SARCOCYSTIS GIGANTEA: STUDIES ON SPOROCYST PRODUCTION, EXCYSTATION AND VIABILITY

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ABSTRACT

Recent advances in knowledge about the sporozoan genus *Sarcocystis* (Protozoa: Apicomplexa) are reviewed and studies on the production, excystation and viability of sporocysts of *Sarcocystis gigantea*, undertaken.

Investigation of a sedimentation/floatation procedure for the mass recovery of *S. gigantea* sporocysts from cat faeces showed that the greatest yields were obtained when a proportion of faeces to floatation medium of 5% and a centrifugal force of 6000 x g for at least 5 min were used. Ninety-six percent of the sporocysts recovered were obtained from the first centrifugation in aqueous NaCl solution, specific gravity 1.2. Although neither sieving nor additional washing of homogenised samples prior to floatation significantly affected sporocyst recovery both reduced the amount of debris present. A considerable reduction in the amount of debris resulted from feeding infected cats on tinned fish rather than tinned meat. The addition of CCl_4 to the NaCl solution also improved sporocyst purity but with a marked reduction in the numbers recovered.

A technique for determining the concentration of sporocysts in faeces, using a modification of the mass recovery procedure and a haemocytometer, was developed. This was shown to be more accurate and reliable than the McMaster method for performing faecal sporocyst counts. It resulted in a mean sporocyst recovery of 75.5% and was used to obtain information about patterns of sporocyst excretion and numbers of *S. gigantea* sporocysts shed by 28 experimentally infected cats.

In all cats, sporocyst excretion commenced 10 or 11 days post-infection(PI). Peak production occurred between 13

and 22 days PI, in most instances on days 17 and 18. Peak numbers (rounded) ranged from 550 to 260,000 (mean = 53,000) sporocysts per gram of faeces or from 38,000 to 6.6. million (mean = 1.7 million) sporocysts per day.

The number of days sporocysts were shed ranged from 26 to at least 60 days PI but in 26 of the 28 infections examined, more than 80% of the total sporocyst yield was produced within 30 days of infection. The total numbers of sporocysts produced by individual cats over the patent period ranged from 164,000 to 56.6 million (mean = 12.7 million). These numbers tended to increase with increasing infective dose and to be greater in those cats receiving multiple rather than equivalent single doses. Neither the sex of the cat, nor experience of one or two previous infections, had any significant effect on the numbers of sporocysts shed.

Studies on the *in vitro* excystation of *S.gigantea* sporocysts revealed that pretreatment before exposure to trypsin and bile was an essential pre-requisite. However, in contrast to *S. tenella* and *S. capracanis*, incubation in cysteine hydrochloride under CO_2 was largely unsuccessful for excysting *S. gigantea:* of the pretreatments tested only exposure to sodium hypochlorite proved effective.

Excystation from sodium hypochlorite-pretreated *S. gigantea* sporocysts took place in trypsin and bile between temperatures of 30° and 43°C and occurred rapidly at 39°C. While the presence of bile or bile salts was essential for this process, that of trypsin was not although more sporocysts excysted in its presence than in its absence. Excystation occurred in the presence of all bile types tested but not when 'Tween 80' was substituted for bile. The highest levels of excystation were recorded when cattle or sheep bile or sodium taurocholate were used and the

lowest when chicken or pig bile were employed. Neither the concentration of sheep bile above 2.5%, nor hydrogen ion concentration (pH range 5.0 to 10.0) appeared to have any marked effect on the level of excystation obtained.

The excystation process for *S. gigantea* was similar to that described for other *Sarcocystis* species and for other coccidian genera that lack sporocyst Stieda bodies. Sporozoites escaped following the collapse of the sporocyst wall and its eventual separation into four elongated pieces.

In vivo studies on excystation of *S. gigantea* indicated that this process was, as *in vitro*, diphasic involving pretreatment and treatment phases. They also tended to support *in vitro* observations that the requirements for the excystation of *S. gigantea* and *S. tenella* sporocysts were quite different. Although the results suggested that for neither species was the pretreatment stimulus likely to be provided by conditions in the rumen alone, exposure to abomasal conditions only, induced moderate levels of excystation in both when they were subsequently treated with trypsin and bile. For *S. gigantea*, 0.25 to 4 hr abomasal exposure was most effective, for *S. tenella* 24 hours. The stimuli necessary to complete the excystation process could, apparently, be provided by 1 hr placement in the duodenum for *S. gigantea* but not for *S. tenella*.

Using *in vitro* excystation as a measure of viability, it was found that at 4°C, *S. gigantea* sporocysts survived considerably better in tap water (85% excystation after 174 days) than in either 2.5% potassium dichromate (15% excystation after 174 days) or 2% sulphuric acid (0% excystation after 5 days). Although they were able to resist 48 hr suspension at room temperature in most

laboratory reagents, disinfectants and anti-coccidial drugs tested, six (sulphuric acid, ammonia, methanol, ethanol, potassium hydroxide, sodium hydroxide, Medol*) had major sporocysticidal properties.

Further investigation with three of these, showed that sporocyst excystation was reduced from 65% to less than 10% following contact with 2.5% sulphuric acid for 1 hr or with 2% ammonia or 4% Medol for 4 hours.

Sporocysts were either killed, or their ability to excyst severely impaired, by heating to 60° and 55°C for 5 and 60 min, respectively, by exposure to ultraviolet radiation at a dose of 4000 ET, or by prolonged storage in water at 24°C. Sporocysts exposed to either constant or intermittent freezing at -18°C suffered a comparatively slow decline in excystation rate with time as did those subjected to desiccation. The duration of survival of desiccated sporocysts was inversely related to relative humidity and after 245 days at 33% RH and temperatures of 15 or 24°C, 60% of such sporocysts excysted.

Studies on the survival of *S. gigantea* sporocysts in faeces outdoors showed that, viability declined most rapidly over the summer months and suggested that they were unlikely to remain infective for more than one year.

Possible associations between the reported findings and both the epidemiology of *S. gigantea* infection and some of the previous unsuccessful or equivocal attempts to experimentally infect sheep with this species, are discussed.

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*Medol: 16% synergistic mixture of five chlorinated phenols.

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CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Sarcocysts and the banana-shaped zoites they contain, first discovered in the muscles of mice by Miescher (1843) and later assigned to the genus *Sarcocystis* by Lankester (1882), are common in many vertebrates. Since their original discovery, such sarcocysts have been recorded in over 190 species of reptiles, birds and mammals (including man) from many geographic areas (Kalyakin and Zasukhin, 1975). Despite their frequent occurrence (Munday *et al.*, 1978, 1979) and their world-wide distribution, both the nature of these organisms and their means of transmission remained a mystery for many years, until a rather similar cyst-forming parasite, *Toxoplasma gondii*, was found to be part of a coccidian life cycle and to be transmitted as a small isosporan oocyst shed in the faeces of cats (Overdulve, 1970; Hutchinson *et al.*, 1970; Sheffield and Melton, 1970; Frenkel *et al.*, 1970; Weiland and Kuhn, 1970). Subsequent investigations, involving tissue culture and transmission experiments (Fayer, 1970, 1972; Rommel *et al.*, 1972; Heydorn and Rommel, 1972a, b; Rommel and Heydorn, 1972), revealed that *Sarcocystis* also shows a close affinity with the coccidian genus *Isospora* and has a two-host cycle. This unravelling of the basic life cycle and the new-found ability to experimentally transmit infection, stimulated considerable further research on *Sarcocystis*. Over the last decade, this has led to a revision of many of the previously held notions about these parasites. Much of the earlier work has been reviewed by Markus *et al.* (1974), Tadros and Laarman (1976), Dubey (1976), Markus (1978) and Collins (1980b).

In this Chapter, recent advances in the knowledge of the genus *Sarcocystis* are reviewed and information specifically concerning *Sarcocystis* infections in sheep is examined. Further literature relating directly to the experimental work described in this thesis is reviewed in the appropriate Chapters.

1.2 THE GENUS *SARCOCYSTIS*

1.2.1 Life cycle

Traditionally, the coccidia were considered to be highly host-specific and to parasitise only the intestinal tract. Their common features were thoughtto include: life cycle in one host (homoxenous), asexual reproduction (merogony or schizogony) within host cells, followed by sexual differentiation (gametogony) into male microgametes and female macrogametes that gives rise to oocysts that are shed in the faeces. By a process of sporogony or sporulation a variable number of sporocysts containing one or more sporozoites are formed within the oocyst which typically serves as the only source of infection for all potential hosts (Kheysin, 1972; Levine, 1973).

The life cycle of *Sarcocystis* shows a considerable departure from this 'conventional' pattern in that it follows an obligatory two-host (heteroxenous) cycle with sexual reproduction taking place in a carnivorous definitive host (predator or scavenger) and asexual reproduction taking place in a herbivorous or omnivorous intermediate prey-host (Fig 1.1).

(a) Infection and development in definitive hosts

A number of animals including snakes, owls, kestrels, ferrets, weasels, stoats, fishers, skunks, badgers, racoons, opossums, cats, bobcats, mountain lions, dogs, coyotes,

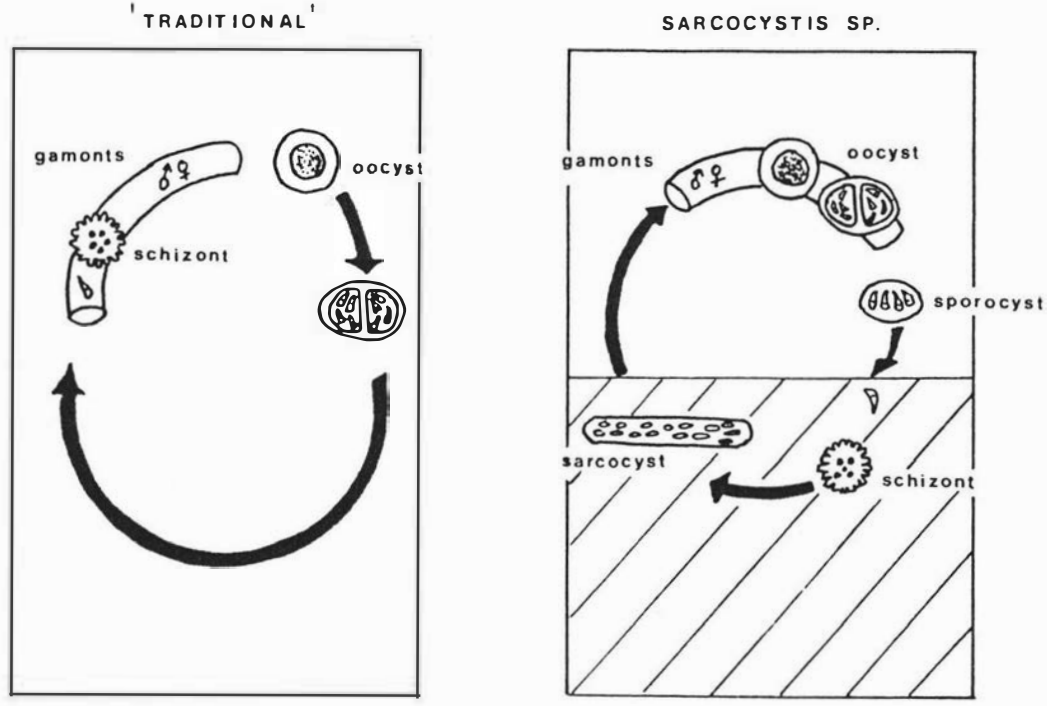

Fig. 1.1 Schematic representation of the 'traditional' or conventional (homoxenous) coccidian life cycle and that of the two-host (heteroxenous) coccidian *Sarcocystis* sp. For *Sarcocystis* sp. clear area represents cycle in definitive host, cross-hatched area cycle in intermediate host

jackals, foxes, wolves, monkeys, baboons and man act as definitive hosts for species of *Sarcocystis* (Levine and Tadros, 1980; Cawthorn *et al.*, 1981, 1983; Dubey, 1982e). Development within these hosts is restricted to the small intestine and is limited to gametogony and sporogony: there is little evidence of invasion of extra-intestinal tissues (Rommel *et al.*, 1974; Fayer, 1974; Munday *et al.*, 1975; Ruiz and Frenkel, 1976; Zaman and Colley 1975; Dubey *et al.*, 1982a) although sporocysts of *S. cruzi* have been found in the mesenteric lymph nodes of dogs (Shimura *et al.*, 1981.

Infection is acquired by ingestion of mature sarcocysts present in infected intermediate host muscle. Following ingestion, the banana-shaped zoites (bradyzoites) (Fig. 1.2) within the sarcocyst are released and, by means of successive thrusts with their anterior ends or after a series of spinning rotations (Dubremetz *et al.*, 1975), penetrate the epithelium of the small intestine. Entry into the epithelium is usually through goblet cells (Ruiz and Frenkel, 1976; Dubey, 1982a; Dubey *et al.*, 1982a). Here, or frequently subepithelially in the lamina propria, within a parasitophorous vacuole, the bradyzoites round up and begin to differentiate into micro- and macrogamonts (Mehlhorn and Scholtyseck, 1974; Markus *et al.*, 1974; Munday *et al.*, 1975; Dubey, 1982a; Dubey *et al.*, 1982a). In the male gamont there is firstly a phase of mitotic division followed by formation of microgametes (Scholtyseck *et al.*, 1971, 1972). The slender microgametes, each enclosed by a single unit membrane, consist of an elongated nucleus, a tubular mitochondrion, several microtubules and either two (Vetterling *et al.*, 1973) or three (Mehlhorn and Heydorn, 1979) flagella. There is no nuclear division in macrogamonts and each macrogametocyte, therefore, contains a single spherical macrogamete.

The microgametes are liberated from the microgametocytes

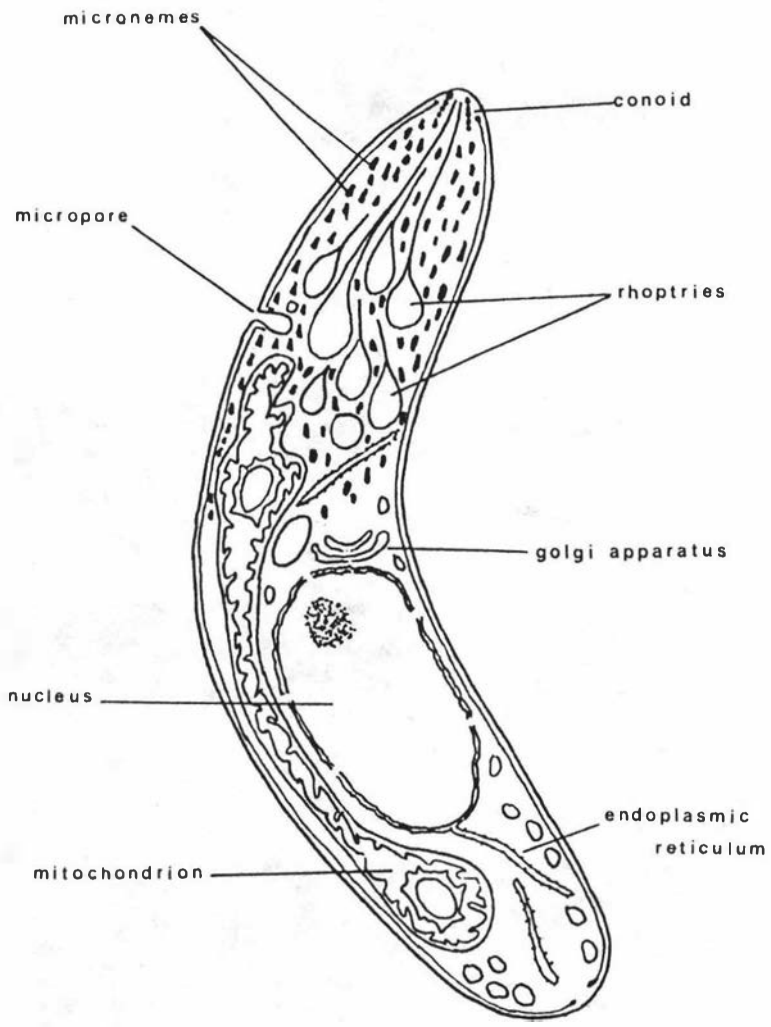

Fig. 1.2 Bradyzoite of *Sarcocystis* sp.

and fertilisation proceeds by fusion of the microgamete plasmalemma with that of the macrogamete and the subsequent entry of the microgamete nucleoplasm into the macrogamete cytoplasm (Sheffield and Fayer, 1980). The fertilised macrogamete (zygote) begins to form an oocyst wall and commences sporulation.

In all *Sarcocystis* spp. so far studied, sporulation takes place within the parasitised cell of the definitive host. This contrasts with the situation in most other species of coccidia in which oocysts are usually passed undeveloped into the external environment where sporulation is completed (Kheysin, 1972; Levine, 1973).

In *Sarcocystis* spp. the first sporulation division results in the formation of two densely basophilic nuclei at opposite poles of the cytoplasm. These two nuclei then divide again, accompanied by fission of the cytoplasmic mass, to form two sporocyst progenitors. The third nuclear and cytoplasmic division within each sporocyst leads to the formation of four sporozoites and a granular residuum (Munday *et al.*, 1975; Mehlhorn and Heydorn, 1978; Colwell and Mahrt, 1983). The completely sporulated oocyst is, therefore, of the isosporan type containing two sporocysts each of which contains four sporozoites (Fig. 1.3). Although the oocyst wall of *Sarcocystis* spp. consists of five layers (Vetterling *et al.*, 1973), it is very delicate and easily ruptured. Consequently, intact oocysts are rarely found in definitive host faeces, free sporocysts being the stage usually excreted.

There are some variations in the prepatent periods (i.e. the time between infection and the first shedding of sporocysts) of *Sarcocystis* infections in definitive hosts. In homoiothermic hosts these are generally of 5 to 25 days duration (Mehlhorn and Heydorn, 1978) but in poikilothermic hosts they may extend to 4 or 5 months with low

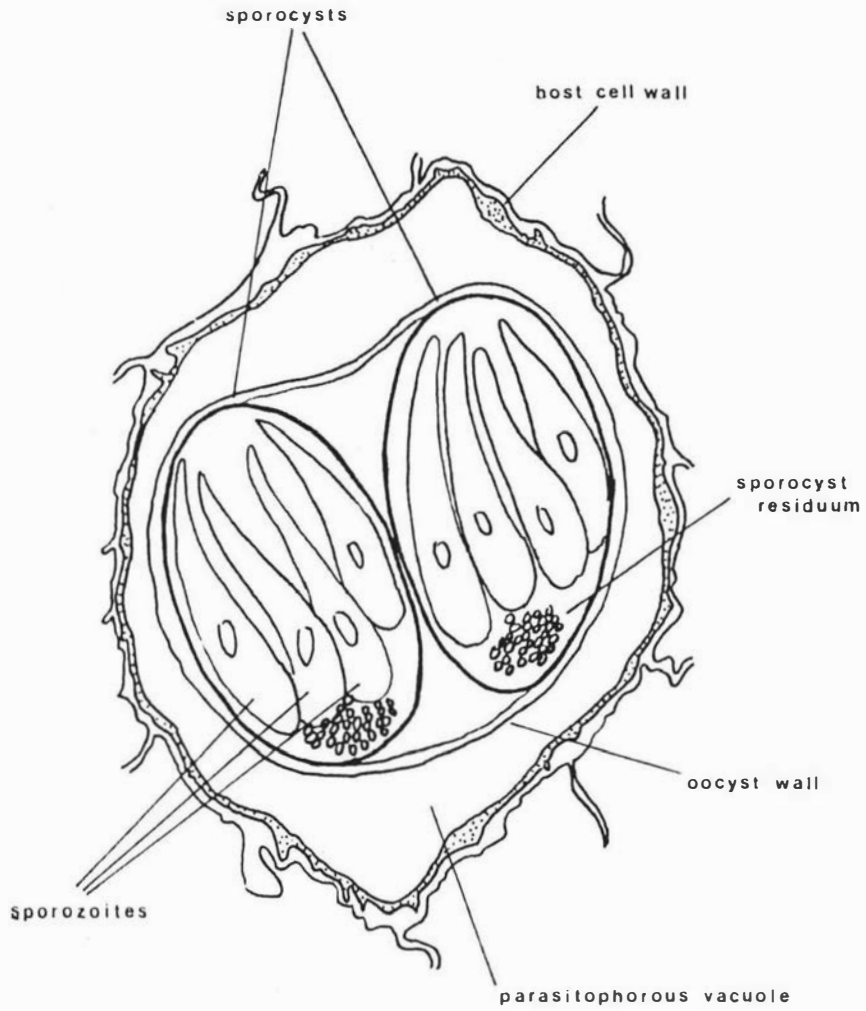

Fig. 1.3 Diagrammatic representation of sporulated oocyst of *Sarcocystis* sp. in definitive host intestinal cell

ambient temperatures or at times of host hibernation (Bledsoe, 1979).

(b) Infection and development in intermediate hosts

The development of various species of *Sarcocystis* has been investigated in some detail in a number of intermediate hosts including cattle (Fayer and Johnson, 1973, 1974; Fayer, 1977a; Pacheco *et al.*, 1978; Dubey *et al.*, 1980; Dubey, 1981a, 1982a, b), sheep (Munday *et al.*, 1975; Mehlhorn *et al.*, 1975a; Heydorn and Gestrich, 1976; Speer and Dubey, 1981; Dubey *et al.*, 1982a), pigs (Heydorn, 1977a, Heydorn and Ipczysnki, 1978; Heydorn *et al.*, 1981b; Barrows *et al.*, 1982a, b), goats (Fischer, 1979; Collins and Charleston, 1979; Aryeetey *et al.*, 1980; Heydorn and Haralambidis, 1982; Heydorn and Unterholzner, 1983), horses (Fayer and Dubey, 1982) and rodents (Zaman and Colley, 1975; Ruiz and Frenkel, 1976; Cerna *et al.*, 1978; Bledsoe, 1980b; Brehm and Frank, 1980; Beaver and Maleckar, 1981; Cawthorn *et al.*, 1983). Within such hosts, development comprises essentially two different phases. The first involves a series of transient asexual proliferative cycles, the second, a more persistent multiplicative phase limited mainly to muscle fibres in which the bradyzoite-filled sarcocysts are ultimately formed. Within this basic pattern there are species differences in the location, timing and number of the initial multiplicative cycles. In the most thoroughly studied species, *S.cruzi* of cattle, there are at least three such cycles, the first two occurring in almost all organs of the body, the third taking place in the blood stream (Fayer, 1979; Dubey, 1982a, d). For most other species of *Sarcocystis* no more than two such generations have been observed with one often being restricted to a particular organ such as the liver (Ruiz and Frenkel, 1976; Cerna and Louckova, 1976; Heydorn, 1977a; Heydorn and Ipczynski, 1978; Heydorn *et al.*, 1981b), lungs (Zaman and Colley, 1975; Beaver and Maleckar, 1981) or mesentery and gut (Dubey,

1982b, 1983c), depending on the species concerned.

Infection is initiated by the ingestion of sporocysts shed in definitive host faeces. Such sporocysts are not infective for the definitive host (Fayer, 1974; Rommel *et al.*, 1974; Fischle, 1973; Bledsoe, 1980a). However, some omnivores, such as man and other primates, may act as either definitive (Heydorn *et al.*, 1975, 1976; Heydorn, 1977a) or intermediate hosts (Mehlhorn *et al.*, 1976, 1977; Jeffrey, 1974; Beaver *et al.*, 1979) for different species of *Sarcocystis*.

Following their ingestion by the appropriate intermediate host, the sporozoites excyst from the sporocyst in response to certain gut stimuli; these are examined in Chapters 4 and 5. The route and manner of transport of the sporozoites from the gut lumen to the site of the first proliferative generation (merogony or schizogony) is unknown although there is some evidence to suggest that for the bovine species *S. cruzi* and *S. hirsuta*, they may be transported via the portal blood in leucocytes (Dubey, 1981a, 1982a, b). In *S. cruzi* infections, the first generation meronts are found in endothelial cells of small and medium sized arteries in many organs 7 to 26 days post-infection. Entry of the sporozoites into the host endothelial cells has not been observed and may, therefore, take place by either active penetration or by phagocytosis (Dubey *et al.*, 1980). Within the cytoplasm of these host cells, the sporozoites round up to form an ovoid uninucleate meront which begins to multiply by endopolygeny, a process in which many daugher cells are formed within one mother cell. In *S. cruzi* infections, mature first generation meronts may contain between 100 to 350 merozoites (Dubey *et al.*, 1980). Second generation *S. cruzi* meronts are found in endothelial cells of capillaries 19 to 46 days after infection (Dubey, 1982a). Multiplication in second generation meronts is again by endopolygeny with each meront containing to 4 to 37 merozoites (Dubey, 1982a).

The third transient proliferative generation of *S. cruzi* takes place in the blood stream. Here, second generation merozoites, either free in the blood, or more frequently, within lymphocytes and monocytes, multiply by a process resembling endodyogeny (a type of asexual reproduction in which two daughter cells develop within a single mother cell) 24 to 46 days after infection (Fayer, 1979; Dubey, 1982a).

From about 40 days after infection, third generation merozoites of *S. cruzi* begin to enter muscle fibres and start the final stage of asexual reproduction leading to the development of the sarcocyst proper. Such sarcocysts initially consist of a single merozoite which rounds up to form a metrocyte. The metrocyte, which is surrounded by a unit membrane, lies within a parasitophorous vacuole. This unit membrane becomes strengthened by an underlying layer of osmiophilic material to form the primary cyst wall. As the cyst wall develops, internal vesicle-like invaginations, and in some species, external protrusions which develop into villus-like structures, are formed (Mehlhorn and Heydorn, 1978). The whole development of the cysts of the sarcosporidia takes place within the interior of muscle fibres. A typically fully developed sarcocyst, therefore, is bounded on the outside by the remnants of the original muscle fibre but in one species of *Sarcocystis*, *S. gigantea*, the parasitised fibre is surrounded by an additional thick layer of host connective tissue considered to be a secondary cyst wall (Mehlhorn *et al.*, 1976).

Growth is initially by multiplication of the metrocytes by means of endodyogeny. At some stage these daughter metrocytes give rise to bradyzoites by a similar process. The growth of the sarcocyst and the production of the bradyzoites is assumed to be continuous with metrocytes being present at the periphery of even the largest cysts. Only when the sarcocyst contains bradyzoites is it mature

and infective for the definitive host: meronts and metrocytes are not infective (Ruiz and Frenkel, 1976; Leek *et al.*, 1977; Heydorn and Gestrich, 1976). The time taken for the sarcocysts to attain infectivity has been investigated in only a few species of *Sarcocystis* but is commonly in the vicinity of 65 to 70 days after infection. (Ruiz and Frenkel, 1976; Sheffield *et al.*, 1977; Fischer, 1979; Dubey, 1982a, b; Dubey *et al.*, 1982a; Fayer and Dubey 1982). However, it may take as little as 58 days for *S. miescheriana* of pigs (Erber and Geisel, 1979) or, apparently, as much as 8 to 14 months for *S. gigantea* of sheep (Munday, 1978).

The ingestion of sporocysts was, until relatively recently, considered to be the only means by which intermediate hosts could become infected with *Sarcocystis* spp. Although there is some circumstantial evidence to suggest that these infections may be congenitally acquired (Cunningham, 1973; Munday and Black, 1976; Hong *et al.*, 1982; Dubey and Bergeron, 1982; Vickers and Brooks, 1983; Jolly *et al.*, 1983), attempts to demonstrate transplacental transmission experimentally have generally been unsuccessful (Fayer *et al.*, 1976; Stalheim *et al.*, 1976; Barnett *et al.*, 1977; Leek and Fayer, 1978a). The recognition, however, that in at least some species of *Sarcocystis*, a parasitaemia of merozoites may occur during the initial multiplicative phase of infection (Zaman and Colley, 1975; Gestrich *et al.*, 1975a; Heydorn and Gestrich, 1976; Heydorn, 1977a; Cerna, 1977) has led to some attempts to transmit infection between intermediate hosts by means of either blood transfusion or, because it often contains blood, colostrum. An attempt to transmit *S. cruzi* from cows to calves via colostrum failed (Fayer *et al.*, 1982b) but successful transmission by blood transfusion either intravenously of *S. cruzi* of cattle, *S. tenella* of sheep, *S. suihominis* of pigs (Fayer and Leek, 1979) and *S. capracanis* of goats (Dubey *et al.*, 1981) or intraperitoneally of *S. dispersa* of mice (Cerna, 1983), has been achieved. In addition, transmission between rodent intermediate hosts

has been obtained using inocula of merozoites, isolated from liver meronts, administered intraperitoneally with *S. sebeki* (Tadros and Laarman, 1979) and both intraperitoneally and orally with *S. idahoensis* (Bledsoe, 1980a). However, similar attempts to transmit bradyzoites of *S. idahoensis* between intermediate hosts by either of these two routes failed (Bledsoe, 1980a) as did attempts to transmit bradyzoites of *S. putorii* orally (Tadros, 1976).

1.2.2 Transport hosts, dissemination of infection and epidemiology

The role of transport hosts (which may be distinguished from intermediate hosts by the absence of any multiplication within them) in the dissemination of *Sarcocystis* infection is largely unknown. In view of the recent findings relating to transmission between intermediate hosts outlined above, it is possible that both haematophagus arthropod vectors and, at least amongst rodents, cannibalism, could play a part in the dissemination of infection, but the significance of these routes under natural conditions remains speculative. The possible role of vectors in disseminating sporocysts, however, is less uncertain. Thus both flies (Markus, 1980) and cockroaches (Smith and Frenkel, 1978) are capable of transporting viable sporocysts of *Sarcocystis*. In addition, earthworms may act as carriers of *Toxoplasma* oocysts (Frenkel *et al.*, 1975) and there seems to be no reason to suppose that they would not be equally capable of distributing sporocysts of *Sarcocystis* spp. Furthermore, the ability of a proportion of viable sporocysts to pass unexcysted through the gut of their intermediate hosts (Box, 1983) may also aid in their dispersal.

In some definitive and domesticated intermediate hosts in particular, the prevalence of *Sarcocystis* infection has frequently been found to be high (Tables 1.1, 1.2). Because of the obligatory two-host nature of the sarcosporidian life

TABLE 1.1 REPORTED PREVALENCES OF *SARCOCYSTIS* SPP. IN DEFINITIVE HOSTS

Host	No examined	% infected	Locality	Author
Cat	516	0.2	Kansas	Dubey, 1973
"	1000	0.2	Ohio	Christie *et al.*, 1976
"	86	1.8	Tasmania	Gregory & Munday, 1976
"	694	4.3	Germany	Walter, 1979
"	508	16.9	New Zealand	McKenna & Charleston, 1980a
"	308	4.6	Germany	Rommel *et al.*, 1982
"	71	1.4	Sydney	Collins *et al.*, 1983
Dog	500	1.8	Ohio	Streitel & Dubey, 1976
"	156	34.0	Wales	Farmer *et al.*, 1978
"	66	39.3	Wales	Leguia & Herbert, 1979
"	481	58.8	New Zealand	McKenna & Charleston, 1980b
"	69	75.0	Wales	Balmer *et al.*, 1982
"	690	26.3	Victoria	Blake & Overend, 1982
"	110	20.9	Sydney	Collins, *et al.*, 1983
Fox	146	9.6	Bulgaria	Golemansky, 1975
"	41	17.0	Wales	Farmer *et al.*, 1978
"	20	25.0	Wales	Leguia & Herbert, 1979
"	198	10.1	Montana	Dubey, 1982e
Coyote	150	14.0	Utah	Fayer & Johnson, 1975b
"	169	52.7	Montana	Dubey *et al.*, 1978
Bobcat	61	3.2	Montana	Dubey, 1982e
Mountain lion	12	16.6	Montana	Dubey, 1982e
Fisher	6	16.6	Montana	Dubey, 1982e

TABLE 1.2 REPORTED PREVALENCES OF *SARCOCYSTIS* SPP. IN DOMESTICATED INTERMEDIATE HOSTS

Host	No. examined	% infected	Locality	Author
Sheep	550	40.7	Hanover	Fidiarakis, 1973
"	6120	99.2	Iran	Afshar *et al.*, 1974
"	404	>90.0	Tasmania	Munday, 1975
"	1095	57.3	Michigan	Seneviratna *et al.*, 1975a
"	719	73.5	Jordan	Sherkov *et al.*, 1977
"	500	85.4	Bavaria	Boch *et al.*, 1979
"	300	85.8	Wales	Leguia & Herbert, 1979
"	17	65.0	Argentina	Bertero *et al.*, 1980
"	467	69.0	Spain	Diez-Banos, 1980
Cattle	631	54.8	Madras	Shukala & Victor, 1974
"	238	90.0	Belorussia	Bogush & Pyshko, 1974
"	458	86.9	Bulgaria	Meshkov, 1975
"	287	>90.0	Tasmania	Munday, 1975
"	155	40.0	Michigan	Seneviratna *et al.*, 1975a
"	118	93.3	Sri Lanka	Seneviratna *et al.*, 1975b
"	630	92.8	Venezeula	GoDoy *et al.*, 1977
"	1020	98.7	Germany	Laupheimer, 1978
"	1000	77.0	Germany	Drost and Brackman, 1978
"	88	76.1	Fiji	Raju & Munro, 1978
"	511	87.4	Austria	Hinaidy *et al.*, 1979
"	79	81.0	Norway	Bratberg & Landsverk, 1980
"	30	100.0	Argentina	Bertero *et al.*, 1980
Goat	66	7.6	Sri Lanka	Seneviratna *et al.*, 1975b
"	60*	28.0	New Zealand	Collins & Charleston, 1979
Pig	4285	11.3	Denmark	Greve, 1974
"	2823	67.0	Belorussia	Bogush & Pyshko, 1974
"	451*	68.0	Germany	Drost, 1974
"	169	4.3	Tasmania	Munday, 1975
"	103	6.8	Michigan	Seneviratna *et al.*, 1975a
"	1175	18.7	Sth Germany	Boch *et al.*, 1978
"	72644	0.2	West Germany	Heydorn *et al.*, 1978
"	1000	41.9	East Germany	Heydorn *et al.*, 1978
"	236	3.4	Ohio	Dubey, 1979
"	50*	10.0	New Zealand	Collins & Charleston, 1979
"	26	58.0	Argentina	Bertero *et al.*, 1980
"	168	16.6	Georgia	Prestwood *et al.*, 1980
"	192*	32.0	Sth East USA	Barrows *et al.*, 1981
Horse	90	23.3	West Germany	Rommel & Geisel, 1975
"	200	15.5	West Germany	Erber & Geisel, 1981
"	275	32.4	Austria/ Russia	Hinaidy & Loupal, 1982

* = Feral animals

cycle, the prevalence of infection in either host population must, to some extent, be interdependent. However, a single infected intermediate host may provide a source of infection for a number of definitive hosts while each infected definitive host may provide sufficient environmental contamination to infect many intermediate hosts. It needs to be borne in mind also that, because of subsequent asexual multiplication, the ingestion of relatively few sporocysts may result in large numbers of sarcocysts in intermediate host muscles. Using the figures provided by Dubey (1982a) and Dubey *et al.* (1980) (see page 9), for example, it can be calculated that ingestion of a single *S. cruzi* sporocyst containing 4 sporozoites, could result in the development of between 3,200 and 103,600 sarcocysts.

Apart from the mere presence of the appropriate hosts, a number of other factors are also likely to influence the prevalence of infection. These include the numbers of sporocysts shed by the definitive hosts, the densities and age structures of the two host populations concerned, their frequencies of contact, their suceptibility to infection and the influence of climate and season on host biology and sporocyst viability.

Information concerning the numbers of sporocysts shed by definitive hosts is very limited (see Chapter 3). Faecal sporocyst counts in naturally infected cats and dogs have been found to be generally low (McKenna and Charleston, 1983), but in experimental infections total sporocyst outputs, over 34 to 60 day intervals, of up to 13 million in foxes (Ashford, 1977) and 90 million in dogs (Ford, 1974), have been recorded.

A number of studies have shown that age, seasonal, climatic or environmental factors may be associated with variations in the prevalence of infection in intermediate hosts (Fidiarakis, 1973; Bogush and Pyshko, 1974; Drost, 1974; Greve, 1974;

Munday, 1975; Seneviratna, 1975a, b ; Sherkov *et al.*, 1977; Drost and Brackman, 1978; Diez-Banos, 1980; Barrows *et al.*, 1981; Boch and Erber, 1981). Although some of these variations may be due to differences in management practices (Meshkov, 1975; Dubey, 1979), some may also be related to host factors or to the biology of the parasite species itself. In older hosts, for instance, the prevalence of infection is likely to be higher than in younger hosts simply because they have had greater opportunity for contact with sporocysts. In other cases, an apparently similar effect may arise through the slow growth of the parasite, as evidently occurs in some macroscopically visible sarcocysts (Munday, 1975), while seasonal variations may result from the destruction of cysts by host reaction (Seneviratna, 1975a; Collins, 1980a).

The influence of such factors on the prevalence of infection in definitive hosts is less well documented with only one study (McKenna and Charleston, 1983) involving naturally infected cats and dogs, being reported. The results of this investigation suggest that none of the factors examined (sex, age, season, environment) apart from possibly host age in cats, had any apparent influence on the prevalence of sporocyst shedding.

1.2.3 Resistance and survival of infective stages

An important consideration with regard to both the epidemiology and successful transmission of *Sarcocystis* spp. must be the ability of their infective stages to survive until ingested by the appropriate hosts. Although such abilities will most obviously be required by those stages shed directly into the external environment (i.e. sporocysts), they may also be of some relevance to other life cycle stages. Thus the ability of sarcocysts in muscle to maintain their viability for some time after the death of the intermediate host is likely to increase the possibility of perpetuating infection via scavenging carnivores. Similarly, the

capacity to withstand procedures involved in meat processing would also be advantageous for the spread of infection both by cats and dogs and by man.

A number of studies on the survival of sarcocysts in meat have indicated that some species of *Sarcocystis* may indeed possess such abilities. For instance, *S. gigantea* cysts isolated from sheep meat, have been shown to retain their infectivity for between 13 and 20 days when stored at temperatures of 10 and 4°C respectively (Collins and Charleston, 1980) while cysts of *S. hirsuta* in beef may remain viable for 18 to 20 days at 2 to 4°C (Gestrich and Heydorn, 1974; Golubkov, 1978). Similarly, raw beef purchased from retail stores has been found to contain viable sarcocysts of *S. cruzi* and *S. hominis* (Fayer, 1975; Leek and Fayer, 1978b). Storage at 20°C, on the other hand, destroyed sarcocyst viability in beef within 4 or 5 days (Golubkov, 1978) while the processing of such meat into salted beef, bologna, or frankfurters, was also deleterious to *Sarcocystis* infectivity (Golubkov, 1978; Leek and Fayer, 1978b). While cooking to temperatures of between 60-75°C has been shown to destroy *Sarcocystis* infectivity in both beef and sheep meat (Gestrich and Heydorn, 1974; Golubkov, 1978; Fayer, 1975; Collins and Charleston, 1980), freezing appears to be much more variable in its effect. Exposure to -20°C for 3 days or -6 to -8°C for 24 hr renders beef non-infective for cats (Gestrich and Heydorn, 1974; Golubkov, 1978) whereas *S. gigantea* cysts have been found to remain infective for the same definitive host even after storage for 60 days at -14°C (Collins and Charleston, 1980). The reasons for such anomalies are not clear but may possibly relate to the rate of freezing, or species differences in response. There is a need for a more critical evaluation of these effects.

Similar investigations, not only of their resistance to physical factors but to some chemical agents as well, have also been undertaken for sporocysts of several *Sarcocystis*

spp. (see Chapter 6). As one might expect of a life cycle stage required to spend a large part of its existence in the external environment, such sporocysts have generally been found to exhibit considerable resistance to both (Leek and Fayer, 1979; Bergler *et al.*, 1980; Heydorn, 1980; Barutzki *et al.*, 1981). Survival under field conditions, however, has been little investigated.

1.2.4 Taxonomy, nomenclature and problems of identification

Prior to the elucidation of their life cycles, *Sarcocystis* spp. were known only by the cysts they produced in the musculature of their intermediate hosts. With the discovery of their coccidian nature and the traditional practice of naming coccidian species strictly on the basis of oocyst and sporocyst morphology, all these protozoans could have been placed in the genus *Isospora*. However, since any such alteration of generic name would have led to considerable confusion, as would adoption of different names for different life cycle stages (e.g. *Isospora* for sporocysts shed by definitive hosts and *Sarcocystis* for cysts in intermediate hosts), it has been generally accepted that the generic name *Sarcocystis* should be retained for both intermediate and definitive host forms (Levine, 1977; Dubey, 1977; Frenkel, 1977).

Originally it was though that each intermediate host species was parasitised by only one sarcosporidian and that the differing cysts seen in such hosts were developing stages of only one cyst type. Therefore, cysts in a particular host species were given a common name e.g. sarcocysts in sheep were referred to as *S. tenella*, those in cattle as *S. fusiformis* and those in pigs as *S. miescheriana*. However, following the discovery of their life cycles and consequent transmission experiments, it was soon found that several *Sarcocystis* spp. form cysts in the same intermediate host. Thus at least three different

species of *Sarcocystis* are now known to each infect sheep, cattle and pigs (Levine and Tadros, 1980; Collins *et al.*, 1979; Moore, 1980). These discoveries and the difficulties in relating such new found sarcocysts to the 'species' originally described in these hosts, led Heydorn *et al.* (1975) to suggest that many nomenclatural problems could be solved by ignoring all earlier names and starting afresh. They proposed that the specific names of the sarcosporidia be made up of a sequential combination of the generic names of their intermediate and definitive hosts. Thus *S. bovicanis* was suggested for the bovine species of *Sarcocystis* (= *S. cruzi*, Levine and Tadros, 1980) completing its life cycle in dogs and *S. bovifelis* (=*S. hirsuta*, Levine and Tadros, 1980) for the species cycling between cattle and cats.

Logical and attractive as this proposal might appear, it is unacceptable on two counts. Firstly, the host specificity of several *Sarcocystis* spp. is less precise than originally supposed. This lack of host specificity for the definitive host has been long known. *Sarcocystis cruzi*, for example, may be transmitted by wolves, foxes, coyotes and racoons as well as dogs (Heydorn *et al.*, 1975; Fayer *et al.*, 1976), prompting Heydorn *et al.* (1975) to suggest that under their system the generic name of the most epidemiologically important definitive host (in this case, the dog) be used. What was not known at that time but is becoming increasingly apparent now, is that some species of *Sarcocystis* can also infect a number of different intermediate hosts. Thus *S. debonei* can utilise seven genera (three orders) of birds as intermediate hosts (Box and Duszynski, 1978; Box and Smith, 1982). Similarly a species of *Sarcocystis* from Cervidae can also develop in two genera of Bovidae (Erber, 1980; Crum *et al.*, 1981) and a *Sarcocystis* spp. from bison may also infect cattle (Fayer *et al.*, 1982a). Furthermore, different species of *Sarcocystis* are now known to utilise both the same intermediate and definitive hosts (Collins

et al., 1979; Heydorn and Unterholzner, 1983). Thus two dog-derived *Sarcocystis* spp. (*S. capracanis*, *S. hircicanis*) are found in goats and two cat-transmitted species (*S. gigantea*, *S. medusiformis*) occur in sheep.

Secondly, under the rules of the International Commission of Zoological Nomenclature, the proposal of Heydorn *et al.* (1975) to dispense with all the 'old' specific names of *Sarcocystis*, appears to be unacceptable (Melville, 1980). This conclusion has not been universally accepted (Frenkel *et al.*, 1979, 1980). Consequently the nomenclature of the sarcosporidia is confused with different names frequently being used for the same species by different authors. Recently, attempts were made to clarify this situation by Levine and Tadros (1980) and in this thesis the names used to identify the various *Sarcocystis* spp. are as suggested by them.

In addition to nomenclatural problems, some difficulties may also be encountered in trying to identify precisely either the cystic or sporocystic stages of the various *Sarcocystis* spp., particularly in naturally acquired infections. In domesticated intermediate hosts the numbers of *Sarcocystis* spp. harboured are usually few and these can often be distinguished by their gross or microscopic appearance and by their site or predilection. Sarcocysts of *S. tenella* of sheep, for instance, occur predominantly in the heart (Bratberg *et al.*, 1982) whereas those of *S. gigantea* are most often located in the oesophagus (Levin and Ivens, 1981). Such sarcocysts may be microscopic or macroscopic in size when fully mature (Munday and Rickard, 1974; Tadros and Laarman, 1976; Collins *et al.*, 1976) and may be able to be distinguished electrophoretically (Atkinson and Collins 1981) or by staining (Moore, 1980). In some species the cyst wall may appear to be thick or thin under light microscopy (Gestrich *et al.*, 1975a; Mehlhorn *et al.*, 1975a, b; Dubey 1983a) and possess protrusions of

characteristic length and thickness (Erber, 1977; Boch *et al.*, 1978, 1979; Hinaidy *et al.*, 1979). However, although ultrastructural studies (Mehlhorn *et al.*, 1975a, b; 1976; Gestrich *et al.*, 1975b; Bergman and Kinder 1975; Zaman and Colley 1975; Sheffield *et al.*, 1977; Collins *et al.*, 1979) have shown that several species of *Sarcocystis* have distinctive cyst wall structures, some do not (Mehlhorn *et al.*, 1976). There is also a serious lack of documentation of changes in appearance of cyst walls with age in known single infections. Accordingly, cyst wall morphology cannot be used for absolute species differentiation. Its value as a criterion for species identification is a relative one and may be used mainly to differentiate between the various cysts in the same intermediate host (Mehlhorn and Heydorn, 1978; Tadros and Laarman, 1978).

Somewhat similar problems may also be encountered in trying to identify the sporocysts of the various *Sarcocystis* spp. since all are structurally similar. All contain four sporozoites and a granular residuum and all lack Stieda bodies. Although there are some differences in size (Levine, 1977; Levine and Ivens, 1981) which may enable recognition in pure experimental infections, many of these differences are so slight as to make the identification of sporocysts in naturally acquired, and hence frequently mixed infections, extremely difficult if not impossible (McKenna and Charleston, 1980a, b).

1.2.5 Pathogenicity

(a) Pathogenicity for intermediate hosts

For many years *Sarcocystis* infections in intermediate hosts were considered to be of little importance. The main cause for concern lay in the disfigurement produced by macroscopically visible sarcocysts in meat intended for human consumption. More recently, some heavy muscle infections with sarcocysts

have been associated with locomotor impairment and lameness in mice (Ruiz and Frenkel, 1976), fowls (Munday *et al.*, 1977) and cattle (Landsverk, 1979), muscular soreness and weakness in man (Beaver *et al.*, 1979), ocular disturbances in cattle (Juyal *et al.*, 1982), myocarditis in cattle and sheep (Landsverk, *et al.*, 1978; Bratberg and Landsverk, 1980; Munday, 1981) and myositis in fowls, ducks, sheep and goats (Munday *et al.*, 1977; Wobeser and Cawthorn, 1982; Collins, 1980a; Landsverk *et al.*, 1978). With the ability to experimentally infect animals, greater attention has been focused on the more acute clinical effects induced by transient proliferative generations preceding muscle invasion which, for some species of *Sarcocystis*, have been shown to be highly pathogenic. Thus calves and bison experimentally infected with large numbers of *S. cruzi* sporocysts have been found to suffer anaemia, anorexia, pyrexia, cachexia and weight loss frequently resulting in death within 26 to 33 days (Fayer and Johnson, 1973, 1974, 1975a; Johnson *et al.*, 1975; Gestrich *et al.*, 1975a; Jungman *et al.*, 1977; Fayer and Lynch, 1979, Fayer and Prasse, 1981; Dubey, 1982c, d; Dubey *et al.*, 1982b). Similar clinical symptoms and death have also been recorded in lambs infected with *S. tenella* sporocysts (Gestrich *et al.*, 1974, 1975a; Munday *et al.*, 1975; Heydorn and Gestrich, 1976; Leek *et al.*, 1977; Dubey *et al.*, 1978; Erber,1982; Erber and Burgkart, 1981), goats infected with *S. capracanis* sporocysts (Fischer, 1979; Collins and Charleston, 1979; Dubey *et al.*, 1981; Heydorn and Haralambidis, 1982), pigs infected with either *S. miescheriana* (Erber *et al.*, 1978; Erber and Geisel, 1979; Boch *et al.*, 1980a; Zielasko *et al.*, 1981; Barrows, 1981; Barrows *et al.*, 1982b) or *S. suihominis* sporocysts (Heydorn, 1977a) and mule deer infected with *S. hemionilatrantis* sporocysts (Koller *et al.*, 1977; Hudkins and Kistner, 1977). In addition, experimental infections of some of the above species of *Sarcocystis* have been found to cause abortion in cattle, sheep, goats and pigs (Fayer *et al.*,

1976; Stalheim *et al.*, 1976; Leek and Fayer, 1978a; Munday, 1981; Fischer, 1979; Dubey, 1981b; Erber *et al.*, 1978; Barrows, 1981), to decrease milk yield in lactating cows (Fayer *et al.*, 1983) and to modify the susceptibility of goats to other infectious agents (Dubey, 1983e).

The precise causes of such manifestations of acute sarcosporidiosis are unknown. Generally, however, severe clinical illness and death have been found to be associated with second generation merogony and it seems to be commonly accepted that the haemorrhage associated with the disruption of host endothelial cells occasioned by the discharge of such merozoites may be primarily responsible. Whether or not other factors, such as the release of cell breakdown products, may also contribute to the pathogenesis of infection is unclear, although the release of a toxin does not appear to play a part in infected goats (Dubey *et al.*, 1981). The cause of *Sarcocystis*-induced abortion is also unknown but, because infection has only rarely been observed in foetal placentae or tissues, it is likely to be primarily a consequence of maternal infection with the foetus being an 'innocent victim' (Dubey *et al.*, 1982b).

In contrast to second generation, first generation merogony has, except for fever, rarely been associated with pathogenic effects, although deaths have been recorded during this stage of parasitic development in goats (Dubey *et al.*, 1981). The reasons for these differences are unclear but may possibly relate to the greater numbers of second as compared to the numbers of first generation meronts and/or to the increased host sensitivity to merozoites following experience of the first proliferative phase.

Not all species of *Sarcocystis*, however, appear to be as pathogenic for their intermediate hosts as those mentioned above. Large infective doses of *S. fayeri* and *S. gigantea*

sporocysts, for example, are apparently only mildly or non-pathogenic for horses and sheep respectively (Fayer and Dubey, 1982; Gestrich *et al.*, 1974; 1975a) as are those of *S. hircicanis* of goats (Heydorn and Unterholzner, 1983). As well cattle infected with either *S. hirsuta* or *S. hominis* show little clinical reaction (Gestrich *et al.*, 1975a; Dubey 1983c).

For many species of *Sarcocystis*, the severity of the disease appears to be dose related (Leek *et al.*, 1977; Heydorn 1977a; Collins and Charleston, 1979; Dubey, 1982b; Dubey *et al.*, 1981, 1982b; Munday, 1981; Erber, 1982; Heydorn and Haralambidis, 1982; Barrows *et al.*, 1982b). Thus while large doses of sporocysts may cause severe disease and rapid death, small doses may produce only mild or subclinical effects. In some cases, however, single experimental infections with as few as 1800 or 5000 sporocysts have been shown to have a significant effect upon the liveweight gain of pigs (Zielasko *et al.*, 1981) and lambs (Munday, 1979a).

Although in many of the above experiments, animals have been inoculated with greater numbers of sporocysts than they might reasonably be expected to encounter under natural conditions, field outbreaks of acute sarcosporidiosis, including abortion, have been reported. Most of these cases have involved cattle (Corner *et al.*, 1963; Meads,1976; Frelier, *et al.*, 1977, 1979; Clegg *et al.*, 1978; Landsverk 1979; Giles *et al.*, 1980; Collery and Weavers, 1981; Dubey and Bergeron, 1982; Hong *et al.*, 1982) but natural infections have also been associated with lymphadenopathy and myocarditis in an adult ewe (Landsverk *et al.*, 1978), polyarteritis nodosa in lambs (Landsverk and Bratberg, 1979) and both diarrhoea and protozoal myeloencephalitis in horses (Schumacher *et al.*, 1981; Simpson and Mayhew, 1980).

(b) Pathogenicity for definitive hosts

In contrast to the situation in intermediate hosts, there is little evidence of *Sarcocystis* infection causing clinical disease in definitive hosts although there have been occasional reports of intestinal disorders. Thus abdominal discomfort has been reported in badgers fed *S. campestri* - infected squirrel (Cawthorn *et al.*, 1983) while enteritis and diarrhoea have been observed in dogs fed beef, pork, mutton, goat meat or camel intestinal tissue infected with *Sarcocystis* spp. (Gorobov, 1975; Heydorn and Haralambidis, 1982; Kuraev, 1981). Furthermore, Craige (1976) considers that *Sarcocystis* infections in dogs may not only be associated with chronic intestinal disorders but also with 'obscure signs of toxicosis, anorexia, nausea, fever and even nervous disorders', although he has provided no evidence to support these contentions.

Similar symptoms of nausea, abdominal pain and diarrhoea have also been recorded in man 3 to 6 hr after eating uncooked *Sarcocystis* - infected beef (Heydorn, 1977b) with even more severe symptoms occurring after the ingestion of similarly infected raw pork. In one person, almost continuous vomiting occurred; another had dyspnoea and a rapid pulse lasting up to 48 hr. In contrast, Aryeetey and Piekarski (1976) observed no clinical symptoms in five people excreting sporocysts after being fed raw minced beef. It is possible, therefore, that humans who exhibited symptoms in Heydorn's (1977b) study may have suffered an immediate hypersensitivity reaction since they were all habitual raw meat eaters (Fayer *et al.*, 1979).

1.2.6 Immunity

(a) Immunity in intermediate hosts

Because of what was once considered to be their largely

benign nature and the fact that there is ordinarily little, if any, cellular reaction around them (Levine, 1973) the possibility that there may be some degree of immunity to sarcocysts in intermediate hosts has, until relatively recently, received little attention. In 1975, however, Gestrich *et al.* (1975b) suggested that the numbers of intact *S. hirsuta* cysts in calves may decrease with time. Since then, a similar 'self-cure' phenomenon has been observed for a number of other *Sarcocystis* spp. including *S. miescheriana* in pigs (Erber and Geisel, 1979; Barrows *et al.*, 1982a, b), *S. capracanis* in goats (Collins, 1980a; Dubey, 1983e), *S. equicanis* and *S. fayeri* in horses (Erber and Geisel, 1981), *S. tenella* in sheep (Erber, 1982) and *S. muris* and *S. dispersa* in mice (Hoffer *et al.*, 1982). Although such a phenomenon, which has been found to commence around the third month post-infection in goats (Dubey, 1983e) and as early as 38 days after infection in pigs (Barrows *et al.*, 1982a), has been suggested as being associated with an 'overcrowding' effect following massive infections (Hoffer *et al.*, 1982), the occurrence of mononuclear cells, chiefly lymphocytes, around degenerating sarcocysts suggests that it is an immunologically-mediated response (Dubey, 1983e).

These findings have led to a number of recent attempts to immunise intermediate hosts against *Sarcocystis* infections by orally inoculating them with subclinical doses of sporocysts. So far, such 'vaccination' attempts have been shown to afford protective immunity against infections with *S. miescheriana* (Erber and Geisel, 1979; Zielasko *et al.*, 1981; Schneider and Rommel, 1983), *S. capracanis* (Dubey, 1981c, 1983), *S. muris* (Leier *et al.*, 1982; Hoffer *et al.*, 1982) and *S. dispersa* (Hoffer *et al.*, 1982). There is also some evidence to suggest that prior infection with sporocysts may provide some protection against *S. cruzi* in calves (Mehlhorn *et al.*, 1975b) although acute infections with this parasite may, according to Frelier (1980), be accompanied by a lymphoid abnormality which could result in

immunosuppression.

The degree of protection afforded by such procedures is affected by a number of factors including the immune status of the host, the numbers of sporocysts given, the species of *Sarcocystis* used and the interval between 'vaccination' and challenge. Single infections with 10 sporocysts per animal, for example, have been found not to protect pigs or goats against subsequent challenge with lethal doses whereas a continuous infection with 50 sporocysts thrice weekly in pigs or a single dose of 1000 sporocysts in both pigs and goats, have (Zielasko *et al.*, 1981; Dubey, 1981c; Schnieder and Rommel, 1983). The protection provided by such immunisation applies only to the species used in induce it: there is no evidence to suggest that any cross-immunity is provided against other species of *Sarcocystis* infecting the same intermediate host (Erber and Geisel, 1979; Munday, 1981; Hoffer *et al.*, 1982). Nor does it seem likely that immunisation of the dam will provide any protection to their offspring (Munday, 1982); indeed in goats at least, immunity to *Sarcocystis* appears to be impaired during pregnancy (Dubey, 1983d).

In goats, protection may be provided in as little as 24 to 60 days after 'vaccination' and may persist for at least 274 days (Dubey, 1981c, 1983e). Mice infected with *S. muris*, on the other hand, are only partially protected 60 days later with complete immunity not being observed until 90 days after 'vaccination' (Hoffer *et al.*, 1982). Conversely, 'vaccination' with *S. miescheriana* provides little protective immunity against acute disease beyond 80 days in pigs (Weber *et al.*, 1983).

Precisely which stage of the life cycle is responsible for inducing such immunity and what stage is most affected by it, is largely unknown. It would seem most likely, however, that this may be induced during the vascular phase of development when there is a large release of parasite antigen

in the form of merozoites into the circulation. Presumably, once merozoites have entered the muscle fibres they are no longer in such close contact with the immune system. Certainly, in goats, the numbers of sarcocysts in tissues cannot be relied on as indicators of protective immunity (Dubey, 1983e). For *S. muris*, recent work suggests that three antigenic polypeptides associated with the surface of bradyzoites may be responsible for eliciting a humoral immune response and the possible induction of protective immunity (Abbas and Powell, 1983).

In goats, meronts or immature sarcocysts were not found in 'vaccinated' animals killed 7 to 67 days post-challenge suggesting that the challenge inoculum of *S. capracanis* was destroyed at an early stage of development, probably during the first proliferative generation (Dubey, 1983e). However, in another study, a challenge dose of *S. capracanis* sporocysts were found to complete their entire development in previously 'vaccinated' goats (Dubey, 1981c). Obviously there is scope for much more work in this area.

(b) Immunity in definitive hosts

As with other coccidia, immunity to *Sarcocystis* infections in their definitive hosts could be assessed by the pattern and degree of sporocyst shedding following challenge infection. Indeed, several studies have demonstrated that for cats, dogs and man, reinfection with *Sarcocystis* is easily achieved (Rommel *et al.*, 1972; Heydorn and Rommel, 1972a; Fayer, 1974; Markus, 1974; Ruiz and Frenkel, 1976; Tadros and Laarman, 1976; Fayer and Kradel, 1977; Aryeetey and Piekarski, 1976). These findings and the observations of Fayer (1974) and Munday *et al.* (1975) that they elicit little cellular reaction in the intestines of dogs, has resulted in the generally held view that *Sarcocystis* infections induce little, if any, acquired immunity in their

definitive hosts (Markus, 1978). Whether or not this view is entirely correct, however, is open to question since others (Speer *et al.*, 1980) have found that reinfection of coyotes with *S. hemionilatrantis* caused a marked mononuclear cellular infiltration, hyperaemia and vascular changes in the lamina propria. Furthermore, in none of the above studies were quantitative estimates of sporocyst yields made and it is entirely possible that at least a partial immunity, resulting in a diminution of sporocyst shedding, may be conferred by previous infection.

1.2.7 Diagnosis

(a) Diagnosis in intermediate hosts

The diagnosis of chronic sarcosporidiosis (i.e. the long-standing clinically inapparent cystic stage) in intermediate hosts usually presents little difficulty. Various methods, ranging from visual inspection with the naked eye, to the use of trichinoscopes, histological techniques and muscle digestion procedures on either biopsy or necropsy material can be used to detect the presence of sarcocysts in such hosts. The reliability of the detection procedure depends on the particular method employed with muscle digestion likely to be the most sensitive (Box and Duszynski, 1977; Collins *et al.*, 1980; Diez-Banos, 1980; Nevole and Lukesova, 1981; Bratberg *et al.*, 1982). The diagnosis of acute clinical sarcosporidiosis, on the other hand, may pose considerable problems. The clinical signs exhibited in such cases (anorexia, weight-loss, fever, anaemia) are non-specific and the stage of the parasite life cycle responsible for acute illness (second generation merogony) is relatively short-lived. Furthermore, even when detected, the meronts of *Sarcocystis* may be confused with tachyzoites and cysts of *Toxoplasma gondii* although these may be distinguished either by special staining techniques (Ryblatovskii and Kislyakova, 1973; Dubey *et al.*, 1981) or

or by ultrastructural study (Dubey *et al.*, 1982b).

The diagnosis of *Sarcocystis*-induced abortion may also present a number of difficulties since evidence of infection is rarely demonstrable in placental or foetal tissues (Fayer *et al.*, 1976; Stalheim *et al.*, 1976, 1980; Barnett *et al.*, 1977; Leek and Fayer, 1978a). Barnett *et al.* (1977), however, have found that merozoites of *S. cruzi* may be revealed readily in maternal caruncles from aborted cows by an immunofluorescent stain. Since a caruncle can be collected when the animal is examined after abortion and does not require the sacrifice of the dam, it would be of interest to know if the same technique is equally applicable to suspected cases of *Sarcocystis*-induced abortion in other ruminant intermediate hosts.

A number of haematological and serological changes have been examined as possible aids for the diagnosis of sarcosporidiosis in intermediate hosts (Mahrt and Fayer, 1975; Munday, 1975; Aryeetey and Piekarski, 1976; Lunde and Fayer, 1977; Fayer and Lunde, 1977; Bordjochi *et al.*, 1978; Tadros *et al.*, 1979; Boch *et al.*, 1979; Baetz *et al.*, 1980; Cerna *et al.*, 1981; Prasse and Fayer, 1981; Reiter *et al.*, 1981; Weiland *et al.*, 1982) while the value of an intradermal test has been investigated in buffalo (Tongson and Mesiona, 1981). Although a number of these aids, particularly such serological procedures as the indirect fluorescent antibody test (IFAT), the indirect haemagglutination test (IHA) and the enzyme-linked immunosorbent assay (ELISA), either singly or in combination, have been shown to be useful in demonstrating the occurrence of *Sarcocystis* infections in domesticated hosts (see Weiland *et al.*, 1982, for review), not all have been evaluated in terms of usefulness in cases of clinical outbreaks. Nevertheless, IHA titres of 1:1458 or greater, have been proposed as suggestive of acute sarcosporidiosis in cattle (Lunde and Fayer, 1977; Frelier *et al.*, 1979). Recent evidence also suggests that

the double antibody sandwich immuno-enzymatic assay (DAS-ELISA) may be of sufficient specificity and sensitivity, not only to facilitate the immunodiagnosis of clinical sarcosporidiosis, but also infections which are subclinical in nature during the acute phase of infection, at least in mice and pigs (O'Donoghue and Weyreter, 1983). Whether similar assays can be used to detect circulating antigens in other *Sarcocystis*-infected hosts, however, requires further investigation.

(b) Diagnosis in definitive hosts

There have been few investigations concerning the serological reactions of *Sarcocystis*-infected definitive hosts. What few have been undertaken, suggest that any rise in antibody levels is likely to occur too late after infection and to be of insufficient magnitude and duration to be of any diagnostic value (Markus, 1974; Ruiz and Frenkel, 1976; Lunde and Fayer, 1977; Boch *et al.*, 1980b; Weiland *et al.*, 1982). The detection of *Sarcocystis* infection in such hosts is, therefore, primarily dependent upon demonstrating the presence of sporocysts either in faeces by floatation procedures or in intestinal tissues by digestion techniques (Dubey, 1980; Prestwood *et al.*, 1980; Box and Smith, 1982).

1.2.8 Treatment

(a) Treatment of intermediate hosts

A number of drugs have been shown to be useful for the control of experimentally induced clinical sarcosporidiosis in domesticated intermediate hosts. These include amprolium in sheep (Leek and Fayer, 1980) and calves (Fayer and Johnson, 1975a), salinomycin in sheep (Leek and Fayer, 1983) and halofuginone in sheep and goats (Heydorn *et al.*, 1981a). In addition, zoalene, Bay g 7183 and a mixture of sulfa-

quinoxaline with pyrimethamine have been found to completely eliminate the merogonic stages of *S. muris* present in the liver of mice 11 to 17 days after infection (Rommel *et al.*, 1981).

Most of these drugs are essentially prophylactic in effect and must be administered continuously over a number of days, commencing at the time of exposure or shortly before, if they are to be effective. One of them, halofuginone, has also been found to be effective as a therapeutic treatment against lethal infections of *S. capracanis* in goats and *S. tenella* in sheep but, because of its inherent toxicity, needs to be used with caution (Heydorn *et al.*, 1981).

(b) Treatment of definitive hosts

Treatment of infection in definitive hosts has been little investigated although Boch *et al.* (1980b) found that spiramycin, clindamycin and sulphonamides were unsuccessful in the treatment of experimentally infected dogs. Since coccidiostats usually act only against meronts and merozoites and not against gametes (Levine, 1973) this result may not be altogether surprising.

1.3 SARCOCYSTS AND SARCOSPORIDIOSIS OF SHEEP

Although it was once thought that the domestic sheep *(Ovis aries)* harboured only one *Sarcocystis* species, it is now known that three or possibly four species may infect this host. Two of these, *S. gigantea* and *S. medusiformis*, produce macroscopically visible sarcocysts ('macrocysts') and utilise the cat as the only known definitive host (Collins *et al.*, 1976, 1979). A third, *S. tenella*, forms sarcocysts which are visible only microscopically ('microcysts', 500 x 60 - 100 μm) and may utilise the dog *(Canis familiaris)*, coyote *(C. latrans)* and possibly the jackal *(C. aureus)* and

red fox *(Vulpes vulpes)* as means of transmission (Ford, 1975; Ashford, 1977; Dubey *et al.*, 1978; Meshkov, 1979; Bratberg *et al.*, 1982; Dubey, 1983b). Recently, evidence has been provided suggesting that a further microscopically visible sarcocyst may develop in sheep following the ingestion of sporocysts shed by dogs. It seems likely, therefore, that what are currently referred to as *S. tenella* infections may, in fact, consist of a mixture of two species: one produces sarcocysts with finger-like protrusions (2-4 μm long and 0.6-0.9 μm wide) forming palisade-like cyst walls while the other has sarcocysts with wavy cyst walls bearing delicate hair-like protrusions (5-11 μm long and < 0.5 μm wide) (Boch *et al.*, 1979; Erber, 1982).

Both macroscopically visible cat-derived sarcocysts may measure up to 1 cm or more in length. However, they differ markedly in their relative widths and may have different sites of predilection. *Sarcocystis medusiformis* cysts tend to be slender, taking the form of tapering cylinders and are most frequently seen in carcase meat; those of *S. gigantea* are fat, bluntly-rounded tubes, most commonly observed in the oesophagus (Collins *et al.*, 1976, 1979). Under light microscopy, *S. gigantea* cysts have a clearly demonstrable PAS-positive outer secondary cyst wall, those of *S. medusiformis* do not (Moore, 1980). Ultra-structurally, *S. gigantea* cysts have irregular placentiform villi with blister-like invaginations on all villar and intervillar surfaces. Villi of *S. medusiformis*, on the other hand, are roughly conical and regular in shape and have snake-like filaments with slightly swollen ends arising from them (Collins *et al.*, 1979).

Apart from differences in their size, shape and cyst wall morphologies, *S. gigantea*, *S. medusiformis* and *S. tenella* can all be differentiated on the basis of the electro-phoretic mobility of their cyst enzymes (Atkinson and Collins,

1981). In addition, sporocysts derived from dogs fed sheep microcysts differ in size from those obtained from cats fed sheep macrocysts, sporocysts of the former measuring 14-15 x 9-10 μm, those the latter, 11-14 x 8-9 μm (Levine and Ivens, 1981). The dimensions of sporocysts derived from either *S. gigantea* or *S. medusiformis* infections, however, are not significantly different (Collins *et al.*, 1979).

Infections with *Sarcocystis* are common in sheep throughout the world (see Table 1.2) with microcysts tending to be more frequently recorded than macrocysts. Afshar *et al.* (1974), for example, found that 99.1% and 12% of 6120 sheep in Iran were infected with micro- and macrocysts respectively, while Bratberg *et al.* (1982) found that, of 198 sheep examined in Norway, 65.1% were infected with *S. tenella* and 18.2% with *S. gigantea*. This is not to suggest that macrocysts occur only infrequently in these hosts; indeed Collins (1980b) recorded a prevalence of over 70% in 1215 adult sheep in New Zealand. Rather such apparent differences in prevalence appear to be related to the age structure of the host populations examined, with macrocysts only rarely being recorded in animals under 1 year of age. Thus Munday (1975) observed macrocysts in only 0.6% lambs compared to prevalences of 8.8% and 66% in sheep 1 to 4 years and over 4 years of age respectively, while Bratberg *et al.* (1982) found 0% of lambs, 11.6% of yearlings and 30% of ewes infected with *S. gigantea*. Although microcysts also tend to be more frequently observed in older than younger sheep, such infections may be widespread in lambs with prevalences of 25% and 87% being recorded in animals of less than 4 to 5 months and less than 12 months of age respectively (Bratberg *et al.*, 1982; Munday, 1975).

Precisely why macrocysts should be so rare in young sheep is not entirely clear. Collins (1980b) has suggested that *S. gigantea* sporocysts may need a period of time in a mature

rumen before becoming infective so that young lambs with minimal rumen function may not be susceptible. Munday (1975), on the other hand, considers that any disparities in the prevalence of micro- and macrocysts in young sheep might be related to differences in the rate of growth and development of the *Sarcocystis* spp. concerned.

Of the species of *Sarcocystis* infecting sheep, the complete life cycle and developmental sequence of only one of them, *S. tenella*, has been reported in any detail (Munday *et al.*, 1975; Heydorn and Gestrich, 1976; Ashford, 1977; Speer and Dubey, 1981; Dubey *et al.*, 1982a). Some studies of the developmental cycles of *S. gigantea* in both the definitive and intermediate host have also been undertaken but similar investigations for *S. medusiformis* have not. For *S. tenella*, the developmental cycle in the definitive host follows that outlined in Section 1.2.1 (a) with gametogony and sporogony in the intestinal epithelium and lamina propria resulting in the excretion of sporulated sporocysts 9 to 15 days after infection (Munday *et al.*, 1975; Ashford, 1977; Dubey *et al.*, 1982a). In the intermediate host, two generations of merogony precede muscle invasion: first generation meronts occur in mesenteric arteries, intestinal blood vessels and occasionally in other organs 9 to 21 days after infection (DAI) and second generation meronts occur in capillaries of most organs 16 to 40 DAI. Muscle invasion by second generation merozoites commences as early as 19 DAI with immature sarcocysts first being found 35 DAI, attaining infectivity 40 days later (Dubey *et al.*, 1982a).

Although the sporogonous phase of development of *S. gigantea* in the cat definitive host appears to be similar to that of other *Sarcocystis* spp. (Mehlhorn and Scholtyseck, 1974; Becker *et al.*, 1979), the preceding sequence of events (gametogony) has not been studied. Similarly, details of the early reproductive cycle in the intermediate host have not been reported. Indeed, attempts to experimentally induce

S. gigantea infections in sheep have either been totally unsuccessful (Gestrich *et al.*, 1975a; Collins, 1980b) or largely equivocal (Munday and Rickard, 1974; Munday, 1978; Cole, 1982). Gestrich *et al.* (1975a), for example, were able to demonstrate neither sarcocysts nor infectivity in muscle from lambs dosed with 1.2×10^6 *S. gigantea* sporocysts up to 8 months previously while Collins (1980b) was unable to find any evidence of infection in 30 lambs given up to 16 million sporocysts. Collins (1980b) offered a number of possible explanations for these failures including the non-susceptibility of the lambs, the non-viability of the sporocysts used, or that, unlike other species of *Sarcocystis*, *S. gigantea* sporocysts require some form of prior 'conditioning' before attaining infectivity. The first two suggestions he largely discounted but considered that a sporocyst 'conditioning' requirement, such as might be provided by previous passage through the intestinal tract of either the intermediate or some transport host, or exposure to environmental agencies, might be a possibility. Munday (1978, 1979b), on the other hand, considered that the results of his investigations largely precluded the necessity for any pre-requisite sporocyst 'conditioning' - a conclusion not totally accepted by Collins *et al.* (1978) - and suggested instead, that any difficulties in demonstrating the infectivity of *S. gigantea* sporocysts were more likely to be due to the slow development of their sarcocysts in sheep and the long interval (8-14 months) required for them to attain maturity. While there is some support (Cole, 1982) for Munday's (1978, 1979b) suggestion that *S. gigantea* cysts may take many months to attain infectivity, this alone does not totally explain the paucity of meronts and immature sarcocysts found in experimentally infected animals before this stage is reached. It is apparent, therefore, that considerably more work will be required before this matter can be satisfactorily resolved.

Unlike *S. tenella*, which has been shown to be highly pathogenic for sheep (see Section 1.2.5 (a)), *S. gigantea* is generally considered to be non-pathogenic for its intermediate host (Gestrich *et al.*, 1974, 1975a; Levine and Ivens, 1981) while the pathogenicity of *S. medusiformis* is unknown. Because of the equivocal results obtained in transmission experiments outlined above, the view that *S. gigantea* is largely benign for sheep must be accepted with some reservation. However, the main importance of macrocystic *Sarcocystis* infections in sheep lies in the fact that the large visible sarcocysts are aesthetically undesirable and most importing countries will not accept meat containing such lesions. Accordingly, carcasses with visible sarcocysts are detained at the end of the meat chains and trimmed or, if infestation is extensive, condemned as unfit for human consumption, a procedure, which in 1974 was estimated to cost the New Zealand meat industry $3 million (Collins, 1974).

CHAPTER 2

THE RECOVERY AND ENUMERATION OF *SARCOCYSTIS GIGANTEA* SPOROCYSTS FROM AND IN CAT FAECES

2.1 INTRODUCTION

In any programme of parasitological research the mass recovery of infective stages (e.g. eggs and oocysts) in a relatively pure form is essential for transmission experiments or for a study of the stages themselves. In addition, the development of accurate and sensitive quantitative methods for determining the concentration of such organisms in faeces is necessary in order to monitor the course of infections in infected hosts.

The techniques employed in such procedures usually involve the recovery, concentration and/or dilution of eggs and oocysts free from faecal debris by a process of homogenisation and/or sieving followed by floatation or sedimentation or both. The efficiency of these procedures has been found to be influenced by such variables as sieve mesh size (O'Grady and Slocombe, 1979; Castelino and Herbert, 1972), the specific gravity and type of floatation medium employed (Parfitt, 1969; O'Grady and Slocombe, 1979), the duration of floatation and sedimentation (Egwang and Slocombe, 1982) and the species of parasite involved (O'Grady and Slocombe, 1979).

For *S. gigantea*, the recovery from and enumeration of sporocysts in faeces are likely to pose a number of problems. The sporocysts are small (13.0 ± 0.06 x 8.34 ± 0.08 µm, n = 50), likely to be of low specific gravity and consequently difficult to separate from light faecal particles, including undigested fat, which appears to be common in cat faeces (pers. obs.). In addition, previous observations suggest that the numbers of sporocysts shed

by infected cats are likely to be few (Rommel *et al.*, 1972; Mehlhorn and Scholtyseck, 1974). It was considered important, therefore, that any recovery or counting techniques should be of optimum efficiency and sensitivity if sporocysts were neither to be overlooked nor harvested in such low numbers that further work with them would have to be curtailed. Accordingly, the following study was undertaken to evaluate the effect of various factors on the recovery of *S. gigantea* sporocysts with the twin objectives of applying this information to devising an appropriate mass recovery procedure and a reliable and accurate means of determining their concentration in faeces.

2.2 MATERIALS AND METHODS

2.2.1 Sporocysts

The sporocysts used in all experiments were obtained from experimentally infected cats. The cats, which were raised and maintained free of extraneous *Sarcocystis* infections on a diet of tinned (i.e. sterilised) food, were infected by feeding them *S. gigantea* cysts excised from the oesophagi of naturally infected sheep freshly obtained from a local Freezing Works. The sporocysts from some of these cats were recovered by a crude sugar floatation technique and stored in tap water as a standard suspension of sporocysts and faecal debris at 4°C until use. An estimate of the sporocyst concentration in this suspension was obtained by mixing it with a magnetic stirrer while removing five 1 ml samples and counting the sporocysts using a haemocytometer. These counts revealed a mean ± standard error concentration of 111,322 ± 4247 sporocysts per ml and, unless otherwise specified, these numbers were used in all experiments.

2.2.2 Evaluation of procedures

All experiments were conducted on a small scale, primarily because of the modest numbers of sporocysts available but also because it was considered that such an approach would allow more precise control of the variables involved.

The study was divided into two main parts. The first was concerned with evaluating the effect of various factors on the recovery and extraction of sporocysts from faeces, the second with determining the number of sporocysts in faeces. For the most part, these evaluations were based on percentage recoveries from samples containing known numbers of sporocysts. Initially, these samples consisted of 1 ml aliquots of the standard suspension alone but subsequently samples of *Sarcocystis*-free cat faeces to which sporocysts had been added, were used. In one experiment, faeces from an experimentally infected cat containing unknown numbers of sporocysts were employed.

2.2.3 General recovery procedure

The precise details of the recovery procedure varied according to the experiment but unless otherwise indicated involved the following sequence:

1) Replicate volumes of a well-mixed sporocyst/faeces suspension in water were placed in graduated tubes and sedimented by centrifugation.

2) The supernatant in each tube was removed to leave a volume of 1 ml and 9 ml of floatation medium added to make a total volume of 10 ml. The tubes were then vigorously shaken and centrifuged again.

3) Following centrifugation, the sporocysts were collected from the surface of the floatation medium

by removing the top 1 ml from each tube using pasteur pipettes attached to flasks connected to water vacuum pumps.

4) The contents of the flasks, which were rinsed with 13 ml of water, were then placed in clean tubes and centrifuged again.

5) The top 13 ml of supernatant in each tube was carefully removed leaving a final volume of 1 ml of sediment containing the recovered sporocysts for counting.

The specific gravity of the floatation medium and the type of floatation medium employed varied according to the experiment as did the duration and force of centrifugation. However, in all experiments the duration and force of the centrifugations were kept the same for both the sedimentation and floatation phases.

2.2.4 Counting of sporocysts

Unless otherwise indicated, the numbers of recovered sporocysts in the 1 ml of sediment of each sample were estimated by examining six 0.9 µl replicates in a haemocytometer (Improved Neubauer) at a magnification of 400 X. The total number of sporocysts recovered was then determined according to the formula:

$$\text{total sporocysts} = V \frac{(10{,}000 \times \bar{X})}{9}$$

where V = final volume of the sample and $\bar{X}$ = the mean number of sporocysts in the six 0.9 µl replicates.

2.2.5 Statistical analysis

Where appropriate and unless stated otherwise, results were

examined for statistical significance using a one-way analysis of variance. Tables for these data are given in Appendix I.

2.3 EXPERIMENTAL PROCEDURES AND RESULTS

All results given represent the means of at least four experiments ± the standard error

2.3.1 Recovery of sporocysts from faeces

A. Using sporocysts in crude suspension

(i) Effect of specific gravity of floatation medium

Sucrose solutions with specific gravities (SG) ranging from 1.025 to 1.30 were added to 1 ml aliquots of the standard sporocyst suspension. All samples were treated as described in Section 2.2.3 at a centrifugal force of 1500 x g for 5 min and percentage recoveries determined.

The results (Fig. 2.1) demonstrated an association between percentage recovery and specific gravity with the greatest numbers of sporocysts being recovered when sucrose solutions of between 1.175 and 1.25 SG were used.

(ii) Effect of type of floatation medium

Solutions of sucrose, $MgSO_4$, $ZnSO_4$, NaCl and $NaNO_3$, all at SG 1.2, were compared for their efficiency in recovering sporocysts from samples of the standard suspension using the same procedures as in the previous experiment.

No significant differences ($P > 0.05$, Appendix Ia) were recorded (Fig. 2.2).

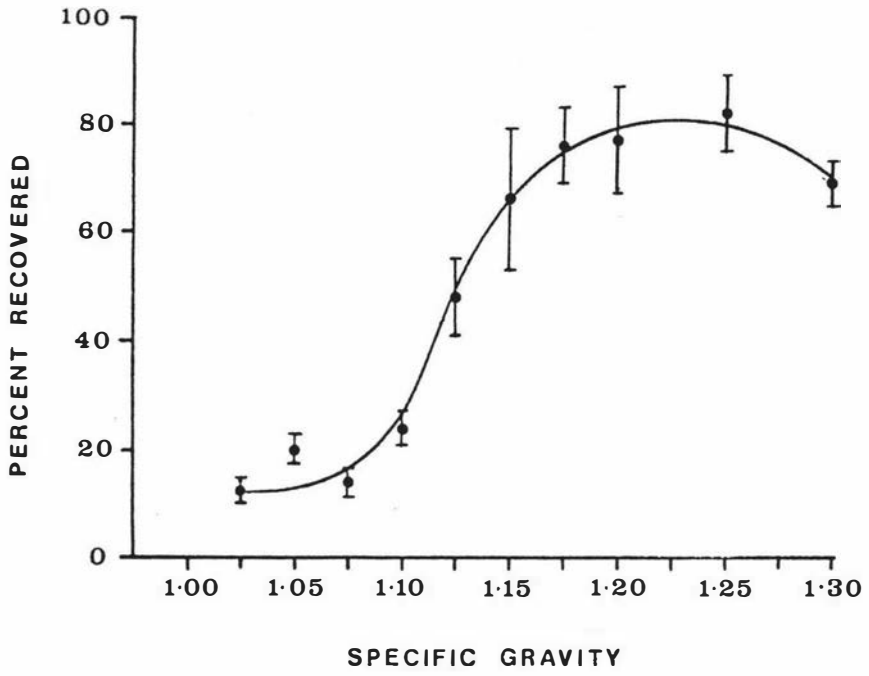

Fig. 2.1 Effect of specific gravity of floatation medium on the recovery of *S. gigantea* sporocysts

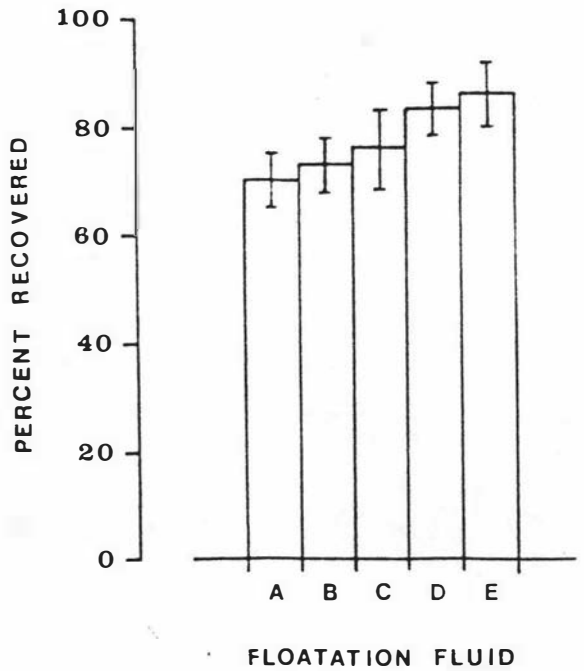

Fig. 2.2 Effect of type of floatation medium on the recovery of *S. gigantea* sporocysts. A = sucrose, B = $ZnSO_4$, C = $MgSO_4$, D = NaCl, E = $NaNO_3$ (all at 1.2 SG)

(iii) Effect of duration of centrifugation

Using the same procedures as in the previous experiments, aliquots of the standard suspension in NaCl solution of 1.2 SG were centrifuged at 1500 x g for periods of between 1 and 11 min as shown in Fig. 2.3.

Differences between the numbers of sporocysts recovered after different centrifugation periods were highly significant ($P < 0.001$, Appendix Ib). In general, centrifugation times of less than 5 min were associated with lower recoveries than were those of 5 min or more (Fig. 2.3).

(iv) Effect of centrifugal force

The effect of differing forces of centrifugation between 500 and 10,000 x g (see Fig. 2.4) for periods of 5 min on sporocyst recoveries were examined using the same procedures and materials as in the previous experiment.

The results, which are presented in Fig. 2.4, showed that the greatest recoveries tended to be obtained when a centrifugal force of 6000 x g was employed with lower recoveries below and above this figure. However, these differences were not statistically significant ($P > 0.05$, Appendix Ic).

(v) Effect of addition of CCl_4 to floatation medium

Despite the fact that the standard suspension had undergone some crude purification (see Section 2.2.1), sporocysts recovered from this material were still accompanied by unacceptably large amounts of debris. It was decided to investigate what effect the addition of an oil and fat solvent, such as carbon tetrachloride (CCl_4), would have on sporocyst cleanliness and recovery.

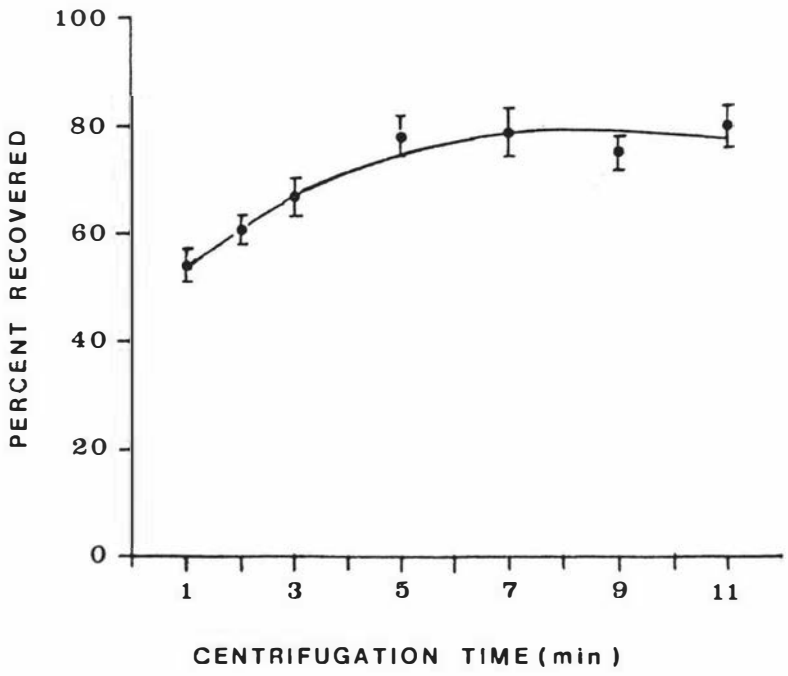

Fig. 2.3 Effect of duration of centrifugation on the recovery of *S. gigantea* sporocysts

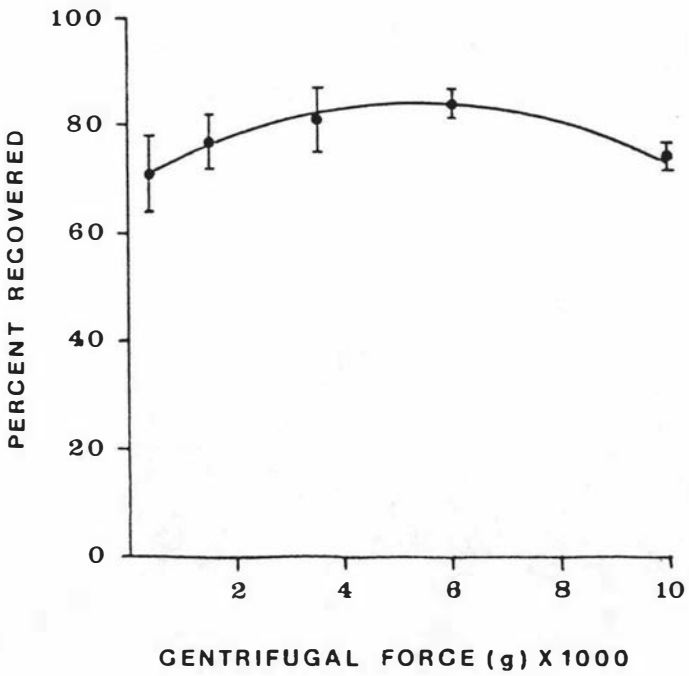

Fig. 2.4 Effect of centrifugal force on the recovery of *S. gigantea* sporocysts

Accordingly, solutions of NaCl (SG 1.2) containing 0, 1, 2.5, 5.0 or 10% CCl_4 were added to aliquots of the standard suspension and centrifuged at 6000 x g for 5 min.

The presence of CCl_4 lead to the formation of a 'plug' of faecal debris at the interface of the solvent and the NaCl solution during centrifugation (Plate 2.1). Consequently this resulted in much cleaner final preparations than obtained using NaCl solution alone (Plate 2.2). However, this improvement in a cleanliness was at the expense of a marked reduction in the numbers of sporocysts recovered (Fig. 2.5). Even at concentrations of 1% CCl_4, only 40% of the numbers obtained using NaCl solution alone were recovered.

B. Using sporocysts added to faeces

Experiments in the previous section were considered to be of a preliminary nature only, undertaken to examine some of the more fundamental factors affecting the recovery of sporocysts which were already largely free of faecal material. These experiments indicated that this could best be achieved using NaCl solution (SG 1.2) and centrifugation at 6000 x g for 5 min. In the next section the knowledge thus gained was employed in an examination of some of the other factors influencing sporocyst recoveries which were likely to be occasioned by their presence in unprocessed faeces.

(i) Effect of sieving

Preliminary observations showed that the counting of sporocysts recovered from faeces which had not initially undergone some form of homogenisation and sieving was impossible. They also showed that prior passage of

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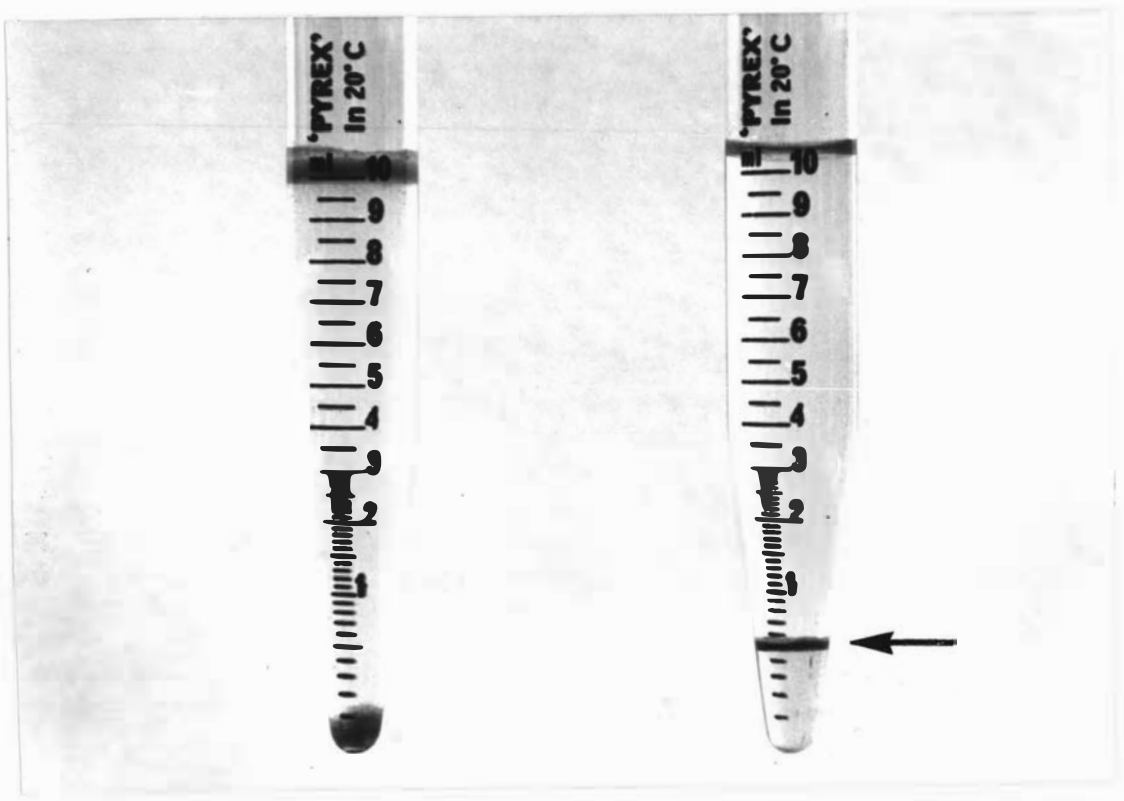
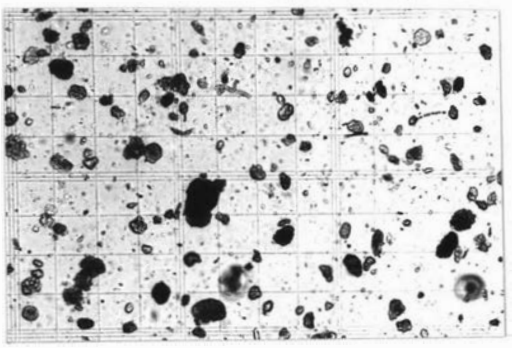
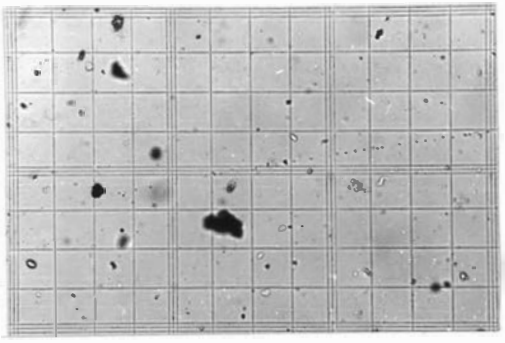
Plate 2.1 Dispersion of faecal debris following centrifugation in NaCl; left - NaCl solution only, right - NaCl solution plus 5% CCL_4 (note plug of faecal debris at the interface of CCL_4 and NaCl solution, arrow)

Plate 2.2 Comparison of amount of faecal debris as seen in haemocytometer following recovery using NaCl solution only (left) or NaCl solution plus 5% CCL_4 (right) (x 250)


'PYREX'
In 20° C
'PYREX'
In 20° C





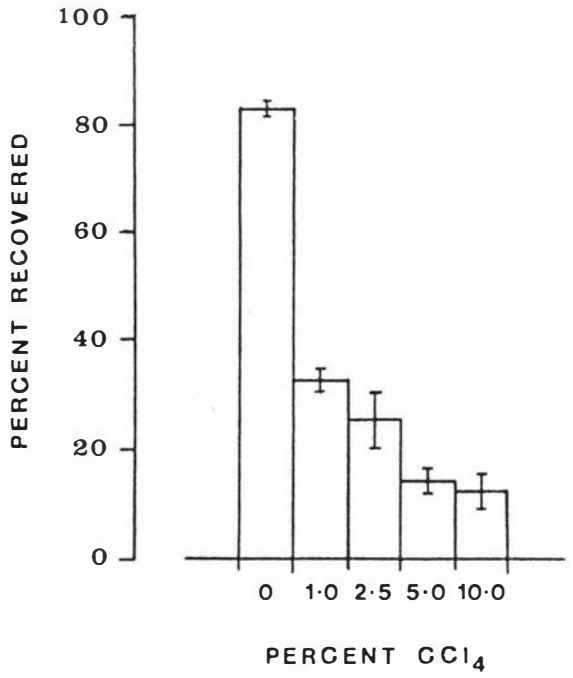


Fig. 2.5 Effect of CCl_4 concentration in floatation medium on the recovery of *S. gigantea* sporocysts

homogenised faeces through a 60 mesh* (aperture 250 μm) sieve alone resulted in the subsequent presence of unacceptably large amounts of coarse debris whereas attempted passage through a 300 mesh* (aperture 53 μm) sieve alone resulted in total sieve blockage. A standard sieving procedure, involving passage through a 60 mesh sieve, followed by passage of the subsequent filtrate through a sieve of 300 mesh size was, therefore, adopted.

In any sieving procedure there is always the possibility that eggs or oocysts may be retained, although it seems reasonable to assume that the degree of entrapment may

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*Endecotts Laboratory Test Sieve

be modified by the volumes of water used to wash such material through. Accordingly, an experiment was conducted to examine the effect of these procedures on the recovery of *S. gigantea* sporocysts.

Twenty four 5 g samples of freshly collected sporocyst-free cat faeces were made up to volumes of 49 ml with tap water. To each sample was added 1 ml of standard sporocyst suspension at one of two points in the procedure. In 18 of the samples (groups B, C and D) it was added at the beginning of the procedure i.e. before homogenisation and sieving had taken place while in the remaining 6 samples (group A) it was added to the filtrate immediately after sieving as a control. Homogenisation of all samples was achieved using an electric beater for 2 min. In two of the three groups of samples to which sporocysts had been added at the beginning of the procedure an additional 50 (group C) or 100 (group D) ml of water were used to wash material through the sieves. In the third such group (B) and the control group (A), no additional water was used. The filtrates of these samples were adjusted to final volumes of 100, 150, 50 and 50 ml, respectively and a single one-fifth aliquot (potentially containing approx. 22,264 ± 849 sporocysts) removed from each. These subsamples were then treated as previously described and percentage recoveries determined.

Sporocyst recoveries in this investigation tended to be lower and more variable than those obtained in previous experiments. The results (Table 2.1 and Appendix Id) suggested that this was more likely to be a consequence of the greater amounts of faecal debris rather than any entrapment during sieving since similar numbers of sporocysts were recovered regardless of whether they were added before or after this step.

(ii) Effect of additional washing prior to floatation

It was observed in the previous experiment, that there were variations in the proportions of faecal debris present in subsample filtrates. It was also observed that there appeared to be an inverse relationship between the proportions of such material and the numbers of sporocysts recovered, suggesting that large amounts of debris may impede sporocyst levitation. It was decided, therefore, to investigate what effect, if any, additional washing in water prior to floatation would have on reducing this debris and enhancing sporocyst recovery.

Six 5 g samples of sporocyst-free cat faeces to which sporocyst suspension was added were each made up to 50 ml with tap water. Each sample was homogenised and sieved as previously described and the filtrates adjusted to final volumes of 50 ml. From each filtrate of each sample, two or three representative one-fifth subsamples were removed, sedimented by centrifugation and their supernatants removed leaving final volumes of 1 ml. Two of the subsamples were subjected to further washing achieved by resuspending the 1 ml of sediment in an additional 9 ml of tap water and sedimenting again by centrifugation. In one subsample this further washing was performed only once and in the second twice. The third subsample was not subjected to any additional washing. All subsamples were then mixed with NaCl solution and sporocyst recoveries determined as already outlined.

This experiment demonstrated that although additional washing prior to floatation may have resulted in some minor reductions in faecal debris (subjective judgement) this did not result in any statistically significant ($P > 0.05$, Appendix Ie) improvement in sporocyst recover-

TABLE 2.1 EFFECT OF SIEVING AND WATER VOLUME ON THE RECOVERY OF *S. GIGANTEA* SPOROCYSTS FROM CAT FAECES

	Group*/Percent recovery			
Sample No	A	B	C	D
1	70.7	63.6	89.8	64.9
2	93.6	62.8	73.6	60.7
3	63.2	79.0	74.4	67.8
4	50.7	68.2	56.6	72.0
5	36.6	39.1	40.8	53.2
6	75.7	60.7	59.9	71.5
Mean ± SE	65.1 ± 8.1	62.2 ± 5.3	65.9 ± 7.0	65.0 ± 2.9

*A - Sporocysts added after sieving, water to faeces (ml/g)= 50:5
B - " " before " " = 50:5
C - " " before " " =100:5
D - " " before " " =150:5

TABLE 2.2 EFFECT OF THE NUMBER OF WASHES* PRIOR TO FLOATATION ON THE RECOVERY OF *S. GIGANTEA* SPOROCYSTS FROM CAT FAECES

	No. of Washes/Percent recovery		
Sample No	x1	x2	x3
1	61.6	84.0	ND
2	59.1	79.9	ND
3	78.2	60.7	64.9
4	57.4	70.7	81.5
5	88.2	80.7	69.0
6	59.9	68.2	65.7
Mean ± SE	67.4 ± 5.2	74.0 ± 3.7	70.3 ± 3.8

*Suspension and centrifugal sedimentation in water
ND = not done

ies (Table 2.2).

(iii) Effect of host diet

Faeces used in the preceding experiments were obtained from cats maintained on a diet of tinned meat*. As mentioned previously, the filtrates of such samples were frequently characterised by excessive amounts of debris. It was considered that an alternative diet could, conceivably, result in a reduction of such material and an experiment was conducted to examine this possibility.

Four 12 week-old kittens, raised and maintained *Sarcocystis*-free were divided into two equal groups and housed separately. One group was maintained exclusively on a diet of tinned meat* the other on a diet of tinned fish**. Two weeks after the kittens had been on their respective diets, the total daily faecal output of both groups was collected on four separate occasions. On each occasion the faeces of each group was thoroughly mixed, a 5 g sample removed and sporocyst suspension added. Each sample was then treated as described in the previous experiment except that only a single one-fifth subsample subjected to one sedimentation prior to floatation was used.

Although there were some variations between them, the amounts of faecal debris in the final preparations of samples from kittens maintained on a fish diet were consistently less than in those from kittens on a meat diet (Plate 2.3). Recovery of sporocysts from faeces of

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*Felix Meat: Beef and/or mutton by products, ground bone, cereals, polyunsaturated vegetable oil, minerals and vitamins, estimated crude fat content 8-10% (Man. specifications).

**Felix Fish: Whole fish, cereals, polyunsaturated vegetable oil, minerals and vitamins, estimated crude fat content 2-3%.

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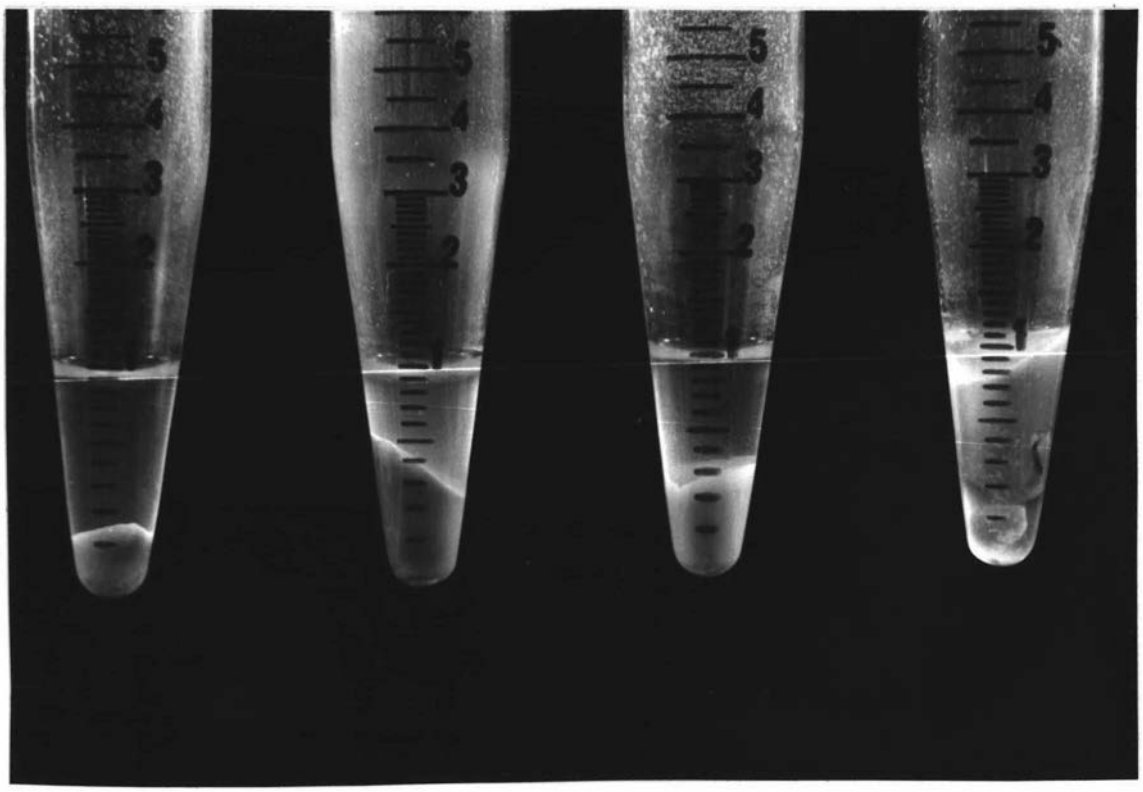
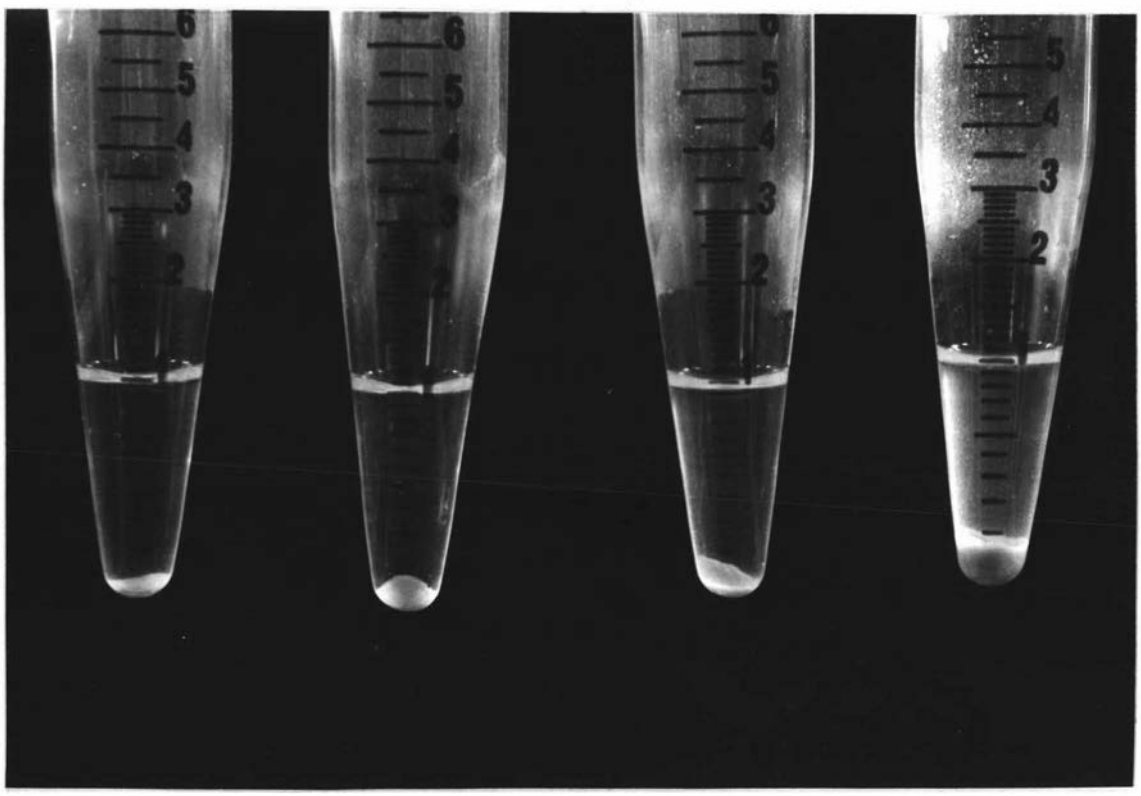
Plate 2.3 Comparison of the amount of debris present in final preparations of four faecal samples from kittens maintained on tinned meat (upper) and tinned fish (lower) diets





kittens fed on the former diet also tended to be better (Table 2.3) but these differences were not statistically significant (t-test; $P > 0.05$, $t = 0.858$, 6 df).

(iii) Effect of number of centrifugations in floatation medium

Sporocysts were added to six 5 g samples of sporocyst-free faeces obtained from cats fed tinned fish. The samples were homogenised and sieved and a representative one-fifth filtrate subsample removed from each, sedimented once by centrifugation and reduced to a final volume of 1 ml. These were then suspended in 9 volumes of NaCl solution (SG 1.2), centrifuged and the sporocysts recovered from the top 1 ml in the usual manner. The remaining 9 ml were then resuspended and the volume restored with further NaCl solution and a second and third centrifugation and recovery carried out on each.

The results (Table 2.4) showed, on average, that 96.6% of the sporocysts recovered from all four samples were obtained from the first centrifugation in NaCl solution with only small proportions being recovered from subsequent centrifugations.

(iv) Effect of proportion of faeces to floatation medium

In all previous experiments in this section, the equivalent of 1 g of sieved faecal material was made up to 10 ml with 9 ml of NaCl solution for the floatation phase i.e. 10% faeces. It was decided to investigate, therefore, if a lower proportion of faeces would result in a higher sporocyst yield.

Six 5 g samples of sporocyst-free cat faeces (on tinned fish diet) to which sporocysts had been added, were homogenised and sieved. From each, two one-fifth filtrate

TABLE 2.3 EFFECT OF HOST DIET ON THE RECOVERY OF *S. GIGANTEA* SPOROCYSTS FROM CAT FAECES

	Diet/Percent recovery	
Collection day	Fish	Meat
1	65.7	49.1
2	61.6	74.0
3	69.0	60.7
4	65.7	59.9
Mean ± SE	65.5 ± ±.5	60.9 ± 5.1

TABLE 2.4 EFFECT OF NUMBER OF CENTRIFUGATIONS IN NaCl SOLUTION ON THE RECOVERY OF *S. GIGANTEA* SPOROCYSTS FROM CAT FAECES

	Centrifugation No/Percent recovery			
Sample No	1st	2nd	3rd	All three
1	54.1	3.7	-	57.8
2	64.9	0.8	0.4	66.1
3	70.7	3.3	0.4	74.4
4	79.0	1.2	-	80.2
Mean ± SE	67.2 ± 5.3	2.3 ± 0.8	0.2 ± 0.1	69.6 ± 4.9

subsamples (A and B) were removed and centrifuged. Subsample A was reduced to a final volume of 1 ml and 9 ml of NaCl solution added. Subsample B was reduced to 2 ml and 18 ml of NaCl solution added. Both subsamples, therefore, were suspended in 9 volumes 1.2 SG NaCl solution but in subsample A the faeces proportion was 10% (equivalent of 1 g of faeces in 10 ml while in subsample B it was 5% (equivalent of 1 g of faeces in 20 ml).

The results, which are presented in Table 2.5, showed a significantly higher percentage recovery when a faeces proportion of 5% was employed (paired t-test; $P < 0.05$, $t = 2.637$, 5 df).

2.3.2 Enumeration of sporocysts in faeces

Estimates of the intensity of helminth and coccidial infections are frequently measured by the numbers of eggs or oocysts they produce in faeces. Such estimates are usually based on counts in small, representative faecal samples and are normally expressed in terms of their concentration per gram. A number of procedures have been described for obtaining such estimates, of which the McMaster technique is the most commonly employed. In this procedure a specified weight of faeces (e.g. 1.7 g) is mixed with a known volume of diluent (50 ml; usually saturated aqueous NaCl) and a representative sample introduced into a special chamber which consists of two parallel glass slides 0.15 cm apart. On the underside of the top slide are two ruled grids each having an area of 1 sq cm. The heavy faecal debris falls to the bottom of the chamber and the eggs and/or oocysts float free to the underside of the cover glass where they can be counted. Their number per gram of faeces can be calculated according to the formula:

TABLE 2.5 EFFECT OF PROPORTION OF FAECES TO FLOATATION MEDIUM ON THE RECOVERY OF *S. GIGANTEA* SPOROCYSTS FROM CAT FAECES

	Faeces proportion/Percent recovery	
Sample No	1:10	1:20
1	64.5	83.2
2	79.0	82.5
3	76.3	87.4
4	71.4	90.8
5	51.3	80.4
6	69.3	65.2
Mean ± SE	68.6 ± 4.1	81.6 ± 3.6

$$\text{No. per g} = \frac{\text{No. counted x dilution}^{(\text{ml}/\text{g})}}{\text{depth of chamber x grid area}}$$

Using the dilutions outlined above and examining both grids, each egg or oocyst counted represents 100 per g.

Similar estimates of the concentrations of *S. gigantea* sporocysts could also be obtained using the procedure outlined in the previous experiments. Thus starting with an initial sample of 5 g of faeces and processing a one-fifth subsample, the number of sporocysts recovered by this means would provide an indication of their concentration per gram. If the volume of the final preparation was 1 ml and six 0.9 µl replicates were examined in a haemocytometer, then it can be seen from the formula given in 2.2.4 that each sporocyst counted would represent 185.2 per g. However, by doubling the number of 0.9 µl replicates examined, or more logically, halving the volume of the final preparation, then a similar sensitivity to that of the McMaster technique could be obtained (i.e. each sporocyst counted = 92.6/g).

In assessing the usefulness of any faecal analysis procedure, two criteria are of prime importance, namely, the reliability and accuracy of the technique and the time and effort required. The McMaster technique is easily and rapidly performed while that of the floatation-haemocytometer technique, described here, is long and tedious. It was decided, therefore, to compare the accuracy of these two techniques for counting *S. gigantea* sporocysts in cat faeces.

(i) Comparison of counting techniques

Initial attempts to use the McMaster technique revealed two major problems. Firstly, the top side of the chamber was too thick to permit the use of an objective lens of sufficient magnification to enable the sporocysts to be seen. Secondly, the large amount of faecal debris floating up with the sporocysts obscured their presence. Accordingly, the

following modifications were made:

1) The top side of the chamber was removed and replaced by two fine cover glasses on which were etched 1 cm sq. grids.

2) Following mixing of the faecal sample and prior to the introduction of a representative aliquot into the chamber, the sample was sieved.

Known numbers of sporocysts were added to eight 5 g samples of faeces from *Sarcocystis*-free cats maintained on a diet of tinned fish. To four of these samples sporocysts were added to produce concentrations of approximately 22,264 ± 849 sporocysts per gram (spg) and to the other four, 2,226 ± 85 per g. Water was added to all samples which were then homogenised and sieved in the usual manner (see Section 2.3.1 B (i)). From each filtrate of each sample, two one-fifth subsamples A and B (each containing the equivalent of 1 g of faeces) were removed, sedimented and reduced to final volumes of 2 and 3 ml, respectively. The number of sporocysts in subsample A was determined by adding 18 ml of NaCl solution (1.2 SG) and proceeding according to the floatation-haemocytometer technique while the number of sporocysts in subsample B was determined using the McMaster procedure. For the latter purpose the 3 ml of faecal sediment was thoroughly mixed with 27 ml of NaCl solution (SG 1.2) and a representative aliquot introduced into the McMaster chamber. In both cases, therefore, the faecal sediment was suspended in 9 volumes NaCl solution (SG 1.2) with similar faeces to NaCl solution proportions (5 and 3.33%, respectively) and comparable sensitivities (each sporocyst counted = 100 spg McMaster, 92.6 spg floatation-haemocytometer).

The results are presented in Table 2.6. The numbers estimated by both procedures were always lower than the actual counts. At both levels of concentration, the float-

TABLE 2.6 FAECAL SPOROCYST COUNTS OBTAINED USING THE FLOATATION-HAEMOCYTOMETER AND THE MODIFIED McMASTER METHOD IN CAT FAECES AT TWO LEVELS OF SPOROCYST CONCENTRATION*

Sporocyst concentration	Sample No.	Counting method/sporocyst counts* McMaster	Floatation-haemocytometer
22,264 ± 849	1	7,800	18,705
	2	9,800	16,853
	3	6,600	14,631
	4	4,400	13,520
	Mean ± SE	7,150 ± 1,130	15,927 ± 1,156
2,226 ± 84.9	1	1,000	1,852
	2	800	1,019
	3	700	1,574
	4	700	1,482
	Mean ± SE	800 ± 71	1,482 ± 173

*Sporocysts per gram (spg) of faeces

ation-haemocytometer technique was significantly better than that of the McMaster procedure (paired t-test; $P < 0.01$, $t = 10.63$; $P < 0.05$, $t = 4.384$, 3 df at concentrations of 22,264 and 2,226/g, respectively).

(ii) Errors within counting technique

The floatation-haemocytometer technique described above has two main sources of potential sampling error: the error introduced in removing a one-fifth subsample from the homogenised and sieved sample and the errors associated with examining only small volumes (six 0.9 µl) of the final preparation (500 µl). It was decided to investigate the significance of such potential errors in the following experiment.

Eight 5 g faecal samples from *Sarcocystis*-free cats maintained on a tinned fish diet were divided into two lots of four. To one lot, sporocysts were added to produce concentrations of approximately 3065 ± 236 spg and to the other 12,668 ± 612 per g. From the filtrates of each homogenised and sieved sample, two one-fifth subsamples were removed and processed in the usual manner. These subsamples were reduced to final volumes of 500 µl and for each, the numbers of sporocysts in each of two six 0.9 µl replicates determined. Data from both lots of four samples were then each subjected to a 3-level nested analysis of variance.

The results (Tables 2.7 and 2.8) showed no significant differences ($P > 0.05$, Appendix If, g) between subsamples within samples, nor between replicates within subsamples, at either of the two levels of infection.

(iii) Reliability of faecal sampling method

Estimates of the numbers of *S. gigantea* sporocysts per gram could be obtained by processing all the faeces available by

TABLE 2.7 SOURCES OF SAMPLING ERROR WITHIN THE FLOATATION-HAEMOCYTOMETER COUNTING TECHNIQUE. FIGURES REPRESENT ACTUAL NUMBERS COUNTED: EXPECTED COUNTS 3065 ± 236 SPG

Samples	A				B				C				D			
Subsamples	A_1		A_2		B_1		B_2		C_1		C_2		D_1		D_2	
Replicates	A'_1	A''_1	A'_2	A''_2	B'_1	B''_1	B'_2	B''_2	C'_1	C''_1	C'_2	C''_2	D'_1	D''_1	D'_2	D''_2
	7	4	4	9	6	5	7	3	6	7	2	2	5	5	4	5
	10	6	4	8	3	4	4	0	4	3	4	5	4	2	3	4
Readings	2	2	7	4	3	6	7	3	0	5	2	3	5	3	5	4
	3	4	7	5	7	5	4	6	4	7	5	3	3	4	9	1
	1	4	4	2	5	3	3	6	2	5	5	3	4	6	4	6
	5	6	1	6	4	4	2	6	3	1	5	2	5	4	3	8
Replicate sums	28	26	27	34	28	27	27	24	19	28	23	18	26	24	28	28
Subsample sums	54		61		55		51		47		41		50		56	
Sample sums	115				106				88				106			
Sample mean	28.8				26.5				22.0				26.5			
Conversion factor	92.6				92.6				92.6				92.6			
Sporocysts/g (sample means)	2,662				2,454				2,037				2,454			

TABLE 2.8 SOURCES OF SAMPLING ERROR WITHIN THE FLOATATION-HAEMOCYTOMETER COUNTING TECHNIQUE. FIGURES REPRESENT ACTUAL NUMBERS COUNTED: EXPECTED COUNTS 12,668 ± 612 spg

Samples	A				B				C				D			
Subsamples	A_1		A_2		B_1		B_2		C_1		C_2		D_1		D_2	
Replicates	A'_1	A''_1	A'_2	A''_2	B'_1	B''_1	B'_2	B''_2	C'_1	C''_1	C'_2	C''_2	D'_1	D''_1	D'_2	D''_2
	21	18	22	22	22	13	12	15	35	21	15	22	18	18	24	19
	17	13	17	19	20	19	15	19	18	21	12	20	18	17	12	9
Readings	16	20	18	18	15	11	20	21	16	23	21	16	18	24	17	22
	14	16	16	16	16	12	21	18	20	26	25	15	12	19	16	14
	20	13	16	11	15	21	16	22	13	15	13	17	17	19	15	14
	17	24	17	17	19	9	15	12	19	16	17	20	16	11	17	11
Replicate sums	105	104	106	103	107	85	99	107	121	122	103	110	99	108	101	89
Subsample sums	209		209		192		206		243		213		207		190	
Sample sums	418				398				456				397			
Sample means	104.5				99.5				114				99.3			
Conversion factor	92.6				92.6				92.6				92.6			
Sporocysts/g (sample means)	8,667				9,214				10,556				9,191			

the floatation-haemocytometer technique and then dividing the number of sporocysts recovered by the weight of the original material. Alternatively, similar estimates could be obtained by processing only a 1 g sample. In practice it has been found more convenient to start with an initial 5 g sample and further process a one-fifth (i.e. 1 g) subsample as in the previous experiment. In this experiment care was taken to ensure that each lot of 5 g samples contained equivalent numbers of sporocysts by adding the same numbers to each. In acquired infections, however, sporocysts may not be evenly distributed throughout the faecal mass and the numbers in a 5 g sample taken from it may not, therefore, be representative of their concentration in the whole. The following experiment was undertaken to investigate this possibility.

One day's total faecal output (86 g) from an 8 month-old cat, infected 13 days previously with *S. gigantea* cysts, was thoroughly mixed for 3 min using a bowl and spatula and six 5 g samples removed. Each sample was then processed as previously described and estimates of their sporocyst concentrations per gram so obtained, examined for statistically significant differences.

The results, which are presented in Table 2.9, showed that there were no significant differences ($P > 0.05$, Appendix Ih) between them.

2.4 DISCUSSION

A number of authors have described laboratory methods for isolating coccidia from faeces (Davies *et al.*, 1963; Davis, 1973; Levine, 1973; Ryley *et al.*, 1976). Although all will result in the recovery of some oocysts, it seems reasonable to assume that the efficiency of the method employed will vary according to the nature of the host faeces and the size

TABLE 2.9 VARIATIONS OF *S. GIGANTEA* SPOROCYST COUNTS BETWEEN SIX 5 GRAM SAMPLES OBTAINED FROM A SINGLE FAECAL MASS

Sample	A	B	C	D	E	F
	25	14	21	15	16	23
	21	22	27	28	22	23
	26	20	27	14	25	17
Readings	19	28	24	18	29	22
	18	25	25	25	10	28
	18	31	14	21	21	15
Total	127	140	138	121	123	128
Conversion factor	92.6	92.6	92.6	9.26	92.6	92.6
Sporocysts/g	11,760	12,964	12,779	11,205	11,390	11,853

and specific gravity of the oocysts of the species of coccidia concerned.

In this study, each stage of a floatation procedure for the recovery of *S. gigantea* sporocysts from cat faeces was investigated in some detail. One of the main objectives was to arrive at the most efficient system available. The results obtained in this study indicate that the following procedure is likely to result in the greatest yields for the least effort:

1) Add water to faeces in the proportion of 10 ml for each 1 g to be processed, homogenise for 2 min with an electric beater and strain through a 60 then a 300 mesh sieve (apertures 250 and 53 μm, respectively).

2) Discard the material retained by the sieves and centrifuge the filtrate at 6000 x g for 5 min.

3) Remove and discard the supernatant so that the sediment filtrate is reduced to a volume equal to twice that of the weight of faeces processed (i.e. 1 ml of sedimented filtrate contains 0.5 g of faeces).

4) To each 1 ml of sedimented filtrate add a further 9 ml of NaCl solution (SG 1.2) and mix well.

5) Centrifuge as in (2) above and remove from the top a volume equal to that of the sedimented filtrate in step 3 (i.e. the top 10%) using a flask fitted to a water vacuum pump and discard the rest.

6) To each 1 ml of recovered material add a further 13 ml of water, centrifuge again as above, remove and discard the supernatant. The sporocysts are present in the sediment.

The present results suggest that using this procedure, a mean percentage recovery of 75.5% might be expected from faeces with estimated sporocyst concentrations ranging from 2,226 to 22,264 per g (data from Tables 2.5 to 2.8 inclusive). This compares favourably with other commonly used floatation techniques (coverslip floatation, gravity pan floatation, zonal gradients and linear gradients) which have been found to recover 40.5 to 60.7% of oocysts present in chicken and turkey faeces (Vetterling, 1969). As in Vetterling's study, the present findings are based on recoveries from relatively small faecal samples and it is recognised that lower returns are likely to be obtained when larger amounts of faecal material are processed. The writer has generally preferred to process approximately 150-200 g (4-5 days' faecal output from a single 12 to 18 week-old kitten) on any one occasion. Using these sorts of quantities and maintaining a strict adherence to the proportions and concentrations specified in the procedure outlined above, yields in the vicinity of 60-70% of the estimated total numbers of sporocysts were maintained. Other techniques for processing larger amounts of faeces have reported better recoveries than this. Vetterling (1969), for example, described a continuous flow differential density procedure in which he reported an 85.4% recovery of oocysts of fowl coccidia. However, this technique uses equipment not readily available to the author and is also primarily designed to avoid the need for sieving; a process which has been found to be an essential and not at all disadvantageous procedure for the recovery of *S. gigantea* sporocysts.

Although sporocysts isolated as described above are far from pure, their cleanliness has been found to be adequate for further experimental work including studies on excystation. The amount of faecal debris may be considerably reduced by maintaining infected kittens on a tinned fish rather than a tinned meat diet and some further improvement may also be

achieved by additional washing of faecal suspensions prior to floatation. However, the degree of improvement accomplished by this latter means is so small as to be unjustifiable in view of the extra time and effort required. A much more significant improvement in purity can be attained by adding CCl_4 to the NaCl solution but only at the expense of a very substantial reduction in the numbers of sporocysts recovered. In addition, the possibility that this solvent may interfere with the physical or chemical nature of the sporocyst wall must also be considered.

The recommended floatation medium is NaCl solution at a specific gravity of 1.2. Even prolonged exposure to this solution has little effect on oocyst viability (Ryley and Ryley, 1978) and the short period of contact required by the present procedure has no apparent effect on the ability of *S. gigantea* sporocysts to excyst (see Chapter 4). Other floatation media tested in this study could also probably be used, at the same specific gravity, with similar degrees of success. However, sucrose solution is sticky and unpleasant to handle while the other chemicals investigated are more expensive than NaCl.

The procedure described can also be used to determine the concentration of sporocysts in faeces starting with a 5 g sample and subsequently processing a one-fifth (i.e. 1 g) subsample after step 1. By reducing the final volume of this subsample to 500 µl in step 6 and counting the number of sporocysts in six 0.9 µl replicates using a haemocytometer, then an estimate of the number of sporocysts per gram can be obtained by multiplying the total number counted by 92.6. This procedure is considerably more time consuming than the commonly employed McMaster method but is more accurate. This is rather surprising since others (Levine *et al.*, 1960; Dorney, 1964; Egwang and Slocombe, 1981) have found the McMaster technique to be generally superior to most tested for the counting of eggs and oocysts in faeces.

The reasons for this apparent disparity are not entirely clear but probably relate, at least in part, to the small size of the sporocysts under study and the nature of cat faeces. Even after sieving, such samples still contain large amounts of fine faecal debris and are frequently excessively turbid. Because of this and because of the depth of the McMaster chamber, light penetration is poor so that many of the sporocysts present in these preparations could either be obscured or overlooked. When counting sporocysts by the floatation-haemocytometer technique, however, much of the faecal debris has already been eliminated and because only 0.1 mm depth of material is examined, light penetration is not a problem. In addition, any uncertainities in identification can be resolved by the use of a higher magnification objective lens, a process which is not possible with the McMaster chamber even when the top slide is replaced with a fine cover glass.

CHAPTER 3

PRODUCTION OF *SARCOCYSTIS GIGANTEA* SPOROCYSTS BY EXPERIMENTALLY INFECTED CATS

3.1 INTRODUCTION

Studies on the life cycle of various species of *Sarcocystis* have shown that development in definitive hosts is restricted to sexual reproduction (gametogony) and sporogony in the small intestine (Heydorn and Rommel, 1972a, b; Fayer, 1974; Mehlhorn and Scholtyseck, 1974; Rommel *et al.*, 1974; Munday *et al.*, 1975; Zaman and Colley, 1975; Ruiz and Frenkel, 1976; Heydorn and Haralambidis, 1982; Hilali *et al.*, 1982; Dubey, 1982a). This is unlike most other species of coccidia where asexual reproduction (schizogony) precedes gametogony and sporogony takes place outside the host (Kheysin, 1972; Levine, 1973). Zoites within sarcocysts in the intermediate host are, therefore, pro-gamonts which, when ingested by the definitive host, differentiate into female macrogametocytes and male microgametocytes. Gametogony typically takes place in the lamina propria of the small intestine with each macrogametocyte producing a single macrogamete and each microgametocyte several microgametes. Fertilisation of each macrogamete by a microgamete gives rise to an oocyst which sporulates within the lamina propria to produce two sporocysts each containing four sporozoites. Following sporulation free, mature sporocysts are excreted in the faeces.

There have been few studies concerning sporocyst production in *Sarcocystis*-infected definitive hosts but it is commonly asserted that sporocyst shedding may continue for a long period and that re-infection is easily achieved (Rommel *et al.*, 1972; Heydorn and Rommel, 1972a; Fayer, 1974; Ruiz

and Frenkel, 1976). Unfortunately studies on which such assertions are based have been largely of a qualitative nature and have provided little information regarding the numbers of sporocysts shed.

As part of the present study on *S. gigantea*, information concerning sporocyst excretion by cats was sought. This information was required, not only to provide basic epidemiological data, but also to determine the best means of obtaining maximum yields of sporocysts for experimental use. Specifically,it was hoped to obtain information about the patterns of sporocyst excretion and numbers of sporocysts shed and how these might be affected by such factors as the size of the infective dose, the mode of infection and any previous experience of infection.

3.2 MATERIALS AND METHODS

3.2.1 Experimental animals

Cats aged from 6 to 44 weeks were used. These were obtained from litters raised with their mothers in isolation. At weaning, kittens were removed and, except in one preliminary experiment, housed individually and fed on a diet of tinned fish and water.

Throughout the course of the study and prior to experimental infection, the faeces of all kittens were examined for extraneous coccidia. Apart from *Isospora felis* oocysts, which were recovered from most kittens at some time, no other coccidians were detected. No experiment was started until excretion of these oocysts had ceased.

3.2.2 Infection of cats

Cats were infected by feeding them intact cysts of *S. gigantea* excised from the oesophagi of naturally infected sheep obtained from a local Freezing Works. Infection was achieved

by incorporating a known weight of cysts in food. Unless stated, cysts were fed to kittens on the same day that they were collected, otherwise they were stored at 4°C in physiological saline.

3.2.3 Detection and counting of sporocysts

Following infection, the total faecal output of each cat was collected daily, weighed, thoroughly mixed and a representative 5 g sample removed. Up to the detection of the first sporocysts, a one-fifth subsample of this sample was processed as described in Chapter 2 except that floatation was carried out using a sucrose solution (SG = 1.2) and sporocysts collected using a standard coverslip recovery procedure. At detection of the first sporocysts shed and from then on, counts of the number of sporocysts per gram of faeces were performed using the floatation-haemocytometer technique (Chapter 2). By multiplying the concentration of sporocysts per gram by the total weight of faeces produced each day it was possible to obtain an estimate of the daily production of sporocysts shed by each cat. By summation of daily sporocyst productions the total sporocyst yield of each infection was calculated.

3.3 EXPERIMENTAL PROCEDURES AND RESULTS

3.3.1 Individual experiments on factors affecting sporocyst yields

(i) Preliminary observations

In order to obtain some indication of sporocyst output and the likely period of observation required, two preliminary investigations were conducted. The first involved four 6 month-old cats housed together (cats 79/1), the second, a single 6 to 7-week old kitten (cat 79/2). In the first investigation a total of 4 g of cysts (shared by all 4 cats)

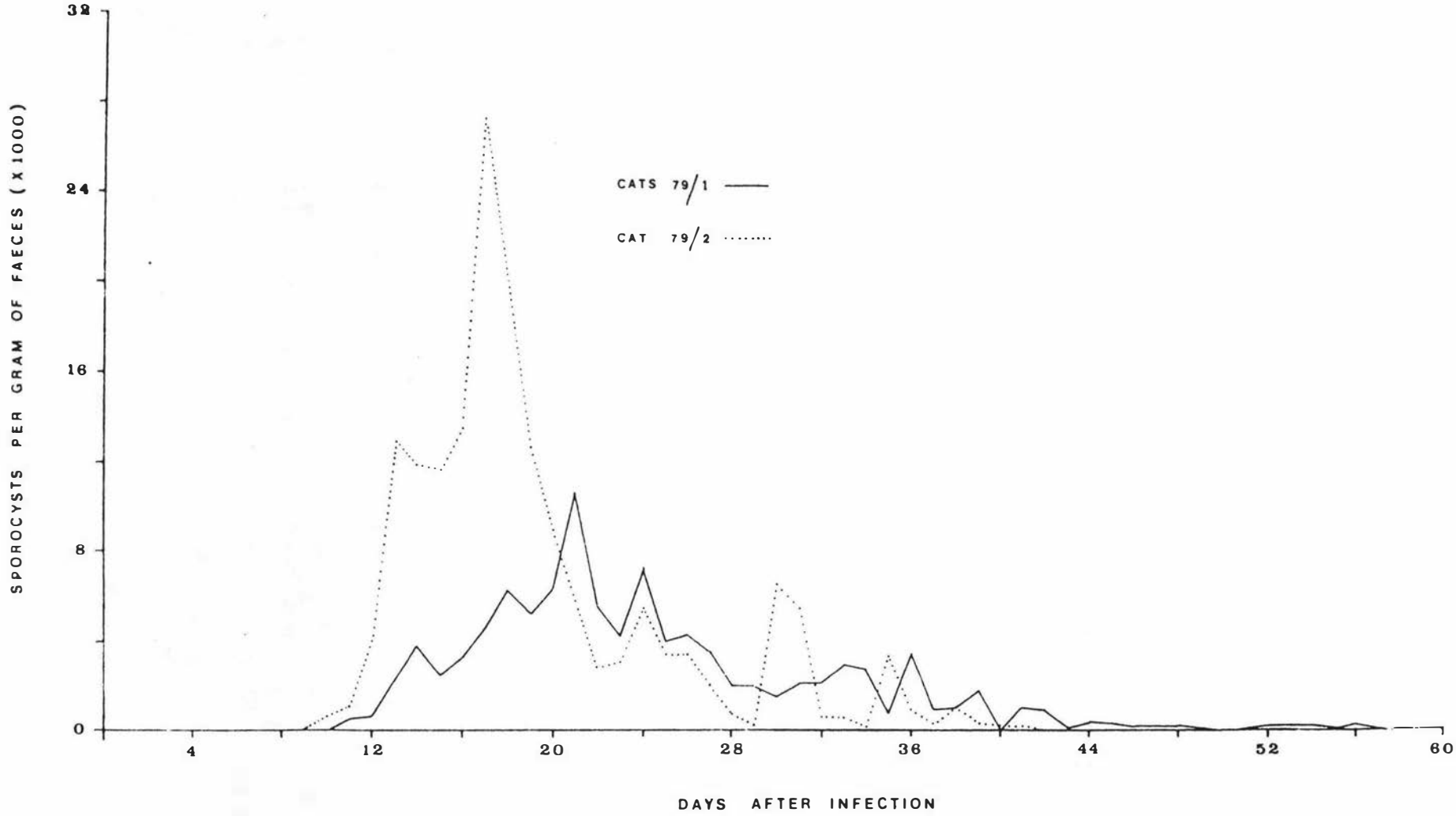


Fig. 3.1 Patterns of sporocyst excretion by *S. gigantea*-infected cats: preliminary investigations

was fed on a single occasion, in the second 3 g. The course of these infections was monitored as described (Section 3.2.3), continuing until no sporocysts were detected in faeces for 10 consecutive days. Because four cats were housed together in the first investigation (cats 79/1), their faeces were pooled and treated as one.

In both investigations the patterns of sporocyst shedding were similar (Fig. 3.1). Sporocyst shedding began 10 or 11 days after infection, rose to a peak 7 to 10 days later and then declined to lower levels until shedding ceased at 41 and 56 days post-infection (PI) in cat 79/2 and the group of four (79/1), respectively. Over the period of shedding, the four cats (79/1) produced a total of 25,078,532 or an average of 6,269,633 sporocysts each and cat 79/2, 7,694,263 sporocysts.

As a result of this study, a standard observation period of 60 day PI was adopted for all future experiments.

(ii) Effect of size of infective dose

Again, two experiments were performed. In the first (Expt. (a)), six kittens (6-7 weeks old)were allocated to three groups of two and each kitten in each pair was fed on a single occasion, either 1.5, 4.5 or 7.5 g of cysts from a common pool. The second experiment (Expt. (b)) was similar to the first except that the cysts were from a different common pool and each kitten (9-10 weeks old) in each pair received either 3, 6, or 9 g of cysts.

The results are presented in Figs. 3.2, 3.3 and Table 3.1. One of the kittens in Expt. (a) (cat 79/4), receiving 7.5 g of cysts, died 14 days after infection of unknown causes. All six kittens in Expt. (b) shed considerably more sporocysts than those in Expt. (a) with peak shedding reaching higher levels, and being maintained for longer periods.

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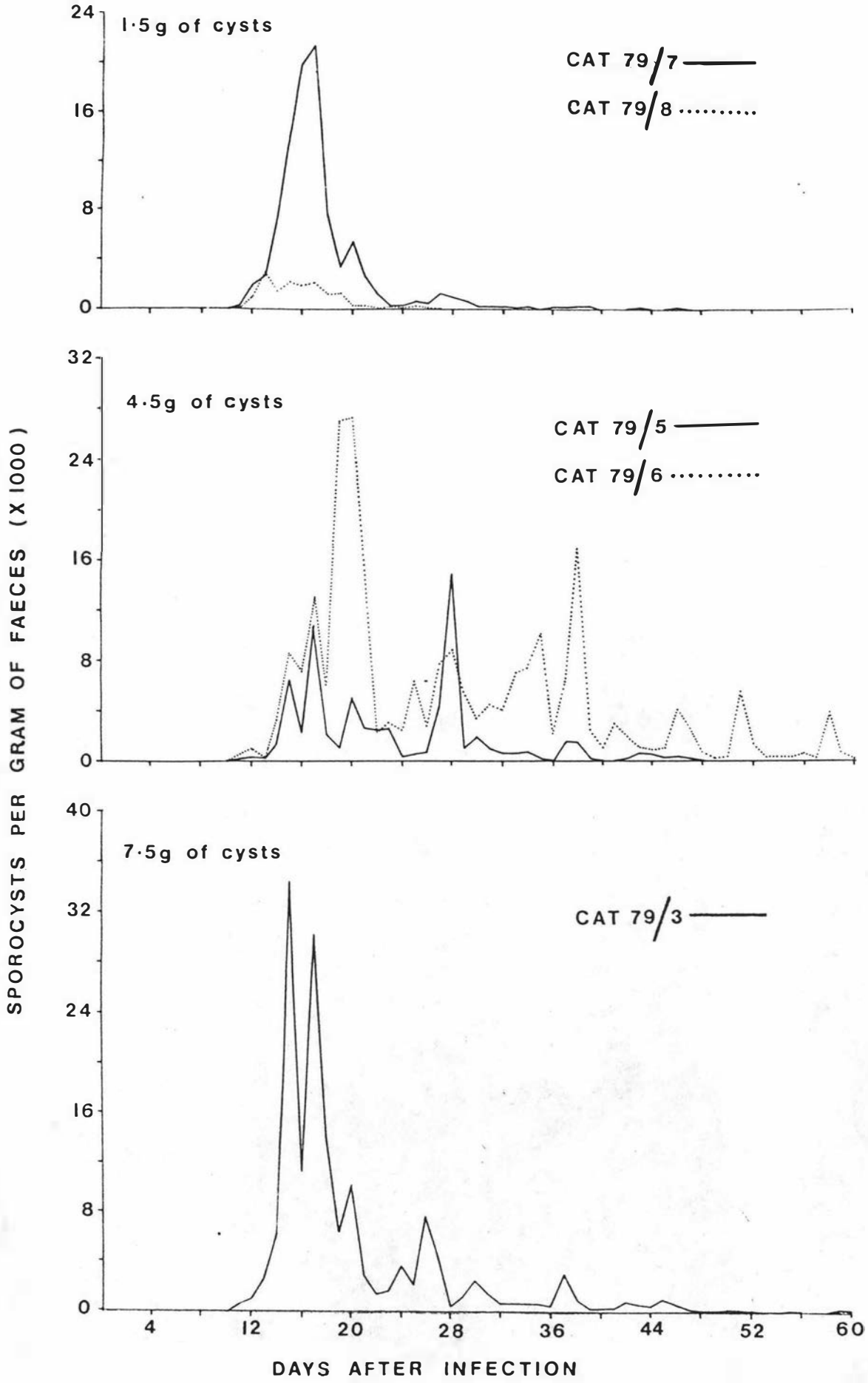
Fig. 3.2 Effect of infective dose size on sporocyst excretion by *S. gigantea*-infected cats: Expt. (a)


1·5g of cysts
CAT 79/7
CAT 79/8
4·5g of cysts
CAT 79/5
CAT 79/6
7·5g of cysts
CAT 79/3
SPOROCYSTS PER GRAM OF FAECES (X 1000)
DAYS AFTER INFECTION
0
8
16
24
32
40
4
12
20
28
36
44
52
60

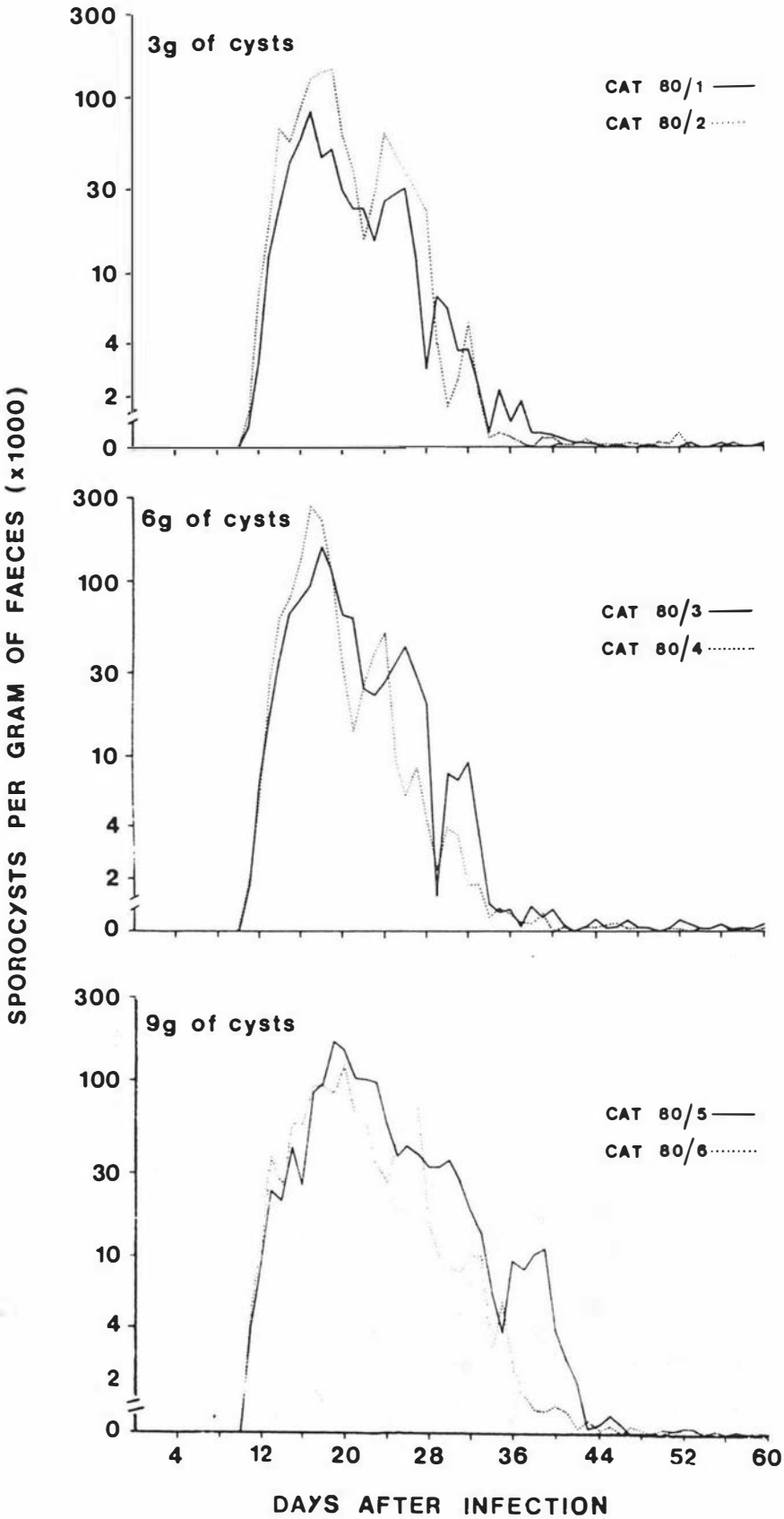


Fig 3.3 Effect of infective dose size on sporocyst excretion by *S. gigantea*-infected cats: Expt. (b)

TABLE 3.1 EFFECT OF INFECTIVE DOSE SIZE ON SPOROCYST PRODUCTION BY *S. GIGANTEA*-INFECTED CATS

	Cat No.	Infective dose (g of cysts)	Total sporocyst yield	Mean total sporocyst yield
	79/7	1.5	3,452,194	2,014,082
	79/8	"	575,969	
Expt. (a)	79/5	4.5	1,747,208	4,923,162
	79/6	"	8,099,115	
	79/3	7.5	6,389,645	-
	79/4*	"	-	
	80/1	3.0	12,881,036	19,481,313
	80/2	"	26,081,589	
Expt. (b)	80/3	6.0	27,861,688	29,586,283
	80/4	"	31,310,878	
	80/5	9.0	56,648,949	40,816,299
	80/5	"	24,983,648	

*Died 14 days post-infection

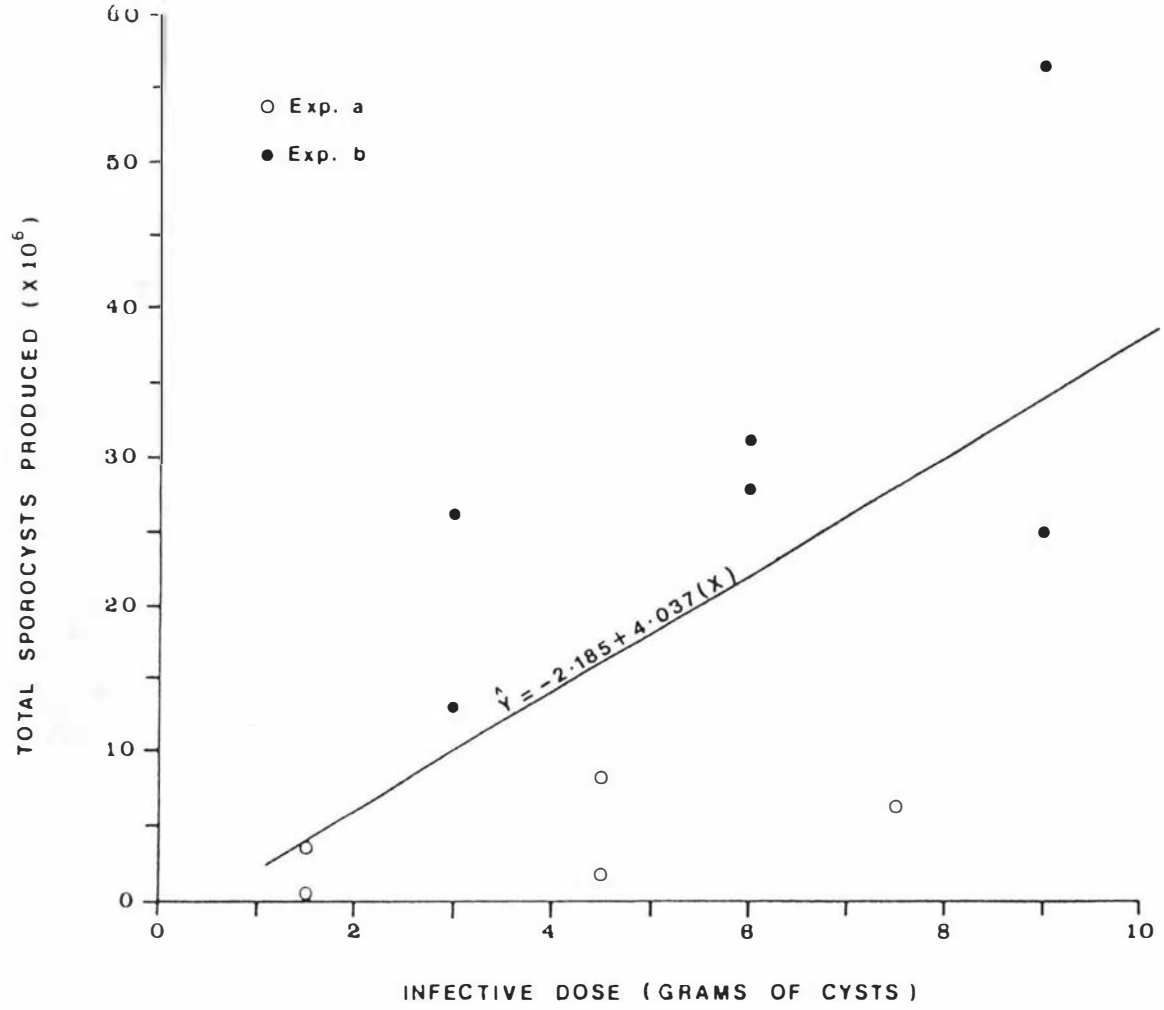


Fig. 3.4 Regression of infective dose size against total sporocysts produced by cats in experiments (a) and (b)

Within each experiment there was a tendency for greater sporocyst yields to be associated with higher infective doses. A common regression analysis for both experiments was computed with 9 degrees of freedom (Fig. 3.4). The r^2 value for this regression was 0.4087 indicating that about 41% of the variation in total sporocyst production was due to the size of the infective dose. The slope of the regression line was significant at the 5% level ($t = 2.4942$).

(iii) Effect of previous experience

It is commonly believed that re-infection with *Sarcocystis* is easily achieved but whether re-infection of hosts with previous experience of *Sarcocystis* results in any subsequent diminution in the numbers of sporocysts shed is unknown. An experiment was undertaken to investigate this possibility.

Six kittens, 6 to 7 weeks of age, were allocated to three groups of two. The first pair of kittens (cats 79/5, 79/6) were each infected on days 0, 90 and 195, the second (cats 79/9, 79/10) on days 90 and 195 and the third (cats 79/11, 79/12) on day 195 of the experiment. On each occasion each kitten was fed 4.5 g of cysts from a common pool. By this means it was possible to compare sporocyst shedding in kittens infected on one (6 kittens), two (4 kittens) or three (2 kittens) separate occasions.

All six kittens shed sporocysts on each occasion that they were infected and, although there was some variability in their patterns of excretion, there was no evidence to suggest that previous experience had any effect on either the length of the prepatent (i.e. the time between infection and the commencement of shedding) or the patent period (i.e. duration of shedding) (Fig. 3.5). The results also indicated that experience of one or two previous infections had no significant effect ($P > 0.05$) on the numbers of

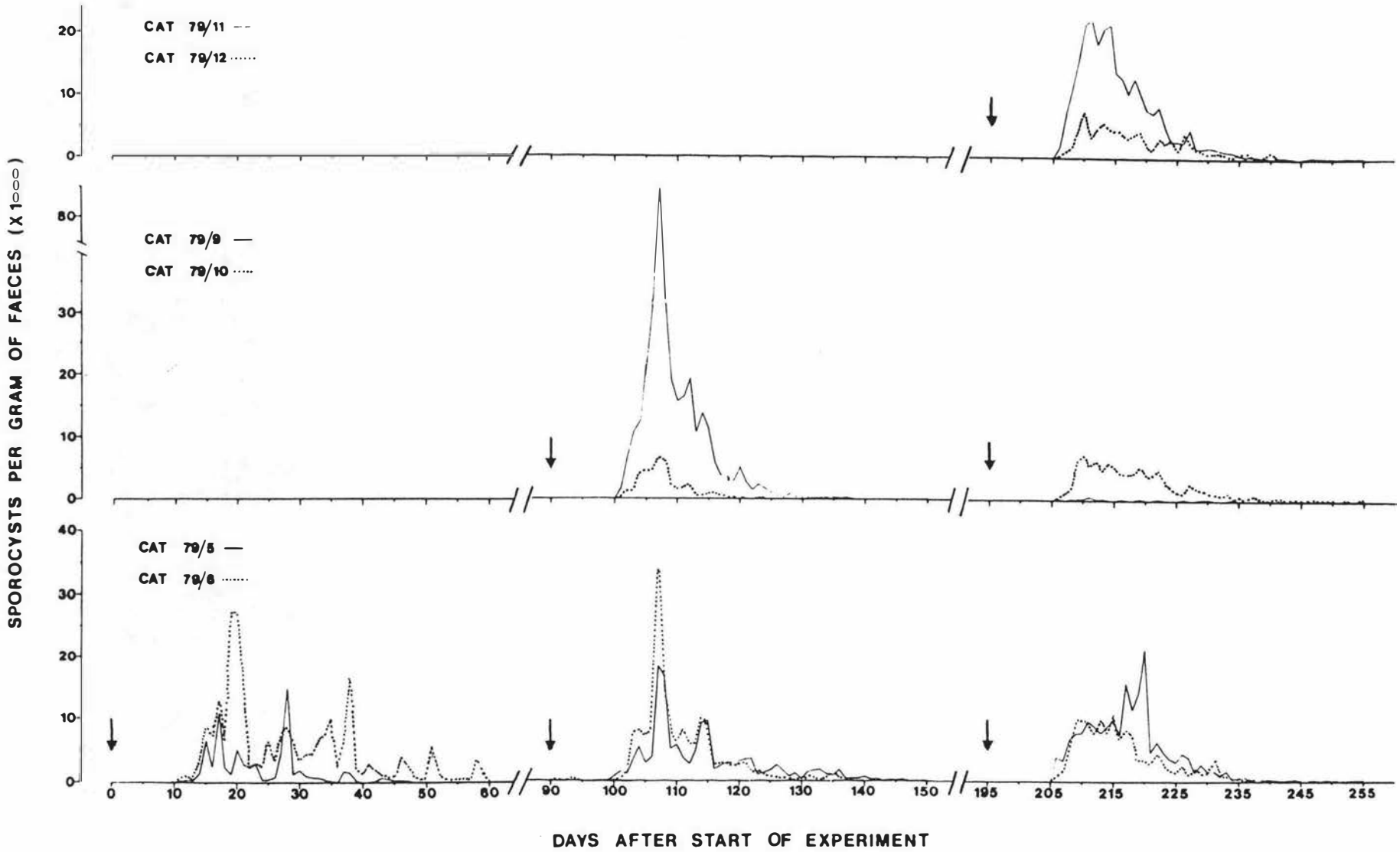


Fig. 3.5 Effect of previous experience on sporocyst excretion by *S. gigantea*-infected cats. Arrows indicate times of infection

TABLE 3.2 EFFECT OF PREVIOUS EXPERIENCE ON SPOROCYST YIELDS IN *S. GIGANTEA*-INFECTED CATS

	Infection day/Total sporocysts produced		
Cat No	0	90	195
79/5	1,747,208	7,918,054	11,575,098
79/6	8,099,115	11,368,553	9,860,371
Mean	4,923,162	9,643,304	10,717,735
79/9		19,800,171	164,275
79/10		3,058,192	7,454,387
Mean		11,429,182	3,809,331
79/11			16,464,650
79/12			6,739,885
Mean			11,602,268

TABLE 3.3 ANALYSIS OF VARIANCE OF DATA IN TABLE 3.2

Source of variation	df	SS	MS	F
Between infections	2	$2.601537679 \times 10^{13}$	$1.30076884 \times 10^{13}$	0.3509 ns
Within infections	9	$3.336161778 \times 10^{14}$	$3.70684642 \times 10^{13}$	
Total	11	$3.596315546 \times 10^{14}$		

F.05 (2,9) = 4.26; ns = not significant

sporocysts shed (Tables 3.2 and 3.3).

(iv) Single versus multiple infective doses

In previous experiments kittens were infected by feeding them cysts in a single meal. It was decided, therefore, to compare sporocyst shedding in cats infected in this manner with those receiving equivalent infective doses spread over a short period of days.

Six kittens 15-16 weeks old, were divided into two groups of three. All three kittens in one group each received 5 g of cysts on a single occasion, the other three each received 1 g of cysts per day for 5 consecutive days as indicated in Table 3.4. The cysts were all taken from the same common pool.

The results are presented in Fig. 3.6 and Table 3.5. Although the patterns of sporocyst excretion in all six kittens were somewhat similar, the duration of shedding in those receiving multiple infections tended to be longer (average 47 days) than those receiving single infections (42 days). Similarly, the total numbers of sporocysts produced by animals in the former group also tended to be greater; kittens infected on five separate occasions producing on average, three times as many as those infected once. These differences, however, were not significantly different (t-test; $P > 0.05$, $t = 2.108$, 4 df).

3.2.2 Accumulated data on sporocyst production

The preceding experiments provide information regarding sporocyst production in cats of various ages, sexes and experience subjected to differing levels and modes of infection. These experiments involved a total of 28 individual infections (all cats except 79/1). In the

TABLE 3.4 EXPERIMENTAL PLAN: MULTIPLE VERSUS SINGLE INFECTIVE DOSES

	Infection day/Infection dose*					
Cat No.	0	1	2	3	4	Total
80/7	1	1	1	1	1	5
80/9	1	1	1	1	1	5
80/11	1	1	1	1	1	5
80/8	-	-	5	-	-	5
80/10	-	-	-	-	5	5
80/12	5	-	-	-	-	5

*Weight of cysts (grams)

TABLE 3.5 EFFECT OF MULTIPLE VERSUS SINGLE INFECTIVE DOSES ON SPOROCYST YIELD IN *S. GIGANTEA*-INFECTED CATS

Infective dose	Cat No.	Total sporocysts produced
1 g x 5	80/7	13,297,368
	80/9	19,107,685
	80/11	6,871,236
Mean		13,092,096
5 g x 1	80/8	7,915,384
	80/10	1,929,509
	80/12	4,542,860
Mean		4,759,918

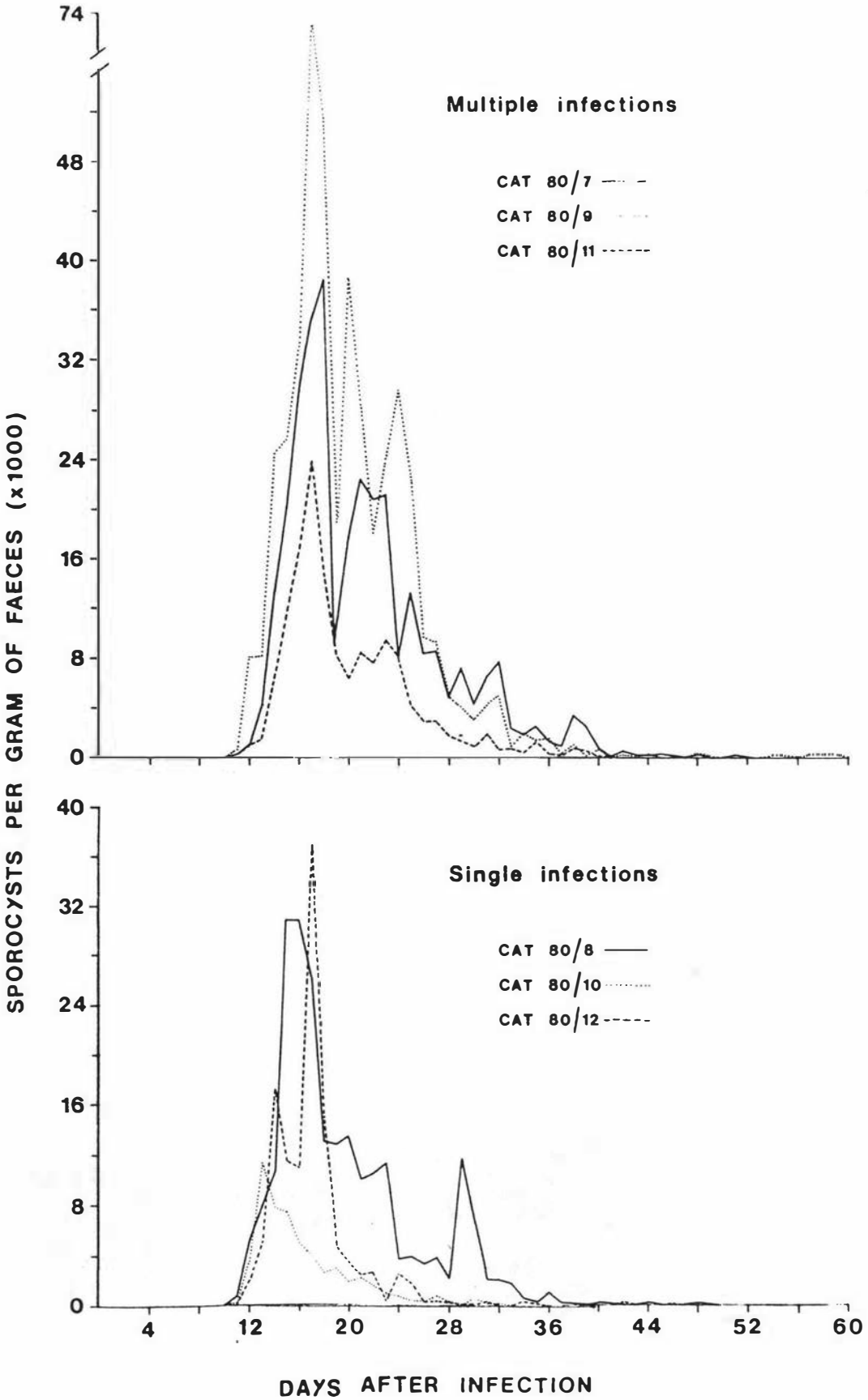


Fig. 3.6 Sporocyst shedding by *S. gigantea*-infected cats given single or multiple infective doses

following section accumulated data from these infections are further analysed to provide additional information regarding sporocyst production by *S. gigantea* infected cats.

(i) General patterns of sporocyst shedding and production

Data relating to the pattern and number of sporocysts shed in the 28 infections are summarised in Figs. 3.7, 3.8 and 3.9. These reveal a fairly consistent pattern of sporocyst shedding although there were considerable variations in the numbers shed. In all cats, sporocyst excretion commenced either 10 or 11 days after infection. Invariably, however, sporocysts produced on day 10 were improperly sporulated. Small proportions of such abnormal sporocysts were occasionally present on day 11 also but by day 12 virtually all sporocysts were fully developed. Peak production occurred between 13 and 22 days PI but in most instances peak numbers were shed on days 17 and 18. Peak numbers ranged from 556 to 260,021 (mean 53,066) sporocysts per g of faeces or from 37,919 to 6,604,372 (mean 1,719,536) sporocysts per day.

The number of days sporocysts were shed ranged from 26 to at least 60 days PI with most cats still shedding at the termination of the experiments (60 days PI). The total number of sporocysts produced in individual infections over these periods ranged from 164,275 to 56,648,949 (mean 12,706,924).

Although the patent period in most infections was usually long, sporocysts were not always shed every day within this period. In fact, sporocyst shedding every day of the patent period occurred in only 5 of the 28 infections. In most cases consistent daily shedding took place up to day 30 PI, thereafter sporadic shedding of only small numbers

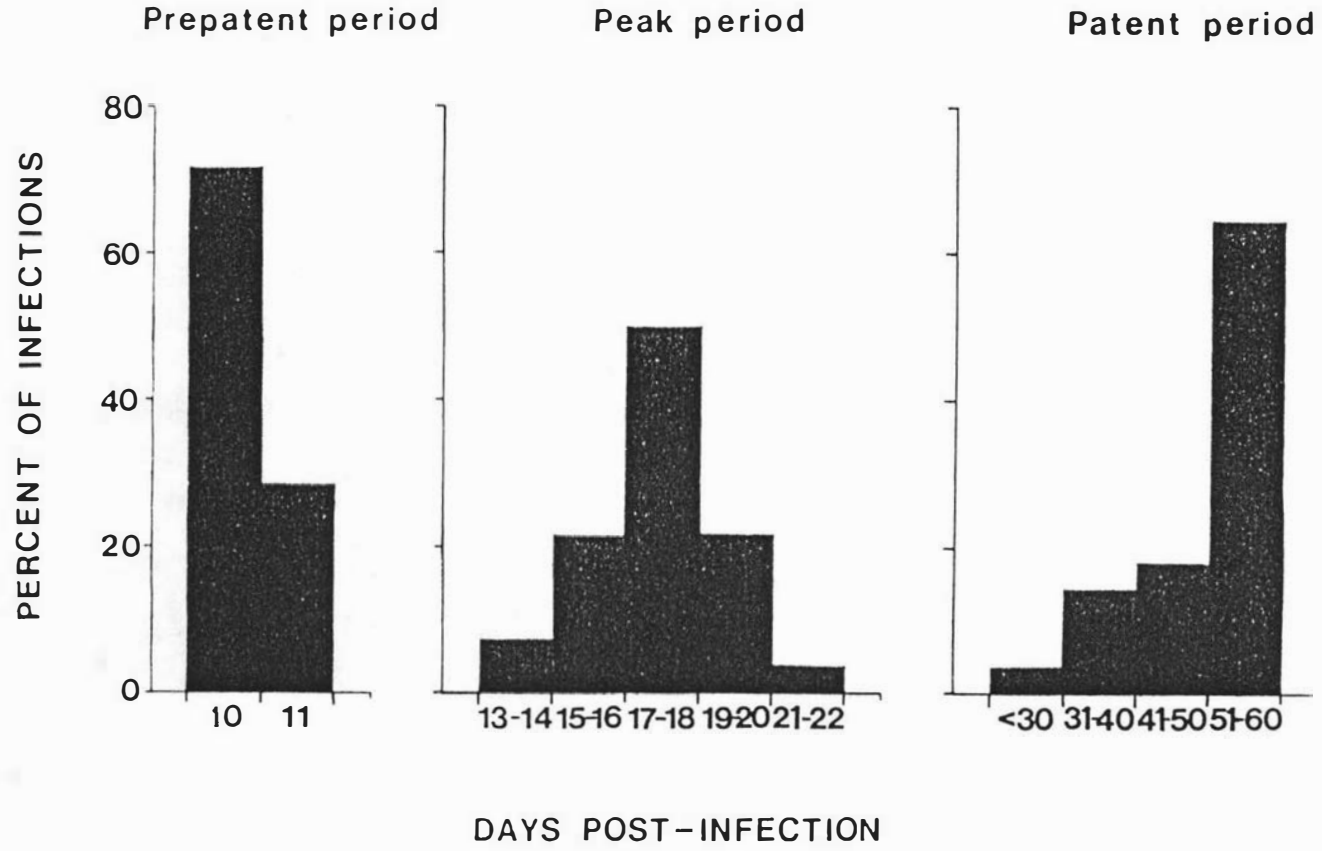


Fig. 3.7 Frequency distributions of prepatent, peak and patent periods of *S. gigantea*-infected cats (data from 28 infections)

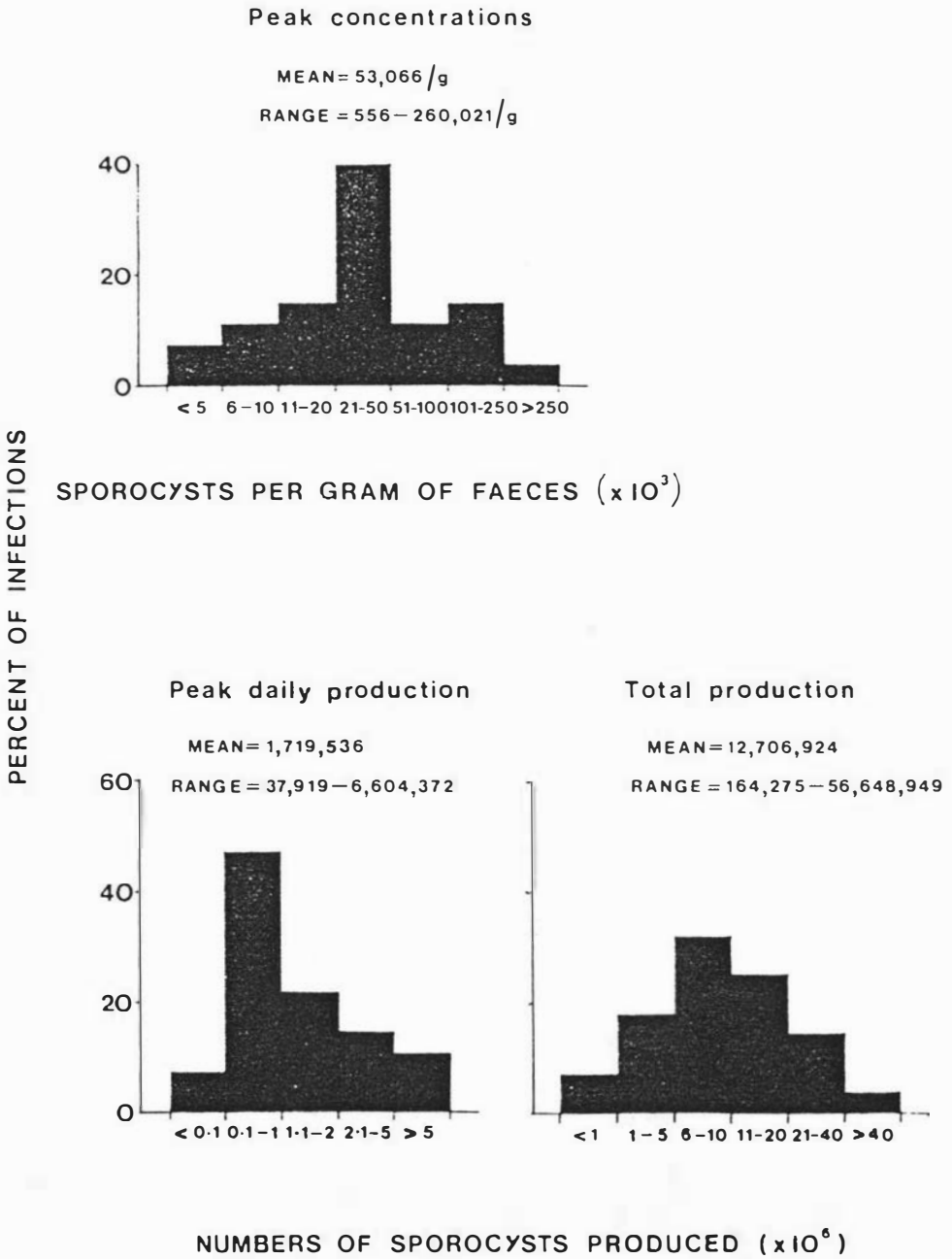


Fig. 3.8 Frequency distributions of sporocyst production by *S. gigantea*-infected cats (data from 28 infections)

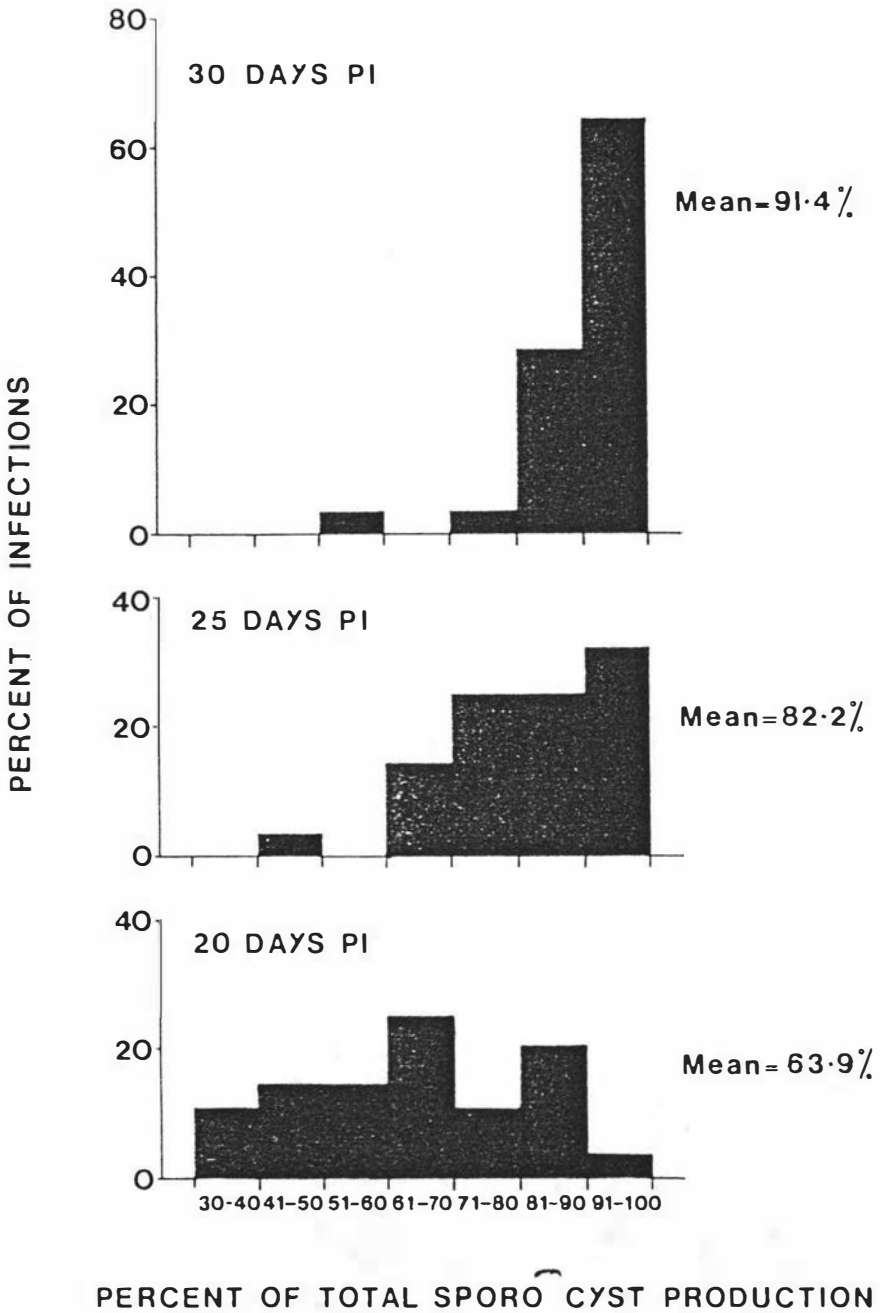


Fig. 3.9 Frequency distributions of percentages of total sporocyst yields in 28 *S. gigantea* infections at 20, 25 and 30 days post-infection

of sporocysts usually occurred. This is illustrated by the fact that in 26 of the 28 infections more than 80% of the total sporocyst yields were recovered within the first 30 days following infection.

(ii) Efficiency of infections

In parasitological investigations, the establishment of experimental infections is often expressed in terms of percentage efficiency. Thus in parasitic nematode infections, the number of adult worms present in hosts may be given as a percentage of the number of infective larvae administered. Comparable estimates of sporocyst production efficiency could be made if the numbers of zoites administered and the proportion likely to produce macrogametocytes were known. Accordingly, an attempt was made to estimate the production efficiency for the *S. gigantea* infections described above.

Zoites from *S. gigantea* cysts were liberated by placing 0.5 g of intact cysts in 10 ml of 0.1% HCl. The number of zoites in 1 g of cysts was then calculated using a Neubauer chamber and a 1:10 dilution of this material according to standard haemocytometer methods (Dacie and Lewis, 1975). These calculations showed that 1 g of cysts contained approximately 1670×10^6 zoites.

The proportion of *S. gigantea* zoites which will differentiate into macro- and microgametocytes is not known but the simplest assumption that they would produce 50% of each seems unlikely to be correct. Since each microgametocyte is capable of producing sufficient microgametes to fertilise several macrogametes, it is more likely that considerably more zoites differentiate into macro- rather than microgametocytes; a likelihood which is supported by the general paucity of microgametocytes found in experimental *Sarcocystis* infections (Mehlhorn and Heydorn, 1978).

There is also no information available regarding the number of microgametes produced by microgametocytes of *S. gigantea* but those of *S. suihominis* have been found to produce 20 to 30 each (Mehlhorn and Heydorn, 1979), those of *S. debonei* 13 to 17 (Box and Duszynski, 1980) and those of *S. cruzi* 3 to 11 (Dubey, 1982a). If similar numbers are produced by microgametocytes of *S. gigantea*, then it may be reasonable to assume that as many as 90% of zoites may differentiate into macrogametocytes. On this basis, 1 g of *S. gigantea* cysts containing 1670 x 10^6 zoites would produce 1503 x 10^6 macro- and 167 x 10^6 microgametocytes. Assuming maximum efficiency, each microgametocyte would need to produce (1503 x 10^6 ÷ 167 x 10^6) 9 microgametes to ensure all macrogametocytes were fertilised, a figure well within the ranges cited above. If 1 g of cyst material is capable of producing 1503 x 10^6 macrogametocytes and 100% fertilisation is assumed, such an infective dose could theoretically produce 1503 x 10^6 oocysts or 2 x 1503 x 10^6 sporocysts. Accordingly, the percentage efficiency of any infective dose may be obtained from the formula:

$$\% \text{ efficiency} = \frac{\text{Total No. sporocysts produced}}{X \times 3006 \times 10^6} \times \frac{100}{1}$$

where X = the weight of the infective dose in grams. Calculations on this basis for the 28 individual infections showed efficiencies ranging from 0.001% to 0.289% with a mean of 0.085%[1].

There appeared to be little association between these measurements and any of the parameters examined in the

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[1] Since completion of this study, a recent paper (Dubey, 1983f) has revealed that approximately 61% of the zoites of *S. hirsuta* muscle cysts differentiate into macrogametocytes in the cat intestine. If this same proportion is applied to *S. gigantea* cysts, then efficiencies ranging from 0.002% to 0.427% (mean 0.126%) are obtained.

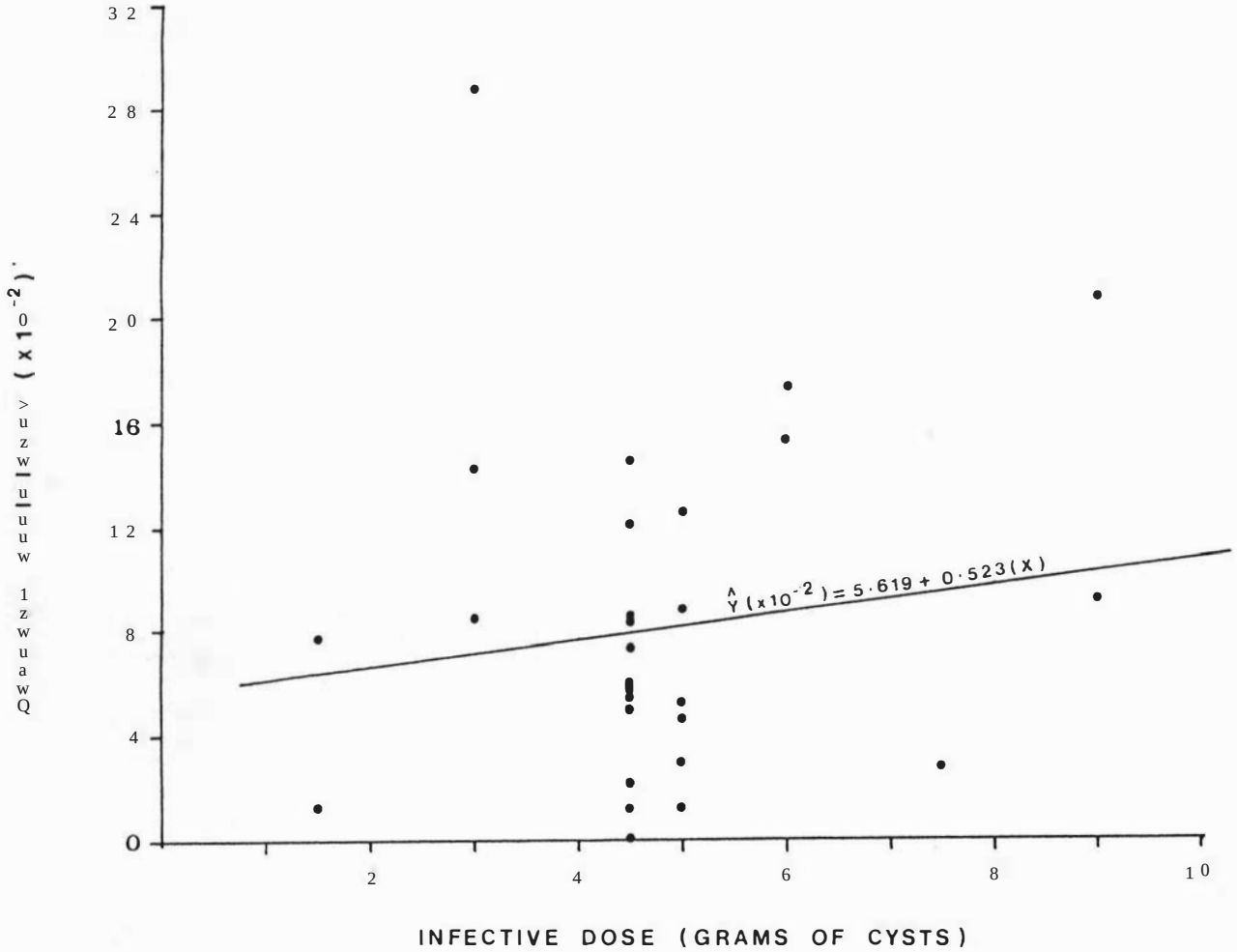

Fig. 3.10 Regression of production efficiency against infective dose size in 28 individual *S. gigantea* infections

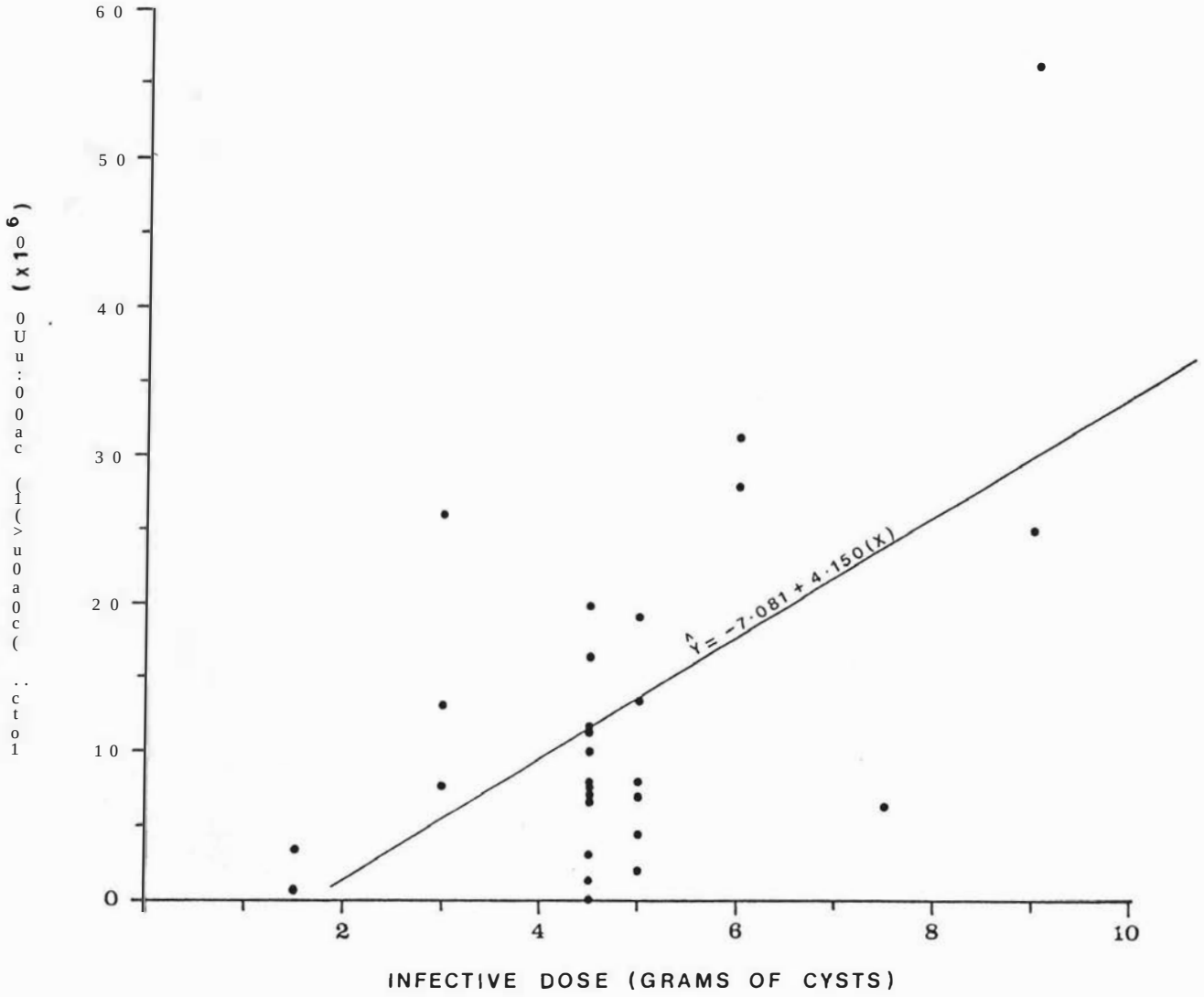

Fig.3.11 Regression of total sporocysts produced against infective dose size in 28 individual *S. gigantea* infections

study although there was an apparent, but non-significant ($P > 0.05$, $t = 2.107$, 4 df), tendency for efficiency to be greater with multiple rather than with single infective doses. A regression analysis of efficiency against infective dose size for the 28 infections revealed a non-significant slope ($P > 0.05$, $t = 0.730$, 26 df) and an r^2 value of only 0.01919 (Fig. 3.10). Interestingly, a comparable regression for total sporocysts yielded against size of infecting dose produced an r^2 value of 0.3396 with a highly significant ($P < 0.01$, $t = 3.656$) slope of the regression line (Fig. 3.11).

(iii) Effect of sex of hosts

Data relating to the numbers of sporocyst shed in the 28 infections were examined for any effects of host sex. The results (Table 3.6) showed that although the mean total numbers of sporocysts shed by males tended to be greater than those shed by females, these differences were not statistically significant ($P > 0.05$, $t = 0.782$, 26 df).

3.4 DISCUSSION

One of the characteristics of 'conventional' coccidial infections is that they are self-limiting in time. The duration of this period is determined primarily by the number of asexual generations, the length of transition from asexual to sexual generation and by the duration of gametogenesis. Thus, in homoiothermic animals, the length of the prepatent period of each coccidial species is relatively constant and while the duration of patency may be influenced by host factors, it is also largely influenced by the developmental cycle of the parasite (Kheysin, 1972).

In *Sarcocystis* spp. infections, gametogony proceeds very rapidly with development to the zygote stage usually being

TABLE 3.6 EFFECT OF HOST SEX ON SPOROCYST YIELD IN *S. GIGANTEA*-INFECTED CATS

Sex	No of infections	Infective dose* (g of cysts)	Sporocyst yield*
Male	21	4.88 ± 0.35	13,748,299 ± 2,851,660
Female	7	4.43 ± 0.77	9,582,796 ± 3,317,589
Total	28	4.77 ± 0.32	12,706,924 ± 2,291,227

*Mean ± SE

completed 18 to 48 hr after infection (Becker *et al.*, 1979; Box and Duszynski, 1980). Despite the rapidity of this development both the prepatent and patent periods of *Sarcocystis* spp. have generally been found to be comparatively long. In the present study, for example, sporocyst shedding was first detected 10 or 11 days after infection and was found to continue for 60 days or more post-infection. Other workers have recorded similar findings for this species with prepatent and patent periods ranging from 11 to 13 and 8 to 76 days respectively (Rommel *et al.*, 1972; Mehlhorn and Scholtyseck, 1974; Bratberg *et al.*, 1982).

Although the patent period of *S. gigantea* was usually found to be long, the present results clearly indicate that the harvesting of sporocysts for experimental purposes is likely to be most profitable between post-infection days 12 to 30. The results also suggest that naturally infected cats could provide foci of heavy sporocyst concentrations during this period with shedding for another 30 days or more, aiding the dispersal of sporocysts over large areas of roaming cats. Whether similar patterns of sporocyst excretion are exhibited by other definitive hosts infected with other species of *Sarcocystis* is not clear, although there is some evidence that they are (Ford 1974; Fayer, 1977b).

The reasons for the relatively long prepatent and patent periods in *Sarcocystis* infections are not completely understood. One possible explanation put forward for extended patency is that zoites from muscle cysts 'wait' somewhere within the definitive host and develop successively into gametocytes (Mehlhorn and Heydorn, 1978). More recent findings, however, indicating the absence of all developmental stages other than oocysts in five species of *Sarcocystis* 22 hr after infection (Becker *et al.*, 1979) appear to rule out this explanation. A second and more likely suggestion, is that the deep location of

gametocytes and oocysts within the intestinal tissues results in their gradual release into the lumen. This location may also explain the excretion of free mature sporocysts in faeces (Tadros and Laarman, 1976). Thus, unlike oocysts of conventional coccidia which are found in the epithelial layer of the intestine and are, therefore, readily released, those of *Sarcocystis* are temporarily trapped. This temporary entrapment in the lamina propria, the oxygen level of this richly vascular tissue and the body temperature of the host, allow the *Sarcocystis* oocysts to sporulate.

The means by which the sporocysts make their way to the lumen of the intestine is also unknown. The simplest explanation is that this is brought about passively by the contraction of the fine muscular strands within the lamina propria during peristalsis. This could force the oocysts to the tips of the intestinal villi where cellular replacement of the epithelial layer is most active (Tadros and Laarman, 1976). Since the oocyst wall of *Sarcocystis* spp. appears to be very delicate (Mehlhorn and Scholtyseck, 1974) this mechanical force may also be responsible for the release of the sporocysts from the oocyst. Obviously, the interval between infection and the first appearance of sporocysts in faeces will depend to a large extent on the time required for sporulation and the period necessary to complete migration from the lamina propria. But this does not entirely explain why unsporulated oocysts are not detected in faeces earlier in the course of infection. Certainly no such oocysts were recovered at any stage in the present investigation. It is possible, of course, that unsporulated oocysts were being continuously shed but destroyed by the hypertonic intestinal contents, because of the delicate nature of the oocyst wall.

Information relating to total sporocyst output is of

particular interest. Previous workers (Rommel *et al.*, 1972; Mehlhorn and Scholtyseck, 1974) have suggested that sporocyst production in cats infected with *S. gigantea* is generally poor. These opinions, however, are based on subjective impressions only and are not supported by the present findings. Indeed, a comparison between the present results and those of other studies (Table 3.7) suggests that cats are capable of excreting as many sporocysts as other hosts. It is recognised, nonetheless, that such comparisons may not be strictly valid because of the different species of *Sarcocystis* involved and the different and usually undetermined amounts of infective material administered.

There were considerable variations between the total numbers of sporocysts shed by cats in this investigation. These variations were not only limited to differences between animals in different experiments but were also apparent both between cats receiving the same infective doses from the same common pools (Tables 3.2, 3.5) and between the same cats infected on different occasions (Table 3.5). Notwithstanding, total sporocyst yields tended to increase with increased dose size and to be greater in those cats receiving multiple rather than single infective meals. Similar results to the former finding have been reported for conventional coccidia but the number of oocysts yielded per oocyst administered (efficiency of infection) has usually been found to decline at high dose levels (Kheysin, 1972). Interestingly, this phenomenon, which was not observed in the present study, is less pronounced in species with shorter developmental cycles than in those with longer ones (Kheysin, 1972). The size of the infective dose resulting in maximum oocyst production, above which efficiency begins to decline, also varies with species (Kheysin, 1972). It is possible, therefore, that the dose levels employed in the present

TABLE 3.7 SPOROCYST PRODUCTION IN EXPERIMENTALLY INFECTED DEFINITIVE HOSTS

Species	Intermediate host	Definitive host (and No.)	Exp. period (days)	Sporocyst yield (millions)	Reference
S. tenella	sheep	dog (2)	60	90 (each)	Ford, 1974
S. tenella?	sheep	fox (1)	34	13	Ashford, 1977
S. cruzi	cattle	dog (21)	40-60	0.8-20	Fayer, 1977
S. gigantea	sheep	cat (28)	60	0.16-56	Present study

study far exceed those necessary to attain optimum efficiency in *S. gigantea* infections. This might account for the extremely low efficiencies recorded here.

The decrease in efficiency of conventional coccidial infections at high dose levels has been variously attributed to 'over population' of the intestinal epithelium and/or to the rapid development of immunity following ingestion of a massive amount of antigenic material (Kheysin, 1972; Levine, 1973). Unlike conventional coccidia, however, *Sarcocystis* infections are considered to confer little immunity to their definitive hosts as evidenced by the lack of cellular reaction induced (Fayer, 1974; Munday *et al.*, 1975) and their apparent ability to reinfect their hosts repeatedly (Rommel *et al.*, 1972; Fayer, 1974; Ruiz and Frenkel, 1976). This claim is further and considerably strengthened by the findings of the present study in which previous experience of infection was found not to result in any diminution of sporocyst production.

In some eimerian coccidia it is schizogony which is considered to stimulate protective immunity with gametogony reputedly having little immunising effect (Rose, 1973). It is tempting, therefore, to speculate that both the evident lack of protective immunity and the apparent absence of an inverse relationship between dose size and efficiency in *S. gigantea* infected cats may be related to the absence of schizogony.

CHAPTER 4

IN VITRO EXCYSTATION OF *SARCOCYSTIS GIGANTEA* SPOROCYSTS

4.1 INTRODUCTION

Following ingestion of sporulated oocysts, one constraint governing the further development of coccidial infections must be the liberation of their sporozoites. For most species of conventional coccidia this process of excystation has usually been found to take place in two phases. The first, or pretreatment phase, affects the permeability of the oocyst wall allowing entry of the second, or treatment phase stimuli, which act upon the sporocysts and possibly the sporozoites. Trypsin and bile are recognised as the most important second phase stimuli and are normally encountered in the small intestine (Jackson, 1962). The requirements for the initial or pretreatment phase, however, appear to differ for coccidia infecting different hosts. Thus for some species of poultry coccidia, mechanical breakage of the oocyst wall, such as may occur in the gizzard, has been found necessary (Doran and Farr, 1962; Farr and Doran, 1962) whereas for coccidia infecting ruminants, chemical changes induced by conditions in the rumen may be required (Jackson, 1962).

The permeability of the oocyst wall may be enhanced *in vitro* by incubation in a reducing medium, usually cysteine hydrochloride, under CO_2 (Jackson, 1962; Hibbert and Hammond, 1968; Bunch and Nyberg, 1970). A similar effect may also be achieved by removal of the outer wall layers using sodium hypochlorite (Speer *et al.*, 1973; Nyberg and Knapp, 1970) and both techniques have been used

to study the excystation process of a number of species of coccidia. With the former procedure the exposure time necessary to achieve the optimum effect has been found to vary from species to species (Bunch and Nyberg, 1970).

Unlike those of conventional coccidia, sporocysts of *Sarcocystis* spp. are not protected by an oocyst wall. It might seem reasonable to assume, therefore, that pretreatment is not an essential prerequisite for the excystation of members of this genus. That this assumption may be incorrect, is suggested by the study of Fayer and Leek (1973) who found that a pretreatment stimulus similar to that employed with conventional ruminant coccidia was necessary to induce *S. cruzi* sporozoites to excyst.

Apart from *S. cruzi*, the excystation requirements of other *Sarcocystis* spp. have not been studied in any detail. The present investigation was undertaken to examine the effect of various factors on the *in vitro* excystation of *S. gigantea* sporocysts with the ultimate aim of using this procedure to determine sporocyst viability and survival.

4.2 MATERIALS AND METHODS

4.2.1 Sporocysts

Sporocysts of *S. gigantea* excreted in the faeces of experimentally infected cats were recovered as previously described (Chapter 2). Those of *S. tenella* and *S. capracanis* were obtained by similar means from dogs fed naturally infected sheep hearts and goat meat, respectively. Unless otherwise stated, all sporocysts used in these experiments were stored in aqueous 2.5% potassium dichromate at 4°C for

1 to 2 months before use.

4.2.2 CO_2 pretreatment

Sporocysts were washed free of storage solution by suspension and centrifugation in four changes of tap water and one of distilled water. Equal volumes of sporocyst suspension in distilled water and freshly prepared 0.04 M solutions of cysteine hydrochloride, sodium dithionite or ascorbic acid, were placed in 100 ml Erlenmeyer flasks, giving final concentrations of 0.02M. The flasks, fitted with inlet and outlet valves, were flushed for 2 min with CO_2. They were then sealed and incubated at 39°C in a water bath. Following incubation, the sporocysts were washed twice with distilled water and twice with Ringer's solution in which they were finally suspended.

4.2.3 Pepsin-HCl pretreatment

One ml aliquots of cleaned sporocysts in distilled water were made up to 10 ml with either 5, 20 or 100% solutions of a pepsin-HCl mixture and incubated under air at 39°C. The pepsin-HCl mixture consisted of 1.5 g of pepsin (1:10,000, BDH Chemicals Ltd) and 10 ml of HCl in 600 ml of water. After incubation, sporocysts were washed free of pepsin-HCl solution and suspended in Ringer's as above.

4.2.4 Sodium hypochlorite pretreatment

Samples of sporocysts in distilled water were suspended in 9 volumes of 0.5% sodium hypochlorite (final concentration = 0.45%) and placed in an ice bath. After the required interval they were washed free of sodium hypochlorite with five changes of tap water, one of distilled water and two of Ringer's solution.

4.2.5 Treatment with excysting medium

Pretreated and non-treated sporocysts suspended in Ringer's solution were added to equal volumes of freshly prepared excysting medium consisting of bile, trypsin (1:300 ICN Pharmaceuticals Inc.) and buffer and incubated in a water bath. The interval and temperature of incubation varied according to the experiment, as did the concentration of trypsin and bile, the type of bile and the buffer. Natural biles used in these experiments were pooled from at least five animals and were frozen until use. All buffers were prepared following the methods of Gomori (1955).

4.2.6 Determination of excystation

Wet mount slides were prepared after the appropriate incubation period in excysting medium and examined microscopically (Magnification x 640) for excysted sporocysts. At each observation interval, 100 randomly selected sporocysts were counted to determine the percentage excysted. Observations showed that excysted sporocysts of *S. gigantea* frequently separated into four elongated pieces (see Section 4.3.3). Determination of the number of sporocysts excysted was made by counting the number of such 'pieces' and dividing by four.

4.3 EXPERIMENTAL PROCEDURES AND RESULTS

All results represent the means of at least four experiments ± the standard error.

4.3.1 Studies on pretreatment stimuli

(i) Effect of pretreatment on potassium dichromate-stored sporocysts

Sporocysts of *S. gigantea* washed free of potassium dichromate

and suspended in distilled water were divided into 14 aliquots. Four aliquots were pretreated with CO_2 and cysteine hydrochloride for 1, 4, 8 or 24 hr, six were pretreated with 5, 20 or 100% solutions of pepsin-HCl mixture for 1 or 6 hr and three were pretreated with sodium hypochlorite for 15, 30 or 60 min. The final aliquot was not subjected to any pretreatment. All pretreated and non-pretreated sporocysts suspended in Ringer's solution were added to excysting medium consisting of 5% (v/v) ovine bile, 0.25% (w/v) trypsin (final concentrations) and tris buffer (pH 7.5). They were then incubated at 39°C for 6 hr after which the percentage excystation was determined.

The results (Table 4.1) showed that only sodium hypochlorite pretreatment resulted in any significant degree of excystation with the level of excystation tending to increase with increasing duration of exposure. Following CO_2-cysteine hydrochloride pretreatment, only low levels of excystation were attained. No excystation was recorded in pepsin-HCl pretreated or non-pretreated sporocysts.

(ii) Effect of pretreatment on water-stored sporocysts

The failure of the sporocysts to respond to any significant extent to cysteine hydrochloride and CO_2 was unexpected since this form of pretreatment has been used to excyst oocysts of many conventional coccidia as well as sporocysts of several *Sarcocystis* spp. including *S. cruzi* of cattle (Fayer and Leek, 1973), *S. meischeriana* and *S. suihominis* of pigs, *S. capreolicanis* of roe deer (Bergler *et al.*, 1980) and *S. debonei* of cowbirds and grackles (Box *et al.*, 1980). Apparently one of the effects of storing oocysts of conventional coccidia in potassium dichromate is that the outer wall layers become

TABLE 4.1 EFFECT OF VARIOUS PRETREATMENTS ON THE EXCYSTATION OF POTASSIUM DICHROMATE-STORED *S. GIGANTEA* SPOROCYSTS

Pretreatment	Concentration (final)	Duration (hrs)	Percent excystation*
None	-	-	-
Sodium hypochlorite	0.45%	0.25	22.6 ± 1.9
		0.5	49.1 ± 2.1
		1	56.3 ± 3.0
Cysteine hydrochloride and CO_2	0.02 M	1	3.7 ± 1.1
		4	5.8 ± 1.3
		8	4.6 ± 1.1
		24	7.3 ± 1.1
Pepsin-HCl	4.5%	1	-
		6	-
	18%	1	-
		6	-
	90%	1	-
		6	-

*Mean ± SE, - = < 1

'tanned' thereby rendering them less permeable (Jackson, 1962). It was considered possible that a similar phenomenon may have occurred with the sporocysts of *S. gigantea* resulting in a reduced effectiveness of the CO_2-cysteine hydrochloride pretreatment. Accordingly, the above series of experiments was repeated using sporocysts which had not been in contact with any form of preservative. Additionally, other reducing solutions (sodium dithionite, ascorbic acid) were substituted for cysteine hydrochloride under CO_2 while in one experiment, 8 hr exposure to cysteine hydrochloride-CO_2 followed immediately by 6 hr exposure to pepsin-HCl, was tried.

The results, presented in Table 4.2, followed a pattern similar to that previously observed. Again cysteine hydrochloride-CO_2 pretreatment was largely unsuccessful as was the use of other reducing solutions under CO_2 with only sodium hypochlorite exposure inducing any significant degree of excystation. The levels of excystation attained in these sporocysts following sodium hypochlorite pretreatment were much greater and appeared less dependent upon exposure period than were those of sporocysts stored in potassium dichromate.

(iii) Effect of floatation medium on pretreatment requirements

Sporocysts used in the previous experiments were recovered from cat faeces using NaCl (SG = 1.2). The continuing failure to obtain the anticipated degree of excystation following cysteine hydrochloride-CO_2 pretreatment (despite the absence of exposure to any preservative) raised the possibility that this contact with NaCl may have damaged them in some way. An experiment was conducted to examine this possibility.

TABLE 4.2 EFFECT OF VARIOUS PRETREATMENTS ON THE EXCYSTATION OF WATER-STORED *S. GIGANTEA* SPOROCYSTS

Pretreatment	Concentration (final)	Duration (hrs)	Percent excystation*
None	-	-	-
Sodium hypochlorite	0.45%	0.25	80.6 ± 1.3
		0.5	82.1 ± 1.4
		1	83.8 ± 2.1
Cysteine hydrochloride and CO_2	0.02 M	1	1.6 ± 0.8
		4	5.5 ± 0.7
		8	13.0 ± 1.3
		24	13.0 1 1.0
Ascorbic acid and CO_2	0.02 M	4	1.4 ± 0.4
		24	1.9 ± 0.6
Sodium dithionite and CO_2	0.02 M	4	-
		24	-
Pepsin-HCl	4.5%	1	-
		6	-
	18%	1	-
		6	1.7 ± 0.5
	90%	1	-
		6	-
Cysteine hydrochloride and CO_2 plus Pepsin-HCl	0.02 M 18%	8	-
		6	7.0 ± 1.3

*Mean ± SE, - = < 1

Faecal samples from four cats infected with *S. gigantea* were thoroughly mixed and divided into two lots. Sporocysts were recovered from both lots by the methods previously described (Chapter 2) except that in one case NaCl was used as the floatation medium and in the other, sugar solution (SG = 1.2). Both lots of recovered sporocysts, which were stored in tap water until use, were then exposed to either sodium hypochlorite for 30 min or cysteine hydrochloride-CO_2 for 8 hr. Following these pretreatments they were incubated in excysting medium as already described (Section 4.3.1 (i)).

The results (Table 4.3) showed that cysteine hydrochloride-CO_2 pretreatment was ineffective both with NaCl-and sugar-recovered sporocysts. Sporocysts pretreated with sodium hypochlorite, on the other hand, excysted with equal facility regardless of the floatation medium used.

(iv) Effect of pretreatment on *S. tenella* and *S. capracanis* sporocysts

In an effort to eliminate the possibility that the preceding results may have been due to some inapparent deficiency in technique, another series of experiments using sporocysts of the dog transmitted ovine *(S. tenella)* and caprine *(S. capracanis) Sarcocystis* species was undertaken. These sporocysts, maintained free of contact with any form of preservative, were subjected to a range of pretreatments similar to those used in experiment (i). All sporocysts were then incubated at 39°C for 6 hr in the same excysting medium as that used previously except that in the case of *S. capracanis*, goat bile was substituted for ovine bile.

The results, which are presented in Table 4.4, showed that, in contrast to *S. gigantea*, the highest levels of excystation with both *S. tenella* and *S. capracanis* sporocysts were obtained following cysteine hydrochloride-CO_2 pretreatment.

TABLE 4.3 EFFECT OF FLOATATION MEDIUM ON THE PRETREATMENT REQUIREMENTS OF *S. GIGANTEA* SPOROCYSTS

Pretreatment	Concentration (final)	Duration (hours)	Percent excystation*	
			NaCl recovered	Sugar recovered
Sodium hypochlorite	0.45%	0.5	86.7 ± 1.0	89.8 ± 0.6
Cysteine hydrochloride and CO_2	0.02 M	8	17.4 ± 1.5	18.3 ± 2.0

*Mean ± SE

TABLE 4.4 EFFECT OF VARIOUS PRETREATMENTS ON THE EXCYSTATION OF *S. TENELLA* AND *S. CAPRACANIS* SPOROCYSTS

Pretreatment	Concentration (final)	Duration (hours)	Percent excystation*	
			S. tenella	*S. capracanis*
None	-	-	7.5 ± 2.2	4.0 ± 1.2
Sodium hypochlorite	0.45%	0.5	16.3 ± 0.7	11.5 ± 2.8
		1	15.3 ± 3.0	9.8 ± 2.8
Cysteine hydrochloride and CO_2	0.02M	8	48.2 ± 0.6	27.0 ± 2.5
		24	69.7 ± 2.7	47.1 ± 2.5
Pepsin-HCl	18%	6	14.9 ± 2.0	2.8 ± 0.1
	90%	6	13.1 ± ±.2	4.0 ± 0.6

*Mean ± SE

4.3.2 Studies on treatment stimuli

The following series of experiments were carried out on *S. gigantea* sporocysts which had been stored in potassium dichromate and subjected to 30 min pretreatment with sodium hypochlorite.

(i) Effect of temperature

Pretreated sporocysts were suspended in excysting medium (final concentrations; 5% ovine bile, 0.25% trypsin, tris buffer pH 7.5) and incubated at temperatures of 21, 25, 30, 35, 39 or 43°C for 1 and 6 hr.

Similar levels of excystation were recorded at both incubation periods at all temperatures tested (Fig. 4.1). Excystation did not take place at 21 and 25°C and only at low levels at 30°C (4.4 ± 1.3% 1 hr, 8.0 ± 3.3% 6 hr). At 35°C the level of excystation increased markedly (36.1 ± 3.3% 1 hr, 37.6 ± 2.0% 6 hr) and tended to increase further, albeit at a slower rate, at 39 and 43°C. As a result of these findings, percentage excystation in all further experiments in this Section was, unless otherwise indicated, determined after 1 hr incubation at 39°C.

(ii) Effect of hydrogen ion concentration

Pretreated sporocysts were incubated in excysting media containing 5% ovine bile and 0.25% trypsin (final concentrations) buffered to one of eight pH levels. The buffers used and their pH ranges were: tris maleate (tris (hydroxymethyl) aminomethanemaleate), pH 5.0 to 6.5; tris (tris (hydroxymethyl) aminomethane), pH 7.0 to 8.5 and sodium carbonate-bicarbonate, pH 9.0 to 10.0.

Hydrogen ion concentration appeared to have little influence and percentage excystation was found to be relatively

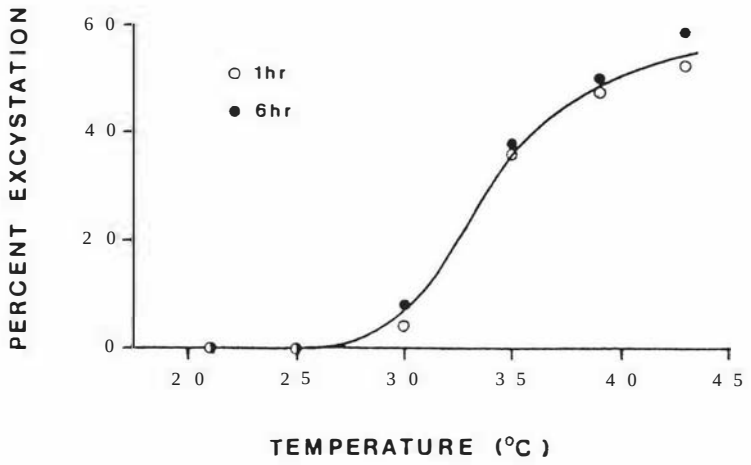

Fig. 4.1 Effect of treatment temperature on the excystation of *S. gigantea* sporocysts (means)

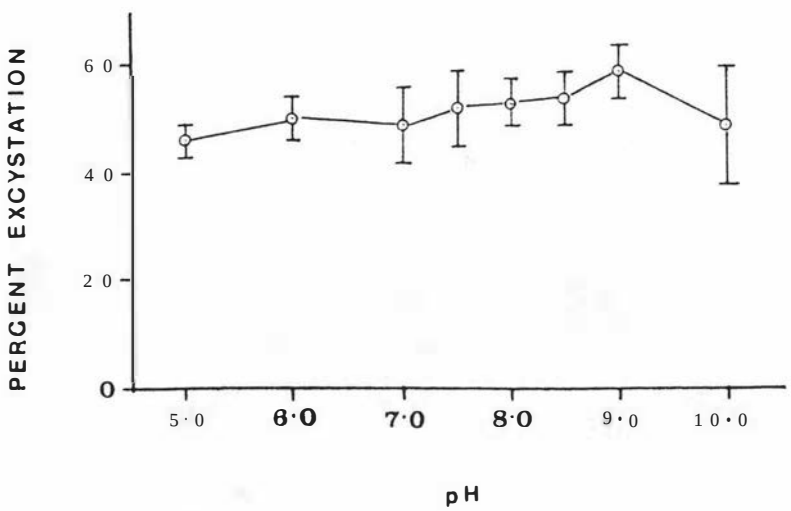

Fig. 4.2 Effect of hydrogen ion concentration on the excystation of *S. gigantea* sporocysts (mean ± SE)

constant at the pH levels tested (Fig. 4.2).

(iii) Effect of bile type or substitute

Sporocysts were incubated in excysting media consisting of 0.25% trypsin (final concentration) and tris buffer (pH 7.5), made up with one of eight different bile types or substitutes. Sheep, cattle, dog, cat, chicken and pig bile were used at final concentrations of 5%. Sodium taurocholate (BDH Chemicals Ltd) was used at a final concentration of 0.75% (w/v) and in one series of experiments, polyoxyethylene sorbitan monolaurate ('Tween 80', Difco Laboratories) at a final concentration of 0.5 x 10^{-5}% (v/v), was substituted for bile.

The results (Fig. 4.3) revealed that excystation occurred in the presence of sodium taurocholate and all bile types but not when 'Tween 80' was substituted for bile. However, not all biles were equally effective. The highest levels of excystation were recorded whenever sodium taurocholate (54.4 ± 3.1%), cattle (59.0 ± 2.3%) or sheep (51.5 ± 1.2%) bile were used and the lowest when chicken (5.1 ± 1.8%) and pig (5.5 ± 0.4%) bile were employed.

(iv) Effect of bile and trypsin concentration

Pretreated sporocysts were added to excysting media consisting of tris buffer (pH 7.5) and, at final concentrations, either 0.25% trypsin and 0, 0.5, 2.5, 5, 10 and 25% ovine bile, or 5% ovine bile and 0, 0.5 and 1% trypsin.

Excystation occurred only when bile was present (Fig. 4.4). At concentrations of 0.5% bile, only 9.8 ± 0.6% of sporocysts excysted. This increased to 43.0 ± 1.4 and 48.4 ± 0.9% respectively, when 2.5 and 5% bile were used

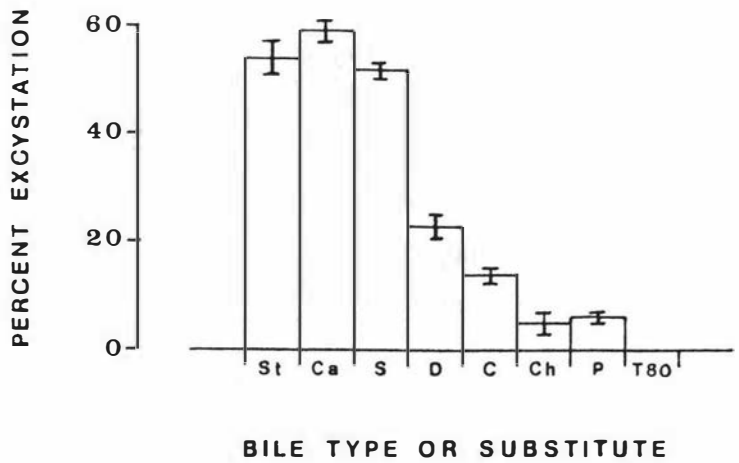

Fig. 4.3 Effect of bile type or substitute on the excystation of *S. gigantea* sporocysts (mean ± SE). St = sodium taurocholate, Ca = cattle bile, S = sheep bile, D = dog bile, C = cat bile, Ch = chicken bile, P = pig bile, T80 = 'Tween 80'

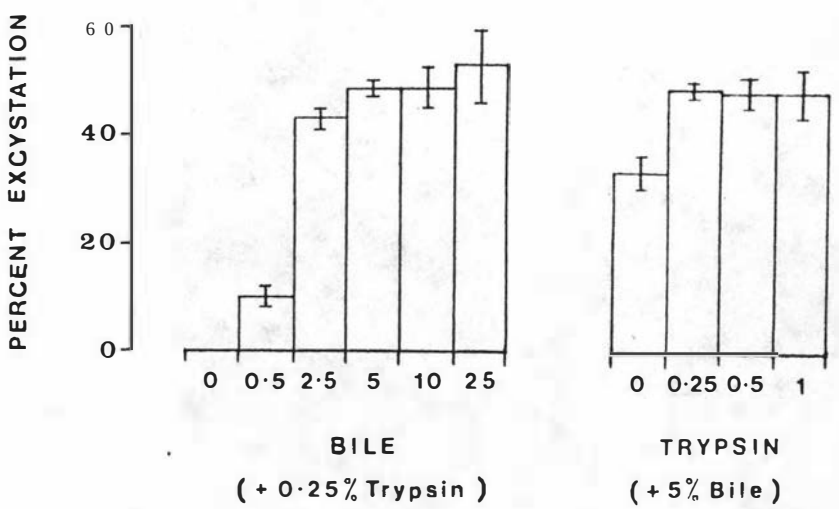

Fig. 4.4 Effect of bile and trypsin concentration on the excystation of *S. gigantea* sporocysts (mean ± SE)

but concentrations above 5% appeared to have little further effect.

Trypsin appeared to be less important than bile since excystation occurred in its absence. However, more sporocysts excysted when trypsin was present than when it was not although above 0.25%, concentration also appeared unimportant.

(v) Effect of discontinuous presence of excysting medium

Jackson (1962), working with the ovine coccidian *Eimeria arloingi (= E. ovina/E.weybridgensis)* found that although both trypsin and bile were essential for excystation it could proceed if oocysts were first treated with bile, washed clean and then treated with trypsin. Others have suggested that sporocysts may be 'preconditioned' by bile (Doran and Farr, 1962) and that further attack on the sporocyst wall may be aided by enzyme secretion by the sporozoites themselves (Doran, 1966). The next experiment was undertaken to determine if the continuous presence of excysting medium was essential for the excystation of *S. gigantea* sporocysts.

Pretreated sporocysts, suspended in 5% ovine bile, 0.25% trypsin (final concentrations) and tris buffer (pH 7.5) were incubated at 39°C for 5, 10, 15 or 30 minutes. After each incubation interval three samples were removed and treated as follows:

1) Sample A was examined immediately.
2) Sample B was washed free of excysting medium with three changes of Ringer's solution and then examined.
3) Sample C was washed free of excysting medium in the same manner as sample B and then incubated for a further 1 hr in Ringer's solution at 39°C before examination.

These three samples were necessary since previous observations indicated that repeated washing with Ringer's solution led to a considerable differential loss of excysted sporocysts. Thus, at each interval of incubation, sample A provided an estimate of the percentage excystation attained in excysting medium alone while the difference between the percentage excystation in samples C and B reflected the degree of excystation due solely to a further 1 hr incubation in Ringer's.

The results, which are presented in Fig. 4.5, showed that following prior exposure to excysting medium, a subsequent 1 hr incubation in Ringer's induced, on average, only an additional 9.5 ± 0.8% of sporocysts to excyst.

4.3.3 The excystation process

To study the excystation process of *S. gigantea*, sporocysts were pretreated for 30 min with sodium hypochlorite and suspended in Ringer's solution as previously described. One drop of this suspension was placed on a slide and to this drop was added a drop of excysting medium (final concentrations of 5% ovine bile and 0.25% trypsin in tris buffer pH 7.5). A coverslip was placed over the preparation and sealed with petroleum jelly. The slide was then examined using a microscope fitted with a warm stage set at 39°C.

Sporocysts appeared unaltered by the pretreatment regime (Plate 4.1A). Following exposure to excysting medium sporozoites within some sporocysts began to move sluggishly by means of weak flexing actions. In a few cases, these fitful movements were accompanied by the appearance of a small hair-like fracture across the equatorial midline of the sporocyst. Suddenly, in many instances without prior warning, the sporocyst ruptured about the equatorial midline

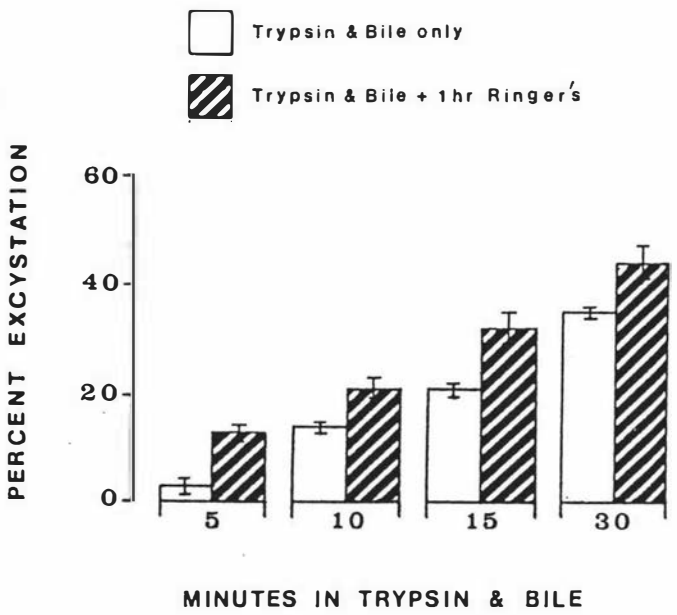

Fig. 4.5 Effect of the discontinuous presence of excysting medium on the excystation of *S. gigantea* sporocysts (Mean ± SE)

on one side. Almost simultaneously the sporocyst walls appeared to partially collapse inwards along their longitudinal perimeters with the sporozoites and sporocyst residuum being forcibly expelled through the area of the initial rupture (Plate 4.1B). The degree of movement exhibited by the expelled sporozoites varied greatly: in most cases little was apparent apart from an occasional bending and flexing. In some instances, however, the sporozoites appeared to 'adhere' to the cover-slip by one end and rotate the free end. Ten liberated sporozoites in an elongated state measured 8.15 ± 0.0 x 2.3 ± 0.8 µm.

Initially, the empty sporocyst appeared as two contiguous triangles sharing a common midline base (Plate 4.1C) but usually the whole structure subsequently and quickly collapsed

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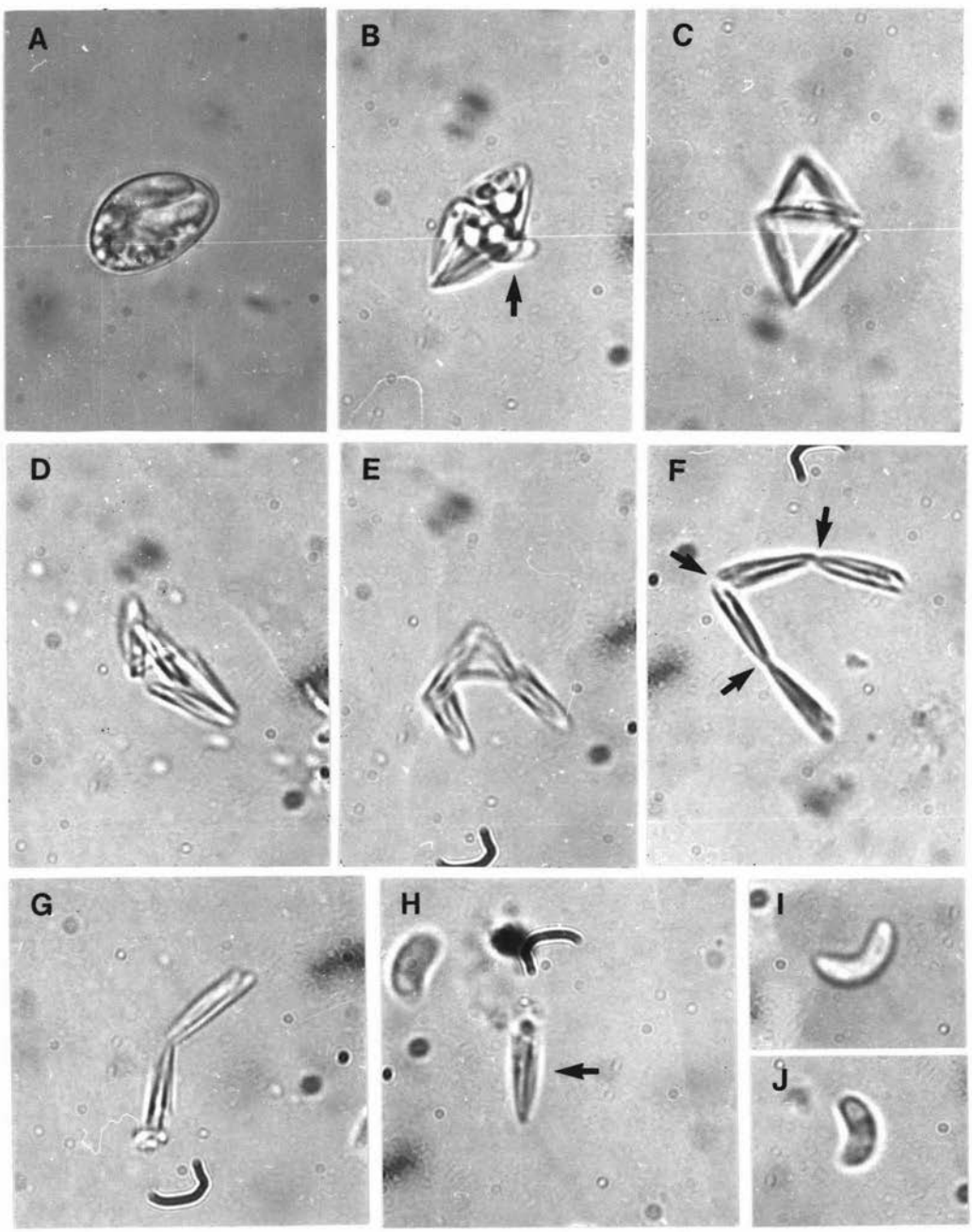
Plate 4.1 Photomicrographs of the excystation process of *S. gigantea* (x 1250). A. Appearance of sporocyst following pretreatment with sodium hypochlorite. B. Commencement of excystation following exposure to excysting medium; note escaping sporozoite (arrow). C. Completely excysted and partially collapsed sporocyst. D. Completely collapsed sporocyst. E. Commencement of separation of sporocyst into four elongated pieces. F. String of four elongated pieces of sporocyst wall linked at narrow pivotal points (arrows). G. Pair of elongated pieces of sporocyst wall. H. Single elongated piece of sporocyst wall (arrow). I. and J. Excysted sporozoites


A
B
C
D
E
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inwards (Plate 4.1D). Frequently the sporocyst wall separated either at the site of the midline fracture or at one of the 'poles' (Plate 4.1E) to form a string of four elongated pieces linked at pivotal points (Plate 4.1F). Commonly, further separation into pairs (Plate 4.1G) or single (Plate 4.1H) elongated pieces took place.

4.4 DISCUSSION

The results of the present study clearly indicate that, despite the absence of a protective oocyst wall, pre-treatment prior to incubation in trypsin and bile is necessary for the *in vitro* excystation of *S. gigantea* sporocysts. Similar results have been reported for *S. cruzi* sporocysts (Fayer and Leek, 1973) but not for those of other closely related cat and dog transmitted coccidia such as *Toxoplasma gondii* (Christie *et al.*, 1978), *Isospora felis*, *I. rivolta* (McKenna and Charleston, 1982) and *I. canis* (Bunch and Nyberg, 1970; Speer *et al.*, 1973). Apart from these species, coccidia from most other hosts have been found to require some form of pretreatment for excystation to occur and, in the case of those infective for ruminants, this has most frequently been achieved *in vitro* by exposure to cysteine hydrochloride and CO_2.

In the present investigation, pretreatment with cysteine hydrochloride or other reducing agents under CO_2 was found to be largely ineffective with *S. gigantea* sporocysts. For this species, sporocyst excystation was only markedly affected by exposure to sodium hypochlorite. For *S. tenella* and *S. capracanis*, on the other hand, cysteine hydrochloride-CO_2 pretreatment was found to be more effective than sodium hypochlorite. Although other pretreatment regimes have not been investigated, cysteine hydrochloride under CO_2 has also been found to be very effective with *S. cruzi* sporocysts (Fayer and Leek, 1973) and has been used to excyst

a number of other *Sarcocystis* spp. (Bergler *et al.*, 1980; Box *et al.*, 1980).

In view of these findings, the failure of this form of pretreatment to induce any significant degree of excystation in *S. gigantea* is rather puzzling. Certainly the present results indicate that this lack of effectiveness was not due to the means of sporocyst recovery used, their method of preservation, or to any deficiencies in the technique employed. Rather one is forced to the conclusion that this failure is associated with the nature of the species itself and, in particular, the physical or biochemical structures of the sporocyst wall. Lipid does not dissolve in hypochlorite (Ryley, 1973) whereas cysteine hydrochloride-CO_2 pretreatment has the apparent effect of unmasking lipid-shielded disulphide bridges in the oocyst wall and reducing them to sulphydryl groups, thereby causing a change in permeability(Jensen *et al.*, 1976; Jolley *et al.*, 1976). Differences in the degree of excystation attained with these two forms of pretreatment may, therefore, suggest that the sporocyst walls of *S. gigantea* are, in contrast to those of *S. tenella* and *S. capracanis*, largely devoid of lipid and disulphide bonds.

Quite apart from these considerations, however, the comparative failure of *S. gigantea* sporocysts to respond to any form of pretreatment other than sodium hypochlorite is even more perplexing, because the stimuli necessary to achieve excystation *in vitro* are generally considered to be essentially similar to those required *in vivo* (Jackson, 1962). Since it is difficult to imagine conditions in the gastrointestinal tract of sheep resembling suspension in sodium hypochlorite, it might be argued that the results obtained in the present study do not represent 'true' excystation at all. It could be suggested, for example, that

the sporozoites within the sporocyst are surrounded by a fluid of high concentration which may be bounded by inner permeable and outer impermeable, sporocyst wall layers. Exposure to sodium hypochlorite might have the effect of removing this outer layer, in the same manner as has been observed with oocysts of other coccidia species (Monné and Honig, 1954; Jackson, 1964; Nyberg and Knapp,1970; Roberts *et al.*, 1970) exposing the more permeable layer beneath. Thus when these 'stripped' sporocysts are exposed to excysting medium there is an influx of fluid into the sporocyst by osmosis. The resulting build up of pressure within, might then lead to the rupture of the sporocyst wall and the passive expulsion of the sporozoites.

That this model is inappropriate is evident from the fact, that following pretreatment with sodium hypochlorite, no excystation will occur unless the sporocysts are subsequently treated with excysting medium containing bile or bile salts. Furthermore, little excystation will occur unless this treatment phase takes place at temperatures of greater than 35°C, using sodium taurocholate, cattle or sheep bile, and in the latter case, at concentrations of 2.5% or more. Thus, although hydrogen ion concentration appeared unimportant for this process, it would seem that the events taking place during the treatment phase are essentially chemical ones, involving bile, bile salts, or some component of them.

Because of the precise host-specificity of *S. gigantea*, the ability to excyst, at least to some extent, in such a wide range of bile types was unexpected. This was particularly so in the case of cat bile since sporocysts of *Sarcocystis* spp. are not infective for their definitive hosts (Fayer, 1974; Rommel *et al.*, 1974). Obviously if they were and since they are fully sporulated when released,

subsequent exposure to digestive enzymes during the natural course of intestinal development in these hosts (see Chapter 3) could lead to a continuously recycling infection. That this does not occur in the dog definitive host of *S. cruzi* could be explained by the inability of canine bile to induce excystation in this species (Fayer and Leek, 1973). For *S. gigantea*, however, an alternative explanation is required and this is most likely to centre around the absence of the necessary pretreatment stimulus.

Although the ability of *S. gigantea* sporocysts to excyst in a diversity of bile types might be attributable to peculiarities associated with sodium hypochlorite pretreatment, other workers have also found that bile from 'abnormal' hosts may be used to excyst coccidia. Doran and Farr (1962), for example, were able to excyst the chicken coccidian *Eimeria acervulina* using bovine bile, while Lotze and Leek (1963) observed excysted sporozoites of a number of sheep coccidia species in the intestinal contents of such diverse hosts as rabbits, rats, hamsters, chickens and turkeys. In addition, *S. capracanis* sporocysts have been excysted with equal facility using either caprine or ovine bile (pers. obs.). The above findings imply that host-specificity, or the ability to infect a particular host, is likely to be related to factors other than merely the ability to excyst in that host.

As well as attacking the sporocyst wall directly, bile is also considered to be primarily responsible for stimulatin the coccidial sporozoites into activity (Farr and Doran 1962; Roberts *et al.*, 1970). Consequently it is conceivable that any final attack on the sporocyst wall results from, or is aided by, enzyme secretion by the sporozoites themselves (Doran, 1966). In the present study, only small increases in levels of excystation were recorded after 1 hr incubation in Ringer's solution following exposure to

excysting medium. This suggests that if enzyme secretion by the sporozoites does play a part in the excystation of *S. gigantea*, then it is only a small one.

While bile or bile salts were essential for the excystation process, trypsin was not. This is also the case with *S. cruzi* (Fayer and Leek, 1973), *S. tenella* (pers. obs.) and other coccidia of cats, dogs, chickens and sheep (Christie *et al.*, 1978; McKenna and Charleston, 1982; Speer *et al.*, 1973; Hibbert and Hammond, 1968) but not for those of cattle which appear to be more dependent upon the presence of trypsin than bile (Hibbert and Hammond, 1968; Hibbert *et al.*, 1969).

In addition to differences in the stimuli required to induce excystation, the mechanics of this process have also been found to vary between coccidian species. Generally, these can be grouped into two distinct patterns according to whether the sporocysts have Stieda bodies or not. In those species which have Stieda bodies, the sporozoites escape through the gap created by the dissolution of this structure (Doran and Farr, 1962; Nyberg and Hammond, 1964; Duszynski and Brunson, 1972, 1973; Speer and Duszynski, 1975) whereas in those species which do not, the sporocyst walls collapse and the sporozoites escape haphazardly (Speer *et al.*, 1973, 1976; Duszynski and File, 1974; Duszynski and Speer, 1976; Christie *et al.*, 1978; McKenna and Charleston, 1982). The excystation process of *S. gigantea* obviously falls into the latter group and in this respect shows a similarity to that described for *S. cruzi* (Fayer and Leek, 1973) and *S. debonei* (Box *et al.*, 1980). Electron microscopic study has revealed a series of ridges dividing the surface of *S. debonei* sporocysts into four plates (Box *et al.*, 1980). These ridges, which represent the margins of the plates, consist of lip-like thickenings of the inner sporocyst wall separated by thin

strips of electron-dense material. Excysting medium apparently acts upon this material causing a separation and curling inwards of the plates and the eventual collapse of the whole sporocyst into four longitudinal pieces. Similar lip-like thickenings have been observed in *S. gigantea* sporocysts (Mehlhorn and Scholtyseck, 1974). Accordingly, the similarity of the excystation process of this species to that of *S. debonei* might be expected.

CHAPTER 5

IN VIVO EXCYSTATION OF *SARCOCYSTIS GIGANTEA* AND *S. TENELLA* SPOROCYSTS

5.1 INTRODUCTION

Both *S. gigantea* and *S. tenella*, which are transmitted by cats and dogs respectively, infect sheep. Studies on the *in vitro* excystation of their sporocysts (Chapter 4) showed that, as with oocysts of most other coccidia, excystation was a two-phase process. The first pretreatment phase rendered the sporocyst walls permeable to the second, treatment phase stimuli which were bile or bile salts and, to a lesser extent, trypsin.

For conventional coccidia infective for ruminants, the pretreatment stimuli are generally considered to be provided in the rumen, those of the treatment phase in the small intestine (Jackson, 1962; Ryley, 1973; Long and Speer, 1977). It is claimed that these pretreatment conditions can be artifically simulated by incubating oocysts in cysteine hydrochloride under an atmosphere of CO_2 (Jackson, 1962; Hibbert and Hammond, 1968; Bunch and Nyberg, 1970).

In the previous Chapter it was observed that although cysteine hydrochloride - CO_2 pretreatment was effective in initiating excystation of *S. tenella* sporocysts it was not with those of *S. gigantea*. Excystation of sporocysts of the latter species could, however, be achieved by previously exposing them to a weak solution of sodium hypochlorite. These differences in pretreatment requirements were unexpected, not only because the two species share the same intermediate host, but also because

conditions comparable to those provided by sodium hypochlorite, seemed unlikely to be encountered in the gastrointestinal tracts of sheep. The work in this Chapter was undertaken in an effort to elucidate the factors responsible for the excystation of *S. gigantea* and *S. tenella in vivo*.

5.2 MATERIALS AND METHODS

5.2.1 Sporocysts

Sporocysts of *S. gigantea* were recovered from the faeces of experimentally infected cats as previously described (Chapter 2). Those of *S. tenella* were obtained by similar means from dogs fed naturally infected sheep hearts. Sporocysts of both species were stored at 4°C in tap water until use: those of *S. gigantea* for a period of 8 to 10 months, those of *S. tenella* for 4 to 5 months.

5.2.2 Sheep

Three 18 month-old Romney sheep, raised and maintained indoors on a diet of hay and pelleted concentrates, were used. Each was surgically fitted with either a permanent rumen, abomasal or duodenal cannula (the latter 15 to 20 cm distal to entry of the bile duct) and held in metabolism cages.

6.2.3 *In vitro* pretreatment and treatment

Sporocysts were subjected to sodium hypochlorite or CO_2 pretreatment and washed by procedures previously described (Chapter 4). Sodium hypochlorite was used at a final concentration of 0.45%, cysteine hydrochloride at 0.02M and

ruminal and abomasal fluids at final concentrations of 90%. The latter two fluids, obtained from the cannulated sheep, consisted of the supernatants of freshly collected samples which were crudely sieved (sieve aperture 250 μm) and centrifuged at 400 x g for 5 minutes.

Following pretreatment, sporocysts were incubated at 39°C in either excysting medium or small intestinal fluid freshly collected via the duodenal cannula. The excysting medium was prepared fresh for each experiment and consisted of 5% (previously frozen) ovine bile and 0.25% trypsin (1:300 ICN Pharmaceuticals Inc) (final concentrations) in tris buffer, pH 7.5 to 7.8. Small intestinal fluid was used at a final concentration of 90% and consisted of a supernatant extract obtained by procedures similar to those described above. Unless otherwise stated, all sporocysts were incubated in excysting medium or small intestinal fluid for 1 hr.

5.2.4 *In vivo* pretreatment and treatment

Sealed sachets of 10 mm wide dialysis tubing (molecular weight cut-off 12000-14000; Selbys Scientific Ltd) containing sporocysts were introduced into the rumen, abomasum or duodenum through the respective cannulae and anchored in place by means of fine nylon line. The sachets were left in place in the rumen and abomasum for varying periods according to the experiment but in the duodenum the period was always 1 hr. Following the appropriate period of exposure, the sporocysts were recovered from the dialysis sachets and either washed twice with distilled water and examined, or washed a further two times with Ringer's solution and incubated in excysting medium before examination.

5.2.5 Determination of excystation

Determination of percentage excystation in all experiments,

each of which was repeated once, was as previously described (Chapter 4).

5.3 EXPERIMENTAL PROCEDURES AND RESULTS

5.3.1 Laboratory experiments

(i) Effect of various artificial and 'natural' pretreatments using excysting medium

In order to determine the viability of the sporocysts under test and to obtain some indication of the site and likely period of pretreatment stimulus required in the sheep host, a preliminary investigation using 'natural' organ fluids and artificial pretreatments, was undertaken in the laboratory. As shown in Table 5.1, eight samples of sporocysts of each species were used. One sample was pretreated with either CO_2 and cysteine hydrochloride for 24 hr or sodium hypochlorite for 30 min, four were pretreated with CO_2 and either ruminal or abomasal fluid for 1 or 24 hr and two were pretreated with CO_2 and ruminal fluid for 24 hr followed immediately by 1 or 24 hr exposure to abomasal fluid and CO_2. The final sample was not subjected to any pretreatment. All sporocysts were then incubated in excysting medium and the percentage excystation determined.

The results (Table 5.1) showed that, despite their apparent viability, as evidenced by the levels of excystation attained following artificial pretreatment, attempts to induce excystation in both *S. gigantea* and *S. tenella* sporocysts using 'natural' pretreatments, were largely unsuccessful.

(ii) Effect of various artificial and 'natural' pretreatments using small intestinal fluid

It was considered possible that the above results may have been due to the inability of the excysting medium to provide

TABLE 5.1 EFFECT OF VARIOUS ARTIFICIAL AND 'NATURAL' LABORATORY PRETREATMENTS ON THE EXCYSTATION OF *S. GIGANTEA* AND *S. TENELLA* SPOROCYSTS FOLLOWING THEIR SUBSEQUENT EXPOSURE TO EXCYSTING MEDIUM*

Pretreatment	Duration (hours)	Percent excystation**			
		S. gigantea		*S. tenella*	
		Expt. (a)	Expt. (b)	Expt. (a)	Expt. (b)
None	-	5.5	2.5	3.7	2.0
Sodium hypochlorite	0.5	95.1	94.7	ND	
Cysteine hydrochloride and CO_2	24	ND		54.9	45.9
Ruminal fluid and CO_2	1	3.2	4.6	5.9	1.0
	24	-	-	3.0	-
Abomasal fluid and CO_2	1	-	-	7.7	1.1
	24	-	-	8.4	2.0
Ruminal fluid/CO_2 plus Abomasal fluid/CO_2	24	-	-	1.0	1.0
Ruminal fluid/CO plus Abomasal fluid/CO_2	24	1.7	2.2	9.6	15.1

* 1 hr at 39°C

** ND = not done, - = < 1

the appropriate second phase stimuli following 'natural' pretreatments. Consequently the series of experiments was repeated using small intestinal fluid in place of excysting medium.

The results (Table 5.2) showed that the use of small intestinal fluid following 'natural' pretreatment did not improve on the excystation level attained with either species. Indeed, this fluid was found to be less successful than excysting medium following artificial pretreatment as well, particularly with *S. tenella* where it was completely ineffective. An attempt was made to induce excystation in this species by extending the period of exposure to intestinal fluid by a further 5 hr following cysteine hydrochloride-CO_2 pretreatment. This was also completely unsuccessful.

5.3.2 Sheep experiments

(i) Using sporocysts suspended in water or Ringer's solution

Two investigations were conducted: the first was to determine the importance of the rumen and/or abomasum as the source of any pretreatment stimulus, the second the effectiveness of the duodenum in providing the treatment phase.

In the first investigation, sporocysts of both species, suspended in distilled water within dialysis tubing sachets, were introduced into either the rumen, abomasum, or sequentially both, for varying periods as shown in Table 5.3. Following the appropriate exposure period in these organs the sporocysts were either examined immediately or after further incubation in excysting medium.

In the second investigation, the percentage excystation of sporocysts suspended in Ringer's solution within dialysis sachets, and which had previously been subjected to either

TABLE 5.2 EFFECT OF VARIOUS ARTIFICIAL AND 'NATURAL' LABORATORY PRETREATMENTS ON THE EXCYSTATION OF *S. GIGANTEA* AND *S. TENELLA* SPOROCYSTS FOLLOWING THEIR SUBSEQUENT EXPOSURE TO SMALL INTESTINAL FLUID*

Pretreatment	Duration (hours)	Percent excystation**			
		S. gigantea		*S. tenella*	
		Expt. (a)	Expt. (b)	Expt. (a)	Expt (b)
None	-	-	-	-	-
Sodium hypochlorite	0.5	85.6	71.4	ND	
Cysteine hydrochloride and CO_2	24	ND		1.0	-
Ruminal fluid and CO_2	1	-	-	-	1.0
	24	-	-	-	1.0
Abomasal fluid and CO_2	1	-	-	2.0	1.0
	24	-	-	3.9	4.7
Ruminal fluid/CO_2 plus Abomasal fluid/CO_2	24 / 1	-	-	1.0	-
Ruminal fluid/CO_2 plus Abomasal fluid/CO_2	24 / 24	-	-	-	5.0

* 1 hr at 39°C

** ND = not done, - = < 1

sodium hypochlorite, cysteine hydrochloride-CO_2, or no pretreatment, were determined after 1 hr placement in the duodenum.

In no instance was excystation recorded in sporocysts placed in either the rumen, abomasum or both, unless they were subsequently treated with excysting medium, or in non-pretreated sporocysts placed in the duodenum.

With *S. gigantea*, the highest levels of excystation were recorded in sodium hypochlorite pretreated sporocysts placed in the duodenum (Table 5.3). Moderate but vary- levels of excystation were also evident in sporocysts treated with excysting medium following 0.25 to 4 hr placement in the abomasum or subjected to 1 hr abomasal exposure following 24 hr prior location in the rumen. However, little excystation occurred in *S. gigantea* sporocysts held solely in the rumen for any interval or in those held in the abomasum for 24 hr with or without prior contract with the rumen.

Ruminal pretreatment alone also appeared to have little effect on the excystation of *S. tenella* sporocysts when subsequently treated with excysting medium (Table 5.3). For this species the highest levels of excystation were recorded in sporocysts incubated in excysting medium after being held in the abomasum for 24 hr or after being held in this organ for 1 hr following 24 hr prior exposure to the rumen. Little excystation was evident in *S. tenella* sporocysts pretreated with cysteine hydrochloride and CO_2 and then placed in the duodenum.

(ii) Using sporocysts suspended in organ fluids

Although the levels of excystation induced in sporocysts placed in the rumen or abomasum in the above experiment were, for the most part, considerably higher than those

TABLE 5.3 EFFECT OF RUMINAL, ABOMASAL OR DUODENAL EXPOSURE ON THE *IN VIVO* EXCYSTATION OF *S. GIGANTEA* AND *S. TENELLA* SPOROCYSTS WHILE SUSPENDED IN DISTILLED WATER OR RINGER'S SOLUTION

'Pretreatment'	Duration (hours)	'Treatment' *	Percent excystation**			
			S. gigantea		*S. tenella*	
			Expt. (a)	Expt. (b)	Expt. (a)	Expt (b)
Sodium hypochlorite	0.5	Duodenum	73.3	74.3	ND	
Cysteine hydrochloride and CO_2	24	"	ND		-	4.0
	1	EM	3.5	6.0	4.0	1.0
Rumen	24	"	7.0	1.0	4.0	6.7
	48	"	5.7	-	8.0	10.0
	0.25	"	25.4	3.3	4.7	8.0
	1	"	37.3	47.8	8.0	3.0
Abomasum	4	"	39.7	34.7	6.0	11.0
	24	"	1.2	-	21.0	28.7
Rumen	24					
plus		"	20.9	23.2	26.0	22.9
Abomasum	1					
Rumen	24					
plus		"	1.0	1.7	3.0	10.0
Abomasum	24					

* EM = excysting medium

**ND = not done, - = < 1

obtained by incubating them in fluids from these organs in the laboratory, they were still less than anticipated. It was suspected that this might have been due to the dialysis sachets preventing some of the enzymes or molecules in these organs from reaching the sporocysts. Accordingly, the previous experiments were repeated, but on this occasion the sporocysts within dialysis sachets were suspended in 90% concentrations of the fluids from the organs in which they were to be placed.

The results (Table 5.4) revealed that far from providing any marked improvement, this procedure(except possibly for *S. tenella* sporocysts sequentially exposed for 24 hr in both the rumen and abomasum) tended to result in even less excystation than when the sporocysts were suspended in water or Ringer's solution.

5.4 DISCUSSION

The results of the present study are disappointing in that they appear to have added little to our knowledge regarding the factors responsible for the excystation of *S. gigantea* and *S. tenella* in sheep. They do, however, permit a limited number of tentative observations to be made. First, they tend to confirm previous observations (Chapter 4) that the excystation requirements of *S. gigantea* and *S. tenella* may be different. Second, they tend to suggest, at least as far as the former species is concerned, that the excystation process *in vivo* is, like the situation *in vitro*, essentially a diphasic one involving a pretreatment and a treatment stimulus.

For both species, the present results suggest that any pretreatment phase is unlikely to be provided by conditions in the rumen alone. This is somewhat surprising since it appears to be generally accepted that the primary stimulus for coccidial excystation in ruminants is provided by this

TABLE 5.4 EFFECT OF RUMINAL, ABOMASAL OR DUODENAL EXPOSURE ON THE *IN VIVO* EXCYSTATION OF *S. GIGANTEA* AND *S. TENELLA* SPOROCYSTS WHILE SUSPENDED IN ORGAN FLUIDS

'Pretreatment'	Duration (hours)	'Treatment' *	Percent excystation**			
			S.gigantea		*S. tenella*	
			Expt. (a)	Expt. (b)	Expt. (a)	Expt. (b)
Sodium hypochlorite	0.5	Duodenum	45.0	46.1	ND	
Cysteine hydrochloride and CO_2	24	"	ND		-	2.0
	1	EM	5.0	5.3	4.0	4.0
Rumen	24	"	14.3	8.7	2.8	3.0
	48	"	-	-	3.0	4.5
	0.25	"	8.5	21.3	11.0	17.1
	1	"	13.7	4.8	9.0	7.0
Abomasum	4	"	-	7.3	2.0	8.0
	24	"	-	-	8.0	31.8
Rumen	24					
plus		"	1.2	-	1.0	4.0
Abomasum	1					
Rumen	24					
plus		"	1.0	-	22.7	17.0
Abomasum	24					

*EM = excysting medium

**ND = no done, - = < 1

organ (Ryley, 1973; Long and Speer, 1977). A review of the literature reveals that support for this view is based on remarkably little evidence. The source of this hypothesis appears to be the work of Jackson (1962) who observed that a high level of excystation could be obtained in oocysts of the ovine coccidian *Eimeria arloingi (= E.ovina/E.weybridgensis)* by first introducing them into the rumen while enclosed in a 'cellophane sac' and then incubating them in a solution of trypsin and sheep bile. Attempts to provide this initial stimulus *in vitro* using ruminal fluid gave variable and generally unsatisfactory results unless incubated under an atmosphere of CO_2. Jackson (1962) also noted that the addition of three chemically unrelated reducing agents (sodium dithionite, ascorbic acid, cysteine hydrochloride) each enhanced the effectiveness of the stimulus but concluded that the contribution of CO_2 was of primary importance.

Since then, other workers have also used similar *in vitro* procedures to excyst coccidia species of cattle and sheep (Nyberg and Hammond, 1964; Hibbert and Hammond, 1968; Hibbert *et al.*, 1969; Bunch and Nyberg, 1970; Fayer and Leek, 1973). Although each of these groups employed a reducing agent (cysteine hydrochloride) in the pretreatment phase of this process, the major emphasis continued to be placed on the importance of CO_2 and, because this gas is present in high concentrations in the rumen (Phillipson, 1977), the assumption of the vital role of this organ in the excystation of ruminant coccidia appears to have been perpetuated. More recent evidence, however, indicates that, for some coccidia species, the contribution of the reducing agent may be just as important as that of CO_2 in providing the initial stimulus for *in vitro* excystation (Jolley and Nyberg, 1974; Jolley *et al.*, 1976; Jensen *et al.*, 1976): this view may be supported by results obtained in the previous Chapter where, although none were particularly effective, neither sodium dithionite nor ascorbic acid were as successful as cysteine hydrochloride

in combination with CO_2 in inducing *S. gigantea* sporocysts to excyst.

Apart from Jackson's (1962) study, few other investigations into the *in vivo* excystation of ruminant coccidia have been undertaken, but those which have been provide little further support for the essential importance of the rumen in this process. Thus, Hammond *et al.*, (1954) and Hammond and Nyberg (1964) were able to induce *Eimeria bovis* infections by injecting oocysts into the abomasum which, in the study of Hammond *et al.* (1954), resulted in as severe infections in calves as those produced by oocysts administered orally.

Similar indications of the possible importance of prior exposure to the abomasum rather than the rumen tend to be provided by the present results as well. As stated previously, little excystation was subsequently observed in sporocysts of either species subjected solely to ruminal pretreatment for any period. Contact with the abomasum alone, on the other hand, induced moderate levels of excystation in both: for *S. gigantea* following 0.25 to 4 hr exposure; for *S. tenella* after 24 hr. Conversely, 24 hr placement in the abomasum appeared inhibitory for *S. gigantea* sporocysts whereas for those of *S. tenella*, short exposure to this organ induced little excystation unless they had previously been placed in the rumen. Since natural transition through the abomasum in sheep is likely to be of limited duration (Phillipson, 1977) these latter findings may be of more relevance to the *in vivo* excystation of *S. tenella* than *S. gigantea*. They may, for instance, indicate that pretreatment of the former species requires sequential passage through both the rumen and the abomasum.

At least following artificial pretreatment, the stimulus necessary to complete the excystation process may, apparently,

be provided by conditions in the duodenum for *S. gigantea* though not for *S. tenella*. These disparities are difficult to reconcile, particularly since excysting medium was effective with both. It is appreciated that the concentrations of trypsin and bile are more likely to be maintained at optimum levels in this medium than in small intestinal fluid where the proportions of primary and secondary bile salts may vary considerably at any particular time. However, such variations do not satisfactorily explain the consistency of results observed in all laboratory and sheep experiments.

Initially, it was considered possible that the failure of pretreated *S. tenella* sporocysts to respond to small intestinal fluid in the laboratory may have been related to the limited incubation period (1 hr) used. But the inability to induce excystation by extending this incubation by a further 5 hr excluded this possibility. Furthermore, although passage through the intestine of adult sheep requires approximately 21 hr, transit through the small intestine is only likely to occupy about 3 of these with the remainder being spent in the colon/caecum (Phillipson, 1977). According to Phillipson (1977) proteolytic activity in caecal contents is stronger than in those of the rumen. In view of this and in view also of the current results, is it possible that completion of excystation for *S. tenella* occurs in the colon/caecum? Obviously this is something which cannot be answered here but may be worthy of further investigation.

The results of the present study illustrate all too well the difficulties and dangers of attempting to equate findings pertaining to *in vitro* excystation with those which may be required *in vivo*, as well as the inherent problems associated with attempting to investigate the latter. It is apparent, for example, that conditions which may be present in either the ovine rumen or abomasum cannot simply be simulated by incubating fluids from these organs under CO_2. Similarly,

the placement of sporocysts within selected organs of experimental hosts cannot be expected to represent the range of conditions likely to be encountered by those traversing the whole gastrointestinal tract following oral ingestion. It seems likely too, that the means of retaining sporocysts in such experiments may 'insulate' them from the various forces operating within these organs, a problem which evidently cannot be surmounted merely by their suspension in organ fluids within dialysis sachets.

With *S. tenella*, an appropriate line of further investigation may be to inject known lethal numbers of sporocysts into various parts of the gastrointestinal tract. Differences in the degree of clinical disease induced may thus provide some indication of the relative importance of locations within it in causing sporocysts to excyst. With *S. gigantea*, however, this approach is unlikely to be successful because of the apparently innocuous nature of such infections and their long development period (see Sections 1.2.5a, 1.3). Despite these difficulties further investigations along these lines are obviously required if a better understanding of the factors responsible for *in vivo* excystation of *Sarcocystis* spp. are to be obtained.

CHAPTER 6

RESISTANCE AND SURVIVAL OF *SARCOCYSTIS GIGANTEA* SPOROCYSTS

6.1 INTRODUCTION

From an epidemiological viewpoint, the role of the exogenous stages in the coccidian life are of central importance in the transmission of infection. Obviously such stages must be able to maintain their viability, in what may frequently be harsh environments, until ingested by their hosts.

For most species of conventional coccidia, these exogenous stages are oocysts; for members of the genus *Sarcocystis* they are free sporocysts (Frenkel, 1974; Markus, 1978; Mehlhorn and Heydorn, 1978). Coccidial oocysts are normally passed into the external environment undeveloped and must complete sporulation before becoming infective; this requires exposure to the appropriate conditions of temperature, moisture and oxygen (Kheysin, 1972; Levine, 1973). Once sporulation is achieved they are normally regarded as hardy, long-lived resting stages, capable of withstanding the action of many chemical and physical agents (Kheysin, 1972; Hammond, 1973; Levine, 1973; Ryley, 1973).

Compared with oocysts of conventional coccidia, the longevity and resistance of sporocysts of *Sarcocystis* spp. have been little studied. Since the resistance exhibited by the former coccidians is generally considered to be provided by the relatively thick and impervious oocyst wall (Kheysin, 1972; Levine,1973), it seems reasonable to suppose that the ability of free sporocysts

to survive in the external environment might be somewhat less. On the other hand, because sporulation takes place within the host (Dubey, 1976; Markus, 1978; Mehlhorn and Heydorn, 1978) sporocysts of *Sarcocystis* spp. have an apparent advantage by being independent of influences of the external environment on sporogony, although it has been suggested that their prolonged maintenance at host body temperatures may, in fact, diminish their viability and infectivity (Tadros and Laarman, 1976).

The ultimate test of the viability of any infectious agent must be its ability to induce infection in susceptible hosts. Plainly, such test procedures can be expensive and time consuming, particularly in cases involving sporocysts of *Sarcocystis* spp. where the difficulties in maintaining intermediate hosts free of extraneous infection during the long development period, are likely to be prohibitive. Accordingly, alternative methods have frequently been adopted to determine viability, such as the presence or absence of infective larval motility (Rose, 1955; Conder, 1978), the ability of nematode eggs to develop or hatch (Waller and Donald 1970; Parkin, 1976; Wharton, 1979), or the ability of coccidial oocysts to sporulate (Ito *et al.*, 1975a, b).

For sporocysts of *Sarcocystis* spp. such an *in vitro* procedure is likely to be most appropriately provided by the ability of their sporocysts to excyst. Obviously sporocysts which cannot excyst cannot induce infection although it is recognised that the mere ability to do so may not necessarily be indicative of their infectivity either. It could be argued that any assessment of viability based upon the ability to excyst may, in fact, err on the side of safety. Thus although in using this

criterion there will undoubtedly be occasions when sporocysts are classified as being viable when they are not infective, there is considerably less possibility of making the more serious error of identifying sporocysts as being non-infective when they are.

With this proviso in mind, the following study was undertaken to determine the viability and survival of *S. gigantea* sporocysts after their exposure to various chemical, physical and environmental influences using *in vitro* excystation as the measure of viability.

6.2 MATERIALS AND METHODS

6.2.1 Sporocysts

Sarcocystis gigantea sporocysts were recovered from cat faeces as previously described (Chapter 2) and stored at 4°C in tap water until use.

6.2.2 Excystation

Sporocysts were subjected to 0.5 hr pretreatment with 0.45% sodium hypochlorite (final concentration) and then incubated at 39°C for 1 hr in excysting medium consisting of 5% ovine bile and 0.25% trypsin (final concentrations) in tris buffer, pH 7.5 to 7.8. Percentage excystation was then determined as stated previously (Chapter 4).

6.2.3 Establishment and monitoring of relative humidities

Three different relative humidities, maintained at constant temperatures, were established in desiccators using saturated solutions (Winstone and Bates, 1960). The solutions, selected for their likely lack of toxicity and their ability to produce relative humidities of 85%,

55% and 33%, consisted of sludges of sucrose, glucose and magnesium chloride, respectively. At frequent intervals before and during the investigation, humidity levels within the desiccators were checked by matching cobalt thiocyanate test papers (BDH Chemicals Ltd) against permanent colour standards (Lovibond Comparator Disk, Tintometer Ltd) according to the procedures of Solomon (1957). No measurable deviations from the expected values were recorded on any occasion.

6.2.4 Ultraviolet (UV) irradiation

Ultraviolet irradiation was applied using a Clemco CUVG 30 T8, 30 watt tube (Gelman Clemoc Pty, Ltd) rated by the manufacturer to deliver approximately 95% of its output at 254 nM. Intensities were measured as microwatts per sq cm ($\mu W/cm^2$) using a J-225 shortwave UV meter (Blak-Ray Lamp, Ultraviolet Products Inc). Variations in intensity of UV output were minimised by using a standard warm-up period of 10 min before beginning irradiation.

The effect of UV irradiation on parasitic organisms is dependent on the total energy (ET) delivered (ET = intensity x time = μW-min/cm^2) (Tromba, 1978). Irradiation doses in the present study, therefore, were measured in these terms with exposure times being held to 10 min or less to eliminate any possible effects of temperature variations that might occur during extended periods of treatment.

6.2.5 Chemicals, disinfectants, coccidiostats and anthelmintics

Freshly prepared solutions were employed on all occasions. Disinfectants were used at concentrations recommended by

the manufacturers, those of coccidiostats/anthelmintics at 100, 500 or 1000 parts per million (ppm) of active ingredient. The solubility of some of these drugs was increased by the addition of a few drops of HCl.

The manufacturers, distributors and active ingredients of the disinfectants, coccidiostats and anthelmintics used, are given in Appendix II.

6.3 EXPERIMENTAL PROCEDURES AND RESULTS

6.3.1 Laboratory experiments on sporocyst resistance and survival

(i) Effect of storage medium and duration

After determination of their percentage excystation, suspensions of sporocysts, approximately 2 weeks old, were divided into three aliquots. Each aliquot, suspended in a 5 cm depth of either tap water, 2.5% potassium dichromate, or 2% sulphuric acid, was then stored at 4°C. Periodically during the course of this storage, two samples of sporocysts were recovered from each aliquot. These were thoroughly washed by suspension and centrifugation in six changes of tap water and one of distilled water and then tested for viability.

The results, which are presented in Fig. 6.1 as mean percentage excystation, showed that sporocysts stored in tap water survived considerably better than did those stored in either sulphuric acid or potassium dichromate. After 174 days, excystation levels of 84.8% and 14.5% respectively, were recorded in tap water and potassium dichromate stored sporocysts whereas no excystation was observed after 5 days storage in sulphuric acid.

(ii) Effect of chemicals and disinfectants

Two investigations were conducted: the first was in the nature of a preliminary screen, undertaken to identify solutions possessing potential sporocysticidal properties; the second was to determine the minimum period of contact required by such solutions to destroy sporocyst viability.

In the first investigation, 8 to 10 week-old sporocysts were suspended in a range of chemicals and disinfectants of various concentrations for 48 hr at room temperature. It was considered that only those solutions capable of virtually eliminating sporocyst viability after this period warranted further investigation. Following this exposure, the sporocysts were washed as described above and their percentage excystation determined. Each experiment was repeated once and was accompanied on both occasions by a control sample of sporocysts suspended in distilled water.

The results of these preliminary investigations are presented in Table 6.1. They identify six chemicals (sulphuric acid, ammonia, methanol, ethanol, sodium hydroxide and potassium hydroxide) and one disinfectant (Medol) as having major sporocysticidal properties. However, because of either the high concentrations required (e.g. methanol, ethanol) or difficulties associated with assessing their effectiveness due to the total disappearance of sporocysts(e.g. sodium and potassium hydroxide), only three (2.5% sulphuric acid, 2% ammonia, 4% Medol) were investigated further.

This second investigation followed the same procedures as before, except that on this occasion exposure to each solution was limited to 0, 0.25, 0.5, 1, 2, 4, 6 or 12 hr

TABLE 6.1 EFFECT OF 48 HOURS SUSPENSION IN VARIOUS CHEMICALS AND DISINFECTANTS ON THE EXCYSTATION OF *S. GIGANTEA* SPOROCYSTS

Chemical/Disinfectant	Concentration (%)	Percent excystation** Expt. (a)	Expt. (b)
Control	-	81.0	79.2
Acetic Acid	2.5	66.1	74.1
Hydrochloric Acid	2.5	64.0	73.0
Nitric Acid	2.5	57.0	44.8
	0.5	46.0	50.1
Sulphuric Acid	1.0	8.0	14.0
	2.5	-	-
Formalin	10.0	53.1	60.0
Hydrogen peroxide	3.0	70.0	83.1
Lugol's Iodine	5.0	74.1	79.0
	0.5	71.0	84.1
Ammonia solution	2.0	1.0	-
	10.0	-	1.5
Methanol	10.0	74.8	63.7
	90.0	-	-
Ethanol	10.0	68.8	62.6
	90.0	-	2.2
Iso-propyl alcohol	90.0	58.5	57.0
	0.5	28.0	40.9
Sodium hydroxide	2.5	12.0	6.7
	5.0	*	*
	0.5	89.1	82.3
Potassium hydroxide	2.5	12.2	9.0
	5.0	*	*
'Dettol'	5.0	63.0	63.2
'Stericide'	5.0	65.3	70.1
'Hibitane'	5.0	71.0	69.2
'Iosene'	2.0	80.4	78.4
'Mucocit'	1.5	85.0	69.2
'Medol'	2.0	23.9	18.2
	4.0	2.0	1.7

*Number of sporocysts remaining after exposure too small to permit determination of percentage excystation.

** - = < 1.

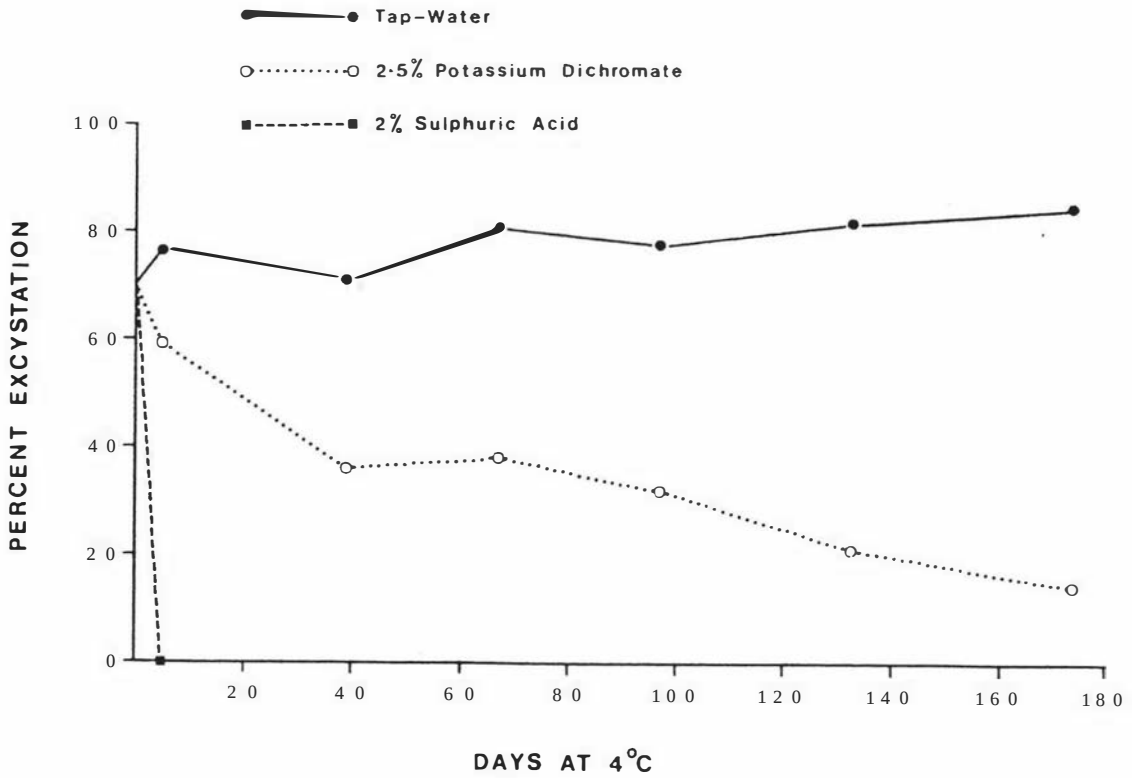

Fig. 6.1 Effect of storage medium and duration on the excystation of *S. gigantea* sporocysts

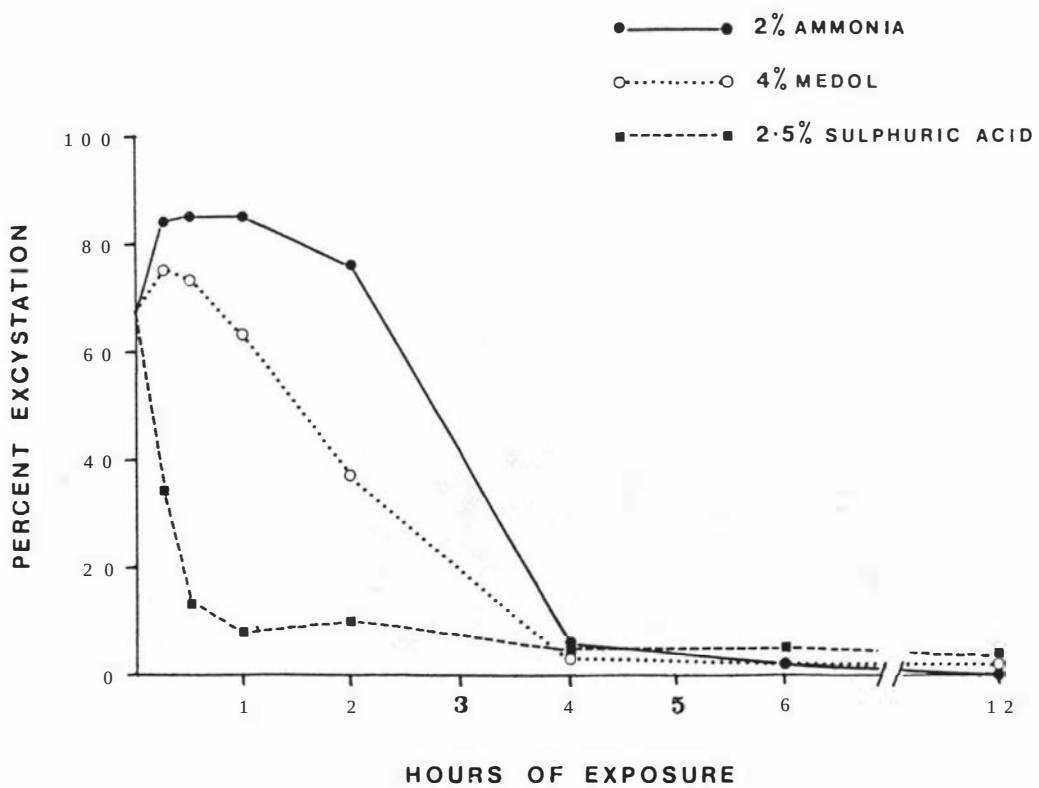

Fig. 6.2 Effect of exposure period and chemicals on the excystation of *S. gigantea* sporocysts

with four replicated samples being tested at each period.

The results (Fig. 6.2) showed that although sporocyst excystation was reduced to very low levels by 4 hr contact with all three solutions, sporocyst viability declined most rapidly in sulphuric acid followed by Medol and ammonia.

(iii) Effect of coccidiostats and anthelmintics

Many anthelmintics, particularly those of the benzimidazole group (e.g. thiabendazole) are known to be ovicidal (Egerton, 1969) and in recent years the ability of nematode eggs to embryonate and hatch when incubated in such solutions has been used as a simple screen for potential efficacy (Coles and Simpkin, 1977; Le Jambre, 1978). While a comparable test has not been used for coccidiostats, a number of them have been found to inhibit oocyst sporulation and infectivity (Ajayi, 1976; Joyner and Norton, 1977; Ruff *et al.*, 1978). In addition, the anticoccidial drug amprolium has been successfully used to reduce the severity of experimental infections of *S. cruzi* in cattle and *S. tenella* in sheep (Fayer and Johnston, 1975a; Leek and Fayer, 1980). In the latter experiments, drug administration started at the time of infection or on the day before, and it is conceivable, therefore, that at least part of the benefit of these treatments may have derived either from the effect of amprolium on the ingested sporocysts themselves or on their recently excysted sporozoites. Accordingly, the following study was undertaken to examine what effect, if any, exposure of *S. gigantea* sporocysts to coccidiostats and the anthelmintic thiabendazole would subsequently have on their ability to excyst.

The investigation was conducted in essentially the same manner as that described previously with 7 to 9 week-old

sporocysts being suspended in the drug under test for 48 hr at room temperature, washed and then subjected to excystation procedures. Again, each experiment was repeated once and was accompanied on both occasions by a control sample of sporocysts suspended in distilled water.

The results (Table 6.2) revealed that none of the drugs used had any marked inhibitory effect on the ability of sporocysts to excyst.

In an effort to determine whether this failure may have been due to the inability of the drugs to gain access to the sporozoites, a further experiment was conducted (with appropriate controls) in which sporocysts were exposed to Amprol (at 1000 ppm) for 48 hr either in combination with a 5% solution of dimethyl sulfoxide (DMSO) (BDH Chemicals Ltd) or following their prior pretreatment with 0.45% sodium hypochlorite for 0.5 hr.

The results (Table 6.3) suggested that although exposure to DMSO or sodium hypochlorite may have had some success in penetrating or increasing the permeability of the walls of the sporocysts, the additional presence of Amprol in combination with these treatments had little further effect on the viability of the sporozoites they contained.

(iv) Effect of heating

Samples of 10 to 13 week-old sporocysts suspended in 1 ml volumes of distilled water and maintained in an ice bath for 10 to 15 min were exposed to temperatures of 50, 55 or 60°C for periods of 0, 5, 10, 15, 30 or 60 min. Following these treatments, the sporocysts were immediately returned to the ice bath, again for 10 to 15 min, before being subjected to the usual excystation procedure. Four replicated samples were tested at each temperature and period.

TABLE 6.2 EFFECT OF 48 HOURS SUSPENSION IN VARIOUS COCCIDIOSTATS AND ANTHELMINTICS ON THE EXCYSTATION OF *S. GIGANTEA* SPOROCYSTS

Coccidiostat/ anthelmintic	Concentration (ppm)	Percent excystation Expt.(a)	Expt. (b)
Control	-	85.1	75.4
Daraprim	100	78.1	82.8
	500	85.0	76.6
	100	80.9	81.0
Sulphadimidine	500	81.0	75.4
	1000	83.5	73.2
	100	84.2	78.2
Toltro	500	74.8	78.8
	1000	85.7	77.8
	100	86.0	67.9
Eftolon	500	69.1	71.4
	1000	79.4	79.4
	100	80.5	70.7
Amprol	500	57.4	73.8
	1000	79.4	75.6
	100	75.1	70.4
Emtryl Soluble	500	63.6	69.4
	1000	76.3	70.7
	100	75.5	57.8
Equizole	500	67.4	78.0
	1000	76.0	73.4

TABLE 6.3 EFFECT OF TREATMENTS TO ENHANCE THE PENETRATION OF AMPROL* ON THE EXCYSTATION OF *S. GIGANTEA* SPOROCYSTS

Treatment	Percent excystation	
	Expt. (a)	Expt. (b)
48 hrs exposure to distilled water	85.1	75.4
48 hrs exposure to Amprol	79.4	75.6
48 hrs exposure to Amprol after 0.5 hrs exposure to 0.45% sodium hypo.	48.4	61.8
48 hrs exposure to Amprol and 5% DMSO	49.6	54.2
48 hrs exposure to 5% DMSO	39.2	42.9
48 hrs exposure to distilled water after 0.5 hrs exposure to 0.45% sodium hypo.	50.7	48.7

*Concentration = 1000 ppm.

The results, given in Fig. 6.3, showed that sporocyst viability was destroyed by exposure to temperatures of 60°C for 5 min or 55°C for 60 min but not at 50°C for any of the periods tested.

(v) Effect of freezing and thawing

After determination of their excystation level, sporocysts approximately 6 months-old suspended in tap water were dispensed as 1.5 ml aliquots into 132 clear polystyrene tubes (Smith Biolab Ltd). These tubes were then randomly allocated to one of three groups maintained as follows: One group was kept at 4°C as a control, one was frozen at -18°C and kept constantly at this temperature while the third was subjected to alternative 24 hr periods at -18°C and room temperature. Periodically, four tubes were removed from each group, allowed to stand at room temperature for approximately 2 hr and the viability of the sporocysts they contained, tested.

As shown in Fig. 6.4, sporocysts kept at 4°C maintained a high level of viability but those subjected to either constant or intermittent freezing suffered a relatively slow decline in excystation rate with time. Initially, the rate of this decline was more rapid in sporocysts kept constantly frozen rather than intermittently. After 170 days, however, there was little difference between them and at the end of the experiment on day 243, excystation levels of 8.7 ± 0.8% and 2.6 ± 0.8% respectively, were recorded in constantly and intermittently frozen sporocysts.

(vi) Effect of ultraviolet (IV) irradiation

One ml aliquots of sporocyst suspension (6 months-old) and

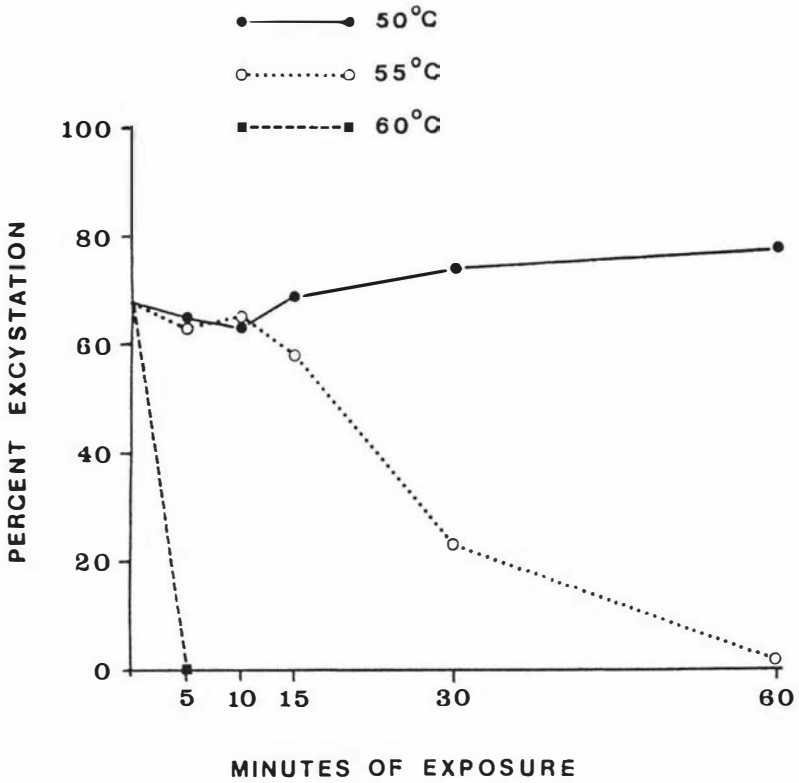

Fig. 6.3 Effect of heating on the excystation of *S. gigantea* sporocysts

4 ml of tap water were placed in 8 cm diameter glass petri dishes (depth of suspension 2 mm). Accompanied by gentle rotation to ensure adequate mixing, these were then subjected to UV irradiation at room temperature with four replicated samples being irradiated at a range of doses from 0 to 4000 ET's (μW-min/cm²).

Irradiation at 100 and 500 ET had little effect on sporocyst excystation (Fig. 6.5). At 1000 ET, the excystation level had declined from 72.4 ± 2.6% in untreated controls to 59.2 ± 2.7% and at 1500 ET only 21.9 ± 4.0% of sporocysts excysted. Thereafter, the excystation level continued to decline, albeit at a slower rate, with increasing irradiation dose and at 4000 ET no

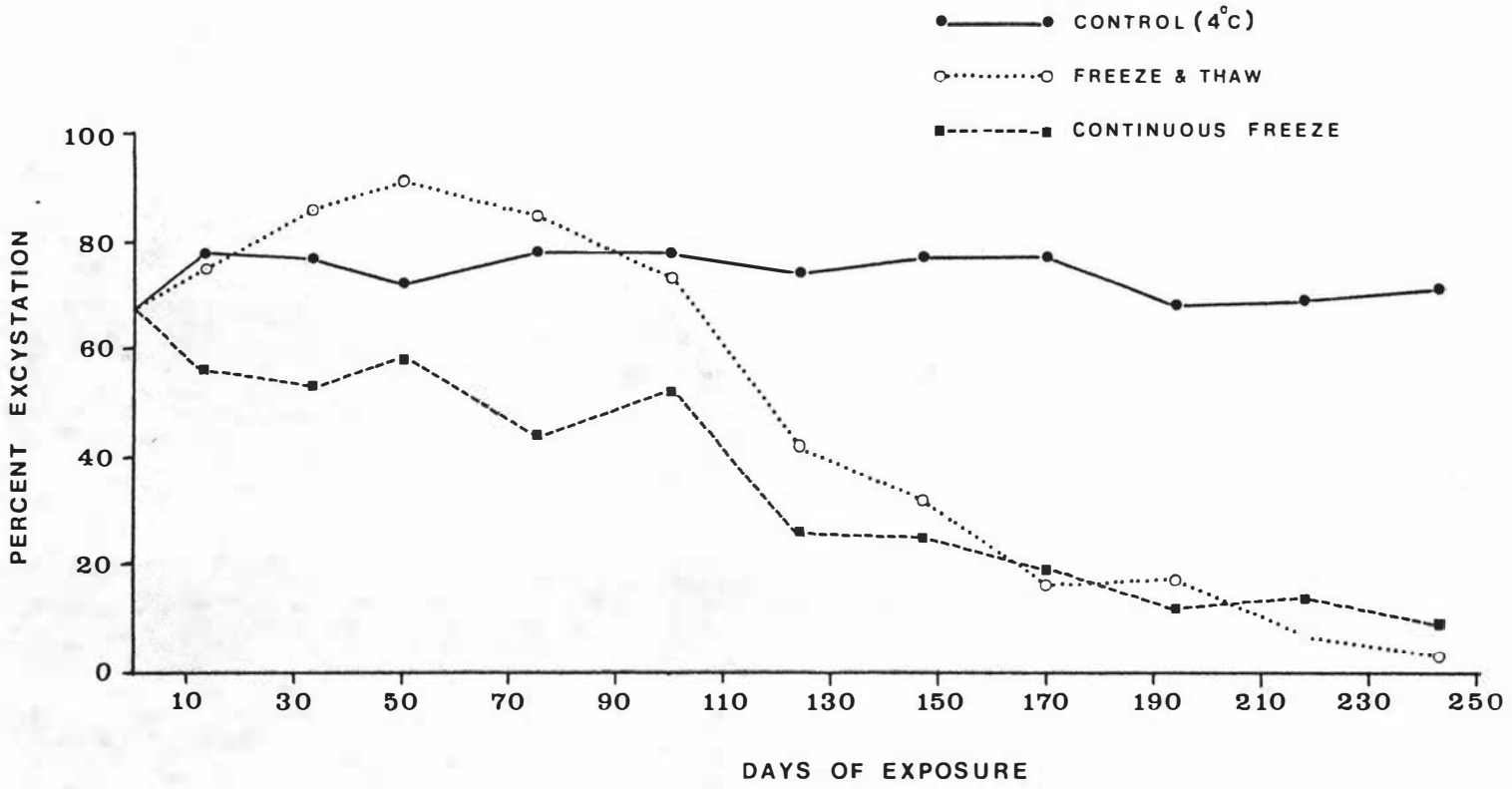

Fig. 6.4 Effect of freezing and thawing on the excystation of *S. gigantea* sporocysts

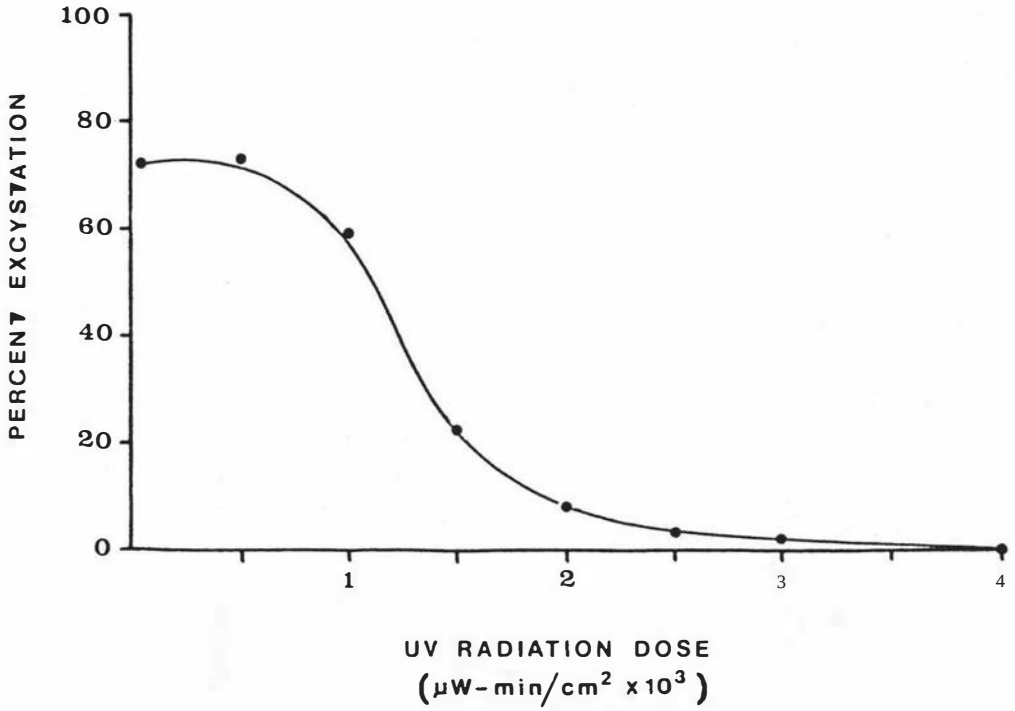

Fig. 6.5 Effect of ultraviolet irradiation on the excystation of *S. gigantea* sporocysts

excystation was recorded.

(vii) Effect of desiccation

Tap water suspensions (2.5 ml) of 6 month-old sporocysts (their excystation level being determined beforehand) were placed in 144 polystyrene tubes. One hundred and eight of the tubes were centrifuged, their supernatants removed, leaving a sediment of sporocysts, faecal debris and surface water in each. After standing for 48 hr at room temperature to allow this surface water to evaporate, they were divided into groups of 36 and randomly allocated to one of three relative humidities (85%, 55% or 33%) at 24°C. The remaining 36 non-centrifuged tubes kept half

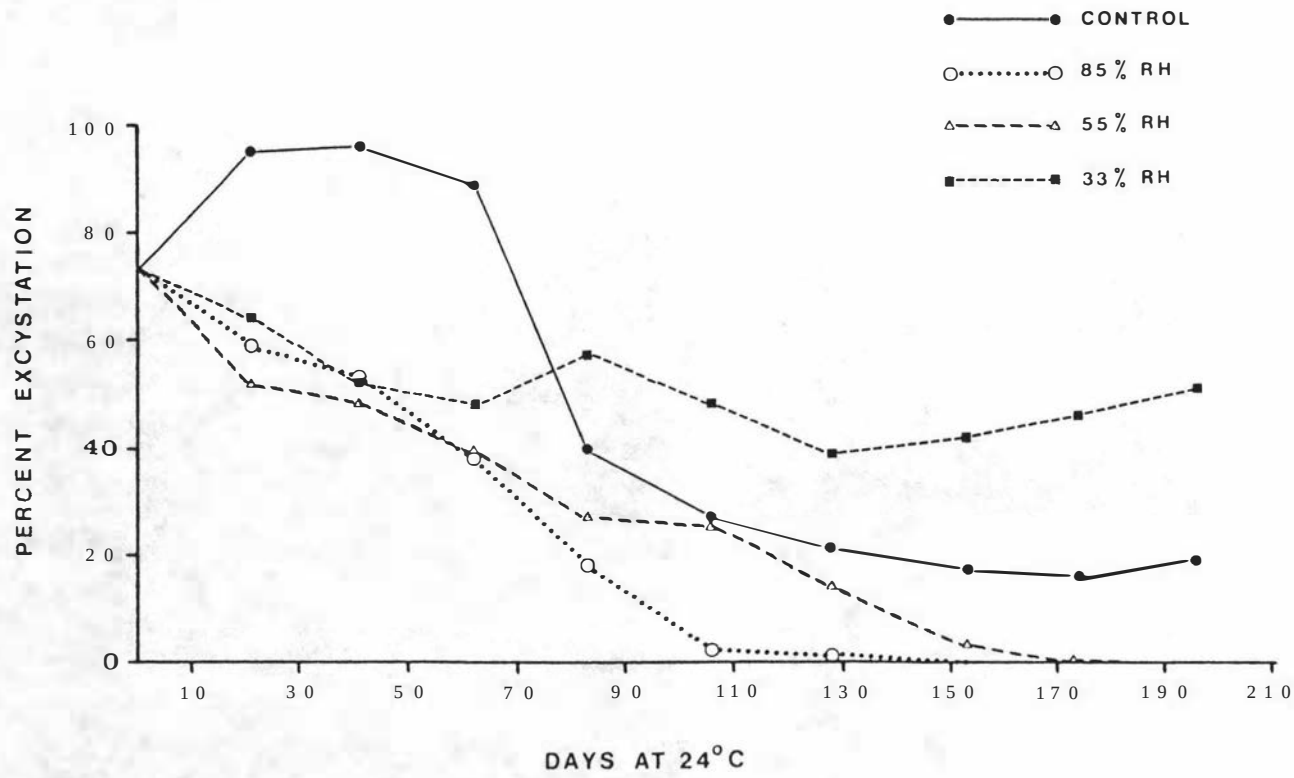

Fig. 6.6 Effect of desiccation on the excystation of *S. gigantea* sporocysts (using polystyrene tubes at 24°C)

full of water, were maintained at the same temperature as undesiccated controls. At 20 to 30 day intervals, four tubes were removed from the control and each of the desiccated groups. Sporocysts in the latter three groups were then rehydrated and all samples allowed to stand at room temperature for 2 hr before their percentage excystation was determined.

The results (Fig. 6.6) showed that *S. gigantea* sporocysts were capable of surviving desiccation for considerable periods with the duration of survival being inversely related to the relative humidity (RH) at which they were maintained. Because of a marked decline in the viability of the control sporocysts however, this latter result was regarded with some scepticism. Initially, it was considered possible that some agent on or in the polystyrene tubes may have combined with the water in the controls and the greater amounts of moisture available at higher relative humidities, to produce toxic solutions. The results of a partial repeat of the above experiment (Fig. 6.7) using on this occasion glass-tubes and 12 month-old sporocysts, however, suggested that this was not the case. Accordingly, a third experiment was conducted in which 12 month-old sporocysts, either held under water, or desiccated and exposed to relative humidities of 33% or 85% in polystyrene tubes, were maintained at temperatures of 15 or 24°C.

The results are presented in Fig. 6.8. Apart from some minor differences, the patterns of survival of desiccated sporocysts in this experiment were basically similar to those observed previously with viability being maintained at higher levels for longer intervals at 33% RH rather than 85% RH at both temperatures tested. The survival of sporocysts maintained in water, however, was considerably better at 15°C than at 24°C.

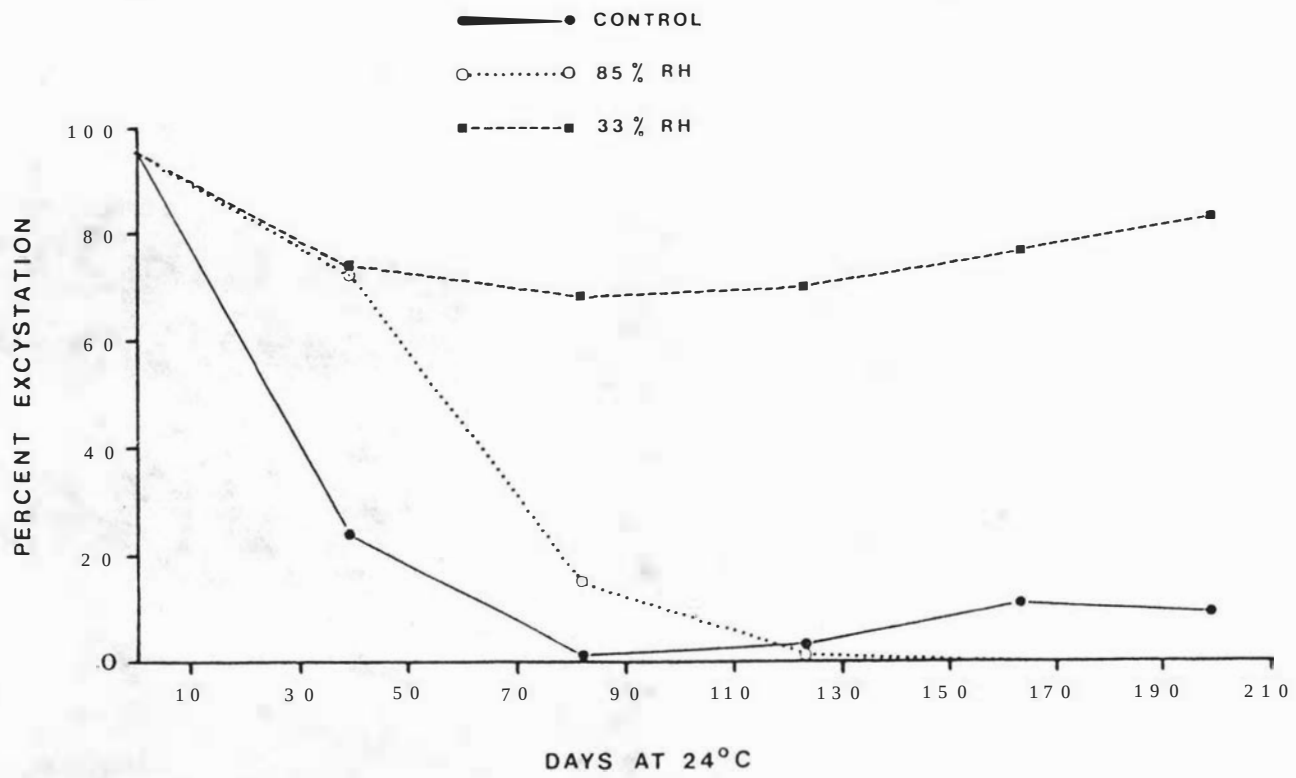

Fig. 6.7 Effect of desiccation on the excystation of *S. gigantea* sporocysts (using glass tubes at 24°C)

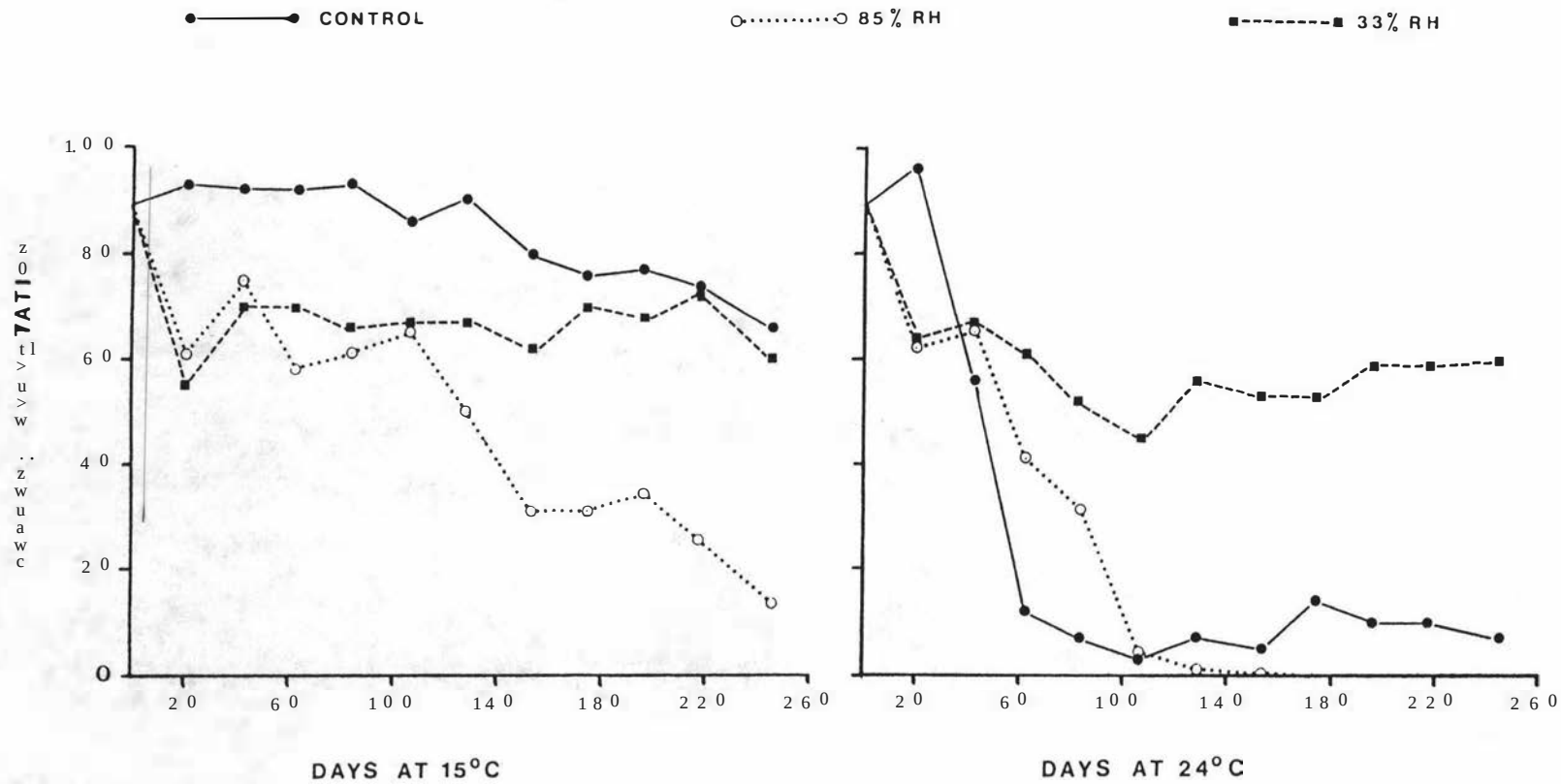

Fig. 6.8 Effect of temperature and desiccation on the excystation of *S. gigantea* sporocysts

6.3.2 Outdoor experiments on sporocyst survival

Studies on the outdoor survival of oocysts and sporocysts are beset by a number of difficulties. Cat faeces are frequently superficially buried (Frenkel *et al.*, 1975). Ideally, therefore, such studies should involve the use of similarily placed samples from which sporocysts are periodically recovered and tested for viability. The difficulty with this approach is that, as a result of the dispersive action of environmental agents such as rain, wind, earthworms and insects, the concentration of sporocysts in faecal samples is likely to diminish with time. This means that eventually the numbers recovered are likely to be too small for adequate testing. Placement of faecal samples out-of-doors in containers such as petri dishes, on the other hand, although ensuring less loss, would provide the sporocysts with unnatural protection against some environmental influences, particularly if the containers were sealed, or alternatively, expose them to a risk of artifical flooding, if they were not. In the present study attempts were made to strike a reasonable compromise between these considerations in the following manner.

Plugs of cotton wool were placed in the bases of polystyrene tubes in which single air holes had previously been drilled (Fig. 6.9). These tubes, measuring 7.5 cm x 1 cm were then half-filled with *Sarcocystis*-free cat faeces and 0.5 ml of concentrated sporocyst suspension added. After standing for 48 hr at room temperature to allow excess water to evaporate, the tubes were completely filled with faeces and capped with terylene gauze covers. They were then placed, open-ends down, in one of four soil-filled plastic containers so that their ventilated bases projected approximately 1.5 cm above the soil surface. Each container, which was provided with a terylene gauze-covered drain hole, was sunk into the ground (Fig. 6.9). Two

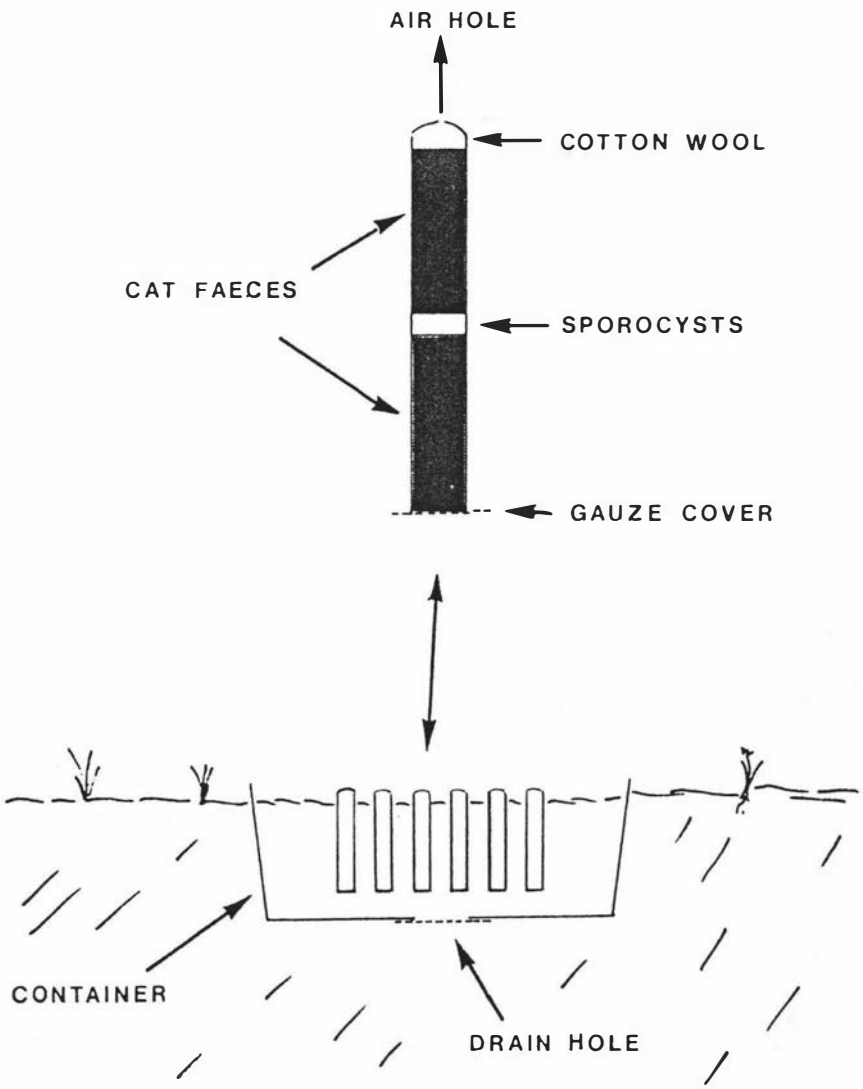

Fig. 6.9 Procedures adopted in studies on the outdoor survival of *S. gigantea* sporocysts

containers were placed in a shaded site located at the eastern end of a building and two were placed out in the open. By this means it was hoped to simulate conditions likely to be encountered by sporocysts in buried faeces. The orientation of the tubes and the presence of the air hole would, it was hoped, allow free exchange of moisture between the samples and the surrounding soil, the gauze covers preventing their removal by earthworms.

At the start of the investigation and at approximately monthly intervals thereafter, one randomly selected tube was removed from each of the four containers and the sporocysts recovered from them subjected to the usual excystation procedure. Two experiments, extending over 12-month periods, were conducted on separate occasions. Daily rainfall and temperature fluctuations for both were recorded at a meterological station located 1 km from the test site.

The results of the first experiment (Expt. (a)) which began in May 1981, using sporocysts approximately 10 months-old, are presented in Fig. 6.10. Although there were considerable variations between the viability of sporocysts recovered from individual tubes on some occasions, there was no obvious relationship between these variations and the particular containers from which they were removed. Therefore, the results in Fig. 6.10 are presented as mean figures for the four replicates. These showed that sporocyst viability was maintained at high levels for the first 28 weeks of the investigation. After nearly 32 weeks the ability of sporocysts to excyst had declined markedly and by 35 weeks virtually no excystation was detected.

The marked decline in sporocyst viability coincided with the beginning of the summer months in December. It was

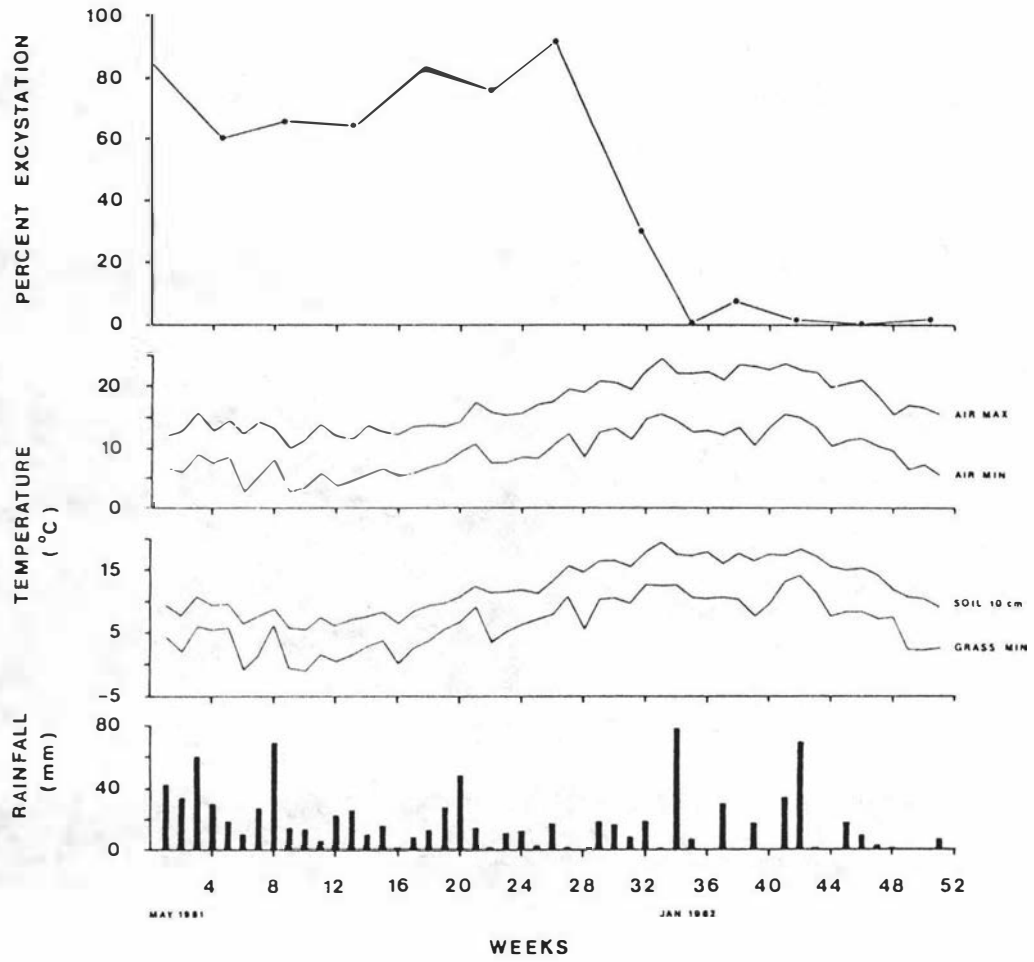

Fig. 6.10 Climatic data and outdoor survival of *S. gigantea* sporocysts in experiment (a) (commenced May, 1981)

unclear, however, if this decline was associated with the rise in temperatures recorded over this period or was more simply related to the length of time the sporocysts had been maintained out-of-doors. Therefore, a second experiment (Expt. (b)) was undertaken in an attempt to resolve this matter. This experiment started in September 1982. Unfortunately sporocysts comparable in age to those employed previously were not available and it was necessary to use sporocysts 2 to 3 months-old on this occasion.

The results are presented, as in the first investigation, as mean percentage excystation for the four replicates (Fig. 6.11). Examination of this figure shows that although summer temperatures were generally lower than those recorded in the previous year, sporocyst viability again declined most rapidly over the December-January period. This decline which occurred 13 to 17 weeks after the start of the experiment, was less marked than that observed in the first investigation with sporocyst viability persisting at moderate to low levels until the study was terminated, although declining steadily throughout.

6.4 DISCUSSION

Knowledge of the resistance and survival of the infective stages of parasitic organisms is obviously of some practical importance. Not only is this information required to understand the epidemiology of such infections and to devise appropriate prophylactic procedures, but also to maintain the viability of laboratory cultures. Studies on coccidian species often require the use of oocysts and sporocysts that have been stored for several weeks or months after collection. Normally these are recovered from faeces and placed in aqueous solutions of

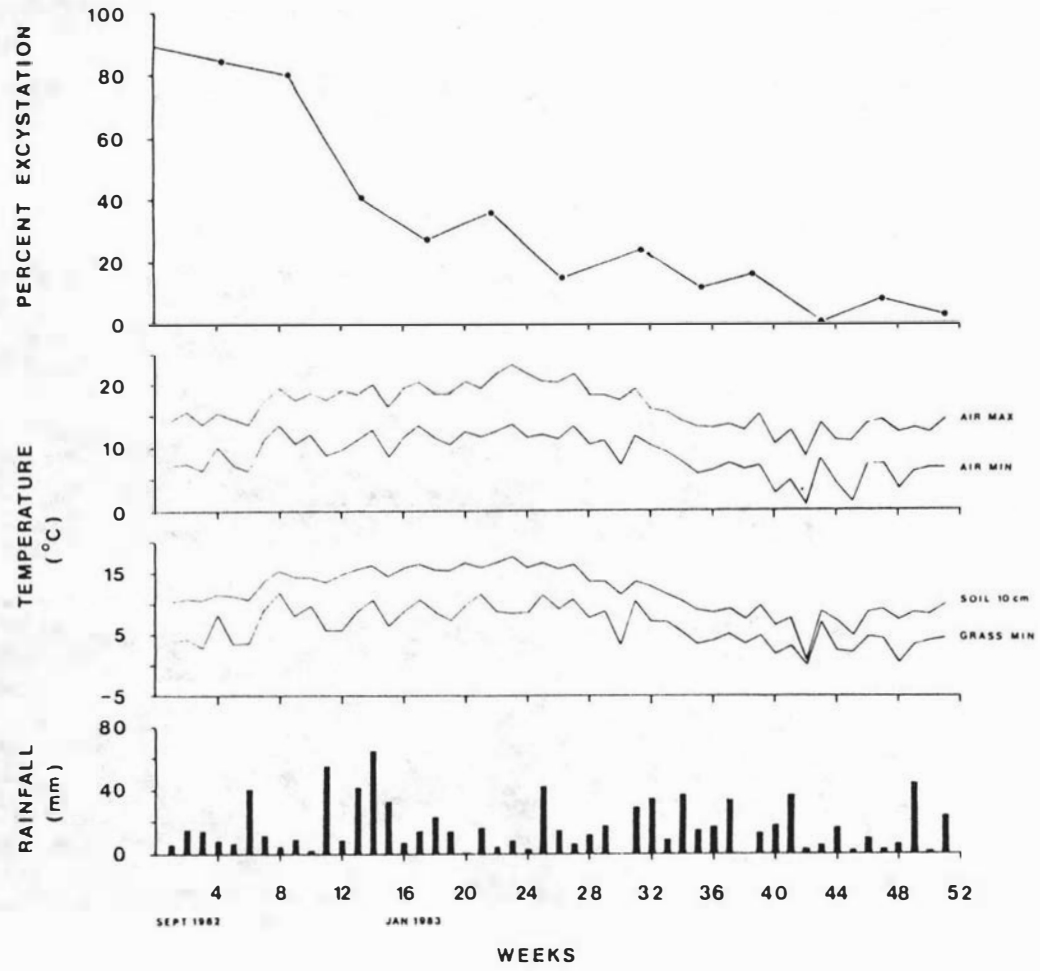

Fig. 6.11 Climatic data and outdoor survival of *S. gigantea* sporocysts in experiment (b) (commenced September, 1982)

2.5% potassium dichromate or 2% sulphuric acid. The primary purpose of these solutions is to retard the growth of bacteria and fungi which may compete with the oocysts for the oxygen vital for sporulation. However, they are also frequently employed as preservatives both for sporulated oocysts of *Eimeria* and *Isospora* (Ryley, 1973) and for sporocysts of *Sarcocystis* spp. (Ruiz and Frenkel, 1976; Ashford, 1978; Box and Duszynski, 1978; Duszynski and Box, 1978; Munday 1979a; Bledsoe 1980b; Cawthorn *et al.*, 1981).

For many species of coccidia, particularly those of the genus *Eimeria*, potassium dichromate has been found to maintain oocyst viability for at least two years (Kheysin, 1972). For others, including two closely related to the genus *Sarcocystis*, namely *Toxoplasma gondii* and *Isospora rivolta*, storage in this medium may lead to a reduction in the permeability of the oocyst wall with better survival being achieved in sulphuric acid (Jackson, 1962; Dubey *et al.*, 1970a; McKenna and Charleston, 1982).

In the present investigation neither of these solutions was found to be satisfactory for the preservation of *S. gigantea* sporocysts. This was particularly true of sulphuric acid which, at 4°C, was found to destroy sporocyst viability within 5 days. Indeed, at room temperature, contact with a 2.5% concentration of this solution resulted in a considerable reduction in the ability to excyst in only a matter of minutes.

The effect of potassium dichromate and sulphuric acid preservation on the viability of *Sarcocystis* spp. sporocysts has been previously reported by Leek and Fayer (1978). Using *S. cruzi* sporocysts, they obtained somewhat similar results to those recorded here, prompting them to suggest that the comparative vulnerability of the exogenous stages

of *Sarcocystis* spp. to such media may be attributable to the absence of an oocyst wall. That this hypothesis represents an over-simplification, is evident from the ability of sporocysts of other *Sarcocystis* spp. to induce infection following their storage in sulphuric acid (Ruiz and Frenkel, 1976; Ashford, 1978; Bledsoe, 1980b). Furthermore, although *S. meischeriana* sporocysts appear to be more susceptible to some commercial disinfectants than do sporulated oocysts of some *Eimeria* and *Isospora* spp. those of *S. muris* do not (Barutzki *et al.*, 1981). Conversely, despite their greater tolerance to sulphuric acid, *Toxoplasma* oocysts are evidently no more refractory to ammonia than are sporocysts used in the present study (Dubey *et al.*, 1970b; Ito *et al.*, 1975a). It would appear, therefore, that variations in the ability of such stages to withstand chemical assault are more likely to be related to differences in the structural and/or biochemical nature of their surrounding envelopes rather than the mere presence or absence of an oocyst wall.

From the present results it is apparent that *S. gigantea* sporocysts are relatively impervious to a wide range of such agents. They were able to resist exposure to most laboratory reagents and disinfectants as well as a number of anti-coccidial drugs. Unfortunately comparable studies have not been undertaken with sporocysts of other *Sarcocystis* spp. and so it is difficult to determine which of the present findings are likely to be of generic and which of specific relevance only. It is evident nevertheless that there may be considerable differences in the abilities of sporocysts of various *Sarcocystis* spp. to withstand at least some of these agents.

The same properties which may lead to variations in the sensitivities of the exogenous stages of coccidian species to chemical assault are also likely to influence their susceptibilities to physical agents as well. Thus Bergler

et al. (1980) found that while there were some differences between them, sporocysts of *S. meischeriana*, *S. suihominis* and *S. capreolicanis* were better able to resist freezing and thawing, lower relative humidities and higher temperatures, than were oocysts of *T. gondii* and some species of *Eimeria* and *Hammondia*. Heydorn (1980), on the other hand, observed that although *S. cruzi* sporocysts were largely refractory to freezing they were relatively susceptible to heating and desiccation.

Because of differences in the temperatures, relative humidities and intervals of exposure employed, it is difficult to make any direct comparisons between the above findings and those obtained in the present study. Even so it is apparent, that while the viability of *S. gigantea* sporocysts may be destroyed by heating and ultraviolet light, their resistance to freezing and particularly, desiccation, can only be considered as remarkable.

For many species of coccidia, apparently including some members of the genus *Sarcocystis*, the ability of oocysts and sporocysts to survive desiccation is frequently measured in days being directly proportional to the relative humidity to which they are exposed (Kheysin, 1972; Hammond, 1973; Bergler *et al.*, 1980). In the present study, *S. gigantea* sporocysts were evidently able to survive desiccation for weeks and months with the duration of survival being inversely related to the relative humidity at which they were maintained. Although this may appear unusual it is by no means unique. Indeed, similar behaviour has been observed with the eggs of the plant nematode *Globodera (Heterodera) rostochiensis* (Ellenby, 1968) hatched and unhatched infective larvae of *Nematodirus battus* (Parkin, 1976) and infective larvae of *Trichostrongylus colubriformis* (Wharton, 1982). The precise means by which such organisms are better able to withstand prolonged

desiccation at lower rather than higher relative humidities are unknown. Ellenby (1968), however, has suggested that for *G. rostochiensis*, it may be associated with a decrease in the permeability of the egg shell as it dries. This is not to suggest that water loss is completely prevented by such a phenomenon but that it is reduced to a very slow rate indeed.

The present results indicate that the sporocyst walls of *S. gigantea* may possess similar properties, properties which not only aid the survival of their sporozoites at times of severe desiccation but possibly increase their tolerance of high environmental temperatures as well. Thus in contrast to their resistance to low and freezing temperatures, *S. gigantea* sprocysts appeared relatively more susceptible to heat. These were either killed by heating to 60 or 55°C for 5 and 60 min respectively, or severely impaired by prolonged exposure to 24°C under water. Desiccated sporocysts, particularly those maintained at 33% relative humidity, appeared to survive reasonably well at 24°C. The reasons for these differences in survival at this temperature are unknown. It is possible that the evaporation of water from desiccated sporocysts was sufficient to aid in their cooling. Alternatively and perhaps more likely, because oxygen is used by oocysts in respiration (Kheysin, 1972) and because the respiratory demands of sporozoites and aerobic bacteria likely to flourish might be expected to increase at higher temperatures, sporocysts stored under water might have died of an oxygen deficit.

That the death of these sporocysts was unlikely to be directly related to temperature effects might be indicated by their surprising ability to survive at 50°C for at least 1 hr; a temperature which has previously been found to result in a 50% mortality of oocysts of a number of

coccidial species within 0.36 to 29 min (Kheysin, 1972). It has been suggested that differences in the sensitivity of oocysts to high temperatures may be associated with the varying thicknesses of their walls (Kheysin, 1972). However, for *S. gigantea*, bradyzoites within isolated muscle cysts appear to survive equally as well as sporozoites within sporocysts at 50°C (Collins, 1980b) suggesting that survival at such temperatures is more likely to depend on the resistance of the zoites rather than the thickness of their enclosing cyst walls.

Temperature and desiccation are, in part, a function on sunlight. However, no work has been undertaken to determine what effect the UV radiation component of sunlight has on the survival of sporocysts of *Sarcocystis* spp. In the present study, UV irradiation was found to be extremely deleterious for *S. gigantea* sporocysts with their ability to excyst being totally eliminated at a radiation dose of 4000 ET. Using a figure of 55 $\mu W/cm^2$ provided by Wright and McAlister (1934) for the intensity of shortwave UV in solar radiation at wavelengths of 297 to 313 nM, the present results suggest that *S. gigantea* sporocysts might be rendered non-infective by only 1.2 hr exposure to sunlight. While this exposure period might seem very brief it should be pointed out that embryonated eggs of *Ascaris suum*, generally considered to be very resistant to adverse environmental conditions and capable of surviving for 5 years or more (Soulsby, 1982), may be killed by 5.5 to 9 hr exposure to sunlight (Spindler, 1940).

In attempting to correlate the effects of physical factors on the survival of *S. gigantea* sporocysts obtained in the laboratory with those existing in the field, it can be seen that certain sets of conditions are more likely to be favourable than others. In general it would appear that

such sporocysts may be better able to retain their viability in either cool or hot-dry conditions than in hot-wet ones.

Laboratory studies carried out with sporocysts isolated from faeces, avoid some of the complexities and difficulties of interpretation imposed by the faecal environment. Moreover, such sporocysts were mainly subjected to only one variable influence whereas those maintained out-of-doors are likely to experience a number of these factors operating at the same time, as well as biological agents. Despite these differences, the pattern of survival of sporocysts maintained out-of-doors equated reasonably well with laboratory results inasmuch as viability tended to decline most rapidly over the summer months when temperatures were high and solar radiation was at its maximum. While this general correlation may provide some satisfaction it is recognised that the procedures adopted in the outdoor experiments must have had some influence on their outcome. It is obvious, for example, that the containment of sporocysts in buried polystyrene tubes must have provided considerable protection from ultra-violet light while at the same time exposing them to higher relative humidities and greater degrees of putrefaction than are likely to be experienced by those liberated from faeces deposited naturally. Accordingly, extrapolation from the present findings to the natural course of events in the field needs to be made with considerable caution.

CHAPTER 7

GENERAL DISCUSSION

It is now over 140 years since sarcocysts were first discovered in the muscles of mice by Miescher (1843). Since that time, progress towards an understanding of the identity, biology and importance of these parasites has been considerable, particularly over the last 10 or 12 years. To a large extent much of this work has, understandably, been directed towards documenting and describing those species infecting domestic livestock, examining their prevalences and distributions and elucidating their life cycles in terms of developmental sequence, host range and pathogenicity. Considerably less attention, however, has been focused on the epidemiology of these infections or on examining some of the more fundamental factors governing parasite transmission such as sporocyst production, viability and longevity.

In New Zealand, sarcosporidiosis is of considerable concern to the sheep industry, with *S. gigantea* being the most economically important species (Collins, 1980b). Despite this and despite the fact that it was the first species of *Sarcocystis* shown to produce intestinal infection in a carnivore (Rommel *et al.*, 1972), comparatively little is known about it. Attempts to infect sheep experimentally have either been unsuccessful or largely equivocal, while development within the definitive host has been investigated to only a limited extent (see Section 1.3).

The inability to readily infect the intermediate host with *S. gigantea* experimentally has frustrated research on this parasite. It has meant, for example, that the path-

ogenicity of this species for sheep remains largely uncertain, while the feasibility or otherwise of instituting potential control measures such as immunisation or chemical prophylaxis is unknown. In addition, it has led to some speculation that *S. gigantea* sporocysts may not be immediately infective for sheep and has drawn attention to the need for some means of determining and confirming their viability (Collins, 1980b).

For this and other species of *Sarcocystis* it was considered that an appropriate measure of sporocyst viability and infectivity was likely to be provided by their ability to excyst. Accordingly, one of the main objectives of the present study was the development of a technique which would enable *S. gigantea* sporocysts to be excysted in the laboratory. Obviously, to achieve this objective required investigation of the various factors affecting excystation *in vitro*. But before doing so, it was felt necessary to ensure that the work was unlikely to be curtailed by a shortage of infective material, previous observations having suggested that the numbers of sporocysts shed by infected cats were likely to be low (Rommel *et al.*, 1972; Mehlhorn and Scholtyseck, 1974).

The study described in this thesis, therefore, comprised four separate, integral parts. The first, was aimed at developing a procedure for the mass recovery of sporocysts from faeces and devising a reliable and accurate means of performing faecal sporocyst counts. The second was directed towards making use of the latter technique to determine the pattern of sporocyst shedding and the numbers of sporocysts shed by infected cats, and thus the best means of ensuring maximum yields. The third was directed towards determining the most effective procedure for the *in vitro* excystation of *S. gigantea* sporocysts and

examining how this might equate with excystation *in vivo*, and the fourth was aimed at making use of *in vitro* excystation as a measure of sporocyst viability and survival under various conditions.

Although excystation *in vitro* has been reported for a number of *Sarcocystis* species, including *S. cruzi*, *S. miescheriana*, *S. capreolicanis*, *S. suihominis* and *S. debonei* (Fayer and Leek, 1973; Bergler *et al.*, 1980; Box *et al.*, 1980), only for the first has any attempt been made to examine the effect of any of the various factors involved. For *S. cruzi*, high levels of excystation occurred when sporocysts were exposed to trypsin and bile following their pretreatment with cysteine hydrochloride and CO_2 (Fayer and Leek, 1973). Since then, the same procedure has been used for the excystment of the other *Sarcocystis* species listed above, with no attempt being made to ascertain if it was the most appropriate for the particular species concerned.

In the present investigation, cysteine hydrochloride-CO_2 pretreatment was found to be largely ineffective for the *in vitro* excystation of *S. gigantea*. For this species, a significant degree of excystation was attained only following the pretreatment of sporocysts with sodium hypochlorite. Since alternative forms of pretreatment have not been examined for other *Sarcocystis* spp., it is possible that sodium hypochlorite exposure might be highly successful for their *in vitro* excystation also; however, the present results certainly indicate that this is not so for *S. tenella* or *S. capracanis*.

Sporocysts of *S. tenella* and *S. capracanis* are both infective for ruminants and are transmitted by dogs as are those of *S. cruzi*. *Sarcocystis gigantea*, on the other hand, is the first cat-transmitted species of

Sarcocystis with a ruminant intermediate host for which *in vitro* excystation has been examined. It is possible, therefore, that any disparity between the pretreatment requirements of *S. gigantea* sporocysts and those of *S. tenella* and *S. capracanis* (and perhaps *S. cruzi* also) may be associated with some idiosyncrasy of cat transmission. Alternatively, because the excystation requirements of other cat-derived *Sarcocystis* spp. are unknown, it may be associated with the nature of the species itself, possibly the physical and biochemical structure of their sporocyst walls.

In the course of the study, attempts were made to compare the ultrastructure of intact *S. gigantea* and *S. tenella* sporocysts recovered from faeces. But, despite efforts to facilitate the process, either by immersing them in liquid nitrogen (Beesley and Latter, 1982) or by sonication, considerable difficulty was encountered in obtaining their infiltration by fixatives and embedding media. Because of such difficulties and lack of time, these attempts were placed in abeyance.

The ultrastructure of oocysts and sporocysts has been described for few coccidia species, and, in view of the difficulties described, it may not be too surprising that those few studies which have been undertaken have most often involved oocysts and sporocysts undergoing excystation in which media penetration is assured (Roberts *et al.*, 1970; Speer *et al.*, 1973, 1976; Christie *et al.*, 1978; Box *et al.*, 1980). Not all such studies, however, have found it necessary to use excystment as a means of circumventing the impervious barriers likely to be presented by the oocyst and sporocyst walls. Indeed, where oocysts and sporocysts of *Sarcocystis* spp. have been examined either *in situ* within the intestinal mucosa (Mehlhorn and Scholtyseck, 1974; Colwell and Mahrt, 1983) or in tissue culture (Vetterling *et al.*, 1973; Becker *et al.*,

1979), resistance to fixatives and embedding medium appears not to have been a problem. The reasons for the apparent differences in the infiltrability of these specimens and those used in the present study are not known. It is possible that they may simply be related to differences in the sporocyst wall permeabilities of the various *Sarcocystis* spp. concerned. Alternatively, it is also possible that structural changes in the sporocyst wall may occur after their formation and release from host cells; a suggestion which has previously been made with respect to the oocyst walls of other coccidia (Speer and Duszynski, 1975).

The stimuli necessary to achieve excystation *in vitro* are generally considered to be similar to those required *in vivo* (Jackson, 1962). Because of this, it is tempting to draw a connection between the apparent contrariety and 'artificiality' of the pretreatment stimulus required to induce the *in vitro* excystation of *S. gigantea* sporocysts with the difficulties encountered by some in attempting to infect sheep experimentally with them. However, the fact that such sporocysts were found to excyst at least to a modest level *in vivo* (Chapter 5), casts doubt on the validity of such a connection. Furthermore, the lack of success in obtaining the *in vivo* excystation of *S. tenella* sporocysts (with which experimental infections, including clinical disease, can be readily established, see Section 1.2.5(a)), indicates that any attempt to equate findings pertaining to *in vitro* excystation with infectivity needs to be approached with caution.

Excystation *in vivo* and its relationship to *in vitro* excystation warrants further investigation. *In vivo* excystation has been little studied but it is generally assumed to be primarily dependent upon the bile/trypsin stimulus, as it is *in vitro*. The failure of *S. tenella*

sporocysts to excyst in either the duodenum or in small intestinal fluid from sheep (see Chapter 5), appears to question the essential importance of these stimuli for excystation *in vivo* as does the ability of sporocysts and oocysts of this and other coccidia species to induce infection when administered parenterally (Charleston, 1982). Moreover, independence from a bile/trypsin stimulus might better accord with the capacity of oocysts of the closely related coccidian, *Toxoplasma gondii*, to infect such a remarkable diversity of hosts (Dubey *et al.*, 1970a).

The use of excystation *in vitro* as a measure of the viability of sporocysts of *Sarcocystis* species is not new. Understandably, however, in view of the difficulties of maintaining intermediate hosts free of extraneous *Sarcocystis* infection, there have been few attempts to ascertain how this measure equates with infectivity. In one such study, (Heydorn, 1980), it was found that the results of excystation of *S. cruzi* sporocysts *in vitro* did not always correspond to the level of infection obtained. In another, Bergler *et al.* (1980), found that the ability of *S. miescheriana* and *S. suihominis* sporocysts to excyst provided a clear indication of their infectivity. While both these studies may provide some evidence to suggest that not all sporocysts which are capable of excysting are necessarily infective, they also lend strong support to the view that those which are incapable of doing so, are not.

Notwithstanding the possibility of species variation and the fact that a different excystation technique was employed, information regarding sporocyst survival obtained in the present study should be interpreted with this in mind. On this basis, sporocysts of *S. gigantea* may safely be assumed to be completely non-infective only following their suspension either in 2.5% sulphuric acid or 90% methanol for

48 hr, their exposure to ultraviolet irradiation at a dose of 4000 ET, or their heating to 60°C for at least 5 minutes. That is not to suggest that sporocyst viability and infectivity were not severely impaired by other forms of chemical and physical treatment examined. Prolonged storage in such standard coccidial oocyst preservatives as 2.5% potassium dichromate, for example, obviously had a deleterious effect on the viability of *S. gigantea* sporocysts as did their lengthy subjection to desiccation and freezing.

Sporocysts used in the latter part of the study had previously been stored in tap water at 4°C for periods ranging from 2 weeks to 12 months. Since there is some evidence to suggest that older *S. cruzi* sporocysts may be more sensitive to physical insult than are younger ones (Heydorn, 1980), the possibility cannot be excluded that somewhat different results might have been obtained in the current study had different aged sporocysts been used. In general, however, the viability of *S. gigantea* sporocysts appeared to be unimpaired by lengthy storage in water.

In reports of attempts to infect sheep experimentally with *S. gigantea*, information concerning the age and means of preservation of those sporocysts used has not always been provided. As a result, the relevance of the discovered vulnerability of *S. gigantea* sporocysts to storage in potassium dichromate and sulphuric acid to unsuccessful attempts to induce infection, is not entirely clear. However it is notable that the failures reported by Collins (1980b) involved the use of sporocysts stored in tapwater at 4°C for no more than 3 weeks.

The reasons for the apparent difficulties associated with efforts to infect sheep experimentally with *S. gigantea* remain unresolved. Nevertheless, *S. gigantea* is obviously

a highly successful parasite as is evident from the frequent occurrence of naturally acquired infections in sheep both in this country and elsewhere (see Section 1.3). In spite of the problems of interpretation regarding sporocyst viability previously referred to, many of the findings obtained in this study may help explain why infection is so common. Cats infected with *S. gigantea* are evidently capable of excreting large numbers of sporocysts for considerable periods. Moreover, such animals may be infected repeatedly with undiminished sporocyst shedding taking place on each occasion. Given the apparent resistance and longevity of these sporocysts, the widespread and common occurrence of both sheep and cats in New Zealand and the ability of sarcocysts in sheep meat to survive at ambient and freezing temperatures (Collins 1980b), then a high prevalence of infection might be expected.

Much work remains to be done. There is a need to define more precisely where, when and how infection is acquired by sheep, to determine the relative importance of domestic and feral cats as sources of infection and to devise possible control measures. The role and contribution of transport hosts in the dissemination of infection also requires investigation but the greatest need is for further studies on the development of *S. gigantea* in its intermediate host.

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APPENDIX I

Analysis of variance tables for data in Chapter 2. The use of one, two or three asterisks after results indicates a probability of less than 0.05, 0.01 or 0.001 respectively; ns = not significant

(a) Effect of type of floatation medium on the recovery of *S. gigantea* sporocysts

Source of variation	df	SS	MS	F
Between samples	4	859812772.2	214953193.2	1.53ns
Withim samples	15	2111294076.0	140752938.0	
Total	19	2971106849.0		

F .05 (4,15) = 3.06

(b) Effect of duration of centrifugation on the recovery of *S. gigantea* sporocysts

Source of variation	df	SS	MS	F
Between samples	6	3013179822	502196637.1	20.25**
Within samples	21	520770715.6	24798605.5	
Total	27	3533950538		

F .001 (6,21) = 5.88

(c) Effect of centrifugal force on the recovery of *S. gigantea* sporocysts

Source of variation	df	SS	MS	F
Between samples	4	501086134	125271533.6	1.56ns
Within samples	15	1205508427	80367228.8	
Total	19	1706594561		

F .05 (4,15) = 3.06

(d) Effect of sieving and water volume on the recovery of *S. gigantea* sporocysts

Source of variation	df	SS	MS	F
Between samples	3	2233316.9	744438.99	0.07ns
Within samples	20	225579142.4	11278957.12	
Total	23	227812459.3		

F .05 (3,20) = 3.10

(e) Effect of additional washing before floatation on the recovery of *S. gigantea* sporocysts

Source of variation	df	SS	MS	F
Between samples	2	6610010.82	3305005.41	0.62ns
Within samples	13	68697236.93	5284402.84	
Total	15	75307247.75		

F .05 (2,12) = 3.89

(f) Sources of sampling error within counting technique (expected counts = 3065 ± 236 spg)

Source of variation	df	SS	MS	F
Between samples	3	16.03	5.34	3.73ns
Between subsamples within samples	4	5.71	1.43	0.79ns
Between replicates within subsamples	8	14.42	1.80	0.44ns
Within replicates	80	328.83	4.11	
Total	95	364.99		

F .05 (3,4) = 6.59, F .05 (4,8) = 3.84, F .05 (8,70) = 2.07

(g) Sources of sampling error within counting technique (expected counts = 12,688 ± 612 spg)

Source of variation	df	SS	MS	F
Between samples	3	95.1	31.7	2.198ns
Between subsamples within samples	4	57.68	14.42	1.661ns
Between replicates within subsamples	8	69.42	8.68	0.511ns
Within replicates	80	1358.5	16.98	
Total	95	1580.7		

F .05 (3,4) = 6.59, F .05 (4, 8) = 3.84, F .05 (8,70) = 2.07

(h) Reliability of faecal sampling method

Source of variation	df	SS	MS	F
Between samples	5	50.92	10.18	0.36ns
Within samples	30	857.83	28.59	
Total	35	908.75		

F .05 (5, 30) = 2.53

APPENDIX II

Active ingredients and manufacturer/distributor of disinfectants, coccidiostats and anthelmintics used in Chapter 6.

A. DISINFECTANTS

Dettol (4.8% chloroxylenol): Reckitt and Coleman (NZ) Ltd.

Hibitane (1% chlorohexidine digluconate): ICI Tasman Ltd.

Iosene (12% phosphoric acid and not less than 1.7% available iodine): Ciba-Geigy (NZ) Ltd.

Medol (16% synergistic mixture of five chlorinated phenols; 3:5 - dimethyl 4-chlorophenol, 2-benzyl 4-chlorophenol, 2-hydroxy diphenyl-sodium, 3-methyl 4-chlorophenol, sodium pentachlorophenate): Laboratory Services Ltd.

Mucocit ("phenolic based disinfectant combined with wetting agent"): Chemavet Distributors.

Stericide (2.4% o-benzyl-p-chlorophenol): Westwood Products.

B. COCCIDIOSTATS

Amprol (12% amprolium): Merck, Sharp and Dohme (NZ) Ltd.

Daraprim (pyrimethamine): Burroughs Wellcome and Co (Aust) Ltd.

Eftolon (25% sulphaphenazole): Pfizer Laboratories Ltd

Emtryl Soluble (40% dimetridazole): May and Baker

Sulphadimidine (sulphadimidine): National Dairy Association (NZ) Ltd.

Toltro (3.63% diaveridine, 14.5% sulphaquinoxaline): Westwood Products

C. ANTHELMINTICS

Equizole (33.3% thiabendazole): Merck Sharp and Dohme (NZ) Ltd.

D. OTHER

Lugol's Iodine (10% potassium iodide, 0.5% powdered iodine).