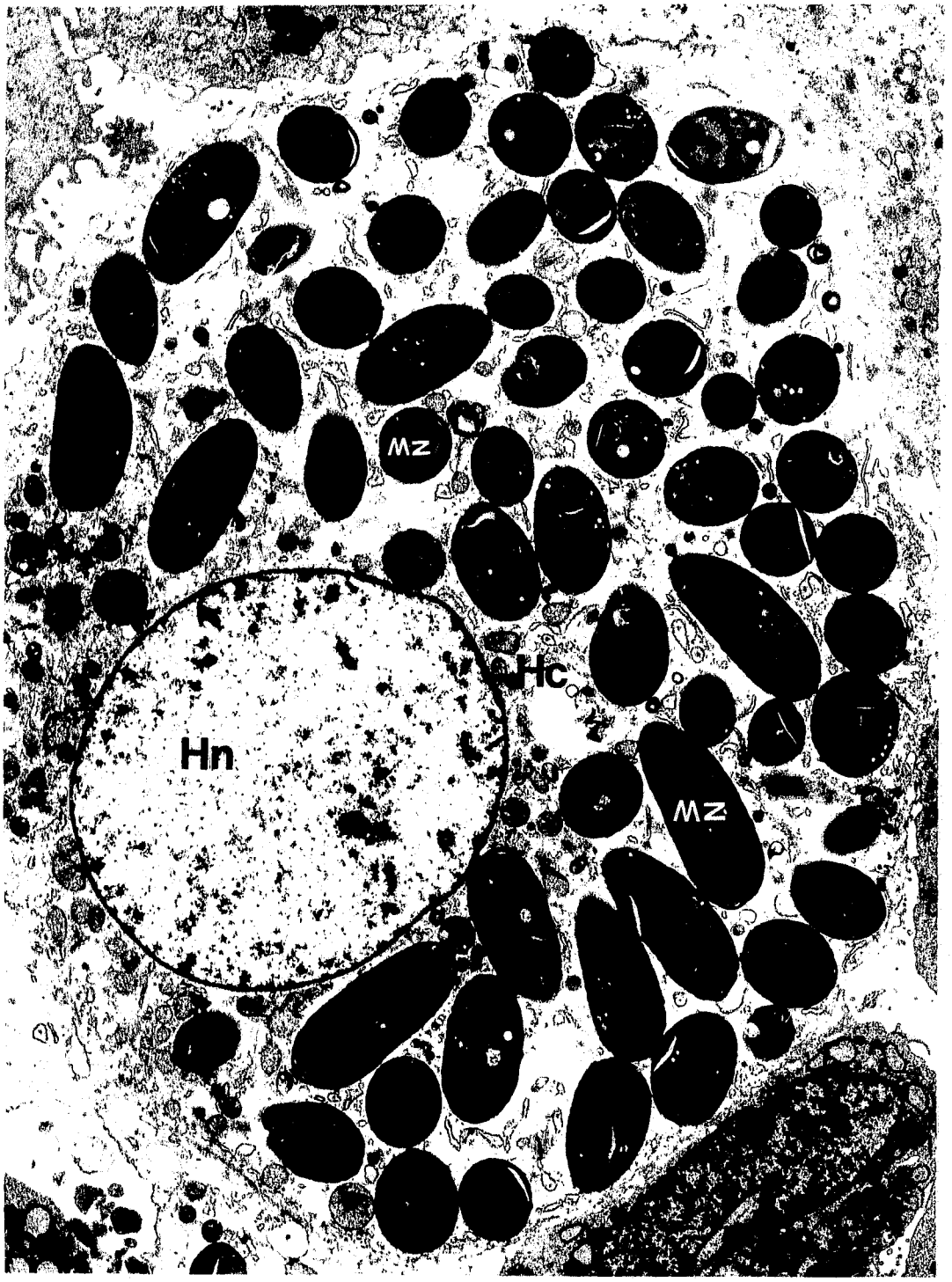
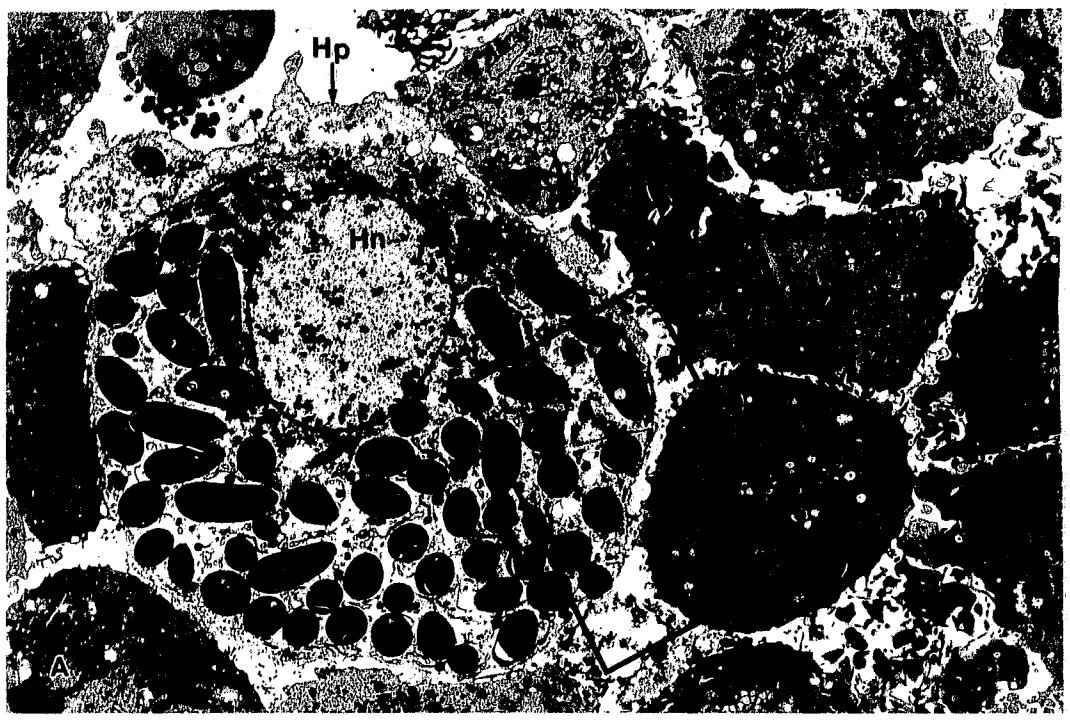

Mz
Hc
Hn
Mz

Fig. 6.20

A) Rat heart myoblast harbouring mature *S. cruzi* schizont. Compare electron density of host cell with that of surrounding uninfected cells (x2500).

B) Higher magnification of boxed area in A). Note difference in ultrastructural organization of infected cell and apparent leaching of host cytoplasm (x8100).


Hp
Hn
A

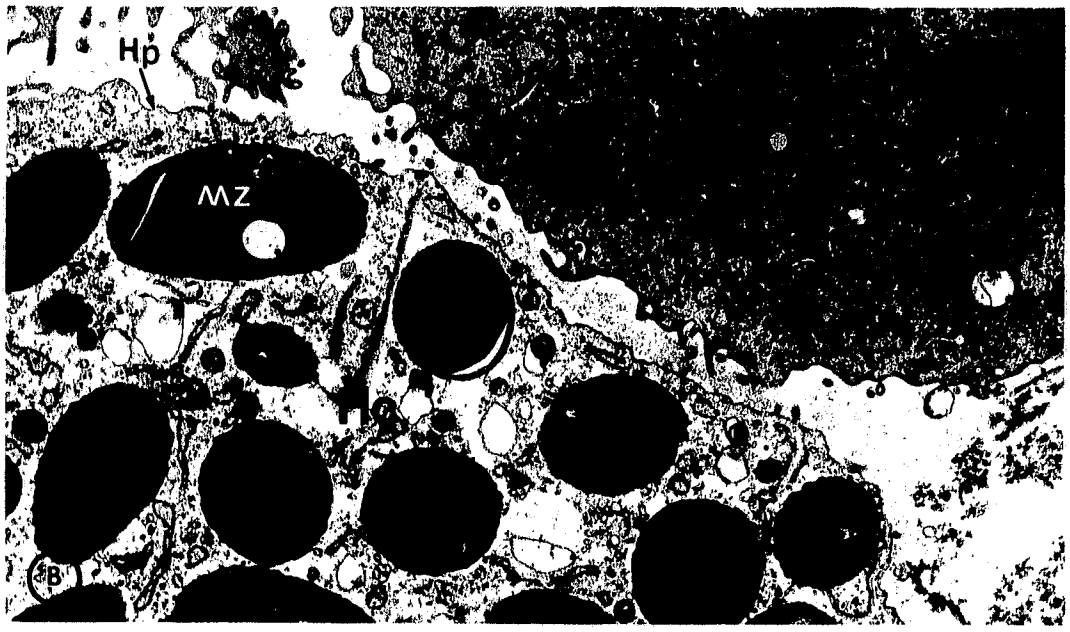
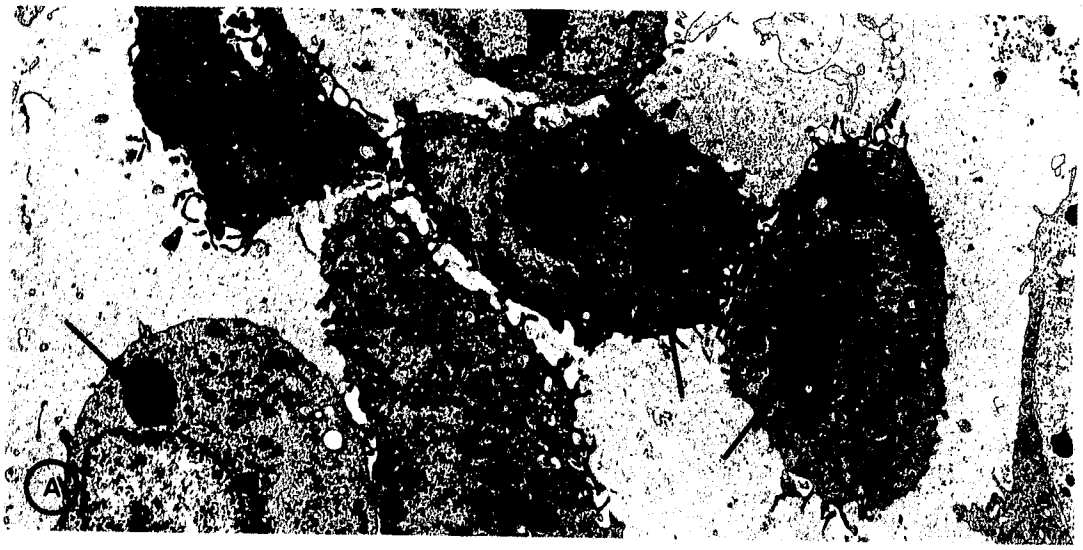
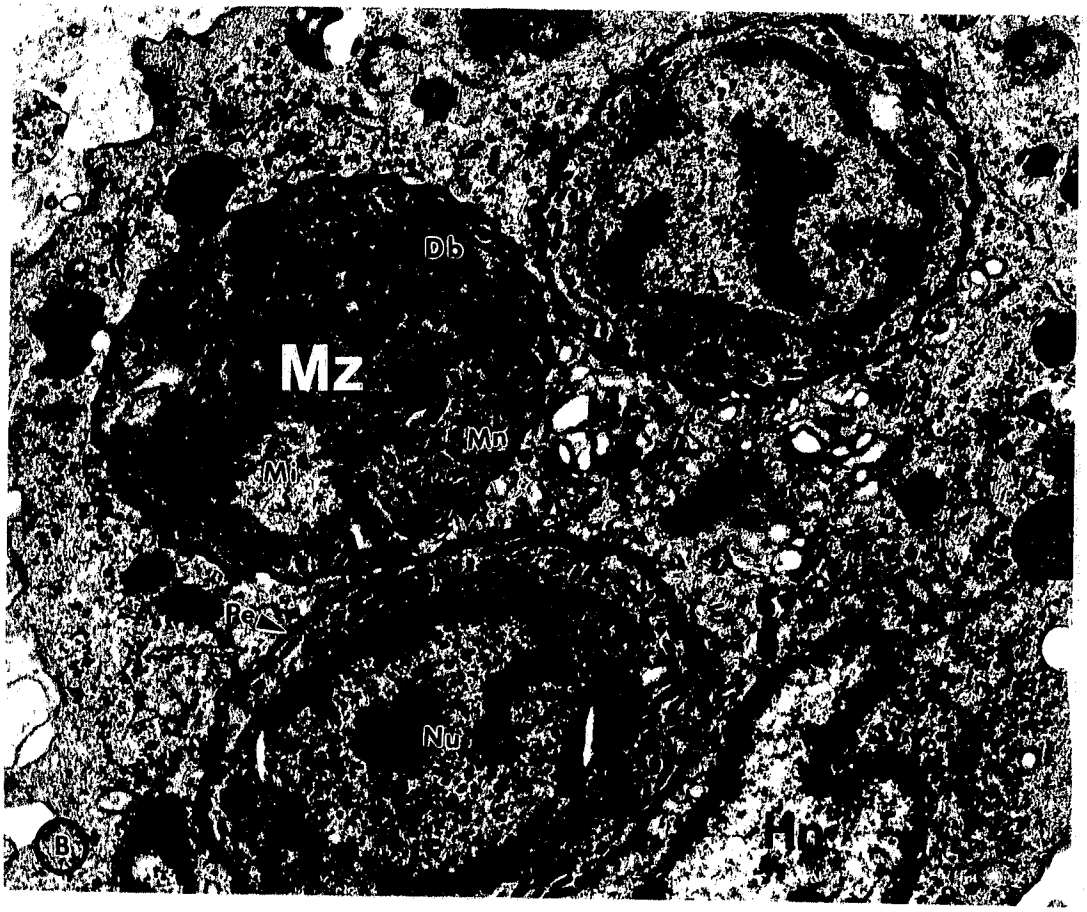

Hp
MZ
B

Fig. 6.21

A) *Sarcocystis cruzi*-infected bovine pulmonary artery endothelium, 10 wk post sporozoite inoculation. Note longitudinal and cross sections of merozoites (arrows) (x4600).

B) Higher magnification of merozoite cluster visible in A). Pathological change in host cell cytoplasm is not evident (x25,500).


A


Db
Mz
Mu
Mi
Pe
Nu
Bx

numbers of merozoites in L-6s and H9C2s as centrally located cells and parasite stages would not thrive. Very heavy cell growth observed in VERO monolayers may have had a similar effect on a smaller scale.

In vitro experiments demonstrated endothelial cells infected with *S. cruzi* sporozoites become surrounded by noninfected cells (Speer *et al.*, 1986b). Forces which attract these cells and their function are not known. Dubey *et al.* (1989) suggest the "satellite" cells transform into capillary-like structures that augment merozoite production and release. Although capillary-like tubes formed in CPAs, they were not always associated with regions of high meront density. Specific or directed cell aggregation was not seen in other cell lines. Perhaps this phenomenon is a function of the endothelial nature of the CPA culture.

Speer and Dubey (1986) observed *in vitro S. cruzi* sporozoite development as early as 14 DPI. Although merogony was not identified by LM until 24 DPI (CPA) and 33 DPI (H9C2) in this experiment, state of maturity (i.e. almost fully mature meronts) indicated development had progressed for sometime. Moreover, monolayers became extremely fragile 17 DPI (20 DPI H9C2) corresponding with meront maturation previously reported (Speer *et al.* 1986). First appearance of merozoites at 30 DPI in CPAs was similar to Andrews *et al.* (1990) result for *S. cruzi* i.e. merozoites first produced 28 DPI. Delay in visualization of meronts (and likely merozoites) occurred because much debris from the initial inoculum remained, obscuring the smaller, earlier immature stages. Replication of tissue culture cells probably served to further "anchor" this material, thus a shorter

incubation is indicated. From Chapter V results (section 5.3.2), a 2 hour incubation period for sporozoite penetration should be sufficient. Additionally, new sporozoite purification techniques could be implemented (Ono *et al.*, 1991; Ndiritu *et al.*, 1993).

Numbers of *S. cruzi* merozoites harvested were significantly less than those reported for *S. hirsuta* using the same technique (Cawthorn *et al.*, 1989). However, merozoite counts do not reflect actual numbers produced because most merozoites actively reentered host cells. Those that do not undergo subsequent development may be "trapped" in the monolayer and are thus inaccessible counting. This occurred with L-6 and VERO cell lines since merozoites were obvious at TEM. Moreover, in H9C2s, merozoites were often seen at the host cell surface although they were not removed by the harvesting technique. The fragility of monolayers would only permit very gentle rocking of flasks when collecting merozoites. This reduced the harvesting efficiency and resulted in fewer numbers of merozoites floating free in medium. Given these confounders, interpretation of relative merozoite production in Table V is difficult. Endothelial cells are obviously the more efficient producers *in vitro,* yet *in vivo* conditions could actually favour a myoblast/muscle cell type.

Delays in establishing the vascular phase of *S. cruzi in vitro* have been attributed, in part, to high host cell specificity (Speer and Burgess, 1987). Speer *et al.* (1986) found that S. *cruzi* sporozoites would penetrate but not develop in bovine kidney cells and mouse macrophages, 2 of 4 cell lines tested. In contrast,

these results show nonspecificity with merozoite production occurring in different species and cell types. Thus, range of host site or cell specificity is not as narrow as previously assumed. Perhaps, as Long and Speer (1977) suggest for *Eimeria* spp., site specificity is primarily conferred by *in vivo* factors. Limited *in vitro* development of *S. tenella* (sheep intermediate host) in bovine but not ovine monocytes (Speer *et al.*, 1986a) underscores the fact that there is much discrepancy between current *in vitro* culture systems and acceptable environments for *Sarcocystis* multiplication *in vivo*. Similarly, *S. cruzi* developed in monkey kidney cells in the experiment herein, yet Speer *et al.* (1986b) observed *S. cruzi* penetration of bovine kidney cells without subsequent asexual development. Factors that dictate an acceptable host environment are still unclear. In light of these experiments and controversy in the literature concerning host cell type, it is premature to assume asexual replication occurs exclusively in endothelial cells. If *S. cruzi* host cell specificity is broader there may be significant implications for pathogenesis, treatment, or control of disease.

Sarcocystis spp. sarcocysts form in striated or cardiac muscle from merozoites of end-stage schizogony (Cawthorn and Speer, 1990). Myoblast and muscle cell lines were selected for culture, in part, for their potential for cyst formation; however, none were evident at termination of the experiment. Although sarcocysts are generally fully formed and infectious 75 DPI (Dubey *et al.*, 1989), the 70 day experimental period may not have been long enough to visualize sarcocysts if present. Apparently *S. cruzi* can be cultured indefinitely provided

cells that support development of schizonts are available to merozoites (Andrews *et al.*, 1990). Extending the culture period (or conducting subcultures) of H9C2s, the more efficient merozoite producing myoblast line, might permit development of sarcocysts. Since the central nervous system also supports *S. cruzi* sarcocyst formation (Dubey, 1982a), subculture of merozoites to neural cells in tissue culture is a further option.

Transmission EM results were disappointing because of difficulty locating parasite stages in tissue pellets. In CPA and H9C2 cell lines, extensive sectioning of blocks was required to visualize merogony and still, earliest developmental stages were not found. Similarly, extensive sectioning of L-6 and VERO blocks did not reveal merogony although it had occurred as evidenced by the presence of merozoites. The latter were easily differentiated from sporozoites by their smaller size and absence of rhoptries and crystalloid bodies. Sporozoites also had abundant amylopectin granules which were not obvious in merozoites.

To facilitate location of parasite stages in EM blocks, initial sporozoite inoculum could be increased. Presumably, this would increase numbers of meronts produced and thus, increase chances of finding parasite upon random sectioning. However, a relatively low upper limit on increases in inocula exists because of fragility of monolayers. Too high a sporozoite inoculum can overwhelm cells initially or delayed destruction may occur coincident with meront maturation or rupture or reinvasion by merozoites. Additionally, high numbers of sporozoites may be difficult to obtain. In light of these difficulties, an *in situ* fixation and

embedding technique is recommended provided areas of merogonous activity can be relocated in culture flasks. Although this is more expensive, utilizing more processing supplies; it allows quick location of specimens of interest, ultimately saving on labour costs. Moreover, precise *in vitro* ultrastructure is preserved since trypsinization of cells is eliminated. Preliminary testing of Spoerri's *et al*. (1980) *in situ* technique with *S. cruzi*- infected H9C2s has shown useful results.

CHAPTER VII - SUMMARY

7.1 Summary of chapter IV - Comparative excystation of *S. cruzi* and *S. hirsuta*.

Modifications to the excystation protocol of Cawthorn *et al.* (1986) resulted in significantly reduced excystation rates. Moreover, I was unable to duplicate their earlier results. Clearly, stimuli responsible for excystation require further elucidation and characterization before mechanisms can be determined. The modified protocol did release viable sporozoites and was conducive to subsequent EM processing; however, elutriation was not an efficient method for sporocyst purification as much debris remained. Incorporation of proteinase K is recommended for *Sarcocystis* spp. excystation in light of a) higher ERs and clean preparations obtained by Ndiritu *et al.* (1993) and b) its ability to produce undamaged sporocysts and sporozoites for EM and *in vitro* studies, reported herein.

Electron microscopic study revealed that the process and ultrastructure of excystation in *S. cruzi* and *S. hirsuta* are similar. Neither sporocysts nor sporozoites of these two *Sarcocystis* spp. were sufficiently morphologically distinct for rapid, reliable species differentiation. However, significant differences ($P=0.000$) in length, area, and perimeter exist, sporocysts and sporozoites of *S. cruzi* being larger than *S. hirsuta*. Further, a review of the literature on excystation in other *Sarcocystis* spp. indicates identical ultrastructure of sporocysts.

7.2 Summary of chapter V - Host cell invasion by sporozoites of *S. cruzi*

The tissue culture and processing technique of Kingsley and Cole (1988) for scanning and transmission electron microscopy was evaluated. Their technique was significantly modified for experiments examining ultrastructure of host cell invasion by *S. cruzi* (sporozoite stage) *in vitro*. Incorporation of a staining procedure and a direct embedding technique facilitated subsequent TEM processing. This technique cannot be recommended for SEM of apicomplexans unless uniform, heavily infected monolayers are used.

Peak invasion for bovine pulmonary artery endothelial cells and rat heart myoblasts was determined to be 15 min and 1 h post sporozoite inoculation, respectively. Sporozoites appeared to penetrate cells by invagination of host cell membranes rather than by membrane rupture. Host cell surface projections and extension of microvilli indicative of a phagocytic process were not evident around sporozoites although both cells types contained phagocytized sporocysts. Although *S. cruzi* SZs were active invaders, host cell participation could not be ruled out. Loss of density of rhoptries of intracellular SZs compared to extracellular SZs indicated their involvement in host cell penetration and post invasion phenomena. Limited observations of granules of intermediate electron density located at the periphery of sporozoites may represent: a) dense granule exocytosis or b) a new population of granules recently identified in *S. muris* (Entzeroth *et al*., 1991). Membranous material surrounding some sporozoites and merozoites was suggestive of parasitophorous vacuole formation. Heavy

parasitization of invaded cells may reflect increased susceptibility to subsequent invasion after initial SZ entry. Both host and parasite factors such as membrane receptor alterations and chemoattractants could be involved.

Larger sporozoite inocula (> 5 X 10^4) and fixations at the time intervals pinpointed in this study are necessary to observe zoites during and immediately post invasion. Additionally, immunoelectron microscopy could be explored to further elucidate the role(s) of organelles implicated in invasion herein.

7.3 Summary of chapter VI - *In vitro* development of the merogonous phase of *S. cruzi*

In vitro asexual development of *S. cruzi* sporozoites occurred in each of four host cell lines tested. Light microscopy revealed maturing meronts in bovine pulmonary artery endothelial cells (CPA) and rat heart myoblasts (H9C2) at 24 and 33 DPI, respectively. Merozoites were evident in CPAs and H9C2s until termination of experiments at 70 DPI although the number of schizogonous generations was not determined. Electron microscopy (EM) was required to confirm schizogony in African green monkey kidney (VERO) and rat skeletal muscle myoblast (L-6) cell lines. Early schizogonous stages were frequently obscured by excess debris from the sporozoite inoculation media. Reduction of inital sporozoite incubation period from 24h to 2h is recommended to reduce debris.

Suitability of host cell type was assessed by quantification of merozoite production. Endothelial cells were most productive; however, harvesting efficiency of Speer and Dubey's (1986) technique was reduced by heavy growth of cells or

monolayer contraction in all other tissue culture lines. Thus, *in vitro* results cannot be extrapolated to *in vivo* development. Inoculation of 2×10^5 sporozoites per 25 cm^2 tissue culture flask resulted in difficulty locating parasite stages in ultrathin sections; therefore, *in situ* EM processing is recommended when areas of merogonous activity can be prelocated in culture vessels. Sarcocyst formation was not apparent in cell lines tested; however, extension of the culture period or subcultures of H9C2s are suggested to visualize this stage of the life cycle.

In contrast to earlier reports, *S. cruzi* was *not* found to have a high host cell specificity *in vitro*. Schizogony and merozoite production occured in various host species and cell types. Therefore, range of host site or cell specificity is broader than previously assumed; implications for pathogenesis, treatment, or control of sarcocystosis must be considered.

VIII. REFERENCES

ADAMS JH, HUDSON DE, TORII M, WARD GE, WELLEMS TE, AIKAWA M, MILLER LH. The Duffy receptor family of *Plasmodium knowlesi* is located within the micronemes of invasive malaria merozoites. Cell 1990; 63: 141-153.

AIKAWA M, MILLER LH, JOHNSON J, RABBEGE J. Erythrocyte entry by malaria parasites. A moving junction between erythrocyte and parasite. Journal of Cell Biology 1978; 77: 72-82.

ANDREWS CD, FAYER R, DUBEY JP. Continuous *in vitro* cultivation of *Sarcocystis cruzi*. Journal of Parasitology 1990; 76: 254-255.

BACHERE E, CHAGOT D, GRIZEL H. Cell separation by centrifugal elutriation. American Fisheries Society Special Publication 1988; 18: 281-285.

BAETZ AL, LEITING SE, BRYNER J, BARNETT D. Prepartum changes of plasma concentrations of prostaglandin F and 13,14-dihydro-15-ketoprostaglandin metabolites in pregnant animals exposed to *Sarcocystis cruzi* or *Campylobacter fetus*. American Journal of Veterinary Research 1981; 42: 22-24.

BALL SJ, PITTILO RM. Structure and ultrastructure. In: Long PL ed. Coccidiosis of man and domestic animals. Boca Raton: CRC Press, 1990: 17-43.

BANDONI SM, DUSZYNSKI DW. A plea for improved presentation of type material for coccidia. Journal of Parasitology 1988; 74: 519-523.

BANNISTER LH, MITCHELL GH. The fine structure of secretion by *Plasmodium knowlesi* merozoites during red cell invasion. Journal of Protozoology 1989; 36: 362-367.

BANNISTER LH, MITCHELL GH, BUTCHER GA, DENNIS ED. Lamellar membranes associated with rhoptries in erythrocytic merozoites of *Plasmodium knowlesi*: a clue to the mechanism of invasion. Parasitology 1986; 92: 291-303.

BARTA JR. Phylogenetic analysis of the class Sporozoea (phylum Apicomplexa Levine, 1970): evidence for the independent evolution of heteroxenous life cycles. Journal of Parasitology 1989; 75: 195-206.

BECKER B, MEHLHORN H, HEYDORN AO. Light and electron microscopic studies on the gamogony and sporogony of 5 *Sarcocystis* species *in vivo* and in tissue cultures. Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. Abt. 1. Originale. 1979; A 244: 235-239.

BOTTNER A, CHARLESTON WAG, HOPCROFT D. The structure and identity of macroscopically visible *Sarcocystis* cysts in cattle. Veterinary Parasitology 1987; 24: 35-45.

BOX ED, MARCHIONDO AA, DUSZYNSKI DW, DAVIS CP. Ultrastructure of *Sarcocystis* sporocysts from passerine birds and opossums: comments on classification of the genus *Isospora*. Journal of Parasitology 1980; 66: 68-74.

BREUER WV. How the malaria parasite invades its host cell, the erythrocyte. International Review of Cytology 1985; 96: 191-238.

BRIGGS M, FOREYT W. Sarcocystosis in cattle. The Compendium on Continuing Education for the Practicing Veterinarian 1985; 7: S396-S400.

BURGESS DE, SPEER CA, REDUKER DW. Identification of antigens of *Sarcocystis cruzi* sporozoites, merozoites, and bradyzoites with monoclonal antibodies. Journal of Parasitology 1988; 74: 828-832.

CANADIAN COUNCIL ON ANIMAL CARE. Guide to the care and use of experimental animals. 2 Vols. Ottawa, Ontario: CCAC 1980-1984.

CARRIGAN MJ. An outbreak of sarcocystosis in dairy cattle. Australian Veterinary Journal 1986; 63: 22-24.

CAWTHORN RJ, GAJADHAR AA, BROOKS RJ. Description of *Sarcocystis rauschorum* sp. n. (Protozoa: Sarcocystidae) in snowy owls (*Nyctea scandiaca*). Canadian Journal of Zoology 1984; 62: 217-225.

CAWTHORN RJ, MARKHAM RJF, HITT ND, DESPRES D. *In vitro* cultivation of the vascular phase of *Sarcocystis hirsuta* (Apicomplexa). Canadian Journal of Zoology 1989; 68: 1068-1070.

CAWTHORN RJ, REDUKER DW, SPEER CA, DUBEY JP. *In vitro* excystation of *Sarcocystis capracanis*, *Sarcocystis cruzi* and *Sarcocystis tenella* (Apicomplexa). Journal of Parasitology 1986; 72: 880-884.

CAWTHORN RJ, SPEER CA. *Sarcocystis*: infection and disease of humans, livestock, wildlife, and other hosts. In: Long PL, ed. Coccidiosis of man and domestic animals. Boston: CRC Press, 1990: 91-120.

CERNA Z, SENAUD J. Sur un type nouveau de multiplication asexuee d'une Sarcosporidie, dans le foie de la souris. Comptes Rendus Hebdomadaires des Seances. Academie des Sciences. Serie (D) (Paris) 1977; 285: 347-349.

CHIAPPINO ML, NICHOLS BA, O'CONNOR GR. Scanning electron microscopy of *Toxoplasma gondii*: parasite torsion and host-cell responses during invasion. Journal of Protozoology 1984; 31: 288-292.

CHOBOTAR B, SCHOLTYSECK E. Ultrastructure. In: Long PL, ed. The biology of the coccidia. Baltimore: University Park Press, 1982: 101-165.

CHRISTIE E, PAPPAS PW, DUBEY JP. Ultrastructure of excystment of *Toxoplasma gondii* oocysts. Journal of Protozoology 1978; 25: 438-443.

CLEGG FG, BEVERLEY JKA, MARKSON LM. Clinical disease in cattle in England resembling Dalmeny disease associated with suspected *Sarcocystis* infection. Journal of Comparative Pathology 1978; 88: 105-114.

CLUBB SL, FRENKEL JK. *Sarcocystis falcatula* of opossums: transmission by cockroaches with fatal pulmonary disease in psittacine birds. Journal of Parasitology 1992; 78: 116-124.

COLLERY P. The pathogenesis of acute bovine sarcocystosis I. Clinical signs and anaemia. Irish Veterinary Journal 1987; 41: 273-280.

COLLERY P. The pathogenesis of acute bovine sarcocystosis IV. The effects of corticosteroid therapy on the course of the anaemia. Irish Veterinary Journal 1989; 42: 85-92.

COLLERY P, WEAVERS E. An outbreak of sarcocystosis in calves in Ireland. Irish Veterinary Journal 1981; 35: 159-162.

CORNER AH, MITCHELL D, MEADS EB, TAYLOR PA. Dalmeny disease. An infection of cattle presumed to be caused by an unidentified protozoan. Canadian Veterinary Journal 1963; 4: 252-264.

CRUM JM, FAYER R, PRESTWOOD AK. *Sarcocystis* spp. in white-tailed deer. I. Definitive and intermediate host spectrum with a description of *Sarcocystis odocoileocanis* n. sp. Journal of Wildlife Diseases 1981; 17: 567-579.

DAUGSCHIES A, ROMMEL M, HOPPEN H-O. Prostanoids during acute sarcocystosis in growing pigs. Parasitology Research 1989; 76: 115-118.

DAWES CJ. Biological techniques in electron microscopy. New York: Barnes & Noble, Inc., 1971.

D'HAESE J, MEHLHORN H, PETERS W. Comparative electron microscope study of pellicular structures in coccidia (*Sarcocystis*, *Besnoitia* and *Eimeria*). International Journal for Parasitology 1977; 7: 505-518.

DORAN DJ. Behaviour of coccidia *in vitro*. In: Long PL, ed. The biology of the coccidia. Baltimore: University Park Press, 1982: 229-285.

DOUGLASS TG, SPEER CA. Enhancement by p-phenylenediamine of osmium for staining coccidia in cell culture. Journal of Parasitology 1983; 69: 621-622.

DUBEY JP. A review of *Sarcocystis* of domestic animals and of other coccidia of cats and dogs. Journal of the American Veterinary Medical Association 1976; 169: 1061-1078.

DUBEY JP. Coyote as a final host for *Sarcocystis* species of goats, sheep, cattle, elk, bison and moose in Montana. American Journal of Veterinary Research 1980; 41: 1227-1229.

DUBEY JP. Early developmental stages of *Sarcocystis cruzi* in calf fed sporocysts from coyote feces. Journal of Protozoology 1981; 28: 431-433.

DUBEY JP. Development of the ox-coyote cycle of *Sarcocystis cruzi*. Journal of Protozoology 1982a; 29: 591-601.

DUBEY JP. Development of the ox-cat cycle of *Sarcocystis hirsuta*. Proceedings of the Helminthological Society of Washington 1982b; 49: 295-304.

DUBEY JP. Microgametogony of *Sarcocystis hirsuta* in the intestine of the cat. Parasitology 1983a; 86: 7-9.

DUBEY JP. Clinical sarcocystosis in calves fed *Sarcocystis hirsuta* sporocysts from cats. Veterinary Pathology 1983b; 20: 90-98.

DUBEY JP. Immunity to sarcocystosis: modifications of intestinal coccidiosis and disappearance of sarcocysts in dairy goats. Veterinary Parasitology 1983c; 12: 23-27.

DUBEY JP, BERGERON JA. *Sarcocystis* as cause of placentitis and abortion in cattle. Veterinary Pathology 1982; 19: 315-318.

DUBEY JP, FAYER R. Zoonoses in practice - sarcocystosis. British Veterinary Journal 1983; 139: 371-377.

DUBEY JP, FAYER R, SEESEE FM. *Sarcocystis* in feces of coyotes from Montana: prevalence and experimental transmission to sheep and cattle. Journal of the American Veterinary Medical Association 1978; 173: 1167-1170.

DUBEY JP, FAYER R, SPEER CA. Experimental *Sarcocystis hominis* infection in cattle: lesions and ultrastructure. Journal of Parasitology 1988; 74: 875-879.

DUBEY JP, SPEER CA. *Sarcocystis canis* n. sp. (Apicomplexa: Sarcocystidae), the etiologic agent of generalized coccidiosis in dogs. Journal of Parasitology 1991; 77: 522-527.

DUBEY JP, SPEER CA, CALLIS G, BLIXT JA. Development of the sheep-canid cycle of *Sarcocystis tenella*. Canadian Journal of Zoology 1982; 60: 2464-2477.

DUBEY JP, SPEER CA, DOUGLASS TG. Development and ultrastructure of first-generation meronts of *Sarcocystis cruzi* in calves fed sporocysts from coyote feces. Journal of Protozoology 1980; 27: 380-387.

DUBEY JP, SPEER CA, EPLING GP. Sarcocystosis in newborn calves fed *Sarcocystis cruzi* sporocysts form coyotes. American Journal of Veterinary Research 1982; 43: 2147-2164.

DUBEY JP, SPEER CA, FAYER R. Sarcocystosis of animals and man. Boca Raton, Florida: CRC Press, Inc., 1989.

DUBREMETZ JF. Common features of host cell invasion by sporozoa. Annales de Parasitologie Humaine et Comparee 1990; 65 suppl. I: 23-25.

DUBREMETZ JF, DISSOUS C. Characteristic proteins of micronemes and dense granules from *Sarcocystis tenella* zoites (Protozoa, Coccidia). Molecular and Biochemical Parasitology 1980; 1: 279-289.

DUBREMETZ JF, TORPIER G. Freeze fracture study of the pellicle of an eimerian sporozoite (Protozoa, Coccidia). Journal of Ultrastructural Research 1978; 62: 94-109.

DUSZYNSKI DW, SPEER CA. Excystation of *Isospora arctopitheci* Rodhain, 1933 with notes on a similar process in *Isospora bigemina* (Stiles, 1891) Luhe, 1906. Parasitology Research 1976; 48: 191-197.

DYKOVA I, LOM J. Fish coccidia: critical notes on life cycles, classification and pathogenicity. Journal of Fish Diseases 1981; 4: 487-505.

ELSASSER TH, HAMMOND AC, RUMSEY TS, FAYER R. Perturbed metabolism and hormonal profiles in calves infected with *Sarcocystis cruzi*. Domestic Animal Endocrinology 1986; 3: 277-287.

ELSASSER TH, RUMSEY TS, HAMMOND AC, FAYER R. Influence of parasitism on plasma concentrations of growth hormone, somatomedin-C, and somatomedin-binding proteins in calves. Journal of Endocrinology 1988; 116: 191-200.

ELY RW, FOX JC. Elevated IgG antibody to *Sarcocystis cruzi* associated with eosinophilic myositis in cattle. Journal of Veterinary Diagnostic Investigation 1989; 1: 53-56.

ENTZEROTH R. Electron microscope study of merogony preceding cyst formation of *Sarcocystis* sp. in Roe Deer (*Capreolus capreolus*). Parasitology Research 1983; 69: 447-456.

ENTZEROTH R. Electron microscope study of host-parasite interactions of *Sarcocystis muris* (Protozoa, Coccidia) in tissue culture and *in vivo*. Parasitology Research 1984; 70: 131-134.

ENTZEROTH R. Invasion and early development of *Sarcocystis muris* (Apicomplexa, Sarcocystidae) in tissue cultures. Journal of Protozoology 1985; 32: 446-453.

ENTZEROTH R, DUBREMETZ JF, HODICK D, FERREIRA E. Immunoelectron microscopic demonstration of the exocytosis of dense granule contents into the secondary parasitophorous vacuole of *Sarcocystis muris* (Protozoa, Apicomplexa). European Journal of Cell Biology 1986; 41: 182-188.

ENTZEROTH R, KONIG A, DUBREMETZ JF. Monoclonal antibodies identify micronemes and a new population of cytoplasmic granules cross-reacting with micronemes of cystozoites of *Sarcocystis muris*. Parasitology Research 1991; 77: 59-64.

FAYER R. *Sarcocystis*: development in cultured avian and mammalian cells. Science 1970; 168: 1104-1105.

FAYER R. Gametogony of *Sarcocystis* sp. in cell culture. Science 1972; 175: 65-67.

FAYER R. Development of *Sarcocystis fusiformis* in the small intestine of the dog. Journal of Parasitology 1974; 60: 660-665.

FAYER R. Effects of refrigeration, cooking and freezing on *Sarcocystis* in beef from retail food stores. Proceedings of the Helminthological Society of Washington 1975; 42: 138-140.

FAYER R. The first asexual generation in the life cycle of *Sarcocystis bovicanis*. Proceedings of the Helminthological Society of Washington 1977a; 44: 206-209.

FAYER R. Production of *Sarcocystis cruzi* sporocysts by dogs fed experimentally infected and naturally infected beef. Journal of Parasitology 1977b; 63: 1072-1075.

FAYER R. Multiplication of *Sarcocystis bovicanis* in the bovine blood stream. Journal of Parasitology 1979; 65: 980-982.

FAYER R. *Sarcocystis*: a clinical entity in bovine practice. Proceedings of the American Association of Bovine Practitioners 1982; 14: 117-119.

FAYER R, ANDREWS C, DUBEY JP. Lysates of *Sarcocystis cruzi* bradyzoites stimulate RAW 264.7 macrophages to produce tumor necrosis factor (cachectin). Journal of Parasitology 1988; 74: 660-664.

FAYER R, DUBEY JP. Protective immunity against clinical sarcocystosis in cattle. Veterinary Parasitology 1984; 15: 187-201.

FAYER R, DUBEY JP. Bovine sarcocystosis. Compendium on Continuing Education for the Practising Veterinarian 1986; 8: F131-F142.

FAYER R, DUBEY JP, LEEK RG. Infectivity of *Sarcocystis* spp. from bison, elk, moose and cattle for cattle via sporocysts from coyotes. Journal of Parasitology 1982; 68: 681-685.

FAYER R, ELSASSER TH. Bovine sarcocystosis: how parasites negatively affect growth. Parasitology Today 1991; 7: 250-255.

FAYER R, JOHNSON AJ. Development of *Sarcocystis fusiformis* in calves infected with sporocysts from dogs. Journal of Parasitology 1973; 59: 1135-1137.

FAYER R, JOHNSON AJ. *Sarcocystis fusiformis*: development of cysts in calves infected with sporocysts from dogs. Proceedings of the Helminthological Society of Washington 1974; 41: 105-108.

FAYER R, JOHNSON, AJ. *Sarcocystis fusiformis* infection in the coyote (Canis latrans). Journal of Infectious Diseases 1975; 131: 189-192.

FAYER R, JOHNSON AJ, HILDEBRANDT PK. Oral infection of mammals with *Sarcocystis fusiformis* bradyzoites from cattle and sporocysts from dogs and coyotes. Journal of Parasitology 1976; 62: 10-14.

FAYER R, JOHNSON AJ, LUNDE M. Abortion and other signs of disease in cows experimentally infected with *Sarcocystis fusiformis* from dogs. Journal of Infectious Diseases 1976; 134: 624-628.

FAYER R, LEEK RG. Excystation of *Sarcocystis fusiformis* sporocysts from dogs. Proceedings of the Helminthological Society of Washington 1973; 40: 294-296.

FAYER R, LEEK RG. The effects of reducing conditions, medium, pH, temperature, and time on *in vitro* excystation of *Cryptosporidium*. Journal of Protozoology 1984; 31: 567-569.

FAYER R, LEEK RG, LYNCH GP. Attempted transmission of *Sarcocystis bovicanis* from cows to calves via colostrum. Journal of Parasitology 1982; 68: 1127-1129.

FAYER R, LYNCH GP. Pathophysiological changes in urine and blood from calves experimentally infected with *Sarcocystis cruzi*. Parasitology 1979; 79: 325-336.

FAYER R, LYNCH GP, LEEK RG, GASBARRE LC. Effects of sarcocystosis on milk production of dairy cows. Journal of Dairy Science 1982; 66: 904-908.

FAYER R, PRASSE KW. Hematology of experimental acute *Sarcocystis bovicanis* infection in calves. I. Cellular and serologic changes. Veterinary Pathology 1981; 18: 351-357.

FERGUSON DJP, BIRCH-ANDERSEN A, HUCHISON WM, SIIM J. The ultrastructure and distribution of micropores in the various developmental forms of *Eimeria brunetti*. Acta Pathologica Scandinavica 1977; 85: 363-373.

FILHO M, MIRAGLIA T. Histochemical observations on the *Sarcocystis fusiformis* cysts. Acta Histochemica 1977; 59: 160-167.

FINK WL. Microcomputers and phylogenetic analysis. Science 1986; 234: 1135-1139.

FORD GE. Prey-predator transmission in the epizootiology of ovine sarcosporidiosis. Australian Veterinary Journal 1974; 50: 38-39.

FORD GE, FAYER R, ADAMS M O'DONOGHUE PJ, DUBEY JP, BAVERSTOCK PR. Genetic characterisation by isoenzyme markers of North American and Australian isolates of species of *Sarcocystis* (Protozoa: Apicomplexa) from mice, sheep, goats and cattle. Systematic Parasitology 1987; 9: 163-167.

FOREYT WJ, PARISH SM, LEATHERS CW. Bovine sarcocystosis: how would you handle an outbreak? Veterinary Medicine 1986; 81: 275-279.

FRELIER PF. Experimentally induced bovine sarcocystosis: correlation of *in vitro* lymphocyte function with structural changes in lymphoid tissue. American Journal of Veterinary Research 1980; 41: 1201-1207.

FRELIER PF, LEWIS RM. Haematologic and coagulation abnormalities in acute bovine sarcocystosis. American Journal of Veterinary Research 1984; 45: 40-48.

FRELIER P, MAYHEW IG, FAYER R, LUNDE MN. Sarcocystosis: a clinical outbreak in dairy calves. Science 1977; 195: 1341-1342.

FRELIER PF, MAYHEW IG, POLLOCK R. Bovine sarcocystosis: pathologic features of naturally occurring infection with *Sarcocystis cruzi*. American Journal of Veterinary Research 1979; 40: 651-657.

FRENKEL JK. *Besnoitia wallacei* of cats and rodents: with a reclassification of other cyst-forming isosporid coccidia. Journal of Parasitology 1977; 63: 611-628.

FRENKEL JK. A review of sarcocystosis of animals and man. Parasitology Today 1990; 6: 367.

FRENKEL JK, HEYDORN AO, MEHLHORN H. Protozoan nomina dubia: to arbitrarily restrict or replace. The case of *Sarcocystis* spp. Journal of Parasitology 1984; 70: 831-815.

FRENKEL JK, MEHLHORN H, HEYDORN AO. Beyond the oocyst: over the molehills and mountains of coccidialand. Parasitology Today 1987; 3: 250-252.

FRENKEL JK, HEYDORN AO, MEHLHORN H, ROMMEL M. Sarcocystinae: *nomina dubia* and available names. Parasitology Research 1979; 58: 115-139.

FRIESEN DL, CAWTHORN RJ, SPEER CA, BROOKS RJ. Ultrastructural development of the sarcocyst of *Sarcocystis rauschorum* (Apicomplexa: Sarcocystidae) in the varying lemming *Dicrostonyx richardsoni*. Journal of Parasitology 1989; 75: 422-427.

GILES RC, TRAMONTIN R, KADEL WL, WHITAKER K, MIKSCH D, BRYANT DW, FAYER R. Sarcocystosis in cattle in Kentucky. Journal of the American Veterinary Medical Association 1980; 176: 543-548.

GRANSTROM DE, RIDLEY RK, BAOAN Y, GERSHWIN LJ, NESBITT PM, WEMPE LA. Type I hypersensitivity as a component of eosinophilic myositis (muscular sarcocystosis) in cattle. American Journal of Veterinary Research 1989; 50: 571-574.

GUT J. Effectiveness of methods used for the detection of sarcosporidiosis in farm animals. Folia Parasitologica (Praha) 1982; 29: 289-295.

HASSELMANN GE. Alteracoes pathologicas do myocardio na sarcosporideose. Bulletin. Institute Brasil Science 1926; 2: 319-321.

HERBERT IV, SMITH TS. Sarcocystosis. Parasitology Today 1987; 3: 16-21.

HEYDORN AO, GESTRICH R, MEHLHORN H, ROMMEL M. Proposal for a new nomenclature of the Sarcosporidia. Parasitology Research 1975; 48: 73-82.

HEYDORN AO, ROMMEL M. Beitrage zum lebenszyklus der sarkosporidien. II. Hund und katze als ubertrager der sarkosporidien des rindes. Berliner und Muenchener Tieraerztliche Wochenschrift 1972; 85: 121-123.

HILL JE, CHAPMAN WL, PRESTWOOD AK. Intramuscular *Sarcocystis* sp. in two cats and a dog. Journal of Parasitology 1988; 74: 724-727.

HOARE CA. Studies on some new ophidian and avian coccidia from Uganda, with a revision of the classification of the Eimeriidae. Parasitology 1933; 25: 359-388.

HONG CB, GILES RC, NEWMAN LE, FAYER R. Sarcocystosis in an aborted bovine fetus. Journal of the American Veterinary Medical Association 1982; 181: 585-586.

JANTZEN B, ENTZEROTH R. Exocytosis of dense granules of cyst merozoites (cystozoites) of *Sarcocystis cuniculi* (Protozoa, Apicomplexa) in cell cultures. Parasitology Research 1987; 73: 472-474.

JENSEN R, ALEXANDER AF, DAHLGREN RR, JOLLEY WR, MARQUARDT WC, FLACK DE, BENNETT BW, COX MF, HARRIS CW, HOFFMANN GA, TROUTMAN RS, HOFF RL, JONES RL, COLLINS JK, HAMAR DW, CRAVANS RL. Eosinophilic myositis and muscular sarcocystosis in the carcasses of slaughtered cattle and lambs. American Journal of Veterinary Research 1986; 47: 587-593.

JENSEN JB, EDGAR SA. Possible secretory function of the rhoptries of *Eimeria magna* during penetration of cultured cells. Journal of Parasitology 1976; 62: 988-992.

JOHNSON AJ, FAYER R, HILDEBRANDT PK. The pathology of experimental sarcosporidiosis in the bovine. Laboratory Investigation 1974; 30: 377-378.

JOHNSON AJ, HILDEBRANT PK, FAYER R. Experimentally induced *Sarcocystis* infection in calves: pathology. American Journal of Veterinary Research 1975; 36: 995-999.

JOHNSON AM, BAVERSTOCK PR. Rapid ribosomal RNA sequencing and the phylogenetic analysis of protists. Parasitology Today 1989; 5: 102-105.

JOHNSON AM, MURRY PJ, ILLANA S, BAVERSTOCK PJ. Rapid nucleotide sequence analysis of the small subunit ribosomal RNA of *Toxoplasma gondii*: evolutionary implications for the Apicomplexa. Molecular and Biochemical Parasitology 1987; 25: 239-246.

JOINER KA. Rhoptry lipids and parasitophorous vacuole formation: a slippery issue. Parasitology Today 1991; 7: 226-227.

KIBENGE FSB, CAWTHORN RJ, DESPRES D, MCKENNA PK, MARKHAM RJF. Development of genomic probes to *Sarcocystis cruzi* (Apicomplexa). Veterinary Parasitology 1991; 40: 9-20.

KINGSLEY RE, COLE NL. Preparation of cultured mammalian cells for transmission and scanning electron microscopy using aclar film. Journal of Electron Microscopy Technique 1988; 10: 77-85.

KOUDELA B, STEINHAUSER L. Evaluation of vitality of sarcocysts in beef by the DAPI fluorescence test. Acta Veterinaria (Brno) 1984; 53: 193-197.

KUHN J. Untersuchungen uber die trichinenkrankheit der schweine. Mitteilungen Landwirtsch 1865; 1.

LABBE A. Sporozoa. In: Schulze FE, Butschli O, eds. Tierreich. Eine Zusammenstellung und Kennzeichnung der Rezenten Tierformen, Vol. 5. Berlin: Friedlander & Sohn, 1899.

LANDSVERK T. An outbreak of sarcocystosis in a cattle herd. Acta Veterinaria Scandinavica 1979; 20: 238-244.

LANKESTER ER. On *Drepanidium ranarum*, the cell parasite of the frog's blood and spleen (Gaule's Wurmchen). Quarterly Journal of Microscopical Science 1882; 22: 53-56.

LEEK RG. Infection of sheep with frozen sporocysts of *Sarcocystis ovicanis*. Proceedings of the Helminthological society of Washington 1986; 53: 297-298.

LEEK RG, FAYER R. Survival of sporocysts of *Sarcocystis* in various media. Proceedings of the Helminthological Society of Washington 1979; 46: 151-154.

LEGER L, DUBOSCQ O. *Selenococcidium intermedium* L. et D. et la systematique des sporozoaires. Archives de Zoologie Experimentale et Generale (5e serie) 1910; 5: 187-193.

LERICHE MA, DUBREMETZ JF. Exocytosis of *Toxoplasma gondii* dense granules into the parasitophorous vacuole after host cell invasion. Parasitology Research 1990; 76: 559-562.

LEUCKART R. Die parasiten des menschen, 2nd ed. Leipzig, Germany: G.F. Winter, 1879.

LEVINE ND. Taxonomy of the Sporozoa. Journal of Parasitology 1970; 56: 208-209.

LEVINE ND. Taxonomy and life cycles of coccidia. In: Long PL, ed. The biology of the coccidia. Baltimore: University Park Press, 1982: 1-33.

LEVINE ND. Apicomplexa: *Sarcocystis*, *Toxoplasma*, and related protozoa. In: Veterinary Protozoology. Ames: Iowa State University Press, 1985: 233-258.

LEVINE ND. The taxonomy of *Sarcocystis* (Protozoa, Apicomplexa) species. Journal of Parasitology 1986; 72: 372-382.

LEVINE ND. The protozoan phylum Apicomplexa. Boca Raton, Florida: CRC Press, 1988.

LEVINE ND, BAKER JR. The *Isospora* - *Toxoplasma* - *Sarcocystis* confusion. Parasitology Today 1987; 3: 101-105.

LEVINE ND, TADROS W. Named species and hosts of *Sarcocystis* (Protozoa: Apicomplexa: Sarcocystidae). Systematic Parasitology 1980; 2: 41-60.

LONG PL, JOYNER LP. Problems in the identification of species of *Eimeria*. Journal of Protozoology 1984; 31: 535-541.

LONG PL, SPEER CA. Invasion of host cells by coccidia. In: Parasite Invasion. Oxford: Blackwell Scientific, 1977: 1-6.

LUMB R, SMITH K, O'DONOGHUE PJ, LANSER JA. Ultrastructure of the attachment of *Cryptosporidium* sporozoites to tissue culture cells. Parasitology Research 1988; 74: 531-536.

LUNDE MN, FAYER R. Serologic tests for antibody to *Sarcocystis* in cattle. Journal of Parasitology 1977; 63: 222-225.

LYCKE E, CARLBERG K, NORRBY R. Interactions between *Toxoplasma gondii* and its host cells: function of the penetration-enhancing factor of *Toxoplasma*. Infection and Immunity 1975; 11: 853-861.

MAHAFFEY EA, GEORGE JW, DUNCAN JR, PRASSE KW, FAYER R. Hematologic values in calves infected with *Sarcocystis cruzi*. Veterinary Parasitology 1986; 19: 275-280.

MAHRT JL, FAYER R. Hematologic and serologic changes in calves experimentally infected with *Sarcocystis fusiformis*. Journal of Parasitology 1975; 61: 967-969.

MARKUS MB. Flies as natural transport hosts of *Sarcocystis* and other coccidia. Journal of Parasitology 1980; 66: 361-362.

MARTIN SW, MEEK AH, WILLEBERB P. Veterinary Epidemiology. Ames, Iowa: Iowa State University Press, 1987.

MASUROVSKY EB, BUNGE RP. Aclar film in biological electron microscopy. Journal of Electron Microscopy Technique 1989; 12: 172-173.

MATUSCHKA FR. Reptiles as intermediate and/or final hosts of Sarcosporidia. Parasitology Research 1987; 73: 22-32.

MATUSCHKA FR, BANNERT B. Recognition of cyclic transmission of *Sarcocystis stehlinii* n. sp. in the Gran Canarian giant lizard. Journal of Parasitology 1989; 75: 383-387.

MCKENNA PB, CHARLESTON WAG. The excystation of *Sarcocystis gigantea* and other coccidia. New Zealand Journal of Zoology 1982; 9: 47-48.

MCKENNA PB, CHARLESTON WAG. The *in vivo* excystation of *Sarcocystis gigantea* and *S. tenella* sporocysts. Veterinary Parasitology 1991; 39: 1-11.

MEADS EB. Dalmeny disease - another outbreak - probably sarcocystosis. Canadian Veterinary Journal 1976; 17: 271.

MEHLHORN H, FRENKEL JK. Ultrastructural comparison of cysts and zoites of *Toxoplasma gondii*, *Sarcocystis muris*, and *Hammondia hammondi* in skeletal muscle of mice. Journal of Parasitology 1980; 66: 59-67.

MEHLHORN H, HARTLEY WJ, HEYDORN AO. A comparative ultrastructural study of the cyst wall of 13 *Sarcocystis* species. Protistologica 1976; 12: 451-467.

MEHLHORN H, SCHOLTYSECK E. Licht- und elektronenmikroskipische Untersuchungen an Entwicklungsstadien von *Sarcocystis tenella* aus der Darmwand der Hauskatze. I. Die oocysten und sporocysten. Parasitology Research 1974; 43: 251-270.

MEHLHORN H, SENAUD J, HEYDORN AO, GESTRICH R. Comparaison des ultrastructures des kystes de *Sarcocystis fusiformis* Railliet, 1897 dans la musculature du boeuf, apres infection naturelle et après infection experimentale par des sporocystes d'*Isospora hominis* et par des sporocystes des grandes formes d'*Isospora bigemina* du chein et du chat. Protistologica 1975; 11: 445-455.

MELVILLE RV. *Nomina dubia* and available names. Parasitology Research 1980; 62: 105-109.

MELVILLE RV. Reply to Frenkel, Mehlhorn, and Heydorn on protozoan *nomina dubia*. Journal of Parasitology 1984; 70: 815.

MICHEL R, SCHUPP K, RAETHER W, BIERTHER FW. Formation of a close junction during invasion of erythrocytes by *Toxoplasma gondii in vitro*. International Journal for Parasitology 1980; 10: 309-313.

MIESCHER F. Uber eigenthumliche schlauche in den muskelm einer hausmaus. Bericht der Verhandlungen der Naturforschenden Gesellschaft 1843; 5: 198-202.

MILLONIG G. Advantages of a phosphate buffer for OsO_4 solutions in fixation. Journal of Applied Physiology 1961; 32: 1637.

MOULE L. Des sarcosporidies et de leur frequence, principalement chez les animaux de boucherie. Mem Soc Sci Arts Vitry-le-Fr 1888; 14: 3.

MUNDAY BL, BLACK H. Suspected *Sarcocystis* infections of the bovine placenta and fetus. Parasitology Research 1976; 51: 129-132.

NAKAMURA K, SHOYA S, NAKAJIMA Y, SHIMURA K, ITO S. Pathology of experimental acute sarcocystosis in a cow. Japanese Journal of Veterinary Science 1982; 44: 675-679.

NDIRITU W, CAWTHORN RJ, KIBENGE FSB. Use of proteinase K in the excystation of *Sarcocystis cruzi* sporocysts for *in vitro* culture and DNA extraction. Veterinary Parasitology, in press.

NICHOLS BA, CHIAPPINO ML. Cytoskeleton of *Toxoplasma gondii*. Journal of Protozoology 1987; 34: 217-226.

NICHOLS BA, O'CONNOR GR. Penetration of mouse peritoneal macrophages by the protozoan *Toxoplasma gondii*. New evidence for active invasion and phagocytosis. Laboratory Investigations 1981; 44: 324-335.

O'DONOGHUE PJ, ADAMS M, DIXON BR, FORD GE, BAVERSTOCK PR. Morphological and biochemical correlates in the characterization of *Sarcocystis* spp. Journal of Protozoology 1986; 33: 114-121.

ONO K, HORN K, HEYDORN AO. Purification of *in vitro* excysted *Sarcocystis* sporozoites by passage through a modified DE 52 anion-exchange column. Parasitology Research 1991; 77: 717-719.

OVERSTREET RM, HAWKINS WE, FOURNIE JW. The coccidian genus *Calyptospora* n. g. and family Calyptosporidae n. fam. (Apicomplexa), with members infecting primarily fishes. Journal of Protozoology 1984; 31: 332-339.

PACHECO ND, FAYER R. Fine structure of *Sarcocystis cruzi* schizonts. Journal of Protozoology 1977; 24: 382-388.

PACHECO ND, SHEFFIELD HG, FAYER R. Fine structure of immature cysts of *Sarcocystis cruzi*. Journal of Parasitology 1978; 64: 320-325.

PAK SP. Occurrence of *Sarcocystis* in *Vulpes corsac* (in Russian), in X Vses. Konferenz Prirodnogo Ochagovost Boleznei. October 9 to 11. Dushanbe Tezisy dokladov 1, Alma-Ata, U.S.S.R., Nauka Kaz. S.S.R., 133, 1979.

PERKINS ME. Rhoptry organelles of apicomplexan parasites. Parasitology Today 1992; 8: 28-32.

PETZ LD, GARRATTY G. Management of autoimmune hemolytic anemias. In: Acquired immune hemolytic anemias. New York: Churchill Livingstone, 1980: 392-440.

POCHE F. Das system der Protozoa. Archive fur Protistenkunde 1913; 30: 125-321.

POHL U, DUBREMETZ JF, ENTZEROTH R. Characterization and immunolocalization of the protein contents of micronemes of *Sarcocystis muris* cystozoites (Protozoa, Apicomplexa). Parasitology Research 1989; 75: 199-205.

PORCHET-HENNERE E. Quelques precisions sur l'ultrastructure de *Sarcocystis tenella*: I. L'endozoite (après coloration negative). Journal of Protozoology 1975; 22: 214-220.

PORCHET-HENNERE E. Étude ultrastructurale de la schizogonie chez la coccidie *Globidium gilruthi*. Protistologica 1977; 13: 31-52.

PORCHET-HENNERE E, NICOLAS G. Are rhoptries of coccidia really extrusomes? Journal of Ultrastructure Research 1983; 84: 194-203.

PORCHET E, TORPIER G. Étude du germe infectioux de *Sarcocystis tenella* et *Toxoplasma gondii* par la technique du cryodecapage. Parasitology Research 1977; 54: 101-124.

PRASSE KW, FAYER R. Hematology of experimental acute *Sarcocystis bovicanis* infection in calves. II. Serum biochemistry and hemostasis studies. Veterinary Pathology 1981; 18: 358-367.

RAILLIET A, LUCET A. Note sur quelques espèces de coccidies encore peu étudiees. Bulletin. Societe Zoologique de France 1891; 16: 246.

ROBERTS WL, SPEER CA, HAMMOND DM. Penetration of *Eimeria larimerensis* sporozoites into cultured cells as observed with the light and electron microscopes. Journal of Parasitology 1971; 57: 615-625.

ROMMEL M. Sarcocystosis of domestic animals and humans. In Practice Sept. 1985; pp. 158-160.

RUSSEL DG. Host cell invasion by Apicomplexa: an expression of the parasite's contractile system? Parasitology 1983; 87: 199-209.

RYLEY JF. Cytochemistry, physiology and biochemistry. In: Hammond DM, Long PL, eds. The coccidia: *Eimeria*, *Isospora*, *Toxoplasma*, and related genera. Baltimore: University Park Press, 1973: 145-181.

SCHOLTYSECK E. Ultrastructure. In: Hammond DM, Long PL, eds. The coccidia: *Eimeria*, *Isospora*, *Toxoplasma*, and related genera. Baltimore: University Park Press, 1973: 81-144.

SCHOLTYSECK E. Fine structure of parasitic protozoa. Heidelberg: Springer-Verlag, 1979.

SCHOLTYSECK E, MEHLHORN H, FRIEDHOFF. The fine structure of the conoid of Sporozoa and related organisms. Parasitology Research 1970; 34: 97-127.

SENAUD J, CHOBOTAR B, SCHOLTYSECK E. Role of the micropore in nutrition of the Sporozoa: Ultrastructural study of *Plasmodium cathemerium, Eimeria ferrisi, E. stiedai, Besnoitia jellisoni*, and *Frenkelia* sp. Tropenmedizen und Parasitologie 1976; 27: 145-159.

SHEFFIELD HG, FAYER R. Fertilization in the coccidia: fusion of *Sarcocystis bovicanis* gametes. Proceedings of the Helminthological Society of Washington 1980; 47: 118-121.

SINDEN RE. A cell biologist's view of host cell recognition and invasion by malarial parasites. Transactions of the Royal Society of Tropical Medicine and Hygiene 1985; 79: 598-605.

SMITH DD. The Sarcocystidae: *Sarcocystis*, *Frenkelia*, *Toxoplasma*, *Besnoitia*, *Hammondia*, and *Cystoisospora*. Journal of Protozoology 1981; 28: 262-266.

SMITH DD, FRENKEL JK. Cockroaches as vectors of *Sarcocystis muris* and of other coccidia in the laboratory. Journal of Parasitology 1978; 64: 315-319.

SOMMERVILLE RI, ROGERS WP. Excystation of coccidia. In: The nature and action of host signals. Advances in Parasitology 1967; 26: 240-293.

SPEER CA. The coccidia. In: Jensen JB, ed. *In vitro* cultivation of protozoan parasites. Boca Raton: CRC Press, Inc., 1983: 1-64.

SPEER CA, BURGESS DE. *In vitro* cultivation of *Sarcocystis* merozoites. Parasitology Today 1987; 3: 2-3.

SPEER CA, BURGESS DE. *In vitro* development and antigen analysis of *Sarcocystis*. Parasitology Today 1988; 4: 46-49.

SPEER CA, CAWTHORN RJ, DUBEY JP. *In vitro* cultivation of the vascular phase of *Sarcocystis capracanis* and *Sarcocystis tenella*. Journal of Protozoology 1986a; 33: 486-490.

SPEER CA, DUBEY JP. Vascular phase of *Sarcocystis cruzi* cultured *in vitro*. Canadian Journal of Zoology 1986; 64: 209-211.

SPEER CA, HAMMOND DM, MAHRT JL, ROBERTS WL. Structure of the oocyst and sporocyst walls and excystation of sporozoites of *Isospora canis*. Journal of Parasitology 1973; 59: 35-40.

SPEER CA, MARCHIONDO AA, DUSZYNSKI DW, FILE SK. Ultrastructure of sporocyst during excystation of *Isospora endocallimici*. Journal of Parasitology 1976; 62: 984-987.

SPEER CA, WHITMIRE WM, REDUKER DW, DUBEY JP. *In vitro* cultivation of meronts of *Sarcocystis cruzi*. Journal of Parasitology 1986b; 72: 677-683.

SPEER CA, WONG RB, BLIXT JA, SCHENKEL RH. Capping of immune complexes by sporozoites of *Eimeria tenella*. Journal of Parasitology 1985; 71: 33-42.

SPOERRI PE, DRESP W, HEYDER E. A simple embedding technique for monolayer neuronal cultures grown in plastic flasks. Acta Anatomica 1980; 107: 221. As cited in Hayat MA. Basic techniques in transmission electron microscopy. New York: Academic Press, Inc., 1986.

SPURR AR. A low viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructure Research 1969; 26: 31-43.

SRIVASTAVA PS, SINHA SRP, JUYAL PD, SAHA AK. Host resistance and faecal sporocyst excretion in dogs exposed to repeated infection with *Sarcocystis levinei*. Veterinary Research Communications 1987; 11: 185-190.

STACKHOUSE LL, CAWTHORN RJ, BROOKS RJ. Pathogenesis of infection with *Sarcocystis rauschorum* (Apicomplexa) in experimentally infected varying lemmings (*Dicrostonyx richardsoni*). Journal of Wildlife Diseases 1987; 23: 566-571.

STALHEIM OHV, FAYER R, HUBBERT WT. Update on bovine toxoplasmosis and sarcocystosis, with emphasis on their role in bovine abortions. Journal of the American Veterinary Medical Association 1980; 176: 299-302.

STOCKDALE PGH, SHEARD A, CAWTHORN RJ. Excystation of *Isospora brachyrhynchi* Cawthorn and Wobeser, 1985 *in vitro*. Canadian Journal of Zoology 1985; 63: 2646-2648.

STOTISH RL, SIMASHKEVICH PM, WANG CC. Separation of sporozoites, sporocysts and oocysts of *Eimeria tenella* by centrifugal elutriation. Journal of Parasitology 1977; 63: 1124-1126.

STROHLEIN DA. Contributions to the biology of experimental *Sarcocystis suicanis* infection in domestic swine. PhD Thesis, University of Georgia College of Veterinary Medicine, 1986.

STROHLEIN DA, PRESTWOOD AK. *In vitro* excystation and structure of *Sarcocystis suicanis* Erber, 1977, sporocysts. Journal of Parasitology 1986; 72: 711-715.

SWITZER JW, JAIN NC. Autoimmune hemolytic anemia in dogs and cats. Veterinary Clinics of North America 1981; 11: 405-420.

TAIT A, SACKS DL. The cell biology of parasite invasion and survival. Parasitology Today 1988; 4: 228-234.

TENTER AM, JOHNSON MR, ZIMMERMAM GL. Differentiation of *Sarcocystis* species in European sheep by isoelectric focusing. Parasitology Research 1989; 76: 107-114.

TOMBES AS. Surface morphology of Giardia cysts recovered from a variety of hosts. In: Jakubowski W, Hoff JC, eds. Waterborne transmission of giardiasis. EPA 600/9-79-001, pp.22-37, June 1979.

TORII M, ADAMS JH, MILLER LH, AIKAWA M. Release of merozoite dense granules during erythrocyte invasion by *Plasmodium knowlesi*. Infection and Immunity 1989; 57: 3230-3233.

VETTERLING JM, PACHECO ND, FAYER R. Fine structure of gametogony and oocyst formation in *Sarcocystis* sp. in cell culture. Journal of Protozoology; 1973; 20: 613-621.

WANG CC. Biochemistry and physiology of coccidia. In: Long PL, ed. The biology of the coccidia. Baltimore: University Park Press, 1982: 167-228.

WEAKLEY BS. A beginner's handbook in biological transmission electron microscopy. Edinburgh: Churchill Livingstone, 1981.

WEATHERSBY AB. *Plasmodium fallax*: the invasion of host cells by exoerythrocytic merozoites in tissue culture. Journal of Parasitology 1987; 73: 77-79.

WENYON CM. Protozoology. A manual for medical men, veterinarians and zoologists. Bailliere, London: Tindall and Cassel Ltd., 1926.

WERK R. How does *Toxoplasma gondii* enter host cells? Reviews of Infectious Diseases 1985; 7: 449-457.

WILLIAMS RB. Hapantotypes: a possible solution to some problems of parasite nomenclature. Parasitology Today 1986; 2: 314-316.

WOODMANSEE DB, POWELL EC, POHLENZ JFL, MOON HW. Factors affection motility and morphology of *Cryptosporidium* sporozoites *in vitro*. Journal of Protozoology 1987; 34: 295-297.

IX. APPENDIX A

AMERICAN TYPE CULTURE COLLECTION - CERTIFIED CELL LINES (CCL) AND CELL REPOSITORY LINES (CRL) USED IN EXPERIMENTS REPORTED HEREIN

African green monkey kidney (VERO) (CCL 81)

Origin:	Normal, adult African green monkey (*Cercopithecus aethiops*)
Medium:	95% Medium 199, 5% fetal bovine serum (FBS), 50 ug and 50 U per ml of penicillin and dihydrostreptomycin (Penstrep), respectively
Subculture:	Rinse with Puck's saline (Appendix C); add 0.25% trypsin for 1-2 min; recommended splitting ratio 1:2 to 1:6
Comments:	Fibroblast-like morphology; rapid cell replication

Bovine pulmonary artery endothelium (CPA) (CCL 207)

Origin:	Isolated from main pulmonary artery of a normal, young, unweaned bovine (*Bos taurus*) calf
Medium:	80% Medium 199, 20% FBS, antibiotic-free
Subculture:	Rinse with Ca^{++}, Mg^{++} free Puck's saline; add 3 ml SMATV (Appendix C) for 1-2 min; splitting ratio 1:3 to 1:4
Comments:	Endothelial morphology

Rat heart myoblast (H9C2) (2-1) (CRL 1446)

Origin:	A subclone of an original clonal cell line derived from embryonic BD1X rat heart tissue
Medium:	90% Dulbecco's Modified Eagle's Medium, 10% FBS, Penstrep
Subculture:	Add 0.25% trypsin for several minutes; split in 1:12 ratio
Comments:	Slow growing cell line; forms multinucleated myotubes in culture; myoblastic component depletes overtime

Rat skeletal muscle myoblast (L-6) (CRL 1458)

Origin:	Isolated from primary cultures of rat thigh muscle maintained for the first two passages in the presence of methyl cholanthrene.
Medium:	90% Dulbecco's Modified Eagle's Medium, 10% FBS, Penstrep
Subculture:	Add 0.25% trypsin for 1-2 min; split in 1:12 ratio
Comments:	Fast growing cell line; myoblasts fuse to form multinucleated and striated muscle fibers; fusion declines with subcultivation

X. APPENDIX B

ELECTRON MICROSCOPY PROCESSING SCHEDULES

Routine TEM Processing

Note:	Process in fume hood and fill vials unless otherwise specified.
Fix:	2% glutaraldehyde in 0.1 M phosphate buffer (pH 6.9, 472 mosm) x 1-2 h to overnight at room temperature or in refrigerator.
Wash:	0.1 M phosphate buffer 2-5 x 10 min on rotator
Post fix:	- 1.0% osmium tetroxide in 0.1 M phosphate buffer x 1-2 h in refrigerator - Use only 2-2.5 ml per vial - Mix epon and turn on polymerizing oven to 70 °C
Note: specified	All further changes are done on a rotator unless otherwise
Wash:	Distilled water x 10 min

Dehydrate:		
	50% ethanol	1 x 10 min
	70% ethanol	2 x 10 min
	95% ethanol	2 x 10 min
	100% ethanol	2 x 15 min

Clear:		
	Propylene oxide (PO)	10 min on rotator
		10 min not on rotator

Infiltrate:		
	50:50 epon/araldite mix to PO	30 min
	75:25 epon/araldite mix to PO	30 min
	Pure epon/araldite	60 min

Embed:	Put tissue in labelled molds (yield is approximately 20 blocks per vial) and fill 2/3 full with pure epon
Polymerize:	In vacuum oven overnight at 65-70 °C. Check polymerized blocks for proper hardness i.e. should not be able to score them with fingernail.

Processing with Spurr's low-viscosity medium (Spurr, 1969)

Process as above until the clearing stage (propylene oxide is not necessary when dehydration occurs in ethanol). Procede with the following changes:

a) To the last change of ethanol add an equal amount of Spurr's resin and place on rotator for 30 min.

b) Add another equal quantity of resin and rotate 30 min.

c) Drain mixture from vials and add pure resin.

d) Change resin at end of day and leave at room temperature overnight.

e) Next day, change resin and polymerize at 70 °C for 8 h or more.

Routine SEM Processing

Fixation:	2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 6.9, 510 mosm) x overnight at 4°C	
Wash:	0.1 M phosphate buffer (pH 7.4, 230 mosm) 3 x 10 min	
Secondary fix & wash:	Not necessary	
Dehydration:	30% ethanol	1 x 10 min
	50% ethanol	1 x 10 min
	70% ethanol	2 x 10 min
	95% ethanol	2 x 10 min
	100% ethanol	1 x 10 min
	100% ethanol	1 x 10 min in refrigerator
	100% ethanol	- load into critical point dryer
		- cool critical point dryer

Critical point dry

Attach to SEM stubs with conductive carbon paint (colloidal graphite)

Sputter coat with gold

Store in vacuum dessicator

XI. APPENDIX C

ALPHABETICAL LISTING OF FORMULATIONS FOR ELECTRON MICROSCOPY[a]

Epon embedding medium:

Mix: 20 ml Araldite 502, 25 ml Epon 812, and 60 ml DDSA (dodecenyl succinic anhydride); stir covered 30 min. Harden with 2.4 ml DMP-30 (2,4,6-tri-dimethylaminomethyl phenol) and restir 30 min. Debubble in vacuum desiccator, and freeze for storage.

Lead citrate:

Dissolve: 1.5 g lead nitrate, 1.5 g lead acetate, 1.5 g lead citrate, and 3.0 g sodium citrate in 90 ml distilled H_20 at 40 C. Add 24 ml fresh 1N NaOH and 40 ml distilled H_20. Refrigerate for storage.

Millonig's modified buffer[b]:

Stock solutions

$NaH_2PO_4 - H_2O$	2.26 g/100 ml H_2O	41.50 ml
NaOH	2.52 g/100 ml H_2O	8.50 ml
Glucose	5.40 g/100 ml H_2O	5.00 ml
$CaCl_2$	1.00 g/100 ml H_2O	0.25 ml

		55.24 ml buffer

Millonig's PBS

$NaH_2PO_4 - H_2O$	1.80 g
$Na_2HPO_4 - 7H_2O$	23.25 g
or	
$Na_2HPO_4 - 12H_2O$	31.05 g
NaCl	5.00 g
Distilled H_2O to	1000 ml

[a]All EM supplies from J.B. EM Services Inc., Pointe-Claire-Dorval, Quebec. Alternate supplier Marivac Limited, Halifax, Nova Scotia.
[b]Millonig (1961) as modified by Dawes (1971).

Phosphate buffer (standard):

Stock solution A

0.2 M sodium phosphate monobasic (NaH_2PO_4 H_2O) — 27.6 g/l

Store in refrigerator < 2 months

Stock solution B

0.2 M sodium phosphate dibasic (Na_2HPO_4) — 28.4 g/l

Store room temperature < 2 months

Buffer solution:

19 ml stock A
81 ml stock B
100 ml distilled H_2O
---
200 ml of 0.1 M phosphate buffer (pH 7.3-7.5)

Puck's saline:

0.4 g KCl
8.0 g NaCl
0.35 g $NaHCO_3$
1.0 g glucose

- Deionized distilled H_2O to 1 liter
- Filter sterilize

Speer's Modified ATV Solution (SMATV):

8.0 g NaCl
0.4 g KCl
0.6 g $NaHCO_3$
1.0 g glucose
1.0 g trypsin
0.2 g versene or EDTA (ethylene diaminetetraacetate)

- Deionized distilled H_20 to 1 liter
- Filter sterilize

Spurr's embedding medium:

10.0 g ERL - 4206 (Vinylcyclohexene dioxide)
6.0 g DER - 736 (Diglycidyl ether of polypropylene glycol)
26.0 g NSA (Nonenylsuccinic anhydride)
0.4 g DMAE (Dimethylaminoethanol)

Uranyl acetate:

Saturated solution in 50% ethanol (approximately 5% uranyl acetate) centrifuged at 450 xg for 10 min. NB: Light sensitive.