STUDIES ON PARASITIC PROTOZOA OF THE GENUS *SARCOCYSTIS*

A thesis presented in partial fulfilment
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ABSTRACT

Earlier investigations into the biology of *Sarcocystis* are briefly reviewed; information reported since 1972 is reviewed in detail.

The relative efficiency of haemagglutination (HAT, macro and micro systems), complement fixation (CFT, macro and micro systems) and the indirect fluorescent antibody test (IFAT) was studied using macrocysts (*S. gigantia*) from sheep oesophagi as antigen. In the HAT, macro system titres were always higher than micro system titres. Hyperimmunised rabbits had higher titres than hyperimmunised sheep. Fifteen of 24 naturally infected sheep had negative titres. The macro CFT gave comparable results: the micro CFT was affected by persistent anticomplementary factors in sheep serum. The IFAT was both sensitive and repeatable. In all test systems, *Sarcocystis* antibody titres were minimal in infected adult sheep and in pasture-raised lambs. The value of serology in surveys of prevalence and in diagnosis of sarcocystosis is discussed.

Two types of macrocyst were found in skeletal muscle of sheep at slaughter: 'fat' cysts resembled oesophageal cysts (*S. gigantia*) grossly and in ultrastructure of the wall; 'thin' cysts (*S. medusiformis* n. sp.) were narrower and ultrastructurally distinct. The relative prevalences of the three sheep macrocysts were independent.

Fat and thin macrocysts were transmitted to cats and similarly sized sporocysts produced. *S. gigantia* sporocysts failed to infect lambs; reasons for this are discussed.

Survival of *S. gigantia* macrocysts was studied using an oxygen electrode and by cat feeding. Macrocysts were viable after 10 minutes at 52.5^{o}C but not after 20 minutes at 55^{o}C or 10 minutes at

60°C. Macrocysts survived 60 days at -14°C, cysts stored at 10°C for 13 days and 4°C for 30 days metabolised vigorously. Sheep meat should be exposed to 60°C for at least 20 minutes to render it non-infective for cats.

Using muscle digestion and histology, *Sarcocystis* spp. were found in (%; number examined); feral goats (28;60), red deer (30;50), wild pig (10;50), norway rat (84;50), mouse (8;50) and rabbit (16;50); none in 62 opossums and 8 wallabies. A goat species was transmitted to dogs (sporocysts 13.6±0.69x9.25±0.55), a rabbit species to cats (sporocysts 12.5±0.31x9.29±0.45) and one in rats to cats (sporocysts 10.59±0.52x7.87±0.41). Appropriate sporocysts failed to infect laboratory rats or rabbits.

A survey showed that feral cats inhabit and breed in a variety of terrains in most parts of New Zealand. The commonest foods eaten were rabbit (22% total reports), opossum (18%), sheep (16.6%) and birds (14.5%).

The development and pathogenesis of a dog-derived species was studied in goats. Doses of 5×10^6 sporocysts caused death at 18 and 19 days after infection; necropsy revealed extensive petechial haemorrhages. Schizonts occupied endothelial cells, especially in renal glomeruli. 6×10^5 sporocysts caused death at 24 and 34 days; lesser doses caused pyrexia, anaemia, anorexia and stunting. Sarcocysts were found in muscle fibres at 34 days, appeared mature at 80 days and were infective for dogs at 129 days. Changes in levels of Hb, PCV, TP, SGOT and *Sarcocystis* antibodies were shown. Four sheep given sporocysts were not infected.

The potential importance of sarcocystosis in animal production and the need for further research is discussed.

PREFACE

Sarcosporidia have been reported in the muscles of a wide variety of hosts, especially farm animals, for more than a century.

In New Zealand, slaughtered adult sheep are frequently seen to be infected with sarcocysts and carcases have to be detained for trimming or occasionally condemned. The presence of *Sarcocystis* macrocysts in mutton adds several million dollars a year to the operating costs of the meat industry.

It has been shown experimentally that *Sarcocystis* infections in ruminants can cause mild to severe illness, even death, and abortions of pregnant animals. The importance of naturally acquired infection to farm animal production in this country is unknown.

The studies described in this thesis were contemporary with the rapid expansion in knowledge of *Sarcocystis* species after 1972 and the aim of the research was to provide basic information on these parasites in New Zealand.

ACKNOWLEDGEMENTS

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CONTENTS

INDEX OF FIGURES

INDEX OF TABLES

TERMINOLOGY

The following terms are used in the text:

bradyzoite, n.	Non-proliferative cell found in mature sarcocysts. Resists pepsin:HCl digestion. (syn: cystozoite, meront, spore).
cyst, n.	Abbr. sarcocyst (macro-, micro).
definitive host, (with respect to *Sarcocystis* spp.)	The host in which gamogony occurs, a carnivorous mammal, bird or reptile.
endodyogeny, n.	The formation of two daughter cells within a mother cell. See Figure 2.6
endopolgeny, n.	The synchronous formation of many daughter cells within a mother cell. See Figure 2.6.
gamogony, n.	Development of gamonts to macro- and micro-gametes and fusion to form a zygote. (syn: gametogony, sexual reproduction).
gamont, n.	Transient stage found in intestinal cells of definitive host, gives rise to macro- or microgametes.
intermediate host (with respect to *Sarcocystis* spp.)	The host in which schizogony occurs and sarcocysts develop.
macrocyst, n. (hence macrocystic, a.)	Mature sarcocyst with a diameter greater than that of a muscle fibre; may be visible without magnification.
macrogamete, n.	'Female' gamete, non-motile, intracellular. Found only in definitive host.
metrocyte, n.	Proliferative cell found in mature and immature sarcocysts, gives rise to bradyzoites, not resistant to pepsin:HCl.
microcyst, n. (hence microcystic, a.)	Mature sarcocyst with diameter less than or equal to that of a muscle fibre.
microgamete, n.	'Male' gamete, motile, flagellate, briefly extracellular (?), fuses with macrogamete to form zygote. Only in definitive host.

oocyst, n.	Resistant infective stage in life cycle of classical coccidia, but only a transient stage in *Sarcocystis* life cycle: breaks down *in situ* to release two sporocysts.
sarcocyst, n.	Long-lived, resistant cyst stage in *Sarcocystis* life cycle, intracellular in muscle. Comprises bradyzoites and metrocytes surrounded by a distinctive wall.
sarcocystosis, n.	Infection of an intermediate or definitive host, with a species of *Sarcocystis*.
sarcosporidia, n.	An old term for *Sarcocystis* species, still useful for referring collectively to sarcocysts in muscle.
schizogony, n.	Division of a schizont to form schizozoites, occurs only in intermediate host. See endopolygeny.
schizont, n.	Transient dividing stage in intermediate host, gives rise to schizozoites by endopolygeny.
schizozoite, n.	Product of schizogony, extra-cellular briefly, invasive, gives rise to schizont or metrocyte de-pending on stage of life cycle. (syn: merozoite).
spore, n.	An outdated term, replaced by bradyzoite.
sporocyst, n.	Resistant infective stage produced by definitive host, passed in faeces. Contains four sporozoites and a residuum.
sporogony, n.	Development of the cytoplasmic mass (sporont) in the oocyst to sporocysts and sporozoites. (syn: sporulation).
(The) Sporozoa, n.	All the protozoa in the Class Sporozoasida (Sub-phylum Apicomplexa).
sporozoal, a.	Referring to a member of the Class Sporozoasida, or features of such protozoa.
sporozoan, n.	A member of the Class Sporozoasida.
sporozoite, n.	Invasive cell, enclosed in sporocyst, released in excystment.

trophozoite, n.	Invasive, multiplicative stage (cf. *Toxoplasma*).
ultimate schizogony	The phase of schizogony immediately preceding the invasion of muscle.
zoite, n.	Shortened form of bradyzoite, trophozoite, sporozoite, etc.
zygote, n.	Product of fusion between macro- and microgametes, intracellular, gives rise to oocyst.

CHAPTER 1

PRELIMINARY INVESTIGATIONS: 1843 to 1972

1.1 Introduction

Sarcocystis was first described by Miescher in 1843 (cited by Scott, 1943a) from the muscles of a mouse; subsequently many species were reported from a wide variety of hosts. During the period of more than one hundred years that followed Miescher's discovery, many attempts were made to transmit these parasites. However, so little progress was made that *Sarcocystis* species remained, until recently, in the taxon: 'Protozoa of uncertain classification' with *Toxoplasma gondii*, *Besnoitia* species and similar 'enigmatic' organisms.

When the ultrastructure of these parasites was examined, their true relationships became clear; new interest in *Sarcocystis* was stimulated leading to the discovery of the life cycle in 1972. Thus it is possible to divide the history of investigations on *Sarcocystis* into the period which preceded the application of electron microscopy to sporozoa and the shorter, subsequent period in which most of the advances have been made.

1.2 The pre-electron microscopy period

Much of the research carried out in this period was reviewed by Scott (1915, 1918, 1920, 1930, 1943a, b) and, therefore, will be dealt with relatively briefly. Following Miescher's discovery, similar structures, 'Miescher's tubes', were found in other hosts. Lankester (1882, cited by Scott, 1930) named the genus *Sarcocystis*. For many years the discovery of new species depended more on chance than design;

methodical examination of muscle from potential hosts has occurred only recently. Kalyakin and Zasukhin (1975) recorded 60 'species' of *Sarcocystis* in approximately 109 mammal, 72 bird and 11 reptile species. The validity of many of these 'species' is uncertain as they were named in the absence of knowledge of their biology.

Between 1843 and 1972 many unsuccessful attempts were made to transmit sarcosporidia; however, several features posed problems for the early investigators. Firstly, sarcocysts comprised single-celled organisms surrounded by a thick wall. The so-called 'spores' (the bradyzoites) did not appear to be able to escape and there was no evidence that cysts ruptured spontaneously. Secondly, spores freed from the cysts were largely inert and exhibited, at most, feeble movements in artificial media. Attempts to culture spores *in vitro* were unsuccessful (Scott, 1943a). Thirdly, the structure of a sarcocyst bore little resemblance to that of other protozoal cysts and, fourthly, whilst the means of transmission was obscure, investigations of domestic animals, in particular revealed that the prevalence of infection was high under a wide variety of environmental conditions. A problem that regularly impeded experiments in transmission was the inability to rear and maintain experimental animals free of extraneous *Sarcocystis* infection: to prevent infection in control animals it was necessary to know how transmission occurred!

Scott (1915, 1918, 1920, 1930, 1943a, b) was highly critical of much of the earlier research on transmission. However, his experiments were no better designed than those of his predecessors and his conclusions equally invalid. For many years after Scott's work there was little interest in *Sarcocystis*. Spindler and Zimmerman (1945) claimed to have cultured an *Aspergillus* species from sarcocysts from pigs and,

following a number of inadequately controlled transmission experiments, concluded that *Sarcocystis* spp. were not protozoa, but fungi. Further poorly designed experiments supported this conclusion (Spindler, Zimmerman and Jaquette, 1946). When Wang (1950) found variations in the structure of the walls of sarcocysts in cattle he suggested that, based on the findings of Spindler and Zimmerman (1945), only one species of *Sarcocystis* exists capable of infecting many hosts and giving rise to cysts of differing morphology. Further inadequately designed experiments on transmission were reported, some after the life cycle had been finally elucidated (Panasyuk *et al.*, 1971; Purohit and D'Souza, 1973; Afaf and Shommein, 1974).

1.3 Early investigations of Ultrastructure

Initial examination of the ultrastructure of *Sarcocystis* (Ludvik, 1958, 1960, 1963) revealed that the spore possessed a conoid, polar ring and sub-pellicular microtubules similar to those found in *Toxoplasma*. The cyst walls of *S. tenella*, *S. miescheriana* and *S. muris* were found to differ, particularly with respect to the morphology of their external surfaces. It was thought that the cyst wall was parasitic in origin and the projections from its surface functioned in nutrition. Ludvik's discovery of the basic similarity of *Toxoplasma* and *Sarcocystis* spores was of major significance, as it pointed to the possibility of their having similar life cycles. Further ultrastructural studies of *Sarcocystis* confirmed and extended Ludvik's work (Senaud and de Puytorac, 1961, 1962; Senaud, 1963; Simpson, 1966; Zeve, Price and Herman, 1966). The structure of *Sarcocystis* is discussed more fully in Section 2.5.

1.4 Elucidation of the life cycle

Fayer (1970, 1972) reported the development of *Sarcocystis* in cell culture. Spores, removed from sarcocysts in grackles *(Quiscalus quiscula)* were inoculated into cultures of various avian and mammalian cells. After one hour the organisms were seen to move by "flexing, gliding and pivoting" and by three hours after inoculation had entered cells. Macro- and microgametes were developed within 24 hours and oocysts by 48 hours; but sporulation did not occur when the cultures were left at room temperature for several days. Thus it was established that sexual reproduction (gamogony) follows the release of spores from cysts but not where or when gamogony takes place.

The basic life cycle of *Sarcocystis* was elucidated by Rommel, Heydorn and Gruber in 1972. The morphological similarity between *Sarcocystis* and *Toxoplasma*, the fact that reproduction by endodyogeny occurred in both genera and the discovery that *Toxoplasma* cycled through the cat (Hutchinson *et al.*, 1970, 1971) pointed to the possibility that *Sarcocystis* also involved carnivores as definitive hosts in its life cycle.

The first attempt by Rommel *et al.*, (1972) at transmission of *Sarcocystis* failed: cats fed minced sheep oesophagus passed only oocysts of *T. gondii* in their faeces. In order to avoid transmitting this parasite again, a second group of cats was given macrocysts dissected from sheep oesophagi, and sporulated sporocysts of approximately 8.0 by 12.5 µm diameter were passed in the faeces. The dog and cat were later found to be definitive hosts for separate species in cattle (Heydorn and Rommel, 1972a), and man for one species in cattle and one in pigs (Rommel and Heydorn, 1972). One hundred and twenty

nine years after the first description of a *Sarcocystis* species the discovery of the life cycle enabled these parasitic protozoa to be more adequately investigated.

CHAPTER 2

CONTEMPORARY STUDIES; PRESENT KNOWLEDGE

2.1 Introduction

Since 1972 there have been many investigations of *Sarcocystis* species. The large amount of information that has accumulated during this relatively brief period does not lend itself to a chronological review. Instead it is summarised under various general headings.

2.2 Nomenclature

At present the taxonomy of *Sarcocystis* species is confused. To some extent this is a result of the recent expansion in knowledge, but there are other causes.

The genus *Isospora* was established to encompass classical coccidia with direct life cycles in which one stage is an oocyst with two sporocysts each enclosing four sporozoites. Because of the new knowledge this taxon may have to be abandoned or the criteria for inclusion in it, changed. Some *Isospora* species are now known to have optionally indirect life cycles and, in addition, the 'typical isosporan oocyst' has been found to occur in the life cycles of *Toxoplasma*, *Sarcocystis*, *Besnoitia* and *Frenkelia* species, none of which could be considered as classical coccidia. If occurrence of an isosporan oocyst in the life cycle is to be the main criterion for inclusion in the genus *Isospora*, then the other generic names are unnecessary. On the other hand it is difficult to see which other features of the biology of these diverse organisms could be used in their classification. Various modifications of generic names have been suggested by Frenkel (1974, 1977), Dubey (1976) and Levine (1977); their suggestions are premature and only add

to the confusion.

Specific names pose further problems. On most occasions in the past new specific names were created whenever the genus was found in a new host species. Less frequently the same specific name was used for sarcocysts in several host species, or no specific name was given at all. The discovery that hosts may harbour more than one species of *Sarcocystis* and that *Sarcocystis* species tend to be host-specific has made it necessary to create new names and redefine existing ones.

Heydorn *et al.*, (1975) suggested that, for simplicity, all *Sarcocystis* species should have names formed from the generic names of the obligatory hosts in their life cycles. Hence *S. bovicanis* cycles between cattle *(Bos taurus)* and dogs *(Canis familiaris)*; *S. ovifelis* between sheep *(Ovis aries)* and cats *(Felis catus)*. There are at least two objections to this proposal. If more than one species of *Sarcocystis* cycle through the same definitive and intermediate hosts - there are two cat-derived species in sheep and two dog-derived species in the horse, for example (see Appendix 1 table 1 for *Sarcocystis* spp. in which transmission has been investigated) - then this system of nomenclature is inadequate. Furthermore, using host generic roots, it is possible to create only one specific name, which is insufficient if two or more *Sarcocystis* species are found to link the same two host genera. In spite of these inherent deficiencies, the system has been used in the naming of several recently discovered species and in providing synonyms for others. It will be for the International Commission on Zoological Nomenclature to decide as to which specific names, new or old, are to be used in the future.

In the present taxonomic confusion it would be presumptuous to try to establish priorities with regard to the names of the species covered by this review; the alternative, of providing synonyms for each specific

name used, would be equally unacceptable. Furthermore, although *Sarcocystis* organisms with similar features have been investigated in several countries, no attempt has yet been made to show that they are in fact the same species. In the circumstances it was decided, for the sake of clarity, to avoid the use of specific names altogether in this thesis and to refer to *Sarcocystis* species only in terms of their hosts. Hence, for example, 'a cat:mouse sp.' is preferred to *'S. muris'* and 'a dog:sheep sp.' to *'S. ovicanis* (syn. *S. tenella)'*. The two cat-derived species in sheep are referred to as cat:sheep 1 sp. (the oesophageal and fat carcase macrocyst form) and cat:sheep 2 sp. (the thin carcase macrocyst form) (see Chapter 4). When a specific name has been employed in a paper with no clear indication as to the hosts involved in the life cycle, the name is retained *in parenthesis* eg: *'S. fusiformis'*. Similarly 'species' is used when the specific identity of an organism is in doubt.

2.3 Host distribution and prevalence

a. Host distribution

Sarcosporidia have been reported from many host species (Kalyakin and Zasukhin, 1975). Appendix 1 table 1 lists those forms where both hosts are known and indicates where the whole or only part of the life cycle has been demonstrated. The summary reveals that a large number of cycles can involve domestic dogs or cats; a situation which may be explained by the worldwide occurrence of both indigenous and introduced forms of these hosts. Small *Felis* species are to be found in most countries in a wide variety of habitats. Many are closely related to *F. catus* and perhaps are also capable of serving as definitive hosts in those life cycles known to involve the domestic cat. Dogs, wolves, foxes and coyotes, also have an extensive distribution.

All the species of *Sarcocystis* investigated have indirect life cycles with carnivores as definitive hosts. Transmission frequently involves predation - of rats by snakes or mice by cats, for example. However, in many cycles, transmission is more likely to result from scavenging. Where the intermediate hosts are large species e.g. cattle, sheep, and pigs, only very young animals that are uninfected or harbouring only immature sarcocysts are likely to be preyed upon by small felids or canids. Therefore the scavenging of the carcases of adults is likely to be the main natural means of transmission of the species in farm animals. Cats are capable of eating a wide variety of foods including the carcases of domesticated and wild animals (Collins and Charleston, 1979a).

Sarcocystis species appear to be host-specific particularly with regard to their intermediate hosts, which may point to a long period of evolution for this genus, allowing close adaptation to their hosts. However, it would be premature to discuss this aspect of distribution as host specificity has not been fully investigated in any species.

b. Prevalence in intermediate hosts

Various methods have been used to survey the prevalence of sarcosporidia including visual inspection of carcases, 'trichinoscopy', histological examination of muscle and *in vitro* digestion of muscle. All of these procedures have their limitations and are likely to underestimate the true prevalence to varying degrees. Thus inspection of animal carcases can detect only macroscopic cysts on the exposed muscle surfaces. Trichinoscopy, while able to detect smaller cysts, can be applied to only a small fraction of the total musculature. Sarcocysts of all sizes are readily detected in histological sections but the amount of tissue that can be scanned is small. Digestion techniques

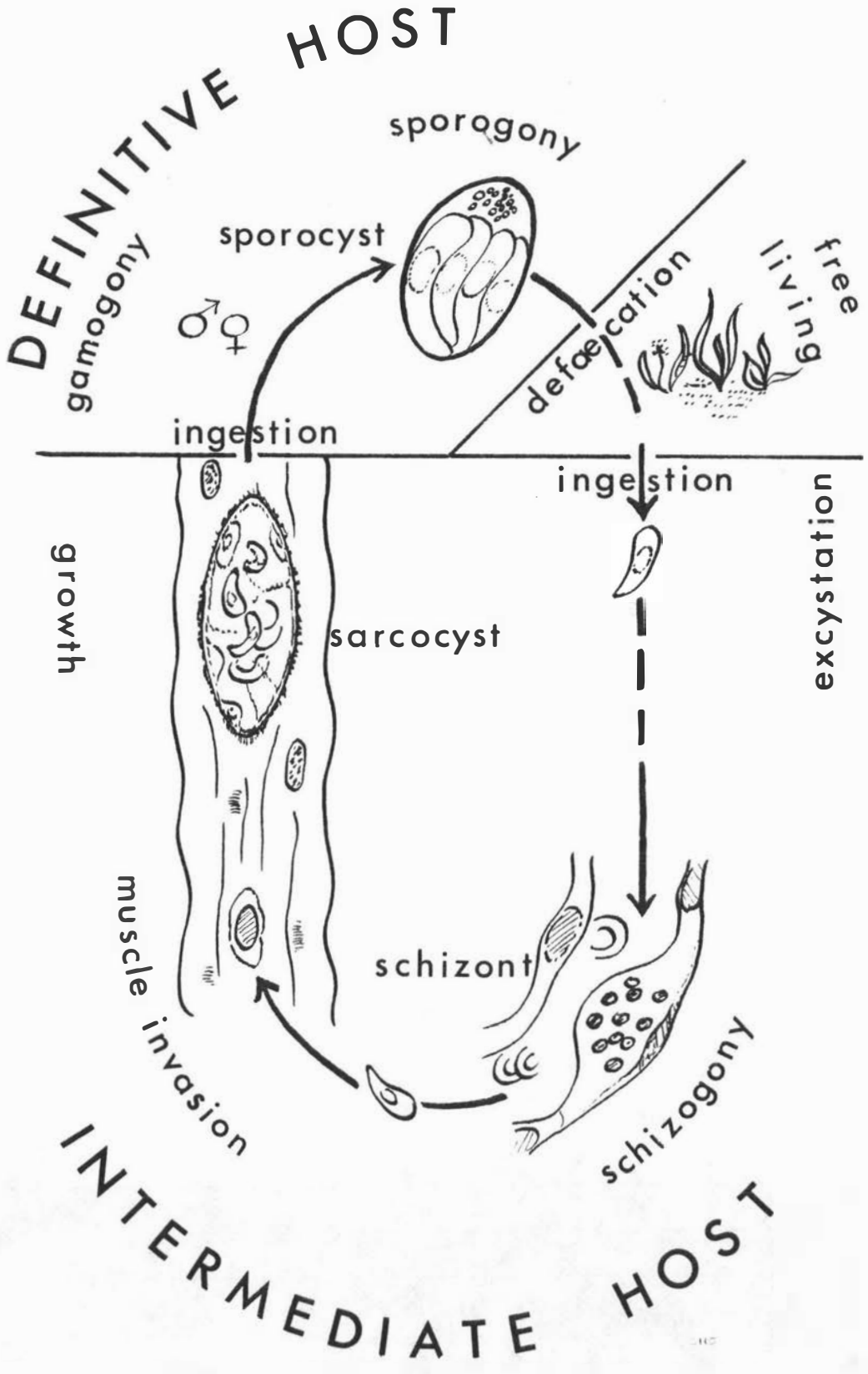


FIGURE 2.1

The life cycle of Sarcocystis

enable larger amounts of tissue to be examined but the proportion of the total musculature of a large animal that can be processed is still limited.

A muscle digestion technique developed for the detection of *Toxoplasma* bradyzoites in muscle and used to survey farm animals (Jacobs and Melton, 1957; Jacobs, Remington and Melton, 1960) has been adapted for the detection of *Sarcocystis* (Seneviratna, Edward and De Giusti, 1975). Muscle digestion was reported to be more efficient than a muscle-press technique in detecting microscopic cysts in cowbirds *(Molothrus ater)* (Box and Duszynski, 1977). Surveys, using muscle digestion, have revealed prevalences of 62.0, 75.3 and 12.7 per cent in adult cattle, sheep and pigs respectively (Seneviratna *et al.*, 1975) and 93 per cent in grackles *(Quiscalus quiscula)* (Fayer and Kocan, 1971) in the U.S.A. Drost and Graubmann (1973, 1975) found 100 per cent prevalence in deer and 70 per cent in wild and domestic pigs in Germany using trichinoscopy and histology. In a survey of meat animals in Tasmania, Australia, using a complement fixation test (CFT) Munday, (1975) found that 100 per cent of lambs had *Sarcocystis* antibodies; although less than one per cent were visibly affected at slaughter. Sixty-six per cent of adult sheep, four or more years of age, had macroscopically visible sarcocysts. In contrast only 7.6 per cent were visibly infected in a survey of aged sheep in South Australia (Munday, 1975). It was suggested that a difference in climate or in the density of definitive hosts could have been responsible for the difference in prevalence.

c. Prevalence in the definitive host

Little published information is available on the frequency of occurrence of sporocysts in the faeces of dogs, cats or humans. Streitel and Dubey, (1976) examined the faeces of 500 stray dogs in Columbus,

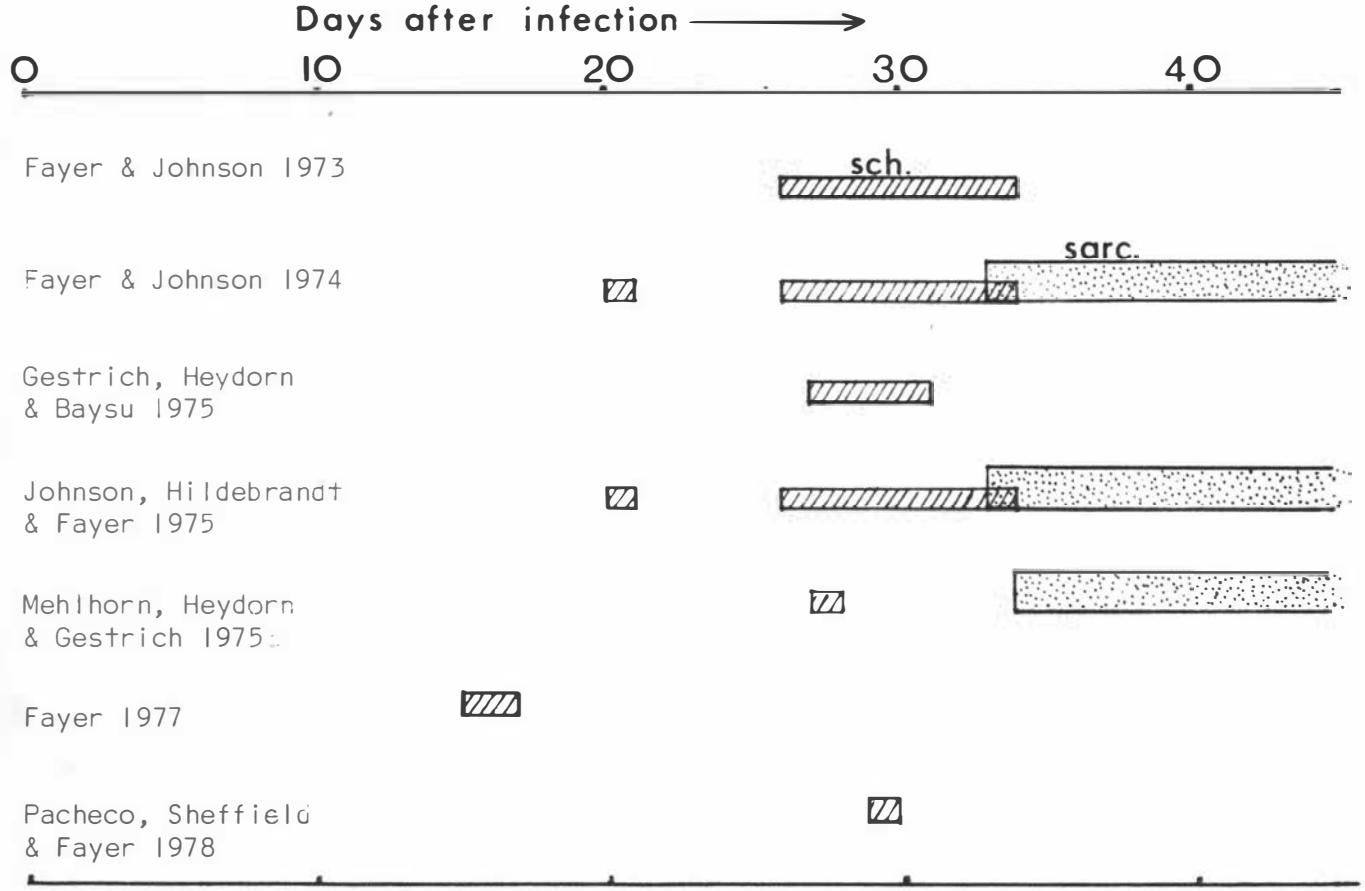


FIGURE 2.2

DOG:OX SPECIES; CHRONOLOGY OF EARLY DEVELOPMENT IN THE INTERMEDIATE HOST

Ohio, U.S.A., for sporocysts: 1.8 per cent of the samples were positive. Of 1000 stray cats in the same location only 0.2 per cent were positive (Christie, Dubey and Pappos, 1976). This is in contrast to the prevalence found in dogs and cats in New Zealand (McKenna, 1978): 58.8 per cent of 481 dogs and 16.9 per cent of 508 cats had sporocysts in their faeces. Since only a single sample was examined from each animal it is likely that the prevalences recorded considerably underestimated the numbers of infected animals.

2.4 Life cycle

a. Introduction

Although the basic life cycle of *Sarcocystis* is known (see Figure 2.1), some phases have not yet been fully investigated. The following sections discuss current knowledge of the development of *Sarcocystis* in the intermediate and definitive hosts.

b. Infection of the intermediate host

Sporozoites leave the sporocyst, i.e. excyst, in response to certain intestinal constituents. Excystment in a dog:ox sp. took place *in vitro* after a two-phase stimulus (Fayer and Leek, 1973): first a so-called 'pretreatment' phase of a carbon dioxide:air mixture in cysteine hydrochloride, followed by treatment with a bile:trypsin mixture. Up to 18 hours of pretreatment was necessary to achieve 90 per cent excystation. McKenna (pers. comm.), using a similar technique, achieved excystment in a dog:sheep and a dog:goat sp. (Collins and Crawford, 1978). However, sporocysts of the cat:sheep 1 sp. failed to respond to these stimuli (see also Chapter 5). The findings of Fayer and Leek (1973), and of McKenna point to excystment of certain species in ruminants taking place in the small intestine following pretreatment in the rumen and/or abomasum. The next stage in infection has not been studied.

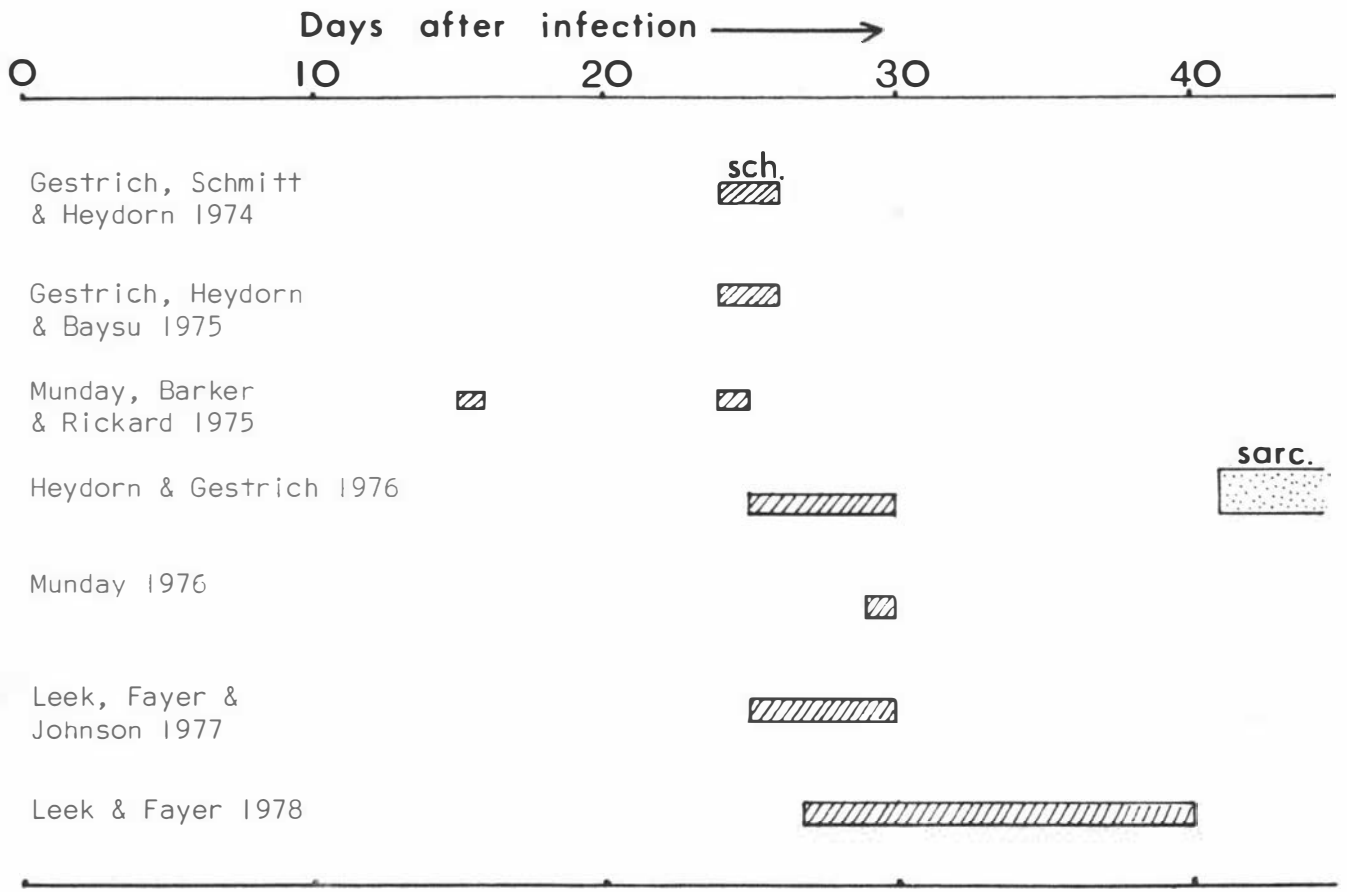


FIGURE 2.3

DOG:SHEEP SPECIES; CHRONOLOGY OF EARLY DEVELOPMENT IN THE INTERMEDIATE HOST

Presumably sporozoites, like bradyzoites, enter mucosa by penetrating epithelial cells, then move into the lamina propria. Later they must enter the circulation and be carried to the site of their next phase of development.

c. Schizogony

Figures 2.2, 2.3 and 2.4 summarise what is known of the time sequence of development, following infection of the intermediate host, in the life cycles of various species of *Sarcocystis*. Dog-derived species in cattle and in sheep have been most investigated and it is assumed that the information incorporated in Figures 2.2 and 2.3 refers to only two distinct species, although this has yet to be demonstrated.

In the period between invasion of the intermediate host's tissues by excysted sporozoites and the development of sarcocysts in muscle one or more phases of schizogony occur. Fayer and Johnson (1973, 1974) found schizonts in the endothelial cells of calves infected 26 to 33 days previously with dog:ox sp. sporocysts (see Figure 2.2). Schizogony has since been reported in several other *Sarcocystis* life cycles (see Figures 2.3, 2.4) but the timing appears to be different for each species. Since there is evidence for schizogony occurring more than once in the pre-muscle phase, the schizogony immediately preceding muscle invasion will be termed the 'ultimate schizogony'. A 'penultimate schizogony' was reported in small and medium sized arteries of calves infected with a dog:ox sp. (Fayer, 1977) and in the liver in a man:pig sp. (Heydorn, 1977; Heydorn and Ipczynski, 1978). A slight temperature rise, approximately 15 days after infection of sheep with dog:sheep sp. sporocysts, points to the likelihood of a penultimate schizogony in this species also (Leek, Fayer and Johnson, 1977; Leek and Fayer, 1978). Although the time sequence in development of the man:pig sp. (see Figure 2.4) appears to allow for only two phases of

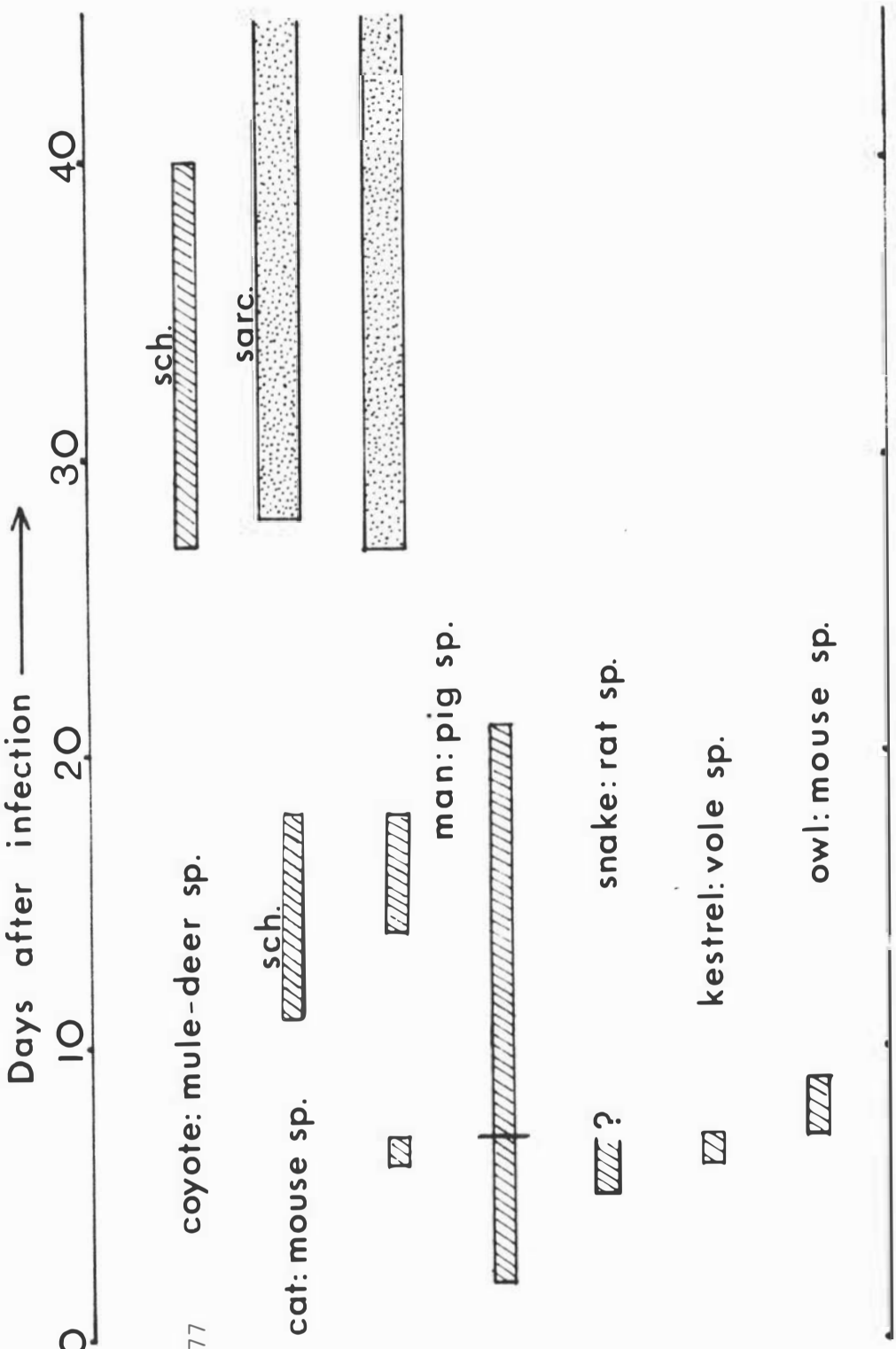


FIGURE 2.4

VARIOUS *SARCOCYSTIS* SPECIES; CHRONOLOGY OF EARLY DEVELOPMENT IN THE INTERMEDIATE HOST

schizogony, it is possible that three phases occur in species infecting ruminants (see Chapter 9). This aspect of the life cycle needs further investigation. With few exceptions the ultimate schizogony usually takes place in endothelial cells of small blood vessels. However, the ultimate schizogony was reported to occur in the liver in a kestrel:vole sp. (Cerna and Louckova, 1976) a cat:mouse sp. (Ruiz and Frenkel, 1976) and an owl:mouse sp. (Munday, 1977). Schizonts of a snake:rat sp. were found only in the lungs of rats 5 to 12 days after infection (Zaman and Colley, 1975).

d. Development in muscle

It is presumed that schizozoites, developed in the ultimate schizogony, enter muscle soon after their release from schizonts. However, there is only indirect evidence for this sequence of events: young sarcocysts have been found in muscle fibres soon after the end of schizogony and there is no evidence of schizozoites developing into stages other than sarcocysts. Several reports describe finding sarcocysts at the same time as schizonts following single experimental infections (Fayer and Johnson, 1974; Johnson, Hildebrandt and Fayer, 1975; Munday, Barker and Rickard, 1975). But since none of these experiments involved specific pathogen-free animals the coexistence of naturally acquired infection cannot be excluded. Furthermore, it is likely that after experimentally dosing ruminants with sporocysts some are delayed in the rumen and that the timing of the ultimate schizogony is therefore asynchronous.

How schizozoites enter fibres is not known. Penetration of the sarcolemma may occur directly or the interior of the fibre may be reached through the membrane of a peripherally placed nucleus or a satellite cell. The latter are known to function as myoblasts and are capable of being incorporated into fibres as additional nuclei during

growth of muscle (Ham, 1969).

A sarcocyst initially consists of a single metrocyte surrounded by the unit membrane of a parasitophorous vacuole (Mehlhorn, Hartley and Heydorn, 1976; Pacheco, Sheffield and Fayer, 1978). Growth is by multiplication of metrocytes and expansion mainly in a longitudinal direction. The unit membrane increases in thickness and external projections develop (see Section 2.5f). At some stage metrocytes give rise to intermediary cells that, after an unknown number of divisions, become bradyzoites (spores). Only at this stage is the sarcocyst mature and infective for a definitive host. The time taken to reach maturity has not been specifically investigated in any species, but some indication of the length of this period may be gained from reports of transmission and of recognition of bradyzoites in sarcocysts. Based solely on the criterion of infectivity for the definitive host, sarcocysts in two species are mature by about 70 days after infection (see Table 2.1).

Metrocytes give rise to bradyzoites by endodyogeny (Senaud, 1967) (see Figure 2.6). The growth of sarcocysts, and production of bradyzoites, is assumed to be continuous: metrocytes are present at the periphery of even the largest cysts.

Sarcocysts, depending on the species, are either microcysts or macrocysts. The former do not expand much beyond the width of a single muscle fibre and grow mainly in length. Macrocysts expand in width and length and may achieve diameters of two centimetres or more although still enclosed by the remains of the infected muscle fibre. Large macrocysts have centres devoid of cells, but contain a milky fluid thought to be the remains of bradyzoites that have died from lack of nutrition. The structure of the sarcocyst wall, metrocyte and bradyzoite are described in Section 2.5.

TABLE 2.1

DEVELOPMENT OF THE SARCOCYST; MATURATION TIME

Sarcocystis species	maturation time (days)	evidence for maturity: bradyzoites present	evidence for maturity: transmission achieved	References
dog:ox	54	+	nd	Fayer and Johnson 1974
dog:ox	62	+	nd	Gestrich, Heydorn and Baysu 1975
dog:ox	76	+	nd	Mehlhorn, Heydorn and Gestrich 1975a
dog:ox	60	+	nd	Fayer, Johnson and Hildebrandt 1975
dog:sheep	150	nd	+	Ford 1974
dog:sheep	70	nd	+	Ford 1975
dog:sheep	81	+	nd	Mehlhorn, Heydorn and Gestrich 1975b
dog:sheep	104	+	nd	Munday, Barker and Richard 1975
dog:sheep	63	+	nd	Heydorn and Gestrich 1976
dog:sheep	81	+	nd	Heydorn and Gestrich 1976
dog:sheep	67	+	nd	Leek, Fayer and Johnson 1977
cat:ox	62	+	nd	Gestrich, Heydorn and Baysu 1975
cat:ox	98	+	nd	Gestrich, Mehlhorn and Heydorn 1975
cat:mouse	116	nd	+	Ruiz and Frenkel 1976
cat:mouse	42	+	nd	Ruiz and Frenkel 1976
cat:mouse	78	+	+	Sheffield, Frenkel and Ruiz 1977
cat:mouse	75	nd	+	Smith and Frenkel 1978
cat:rat	180	+	nd	Ashford 1978
human:ox	62	+	nd	Gestrich, Heydorn and Baysu 1975
human:ox	62	+	nd	Heydorn, Mehlhorn and Gestrich 1975
human:pig	56	+	nd	Heydorn 1977b
owl:mouse	67	+	nd	Cerna 1976
owl:mouse	42	+	nd	Munday 1977
kestrel:vole	51	+	nd	Cerna and Loucka 1976

e. Development in the definitive host

Release of bradyzoites from sarcocysts may occur during mastication but is more likely to follow contact with stomach secretions. Macrocysts (cat:sheep 1 sp.) from sheep oesophagi ruptured in one to two minutes when exposed to a pepsin:HCl digest medium (Collins, unpubl. obs.). Bradyzoites invade the mucosa of the small intestine; the stimulus for invasion is unknown. Scott (1943a) found that bradyzoites were stimulated to move by contact with bile; however, Fayer (1970, 1972) reported that invasion of cells *in vitro* took place in the absence of digestive substances. Bradyzoites penetrate goblet cells under the epithelium (Ruiz and Frenkel, 1976); the infected cells may migrate into the lamina propria (Mehlhorn and Scholtyseck, 1974). Gamogony takes place mainly in the tips of the villi (Heydorn and Rommel, 1972b; Fayer, 1974; Munday, Barker and Rickard, 1975) and macro-gametocytes are more numerous than micro-gametocytes (Munday, Barker and Rickard, 1975; Ruiz and Frenkel, 1976). Flagellate micro-gametes fuse with macro-gametes, the zygote produces an oocyst wall and the contents develop into sporoblasts and then sporocysts. Sporulation occurs *in situ* in a few days. The sequence of development in various species, is summarised in Table 2.2.

There is no evidence of invasion of extra-intestinal tissues, although only one experiment to investigate this possibility has been reported (Rommel *et al.*, 1974). In the *Sarcocystis* life cycle, bradyzoites develop directly into gametocytes, in contrast to the *Toxoplasma* life cycle where gamogony is preceded by schizogony (Ferguson *et al.*, 1974). This raises interesting questions as to when the reduction division occurs in the cycle and whether or not bradyzoites are predetermined in their development to macro- or micro-gametocytes.

Sporulation (sporogony) takes place at mammalian body temperature;

oocysts developed in cell culture failed to sporulate at room temperature (Fayer, 1972). However, in species infècting poikilothermic hosts sporulation must occur at lower temperatures (Rzepczyk, 1974; Zaman and Colley, 1975). Sporocysts are probably released from the mucosa by the breakdown of infected cells. The shedding of sporocysts is therefore sporadic, with an early peak of production followed by a long period of intermittent shedding. In experimental infections it is not unusual for some sporocysts to be passed before they are sporulated (Collins, unpubl. obs.).

Definitive hosts may continue to shed sporocysts for as long as several months after a single infection, but it is doubtful if 'old' sporocysts, i.e. those that have been retained in the intestinal mucosa for several months, are viable (Ashford, 1978).

2.5 Morphology

a. Macrogamete

Young macrogametes *in vitro* were approximately 8.0 x 4.5µm in diameter and had prominent nuclei (Vetterling, Pacheco and Fayer, 1973). Macrogametes were surrounded by a pellicle composed of two unit membranes and large granules were present at the periphery. Less detailed descriptions of similar structures *in vivo* have been given by others (Heydorn and Rommel, 1972b; Fayer, 1974; Munday, Barker and Rickard, 1975; Ruiz and Frenkel, 1976).

b. Microgamete

Vetterling *et al.*, (1973) found young microgametocytes, *in vitro*, of approximately 4.4 to 6.5µm by 3.5 to 5.0µm diameter. Flagellate microgametes, released into the parasitophorous vacuole, were slender, comma-shaped and about 3µm in length. The two flagella, originating from anterior basal bodies, extended posteriorly. Microgametocytes have been found and described only infrequently *in vivo* (Heydorn

TABLE 2.2

DEVELOPMENT OF *SARCOCYSTIS* SPECIES IN THE DEFINITIVE HOST: CHRONOLOGY

Sarcocystis species	dog: ox	dog: sheep	dog(fox): sheep	cat: ox	cat: mouse	ex grackle *in vitro*
free bradyzoites				4hr		
bradyzoites in cells			16hr	8hr	9hr	3hr
gametocytes		24hr		24hr	12hr	24hr
gametes	2-13d			3d		30hr
zygotes			2½d		1d	
oocyst wall	7-13d	4d		4d	2d	42-48hr
sporoblast				5d	3d	
sporocyst wall					4d	
sporulated sporocysts	10d	7d			5d	
	Fayer 1974	Munday, Barker & Rickard 1975	Ashford 1977	Heydorn & Rommel 1972b	Ruiz & Frenkel 1976	Fayer 1970, 1972

and Rommel, 1972b; Fayer, 1974; Munday, Barker and Rickard, 1975; Zaman and Colley, 1975; Ruiz and Frenkel, 1976).

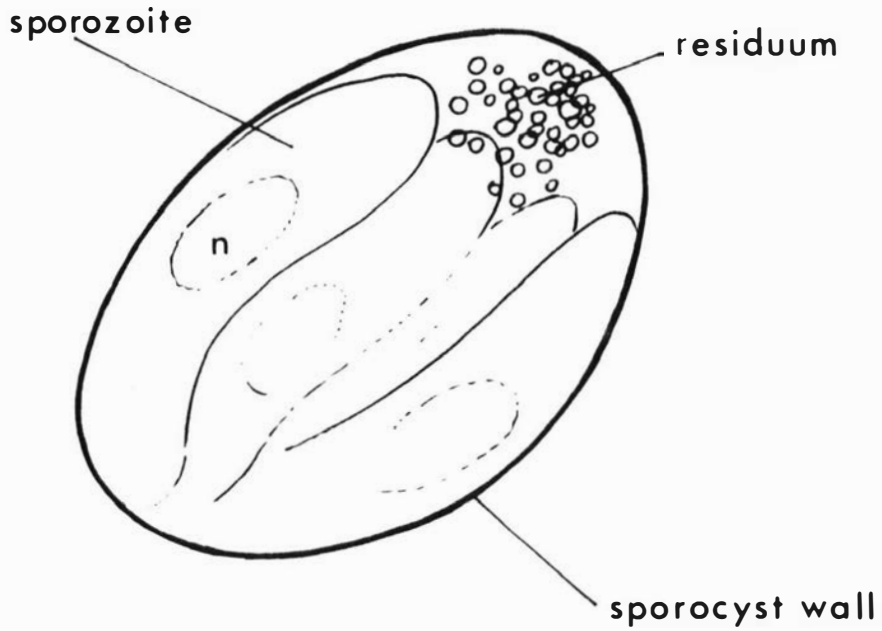
c. Oocyst

The oocyst wall develops from peripheral granules and vesicles in the zygote (Vetterling *et al.*, 1973), and consists of five layers in addition to the two membranes of the pellicle. Development of the oocyst wall is reported to occur at widely differing times after infection according to species (see Table 2.2)but this phase of the life cycle needs more detailed study.

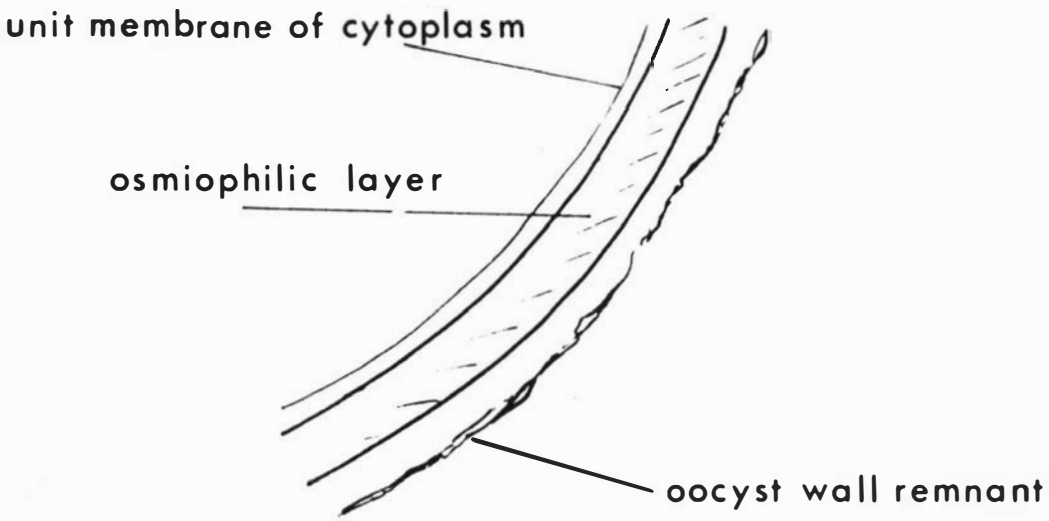
d. Sporocyst

Sporocysts of different species of *Sarcocystis* are remarkably similar in shape, being ellipsoidal with one side slightly flattened (see Figure 2.5). The dimensions of sporocysts differ with species but the ranges frequently overlap. The dimensions of sporocysts described since 1972 are compared in Appendix 2 tables 1 and 2. Apparent differences in dimensions of sporocysts of the same species, as recorded by various workers, probably resulted from the use of different measuring techniques and possibly also from the influence of the media in which the sporocysts were stored.

The sporocyst wall has been little studied. It is possible that there are differences in the physico-chemical structure of the walls in each species that could be involved in host specificity and also affect excystment *in vitro* (see Section 2.4b). Mehlhorn and Scholtyseck (1974a) reported that sporocysts, in the intestines of cats infected with cat: sheep 1 sp. macrocysts, had walls composed of a 50 to 60 nm thick osmiophilic layer and an outer granular layer, thought to be a relic of the oocyst wall (see Figure 2.5).



Sporocyst



Section of wall

FIGURE 2.5

THE MORPHOLOGY OF *SARCOCYSTIS*; THE SPOROCYST

Sporulated sporocysts contain four sporozoites and a granular residuum (see Figure 2.5). There are no descriptions of the ultrastructure of *Sarcocystis* sporozoites; presumably they resemble coccidian sporozoites and contain the usual sporozoan organelles. The structure and chemical nature of the residuum is also unknown. The residuum granules, which may form a compact rosette or be scattered throughout half of the sporocyst, perhaps serve as food reserves, or function in osmoregulation or even in buffering the effects of external electrolytes.

e. Schizonts, schizozoites and schizogony

Four different structures have been described from the period preceding invasion of muscle.

(i) Schizont type 1

This structure, found in the endothelial cells of small blood vessels during the ultimate schizogony, is associated with the pathogenic phase of sarcocystosis. Up to 200 schizozoites may be present in a schizont, sometimes arranged in a palisade or rosette formation. This type of schizont gives rise to schizozoites approximately 3 to 4 µm long with a nucleus about 2 µm in diameter (Fayer and Johnson, 1973, 1974; Markus, Killick-Kendrick and Garnham, 1974; Johnson, Hildebrandt and Fayer, 1975; Munday, Barker and Rickard, 1975; Zaman and Colley, 1975; Ruiz and Frenkel, 1976; Fayer, 1977; Leek, Fayer and Johnson, 1977; Munday, 1977; Heydorn and Ipczynski, 1978; Leek and Fayer, 1978).

(ii) Schizont type 2

A smaller structure than the above, containing smaller schizozoites (2 x 1 µm), was described by Markus *et al.*, (1974) in a kidney of a calf infected with dog:ox sp. sporocysts. Small schizonts containing small schizozoites were also found in the lungs of rats infected with snake:rat sp. sporocysts (Zaman and Colley, 1975).

(iii) Basophilic mononuclear organisms

Fayer and Johnson (1974) described finding "mononuclear basophilic organisms" in organs of calves infected with dog:ox sp. sporocysts at 27 and 33 days after infection. The dimensions of these structures and the cell type in which they occurred were not reported. One similar structure was found by Munday *et al.*, (1974) in endothelium in a lymph node.

(iv) Large zoites in blood

Zoites with mean dimensions of 7.85 x 2.48 µm (n = 25) were found in the blood of rats 4 to 9 days after infection with a snake:rat sp. (Zaman and Colley, 1975). These organisms were present at the same time as type 2 schizonts.

The relationship of these four structures to each other in the life cycle is not certain. Undoubtedly type 1 schizonts are involved in the ultimate schizogony and give rise to schizozoites approximately 4 µm in length. The type 2 schizont found by Markus *et al.*, (1974), in the tubules only of a kidney that also harboured type 1 schizonts in glomeruli, was thought to be that of "a different generation." Basophilic mononuclear organisms may have been early schizonts: they resemble structures identified as immature schizonts during a study of the life cycle of a dog:goat sp. (see Chapter 9). The large zoites seen by Zaman and Colley (1975) are difficult to place in relation to other stages in the life cycle: they apparently did not derive from the schizonts found in the lungs of the same animals.

Pacheco and Fayer (1977) carried out the only study of ultrastructure during schizogony. They described schizogony as being endopolygeny - the formation of many daughter cells within one mother cell (see Figure 2.6). Schizonts had a pellicle formed of two unit membranes

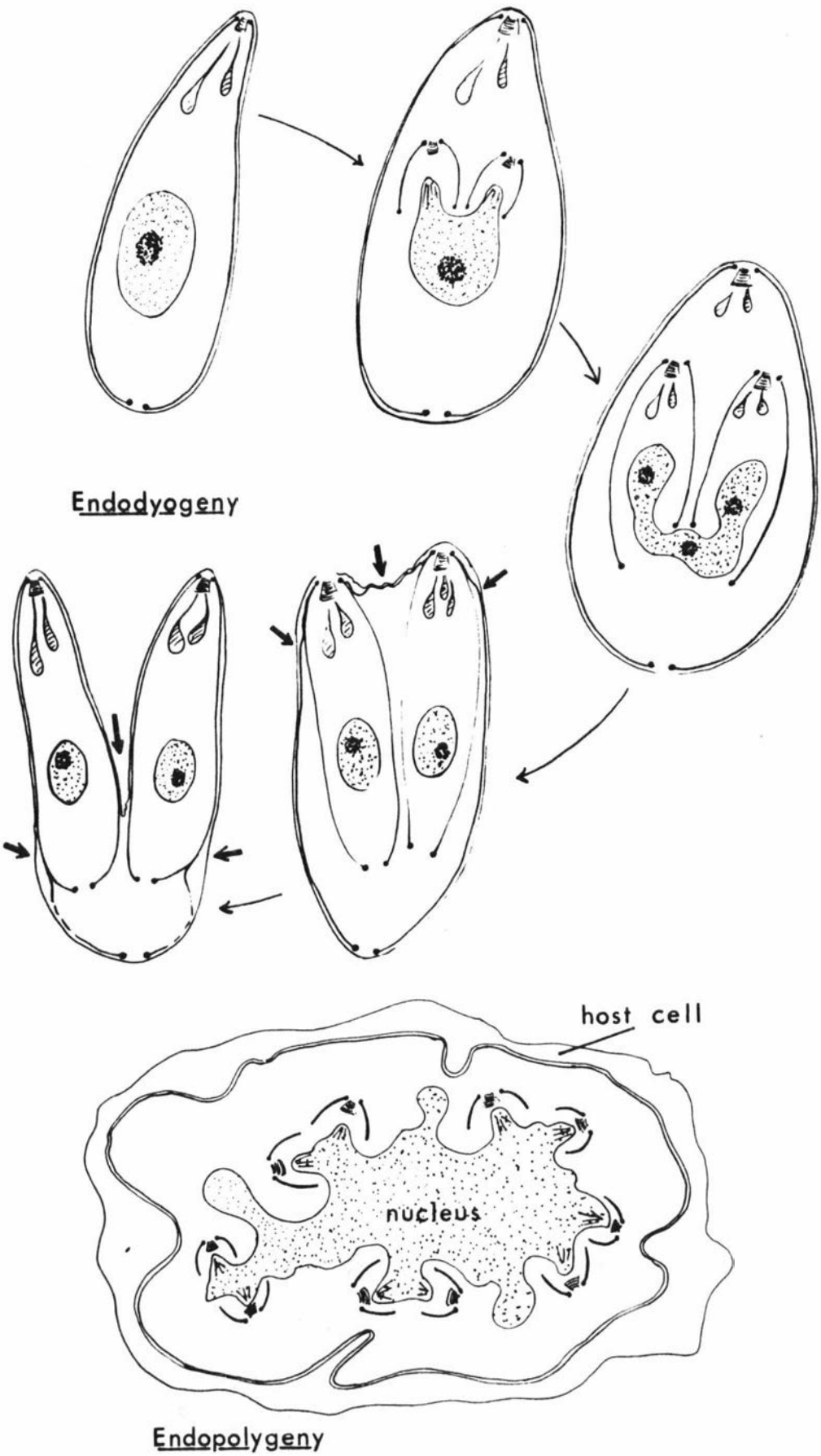


FIGURE 2.6

ASEXUAL REPRODUCTION IN *SARCOCYSTIS*

but were not surrounded by a parasitophorous vacuole. Free schizozoites have been discovered infrequently in histological sections; Pacheco and Fayer (1977) reported that mature schizozoites resembled bradyzoites in shape and possessed similar organelles.

f. The sarcocyst wall

A young sarcocyst consists of metrocytes surrounded by fine granular ground substance and enclosed by the unit membrane of the parasitophorous vacuole. The sarcocyst wall is formed from this unit membrane on the inside of which dense ground substance is deposited. The early cyst wall has small regular protruberances externally (Pacheco, Sheffield and Fayer, 1978). In the development of the dog: sheep sp. sarcocyst, villi have developed from the protruberances by 81 days after infection (Mehlhorn, Heydorn and Gestrich, 1975b). A study of the development of dog:ox sp. sarcocysts (Mehlhorn, Heydorn and Gestrich 1975a) showed that growth of walls took place between villi and, after 76 days, villi were folded horizontally to the wall surface.

The ultrastructure of the mature sarcocyst wall is of considerable interest in understanding the host:parasite relationship and in the identification of certain species. Cyst walls of various species of *Sarcocystis* have been described by many workers (Mehlhorn and Scholtyseck, 1973; Heydorn *et al.*, 1974; Scholtyseck, Mehlhorn and Muller, 1974; Bergmann and Kinder, 1975; Gestrich, Mehlhorn and Heydorn, 1975; Kaliner, 1975; Mehlhorn, Heydorn and Gestrich, 1975a, b; Zaman and Colley, 1975; Mehlhorn, Hartley and Heydorn, 1976; Viles and Powell, 1976; Erber, 1977; Kan and Dissanaike, 1977; Sheffield, Frenkel and Ruiz, 1977; and Pacheco, Sheffield and Fayer, 1978). A typical sarcocyst is bounded on the outside by the sarcolemma of the original muscle fibre (see Figure 2.7). This encloses the remnants of the cytoplasm and organelles of the

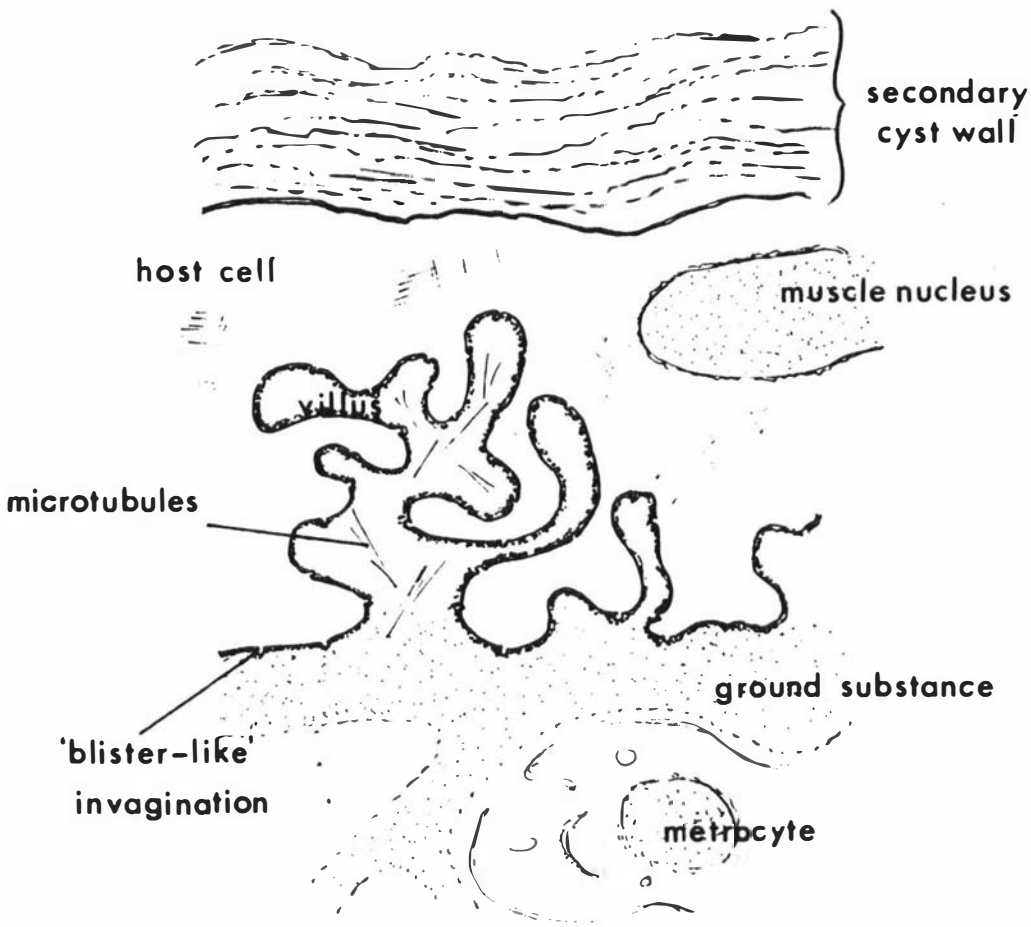
fibre in close contact with the villi. Villi have a variety of shapes depending on the species, are lined with a dense ground substance and enclose bundles of microtubules. The surfaces of the villi and intervillar wall may be pierced by invaginations that, together with the microtubules, probably function in nutrition. The inner layer of the wall is a thick band of ground substance that also extends as septae into the centre of the cyst to form compartments in which metrocytes and developing bradyzoites are found. Sarcocysts of the cat:sheep 1 sp. only are surrounded by an additional layer - the secondary wall - which is composed of host-derived collagenous connective tissue (see Figure 2.7). The cyst walls of various species of *Sarcocystis* were compared in a paper by Mehlhorn, Hartley and Heydorn (1976), who showed that it is not always possible to identify species solely by wall structure.

g. Metrocyte

The ultrastructure of the metrocyte in the early sarcocyst has not been studied, but it probably resembles that of a metrocyte in a mature cyst (see Figure 2.8). This stage is bounded by a pellicle composed of a double unit membrane invaginated occasionally by micropores. All the organelles found in bradyzoites are present in metrocytes including the conoid (Heydorn, Mehlhorn and Gestrich, 1975). Metrocytes are found in close contact with the ground substance of the sarcocyst wall. Bradyzoites develop from metrocytes by an unknown number of phases of endodyogeny (see Figure 2.6).

h. Bradyzoite

This, the spore of the earlier workers, and since given several other names, has been studied on many occasions. Bradyzoites are curved cylindrical cells with tapered ends. One end is more pointed than the other end and is termed anterior. Size varies to some extent with species and has been recorded many times (Kalyakin and Zasukhin,



Sarcocyst wall (Cat: sheep1 sp.)

FIGURE 2.7

THE MORPHOLOGY OF *SARCOCYSTIS;* THE SARCOCYST WALL

1975). However, the curved shape of bradyzoites makes accurate measurement of length difficult and, therefore, dimensions are of little value in identifying species. The detailed structure of a typical bradyzoite is shown in Figure 2.8. The pellicle, approximately 400 A^{O} thick, comprises an inner double and outer single unit membrane. At the extremities the inner membrane is absent as it ends at the anterior and posterior polar rings. Twentytwo subpellicular microtubules, that are thought to function in movement and/or support (Hammond and Long, 1973), run longitudinally between the polar rings. Micropores penetrate the pellicle in a few places and are thought to have a nutritional function. Metrocytes and bradyzoites of the man:ox sp. were described by Heydorn *et al.* (1975) as ingesting vesicles lying nearby. However, the association they found could equally well have been interpreted as excretion.

The anterior end encloses a conoid and related structures that are similar to those of many other sporozoa (Scholtyseck, Mehlhorn and Freidhoff, 1970). A conoid is a truncated cone of spirally arranged fibrils 260-300 A^{O} in diameter with an open top encircling the ends of the rhoptries. The polar ring and conoid are not connected and the latter may be found anterior or posterior to the former. Rhoptries are organelles with a glandular appearance and are thought to function with the conoid in cell penetration. Micronemes, convoluted cord-like structures of 600-900 A^{O} diameter packed around the rhoptries in the anterior third of the cell, are also thought to function in the invasion of cells. The globular nucleus lies in the posterior half of the bradyzoite surrounded by numerous polysaccharide granules (Mehlhorn and Scholtyseck, 1974b) and other organelles. Adenosine triphosphatose has been found in the endoplasmic reticulum, perinuclear space and between

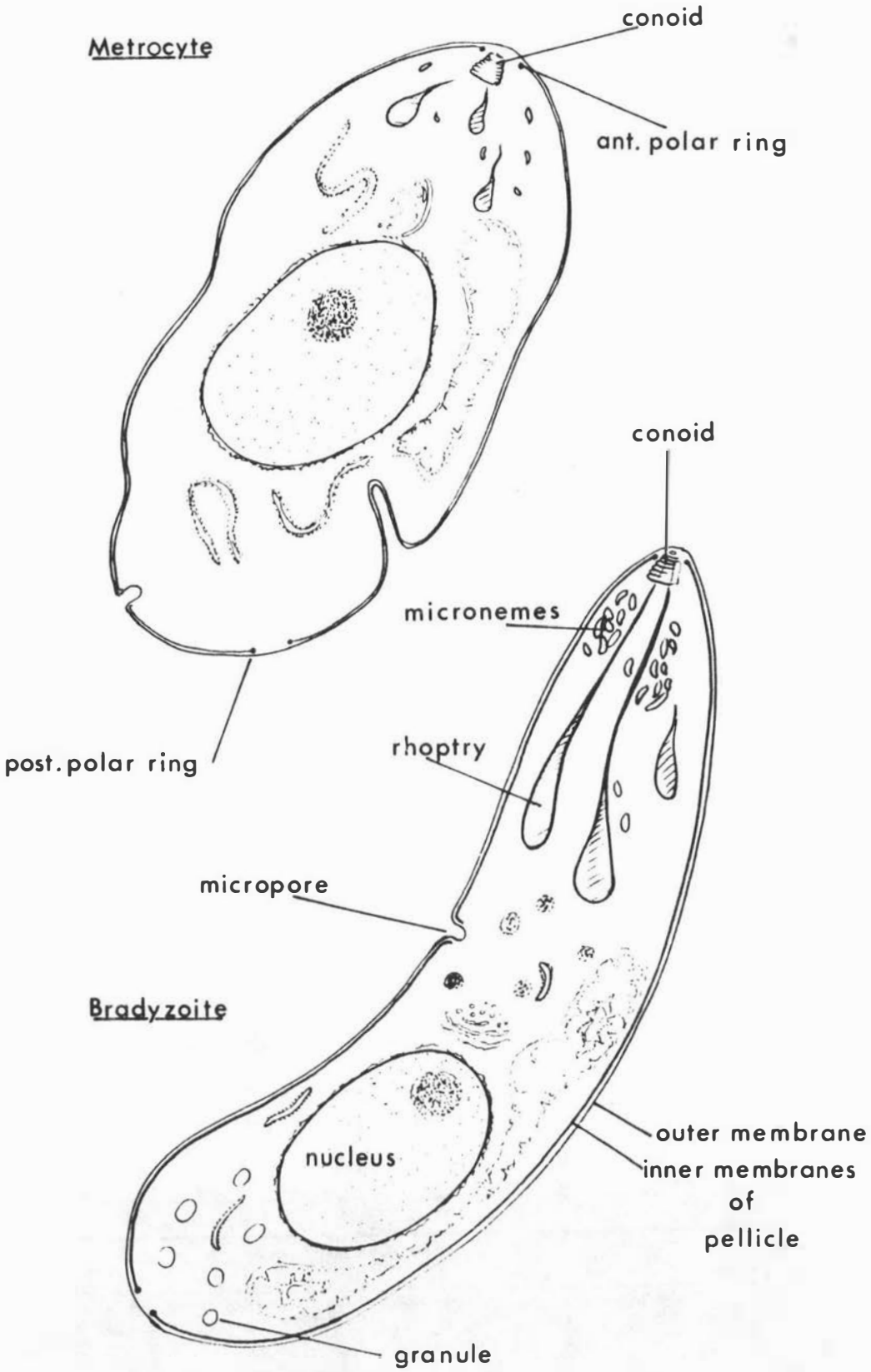


FIGURE 2.8

THE MORPHOLOGY OF *SARCOCYSTIS*; THE METROCYTE AND BRADYZOITE

the inner membranes of the pellicle (Mehlhorn and Scholtyseck, 1974b); its absence from mitochondria was ascribed to the relatively low energy requirements of this stage. Alkaline phosphatase activity was present on the outside of the pellicles of both bradyzoite and metrocyte and on the inner surface of the primary cyst wall. Study of the cytochemistry of the conoid and associated structure might provide useful information on their function.

2.6 Sarcocystosis

a. Introduction

Until recently infection with a *Sarcocystis* species was regarded as of little pathological significance. However, Fayer and Johnson, (1973, 1974) showed that in certain circumstances *Sarcocystis* spp. can be highly pathogenic to intermediate hosts. Illness may be associated with the ultimate schizogony and its severity varies directly with the size of the infective dose of sporocysts. Since there is no clear dividing line between pathogenic and non-pathogenic infection, the term sarcocystosis is used here for all infections with *Sarcocystis* spp. The pathogenesis, gross and clinical pathology, symptoms, diagnosis and treatment of sarocystosis are discussed in the following sections.

b. Pathogenesis and pathology

Illness and death associated with the ultimate schizogony in endothelial cells have been reported from experimental infections with several species of *Sarcocystis* (see Table 2.3 and Figures 2.9 and 2.10) and from one presumed natural infection (Corner *et al.*, 1963). The cause of the illness is not fully understood. It is presumed that when mature schizonts discharge their contents, the disruption of host endothelial cells is accompanied by haemorrhage into surrounding tissues. Whether or not other factors, such as the release of cell breakdown

TABLE 2.3

SARCOCYSTOSIS; SYMPTOMS IN ACUTE DISEASE

Sarcocystis species	dog:ox	dog:ox	dog:ox	dog:ox	? :ox	? :ox	dog:sheep	dog:sheep	dog:sheep	dog:sheep	coyote: mule-deer
temperature rise				+	+	+	+		+	+	+
inappetance	+	+	+	+	+		+		+	+	+
loss of weight	+	+	+	+	+			+	+	+	+
anaemia	+	+	+	+	+	+		+	+		
hypersalivation				+	+						
increased heart rate	+										
dyspnoea										+	
muscular tremors				+	+					+	
neurological symptoms		+		+						+	
hair loss				+	+						
oedema					+						
diarrhoea					±						
abortion				+	+					+	
recumbency	+	+	+	+	+					+	
death	+	+	+	+	+	+	+		+	+	+

Fayer & Johnson 1973

Johnson, Hildebrandt & Fayer 1975

Mahrt & Fayer 1975

Fayer, Johnson & Lunde 1976

Corner *et al.* 1963

Meads 1976

Gestrich, Schmitt & Heydorn 1974

Munday, Barker & Rickard, 1975

Leek, Fayer & Johnson 1977

Leek & Fayer 1978a

Hudkins & Kistner 1977

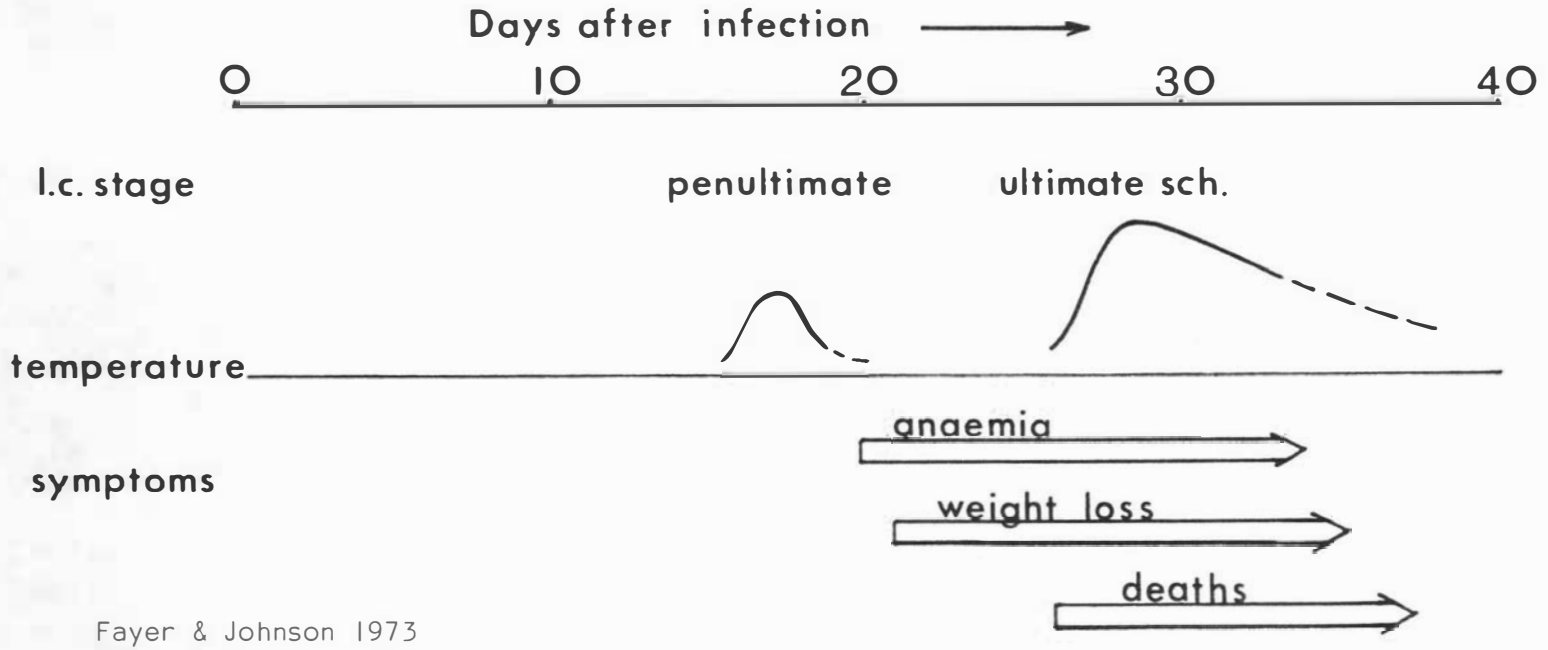


FIGURE 2.9

SARCOCYSTOSIS, DOG:OX SPECIES; CHRONOLOGY OF SYMPTOMS COMPILED FROM SEVERAL SOURCES

Fayer & Johnson 1973
Fayer & Johnson 1974
Fayer & Johnson 1975
Gestrich, Heydorn & Baysu 1975
Johnson, Hildebrandt & Fayer 1975
Mahrt & Fayer 1975
Fayer, Johnson & Lunde 1976
Fayer 1977
Lunde & Fayer 1977

products or parasite toxins, also contribute to the pathogenesis is unknown. Also it is not understood how heavy infection of a pregnant animal can cause foetal death and abortion.

Necropsy of animals at the peak of the schizogonic crisis reveals petechial haemorrhages in all organs; petechiae are especially obvious in the mesenteries, on the serosal surfaces and in subcutaneous muscles. Frequently the heart is severely affected and may appear uniformly dark red from the confluence of haemorrhages and from congestion. Histological examination reveals petechiae in all tissues, but especially in those well supplied with small blood vessels. Schizonts are present in endothelial cells and are most plentiful in glomeruli (see Chapter 9). Affected tissues are diffusely infiltrated with mononuclear cells (Fayer and Johnson, 1973, 1974; Johnson, Hildebrandt and Fayer, 1975; Leek, Fayer and Johnson, 1977) (see also Chapter 9).

The dog:ox, dog:sheep, man:pig and cat:pig spp. have all been described as causing severe illness. The pathogenic effects of a dog:goat sp. are described in Chapter 9. Infections of cattle with the man:ox and cat:ox spp. were reported as causing only mild symptoms (Gestrich, Heydorn and Baysu, 1975) and reports of infections with the cat:mouse and cat:rat spp. did not mention any pathogenic effects (Ruiz and Frenkel, 1976; Ashford, 1977).

The possibility of there being differences in pathogenicity between *Sarcocystis* species has not been adequately investigated. To make such comparisons it will be necessary to be able to relate infective doses on the basis of sporocysts per unit weight of the host and also to measure the number of sporocysts that fail to establish and pass out unchanged in the faeces.

c. Symptoms and clinical pathology

In experimentally induced severe sarcocystosis there is little noticeable change in health before the schizogonic crisis. A small,

Gestrich, Schmitt & Heydorn 1974
Gestrich, Heydorn & Baysu 1975
Heydorn & Gestrich 1976
Munday 1976
Leek, Fayer & Johnson 1977
Leek & Fayer 1978

FIGURE 2.10

SARCOCYSTOSIS, DOG: SHEEP SPECIES; CHRONOLOGY OF SYMPTOMS COMPILED FROM SEVERAL SOURCES

transient rise in temperature has been recorded in infections with two species, probably associated with a penultimate schizogony (see Figures 2.9 and 2.10). The schizogonic crisis is marked by pyrexia, anorexia, anaemia, weight loss and depression (see Table 2.3), death may follow quickly or after a period of recumbency. Animals that survive, slowly return to normal health. However, recovery may be accompanied by stunting, hair loss and neuromuscular symptoms (Fayer, Johnson and Lunde, 1976; Leek and Fayer, 1978). Animals affected during pregnancy may abort (Corner *et al.*, 1963; Fayer, Johnson and Lunde 1976, Leek and Fayer, 1978). The chronology of symptoms in infections with the dog:ox and dog:sheep spp. is shown in Figures 2.9 and 2.10.

The acute anaemia that results, presumably, from the extensive petechial haemorrhages, is reflected by a rapid fall in the number of circulating erythrocytes, packed cell volume and haemoglobin concentration (see Table 2.4). A fall in the total serum protein has been recorded in some infections (Leek, Fayer and Johnson, 1977; Leek and Fayer, 1978a). Serum enzymes (see Table 2.4) were monitored by Fayer and Johnson (1975) and Mahrt and Fayer (1975); the levels rose significantly from about four weeks after infection. However, such changes are not diagnostic of sarcocystosis and merely reflect tissue damage.

d. Diagnosis

Diagnosis of severe sarcocystosis is difficult and has to be based largely on circumstantial evidence; no specific test has been developed. The problem, in a particular circumstance, is in determining whether or not clinical illness or ill-thrift has been caused by the ingestion of large numbers of sporocysts over a short period of time. Therefore diagnosis may have to rely mainly on history, suppor-

TABLE 2.4

Sarcocystis species	total rbc	Hb conc	PCV	total WBC	diff. WBC	serum protein	CPK	LDH	SGOT	
dog:ox	nd	nd	dec	nd	nd	nd	incr	incr	incr	Fayer & Johnson 1975
dog:ox	dec	dec	dec	nc	neutrophils incr lymphocytes dec	not signif.	incr	incr	incr	Mahrt & Fayer 1975
dog:ox	dec	nd	dec	nd	nd	nd	nd	nd	nd	Fayer, Johnson & Lunde 1976
?:ox	dec	dec	dec	incr	nd	nd	nd	nd	nd	Corner *et al.* 1963
dog:sheep	dec	dec	dec	nd	nd	dec	nd	nd	nd	Leek, Fayer & Johnson 1977
dog:sheep	dec	dec	dec	nd	nd	dec	nd	nd	nd	Leek & Fayer 1978

incr. increase dec. decrease nd not done nc no change

ted by the results of haematological and serological examination. Additional evidence might be provided by muscle biopsy or necropsy of a severely affected animal. The presence of sarcocysts in muscle, although evidence of infection with a *Sarcocystis* species, at some time in the past, is very common, and not an indication of recent ingestion of sporocysts.

Long-standing *Sarcocystis* infections are associated with only low levels of antibodies (see Chapter 3), whilst the ingestion of pathogenic doses of sporocysts results in rising levels of immunoglobulins (Fayer and Lunde, 1977) (see Chapters 3 and 9). It is possible, therefore, that a presumptive diagnosis of severe sarcocystosis on the basis of history and clinical signs could be confirmed by detecting rising levels of *Sarcocystis* antibodies. The application of serological tests to the diagnosis of sarcocystosis is discussed more fully in Chapter 3.

e. Treatment

There is only one report of the use of drugs in experimental sarcocystosis (Fayer and Johnson, 1975). Calves dosed with dog:ox sp. sporocysts were given amprolium at a dose rate of 100 mg/kg body weight daily between 0 and 30 days after infection. The unmedicated infected group gained weight more slowly than the medicated infected group; most weight was gained by the uninfected controls. It was thought that medication reduced the severity of the disease. But, since treatment started on the day of infection, the medication could have affected the parasites in the intestine before they invaded the mucosa and the results may merely reflect a difference in dose rate. Research is undoubtedly needed on treatment of acute sarcocystosis, but medication to kill sarcocysts in muscle is probably neither practical nor

desirable as degenerating parasites would undoubtedly result in myositis.

f. Sarcocystosis in the definitive host

There is no evidence that the development of *Sarcocystis* in the intestines of carnivores has any clinical effect. However, Heydorn (1977a) found that humans who ate large quantities of infected raw beef (dog:ox and man:ox spp.) and pork (man:pig sp.) became ill after six hours. Symptoms included nausea, colic and diarrhoea lasting up to 36 hours. The infected pork caused more severe symptoms, including continual vomiting and dyspnoea.

g. Epidemiology

No coordinated studies of the epidemiology of sarcocystosis have been made. Separate aspects of the subject are covered in reports of prevalence of infection in certain intermediate and definitive hosts (see Section 2.3 and Chapters 4 and 7), of survival of sarcocysts in muscle (see Chapter 6) and a study of the distribution of feral cats (see Chapter 8). In addition there is some information available on the survival of sporocysts incorporated in reports of life cycle studies; these are summarised in Table 2.5. Some sporocysts survived storage in frozen faeces (Fayer and Johnson, 1975), others in 2% sulphuric acid (Ruiz and Frenkel, 1976; Ashford, 1978; Smith and Frenkel, 1978).

Survival under field conditions has not been investigated. Some sporocysts fail to excyst after ingestion and may be passed apparently undamaged in the faeces (Collins, unpubl. obs.). The viability of such 'passaged' sporocysts is not known. Failure to take this potential source of infection into account can lead to spurious results in transmission experiments (Collins, Charleston and McKenna, 1978; Munday,

1978). Smith and Frenkel (1978) showed that sporocysts of the cat: mouse sp. were still infective for mice after 20 days in the intestine of the American cockroach *(Periplaneta americana)*. Transport hosts may prove to be important in infection of grazing animals.

Host resistance to reinfection has received little direct attention. Reinfection of the definitive host can occur with no diminution in sporocyst production (McKenna, pers. comm.). The role of circulating antibodies in the intermediate host is not known and the possibility that cell-mediated immunity controls the establishment and survival of sarcocysts has also not been explored (see Chapter 9).

TABLE 2.5

SPOROCYST STORAGE; MEDIA AND TIME

Sarcocystis species	medium	storage time	temp. °C	reference
dog:ox	faeces	2-7 mth	below 0	Fayer & Johnson 1975
dog:ox	tap water	1-6 mth	4	Gestrich, Heydorn & Baysu 1975
dog:ox	not stated	less than 60d	not stated	Mahrt & Fayer 1975
dog:ox	tap water	1-6mth	4	Mehlhorn, Heydorn & Gestrich 1975a
dog:ox	not stated	less than 60d	not stated	Fayer, Johnson & Hildebrandt 1976
dog:ox	tap water	less than 3 mth	5	Fayer & Lunde 1977
dog:sheep	not stated	less than 2 wks	not stated	Ford 1975
dog:sheep	tap water	1-6 mth	4	Gestrich, Heydorn & Baysu 1975
dog:sheep	tap water	not stated	not stated	Mehlhorn, Heydorn & Gestrich 1975b
dog:sheep	not stated	2 wks to 3 mth	7-10	Leek, Fayer & Johnson, 1977
dog:sheep	not stated	4½ mth	7-10	Leek & Fayer 1978
cat:ox	tap water	1-6 mth	4	Gestrich, Heydorn & Baysu 1975
cat:ox	tap water	1-6 mth	4	Gestrich, Mehlhorn & Heydorn 1975
cat:mouse	2% H_2SO_4	not stated	not stated	Ruiz & Frenkel 1976
cat:mouse	2% H_2SO_4	119d	21	Smith & Frenkel 1978
cat:mouse	faeces	20d	RT	Smith & Frenkel 1978
cat:rat	2% H_2SO_4	not stated	not stated	Ashford 1978
human:ox	tap water	1-6 mth	4	Gestrich, Heydorn & Baysu 1975

CHAPTER 3

SEROLOGY

3.1 Introduction

The serological diagnosis of sarcocystosis in intermediate hosts has been attempted using haemagglutination (HAT), complement fixation (CFT) and indirect fluorescent antibody (IFAT) tests (see Table 3.1).

HATs have been used only in experimental infections in cows (Fayer, Johnson and Lunde, 1976) and calves (Lunde and Fayer, 1977) with a dog:ox species. Bradyzoites prepared from beef heart by peptic digestion were used as antigen. The HAT titres rose steadily and were at their highest at 80 to 100 days after infection when sampling stopped.

CFTs have been used to diagnose natural *Sarcocystis* infection in sheep (Awad, 1958), to determine the prevalence of infection in sheep and cattle (Munday, 1975), and to confirm experimental infections of sheep (Munday and Courbould, 1974; Munday and Rickard, 1974; Ford, 1975; Munday, Barker and Rickard, 1975; Munday, 1976). Bordjochki *et al.* (1978) compared the efficiency of a CFT with that of an IFAT in detecting antibodies in rabbits immunised with four 'species' of *Sarcocystis* and found that the CFT, using "macrocystic *S. tenella* antigen" was capable of detecting homologous antibodies but not those formed against other 'species'. The results of all these studies are somewhat inconclusive as there were either deficiencies in the experimental design (Awad, 1958; Bordjochki *et al.* 1978) and in the preparation of reagents (Munday and Courbould, 1974) or failure to establish the specificity of the reaction and the significance of the titres recorded (Munday and Courbould, 1974; Munday and Rickard, 1974;

TABLE 3.1

SEROLOGICAL TESTS USED IN STUDIES ON *SARCOCYSTIS*

Host animal tested	infecting species	test used	source of antigen	reference
cows	dog:ox	HAT	beef heart	Fayer, Johnson & Lunde, 1976
calves	dog:ox	IFAT	beef oesophagus	Nedjari *et al.* 1976
calves	dog:ox	HAT	beef heart	Fayer & Lunde 1977 Lunde & Fayer 1977
sheep	cat:sheep	CFT	Cat:sheep 1 sp. Macrocysts	Munday & Rickard 1974
sheep	dog:sheep	CFT	"	Munday & Rickard 1974
sheep	cat:sheep	CFT	"	Munday *et al.* 1975
sheep	cat:sheep	CFT	"	Ford 1975
sheep	'ovine spp.'	CFT	"	Munday 1975
cattle	'bovine spp.'	CFT	"	Munday 1975
humans	human:ox	IFAT	*'S. fusiformis'*	Tadros *et al.* 1974
humans	human:ox	(micro agar (precipn., (IFAT	('ovine sarco- (cysts' or 'bo- (vine sarco- (cysts'	Aryeetey and Piekarski 1976
rats	cat:sheep 1	(micro agar (precipn., (IFAT	('ovine sarco- (cysts' or 'bo- (vine sarco- (cysts'	Aryeetey and Piekarski 1976
mice	cat:mouse	IFAT	cat:mouse sp.	Ruiz & Frenkel 1976
mice	cat:mouse	IFAT	cat:mouse sp.?	Smith & Frenkel 1978
cats	cat:mouse	IFAT	cat:mouse sp.	Ruiz & Frenkel 1976

Ford, 1975; Munday, Barker and Rickard, 1975; Munday, 1976).

The IFAT has been used to detect antibodies against numerous parasites including species of *Sarcocystis* (Andrade and Weiland, 1971; Tadros *et al.*, 1974; Ruiz and Frenkel, 1976; Bordjochki *et al.*, 1978; Smith and Frenkel, 1978). The test has been performed using either homologous antigen, in the form of bradyzoites released from muscle by digestion (Ruiz and Frenkel, 1976; Smith and Frenkel, 1978), or heterologous antigen. Thus bradyzoites from macrocysts of the cat: sheep 1 sp. were used to detect antibodies to *'S. fusiformis'* in humans (Tadros *et al.*, 1974). Few investigations of cross-reactivity, at either the generic or specific level, have been made. Andrade and Weiland (1971) showed that no cross-reaction occurred between *Sarcocystis*, *Toxoplasma*, *Isospora* and *Eimeria*. In a study of a cat:mouse sp., no cross-reaction was seen between *Sarcocystis* and *Toxoplasma* but some cross-reaction was seen between *Sarcocystis* and *Besnoitia* (Ruiz and Frenkel, 1976). Bordjochki *et al.* (1978) reported that the test could not distinguish between various 'species' of *Sarcocystis*.

Preliminary data obtained during this study showed that antibodies, detectable in agar diffusion studies, were produced by rabbits and sheep immunised with antigen prepared from cat:sheep 1 sp. macrocysts. Sera from hyperimmune rabbits gave a greater number of precipitation lines than did sera from hyperimmune sheep. Naturally infected sheep, i.e. animals with oesophageal macrocysts at slaughter, had little, if any, antibody detectable by this technique.

This chapter describes a study of the relative efficiencies of the HAT, CFT and IFAT in detecting *Sarcocystis* antibodies in natural and experimental infections. Sera from hyperimmunised rabbits and sheep,

sheep raised worm-free indoors, lambs grazed on pasture for varying periods and naturally-infected adult sheep were examined. As the investigations were carried out over several years the sera studied were not the same for each test and the results, therefore, are not comparable on an individual serum basis but only according to categories of sera.

In addition to examining various sera with the IFAT the subjective nature of the test prompted an assessment of its repeatability.

3.2 Materials and methods

Haemagglutination test (HAT)

Two systems were studied: one employed 500 µl volumes of reagents and is referred to as a macro system, the other, using 25 µl volumes is referred to as a micro system.

a. HAT, Macro system

(i) Preparation of antigen

Whole macrocysts (cat:sheep 1 sp.) removed from sheep oesophagi were washed three times in phosphate buffered saline, pH7.2, 0.01M (PBS) (see Appendix 3a), dried on filter paper and weighed. A 10 per cent suspension (W/V) of cysts was made in PBS, homogenised and centrifuged at 2,000g for 10 minutes. The supernatant was collected, dialysed against PBS for 24 hours and stored in 2 ml aliquots at -14^{o}C. This stock antigen was diluted 1:400 in PBS before use as it was found that at concentrations of 1:1000 or greater, the preparation caused clumping of sheep erythrocytes.

(ii) Preparation of hyperimmune sheep and rabbits

Sheep were given 4 injections of the stock antigen at weekly intervals and bled one week after the final injection. The first injection comprised 1 ml of antigen and an equal volume of Freund's

complete adjuvant* given intramuscularly; the second and third injections contained Freund's incomplete adjuvant*. A final injection of 5 ml of stock antigen without adjuvant was given intraveously. Rabbits were given four intramuscular injections at weekly intervals. The first injection comprised 500 µl of antigen diluted 1:100 with PBS and an equal volume of Freund's complete adjuvant; the next three injections incorporated Freund's incomplete adjuvant. Concentrations of antigen greater than 1:100 were found to cause death in rabbits on repeated injection.

(iii) Preparation of sensitised erythrocytes

Sheep blood was collected into Alsever's solution (see Appendix 3b) and stored at 4°C. For the test, blood was centrifuged at 2,000g for 5 minutes and the harvested erythrocytes washed twice in PBS. 1ml of packed, washed erythrocytes was suspended in 20ml of 1:20,000 tannic acid in PBS and incubated at 37°C for 10 minutes, with frequent, gentle mixing; samples showing panagglutination were discarded. The tanned erythrocytes were washed three times with PBS, and 1ml of packed erythrocytes was incubated at 37°C for 30 minutes with 20ml of 1:400 antigen. The sensitised erythrocytes were washed three times with PBS, once with 0.02 per cent gelatine in PBS (GPBS) and finally resuspended in 20ml GPBS to give a 5 per cent erythrocyte suspension.

(iv) Preparation of sera

Test sera were diluted 1:5 in GPBS and incubated with 1ml of packed washed erythrocytes at RT for 30 minutes to remove antibodies to ovine erythrocytes.

* Difco, Detroit, U.S.A.

(v) Test procedure

Doubling dilutions of sera, starting at 1:50, were made in 500 µl volumes of GPBS in haemagglutination plates*. 500 µl of sensitised erythrocyte suspension was then added to each well and the contents mixed. The tray was incubated at RT for 3 hours. The titre was taken as the highest dilution showing complete agglutination. Tanned and untanned erythrocyte controls were included in each test.

(vi) Sera tested

The sera tested were from:

1. Two adult rabbits hyperimmunised with cat:sheep1 sp. macrocyst antigen and designated HR/1 and HR/2.
2. Nine adult sheep hyperimmunised with cat:sheep 1 sp. antigen and designated HS/1, HS/2 etc.
3. Seven lambs raised indoors, worm-free, on artificial foods, 5 months old and designated WF/1, WF/2 etc.
4. Twenty-four adult sheep found to have oesophageal macrocysts (cat:sheep 1 sp.) at slaughter and designated S/1, S/2 etc.

b. HAT, Micro system

(i) Preparation of antigen, as described above.

(ii) Preparation of sensitised erythrocytes, as described above.

(iii) Preparation of sera, as described above.

(iv) Test procedure

The test was carried out in 25 µl volumes in round bottomed microtitre plates+ (type 24 AR), incubation being at RT for 3 hours. Controls, as described for the macro test, were incorporated in each test. The titre was taken as the highest dilution showing complete haemagglutination.

*MRC Influenza haemagglutination plates

+Cooke Microtitre System, Dynatech Laboratories Inc., U.S.A.

(v) Sera tested

The sera tested in the micro system were those tested in the macro system.

Complement fixation test (CFT)

Two assay systems were investigated: a standard tube test and a microtitre system.

c. CFT, standard tube test

(i) Preparation of antigen

The antigen was treated to remove components that caused clumping of erythrocytes. 1ml of stock antigen, prepared as for the HAT, was diluted to 1:40 in PBS, mixed with 1ml of packed, washed sheep erythrocytes and held at RT for 30 minutes. The erythrocytes were removed by centrifugation at 2,000g for 5 minutes. Supernatants showing evidence of haemolysis were discarded.

(ii) Preparation of erythrocytes

A 4 per cent suspension of packed, washed sheep erythrocytes, collected and prepared as for the HAT, was made in calcium/magnesium saline (CMS) (see Appendix 3c). Equal volumes of erythrocyte suspension and an anti-sheep erythrocyte antiserum*, diluted 1:2000 in CMS as recommended by the manufacturer, were mixed to give a 2 per cent suspension of sensitised erythrocytes.

(iii) Complement

Guinea-pig complement+ was diluted 1:40 in CMS as recommended by the manufacturer.

(iv) Test procedure

Doubling dilutions of sera were made in CMS in disposable polystyrene tubes using 500 µl volumes. 500 µl of antigen 1:40 and

* 'Haemolysin', Central Serum Laboratories, Australia

+ Dried Preserved Guinea-Pig Serum, Wellcome Research Laboratories, U.K.

500 µl of complement 1:40 were added to each tube and the tubes incubated at 37°C for 30 minutes. 500 µl of sensitised erythrocyte suspension were then added and the tubes reincubated at 37°C for a further 30 minutes. The tubes were then centrifuged at 2,000g for 1 minute and the supernatants compared with that of a tube showing 50 per cent haemolysis (prepared by lysing 1 volume of sensitised erythrocytes in 7 volumes of distilled water). The serum dilution showing 50 per cent haemolysis was taken as the titre. Control tubes, in which either the antigen or the serum was replaced with an equal volume of CMS, were included in all tests.

(v) Sera tested

The sera tested were from:

1. Three rabbits hyperimmunised with cat:sheep 1 sp. antigen, and designated HR/3, HR/4 and HR/5.
2. Three adult sheep hyperimmunised with cat:sheep 1 sp. antigen, and designated HS/10, HS/11 and HS/12.
3. Three lambs, 7 months old, raised worm-free indoors on artificial foods and designated WF/8, WF/9 and WF/10.
4. Thirty-two adult sheep found to have oesophageal macrocysts (cat:sheep 1 sp.) at slaughter and designated S/25, S/26 etc.

All sera were heat-inactivated at 56°C for 30 minutes before use.

d. CFT, Microtitre system

(i) Preparation of antigen, as described above.

(ii) Preparation of erythrocytes, as described above.

(iii) Complement.

A number of sera tested in the microtitre system were seen to be

anticomplementary. In an attempt to overcome this, complement was used at a dilution of 1:10 rather than 1:40.

(iv) Preparation of sera

Sera showing anticomplementary effects were heat treated at 62°C for 30 minutes (Munday, pers. comm.), followed by absorption three times with bentonite clay (Adler, 1960). Approximately equal volumes of clay and serum were mixed and held at RT for 30 minutes. The clay was removed by centrifugation at 2,000g for 5 minutes.

(v) Test procedure

Doubling dilutions of sera were made in CMS in round-bottomed microtitre plates (type 24 AR) using 25 µl volumes. 25 µl volumes of complement and of antigen were added to each well and the contents mixed by rotation of the plate. After incubation at 37°C for 30 minutes 25 µl of sensitised erythrocytes were added to each well and the plates reincubated at 37°C for a further 30 minutes. In an attempt to improve the sensitivity, the test was repeated on 48 sera and the second warm incubation was replaced with cold incubation at 4°C overnight (Kabat and Mayer, 1961). After incubation the plates were centrifuged at 600g for 15 minutes and the titre was taken as the highest dilution of serum showing no haemolysis. Serum, antigen and complement controls were included in each test.

(vi) Sera tested

Sera tested were from:

1. Four adult sheep hyperimmunised with cat:sheep 1 sp. antigen and designated HS/13, HS/14, HS/15 and HS/16.
2. Twenty-seven lambs, 5 months old, raised worm-free indoors on artificial foods and designated WF/11, WF/12 etc.

3. The same worm-free lambs as in 2 above put out to pasture and tested weekly for 10 weeks. The sera collected one week after turning out were designated WF/11/P1, WF/12/P1 etc., those collected two weeks after turning out, WF/11/P2, WF/P12/P2 etc.

4. Twenty-six lambs pasture-raised from birth and tested when 3 months old, designated PR/1, PR/2 etc.

e. Indirect fluorescent antibody test (IFAT)

(i) Preparation of IFAT slides

Bradyzoites, harvested from cat:sheep 1 sp. macrocysts were washed three times in PBS, fixed for 30 minutes at RT in 2 per cent formol saline, washed again and resuspended in PBS. After adjustment of the concentration of bradyzoites to approximately 100 per field at 100 x magnification, 50 µl volumes were placed in wells on microscope slides. The wells were made by inscribing 5mm diameter circles on the glass with a diamond pen. Slides were air-dried and stored at -14°C. Slides could be stored in this way for at least 6 months.

(ii) Fluorescent conjugate

Fluorescein-labelled rabbit anti-sheep immunoglobulin* was diluted 1:60 in PBS. This dilution provided a clearer endpoint than did the 1:20 to 1:40 range of dilution recommended by the manufacturers.

(iii) Test Procedure

IFAT slides were removed from storage and dried in an incubator at 40°C for 20 minutes prior to use. Doubling dilutions of the test sera in PBS were made in microtitre plates and 50 µl volumes of each dilution placed in sequence in the prepared wells. Slides were incubated in a moist atmosphere at RT for 30 minutes, then washed in PBS

* Wellcome Laboratories, U.K.

for 30 minutes with three changes. Sufficient conjugate was added to cover each of the wells, the slides reincubated at RT for 30 minutes and then washed in PBS for 30 minutes with three changes. After the addition of mountant (10 per cent glycerol in PBS, pH7.0) and cover slips, the slides were examined by fluorescent microscopy using a 12 V, 100 W quartz halogen lamp and a 30.8 x 1 FITC3 exciting filter*. One well on each slide acted as a conjugate control in which serum was replaced by PBS; a second well acted as a serum control in which conjugate was replaced by PBS. Each test included known positive and negative sera.

(iv) Determination of titres

Since it was found that fluorescence was not evenly distributed over a well and that bradyzoites tended to clump together at the centre, only peripherally situated bradyzoites were used to determine the titre. Wells where the majority of the peripheral bradyzoites were surrounded by complete rings of fluorescence were designated positive (see Figure 3.1a).

Wells where most of the bradyzoites had incomplete rings of fluorescence or only a 'cap' at one end were termed negative (see Figure 3.1b). The titre was taken as the highest dilution of the serum giving a positive reaction.

(v) Sera tested

Sera tested were from:

1. Three adult sheep hyperimmunised with cat:sheep 1 sp. antigen, as for the HAT, and designated HS/17, HS/18 and HS/19.
2. One adult sheep hyperimmunised with cat:sheep 2 sp. antigen and designated HS/20.

* Immunopan, Reichert, Austria.

3. Ten caesarean-derived, colostrum-free lambs designated CF/1 CF/2 etc.

4. Twenty-four lambs raised worm-free indoors on artificial foods, bled when 1 to 2 weeks old and designated WF/38, WF/39 etc.

5. Nineteen lambs, born and raised on pasture, bled when one month old and then monthly for the following five months and designated PR/27/1, PR/28/1 etc., at one month and PR/27/2, PR/28/2 etc., at two months and so on.

6. Twelve adult sheep found to have oesophageal macrocysts (cat: sheep 1 sp.) at slaughter and designated S/57, S/58 etc.

(vi) Repeatability test

Six sheep sera with IFAT titres ranging from negative to greater than 1:256 were coded 1 to 6 and tested twice blind by two observers (G.H.C. and J.W.).

3.3 Results

a,b. HAT, macro and micro systems

Table 3.2 presents the HAT titres recorded in both macro and micro systems. Titres attained in the macro system were always higher than those given by microtitration. Sera from hyperimmune rabbits gave higher titres than did those from hyperimmune sheep. Lambs raised worm-free gave maximum titres of 1:100 by the macro system, while 15 of 24 naturally infected sheep (62.5 per cent) had negative titres and the remainder had titres ranging from 1:50 to 1:400. Sera from worm-free lambs and the infected adult sheep were negative in the micro test.

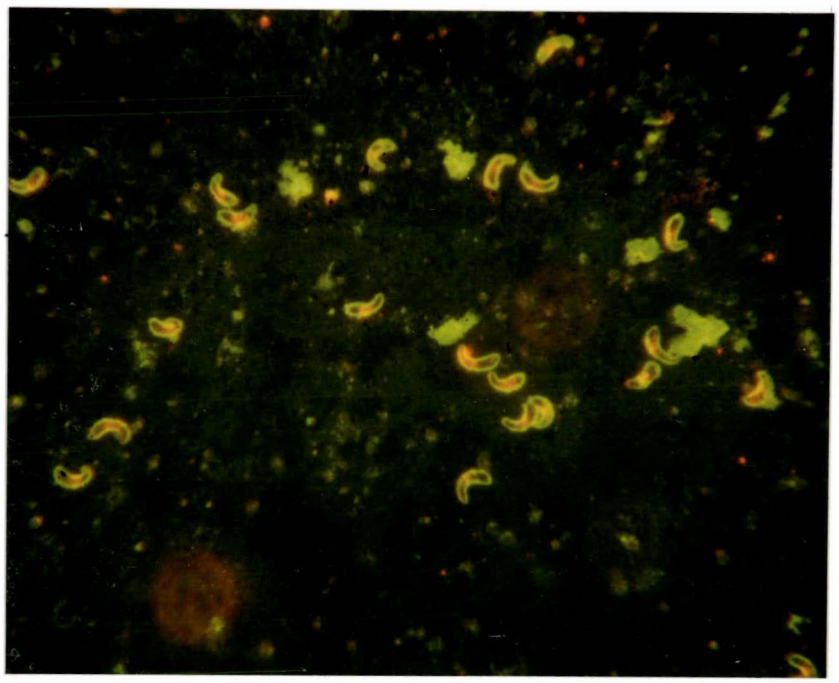
c. CFT, standard tube test

Antisera from hyperimmunised rabbits gave higher titres than did antisera from hyperimmune sheep (see Table 3.3). The sera of three lambs raised indoors and presumably not exposed to infection were

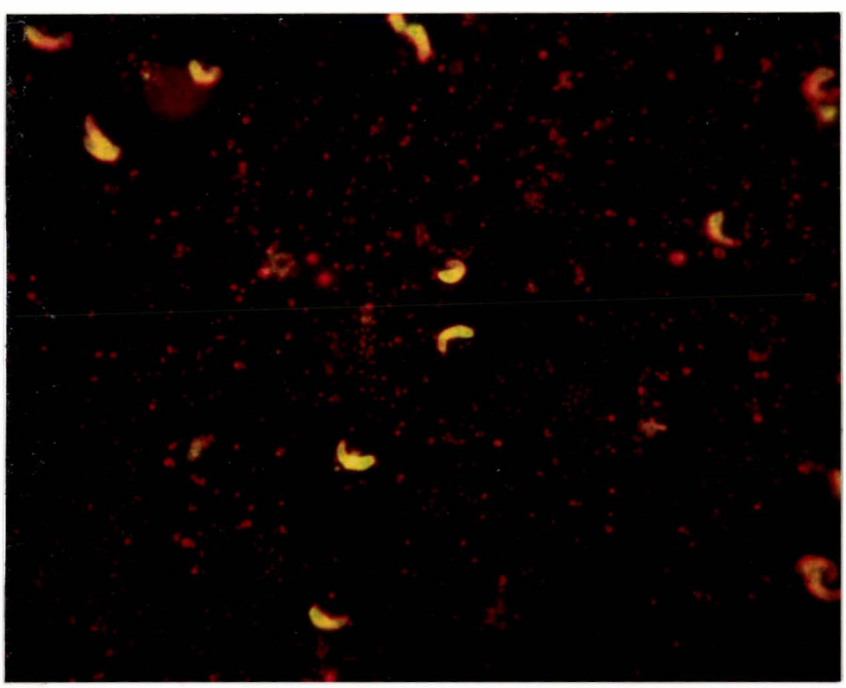
FIGURE 3.1 PHOTOMICROGRAPHS OF *SARCOCYSTIS* BRADYZOITES (X500) IN THE INDIRECT FLUORESCENT ANTIBODY TEST.

a. Positive well; bradyzoites with complete rings of fluorescence.

b. Negative well; bradyzoites with no peripheral fluorescence.



a



b

TABLE 3.2

HAEMAGGLUTINATION TEST TITRES

Serum	Source	titre macro	titre micro
HR/1	hyperimmunised rabbit	51,200	1,600
HR/2	hyperimmunised rabbit	12,800	800
HS/1 to HS/9	9 hyperimmunised sheep	5/800* 3/1600 1/3200 2/6400	6/100 1/200 2/400
WF/1 to WF/7	7 worm-free lambs	5/0 1/50 1/100	7/0
S/1 to S/24	24 naturally infected adult sheep	15/0 3/50 4/100 1/200 1/400	24/0

* number of animals/titre

TABLE 5.5

COMPLEMENT FIXATION, STANDARD TUBE TEST TITRES

Serum	Source	Titre
HR/3	hyperimmune rabbit	128
HR/4	hyperimmune rabbit	64
HR/5	hyperimmune rabbit	128
HS/10	hyperimmune sheep	16
HS/11	hyperimmune sheep	16
HS/12	hyperimmune sheep	32
WF/8	worm-free lamb	0
WF/9	worm-free lamb	0
WF/10	worm-free lamb	0
S25 to S57	32 naturally infected sheep	21/0* 5/2 3/4 3/8

* number of animals/titre

negative. Twenty-one of 32 (62.5 per cent) adult sheep infected with the cat:sheep 1 sp. were negative to the test, the maximum titre of 1:8 being recorded in only 3 (9.4 per cent) animals. The controls showed that the test was not influenced by anticomplementary factors.

d. CFT, microtitre test

Hyperimmune sheep had titres of 1:16, the maximum titre recorded in the system used (see Table 3.4). Worm-free lambs, raised indoors, were negative to the test. During 10 weeks on pasture 8 of these animals (29.6 per cent) developed positive titres: the maximum titre reached was 1:4. Of 26 lambs raised from birth on pasture for three months, 5 (19.2 per cent) had positive titres.

Anti-complementary effects were seen in tests using sera heated at 56°C for 30 minutes and incubated at 37°C for 30 minutes. Attempts to remove the anti-complementary factors from sera by heating at 62°C for 30 minutes were unsuccessful. However, absorption of these anti-sera three times with bentonite overcame this problem provided warm incubation (37°C for 30 minutes) was used. When heat-treated, bentonite -absorbed sera were tested using incubation at 4°C overnight, anti-complementary effects were again seen. The use of complement at a dilution of 1:10 did not overcome this effect and invariably reduced positive titres by two dilutions.

e. IFAT

The titres of sera from the various groups of sheep tested are given in Table 3.5. The sera from 10 caesarean-derived, colostrum-free lambs were negative as were 4 of 24 sera (16.7 per cent) from animals raised worm-free indoors for 1 to 2 weeks. Ten (41.7 per cent) of these lambs had titres of 1:8 or more, the maximum titres being 1:16.

TABLE 3.4

COMPLEMENT FIXATION: MICROTITRE TEST TITRES

Serum	Source	Titre
HS/13	hyperimmune sheep	16
HS/14	hyperimmune sheep	16
HS/15	hyperimmune sheep	16
HS/16	hyperimmune sheep	16
WF/11 to WF/38	27 worm-free lambs	27/0*
WF/11/1 to WF/38/1	27 worm-free lambs after 1 week on pasture	27/0
WF/11/2 to WF/38/2	" " 2 weeks	27/0
WF/11/3 to WF/38/3	" " 3 weeks	27/0
WF/11/4 to WF/38/4	" " 4 weeks	25/0 2/2
WF/11/5 to WF/38/5	" " 5 weeks	25/0 2/2
WF/11/6 to WF/38/6	" " 6 weeks	23/0 4/2
WF/11/7 to WF/38/7	" " 7 weeks	25/0 1/2 1/4
WF/11/8 to WF/38/8	" " 8 weeks	23/0 4/2
WF/11/9 to WF/38/9	" " 9 weeks	25/0 1/2 1/4
WF/11/10	" " 10 weeks	27/0
PR/1 to PR/26	26 pasture raised lambs 3mths old	21/0 3/2 1/4 1/8

* number of animals/titre

TABLE 3.5

INDIRECT FLUORESCENT ANTIBODY TEST TITRES

Serum	Source	Titre
HS/17	hyperimmune sheep (cat:sheep 1 sp.)	>256
HS/18	" " "	>256
HS/19	" " "	>256
HS/20	" " " 2 sp.)	128
CF/1 to CF/10	caesarean-derived, colostrum-free lambs	10/0*
WF/38 to WF/62	24 worm-free lambs	4/0 5/2 5/4 4/8 6/16
PR/27/1 to PR/48/1	19 lambs pasture raised, 1 month old	18/0 1/2
PR/27/2 to PR/48/2	" " " 2 months old	19/0
PR/27/3 to PR/48/3	" " " 3 months old	14/0 4/2 1/4
PR/27/4 to PR/48/4	" " " 4 months old	12/0 4/2 3/4
PR/27/5 to PR/48/5	" " " 5 months old	14/0 5/2
PR/27/6 to PR/48/6	" " " 6 months old	19/0
S57 to S69	12 naturally infected adult sheep	6/0 4/2 2/4

* number of animals/titre

TABLE 3.6

INDIRECT FLUORESCENT ANTIBODY TEST;

TEST OF REPEATABILITY WITH TWO OPERATORS

Serum Code No.	test 1 (G.H.C.)	test 2 G.H.C.)	test 3 (J.W.)	test 4 (J.W.)
1	256	256	128	256
2	-ve	-ve	-ve	-ve
3	64	64	64	64
4	128	128	64	128
5	2	-ve	-ve	-ve
6	16	8	16	16

Pasture raised lambs generally had low or negative titres. Titres developed in 14 out of 19 animals (73.7 per cent) during the first 5 months but by 6 months all were negative. Twelve naturally infected sheep also had low (maximum 1:4) or negative titres. By comparison three sheep hyperimmunised with cat:sheep 1 sp. antigen had titres greater than 1:256 and one sheep given cat:sheep 2 sp. antigen had a titre of 1:128.

Table 3.6 records the results of 4 tests by two observers on 6 sera; titres recorded differed by no more than one dilution indicating an acceptable degree of repeatability of the test when performed by experienced workers.

3.4 Discussion

The serology of sarcocystosis has received little attention and hence knowledge of the immune response to *Sarcocystis* species is fragmentary. The results of the studies described in this chapter have not greatly changed this situation but are a basis for further studies.

The haemagglutination test was found to be satisfactory for the detection of *Sarcocystis* antibodies provided large volumes of reagents were used; the change to a microtitre system resulted in reduced sensitivity. Naturally infected sheep gave only low titres, even in the macro system, and since the test is least specific at low titres it is unlikely to be of value in the detection of *Sarcocystis* antibodies in surveys of farm animals. The HAT is most specific when antibody levels are high and hence the test is likely to be useful in measuring the rise in *Sarcocystis* antibodies that follows the intake of sporocysts by an intermediate host. HATs have been used in experimental infections in cattle, where titres of 1:100,000 were seen at 105 days (Fayer, Johnson and Lunde, 1976) and 1:39,000 at 90 days (Lunde and Fayer, 1977) after infection. However the possibility that cross-reaction may occur

between *Sarcocystis* and related organisms in the HAT has not been investigated and this aspect will need clarification before the test can be used in the diagnosis of sarcocystosis.

The sensitivity of the CFT was not affected by the change from a macro to a microtitre system. However, when smaller volumes of reagents were used, many sera showed anticomplementary effects. This difficulty was partially overcome by absorbing sera with bentonite before testing, but was not completely eliminated, as the anticomplementarity reappeared when a longer, cold incubation method was substituted for the shorter, warm incubation method. Anticomplementary effects were not removed by heat treatment at 62°C nor satisfactorily compensated for by increasing the complement concentration.

It is surprising that no mention is made of the problem of anticomplementary effects in sheep sera in the CFT studies of Munday and Courbould (1974) and subsequently in several other studies (see Table 3.1). The reliability of their test system is also questionable for other reasons. Firstly, the test employed antigen prepared from sheep oesophageal macrocysts by a process that included boiling an extract of cysts for 20 minutes; a procedure that does not yield an effective antigen (Collins, unpubl. obs.). Secondly, there is no indication of how the diagnostic titre for *Sarcocystis* infection was established and, thirdly, the specificity of the test is unknown; Munday (1975) used the test, without a change of antigen, to determine the prevalence of *Sarcocystis* 'species' in both cattle and sheep.

The indirect fluorescent antibody test was found to be a convenient test and the results were repeatable. Some difficulty was experienced initially in interpreting the results. The distribution of bradyzoites within wells was usually uneven, and organisms that were crowded at the centre took up less stain than did those more widely

placed at the periphery. These latter were more consistent in their staining and were therefore used when determining the titre. When fluorescent conjugate was used in the test at the dilution recommended by the manufacturer, the brightness of the fluorescence interfered with determination of the end point. Further dilution of the conjugate made it easier to differentiate between positive and negative walls. Reading an IFAT is, to some extent, subjective, but it was found that experienced operators could produce comparable results.

The results from all three serological tests showed a similar pattern in that hyperimmune sheep had high titres; naturally infected and grazed animals had low or negative titres. Indoor reared animals that were presumed to be uninfected were mostly negative; the occasional low positive titre could be ascribed to maternal antibodies acquired in colostrum. Sera from adult sheep with numerous oesophageal macrocysts recorded only minimal levels of antibodies and it is doubtful, therefore, if any of these tests will be suitable for the detection of long established, naturally acquired *Sarcocystis* infections. A negative antibody titre is obviously not a reliable indicator of the absence of infection.

While hyperimmune sheep sera showed higher titres than did sera from naturally infected sheep they were consistently lower than the titres of sera from hyperimmune rabbits. These findings are in agreement with the results of a preliminary agar diffusion study, where sera from hyperimmune rabbits gave five or more precipitation lines against cat:sheep 1 sp. macrocyst antigen, whereas hyperimmune sheep sera produced at most two lines. Presumably as a result of adaptation to its intermediate host, the cat:sheep 1 sp. possesses few antigens that are not also found in the sheep.

The development of a species of *Sarcocystis* in an intermediate host is divisible into three phases. Firstly, following excystment, sporozoites invade host tissues and a relatively small amount of parasite antigen comes into brief contact with the host's immune system. Subsequently, one or more phases of schizogony release increasing amounts of antigen into the circulation, culminating in the discharge of schizozoites after the ultimate schizogony. Finally, the invasion of muscle fibres and development within the sarcocyst wall causes the parasite to be sequestered from the immune system. Thus it is likely that the host's immune response is activated by infection and early development but the main antibody production is in response to the products of the ultimate schizogony. This is supported by reports of experimental infections in which titres of *Sarcocystis* antibodies rose from between four and five weeks after infection (Munday, Barker and Rickard, 1974; Fayer and Lunde, 1977; Lunde and Fayer, 1977). It is possible that each phase of the life cycle involves the occurrence or exposure of different parasite antigens; the sporozoite may be antigenically different from the schizozoite and the bradyzoite. If this is so, it could explain why schizozoites are capable of surviving outside host cells as late as four weeks after the initial infection, before they enter muscle fibres. Also, if antigenic variation does occur, tests employing bradyzoites as antigen are inappropriate for the detection of antibodies to the products of schizogony.

The diagnosis of sarcocystosis as a cause of economic disease in farm animals would be aided by the development of a suitable serological test. Since disease appears more likely to be caused by the ingestion of large numbers of sporocysts over a short space of time rather than the sporadic intake of small numbers, a diagnostic test will need

to show that antibody levels rose following the onset of illness at the ultimate schizogony. The IFAT and HAI could be used for this purpose and it would be necessary to test at least two serum samples, separated by perhaps two weeks, to show a rising titre. However, both tests need further development and evaluation before they are employed in field outbreaks. The possibility of cross-reactivity between *Sarcocystis* and related genera, and between individual *Sarcocystis* species in these tests needs examination. It will also be necessary to study the quantitative relationship between the numbers of sporocysts ingested and the antibody titres produced. At present we are still a considerable way from the development of a diagnostic test for sarcocystosis.

CHAPTER 4

SHEEP MACROCYST STUDIES 1: FAT AND THIN CYSTS

4.1 Introduction

Adult sheep slaughtered at meat works in New Zealand are commonly infected with macrocysts (macroscopically visible sarcocysts) (see Table 4.1). Sarcocysts are seen most commonly in oesophageal muscle (see Figure 4.1) but are of no importance to meat hygiene; less frequently macrocysts are visible in the carcase (see Figure 4.2 T and F) and have to be trimmed away. Extensive infection may necessitate condemnation of the entire carcase. Despite the importance of *Sarcocystis* to the meat industry, and the frequency with which macrocysts occur, no studies have been made of their prevalence or identity. During preliminary investigations it was noted that two forms of macrocyst were present in carcases: a thin tubular cyst with tapered ends and a wider form with bluntly rounded ends (see Figure 4.2 T and F). These were provisionally designated 'thin' and 'fat' respectively as their relationship was unknown (Collins, Charleston and Moriarty, 1976). The presence of two distinct populations of macrocysts - thin and fat - in carcase muscle was confirmed by a study of their dimensions.

The existence of more than one form of macrocyst in sheep had not been reported previously and the question arose as to the relationship between thin and fat macrocysts and oesophageal macrocysts. This was explored by studying the relative prevalence of the three cyst types in adult sheep after slaughter and by comparing the ultrastructure of their cyst walls.

4.2 Materials and methods

a. Measurement of carcase macrocyst dimensions

Pieces of muscle, trimmed from the abdominal wall or diaphragm

TABLE 4.1

PREVALENCE OF *SARCOCYSTIS* IN SHEEP DETECTED AT MEAT WORKS IN NEW ZEALAND

(From figures supplied by the Meat Division of the Ministry of Agriculture and Fisheries).

Year	Total Kill	Incidence of *Sarcocystis*	%	Condemned for *Sarcocystis*	%
Oct 1974 to May 1975	Sheep 6,086,426	1,012,787	16.640	9,999	0.099
	Lambs 24,007,932	776	0.003	18	0.000
1975 to 1976	Sheep 6,591,753	372,343	5.649	6,636	0.100
	Lambs 25,961,234	249	0.001	3	0.000
1976 to 1977	Sheep 6,960,116	396,002	5.690	7,569	0.109
	Lambs 25,417,157	345	0.001	9	0.000

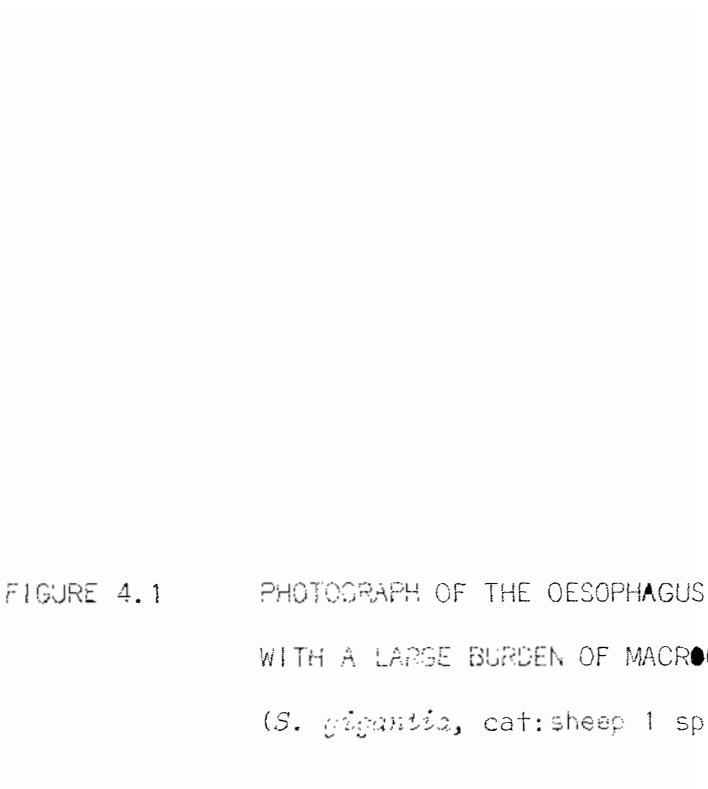


FIGURE 4.1 PHOTOGRAPH OF THE OESOPHAGUS OF AN ADULT SHEEP WITH A LARGE BURDEN OF MACROCYSTS (*S. gigantia*, cat:sheep 1 sp.)

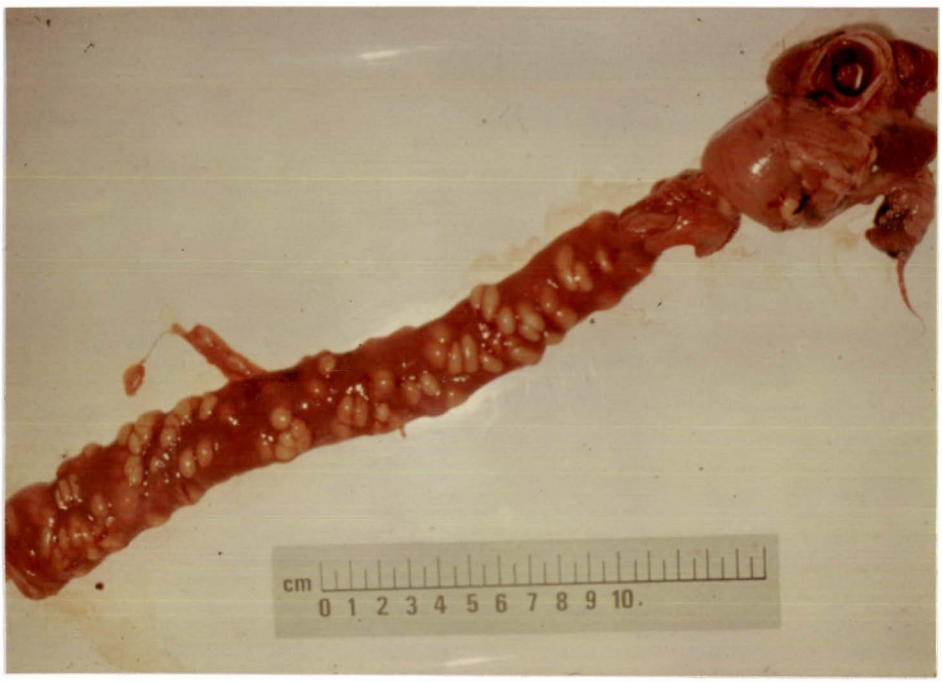
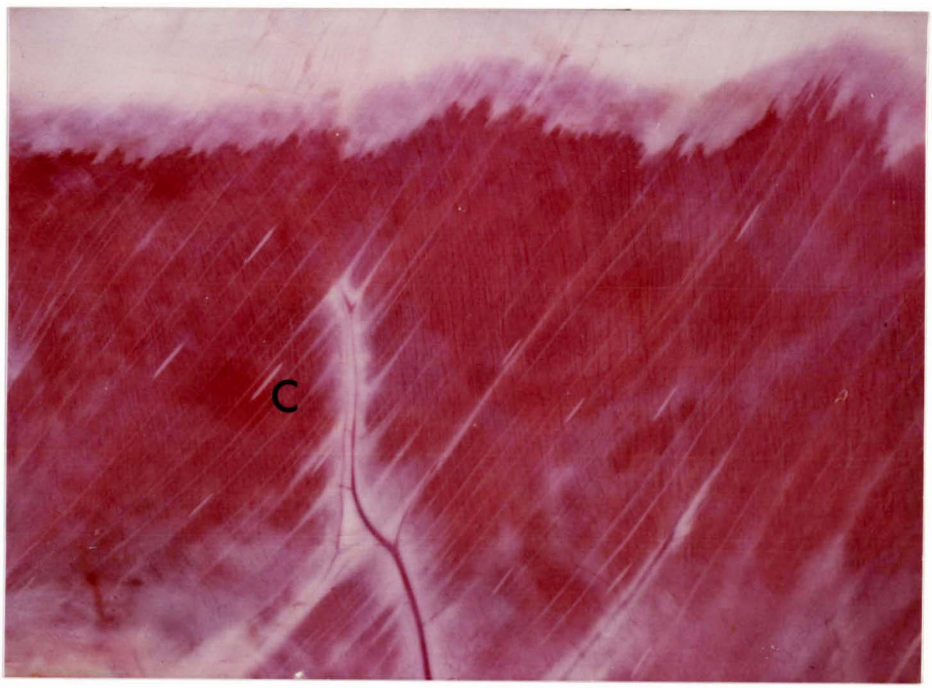
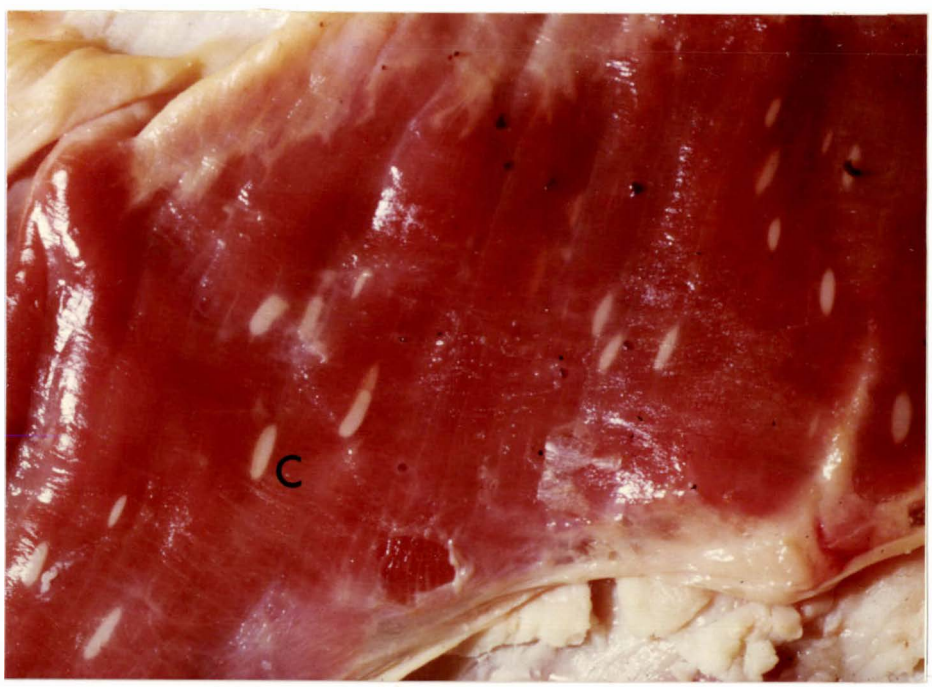

cm
0 1 2 3 4 5 6 7 8 9 10

FIGURE 4.2 PHOTOGRAPHS OF MACROCYSTS IN THE MUSCLE OF THE DIAPHRAGM OF ADULT SHEEP (x approximately 0.5)
T. Thin carcase macrocyst (*S. medusiformis* n.sp., cat:sheep 2 sp.). F. Fat carcase macrocyst ('*S. gigantia*', cat:sheep 1 sp.)



T



F

of ewe carcases and containing one or more macrocysts, were obtained from a local meat works. The meat was stored at 4°C and examined within 24 hours of collection. The dimensions (length L and width W) of 503 cysts unselected for type found on the surfaces of trimmings were measured to the nearest 0.1mm using 6 x magnification.

b. Prevalence of oesophageal and carcase macrocysts

1215 adult sheep were examined after slaughter at a local meat works for the presence of oesophageal and carcase macrocysts.

An assistant stood next to the process chain and examined every third oesophagus for macrocysts. He recorded his findings on a label that was then attached to the corresponding carcase. Viscera and carcase passed along the chain side by side to where the author stood. The carcase was checked for the presence of fat and/or thin cysts and the result recorded on the label which was removed and placed in a container. The time available for examination, despite examining only every third carcase, rarely exceeded 10 seconds. This allowed only a rapid scan of the surfaces of the pleural and abdominal cavities and the external surfaces of the left and right sides of each carcase. Approximately 200 sheep were examined on each of 6 occasions over a period of three months. The information on the labels was accumulated and analysed.

c. Electron microscopy of cyst walls

Macrocysts were dissected from oesophagi and carcase trimmings and five specimens, of approximately equal length, of each type of cyst were fixed in glutaraldehyde, post-fixed in osmium tetroxide, embedded in epoxy resin*, sectioned and examined by electron microscopy (Griffin, 1972).

* 'FLUKA', Chemische Fabrik, Buchs SG, Switzerland.

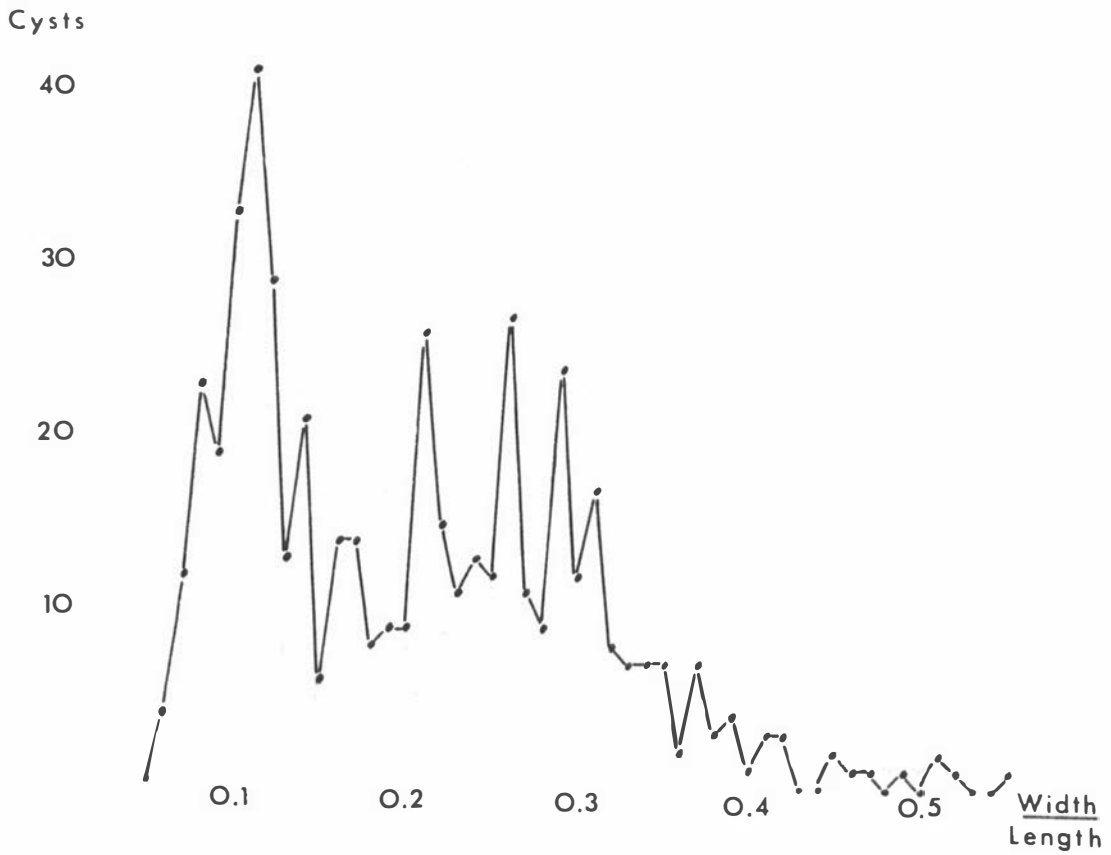


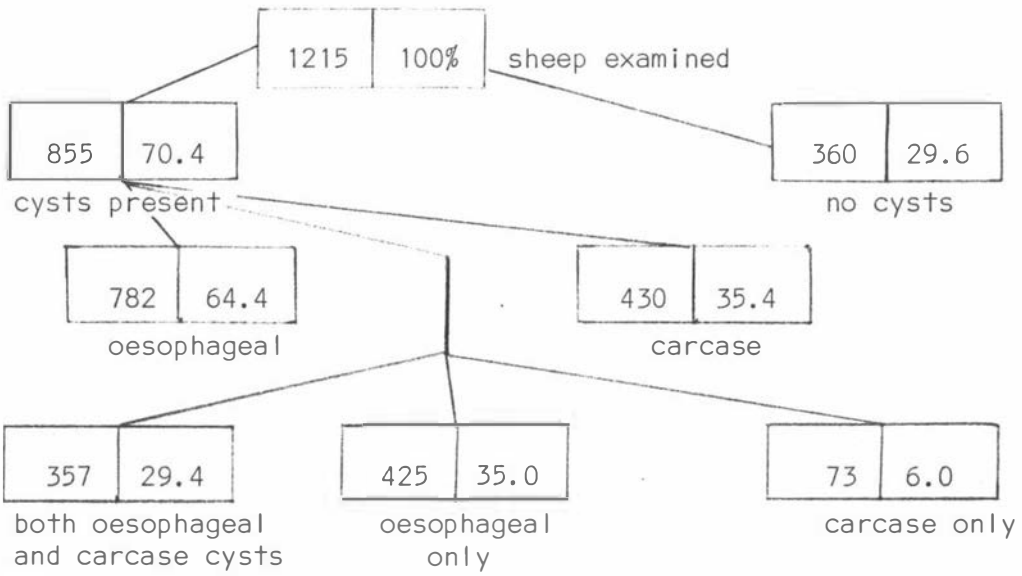
FIGURE 4.3

THE FREQUENCY DISTRIBUTION OF THE RATIO BETWEEN CYST AXES (R = W/L) OF 503 CARCASE MACROCYSTS

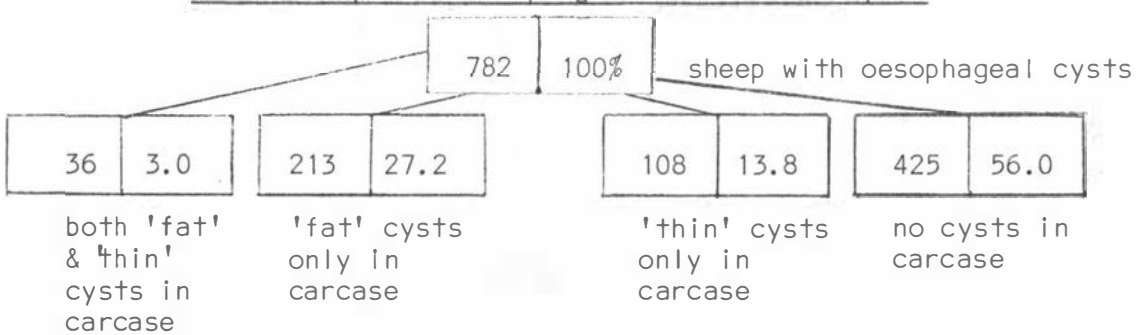
FIGURE 4.4 DIAGRAM SHOWING THE FREQUENCY DISTRIBUTION OF MACROCYSTS IN 1215 ADULT SHEEP AT SLAUGHTER

The presence or absence of oesophageal cysts is compared with the presence or absence of fat and thin carcase macrocysts.

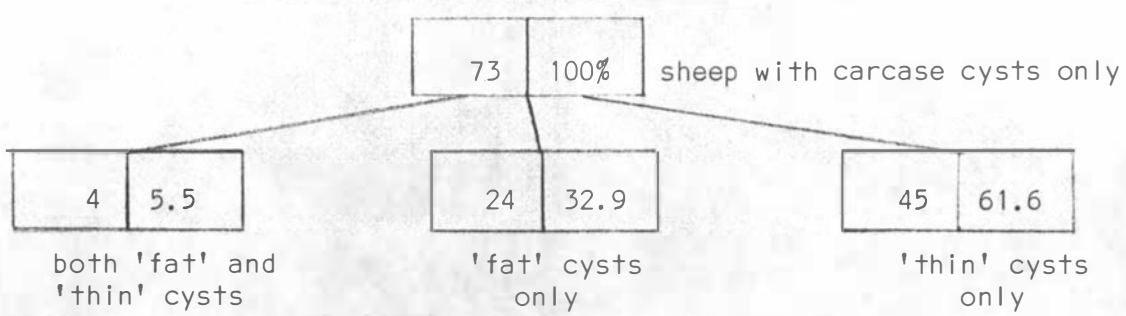
Macrocysts in adult sheep



Adult sheep with oesophageal and carcase cysts



Adult sheep with only carcase cysts



4.3 Results

a. Carcase macrocyst dimensions

Figure 4.3 records the frequency distribution of the ratio between the lengths of the cyst axes (ratio = $\frac{W}{L}$) of 503 carcase macrocysts. The data show that there are two populations of macrocysts with distinct shapes in sheep carcases. Thin cysts apparently grow mainly in length, fat cysts in both dimensions.

b. Macrocyst prevalence

The presence or absence of oesophageal, thin and fat macrocysts in 1215 sheep is shown diagrammatically in Figure 4.4. Statistical analysis of the results, by X^2 test using Yate's correction, revealed no significant difference in the distribution of either thin or fat cysts whether oesophageal cysts were present or not.

c. Macrocyst wall ultrastructure

The structure of the wall of fat cysts was found to be similar to that of oesophageal cysts (see Figure 4.5 O and F. Both possess villi that are irregular in shape and blister-like invagination on all the villar and inter-villar surfaces. A secondary cyst wall of collagen is present in both types of macrocyst (see Figure 2.7).

The thin cyst wall is distinctly different; villi are roughly conical and regular. Both the villi and the intervillar wall give rise to snake-like filaments of regular diameter but with slightly swollen ends, (see Figure 4.5 T); the filaments branch occasionally. Blister-like invaginations are confined to the intervillar surface and the surfaces of the proximal parts of the villi. There is no collagenous secondary wall.

4.4 Discussion

The study of cyst dimensions confirmed the observation that two forms of macrocyst may be found in sheep carcases. The method of

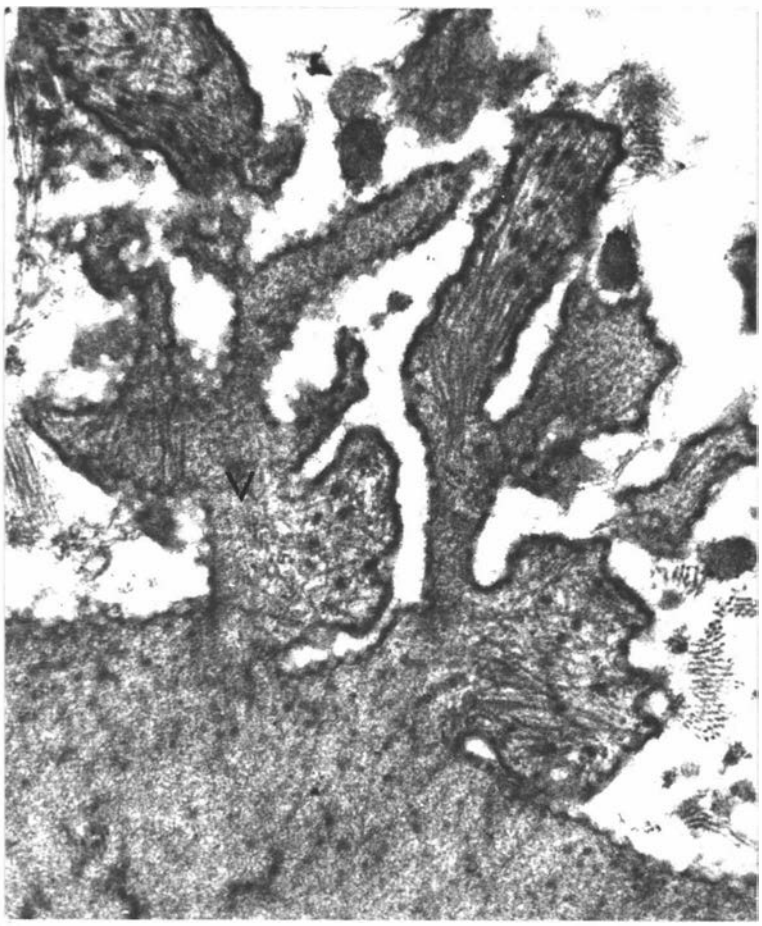
FIGURE 4.5 ELECTRON MICROGRAPHS OF THE WALLS OF *SARCOCYSTIS* MACROCYSTS IN SHEEP (X 22,500).

O. Oesophageal macrocysts (*'S. gigantia'*, cat: sheep 1 sp.) (V = villus).

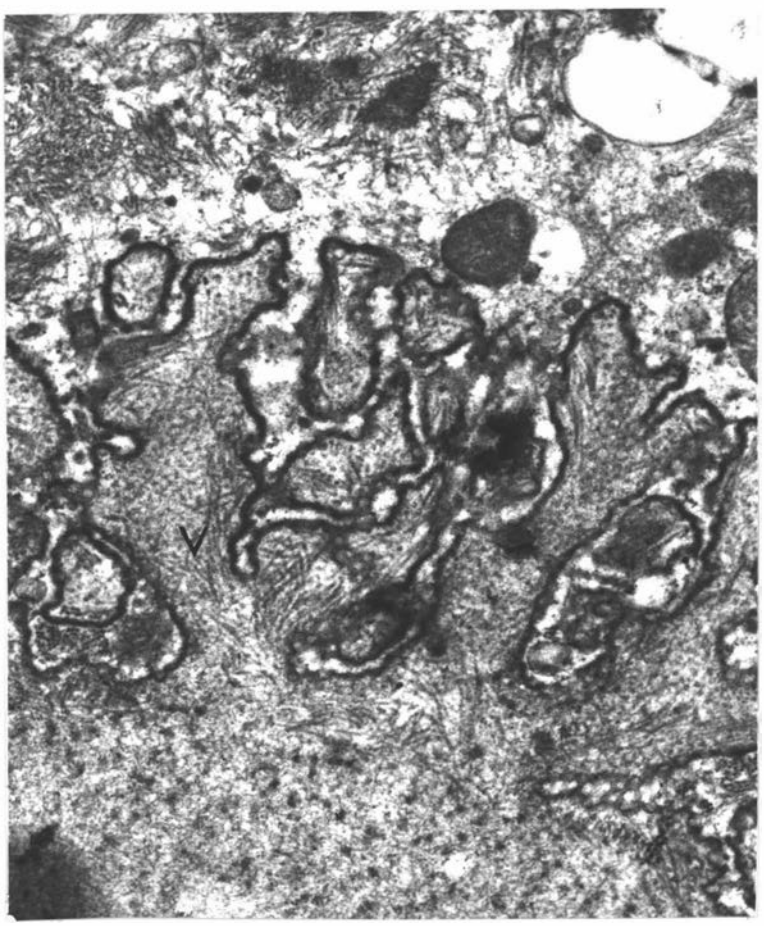
F. Fat carcase macrocyst (*'S. gigantia'*, cat: sheep 1 sp.) (V = villus).

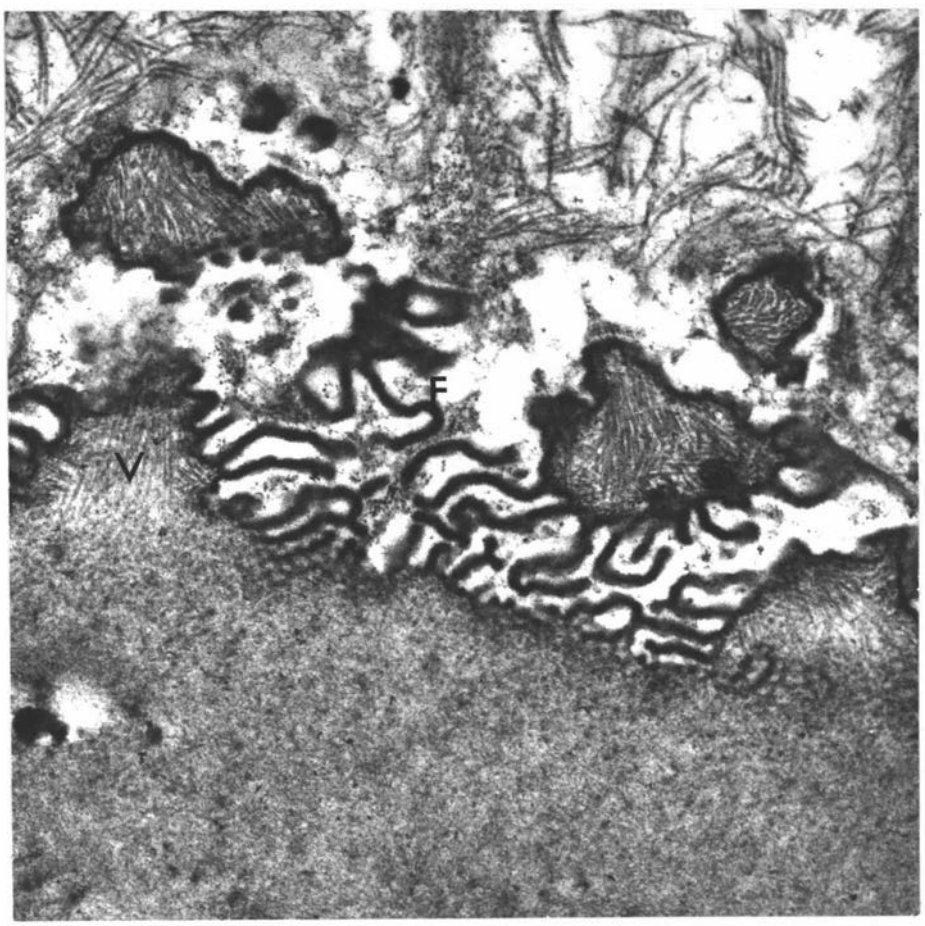
T. Thin carcase macrocyst (*S. medusiformis* n.sp., cat:sheep 2 sp.) (F = filament; V = villus).

O



F




T
F
V

measurement was not sensitive enough to detect significant differences in the dimensions of the smallest cysts examined, hence the graph (see Figure 4.3) shows a small overlap between the two populations. The possibility that thin cysts are an earlier form of the fat cysts and that growth takes place, initially in length, later in both dimensions may be discounted by the fact that thin cysts can be as long as fat cysts.

A high proportion of adult sheep - approximately 70 per cent - were found to be infected with macrocysts. However, considering the limited time available for examination and the fact that only superficial cysts, that were large enough to be readily seen, could be recorded, it is obvious that the real prevalence was much higher and probably close to 100 per cent. McNab and Robertson (1972), in a survey of the prevalence of *Cysticercus ovis* in sheep carcases found that only one in five to ten infected animals had superficially visible cysts.

Statistical analysis showed that the presence or absence of oesophageal cysts was not correlated with that of thin or fat cysts in the carcase: infection had occurred randomly. There was no evidence that the presence of either of the carcase cyst types caused any reduction in the prevalence of the other; although it is possible that the failure to show a significant correlation in occurrence between the cyst types resulted from the lack of sensitivity of the method of detection used in the survey.

The study of cyst wall ultrastructure confirmed the presence of two distinct macrocystic species in skeletal muscle. The fat cyst appears to be the same species as the oesophageal cyst. However, the common identity of these two forms will only be fully proved by showing that sporocysts, derived from feeding either oesophageal or fat cysts to cats, give rise to both forms in sheep.

The structure of the thin cyst wall has not been described previously. Bergmann and Kinder (1975) investigated the structure of sarcocysts in sheep and compared 'thick' and 'thin' walled forms: the sarcocysts of *'S. tenella'* (cat:sheep 1 sp.) and *'S. ovicanis'* (dog:sheep sp.) respectively. No other type of cyst was described. A paper comparing the ultrastructure of the cyst walls of various species by Mehlhorn, Hartley and Heydorn (1976) also contained only two ovine species. At present it is generally accepted that, if mature sarcocysts have walls with distinctly different ultrastructures, they belong to different species. But the converse is not necessarily true (Mehlhorn, Hartley and Heydorn, 1976).

There is no doubt, therefore, that the thin macrocyst is a distinct species; not only does it have a different shape and wall ultrastructure but there are also differences in the electrophoretic mobility of certain enzymes (phosphoglucomutase (PGM) and 6 - phosphogluconate dehydrogenase (6 PGD)) between fat and thin cysts (Atkinson and Collins, in press). The discovery of a further species in sheep poses a problem of nomenclature. If as seems possible, Railliet (1886b) gave the name *S. tenella* (formerly *Miescheria tenella*) to the 'delicate walled' microcysts found by Moulé (1886c) and *Babiania* (now *Sarcocystis*) *gigantia* to the macrocysts in the oesophagus and carcase then the cat:sheep 2 sp. is without a name. It is proposed that the thin macrocystic species be named *S. medusiformis* n.sp. in recognition of the distinctive structure of its cyst wall (Collins, Atkinson and Charleston, 1979).

CHAPTER 5

SHEEP MACROCYST STUDIES 2: TRANSMISSION

5.1 Introduction

The dog:sheep sp. is the only species of *Sarcocystis* occurring in sheep that has been transmitted through a complete life cycle (Ford, 1975). Although the cat:sheep 1 sp. was involved in the elucidation of the *Sarcocystis* life cycle (Rommel, Heydorn and Gruber, 1972) transmission of this species to sheep has yet to be demonstrated unequivocally.

In this chapter the transmission of the thin macrocyst species to its definitive host is reported and the sporocysts of this species are compared with those of the cat:sheep 1 sp. Attempts to infect lambs with cat:sheep 1 sp. sporocysts and study the early development of this species in sheep are also described.

5.2 Materials and methods

a. Transmission studies in dogs and cats

i) Experimental animals

Conventionally reared kittens and puppies were obtained at weaning and maintained free of *Sarcocystis* on processed food.

ii) Macrocysts

Fat and thin cysts were excised from mutton trimmings taking especial care to obtain cysts free of muscle that might be infected with dog:sheep sp. microcysts. Macrocysts were fed within 6 hours of collection either in gelatine capsules or incorporated in food.

iii) Examination of faeces

All the faeces of all the experimental animals were collected daily and examined for sporocysts (see Appendix 4a(i) for

method) for a period of 2 weeks before and 4 weeks after dosing. Sporocysts were measured at 100 x magnification with a micrometer eyepiece (see Appendix 4c).

iv) Experimental procedure

Kittens and puppies were given macrocysts in 8 separate experiments as summarised in Table 5.1.

b. Transmission studies in sheep

i) Sporocysts

Kittens, raised free of *Sarcocystis* infection and examined for sporocysts (see Appendix 4a (i) and (ii)) for two weeks prior to dosing were fed cat:sheep 1 sp. macrocysts dissected from the oesophagi of sheep. Sporocysts were harvested (see Appendix 4a (i), (ii) and (iii)) and stored in tap water at 4°C for up to two weeks before use. Counting sporocysts was carried out by the method described in Appendix 4b.

ii) Experimental animals

Lambs were raised indoors from birth on calf milk replacer* and at the time of infection were helminth-free but not coccidia-free. The animals were infected at various ages (detailed below).

iii) Haematology

Unclotted blood was examined, by standard procedures, for haemoglobin concentration (Hb), packed cell volume (PCV) and hence the mean corpuscular haemoglobin concentration (MCHC), total serum protein (TP), total leucocytes (WBC) and differential leucocyte counts (diff. WBC).

* 'ANCALF', N.Z. Cooperative Dairy Company, Palmerston North, N.Z.

iv) Serology

Serum from clotted blood was examined for *Sarcocystis* anti-bodies by CFT (see Chapter 3, CFT, standard tube test for method) and IFAT (see Chapter 3, IFAT, for method).

v) Histopathology

Lambs killed at intervals (see below for details) were immediately necropsied. Samples were taken of heart, lung, liver, kidney, spleen, brain, spinal cord, oesophagus (3 levels), reticulum, abomasum (cardiac and pyloric regions), small intestine (at 1m intervals), caecum, colon, mesenteric lymph node, diaphragm, thigh muscle and bone marrow. Tissues were fixed in 10 per cent formol saline or in Bouin's solution (gastro-intestinal tissues), embedded in paraffin wax and at least two sections approximately 6 µm thick stained with haematoxylin and eosin were examined.

vi) Examination of tissues by an indirect fluorescent antibody techinque

In Experiment 3 only (see below), samples of tissue (from the organs listed above) approximately 10 x 10 x 2mm were removed soon after death, frozen in pentane-liquid nitrogen and stored at -40^{o}C over solid carbon dioxide. Sections, cut approximately 2 µm thick in a cryostat, were air dried, fixed in methanol, processed as for the IFAT (see Chapter 3 for method) and examined by fluorescent microscopy. At least 5 sections were examined from each tissue block. The tissues of the control lambs and standard IFAT slides prep-

ared with cat:sheep 1 sp. bradyzoites (see Chapter 3) served as negative and positive controls respectively.

vii) Experimental procedure

Experiment 1: Eighteen lambs, 6 months old were used in the experiment. Fourteen were each given approximately 8.3×10^5 sporocysts by stomach tube; 4 were left undosed as controls. All the lambs were bled 6 and 3 days before and on the day of dosing, then daily until killed. One dosed lamb was killed daily starting on day 1 and control lambs on days 1, 5, 10 and 14. Haematological, serological and pathological examinations were carried out as described above.

Experiment 2: Nine lambs, 3 months old were given various doses of sporocysts and killed at intervals as shown below. The faeces of one lamb (lamb 3*) were examined for sporocysts (see Appendix 4a(i) and (ii)) daily for 5 days after dosing. Two lambs (lambs 8^+ and 9^+) were injected intramuscularly with 2.0 mg dexamethasone$^{\phi}$ on day 0 and at 2 day intervals following as an immunosuppressant. Two lambs were not dosed with sporocysts and served as controls.

lamb	dose of sporocysts	killed and examined
1	5×10^6	day 1
2	5×10^6	2
3*	16×10^6	5
4	5×10^6	8
5	5×10^6	8
6	5×10^6	13

ϕ DEXADRESON, Intervet Laboratories Ltd., U.K.

lamb	dose of sporocysts	killed and examined
7	5×10^6	13
8+	1.2×10^6	29
9+	1.2×10^6	50
10	0	1
11	0	50

Haematological and pathological examinations were carried out as described above. No serological test was performed.

Experiment 3: Seven lambs, less than one week old, were each given 1×10^6 sporocysts by stomach tube. Two lambs were left undosed as controls. Dosed lambs were killed 20 and 23 hours, 2, 5, 14, 21 and 29 days after infection. Control lambs were killed at 14 and 29 days. One lamb, killed at 23 hours, received sporocysts that had been pre-treated with 0.5 per cent sodium hypochlorite solution for 50 minutes in an attempt to render the sporocysts more susceptible to the excystment stimulus (McKenna, pers. comm.). At necropsy, samples were removed for histology as described above and further samples were frozen and examined by an indirect fluorescent antibody technique (see above). No haematological or serological examinations were carried out.

5.3 Results

a. Transmission in dogs and cats

All the kittens fed thin macrocysts excreted sporocysts in their faeces starting 12 to 22 days after infection (see Table 5.1). The dimensions of the sporocysts are given in Table 5.2. Both kittens given fat macrocysts produced sporocysts. The dimensions of sporocysts from kittens fed thin or fat cysts were not significantly different

TABLE 5.1

TRANSMISSION EXPERIMENTS: RESULTS OF FEEDING FAT OR THIN CARCASE MACROCYSTS FROM SHEEP TO KITTENS AND PUPPIES

Expt.	test animals	infective dose 'fat' cysts	infective dose 'thin' cysts	result
1	2 kittens	0	15 each	+ve
	2 kittens	0	0	-ve
2	2 kittens	0	10 each	+ve
	2 kittens	0	0	-ve
3	1 kitten	0	25	+ve
	3 kittens	0	0	-ve
4	2 kittens	20 each	0	+ve
	1 kitten	0	0	-ve
5	2 puppies	0	30 each	-ve
	1 puppy	0	0	-ve
6	3 puppies	0	20 each	-ve
	1 puppy	0	0	-ve
7	4 puppies	4 each	0	-ve
	7 puppies	0	0	-ve
8	7 puppies	4 each	0	-ve
	3 puppies	0	0	-ve

+ve = sporocysts passed in faeces
-ve = no sporocysts passed in faeces

TABLE 5.2

TRANSMISSION EXPERIMENTS: DIMENSIONS OF SPOROCYSTS FROM KITTENS FED FAT OR THIN CARCASE MACROCYSTS FROM SHEEP

Expt.	Sporocyst dimensions (µm) range	mean ± SD	n=
1 (thin cysts)	10.3-12.5 x 7.3-8.8	12.0±0.6 x 7.7±0.4	50
2 (thin cysts)	10.5-13.0 x 7.3-8.3	12.1±0.6 x 7.7±0.4	25
3 (thin cysts)	12.3-12.8 x 7.8-8.8	12.5±0.2 x 8.2±0.4	25
4 (fat cysts)	10.5-13.0 x 7.3-8.3	12.2±0.6 x 7.7±0.3	25

(see Table 5.2). No puppies or control kittens produced sporocysts.

b. Transmission in sheep

No significant gross or microscopic lesions that could be ascribed to *Sarcocystis* infection and no organisms were found in any animal except for lamb number 8 in Experiment 2. This lamb had several immature sarcocysts of 8.0 to 42.0 µm diameter in the striated muscle of the oesophagus. One section contained 9 sarcocysts. All of the sarcocysts appeared to contain only metrocytes and the species was not identified. The haematological values of the lambs in Experiments 1 and 2 stayed normal throughout and no increase in *Sarcocystis* antibodies occurred in Experiment 1. No fluorescence that could be associated with organisms was seen in sections from the lambs of Experiment 3; the bradyzoites on the IFAT slides showed peripheral fluorescence.

Sporocysts, of similar size to those in the experimental dose, were found in the faeces of lamb 3 on days 1 and 2 of Experiment 2.

5.4 Discussion

The studies showed that both the fat and thin macrocyst species in sheep cycle through cats and that the sporocysts are similar. The dimensions of the sporocysts are also similar to those reported for the cat:sheep 1 sp. (see Appendix 2 table 1). Therefore, at present, it is only possible to identify the thin macrocyst species (*S. medusiformis* n. sp., cat:sheep 2 sp.) by its shape, cyst wall ultrastructure and the electrophoretic mobility of certain enzymes (Atkinson and Collins, in prep.).

The transmission of the fat macrocyst species through cats and the production of sporocysts with dimensions of approximately 12.2 x 7.7 µm is further evidence for it being the same species as the oesophgeal form. However, their common identity, as was discussed in

Chapter 4, will only be fully proved by their transmission to sheep.

In three experiments, 30 lambs aged between a few days and 6 months were given doses of up to 16 million sporocysts and no evidence of infection was found. The one lamb found to have sarcocysts in its oesophagus 29 days after infection was, because of the size and degree of development of these cysts, probably infected before the start of the experiment. The lambs used in these experiments were maintained in conditions that could not exclude the possibility of extraneous infection.

Other workers have failed to show unequivocally that cat:sheep 1 sp. sporocysts are infective for sheep (Munday and Rickard, 1974; Gestrich, Heydorn and Baysu, 1975; Munday, 1976). Sporocysts of the cat:sheep 1 sp. have been reported as being 'non-pathogenic' to sheep (Gestrich, Schmitt, and Heydorn, 1974;) it is likely that in this instance also, infection did not occur. Munday (1978) claimed to have infected sheep born and raised on a cat-free island with this species, but his conclusions were invalid (Collins, Charleston and McKenna, 1978) as the undosed control sheep were found to be infected.

There are two possible explanations for the lack of success in the author's studies: either the lambs were not susceptible to infection or the sporocysts were not infective. The first possibility is not easy to sustain: lambs of various ages between birth and six months, were challenged with a wide range of doses of sporocysts and yet failed to become infected. It is also difficult to believe that the sporocysts could have been non-viable: sporocysts of other species harvested and stored in the same way readily produce infection.

The most likely explanation for the failure of the experimental infections is that cat:sheep 1 sp. sporocysts differ fundamentally from those of other species in that they are not immediately infective. In support of this hypothesis it has been found that less than 20 per cent of cat:sheep 1 sp. sporocysts excyst in conditions that cause up to 80 per cent excystment in other species, unless pretreated with sodium hypochlorite solution (McKenna, pers. comm). Presumably this artificial treatment renders the sporocyst wall pervious to the excystment stimulus. When and where, under natural conditions, similar 'conditioning' takes place is not known. It is possible that cat:sheep 1 sp. sporocysts have to be passed through an entire gastro-intestinal tract before they are infective to a second animal. Sporocysts were found apparently unharmed in the faeces of one lamb in the studies described above and 'passaged' sporocysts probably caused infection of control sheep in Munday's (1978) experiment. Alternatively, sporocysts may need a period of time in a mature rumen, subjected to chemical and/or mechanical damage, before they become infective. If so, lambs with minimal rumen function would not be susceptible to infection. This possibility is supported by the finding that oocysts of certain eimerian coccidia of cattle were more infective and gave rise to higher levels of oocyst production if administered to calves in dry rather than liquid feed (Smith and Davis, 1965). Further alternative forms of 'conditioning' might be provided by transport hosts (Smith and Frenkel, 1978) or by the external environment. Whatever the cause of the apparent lack of infectivity of cat:sheep 1 sp. sporocysts, it is difficult to reconcile these findings with the high prevalence of the species in sheep (see Chapter 4).

CHAPTER 6

THE EFFECT OF TEMPERATURE ON THE VIABILITY OF SARCOCYSTS

6.1 Introduction

The sarcocyst is a long-lived resting stage in the life cycle of *Sarcocystis*, capable of further development only if eaten by the appropriate intermediate host. Since many species, especially those in the larger farm animals, must rely on scavenging, rather than predation by carnivores for transmission it would be advantageous for sarcocysts to be able to survive not only the death of the host but also the decomposition of tissues that follows. Survival of sarcocysts in carcases and in processed meat is likely to be an important factor in the epidemiology of sarcocystosis.

Any future attempt to reduce the prevalence of sarcocystosis in farm animals is likely to involve the treatment of meat to render it non-infective for dogs and cats. Successful treatment will have to be based on knowledge of how sarcocysts are affected by various external factors. For parasitic protozoa a change in temperature from what is physiologically normal is likely to be deleterious and the effect a function of both the difference in temperature and the exposure time. The following experiments were designed to show the time-temperature regime that would kill or render *Sarcocystis* bradyzoites non-infective. Treatments were applied to macrocysts of the cat:sheep 1 sp. and their metabolism was monitored. Infectivity was subsequently tested by feeding treated cysts to cats.

6.2 Materials and methods

a. Oxygen electrode studies

i) The oxygen electrode - principle

The Rank Oxygen Electrode* (see Figure 6.1A) is used to monitor the oxygen uptake of small organisms suspended in a liquid medium. Oxygen, diffusing from the medium, passes across a thin teflon membrane and is reduced at a platinum electrode. A potential is maintained through an outer silver electrode and an electrolyte bridge - saturated KCl solution (see Figure 6.1B). As the medium is constantly stirred and maintained at a constant temperature the current generated is directly proportional to the amount of oxygen in the medium. Metabolising organisms remove oxygen and this is monitored on a potentiometric chart recorder.

ii) Method of use

The apparatus was set up with water circulating at 40^{o}C. The chart recorder was adjusted at the beginning of an experiment, and before testing each sample, by establishing base lines using saline 100 per cent saturated with oxygen and saline depleted of oxygen by the addition of sodium dithionite. The teflon membrane and electrolyte were changed frequently to maintain maximum sensitivity. Each sample of cysts was tested, after reestablishing the base lines, by placing it in the chamber with saline saturated with oxygen, allowing one minute for temperature equilibration, then recording uptake for 5 minutes. Since electrode conditions were similar during each test, it was possible to compare the oxygen uptake rates of the samples.

* Rank Bros., Cambridge, U.K.

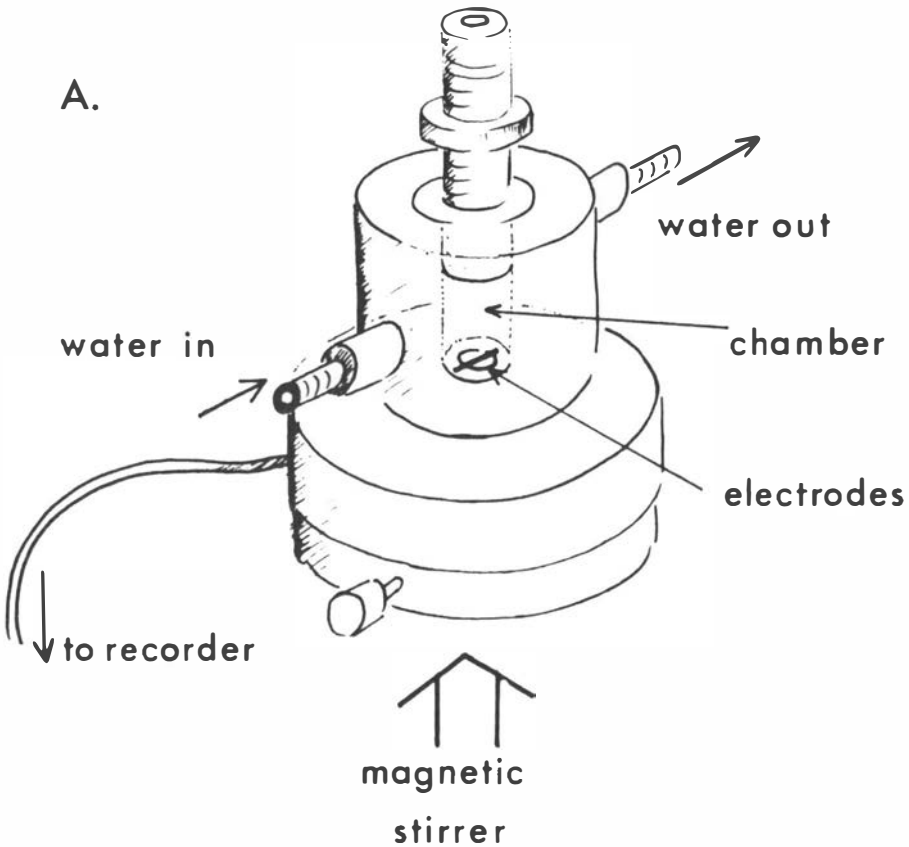
Facing page 92

FIGURE 6.1 THE RANK OXYGEN ELECTRODE

A. Diagram showing main features of the apparatus

B. Diagram showing the electro-chemical reactions that occur when the apparatus is in use (adapted from a figure in Delieu and Walker, 1972).

The Rank Oxygen Electrode



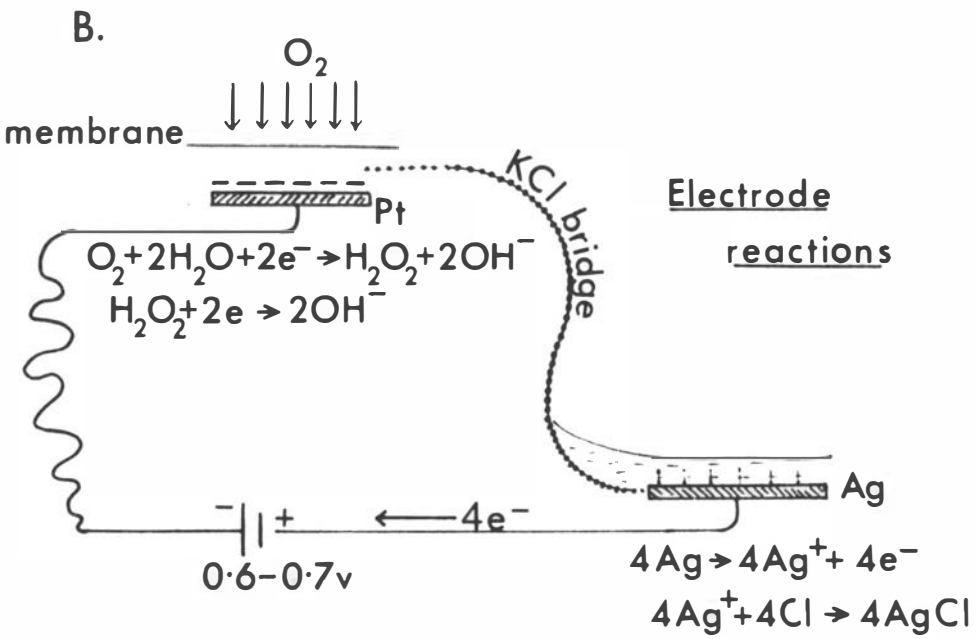


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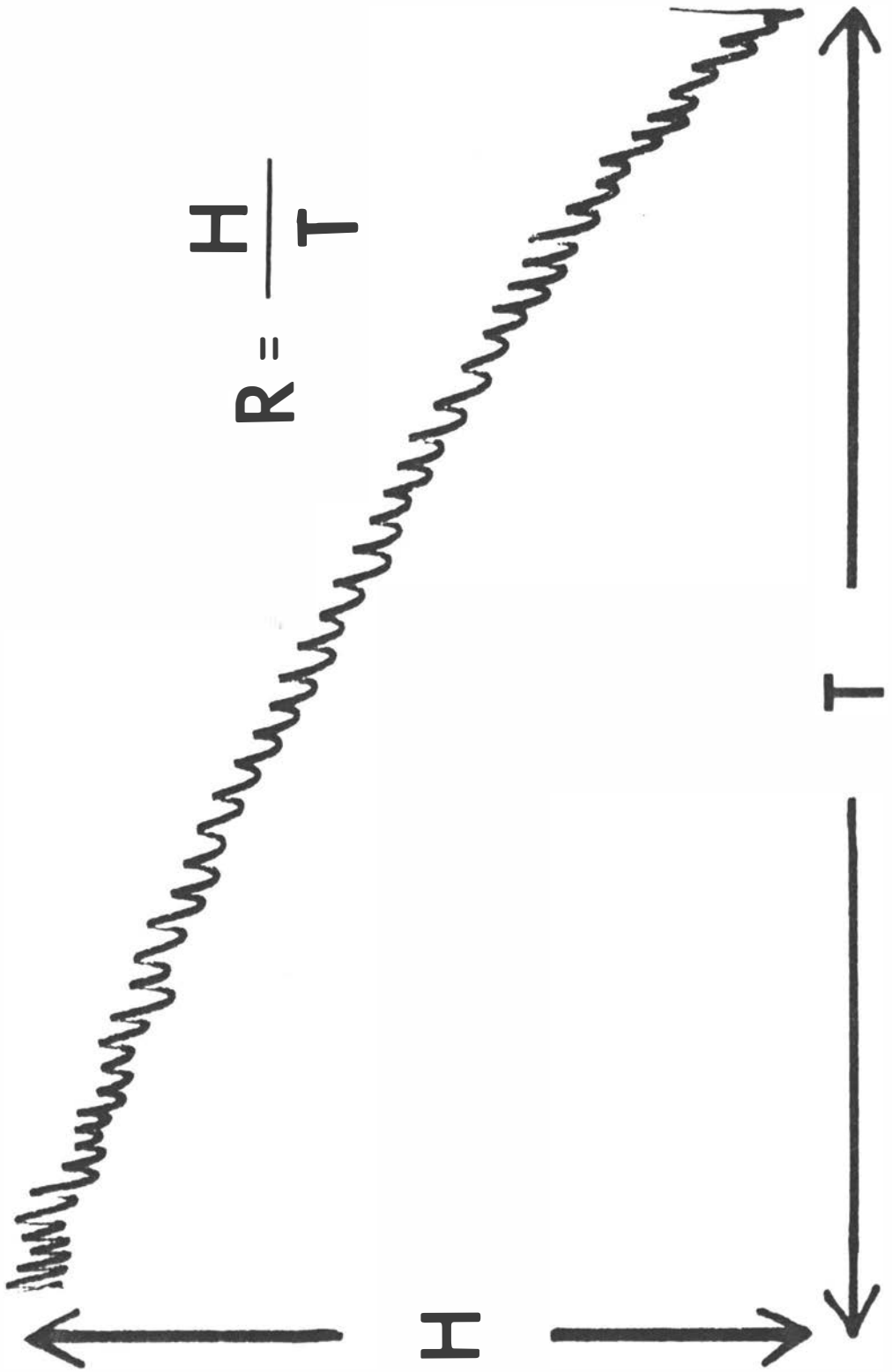
FIGURE 6.2 CALCULATION OF THE OXYGEN UPTAKE RATE

Diagram showing how the rate of oxygen uptake (R) was calculated from a typical trace.

H = lateral movement of the recorder pen

T = longitudinal movement of the recorder pen with the chart moving at a constant rate


R = H/T
H
T

The oxygen uptake rate was calculated from $R = \frac{H}{T}$ where H is the lateral and T the longitudinal movement of the trace (see Figure 6.2).

iii) Preparation of sarcocysts

Macrocysts of the cat:sheep 1 sp. were removed, within 3 hours of death, from the oesophagi of ewes slaughtered at a local meat works, washed three times in physiological saline and stored in saline at 4°C for up to 6 hours before testing. In some experiments macrocysts were left *in situ* and treatments applied to whole infected oesophagi.

iv) Heating

One gram samples of macrocysts were placed in 5 ml volumes of physiological saline in glass tubes and held at temperatures of 47.5, 50.0, 52.5, 55.0, 57.5, and 60.0°C for 5, 10, 15, 20, 30, 40 and 60 minutes in a water bath. The cysts were then removed from the saline and, without delay, placed in the oxygen electrode chamber and the oxygen uptake recorded. Five replicate samples were tested at each temperature and time.

v) Storage at ambient and below ambient temperatures

One gram samples of cysts were held in 5 ml physiological saline in glass tubes plugged with cotton wool at 4 and 10°C, and RT for 6, 13, 20 and 27 days, then examined for oxygen uptake. Two replicate samples were tested at each temperature and time. Whole infected oesophagi were held in glass jars at the same temperatures and cysts were removed weighed and tested at the same times as above.

vi) Freezing

Infected oesophagi in glass jars, were held at -14°C and the cysts tested at monthly intervals for 7 months. On the

day of examination, oesophagi were thawed at RT for 2 hours, cysts were dissected out, weighed and two replicate samples tested.

vii) Control samples

Five one gram samples of cysts were boiled for 2 minutes and tested for oxygen uptake.

b. Cat feeding studies

i) Experimental animals

Kittens, bred from caged cats fed solely on processed foods, were shown to be free of infection with *Sarcocystis* by checking their faeces for sporocysts (see Apendix 4a (i) and (ii)) for 2 weeks before the experiment.

ii) Experimental procedure

Three 5g samples of macrocysts, obtained and prepared as above, were heated at each of the temperatures and times shown in Table 6.4a. Three kittens each received a 5g sample of treated cysts incorporated in food and were placed together in a single cage. A second group of three kittens was left undosed as controls. All the faeces from each cage were examined for sporocysts (see Appendix 4a (i) and (ii)) daily for three weeks. Treated kittens that produced no sporocysts were killed on day 22 and the small intestine examined for sporocysts by the method given in Appendix 5d.

Infected oesophagi, maintained at -14^{o}C as above, were thawed at the times shown in Table 6.4b. Three kittens maintained in the same cage received 5g samples of thawed cysts; three other kittens were left undosed as controls. All the kittens were tested for sporocysts as before.

°C		exposure time 5 min	10	15	20	30	40	60
47.5	range	0.41-0.95	0.49-0.96	0.56-0.95	0.61-0.90	0.61-0.98	0.83-0.95	0.86-1.06
	mean	0.68	0.65	0.72	0.77	0.81	0.88	0.91
50.0	range	0.52-0.89	0.56-0.93	0.56-1.07	0.64-1.04	0.84-1.11	0.73-1.13	0.68-1.16
	mean	0.72	0.75	0.81	0.85	0.97	0.98	0.92
52.5	range	0.54-1.06	0.84-1.27	0.83-1.22	0.70-1.05	0.68-1.01	0.70-0.85	0.31-0.35
	mean	0.77	1.00	1.03	0.86	0.83	0.78	0.33
55.0	range	0.75-1.03	0.37-0.79	0.17-0.45	0.23-0.40	0.20-0.33	0.26-0.33	0.23-0.35
	mean	0.90	0.56	0.30	0.30	0.28	0.30	0.28
57.5	range	0.27-0.52	0.23-0.43	0.18-0.44	0.20-0.29	0.15-0.25	0.13-0.20	0.10-0.17
	mean	0.37	0.31	0.31	0.24	0.21	0.17	0.14
60.0	range	0.20-0.40	0.17-0.30	0.09-0.21	0.13-0.29	0.10-0.24	0.06-0.16	0.09-0.16
	mean	0.30	0.25	0.14	0.22	0.18	0.13	0.14

TABLE 6.1

OXYGEN UPTAKE RATES; HEATED CYSTS, FIVE REPLICATES

FIGURE 6.3 THE EFFECT OF HEAT ON THE OXYGEN UPTAKE RATE OF SHEEP OESOPHAGEAL MACROCYSTS

(Rate = mean oxygen uptake rate of 5 replicate samples of cysts; Time = period of exposure to test temperature).

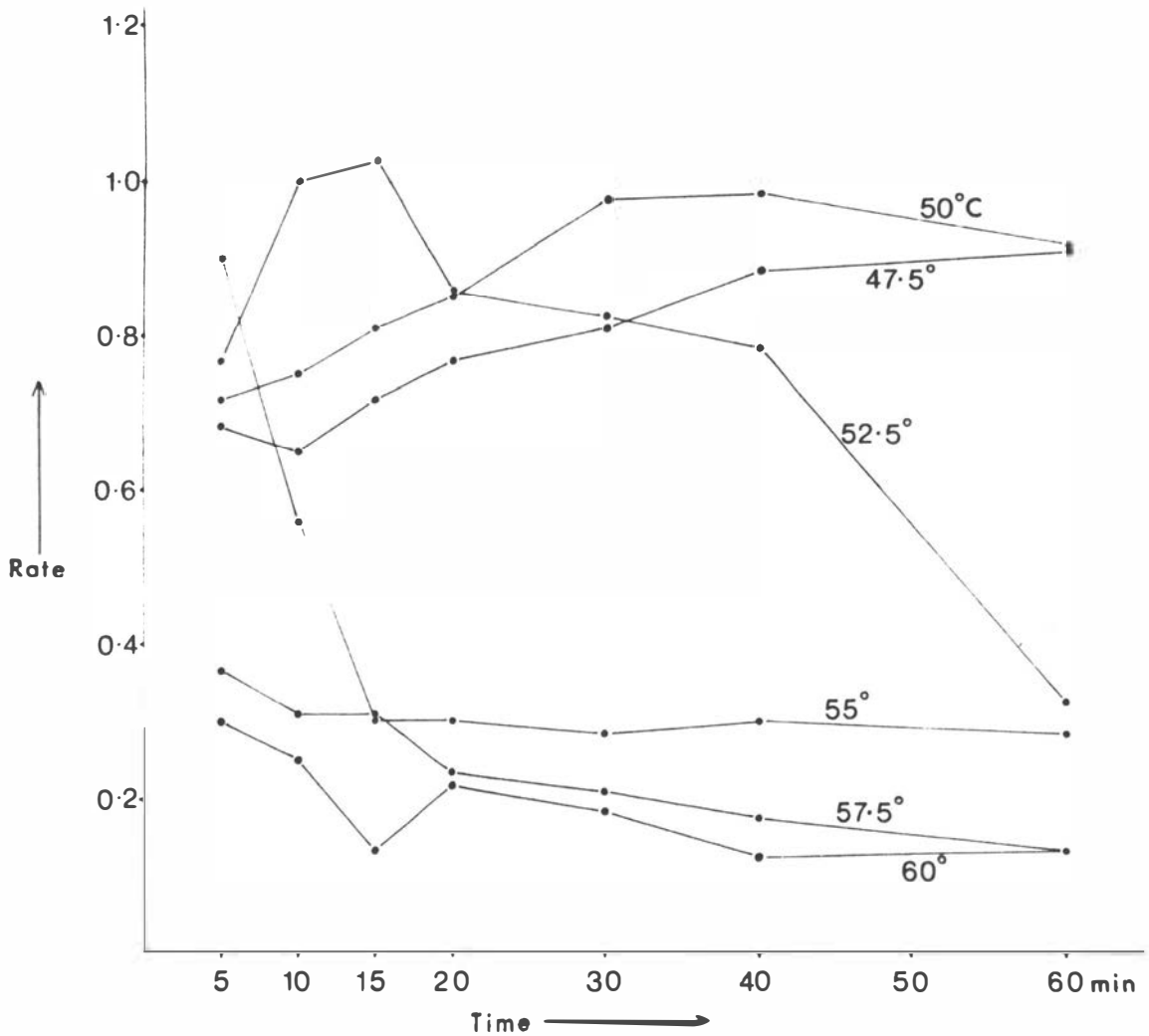

1·2
1·0
0·8
0·6
0·4
0·2
Rate
50°C
47·5°
52·5°
55°
57·5°
60°
5
10
15
20
30
40
50
60 min
Time

TABLE 6.2

OXYGEN UPTAKE OF SHEEP OESOPHAGEAL MACROCYSTS STORED AT AMBIENT AND BELOW AMBIENT TEMPERATURES

	6 days	13	20	27
RT	minimal	minimal	not done	not done
10°C	strong	strong	minimal	minimal
4°C	strong	strong	strong	minimal

a. Cysts in saline

	6 days	13	20	27
RT	autolysed not done	not done	not done	not done
10°C	strong	strong	autolysed not done	not done
4°C	strong	strong	strong	autolysed not done

b. Cysts in oesophagi

The range of temperatures and times tested by cat feeding was limited by the number of cats available for study.

6.3 Results

a. Oxygen electrode studies

The oxygen uptake rates of 210 samples of heat-treated cysts are recorded in Table 6.1 and Figure 6.3. At 47.5°C the longer the cysts were treated, the greater was the rate of oxygen uptake. Cysts treated at 50°C also showed an increase in rate with time but a small decline occurred after 30 minutes. At 52.5°C the rate rose rapidly to a peak at 15 minutes then declined to a low at 60 minutes. Cysts treated at 55°C showed a high oxygen uptake rate only at 5 minutes thereafter the rate declined rapidly to a level that was comparable with that seen in cysts treated at 57.5 and 60.0°C.

Cysts held at room temperature, in saline or oesophagi, disintegrated in less than a week. At lower temperatures cysts maintained their integrity longer, but all had decomposed by 27 days. Since it was found impossible to transfer all of each sample of cysts to the electrode or remove intact cysts from autolysing muscle, samples were not of equal weight. Hence it was not possible to compare rates of oxygen uptake but only to record the uptake of oxygen as 'strong' - oxygen rapidly taken up by the organisms in the electrode chamber, or 'minimal' - little or no oxygen taken up. Cysts stored in saline at room temperature showed minimal oxygen uptake at 6 days, whilst at 10°C the rate of uptake declined after 13 days and at 4°C after 20 days (see Table 6.2a). Oesophagi kept at room temperature autolysed quickly so that it was impossible to remove cysts for testing at 6 days. However cysts were still intact after 13 days at 10°C and

TABLE 6.3

OXYGEN UPTAKE RATES OF SHEEP OESOPHAGEAL MACROCYSTS STORED AT -14°C

Storage time	1 mth	2	3	4	5	6	7
Sample 1	0.53	0.44	0.35	0.48	0.41	0.42	0.38
Sample 2	0.48	0.54	0.45	0.40	0.50	0.49	0.32
Mean rate	0.51	0.49	0.40	0.44	0.45	0.46	0.35

after 20 days at 4°C and these cysts took up oxygen vigorously (see Table 6.2b).

Oxygen uptake rates of cysts that had been held at -14°C were intermediate between those of cysts heated to 47.5 and 57.5°C and declined slowly over 7 months (see Table 6.3).

The control cysts, after boiling for 2 minutes, had a mean oxygen uptake rate of 0.04.

b. Cat feeding studies

The results of feeding treated cysts to kittens are given in Table 6.4a and b. Treatment at 60°C for 10 minutes or 55°C for 20 minutes rendered cysts non-infective. Cysts heated at 52.5°C for 10 minutes or stored frozen for 60 days were still infective for kittens. The control kittens remained negative throughout the experiment.

6.4 Discussion

The results of the heating experiments are best explained in terms of change in the metabolic rate of the parasite. Metabolism was apparently stimulated by temperatures a little above normal body temperature (47.5°C), the effect increasing with time (see Figure 6.3). At higher temperatures stimulation was more marked but presumably the rate of oxygen uptake fell when metabolites were exhausted. At 57.5 and 60.0°C there was, apparently, a failure of metabolism, probably resulting directly from damage to cell proteins. Cysts treated at these temperatures took up oxygen at a low constant rate that probably indicates cell death. This assumption is supported by the finding that cysts heated at 55 and 60°C for 20 minutes were not infective for kittens. The difference in rate of oxygen uptake between cysts treated in this way and the control cysts is probably due to oxidative processes that took place in the cytoplasm of the dead treated cells

even in the absence of functional metabolic enzymes.

Cysts that were capable of utilising oxygen at 50 and 52.5°C were also capable of infecting kittens; exposure to a temperature of 17 degrees higher than physiologically normal appeared to have no effect on viability. These findings are comparable with those of other workers (Gestrich and Heydorn, 1974; Fayer, 1975; Leek and Fayer, 1978b) although their studies were of limited value as treatments were applied to sarcocysts enclosed in beef obtained at various times after slaughter. The same workers all reported that *Sarcocystis* in meat was killed by freezing, whereas these studies show that infectivity of the cat:sheep 1 sp. is retained for two months at -14°C and probably longer if the ability to take up oxygen equates with viability. The life of sarcocysts is thus prolonged by freezing although not for as long as 516 days (see Table 6.4b). Low non-freezing temperatures also prolong survival of the parasites: cysts stored at 4°C for 3 weeks still metabolized vigorously. The survival time of bradyzoites in meat may be considerably longer than is indicated by the results of these experiments. Even after cysts had completely disintegrated in decomposing muscle it was still possible to find apparently intact bradyzoites.

The results of these studies show that, in cool climates, carcases of sheep will remain infective for cats for several weeks and that the period of infectivity is extended by freezing or near freezing temperatures. Where mutton is fed to cats it can be recommended that if all parts of the meat are exposed to 60°C or more for at least 20 minutes this will render it non-infective.

TABLE 6.4

THE RESULTS OF FEEDING HEAT TREATED OR FROZEN SHEEP OESOPHAGEAL MACROCYSTS TO CATS

	10 min.	20 min.
47.5°C	+	+
50.0	not done	+
52.5	+	not done
55.0	not done	-
60.0	-	-

a. Heat-treated cysts

	16 days	60 days	516 days
-14°C	+	+	-

b. Frozen cysts

+ = Sporocysts found in faeces
- = Sporocysts absent from faeces and small intestine

CHAPTER 7

THE PREVALENCE AND TRANSMISSION OF *SARCOCYSTIS* SPECIES IN WILD AND FERAL ANIMALS

7.1 Introduction

Sarcosporidia have been found in the muscles of many animals and birds throughout the world (Kalyakin and Zasukhin, 1975). Whilst the taxonomic identity of many of these forms is still uncertain, the life cycles of nearly 30 distinct species have been fully or partially demonstrated (see Appendix 1 table 1). Wherever permanent associations of carnivores and ground-feeding herbivores or omnivores exist there are likely to be species of *Sarcocystis* cycling between them.

New Zealand is unique in that, apart from the native bats, all the land mammals present were introduced. Hence all the *Sarcocystis* species found here must have been introduced along with both their intermediate and definitive hosts. There are only a few carnivorous vertebrate species in this country: the domestic dog *(Canis familiaris)*, domestic /feral cat *(Felis catus)*, stoat *(Mustela erminea)*, weasel *(M. nivalis)* and ferret *(Putorius putorius furo)*. Other mammals such as the hedgehog *(Erinaceus europeus)* and the feral pig *(Sus scrofa)* are also occasional eaters of carrion. Birds such as the New Zealand Falcon *(Falco novaeseelandiae)* or the Australian Harrier *(Circus approximans)* might also be considered as potential definitive hosts in cycles yet to be investigated.

No survey of the prevalence or distribution of *Sarcocystis* spp. in wild or feral animals has ever been carried out in New Zealand, although sarcocysts have been observed during routine histological examinations. Information on the prevalence and transmission of

Sarcocystis species in wild and feral animals could be of importance in understanding the epidemiology of sarcocystosis in domesticated animals since the same definitive hosts are likely to be involved.

7.2 Materials and methods

a. Examination of muscle

i) Collection of samples

Samples of muscle were obtained from the carcases of wild and feral animals (see Table 7.1). Approximately 50g of muscle were removed from the larger specimens, lesser amounts from rats and mice. Where possible, the samples comprised muscle taken from several sites in the carcase of each animal, but sometimes circumstances restricted sampling to one site only. Mouse samples consisted of all the muscle that could be removed from both hind legs, the pelvis and abdomen.

ii) Digestion procedure

A 20g subsample (2g for rats and mice) of muscle was chopped into pieces less than 5mm thick and digested for 2 hours at 40^{o}C in a pepsin:HCl digest medium (Seneviratna, Edward and De Giusti, 1975). The medium comprised:

pepsin	1.3g
sodium chloride	2.5g
hydrochloric acid (conc.)	3.5ml
distilled water to	500ml

The muscle digest was first sieved (mesh aperture 0.5mm) to remove large particles, then centrifuged at 2000g for 5 minutes. The supernatant was discarded and the deposit resuspended in 2ml of physiological saline. 0.25ml of the suspension was examined systematically at 400 x magnification

for ten minutes, or less if the sample was found to be positive. Bradyzoites of *Sarcocystis* spp. are recognised by their distinctive banana-like shape, smooth walls and translucent contents.

iii) Histology

A second sub-sample of muscle was fixed in 10% formol saline embedded in paraffin wax and two 6 µm sections, cut approximately 100 µm apart and stained with haematoxylin and eosin, were examined for sarcocysts.

b. Transmission studies: intermediate to definitive host

i) Experimental animals

Newly weaned kittens and puppies were shown to be free of infection with *Sarcocystis* species, by examining their faeces daily for two weeks prior to the experiment (see Appendix 4a (i) and (ii)).

ii) Experimental procedure

Muscle from three host species (see Table 7.2), shown to be infected by pepsin:HCl digest, and stored at 4^{o}C, was fed to kittens and puppies within 48 hours of collection. The infected muscle was chopped, mixed with tinned food and fed *ad libitum* during the 6 hours following a 24 hour fast. One undosed animal served as a control for each experimental feeding. All the faeces of the dosed and control animals were examined for sporocysts daily for three weeks after the experimental feeding (see Appendix 4a (i) and (ii)). Samples of sporocysts produced were measured as described in Appendix 4c.

c. Transmission to intermediate hosts

i) Experimental animals

Conventional laboratory rats and rabbits reared and maintained on processed foods were used; *Sarcocystis* species have never been found in stock obtained from this source.

ii) Sporocysts

Sporocysts extracted from the faeces of cats (see Appendix 4a (i), (ii) and (iii)) were stored for up to 2 weeks in tap water at 4°C until used.

iii) Experimental procedure

Rats and rabbits were given various doses of the appropriate sporocysts (see Table 7.3) in 2ml water by stomach tube on Day 0. Two rats and two rabbits were left undosed as controls. The experimental animals were examined daily for obvious signs of illness. Dosed animals were killed at intervals (see Table 7.4) and samples of muscle from heart, diaphragm, oesophagus, tongue, abdomen and thigh were examined by *in vitro* digestion (for method see above) and histology (see above). The control animals were killed and examined on day 159.

7.3 Results

a. Examination of muscle

The results of the survey are summarised in Table 7.1. Infection was detected more frequently by muscle digest than by histology; the muscle digest techinque detected all infection found by histology. However, no direct comparison of the efficiency of the two methods was made as the sample sizes were not comparable. The prevalence recorded

in Table 7.1 is that derived from muscle digestion.

Sarcocystis species were found in goats, red deer, pigs, rats, mice, and rabbits. The infection in goats, red deer, pigs and rabbits were microcystic; macrocysts (see Section 2.4d) were present in rats and mice.

b. Transmission to definitive hosts

A *Sarcocystis* species found in feral goats was transmitted to dogs and species in rabbits and rats, to cats (see Table 7.2). The dimensions of the sporocysts produced are given in Table 7.3. No control kittens or puppies produced sporocysts.

c. Transmission to intermediate hosts

No *Sarcocystis* infection was found by muscle digest or histology in either dosed or control animals (see Table 7.4). No animals showed signs of illness during the period of the studies.

7.4 Discussion

These studies revealed that *Sarcocystis* species are present in several wild and feral mammals in New Zealand and that at least three of these species are transmitted by cats or dogs. However, the value of the prevalence data is limited; only relatively small numbers of animals from a few areas were examined. Wide differences in prevalence between areas are possible, the variation depending on the degree of association between intermediate and definitive hosts. In addition, the small proportion of the total musculature of each animal that was examined must have resulted in the actual prevalences being underestimated.

The samples of goat muscle were obtained from Hawke's Bay, an area where feral goats are frequently found grazing farmland with sheep. The high prevalence in goats could result from farmers using

TABLE 7.1

SARCOCYSTIS SPP. IN WILD AND FERAL ANIMALS; PREVALENCE

Species examined	Number examined	Prevalence %	Origin of animals
Goat (feral) *Capra hircus*	60	28	Farmland, Hawkes Bay area
Red deer *Cervus elaphus*	50	30	Rotorua area
Pig (feral) *Sus scrofa*	50	10	Various areas of North Island
Rat *Rattus norvegicus*	50 (40 / 10)	84 (100 / 20)	Rubbish dumps, Palmerston North area Bush areas in farmland Palmerston North area
Mouse *Mus musculus*	50	8	Farmland, Palmerston North area
Rabbit *Oryctolagus cuniculus*	50	16	Farmland, Palmerston North area
Opossum *Trichosurus vulpecula*	62	0	Bush, Hawkes Bay area
Scrub wallaby *Wallabia rufogrisea*	8	0	Waimate, South Island

TABLE 7.2

SARCOCYSTIS SPP. IN WILD AND FERAL ANIMALS; TRANSMISSION

Intermediate host	Infected muscle fed to No. of dogs	No. of cats	Results
Goat *C. hircus*	4		Sporocysts produced from day 9
		2	-ve
Rabbit *O. cuniculus*	3		-ve
		3	Sporocysts produced from day 14
Rat *R. norvegicus*	3		-ve
		4	Sporocysts produced from day 11

Intermediate host	Definitive host	Sporocyst dimensions range (µm)	mean (µm) and s.d.	n=
Goat *Capra hircus*	dog *Canis familiaris*	12.50-15.00x8.00-10.00	13.46±0.69x9.25±0.55	25
Rabbit *Oryctolagus cuniculus*	cat *Felis catus*	11.25-13.00x8.75-10.00	12.5±0.31x9.29±0.45	40
Rat *Rattus norvegicus*	cat *F. catus*	10.00-11.75x7.5-9.0	10.59±-0.52x7.87±0.41	25

TABLE 7.3

SARCOCYSTIS SPP. IN WILD AND FERAL ANIMALS; SPOROCYST DIMENSIONS

raw goat meat as food for working dogs, despite it being illegal to do so (see The Hydatids Act, 1968, amended 1972). Also, even if infected goat meat is frozen and stored at -10°C for 7 or more days to kill any *Cysticercus ovis* that may be present as is recommended in the Hydatids legislation, the meat may still harbour viable sarcocysts (see Chapter 6). Further studies of the dog:goat sp. are described in Chapter 9.

Sarcocystis species have been reported in red deer in other parts of the world (Drost and Graubman, 1975; Kalyakin and Zasukhin, 1975) but not transmitted to a definitive host. Hudkins and Kistner (1977) reported transmission of a species in mule deer *(Odocoileus hemionus hemionus)* to coyotes *(Canis latrans)*. Transmission of the species found in this country was not attempted. As there are no large carnivores here, transmission is probably by way of feral cats or the dogs of hunters and reiies on scavenging rather than predation.

Three species of *Sarcocystis* have been found in pigs overseas, with man, dog and cat as the respective definitive hosts (see Appendix 1 table 1). The source (or sources) of the infection found in feral pigs in this country has yet to be demonstrated. It is likely that at least two species are present; the form found in feral pigs in the survey was microcystic but macrocysts have been seen in this species on other occasions (Collins, unpubl. obs.).

The higher prevalence of infection in rats from rubbish dumps, compared with that in the smaller sample from farmland, is probably the result of a closer association between cats and rats. All rubbish dumps in the Palmerston North area support large populations of both hosts (Collins, unpubl. obs.). It is not easy to understand why cat: rat sp. sporocysts failed to infect laboratory rats. Ashford (1978) reported transmission of a cat:rat sp. to rats; this may have been the same species as was found in the survey as the sporocysts he described

TABLE 7.4

TRANSMISSION STUDIES IN RATS AND RABBITS

Experimental animal	*Sarcocystis* species	dose of sporocysts	animals killed, days after infection	result
Rat 1	cat:rat	5×10^3	88	-ve
2	"	10×10^3	112	-ve
3	"	5×10^3	159	-ve
4	"	10×10^3	159	-ve
Rabbit 1	cat:rabbit	3×10^4	46	-ve
2	"	2×10^4	61	-ve
3	"	2×10^4	75	-ve
4	"	6×10^4	90	-ve
5	"	6×10^4	110	-ve
6	"	6×10^4	140	-ve

had similar dimensions. Transmission of the species found in New Zealand needs further study not only to determine the conditions in which laboratory rats can be infected with sporocysts from cats but also to see whether or not this species is infective for the other two rat species present in this country, *R. rattus* and *R. exulans*.

The finding of a species of *Sarcocystis* in mice had been anticipated: McKenna (1978) found sporocysts in the faeces of 16.9 per cent of 508 cats and pooled sporocysts gave rise to sarcocysts in laboratory mice. The life cycle of a cat:mouse sp. found in the USA (Ruiz and Frenkel, 1976) resembles that of the New Zealand species (McKenna, pers. comm.).

The rabbits examined in the survey came from farmland reported to also harbour feral cats (Costello, pers. comm.). The association between cats and rabbits is well established in New Zealand; rabbits commonly form the major part of the diet of feral cats (Collins and Charleston, 1979) (see Chapter 8). The relatively low prevalence in rabbits found in the survey could be ascribed to cats preying mainly on young rabbits (Gibb, Ward and Ward, 1969), that are less likely to harbour mature sarcocysts. Munday *et al.* (1978) in a survey of *Sarcocystis* and related species in Australian mammals, found by histology that 21.1% of 109 rabbits were infected with sarcocysts. Once again it is not known why cat:rabbit sp. sporocysts failed to give rise to sarcocysts in laboratory rabbits. A species found in the American cottontail rabbit *(Sylvilagus floridanus)* was transmitted to cats (Crum and Prestwood, 1977; Fayer and Kradel, 1977) but could not be further transmitted to European rabbits (Fayer and Kradel, 1977). The sporocysts of this species had slightly different dimensions from those given above (see Appendix 2 table 2).

The failure to find infection in two marsupial species is not surprising. It is unlikely, given the host specificity of *Sarcocystis* species and the lack of marsupial carnivores in this country, that any wild marsupials in New Zealand are infected. Despite the small sample examined, the result probably reflects the true situation. An Australian survey (Munday *et al.*, 1978) failed to find *Sarcocystis* spp. in 155 opossums *(T. vulpecula)*; one animal had an unidentified cyst in an extrinsic eye muscle.

CHAPTER 8

THE FERAL CAT AND *SARCOCYSTIS*

8.1 Introduction

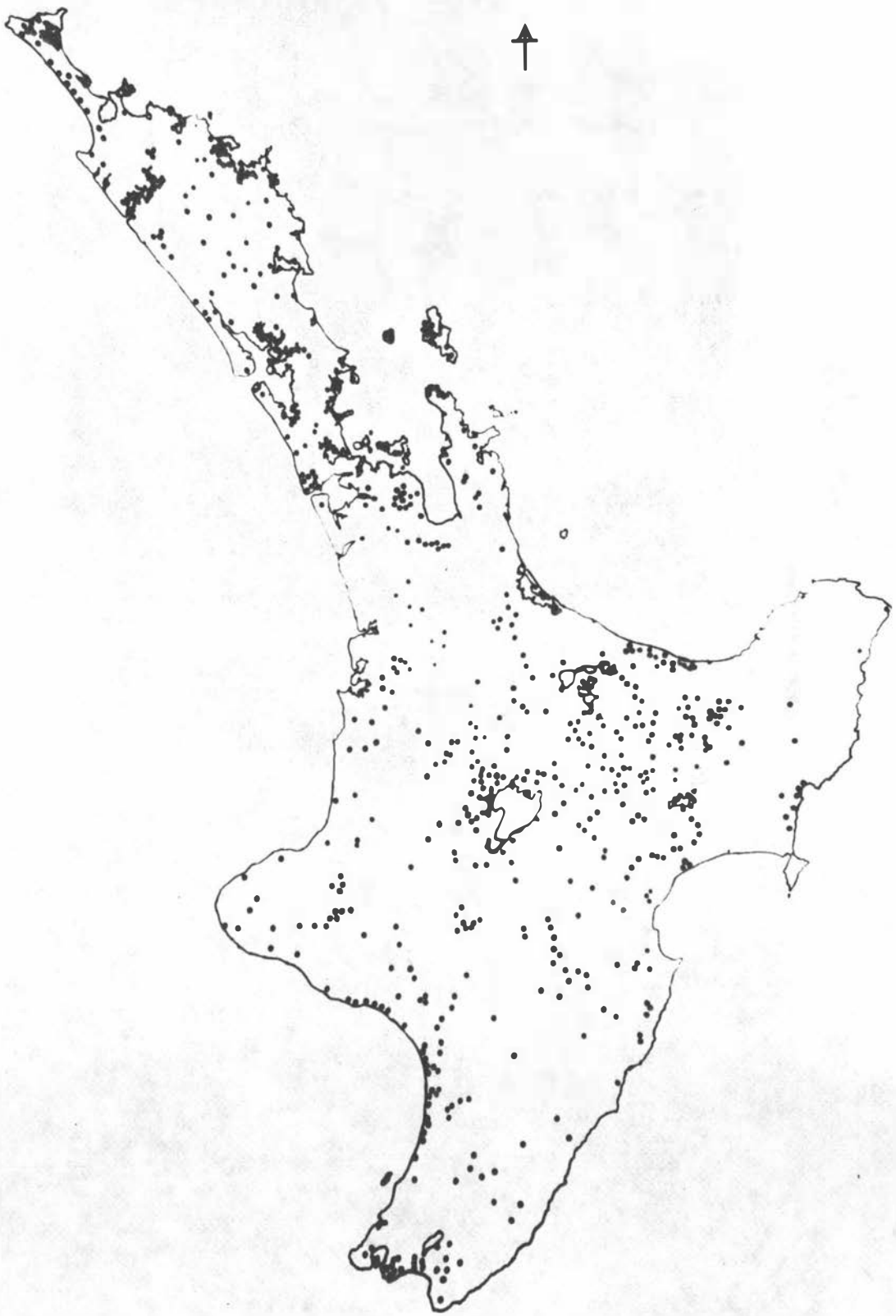
The domestic cat, *Felis catus*, is involved in more *Sarcocystis* life cycles than any other carnivore, (see Appendix 1 table 1). Given the widespread distribution of felids, it is likely that elucidation of further life cycles will not change this situation. In life cycles involving the cat, the intermediate host must form part of its diet and the prevalence of infection in the intermediate host will be, to some extent, a measure of the closeness of the association of the two hosts.

Infection of sheep with macrocystic species derived from cats, is very common in New Zealand (see Table 4.1) and it is doubtful whether any farming areas are free of it. Hence cats must be commonly associated with sheep and infected mutton an important item in their diet. Cats, both domestic and feral, are commonly found near houses and farms; the question is how important are feral cats in the transmission of infection to sheep in areas remote from human habitation?

Feral cats were reported as being numerous in South Auckland by 1843 (Dieffenbach, quoted by Wodzicki, 1950) in Canterbury by 1860 (Butler, quoted by Davidson 1965) and, later, wherever rabbits were abundant (McLean, 1966). Wodzicki (1950) carried out the only survey of feral cats. He found that "wild cats are fairly continuously distributed over both islands in varying degrees of density." However, since he obtained his information solely from stock inspectors, the survey did not cover the more remote areas of the country.

To record the distribution of feral cats over the whole country, paying particular attention to unpopulated areas, would be a monumental

FIGURE 8.1 FERAL CAT SURVEY; MAP OF SIGHTINGS OF FERAL CATS IN THE NORTH ISLAND OF NEW ZEALAND.



task. The alternative, given the resources available, was to question as many people as possible who frequent the more remote parts of the country and thereby establish whether or not feral cats are widespread. It was assumed that if cats were seen many kilometres from the nearest human habitation they were self-sufficient and hence justifiably termed 'feral'.

8.2 Materials and methods

Information on feral cats was collected by questionnaire and through correspondence. The questionnaire (see Appendix 5 figures 1 and 2) was divided into two parts and asked for

a. general observations on feeding and food, breeding and population trends of feral cats.

b. specific sightings of feral cats, with details of location, terrain and distance from human habitation.

It was sent through various government organisations (see Appendix 5 table 1) to a wide variety of potential observers many of whom were working regularly in areas remote from human habitation. Letters inviting submissions were placed in farming journals and magazines that cater for people whose leisure activities take them into unpopulated areas.

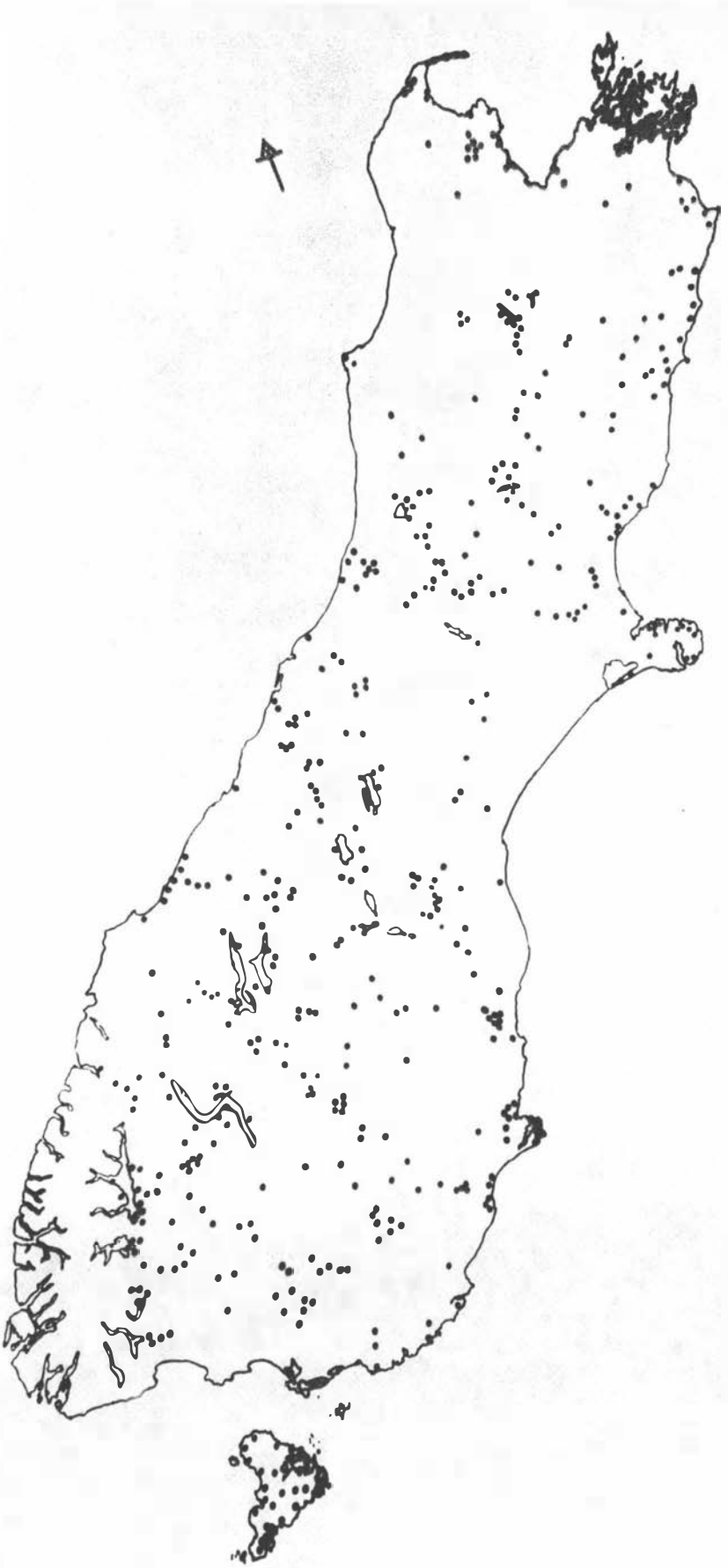
8.3 Results

a. General observations

Replies were received between September 1977 and August 1978, with the majority arriving in the first four months. Information was received from 409 correspondents with a wide variety of occupations (see Table 8.1) and recorded on punchcards. 54 of 409 (13.2 per cent) reported that they had not seen feral cats: 355 (86.8 per cent) reported seeing feral cats. 251 of the 355 (70.7 per cent) who had seen feral cats also reported that there was evidence of breeding occurring

Facing page 119

FIGURE 8.2 FERAL CAT SURVEY; MAP OF SIGHTINGS OF FERAL CATS IN THE SOUTH ISLAND OF NEW ZEALAND.



- kittens or pregnant females were seen, for example. 251 observers (not necessarily the same observers as before) reported cats feeding on a variety of foods (see Table 8.2).

114 of 355 observers (32.1 per cent) thought feral cats were "more common" and 36 (10.1 per cent) "less common" than previously. 205 observers (57.7 per cent) answered "don't know" or "static" or failed to complete this section.

b. Specific sightings

The locations of 950 sightings of feral cats were provided by observers and plotted on an outline map of New Zealand. As replies sometimes involved multiple sightings the following convention was adopted in plotting the locations:

a single sighting: one point on the map at the actual location.

several sightings in the same area: two adjacent points. Comments such as: "many cats seen in the area" or: "the area has a large cat population" were recorded as three adjacent points. A total of 1082 points were mapped (see Figures 8.1 and 8.2). Feral cats were seen in all categories of terrain (see Table 8.3).

In 309 of 950 (32.5 per cent) specific sightings there was evidence of breeding; this is correlated with terrain in Table 8.4. Over two thirds of all sightings were made more than 2 kilometres away from human habitation and nearly one third more than 10 kilometres away (see Table 8.5).

c. Replies from Agricultural Pest Destruction Boards

Observers working for Agricultural Pest Destruction Boards mostly replied in general terms and described the occurrence of feral cats according to board areas. Pest destruction officers are responsible for, and acquainted with, large areas of country; many of their duties

TABLE 8.1

FERAL CAT SURVEY; OBSERVERS - OCCUPATION/INTEREST

Occupation/Interest	Number	% Observers
Pest destruction shooting/trapping	62	15.2
Forestry	102	24.9
Wild life management	52	12.7
Farming	132	32.3
National Parks	15	3.7
Tramping	2	0.5
Other	44	10.8
TOTAL	409	100%

TABLE 8.2

TYPES OF FOOD SEEN EATEN BY FERAL CATS

Type of food eaten	Number of times food reported	% Total reports
Rabbit	115	22.0
Opossum	94	18.0
Sheep	87	16.6
Bird	76	14.5
Hare	45	8.6
'Human refuse'	24	4.6
Deer	19	3.6
Pig	11	2.1
Rat	10	1.9
Cattle	7	1.3
Fish	7	1.3
Goat	7	1.3
Mouse	7	1.3
Frog	3	0.6
Hedgehog	3	0.6
Insect	3	0.6
Cat, 'dead stock', lizard, 'placenta', wallaby	1 each	0.2
	523	

TABLE 8.3

SIGHTINGS OF FERAL CATS ACCORDING TO TYPES OF TERRAIN

Terrain	Number of sightings	% Sightings (950)
Grassland	341	35.9
Tussock	196	20.6
Scrub	371	39.1
Native bush/forest	432	45.5
Plantation	170	17.9

TABLE 8.4

SIGHTINGS OF FERAL CATS; BREEDING VERSUS TERRAIN

Terrain	Sightings with evidence of breeding	% Total sightings for terrain
Grassland	137/341	40.2
Tussock	75/196	38.3
Scrub	134/371	36.1
Bush	144/432	33.3
Plantation	76/170	44.7

are carried out at night. Hence because of their experience and the nature of their work their observations are worthy of special mention. Table 8.6 lists the board areas reported to have widespread and permanent populations of feral cats.

8.4 Discussion

Feral cats were sighted in many parts of the country and, as the sightings appear to be distributed randomly, it can be assumed that cats are likely to be present nearly everywhere. It would be unwise to draw any conclusions about the area density of cats from the distribution of sightings. The maps are probably more a record of the presence of interested observers than of cats, as may be deduced from the lack of sightings in Fiordland and East Cape. Given the natural shyness of feral cats and their mainly crepuscular activity, the number of sightings is likely to be a gross underestimate of the actual population.

Feral cats appear to be able to survive and breed in a wide variety of habitats. Many observers exceeded their brief with regard to the questions on terrain in the questionnaire and described habitats as diverse as coastal sand dunes, river beds, dense rainforest, sheep pasture and mountain country up to the snow line. Cats have been seen at 3000mabove sea level in the Avoca Valley, Canterbury (Clarke pers. comm.). Considering that many sightings occurred a long way from human habitation, and that breeding was reported from all types of terrain it is likely that breeding populations of feral cats are present in most parts of the country. Little value can be placed on the fact that more observers thought that feral cats were "more common": an even larger number were non-committal. The form of the question was such that only subjective answers based more on belief than knowledge

TABLE 8.5

SIGHTINGS OF FERAL CATS ACCORDING TO DISTANCE FROM HUMAN HABITATION

Distance	Number of sightings	% Sightings (950)
< 2 km	397	41.8
2-10 km	478	50.3
> 10 km	277	29.2
Unknown distance	11	1.2

could be elicited.

The number of sightings in an area is no measure of the actual population for the reasons discussed above. However, the results of two other studies help to show the likely density of cats on farmland. Scientists of the Ministry of Agriculture and Fisheries Rabbit Research Section are monitoring changes in populations of rabbits and other mammals in areas of North Canterbury. Set routes are covered by motor cycle and counts made on four successive nights, weather permitting, three or four times a year under standardised conditions. During 1977 and 1978, in 173 runs over a mean distance of 24.7km, a mean of 0.038 cats was seen*. Based on an estimated searched swath area of 6 to 8ha per kilometre covered, the cat density in these areas was calculated as 5 to 6 cats per 1000ha.

Agricultural Pest Destruction Board officers carried out a nine month survey of a 500,000 acre block in Hawke's Bay in 1969 and estimated that there were 3 cats per 1000 acres (7.4 per 1000ha). The results of these surveys are remarkably similar. If it is assumed that there are perhaps 8 cats per 1000ha evenly distributed throughout the country (268,000 km^2 approx) there are 214,400 cats present, or one cat per 250 sheep. Taking into account the large number of cats living in and near human habitation it is possible that in some areas the actual ratio is as high as 1 to 100.

There have been many studies of the diet of cats, domestic, feral and wild *(F. silvestris)*. Small mammals, especially rabbits, rats and mice, are preferred but a wide variety of other organisms may be eaten including lizards, frogs and insects (Coman and Brunner 1972; Condé *et al.*, 1972; Heidemann, 1973; Fitzgerald and Karl, 1979). Cats on Little Barrier Island, New Zealand, thrive on seabirds and rats

* Information supplied by Williams, J.M., Broad, T.M., Robson, D.L., Rabbit Research Station, M.A.F., Christchurch.

TABLE 8.6

AGRICULTURAL PEST DESTRUCTION BOARD AREAS
WITH EXTENSIVE PERMANENT CAT POPULATIONS

North Island	South Island
Mangonui Co.	Marlborough
Hokianga Co.	Kaikoura
Bay of Islands Co.	Amuri
Otamatea Co.	Selwyn
Coromandel Co.	Ashley
Waikato Co.	Plains
Te Puke	Banks Peninsula
Rotorua-Taupo	Ashburton
Wangamomona	Pukaki
Central King Country	Mackenzie
Hawera Co.	Upper Clutha
Western	Lindis Hawea
Taihape	Kurow
Hawke's Bay	Tokarahi
Ruahine	Papakaio
Manawatu	Otepopo
Wairarapa	Whakatipu
	Alexandra
	East Otago
	Mid Whakatipu
	Northern Southland
	Roxburgh
	Waipori
	Mount Wnedon
	Western Southland
	Mid Southland
	Southern Southland

(Marshall, 1961).

The results of this survey confirm that cats have a varied diet: they kill a variety of small prey species but are also undiscriminating scavengers capable of eating the remains of any animal. The size of the territory of a feral cat depends both on the availability of food and the presence of other cats. Young cats have to migrate when they are mature and when food is scarce adults leave their territories to look for new sources (Corbett, pers. comm.). Since cats are mobile it is inevitable that some of the carcases of sheep that die on farmland will be found and eaten, ensuring the transmission of the cat:sheep spp. of *Sarcocystis*.

CHAPTER 9

EXPERIMENTAL INFECTION OF GOATS WITH A DOG:GOAT SPECIES

9.1 Introduction

There are few records of *Sarcocystis* species in goats. Scott (1943 a, b) cites six authors as having reported caprine sarcosporidia. Kalyakin and Zasukihn (1975) list only two reports of infection in goats. The transmission of a microcystic form in feral goats to dogs (Collins and Crawford, 1978) (see Chapter 7) provided the opportunity to complete the life cycle of this parasite and to study its development and pathogenicity in goats. The dog:goat sp. was found to be especially suitable for the study of sarcocystosis in ruminants: both dogs and goats free of *Sarcocystis* were readily available and, as housing was available on a sheep farm many kilometres from the nearest feral goat population, the environment of the experimental goats was predictably free of dog:goat sp. sporocysts.

The aims of the investigations were to study the development of the dog:goat sp. in goats and to monitor any illness resulting from the ingestion of sporocysts.

9.2 Materials and methods

a. Experimental animals

Thirteen goats and four sheep were reared indoors from birth on calf milk replacer*. The animals were less than one month old at the start of the experiment.

b. Sporocysts

Sporocysts of the dog:goat sp. were harvested from the faeces

* 'ANCALF', N.Z. Cooperative Dairy Company, Palmerston North, N.Z.

of dogs, infected as described in Chapter 7, and were stored in tap water at 4°C for up to two weeks before use.

c. Experimental procedure

Eleven goats and four sheep were given sporocysts by stomach tube, in two separate experiments, in the doses shown in Table 9.1. In the first experiment three goats, designated 1, 2 and 3, were given large doses of sporocysts (see Table 9.1) and necropsied on days 18 and 19. In the second experiment, eight goats (numbers 4 to 11) and four sheep (numbers 1 to 4) were given various doses of sporocysts and necropsied at intervals (see Table 9.1.). Two control goats (numbers 12 and 13) were left undosed and kept in isolation for two weeks before being housed with the infected animals in a clean pen. Isolation was necessary to prevent the control goats becoming infected by sporocysts passed in the faeces of the dosed animals (see Chapter 5). In both experiments the rectal temperatures of all animals were recorded daily and the animals were checked twice daily for signs of illness.

d. Haematology

All the animals in the second experiment were bled on day 0 and then twice weekly up to day 45 or death, if earlier. Blood was collected into 10 ml vacuum tubes containing ethylenediaminetetraacetic acid (EDTA*) and examined by standard techniques for haemoglobin concentration (Hb), packed cell volume (PCV), and the mean corpuscular concentration (MCHC) was calculated; the total leucocyte count (WBC), differential leucocyte count (diff. WBC), total serum protein (TP), protein fractions and the levels of the serum enzymes:glutamic transaminase (SGOT) and lactic dehydrogenase (LDH) were estimated.

e. Serology

Goats 4 to 13 and sheep 1 to 4 were bled twice weekly up to day 63, or death, if earlier, and all surviving animals at day 129. Blood was

* Venoject, Terumo Corporation, Japan.

TABLE 9.1

EXPERIMENTAL INFECTION OF GOATS AND SHEEP WITH DOG:GOAT SP. SPOROCYSTS

Experimental animal	Dose of sporocysts	Days after infection	Summarised results
Goat 1	5×10^6	18 k	Schizonts in endothelial cells, all tissues. Many schizonts in renal glomeruli
2	5×10^6	19 d	As above
3	5×10^6	19 k	As above
4	6×10^5	34 k	Early sarcocysts comprising 1 to 5 metrocytes in muscle fibres. Single schizont in tongue.
5	6×10^5	24 d	3 schizonts in heart only
6	15×10^4	129 k	Sarcocysts in all muscles
7	15×10^4	129 k	As above
8	3×10^4	129 k	As above
9	3×10^4	80 k	Sarcocysts in all muscles
10	15×10^3	34 d	Occasional metrocytes in muscle fibres
11	15×10^3	93 k	Sarcocysts in all muscles
12	Not dosed	42 k	No infection in any tissues
13	Not dosed	143 b	No infection in abdominal or thigh muscles
Sheep 1	6×10^5	42 k	No infection in any tissues
2	6×10^5	129 k	As above
3	15×10^4	129 k	As above
4	15×10^4	129 k	As above

k = killed d = died b = biopsied

collected in 10 ml plain vacuum tubes and the serum stored at -14°C until tested. *Sarcocystis* antibodies were estimated by an IFAT (see Chapter 3 for method) using dog:goat sp. bradyzoites harvested by pepsin:HCl digestion (see Chapter 7 for method) from the muscle of goats 6, 7 and 8 killed on day 129.

f. Pathology

Necropsies were carried out on goats 1 and 12 and sheep 1 to 4 and samples of tissues prepared for histology. Tissue samples were removed from tongue, oesophagus, lung, heart, liver, spleen, kidneys, abomasum, small intestine (five samples taken at 1m intervals starting at the pylorus), caecum, colon, mesenteric and popliteal lymph nodes, brain, spinal cord, bone marrow and muscle of the diaphragm, abdominal wall and hind leg. Biopsy samples of muscle were removed, under general anaesthesia, from the abdominal wall and hind leg of control goat 13 on day 140. All tissues were fixed in 10 per cent formol saline, blocks were cut and embedded in paraffin wax and two 6µm sections, cut approximately 100 µm apart were stained with haematoxylin and eosin.

g. Life cycle stages

Schizonts present in the renal glomeruli of goats 1, 2 and 3 were studied in sections of kidney stained with haematoxylin and eosin. Schizonts were classed as 'mature' when they enclosed many separate nuclei and 'immature' when complete nuclear separation had not taken place. The numbers of mature and immature schizonts were counted in samples of 50 glomeruli cut in equatorial section from each goat.

Using sections of kidney from goat 3, samples of schizonts and of glomeruli were measured at 400 x magnification with a micrometer eyepiece and the total number of schizonts present in both kidneys estimated using the formula (Hendy, pers. comm.):

$$N = 2/3 \ \frac{R}{r} \ n$$

where N = mean number of schizonts/glomerulus

R = mean radius of a glomerulus cut in equatorial section

r = mean radius of a schizont cut at maximum diameter

n = mean number of schizonts/glomerulus cut in equatorial section.

The total number of schizonts in the glomeruli of two kidneys was estimated as:

$$2 \ (N \times 5 \times 10^5)$$

The figure of 5×10^5 for the total number of glomeruli in a goat kidney was obtained by extrapolation from figures for pig, ox and dog (Dukes, 1955).

Muscle sections from goats 4 and 10 and control goat 12 were stained with celestine blue:haematoxylin and eosin, a double nuclear stain, as this was found to be especially useful in the differentiation of young sarcocysts from muscle nuclei.

Using sections of striated muscle, cut longitudinally, from goat 4, an unselected sample of 100 young sarcocysts was examined and a mean number of metrocytes per sarcocyst calculated. A further unselected sample of 100 young sarcocysts were examined with a micrometer eyepiece at 1000 x magnification and classed as situated "adjacent to" (separated by less than 1 µm) or "not adjacent to" (separated by more than 1 µm) a nucleus, in an attempt to confirm, or otherwise the observation that young sarcocysts tended to lie in association with muscle nuclei.

Samples of muscle from goats 6, 7 and 8 were fixed in glutaraldehyde, post-fixed in osmium tetroxide, embedded in epoxy resin*, sectioned and the ultrastructure of the walls of sarcocysts present in the sections examined by electron microscopy (Griffin, 1972).

h. Muscle digest

Samples of muscle from the diaphragm, abdominal wall and hind leg of sheep 2, 3 and 4 killed on day 129 and biopsy samples from control goat 13 were examined for *Sarcocystis* bradyzoites by pepsin: HCl digestion (see Section 7.2 for method).

i. Dog feeding

In order to complete the life cycle and to determine whether or not sarcocysts were mature 129 days after infection, samples of muscle from goats 6, 7 and 8 were combined and fed to three dogs. One dog not fed goat meat served as a control. All the faeces were checked for sporocysts, daily for two weeks prior to feeding to ensure they were free of *Sarcocystis* infection and for four weeks after feeding (see Appendix 4a (i) and (ii)).

9.3 Results

a. Clinical observations

Goats 1, 2 and 3, given 5×10^6 sporocysts, showed no signs of illness until day 17 when they became lethargic and stopped eating. Goats 1 and 3 became recumbent and were killed on days 18 and 19 respectively; goat 2 was found dead on day 19. The temperatures of all three goats rose steadily from two days after dosing (see Figure 9.1). The mean daily temperature of goats 1, 2 and 3 showed a

* 'FLUKA', Chemische Fabrick, Buchs SG, Switzerland.

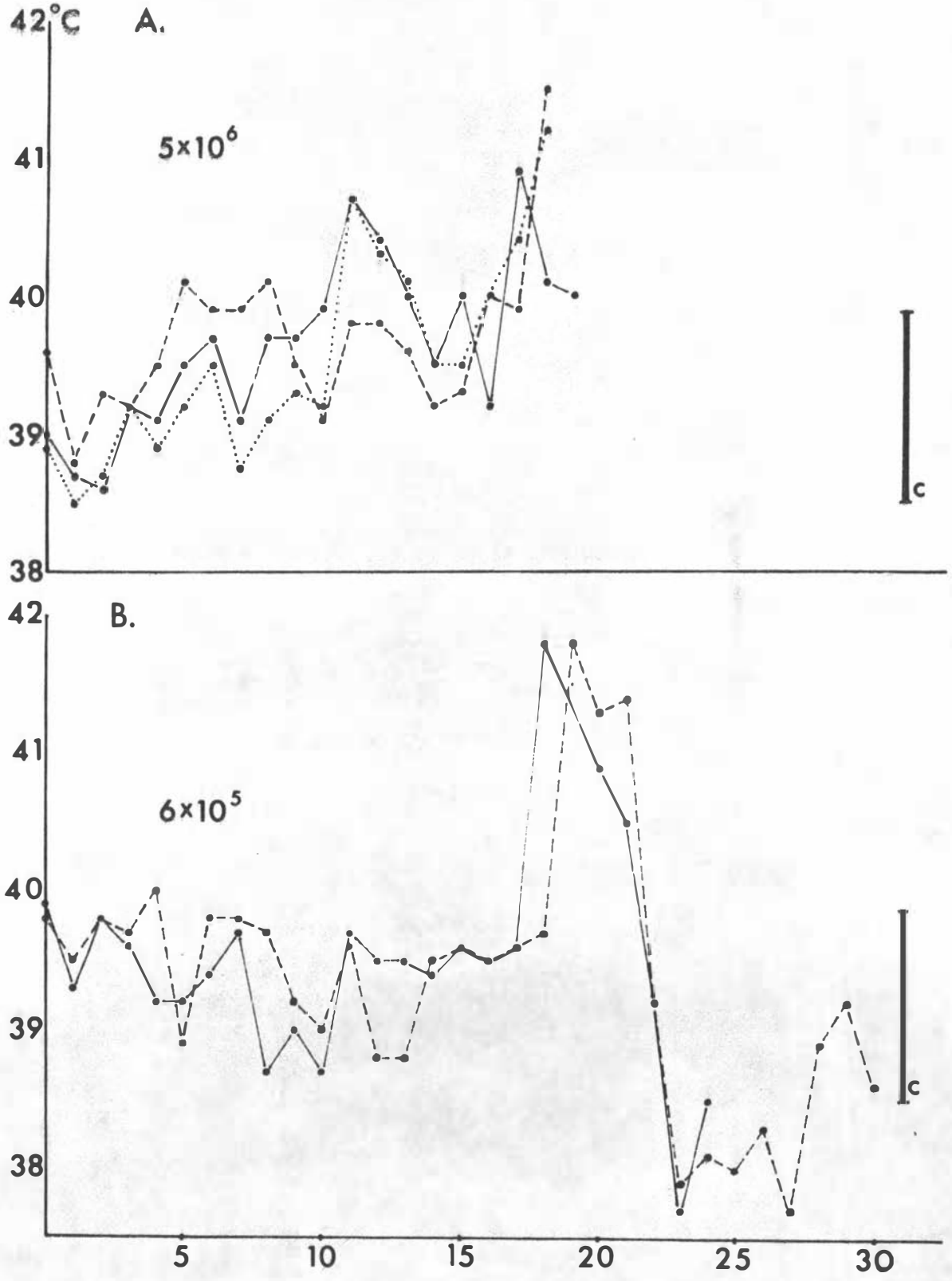
Facing page 136

FIGURE 9.1 RECTAL TEMPERATURES RECORDED IN GOATS GIVEN DOG:GOAT SP. SPOROCYSTS

(A: goats 1,2,3 given 5×10^6 sporocysts;

B: goats 4 and 5 given 6×10^5 sporocysts.

Bar c at right hand corner = mean temperature of control goats 12 and 13 ± 2 x SD.)


42°C
A.
5×10^6
41
40
39
38
C
B.
6×10^5
42
41
40
39
38
C
5
10
15
20
25
30
days after infection

Facing page 137

FIGURE 9.2 MEAN RECTAL TEMPERATURES OF GOATS GIVEN 5×10^6 DOG:GOAT SP. SPOROCYSTS (Arrows Indicate phases of schizogony; ? and ?? are phases proposed to coincide with the rises in temperature; u.sch. = ultimate schizogony. Bar c at right hand side = mean temperature of control goats 12 and 13 ± 2 x SD).

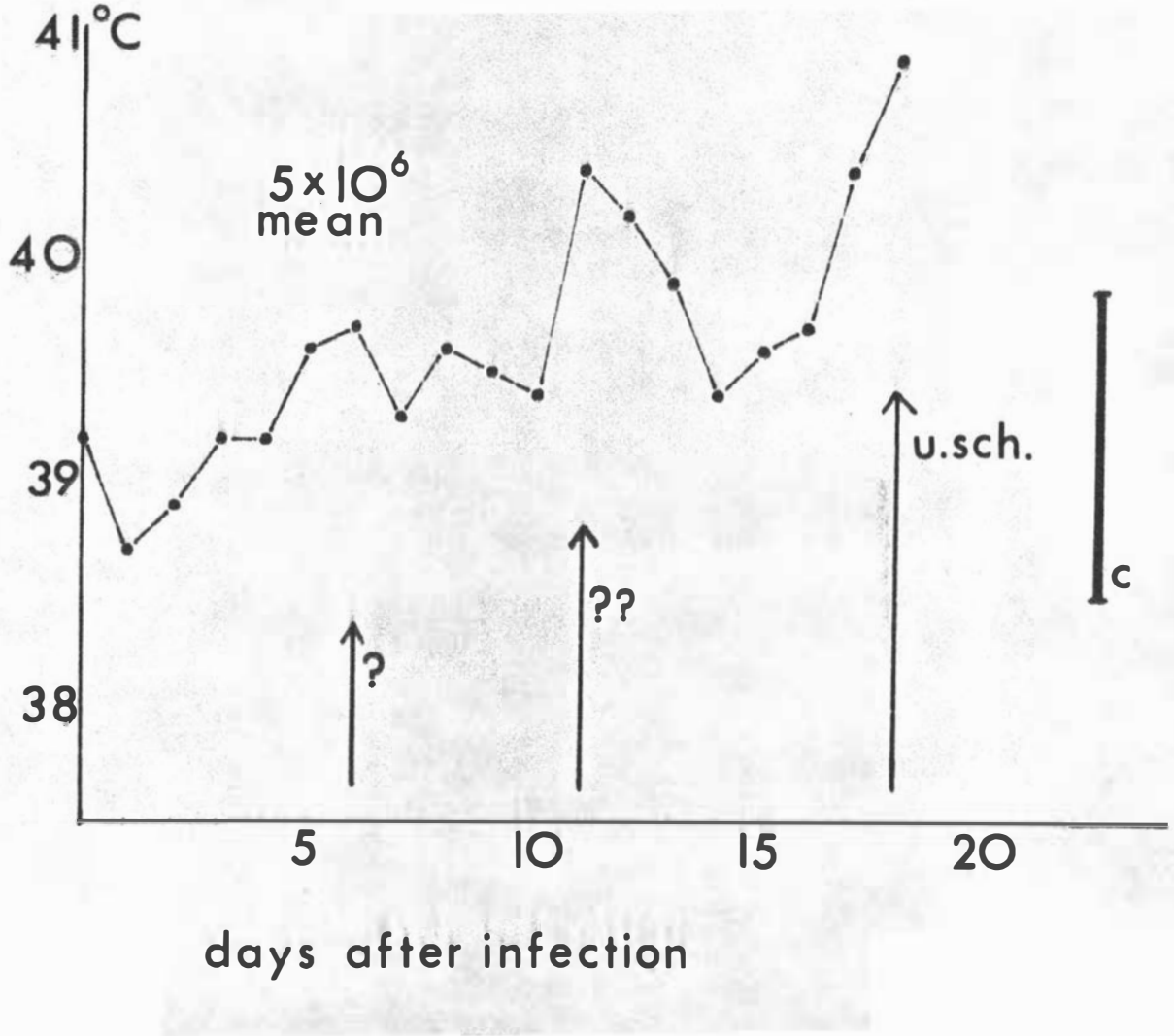

41°C
40
39
38
5x10⁶
mean
?
??
u.sch.
c
5
10
15
20
days after infection

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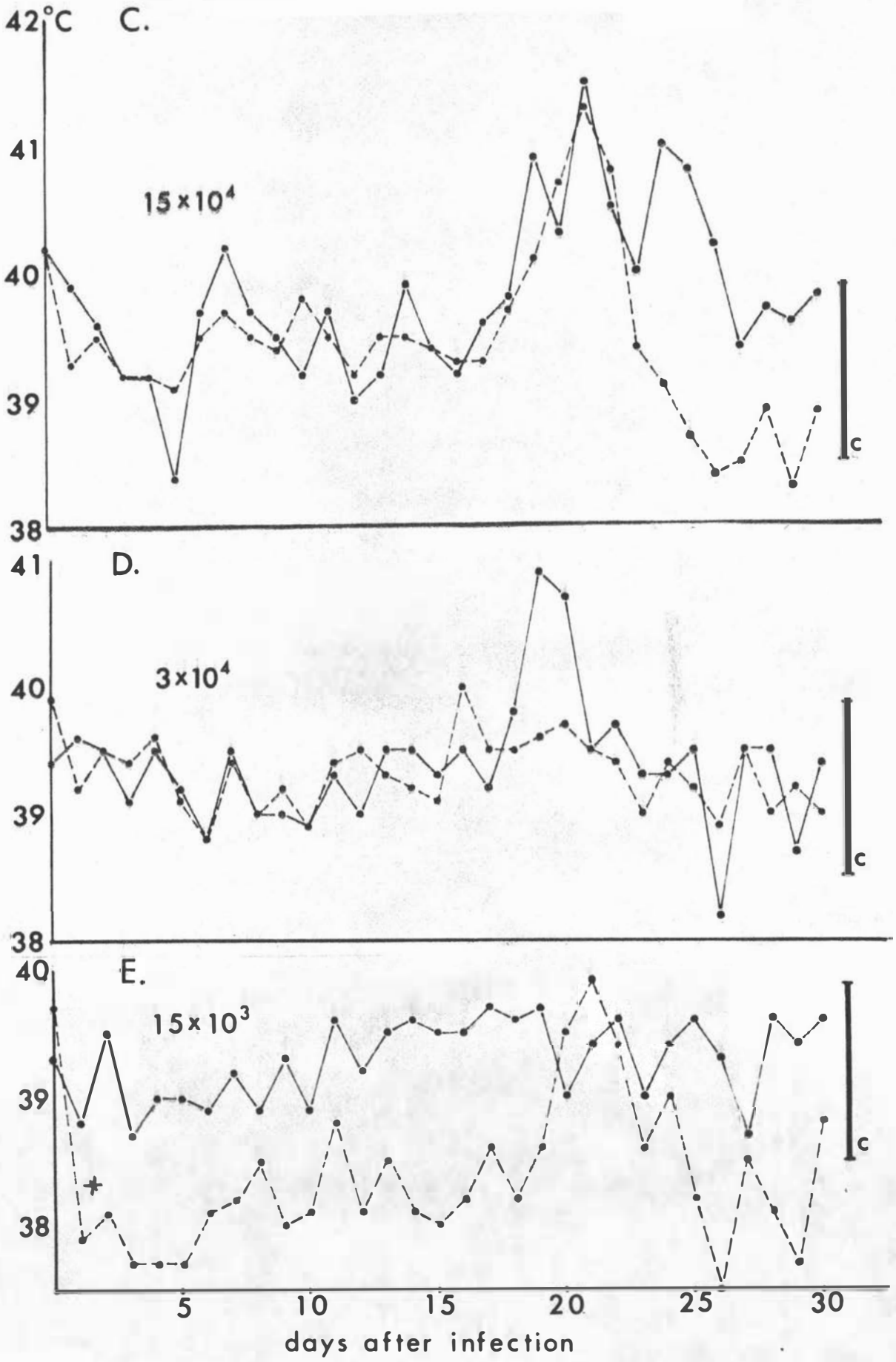
FIGURE 9.3 RECTAL TEMPERATURES RECORDED IN GOATS GIVEN DOG:GOAT SP. SPOROCYSTS.

(C: goats 6 and 7 given 15×10^4 sporocysts;

D: goats 8 and 9 given 3×10^4 sporocysts;

E: goats 10 and 11 given 15×10^4 sporocysts;

Bar c at right hand side = mean temperature of control goats 12 and 13 ± x2SD; * see text).


42°C
C.
41
15×10⁴
40
39
38
c
41
D.
40
3×10⁴
39
38
c
40
E.
15×10³
39
38
c
5
10
15
20
25
30
days after infection

Facing page 139

FIGURE 9.4 HAEMOGLOBIN LEVELS (grams per decilitre) RECORDED IN GOATS GIVEN DOG:GOAT SP. SPOROCYSTS.
(B: goats 4 and 5 given 6×10^5 sporocysts;
C: goats 6 and 7 given 15×10^4 sporocysts;
D: goats 8 and 9 given 3×10^4 sporocysts;
E: goats 10 and 11 given 15×10^3 sporocysts.
Bar c at right hand side = mean haemoglobin level of control goats 12 and 13 ± 2 x SD;
* see text).

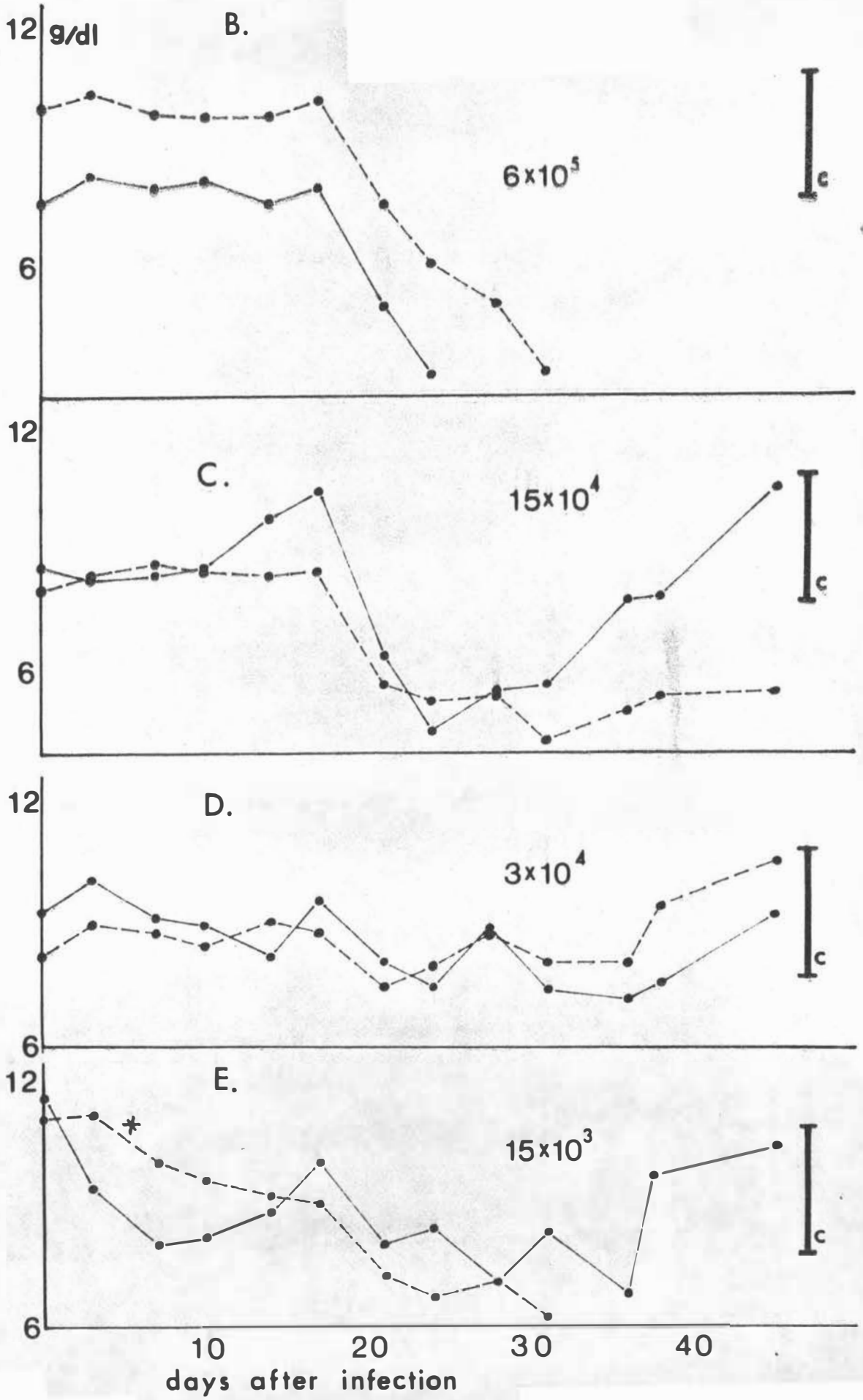

12 g/dl
B.
6x10^5
c
6
12
C.
15x10^4
c
6
12
D.
3x10^4
c
6
12
E.
*
15x10^3
c
6
10
20
30
40
days after infection

FIGURE 9.5 PACKED CELL VOLUMES RECORDED IN GOATS GIVEN DOG:GOAT SPOROCYSTS

(B: goats 4 and 5 given 6×10^5 sporocysts;

C: goats 6 and 7 given 15×10^4 sporocysts;

D: goats 8 and 9 given 3×10^4 sporocysts;

E: goats 10 and 11 given 15×10^3 sporocysts.

Bar c at right hand side = mean packed cell volume of control goats 12 and 13 ± 2 x SD;

* see text).

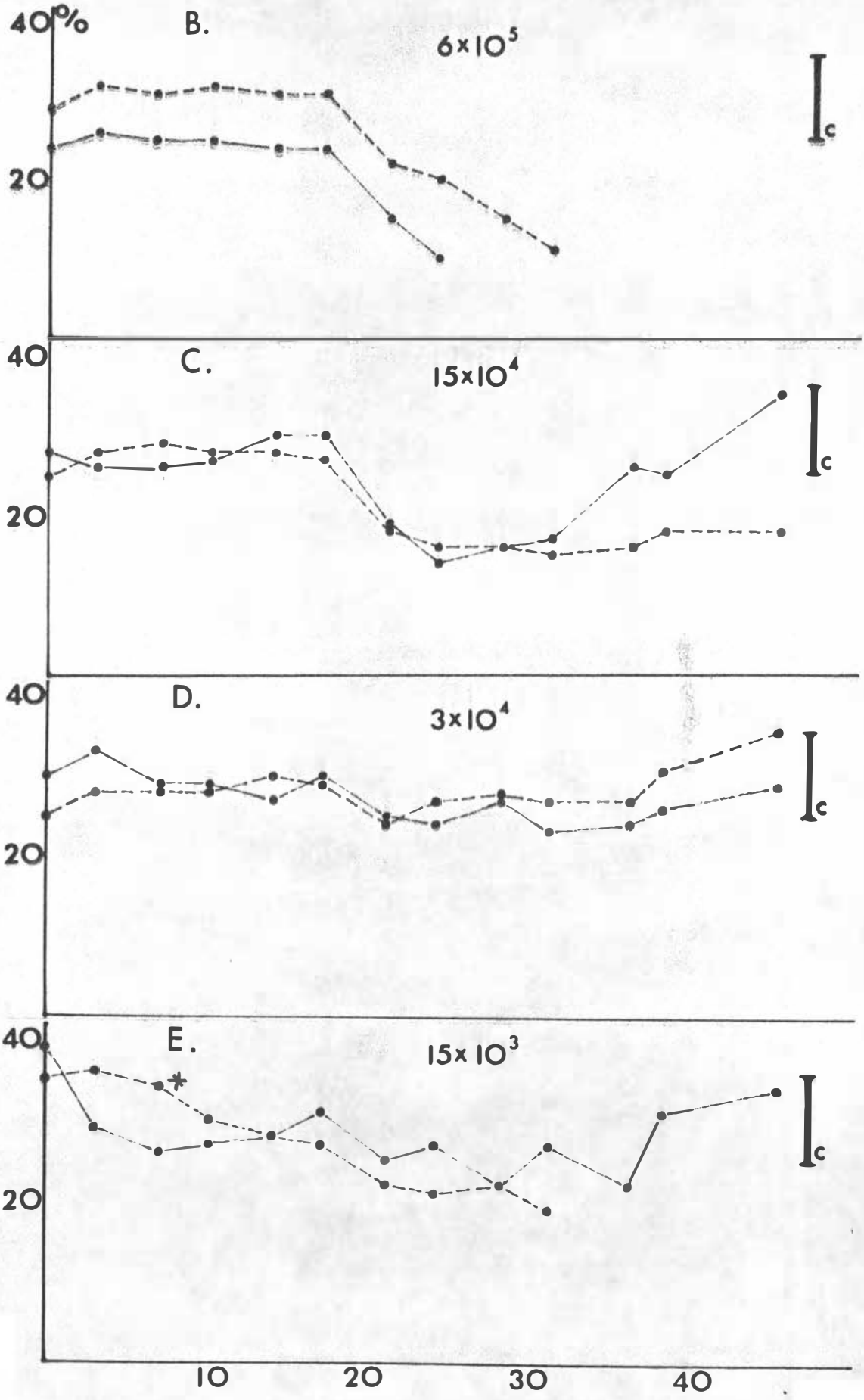
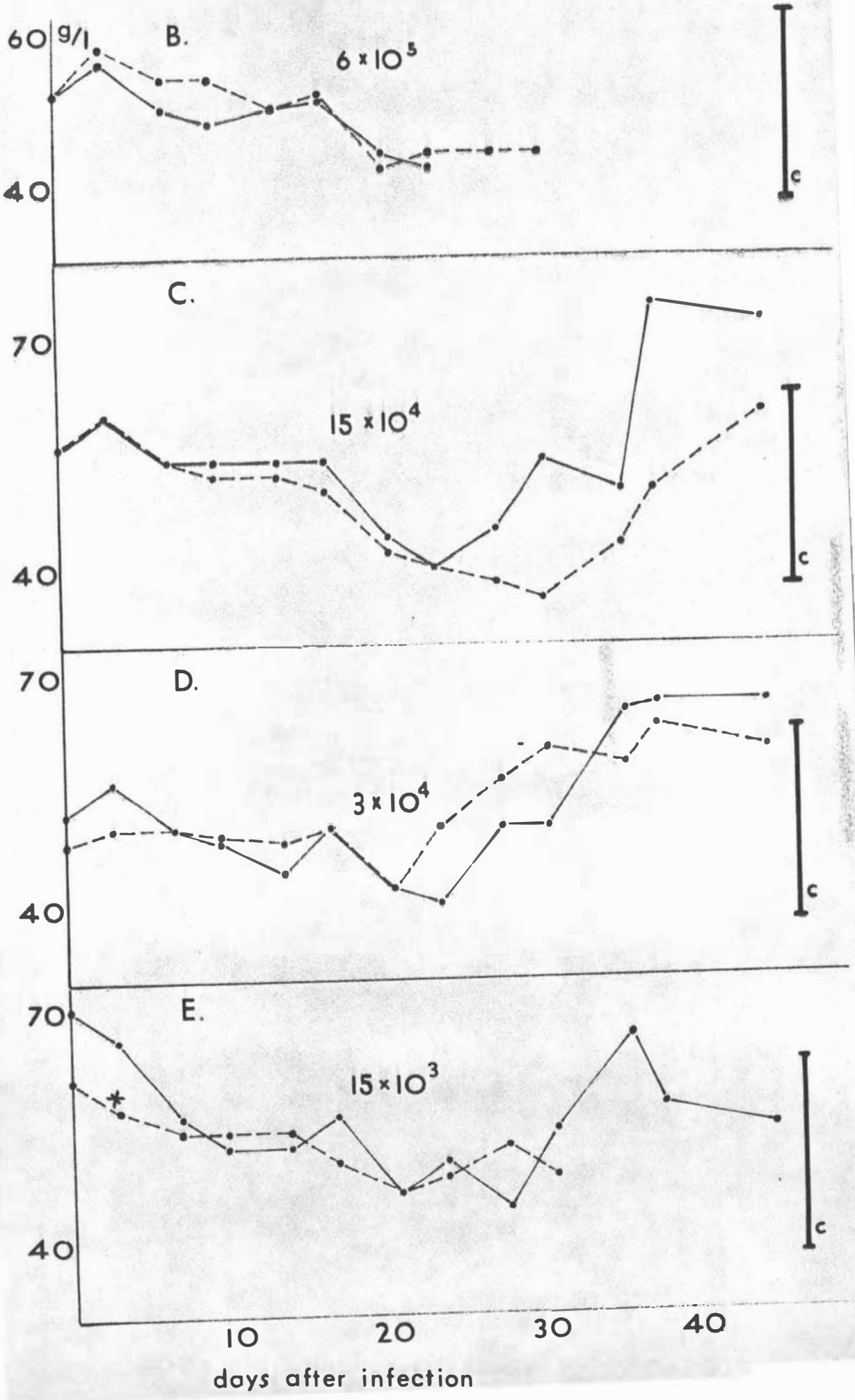

40%
B.
6x10^5
20
c
40
C.
15x10^4
20
c
40
D.
3x10^4
20
c
40
E.
15x10^3
20
c
10
20
30
40
days after infection

FIGURE 9.6 TOTAL SERUM PROTEIN LEVELS (grams per litre) IN GOATS GIVEN DOG:GOAT SP. SPOROCYSTS (B: goats 4 and 5 given 6×10^5 sporocysts; C: goats 6 and 7 given 15×10^4 sporocysts D: goats 8 and 9 given 3×10^4 sporocysts; E: goats 10 and 11 given 15×10^3 sporocysts. Bar c at right hand side = mean total serum protein level of control goats 12 and 13 ± 2 x SD; * see text).


60 g/l
B.
6 x 10^5
40
C.
70
15 x 10^4
40
D.
70
3 x 10^4
40
E.
70
15 x 10^3
40
C
10
20
30
40
days after infection

progressive rise from day 1 and also three distinct peaks at days 6, 11 and 18 (see Figure 9.2).

Goats 4 and 5 given 6×10^5 sporocysts showed symptoms of dullness, weakness and inappetance from day 19 and were killed when they became recumbent on days 34 and 24 respectively. The temperatures of these two goats stayed within the normal range until they rose abruptly on days 18 and 19, This was followed by a drop to sub-normal temperatures from day 22 (see Figure 9.1).

Doses of less than 6×10^5 sporocysts resulted in illness marked by inappetance, weakness and a small rise in temperature between days 18 and 25 (see Figure 9.3); this was followed by a recovery to apparently normal health. However, goats that received 15×10^4 and 3×10^4 sporocysts were considerably retarded in their growth and this stunting was still apparent at day 129. Goat 10 was found dead on day 34 although given only a small number of sporocysts. However, this animal had failed to thrive from birth and atypical changes were recorded in all the blood values during the period of the experiment (see curves marked * in Figures 9.3, 9.4, 9.5, 9.6, 9.7. The cause of death could not be determined

No signs of illness were seen in the control goats or sheep and the temperatures of all these animals remained normal throughout the experiment.

b. Haematology

Hb and PCV levels fell after day 17 in all dosed animals in the second experiment (goats 4 to 11); the severity of the anaemia was related directly to the number of sporocysts given (see Figures 9.4 and 9.5). In the surviving animals recovery to normal levels took

place by 38 days after infection. No change occurred in the MCHC. A fall in TP was seen in all the infected goats from 3 days after infection but levels remained mostly within the normal range; recovery to levels that were similar to or higher than the original levels took place in the surviving goats by day 24 to 31 (see Figure 9.6). SGOT levels rose and peaked at 36 to 38 days, then fell (see Figure 9.7). No change from the normal values were recorded in WBC, diff. WBC, protein fractions or LDH. No detectable change in any blood values from normal was seen in the control goats or sheep.

c. Serology

No rise in IFAT titre was recorded until day 28. The titres of goats 6,7,8,9 and 11 up to day 63 are shown in Figure 9.8. Titres rose continuously and, at day 63, when regular observations ceased, were apparently still rising. At day 129 the titres of goats 6, 7 and 8 were 1 in 1024, 256 and 1024 respectively.

d. Gross pathology

Gross pathological changes were confined to goats 1, 2 and 3, necropsied on days 18 and 19, and goats 4 and 5 on days 34 and 24 respectively. Small haemorrhages were present in large numbers in all tissues on days 18 and 19 and were most obvious in subcutaneous muscle, mesenteries and serosae (see Figure 9.9A). The heart (see Figure 9.9B) and oesophagus were mottled dark red and haemorrhages were visible on the cut surfaces of skeletal muscles. Kidneys were enlarged and mottled.

No haemorrhages were seen at day 24 in goat 5 but serosæ were flecked with brown pigment. At 34 days the gall bladder of goat 4 was enlarged by an accumulation of inspissated bile. No macroscopic lesions were found in goats necropsied on days 80, 93 and 129.

Facing page 144

FIGURE 9.7 SERUM GLUTAMIC OXALOACETIC TRANSAMINASE (SGOT) LEVELS (International units per litre) IN GOATS GIVEN DOG:GOAT SP. SPOROCYSTS

(B: goats 4 and 5 given 6×10^5 sporocysts;
C: goats 6 and 7 given 15×10^4 sporocysts;
D: goats 8 and 9 given 3×10^4 sporocysts;
E: goats 10 and 11 given 15×10^3 sporocysts.
Bar c at right hand side = mean SGOT level of control goats 12 and 13 ± 2 x SD; * see text).

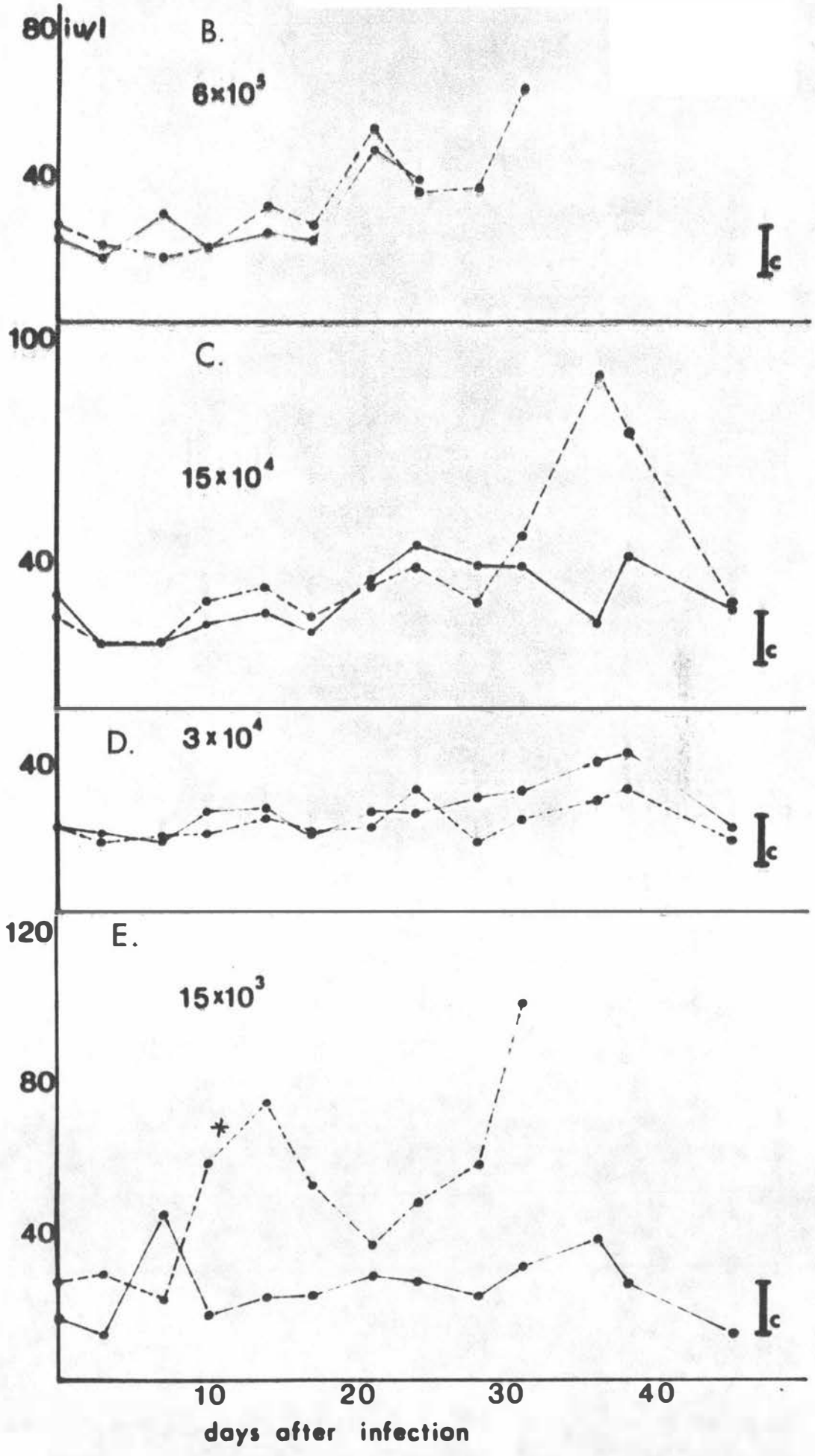
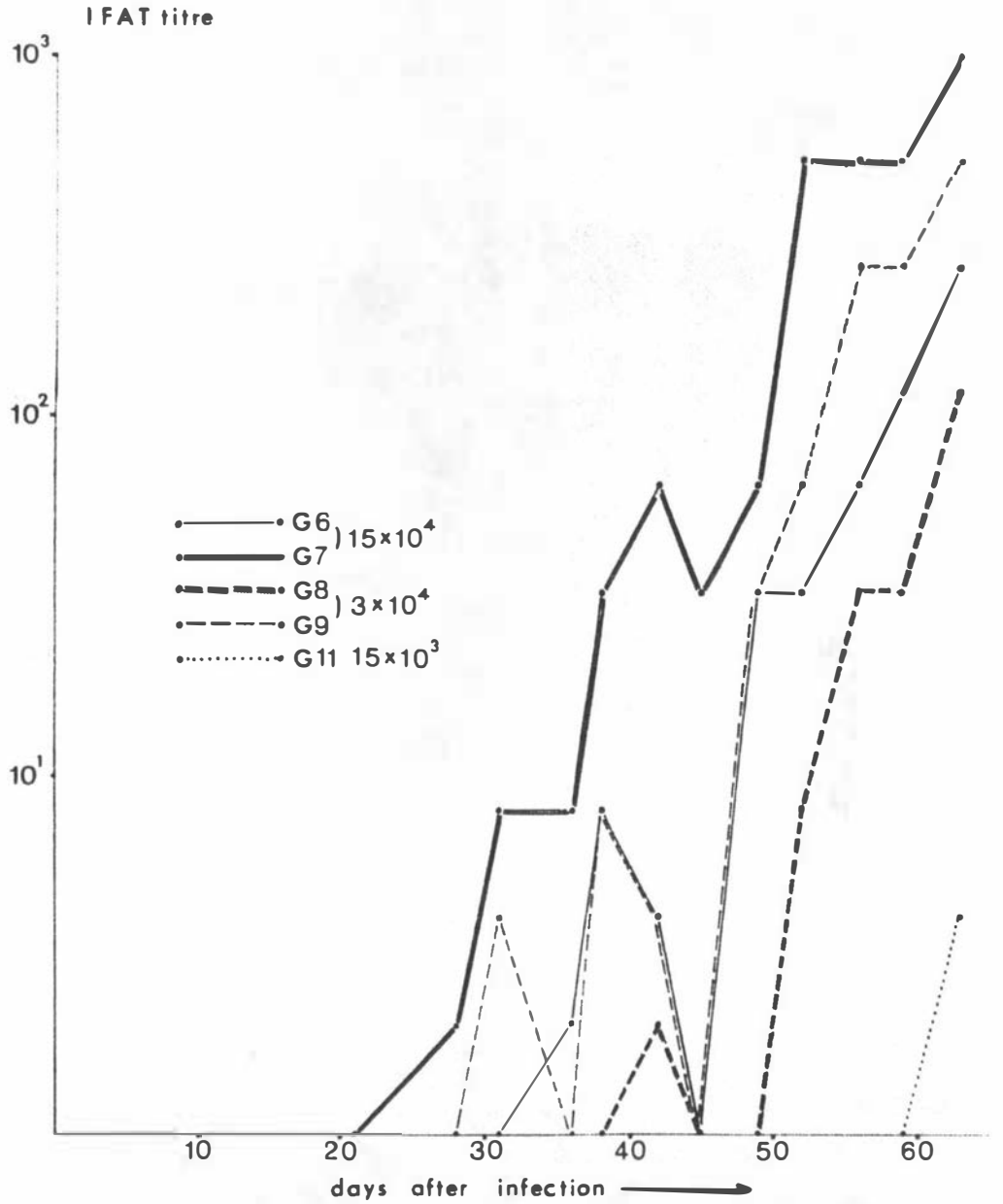

80 iwl
B.
6×10⁵
40
Ic
100
C.
15×10⁴
40
Ic
D. 3×10⁴
40
Ic
120
E.
15×10³
80
40
*
Ic
10
20
30
40
days after infection

FIGURE 9.8 INDIRECT FLUORESCENT ANTIBODY TEST (IFAT) TITRES IN GOATS GIVEN DOG:GOAT SP. SPOROCYSTS (Goats 6 and 7 given 15×10^4 sporocysts; Goats 8 and 9 given 3×10^4 sporocysts; Goat 11 given 15×10^3 sporocysts).


IFAT titre
10^3
10^2
10^1
G6
G7
) 15×10^4
G8
G9
) 3×10^4
G11 15×10^3
10
20
30
40
50
60
days after infection

e. Histopathology

Histological examination revealed small haemorrhages in all tissues examined from the goats necropsied on days 18 and 19. The kidneys showed severe nephrosis and tubules contained proteinaceous material. The renal tissues were diffusely infiltrated with lymphocytes. Schizonts were present in endothelial cells of small and large blood vessels and were especially numerous in the renal glomeruli (see Figure 9.10a).

At 24 days schizonts were not found in any tissue except the heart (see Table 9.1). Haemorrhages were not seen but many small deposits of haemosiderin were present in all tissues, with larger deposits in the spleen. Hypercellularity in the kidney was most marked at this time.

A single schizont was found in a section from the tongue of goat 4 on day 34. Many young sarcocysts were present in striated muscle fibres.

On day 80, sarcocysts up to 370 µm in length were common in muscle fibres and on day 93 sarcocysts were up to 650 µm in length. Sarcocysts were present in all the striated muscles of the goats examined at day 129, although relatively small numbers were found in heart muscle. The muscles of all the goats killed on day 129 were infiltrated with mononuclear cells. No organisms or lesions that could be ascribed to *Sarcocystis* were found in the tissues of the control goats or those of the sheep.

f. Life cycle stages

Both mature and immature schizonts were present in the tissues on days 18 and 19. Mature schizonts were irregular in shape and contained schizozoites approximately 5 µm in length with nuclei of about 2 µm diameter (see Figure 9.10a). Immature schizonts were more

TABLE 9.2

SCHIZONTS IN EQUATORIAL SECTIONS OF RENAL GLOMERULI IN GOATS 1, 2 AND 3 GIVEN 5 x 10^6 DOG:GOAT SP. SPOROCYSTS

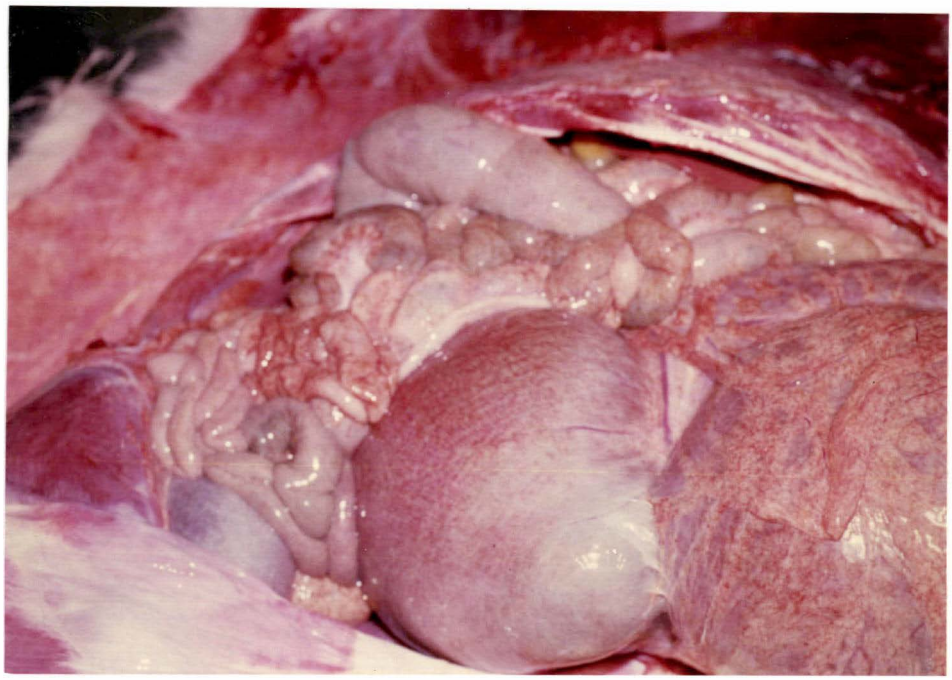
Goat No.	Days after Infection	Mean no. schizonts per glomerular section n = 50	Immature schizonts %	Mature schizonts %
1	18	2.70	85.2	14.8
2	19	6.62	44.4	55.6
3	19	7.08	49.2	50.8

Facing page 148

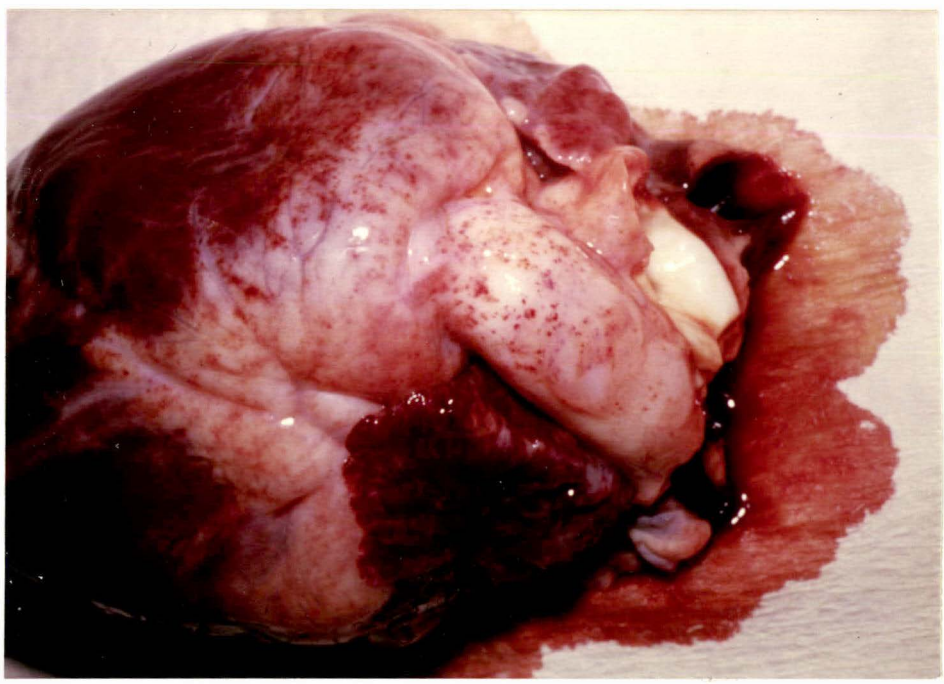
FIGURE 9.9 PHOTOGRAPHS TAKEN DURING THE NECROPSY OF GOAT 3, 19 DAYS AFTER INFECTION WITH DOG:GOAT SP. SPOROCYSTS.

A. Abdominal organs *in situ* showing extensive petechial haemorrhages on the serosae.

B. The heart, showing petechiae.



A



B

regular, basophilic, and were surrounded by a thin eosinophilic membrane (see Figure 9.10 b,c). The mean number of schizonts per glomerular section and the proportions of mature and immature schizonts in the samples examined from goats 1, 2 and 3 are given in Table 9.2. A higher proportion of schizonts were mature on day 19 than on day 18. From the mean number of schizonts per glomerulus it was estimated that, together, the kidneys of goat 3 contained 34×10^6 schizonts. At day 34 young sarcocysts comprised one to five metrocytes (mean 1.99, n = 100) surrounded by a parasitophorous vacuole (see Figure 9.11); single metrocytes measured up to 16.0 x 4.0 µm. 72 of 100 young sarcocysts were found to be in close association with muscle nuclei.

At days 80 and 93 sarcocysts contained bradyzoites and at day 129 muscle containing sarcocysts when fed to dogs gave rise to sporocysts of similar dimensions to those that were used to infect the goats. The control dog remained negative. The life cycle of the dog:goat sp. was thus completed. No bradyzoites were found after digestion of muscle from control goat 13 and sheep 2, 3 and 4.

Study of the ultrastructure of the mature sarcocyst wall showed that this species has relatively short interlocking villi of similar shape and size, with transverse sections approximating to a parallelogram (see Figure 9.12 L and T). Blister-like invaginations similar to those seen in species that infect sheep (see Chapter 4) are confined to the bases of the villi. No secondary cyst wall is present. The wall of the dog:goat sp. is distinctly different in structure from the walls of other *Sarcocystis* species previously described.

9.4 Discussion

Disease in goats caused by the dog:goat sp. of *Sarcocystis* was

Facing page 150

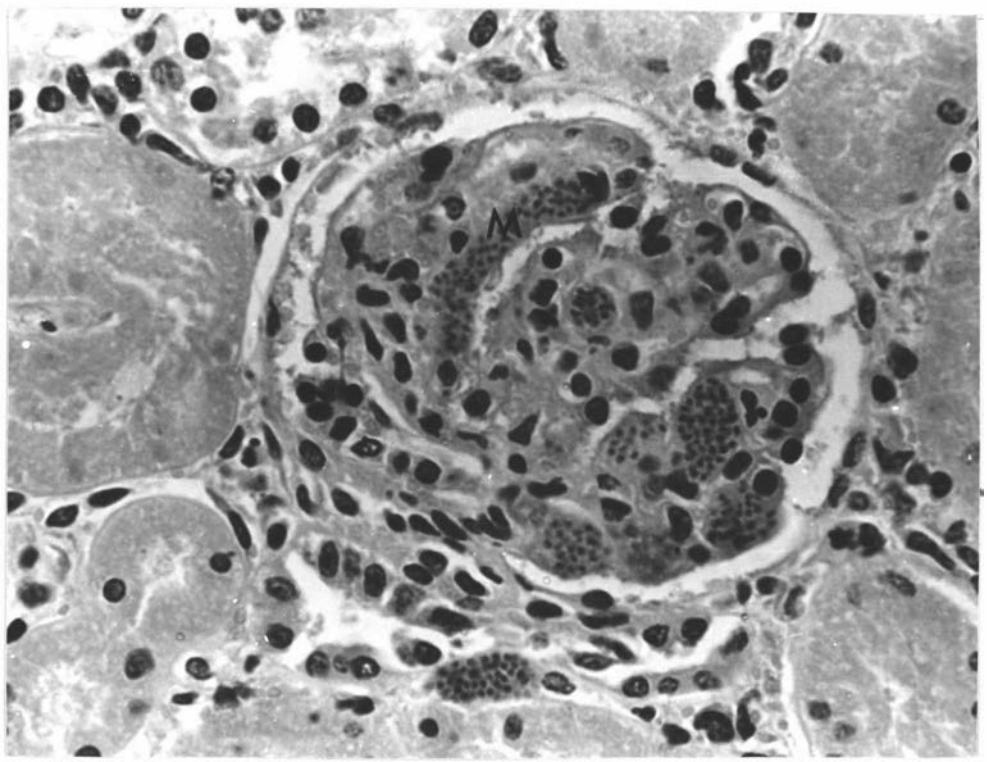
FIGURE 9.10 PHOTOMICROGRAPHS OF SCHIZONTS IN SECTIONS OF KIDNEY 19 DAYS AFTER INFECTION WITH DOG:GOAT SP. SPOROCYSTS

a. Renal glomerulus with mature schizonts (X1000)

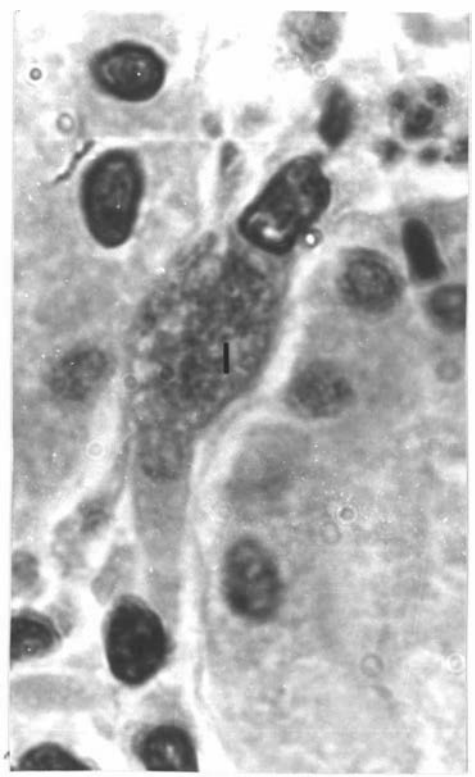
b. Immature schizont (X2,500)

c. Mature and immature schizonts (X2,500)

(I = immature schizont; M = mature schizont).



a



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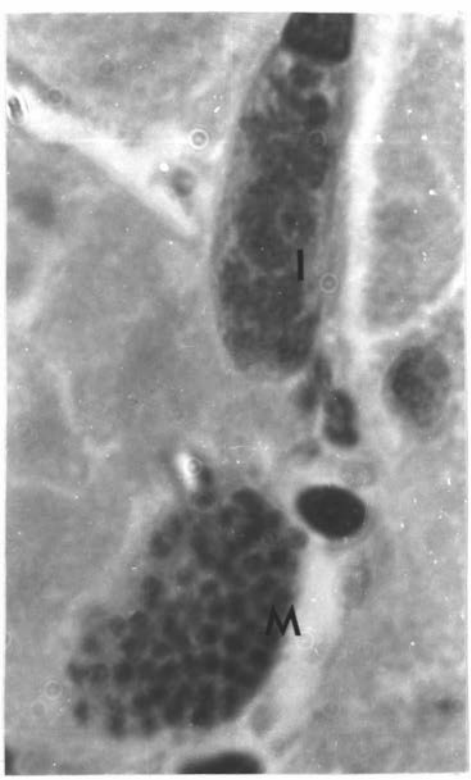


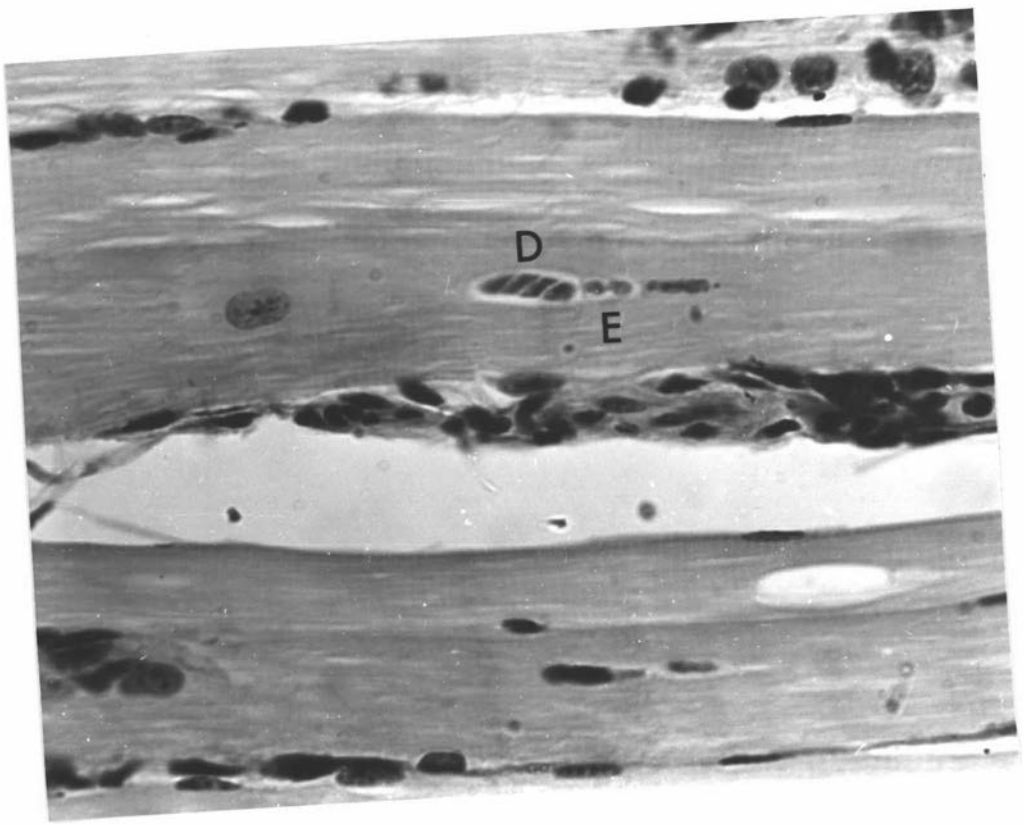
c

associated with similar symptoms to those described for other species in ruminants (see Table 2.1). Illness started and was most severe at the time of the ultimate schizogony (see Figure 9.3); those goats that did not become recumbent and die, subsequently made a slow recovery. No clinical signs were seen at the time when invasion of muscle must have occurred nor during the maturation of the sarcocysts. Both the abrupt rise in temperature and the onset of anaemia were directly associated with the ultimate schizogony.

It would be difficult to ascribe the external signs of illness or the anaemia to any cause other than the destruction of endothelial cells by the release of schizozoites from mature schizonts. The synchronous occurrence of small haemorrhages in all the organs of the body would account for the sudden onset of illness with symptoms of dullness and weakness, for the post-haemorrhagic nature of the anaemia, for the fall in total serum protein without a change in the protein fractions, and for the rise in SGOT levels. The numerous haemorrhages were also the source of the haemosiderin deposits evident at day 24. The only observation that cannot be fully ascribed to the 'schizogonic crisis' is the apparent failure of some of the infected animals to return, subsequently, to a normal pattern of growth. This important aspect of sarcocystosis in goats will receive more detailed investigation in further experiments.

The results from the IFAT showed that *Sarcocystis* antibodies in serum began to rise from about 28 days after infection. This is comparable with results from other experimental infections (Munday, Barker and Rickard, 1975; Fayer, Johnson and Lunde, 1976; Fayer and Lunde, 1977; Lunde and Fayer, 1977). It is likely that the rise in antibody titre occurs in response to the release of large amounts of

FIGURE 9.11 PHOTOMICROGRAPH OF YOUNG SARCOCYSTS IN A MUSCLE FIBRE 34 DAYS AFTER INFECTION WITH DOG:GOAT SP. SPOROCYSTS (X1000).
(One sarcocyst (D) encloses 4 metrocytes seen in longitudinal section; a second (E) encloses 2 metrocytes seen in transverse section).


D
E

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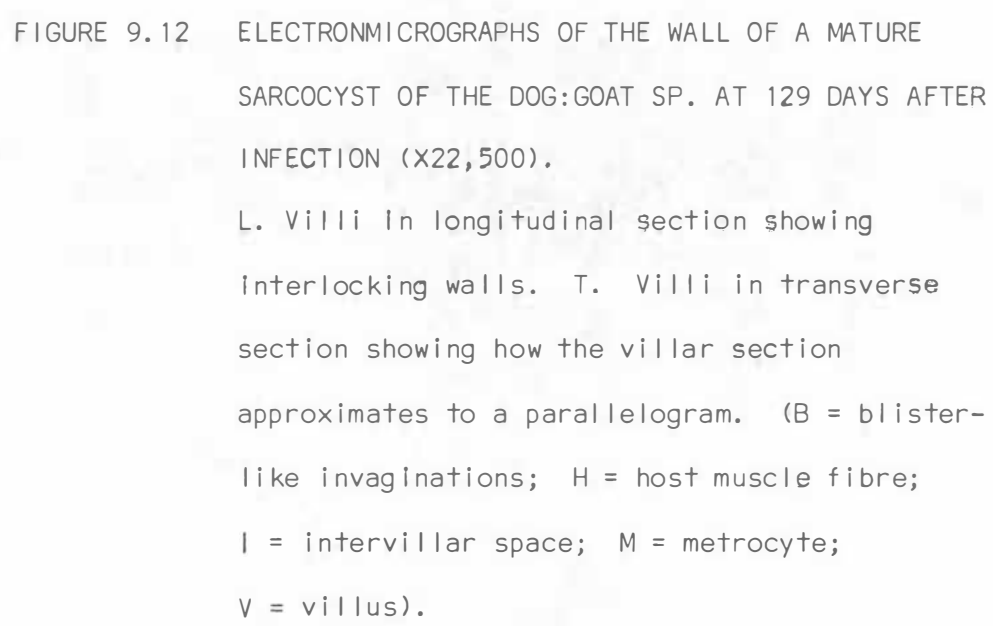
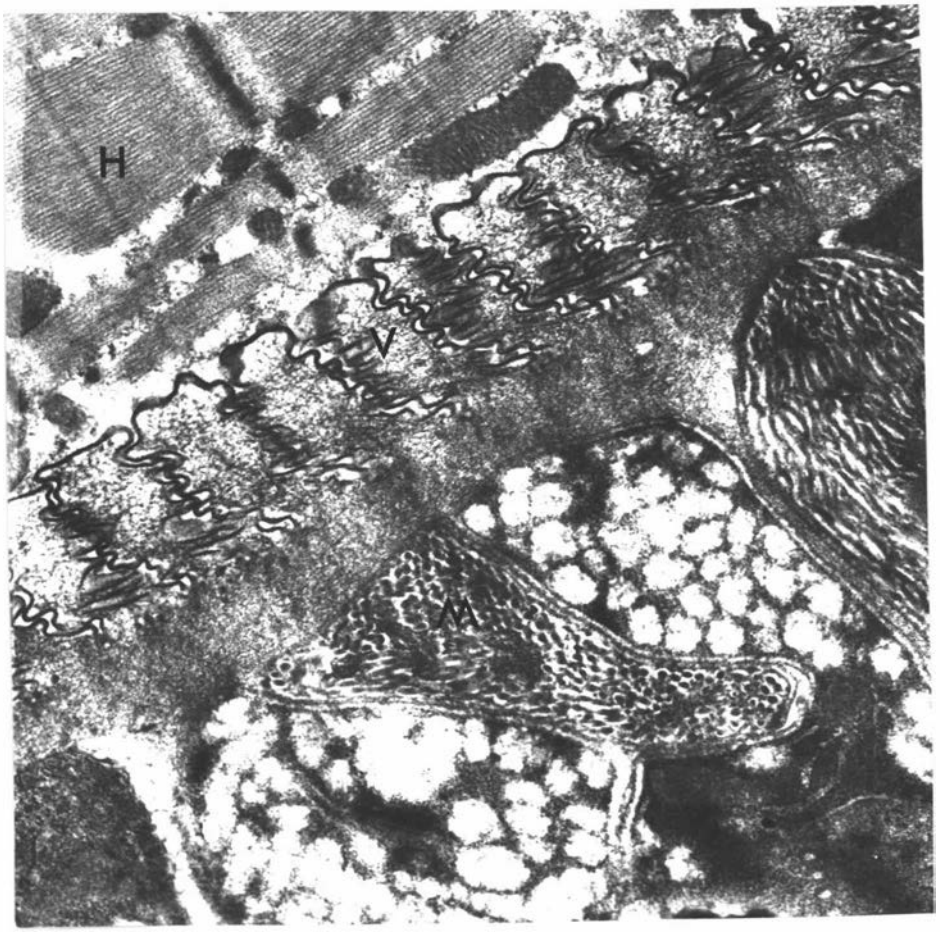


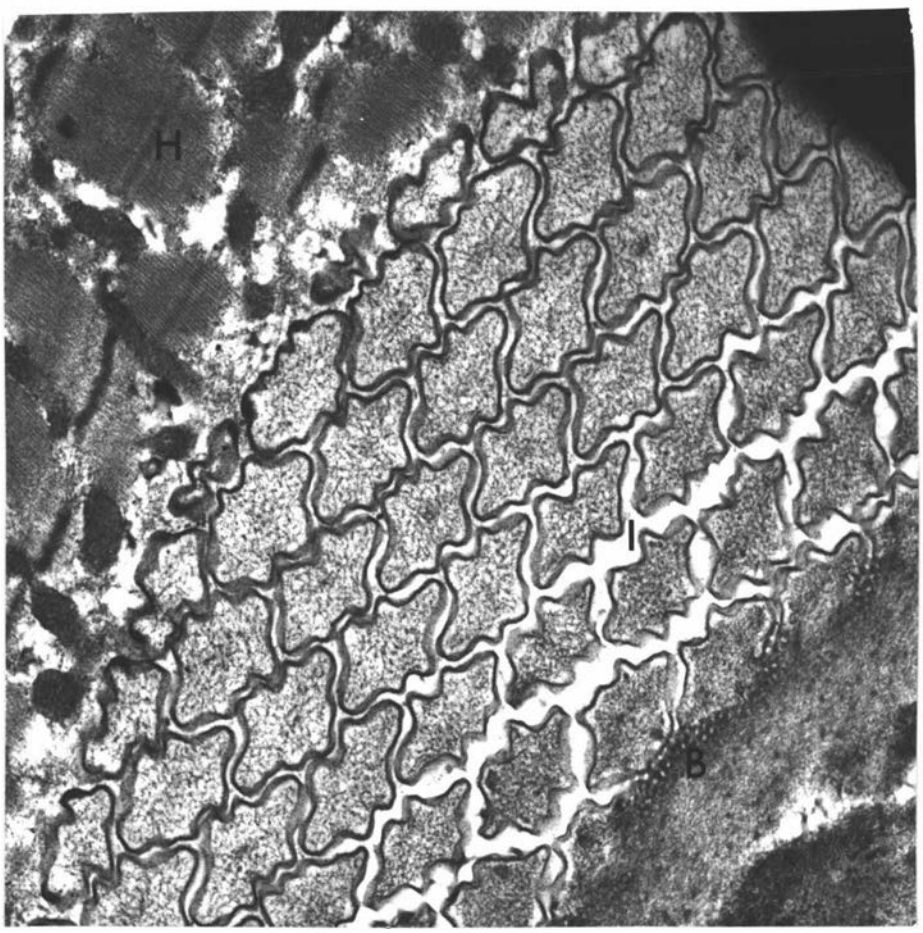
FIGURE 9.12 ELECTRONMICROGRAPHS OF THE WALL OF A MATURE SARCOCYST OF THE DOG:GOAT SP. AT 129 DAYS AFTER INFECTION (X22,500).

L. Villi in longitudinal section showing interlocking walls. T. Villi in transverse section showing how the villar section approximates to a parallelogram. (B = blister-like invaginations; H = host muscle fibre; I = intervillar space; M = metrocyte; V = villus).

L



T

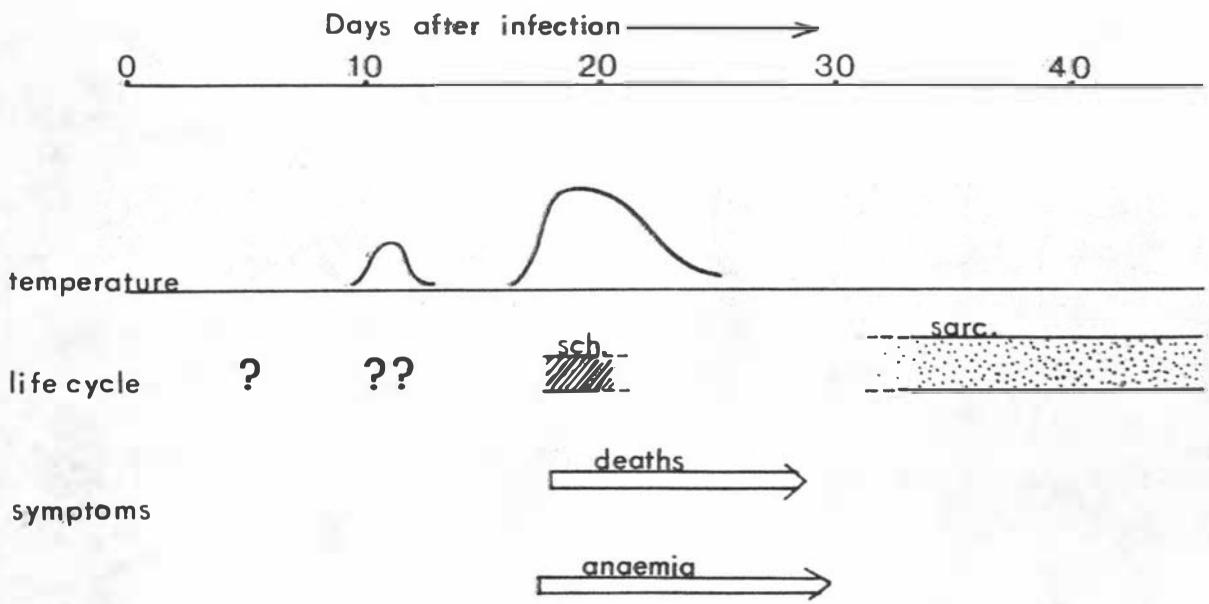


parasite antigen at the ultimate schizogony and the presence of schizozoites in the circulation. Once schizozoites have entered muscle fibres they are, presumably, no longer in close contact with the immune system. However, it is possible that a minority of schizozoites fail to reach their next site of development and their presence, together with a few 'late' schizonts - as seen at day 34- would continue to stimulate the immune system after the majority of parasites have left the circulation.

The macroscopic lesions seen at necropsy of the heavily infected goats (goats 1, 2 and 3) resembled those described in other ruminant species (see Chapter 2). The multiple petechial haemorrhages (see Figure 9.9 a,b) on the surfaces of the thoracic and abdominal organs and the mottling of the heart appear to be characteristic of acute disease in ruminants caused by *Sarcocystis* species. However, these distinctive lesions soon disappear - by 24 days only small flecks of brown pigment were seen on the serosae - and hence they are unlikely to be of much use in diagnosing the condition in the field, unless necropsy is carried out at the time of schizogony.

The lymphoid infiltration of the kidney undoubtedly occurs in response to the presence of schizonts and the hypercellularity is noticeable for some time after the end of schizogony. It is not known if kidney function is permanently impaired by the destruction of endothelial cells in glomeruli during schizogony and this aspect of sarcocystosis certainly warrants further investigation. The lymphoid infiltration of muscle at 129 days could have been the result of an immune response to the presence of large numbers of growing sarcocysts. The possibility that cell mediatedimmunity plays some part in host resistance to *Sarcocystis* will be examined in further experiments.

The development of the dog:goat sp. in the goat is similar to that described for other species and hosts; only the timing is


Days after infection
0
10
20
30
40
temperature
life cycle
?
??
sch.
sarc.
symptoms
deaths
anaemia

different (see Figure 9.13). In this species the ultimate schizogony occurs at 18 and 19 days, which is intermediate in time between that reported for species in cattle and sheep and others in pigs and rats (see Figures 2.2, 2.3 and 2.4). There is evidence that at least one, if not two, phases of schizogony occur before 18 days in the dog:goat sp. The mean temperature curve of goats, 1, 2 and 3 includes three distinct peaks that divide the first 18 days after infection into three approximately equal periods (see Figure 9.2). Presumably the first and second peaks are the results of schizogony as well as the third, and the periods between correspond to the time taken for schizonts to reach maturity. Further support for the occurrence of early schizogony in this species was provided by estimating the total population of schizonts in goat 3. From the mean number of schizonts per glomeruli it was calculated that the glomeruli contained in two kidneys enclosed 34×10^6 schizonts. Assuming, probably conservatively, that there were ten times this number, or 340×10^6 schizonts, in the whole animal, then, as the original infection comprised 5×10^6 sporozoites, a multiplication factor of 17x is necessary to account for the increase in number of organisms; therefore one or more phases of schizogony must have preceded the ultimate schizogony.

The finding of occasional schizonts in tissues at 24 and 34 days might be explained as normal variation in the length of this phase of development. However, the maturation of large numbers of schizonts at 18 and 19 days and the abruptness of the associated illness shows that the parasite is highly synchronous in its development. The occurrence of 'late' schizonts is probably due to delay in the release of sporocysts from the rumen and/or to some sporocysts being passed unchanged in the faeces of dosed animals and infecting the host, after reingestion,

a few days later.

It is assumed that schizozoites, released after the ultimate schizogony, enter muscle fibres soon afterwards. If so, it is curious that no sign of muscle invasion was found on day 24 but only on day 34. Infection of muscle fibres must have occurred between days 24 and 34; further experiments will be necessary to determine the precise time. It should be noted, however, that investigations of the development of protozoal parasites within large mammalian hosts inevitably pose problems in locating the organisms. The size of the parasites relative to that of their hosts makes their detection difficult, although the use of a double nuclear stain, celestine blue: haematoxylin and eosin, helped in this instance. Identification of the earliest muscle stages, and possibly the earliest phase of schizogony, may need the development of more specialised histological techniques.

It is not known how schizozoites enter muscle fibres. Penetration may occur directly through the sarcolemma or by way of a peripherally placed nucleus. The fact that a high proportion of early sarcocysts were found lying in close association with muscle nuclei is an indication that they may play a part in muscle invasion.

Although sarcocysts were shown to be infective by day 129, maturity was almost certainly attained earlier:bradyzoites were seen in sarcocysts at day 80. Infectivity has been reported at about 70 days in other species (see Table 2.1).

There is little doubt that this species of *Sarcocystis* is specific to goats as intermediate hosts. Sheep were not infected with sporocysts identical to those that infected goats and no signs of illness were seen. The distinctive structure of the sarcocyst wall of this species is further evidence that it is not an ovine parasite that is also

capable of infecting goats; and the difference in the timing of the ultimate schizogony also points to its separate identity. Further transmission studies will be necessary before it is certain that goats are the only ruminants in which the species is found.

The dog:goat sp. warrants further study for several reasons. Not only is the parasite readily cycled between dog and goat but both of these hosts are easily reared and maintained under laboratory conditions. Provided studies are carried out in situations where dog:goat sp. sporocysts are not likely to be present in the environment, satisfactory results may be obtained without resorting to the use of specific pathogen-free animals.

Several aspects of the life cycle of this species need further investigation - the early phases of schizogony, the process of invasion of muscle fibres, the time of maturity of sarcocysts and the development of the parasite in the dog.

Sarcocystosis in goats warrants further study as the results could provide better means of diagnosis and treatment in other ruminants. The possibility that low doses of sporocysts can cause ill-thrift and stunting in farm animals needs particular attention as sub-clinical disease caused by *Sarcocystis* could be of considerable economic importance in animal production.

CHAPTER 10

CONCLUSIONS

This decade has seen renewed interest in *Sarcocystis* and, at present, many species are being investigated throughout the world. The diversity of the topics studied, as reported in this thesis, reflects the need for basic knowledge of all aspects of these parasites.

The life cycle of *Sarcocystis* is now well understood in general terms but there are several aspects that still need detailed study. For example, the period between excystment in the small intestine of the intermediate host and the ultimate schizogony is largely unexplored. It is possible that sporozoites after invading the mucosa, pass by way of the portal system to the liver and there undergo schizogony for the first time. Schizogony in the liver soon after excystment has been reported in the man:pig sp. (Heydorn and Ipczynski, 1978). Schizogony may occur as early as 6 days after infection in the dog:goat sp. (see Chapter 9) and the possibility that it takes place in the liver will be examined in further studies.

The period between the ultimate schizogony and the invasion of muscle also warrants investigation: it is necessary to know what happens to schizozoites after their release from schizonts and how they penetrate muscle fibres. It may be that these early phases in development would be better investigated initially in tissue culture using the appropriate types of cells. The life cycles of the *Sarcocystis* species found in ruminants, because of their economic importance, will have to be investigated in detail.

The failure to achieve transmission of the cat:sheep 1 sp. to sheep has frustrated research on the development of this species, and

has raised important new questions. We need to know why the sporocyst is apparently not directly infective for sheep and how it becomes infective. It is difficult to see how the lack of infectivity of the sporocyst of this species can be reconciled with its frequent occurrence in sheep. All other aspects of the epidemiology help to explain its high prevalence in New Zealand: the large sheep population, the widespread distribution of both sheep and cats, the scavenging behaviour of cats, the survival of macrocysts at ambient and freezing temperatures and the survival of sporocysts in water all facilitate transmission. The cat:sheep 1 sp. is, at present, the most economically important species in this country and therefore the problem of sporocyst infectivity urgently needs a solution.

The cat:sheep 2 sp., that gives rise to thin macrocysts in sheep, has yet to be investigated. This species, although apparently of less economic importance than the cat:sheep 1 sp. still warrants careful study. Comparison of the features of the two macrocystic species in sheep will undoubtedly lead to a deeper understanding of the epidemiology of *Sarcocystis* infections in ruminants.

Other species of *Sarcocystis* in New Zealand also need further attention; the definitive hosts of the forms in red deer and wild pigs have yet to be found and there are many other animals to be investigated as intermediate or definitive hosts. Sarcocysts have been seen in the muscles of various birds in this country (Collins, unpubl. obs.); the specific identities of these parasites are unknown. Similarly, although sarcocysts have been found frequently in domestic cattle, pigs and horses (Collins unpubl. obs.) the species have not been identified and their prevalence and economic importance have yet to be investigated.

Sarcocystosis has yet to be shown to be of more than minor importance to animal health. Occasionally, farm animals ingest large

numbers of sporocysts and suffer the effects of the ultimate schizogony; at present it is not known how frequently such episodes occur. The likelihood of a ruminant ingesting a significant number of sporocysts must depend on several factors including the population density and distribution of the definitive host, the population density and management of the intermediate host, the diet of the definitive host, the concentration of sporocysts in its faeces and the survival of sporocysts on pasture. Although it has been shown by various workers that large numbers of sporocysts cause death or severe illness and abortions in pregnant animals, no attempt has been made to establish the minimum number of sporocysts necessary to produce these results. The cause of most of the abortions that occur in cattle and sheep each year in New Zealand is not known; the importance of *Sarcocystis* species in this context urgently needs investigation. Low doses of sporocysts usually result in only mild illness; however, recovery may be accompanied by stunting (see Chapter 9). The possibility that subclinical disease can be caused by 'natural levels of infection' could be of considerable economic importance.

In order to define the importance of sarcocystosis as a cause of both clinical and sub-clinical disease in farm animals, it will be necessary to develop more efficient diagnostic techniques than are available at present. Serological tests such as the HAT and IFAT, although capable of demonstrating rising levels of *Sarcocystis* antibodies, have yet to be shown to be either genus or species specific. Perhaps a biochemical test, able to show that damage has occurred to endothelial cells or that muscle fibres have been invaded, will prove to be more useful in diagnosis of acute disease than serological tests.

If sarcocystosis proves to be an important cause of economic loss

on farms it will be necessary to devise measures for its control. Some reduction in the prevalence of infection in animals on a particular farm might be achieved by the extermination of feral cats and careful feeding of domestic cats and working dogs, but to control sarcocystosis in all New Zealand's farm animals by such means is probably impracticable. An alternative approach may lie in immunisation. The intermediate host undoubtedly mounts an immune response to *Sarcocystis* infection; whether or not the antibodies produced are protective has yet to be established. Study of the dog:goat sp. (see Chapter 9) revealed a cellular response to the sarcocysts in muscle fibres. Further study of the development of *Sarcocystis* species in both laboratory animals and ruminants will be necessary to determine the importance of host resistance in sarcocystosis and whether or not it can be artificially stimulated.

Infection of goats with the dog:goat sp. could serve as a useful experimental model for other ruminant infections. In circumstances where there are no facilities for rearing and maintaining specific pathogen-free animals it is necessary that conventionally reared experimental animals are kept in an environment that is predictably free of infective sporocysts. In New Zealand it would be difficult to find an area reliably free of sporocysts of cattle and sheep species, so that extraneous infection is liable to complicate experiments involving these species. The limited distribution of feral and farmed goats in the country and hence of dog:goat sp. sporocysts means that it should be possible to carry out experiments on goats in both laboratory and field conditions with little risk of extraneous infection. In these circumstances research on *Sarcocystis* in goats is more likely to yield worthwhile information on ruminant sarcocystosis than comparable studies

on sheep and cattle.

The study of *Sarcocystis* is now entering a period of consolidation after the rapid expansion of interest that followed the discovery of the life cycle in 1972. There is little doubt that the unique features of this genus and the multiplicity of species will ensure these organisms are the subjects of veterinary research for a long time to come.

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APPENDICES

APPENDIX 1

TABLE 1

SPECIES OF *SARCOCYSTIS* IN WHICH TRANSMISSION BETWEEN HOSTS HAS BEEN REPORTED

whole l.c. = transmission through the complete life cycle shown i.e. intermediate to definitive host and then to a second intermediate host or further.

part l.c. = transmission from intermediate to definitive host only.

Intermediate host (I.h.)	definitive host (d.h.)	transmission achieved	references
Domesticated spp.			
Ox *Bos taurus*	dog, *Canis familiaris* *C. latrans* *C. lupus* *Vulpes vulpes* *Procyon lotor?*	whole l.c.	Heydorn & Rommel, 1972a Suten and Coman, 1973 Fayer, 1974, Fayer & Johnson, 1975a Dubey & Streitel, 1976
Ox	cat, *Felis catus*	whole l.c.	Heydorn & Rommel, 1972a Suten & Coman, 1973 Markus, Killick-Kendrick & Garnham, 1974 Dubey & Streitel, 1976
Ox	man, *Homo sapiens* *Macaca rhesus* *Papio cynocephalus*	whole l.c.	Rommel & Heydorn, 1972 Heydorn, Gestrich & Janitschke, 1976 Heydorn, 1977a

APPENDIX TABLE 1 continued

Intermediate host (i.h.)	definitive host (d.h.)	transmission achieved	references
Sheep *Ovis aries*	dog *V. vulpes*	whole l.c.	Ford, 1974 Munday & Corbould, 1974 Munday & Rickard, 1974 Rommel *et al.*, 1974 Dubey & Streitel, 1976 Munday, 1976 Ashford, 1977
Sheep	cat 1	part l.c.	Rommel, Heydorn & Gruber, 1972 Mehlhorn & Scholtyseck, 1974a Munday & Rickard, 1974
Sheep	cat 2	part l.c.	See Chapter 5
Goat *Capra hircus*	dog	whole l.c.	Collins & Crawford, 1978 See Chapters 7 & 9.
Buffalo *Bubalus bubalis*	dog	part l.c.	Dissanaike *et al.* 1977 Dissanaike & Kan, 1978
Buffalo	cat	part l.c.	Dissanaike *et al.*, 1977
Horse *Equus caballus*	dog 1	part l.c.	Rommel & Geisel, 1975
Horse	dog 2	part l.c.	Dubey *et al.*, 1977
Pig *Sus scrofa*	dog	part l.c.	Rommel *et al.*, 1974
Pig	cat	whole l.c.?	Golubkovan & Kisliakova, 1974
Pig	man	whole l.c.	Rommel & Heydorn, 1972 Heydorn, 1977 Heydorn & Ipczynski, 1978

APPENDIX 1 TABLE 1 continued

Intermediate host (i.h.)	definitive host (d.h.)	transmission achieved	references
Domestic fowl *Gallus domesticus*	dog	part l.c.	Munday, Humphrey & Kila, 1977
Non-domesticated spp.			
Grant's gazelle *Gazella granti*	dog	part l.c.	Janitschke, Protz & Werner, 1976
Grant's gazelle	cat	part l.c.	Janitschke, Protz & Werner, 1976
Mule deer *Odocoileus hemionius hemionius*	coyote *C. latrans*	whole l.c.	Hudkins & Kistner, 1977
European rabbit *Oryctolagus cuniculus*	cat	part l.c.	See Chapter 7
Cottontail rabbit *Sylvilagus floridanus*	cat	part l.c.	Crum & Prestwood, 1977 Fayer & Kradel, 1977
Cottontail rabbit	raccoon *Procyon lotor*	part l.c.	Crum & Prestwood, 1977
Rat *Rattus norvegicus*	cat	whole l.c.	Ashford, 1978
Rat *R.fuscipes*	snake *Morelia spilotes variegata*	part l.c.	Rzepcyk, 1974

APPENDIX 1 TABLE 1 continued

Intermediate host (i.h.)	definitive host (d.h.)	transmission achieved	references
Rat *R. orientalis*	snake *Python reticulatus*	whole l.c.	Zaman and Colley, 1975
Mouse *Mus musculus*	cat	whole l.c.	Ruiz and Frenkel, 1976 Smith and Frenkel, 1978 McKenna (pers. comm.)
Mouse	owl *Tyto novaehollandiae* *T. alba*	whole l.c.	Cerna, 1976 Munday, 1977
Vole *Microtus arvalis*	kestrel *Falco tinnunculus*	whole l.c.	Cerna and Louckova, 1976
Vole	Weasel *Mustela nivalis*	whole l.c. ?	Tadros & Laarman, 1975
Cowbird *Molothrus ater* grackle *Cassidix mexicanus* *Quiscalus quiscula*	opossum *Didelphis virginiana*	part l.c.	Duszynski & Box, 1978
Duck *Anas acuta*	opossum	part l.c.	Duszynski & Box, 1978

APPENDIX 2

TABLE 1

Sporocyst sizes - species that develop in domesticated intermediate hosts

Sarcocystis species	length (range) µm	width (range) µm	mean µm	n=	Reference
dog:ox	13.9 - 17.0	6.2 - 10.8	15.9± 1.0 x 8.3± 1.1	50	Heydorn and Rommel 1972a
"	14.8 - 17.3	8.7 - 9.9	16.0 x 9.8	20	Mahrt 1973
"	12.7 - 21.6	9.2 - 14.6	15.2 x 10.0	?	Suten & Coman 1973
"	14.2 - 20.6	9.2 - 12.8	15.7 x 9.9	20	Fayer 1974
"	14.0 - 17.0	8.0 - 10.0	15.0 x 9.5	27	Dubey & Streitel 1976
coyote:ox	13.3 - 17.1	9.5 - 11.4	15.7 x 10.6	49	Fayer & Johnson 1975
fox:ox	15.3 - 16.9	10.0 - 10.8	16.0± 0.6 x 10.4± 0.4	15	Rommel *et al* 1974
wolf:ox	14.6 - 16.9	10.0 - 10.8	16.2± 0.8 x 10.7± 0.2	10	"
dog:sheep	?	?	15.0 x 10.0	?	Ford 1974
"	?	?	14.0 x 9.0 approx	?	Munday & Courbould 1974
"	?	?	14.7 x 9.0	?	Munday & Rickard 1974
"	13.1 - 16.1	8.5 - 10.8	14.8± 0.8 x 9.9± 0.7	100	Rommel *et al* 1974
"	14.0 - 15.4	7.7 - 11.2	14.7 x 9.0	30	Munday, Barker & Rickard 1975
"	?	?	about 14.7 x 9.0	?	"
"	13.0 - 15.0	9.0 - 10.0	14.0 x 9.2	11	Dubey & Streitel 1976
"	?	?	15.0 x 10.0	?	Munday 1976
dog:water buffalo	15.0 - 16.0	9.0 - 10.0	?	?	Dissanaike *et al* 1977
"	15.0 - 16.0	9.0 - 10.0	?	?	Dissanaike & Kan 1978
dog:goat	12.5 - 15.0	8.0 - 10.0	13.5 x 9.3	25	Collins & Crawford 1978
dog:horse 1	15.0 - 16.3	8.3 - 11.3	15.2± 0.44 x 10.0±0.34	?	Rommel & Geisel 1975
dog:horse 2	11.0 - 13.0	7.0 -8.5	12.0±0.5 x 7.9±0.5	60	Dubey *et al* 1977
dog:pig	10.8 - 13.8	9.2 - 10.8	12.6±0.6 x 9.6±0.5	50	Rommel *et al* 1974
dog:domestic fowl	10.0 - 13.0	7.3 - 8.5	11.5 x 8.3	?	Munday, Humphrey & Kila 1977
cat:ox	10.8 - 13.9	6.9 - 9.3	12.5±0.8 x 7.8±0.6	50	Heydorn & Rommel 1972a
"	12.5 - 13.8	9.2 - 9.5	?	?	Suten & Coman 1973
"	11.0 - 14.9	8.2 - 10.4	12.9 x 8.7	50	Markus, Killick-Kendrick & Garnham 1974
"	11.0 - 14.0	8.0 - 11.0	12.4 x 8.5	72	Dubey & Streitel 1976
cat:sheep 1	10.8 - 13.9	7.7 - 9.3	12.4±0.8 x 8.5±0.5	50	Rommel, Heydorn & Gruber 1972
"	10.5 - 14.0	8.0 - 9.7	12.4 x 8.7	70	Mehlhorn & Scholtyseck 1974a
"	9.8 - 12.4	6.2 - 8.0	10.9 x 6.8	?	"
" ?	?	?	12.5 x 8.0	?	Munday & Rickard 1974
cat:water buffalo	?	?	13.0 x 8.0	?	Dissanaike *et al* 1977
cat:pig	13.0 - 14.0	7.0 - 8.0	13.0 x 8.0	?	Dubey 1976

Sarcocystis species	length (range) µm	width (range) µm	mean µm	n=	Reference
man:ox	?	?	14.7 x 9.3	50	Rommel & Heydorn 1972
baboon:ox (man:ox sp.)			14.2 x 9.6	15	Heydorn, Gestrich & Janitschke 1976
rhesus monkey: ox (man:ox sp.)			14.9 x 11.0	16	"
man:pig	?	?	12.6 x 9.3	133	Rommel & Heydorn 1972
"	11.6 - 13.9	10.1 - 10.8	13.5±0.5 x 10.5±0.4	50	Heydorn 1977b

APPENDIX 2
TABLE 2

Sporocyst sizes - species that develop in non-domesticated intermediate hosts

dog:grant's gazelle	12.6 - 18.0	8.4 - 12.0	16.1±1.4 x 10.6±1,0	100	Janitschke, Protz & Werner 1976
coyote:mule deer	?	?	14.4 x 9.3	80	Hudkins & Kistner 1977
cat:grant's gazelle	10.5 - 15.6	8.4 - 12.0	13.2±1.2 x 9.2±0.8	100	Janitschke, Protz & Werner 1976
cat:mouse	8.7 - 11.7	7.5 - 9.0	10.3 x 8.5	40	Ruiz & Frenkel 1976
"	?	?	10.8 x 7.8	20	Smith & Frenkel 1978
cat:rat (R.n)	?	?	10.5 x 7.9	50	Ashford 1978
cat:cottontail rabbit	13.2	8.8 - 11.0	13.2 x 9.7	15	Crum & Prestwood 1977
"	13.0 - 16.7	9.3 - 11.1	13.6 x 9.4	55	Fayer & Kradel 1977
raccoon:cottontail rabbit	11.1 - 14.4	8.9 - 11.1	12.8 x 9.1	30	Crum & Prestwood 1977
owl:mouse	11.0 - 14.0	8.0 - 11.0	?	?	Cerna 1976
kestrel:vole ?	(13.0 - 14.0)	(10.0 - 11.0)	?	?	Cerna & Louckova 1976
snake:rat *(R. fuscipes)*	?	?	9.6 x 6.6	?	Rzepczyk 1974
snake:rat *(R.orientalis)*	8.0 - 11.0	7.0 - 10.0	9.1 x 7.7	25	Zaman & Colley 1975
opossum:cowbird, grackles	11.0 - 12.0	7.0 - 8.0	?	25	Duszynski & Box 1978
opossum:duck	10.0 - 12.0	7.0 - 9.0	11.2 x 8.2	25	"

APPENDIX 3

Reagents used in Serology

a. Phosphate buffered saline (PBS)

Na_2HPO_4	1.20g
$NaH_2PO_4H_2O$	0.22g
(or $NaH_2PO_42H_2O$)	(0.25g)
NaCl	8.5g
H_2O	to 1000ml

b. Alsever's solution

dextrose	20.50g
sodium citrate (dihydrate)	8.00g
citric acid (monohydrate)	0.55g
sodium chloride	4.20g
distilled water	to 1000ml

For use mix 1:1 with whole blood

c. Calcium magnesium saline (CMS)

NaCl	8.5g
$MgCl_2,6H_2O$	0.1g
$CaCl_2,2H_2O$	0.04g
distilled water	to 1000ml

APPENDIX 4

METHODS USED IN THE RECOVERY, CLEANING, COUNTING AND MEASUREMENT OF SPOROCYSTS

a. Extraction of sporocysts from faeces

(i) Sieving and washing

The faeces were mixed with approximately five times their volume of water in a blender for 2 minutes at low speed, sieved (mesh aperture 0.5mm) and the liquid retained. The sieve deposit was resuspended in water in the blender and resieved; the liquid was retained. The sieve deposit was then washed in the sieve until the liquid came through clear, then the deposit was discarded. The accumulated sieved washings were allowed to sediment for 24 hours at 4°C, and the supernatant was discarded. The deposit was resuspended in water and sedimentation repeated. After separation from the supernatant the deposit was centrifuged at 2000g for 5 minutes and further supernatant discarded.

(ii) Floatation

The deposit in the centrifuge flasks was resuspended in floatation liquid (NaCl aqueous solution, S.G. 1.2) and centrifuged at 2000g for 5 minutes. The surface layer was removed into a flask by vacuum and retained. The processes of suspension, centrifugation and collection were carried out three times on each sample of faeces and then the contents of the centrifuge flask were discarded. These procedures yielded a preparation that was clean enough to be examined directly for sporocysts and if, necessary, appropriately diluted for transmission studies.

When a small amount of faeces was examined, floatation was carried out in a 100ml glass tube and sporocysts were collected by

placing a cover slip on the top of the upright tube in contact with the surface film, removing it after 5 minutes and placing it on a microscope slide for examination.

If sporocysts were to be stored or measured the following additional processes were used:

(iii) Cleaning

The sporocyst preparation, obtained as above, was resuspended in at least ten times its own volume of water and subjected to centrfugation and floatation once as described above. This process resulted in a preparation suitable for storage. Sporocysts were stored in tap water at $4^{o}C$.

(iv) Cleaning with carbon tetrachloride

To obtain sporocysts relatively free of debris, 1ml of cleaned sporocysts, 25ml floatation liquid and 3ml carbon tetrachloride were shaken together for 5 seconds in a glass tube then centrifuged at 2000g for 5 minutes. The sporocysts were harvested from the surface film.

b. Counting sporocysts in faecal preparations

The preparation was made up to a known volume with tap water and then, whilst mixing the solution, a small sample was removed by pasteur pipette and used to fill one chamber of a haemocytometer. After allowing the contents to sediment for two minutes the sporocysts were counted in all the 9 $\times 1^{2}$mm areas. Ten samples of each preparation were counted. The total number of sporocysts in the preparation was estimated using the formula:

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

where V = volume of preparation

X = mean number of sporocysts per sample

c. Measurement of sporocysts

Sporocysts, prepared and cleaned as described above, were measured, in water, in both axes (maximum length and width) with a micrometer eyepiece at 400 x magnification. Only sporocysts lying with their longitudinal axis parallel to the plane of the slide were measured. Sizes of sporocysts were expressed as the range, mean and standard deviation of the length of each axis.

d. Examination of the small intestine for sporocysts

The small intestine was opened along its entire length and the mucosa scraped off. The scrapings were digested in 1 per cent sodium hypochlorite solution for 30 minutes at $0^{o}C$. The suspension was centrifuged at 2000g for 5 minutes and the supernatant discarded. The deposit was washed twice with PBS by centrifugation then examined at 400 x for sporocysts.

APPENDIX 5

FIGURE 1

FERAL CAT SURVEY: QUESTIONNAIRE - PAGE 1

NAME ..... ADDRESS .....

DATE ....

OCCUPATION ....

---

1. Have you seen feral cats (or signs of cats - droppings, tracks, etc.) in areas where you work or in areas visited during recreation?
   (circle one)
   YES/NO

   Please give details of sightings on next page

2. Have you seen any signs to show that cats are breeding in the wild, e.g. kittens or pregnant females?
   (circle one)
   YES/NO

   If YES, please add a B to the relevant sighting(s) on the next page

3. Have you seen feral cats feeding on the remains of -
   (tick for "yes")

   Sheep
   Pigs
   Deer
   Other Animals
   Give Details

4. Do you think that, in the areas you frequent, feral cats are -

   more common less common don't know (circle one)

   than they were 5 years ago?

5. Have you any further comments on feral cats?

I have seen cats at: (give approximate location, map reference if known)	Type of Country					Distance from Nearest Permanent Human Habitation			
	grassland	tussock	scrub	native bush or forest	plantation	up to 2 km.	2-10 km.	over 10 km.	don't know
1.									
2.									
3.									
4.									
5.									

APPENDIX 5 FIGURE 2 QUESTIONNAIRE PAGE 2

APPENDIX 5 TABLE 1

FERAL CAT SURVEY; NEW ZEALAND GOVERNMENT ORGANISATIONS THAT DISTRIBUTED QUESTIONNAIRES TO THEIR STAFF

Agricultural Pests Destruction Council

Animal Health Division, Ministry of Agriculture and Fisheries

Department of Lands and Survey

Ecology Division, Department of Industrial and Scientific Research

National Park Boards

New Zealand Forest Service

Wildlife Service, Department of Internal Affairs