THE ROLE OF THE CYTOSKELETON IN THE MOTILITY OF COCCIDIAN SPOROZOITES.

by

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A B S T R A C T

Despite the potential importance of motility in the coccidian life cycle, and the life cycles of other members of Apicomplexa, the cellular basis of locomotion has been the subject of only indirect and fragmentary studies. In this research project the motility of Eimeria tenella and Eimeria acervulina sporozoites was examined.

An understanding of morphology is vital to any theory of function so an extensive study of the ultrastructure of these sporozoites was undertaken. This involved techniques such as critical - point drying of whole cells and glycerination in the presence of heavy meromyosin. All procedures included buffers designed specifically to support contractile components and their interrelationships.

A behavioural study of sporozoite motility was carried out and a profile of motile activity presented. "Gliding" was the only locomotive action expressed by these sporozoites and only occurred when the sporozoite was in contact with a substratum. Microtubule inhibitors had no effect on motility or on the microtubules. Microfilament inhibitors stopped "gliding". Sporozoites were labelled with fluorescein - isothiocyanate conjugated cationised ferritin. The label moved backwards relative to the sporozoite as the sporozoite moved forwards relative to the substratum. This activity was also sensitive to microfilament inhibitors. A model of locomotion was postulated, based on a micro-filamentous, capping mechanism.

An examination of the contractile proteins present using indirect immunofluorescent techniques was inconclusive due to non - specific binding of antibodies to the sporozoites. A new fluorescent label for F actin. N.B.D. phallacidin, was used and whilst it demonstrated an extensive stress fibre system in 3T3 fibroblasts it revealed an even distribution of filamentous actin in these sporozoites. Gel electrophoresis of whole cell extracts on SDS-polyacrylamide gels revealed a protein with a molecular weight identical to actin

A model of coccidian sporozoite motility was proposed involving a capping mechanism directed by the subtending cytoskeleton. The model's relevance to host - cell invasion was experimentally examined and was shown to be consistent with current observations in invasion.

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GENERAL INTRODUCTION

The knowledge of the molecular mechanisms of intracellular motility has increased, particularly in recent years, as a consequence of advances in cell biological and biochemical techniques. There are, however, certain areas in which these techniques have not been exploited. One such area is the motility of protozoa belonging to the phylum Apicomplexa. Members of this group are all parasitic, ranging from gregarines to malarial parasites. At present there is not a satisfactory explanation for the motility of a single species in this phylum. This study is particularly concerned with the contractile basis of motility in Coccidian parasites. These protozoa will obviously have experienced different evolutionary pressures from those of free - living protozoa. Nevertheless it is logical to suggest that many areas of their biology will conform to principles demonstrated in free - living protozoa. The invasive stages of Coccidian parasites were examined because of their relatively high degree of motility and because of their availability. Cysts containing sporozoites could be stored for several months in potassium dichromate solution (Chapter 1) and "hatched" when needed.

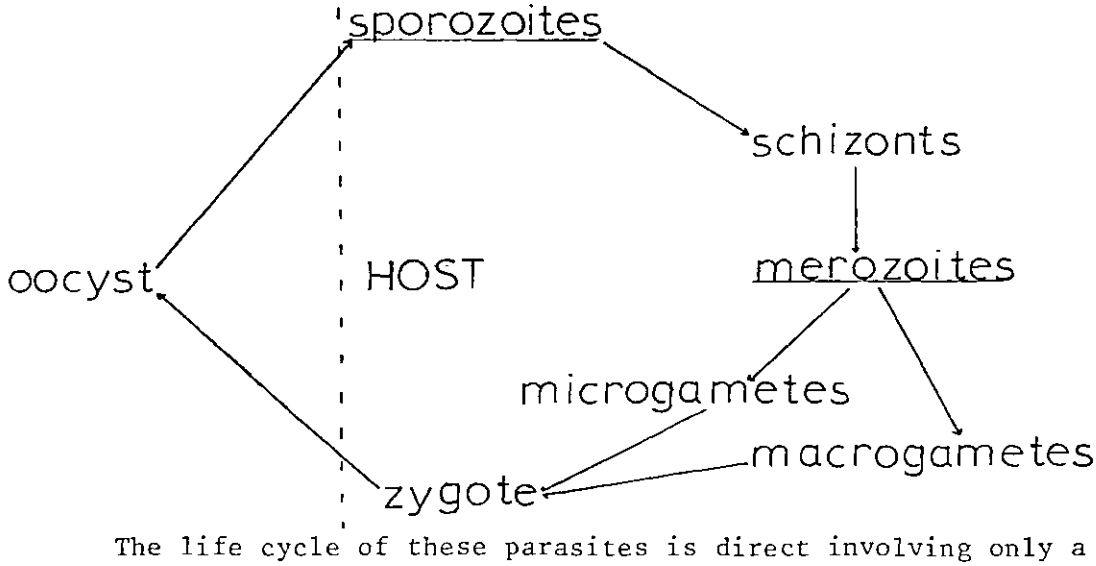
General Biology of Coccidian Parasites

The life cycles and general biology of the parasites examined and presented as background information to illustrate the functions fulfilled by these motile stages and the constraints under which they exist. The coccidia are obligate intracellular parasites with life cycles involving one or two hosts. The classification of this group has recently been revised by Levine, Corliss, Cox, Deroux, Grain, Honigberg, Leedale, Loeblich, Lom, Lynn, Merinfield, Page, Poljansky, Sprague, Varra and Wallace (1980). This system is used throughout this

study and is as follows:-

Phylum	APICOMPLEXA
Class	SPOROZOEA
Subclass	COCCIDIA
Order	EUCOCCIDIA
Suborders	EIMERIINA (includes Eimeria Sarcocystis Toxoplasma spp.)
	HAEMOSPORINA (includes Plasmodium spp.)

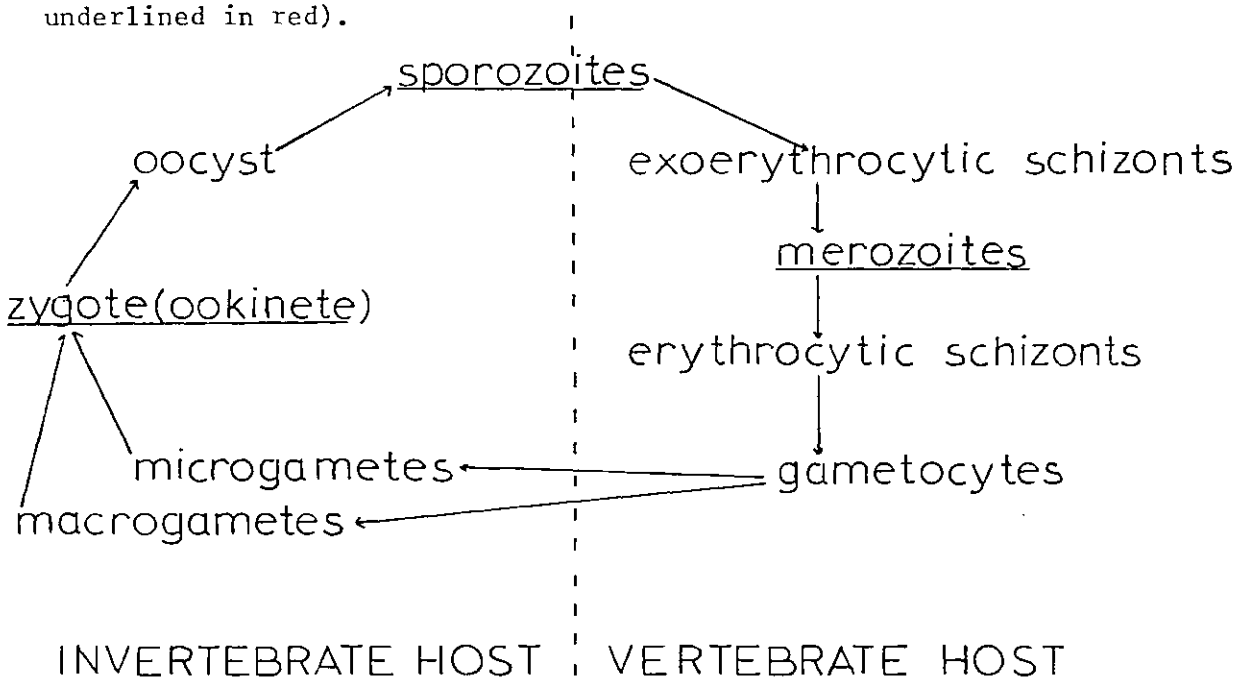
Most of this project was conducted on members of the suborder Eimeriina, although one species of Haemosporina was also examined. The bulk of experiments were conducted on sporozoites of Eimeria acervulina and Eimeria tenella. These two species both parasitise the gut of chickens. A generalised life cycle of these parasites is illustrated below (sporozoites and merozoites, the motile, invasive stages are underlined in red).



The life cycle of these parasites is direct involving only a single host. Infection is initiated by accidental ingestion of the oocyst. The trypsin in the digestion tract triggers the excystation of the sporo-

zoites. The sporozoites invade the gut lining, enter the epithelial cells and multiply by schizogony to form merozoites. Merozoites enter other gut epithelial cells and either multiply to form more merozoites or produce gametocytes. The male and female gametes fuse as a zygote. This zygote is released in the faeces as a resistant, protective cyst ready for ingestion.

In the "higher" Coccidia , such as Haemosporina, the life cycle is more complex and involves two hosts. Plasmodium is the most familiar of this group of parasites. A diagrammatic representation of its life cycle is given below (again the motile, invasive stages are underlined in red).



In these parasites infection is initiated by injection of naked sporozoites in the saliva of a mosquito, into the skin of the vertebrate host. Sporozoites are carried in the blood to the liver, enter the parenchyma cells and grow. They undergo schizogony and release merozoites. Most merozoites enter red blood cells, multiply, are released and invade more red blood cells. Some, however, form gametocytes, these wait in

the blood until taken up by a mosquito; when this happens male microgametes are released, fuse with female gametes and form ookinetes. The ookinete invades the mosquito gut wall and changes into an oocyst. Mature oocysts release sporozoites into the haemocoele, some enter the salivary gland and may be injected into a new host when the mosquito feeds.

The eimeriine parasites possess only two motile, invasive stages whilst malarial parasites have three. It is however the motility of the sporozoites of these groups that form the basis of this project, because of the ready availability of large numbers of these cells. However all these stages show certain behavioural and ultrastructural similarities, (Chapter 2 and 3) that enable numerous generalisations to be made. For ease of discussion the term "'zoite" is used to refer to all, and any, of the motile invasive stages of Coccidia.

This outline of the biology of 'zoites of Apicomplexa is deliberately kept superficial. It is only to acquaint non - parasitologists with the environments inhabited by these cells. For the purpose of this study these 'zoites are motile cells first, and parasites second.

The Biology of Cell Motility

The motile behaviour of amoebae, and other protozoa, has fascinated scientists since the invention of the simple microscope by Leeuwenhoek in the seventeenth century. The movement of animal cells is vital to enable individual cells to respond to their environment. Cell locomotion may be endogenous or exogenous in origin. Most free - living protozoa rely entirely on endogenous motility, locomotion generated by an intracellular system. Many parasitic protozoa however, exhibit both endogenous and exogenous motility. Sporozoites of Eimeria spp. "exploit"

the digestive tract of their host to transport them to the area of potential infection and sporozoites of Plasmodium spp. "use" the blood system to reach the liver. These stages do, however, have the ability to locomote and it is this endogenous locomotion that is the subject of this study.

The growth of cell motility as an area of scientific interest began in the late fifties and early sixties with the sliding filament theories of muscle contraction (Huxley and Hanson 1954) and of flagellar movement (Satir 1968). This was followed by the demonstration of the muscle proteins, actin and myosin in non - muscle cells, where, together with tubulin, they form the contractile and structural elements of most cells. Since then it has been revealed that microfilament and microtubule - based systems are responsible for a wide range of cellular functions from cell division and cytoplasmic streaming to amoeboid locomotion and phagocytosis. The almost universal occurrence of these proteins suggests that nature has, once again been highly conservative. The substructure of actin and tubulin especially has been protected through evolution and differs little between mammalian cells and protist s. Partial amino acid sequencing of echinoderm and avian tubulins revealed identical α subunits, and β subunits that differed by a single amino acid substitution (Luduena and Woodward 1973). Actin shows more changes, about 6% of the residues are different in actins from sources as diverse as Acanthamoeba and rabbit skeletal muscle, though it appears certain sequences are highly conserved and its physical properties remain the same (Korn 1978). Tubulin, actin and myosin are thought to fulfill similar roles in all cells in which they are present. The idea that skeletal and motile functions are similar in all cells is not, however,

new. As long ago as 1835 Dujardin proposed that all cells were composed of a "sarcode" that possessed structural and contractile properties similar to those of muscle. Obviously in any study on cellular motility these systems must be the first considered, however, non - actin or tubulin based motility has been demonstrated in some cells, eg. the ciliate Vorticella (Weis - Fogh and Amos 1972).

This study of the motility of Coccidian sporozoites begins with an examination of the basic ultrastructure of these 'zoites. Particular attention is paid to their cytoskeletal elements because a cell's cytoskeleton usually reveals the polarity of that cell and frequently forms the anchorage for the motile force generating elements. The motile behaviour of these 'zoites is carefully examined, previous studies tended to "assume" that 'zoite locomotion was achieved by a swimming action (reviewed by Jahn and Bovee 1968). It is necessary to record and analyse the locomotion of these parasites before proposing a possible contractile mechanism. This contractile basis of 'zoite motility is studied using anti - mitotic drugs. These experiments are combined with the use of certain surface labelling compounds. Results are used to suggest a model of 'zoite locomotion. The existence of the protein(s) necessary to this model is examined by biochemical and immunological methods.

Finally the relevance of this model to the other function of these 'zoites, host - cell invasion, is examined. Current theories do not adequately explain some of the more recent observations; an alternative model of host - cell invasion is proposed. The implications of this model to current cell motility research and to the field of parasite treatment/control are discussed.

CHAPTER 1.

EXCYSTATION AND PURIFICATION OF EIMERIINE SPOROZOITES

Oocysts of Eimeria acervulina were kindly supplied by Dr. J. Spelman (May and Baker, Ongar, Essex) and those of Eimeria tenella by Dr. R. Williams (Burroughs - Wellcome, Berkhamstead, Berkshire). These sporulated oocysts were stored in 2% potassium dichromate solution until required. It has been demonstrated that Eimeria sporozoites retain infectivity after 2 years storage in this manner (Vetterling and Doran 1969). Sporozoites used in the motility studies carried out in this project were only excysted from oocysts that had been stored in potassium dichromate for less than four weeks. After this time a distinct fall in motile behaviour could be observed.

The sporozoites were excysted, as required, by the following technique adopted from Davis (1973). Oocysts were first washed three times in distilled water using a blood centrifuge which pelleted the oocysts in 5s at 14,000g. They were then resuspended in 14% w/v sodium hypochlorite solution and left for 5 min. at $0^{o}C$ to layer out of solution. The suspension was then overlaid with distilled water and spun for 5s in the blood centrifuge. The cleaned oocysts formed a layer at the water/hypochlorite interface. The oocysts were pipetted off and washed three times in distilled water. This hypochlorite treatment weakens the oocyst wall prior to excystation. The oocysts were then resuspended in Hank's balanced salt solution (HBSS) containing:

	mg/l
$CaCl_2$	140.0
KCl	400.0
KH_2PO_4	60.0
$MgSO_4(H_2O)_7$	200.0
NaCl	8000.0
$NaHCO_3$	350.0
$Na_2HPO_4(H_2O)_7$	90.0

The blood centrifuge tube containing the oocyst suspension was then half filled with Grade 7 Ballotini beads (450 - 500 µm diameter) and shaken in a Whirlimixer until 80 - 90% of the oocysts were seen to have burst. This normally took 30 - 45s and at intervals during the procedure, samples were examined by light microscope. The released sporocysts were then washed in HBSS and resuspended in 0.5% bile salts (Difco) and 0.25% trypsin (Difco) in HBSS at 41°C. Different species of Eimeria exhibit different hatching times, E. acervulina excysted in 15 - 30 min. and E. tenella required 60 - 90 min..

The suspension of sporozoites obtained from the excystation technique contained oocyst and sporocyst shells as well as some unsporulated oocysts, these had to be removed before the sporozoites were used. Two techniques for purification were used. The first is adopted from Wagenbach (1969). Briefly, a 5ml syringe was used to hold a column of glass beads (150 - 200 µm diameter) supported by glass wool. The sporozoite suspension was passed through this column and most of the debris was removed. This technique reduced the total number of sporozoites in suspension by 50 - 70%. A more efficient

and simpler technique involved the passing of the sporozoite suspension through a 10 μm pore polyester bolt cloth filter (Henry Simon, Cheadle Heath, Stockport). This filter was held in a Millipore, Swinnex - type, filter holder attached to a syringe containing the suspension. This purified suspension of excysted sporozoites was used immediately for motility studies because the motile behaviour of the sporozoites decreased markedly with time. Sporozoites intended for other experiments were stored for up to 2 hours in HBSS at $4^{o}C$, this has been demonstrated not to unduly affect the viability and infectivity of these parasites (Millard and Long, 1974).

CHAPTER 2

THE FINE STRUCTURAL CHARACTERISTICS OF THE INVASIVE STAGES OF APICOMPLEXA.

2.1 INTRODUCTION

In the study of any cell function it is vital to possess an understanding of the anatomy of the cell under examination. In the past twenty years with the improvement in electron microscopy, many ultrastructural studies have been carried out on coccidia. In 1970 Levine established the subphylum Apicomplexa on the basis of their ultrastructural findings, this was formalised in 1980 by Levine, Corliss, Cox, Deroux, Grain, Honigberg, Leedale, Loeblish,Lom, Lynn, Merinfield, Page, Poljansky, Sprague, Vavra and Wallace. The primary characteristic of this group is the presence in at least one stage, always the "motile" infective stage, of typical fine structural features. These have been revealed and examined by electron microscopy. The taxonomic, as well as the pathogenic, importance of these invasive stages has resulted in an extensive group of publications on the ultrastructure of the 'zoites of Apicomplexa. This work may be conveniently divided into two sections. The first consists of general fine structure examinations mostly completed by the early 1970s; these form the basis for the taxonomy of the group and are reviewed by Scholtyseck (1973). The second section concerns the more recent studies on the various motile stages of Apicomplexa that have been specifically designed to examine the function, or substructure, of particular areas of these 'zoites.

The initial fine structural examinations of Apicomplexa (Garnham 1963; Garnham, Baker and Bird 1962; Andreassen and Behnke 1968; Colley 1967; MacLaren and Paget 1968; Ryley 1969; Scholtyseck and Piekarski 1965; Sheffield and Hammond 1966; Sheffield, Garnham and Shiroishi 1971; Roberts and Hammond 1970) revealed a certain consistency of basic structure in the motile infective stages. This work is summarised in Figure 2.1, a diagram of a "typical" coccidian 'zoite which was compiled from these publications and from Scholtyseck (1979). The various structures are now discussed. The pellicle (P), which forms the boundary layer of the 'zoite, varies in width between species, it is 40 nm in Eimeria (Vivier, Devauchelle, Petitprez, Porchet - Hennere Prensier, Schrevel and Vinckier 1970; Roberts and Hammond 1970; Scholtyseck Mehlhorn and Friedhoff 1970; Sheffield, Garnham and Shiroishi 1971; Aikawa 1967). It consists of a plasmalemma (PL) and an electron dense space of 15 - 20 nm diameter, with an inner layer consisting of two unit membranes. The plasmalemma is continuous enclosing the entire cell, whereas the inner membrane complex terminates near the anterior end at the polar ring (PR). The inner membranes are also interrupted at the posterior end where they thicken to form a posterior polar ring (PP). This inner membraneous layer is said to originate from the endoplasmic reticulum (Scholtyseck 1979).

In all the motile stages in the life cycle of coccidian parasites, subpellicular microtubules (MT) are present beneath the cell boundary/pellicle. These were beautifully demonstrated in Eimeria ninakohlyakimovae by Roberts and Hammond 1970 and in Sarcocystis tenella by E. Porchet - Hennere 1975. In all 'zoites these microtubules

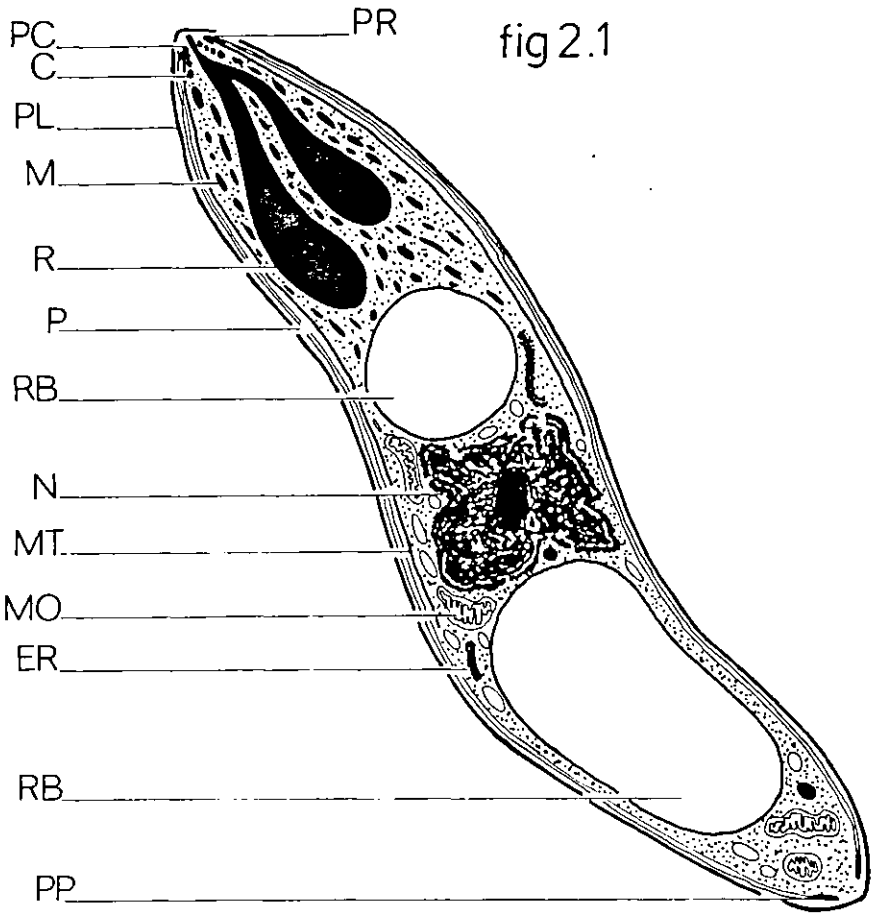


fig 2.1

A diagrammatic representation of a typical coccidian 'zoite. This cell has a complex pellicle (P) consisting of a plasmalemma (PL) and a double inner membrane layer. The double membrane layer terminates near the anterior at the polar ring (PR) and near the posterior at the posterior polar ring (PP). Beneath the pellicle lie subpellicular microtububules (MT) and conoid (C) with preconoidal rings (PC). These parasites possess secretory bodies called micronemes (M) and rhoptries (R). Also present are refractile bodies (RB), a nucleus (N), mitochondria (MO) and rough endoplasmic reticulum (ER).

are distributed around the periphery, running from the anterior end to the nuclear region or on to the posterior refractile body. The number of microtubules varies between species (Scholtyseck 1973). During these earlier studies of ultrastructure it was first postulated that these microtubules, which are present in all the motile stages of Apicomplexa, may be involved in sporozoite motility (Garnham 1966; Jahn and Bovee 1968). The polar ring (PR) which is seen as an osmiophilic thickening is thought to be formed by a thickening of the inner membrane (Scholtyseck 1979). The number of polar rings varies from one to three depending on the species. Lankestrella hylae, E. callospermophili and merozoites of Isospora sp. have two whereas merozoites of Plasmodium sp. possess three (Schmidt, Johnston and Stehbens 1967; Aikawa . 1966,1967; Roberts, Hammond and Speer 1970; Roberts Speer and Hammond 1971). The negatively stained preparations of Eimeria (Roberts and Hammond 1970) and Sarcocystis tenella (Porchet - Hennere 1975) revealed that the subpellicular microtubules were firmly anchored into the polar ring. It was proposed that these rings were involved in the movement of the conoid seen during host cell invasion (Scholtyseck 1973).

In 1954 Gustafson, Agar and Cramer observed a hollow cone - like structure at the apical tip of Toxoplasma gondii merozoites and named it the conoid. Since then,the organelle has been described in many of the Apicomplexa with the exception of the Haemosporidia and the Piroplasms. The conoid is best illustrated in the negative stained preparations of Sarcocystis tenella (Porchet - Hennere 1975). It consists of a truncated hollow cone of spirally arranged fibrillar structures from

26nm to 30nm in diameter. In the different groups of Apicomplexa the conoid varies from 0.15 - 0.4 μm in diameter at the anterior end and 0.2 - 0.5 μm in diameter at the posterior end and the length varies from 0.1 μm to 4 μm. The number of fibrillar elements making up the conoid varies slightly from 6 - 8 depending on species (Scholtyseck 1973). Many different species of coccidia have preconoidal rings (PC) (Scholtyseck, Mehlhorn and Friedhoff 1970; Sheffield and Hammond 1966). This pair of rings is connected to the anterior of the conoid. At the posterior end of the conoid lies the polar ring, there are no observable connections between the polar ring and the conoid (Sheffield, Garnham and Shiroishi 1971; Roberts, Hammond and Speer 1970; Roberts, Speer and Hammond 1971; Roberts and Hammond 1970). Sometimes the polar ring surrounds the posterior of the conoid and sometimes it surrounds the anterior tip. These observations supported the suggestion that the conoid is capable of being protruded and retracted (MacLaren and Paget 1968; Roberts and Hammond 1970).

The paired organelles or rhoptries (R) are seen as electron dense club shaped structures located in the anterior region of the 'zoites of many Sporozoa. These were first reported in Haemamoeba (Plasmodium) gallinaceum by Garnham, Bird and Baker (1960). Though these organelles frequently vary in shape and organisation they have been observed in many different species of Apicomplexa. The anterior region of these organelles are elongate and extend through the conoid. Some authors have observed empty regions of these bodies revealing a membraneous layer (Sheffield and Milton 1968) around the electron dense mass. In 1968 Schrevel demonstrated acid phosphotase activity

in the rhoptries of the gregarine Selenedium. Since then many authors have suggested that the rhoptries have a secretory function involved in the penetration of host cells by these 'zoites (Jensen and Edgar 1976). In 1974 Kilijian purified a histidine - rich protein from the 'zoites of Plasmodium falciparum. Later work (Kilijian and Jensen 1977) demonstrated that this protein interacted with the host cell membrane and enhanced cell invasion. In 1975 Lycke Carlberg and Norrby isolated a protein with similar action from Toxoplasma gondii. They called this protein the penetration factor. Werk, Dunker and Fischer (in press) mimicked the action of this protein using poly - arginine and T. gondii cystozoites. It may be speculated that such membrane - modifying proteins may be secreted by the rhoptries.

The micronemes (M) are small,densely staining ovoid structures. They vary slightly in appearance between species which may account for the early profusion of terms for these organelles ranging from "toxonemes" (Gustafson et al 1954), "cytoplasmastrange" (Scholtyseck and Piedorski 1965) "lankastrellonemes" (Garnham 1962) and finally "convoluted tubules" (Garnham 1963). The situation was saved by Jacobs who coined the term "micronemes"in 1967. The function of these bodies was thought to be secretory and it was suggested that they form a continuous system with the rhoptries (Sheffield 1966). More recent work (Mehlhorn, Senaud, Chobotar and Scholtyseck 1974) on the origin of the rhoptries and micronemes indicates that although they share a common origin they are not interconnected.

The micropore is an organelle formed in the pellicular layer. In cross - section it appears as an invagination of the

outer membrane and a thickened pore through the inner membrane (Scholtyseck and Mehlhorn 1970; Porchet - Hennere 1975). In transverse section the micropore consists of two concentric rings (Scholtyseck 1973). Micropores have been reported in almost every developmental stage of the life cycle of Apicomplexa. It is a feeding organelle, though it does occur in stages that do not feed. It was first described in Plasmodium falciparum by Garnham, Bird, Baker and Bray (1961).

Many coccidian 'zoites also have refractile bodies (RB). Eimeria possess two, one anterior and one posterior to the centrally positioned nucleus. They appear as electron dense areas with no obvious substructure. They vary in size between species. The anterior refractile body disappears shortly after the parasite has entered the host cell (Fayer and Hammond 1967). These bodies were shown to consist of amylopectin by Ryley, Bentley, Manners and Stark (1969), who suggested that, as carbohydrate containing organelles, they may act as nutrient stores in these parasite cells.

These early ultrastructural studies have been invaluable to taxonomic research into this group and have also revealed an extensive picture of the subcellular organisation of these parasites. They demonstrated that certain organelles and structures are present in all the"motile" infective stages of Apicomplexa. All 'zoites possess a trimembraneous pellicle with subtending microtubules associated with a polar ring and rhoptries and micronemes. In the mid 1970s, following several improvements in electron microscopical techniques and the application of a more cell biological approach to

parasitology, researchers began to examine structure with specific functions in mind. Workers began to test their hypotheses by monitoring changes in behaviour and relating them to ultrastructural modifications. In the last five years several experimental papers on coccidian ultrastructure have been published that greatly aid an understanding of these parasites' biology. Some of these papers, especially those with specific relevence to sporozoite motility, form the second part of this review of coccidian ultrastructure.

In 1977 Porchet and Torpier examined the pellicles of Toxoplasma and Sarcocystis 'zoites by freeze fracture. They demonstrated that the outer membrane of the pellicle is continuous and that the inner membrane complex consists of "rectangular flattened vesicles" aligned in longitudinal rows joined in a patchwork fashion. These rows followed a helical path comparable with the spiral of the sporozoite body. Sarcocystis had eleven rows whereas the number varied in Toxoplasma . The membranes of this inner complex were characterised by parallel alignments of particles on some of the faces. These particle arrays appeared to correspond to the number and arrangement of microtubules. A similar series of membrane perturbations had been reported in Plasmodium (Aikawa 1966, 1967). Also in 1977, D'Haese, Mehlhorn and Peters reported that negatively stained preparations of pellicles from Sarcocystis ovifelis, Besnoitia jellesoni and Eimeria falciformis had a mesh - like appearance. All pellicles examined revealed rib - like structures that were highly prominent towards the anterior tip of these 'zoites Further freeze fracture studies were carried out on Eimeria nieschulzi by Dubremetz and Torpier (1978); they confirmed that these 'zoites possessed a

similar set of particles and they mapped the arrangement of these perturbations. The plasmalemma's internal surface had an almost completely random distribution of intramembraneous particles. The inner membranes, however, bore regular arrays of intramembraneous particles on the outer surface of the outer membrane and the inner surface of the inner membrane. It was suggested that these arrays corresponded to the locations of the subpellicular microtubules. The authors proposed that as these structures appeared in these and other 'zoites of Apicomplexa Eimeria tenella (Ryley 1969), Eimeria necatrix (Dubremetz 1976) Plasmodium berghei (Seed, Aikawa, Prior, Krier and Pfister 1973) and Plasmodium lophurae(Aikawa 1966) that this might reflect the function of these stages. These stages are all motile and they all invade host cells.

The trimembraneous pellicle with subtending microtubules is also present in Gregarinida, subphylum of Sporozoa. In this group the presence of microtubules immediately beneath the pellicle has led to extensive research into their function during gregarine motility. The destruction of the microtubules results in the loss of motility (Schrevel, Buissonet and Metais 1974; Stebbings, Boe and Garlick 1974). More recent research has suggested that these protozoa exhibit a sliding microtubule mechanism of a type related to the ciliary axoneme (Mellor and Stebbings 1980). The similarity of the pellicle structure and of the gliding motility expressed by both coccidia and gregarines suggest a related motile mechanism. Heller and Scholtyseck (1971) have reported finding arm - like structures that connected the microtubules with the inner membrane of the pellicle.

These were observed in the developing merozoites of Eimeria stiedae. Similar microtubule/membrane linkages have also been reported in Plasmodium yoelii nigeriensis (Sinden 1978). These arm structures have, however, not been observed in any other thin sectioned preparations or in any negatively stained specimens.

Gregarines also possess other "contractile" elements. In 1980 Hildebrand demonstrated extensive arrays of microfilaments arranged along the longitudinal axis of the trophozoite of Didymophyes gigantea . He incubated the gregarines in glycerol and, by the addition of ATP to the glycerol extracted cells, caused polymerisation of developed myonemes. Such contractile elements have not been reported in any of the 'zoites of Apicomplexa.

In this chapter the cytoskeletal components of Eimeria tenella and Eimeria acervulina were examined by various techniques previously used to demonstrate contractile proteins in other eukaryotic cell types. Two mammalian cell types that have already been extensively studied were used for control purposes. These techniques were designed to visualise the gross organisation of contractile and cytoskeletal components and examine their molecular substructure. The procedures include basic ultrastructural examination, preparations of whole cells with their membranes removed by detergent treatment to reveal the cytoskeleton, and glycerol extracted cells labelled with heavy meromyosin and stained with tannic acid to define the substructure of any contractile elements present. The results of these experiments and their relevance to sporozoite motility are discussed.

2.2 MATERIALS AND METHODS

2.2.1 Procedure for general ultrastructural examination.

Suspensions of sporozoites of Eimeria acervulina and Eimeria tenella prepared as described in Chapter 1. were processed for thin section microscopy by two different methods. In the first procedure the organisms were fixed for 30 mins. at room temperature in a solution of 2.5% glutaraldehyde and 0.6% sucrose in 45 mM phosphate buffer pH 7.57. The stock buffer contained:

KH_2PO_4 6mM

Na_2HPO_4 44mM

The parasites were then washed in stock buffer, with 2% sucrose, and finally postfixed in 1% osmium tetroxide and 2% sucrose in 25mM phosphate buffer for 30 min.. In the second procedure the sporozoites were fixed in 2.5% glutaraldehyde and 2.5% tannic acid (Sigma) in 0.1M cacodylate buffer, pH 6.9, for 60 min. at room temperature. The sporozoites were then washed in 0.1M cacodylate and postfixed in either 1% osmium tetroxide or 1% uranyl acetate in 0.1M cacodylate for 60 min. at room temperature. Parasites processed by both methods were then dehydrated through either an alcohol or acetone series, 20%, 35%, 50%, 60%, 70%, 80%, 90% for 5 min. in each and 100%, three changes for 30 min. each. The samples were then infiltrated in Spurr's resin (Agar Aids) by incubating for 60 min. in 2:1 solvent to resin, followed by 1:2 solvent to resin at room temperature and 60 min. in pure resin at 4°C. The Spurr's resin was then changed and the sample was polymerised at 70°C for 9 hrs. Spurr's resin was used because it infiltrated any oocysts remaining in the sporozoite suspension better than either Araldite or Epon. This made section cutting easier.

Thin sections were then cut and placed directly onto 300 or 400 mesh support grids. The specimens were stained by placing the grid, section side down, on a saturated solution of uranyl acetate in 50% ethanol for 90 min. The grids were then washed in distilled water by running water from a wash bottle down the fine forceps that held the grid, dried and placed on Reynold's lead solution (Reynolds 1963) for 2 min. Both the Reynolds lead and uranyl acetate solutions had been cleaned by centrifugation at 14,000g for 1 min. before use. The grids were washed in 0.02M sodium hydroxide, then in distilled water and finally dried. The sections were examined in a Phillips 300 transmission microscope operated at 60KV for the low power micrographs and at 80 or 100KV for the higher magnifications needed for substructural examinations.

2.2.2 Negative staining of Eimeriine Sporozoites

Sporozoites were negatively stained in phosphotungstic acid by a technique previously employed by Roberts and Hammond (1970). Briefly, purified suspensions of sporozoites in Hank's buffered salt solution were centrifuged for 10s at 14,000g in a blood centrifuge. The pellet was then resuspended in a 1% aqueous solution of phosphotungstic acid, made up by dissolving 0.1 gm of phosphotungstic acid in 10 ml of distilled water and neutralising to pH 7 with 1 M KOH. A drop of the suspension was placed on a formvar - coated support grid and left to settle for 2 - 5 min. The excess stain was then removed by touching the grid against filter paper. The grid was then left to dry at room temperature before being examined in a Phillips 300 transmission electron microscope operated at 60 - 80 KV.

2.2.3 Critical - point drying of whole cell preparations.

A drop of Hank's buffered salt solution containing eimeriine sporozoites was placed on a formvar - coated support grid and left at room temperature for 10 min. This allowed the sporozoites to settle and adhere to the plastic surface. Initially these grids were coated in poly - L - lysine, by soaking the grids in a 0.1% aqueous solution of poly - L - lysine, m.wt. 350,000 (Sigma), for 10 min. and washing in distilled water, to increase the number and strength of the adhesion of the sporozoites to the formvar coat. This, however, was found to be unnecessary as the sporozoites were capable of adhering to the formvar directly and the poly - L - lysine only increased the background debris level. The grids with attached sporozoites were then washed three times in a microtubule/microfilament support buffer adopted from Batten, Aalberg and Anderson (1980) and Schliwa, Weber and Porter (1981). The support buffer contained:

PIPES	50mM
(Piperazine - N,N' - bis {2 - ethane sulphonic acid})	
EGTA	5mM
(Ethyleneglycol - bis - {β - aminoethyl ether} N,N' - tetraacetic acid)	
KCl	30mM
$MgCl_2$	3mM
POLYETHYLENE GLYCOL (6,000)	2%

adjusted to pH 6.9 with 1M KOH. Following washing, the grids were placed in a solution of 0.1 - 5% (v/v) Triton X - 100 in support buffer for 10s to 5 min. at 21°C. Triton X - 100 is a nonionic detergent which removes most of the membrane lipid causing dissolution

of the cell membrane. In an attempt to reduce any proteolytic damage during detergent treatment, various enzyme inhibitors were added to the buffer. These were soya bean trypsin inhibitor (BDH), chymostatin (Sigma) and phenylmethylsulphonyl fluoride (Sigma). They did not improve the number or "intactness" of the microfilaments, microtubules and intermediate filaments observed in the cells processed by this technique. Following detergent digestion, the cells were washed in buffer and fixed in 2.5% glutaraldehyde in buffer for 10 min. at 21^{o}C. Primary fixation was followed by 10 min. secondary fixation in either 1% osmium tetroxide or 1% uranyl acetate, both made up in a balanced salt solution containing 30mM KCl and 3mM Mg Cl_2 adjusted to pH 6.9 with 1M KOH. Postfixation in osmium tetroxide was normally avoided because osmium is known to cause partial dissembly of actin filaments (Pollard, Fujiwara, Niederman and Maupin - Szamier 1976; Wolosewick and Porter 1979). The grids were then dehydrated through an acetone series, 15%, 20%, 50%, 80%, 90% for 2 min. each and three changes of 100% acetone each for 2 min. The grids were then critical - point dried and stored in a vacuum dessicator. The specimens were examined in a Phillips transmission electron microscope operated at 100KV.

The extent to which this technique supported the cytoskeleton of non - muscle cells was examined by processing two mammalian cell types as controls for the sporozoite preparations. These were mouse peritoneal macrophages obtained by peritoneal washout of a freshly killed mouse (Alexander 1975) and mouse 3T3-fibroblasts maintained in

culture in Dulbecco's modified Eagle's medium with 10% foetal calf serum and 20mM HEPES. The cells were prepared for treatment by putting a suspension of cells in a flat - bottomed bijou with formvar coated gold grids at the base. The cells settled and spread on the grids. These control preparations were then processed in the same way as the grids of sporozoites.

2.2.4 Fine structure of glycerol extracted cells

Sporozoites of E. tenella and E. acervulina were pelleted before glycerination, with a 10s spin in a blood centrifuge. Controls of mouse 3T3 fibroblasts were settled onto glass coverslips and glycerinated in situ. Both cell types were then incubated in cold ($4^{o}C$) 50% glycerol, prepared by diluting glycerol with an equal volume of a buffer designed to support contractile proteins, adapted from Taylor (1976) consisting of:

PIPES	5.0mM
EGTA	5.0mM
K Cl	30.0mM
$Mg\ Cl_2$	1.0mM
$Ca\ Cl_2$	2.0mM

adjusted to pH 6.9 with 1M KOH.

This buffer differed from the buffer used in the critical - point drying technique because the presence of glycerol meant that only a reduced buffer capacity was needed and the presence of a trace of Ca^{++} was to support polymerised actin during the prolonged incubation. In some preparations 1.0mM Na_2 ATP was added to the buffer and the preparations were allowed to warm to room temperature

at some stage during the incubation in an attempt to polymerise any G actin to form microfilaments, these preparations showed no apparent change. The cells were incubated in 50% glycerol ($4^{o}C$) for between 2 hr. and 72 hr. This glycerol solution was then replaced with fresh 25% glycerol for 15 min. followed by 5% glycerol for another 15 min., both solutions at pH 6.9 in the same support buffer. The cells were then washed in a balanced salt solution containing 30mM K Cl and 1.0mM Mg Cl_2 before being fixed for 30 min. at $4^{o}C$ in 2.5% glutaraldehyde and 5% tannic acid in the balanced salt solution (pH 6.9). The sporozoites and fibroblasts were then washed and stained for 30 min. in 1% uranyl acetate in the balanced salt solution. The cells were then dehydrated through an acetone series, as for general ultrastructural examinations, and embedded in Spurr's resin. Thin sections were then cut, stained and examined in a Phillips 300 transmission electron microscope operated at 80 - 100 KV.

2.2.5 Heavy meromyosin labelling of glycerol - extracted cells.

Myosin was extracted and purified from rabbit skeletal muscle by a technique adapted from Keilley and Harrington (1959). In summary, rabbit back muscle was dissected out, chilled on ice and minced. It was then extracted for 10 min. in three volumes of 0.5M K Cl in 0.1M $K_2 HPO_4$ at $4^{o}C$. The resulting suspension was centrifuged at 15,000rpm* for 15 min. and the supernatant retained. The pH was readjusted to 6.6 with 0.5M acetic acid and the solution was diluted by a factor of ten with cold distilled water. The precipitate was sedimented in a large capacity centrifuge at 3,000rpm for 20 min. The pellet was redissolved in 2M K Cl and the final salt content was readjusted

* All spins were carried out in an MSE titanium angle rotor head (type 43114-103), which, at 15,000 rpm, developed 18,000g.

to 0.5M K Cl. This was spun for 15 min. at 15,000rpm. The supernatant was retained and diluted to 0.04M K Cl with cold, distilled water. The precipitate was centrifuged down and retained. The pellet was then dissolved in 2M K Cl, readjusted to 0.5M K Cl and then a saturated ammonium sulphate solution, pH readjusted to give pH 7 at a 1:10 dilution, was added to yield a final saturation of 40%. The solution was centrifuged and the pellet was discarded. The ammonium sulphate concentration was increased to 50%, the solution was centrifuged, and the pellet was retained. The pellet of myosin was finally resuspended in 0.5M K Cl and dialysed overnight against 0.5M K Cl and 10mM EDTA to solubilise the myosin.

The myosin was cleaved into light and heavy chains by the technique of Lowey and Cohen (1962). Myosin, at 13.5 mg/ml in 0.5M K Cl in 0.1M phosphate buffer, pH 7.0, was mixed in a ratio of ten volumes to one with trypsin (Sigma, grade 1), 0.5 mg/ml in 0.001M H Cl. Digestion was allowed to proceed at room temperature for 5 min. The reaction was terminated by the addition of one volume of soya bean trypsin inhibitor (BDH), 1.0 mg/ml in distilled water adjusted to pH 7.4, to every volume of trypsin used. Light meromysin and undigested myosin were precipitated by dialysing the mixture against cold 7mM phosphate buffer pH 7.0. The precipitate was pelleted by centrifugation at 100,000g for 1 hour. The supernatant contained the heavy meromyosin (HMM). The heavy meromyosin was store by lyophilisation with 2 mg of sucrose added for each mg of HMM in solution.

Heavy meromyosin was used to label actin filaments in situ. Both the mouse fibroblasts and the eimeriine sporozoites were glycerinated

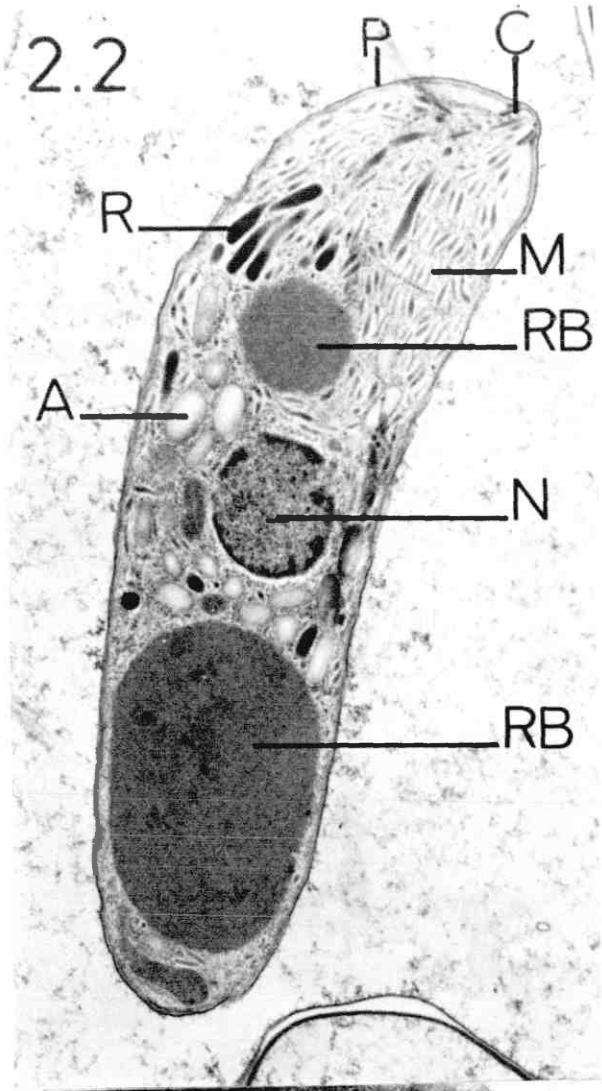
in 50% glycerol in support buffer by the same technique used for glycerol extracted cells. However, the 25% glycerol solution that replaced the 50% glycerol solution, also contained 2 - 4 mg/ml of HMM. The cells were incubated in this solution for 2 - 12 hours at $4^{\circ}C$. The buffer used was the same one used for standard glycerol extraction. It contained no Na_2 ATP because this nucleotide causes the dissociation of the myosin head or HMM from the actin filament. This takes place prior to the bending of the myosin head that results in the sliding of the two filaments relative to each other. The cells were then processed for thin section electron microscopy by the same technique employed on the glycerol extracted cells.

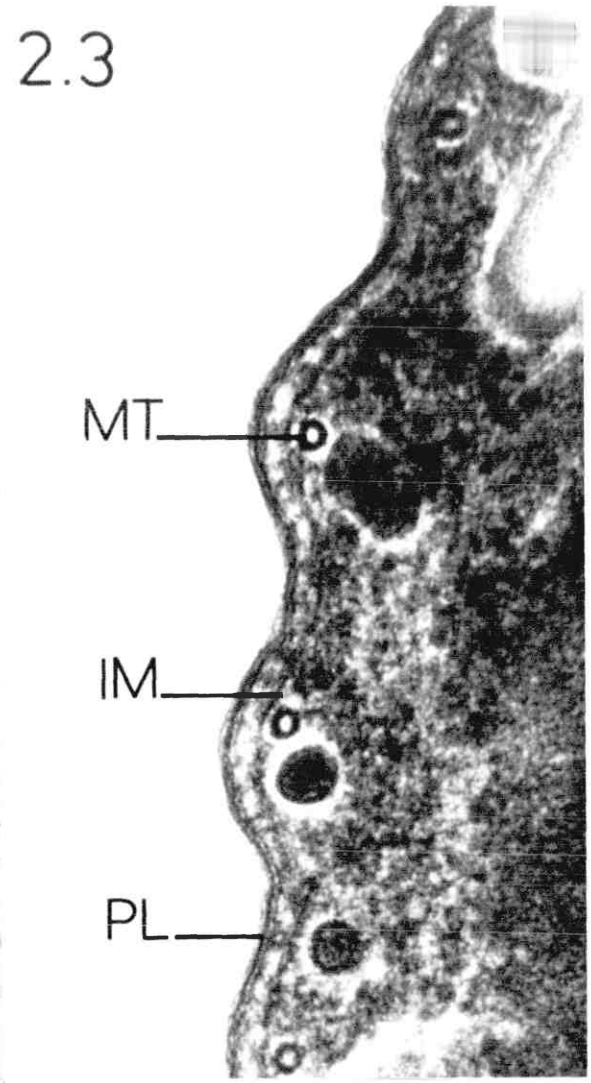
2.3 RESULTS

2.3.1 General ultrastructure of coccidian sporozoites

Despite the extensive ultrastructural research on coccidian sporozoites already published it was necessary to examine the general characteristics of Eimeria tenella and Eimeria acervulina to confirm their structural organisation. The general anatomy of the motile, vermiform sporozoites of Eimeria is illustrated in figure 2.2 This sporozoite of E. tenella shows the cells refractile bodies, the lipid - filled vesicles (Frandsen, 1970) the amylopectin granules (Ryley 1973) and the nucleus. The sporozoite also possesses several organelles that are found in all the motile stages of coccidian parasites. These are the trimembraneous pellicle, conoid, polar rings and associated subpellicular microtubules, the rhoptries and micronemes,and the micropores.

The pellicle consists of an outer unit membrane, the plasmalemma, and an inner layer of two unit membranes (fig. 2.3). The spacing between the plasmalemma and the double inner membrane varies from 18 - 21nm. The plasmalemma is continuous, covering the entire cell. The inner membrane terminates at the anterior polar ring (fig. 2.1 & 2.2) and at the posterior end just short of the posterior polar body (fig. 2.1 & 2.2). The polar rings and the conoid are at the anterior tip of the sporozoite. The hindmost polar ring forms the anchorage site for the subpellicular microtubules (fig. 2.4), whilst the anterior polar ring acts as a "seat" for the conoid. The conoid itself consists of two preconoidal rings with several helically wound microtubules of regular diameter (24nm) (fig. 2.5). Through the


2.2
P
C
R
M
RB
A
N
RB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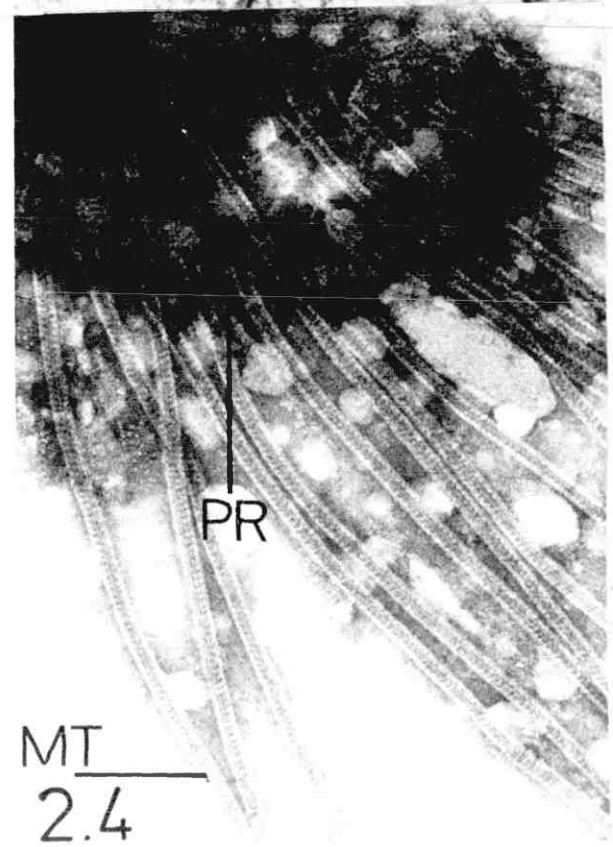
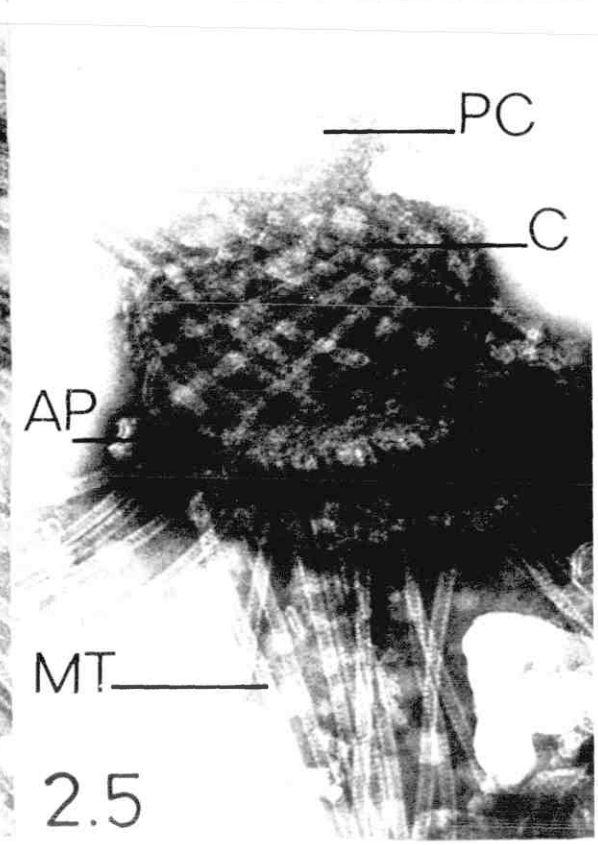
MT
IM
PL


PR
MT
2.4


PC
C
AP
MT
2.5

Figs. 2.2 and 2.3. Electron micrographs of thin sections of Eimeria tenella sporozoites (processed in phosphate buffer).

Fig. 2.2. L.S. revealing the general body organisation, conoid (C), trimembraneous pellicle (P),and rhoptries (R) and micronemes (M), that characterises these invasive stages. Also visible is the nucleus (N), two refractile bodies (RB) and amylopectin granules (A) X 15,000.

Fig. 2.3. T.S. of the sporozoite's pellicle demonstrating the sub-pellicular microtubules (MT) associated with the double inner membrane complex (IM). The plasmalemma (PL) is continuous over the parasite. X 165,000.

Figs. 2.4 and 2.5. Microtubules from Eimeria acervulina sporozoites, negatively stained with phosphotungstic acid.

Fig. 2.4. The microtubules (MT) are anchored at their anterior end to the hindmost polar ring (PR) which lies immediately behind the anterior polar ring with its associated conoid. X 109,000

Fig. 2.5. The conoid (C) consists of two preconoidal rings (PC) with a "basket" of helically wound microtubules seated on the anterior polar ring (AP). X 96,000

centre of the conoid run two straight microtubules; these microtubules show no apparent connection with the conoid or any other part of the microtubular cytoskeleton.

The subpellicular microtubules are anchored to the hindmost polar ring and extend 7 - 10 μm down the sporozoite body. The number of subpellicular microtubules is fairly constant within a species but tends to differ between species, though E. acervulina and E.tenella both have between 23 and 24 microtubules. The microtubules of these sporozoites run directly beneath the trimembraneous pellicle. Sporozoites prepared by the two techniques used for general fine structure examinations reveal microtubules that have no obvious side projections or bridges associated with the membranes. The high magnification electron micrograph of a negatively stained preparation of E. acervulina reveals a distinct periodicity of 8nm in the substructure of these microtubules (fig. 2.6). This technique also failed to demonstrate any side arms associated with these microtubules.

The rhoptries and micronemes are electron dense bodies located in the anterior third of the sporozoite body (fig. 2.1 & 2.2). The rhoptries are large club - shaped organelles that run into and through the conoid (fig. 2.7). The micronemes are small, ovoid, osmiophilic bodies that are,in Eimeria, randomly arranged within the anterior third of the sporozoite (fig 2.1 & 2.2). The micropore consists of an invagination of the outer membrane, the plasmalemma, and two thickened rings formed by the inner bimembraneous layer. The plasmalemma is continuous through the invagination whilst the inner membranes form a pore.

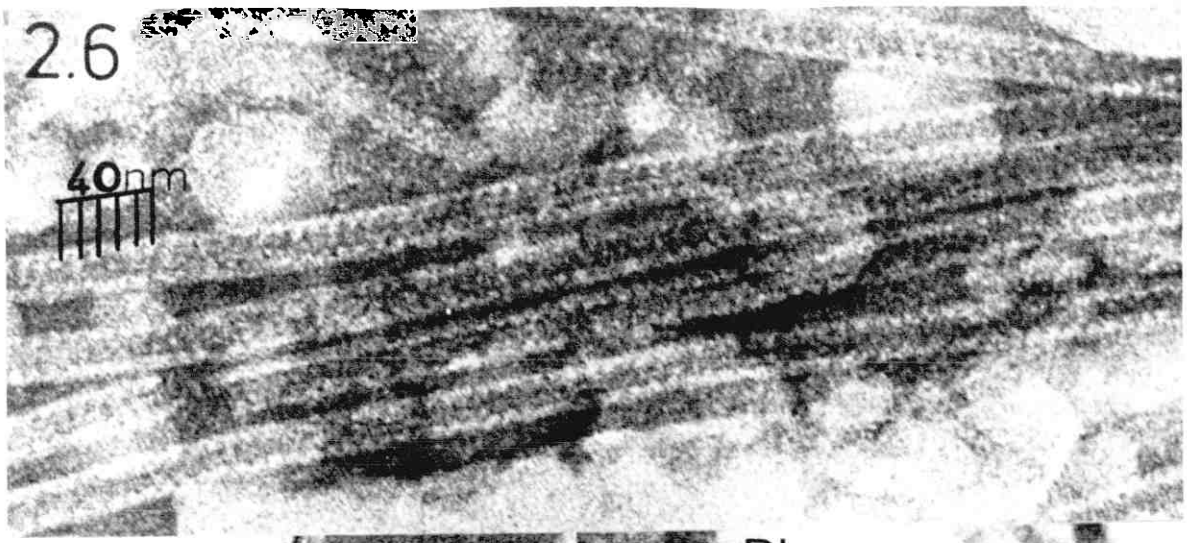
Fig. 2.6. Negatively stained microtubules of E. acervulina. The 8 nm periodicity of the tubulin subunits is clearly visible. X 320,000

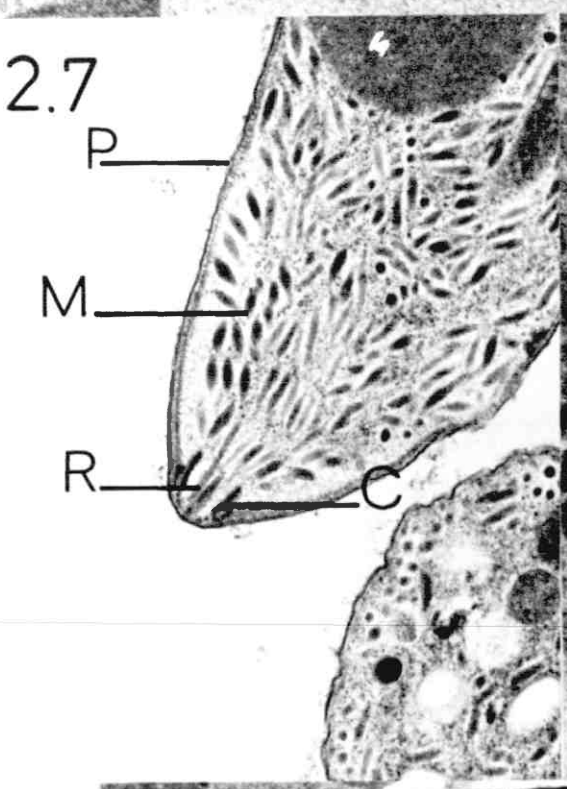
Fig. 2.7 to 2.9. Electron micrographs of thin sections from coccidian 'zoites. (All samples were processed in cacodylate buffer with tannic acid).

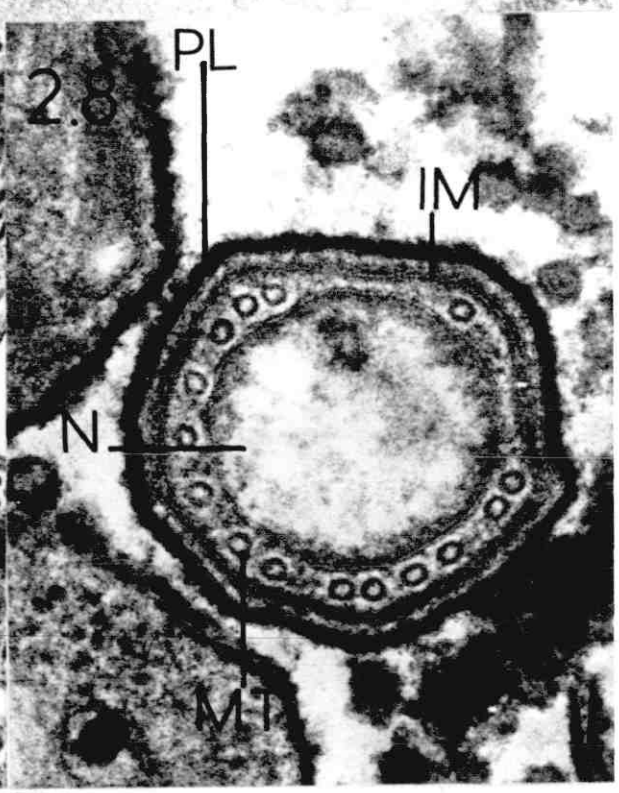
Fig. 2.7. L.S. through the conoid (C) of a sporozoite of E. tenella. The rhoptries (R) can be seen running through the conoid. Also visible are the micronemes (M) and the trimembraneous pellicle (P). X 26,000

Fig. 2.8. T.S. through the nucleus (N) region of a sporozoite of P. yoelii. These cells also possess subpellicular microtubules (MT), a double inner membrane complex (IM) and a plasmalemma (PL) X 112,000

Fig. 2.9. L.S. of a cytozoite of S. ovicanis. These 'zoites have a vermiform shape like that of Eimeria spp. The subcellular organisation with conoid (C), micronemes (M) and nucleus (N) is similar to fig. 2.2. X 18,500


2.6
40nm


2.7
P
M
R
C


2.8
PL
IM
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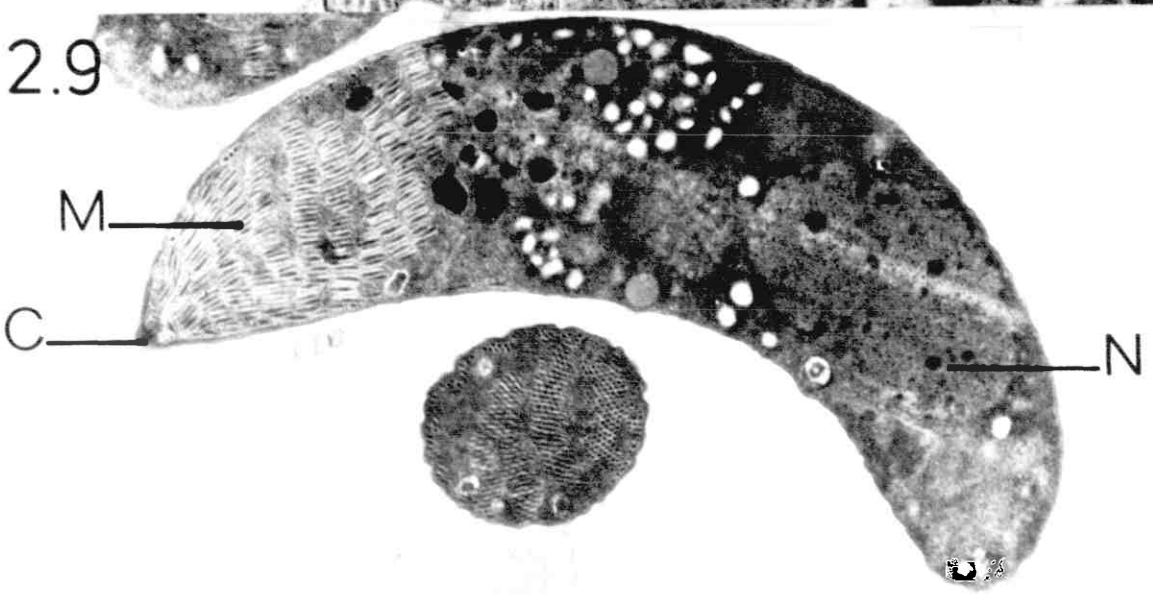
MT


2.9
M
C
N

The structures characteristic of these motile stages of eimeriine parasites, the trimembraneous pellicle, the polar ring and associated microtubules and the rhoptries and micronemes are present in all the other infective stages of the members of the subphylum Apicomplexa. The cross - section of a sporozoite of Plasmodium yoelii (fig. 2.8) reveals the same trimembraneous pellicle with its subtending microtubules. The highly developed surface coat is not usually seen on sporozoites taken directly from the oocyst and kept in serum - free medium. This technique had not previously been used to process malarial parasites and it raises the following two points. Either this surface coat is an artefact generated by tannic acid fixation or it is present in, but not resolved by, previous studies (Sinden 1978). This question merits further attention because of the possible involvement of this surface coat in motility and host cell invasion (Schulman, Oppenheim and Vandenberg 1980; Turner 1981). Another micrograph of a thin section through another member of Apicomplexa, Sarcocystis ovicanis illustrates the remarkably conservative organisation of structure reflected by coccidian 'zoites (fig. 2.9). In Sarcocystis the micronemes are arranged into rows unlike the random distribution seen in Eimeria.

2.3.2 Three dimensional visualisation of the intact cytoskeleton of Eimeria Sporozoites.

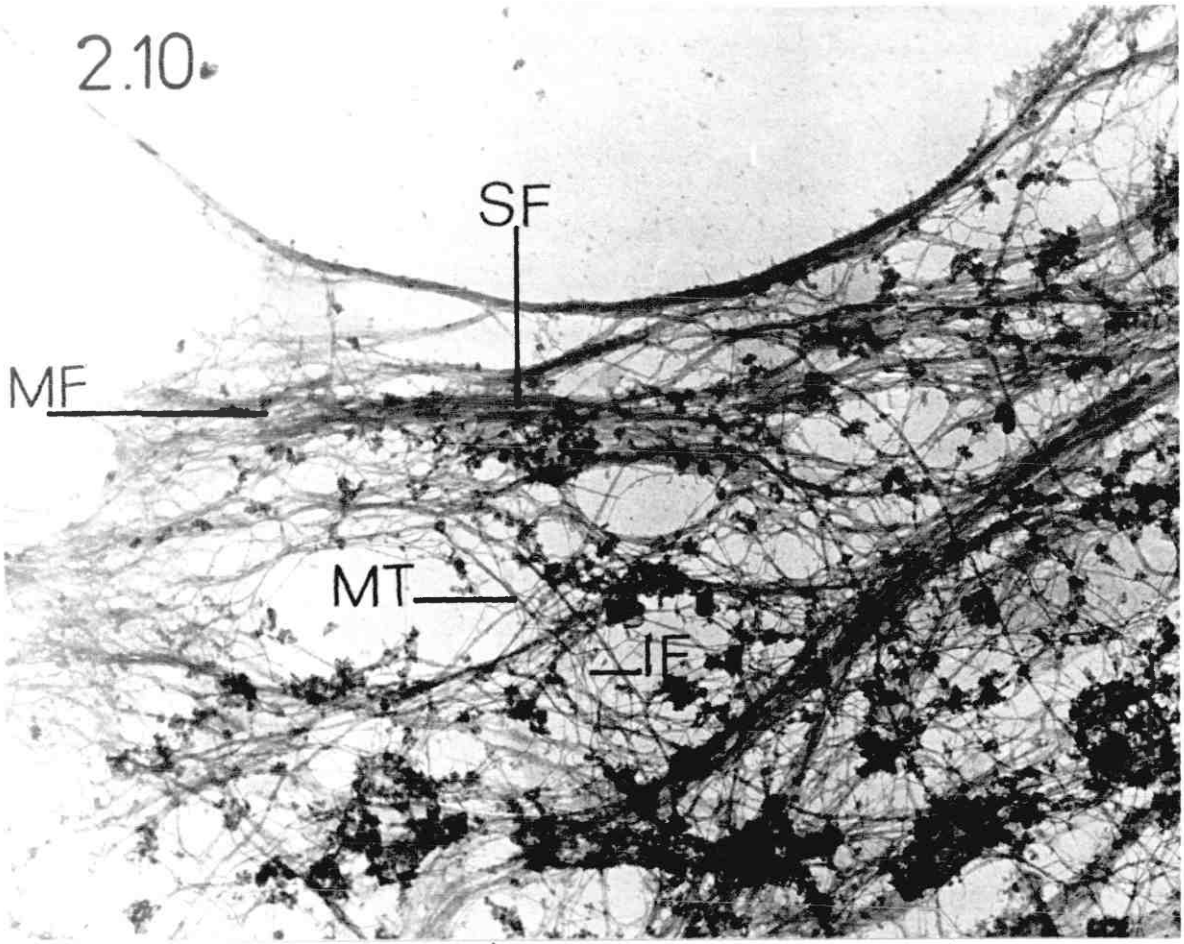
The two mammalian cell types processed as controls were mouse 3T3 fibroblasts and mouse peritoneal macrophages. The fibroblasts revealed an elaborate network of microtubules, intermediate filaments and microfilaments (fig. 2.10). Most of the microfilaments were

Fig. 2.10 and 2.11. Mammalian cells with their plasmalemma removed by detergent treatment, then fixed, stained and critical - point dried.

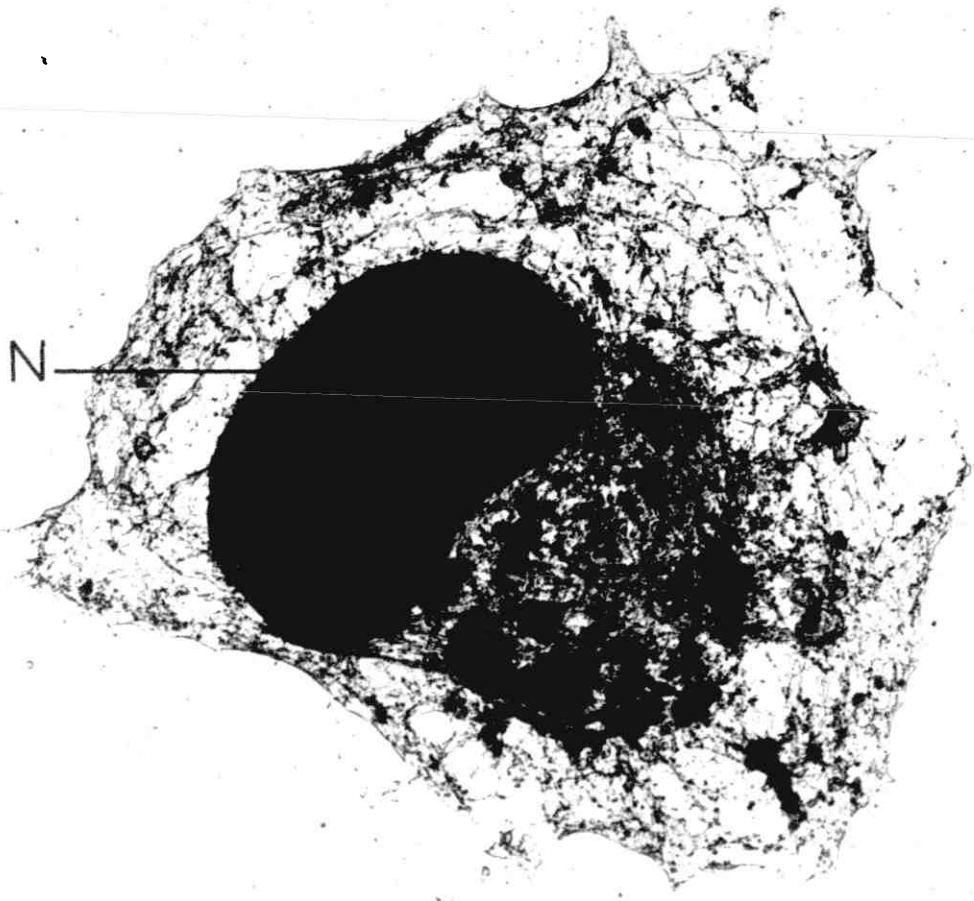
Fig. 2.10. A mouse 3T3 fibroblast, extracted in 0.2% Triton X - 100 and 2% polyethylene glycol in support buffer for 30 s. The cell has an extensive cytoskeleton of microtubules (MT), intermediate filaments (IF) and microfilaments (MF). Most of the microfilaments are arranged in stress fibres (SF). X 22,500

Fig. 2.11. A mouse peritoneal macrophage treated in 0.2% Triton X - 100 and 2% polyethylene glycol in support buffer for 2 min. The nucleus (N) lies within a network of microtubules and individual microfilaments. These cells lack the stress fibre system seen in the fibroblasts. X 4,290

2.10



2.11



arranged in stress fibres extending from cell/substratum adhesion sites into the main cell body. The arrangement of the actin skeleton of fibroblasts has been extensively described from thin - section transmission electron microscopy by Badley, Woods, Smith and Rees (1980). The macrophages, which are extremely plastic cells, were shown to lack this extensive stress fibre system. They do however possess a highly organised cytoskeleton of microtubules, microtrabeculae and individual actin filaments (fig. 2.11). These control cells clearly demonstrated that cells processed by this technique retain their network of microtubules, microfilaments and other cytoskeletal elements.

Sporozoites of E. acervulina processed by detergent digestion and critical - point drying revealed an organised system of sub pellicular microtubules in close association with the cells trimembraneous pellicle (fig. 2.12). The pellicle of these sporozoites was more resistant to digestion by Triton X - 100 than the "simple" plasmalemma of the control cells. This meant that the details of the cytoskeletal elements were obscured by the cytoplasm and organelles retained by the parasite (fig. 2.12). Attempts to increase digestion by prolonged incubation in Triton X - 100 and the use of a stronger detergent, Nonidet P - 40, resulted in damage to the microtubular cytoskeleton (fig. 2.13). Fortunately, during one preparation a batch of "holey" grids was used in error. The holes in the formvar membrane had allowed the cytoplasm and its organelles to drain out through the detergent weakened pellicle and through the holes in the grid. This technique allowed examination

Figs. 2.12 to 2.15. Sporozoites of E. acervulina extracted in detergent, fixed, stained and critical - point dried.

Fig. 2.12. This sporozoite was treated in 2% Triton X - 100 with 2 mM soya bean trypsin inhibitor and 2% polyethylene glycol in support buffer for 15 min. The nucleus (N), conoid (C), collar (CO) and some microtubules (MT) are visible, however, much of the cytoskeleton is obscured by the pellicle. X 15,500

Fig. 2.13. Treatment in 0.5% Nonidet P - 40 in support buffer for 3 min. has disrupted the organisation of the subpellicular microtubules. X 10,500

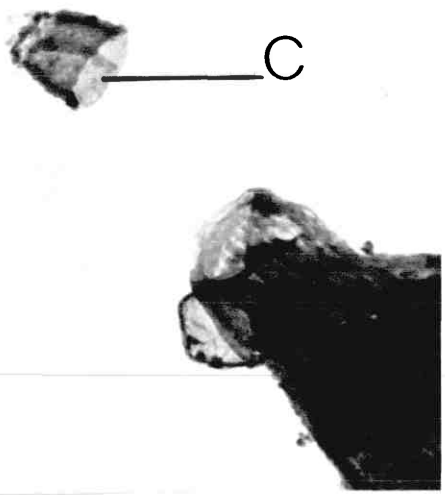
Fig. 2.14. This sporozoite, processed on a holey grid in 2% Triton X - 100 and 2% polyethylene glycol in support buffer for 15 min., has lost much of its cytoplasm through the ruptured pellicle. The conoid (C), collar (CO), micropores (MP) and subpellicular microtubules (MT) are seen clearly. The microtubules are closely associated with the pellicle and follow a helical path extending about half the length of the sporozoite. X 16,400

Fig. 2.15. The conoid (C) has become separated from the rest of the microtubular cytoskeleton (this sample was prepared as in fig. 2.12). X 43,500

2.12

2.13

2.15



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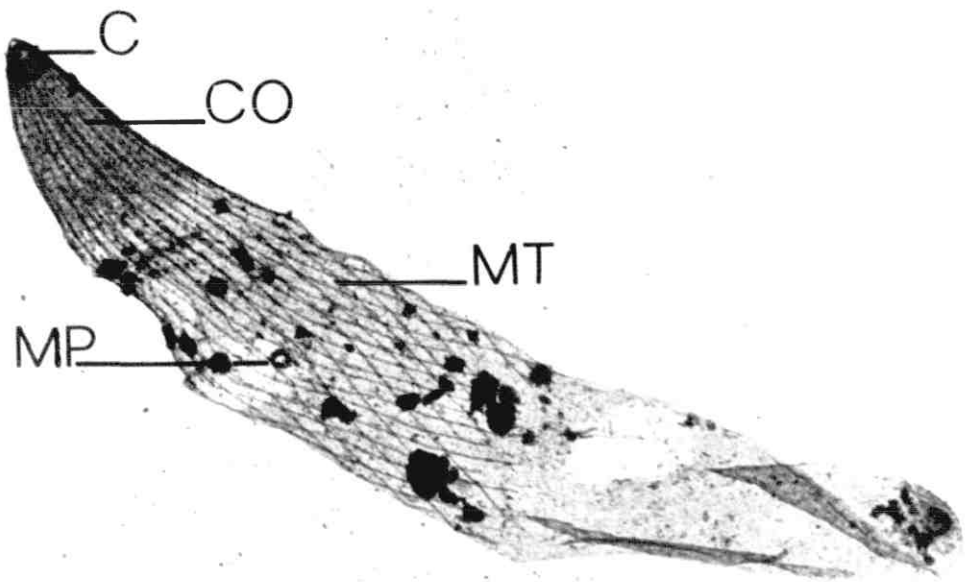


of the three - dimensional organisation of these microtubules within the cell. The sporozoites processed on these grids had a clearly defined microtubular cytoskeleton (fig. 2.14). In E. acervulina these microtubules are firmly anchored in the posterior polar ring. This polar ring was only loosely associated with the conoid which could be easily separated, intact, from the rest of the microtubular cytoskeleton (fig. 2.15). From the posterior polar ring the subpellicular microtubules ran, relatively straight, for 2 - 3 μm through the collar region of the 'zoite (fig. 2.14). On leaving the collar they twisted into a spiral arrangement comparable with the sporozoites helical body shape (fig. 2.14). The microtubules extended for 7 - 10 μm down the body of the sporozoite. The two stereo pairs of critical - point dried sporozoites (fig. 2.16 & 2.17) demonstrated the three dimensional organisation of microtubules within the sporozoite. They underline the close association that exists between the membranes and the subpellicular microtubules. Both figures illustrate the helical arrangement of the microtubules which appears to dictate the characteristic spiral shape of the sporozoite. The rigidity and tensile strength of this tubular skeleton is demonstrated in fig. 2.18 when the microtubules were separated from the pellicular membranes by prolonged detergent treatment. The pellicle had lost its shape whereas the microtubular cytoskeleton had retained its structure. The subpellicular microtubules possessed no obvious side arms or projections and had no terminal caps, or specialised anchorage sites, at their posterior ends (fig. 2.19). The sporozoites of E. acervulina examined by this technique showed 2 - 3 micropores per

Fig. 2.16 and 2.17. Stereoscopic pairs of sporozoites of E. acervulina extracted in 2% Triton X - 100 with 2 mM phenylmethylsulphonyl fluoride in support buffer for 10 min.

Fig. 2.16. This stereoscopic pair illustrates the close association between the subpellicular microtubules and the pellicle. This sporozoite has two micropores (arrowed). X 21,200

Fig. 2.17. This preparation has 3 micropores (arrowed) and a pronounced collar region. The hole in the pellicle through which the cytoplasm has drained is also visible. X 12,500

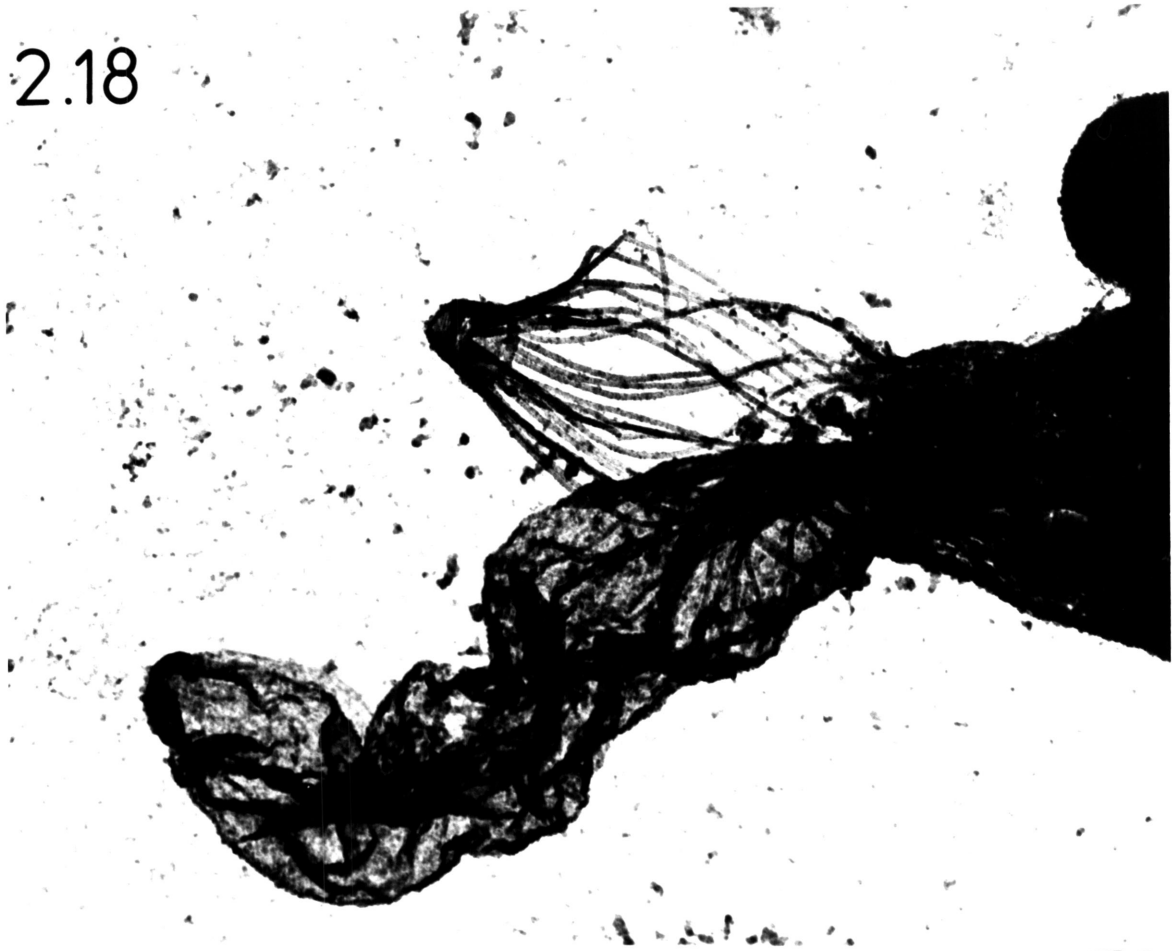
2.16

2.17

Fig. 2.18 and 2.19. Detergent - extracted, critical - point dried, sporozoites of E. acervulina.

Fig. 2.18. This sporozoite was treated in 5% Triton X - 100 in support buffer for 15 min. The microtubular cytoskeleton is intact and retains much of its structural organisation despite being separated from the pellicle. X 25,300

Fig. 2.19. High magnification electron micrographs of a parasite extracted in 2% Triton X - 100 with 2 mM phenylmethylsulphonyl fluoride in support buffer for 10 min. The subpellicular microtubules lack any obvious side projections and terminate without any structural specialisations. The pellicle has a net - like appearance.
X 57,500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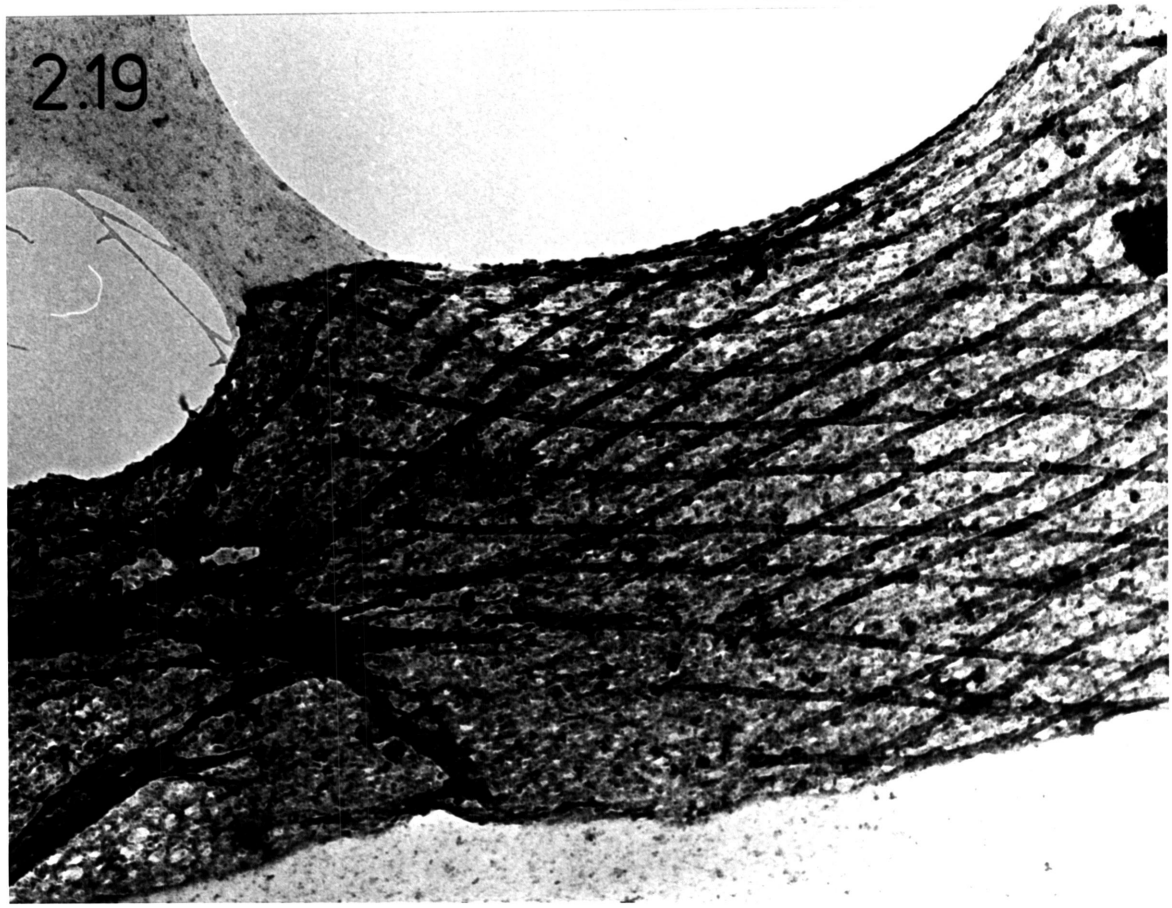

2.19

sporozoite (fig. 2.12, 2.14, & 2.16). The thickenings of the inner membranes to form the pore of the micropore were clearly seen in these sporozoites. Despite careful and extensive examination there was no apparant, and certainly no obvious, microfilamentous system revealed by this technique.

2.3.3 Examination of the substructure of the coccidian cytoskeleton.

Mouse 3T3 fibroblasts had been used in previous research examining actin filaments in cells. As a result of this work these fibroblasts were chosen as control cells to monitor and improve the technique that was then applied to the coccidian sporozoites. Incubation in glycerol resulted in the removal of the cells' cytoplasm and various organelles and revealed the cells' cytoskeleton, free from background material (fig. 2.20). The definition in these preparations was greatly enhanced by the use of tannic acid in the primary fixative solution. The tannic acid behaved as previously reported (La Fountain, Zobel, Thomas and Galbraith 1977) and stained the periphery of the cells' filamentous elements. This meant that the microtubules, intermediate filaments and microfilaments were densely stained and clearly defined (fig. 2.20). The clearest micrographs came from the preparations that were postfixed in uranyl acetate rather than osmium tetroxide.

This technique was then applied to sporozoites of E. tenella and E. acervulina. Incubation in glycerol has an identical effect to that seen in the fibroblasts; all the cytoplasmic "background" was removed. The microtubules were well defined so that the thirteen individual protofilaments could be clearly seen (fig. 2.21). This

Figs. 2.20 to 2.24. Cells extracted in glycerol in a microfilament support buffer.

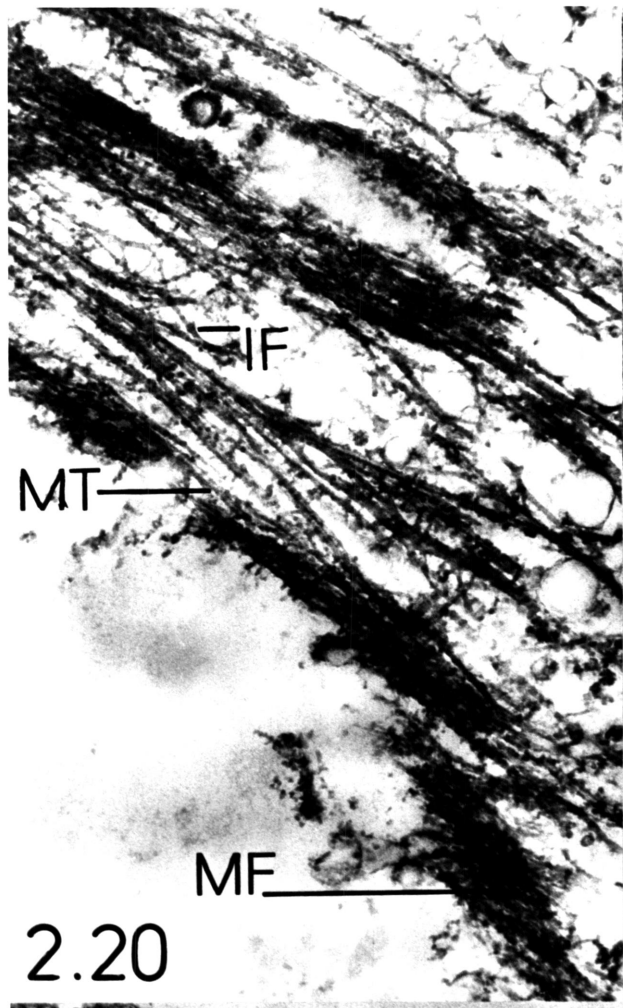
Fig. 2.20. Mouse 3T3 fibroblast. Much of the cytoplasm has been removed by glycerol treatment. The cytoskeletal elements, microtubules (MT), intermediate filaments (IF) and microfilaments (MF) are clearly visible. The arrangement of the cytoskeleton is similar to that seen in the critical - point dried preparations (fig. 2.10). X 38,300

Fig. 2.21. A partially sporulated oocyst of E. tenella. The thirteen protofilament substructure of these microtubules is well defined. X 355,000

Fig. 2.22. A sporozoite of E. acervulina. In this preparation the microtubule has a "peg" (arrowed) on the side closest to the pellicular membranes. X 1,114,000

Fig. 2.23. The pellicle of a sporozoite of E. acervulina, with its plasmalemma (PL) and double inner membrane complex (IM). There are no filamentous structures visible in this preparation. X 245,000

Fig. 2.24. Mouse 3T3 fibroblast extracted in glycerol and incubated in heavy meromyosin. The intermediate filaments (IF) are comparable with those in fig. 2.20. The microfilaments (MF) are decorated with the "arrowhead" formation typical of actin/HMM interaction. X 43,500



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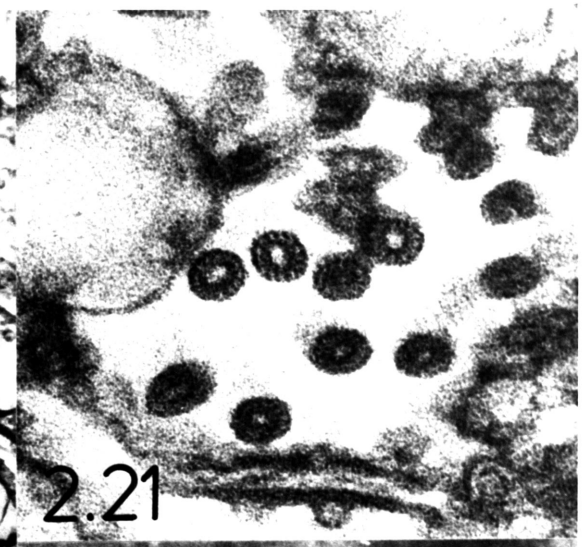

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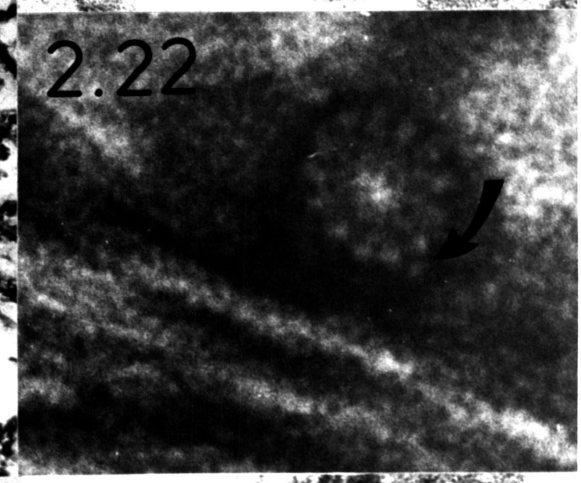

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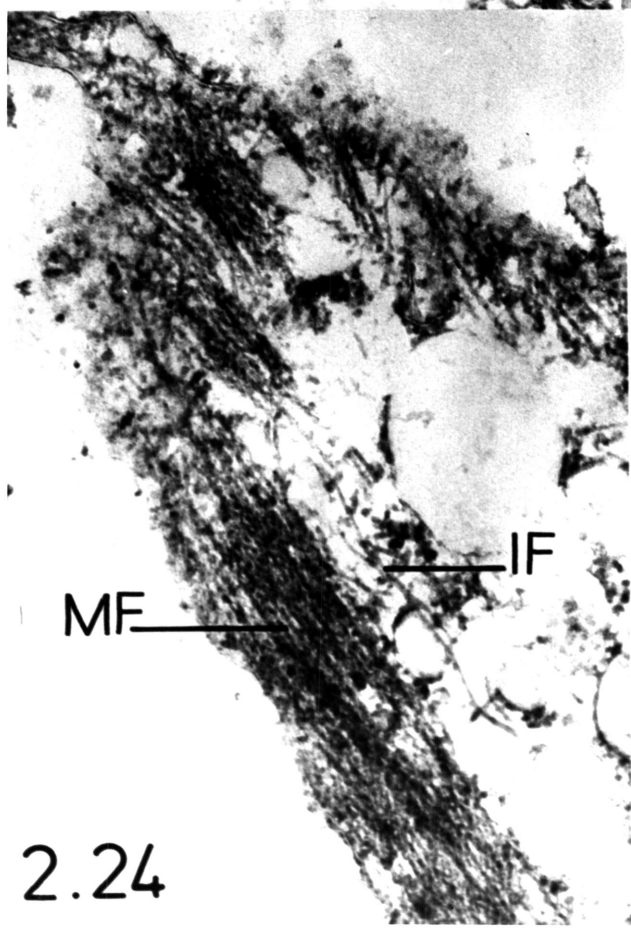

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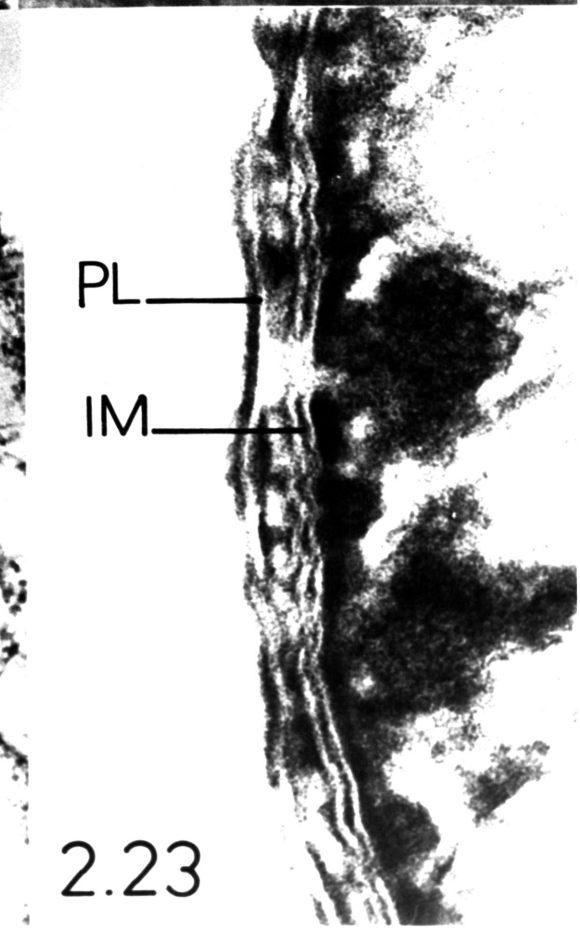


2.23

suggested very strongly that, contrary to earlier speculations (Jensen and Edgar 1976) as to the "normality" of these tubules, their sub-structure was consistant with that demonstrated in microtubules from other sources. In contrast to the other techniques used to examine E. tenella and E. acervulina this technique showed that a few of the microtubules in the thin sections had a peg, or globular protein, associated with the side of the microtubule closest to the membrane (fig. 2.22). However, most of the microtubules examined did not possess this apparent specialisation. The plasmalemma and the underlying double membrane layer were separated by this technique and the trimembraneous nature of the pellicle was clearly demonstrated (fig. 2.23). Despite careful examination, especially of the areas around the microtubules and between the plasmalemma and the inner membranes, no filaments were observed in these specimens.

The heavy meromyosin head of rabbit skeletal myosin was used as a probe in an attempt to label actin filaments in the 3T3 fibroblasts and in the eimeriine sporozoites. The fibroblasts were incubated in glycerol and heavy meromyosin HMM, and on examination, revealed an array of "arrowhead" filaments (fig. 2.24). This was typical of the pattern seen in preparations of HMM decorated actin filaments. When the HMM was added to the preparation of glycerinated sporozoites no such pattern was detected either in the general cell body, in association with the subpellicular microtubules, or, as far as could be seen, between the plasmalemma and the inner membranes.

2.4 DISCUSSION

The basic ultrastructure of the motile, invasive stages of Apicomplexa has been the subject of numerous detailed descriptions, mainly from thin sectioned material. This present study confirmed that the basic ultrastructure of coccidian sporozoites was mirrored by both Eimeria acervulina and Eimeria tenella. Both these motile invasive cells had refractile bodies, amylopectin granules, lipid particles and a nucleus positioned centrally in the sporozoite body. But the organelles that identify these cells as the infective stages of coccidia are the trimembraneous pellicle, the subpellicular microtubules with their polar rings and conoid, and the rhoptries and micronemes.

The pellicle was not closely studied in this project because of the extensive and detailed examinations that had been carried out previously on 'zoites of coccidia, including Eimeria (D'Haese et al 1977; Porchet and Torpier 1977; Dubremetz and Torpier 1978). The findings of all these research workers was consistant with the following description. In negatively stained preparations the pellicle exhibits rib - like structures that are most clearly defined at the anterior tip of the sporozoite. These rib - like structures follow a helical path down the sporozoite body. Freeze fracture studies also revealed organised structures in the cell membrane. The rib - like structures seen in negatively stained preparations were shown to be intramembraneous particles, proteins inserted into the phospholipid bilayer of the unit membrane found in most biological membranes (Bretscher and Raff 1975). The plasmalemma had an almost completely

random distribution of these intramembraneous particles on its inner surface. The inner membranes, which consisted of many joined membrane "plaques" or leaflets, bore regular arrays of intramembraneous particles on the outer surface of the outer membrane and on the inner surface of the inner membrane. These particles were arranged in two dense lines to each rib. At the anterior of the sporozoite this arrangement was very highly ordered and Porchet and Torpier (1977), proposed that there may be involved in the elongation and contraction of the conoid and surrounding area during invasion. It was also suggested, by these and other workers, that the particulate lines corresponded to the locations of the subpellicular microtubules, however, no information was then available as to the overall organisation of microtubules within the complete cell.

The subpellicular microtubules vary in number according to species, E. acervulina and E. tenella have either 23 or 24. They also vary in length according to species (D'Haese et al, 1977), in E. acervulina and E. tenella they are between 7 and 9 μm in length. In thin sectioned preparations of these sporozoites the microtubules are distributed fairly evenly at the margin of the parasite, closely related to the inner membrane of the pellicular complex. Some authors have described arm - like structures linking the microtubules to the pellicle (Heller and Scholtyseck 1971; Sinden 1978). Heller and Scholtyseck found these links in developing merozoites of E. stiedae and in these cells the microtubules are closer to the membranes than in the fully formed merozoites. These structures may be involved in organising structure during this developmental stage. Sinden (1978) reported microtubule membrane links in sporozoites of Plasmodium

yoelii, these were, however, only seen in sporozoites processed by one fixation procedure. The negatively stained preparations of E. tenella and E. acervulina revealed no arm - like structures. The critical - point dried specimens also failed to reveal any side - arms associated with the subpellicular microtubules. Both techniques did, however, reveal the protofilament substructure and its constituent subunits that form the substructure of microtubules. The protofilament substructure was best illustrated by the thin sections from glycerinated preparations stained in tannic acid. These specimens demonstrated the "normal" thirteen protofilament substructure. Some specimens prepared by this technique revealed a peg - like structure associated with the sub-pellicular microtubules on the side nearest the pellicle. The fact that microtubule - associated structures are only seen under certain conditions and only in thin sectioned material makes it difficult to evaluate their function. Obviously arm bearing microtubules are implicated in many motile systems from flagella (Satir 1968) to cytoplasmic streaming (Tucker 1978) but the difficulty in visualising these arm - like structures in these 'zoites tends to suggest that they are not present as regular arrays as in other systems.

The critical - point dried sporozoites revealed much information about the overall organisation of the subpellicular microtubule skeleton. The arrangement had been suggested previously by D'Haese, Mehlhorn and Peters (1977) but this was the first time that it had been demonstrated. The tensile strength of this cytoskeleton is shown in fig. 2.18 where the pellicle was removed from the microtubules yet they still retained their cage - like structure. This suggests that the spiral body shape typical of 'zoites of Apicomplexa is probably dictated by the helical arrangement of microtubules adopted within these sporozoites.

The stereo pairs of the complete sporozoites of E. acervulina reveal the three - dimensional organisation within these cells and also serve to underline the close association that exists between the membranes and their subtending skeleton. It is likely that body shape is controlled by an interaction of these two systems.

All techniques failed to demonstrate a microfilamentous based contractile system in the sporozoites of E. tenella and E. acervulina. Two of these techniques, critical - point drying of whole cells and thin sectioned, glycerinated preparations were used to demonstrate actin filaments in fibroblasts (Buckley 1975; Ishikawa, Bischoff and Holtzer 1969). The decoration of these filaments with the head, the heavy chain, of the myosin molecule demonstrated that they were actin. This incubation in heavy meromyosin resulted in the classic arrowhead labelling of the filaments. Even using this HMM as a probe for actin failed to demonstrate any microfilamentous contractile system in these sporozoites. This does not, however, mean that these motile cells do not possess such a system, it only means that these techniques failed to resolve a microfilamentous system in these parasites.

Members of Apicomplexa have diversified to fill many niches in many hosts. The life cycles adopted by these parasites show many differences. It is therefore startling to find that all these parasites employ motile invasive stages that reveal great structural and organisational similarities. This fact was used by Levine (1970) to create the subphylum Apicomplexa. These similarities demonstrate that the general fine structure of these stages has been highly conserved, or

protected, throughout evolution. This in turn suggests that the structure is important to the successful functioning of these stages. As the functions of these 'zoites are always motility and invasion it would be logical if either motility or invasion, or both, were an expression of the shared characteristics of these parasitic stages.

Sporozoites of E. tenella and E. acervulina possess several structures involved in motility in other cells and these must be the first to be examined as possible motile systems. Microtubules may be regarded as the "prime suspect" because of their role in many types of intracellular motility. Microtubules can be directly implicated in movement, as in flagella (Satir 1968), or can fulfil an anchoring role like the microtubular cytoskeleton visualised in the critical - point dried fibroblasts. The intramembraneous particles found in the pellicle are similar to the membrane proteins of surface modulating assemblies found in many culture cell types (Edelman, Wang and Yahara 1976). These surface modulating assemblies have been shown to be involved in the motility of these cells. Despite the inability to demonstrate microfilaments in these sporozoites, the universality of acto - myosin systems suggests that it cannot yet be dismissed as a possible cause of sporozoite motility. From this ultrastructural foundation it is now possible to study the mechanics of sporozoite motility with some knowledge of the motile system, or systems, that may be implicated.

CHAPTER 3.

A DESCRIPTION OF THE MOTILITY OF EIMERIINE SPOROZOITES.

3.1 INTRODUCTION

This study is confined to the endogenous movement of these parasite cells; that is, movement expressed by an intracellular contractile system. The study of a dynamic process like intracellular motility involves two distinctly different experimental procedures. Firstly morphology; an understanding of the basic anatomy of the cell involved, this was concluded in Chapter 2. Secondly behaviour; a detailed examination of the cell's movement to provide a definitive and quantitative description. By the careful breakdown of motility into a series of individual body postures, the mechanics of movement and hence its possible contractile basis, may be understood.

The 'zoites of Apicomplexa exhibit different degrees of motility, some, like Toxoplasma gondii (Maxwell and Drobeck 1955), are capable of active migration over great distances whilst in others, such as the merozoites of Plasmodium knowlesi (Dvorak, Miller, Whitehouse and Shiroishi 1975), motile behaviour is sporadic, weak and "ineffectual". The original observations and subsequent research into the motility of these parasites has been reviewed by Garnham (1966). This review drew attention to the basic principles of motility in parasitic protozoa in general. Unfortunately the failure of many early workers to optimise temperature or use a suitable support medium, noted by Garnham (1966), led to many misleading observations. However in 1968 Jahn and Bovee published another review, this time complimenting

the existing data on sporozoan motility with observations and diagrams of their own. This work contained definitive and quantitative descriptions of the motility of 'zoites of several different sporozoa. Few of these descriptions have been improved upon since. The authors recognised the behavioural similarities between the gliding motility of 'zoites of Haemosporina and Eimeriina. They suggested that the locomotion of 'zoites of the Apicomplexa was achieved by the propagation of helical waves down the elongate, 'zoite body. Apart from the sporozoites of Coccidia there are other protozoan cells that exhibit a similar gliding motility. One is the ookinete of Plasmodium spp. which is capable of gliding forwards or backwards, following a helical path (Freyvogel 1965, 1967). This motile, invasive stage of Plasmodium also has the trimembraneous pellicle with subtending microtubules and the micronemes and rhoptries, which suggest that this ookinete is a "'zoite" in all but name. This study has so far only discussed the motile behaviour of 'zoites from one subclass of Sporozoea, namely the Coccidia. However, gliding motility, similar to that exhibited by the Coccidia, has also been observed in the other subclass, the Gregarinia. Most gregarines are endoparasites of annelids and arthropods. Trophozoites of these gregarines are highly active, capable of vigorous flexing movements and of prolonged gliding activity. These gregarines also possess a trimembraneous pellicle with many subpellicular microtubules. During gliding, the pellicle is thrown into deep folds running along the anterior - posterior axis (Vavra & Small 1969), such folds have not been observed in the coccidia.

Garnham (1966) noted that the 'zoites capacity for movement

was affected not only by temperature, but also by the presence of certain chemicals in their medium. Since this initial observation, experiments have been conducted on several species of Apicomplexa to find the optimum conditions for motility. In 1970 Speer, Hammond and Kelley reported the stimulation of motility in the merozoites of certain species of Eimeria by the addition of bile salts to their media. Then in 1974, Vanderberg published an extensive study on the motility of sporozoites of Plasmodium berghii, P. cynomolgi and P. falciparum. In this study he described "flexing , waving, thrusting and gliding" movements of these sporozoites. He also noted the marked effect of certain chemicals on the motility of the sporozoites examined. Albumin had a stimulatory effect on motility, whilst the presence of serum globulins in the media inhibited sporozoite movement. This demonstrates that certain factors that may be encountered in the natural environments of these sporozoites modify their motile behaviour. The extent to which the motility of sporozoites of Eimeria may be stimulated is examined by the addition of certain chemicals to suspensions of sporozoites. Early studies and descriptions of 'zoite motility appear based on the presumption that the serpentine nature of sporozoite locomotion is produced by active body flexions. All existing diagrams of locomotions and movement of the Sporozoea consist of tracings from individual film frames of 'zoites viewed from above or below. The two - dimensional nature of these earlier studies results from the acceptance of the active body flexion theory. This acceptance had, effectively, precluded further analysis of 'zoite motility. In this chapter the motility of sporozoites of Eimeria acervulina, E. tenella, E. necatrix, E. nieschulzi was examined. The use of a closed circuit television system linked to a videotape

recorder allowed the movement of these protozoa to be recorded. These recordings were then played back and analysed frame by frame to form a catalogue of body attitudes. These outlines were then used to recreate a three - dimensional model of sporozoite locomotion. It is hoped that this three - dimensional model will provide more complete and functional description of 'zoite motility. The implications of this model and its possible contractile basis are discussed.

3.2 MATERIALS AND METHODS

3.2.1 General

The sporozoites of Eimeria tenella and E. acervulina used in this study were excysted, purified and suspended in fresh Hank's buffered salt solution (HBSS) by the technique described in Chapter 1. Preliminary studies revealed that sporozoites from oocysts stored for over a month in 2% potassium dichromate had suffered a slight decline in motility. For this reason only oocysts, isolated from infected chickens less than a month previous, were excysted for motility studies.

Attempts were made to stimulate the motile behaviour by the addition of various chemicals to the HBSS in which the sporozoites were suspended. The sugars glucose, fructose and sucrose were added at 10mM concentration. These sugars are known to be utilised by sporozoites of E. tenella (Ryley, 1973). Sporozoites of several Eimeria spp. are reported to have had their motile activity increased by the addition of bile salts to the medium (Speer et al, 1970). The effect of a 4% concentration of a crude bile salt extract (Difco) on the motility of E. tenella and E. acervulina was examined. Vanderberg (1974) demonstrated an increase in the motility of Plasmodium sporozoites in the presence of albumin, so its effect on sporozoites of Eimeria was examined by the addition of 1.0 and 5.0% (w/v) bovine serum albumin (Sigma) to the support medium. The possible effects of serum and of certain essential amino acids were examined by resuspending the sporozoites of E. acervulina and E. tenella in Medium 199 with 10% foetal calf serum and observing their motile behaviour. This experiment was necessary as a control for later

studies on the effects of cell membrane/parasite membrane contact on the motility of those sporozoites. In these subsequent experiments on membrane/membrane interactions sporozoites were settled onto monolayers of different culture cells and their motile behaviour was observed and recorded. The culture cell lives available were Chang liver cells, embryonic chick brain cells, mouse peritoneal macrophages, mouse 3T3 fibroblasts and Xenopus epithelial cells.

3.2.2 Analysis of Motility

The motile behaviour of these sporozoites was analysed from videotape recordings. These recordings were taken with a closed circuit television system consisting of:-

Microscope - Wild M20 (phase contrast optics)

Camera - Hitachi CCTV camera (type HV - 17SK)

Timer - Studio 99 video timer (type VTG 33)

Videotape Deck - National Time Lapse VTR (type NV8030)

Television Monitor - Hitachi CCTV (type VM - 173EK)

The videotape deck had a variable speed for recording and for play back; it also had frame - freeze facilities so an individual frame could be displayed on the monitor. The microscope used was fitted with a controlled environment stage with a variable heat setting. The motility studies of Eimeria acervulina , E. tenella and E. necatrix were conducted at 41°C whilst those of E. nieschulzi, Sarcocystis ovicanis and Plasmodium yoelii were at 38°C.

3.2.3 Isolation of other sporozoites of Apicomplexa

Sporozoites of Eimeria necatrix were prepared from sporulated

oocysts by the technique outlined in Chapter 1, though the incubation period in trypsin and bile salt solution was 45 - 60 min. Sporozoites of E. nieschulzi were also prepared from sporulated oocysts by this technique but this time the agitation with the Grade 7 Ballotini beads was reduced to 5s. and the trypsin and bile salt incubation was carried out at 38°C instead of 41°C because these protozoa were rodent not avian parasites.

The cystozoites of Sarcocystis ovicanis were isolated from sheep cardiac muscle by the following technique. Heart muscle from a freshly killed sheep was minced and washed through a muslin cloth filter with HBSS. The suspension in the filtrate was pelleted at 850g for 15 min. in an MSE bench centrifuge. The cystozoites were released by incubating the resuspended pellet in trypsin and bile salt solution, at 38°C, for 10 - 20 min. The cystozoites were purified by either of the techniques outlined in Chapter 1.

Sporozoites of Plasmodium yoelii were isolated from infected mosquitos, Anopheles stephensi by the following technique adapted from Pacheco, Strome, Mitchell, Bowden and Beaudoin (1979). Firstly the mosquitos were ground up, using a mortar and pestle, in 1ml of serum. The mortar was then washed out with 20ml of medium, the media used were M 199, HBSS and Dulbecco's Eagles medium, the resulting suspension was spun at 850g for 15 min. in an MSE bench centrifuge. The pellet was discarded and the supernatant was then spun at 12,500g for 15 min. in a swing - out rotor head (MSE type 43127 - 506) to pellet the sporozoites. The pellet was resuspended in 2ml of medium and layered onto a discontinuous gradient consisting of:-

Top Phase 2ml renografin: 1ml serum: 3ml medium.

Bottom Phase 3ml renografin: 1ml serum: 3ml medium.

(renografin was obtained from E.R. Squibb and Son, Princeton N.J.) This gradient was then spun at 12,500g for 15 min. at 4°C in a swing - out head rotor (MSE type 43127 - 506). The malarial sporozoites were finally washed by suspending in 20ml of medium with 10% serum, either foetal calf or newborn calf, and spun, as above, at 12,500g for 15 min. at 4°C. The pelleted sporozoites were then resuspended and examined.

3.3 RESULTS

3.3.1 General

Preliminary observations suggested that attempts to enhance sporozoite motilty, by the addition of various energy substrates and other chemicals previously reported to have stimulated sporozoite metabolism or motility, did not have any effect on the "vigour" of the movement of sporozoites of E. tenella or E. acervulina. Experiments on these sporozoites demonstrated that although bile salts do initiate motility immediately prior to excystation they do not, as previously reported (Speer et al 1970), stimulate motility further. The addition of extraneous substrates, glucose, sucrose and fructose, has been reported to cause an increase in the metabolism of E. tenella sporozoites (Ryley 1973). The addition of these sugars to the sporozoite support medium did not increase the motile activity of these cells. However, qualitative observations suggested that the duration of motile behaviour was doubled in these preparations. The presence of bovine serum albumin in the medium also failed to affect the motile behaviour of these two coccidia, though it has been reported to increase motility in Plasmodium sporozoites (Vanderberg 1974).

Subsequent experiments consisted of attempts to assess the effect of physical stimuli such as contact with living cells. These experiments were controlled by examining the effect of cell medium containing essential amino acids and 10% foetal calf serum. Sporozoites of E. tenella and E. acervulina suspended in these media did not exhibit any observable modifications to their motile behaviour. Next the effect of contact with a cell membrane was examined. Sporozoites of E.

acervulina and E. tenella were allowed to settle onto monolayers of culture cells. Although these sporozoites invaded all cell types used with impunity, no increase in motility was observed.

3.3.2 The Motility of E. acervulina Sporozoites

Preliminary observations suggest that sporozoites of E. acervulina were more active than those of E. tenella; for that reason this initial study was carried out exclusively on sporozoites of E. acervulina. Initial observations revealed that the motile behaviour of these sporozoites was similar to, although much more vigorous than, that described in various species of Plasmodium (Vanderberg 1974). For ease of description and to underline these behavioural similarities the definitions of Schaudin (1903) used by Vanderberg (1974) are adopted in this profile of sporozoite motility.

Movement: was the change of position of one part of the body relative to another.

Locomotion: was the active passage of the organism from one location to another.

Motility: includes movement or locomotion, or both.

All subsequent experiments were conducted in HBSS unless otherwise stated. The Eimeria sporozoites examined displayed two types of movement, namely pivoting and bending, and one form of locomotion, referred to as gliding.

The bending behaviour involved the relatively slow flexion of the anterior half of the sporozoite towards the "inside curve" of the sporozoite body. This was followed by the almost springlike straightening of the 'zoite (fig. 3.1). This "release", and the

fig3.1

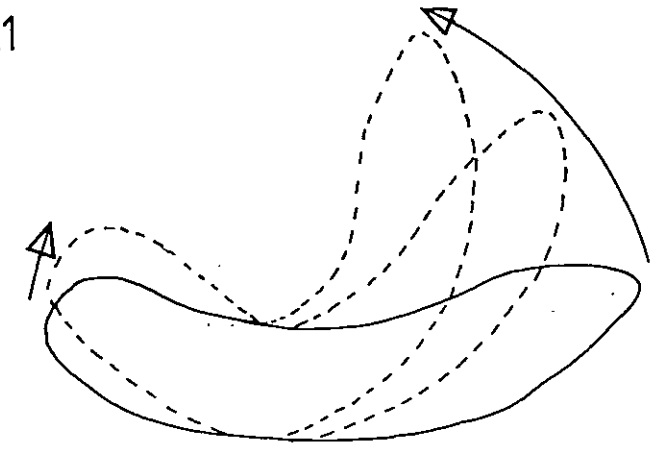


fig 3.2

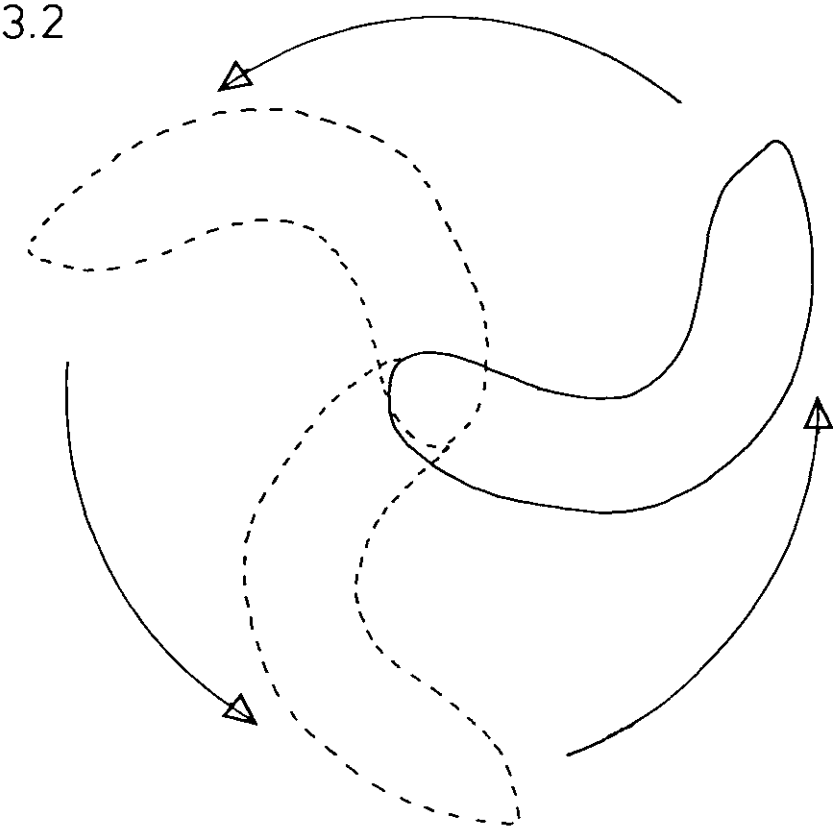


Fig. 3.1 Bending movement of sporozoites of E. acervulina. The anterior half is more flexible than the posterior

Fig. 3.2 Pivoting movement of sporozoites of E. acervulina. The posterior pole of the parasite is associated with the substratum

fact that exhausted sporozoites are relatively straight suggests that bending, and not straightening, is the energy consuming step.

Pivoting was only seen where sporozoites were attached to the substratum by their posterior pole. All sporozoites observed described an anti-clockwise rotation of their anterior pole (N.B. reversed to clock-wise by the microscope optics). This behaviour pattern is illustrated in fig. 3.2. The rotation of the sporozoite body was the same as the unidirectional rotation that occurred during sporozoite locomotion (discussed below).

Sporozoite locomotion, or gliding, proceeded at 4 - 8μm/s. This was achieved by a continuous, smooth, gliding motion. Locomotion was only observed when sporozoites were in contact with the substratum. Initial observations suggested that, as previously proposed, locomotion resulted from the propagation of helical waves passing down the sporozoite body enabling the sporozoite to swim through the medium. Immediately prior to locomotion, the sporozoites adopted a distinctive body posture, they then progressed smoothly through a sequence of body attitudes which terminated with only the posterior pole associated with the substratum. This "unit" of locomotion resulted in a forward displacement of 7 - 10μm. The body length of these sporozoites was 12 - 13μm. These units were usually summated and some sporozoites were observed moving up to 80μm by a continuous series of these unit sequences.

3.3.3 An Activity Profile of Sporozoite Motile Behaviour

The distribution of the different motile behaviour patterns during the life of sporozoites of E. acervulina is shown in fig. 3.3.

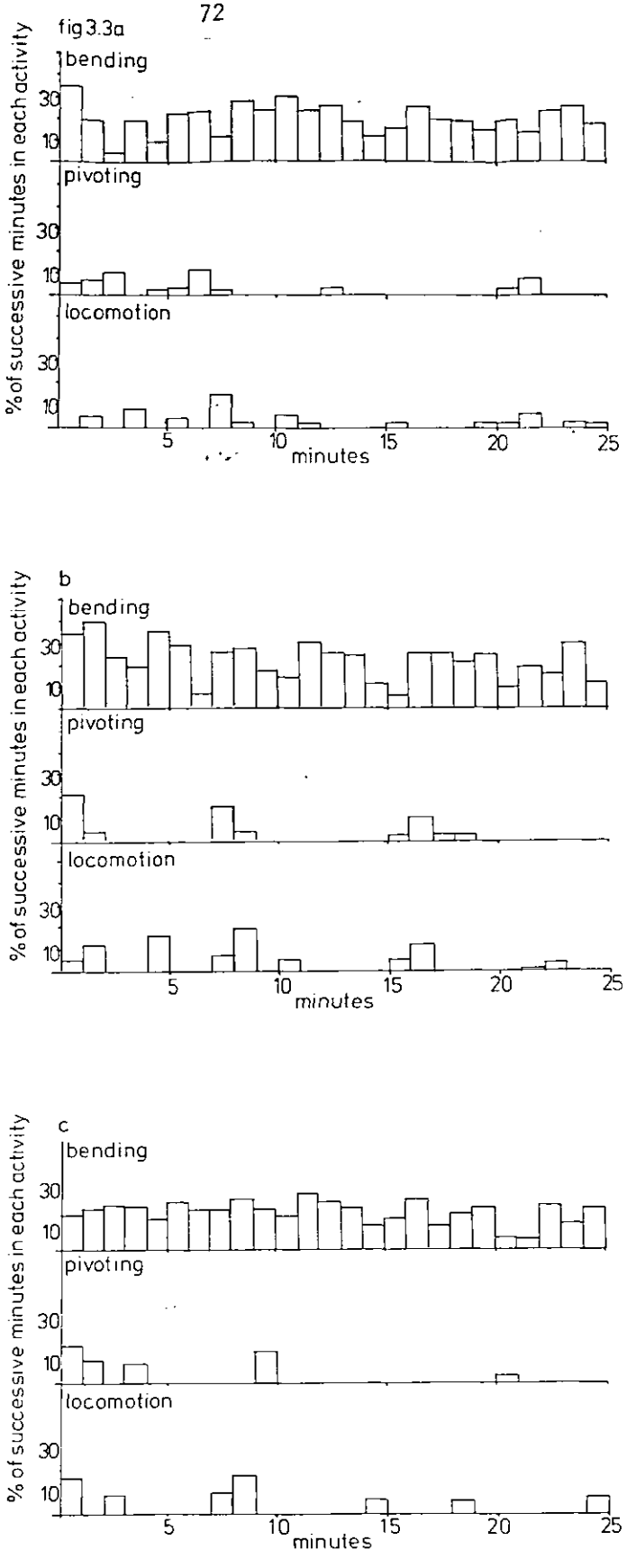


Fig. 3.3 Activity profiles of the motile behaviour of three individual sporozoites of E. acervulina illustrating the time devoted in each successive minute to each activity (bending, pivoting and locomotion).

These histograms represent the behaviour profiles from three individual sporozoites. A sample of twelve sporozoites was examined and, of the twelve histograms produced these three profiles best represented the range of motile behaviour during these sporozoites life. These histograms only deal with the first 25 min. of the sporozoites' life, after this time the level of motility was severely depressed. Motility was observed in sporozoites up to 2 hr. in HBSS at 41^{o}C. Bending behaviour is distributed continuously throughout the first 25 min. of sporozoite life, following excystation. The number of bending movements dropped with time (fig. 3.4a) though the proportion of time spent in this activity remained constant (fig. 3.3). Pivoting, on the other hand, occured sporadically (fig. 3.3). It was most common during the first few minutes following excystation and decreased in frequency against time. The vigour of this activity, measured as the number of complete turns per minute, showed a marked decline against time (fig. 3.4b). Locomotion, like pivoting, was a spasmodic activity, this activity was frequently contiguous with the pivoting behaviour. The frequency of locomotion decreased with time (fig. 3.3) as did the distance moved during each period of locomotion activity (fig. 3.4c). The decrease was expressed in a drop in the speed of locomotion and a shortening of the duration of these motile periods.

3.3.4 Analysis of Sporozoite Locomotion.

The sequence of body postures that constituted a "unit" of locomotion (discussed in 3.3.2) was analysed from videotape recordings. The recordings, normally taken at 5 frames/s, were played back, frame by frame, and photographed from the television monitor. By this method

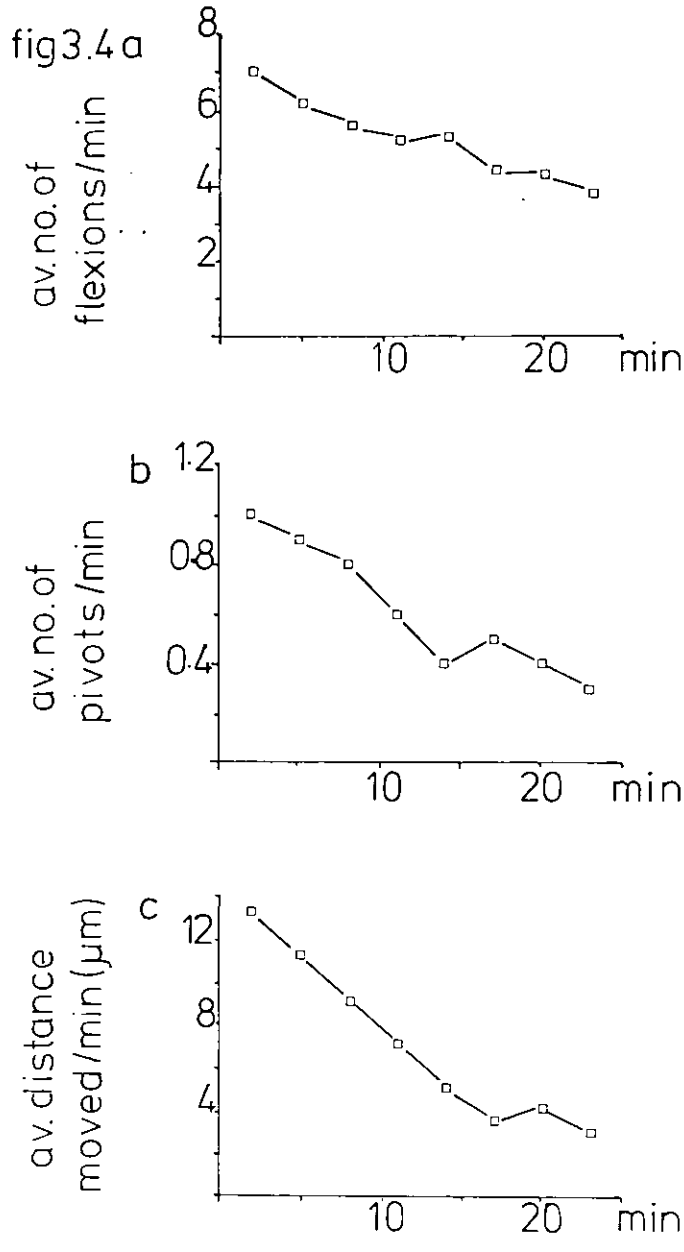


Fig. 3.4 The motile behaviour of E. acervulina sporozoites, in HBSS at 41°C, plotted against time.

a) Flexing or Bending

b) Pivoting

c) Locomotion

the sequence of body postures (fig. 3.5a) and their corresponding outlines (fig. 3.5b) were examined. Careful study and experimentation with plasticine models revealed that the body postures seen during locomotion could be duplicated using a model with a rigid body shape. This rigid model was moved to reproduce the body shapes recorded in fig. 3.5a and fig. 3.5b and then photographed from the vertical (fig. 3.5c) and the horizontal plane. The horizontal, or side, view of the body postures adopted by this model (fig. 3.5d) were then traced to give a series of outlines (fig. 3.5e). These diagrams demonstrate that the sporozoite may adopt a rigid body shape which was rotated along the helical path to reproduce the body attitudes recorded from living sporozoites.

Locomotion was only observed when the sporozoites were in contact with the substratum. In this model an area of the sporozoite remains in contact with the substratum throughout locomotion (fig. 3.6). This area of contact describes a helical path down the outside surface of the rigid, helically curved sporozoite body.

3.3.5 Observations on the Motility of other 'zoites of Apicomplexa.

Although much of this work on 'zoite motility was carried out on sporozoites of *E. acervulina* the behaviour of other motile stages of other species of Apicomplexa was also observed. The sporozoites of several species of *Eimeria*, including *E. tenella*, *E. necatrix* and *E. nieschulzi*, were examined. They each exhibited motility identical to that described for *E. acervulina*. The cystozoites of *Sarcocystis ovicanis*, isolated from sheep heart muscle, were also observed employing

Fig. 3.5. Analysis of the locomotory behaviour of E. acervulina sporozoites from a video - recording of a "unit" of locomotion.

a) Photographs from the T.V. monitor of single frames (at 0.4 s. intervals) that make up a "unit" of locomotion. X 3,100

b) Outlines of the body shapes (taken from six different recordings of locomoting sporozoites). The shaded area represents the path of membrane/substratum contact, explained in fig. 3.5e

c) Plasticine model with fixed body shape revolved to produce the outlines above.

d) The same model photographed from the side.

e) The outlines of these body shapes suggest that the sporozoite remains in contact with the substratum (shaded area) along the outside of its helically coiled body throughout locomotion.

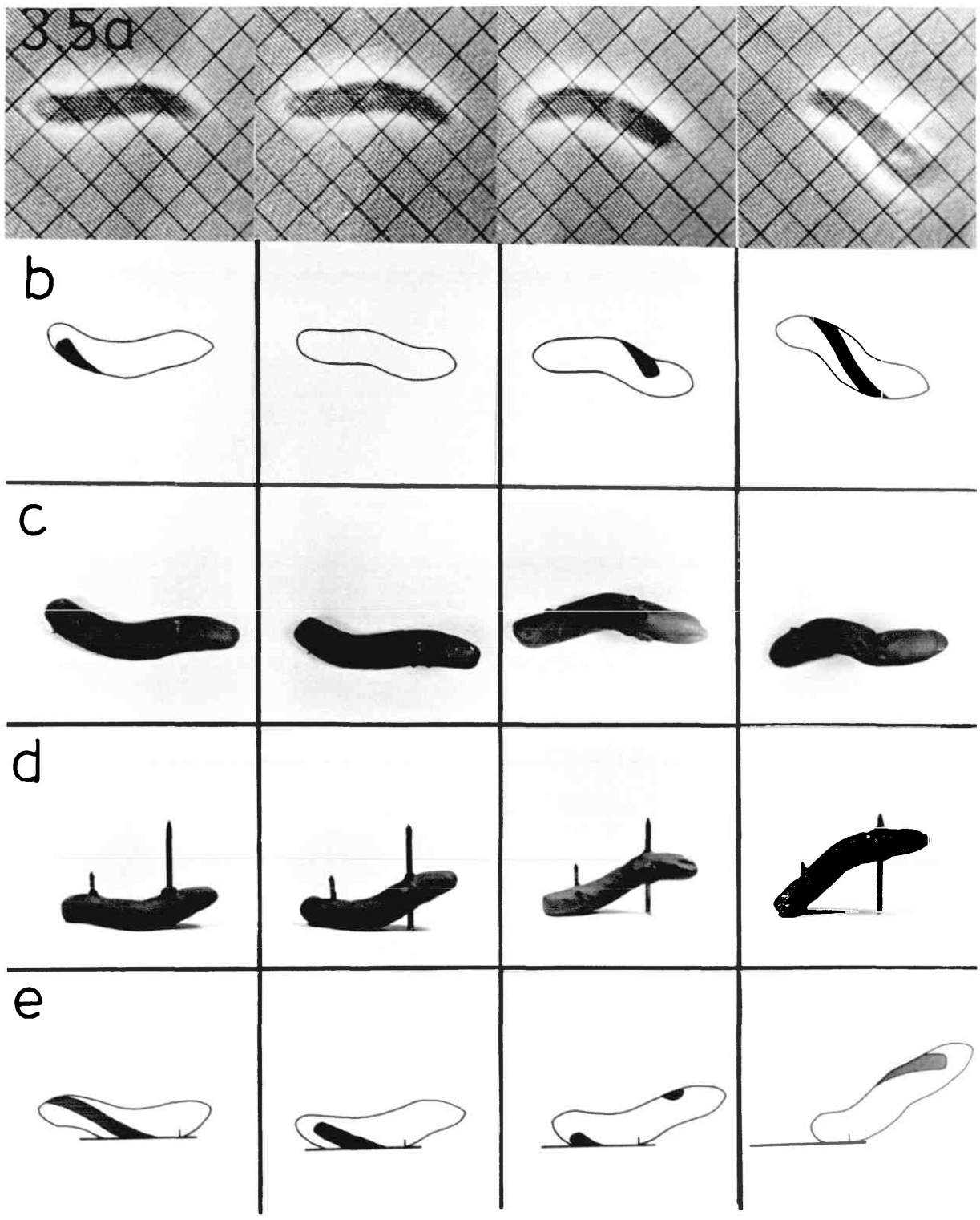
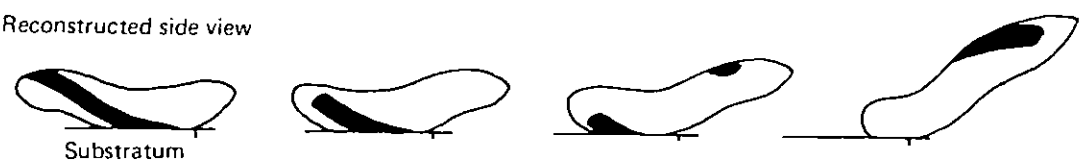

3.5a
b
c
d
e

Fig. 3.6

Actual view from above

Tracings of the postures assumed by the sporozoites of *E. tenella* and *E. acervulina* during gliding, as taken directly from a CCTV system attached to a phase-contrast microscope. (The shaded area represents the total area of contact with the substratum during a single unit of movement.)

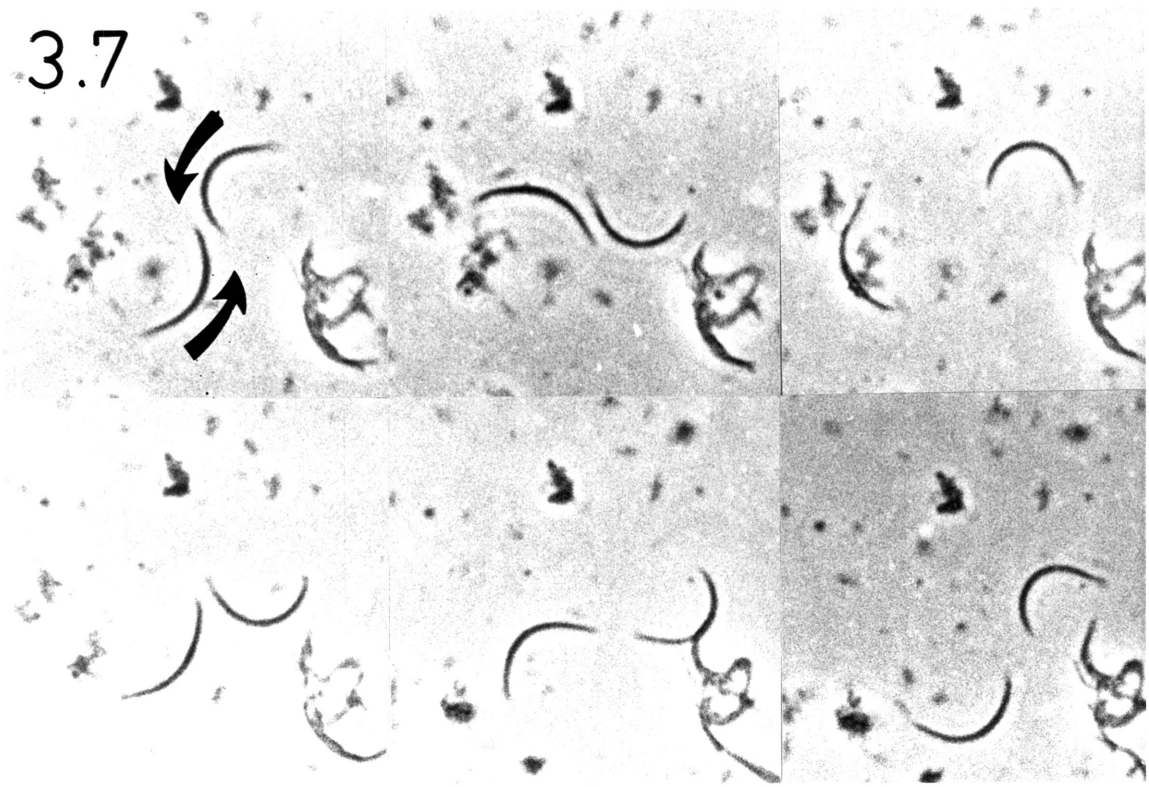
Reconstructed side view



Postures of a rigid sporozoite model, which was rotated along a helical path and successfully recreated the attitudes actually observed *in vivo* The illustrations are of this model viewed from the side, the shaded area again indicates the sporozoite–substratum contact area.

bending, pivoting and gliding motility. Sporozoites of Plasmodium yoelii were isolated from whole mosquitos and suspended in either Medium 199 or Dulbecco's modified Eagle's medium, both with 10% foetal calf serum. These sporozoites exhibited bending, weak pivoting, which appeared to be behaviourally similar to Vanderberg's (1974) description of "waving" movement, and gliding motility. The gliding of these sporozoites did not appear to involve the same rigidly maintained body shape. Some sporozoites were observed gliding around in circles (fig. 3.7). The body shape of these sporozoites obviously did not change during locomotion. This circular gliding behaviour cannot, therefore, be the product of active body flexions. The gliding motility was smooth and continuous, as in the other 'zoites observed, but sporozoites of P. yoelii were capable of moving backwards or forwards. In fact, some sporozoites were observed oscillating over what appeared to be a fixed point on the substratum. This reversal of the gliding motility has not been observed in 'zoites other than those of Plasmodium.

Fig. 3.7. Phase - contrast micrographs of sporozoites of P. yoelii gliding around in circles (arrows indicate direction). The body shape of these cells remained fixed during this behaviour. X 1,250


3.7

3.4 DISCUSSION

Since the original description of sporozoite motility as being "serpentine" (Grasse 1899) it has been accepted, almost without question, that 'zoite locomotion results from the propagation of waves down the 'zoite body (Garnham 1966, Jahn and Bovee 1968). It is only recently that alternative explanations have been put forward (vanderberg 1974). The careful analysis of the body shapes generated during a "unit" of locomotion of E. acervulina sporozoites demonstrates that a rigid body posture may be maintained throughout the locomotion of these parasites. The presence of a contractile system capable of maintaining this rigid body shape is indicated by the bending behaviour witnessed in these sporozoites. The model used to duplicate the recorded body postures had a helically shaped body which was rotated along a spiral path to simulate sporozoite locomotion. Both pivoting and gliding require the maintenance of an intimate association with the substratum and, in E. acervulina, both involve the same unidirectional rotation of the sporozoite body. These similarities suggest that both these forms of motility are the products of the same cellular mechanism. When these sporozoites reach the "end" of a unit of locomotion they are left with only their posterior pole associated with the substratum (fig. 3.6). They usually twist over and re - attach their anterior end to the substratum. The pivoting behaviour of sporozoites may in fact be a "searching" mechanism to maximise the chance of successful attachment and therefore successful locomotion. The fact that pivoting and gliding occur contiguously would support such a connection.

Sporozoite/substratum attachment is obviously an important component of this model of sporozoite locomotion. In the model this attachment is maintained throughout locomotion. The maintenance of such an adhesion site would provide an anchorage point for a contractile system to work against. It is interesting to note that this membrane/substratum contact area describes a helical path down the outside of the sporozoite body (fig. 3.6). This path will obviously be governed by the sporozoite body shape. In the previous chapter it was demonstrated that the subpellicular microtubules, membrane plaques and the intramembraneous particles were arranged in a spiral compatible with the helical shape of the sporozoite. It would be logical for a cell with a spirally arranged cytoskeleton to employ a contractile system that operated along this subtending framework. In most eukaryotic cells it is the microtubules that act as the "bones" that direct and organise the associated contractile proteins.

The other members of Apicomplexa examined in this study were sporozoites of E. necatrix, E. nieschulzi, Plasmodium yoelii and cytozoites of Sarcocystis ovicanis. All these cells possessed a repertoire of motility almost identical to that described in E. acervulina. Some 'zoites showed more and some less activity than E. acervulina, and P. yoelii 'zoites did not rigidly maintain the helical body shape. However the strongest evidence for related motile behaviour, especially the gliding locomotion, comes from Jahn and Bovee's "Locomotion of Blood Protists" (1968). This work contains beautifully definitive descriptions of the motility of 'zoites of many different sporozoa. The parallels between these descriptions

cannot be ignored; for example, this subscript to a motility diagram: "Sequence A consists of tracings (each 60th picture) from 300 pictures of a cinephotomicrographic film of a haemogregarine trophozoite in steady gliding progress along a clockwise helical path, photographed at 2 pictures/second. The organism makes contact with the substratum once with each completion of a helix, and the body glides over each contact point, without interruption, from one end to another".

Close examination of the diagram referred to demonstrates that the outlines of the 'zoite are functionally akin to those of E. acervulina (fig. 3.6). This gliding locomotion has been reported in the motile stages of many species of Plasmodium (Schaudin 1902, Yoeli 1964, Freyvogel 1965 and Chao 1966), in the sporozoites of coccidia (Doran, Jahn and Rinaldi 1962 and Bovee 1965), and the trophozoites of many gregarines (Cox 1965 and MacGregor and Thomasson 1965). The failure of these early workers to visualise the motility of these 'zoites as a three - dimensional phenomena has kept the potential importance of a contractile system based on a sporozoite/substratum interaction hidden.

From this behavioural similarity it is interesting to return to the projections made in Chapter 2. Ultrastructural examinations of sporozoan 'zoites revealed a highly conserved organisation that suggested a common mechanism of motility. The behavioural similarities support this hypothesis. This idea was first expressed by Grasse (1953) who suggested that the flexing movements of all coccidiomorphs were due to myonemes, and their helical progression to be a type of gliding,

in both cases similar to such movements in gregarines. Unfortunately the contractile mechanism(s) responsible for the motility of these cells are not as obvious as the developed myonemes present in some gregarines (Hildebrand 1980). Despite careful ultrstructural examination, the contractile basis of motility remains unknown, but the model of 'zoite locomotion (fig. 3.6) does point to the next area for investigation. If locomotion does involve a membrane/substratum interaction then the membrane must possess certain receptors, or binding sites, that would, in turn, be associated with an intracellular contractile system.

CHAPTER 4.

THE CONTRACTILE BASIS OF SPOROZOITE LOCOMOTION

4.1 INTRODUCTION

In 1966 Garnham stated that the merozoite of Plasmodium must employ a propulsive force to gain entry into the corpuscle. To effect this entry would involve the parasite's contractile system; such a system may also be used in other cellular functions, such as motility. However, despite the obvious potential importance of 'zoite locomotion to subsequent cell invasion and therefore successful infection, this contractile basis of sporozoite locomotion has largely only been the subject of indirect and fragmented studies. In Chapter 3 the possible importance of a membrane associated contractile system is stressed, so in this Chapter the existence of such a mechanism is examined. This study involved the assessment of the roles played by different contractile proteins by modifying their action with various anti - mitotic agents. The ultrastructural effects of these drugs were also monitored. By these techniques, an attempt was made to demonstrate possible linkages between contractile elements of the cytoskeleton and intramembraneous proteins that may, in turn, associate with the substratum, a system similar to that found in mammalian culture cells (Badley, Woods, Smith and Rees 1980). The membranes of sporozoites were examined for these possible binding sites with lectins, antibodies and cationised ferritin.. The mobility of these surface ligands was observed and their contractile basis examined by the use of the anti - mitotic drugs.

The actions of anti - mitotic agents in the study of many

motile systems have produced much information as to the roles played by the different cytoskeletal and contractile elements (Borgers and de Brabander 1975; Thomas 1978). The effects of several of these drugs on the invasive and motile behaviour of sporozoites of Eimeria magna was examined by Jensen and Edgar (1976). They found that these sporozoites were unaffected by the microtubule inhibitors but that the microfilament inhibitor, cytochalasin B, stopped invasion and suppressed motility. The authors speculated that this was due to the abnormal nature of the microtubules which may be "tubular aggregates of contractile elements that are sensitive to cytochalasin B". Unfortunately they did not supplement these light microscope observations with an ultrastructural study. In this study the effects of a range of microtubule inhibitors and microfilament inhibitors on the motility of Eimeria tenella and Eimeria acervulina was examined. These behavioural studies were extended by ultrastructural examination of treated cells to evaluate any detectable structural modifications caused by the drugs.

The model of sporozoite locomotion proposed in Chapter 3 required adhesion between the parasite plasmalemma and the substratum. These potential substratum attachment sites were examined by assaying the binding of different chemicals known to interact with the plasmalemma of other species of Apicomplexa, and other cell types. Lectins are known to associate with the carbohydrate containing proteins exposed on the surface of many different cell types (Sharon 1977). These labels have been used in previous attempts to characterise certain components of the coccidian 'zoite plasmalemma. Sethi, Rahman,

Pelster and Brandis (1977) reported that the lectins they used failed to bind with cytozoites of Toxoplasma gondii. Then in 1980, Turner also reported the failure of lectins to associate, this time with Plasmodium gallinaceum sporozoites in the presence and absence of host serum. Contrary to these, Schulman, Oppenheim and Vanderberg (1980) stated that the binding of some lectins to sporozoites of Plasmodium occured in the presence of host serum. Obviously some disagreement exists as to the binding of lectin to these 'zoites. The interaction of Eimeria tenella and E. acervulina sporozoites with several lectins is reported in this Chapter. Another membrane label that has been used on 'zoites of Apicomplexa are antibodies against certain surface proteins. Such antibodies are reported to coat sporozoites (Cochrane, Aikawa, Jeng and Nussenzweig, 1976) and so may be used as membrane labels. The third membrane binding compound used in this present study was the protein cationised ferritin. This multivalent protein has been used to localise anionic charged sites on the surface of many cells, from protozoa (King and Preston 1977b) to the mammalian oviduct ciliary membrane (Anderson and Hein 1977). Cationised ferritin is visualised by electron microscopy as an electron dense spot 12.5nm in diameter. If conjugated with fluorescein isothiocyanate this label may also be visualised by fluorescent microscopy. The binding of this label to coccidian 'zoites has already been successfully demonstrated by Dubremetz and Ferriera (1978) using Eimeria nieschulzi sporozoites and Sarcocystis tenella endozoites.

These membrane labels were employed so that the mobility of certain intramembraneous components may be demonstrated and examined.

The possible existence of a membrane associated motile system has already been indicated by two previous studies. In 1974 Vanderberg examined the effects of covering sporozoites of Plasmodium with surface binding antibody. He observed that the sporozoites were able to glide in contact with the substratum until they became coated with antibody. The coat inhibited gliding motility. The gliding behaviour returned as the antibody coat was "capped" and shed from the posterior of the sporozoite. In 1978 Dubremetz and Ferreira examined the membrane mobility of two coccidian 'zoites. Both these parasites were capable of capping and shedding the surface bound cationised ferritin. This activity was inhibited by low temperature and by cytochalasin B. These researchers also commented on the close relationship between this capping reaction and sporozoite locomotion.

In this Chapter the contractile basis of the capping and shedding of surface bound labels is closely examined. The effects of a wide range of anti - mitotic agents on capping and locomotion are investigated at light microscope and at electron microscope level. The modifications caused by these agents to capping and sporozoite locomotion are compared. The relationship between these two contractile phenomena is discussed and a theory for a common contractile system is proposed.

4.2 MATERIAL AND METHODS

4.2.1 Microtubule Inhibitor Studies

The effects of varying concentrations of anti - microtubule agents on sporozoite motility and on the subpellicular microtubules were examined. The microtubule inhibitors used were, exposure to low temperature, 0 - 4°C, and the compounds colchicine, griseofulvin, vinblastine sulphate and nocodazole. The mode of action of these drugs has been reviewed by Borgers and de Brabander (1975) and are discussed in section 4.4. Colchicine and griseofulvin are water soluble, these were diluted to concentrations from 0 - 200 µg/ml in Hanks buffered salt solution (HBSS). Vinblastine sulphate was first dissolved in ethanol before being diluted in HBSS to a final drug concentration of 0 - 200 µg/ml and a constant solvent concentration of 1%. Nocodazole was dissolved in dimethyl sulphoxide (DMSO) before being diluted in HBSS. This drug was used in concentrations of 0 - 200 µg/ml and a final solvent concentration of 0.1%. Mouse 3T3 fibroblasts were used as positive controls for the assay of these solutions, the actions of all these drugs caused the fibroblasts to round up. The effect of 1% ethanol and of 0.1% DMSO was tested on monolayers of fibroblasts and on suspensions of eimeriine sporozoites. Neither cell type showed any modification of shape or motility. Sporozoites of *E. tenella* and *E. acervulina* were treated with these drugs by two different procedures. The sporozoites were either resuspended directly into the experimental solution, warmed to 41°C and observed; or they were preincubated in the drug for between 5 min. and 6 hr. at 4°C before being warmed to 41°C and observed. The motile behaviour of

these sporozoites was recorded and analysed by the closed circuit television system discussed in Chapter 3. The sporozoites were observed for 2 h at 41°C.

The effects of these inhibitors on the subpellicular microtubules and on the conoid were examined by the negative staining of suspensions of treated sporozoites. These parasites were stained in phosphotungstic acid, as described in Materials and Methods, Chapter 2, and examined in a Phillips E.M. 300.

4.2.2 Microfilament Inhibitor Studies

The anti - microfilament agents used were the fungal metabolite cytochalasin B and exposure to high magnesium ion concentrations (5 - 15 mM). Cytochalasin B is insoluble in water, so a stock solution was dissolved in DMSO. This stock solution was diluted with HBSS to yield final drug concentrations of 0 - 10 µg/ml. The final concentration of DMSO was adjusted to 0.1%. Sporozoites suspended in a control solution of 0.1% DMSO in HBSS showed no change in their motility. The high magnesium ion concentration was achieved by adding $MgCl_2$ to the HBSS to yield final concentrations from 5 - 15 mM. Sporozoites of E. tenella and E. acervulina were resuspended in the experimental solutions, warmed to 41°C and observed and recorded on the closed circuit television system.

The ultrastructural changes effected by these procedures were examined by thin section transmission electron microscopy. The parasites were fixed and processed in phosphate buffer as described in Materials and Methods Chapter 2. The magnesium ion concentrations were adjusted to experimental levels in the phosphate buffer where relevant.

4.2.3 Surface Labelling of the Sporozoite Plasmalemma

1. Lectins

The presence of lectin receptors in the glycocalyx of sporozoites of *E. tenella* and *E. acervulina* was examined with fluorescein isothiocyanate (FITC) conjugated lectins. The lectins used were concanavalin A (Miles - Yeda) *Ricinus communis* (Sigma) and peanut (Sigma). The lectin binding properties were examined on fixed and unfixed sporozoites. Sporozoites were fixed in 0.2% glutaraldehyde in HBSS. Half this sample was washed and incubated for 10 min. at room temperature in 5% (w/v) bovine serum albumin (BSA), this was to inactivate any residual aldehyde terminal groups. Both samples of fixed sporozoites were then washed three times in HBSS. Samples of unfixed, fixed and fixed/BSA - treated sporozoites were incubated in the appropriate lectin at $4^{o}C$ and $41^{o}C$. The lectins were added to the HBSS to give a final concentation of 25 µg/ml. The parasites were incubated in these lectins for 30 min., washed three times in HBSS, using the blood centrifuge, and resuspended in fresh HBSS prior to examination. All samples were processed at the same temperature throughout.

The specifity of the binding was examined by including the relevant competitive saccharide inhibitor in the lectin solutions of a duplicate set of samples. The saccharides used were, for concanavalin A, α methyl - D - mannoside and for *R. communis* and peanut, D - galactose. The positive control for the lectins and their inhibitors was red blood cells. These preparations demonstrated that the lectins bound to the red blood cells unless the saccharide inhibitors were present. The two saccharides were added to a final concentration of 100 mM. All preparations, parasite and control, were examined under a Leitz Orthoplan

incident fluorescent microscope.

4.2.3 Surface Labelling of the Sporozoite Plasmalemma

2. Antibodies

A surface antibody to sporozoites of E. acervulina was prepared as follows. Sporozoites were excysted and purified by the technique in Chapter 1. The number of sporozoites was adjusted to $1x10^6$. These cells were then fixed in 2.5% glutaraldehyde in HBSS for 15 min.. They were then washed three times and resuspended in a minimal volume of HBSS. This solution was then mixed to form an emulsion with Freund's complete adjuvant yielding a final volume of 0.8 ml. This emulsion was injected, in 0.2 ml doses, directly into the four axial lymph nodes of a New Zealand white rabbit. This dosage was repeated ten days later. The rabbit was bled 27 days after the first dose of antigen. The presence of an antibody that reacted with E. acervulina sporozoites was confirmed by the agglutination of these sporozoites when placed in a solution containing immune serum diluted 1:10 in HBSS. The rabbit was thereafter bled at 7 day intervals for 6 weeks, the blood allowed to clot and the serum was removed. The surface antibody produced did not cross react with E. tenella sporozoites. The rabbit serum did, unfortunately, cause antibody mediated complement lysis, thus killing the sporozoites of E. acervulina. Attempts to deactivate the complement by incubating the serum at $56^{o}C$ for 20 min. failed, obviously this complement component was heat stable.

Further work on this sporozoite agglutinating antibody was done in conjunction with Miss A. O. Wosencroft, a third year undergraduate as part of her final year project. The rabbit immune serum was fractionated

on an Ultragel Ac 34 column (dimensions 60 x 1.2 cm, bed volume 52 x 1.2 cm). The column was equilibrated with 50 mM phosphate buffered saline (PBS) at pH 7.4 and run at a flow rate of 4.7 mls/hr. This column was calibrated with dextran blue, bovine serum albumin and ribonuclease A so the molecular weights of serum fractions could be calculated. Of the fractions collected one, with an estimated molecular weight of 155,000 was able to agglutinate sporozoites without causing lysis. Lysis only occured when other fractions, collected at.M. wts 66,800 at 7,670, were added back to the 155,000 fraction. The properties of this fraction suggest that it is immunoglobulin G.

The presence of this antibody on the surface of the sporozoites of E. acervulina was examined by indirect immunofluorescence. The sporozoites were incubated for 30 min. at 4°C and 41°C in the 155,000 fraction diluted 1:10 in HBSS. These sporozoites were then washed three times in HBSS at the relevant temperature and then incubated in FITC sheep anti - rabbit immunoglobulin (Wellcome) at 1:20 dilution, for 30 min. at the same temperature. The sporozoites were finally washed three times and examined under a Leitz Orthoplan incident fluorescent microscope at either 4°C or 41°C. The possible movement of antibody on the sporozoite surface was examined by processing the sporozoites at 4°C and warming them up to 41°C, in the presence and absence of cytochalasin B, whilst under observation.

4.2.3 Surface Labelling of the Sporozoite Surface

3. Cationised Ferritin

Cationised ferritin has been used to study the anionic sites distributed over cell surfaces. Recently a technique has been developed to conjugate cationised ferritin with the fluorochrome, fluorescein isothiocyanate (FITC) (King and Preston 1977). This allows

the movement of these sites to be followed in living cells by fluorescent microscopy. The cationised ferritin was conjugated as follows; 11.5 mg of cationised ferritin (Miles - Yeda) was dissolved in 1 ml of 50 mM phosphate buffer (pH 7.6). This was then mixed with 1.5 mg of fluorescein isothiocyanate (FITC) on celite (10%) (Sigma) for 30 min. at room temperature. This allowed the FITC to attach to the protein. The excess FITC was removed by passing the suspension down a Sephadex G 25 column. This column was prepared in a large pipetmaster tip (10 ml). The end of the tip was plugged with glass wool before it was filled with Sephadex G 25 equilibrated for 15 min. in the phosphate buffer. The suspension of protein and label was passed down the column which was kept topped up with phosphate buffer. The first fraction through the column was brown. This contained the FITC conjugated cationised ferritin, so it was retained. The labelled eluant was diluted 1:5 with HBSS before use. Sporozoites of E. acervulina and E. tenella were incubated in the label at 4°C and at 41°C for 20 min. They were then washed three times in HBSS at the appropriate temperature in a blood centrifuge. This centrifuge operated at 14,000g and pelleted the sporozoites in a 1 - 2 s. spin. Care had to be taken because the centrifuge could also pellet the FITC - cationised ferritin if spun for too long. The presence of a brown tint in the HBSS meant that the unreacted label had not been pelleted with the sporozoites. Finally the sporozoites were suspended in HBSS at 4°C and 41°C and examined with a Leitz Orthoplan incident fluorescent microscope. Some of the parasites processed at 4°C were warmed to 41°C whilst under observation.

Samples of cationised ferritin labelled sporozoites of E. acervulina were also processed for electron microscopy. In these

experiments unlabelled cationised ferritin was diluted to 0.575 mg/ml in HBSS. Sporozoites were incubated in the label at 4°C and at 41°C for 10 - 20 min. They were then washed until all excess label was removed. The best method was three 10 ml changes of HBSS, at the appropriate temperature, centrifuged at 850 g for 10 min. in an MSE bench centrifuge. This technique was used because the increased dilution of cationised ferritin, which was then spun at a lower speed, produced a clean preparation in which the only cationised ferritin present was that associated with the sporozoites. These precautions prevented any labelling of sporozoites taking place after fixation. Some parasites were processed in HBSS in the presence of cytochalasin B at 41°C. Others were processed at 4°C and warmed to 41°C for 2 min. immediately prior to fixation. All samples were fixed in 2.5% glutaraldehyde in 45 mM phosphate buffer as Materials and Methods, Chapter 2, and processed for thin section transmission electronmicroscopy.

4.3 RESULTS

4.3.1 Effects of Microtubule Inhibitors on Sporozoite Motility and the Cytoskeleton

The drugs and treatments used in an attempt to depolymerise the microtubules of two Eimeria spp. have been used on many other systems. Cold treatment causes the reversible depolymerisation of the microtubules in the axopodia of Heliozoa (Jones and Tucker 1981).

None of the four microtubule inhibitors, colchicine, griseofulvin, vinblastine sulphate and nocodazole, used in this study had any effect on the sporozoites of E. tenella and E. acervulina. The use of high drug concentrations (up to 200 µg/ml) and prolonged incubations (up to 6 h at 4°C) did not affect the motility or shape of these parasites. Neither did treatment at 0 - 4°C.

Negatively stained preparations of sporozoites treated with the above drugs were examined in the electron microscope to determine whether any morphological changes in their microtubular cytoskeleton could be detected. These studies showed that the microtubule length and number (fig. 4.1) and the protofilament and subunit organisation (fig. 4.2) were all identical to those seen in normal untreated sporozoites. Thus, whilst it was not directly proven that the drugs used penetrated the sporozoites, the prolonged incubation times, the cold shock treatment and the success and rapidity of cytochalasin B (section 4.3.2) all suggest that the microtubules were exposed to these drugs but were insensitive to them.

4.3.2 Effects of Microfilament Inhibitors on Sporozoite Motility and the Cytoskeleton

The anti - phagocytic agent cytochalasin B causes the dissociation

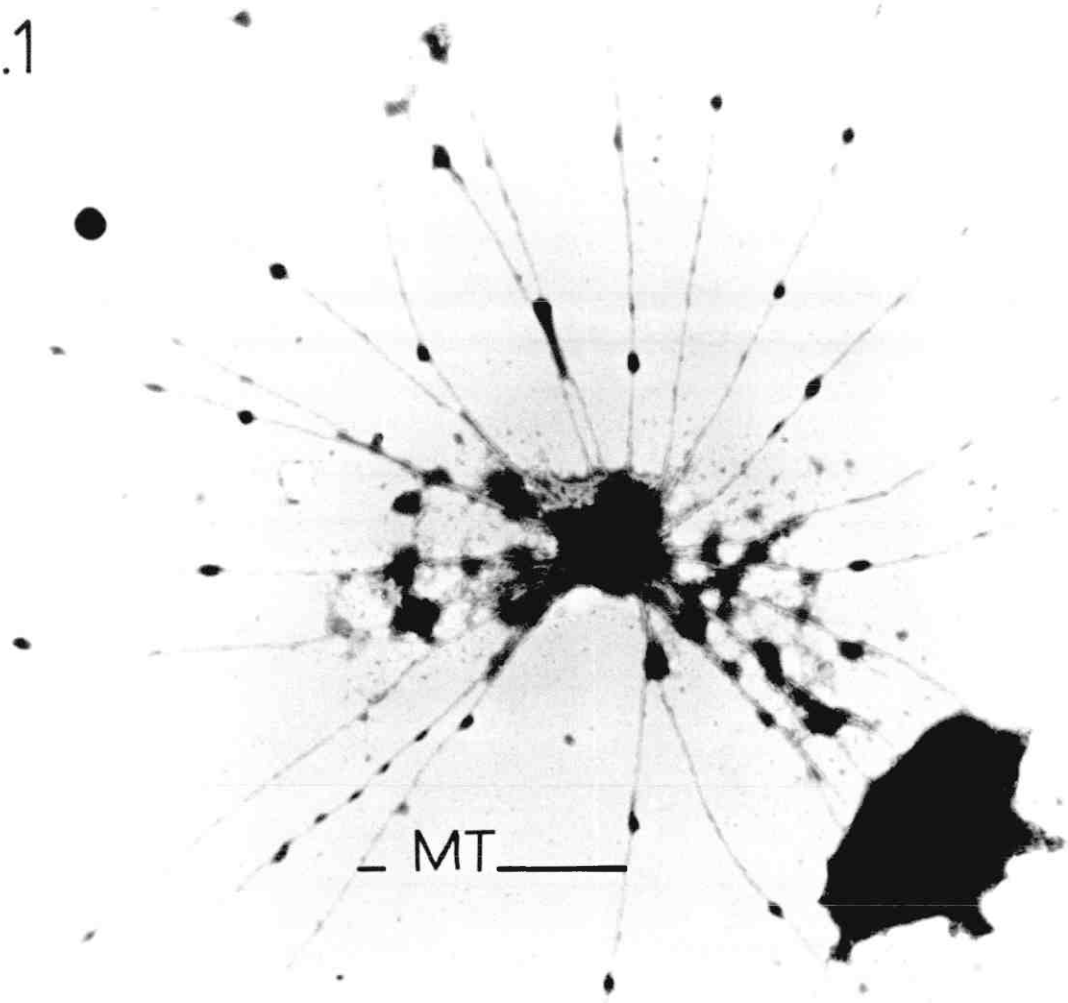
Figs. 4.1, 4.2 and 4.4. Electron micrographs of sporozoites of Eimeria spp.

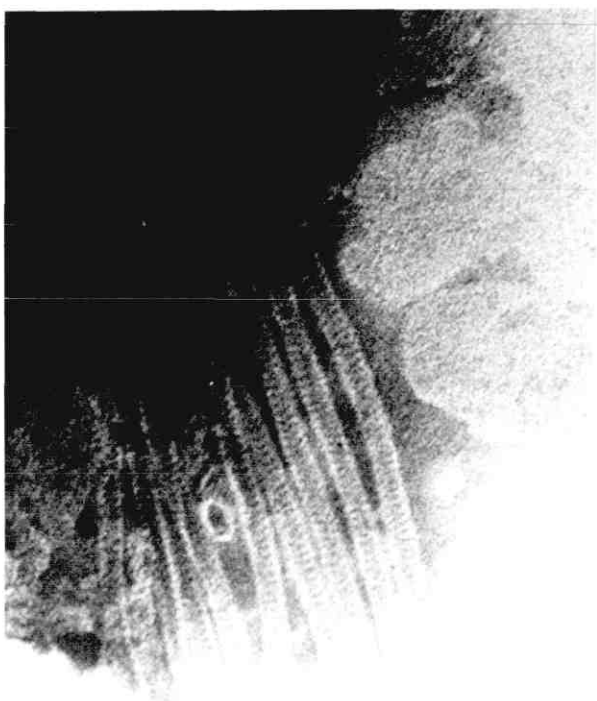
Fig. 4.1. Negatively stained microtubules (MT) from a sporozoite of E. acervulina previously incubated in 100 µg/ml vinblastine sulphate for 6 h at 4°C and 1 h at 41°C. The length and number of microtubules is the same as that of control preparations.
X 14,800

Fig. 4.2. Negatively stained preparation of microtubules from E. acervulina incubated in 100 µg/ml colchicine for 6 h at 4°C and 1 h at 41°C. The subunit periodicity remained unchanged. X 156,000

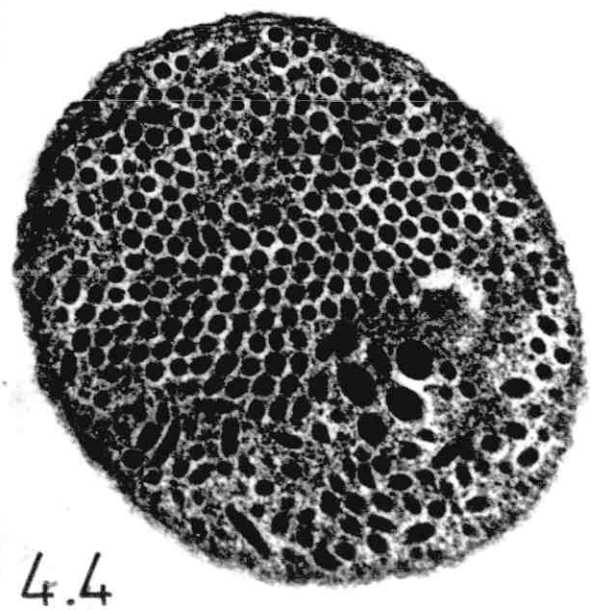
Fig. 4.4. C.S. of a sporozoite of E. tenella pretreated for 1 h in 15 mM $MgCl_2$ in HBSS. There are no detectable ultrastructural changes between these cells and untreated ones.
X 40,300

4.1





4.2



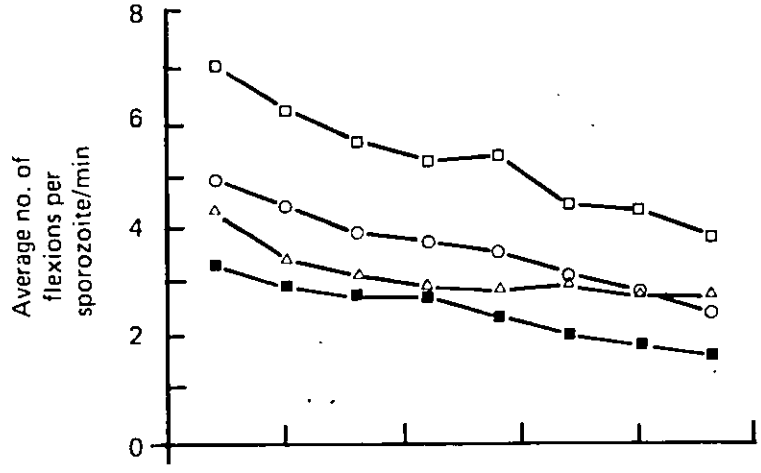
4.4

of F actin by binding on the net gain end of polymerising actin filaments (McLean, Fletcher and Pollard 1980; Brown and Spudich, \٩٨١). It is also known to inhibit glucose transport in some cells. Lin, Lin and Flanagan (1978) demonstrated that this effect was due to the drug binding to the sugar transport proteins in the membrane. This problem of dual action was overcome in two ways. Firstly, cytochalasin B was used in very low concentrations, up to 10 µg/ml, to limit its site of action because it binds preferentially to actin. Secondly these sporozoites were suspended in HBSS so no sugar transport across the plasmalemma should occur. These sporozoites utilise internal carbohydrate reserves (Ryley 1973).

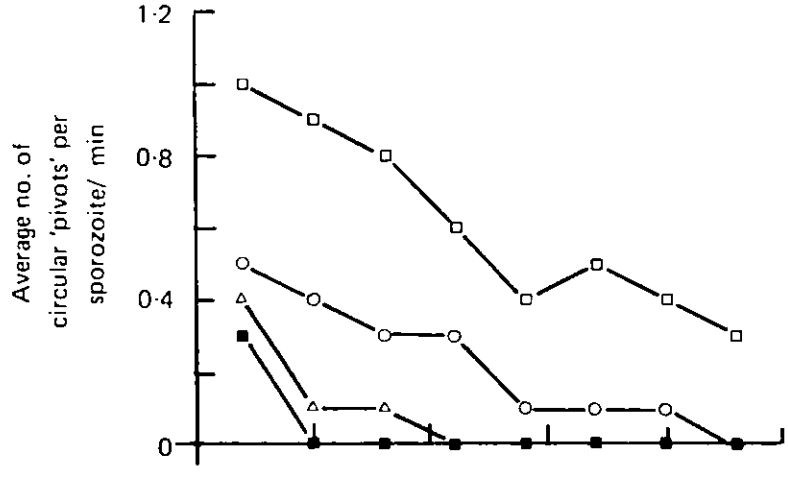
The use of high magnesium ion concentration has been shown to inhibit cytoplasmic streaming in the formaniferan Allogromia (McGee-Russell and Allen 1971). In 1976 Weihing demonstrated that concentrations of Mg^{++}, above 5 mM induced actin to form paracrystals.

The effects of various concentrations of cytochalasin B on the three types of motility expressed by sporozoites of E. acervulina are illustrated in the graphs in fig. 4.3. Each point on each graph represents an averaged value of "motility" from a sample of 50 sporozoites. Each experiment was repeated five times and each time a sample of ten sporozoites was observed, recorded and analysed. These results demonstrated that the pivoting and gliding components of motility were significantly depressed by drug concentrations exceeding 2.5 µg/ml. The bending behaviour was relatively insensitive to the drug. The effect of cytochalasin B on sporozoite motility was virtually instantaneous. Sporozoites suspended in HBSS with 10 µg/ml of cytochalasin B showed little pivoting or gliding after only 5 s. exposure to the drug.

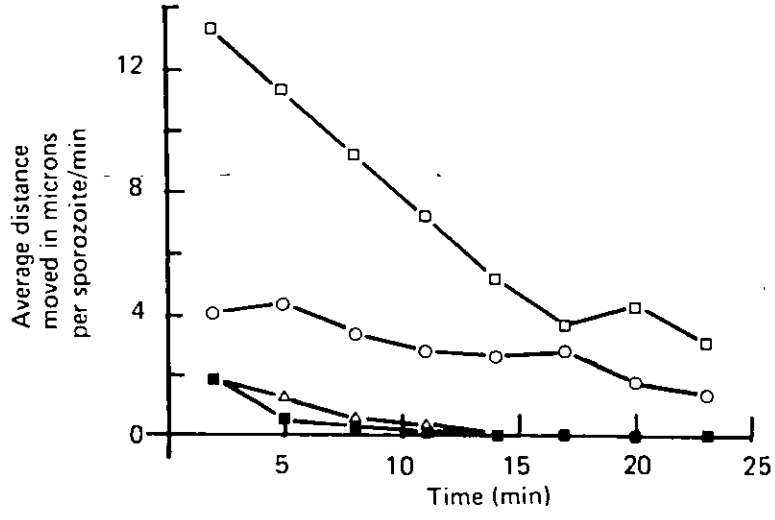
Fig. 4.3



The bending behaviour of *E. acervulina* sporozoites plotted against time. Sporozoites were suspended in HBSS (□) and in varying concentrations of cytochalasin B: 1·0 μg/ml (○); 2·5 μg/ml (△); 5·0 μg/ml (■); at 41 °C. Each reading represents the average value from a sample of 50 sporozoites.



The pivoting behaviour of *E. acervulina* sporozoites suspended in HBSS.



The locomotion of *E. acervulina* sporozoites plotted as distance moved by an average sporozoite against time. The sporozoites were examined in HBSS.

Conversely motility was restored within 5 - 10 s. of washing away the inhibitor with fresh HBSS. The drug cytochalasin B also suppressed gliding and pivoting behaviour in 'zoites of E. tenella, Sarcocystis ovicanis and Plasmodium yoelii. In the presence of the drug these parasites showed only flexing movement. This effect was totally reversible for all cells examined.

Treatment of sporozoites of E. acervulina and E. tenella in 15 mM magnesium ions caused the cessation of all motile behaviour after 20 min. Lower concentrations, 5 mM and 10 mM, took 1 h. and 45 min. respectively to stop motility. The effect of this treatment was not reversible, though some sporozoites treated did recover feeble bending movements when resuspended in fresh HBSS at 41°C. The ultrastructural examination of Mg^{++} - treated sporozoites did not reveal any of the aggregations of filamentous material demonstrated in the ciliate Nassula (Russell, unpublished results). There were no detectable ultrastructural changes in these sporozoites (fig. 4.4).

4.3.3 Labelling of the Sporozoite Plasmalemma

Lectins were used here in an attempt to label any exposed receptor sites on the surface of E. tenella and E. acervulina sporozoites. The lectins used were concanavalin A, peanut and Ricinus communis I agglutinins. R. communis agglutinin was of particular interest because it associates with fibronectin. The protein fibronectin is utilised by many vertebrate cells for cell substratum adhesion, especially mammalian culture cell lines (Badley et al 1980). The results of this experiment are tabulated in fig. 4.5. None of the lectins reacted with either of the two Eimeria spp. examined. The viability and specificity of these lectins were demonstrated using red blood cells and red blood

Fig. 4.5 *Specificity of binding of membrane labels to the surface of* E. acervulina *and* E. tenella

Cell membrane label	Binding reaction with parasite surface		
Lectins	Sporozoites	Sporocysts	Oocysts
Concanavalin A (FITC)	–	–	+
Concanavalin A (FITC) + α-methyl-D-mannoside	–	–	–
R. communis (FITC)	–	–	+
R. communis (FITC) + D-galactose	–	–	–
Peanut (FITC)	–	–	+
Peanut (FITC) + D-galactose	–	–	–
Anionic charge markers			
Cationized ferritin	+	+	+
Cationized ferritin (FITC)	+	+	+

Fixed and unfixed parasites were incubated at 4 and 41 °C with the lectins. Binding specificities are similar in all cases. The oocysts show slight autofluorescence but a marked increase in fluorescence (+) demonstrated binding.

cells with the appropriate saccharide inhibitors. The oocysts and empty oocyst shells of both species of sporozoites reacted positively with all lectins used. These oocysts, which show slight autofluorescence, exhibited a marked increase in fluorescence after incubation with all the FITC conjugated lectins.

The surface antibody raised against glutaraldehyde fixed sporozoites of E. acervulina reacted positively with these sporozoites. The antibody was found to be species specific when added to suspensions of E. tenella and E. nieschulzi. The immunoglobulin G fraction was isolated and identified as part of a final year project undertaken by Miss A.O. Wosencroft. The presence of 1 gG on the surface of E. acervulina sporozoites was visualised by labelling the rabbit anti-sporozoite 1 gG with FITC conjugated sheep anti - rabbit I gG. The technique was used to examine living, untreated, sporozoites (section 4.3.4).

Anionic sites have been demonstrated on the surface of many cells (Quinton and Philpott 1973). The protein cationised ferritin has been extensively used in the localisation and fine structural examination of these charged sites (Dwyer 1975; Grinnel, Tobleman and Hackenbrock 1975). FITC conjugated cationised ferritin reacted strongly with the sporozoites, sporocysts and oocysts of both E. tenella and E. acervulina, this result is tabulated in fig. 4.5.

4.3.4 The Interrelationship between the Mobility of these Membrane Components and Sporozoite Locomotion.

The "capping" reaction or active polarisation of certain intramembraneous proteins to one site on a cell, often prior to shedding, has been observed in many culture cells(Bourguignon and Singer 1977; Bourguignon and Rozek 1980; Edelmen, Wang and Yahara 1976)

and in some protozoa (Barry 1979 ; King and Preston 1977a). Sporozoites of E. acervulina incubated in surface antibody, or in FITC - cationised ferritin, at 4°C were observed capping their fluorescent label to their posterior pole when warmed to 41°C. The capping of cationised ferritin was very swift and most sporozoites examined had shed the label before the medium reached 41°C. If kept at 4°C the sporozoites were covered in a uniform coating of fluorescent label (fig. 4.7a) but on raising the temperature the label was seen to move to the posterior (fig. 4.6a). All sporozoites observed capping had also experienced a forward displacement of 7 - 10 μm, the equivalent to a "unit" of locomotion (Chapter 3). Close observations of these sporozoites revealed that the membrane label "moved" backwards relative to the sporozoite at the same rate as the sporozoite moved forwards relative to the substratum. The substratum and the leading edge of the cap remained static relative to each other. This capping of cationised ferritin, and surface antibody was, like locomotion, inhibited by low temperature (fig. 4.6a) and by cytochalasin B (fig. 4.8a). Capping behaviour, and locomotive behaviour, returned simultaneously on warming or replacement of support medium.

Ultrastructural examination of sporozoites labelled with cationised ferritin confirmed the light microscope observations. Sporozoites incubated at 4°C in cationised ferritin were capable of capping the label (fig. 4.6b), unless kept at 4°C or placed in a solution containing cytochalasin B (fig. 4.8b). The sporozoites kept at 4°C (fig. 4.7b) showed "patching" of the surface label, whilst those incubated in cytochalasin B were covered in an even coat.

Figs. 4.6a, 4.7a and 4.8a. Phase - contrast and fluorescence micrographs of sporozoites of E. acervulina.

Figs. 4.6b, 4.7b and 4.8b. Electron micrographs of longitudinal sections from sporozoites of E. acervulina (processed in phosphate buffer).

Fig. 4.6a. Cells incubated in FITC - labelled cationised ferritin at 4°C and warmed to 41°C. The label is capped to the posterior of the parasite. X 1,900

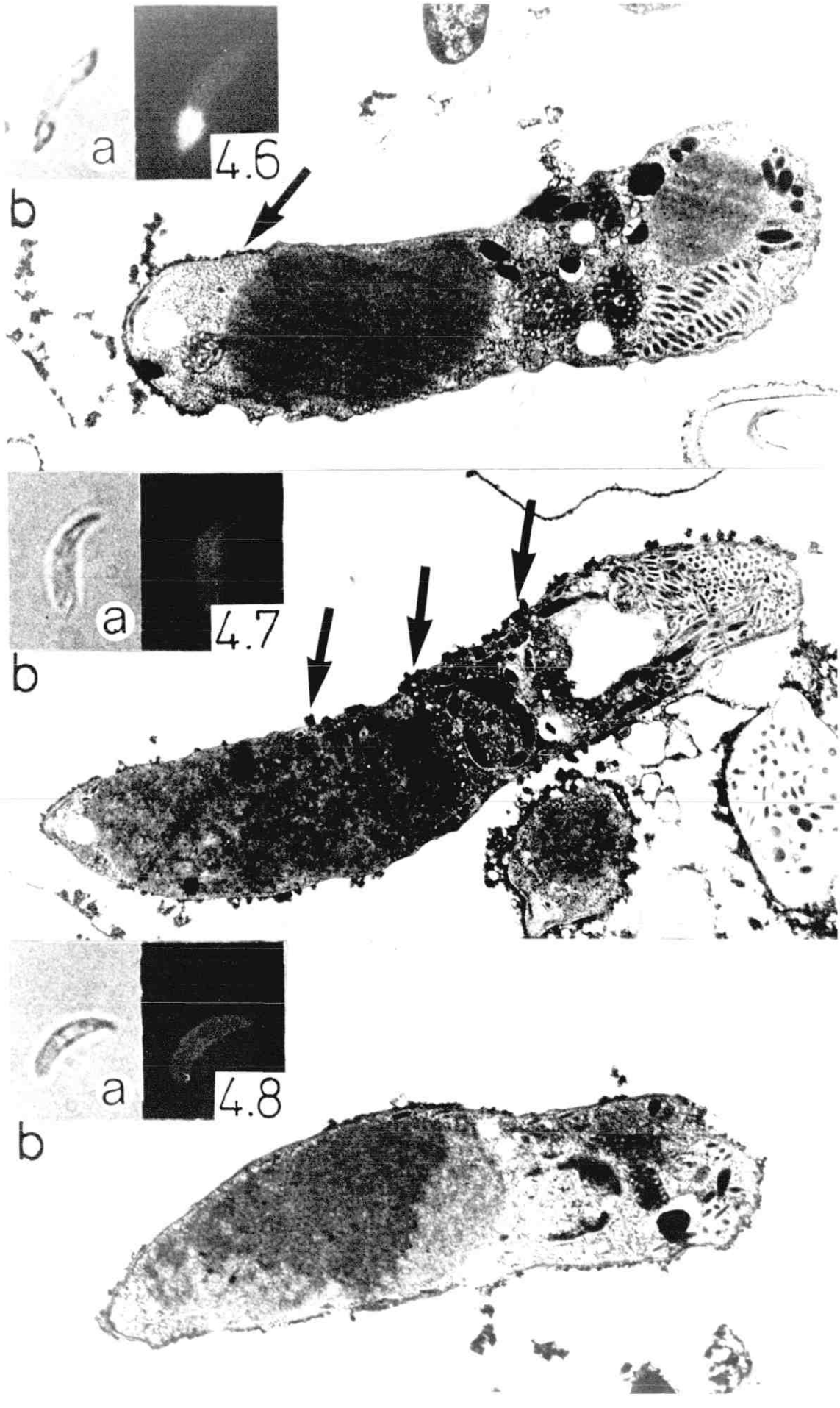
Fig. 4.6b. This sporozoite was incubated in cationised ferritin at 4°C, washed at 4°C, and warmed to 41°C. The label is restricted to the posterior of the cell (arrowed). X 20,200

Fig. 4.7a. Cells incubated in FITC - labelled cationised ferritin at 4°C and washed at 4°C. The label is distributed over the parasite surface. X 1,900

Fig. 4.7b. This sporozoite was incubated in cationised ferritin at 4°C and washed at 4°C. The label is present all over the cell, some patching is evident (arrowed). X 16,400

Fig. 4.8a. Incubated in FITC - labelled cationised ferritin at 41°C in the presence of 10 µg/ml cytochalasin B. The label covers the parasite. X 1,900

Fig. 4.8b. This cell was incubated in cationised ferritin, and washed, at 41°C in HBSS with 10 µg/ml cytochalasin B. The label is evenly over the sporozoite plasmalemma. X 20,400


a
4.6
b
a
4.7
b
a
4.8
b

4.4 DISCUSSION

The experiments used in this Chapter examined the potential roles played by the different components of the sporozoites cytoskeleton, in particular the subpellicular microtubules, and the possible involvement of an actomyosin based contractile system.

The anti - microtubule drugs used were colchicine, vinblastine sulphate, griseofulvin and nocodazole. Colchicine, by binding to the tubulin molecules in solution pushes equilibrium towards depolymerisation and so causes disassembly of microtubules in many cell systems (Wilson, Anderson and Chin 1976). Vinblastine sulphate associates with the growing end of the microtubule and prevents continued assembly. It causes the reorganisation of tubulin into vinblastine - tubulin paracrystals (Bryan 1971). Griseofulvin acts in a similar manner to colchicine though it uses a different binding site on the tubulin dimer (Wilson 1975). Nocodazole is one of the more recent benzimidazole carbamate anti - tumour drugs, it reversibly depolymerises microtubules at very low concentrations (de Brabander, Van der Viere, Aerts, Borgers and Janssen 1978; Zieve, Turnbull, Mullins and McIntosh 1980).

The compounds colchicine, vinblastine sulphate, griseofulvin and nocodazole did not affect the motility of E. tenella or E. acervulina sporozoites. This confirmed the earlier findings of Jensen and Edgar (1976) who demonstrated that the motile and invasive behaviour of Eimeria magna sporozoites was insensitive to the microtubule inhibitors colchicine, colcemid and vinblastine. These authors proposed that, either the microtubules were affected by these drugs but, because they were not involved in sporozoite motility, they had no affect on motile behaviour; or that the microtubular structures were in fact

not microtubules but "tubular aggregates of contractile elements". This present study on E. tenella and E. acervulina suggests that these conclusions may now be revised. Ultrastructural examination of treated cells revealed that the microtubules were unchanged, and that their substructure was consistant with that found in microtubules from different sources (Amos 1977; Amos and Klug 1974). These findings demonstrate that, in these two species of Eimeria at least, the drugs did not affect the microtubules, and that this failure was not due to any detectable abnormality of the subpellicular microtubules. This apparent inability of the drugs to affect the microtubules has three possible explanations. Firstly, related to Jensen and Edgar's proposal, the tubulin that makes up these microtubules may have, through evolution, different amino acid sequencing which could affect the binding characteristics of these drugs. However,to achieve this insensitivity the tubulin would have had to have altered all four different drug binding sites; this,coupled with the universal action of these drugs,suggests not. Secondly, and most simply, the drugs may have failed to penetrate the trimembraneous layer of the sporozoite pellicle. This remains a possibility and it could be tested by using ^{14}C - labelled inhibitors. Thirdly, these microtubules may be insensitive to these drugs because, like the microtubules in cilia and flagella, they are in a fully polymerised and stabilised condition. This insensitivity is an interesting phenomenon but as the drugs were used to assess, by dissolution of the microtubules, the role played by the microtubules in sporozoite motility, this effect was not pursued any further.

Amongst the anti - phagocytic agents used by Jensen and Edgar (1976) was the microfilament inhibitor cytochalasin B. These workers

observed that cytochalasin B completely inhibited host cell invasion of E. magna sporozoites. They also noted that the motile behaviour of these cells was depressed, the sporozoites had ceased to glide and pivot and only showed occasional bending movement. This effect was totally reversible. These early observations were identical to those made during the study of the effects of cytochalasin B on the motility of E. tenella and E. acervulina sporozoites. In fig. 4.3 the effects of a 10 µg/ml concentration of cytochalasin B on sporozoite motility are illustrated. These sporozoites also ceased to glide and pivot and only showed flexing movement. This effect was shown to be fully reversible. Jensen and Edgar suggested that these effects were due to the presence of a microfilament based contractile system. To explain this and the insensitivity of the sporozoites to the anti - microtubular agents, they speculated that the microtubules were "tubular aggregates of contractile elements that are sensitive to cytochalasin B". For the reasons discussed above this is an unlikely hypothesis. However the action of cytochalasin B on the sporozoites of Eimeria spp. does suggest that a microfilament based system is involved in 'zoite motility.

The three types of sporozoite motility, discussed in Chapter 3, show a different effect when exposed to cytochalasin B. Pivoting and gliding are inhibited by similar concentrations (fig. 4.3), this suggests that these two types of motility may have a common contractile mechanism. The speed of this inhibitor may be an indication that this contractile system lies close to the surface of the sporozoite, possibly beneath the plasmalemma. The flexing or bending behaviour of these sporozoites is relatively insensitive to cytochalasin B, this has

several possible explanations. First, drug penetration; if the contractile mechanism is connected to the subpellicular microtubules and associated intramembraneous particles, its relative insensitivity may be due to the difficulty of diffusing across the trimembraneous pellicle. Second, drug specificity; the bending behaviour may not be the expression of an actin based system and the slight drop in activity may be due to inhibition of glucose transport (Lin, et al 1978). Finally, target stability; cytochalasin B operates by binding onto the net gain end of polymerising F actin forcing the equilibrium towards G actin (McLean, Fletcher and Pollard 1980; Brown and Spudich 1981). This drug does not inhibit all actin polymerisation, nor does it depolymerise all actin filaments. A stabilised F actin system would be less sensitive to the drug. These results support the hypothesis that gliding and pivoting motility, at least, are products of an actin based contractile mechanism.

The raising of the magnesium ion concentration has been shown to stop all cytoplasmic streaming in Allogromia (McGee- Russell and Allen 1971) and inhibits contractile vacuole systole and cytoplasmic streaming in the ciliate Nassula (Russell, unpublished results). The molecular basis of this action is the increased polymerisation of actin into paracrystaline form (Weihing 1976) so that it loses its contractile properties. This theory is supported by ultrastructural observations from Nassula. High magnesium ion concentration results in an increase in the filamentous material on the faces of the cytopharyngeal basket associated with cytoplasmic streaming (Russell, unpublished results). Sporozoites of E. tenella and E. acervulina treated in 15 mM $MgCl_2$ lost all their motile behaviour, however, no ultrastructural

changes could be detected in these treated sporozoites. Magnesium ions fulfil many functions in biological systems so it is not possible to state categorically that this inhibition of motile behaviour was due to its effect on actin.

Studies involving the use of lectins to characterise the surface receptors in the glycocalyx of 'zoites of Apicomplexa have revealed an unexpected lack of binding sites (Sethi et al 1977) compared to many other cells (Sharon and Lis 1972, Sharon 1977). In contrast to these findings Schulman et al (1980) demonstrated that although sporozoites of Plasmodium berghei and P. knowlesi had no detectable native glycoproteins on their plasmalemma following incubation in host serum, they could be labelled with concanavalin A and Ricinus communis agglutinin I. However, when this work was repeated on Plasmodium gallinaceum sporozoites (Turner 1980) no binding of lectin could be seen either before or after incubation in host serum. Schulman et al (1980) had employed a rather novel technique that differed from that used by other workers; they had examined the parasites whilst incubating in the FITC conjugated lectin solution. This present study on sporozoites of Eimeria tenella and E. acervulina showed that, like Toxoplasma gondii (Sethi et al 1977), no labelling could be detected on either fixed or unfixed sporozoites. A more recent study by Mauras, Dodeur, Laget, Senet and Bourrillon (1980) using radiolabelled lectins, these are far more sensitive than FITC conjugated lectins, demonstrated that trophozoites of Toxoplasma gondii did bind concanavalin A. The number of receptor sites per cell was only 3×10^4; such low concentrations of receptors would not be resolved by FITC conjugated lectins. This suggests that these 'zoites do possess lectin binding sites but in very low concentrations. It may be possible

to increase the number of sites, in at least some of the 'zoites of Apicomplexa, by incubation in serum, but this has yet to be conclusively demonstrated.

Other techniques were employed to find a membrane label for these sporozoites. The use of antibody as a surface label has been documented in Plasmodium (vanderberg, 1974), surface antibodies have also been used in conjunction with infectivity studies in Eimeria (Long and Rose, 1972). Antibody was therefore prepared against sporozoites of E. acervulina for use as a membrane marker. The protein cationised ferritin has also been used as a membrane binding compound on coccidian sporozoites (Dubremetz and Ferriera 1978). When conjugated with FITC this marker can be visualised with a light/ fluorescence microscope. Both these labels have the advantage that they can be used on living cells so they may be employed to detect membrane motility in these 'zoites.

The movement of surface bound antibody in the 'zoites of Apicomplexa is referred to as the circum sporozoite precipitation reaction. In Plasmodium (Vanderberg, 1974) sporozoites were observed gliding along the substratum until antibody was added to the medium. The antibody coated the sporozoites and stopped gliding. Gliding motility recommenced with the "capping" and shedding of this antibody coat off the posterior end of the sporozoite. The same reaction was observed in sporozoites of E. acervulina, in these experiments the sporozoite surface antibody was labelled with a FITC conjugated second antibody. Sporozoites kept at $4^{o}C$, or in the presence of cytochalasin B could not cap the surface label. However, those sporozoites in fresh HBSS warmed to $41^{o}C$ capped the antibody almost immediately. As with

Plasmodium, sporozoites coated in antibody were immotile but started moving on capping the surface antibody. These experiments demonstrate that these parasites possess a membrane associated contractile system capable of moving certain membrane components to the posterior of the cell. The fate of these components, whether shed or internalised is unknown.

Cationised ferritin was first used as a surface marker on coccidian sporozoites by Dubremetz and Ferriera (1978) in their study on Eimeria nieschulzi sporozoites and Sarcocystis tenella endozoites. They observed that the cationised ferritin bound all over the parasites. This label was subsequently capped to the posterior pole and shed unless the cells were kept at $4^{o}C$ or in cytochalasin B. These workers also noted the close association between gliding and capping. In this present study these experimemts were repeated and extended on sporozoites of E. tenella and E. acervulina. These sporozoites also capped the cationised ferritin unless inhibited by low temperature or by cytochalasin B. Careful observation revealed that the capping and gliding were related. They appeared to be different expressions of the same contractile mechanism. The leading edge of the cap remained static relative to the substratum, whilst the sporozoite moved forwards relative to both the cap and the substratum. Ultrastructural examination revealed that sporozoites labelled with cationised ferritin and kept in cytochalasin B were covered in a uniform coat of label (fig. 4.8b) suggesting the even distribution of anionic sites over the cell. Cationised ferritin labelled sporozoites kept at $4^{o}C$ also had a complete coating of label, but in these cells the label was "patched" (fig. 4.7b). This suggests that the sites were restricted to discrete areas or patches on the

plasmalemma. Sporozoites labelled at 4°C and warmed to 41°C had a plasmalemma clear of cationised ferritin except for a cap at their posterior (fig. 4.6b). These experiments demonstrate the capability of these sporozoites to cap more than one type of membrane label. The differences between labelled sporozoites at 4°C and those in cytochalasin B support the theory of an actin based contractile system. At 4°C actin filaments would be intact and, if connected to membrane receptors, would limit their mobility thus causing patching. But in cytochalasin B the actin filaments would be depolymerised and the receptors free to disperse evenly over the cell surface. A similar reaction has been demonstrated in culture cells where receptors were freed from anchorage to the cytoskeleton allowing crosslinking with succinylated markers (Shetterline, 1980).

The demonstration of a "capping mechanism" in these sporozoites adds further support to the theory of sporozoite motility evolved in Chapter 3, and illustrated in fig. 3.6. This capping reaction shows that these sporozoites possess a contractile mechanism capable of working against a membrane/substratum adhesion site, such as the one proposed in fig. 3.6. This study has resulted in the following model of 'zoite locomotion. The 'zoite is capable of capping a wide range of surface ligands by an organised submembraneous microfilament system. The specificity and availability of these cell surface ligands will control whether the capping reaction results in locomotion (when a substratum binding ligand is involved) or in the capping and shedding of surface bound components (like the circumsporozoite precipitation reaction). Obviously if these parasites are using a surface capping mechanism as a means of locomotion it would be an advantage to adhere

to as many different surfaces as possible. The presence of such non specific ligands as anionic charged sites,would be ideal and would also explain these 'zoites ability to adhere to glass and plastic as well as the many cell types examined (Chapter 3).

The product of these first four chapters is a theory of sporozoite locomotion based on a membrane associated microfilament contractile system. However the experiments designed to test the existence of such a contractile system have produced rather contradictory evidence. The ultrastructural examination of these sporozoites (Chapter 1) failed to demonstrate actin filaments although the same techniques of glycerination and incubation in heavy meromyosin revealed F actin in other cell types. Incubation in high magnesium ion concentrations caused an increased polymerisation of microfilaments in the ciliate Nassula but again failed to demonstrate similar arrays in these parasites. All this evidence is against the existence of an actomyosin based system. However the drug, cytochalasin B, a known inhibitor of F actin suppressed locomotion and capping activity in these cells. This, on the other hand, suggests that there is an actin based contractile system and that this system is associated with the plasmalemma. If such a system is immediately beneath the plasmalemma it may be difficult to visualise by the techniques employed previously. Before a more complete model of locomotion may be assembled it is vital to determine whether or not these parasites contain the protein,actin, required for the proposed contractile system.

CHAPTER 5

IMMUNOLOGICAL AND BIOCHEMICAL ANALYSIS OF CONTRACTILE PROTEINS IN EIMERIINE SPOROZOITES.

5.1 INTRODUCTION

In the previous Chapter it was proposed that capping and 'zoite locomotion are manifestations of a single process. Capping appears to be the result of certain cell surface receptors binding to particulate (or molecular) objects; these objects cannot function as immovable substratum and so are capped to the posterior of the sporozoite. This behaviour has been described in culture cells as an "unsuccessful or abortive cell adhesion, in that the particles adhere to the cell, rather than the cell adhere to the substratum" (Rajaraman, MacSween and Fox, 1978). It is suggested that this longitudinal particle movement on the cell surface membrane is affected by a submembraneous microfilament system resulting in capping, or, if successful substratum adhesion has occured, locomotion.

The similarities between the cap formation seen in many culture cell types and the capping behaviour of these 'zoites suggest that the contractile system employed by these culture cells may resemble that used in sporozoite locomotion. Taylor, Duffus, Raff and de Petris (1971) demonstrated that the capping of surface receptors required energy from cell metabolism. The capping of culture cells did not involve translocation or cell shape change, so the receptors must be in contact with a submembraneous system capable of mediating their systematic movement (Rutishauser, Yahara and Edelman, 1974). Microtubule inhibitors partially reversed this receptor mobility (Yahara and Edelman 1973). These

inhibitors did not affect the actual capping behaviour, and so microtubules appear to be involved only in the regulation of "patch" formation (the grouping of receptors) prior to capping. In 1971 Taylor et al demonstrated that the capping of receptor patches was inhibited by cytochalasin B. More recently Edelman, Yahara and Wang 1976 examined lymphocytes by indirect immunofluorescence using antibodies against contractile proteins. These cells reacted positively with anti - actin and anti - myosin antibodies. In 1976 Edelman, Wang and Yahara proposed that surface modulation was caused by membrane receptors, associated with certain external ligands, interacting with a microfilament based contractile system which was directed, and anchored by a microtubular network.

This proposed connection between microfilaments and certain intramembraneous proteins has been the subject of several studies. In 1975 Albertini and Clarke examined concanavalin A induced caps of ovarian granulosa cells, demonstrating that the microfilaments present beneath this cap were far greater in number than anywhere else in the cell. Pollard and Korn (1973) demonstrated that purified membranes of Acanthamoeba castellani were rich in associated actin. The nature of this actin/membrane attachment is unknown. The proteins previously thought to link actin to membraneous elements, α actinin and vinculin, have been shown to bind to the side, not the tip of actin filaments, and do not attach them to the membrane (Burridge, 1980). It is, however, accepted that actin is in some way associated with certain intramembraneous proteins that act as surface receptors on the external face of the plasmalemma.

The generation of movement by an actin based system has two

possible mechanisms. Firstly, actin - myosin ATPase activity; this involves the two types of filaments sliding over one another (first found in muscle). Secondly, assembly and disassembly of actin bundles, such as the extension of the acrosomal process in Thyone sperm (Tilney 1975). The presence of myosin in the culture cells examined suggests that the former is the more likely. It is proposed that one set of microfilaments is anchored to the membrane ligands, whilst another set is associated with an intracellular structure, such as a microtubule, and that these two sets of microfilaments interact with a type of non - muscle myosin as shown in fig. 5.1 (from Cappuccinelli 1980). Such a contraction generating system would be directed by the "skeleton" of the cell, its microtubular network.

In this Chapter the techniques employed to evaluate the contractile system in these mammalian culture cells are adapted and reapplied to the sporozoites of E. acervulina and E. tenella. The presence and localisation of actin is examined by indirect immunofluorescence, phallatoxin binding to F actin and gel electrophoresis. Attempts were also made to visualise myosin within these cells by indirect immunofluorescence. No attempt was made to detect myosin by gel electrophoresis for two reasons. Firstly, there are several different myosins that have been identified in non - muscle cells (Korn 1978) and these myosins all possess different molecular weights. Secondly, non - muscle cell myosin is more prone to cleavage by SDS and mercaptoethanol, used in the sample buffer for gel electrophoresis, than muscle myosin which retains its characteristic 200,000 dalton structure. Both these properties of non - muscle myosin would make it difficult to identify by its molecular weight.

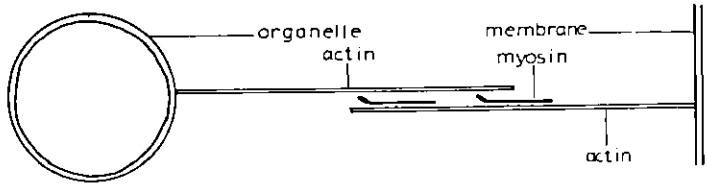


Fig. 5.1 A possible mechanism of contractility in non-muscle cells. (adapted from Cappuccinelli, 1980).

The information gained from this examination of the contractile proteins present in these parasites is used to determine a possible contractile mechanism for sporozoite locomotion and capping, demonstrated in Chapter 4. A molecular model for the contractile basis of this system is proposed.

5.2 MATERIALS AND METHODS

5.2.1 Purification of contractile proteins

1. Actin

Actin was extracted and purified from rabbit skeletal muscle by a technique adapted from Keilley and Harrington(1959). In summary, rabbit skeletal muscle was dissected out, chilled on ice and minced. It was then extracted for 10 min. in three volumes of 0.5 M KCl in 0.1 M K_2HPO_4 at 4°C. The resulting suspension was centrifuged at 15,000 rpm (18,000g) for 15 min. This, and all subsequent centrifugations were carried out in an MSE titanium angle head rotor, type 43114 - 103, in an MSE Superspeed 65. The pellet was resuspended in 1 litre of cold distilled water, the pH was adjusted to pH 8.2 - 8.5 with 1 M sodium carbonate and the suspension was spun at 10,000 rpm (8,000g) for 15 min. The pellet was retained and the procedure repeated until the pellet started to swell, this usually occured after 3 - 4 washes. The pellet was then stirred into 500 ml of acetone (4°C) and left for 20 min. before being filtered off using a Buchner funnel. The filtrate was air dried and stirred into another 500 ml of cold acetone, left for 20 min., filtered and dried. The dry powder was then stirred into 500 ml cold chloroform 4°C, filtered, resuspended in another 500 ml of cold chloroform, filtered and air dried. 10 g of the resulting powder was then extracted in 200 ml of actin extraction buffer containing:-

Tris - HCl	2 mM (pH 8.0 at 25°C)
ATP	0.2 mM
DTT (Dithiothreitol)	0.5 mM
$CaCl_2$	0.2 mM

for 30 min. at 4°C, centrifuged at 15,000 rpm (18,000g) for

15 min. and filtered through glass wool into a measuring cylinder. The filtered supernatant was then adjusted to 50 mM KCl and 2 mM $MgCl_2$ and left to stand at room temperature for 30 min. It was then transferred to the cold (4°C) and left for 90 min. The KCl concentration was then readjusted to 0.8 M and the solution was left to stand at 4°C for another 90 min. It was then centrifuged for 3 hr at 35,000 rpm (90,000g). The supernatant was discarded and the pellet was resuspended in actin extraction buffer before being dialysed against three changes of the same buffer. The monomeric, or G, actin was then clarified by centrifugation at 35,000 rpm (90,000g) for 3 hr. This suspension of G actin could be polymerised into F actin by adjusting the KCl concentration to 50 mM.

2. Tubulin

Tubulin was purified from chick brains by the technique of Burns (personal communication). 100 chick brains were dissected out, and placed in 100 ml of cold microtubule assembly buffer (5°C) consisting of:-

MES	0.1 M
EGTA	2.5 mM
$MgSO_4$	0.5 mM
EDTA	0.1 mM
DTT	0.1 mM

pH adjusted to 6.4 with KOH.

The mixture was then homogenised for 1 s in a blender and then 5 passes in a teflon homogeniser. This suspension was centrifuged at 15,000 rpm (8,000g) as above, for 30 min. at 4°C. The pellet was discarded and the supernatant, which contained tubulin and nucleosidediphosphate kinase (NDPK), was retained. 1 mM of ATP was added to the solution to dissociate the NDPK. The solution was then brought to 38% saturation

with a 100% saturated solution of ammonium sulphate. The 100% saturated ammonium sulphate solution had been adjusted to pH 6.4 with NH_4OH. The protein/ammonium sulphate solution was left to precipitate for 30 min, then centrifuged at 15,000 rpm (18,000g) for 15 min. The pellet was discarded and the supernatant was retained. The concentration of ammonium sulphate was then raised to 50% saturation. This was then centrifuged at 15,000 rpm (18,000g) for 15 min. at $4^{o}C$. The supernatant which contained the NDPK was discarded and the tubulin pellet was retained. The pellet was then redissolved in fresh microtubule assembly buffer ($4^{o}C$) with 0.1 mM GTP and dialysed against the same buffer for at least 2 hr at $4^{o}C$. The solution was then passed down a DEAE column, consisting of Whatman DE 52. At pH 6.4 the tubulin bound to the column. The column was cleared by running through a 0 - 0.75 M NaCl gradient, also in microtubule assembly buffer. The tubulin came off the column as two peaks (fig. 5.2). The first peak consisted of tubulin with 4 sulphydryl groups and some residual NDPK, this tubulin occurs in the cytoplasmic pool. The second peak was retained, this was the tubulin with 8 sulphydryl groups per dimer that came from previously intact microtubules. The tubulin was stored in 50% glycerol at $-20^{o}C$.

3. Myosin

The technique for the extraction and purification of myosin was described previously in Chapter 2, section 2.2.5.

5.2.2 Preparation of Antibodies against Contractile Proteins

The technique used for the raising of antibodies against contractile proteins was similar to that used to produce antibodies against E. acervulina sporozoites, described in Chapter 4, section 4.2.3.

For anti - tubulin antibody; 5 mg of tubulin protein (purified

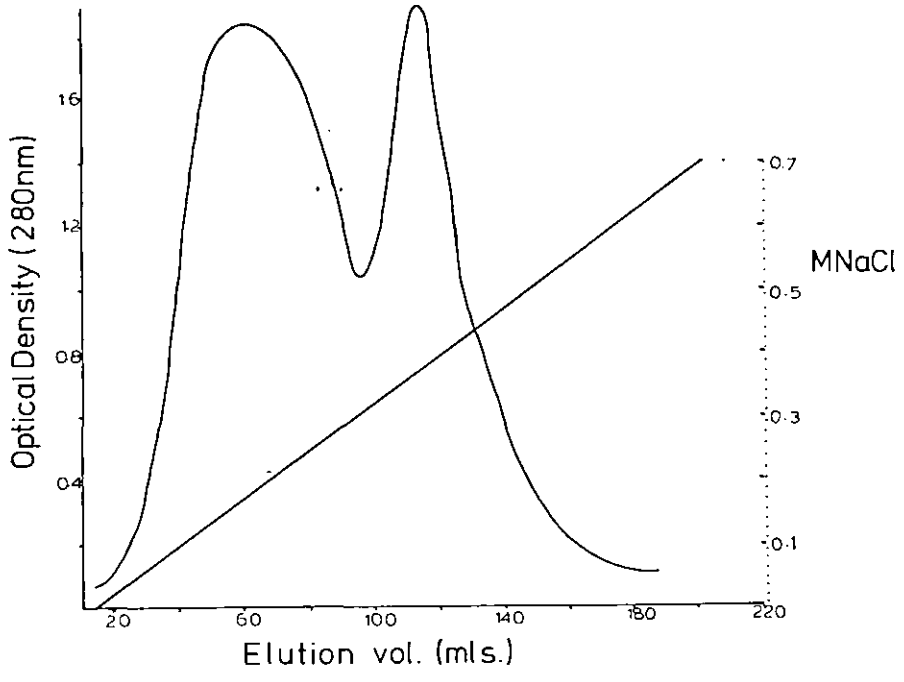


Fig. 5.2 Optical densitometer trace revealing two protein peaks in the NaCl gradient run through the Whatman DE52 column. The second peak contained the 8 S-H tubulin used in subsequent experiments.

as above) was crosslinked with 2.5% glutaraldehyde in 50 mM Tris - HCl (pH 7.0) for 1 hr at 4°C (Weber, Pollack and Bibring 1975). The protein was then washed and resuspended in 50 mM phosphate buffered saline (pH 7.5). This was emulsified 1:1 with Freund's complete adjuvant and injected into the four axial lymph nodes of a New Zealand White rabbit on day one. On day ten this dose was repeated and on day twenty seven the rabbit was bled from its ear. The blood was allowed to clot and the serum was pipetted off. The serum was cleared by centrifugation at 850g for 15 min. in an MSE bench centrifuge. Complement was heat inactivated at 56°C for 20 min. The specificity of the antisera was tested by an Ouchterlony immunodiffusion test against tubulin, bovine serum albumin and actin and by indirect immunofluorescent labelling, described in section 5.2.3, of mouse 3T3 fibroblasts. The microtubular cytoskeleton of these cells has already been extensively examined by this technique (Weber et al 1975).

The preparation of anti - actin and anti - myosin antibodies was identical to the above procedure except 400 µg of unfixed actin or 500 µg of unfixed myosin was injected, instead of the 5 mg of glutaraldehyde - fixed tubulin used in the preceeding method. These quantities had been calculated by previous researchers as being the optimum amount required to produce a large, but specific immune response (Lazarides and Weber 1974; Weber and Groeschel - Stewart 1971).

Although these antibodies all reacted positively with the relevant protein in the immunodiffusion test they had a high level of background interference when used on cell preparations. Additional antibodies were kindly donated by Dr. C. King, University College, London, and by Dr. P. Shetterline, Liverpool University.

5.2.3 Indirect Immunofluorescent Labelling of Contractile Proteins *in situ*.

Cells were prepared by a technique described by Weber *et al* (1975). Mouse 3T3 fibroblasts were grown on coverslips in Dulbecco's modified Eagle's medium; these cells were used as controls. Sporozoites of *Eimeria tenella* and *Eimeria acervulina* were excysted, suspended in fresh HBSS and allowed to settle onto the coverslips for 10 min at room temperature. When these coverslips were washed gently in HBSS the sporozoites remained adhered to the glass whilst the debris and unexcysted material was washed off. The cells were then fixed by placing the coverslips in 3.7% formaldehyde in phosphate buffered saline (pH 7.4) for 5 min at room temperature. The phosphate buffered saline (PBS) contained:-

NaCl	147 mM
KCl	2.7 mM
$Na_2HPO_4(H_2O)_7$	15.2 mM
KH_2PO_4	1.5 mM

The coverslips were then washed in PBS and treated in methanol (-20°C) for 3 min and acetone (-20°C) for 3 min. The coverslips were then either air dried or washed in PBS. The cells were then incubated in the primary antisera. 200 µl of antisera in PBS was placed on each coverslip. The titre of each antisera differed so the dilutions for optimum staining producing clearly defined preparations also differed. The dilutions used were; anti - actin at 1:20 in PBS, anti - tubulin at 1:30 and anti - myosin at 1:50. The cells were incubated in these solutions for 45 min at 37°C in a damp chamber to reduce evaporation. The samples were then washed carefully in PBS and incubated for 30 min at 37°C in the second antibody, fluorescein isothiocyanate conjugated goat anti rabbit γ globulins (Wellcome). This antibody was diluted 1:20 in PBS.

The cells were then washed and mounted in a 1:10 mixture of PBS in glycerol. The coverslips were examined using a Leitz Orthoplan microscope equipped with epifluorescence optics.

This technique produced crisply labelled fibroblasts but there was a background of non - specific fluorescence in the sporozoite preparations. Control preparations of sporozoites were incubated in non - immune rabbit serum and the FITC - anti - rabbit antibody, these revealed non specific binding of sera components. Several different methods of preparation were tried in an attempt to prevent this unwanted fluorescence.

It has been suggested that the aldehyde terminal groups that result from fixation in formaldehyde may attract certain components of the FITC secondary antibody (Weber, Rathke and Osborn 1978). These authors used sodium borohydride ($NaBH_4$) to reduce the aldehyde and hence reduce the background staining. In following their technique the acetone ($-20^{o}C$) step was dropped and cells were incubated in methanol ($-20^{o}C$) for 10 min. They were then washed in three changes of 0.5 mg/ml $NaBH_4$ in PBS for 4 min each at room temperature. They were washed in fresh PBS and incubated in primary and secondary antibody as before.

The problem of non specific binding of secondary antibody was examined by the following method. Some preparations were incubated in non - immune rabbit serum for 15 min at $37^{o}C$ instead of incubation in the primary antisera. These were then processed in secondary antibody, enabling any non specific binding of the secondary label to be detected.

Several different adaptations of the above techniques were attempted to eliminate non specific binding of label but the cleanest preparations of control cells, the 3T3 fibroblasts, were obtained using

the technique described above, from Weber et al (1975).

5.2.4 Visualisation of F actin with Nitrobenzooxadiazole - Phallacidin.

The binding of the fungal toxin phallacidin has been shown to be specific for F actin (Wieland 1968, Wulf, Deboben, Bautz, Faulstich and Wieland 1979). Recently this phallotoxin has been conjugated with the fluorescent label nitrobenzooxadiazole by Barak, Yocum Nothnagel and Webb (1980) (Molecular Probes Inc. 849 J Place, Suite B, Plano, Texas. 75074).

The following procedure was used to prepare both control and experimental cells. The control cells, 3T3 fibroblasts, had been examined previously by this technique (Barak et al 1980). Coverslips of eimeriine sporozoites were prepared as described in section 5.2.3. These coverslips were washed in PBS (section 5.2.3) and fixed in 3.7% formaldehyde in PBS for 10 min at room temperature. The cells were then washed in PBS and extracted in acetone ($-20^{o}C$) for 5 min. The coverslip was then air dried and 200 µl of PBS containing 33 ng of nitrobenzooxadiazole (NBD) - Phallacidin, was carefully pipetted onto the cell side of the coverslip. These preparations were incubated in NBD - Phallacidin for 20 min. at either $4^{o}C$ or room temperature. The coverslips were then washed in PBS and mounted in a 1:1 solution of glycerol and PBS. The coverslips were sealed around the edge with nail varnish and stored at $4^{o}C$ in the dark prior to examination.

The NBD - Phallacidin was stored as a stock solution of 3.3 µg/ml in methanol. The methanol was evaporated off prior to use.

5.2.5 Gel Electrophoresis of Coccidian Sporozoites

The SDS polyacrylamide gels were prepared from the following

solutions by a technique adapted from Laemmli (1970).

Separation Gel

8 mls of 30% acrylamide, 0.75% bis - acrylamide

3.75 mls of 3 M Tris - HCl (pH 8.8)

0.3 mls of 10% sodium dodecyl sulphate

25 µl of TEMED

75 µl of 10% ammonium persulphate (fresh)

dilute to 30 ml with distilled water.

Stacking Gel

1 ml of 30% acrylamide, 0.75% bis - acrylamide

2.5 ml of 0.5 M Tris - HCl (pH 6.5)

50 µl of 10% sodium dodecyl sulphate

6.4 ml of Distilled Water

25 µl of TEMED

25 µl of 10% ammonium persulphate (fresh)

Chamber Buffer

0.025 M Tris - HCl - 0.192 M glycine

0.1% sodium dodecyl sulphate

Sample Buffer

6.25 mM Tris - HCl

4% sodium dodecyl sulphate

20% glycerol

10% mercaptoethanol

Stained with bromophenol blue

The gels were prepared in tubes, by pouring in the separation gel first. This gel was then overloaded with isobutanol and allowed to set. The isobutanol was then shaken off and the stacking gel was poured onto the separation gel; this gel was also overloaded with isobutanol.

After the gel had set this isobutanol was again shaken off. The tube gels were set up in the chamber buffer ready for use.

The samples for gel electrophoresis were boiled for 3 - 5 min. in sample buffer to ensure that all the protein present was in solution. Between 10 - 50 µl of the sample was loaded into each tube gel. The gels were focused at 100 V until the protein front had almost reached the end of the stacking gel. The separation gels were run at 70 V, and 6m Amps per gel.

The gels were then taken out of their tubes, stained overnight in 10% acetic acid, 25% isopropanol, 0.025% coomassie blue and, finally, destained in 10% acetic acid. They were then scanned in a Beckman DU 8 gel scanning attachment.

Sporozoites of Eimeria acervulina and Eimeria tenella prepared and purified by the technique described in Chapter 1 were not pure enough for analysis by gel electrophoresis. The method for purification of sporozoites of Plasmodium (Pacheco et al 1979) was adapted to clean suspensions of coccidian sporozoites. The oocysts were hatched as outlined in Chapter 1 then the suspension was layered onto a discontinuous renografin gradient consisting of:-

Top Phase - suspension of sporozoites etc in HBSS	2 ml.
Middle Phase - renografin 1:2 in HBSS	5 ml.
Bottom Phase - renografin 3:4 in HBSS	5 ml.

This gradient was centrifuged at 12,700 rpm (16,500g) in an MSE Hi - spin 21 with a swing out rotor head type 43124 - 705. The suspension separated out with the intact oocysts and sporocysts at the first (highest) interface, the sporozoites in between the two renografin phases, and the oocyst and sporocyst shells at the base of the tube.

The sporozoites were then washed in two changes of HBSS and pelleted ready for the addition of the sample buffer.

5.2.6 Indirect Immunolabelling of Contractile Proteins after Gel Electrophoresis.

Attempts were made to label certain protein bands on the SDS - acrylamide gels by the technique of Towbin, Staehelin and Gordon (1979). Briefly, samples of sporozoites and purified contractile proteins were run on 10% acrylamide slab gels. The proteins were then transferred by an electro - blot technique to nitrocellulose paper. The gel was then stained in coomassie blue whilst the nitrocellulose sheet was incubated in 3% bovine serum albumin in 10 mM Tris buffer (pH 7.4) saline (0.9%), this buffered saline was used throughout the procedure. The nitrocellulose sheet was washed, and incubated in the primary antibody at 1:50 dilution for 30 min. at room temperature. It was then washed and incubated in a peroxidase conjugated secondary antibody. After extensive washing the blot was "developed" in a 0.01% hydrogen peroxide solution, dried in the air and examined.

5.3 RESULTS

5.3.1 Immunofluorescent Labelling of Contractile Proteins

The 3T3 fibroblasts prepared for immunofluorescence with anti - actin, anti - myosin and anti - tubulin antibodies revealed a highly ordered array of cytoskeletal elements (fig. 5.3). The actin and myosin (fig. 5.3b and c) appeared to be localised in the same areas. This pattern of contractile elements seen in these preparations were identical to that visualised in 3T3 fibroblasts by previous workers (Weber and Groeschel - Stewart 1974; Weber et al 1975; Weber, Bibring and Osborn 1975; Lazarides and Weber 1974). The cells labelled cleanly when processed by the procedures described. The microtubular skeleton and the microfilamentous stress fibre system seen in the preparations were also compatable with the cytoskeletal network exposed in the critical point dried fibroblasts seen in Chapter 2.

Unfortunately all attempts to duplicate these results on sporozoites of E. acervulina and E. tenella were unsuccessful. These preparations were highly fluorescent (fig. 5.4) but this fluorescence was also seen in those preparations incubated in only the FITC conjugated secondary antibody.

5.3.2 NBD - Phallacidin Labelling of F actin in Eimeriine Sporozoites.

It has been demonstrated that the fungal toxin NBD - Phallacidin binds specifically to F actin in fixed and unfixed cells (Wulf et al 1979; Nothnagel, Barak, Sanger and Webb 1981). Monolayers of 3T3 fibroblasts were processed as controls (fig. 5.5). The labelled areas in these cells corresponded to the areas seen in fibroblasts treated in anti - actin antibody, and to the stress fibre bundles revealed in the critical -

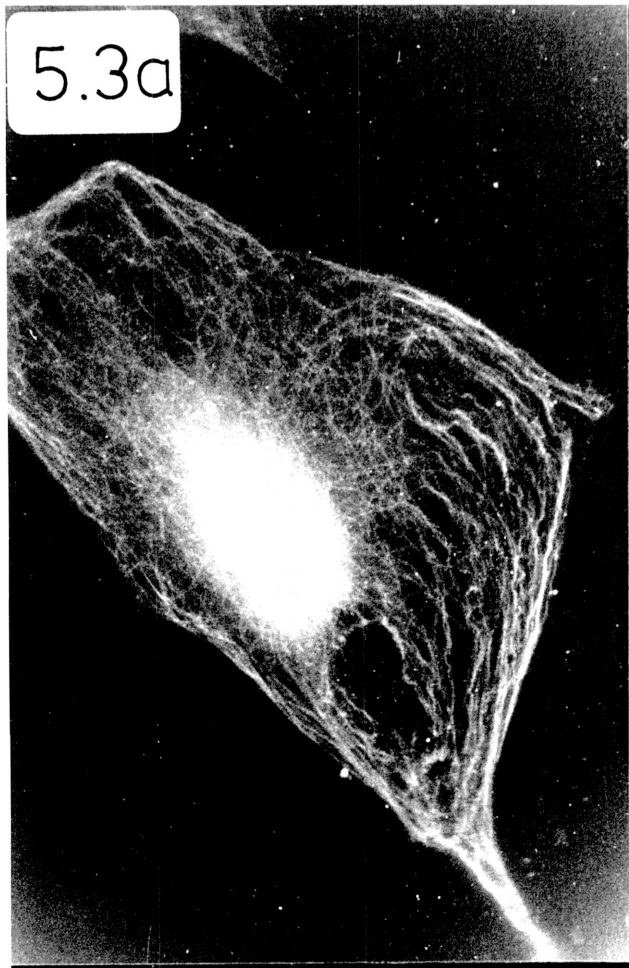
Fig. 5.3. Fluorescence micrographs of mouse 3T3 fibroblasts stained by indirect antibody technique with antibodies against contractile proteins.

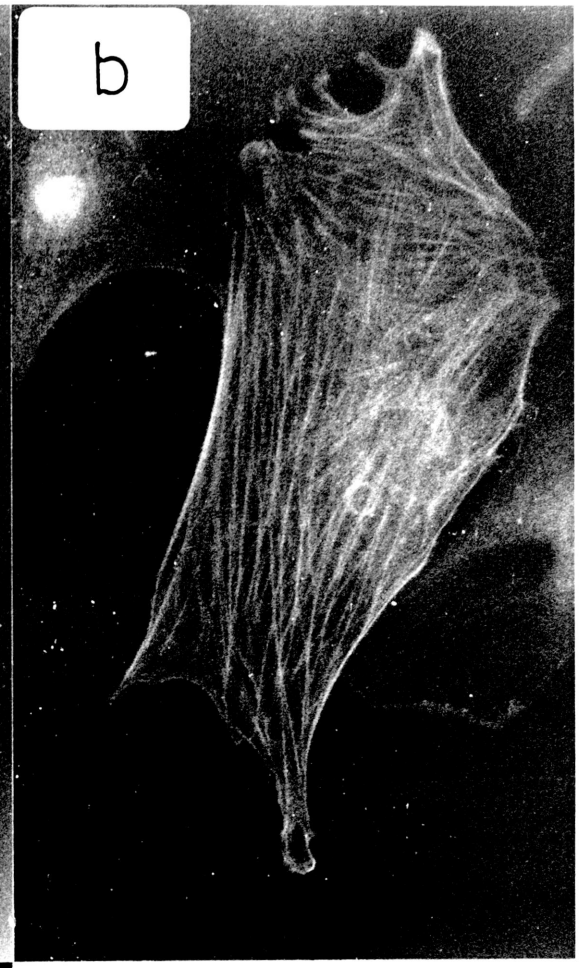
a) Anti - tubulin antibody; the microtubules are revealed running out from the body of the cell. X 875

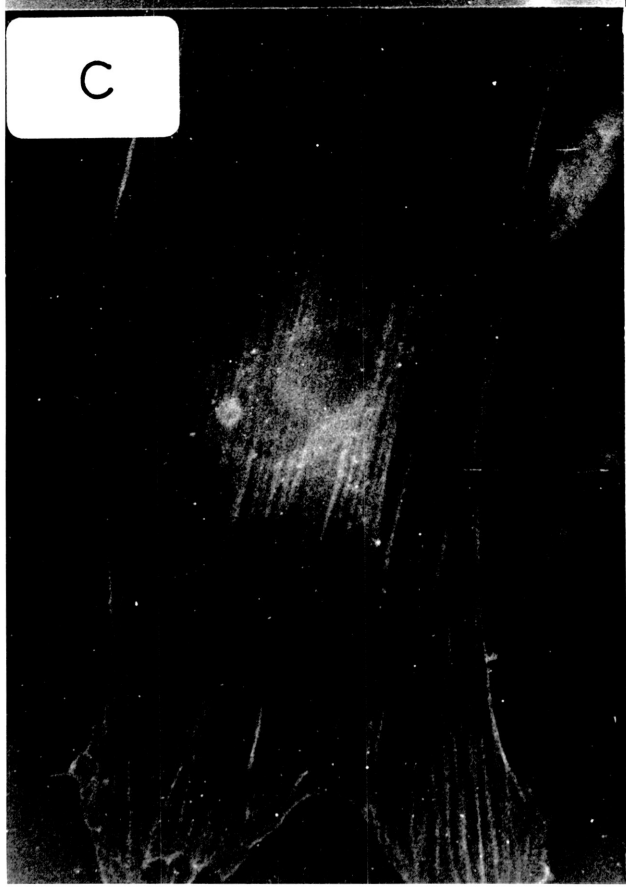
b) Anti - actin antibody; the microfilaments are arranged as stress fibres. X 875

c) Anti - myosin antibody; myosin appears to be associated with the actin stress fibres. X 875

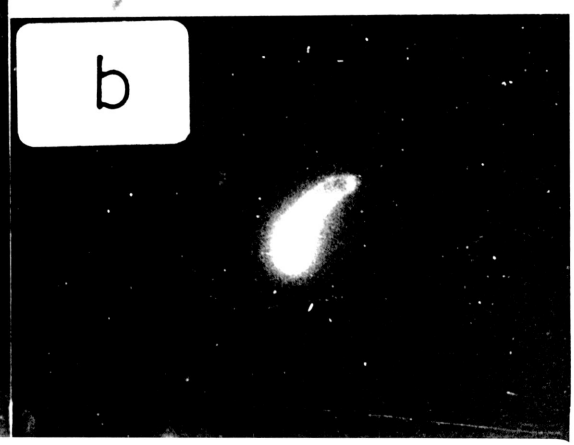
Fig. 5.4 a) Phase - contrast and b) fluorescence micrograph of a sporozoite of E. acervulina processed for indirect antibody labelling of actin. However this fluorescence persisted even in control preparations demonstrating that this binding was non - specific. X 1,500







5.4a

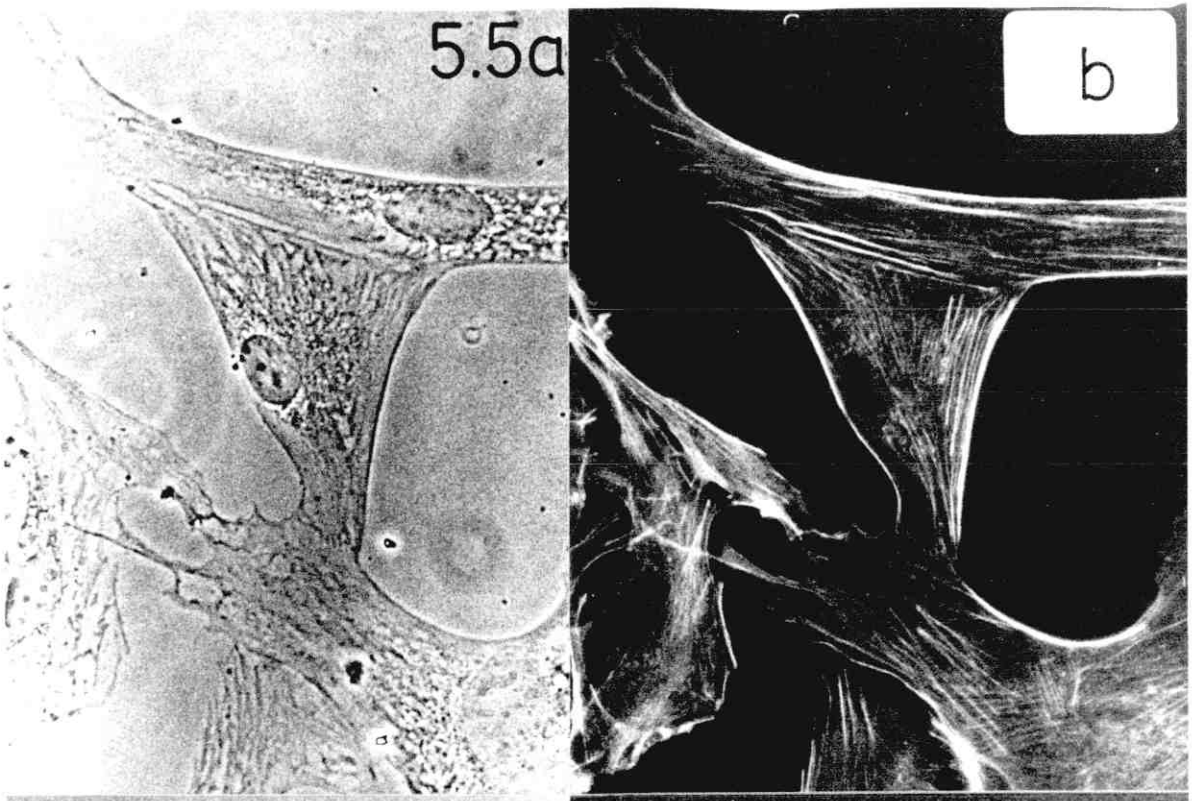


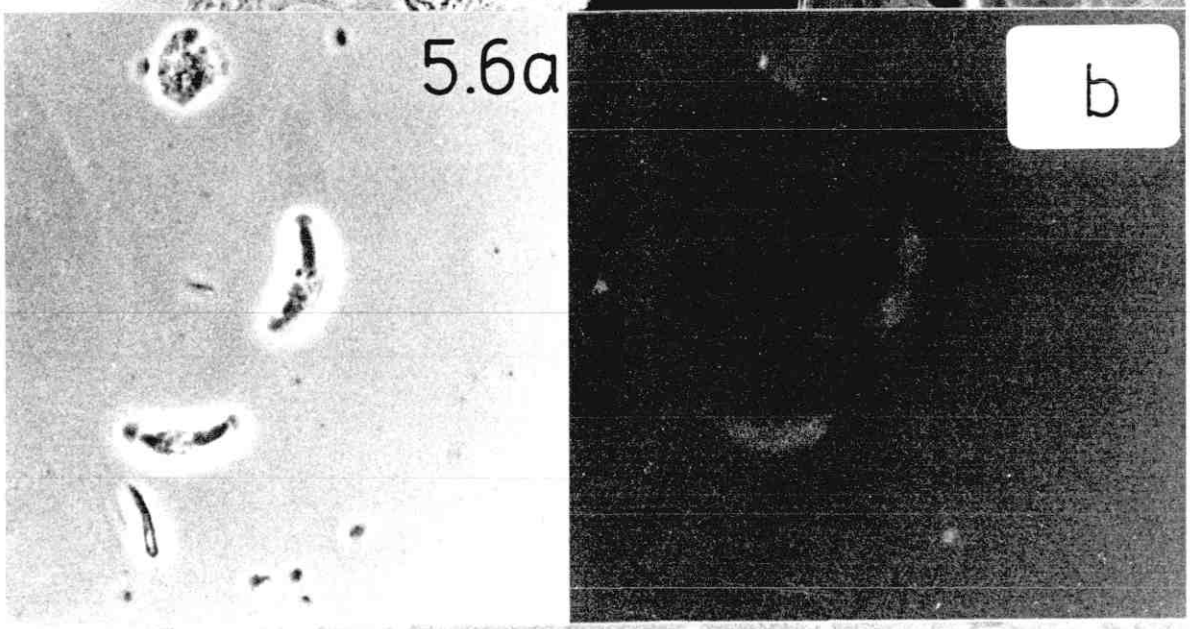
Figs. 5.5 and 5.6. Cells stained with NBD - phallacidin, a compound that binds specifically to F actin.

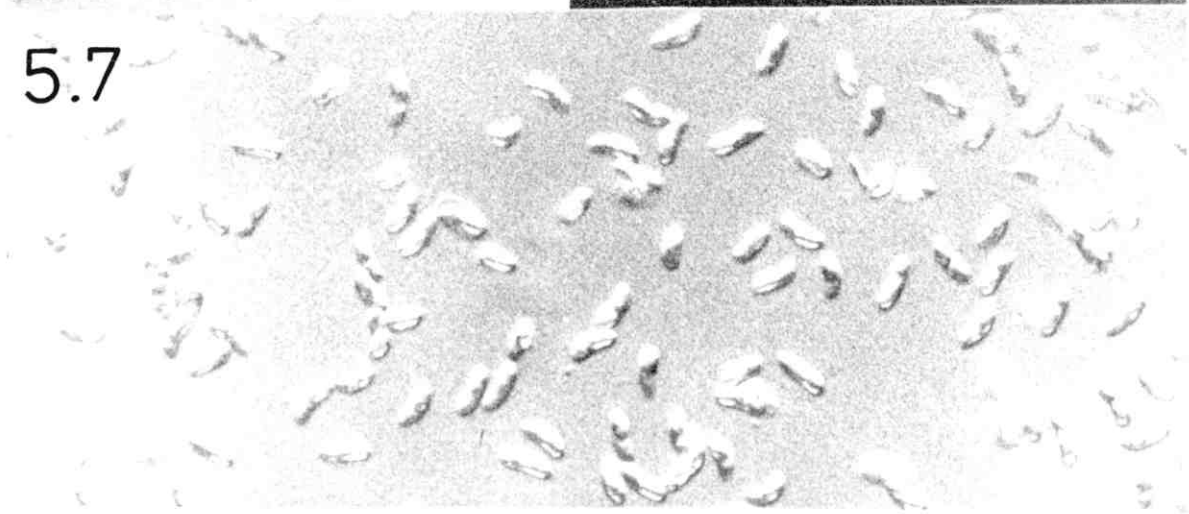
Fig. 5.5 a) Phase - contrast and b) fluorescence micrographs of a mouse 3T3 fibroblast. The F actin label is distributed similarly to the anti - actin antibody (fig. 5.3b). X 530

Fig. 5.6 a) Phase - contrast and b) fluorescence micrographs of sporozoites of E. acervulina. The NBD - phallacidin was evenly distributed over these cells. X 1,800

Fig. 5.7. Nomarski interface - contrast micrograph of a sample of E. acervulina sporozoites purified in a discontinuous renografin gradient. X 750


5.5a
b


5.6a
b


5.7

point dried preparations (Chapter 2). The labelling of these structures was much sharper using NBD - phallacidin than it was using the anti - actin antibody. This could be due to NBD - phallacidin labelling only F actin, so that any background fluorescence caused by a cytoplasmic pool of monomeric actin is eliminated.

Sporozoites of E. tenella and E. acervulina were prepared by the same technique used on 3T3 fibroblasts. These sporozoites labelled evenly over their body (fig. 5.6). The fluorescence from these parasites was not as intense as that seen in the fibroblast preparations. Fragments of oocyst shell and empty sporocysts that were processed along with the sporozoites did not label.

5.3.3 Gel Electrophoresis of Sporozoites of E.tenella and E. acervulina.

Suspensions of sporozoites of E. tenella and E. acervulina were cleaned, prior to use, by centrifugation in a discontinuous renografin gradient. The suspension of sporozoites obtained from this gradient was greater than 99% pure (fig. 5.7). These suspensions were then dissolved in sample buffer and run on the SDS acrylamide gels.

The actin purified from rabbit skeletal muscle was run to examine its purity. The gel was then stained, destained and scanned in a Beckman DU 8 optical densitometer. The resulting trace, fig. 5.8, showed that the actin was pure. Gels were then run of the sporozoites of E. acervulina and of E. acervulina plus rabbit skeletal actin. These gels were also stained, destained and scanned in the optical densitometer. The sporozoite sample had many bands, or peaks (fig. 5.9), and one of these peaks appeared at approximately 42,000 daltons, the molecular

fig5.8

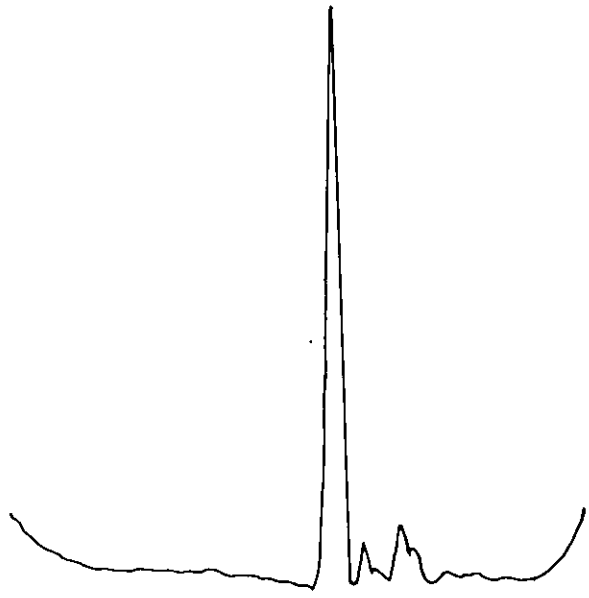


fig5.9

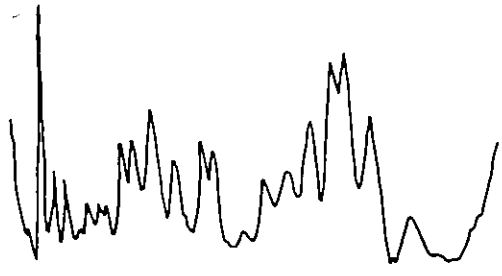
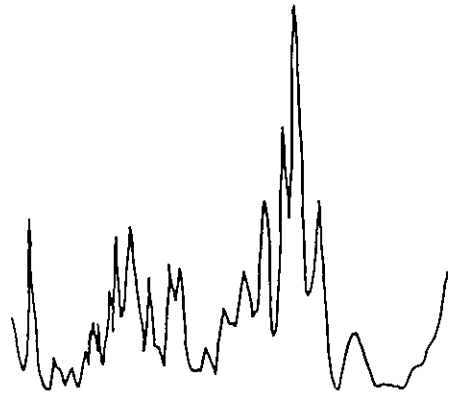


fig5.10



Traces from gel scans of:-

Fig. 5.8 Rabbit skeletal actin

Fig. 5.9 E. acervulina sporozoites

Fig. 5.10 E. acervulina sporozoites and rabbit skeletal actin

weight of the actin. The trace from the gel that was run of sporozoites plus actin showed that this peak at 42,000 daltons was increased by the addition of actin to the suspension (fig. 5.10).

These results demonstrate that these sporozoites contain a protein that co - migrates identically with rabbit skeletal actin.

5.3.4 Indirect Immunostaining of Protein following Gel Electrophoresis.

This technique has been used to examine proteins of various culture cell types (Lane, personal communication). It is known that not all proteins survive electroblot transferance intact and certainly this technique does denature some proteins. Attempts were made to label contractile proteins from extracts of 3T3 fibroblasts, eimeriine sporozoites, and samples of the proteins themselves. These attempts failed due to heavy background staining of the nitrocellulose blot. This technique was not pursued any further.

5.4 DISCUSSION

The aim of this study was to determine which contractile proteins were present in the sporozoites of E. tenella and E. acervulina . The techniques applied were indirect immunofluorescence, NBD - phallacidin F actin binding and gel electrophoresis. Only the last technique had been used previously on 'zoites of Apicomplexa. There have been two previous gel electrophoresis studies on coccidian 'zoites. The first, in 1979 by Dubremetz and Dissous, examined the dense granules and micronemes of 'zoites of Sarcocystis tenella isolated from sheep oesophagi. These authors disrupted the cells, purified the fractions on a discontinuous sucrose gradient and ran each fraction, as well as a sample of intact 'zoites, on SDS polyacrylamide gels. Unfortunately these authors were not interested in the "actin area" of the gel and they used a 43,000 dalton protein to calibrate the gel. This obscured any possible 42,000 dalton band. 'Zoites of Sarcocystis spp. were an obvious choice for gel electrophoresis studies because of the ease in obtaining large numbers of parasites. However, because the cysts were intramuscular, the purification would have to be extensive to eliminate possible contamination with muscle actin.

The other study of the protein content of 'zoites of Apicomplexa was carried out in 1981 by Johnson, McDonald and Neoh on the tachyzoites of Toxoplasma gondii. These parasites were obtained by peritoneal exudate of laboratory mice. Samples of these 'zoites and of two control preparations, non - infected exudate and host cell serum, were run on SDS polyacrylamide gels. The parasite preparations showed protein bands at 41,000 and 43,000 daltons whilst the control exudate had bands at 42,000 and 43,000 daltons. Unfortunately this study cannot be used as

evidence in this present work because, firstly, the authors were not interested in this part of the gel so its calibration may easily be 1,000 daltons out, and secondly, the samples had contamination from other bodies in the exudate. The most common contaminant were red blood cells, these cells are known to contain actin which could easily produce a false result.

This present study on the sporozoites of two species of Eimeria was specifically designed to examine the contractile proteins, particularly actin, present in these parasites. An advantage of using these sporozoites was that they were not isolated from intramuscular cysts nor were the suspensions contaminated by any actin containing cells. This meant that any possible contamination with external actin was removed. The suspension of oocyst was washed extensively during excystation (Chapter 1). The hatched sporozoites were cleaned to remove all oocyst and sporocyst debris in a discontinuous renografin gradient. This procedure produced 99% pure suspension of 'zoites, the only visible contamination was the occasional fragment of oocyst or sporocyst shell. The gel scans (fig.5.8 - 5.10) reveal the presence of an actin - like protein that co - migrates identically with rabbit skeletal actin. It is known that all actins have been highly protected through evolution and that non - muscle actin and rabbit skeletal actin possess the same electrophoretic mobility (Korn 1978). Attempts to positively identify the 42,000 dalton band as actin by indirect antibody labelling of a nitrocellulose sheet previously treated by electroblot protein transfer were unsuccessful.

Although indirect immunofluorescent labelling of contractile proteins in situ successfully demonstrated actin, myosin and tubulin in mouse 3T3 fibroblasts, attempts to repeat these experiments on

coccidian sporozoites were defeated by non - specific binding. However, the sporozoites of E. acervulina and E. tenella both reacted positively with NBD - phallacidin. This fungal toxin is known to bind specifically to F actin (Wulf et al 1979).

The demonstration of a 42,000 dalton protein and the positive reaction of NBD - phallacidin strongly suggests that actin is present in these parasites. The distribution of label following treatment in NBD - phallacidin indicates that actin is present as polymerised microfilaments evenly spread on the 'zoite body. These results enable a model of the force generating mechanism behind sporozoite locomotion to be proposed. It is known that actin associates with proteins embedded in the plasmalemma of some cells (Pollard and Korn 1973; Weihing 1976). Such an association could form the basis of a contractile system capable of the cap formation discussed in Chapter 4. The failure to find any microfilaments in these parasites during the ultr-structural examinations in Chapter 2, could be explained by their situation. If actin filaments are associated with the inner face of the plasmalemma then they will lie between the plasmalemma and the inner membrane complex. This area is usually electron dense and therefore difficult to examine.

The model of the molecular basis of 'zoite locomotion is represented in the diagram fig. 5.11. The sporozoite is attached to the substratum by means of certain surface receptors exposed on the outside of the plasmalemma. The exact nature of these sites is unclear. There may only be those visualised by cationised ferritin, Chapter 4. These anionic sites would be capable of adhering to most surfaces encountered by these cells. The substratum/receptor complex then acts as an anchorage

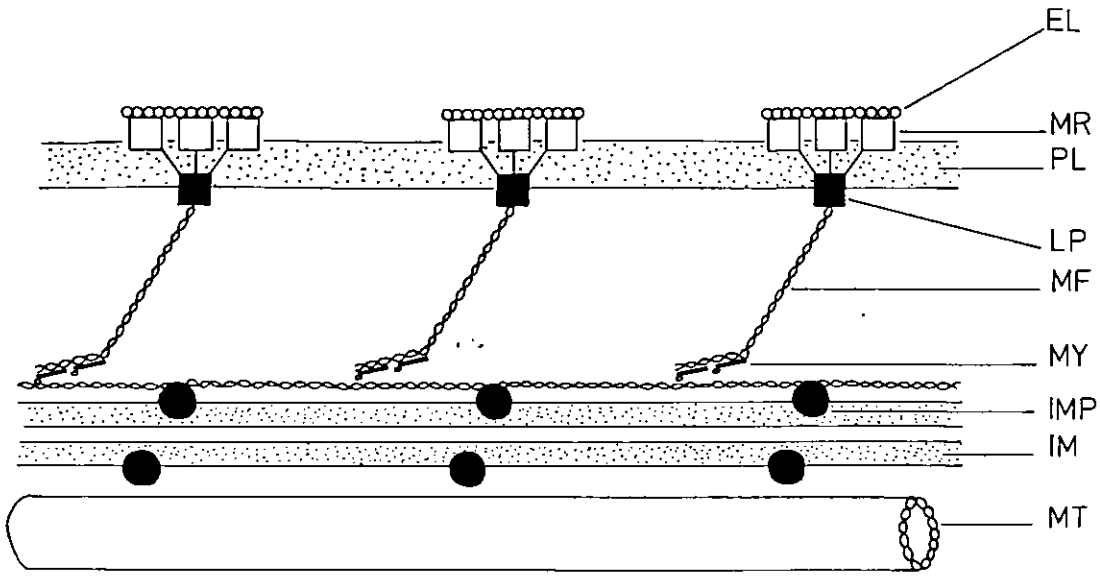


Fig. 5.11 A model of the molecular basis of 'zoite locomotion. The external ligands (EL) associate with membrane receptors (MR) in the plasmalemma (PL). This association causes patching of receptors as they bind to a linkage protein (LP) which is attached to the first set of actin filaments (MF). These microfilaments have single-headed myosin molecules (MY) on their free ends. The myosin interacts with a second set of microfilaments anchored to the intramembraneous particles (IMP) of the inner membrane complex (IM). The linear organisation of these particles is dictated by the subpellicular microtubules (MT).

site against which the contractile system can "pull"; such a system is similar to that proposed for culture cells (Rajaraman,MacSween and Fox 1978). It has been suggested by Klausner, Bhalla, Dragsten, Hoover and Karnovsky (1980) that this linkage between the membrane receptor and the microfilament system is indirect. This would allow the one contractile system to cap many different groups of receptors independently. The receptors would move freely around in the plasmalemma until "transformed" by interaction with an external ligand. Transformation would involve association with the intramembraneous protein linked to the actin filament. The interaction between contractile elements and cell/substratum adhesion sites has been elegantly demonstrated in fibroblasts by Badley, Woods, Smith and Rees (1980). The mobility of these receptors, in particular the anionic sites, has been examined in culture cells by Buteman, Bourguignon and Bourguignon (1981).

The motile force could involve the interaction between the plasmalemma associated microfilaments discussed above, and another set of microfilaments linked to the intramembraneous particles on the outer face of the inner membrane complex. These particles are organised in linear arrays parallel to the direction of locomotion (Porchet and Torpier 1977; Dubremetz and Torpier 1978; Dubremetz, Torpier, Maurais, Prensier and Sinden 1979). The movement may be generated by single headed myosin molecules with their light chain associated with one actin filament whilst their heavy chains "pulled" against the other filament. (fig. 5.11). This swiveling mechanism is caused by the same actin mediated myosin ATPase activity found in muscle. Single headed myosins of this type have been isolated from Acanthamoeba castellani (Pollard and Korn 1973a and b). Myosin is the most likely candidate for such a system because of its common association with actin (Korn 1978) and its

occurrence in capped culture cells (Edelman et al 1976).

It is unlikely that the subpellicular microtubules are actively involved in the propagation of locomotion in these parasites. This would agree with observations made from capping lymphocytes (Unanue and Karnovsky 1974). These workers suggested that the microtubular cytoskeleton fulfills an orientating role that directs membrane mobility. Despite the vast morphological differences between these cells and the sporozoites, the microtubules, as part of the shape determining system in both cells, could fulfill a similar role for capping, and hence locomotion in sporozoites. It is likely that the microtubules are "responsible" for the linear arrangement of intramembraneous particles on the outer and inner surfaces of the inner membrane complex. This arrangement may take place during 'zoite formation. Studies on the developing merozoites of E. stiedae have revealed arm - bearing microtubules in close association with the inner membrane complex. Similar structures have not been seen in fully formed eimeriine 'zoites.

In this Chapter a model to explain the possible molecular basis of locomotion and capping has been proposed. This model should be related to the sequence of events discussed in Chapter 4 to form a complete "description" of 'zoite locomotion. Stated briefly, the 'zoite is capable of capping a variety of membrane receptor types by the submembraneous contractile system proposed above. It is the specificity and availability of these receptors that will control the external manifestation of this single contractile mechanism. It is suggested that, in sporozoites of E.tenella and E. acervulina at least, these parasites adopt a fixed, helically coiled, body shape. This would explain the spiralling nature of sporozoite motility, described in Chapter 3. This rigid body shape

is not, however, adopted by all locomoting sporozoites, for example Plasmodium yoelii (Chapter 3). During the ultrastructural examination of these parasites it was suggested that the conservative nature of 'zoite organisation could reflect a common mechanism of locomotion. This, coupled with the behavioural similarities discussed in Chapter 3 supports the theory that all the invasive stages of parasites of Apicomplexa share a common mechanism of locomotion and therefore a common sub-membraneous contractile system.

The purpose of these 'zoites is the infection of a new host or a new cell. Successful infection is the result of two functions performed by these 'zoites; locomotion to the host cell and invasion through the host cell membrane. The results from the previous Chapters suggest that locomotion in the 'zoites of Apicomplexa has a common basis. It may also be that invasion of the 'zoites of Apicomplexa shares this common basis. Host cell invasion involves the translocation of the extracellular parasite into its host cell. The possible involvement of a contractile system associated with the parasite surface membrane must be examined. The next, and final Chapter, investigates the possible implications that the contractile system, proposed for sporozoite locomotion, has for host cell invasion.

CHAPTER 6.

HOST CELL INVASION: AN EXPRESSION OF THE PARASITE'S CONTRACTILE SYSTEM.

6.1 INTRODUCTION

The life cycle of Eimeriina is direct and infection is spread by means of cysts resistant to desiccation. The sporozoites, usually eight in number, contained in these cysts are the initial stage of a new infection. Successful infection begins when the prospective host ingests a sporulated oocyst. The sporozoites excyst in the digestive tract and invade the gut endothelial lining. In Eimeria spp. they enter the epithelial cells and undergo their first division, schizogony, producing the other invasive stage, the merozoites. Eimeriina are relatively "simple" parasites possessing only two invasive stages, sporozoites and merozoites, in their life cycle. The Haemosporina, which include malarial parasites, have three invasive "'zoite" stages; the sporozoite, the merozoite and the ookinete. The economic, veterinary and medical importance of both groups of parasites has meant that their mode of invasion has been studied extensively. This introduction includes a short review of the research on 'zoite invasion of host cells and it is divided into two parts. The first deals with the invasion process of eimeriine sporozoites. The second examines the research into the interaction between the merozoite of Plasmodium spp. and the red blood cell. The latter system forms the basis of the two most cited theories of 'zoite invasion.

Early researchers suggested that eimeriine 'zoites entered host cells by means of phagocytosis (Doran and Vetterling 1967;

Jones, Yeh and Hirsch, 1972). However cinemicrographic studies of Eimeria larimerensis sporozoites demonstrated that, these parasites at least, adopted a more active entry process (Speer, Davis and Hammond 1971). They noted 'zoites thrusting a slender protruberance into cells immediately prior to invasion. Invasion itself was rapid and appeared to be initiated by an invagination in the host cell membrane. Sporozoites leaving cells were frequently accompanied by a release of cytoplasm, indicating rupturing of host cell membrane.

Ultrastructural studies of coccidian 'zoites in the act of penetrating, and leaving, host cells have been conducted on several different species. In 1971 Roberts, Speer and Hammond examined sporozoites of E. larimerensis fixed after incubation with host cells. Sporozoites were seen to be constricted at their point of entry or exit from the host cells. Some of the host cell membrane was carried into the cells as they entered. Later work by Jensen and Hammond (1975) on sporozoites of E. magna revealed that the host cell membrane was not ruptured during penetration. Parasites were fixed moving into a membrane bound vacuole intimately associated with the parasite plasmalemma. In 1978 a similar study on Isospora canis sporozoites (Jensen and Edgar) confirmed that coccidian 'zoites enter into a vacuole formed by an invaginating host cell membrane. The vacuole was sealed off by short pseudopodia growing over the entry hole. These authors also suggested that because of the microfilaments aggregated in this area of the host cell, constriction at the site of entry was a cell mediated response to penetration.

The initial invagination of host cell membrane immediately prior to invasion, noted by Speer et al (1971), is thought to be induced

by the parasite. Lycke, Carlberg and Norrby (1975) purified a protein of molecular weight 70,000 to 150,000 daltons from 'zoites of Toxoplasma gondii. This protein was called penetration enhancing factor because it caused spontaneous invagination of cell membranes and increased the incidence of invasion. It's suggested that this protein may originate from the rhoptries. Recently this effect has been mimicked by the synthetic polycation poly - arginine (Werk, Dunker and Fischer, in press).

The invasive behaviour of eimeriine 'zoites conforms to the following general description. The parasite attaches to its potential host cell and orientates itself so its anterior pole is in contact with the cell membrane. It then induces initial vacuole formation in the host cell, possibly by secretion of a "penetration enhancing factor" from the rhoptries. The parasite is then moved into this vacuole which becomes closely associated with the parasite membrane. Short pseudopodia form a parasitophorous vacuole sealing the parasite off from the exterior. The method of entry of the parasite into the membraneous invagination is unknown. Present theories explaining this process have evolved from research on the invasion of merozoites of Plasmodium into red blood cells.

Merozoites of Plasmodium spp. are the most studied of all the invasive stages of Apicomplexa. Similarities between this process and the invasive behaviour of other Coccidia have been noted by many researchers (eg Sinden 1978). Merozoite invasion follows a strict sequence (reviewed by Bannister, Butcher and Mitchell 1977). Initially the merozoite adheres to the red blood cell, then, by violent spasmodic distortions, it orientates itself so its apical pole is in contact with the erythrocyte membrane. The parasite then induces the host cell

to invaginate and subsequently passes into this cavity. A circular belt-like junction between merozoite and erythrocyte is maintained through invasion. Passage of the parasite into the vacuole is accompanied by a shedding of parasite cell coat. The merozoite is finally enclosed in its parasitophorous vacuole.

The initial attachment of merozoite to erythrocyte appears to be a function of the parasite's extensive cellular coat (Ladda, Aikawa and Sprinz 1969; Aikawa 1971). This coat is highly structured, consisting of many "T" or "Y" -shaped "bristles" with 20 nm stems. The surface of these parasites has a net negative charge (Bannister et al 1977). The interaction between parasite and host cell surfaces, reviewed by Miller (1977), is a highly ordered and complex event and is undoubtedly involved in triggering host cell invasion (Miller et al 1979). During invasion, this surface coat is shed. It has been suggested that this process is analogous to "capping" (Bannister et al 1977).

Invagination of the erythrocyte membrane occurs opposite the apical complex of the attached parasite. Kilejian (1974,1976) has purified a histidine-rich protein from merozoites of Plasmodium lophurae. She suggested that this protein, known to cause membrane perturbations, is released from the apical complex during invasion. Such a protein would be functionally akin to the penetration enhancing factor found in T. gondii (Lycke et al 1975). The erythrocyte membrane expands inwards forming a rounded parasitophorous vacuole, frequently accompanied by small, membrane - bound vesicles (Bannister, Butcher, Dennis and Mitchell 1975). The area of parasite/erythrocyte interaction becomes thickened into a specialised junction (Miller, Aikawa, Johnson and Shiroishi, 1979; Aikawa and Kilejian 1979). A recent freeze - fracture

study of this complex (Aikawa, Miller, Rabbage and Epstein 1981) revealed a band of intramembraneous particles on the inner face of the outer layer of the erythrocyte membrane. This specialised junction is formed even in the presence of cytochalasin B when the parasite is able to attach to the erythrocyte but unable to invade. In normal preparations formation of this junction is followed by the merozoite entering the parasitophorous vacuole. During entry the junction and apical complex are the only two areas of the parasite in contact with the erythrocyte. As the merozoite enters the vacuole the junction is moved down the parasite.

The merozoite's mode of entry into host cells has been the subject of much speculation. In 1977 Bannister et al suggested that the merozoite enters the red blood cell because the connection between the apical pole and the erythrocyte membrane, maintained throughout invasion, draws the parasite into the vacuole as it is enlarged by the "penetration enhancing" protein. Aikawa, Miller, Johnson and Rabbage (1978) put forward an alternative hypothesis. The movement of the merozoite into the erythrocyte may be caused by a modified "zipper" model. Migration of the circular junction would require attachment of the leading edge and detachment of the trailing edge. By this process the merozoite would be drawn into the erythrocyte. Since these models were first proposed more experimental data has become available. Miller et al 1979, demonstrated that cytochalasin B inhibited invasion of Plasmodium merozoites into erythrocytes. This sensitivity implies an actin - based contractile system is active during invasion. Such a system is not contained in either model. In the "zipper" theory, Aikawa et al (1978) also fail to suggest which cell, host or parasite, consumes energy in

this process. These theories are now, without further modification, unable to accommodate all current observations.

This thesis was devoted to the examination of the contractile basis of locomotion in coccidian 'zoites. The model of this system, proposed in Chapter 5, entails the movement of surface membrane receptors, capable of binding to external ligands, by a submembraneous, actin - based contractile system. In Chapter 6 host - cell invasion is re-examined as a possible expression of the parasites contractile system.

Invasive behaviour of sporozoites of Eimeria acervulina and Eimeria tenella into culture monolayers was studied by light microscopy, and transmission and scanning electron microscopy. The role played by the parasite's contractile system was assessed by immobilising the sporozoites in high magnesium ion concentration prior to incubation with culture cells. The effect of cytochalasin B on host - cell invasion was also examined. An alternative theory of invasion, based on the results of these experiments is proposed.

6.2 MATERIAL AND METHODS

6.2.1 Light Microscope Observations on Host - Cell Invasion by Eimeriine Sporozoites.

Sporozoites of Eimeria acervulina and Eimeria tenella were observed invading monolayers of mouse 3T3 fibroblasts, L 132 human lung cells, Xenopus epithelial cells and mouse peritoneal macrophages. Cell monolayers were prepared by placing a suspension of cells, in the relevant medium, in flat bottomed bijou bottles with a sterile coverslip at the base. Coverslips were washed in HBSS immediately prior to use. Sporozoites were excysted, purified and resuspended in fresh HBSS, as described in Chapter 1. A suspension of sporozoites was pipetted onto the monolayer and allowed to settle for 2 min at 4°C. The coverslip was then placed, cell side up, onto a glass slide and covered by a large coverslip supported around the edges with vaseline. Preparations were examined with a Leitz Orthoplan light microscope equipped with a controlled environment stage maintained at 41°C.

Effects of various treatments and drugs on host cell invasion were monitored. The invasive behaviour of sporozoites preincubated in HBSS with 15 mM $MgCl_2$ for 30 min at 4°C and 41°C was examined at 41°C. The effect on invasion observed while incubating parasites and cells at 41°C in the presence of 10 µg/ml cytochalasin B, known to inhibit gliding and pivoting motility (Chapter 4 Section 3), and 0.1% DMSO was also monitored. Control preparations were also run in 0.1% DMSO in HBSS and revealed no affect on invasion.

Some of the coverslips of different preparations of sporozoites and culture cells, in the presence and absence of motility inhibitors, were processed and stained in giemsa. Following examination of living

cells (as above), coverslips were washed three times in HBSS and air dried using a warm air flow. They were fixed for 1 min in methanol at room temperature and stained for 10 min in 10% giesma in phosphate buffer (50 mM) pH 7.5. Coverslips were finally washed in tap water, dried in air and mounted in Diatex permanent mounting medium.

6.2.2 Ultrastructural Examination of Host Cell Invasion.

The interaction between invading sporozoites and mouse 3T3 fibroblasts was examined by transmission electron microscopy and by scanning electron microscopy.

Samples for thin section electron microscopy (T.E.M.) were prepared as follows. 3T3 fibroblasts were washed and suspended in HBSS. A suspension of 'zoites in HBSS was mixed with the suspension of fibroblasts. The cells were allowed to settle for 2 min at $4^{o}C$ before being warmed to $41^{o}C$. Some cell suspensions contained HBSS with 10 µg/ml cytochalasin B. Samples were taken from each and fixed at 5, 10 and 15 min intervals. Cells were fixed in 2.5% glutaraldehyde, 5% tannic acid in 0.1 M cacodylate buffer, pH 6.9, for 1 hour and pelleted in a blood centrifuge at 14,000 g for 3 s. The material was processed for T.E.M. by the procedure outlined in Chapter 2, section 2.2.1.

Samples of sporozoites invading monolayers of 3T3 fibroblasts were prepared for S.E.M. by the following technique. Monolayers of culture cells were prepared by the same procedure described in section 6.2.1. Suspensions of sporozoites of E. acervulina in HBSS were placed on monolayers, previously washed in HBSS, and allowed to settle for 2 min at $4^{o}C$. Coverslips were incubated at $41^{o}C$ for 5, 10 and 15 min. Following incubation, samples were fixed for 1 hr in 2.5% glutaraldehyde,

5% tannic acid in 0.1 M cacodylate buffer, pH 6.9. Coverslips were then washed in 0.1 M cacodylate buffer and dehydrated through an acetone series, as in section 2.2.3 ending with 3 5 min changes of 100% acetone, critical - point dried, and coated in gold with a Polaron E3000 sputter coater. The specimens were examined in an Hitachi 520 scanning electron microscope.

6.3 RESULTS

6.3.1 Light Microscope Observations

Sporozoites of Eimeria tenella and E. acervulina readily entered all cell types examined. Penetration was usually completed within a second or two. Sporozoites frequently appeared constricted at the site of entry into the cell (fig. 6.1). Both species of sporozoite were capable of gliding smoothly, in association with all cell types examined. In most sporozoites observed, gliding and invasion appeared continuous with each other. Sporozoites frequently entered cells during locomotion, and with no apparent change of speed. Sporozoites were also capable of penetrating the nuclear membrane of cells examined (fig. 6.2).

Few of the sporozoites pretreated in 15 mM $MgCl_2$ in HBSS were able to attach to cells (fig. 6.3), attachment was assayed by drawing fresh medium across the preparation, washing off all parasites that had not adhered to the cell monolayer. None of these sporozoites were observed invading, or inside, cells. Motility of sporozoites treated in this manner (Section 4.2.2) was limited to feeble flexing movements. Sporozoites incubated with culture cells in presence of 10 µg/ml of cytochalasin B also attached in small numbers (fig. 6.4). Again no intracellular 'zoites were observed. These sporozoites were also unable to glide (Section 4.2.2). However replacement of the medium with fresh HBSS enabled the parasites to glide and invade culture cells.

The giemsa stained preparations support these observations. Control preparations of sporozoites and culture cells incubated together in HBSS for 15 min at 41°C reveal between 25 - 40% of the parasites inside cells (fig. 6.5). However, preparations of $MgCl_2$ treated sporozoites and those incubated in the presence of cytochalasin B

Figs. 6.1 to 6.4. Nomarski interference - contrast micrographs of sporozoites of E. acervulina on monolayers of mouse 3T3 fibroblasts maintained at 41°C.

Fig. 6.1. A sporozoite invading a fibroblast. The parasite is blurred because it was moving into the cell as the photograph was being taken. The 'zoite is constricted at its site of entry (arrowed). X 1,750

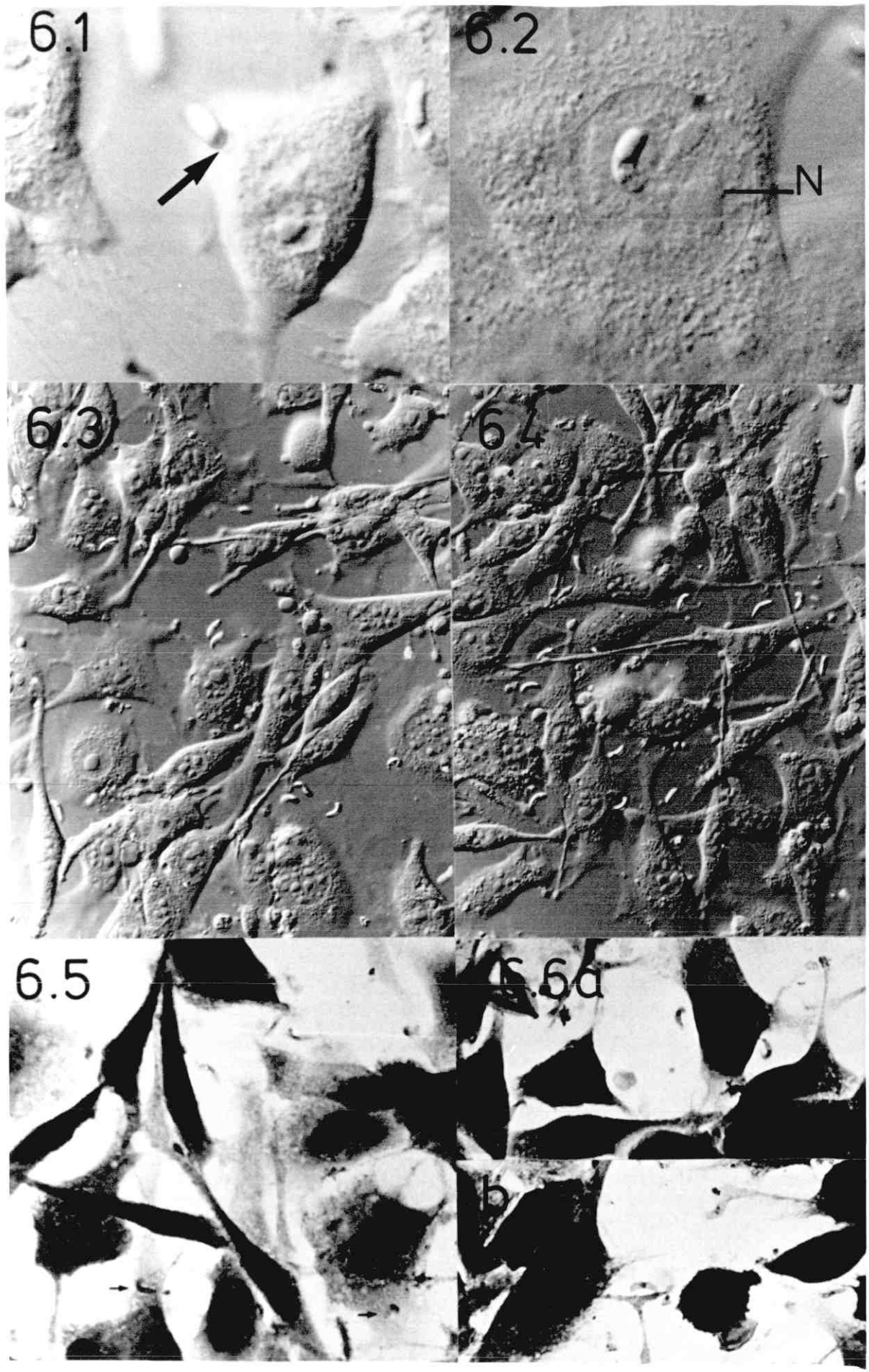
Fig. 6.2. An intra - nuclear sporozoite. X 1,900

Fig. 6.3. Sporozoites pretreated in 15 mM $MgCl_2$ in HBSS for 30 min. at 4°C. "Loose" parasites were removed by washing. No intracellular parasites are seen, although many have adhered to the fibroblasts. X 450

Fig. 6.4. These parasites and fibroblasts were incubated, together, in the presence of 10 µg/ml cytochalasin B. "Loose" parasites were removed by washing. Again, no intracellular parasites are seen although attachment has still occured. X 450

Fig. 6.5. Geimsa stained monolayer of fibroblasts incubated with E. acervulina sporozoites. Intracellular sporozoites are clearly seen in these preparations (arrowed). X 590

Fig. 6.6 a) Sporozoites pretreated in 15 mM $MgCl_2$ or b) incubated in the presence of 10 µg/ml cytochalasin B. No intracellular parasites were observed. However some 'zoites remain attached to the cells. X 590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b

showed very few parasites in association with cells and none inside the cells (fig. 6.6). Most of these sporozoites appear to have been washed off during processing.

6.3.2 Ultrastructural Examination of Invading and Intracellular Sporozoites.

From examinations of living cells in the preceeding section it appears that the invasion of these parasites into culture cells conforms to the following pattern. Firstly, the sporozoites must come in contact with, and attach to, the cell. In cases where 'zoites must invade "static" tissues, eg. the sporozoites of Sarcocystis spp. , the endogenous motility of the parasite is vital to its success. Secondly, the sporozoite invades and enters the cell. Both attachment and invasion were examined by T.E.M. and S.E.M.

Sections from a fibroblast/sporozoite preparation demonstrated that the two cells readily adhered to one another. Attachment is seen as a close (10 - 40 nm) association between the plasmalemmas of the two cells.* Unlike the specialised junction observed in invading merozoites of Plasmodium (Miller et al 1979; Aikawa, Miller, Rabbage and Epstein 1981), no structural modifications were detected at the site of adhesion. Sporozoites are also capable of binding to fibroblasts in the presence of cytochalasin B. Attachment appears the same as in control preparations (fig. 6.8).

Entry of the parasite into culture cells is a fairly rapid event. Electron micrographs of this process demonstrate that the association between parasite and "host" membranes is now more intimate (2 - 5 nm) (fig. 6.9) and extends from anterior to posterior down the sporozoite body. Invading sporozoites proceed into cells down a tunnel

* Fig. 6.7

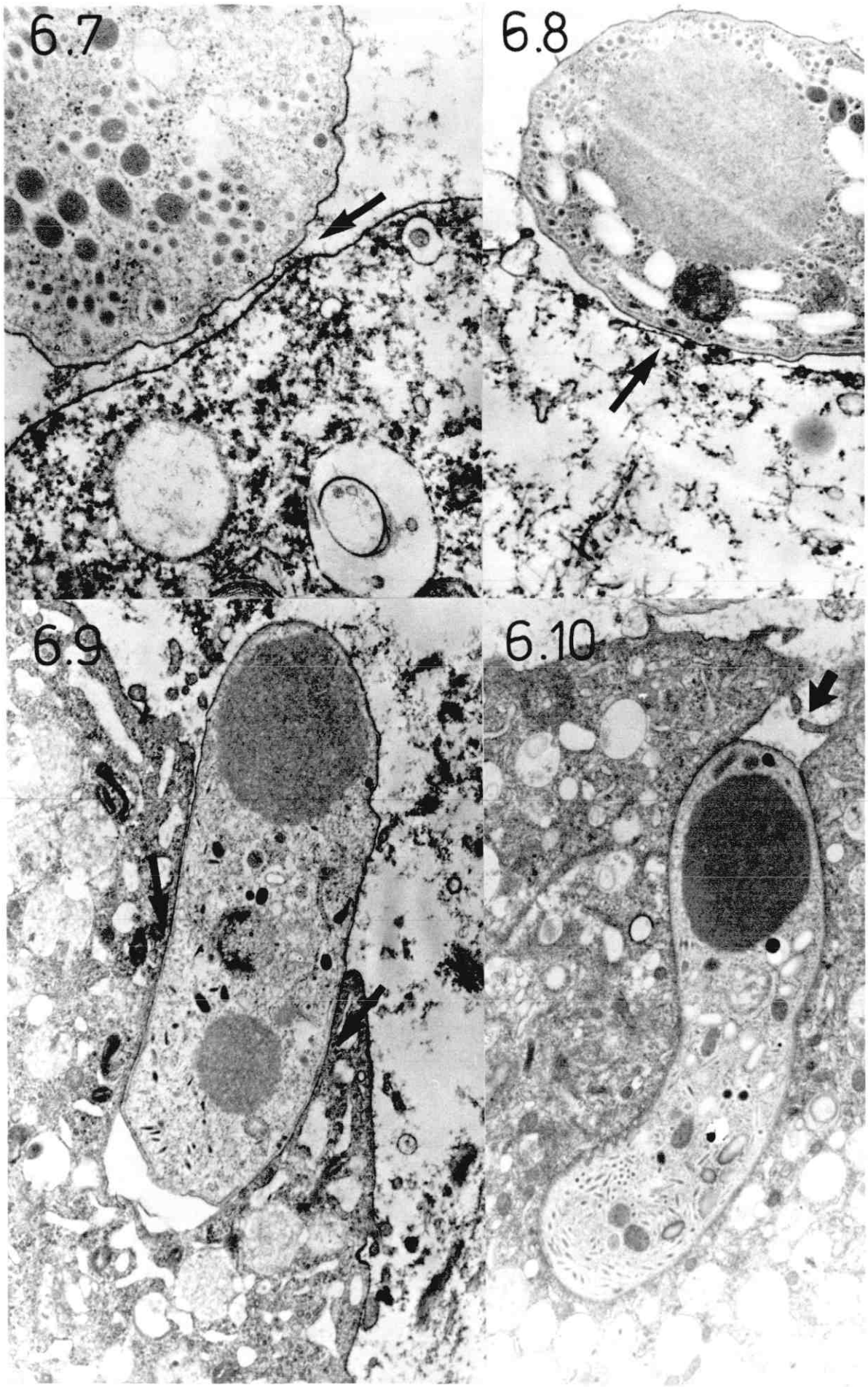
Figs. 6.7 to 6.10. Electron micrographs of eimeriine sporozoites invading mouse 3T3 fibroblasts. (all specimens were processed in cacodylate buffer).

Fig. 6.7. E. tenella sporozoite associated with the surface membrane of a fibroblast. The parasite has "formed" a loose attachment (arrowed) to the fibroblast. X 48,000

Fig. 6.8. Cells incubated in 10 µg/ml of cytochalasin B form a similar attachment site (arrowed). X 34,000

Fig. 6.9. A sporozoite of E. acervulina invading a fibroblast. The parasite/host membrane interaction is now very close (arrowed). The parasite moves/is moved into a vacuole formed by the host cell membrane. X 19,500

Fig. 6.10. This sporozoite of E. tenella lies inside the fibroblast in a "tube" of membrane. Short pseudopodia (arrowed) have begun to close off the mouth of this tube. X 16,500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of membrane (fig. 6.10). Eventually this passage is sealed with short pseudopodia behind the parasite, leaving it enclosed within its own "host" cell membrane sac (fig. 6.11). The posterior pole of intracellular sporozoites is frequently associated with "bubbles" of covering membrane (fig. 6.12). The ultrastructure of extracellular and intracellular sporozoites has no detectable modifications. Sporozoites exit from cells by first "pushing" the host plasmalemma out into an extended cellular process (fig. 6.13) and then bursting out of the plasmalemma and out of its membraneous sac (fig. 6.14). Sporozoites left host cells, as they entered, anterior end first.

Cell invasion by coccidian sporozoites was also examined by scanning electron microscopy. The preparations revealed many sporozoites entering and leaving "host" cells. Invading sporozoites were seen to head deep into the cell body at right angles to the plasmalemma (fig. 6.15). The point of entry appears slightly constricted (fig. 6.15). Sporozoites also invaded cells at shallow angles to the plasmalemma (fig. 6.16), the host/parasite junction is less marked in these specimens. Sporozoites were seen travelling along extremely fine cell processes (fig. 6.17). Exiting parasites pushed out the "host" cell membrane (fig. 6.18) before bursting out, rupturing the host plasmalemma (fig. 6.19).

Figs. 6.11 to 6.14. Electron micrographs of E. acervulina sporozoites and mouse 3T3 fibroblasts (all samples were processed in cacodylate buffer).

Fig. 6.11. Parasites enclosed within their own parasitophorous vacuole (PV).

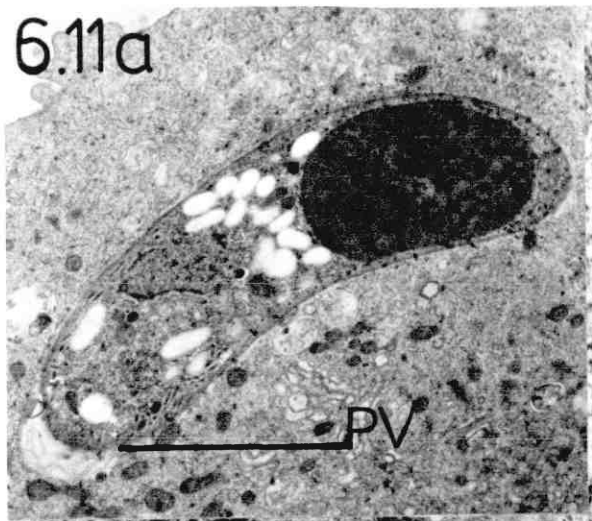
a) L.S. X 12,500

b) C.S. X 31,800

Fig. 6.12. An intracellular sporozoite with "bubbles" of membrane associated with its posterior pole (PP). X 50,100

Fig. 6.13. A 'zoite pushing out the host cell plasmalemma (PL) into an extended process. This was frequently observed when parasites left their "host" cells. X 9,700

Fig. 6.14. This sporozoite is leaving the fibroblast. It has ruptured the culture cell's plasmalemma (PL) and the parasitophorous vacuole membrane. X 19,000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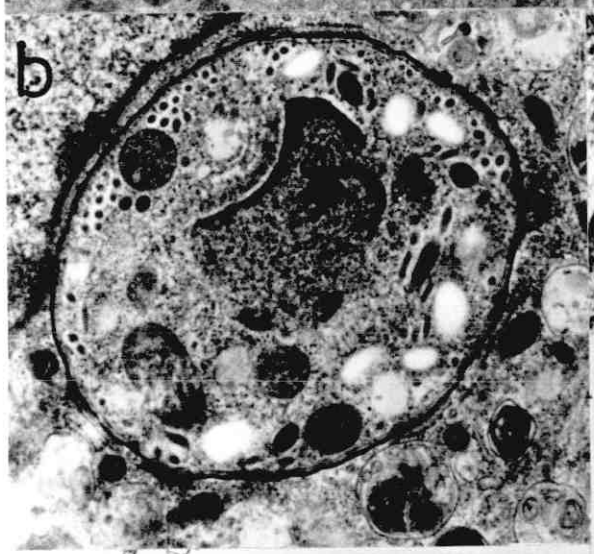
PV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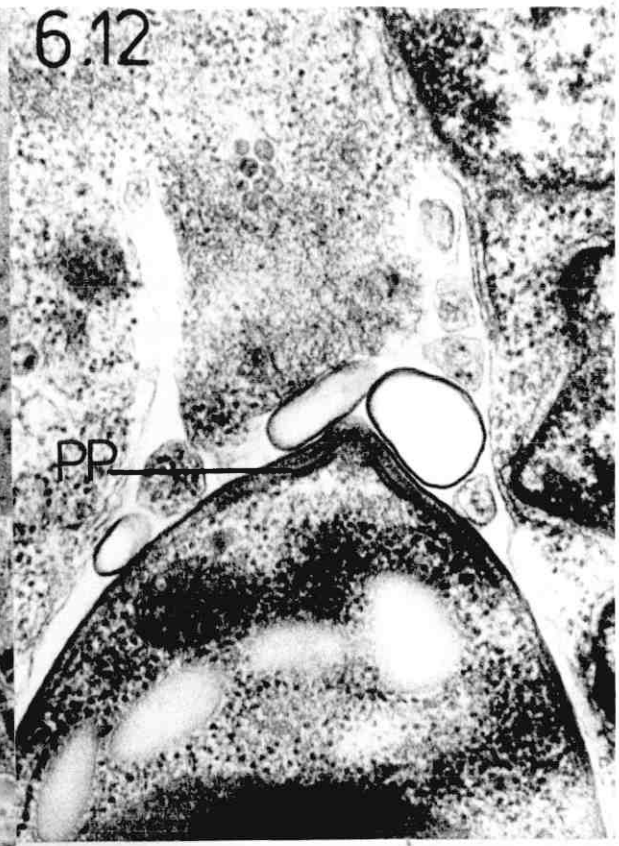
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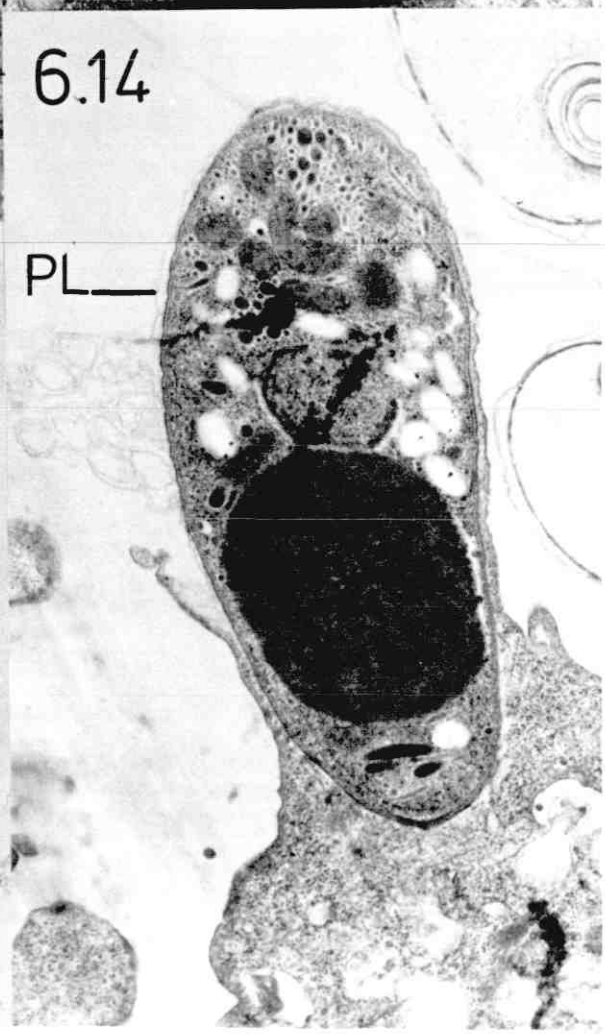
PP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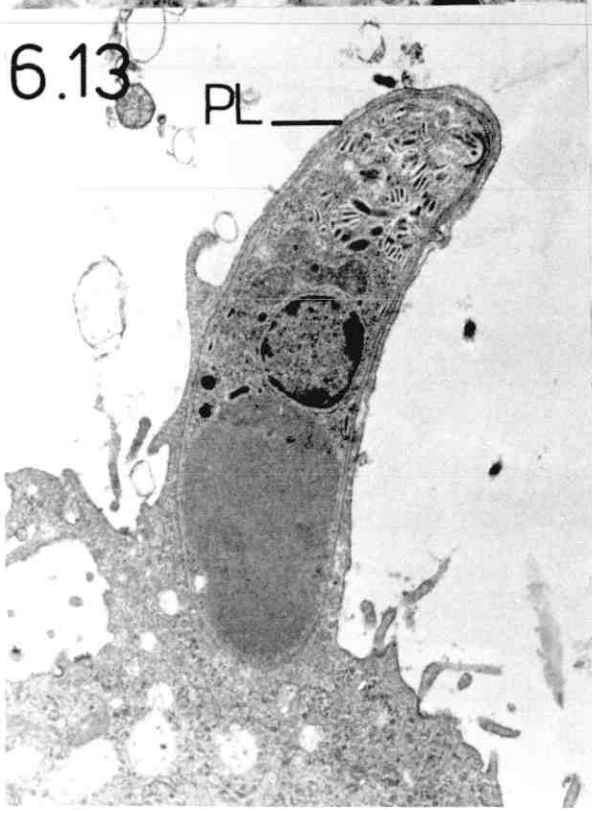
PL


6.13
PL

Figs. 6.15 to 6.19. Scanning electron micrographs of E. acervulina sporozoites on mouse 3T3 fibroblast monolayers.

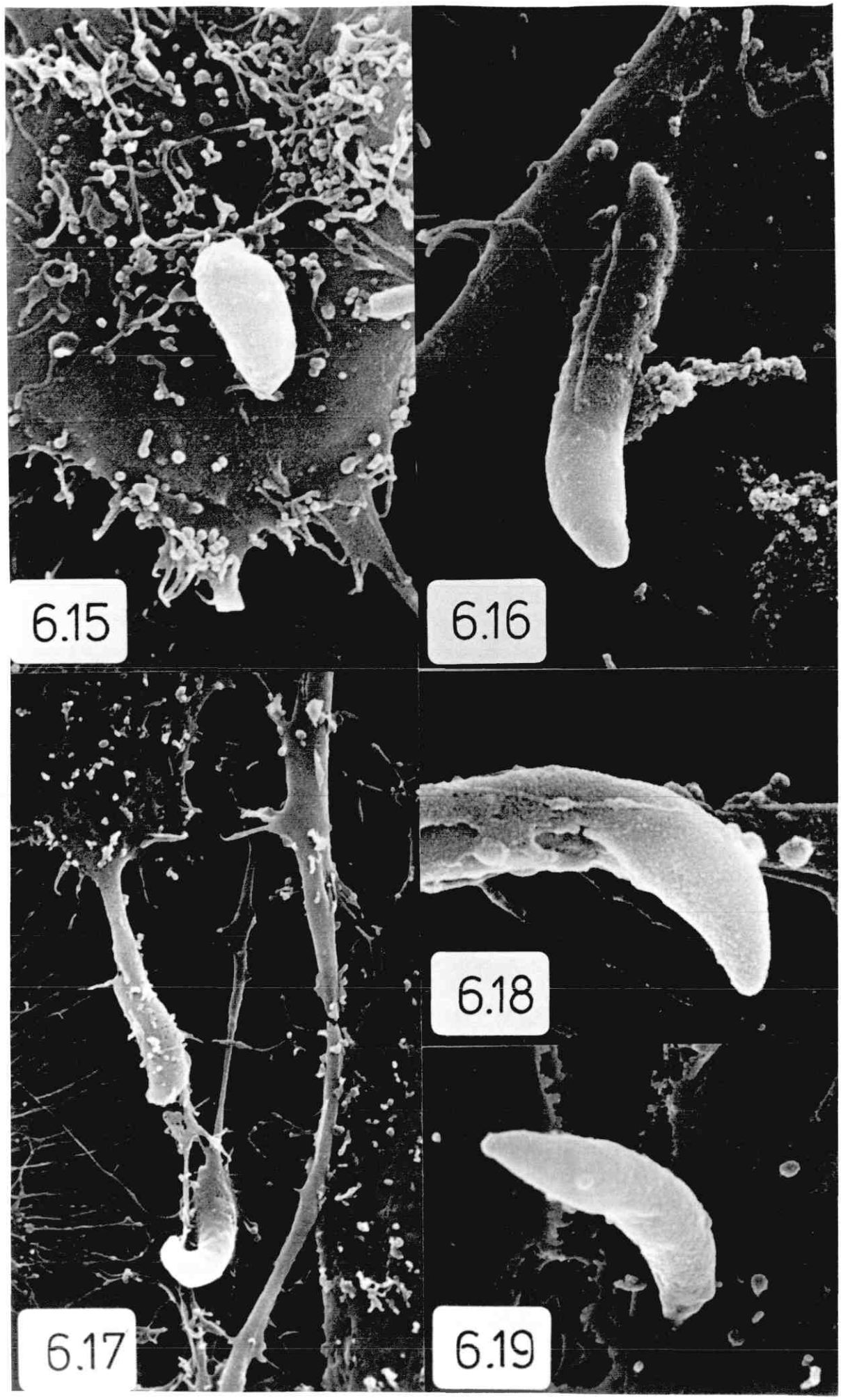
Fig. 6.15. An invading sporozoite. The posterior third of the parasite is still outside the cell. A band of constriction is visible at the point of entry (arrowed). X 7,750

Fig. 6.16. Another invading sporozoite. This time the parasite/host junction is less marked, and no constriction of the 'zoite is detectable. X 10,000

Fig. 6.17. Two 'zoites; one moving along the inside of a fine cell process and another leaving its "host" cell through an adhesion site at the end of a cell process. X 4,900

Fig. 6.18. A parasite "pushing" out the fibroblast plasmalemma (possibly before exiting from the cell). X 13,000

Fig. 6.19. This sporozoite is leaving its "host" cell. The anterior of the 'zoite is now free from the cell surface and parasitophorous vacuole membranes. X 11,000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6.19

6.4 DISCUSSION

For reasons of clarity the discussion is divided into two sections. In the first section chemicals that modify the invasive behaviour of E. tenella and E. acervulina, and related parasites, are examined to assess the possible involvement of the parasite's contractile system in host - cell invasion. An ultrastructural study of these sporozoites invading, and leaving, "host" cells forms the second section. An alternative model of 'zoite invasion is proposed, this model is also relevant to 'zoites leaving host cells and to other invasive stages of Apicomplexa.

The effects of the antimicrofilament agent cytochalasin B on invasive behaviour of eimeriine 'zoites was first examined in 1976 by Jensen and Edgar. They found that this compound prevented cell invasion of Eimeria magna sporozoites into bovine kidney cells. It also stopped pivoting and gliding motility in these parasites. Its action was reversible and invasion recommenced as soon as the cells were resuspended in fresh medium. The authors recognised that they could not separate the effect on 'zoites from the possible effect on culture cells, however, they felt that sporozoite motility was important to successful invasion. Further attempts to inhibit invasion of E. magna 'zoites with the anti-microtubular agents colchicine, colcemid and vinblastine sulphate were unsuccessful. In 1978 similar experiments by Ryning and Remington using cytochalasin D and trophozoites of Toxoplasma gondii produced results comparable to the earlier work. These 'zoites were unable to invade macrophages or bladder tumour cells in the presence of cytochalasin D. But the authors suggested this was due to the drug inhibiting phagocytic activity, induced, even in non-

phagocytic cells, by the parasite. Invasion of the merozoite of Plasmodium knowlesi into red blood cells was also examined in the presence of cytochalasin B (Miller et al 1979). Despite the presence of the drug, parasites were able to attach to erythrocytes and orientate themselves so their apical complex was associated with the erythrocyte membrane, but they also were unable to enter the host cell. Attachment appeared normal and the parasite/host junction consisted of an aggregation of intramembraneous particles on the erythrocyte membrane, as in the control (Aikawa et al 1981). Contrary to these findings Danforth, Aikawa, Cochrane and Nussenzweig (1980) demonstrated that sporozoites of Plasmodium invaded macrophages in the presence of cytochalasin B, but were inhibited by pretreatment in colchicine. Unfortunately this study did not include an ultrastructural examination of treated parasites to demonstrate the action of this anti - mitotic agent. The effects of cytochalasin B on motility of sporozoites of Eimeria tenella and E. acervulina were discussed in Chapter 4. As in all the earlier studies no invasion was observed when parasites were incubated with "host" cells in the presence of cytochalasin B. Ultrastructural examination of parasite/host attachment sites did not reveal any modifications from the control preparations. Invasion recommenced with 'zoite locomotion, however, it is not possible to separate the effects of cytochalasins on parasite motility from their possible effects on parasite induced phagocytosis.

Jensen and Edgar (1976) also demonstrated the ability of E. magna sporozoites to invade cells treated with the metabolic inhibitors sodium fluoride, iodoacetate and 2 - deoxyglucose. These parasites invaded poisoned cells with the same frequency as control cells. This

strongly suggests that it is the parasite cell that is active during invasion. This work was extended by Werk and Bommer (1980) on 'zoites of Toxoplasma gondii. They inhibited oxidative respiration with cyanide, almost completely depressing invasion. However on the addition of excess extraneous glucose (70 mM) the invasive behaviour of the 'zoites was almost totally restored. The action of these metabolic and F actin inhibitors suggest the following; first, an actin based contractile system is involved in parasite/host cell invasion; second, it is the parasite that is the energy consuming during invasion. Therefore it is logical to propose that invasion is an expression of the parasite's contractile system. This conclusion is supported by the inhibition of invasion of sporozoites of E. tenella and E. acervulina following pre-treatment in 15 mM magnesium ion concentration.

Prior to this project little was known of the contractile system of 'zoites of Apicomplexa. However now that some understanding of the membrane associated contractile system has been achieved, and the experiments with metabolic and mitotic inhibitors suggest that it is active during host cell invasion, it is possible to examine the ultrastructure of invasion and suggest a possible contractile basis.

Ultrastructural examination of sporozoites of E. acervulina and E. tenella during invasion of culture cells confirms the observations of previous researchers, reviewed in the introduction to this Chapter. The mode of invasion adopted by these, and other, 'zoites examined follows a strict sequence of events. In summary, the parasite attaches to the "host" cell, orientates itself so its apex is associated with the cell, induces an invagination in the host cell, is moved into the resulting vacuole and is enclosed within this parasitophorous vacuole.

Attachment between host cell and 'zoite appears to be independent of motility because it occurs even in the presence of cytochalasin B. However the number of attached parasites is far fewer in these preparations, so motility is obviously important in maximising the number of possible attachments. Orientation of the 'zoite, especially noticeable in preparations of red blood cells and merozoites, also takes place in the presence of cytochalasin B (Miller et al 1979). It, therefore, cannot be a function of the parasites motile system. It has been suggested that orientation is a function of a gradient of receptors over the body of the merozoite (Bannister 1977). The initial deformation of the host cell membrane has been observed in many different species of Apicomplexa, including Toxoplasma (Aikawa, Komata, Asai and Midorikawa 1977), Eimeria (Jensen and Hammond 1975), Babesia (Rudzinska, Trager, Lewengrub and Gubert 1976) and Plasmodium (Bannister et al 1977). These authors propose that this is a function of membrane disrupting proteins purified from 'zoites of Plasmodium (Kilejian, 1974, 1976) and from 'zoites of Toxoplasma (Lycke et al 1975). Plasmodium merozoites are capable of forming a parasite - sized vacuole by this technique. The 'zoite is then transported from outside the host cell into the parasitophorous vacuole. Closing the vacuole is achieved in Eimeria spp. by the host cell forming short pseudopodia (fig. 6.10) but in Plasmodium merozoites the parasite draws the host cell over its posterior pole as it enters the erythrocyte (Aikawa and Kilejian 1979).

Translocation of the 'zoite, which is attached by its anterior to the "host" cell, into the partially formed parasitophorous vacuole, could involve the parasite's membrane associated contractile system. Existing theories are not consistent with all current observations on

invasion of 'zoites of Apicomplexa into host cells. Both the present theories have been developed to explain the invasion of Plasmodium merozoites into red blood cells. Bannister et al (1977) suggested that movement of the merozoite into the vacuole was a passive event due to vacuole enlargement. Aikawa et al (1978) proposed that this movement was achieved by a modified "zipper" mechanism of junction attachment and detachment. However, neither of these models explain host - cell invasion's sensitivity to cytochalasin B. More recently, Aikawa et al (1981) have stated that the intramembraneous particles at the host/ parasite junction could be anchorage sites for the erythrocyte's actin filaments. It is my opinion that the authors have implied, though not actually stated, that the sensitivity to cytochalasin B is due to the involvment of the host's contractile system in the "zipper" process. Observations on the invasive behaviour of other 'zoites of Apicomplexa suggest different. Werk and Bommer (1980) and Jensen and Edgar (1976) demonstrated on 'zoites of Toxoplasma and Eimeria that it is the parasite cell that is metabolically active during invasion. This is supported by Mack and Vanderberg (1978) who demonstrated that "exhausted" sporozoites of Plasmodium were less infective than those maintained in medium with glucose.

An alternative model of the entry of the parasite into the induced vacuole based on the mobility of certain elements of the parasite's plasmalemma is proposed. Following attachment, orientation and induction of the membrane bound vacuole, the 'zoite moves into the vacuole by capping the parasite/host cell junction down the body. In the Plasmodium merozoite/erythrocyte interaction the vacuole is large enough to accommodate the parasite. However sporozoites of Coccidia

may have to enlargen their vacuole following initial membrane deformation. This would entail the parasite and host cell ligand complex remaining static whilst the parasite, in capping this junction, "pulled" the extra host - membrane required through the junction. This interaction of parasite "pushing" against vacuole could account for the close association between the 'zoite and host cell membrane (fig.6.9). However it is not known if any components of parasite origin are adding to the membrane, which could provide an alternative explanation for the increase in vacuole size during invasion. In this model it is the parasite, not the host cell, that is the energy consuming cell. The directed movement of certain ligands exposed on the parasite surface, exploited during invasion, could also be employed by parasites leaving host cells. Electron micrographs of 'zoites leaving cells (fig. 6.13 and 6.14) reveal the parasites forming a cell process before rupturing the plasmalemma when they exit (in appearance almost an inversion of the invasion process). As these 'zoites possess such non - specific ligands as anionic sites (Chapter 4) they would be capable of binding to the inner surface of the cell plasmalemma. Capping this interaction would enable the parasite to "burst" out of the cell.

The advantage of this model over previous ones is it requires no special modifications to either parasite or host cell. These parasites are known to possess a contractile system capable of capping several different surface receptors to the posterior of the cell. As with locomotion and antibody capping it could be the type of receptors implicated that would control the invasive nature of "locomotion". The continuous nature of gliding and invasion noted in sporozoites of E. tenella and E. acervulina supports the suggestion of a common

contractile mechanism. The possibility that surface receptors mediate invasive behaviour has been demonstrated by Miller et al (1979). Merozoites of Plasmodium attach to Duffy positive and negative erythrocytes, the specialised junction, however, is only seen in Duffy positive/merozoite interactions. Merozoites only invade Duffy positive erythrocytes. Junction formation is insensitive to cytochalasin B and parasites are able to invaginate both Duffy negative and positive erythrocyte membranes. In this case the specificity of invasion appears to be governed by the interaction of membrane receptors.

If this contractile system is universal within the Sporozoa it may indicate a common mechanism of cell "penetration". This group is thought to have evolved from a "primitive gregarine" stem line. Most modern gregarines are extracellular parasites living in the body cavity of their hosts. There does, however, appear to be a tendency towards an intracellular existence, 2 species of Gregarinia, Monocystis and Lankestria have sporozoites that enter host cells, grow and leave as larger trophozoites, without a stage of division. If these parasites, and the extracellular gregarines, possess a membrane associated contractile system it would be a pre-adaptation for an intracellular stage in the life cycle. Very recently King (1981) demonstrated that the trophozoites of Gregarina were capable of transporting beads, bound to certain intramembraneous components, along their longitudinal axis.* It is logical to suggest that this capping ability demonstrated in these gregarines, and in the sporozoites of Eimeria, represents the contractile system, perhaps of locomotion, present in their common ancestor. Therefore, because it is perfectly pre-adapted to an invasive method of infection, this characteristic would be protected through evolution

and present in all modern day Sporozoa. I propose that the "capping" theory of host - cell invasion applies to all the Apicomplexa, including gregarines with an intracellular stage.

* This mobility was cytochalasin B sensitive (King and Lee, unpublished results).

CHAPTER 7

GENERAL DISCUSSION

7.1 THE LOCOMOTION OF 'ZOITES OF APICOMPLEXA

It is rather anomalous that, despite the obvious medical and economic importance of this group of parasites, there are still areas of their biology that have attracted little attention. One such area is the locomotion of their motile invasive stages. In this present study sporozoites of Eimeria tenella and Eimeria acervulina were used as "models" because of their relatively high degree of motility and their ready availability. Whilst the original conception of this project was to investigate the contractile basis of motility as a possibile mechanism of host cell invasion , this study of the locomotory behaviour of these protozoa was, for myself, an "academic" exercise borne out of a strong interest in intracellular motile systems.

Research on intracellular motility usually starts with an ultrastructural examination of the skeletal, or fibrillar, structures of the cell. These cytoskeletal elements always reflect the cell's polarity and are frequently involved in the generation or transmission of motile force. This ultrastructural study (Chapter 2) revealed a highly organised microtubule/membrane complex, represented in fig. 7.1, which acts as the framework for the theory of 'zoite locomotion evolved in Chapter 3, 4 and 5. In summary, sporozoites of Eimeria tenella and Eimeria acervulina adhere to the substratum close to their anterior pole by certain membrane receptors exposed on their plasmalemma. The 'zoite then adopts the spiral shape characteristic of these cells, this shape appears dictated by the microtubule/membrane complex. The substratum - bound receptors are then capped, down the 'zoite body,

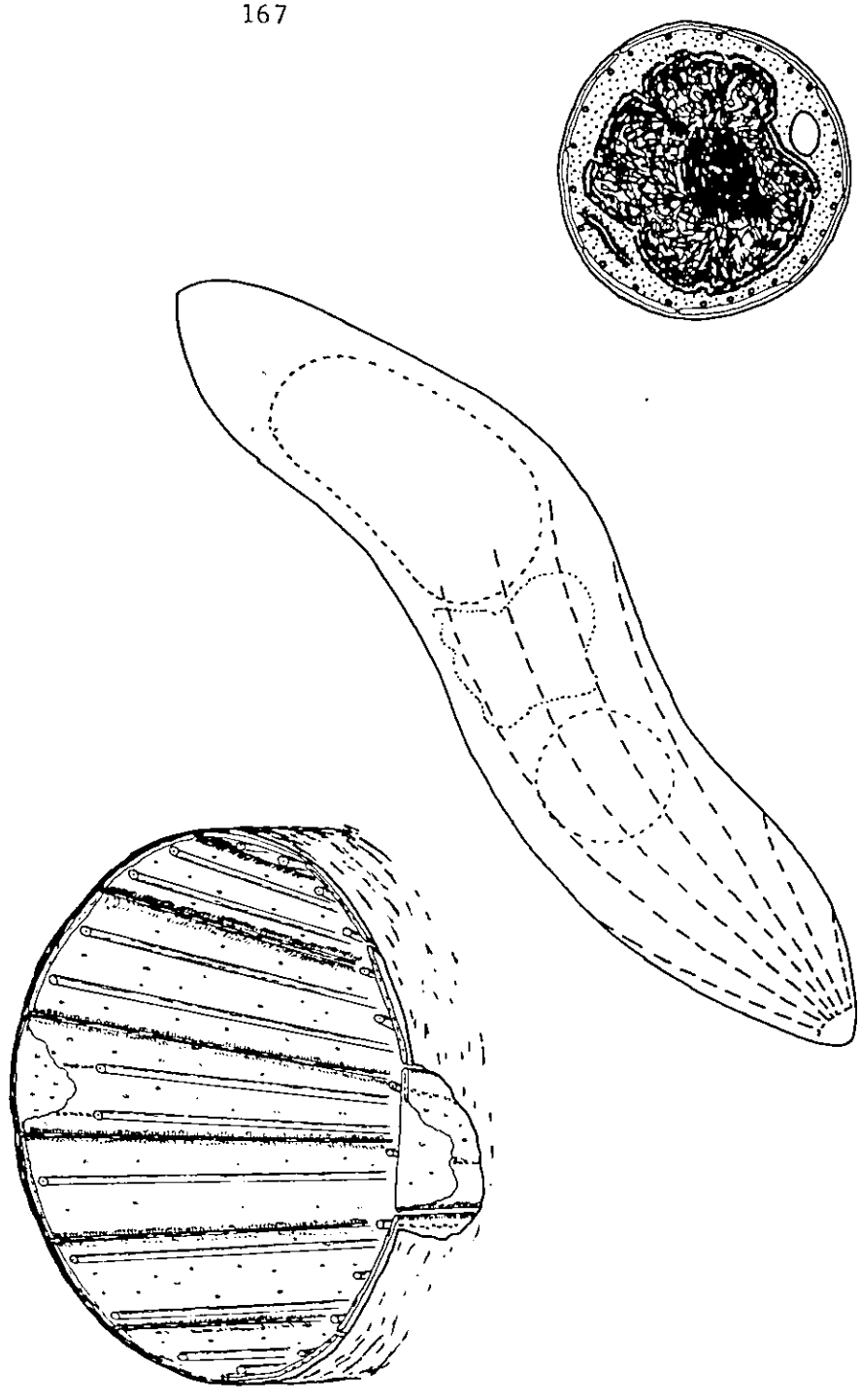

Fig. 7.1 Diagram of the microtubule/membrane cytoskeleton of coccidian 'zoites. The upper diagram is a C.S. through the nuclear region showing the microtubules and trimembraneous pellicle. The middle diagram demonstrates the organisation of the microtubules in an intact cell. The lower sketch is a 3-D representation of the cytoskeleton showing the subpellicular microtubules and the inner membrane leaflets with linear arrays of intramembraneous particles on their inner and outer faces.

driving the cell forwards. Experimental observations, Chapters 4 and 5, suggest that this capping mechanism is achieved by membrane - associated microfilaments interacting with other contractile elements anchored to the cytoskeleton. The organisation of this contractile system is unknown, however, a working model was proposed in fig. 5.11. In this model actin filaments are linked to membrane receptors, via an intermediate link age protein. Their free ends are associated with the tails of single - headed myosin molecules, similar to those from Acanthamoeba (Korn 1978). The myosin heads are free to interact with actin organised by the intramembraneous particles on the inner membrane complex. This second set of actin filaments may be organised as actin cables, similar to those involved in chloroplast transport in Chara (Kersey and Wessells 1976). The actin - mediated ATPase activity of the myosin would pull the first set of actin filaments, and associated membrane receptor(s), to the posterior of the 'zoite. In this model it is the binding characteristics of the membrane receptors that control the external expression of the single contractile system.

The mobility of certain intramembraneous proteins exhibited by many culture cell types appears behaviourally similar to the capping and locomotion of coccidian 'zoites. Rajaraman, MacSween and Fox (1978) suggested that capping in culture cells resulted from binding to particulate objects whilst attempting to bind to an unyielding substratum. From this premise they made the following predictions.

"

a) Cell adhesion to the substratum and capping of membrane bound particles are mutually exclusive and are determined by the nature of the foreign object.

b) Capping and cell locomotion are effected by similar cellular processes.

c) Capping should occur in a direction opposite to cell locomotion.
d) Conditions that affect cell locomotion should also influence the the process of capping. "

These predictions of behaviour, evolved from research into membrane mobility of culture cells, are also applicable to coccidian 'zoites. Therefore the theory of membrane receptor mobility in culture cells is also relevant for capping and locomotion of 'zoites. I suggest that the main difference between the two cell types is simply in the level of organisation of the subtending cytoskeleton. Culture cells are extremely plastic cells, whereas 'zoites have a well - defined shape reflecting a permanent cytoskeleton with fixed polarity. The time - limiting factor in culture cell capping is probably the re - organisation of the contractile and cytoskeletal components. In 'zoites the time - limiting factor would be the time course of the actin - mediated myosin ATPase activity. The permanent cytoskeleton of these parasites would enable a faster reaction. In this respect these parasites are unique because they are highly active, motile organisms that exploit a contractile mechanism first recognised (de Petris and Raff 1972) in slow moving culture cells. The speed and repetitive nature of their capping and locomotion would make coccidian 'zoites a useful model to study certain aspects of the directed mobility of select membrane components.

The similarities between the two cell types allow further comparisons to be drawn. There are two "popular" control mechanisms proposed for regulating receptor mobility in culture cells that may be relevant to 'zoite locomotion. Both theories are highly speculative and could only be experimentally tested with extreme difficulty. Butman,

Bourguignon and Bourguignon (1980) proposed that the binding of external ligand facilitates the release of a membrane - bound enzyme, adenyl cyclase, which raises the cyclic AMP concentration. Cyclic AMP is known to activate myosin light chain kinase which, in turn, would phosphorylate myosin and stimulate acto - myosin ATPase activity. This would result in the translocation of the attached ligand. An alternative model from Klausner, Bhalla, Dragsten, Hoover and Karnovsky (1980) suggested that membrane receptors float freely in the plasmalemma until associated with an external ligand. This association causes the receptors to link up with a calcium - binding protein that is, in turn, linked to the sub-membraneous contractile system. This receptor linkage results in a localised release of Ca^{++} which triggers actin/myosin interaction. The theory of Klausner et al (1980) is attractive in that it allows free movement of different types of membrane receptor until interaction with external ligands results in their displacement by an intracellular contractile system. However, their theory of a Ca^{++} trigger comes from the inhibitory effect of free fatty acids on cap formation. It has since been demonstrated that this inhibition is due to the free fatty acids depleting cellular ATP (Corps, Pozzan, Hesketh and Metcalfe 1980). It is possible to accept the free receptor theory and amalgamate it with the intracellular pathway proposed by Butman et al (1980) to produce a working hypothesis for mobility of membrane receptors. Again, the binding specificity of the receptors controls the systems expression. This interpretation of capping and locomotion is also applicable to 'zoites of Apicomplexa.

So far this discussion has examined the surface modulating mechanisms of culture cells and coccidian 'zoites. There are, however, other cell types capable of similar capping behaviour that have not been

mentioned. Trypanosomes use flagellar locomotion, but they are also able to cap certain membrane components (Barry 1979). The author suggested that this property was used to alter the cell's antigenic character, so avoiding the host's immune response. The "recognised" glycoprotein would be shed with associated antibody. The contractile basis of this behaviour is unknown, although trypanosomid forms of Leishmania appear to have an actin - weight protein associated with their pellicle (Dwyer 1980). Capping of membrane bound glycoproteins has also been observed in the rhizopod Entamoeba histolytica (Pinto da Silva, Martinez - Palomo and Gonzalez - Robles 1975). The contractile mechanism was not examined. There are however, other members of the Rhizopoda capable of similar movement of surface receptors. In 1951 Ray observed Acanthamoeba castellani moving bacteria, adhered to its surface, to the posterior of the cell. This was probably the first reported demonstration of capping. More recently, Naeglaria gruberi's ability to cap cationised ferritin and concanavalin A has been examined (King and Preston 1977a, Preston, O'Dell and King 1975). Capping was found to be concommitant with motility. The authors suggested that capping and locomotion were products of the same contractile mechanism. Unfortunately less information is available on the contractile basis of the membrane mobility of these cell types, however, their capping mechanism does appear at least behaviourally similar with that of culture cells and coccidian 'zoites. Although I'm sure free - living rhizopods possess similar membrane properties (eg. McGee - Russell and Allen 1971), it is interesting that the cells in which capping has been demonstrated are either from a multicelled organism; parasites within a multicelled organism; or free living protozoa capable of existing within a multicelled

organism. This suggests that the directed mobility of membrane components is useful, and an advantageous pre - adaptation, to existence within a multicellular system.

Apart from capping and locomotion, there is another function that these 'zoites perform that may involve the parasite's membrane - associated contractile system. The projected properties of this contractile system are remarkably similar to the recorded data on host - cell invasion of coccidian and malarial parasites.

7.2 HOST - CELL INVASION BY 'ZOITES OF APICOMPLEXA

Host cell invasion by these parasites follows a strict sequence; attachment to the potential host cell, induction of a parasitophorous vacuole, passage of parasite into vacuole and pinching off of vacuole from the cell's plasmalemma. The passage of 'zoite into host - cell is thought to involve the parasite's contractile system. Existing theories of invasion have been developed from research into the merozoite/ erythrocyte interaction of Plasmodium parasites. Neither of these theories, the passive uptake due to vacuolar enlargement (Bannister et al 1977) or the modified "zipper" theory (Aikawa et al 1978) explain all current observations on merozoite invasion.

In the present study the invasive behaviour of Eimeria acervulina and Eimeria tenella was examined. The results were used in proposing an alternative model of invasion, in which movement of parasite into host - cell is achieved by capping the parasite/host junction down the 'zoite's body. Again the external expression of the same membrane - associated contractile system would be dictated by preferential binding of 'zoite membrane receptors. This simple model, with no modifications, fulfills all current observations on host - cell invasion by 'zoites of

Apicomplexa and, I believe, that this method of invasion will prove to be common to all 'zoites of Apicomplexa.

The motile, invasive stages of these parasites are the most hazardous in the parasite's life cycle; a conclusion supported by the number of 'zoites usually produced. These 'zoites are the initial stage of infection of a new cell/host. The potential vulnerability of 'zoites has prompted extensive research into the cellular basis of host - cell invasion. The model of invasion, outlined above, has certain implications as to the future of this research. If host - cell invasion were achieved by a process unrelated to the normal functioning of host cells it may be possible to inhibit invasion without affecting the host. However, if invasion is a function of an actin - based contractile system, as proposed in this study, differential inhibition would be difficult. Some anti - microtubular agents show differing degrees of action between higher and lower eukaryotes (Quinlan, Roobol,Pogson and Gull 1981), but the anti - microfilament drugs, the cytochalasins, appear equally effective on all actins (Thomas 1978). This suggests attempts to prevent invasion by inhibition of the parasite contractile system to be impractical. An alternative method may lie in the apparent control membrane receptors have over the external manifestation of the submembraneous contractile system. The importance of certain receptors on the red blood cell to successful invasion by Plasmodium merozoites has already been demonstrated (Miller, Aikawa, Johnson and Shiroishi 1979; Pasvol, Weatherall, Wilson, Smith and Gilles 1976). The masking of the parasites receptors specific to these host cell sites by the induction of antibodies in the host is another potential area of treatment/control.

This project was largely concerned with 'zoite locomotion. It has, however, been demonstrated that this locomotion may represent the ideal model for studying the contractile basis of the third stage of invasion; translocation of parasite into host.

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