THE ROLE OF POLYAMINES AND RELATED METABOLITES IN THE GROWTH AND MORPHOLOGY OF TWO PISCINE TRYPANOSOMES

BY

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A Thesis Submitted in Partial Fulfilment of the Requirements of Kingston University for the Degree of Doctor of Philosophy

May 1996

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ABSTRACT

In comparison with the vast amount of literature available on the trypanosomes of mammals, relatively little is understood of those of lower vertebrates, including those present in the blood of fishes. Previous studies on fish trypanosomes have been concerned mainly with their life cycles and morphology, but this project presents data on their ultrastructure, polyamine and thiol metabolism and their responses to polyamine biosynthesis inhibitors.

Trypanosoma granulosum from the European eel and *Trypanosoma danilewskyi* from the crucian carp were isolated from the blood of their respective hosts and successfully cultured *in vitro* in an undefined diphasic medium. Several semi-defined and fully-defined media were then tested for their suitability to sustain growth of the parasites. A modified version of SDM-79 successfully provided the growth and conditions necessary for morphological, ultrastructural and biochemical studies of both parasites.

The ultrastructure of bloodstream *T. granulosum* highlighted similarities with some stercorarian trypanosomes. In diphasic medium and modified SDM-79 the bloodstream parasites transformed into forms similar to those reportedly found in their leech vector. Mainly trypomastigote forms were present in modified SDM-79, although epimastigotes, not found in any blood smears were also present. Ultrastructurally, cultured *T. granulosum* was similar to other members of the genus *Trypanosoma*.

Biochemical analyses of the parasites using high performance liquid chromatography, enzyme assays and radiolabelling studies revealed that their polyamine and thiol

metabolisms were unusual amongst trypanosomatids for example the African trypanosomes and *Leishmania* spp. Low levels of intracellular free polyamines, a rapid high affinity saturable uptake system for putrescine, the presence of glutathione as the major intracellular thiol, and the presence of the newly discovered thiol homotrypanothione, suggested that both *T. granulosum* and *T. danilewskyi* were similar to *Trypanosoma cruzi* in their polyamine and thiol metabolism.

Known inhibitors of polyamine metabolism DL-α-difluoromethylornithine (DFMO), methylglyoxal-bis-(guanylhydrazone) (MGBG) and diminazene aceturate (Berenil) were all shown to inhibit growth and to cause drastic alterations to the morphology and ultrastructure of *T. granulosum* (and *T. danilewskyi*). These anti-parasitic actions did not appear to result from polyamine depletion.

Declaration

I hereby declare that whilst registered for a research degree at Kingston University, I have not been a registered candidate or enrolled student at any other academic or professional institution.

I declare that the materials contained in this thesis have not been used in any other submission for an academic award. All the sources of investigation have been duly acknowledged.

This thesis has been composed by myself and is the result of my own investigation.

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May 1996

Acknowledgements

I would like to thank my supervisors Dr. Calli Mistry, Dr. Angela Russell and Dr. Ralph Manly for their guidance and support throughout my research years at Kingston. Their combined dedication and enthusiasm for their individual disciplines has been a constant source of inspiration and the acknowledgements section of my thesis can not do justice to the respect I have for them.

My thanks go to the technical staff of Kingston University for their help during various aspects of my research. Special thanks to Mrs Gurm Sappal, Ms. Jayne Reeves, Mr. Fred Quentin, Mr. Trevor Wilkins and to Mr. Bill Edwards whose technical skills are of the highest quality. I am particularly grateful to Dr. Peter Daszak for his expert TEM tuition and for his contributions to office debate.

I am extremely grateful to the staff of the Parasitology and Vector Biochemistry Unit at the London School of Hygiene and Tropical Medicine (LSHTM) in particular Dr. Karl Hunter, Mr. Mark Ariyanayagam, Dr. Sarah LeQuesne and Dr. Keith Smith for their time, patience and technical help during my many visits to their laboratories. Special thanks also to Professor Alan Fairlamb (LSHTM) for allowing access to his equipment and chemicals and for enduring my constant interrogation.

I would like to acknowledge the School of Life Sciences at Kingston University, in particular Dr. Ralph Manly for funding my research. Also my parents for financing the binding of this thesis and the British Society for Parasitology and Biochemical Society for their generous financial assistance needed to attend several conferences.

I would also like to thank Dr. Indrajit Das (Charing Cross & Westminster Medical School) and in particular Dr. Prakash Mistry (Institute of Cancer Research) for their academic and practical advice on various aspects of my research. Special thanks to Dr. Cristina Cruz for supplying additional parasites in a time of need and Mr. Bill Edwards and Miss Sue Choopani for their excellent translations of foreign manuscripts.

Finally thankyou to my girlfriend Sarah, who for the past three and a half years has been a rock in the quagmire of my PhD research.

This thesis is dedicated to my parents to whom I am indebted, as they have encouraged me throughout my long period of education, at no time wavering in their support of my academic goals.

ABSTRACTS & PUBLICATIONS

Thorborn D. E., Mastri C., Russell A. and Hunter K. J. (1994) The effects of polyamine biosynthesis inhibitors on the growth, morphology and related biochemical events in *Trypanosoma granulosum* from the European eel *Anguilla anguilla* L. *Poster: Spring Meeting (British Society for Parasitology)* 6-8th April.

Mackintosh D., Zubairi S., Russell A. J., Thorborn D. E. and Mastri C. (1994) Insulin stimulates the growth of *Trypanosoma granulosum* in culture. *Poster: Spring Meeting (British Society for Parasitology)* 6-8th April.

Thorborn D. E., Mastri C., Russell A. J. and Hunter K. J. (1994) Polyamine metabolism in fish trypanosomes. *Poster: Biochemical Society (meeting 651).*

Thorborn D. E., Mastri C. and Davies A. J. (1995) Polyamines and thiols in *Trypanosoma granulosum. Poster: Trypanosomiasis and Leishmaniasis Seminar (British Society for Parasitology)* 3-6th September.

Davies A. J., Mastri C., Thorborn D. E. and Mackintosh D. (1995) Experiments with growth of the eel trypanosome, *Trypanosoma granulosum* Laveran and Mesnil 1902, in semi-defined and defined media. *J. Fish Dis.* 18, 599-608.

GENERAL INTRODUCTION

Members of the genus *Trypanosoma* are protists belonging to the **Phylum:** Sarcomastigophora, **Class:** Zoomastigophorea, **Order:** Kinetoplastida and **Family:** Trypanosomatidae (Figure 1.1). The genus was characterised by Gruby in 1843 for parasites present in the blood of frogs. These organisms, or trypanosomes, are identified by having a slender, uni-flagellated body, a kinetoplast close to the posterior end of the cell, and an undulating membrane at some stage in their life cycle. The most frequently studied trypanosomes are those which infect mammals, particularly man, domestic livestock and game animals. Trypanosomes however, also occur in fishes, amphibia, reptiles, birds and invertebrates. Those infecting vertebrates predominantly inhabit blood, lymphatics, cerebro-spinal fluid and sometimes the tissues of their vertebrate host. They are transmitted between hosts during the blood-meal of a blood sucking invertebrate vector. In this invertebrate host, the trypanosome usually completes part of its life cycle prior to being transmitted to another vertebrate host (Kreier and Baker 1987; Mehlhorn and Walldorf 1988; Smyth 1994).

Some species of trypanosomes are serious pathogens of man and other mammals, causing trypanosomiasis, and therefore research has naturally concentrated mainly on these pathogenic species. Successful isolation and culture of these parasites have allowed detailed studies on their biology independent of host-related factors (Baker 1987; Gray *et al.* 1987; Brun and Jenni 1987). Currently there is much data available on the life cycles, morphology, fine structure, pathogenicity, biochemistry, immunology and control measures of these parasites. In comparison, relatively less is understood about the trypanosomes of lower vertebrates. Something is known of their life cycles, morphology

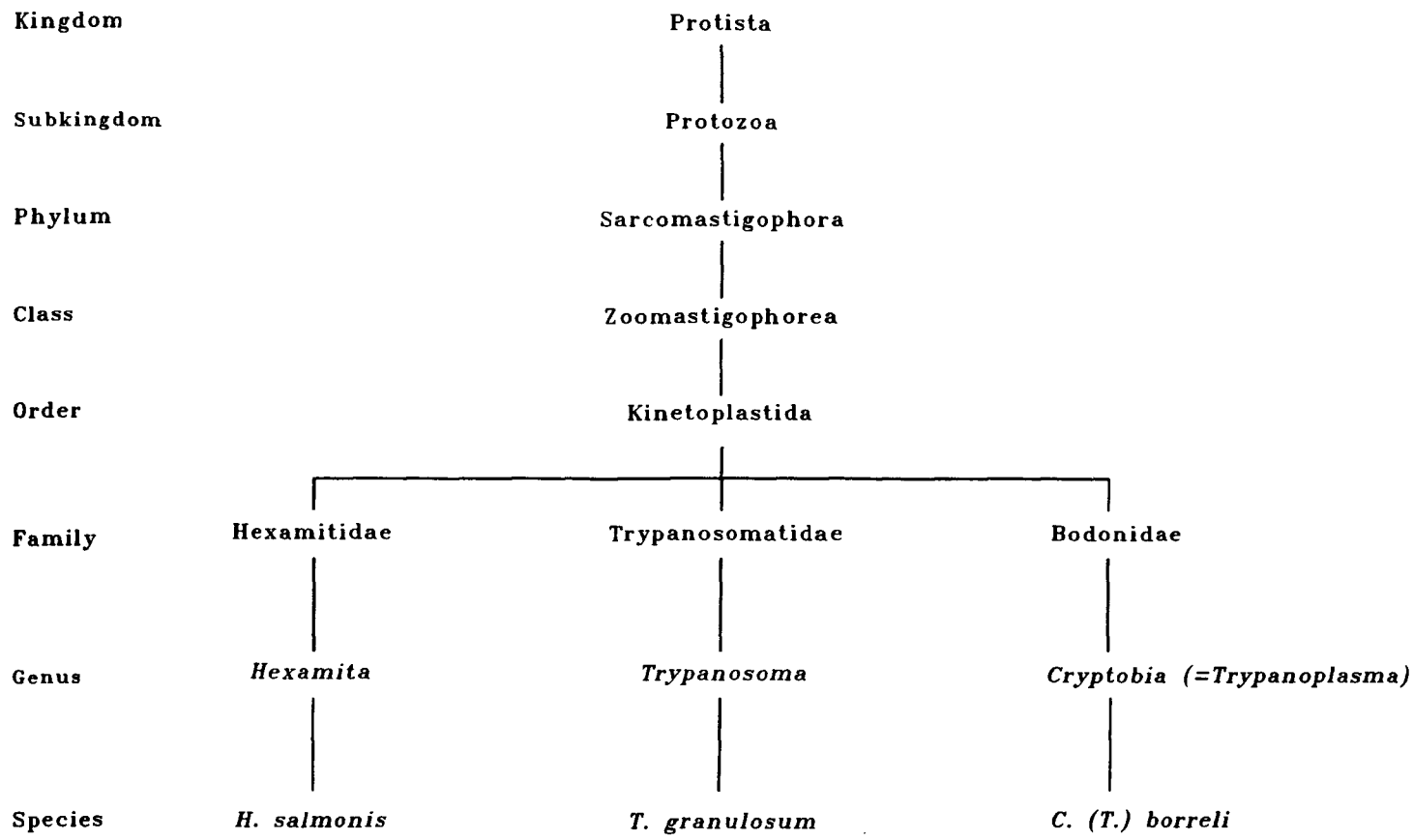


Figure 1 Classification of some species of parasitic protozoa of fishes. Based on Woo (1994).

and fine structure but very little is known of their biochemistry, pathogenicity and immunology.

This research project aims to partially redress the balance, by investigating two little-known trypanosomes of fishes, with special emphasis on *Trypanosoma granulosum* Laveran and Mesnil 1902. It is intended to study their cultivation, biochemistry and the effects of chemotherapeutic agents on the culture forms. To achieve this, the project is divided into two major sections. The parasitology section summarises some of the literature available on the pathogenesis and chemotherapy of mammalian trypanosome infections, and introduces the biology of fish trypanosomes. The biochemistry section highlights certain aspects of polyamine and thiol metabolism, explains why these pathways are important to trypanosomes, and explains the impact these pathways might have on rational drug design. The results gathered from the experimental part of each section are discussed individually and conclusions are drawn from both discussions.

SECTION I

CHAPTER 1: INTRODUCTION

1.1 Mammalian trypanosomes

Microscopical and biochemical techniques have revealed that the life cycle of trypanosomes in their vertebrate host and invertebrate vectors, consists of a series of morphological and physiological changes, comprising shifts in the position of the kinetoplast in relation to the nucleus and the anterior of the cell (Kreier and Baker 1987). These, and other changes, facilitate the development of the parasites in a range of environments and may allow evasion of host immune responses.

There are three pathogenic species of trypanosomes infecting humans: *Trypanosoma brucei gambiense* Dutton 1902, and *Trypanosoma brucei rhodesiense* Stephens and Fantham 1910 which are the causes of African trypanosomiasis, and *Trypanosoma cruzi* Chagas 1909 which causes American trypanosomiasis or Chagas' disease.

Human African trypanosomiasis, or sleeping sickness as it is also known, occurs in 36 African countries following the geographic distribution of the tsetse fly (*Glossina* sp.), the vector for the parasite. The distribution of the disease changes from time to time as efforts are made to control it, but some places remain decimated by epidemics of sleeping sickness (Goodwin 1974; Frenkel *et al.* 1988; Kuzoe 1993; Pépin and Milford 1994).

Gambian sleeping sickness, caused by infection with *T. b. gambiense*, is slow in onset and may last from a few months to several years. The early stages of the disease are characterised by successive peaks in parasitaemia followed by fever and lymphoid

hyperplasia leading to splenomegaly and enlargement of cervical lymph nodes. The trypanosomes then invade the central nervous system and the cerebrospinal fluid causing infiltration of immune cells. This inflammation spreads to the cranial and spinal nerve roots leading to headache, apathy, muscle wasting, somnolence, paralysis, coma and death (Frenkel *et al.* 1988).

Rhodesian sleeping sickness is caused by infection with *T. b. rhodesiense*. The early stages of the disease are similar to the Gambian disease except that enlargement of lymph nodes and pyrexia are more prominent. The disease is swift in onset and is characterised by myocarditis and weight loss, and death usually occurs in the absence of treatment (Frenkel *et al.* 1988; Kuzoe 1993).

Chagas' disease is a significant problem in the South American countries of Brazil, Venezuela and Bolivia, where socioeconomic conditions cause frequent contact between the triatomine vectors of *T. cruzi* and the rural populations (Bryan *et al.* 1994). It is estimated that mortality due to Chagas' disease is around 45,000 deaths per annum (WHO, 1993). According to the 1991-92 World Health Organisation report, *T. cruzi* currently infects an estimated 16-18 million people in South and Central America with a further 90 million people (approximately 25% of the population) at risk of infection.

The life cycle of *T. cruzi* involves obligatory passage through both vertebrate (man and other animals) and invertebrate triatomine hosts. Infectious metacyclic forms of the parasite present in the faeces of the vector bug, enter the vertebrate host either through contamination of a wound, usually from a bite or through penetration of the mucosa of the eye or mouth. Amastigote forms of the parasite multiply in the muscle and fat cells of the

dermis causing the characteristic inflammatory nodule, the Chagoma. The parasite then undergoes intracellular transformation to the trypomastigote form of the parasite responsible for dissemination of the infection. After infection and a subsequent incubation period the acute phase of infection begins, which without treatment results in 2-8% mortality, with the majority of deaths occurring in small children. Following a latent period which may last 10-40 years, about 10-30% of individuals go on to develop the clinical symptoms characteristic of chronic Chagas' disease. In the chronic phase of the disease, lymphatic and haematogenous dissemination, usually accompanied by fever, allows the trypanosomes to spread throughout the body and parasitize many tissues leading to cardiac (cardiomyopathy) and digestive (mega-colon and mega-oesophagus) disturbances, possibly as a result of various neuropathies and immune reactions (Köberle 1974; Kreier and Baker 1987; Dusanic 1991; de Castro 1993).

Trypanosomiasis can also affect wild and domestic animals. The causative agents of the disease in cattle, goats and sheep are *Trypanosoma brucei brucei*, *Trypanosoma congolense* and *Trypanosoma vivax*. Typically, animal trypanosomiasis leads to increased weakness, extreme emaciation and eventually death. The effect of animal trypanosomiasis on the economy of a region is difficult to assess, but it is known that over 25 million doses of trypanocidal drugs are administered each year which may give some indication as to the size of the problem (Raether 1988).

1.1.1 Chemotherapy of mammalian trypanosomiasis

There are only four drugs currently available for treating African trypanosomiasis in humans (see Table 1.1): suramin, pentamidine, melarsoprol and eflornithine (DFMO).

GENERIC DRUG (BRAND)		TARGET INFECTION	POSSIBLE MODE OF ACTION	SIDE EFFECTS	REFERENCES
Suramin	(Moranyl® Bayer 205 Germanin®)	Early stage *T. b. rhodesiense*	Inhibition of glycolytic enzymes	Rare. Anaphylactic shock and proteinuria.	Pépin and Milford 1994; Wéry 1994; Wang 1995.
Pentamidine	(Lomidine®)	Early stage *T. b. gambiense*	Inhibition of polyamine metabolism[1], glycolytic enzymes and disruption of kDNA	Rare. Thrombocytopenia and leukopenia.	Bitonti *et al.* 1986; Pépin and Milford 1994; Newton 1974.
Melarsoprol	(Mel B Arsobal®)	Late stage *T. b. gambiense/T. b. rhodesiense*	Inhibition of pyruvate kinase and glutathione/trypanothione reductase[2]	Severe. Encephalopathy	Cunningham *et al.* 1994; Flynn and Bowman 1969; Pépin and Milford 1994
Eflornithine	(DFMO Ornidyl®)	Early/late stage *T. b. gambiense*	Inhibition of polyamine metabolism[1]	Rare. Thrombocytopenia, anaemia, leucopenia and diarrhoea.	Jänne *et al.* 1991; Doua and Yapo 1993; Pépin and Milford 1994; Schecter and Sjoerdsma 1986;
Nifurtimox	(Lampit®)	Acute *T. cruzi*	Generation of reactive oxygen species and inhibition of nucleic acid synthesis	Severe. Weight loss, ataxia, nystagmus, tremor and adiadococinesia.	Pépin and Milford 1994; Wéry 1994; de Castro *et al.* 1994; Docampo *et al.* 1981; Fairlamb *et al.* 1985; Docampo and Moreno 1986; Fairlamb 1990
Benznidazole	(Rochagan®)	Acute *T. cruzi*	Generation of reactive oxygen species and inhibition of nucleic acid synthesis	Severe. Bone marrow suppression, peripheral neuropathy and chromosomal damage in peripheral lymphocytes.	Gutteridge 1987; Raether 1988; Gorla *et al.* 1988
Diminazene	(Berenil®)	*T. b. brucei*, *T. b. congolense* and *T. vivax*	Inhibition of polyamine metabolism[1] and binding to kinetoplast DNA	Rare.	Pépin and Milford 1994; Peregrine and Mamman 1993; Wang 1995; Raether 1988

Table 1.1 Summary of the targets and effects of antiprotozoal agents. [1] For review of inhibition of polyamine metabolism see section 5.1.6, [2] For review of inhibition of thiol metabolism see section 5.2.1

Until 1990, melarsoprol was the only drug capable of treating late stage *T. b. gambiense* and *T. b. rhodesiense* infections but knowledge of the severe side effects caused by melarsoprol therapy has caused high levels of anxiety amongst members of endemic populations (Pépin and Milford 1994). The recent appearance of DFMO, a remarkably non-toxic drug, has allowed treatment of both early and late stage *T. b. gambiense* infections, but the drug remains ineffective against *T. b. rhodesiense* infections (Schechter and Sjoerdsma 1986; Doua and Yapo 1993). In addition, the plasma half-life of DFMO is so short that a large amount of drug is needed to maintain therapeutic concentrations, making DFMO treatment expensive. Currently there are no safe and cheap alternatives for treating African trypanosomiasis.

Existing chemotherapy for Chagas' disease is highly unsatisfactory. Only two drugs have been used successfully in the treatment of acute American trypanosomiasis namely the nitroheterocyclic compounds nifurtimox (Lampit®) and benznidazole (Rochagan®). However no drugs are available for prophylaxis or the treatment of chronic patients. Both nifurtimox and benznidazole are orally active making administration easy (Gutteridge 1987), however they are both extremely toxic, suggesting the need for the development of a cheap and safe chemotherapy for Chagas' disease.

In endemic areas, *T. cruzi* infection via blood transfusions can be prevented by prior treatment of potentially infected blood with Gentian violet (crystal violet), although the drug has several side effects including microagglutination and rouleaux formation of erythrocytes and patients complain of the violet colour of the blood post-transfusion (Docampo *et al.* 1991). Clinical data suggest that gentian violet would not pass current safety procedures (Hammond *et al.* 1984), therefore much research has centred on the

chemotherapy of bloodstream *T. cruzi*. A series of natural and synthetic drugs have been tested *in vitro* to see whether they have any activity against bloodstream *T. cruzi*. Many of these have been shown to lyse bloodstream *T. cruzi* but they need to be tested further in the presence of mammalian cells and in experimental animals (Hammond *et al.* 1984; de Castro *et al.* 1994).

Treatment of animal trypanosomiasis is currently dependent on the salts of three compounds: diminazene, homidium and isometamidium. Of the three compounds diminazene, marketed by Hoechst as the diaceturate salt (Berenil®), is the most commonly used for the treatment of animal trypanosome infections, due to its high therapeutic index and low incidence of resistance (Peregrine and Mamman 1993; Wang 1995). The use of homidium and isometamidium in the treatment of animal trypanosomiasis has been greatly reduced since the 1970's due to widespread resistance and acute toxicities associated with administration of the drugs (Kinabo 1993; Wang 1995).

1.1.2 Ultrastructure of pathogenic flagellates

The majority of information on the fine structure of pathogenic flagellates comes from the study of sectioned material by transmission electron microscopy (TEM), although scanning electron microscopy (SEM) and X-ray microanalysis have been used to a lesser degree (Vickerman and Tetley 1977). The majority of this work has been performed on the mammalian trypanosomes, and studies using TEM have shown a constancy of basic structure in these flagellates. For instance, a number of flagellated protozoa contain a unique mitochondrion which contains an unusually large amount of deoxyribonucleic acid (DNA) localized in one region of the inner matrix. This mass of DNA is termed

kinetoplast-DNA (k-DNA). This DNA-containing organelle has been given a variety of names. These have included: nucleolus, micronucleus, blepharoplast, centrosome, kinetonucleus, parabasal body and kinetoplast-mitochondrion (Simpson 1972). The presence of this kinetoplast is characteristic of a large group of flagellated protozoa called the Kinetoplastidae. The k-DNA consists of a parallel array of fine fibrils orientated parallel to the long axis of the cell forming a slightly concave disc. This nucleoid is housed in a capsular expansion of the mitochondrial apparatus or chondriome. In all kinetoplastid flagellates the chondriome appears to be a highly branched mitochondrion which contains typical mitochondrial cristae arising from the inner mitochondrial membrane (Vickerman 1974). The DNA is situated in the matrix close to the inner mitochondrial membrane.

The fine structure and cyclical development of the mammalian pathogen *T. b. brucei* is well documented (Vickerman 1971). During this cyclical development, the trypanosome exhibits morphological changes, the most prominent of which is the shift in the position of the kinetoplast in relation to the position of the nucleus and the posterior of the trypanosome. This can be seen by light microscopy as well as by TEM. The bloodstream forms of *T. b. brucei* are pleomorphic. In the slender bloodstream form, the mitochondrion is represented by a single peripheral canal extending throughout the length of the trypanosome, with a few or no cristae. Progression to the stumpy form (through an intermediate form) leads to swelling of the mitochondrial canal and plate-like cristae are found throughout the mitochondrion. After two days growth in a monophasic blood broth medium the bloodstream trypanosomes differentiate into parasites corresponding to the early development forms found in the vector gut (Brown *et al.* 1973).

Electron microscopy has also revealed that bloodstream forms of salivarian (those which develop in the salivary glands of the invertebrate vector) trypanosomes have a surface coat 12-15nm thick covering the plasma membrane (Vickerman 1969). This coat is discarded within 24hrs of entering a culture system or vector. However, in *T. b. brucei* and *T. congolense* the surface coat is regained as the vector epimastigote (a stage characterised by the kinetoplast usually being located alongside the nucleus) transforms into infective metacyclic forms (a stage characterised by a shorter, thinner, body and a kinetoplast close to the posterior of the cell). It has been argued that the surface coat is an adaptation to a bloodstream environment and contains variant antigens (Vickerman 1969). This hypothesis is based on 5 observations: (1) a loss of surface antigenic character is correlated with a loss of coat, (2) the infective metacyclic trypanosome possesses the same surface antigens as the original stumpy bloodstream form which infected the vector, (3) the surface coat and the variant antigens appear to be glycoproteins, (4) antibody specific to the variant will only bind to cells which posses an intact surface coat, (5) a compact surface coat has not been reported in trypanosomes which do not have the capacity for antigenic variation, for example stercorarian (where development takes place in the gut of the invertebrate vector) trypanosomes (Vickerman 1974). It has been suggested that in the insect trypanosomatid, *Crithidia fasciculata*, the cell coat is not a stable morphological character and is produced only in response to the appropriate environmental conditions (Brooker 1976).

Another feature of all species of trypanosomes is the flagellum. Flagella are thin (about 0.2μm thick), whip like structures composed of a central microtubular array (axoneme) surrounded by an outer sheath which is the continuation of the cell membrane (Alberts *et al.* 1989). The axoneme has an arrangement of tubules consistent with the majority of

cilia and flagella, which consists of a central pair of microtubules surrounded by an inner sheath linked radially to nine peripheral doublet microtubules via secondary fibres. The flagellum arises from basal body (kinetosome), an organelle homologous with the centriole and consisting of nine peripheral triplet tubules. Only two of the microtubules of each kinetosomal triplet become one of the doublets in the axoneme (Lom and Dyková 1992). The proximal end of the kinetosome is open whereas the distal end is closed by a partition called the terminal plate, out of which extends the central pair of axoneme microtubules which are absent in the kinetosome (Vickerman 1974). As the flagellum emerges from the flagellar pocket it acquires another structure - the paraxial rod, a network of fortifying microfilaments (Mehlhorn *et al.* 1988) which is linked to two of the doublets through a bifurcated process (Vickerman 1969; Brooker 1971a, 1971b).

An additional single layer of microtubules orientated in a general anterior-posterior direction and situated just below the cell membrane is recognised as a typical feature of all trypanosomatid flagellates. These pellicular microtubules are joined by a number a fine filaments not always visible on a transverse section (Brooker 1971c). It is thought that the function of pellicular microtubules is to allow the deformation and extension of the cell.

1.2 Piscine trypanosomes

In contrast to the number of trypanosomes described from man and domestic animals, approximately 190 species have been recorded from fish (Lom 1979; Woo 1994). Because of their relatively non-pathogenic nature and their apparent readiness to grow in culture, studies on their biology could yield valuable insight into mammalian trypanosome pathogenicity and into the phylogeny of the Trypanosomatidae.

1.2.1 An historical review of the study of piscine trypanosomes

According to Lom and Dykova (1992), fish protozoa were first recorded in China during the Sung Dynasty (964-126 AD). Trypanosomes however, were probably first described in fish in the mid-nineteenth century. Amoeba-like organisms noted in the blood of the trout (*Salmo fario*) (Valentin 1841) and northern pike (*Esox lucius*) (Remak 1842) were remarkably similar to trypanosomes described by Gruby in 1843 (see Laveran and Mesnil 1907). Gros (1845) described "vermicles" in the blood of several species of fishes, including gudgeon (*Gobio gobio*), rockling (*Gaidropsaurus tricirratus*), redfin perch (*Perca fluviatilis*), sterlet (*Acipenser* sp.), burbot (*Lota lota*) and tench (*Tinca tinca*). The parasite found in rockling, was probably the first pleomorphic marine fish trypanosome to be described. Other workers who described fish trypanosomes at this time were Berg (1845), Wedl (1850) and Chaussat (1850).

There were few reports of trypanosomes from fishes after these initial discoveries in the mid-nineteenth century, until the lengthy descriptions of Mitrophanow (1883) of *Haematomonas cobitis* from the giant loach (*Misgurnus fossilis*) and *Haematomonas carassi* from the crucian carp (*Carassius carassius*), which Laveran and Mesnil (1912) thought belonged to the genus *Trypanosoma*. Later, Danilewsky (1885) studied the blood of a number of freshwater fishes, noting two different types of flagellate. These flagellates were also noted by Chalachnikov (1888) in the blood of common carp, northern pike, crucian carp and the ruffe (*Acerina cernua*). Laveran and Mesnil (1901) placed these parasites into two genera. The first parasite found in the pike was placed in the genus *Trypanosoma* and was named *Trypanosoma remaki* after Remak who first saw the parasite. They assigned the second parasite, found in the blood of red-eye (*Scardinius*

erythrophthalmus), to a new genus, *Trypanoplasma*, and named the parasite *Trypanoplasma borelli* (Laveran and Mesnil 1901).

As interest in the study of fish trypanosomes increased, new species for example *Trypanosoma soleae* from the sole (*Solea solea*) (Laveran and Mesnil 1901) and *T. granulosum* from the eel (*Anguilla anguilla*), which was first seen by Sabrazès and Muratet, were described (Laveran and Mesnil 1902a). Brumpt (1906b), reported a further 8 species from freshwater fishes and Lingard (1904) noted seasonal variations in parasitaemia of a number of Indian fishes infected with trypanosomes. More recently *Trypanosoma occidentalis* (Becker 1967), *Trypanosoma phaleri* (Jones and Woo 1990) and *Trypanosoma burresoni* (Jones and Woo 1993) were characterized from North American freshwater fishes.

1.2.2 Transmission and host specificity

Laveran and Mesnil (1902a, b), were among the first to attempt transmission experiments. They inoculated blood from an infected pike into an apparently uninfected pike. The trypanosomes divided in the blood by unequal binary division, reaching a peak parasitaemia about 3-4 weeks post inoculation. Transmission of *T. granulosum* between eels was also successful, but their attempts to transmit the parasite between species were unsuccessful. As a result of these experiments, Laveran and Mesnil suggested that different species of trypanosomes could be classified according to their host specificity, even if morphologically similar. This prompted Lebailly (1905) to study host restrictions in marine fishes. He inoculated blood from a *Trypanosoma limandae* infected dab (*Limanda limanda*), into an eel, but without success. In addition, conger eels (*Conger*

conger) inoculated with blood containing *T. granulosum* from *Anguilla anguilla*, similarly showed no parasitaemia. Other recipients of inoculation with *Trypanosoma platessae*, *Trypanosoma callionymi* and *T. granulosum*, were *Conger conger*, gunnel (*Pholis gunnellus*) and rockling. All recipient fishes remained negative for detectable inoculated parasites, suggesting that host specificity in marine fishes was similar to that demonstrated in freshwater fishes by Laveran and Mesnil (Lebailly 1905). However, Robertson (1911) showed that not all fish trypanosomes were host specific and Needham (1969) demonstrated that trypanosomes could pass between closely related fishes. More recently *T. danilewskyi* was demonstrated to show lack of host specificity, actually changing size in different hosts (Woo and Black 1984), a feature also observed in *T. cobitis* (Letch 1977).

1.2.3 Classification of piscine trypanosomes

Leeches are thought to be the invertebrate vectors of fish trypanosomes, and Brumpt (1906b), described a method for classifying these trypanosomes according to which area of the gut of the leech development took place. Trypanosomes were divided into two groups: firstly those that developed solely in the stomach of the leech, with no invasion of the intestine or proboscis sheath; secondly those that initially developed in the stomach and then migrated to the intestine, where they remained until the parasites migrated to the proboscis sheath for transmission to a host fish. This division presented a difficult method of classification and other methods evolved, including classification according to kinetoplast size. This included trypanosomes with small kinetoplasts, for example *T. scardinii* and *Trypanosoma carassii*, and trypanosomes with large kinetoplasts, for example *T. remaki* and *T. granulosum* (Bykhovskaya-Pavlovskaya *et al.* 1962).

A comparison by Fanthem *et al.* (1942) of a monomorphic trypanosome found in the blood of the Canadian yellow perch (*Perca flavescens*) with *T. percae* from the European perch first described by Brumpt (1906a), revealed similarities, but the Canadian trypanosome was shorter and broader with a relatively short free flagellum. These slight differences and the presence of the trypanosome in a different host, were sufficient for these authors to name the Canadian trypanosome *T. percae* n.var. *canadensis*. The use of average measurements as a chief criterion for classifying trypanosomes was considered an inadvisable method to differentiate between trypanosomes by Laird (1951), although he did suggest an alternative method of classification. Morphometric techniques used in the classification of mammalian trypanosomes were later applied to fish trypanosomes. These measurements included the distance from the posterior end to the kinetoplast, the distance from the posterior end of the nucleus to the kinetoplast, the distance from the anterior edge of the nucleus to the anterior end of the body, the length of the free flagellum, the maximum breadth of the body and the total length of the body (Mackerras and Mackerras 1961). Similar measurements were used by Hasan and Qasim (1962) in their statistical analyses of measurements of *Trypanosoma punctati* from lata fish (*Ophicephalus punctatus*).

As described above and in section 1.2.2, morphology and host specificity are not totally reliable methods for classifying a species of fish trypanosome therefore care must be exercised when identifying a species. Techniques are now available for the determination of the antigenic profiles of different species of haemoflagellates. The use of SDS-PAGE and Western immunoblotting has provided evidence of polypeptide differences amongst *Cryptobia* sp. but as the characteristics for most species of haemoflagellates are unknown their usefulness as taxonomic tools are not clear. They may prove useful however in

determining species of fish trypanosomes and resolve the contradictory early studies of host specificity (Woo and Thomas 1991). In addition, the selective sugar binding specificity of lectins represents a valuable tool for discerning cells composed of different surface sugars and has enabled differentiation between marine and freshwater trypanosomes. However the ability of this technique to differentiate between similar species remains to be determined (Zajícek and Lukes 1992). Enzyme analyses have also been undertaken for strain identification, leading to suggestions that the number of fish trypanosome species should be reduced (Letch 1977; Zajícek 1991a; Zajícek and Pecková 1995).

1.2.4 Piscine trypanosomes: monomorphic or pleomorphic?

Pleomorphism has been observed in a number of fish trypanosomes. Dutton *et al.* (1906) described a trypanosome in the African mudfish (*Clarias angolensis*) which had three distinct forms distinguished by morphometry. Further studies revealed similar pleomorphism in other trypanosome species, including observations by Minchin (1909) of two forms of *T. remaki* in the blood of pike, described previously by Laveran and Mesnil as var. *parva* (small) and var. *magna* (large) on the basis of size. Minchin (1909) also observed three different types of *T. percae* in the blood of the perch, and seven gradations between the smallest and largest *T. granulosum* from the eel, and suggested that the pleomorphism observed in *T. granulosum* strongly indicated different stages of growth in the parasites. In such pleomorphic species, the first trypanosomes to appear in the blood are thought to be "young", followed by "intermediate" and finally the "adult" forms which are thought to persist in chronic infections (Lom and Dyková 1992). Minchin (1909), also described monomorphism in *Trypanosoma tincae* from the tench and *T. abramis* from the

bream (which Minchin described as being morphologically identical to *T. tincae*), although a lack of material may have been responsible for this conclusion. Further studies on *T. remaki* confirmed the above earlier findings of two morphologically distinct forms of the parasite in the blood of the pike (Kudo 1921). Tanabe (1924) observed three different forms of *Trypanosoma cobitis* in the blood of the Japanese loach (*Misgurnus anguillicaudatus*) and Baker (1960) reported two distinct forms of *Trypanosoma mukasai* in several species of East African fish.

1.2.5 Ultrastructure of piscine trypanosomes

Few studies have been made of the ultrastructure of trypanosomes from fishes. Boisson *et al.* (1967) gave a brief description of the fine structure of a trypanosome from the marine fish *Zanobatus schoenleini* and Preston (1969) described the form and function of the cytostome-cytopharynx of the culture forms of *T. raiae* from the skate. Lewis and Ball (1979) were the first to describe the ultrastructure of a freshwater fish trypanosome. They observed *T. cobitis* in their leech vectors (*H. marginata*) and the attachment to the crop wall of the leech by modification of the tip of the trypanosome's flagellum. Studies on bloodstream *T. danilewskyi* revealed the presence of a fully developed cytostome-cytopharyngeal apparatus which at the time had not been found in any trypanosome studied in the haematozoic state (Lom *et al.* 1980).

The fine structure of the epimastigotes of *T. cobitis* in the leech vector was described in detail (Lewis and Ball 1980) and the division of this trypanosome both in the leech vector and in culture were studied (Lewis and Ball 1981a, b). These studies included descriptions of bacteria-like microorganisms in the epimastigote and metacyclic trypomastigote stages

of *T. cobitis* (Lewis and Ball 1981b). More recent studies on the ultrastructure of fish trypanosomes revealed that *T. danilewskyi* from the common carp, has an unusual process of cytokinesis involving disaggregation of k-DNA prior to k-DNA division, a phenomenon not seen in other Kinetoplastida (Paterson and Woo 1984). The fine structure of fish trypanosome kinetoplast DNA was also elucidated in *Trypanosoma cf. carassii* (Zajícek *et al.* 1991).

1.2.6 Vector relationships in the trypanosomatidae of fishes

At the end of the nineteenth century, interest in the intermediate host for fish trypanosomes began. The invertebrate vector of fish trypanosomes was first described by Leydig (1857) (see Lebailly 1905), who noted the presence of trypanosomes in the crop of two genera of leeches, *Piscicola* and *Pontobdella*. Later, Brumpt (1904), inoculated trypanosome-like flagellates from the crop of the leech *Hemiclepsis marginata* into European minnow (*Phoxinus phoxinus*), rudd and stickleback (*Gasterosteus aculeatus*) with negative results. He also noted that the trypanosome infection was not transmitted vertically in *H. marginata*. In addition Brumpt (1906a) described the development of numerous freshwater fish trypanosomes in *H. marginata*, all of which failed to develop in *Piscicola geometra*, suggesting vector specificity.

Extensive studies on the life cycle of fish trypanosomes in their leech vectors and their vertebrate hosts were undertaken by Robertson (1906, 1910, 1911). Robertson (1906) studied the development of *Trypanosoma raiae* in the crop of *Pontobdella muricata* and described the development of trypanosomes from freshwater fishes including bream, redfin perch and rudd in *H. marginata*. She also noted that these trypanosomes multiplied

rapidly in the crop of the leech and underwent morphogenesis. Upon transformation into a slender form, the trypanosomes migrated to the proboscis sheath where they were inoculated into a fish at the next blood meal (Robertson 1911). In the case of *T. granulosum* from the eel, initial multiplication occurred in the stomach of *H. marginata* followed by a further period of development in the intestine (Brumpt 1906a), a seemingly unusual occurrence for freshwater fish trypanosomes (Molyneux 1977), although such progression had been observed in the marine trypanosome, *T. raiae* (Needham 1969). Tanabe (1924) followed the development of trypanosomes from the Japanese loach in the leech *Hirudo nipponica* and Qadri (1962a) studied the life cycle of *T. danilewskyi* from common carp in *H. marginata*. In his review of trypanosome vectors, Molyneux (1977) stated that *H. marginata* can be infected with a number of trypanosomes whereas *P. geometra* has poor vectoral capacity. Interestingly, Laird (1951) found the blood of a number of freshwater fishes to be free of blood parasites, suggesting a lack of suitable vectors in New Zealand's rivers.

1.2.7 The effects of trypanosomal infections on their vertebrate fish hosts

Although most fish trypanosomes are regarded as non-pathogenic, there is evidence that a number may exert quite considerable effects on their hosts. Robertson (1911) reported occasional mortality in fishes harbouring trypanosome infections and recent observations suggest that mortality is more common in fishes infected with trypanosomes than previously thought. A 60-100% mortality was observed in syringe-passaged *T. danilewskyi* strains in goldfish (Lom and Suchánková 1974). This suggested that many wild fishes examined for blood parasites present with chronic infections and a percentage of the natural population have already died. Further laboratory studies on MS or Ma

strains of *T. danilewskyi* introduced into parasite-free carp by intra-peritoneal injection, caused 10-100% mortality, depending on the strain of the trypanosome, and the age of the fish (Lom 1979). High mortality (65%) occurred in juvenile Atlantic cod (*Gadus morhua*) and winter flounder (*Pseudopleuronectes americanus*) experimentally infected with *T. murmanensis*, but deaths decreased with increasing age of the fishes (Khan 1985).

There are several reports describing anaemia resulting from trypanosome infections in fishes. These include skates (*Raja punctata*) infected with *Trypanosoma variabile* (Neumann 1909), perch (Robertson 1911), ling infected with *Trypanosoma lotae* (Smirnova 1970), young Atlantic cod infected with *Trypanosoma murmanensis* (Khan 1977), eels infected with *T. granulosum* (Boon *et al.* 1990), and goldfish infected with *T. danilewskyi* (Islam and Woo 1991a). In goldfish experimentally infected by intraperitoneal infection with *T. danilewskyi*, it was thought anaemia was caused by haemolysin secreted by the parasite and by haemodilution (Islam and Woo 1991a). Other blood changes reported in fishes infected with trypanosomes, included increases in: eosinophils (Neumann 1909), phagocytic leucocytes (Smirnova 1970), abnormal erythrocytes (Tandon and Joshi 1973) and serum albumin levels (Cottrell 1977). Examination of blood smears from an eel infected with *T. granulosum* revealed nuclear degeneration in approximately 45% of erythrocytes, however it was thought unlikely that the degeneration was due to *T. granulosum* infection (Eiras 1983).

Inflammation of the brain, fatty degeneration of organs (Neumann 1909) and damage to the haemopoietic organs (Dyková and Lom 1979) have been observed in fishes harbouring trypanosome infections. In addition, the effects of infections by *T. murmanensis* on condition factor (K-factor), organ somatic indices (SI's) and plasma protein levels of four

species of marine fishes were significantly different in some infected fish groups than in uninfected fishes (Khan 1977; Khan 1985). Studies on goldfish experimentally infected by intraperitoneal injection with *T. danilewskyi* also presented with anorexia, thought to be caused by an increase in interleukin-1 release from phagocytes in response to protozoan infection (Islam and Woo 1991b)

One study of concurrent parasitic infections of fishes showed that cod infected with the parasitic copepod *Lernaeocera branchialis* were disease free, but in the presence of *T. murmanensis* and the copepod, cod were adversely affected (Khan and Lacey 1986). Recently it has been discovered that oestradiol increases the susceptibility of goldfish to *T. danilewskyi* infection (Wang and Belosovic 1994).

1.2.8 *In vitro* cultivation of piscine trypanosomes

Upon inoculation into culture medium, bloodstream trypanosomes transform into stages similar to those found in the leech vector. The precise structure of these stages varies with species and culture conditions (Lom 1979).

The earliest attempts at *in vitro* cultivation of fish trypanosomes were at the beginning of the twentieth century. Initial *in vitro* studies demonstrated that *T. granulosum* could multiply in eel blood (10-19^{0}C) for 8 days (Sabrazes and Muratet 1902). Petrie (1905) and Brumpt (1906a) cultured *T. danilewskyi* and *T. granulosum* respectively in modifications of blood agar (NNN medium) but failed to establish subcultures even with large inocula. Thompson (1908) used a modified NNN medium to cultivate *T. carassii* from goldfish (*Carassius auratus*). He described the various developmental forms of the trypanosome

at 7, 21, 28 and 43 days but still unable to establish subcultures. Ponselle (1913) used a diphasic rabbit-blood medium modified from Novy and MacNeal's medium to successfully culture *T. granulosum* but again failed to maintain subcultures. This medium was also used to culture *T. cobitis* from the Japanese loach but division was only reported 2-3 days after inoculation, again with no subcultures being established (Tanabe 1924).

The first report of continuous culturing of fish trypanosomes was by Qadri (1962b), who maintained *Trypanosoma striati* from the Indian fish *Ophicephalus striatus*, in Shortt's modification of NNN medium for over 16 months. Peterson (1974) cultured *T. percae* on a modified 4N medium, finding detection of light infections easier by cultivation rather than direct examination of the blood of the perches. Lom and Suchankova (1974) also demonstrated the growth of *T. danilewskyi* on diphasic blood agar medium for 3 years.

Recently, modifications of the blood agar used by Ponselle (1913) were used to cultivate *Trypanosoma catostomi* and *T. phaleri* from North American freshwater fishes (Jones and Woo 1991) and *T. granulosum* was successfully cultured on horse blood nutrient agar slopes (Davies *et al.* 1992). Several species of fish trypanosome have been cultured in a monophasic medium which was relatively undefined (Zajícek 1991a, 1991b; Zajícek and Pecková 1995) although *T. danilewskyi* has been cultivated using a fully defined serum free medium (TDL-15) supplemented with insulin, a medium based on a culture medium used in the cultivation of goldfish macrophage cell lines (Wang and Belosovic 1993). Trypanosomes from marine fishes were also grown *in vitro*. These included *T. raiae* which was cultured in Johnson's blood agar medium (Preston 1969) and *Trypanosoma boissoni* which was cultured on Tobie's and NNN medium but better results were obtained using a brain-heart-infusion agar (Ranque 1973).

Fish tissue cultures of the fathead minnow (FHM) and the brown bullhead (BB) grown in MEM medium (containing 10% foetal calf serum) allowed growth of *T. danilewskyi* at 25°C (Smolíková *et al.* 1977). The presence of haemin in this culture system provided the best growth environment for the parasite, but tissue culture medium without the presence of the fish cells could not support the parasite (Smolíková *et al.* 1977).

1.3 Aims of this section

The aim of this part of the project is firstly to isolate *T. granulosum* from the European eel (*Anguilla anguilla*). The use of fully-defined and semi-defined media have not been reported for the cultivation of *T. granulosum*. It is intended to identify a medium which will provide equivalent or superior growth to the currently used undefined horse-blood nutrient agar slopes (Davies *et al.* 1992) which will then be suitable for biochemical studies on the parasite. The growth, morphology and fine structure of the cultured parasite will be determined and discussed. As the fine structure of bloodstream *T. granulosum* remains uncharacterised, it is also intended to present preliminary data on the fine structure of this parasite. In addition, the effects of three anti-trypanosomal drugs, namely DL-α-difluoromethylornithine (DFMO), methylglyoxal bis(guanylhydrazone) (MGBG) and diminazene aceturate (Berenil) on the growth, morphology and fine structure of the cultured parasite will be determined and discussed. The effects on growth of adding polyamines to drug treated cells and aminoguanidine to control cells will also be determined and discussed.

A second parasite, *T. danilewskyi* isolated from crucian carp (*Carassius carassius*), will be cultured in similar conditions to *T. granulosum* allowing comparison of the two fish

trypanosomes. The effects of DFMO, MGBG and Berenil on the growth and morphology of *T. danilewskyi* will be determined and discussed in relation to *T. granulosum* and other trypanosomes.

CHAPTER 2: METHODS

2.1 Chemicals

The following items were purchased from Agar Scientific:

Epoxy (Araldite) Resin Kit, glutaraldehyde, osmium tetroxide, paraformaldehyde, propylene oxide and uranyl acetate.

The following item was purchased from Anglia Scientific:

May-Grünwald

The following items were purchased from BDH Ltd:

4-Aminobenzoic acid, ferrous sulphate, Giemsa, glucose, 3-(n-morpholino)propane sulphonic acid, sodium hydrogen carbonate, sodium pyruvate and taurine.

The following items were purchased from Sigma:

Adenine, adenosine, L-alanine, L-arginine, biotin, bovine haemin, calcium chloride, calcium pantothenate, cobalt sulphate, copper sulphate, diminazene aceturate (Berenil), EDTA, folic acid, glucosamine-Hcl, L-glutamine, glycerol, guanosine, insulin, magnesium sulphate, L-methionine, methylglyoxal-bis-(guanylhydrazone) dihydrochloride (MGBG), nicotinamide, L-phenylalanine, potassium iodide, L-proline, putrescine dihydrochloride, pyridoxamine dihydrochloride, riboflavin, L-serine, sodium chloride, sodium diphosphate, sodium molybdate, spermidine trihydrochloride, spermine tetrahydrochloride, thiamine, L-threonine, thymine, tricaine methanesulphonate, triethanolamine, L-tyrosine, tween

80, zinc sulphate.

The following item was purchased from Tissue Culture Services:

Defibrinated horse-blood

DFMO was a generous gift from Merrell Dow and all other reagents used for cell culture were purchased from Gibco BRL (now Life Technologies Ltd.).

2.2 Collection and housing of fishes

European eels were purchased from an eel farm which had in turn obtained the eels from the River Frome in Dorset, England. Crucian carp were obtained from the National Rivers Authority (NRA), Waltham Cross. The fishes were maintained in freshwater recirculating systems at Kingston University's Victoria Road annexe and kept at a constant temperature (20^0C). Eels were fed periodically on a diet of live earthworms as commercial fish pellets proved unsuccessful. Crucian carp were fed on commercial fish pellets daily.

2.3 Examination of fishes for parasites

Eels 30-70cm long and crucian carp 10.5-14.0cm long, were anaesthetized by immersing them in fresh water containing 0.05%(w/v) MS-222 (tricaine methanesulphonate). Prior to bleeding the fishes, nutrient blood agar slopes were prepared as described below (section 2.5.1.1). At the proposed site of bleeding, the skin of the fish was cleaned by swabbing with 70% ethanol. The eels and carp were then bled from the caudal vein using

a 1ml heparinised syringe and 25G x 1" needle. The fish were revived following bleeding by placing them in fresh water. Once revived the fish were replaced in the freshwater recirculating system. Infected fish were identified by placing a drop of blood onto a slide and examining it for active trypanosomes using phase contrast microscopy.

2.4 Sterilisation of equipment and aseptic technique

Trypanosome cultures were maintained free of microbial contaminants by the use of sterile culture media, plasticware and equipment, and the application of aseptic techniques. All cell culture work was conducted in a Gelaire Class 100 Laminar Air Flow Cabinet equipped with a Bunsen burner. Before use, all glassware was immersed in water containing Decon 90®, rinsed in distilled water and dried in a drying cabinet at 70°C. Sterilisation of bijou bottles, Pasteur pipettes, blow out pipettes, Gilson pipetman tips and glassware was done by autoclaving (121°C, 15 psi) for 20 minutes.

For the preparation of all cell culture media deionised water, autoclaved as above, was used. Labile solutions such as some media and serum were sterilised by filtration through disposable 0.22μm Sartorius® membrane filters. The final precaution against bacterial contamination was the addition of a penicillin/streptomycin mixture to the media (for concentrations see media recipes).

2.5 Preparation of media

2.5.1 Diphasic (Davies *et al.* 1992)

Nutrient agar solution was made by dissolving 2.3g of nutrient agar powder in 100ml deionised water. This solution was sterilised by autoclaving (121^{0}C, 15psi) for 20 minutes. The solution was left at room temperature to cool and before solidifying, defibrinated horse blood (10% final concentration) was added aseptically and mixed. The blood nutrient agar mixture was decanted aseptically into sterile bijou bottles (30ml) and left at an angle of 45^{0}C so that the agar set into slopes. Once the slopes were set and cooled to room temperature, 2ml of Ringer's solution containing penicillin (200units/ml) and streptomycin (200μg/ml) were added aseptically to each slope. The slopes were then left for 24hrs at 4^{0}C. Prior to inoculation the slopes were gently shaken and allowed to warm to room temperature.

Approximately 0.05ml of infected fish blood was aseptically added to each overlay and the cultures were incubated at 20^{0}C for two weeks. Cells were counted daily using an Improved Neubauer haemocytometer and when growth reached approximately 1-2 X 10^7 trypanosomes/ml of overlay, the cells were inoculated onto blood agar slopes or flasks containing a modified SDM-79 (as described below).

2.5.2 Fluid media

2.5.2.1 Preparation of semi-defined media

2.5.2.1.1 Modified SDM-79

The medium described in this section is a modified version of SDM-79 (Brun and Schönenberger 1979) with 7.0g of MEM F-14 (Gibco Bio Cult) omitted because it was no longer available for purchase. From here onwards this medium is termed modified SDM-79.

Medium ingredients /litre :

Medium 199 w/Earle's salts w/L-glutamine w/o $NaHCO_3$	2.2g
MEM amino acids (X50) w/o L-glutamine	8.0ml
MEM nonessential amino acids (X100)	6.0ml
Glucose	1.0g
HEPES (1M)	33.6ml
3-(n-morpholino) propane sulphonic acid (MOPS)	5.0g
$NaHCO_3$	2.0g
Na-pyruvate	100mg
L-alanine	200mg
L-arginine	100mg
L-glutamine	300mg
L-methionine	70mg

L-phenylalanine	80mg
L-proline	600mg
L-serine	60mg
Taurine	160mg
L-threonine	350mg
L-tyrosine	100mg
Adenosine	10mg
Guanosine	10mg
Glucosamine-Hcl	50mg
Folic acid	4mg
4-aminobenzoic acid	2mg
Biotin	0.2mg

The above were dissolved in approximately 950ml of sterile deionised water. To this solution 10ml of stock penicillin (5000iu/ml)/streptomycin (5000μg/ml) solution were added. The pH of the medium was adjusted with 2M NaOH, to pH7.3. Bovine haemin stock solution (2.5mg/ml in 0.05M NaOH) was autoclaved and added at a final concentration of 7.5mg/ltr. The solution was made up to 1000ml with sterilised deionised water and the pH checked. The medium was filter sterilised using 0.22μm filters and stored at 4^{0}C for a maximum of four weeks. Prior to inoculation, an aliquot of the medium was allowed to warm to 20^{0}C and foetal calf serum (final concentration 10%) was added.

The following three modifications to the modified SDM-79 medium described above were also investigated for their suitability for growth of the parasite and were prepared as

described above in the method for SDM-79:

a) SDM-79A: modified SDM-79 with αMEM (with ribo/deoxyribonucleosides, without $NaHCO_3$ to the same concentration as the missing MEM F-14 powder).

b) SDM-79B: modified SDM-79 with αMEM (without ribo/deoxyribonucleosides, without $NaHCO_3$ to the same concentration as the missing MEM F-14 powder).

c) SDM-79C: modified SDM-79 with S-MEM (with Earle's salts [modified], with L-glutamine, without $NaHCO_3$ to the same concentration as the missing MEM F-14 powder).

<u>2.5.2.1.2 Medium B</u> (Brun *et al.* 1981)

Minimal Essential Medium (w/ Earle's salts, w/o L-glutamine)	450ml
MEM nonessential amino acids (X100)	5ml
HEPES	3.58g
Glucose	1.0g
L-glutamine	150mg
Deionised water	45ml

The pH of the medium was adjusted to pH 7.5 with 2M NaOH and the medium was filter sterilised with 0.22μm Sartorius® filters and stored at 4^0C for a maximum of four weeks.

Prior to inoculation, an aliquot of the medium was allowed to warm to 20^{0}C and foetal calf serum (final conc. 15%) was added.

2.5.2.2 Preparation of fully defined media

2.5.2.2.1 Medium D (Dewey and Kidder 1971)

Stock solutions:

NaCl & PO_4 s	**mg/ml**
NaCl	200.0
Na_2HPO_4	62.5
KH_2PO_4	25.0

EDTA & salts	**mg/ml**
Na_4EDTA	70.0
$MgSO_4.7H_2O$	40.0
$FeSO_4.7H_2O$	0.49
$CaCl_2.2H_2O$	0.73
$Na_2MoO_4.2H_2O$	0.101

Vitamins	**mg/ml**
Biotin	0.002
Nicotinamide	0.50
Pyridoxamine.2HCl	0.20

Ca Pantothenate	0.80
Riboflavin	0.20
Thiamine	0.20

Trace metals	**mg/ml**
$ZnSO_4$	68.0
$MnSO_4.H_2O$	77.0
$CoSO_4.7H_2O$	2.4
$CuSO_4.5H_2O$	0.07
KI	0.03

Haemin: 5mg/ml bovine haemin in 50% triethanolamine, diluted to 1mg/ml with deionised water in order to reduce the viscosity.

To make 400ml of Medium D:

Amino acid solution (BME X100)	80ml
Vitamins (no biopterin)	4ml
NaCl & PO_4's	8ml
EDTA & salts	4ml
Tween 80	1.84ml
Glucose	4g
Thymine	16mg
Adenine	20mg
Trace metals	400μl

Biopterin (1ng/ml final conc.)	400μl
Haemin	2ml

Once dissolved the pH of the medium was adjusted to pH8.0 with 2M NaOH and 10ml of stock penicillin (5000iu/ml)/streptomycin (5000μg/ml) solution were added. The medium was then made up to 400ml with deionised water and sterilised by filtration through a 0.22μm Sartorius® filters and stored at 4^{0}C prior to use.

<u>2.5.2.2.2 TDL-15 Medium</u> (Wang and Belosovic 1994)

The medium was composed of 25% Leibovitz's L-15 Medium, 25% Dulbecco's Modified Eagle Medium (w/o sodium bicarbonate) and 50% modified Hank's balanced salt solution (w/o Ca^{2+} and Mg^{2+}), supplemented with the following: 12.5ml of MEM amino acid solution (X50), 12.5ml of MEM non-essential amino acid solution (X100), 12.5ml sodium pyruvate solution, 12.5ml of L-glutamine solution (X100), 10ml MEM vitamin solution (X100), 20ml of nucleic acid precursor solution (2.5mM) consisting of adenosine, cytidine, hypoxanthine, thymidine and uridine, 5mg/litre insulin and 840mg/litre of sodium bicarbonate solution in 1000ml of TDL-15 medium. To this 10ml of stock penicillin (5000iu/ml)/streptomycin (5000μg/ml) solution were added. The medium was then sterilised by filtration through 0.22μm Sartorius® filter and stored at 4^{0}C. A second modified TDL-15 medium was also made where bovine haemin stock solution (2.5mg/ml in 0.05M NaOH) was autoclaved and added at a final concentration of 7.5mg/ltr to the medium.

2.6 Culturing in semi-defined and fully defined media

9ml of the medium to be tested for suitability of trypanosome growth was added to sterile culture flasks (50ml). To the medium was added 1ml of overlay (shaken) from a culture containing 1-2 x 10^7 trypanosomes/ml (final concentration 1-2 x 10^6 trypanosomes/ml) cultured in modified SDM-79 and placed in an incubator at 20^0C. Duplicate counts were made daily of each culture using aseptic technique and an improved Neubauer haemocytometer. All experiments were conducted at least twice and within each experiment, cultures were run in triplicate. In an attempt to reduce the volume of culture media, sterile Leighton tubes were inoculated with the 1.8ml of the same media and 0.2ml of SDM-79 culture.

2.7 The effects of drugs on cell number and morphology of trypanosomes

The effects of DFMO, MGBG and Berenil on the cultured trypanosomes were determined by adding each drug to modified SDM-79 at a final concentration of 0-50mM, finally re-checking the pH of the medium in the presence of the drugs. Cells were counted after two and four days growth and IC_{50} concentrations were determined for each drug. To examine the effects of the drugs on the morphology of the trypanosomes, cells were washed in Dulbecco's PBS, smeared on slides and stained with May-Grünwald Giemsa (section 2.11). Cells were measured with an eyepiece graticule and stage micrometer. Also determined was the effect on the growth of trypanosomes, of adding either putrescine, spermidine or spermine (100μM final concentration) to cells after two days culture in medium containing drugs at their approximate IC_{50} concentrations (0.2mM for MGBG/Berenil and 16mM for DFMO).

2.8 The effect of aminoguanidine on the growth of *T. granulosum*

Aminoguanidine was added to modified SDM-79 at a range of final concentrations from 10^{-4}M to 10^{-6}M. Triplicate cultures were counted in duplicate using an improved Neubauer haemocytometer.

2.9 Storage of cultures in liquid nitrogen (Davies *et al.* 1992)

For the preservation of trypanosomes in liquid nitrogen, 100μl of sterilised glycerol was placed in a 1ml cryogenic ampoule. To the glycerol 900μl of modified SDM-79 containing approximately 10^7 trypanosomes were aseptically added and mixed. The ampoules were sealed, arranged upright and left at 4^0C for 10 minutes followed by 24 minutes in a -20^0C freezer. The ampoules were then lowered into liquid nitrogen gradually over 2hrs (2-3cm every 15 minutes) until the samples were totally frozen. The ampoules were then transferred to a liquid nitrogen refrigerator for storage.

2.10 Recovery of cells from liquid nitrogen storage

The frozen ampoule was thawed in a water bath at 20^0C, the ampoule was then dried and swabbed with 70% ethanol. Using aseptic techniques the cell suspension was placed in a sterile centrifuge tube and centrifuged (1511g, 20^0C, 15 mins). The supernatant was discarded and the cells were resuspended in 1ml of fresh growth medium. These cells were then used to re-establish cultures.

2.11 Staining of blood and culture smears - May-Grünwald Giemsa

Solutions:

a) May-Grünwald 0.25% (w/v) in absolute methanol

b) Giemsa diluted 1:3 with phosphate buffer (pH6.8)

Blood containing trypanosomes was smeared onto clean glass slides, rapidly air dried and fixed in absolute methanol for 10 minutes. Prior to fixation of trypanosomes from culture medium, cells were washed in Dulbecco's PBS (1511g, 4°C, 15mins). Smears were stained in May-Grünwald (solution a) for 1 minute. These were then stained in 50% May-Grünwald (solution a)/water mixture for 1 minute and stained in Giemsa for 60 minutes. The stain was then differentiated in distilled water for 5 seconds and checked under the microscope. The slides were then air dried and examined under oil immersion.

2.12 Transmission electron microscopy

2.12.1 Tissue processing

Phosphate buffer:

Soln A: 2.76g of $NaH_2PO_4.H_2O$ in 100ml deionised water

Soln B: 5.66g of $Na_2HPO_4.7H_2O$ in 100ml deionised water

Solution A was then added to solution B until pH 7.4 (approximately A:B=20:80) was reached.

Karnovsky's fixative:

Stock solution of paraformaldehyde:

8g paraformaldehyde in 100ml water heated to 60-70^{0}C and 12 drops of 1M NaOH were added to clear the solution.

To make 50mls of fixative:

25mls of paraformaldehyde solution plus 5mls of 50% glutaraldehyde made to 50ml volume using 0.1M phosphate buffer.

All cells were fixed with Karnovsky's fixative using the highest grade chemicals according to the following method. Cells were washed twice (1511g, 4^{0}C, 15 mins) in Dulbecco's PBS and resuspended in 5ml Karnovsky's fixative (48hrs, 4^{0}C). Fixed cells were then spun down (1511g, 4^{0}C, 15 mins), resuspended in 0.1M phosphate buffer and transferred to 1.5ml eppendorf tubes. The cells were then washed in buffer (3 X 10mins) and resuspended in 2% osmium tetroxide in 0.1M phosphate buffer for 3 hours. The cells were then briefly washed in deionised water and resuspended in 2% aqueous uranyl acetate for 6 hours. The cells were then taken through the following series of ethanol concentrations:

10% ---------- 30mins

20% ---------- 30mins

30% ---------- 30mins

40% ---------- 30mins

60% ---------- 30mins

70% ---------- 30mins

90% ---------- 30mins

100% ---------- 3 x 20mins

Resin:

The epoxy resin Araldite CY212 was used as the embedding medium throughout the experiments. Fresh resin was used for each batch of samples using the following recipe:

Araldite CY212	100mls
Dodecenyl succinic anhydride (DDSA)	100mls
Dibutyl phthalate	10mls
Benzyl dimethylamine (BDMA)	4mls

BDMA was added after the other chemicals were thoroughly mixed.

The cells were then washed 3 x 10mins in propylene oxide (100%) and transferred through a series of resin:propylene oxide mixtures (1:3, 1:1, 3:1, pure resin - two hours per step) at room temperature so as to allow adequate infiltration of the resin into the cells. The cells were then placed in fresh resin (100%) and polymerised by placing in an oven (60^0C, 48hrs).

2.12.2 Semi-thin sectioning and staining

One μm-thick sections (semi-thin) were cut from all blocks using a glass knife on a

Reichert-Jung Supernova ultramicrotome. These sections were placed on a glass slide and stained with 1% toluidine blue in 1%(aq) borax. The sections were then rinsed in distilled water and viewed under a light microscope. Areas of interest were selected and the resin blockface trimmed to approximately $1mm^2$ around the selected area.

2.12.3 Ultrathin sectioning and staining

Ultrathin sections (80-100nm) were cut on a Reichert-Jung Supernova ultramicrotome using glass knives and were collected on copper grids for staining. Grids were stored in dust-free boxes until required. Sections were stained with 10% uranyl acetate in methanol for 20 minutes followed by washing in absolute methanol (4 x 30 seconds) and blotted dry on filter paper. Sections were then stained with Reynold's lead citrate for 20 minutes (Reynolds 1963) and washed in NaOH (0.02M) for 15 seconds followed by double distilled water (4 x 15 seconds). The weak sodium hydroxide solution was used to prevent the formation of lead carbonate from a lead citrate reaction with carbon dioxide in air. The grids were then blotted dry on filter paper.

Following staining, ultrathin sections were viewed using a Philips EM 301 Advanced Research Microscope with a 25μm objective aperture and an accelerating voltage of 100Kv. Micrographs were taken using Ilford EM film and the film was developed for 3.75 minutes (20^{0}C) in Ilford PQ Universal Developer and fixed in Ilford Hypan Photographic Fixer. Micrographs were printed on Agfa-Gevaert Rapitone paper (grade I-III) using a Durst Laboratory 1000 enlarger and an Agfa-Gevaert Rapidoprint DD 3700 developer.

CHAPTER 3: RESULTS

3.1 *T. granulosum*

3.1.1 Bloodstream *T. granulosum*

3.1.1.1 Morphology of bloodstream *T. granulosum*

In infected eel blood examined by phase-contrast microscopy, *T. granulosum* was observed moving amongst the nucleated erythrocytes. The flagellum, undulating membrane, kinetoplast and nucleus could clearly be seen. In addition a number of cytoplasmic granules were observed.

Examination of May-Grünwald stained blood smears revealed an intensity of 0.096 trypanosomes/1000 erythrocytes. The cells were fusiform with an oval shaped approximately centrally located nucleus spanning the width of the organism. The kinetoplast was located close to the posterior of the cell. The undulating membrane originated close to the kinetoplast and ran forward to terminate at the free flagellum. The cytoplasm appeared densely stained and granules could clearly be seen in the majority of the cells (Plate 3.1). No dividing bloodstream forms were observed. The purpose of examining the stained blood films was to confirm (see below) that the parasites fell within the morphometric parameters already established by others for the identification of *T. granulosum* (Lom and Dyková 1992; Cruz 1995).

Length	59.0±9.0μm
Width at widest point	2.6±0.7μm
Distance from centre of nucleus to posterior (NP)	25.1±5.9μm
Distance from centre of nucleus to kinetoplast (NK)	23.9±6.3μm
Free flagellum (n=8)	22.9±6.2μm
NP/NK	1.1±0.2μm

n=22 except where otherwise stated

3.1.1.2 Ultrastructure of bloodstream *T. granulosum*

Bloodstream forms were scanty and therefore only a few of these trypanosomes were seen by transmission electron microscopy, although many thin sections were examined. Generally, the fine structure of bloodstream *T. granulosum* was similar to that of the culture form of the parasite described below. However, the ultrastructure of the bloodstream form differed in three ways from that of the culture form, although they were fixed by the same method: firstly, the cytoplasm was packed with dense ribosomes, which appeared more prominent than in culture forms; secondly, the mitochondria in the bloodstream forms appeared more prominent and extremely dense compared with those of the cultured forms and they were probably highly branched. In addition the mitochondrial cristae appeared plate-like (Plate 3.2; Plate 3.3); finally, electron dense bodies described in the culture form, and thought to be lipid vacuoles, were uncommon in the bloodstream form, although empty vacuoles which may have represented the position of the bodies were observed (Plate 3.4).

3.1.2 *T. granulosum* cultured in drug-free media

3.1.2.1 Morphology of cultured *T. granulosum*

The morpholgy of *T. granulosum* cultured on blood-agar slopes was not determined due to clumping observed in cells present in the overlay (see plate 3.21). However once introduced into modified SDM-79, cells transformed into a mixture of shorter trypomastigotes, which dominated, and epimastigotes. Cultured trypomastigotes ranged from 70.8±4.2% of the total population after 1 day in culture to 94.4±1.7% by day 6 (Table 3.1) (Plate 3.5). These forms were not only shorter but slimmer than the bloodstream forms, being 28.3±5.8μm in body length and 1.7±0.5μm wide. The length of the free flagellum was 13.0±2.7μm. The position of the kinetoplast varied even amongst trypomastigote forms but most were located approximately half way between the posterior of the cell and the nucleus.

Time of culture / days	Percentage trypomastigotes in modified SDM-79
1	70.8% ±4.2%
2	82.2% ±0.9%
3	80.9% ±1.7%
4	82.7% ±3.5%
5	85.3% ±0.9%
6	94.4% ±1.7%

Table 3.1 Percentage of *T. granulosum* trypomastigotes in modified SDM-79 medium. Results are the mean of duplicate cultures ± standard deviation. Parasites counted at each time point = 150 per culture.

3.1.2.2 Ultrastructure of cultured *T. granulosum*

A plasmalemma surrounded the parasite and beneath it was a single layer of microtubules. Microtubules ran longitudinally and parallel to each other throughout the cell (Plate 3.6; Plate 3.7; Plate 3.8). The cell cytoplasm contained many ribosomes. Also present were roughly circular moderately electron-dense membrane-bound bodies, possibly lipid vacuoles (Plate 3.9). Small and large vacuoles, apparently lacking contents, were also present. Golgi was seen infrequently (Plate 3.10) and membrane bounded vesicles of unknown origin and function were present (Plate 3.15). Nuclei were bounded by a nuclear envelope comprising inner and outer membranes with nuclear pores (Plate 3.9). Peripheral chromatin was particularly prominent (Plate 3.11), and divided nuclei could often be seen in the cell prior to completion of cell division (Plate 3.12). A prominent, electron dense nucleolus of variable shape, containing ribosomes and was usually centrally located, could be seen in most nuclei (Plate 3.11). Occasionally, relatively long fibrous structures of unknown origin and function could be seen close to the nuclei (Plate 3.12; Plate 3.13, Plate 3.14).

The kinetoplast was seen as an elongate organelle consisting of fibrous DNA enclosed by a double membrane continuous with mitochondrial membranes (Plate 3.15). A basal body usually lay adjacent to the kinetoplast (Plate 3.16). Occasionally dividing kinetoplasts could be seen. Kinetoplast division was apparently initiated by elongation involving lateral extension (Plate 3.14). Two kinetoplasts were sometimes observed within the same mitochondrion before completion of cell division (Plate 3.17).

Profiles of mitochondria could be seen throughout the cell (Plate 3.9; Plate 3.13; Plate

3.15; Plate 3.18). However it was not clear whether these represented one branching mitochondrion or whether several individual mitochondria existed. Cristae were plate-like areas of the mitochondrial inner membrane.

Close to the kinetoplast was a deep invagination of the pellicular membrane, the flagellar pocket (Plate 3.16). In this lay the flagellum (Plate 3.12), or sometimes two flagella were seen (Plate 3.11). Transverse sections of the flagellum showed the classical "9+2" arrangement of microtubules. The two central tubules could be seen to be linked radially to the peripheral doublets (Plate 3.19). The characteristic dynein arms of subtubule A could also be seen projecting clockwise (Plate 3.19). Running parallel to the tubules was the paraxial rod (Plate 3.19). Connections could be seen from the peripheral microtubules extending into the paraxial rod (Plate 3.19). Longitudinal sections of the flagellum showed its lattice-like ultrastructure (Plate 3.13; Plate 3.20).

3.1.2.3 Growth of cultured *T. granulosum*

3.1.2.3.1 Growth on nutrient agar slopes

Primary isolates from eel blood, grew well on horse blood nutrient agar slopes. After approximately two weeks post-inoculation, the numbers of parasites reached approximately 1-2x10^7 trypanosomes /ml of overlay. Accurate counting of organisms were difficult due to clumping (Plate 3.21). When parasites from the overlay of the horse blood nutrient agar slopes were inoculated into cultures with plain agar substituted for the nutrient agar, no growth was observed.

3.1.2.3.2 Growth in semi-defined and defined media

Modified SDM-79 provided a rapid growth medium for the parasites. Foetal calf serum from Gibco appeared to stimulate growth better than that from Sigma, which gave poor growth. When Gibco serum was present, parasites reached $1.8 \pm 0.1 \times 10^7$ trypanosomes /ml of medium within seven days (Figure 3.1) with a doubling time of approximately 2 days. Cells were relatively easy to count as little clumping was observed. The cells remained in the stationary phase of growth for at least two further days. In the semi-defined media described as SDM-79A (Figure 3.2), SDM-79B (Figure 3.3) and SDM-79C (Figure 3.4) (in section 2.5.2.1 of methods chapter), growth looked promising during the first 24 hours. In SDM-79A (αMEM with ribo/deoxyribonucleotides) in particular, parasites more than doubled their numbers from inoculation, from 1.5×10^6 to 3.2×10^6 trypanosomes/ml of media during this period. However, after two days growth in SDM79A, SDM79B and SDM79C the numbers of trypanosomes declined. No growth of parasites was observed in semi-defined medium Medium B, or the fully defined media Medium D, MEM, TDL-15 and TDL-15 with added haemin.

When *T. granulosum* was cultured in modified SDM-79 containing the polyamine oxidase inhibitor aminoguanidine, there was a 13% increase in the number of cells after 5 days compared with those cells cultured in modified SDM-79 lacking additional aminoguanidine (Figure 3.5). However there was no significant difference between the number of cells cultured in media containing 10^{-4}M, 10^{-5}M or 10^{-6}M aminoguanidine.

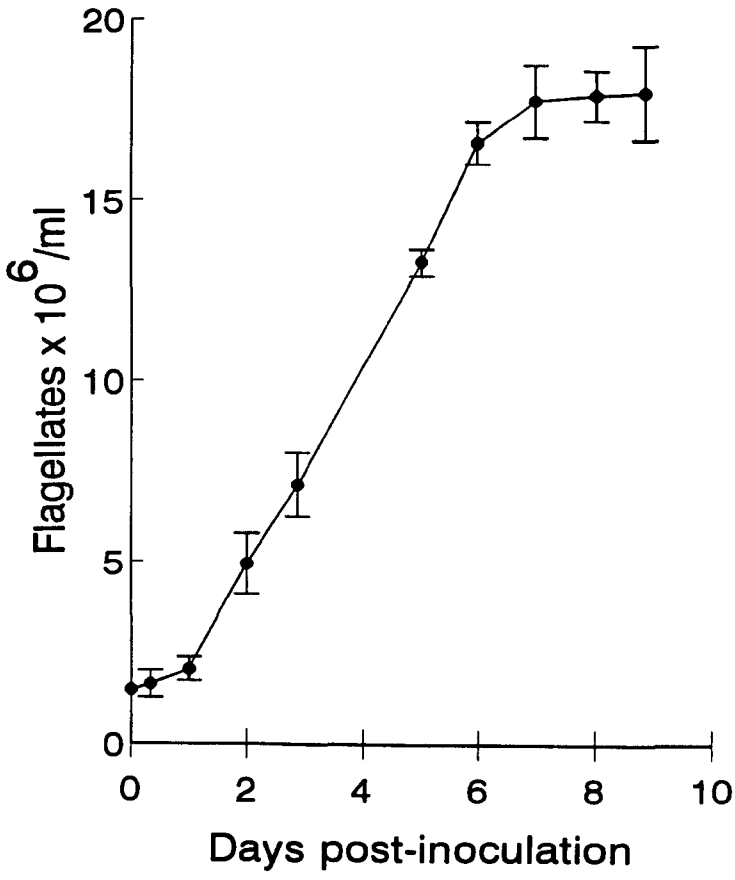


Figure 3.1 Growth of *T. granulosum* in modified SDM-79. Error bars represent standard deviation where n=6.

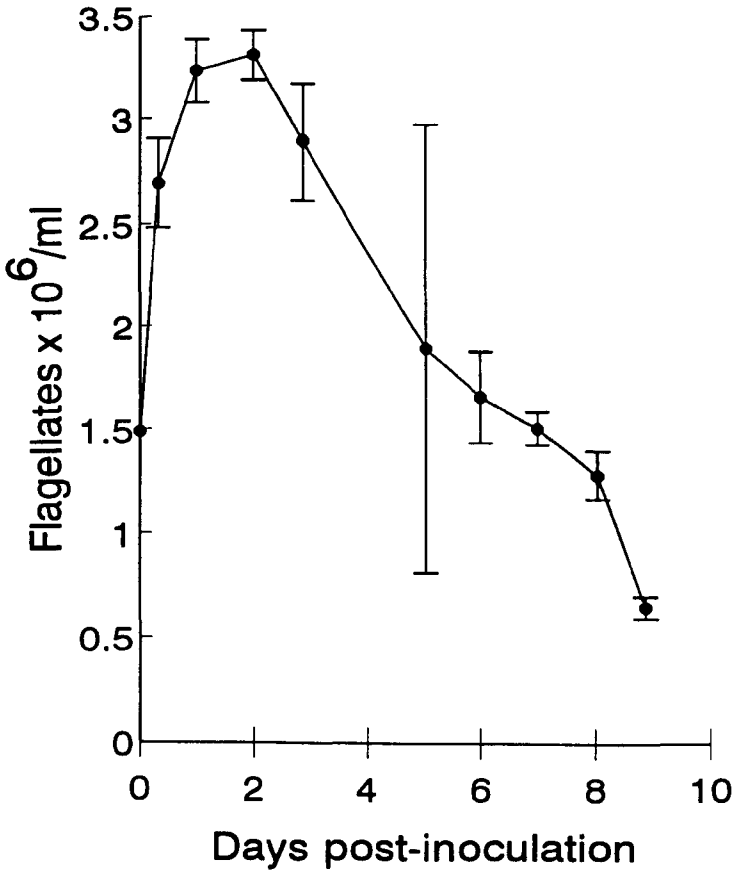


Figure 3.2 Growth of *T. granulosum* in SDM79A. Error bars represent standard deviation where n=6.

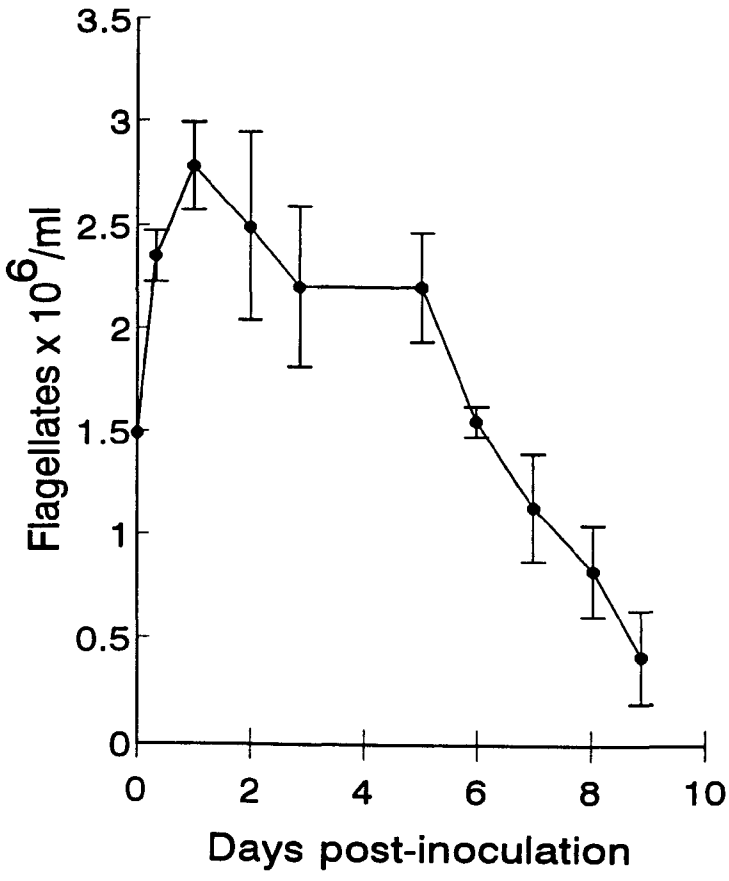


Figure 3.3 Growth of *T. granulosum* in SDM79B. Error bars represent standard deviation where n=6.

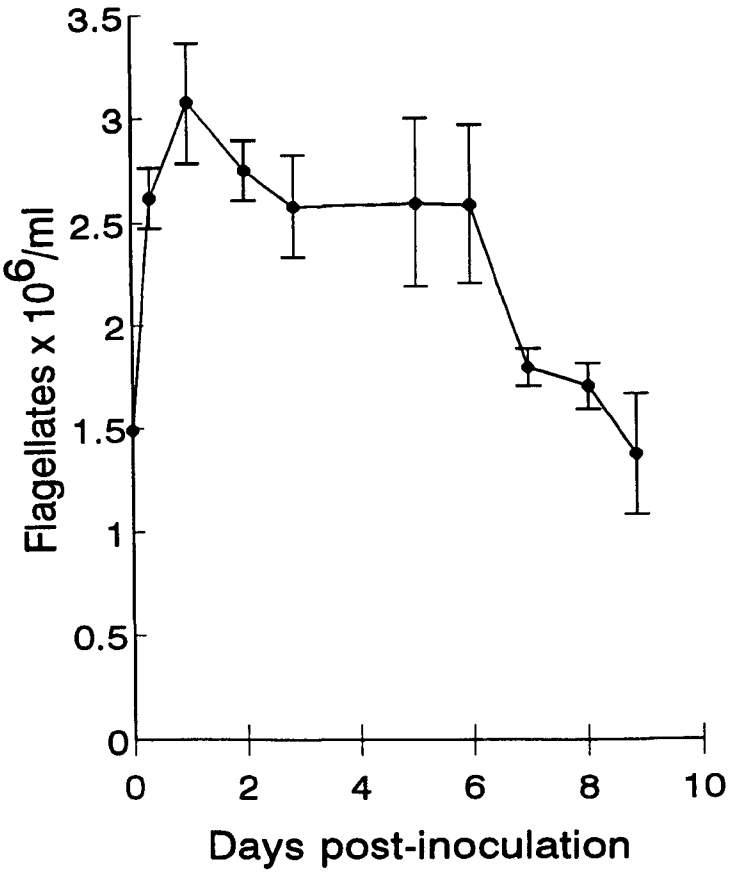


Figure 3.4 Growth of *T. granulosum* in SDM79C. Error bars represent standard deviation where n=6.

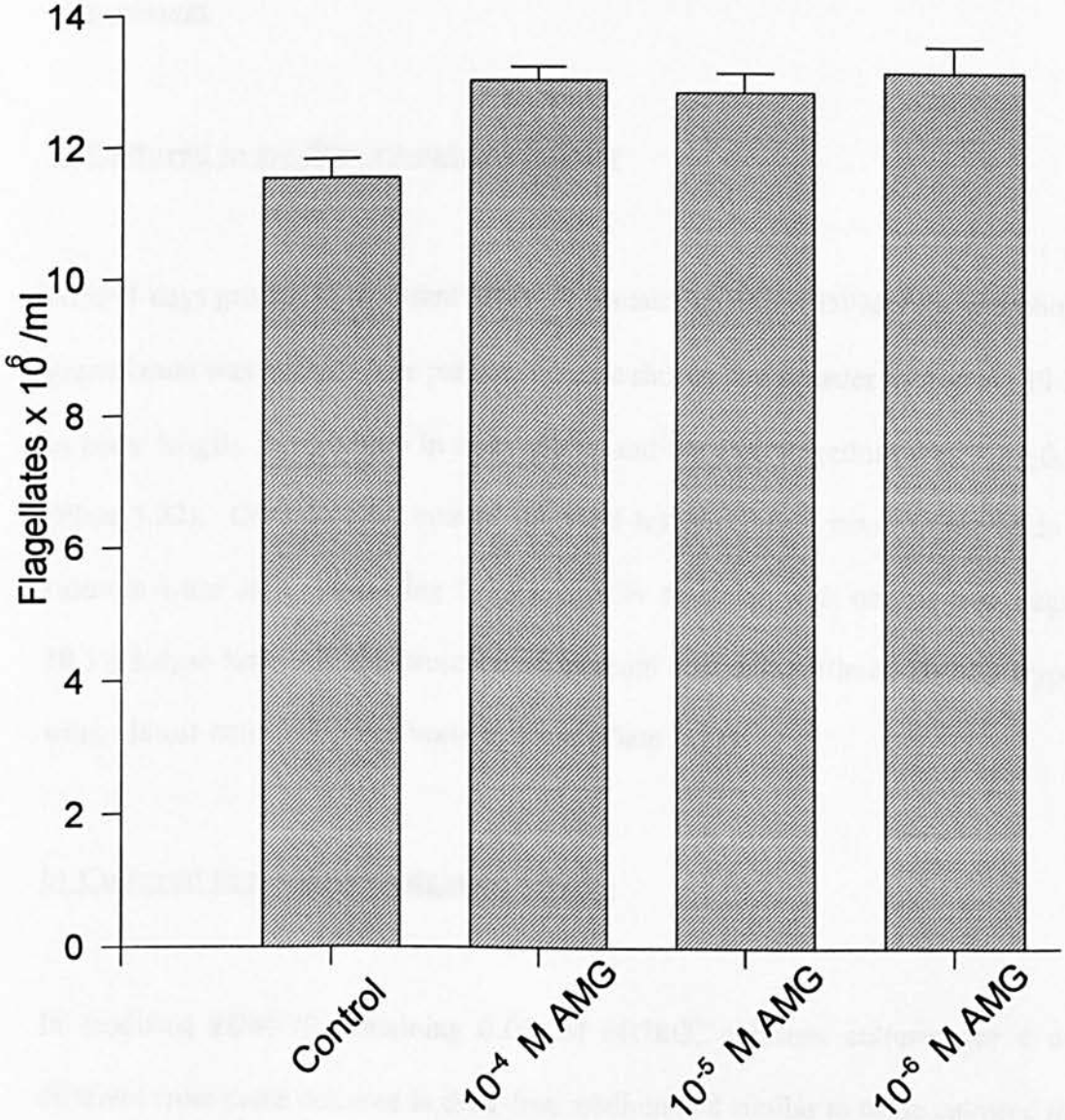


Figure 3.5 Growth of *T. granulosum* in modified SDM-79 containing aminoguanidine. Cells were cultured for 5 days in medium containing a range of concentrations of aminoguanidine. Error bars represent standard deviation where n=3. AMG=aminoguanidine.

3.1.3 *T. granulosum* cultured in medium containing polyamine biosynthesis inhibitors (for review of inhibition of polyamine metabolism see 5.1.6)

3.1.3.1 The effects of polyamine biosynthesis inhibitors on the morphology of *T. granulosum*

a) Cultured in medium containing DFMO

After 4 days growth in modified SDM-79 containing 10mM DFMO the morphology of *T. granulosum* was altered. The parasite became shorter and broader measuring 19.2±3.0μm in body length, 3.3±0.6μm in body width and the free flagellum was 3.3±0.6μm long (Plate 3.22). Occasionally, bizarre spherical trypanosomes, never observed in drug-free medium were seen, measuring 5.7±1.2μm in diameter with one or two flagella up to 18.1±3.4μm long. In the presence of medium containing 50mM DFMO trypanosomes were almost entirely of this bizarre shape (Plate 3.23).

b) Cultured in medium containing MGBG

In modified SDM-79 containing 0.05mM MGBG, parasites cultured for 4 days were different from those cultured in drug-free medium and similar to those cultured in medium containing 0.05mM Berenil. As the concentration of MGBG in the medium was increased, numbers of bizarre trypanosomes similar in size to those described above also increased. In the presence of 1mM MGBG the population was almost entirely of bizarre shape (Plate 3.24).

c) Cultured in medium containing Berenil

In modified SDM-79 containing 0.05mM Berenil, cells cultured for 4 days were shorter ($17.4 \pm 2.6 \mu m$ body length) and wider ($2.5 \pm 0.3 \mu m$ body width) than cells cultured in drug-free medium. Rarely, a bizarre form similar to those observed in cells cultured in modified SDM-79 containing DFMO, were also seen (Plate 3.25). In the presence of 0.2mM Berenil approximately 50% of the population were bizarre forms (Plate 3.26) while the remaining 50% were not significantly different in morphology to those cultured in the presence of 0.05mM Berenil. Numbers of bizarre trypanosomes increased with increased concentrations of Berenil in the medium. In the presence of 1mM Berenil, the cells were almost entirely abnormal (Plate 3.27).

3.1.3.2 The effects of polyamine biosynthesis inhibitors on the ultrastructure of *T. granulosum*

a) Cultured in medium containing DFMO

In the presence of 20mM DFMO cells appeared to be slightly swollen compared with cells cultured in drug-free medium and myelin-like figures were occasionally seen (Plate 3.28). Increased numbers of membrane-bound moderately electron-dense lipid bodies were observed compared with cells from drug-free medium (Plate 3.28). Empty vacuoles of similar shape and size (Plate 3.29) and large cytoplasmic vacuoles of variable size and shape containing membranes (Plate 3.30) were also present. A slight increase in the cytoplasmic ribosome density was noted but nuclei appeared similar to those of cells cultured in drug-free medium. Mitochondria were mostly similar to those in cells cultured

in drug free medium and kinetoplasts did not appear significantly different from normal, although occasionally the mitochondrion surrounding the kinetoplast appeared swollen (Plate 3.30). Occasionally however, the mitochondrial matrix appeared electron-lucent (Plate 3.31). A counter-clockwise arm could be seen in subtubule B in all peripheral doublets within the flagellum of one trypanosome cultured in the presence of 20mM DFMO (Plate 3.32). This structure was not observed in cells cultured in drug-free medium.

Cells cultured with 50mM DFMO exhibited gross ultrastructural differences compared with cells cultured in drug-free medium. Large cytoplasmic vacuoles often occupying half the area of the cell were seen (Plate 3.33). These vacuoles themselves contained smaller vacuoles of unknown origin and function (Plate 3.34; Plate 3.35). The electron dense membrane-bound lipid like bodies seen in drug-free cells and in those cultured with 20mM DFMO, were rarely seen in cells cultured with 50mM DFMO. Mitochondria containing plate-like cristae similar to those found in control and 20mM DFMO treated cells were present throughout the cells (Plate 3.36). Nuclei and kinetoplasts were similar to those observed in cells cultured in drug-free medium and medium containing 20mM DFMO, although mitochondrial membranes surrounding the kinetoplast were occasionally swollen (Plate 3.33). Occasionally coated pits were observed which appeared to be forming coated vesicles (Plate 3.36).

b) Cultured in medium containing MGBG

Cells cultured with 0.2mM MGBG contained large numbers of membrane-bound bodies of similar size and shape to those electron dense bodies described above (Plate 3.37). In

the majority of cells the cytoplasm was packed with ribosomes but occasionally cells contained few ribosomes (Plate 3.38). Several apparently empty membrane-bound or moderately electron dense bodies of similar size and shape to those described above, were observed in most cells (Plate 3.39). Golgi was more prominent in most cells cultured with 0.2mM MGBG than in cells cultured in other medium (Plate 3.40). Several cells were seen to be in the process of flagellar division (Plate 3.41) although dividing nuclei were not observed. Kinetoplast ultrastructure was apparently unaffected by 0.2mM MGBG although this structure was not observed in all cells. Mitochondria were difficult to observe in cells cultured in the presence of 0.2mM MGBG (Plate 3.37) and on the rare occasions that cristae were noted they appeared plate-like (Plate 3.38). Long fibrous structures described in cells cultured without drugs could also be seen in cells cultured in medium containing MGBG (0.2mM) (Plate 3.37; Plate 3.40).

Cells cultured with 1mM MGBG contained large cytoplasmic vacuoles of differing size and shape (Plate 3.42; Plate 3.43), although vacuolation was not as great as in cells cultured with 50mM DFMO. Cells cultured with 1mM MGBG often contained myelin-like figures (Plate 3.44) and membranous whirls (Plate 3.45) of unknown origin, although myelin-like figures were occasionly seen in control cells. Cytoplasmic ribosome density and moderately electron dense membrane-bound bodies were similar to those described above (Plate 3.46). Mitochondria were difficult to observe but they appeared to be branched throughout the cell (Plate 3.46). The shape of cristae within the mitochondria were not determined. Occasionally, partial disruption of the kinetoplast appeared to have occurred, to form a chaotic structure beneath the flagellar pocket (Plate 3.47).

c) Cultured in medium containing Berenil

Cells cultured with 0.2mM Berenil contained many electron-lucid membrane-bound vacuoles often filling a large area of the cell (Plate 3.48). On one occasion a few microtubules underlying the sub-pellicular microtubules were seen (Plate 3.49). In the majority of cells cytoplasmic ribosome density appeared to be similar to that observed in cells cultured in medium containing 0.2mM and 1.0mM MGBG. Large areas of cytoplasmic disruption were present in many cells (Plate 3.50; Plate 3.51) and disaggregated kinetoplasts were present (Plate 3.50; Plate 3.52). An unusual elongation of the nucleus and apparent swelling of the nuclear membrane was also observed in one cell (Plate 3.53). In the presence of 1mM Berenil much of the cell structure was destroyed. Nuclei and kinetoplasts were difficult to discern although the pellicle and flagella were observed (Plate 3.54).

3.1.3.3 The Effects of polyamine biosynthesis inhibitors on growth of *T. granulosum*

As the concentrations of DFMO, MGBG or Berenil in the medium were increased, numbers of trypanosomes decreased 4 days post inoculation (Figure 3.6, 3.7, 3.8), compared to the number of trypanosomes cultured in drug-free medium. In medium containing high (50mM) concentrations of DFMO, more than 90% of the cells had perished 4 days post inoculation (Figure 3.6). The concentration of DFMO that caused 50% mortality (IC_{50}) after 4 days culture was 16 ± 2mM. In media containing 0.5mM MGBG approximately 70% of cells had perished (Figure 3.7), and the IC_{50} was 0.22 ± 0.02mM. Concentrations of MGBG of 1mM and 2mM did not significantly increase

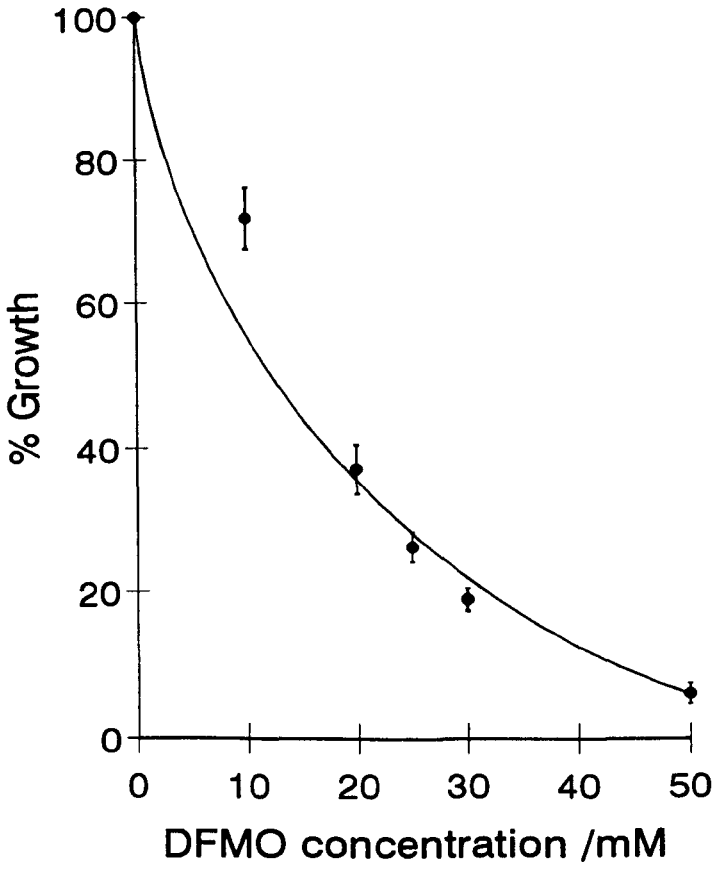


Figure 3.6 The effect of DFMO on the growth of *T. granulosum*. Cells were cultured for 4 days in modified SDM-79 containing a range of concentrations of DFMO. Error bars represent standard deviation where n=3.

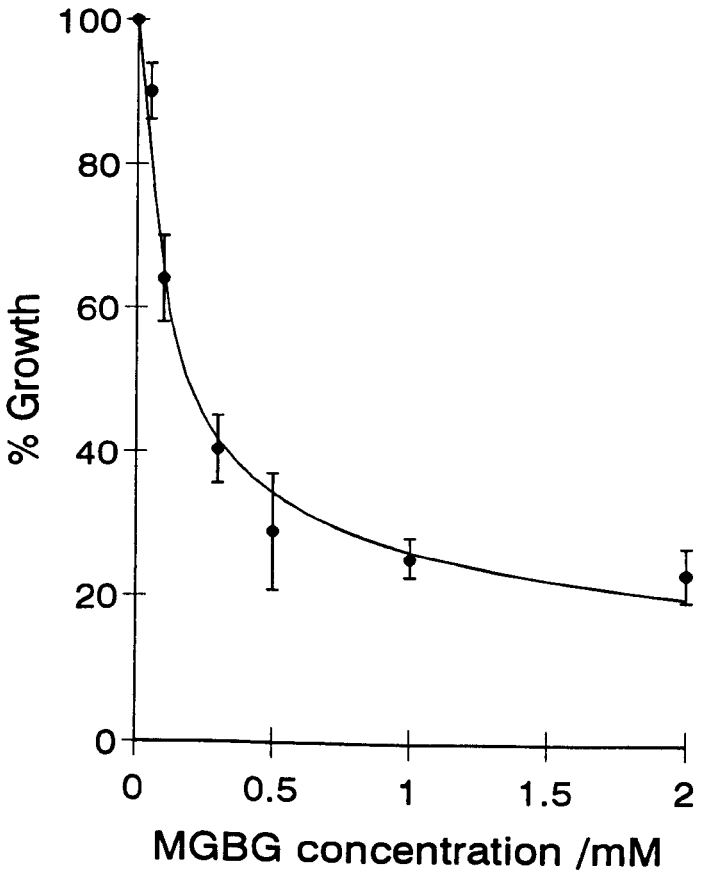


Figure 3.7 The effect of MGBG on the growth of *T. granulosum*. Cells were cultured for 4 days in modified SDM-79 containing a range of concentrations of MGBG. Error bars represent standard deviation where n=3.

cell mortality compared with that seen in cells cultured in medium containing 0.5mM MGBG. In medium containing 1mM Berenil more than 90% of the cells had perished (Figure 3.8) and the IC_{50} was 0.08±0.01mM.

When *T. granulosum* was cultured in modified SDM-79 containing either ornithine or sorbitol added at high concentrations (50mM) to simulate high concentrations of DFMO, there was no significant difference between numbers of cells after 4 days growth compared to cells cultured in modified SDM-79 without additional ornithine or sorbitol.

3.1.3.4 The addition of polyamines to drug treated cells

Addition of putrescine (100μM final concentration) after 2 days growth, to cells cultured in modified SDM-79 containing 16mM DFMO (Figure 3.9) and 50mM DFMO (results not shown) had no significant effect on the growth of trypanosomes by 4 days. However the addition of putrescine (100μM) to cells cultured in drug-free medium 2 days post inoculation, resulted in a significant increase (24%) in the numbers of trypanosomes by 4 days post-inoculation (Figure 3.9).

Addition of spermidine (100μM) or spermine (100μM) to cells at two days in media containing either MGBG (0.2mM) or Berenil (0.2mM) showed a significant increase in the number of cells present at 4 days post-inoculation compared to cells cultured in MGBG or Berenil treated medium without the addition of either spermidine or spermine (Figure 3.10). Addition of spermidine (100μM) to cells cultured in drug-free medium caused a significant increase in the numbers of parasites compared to those cells cultured in medium

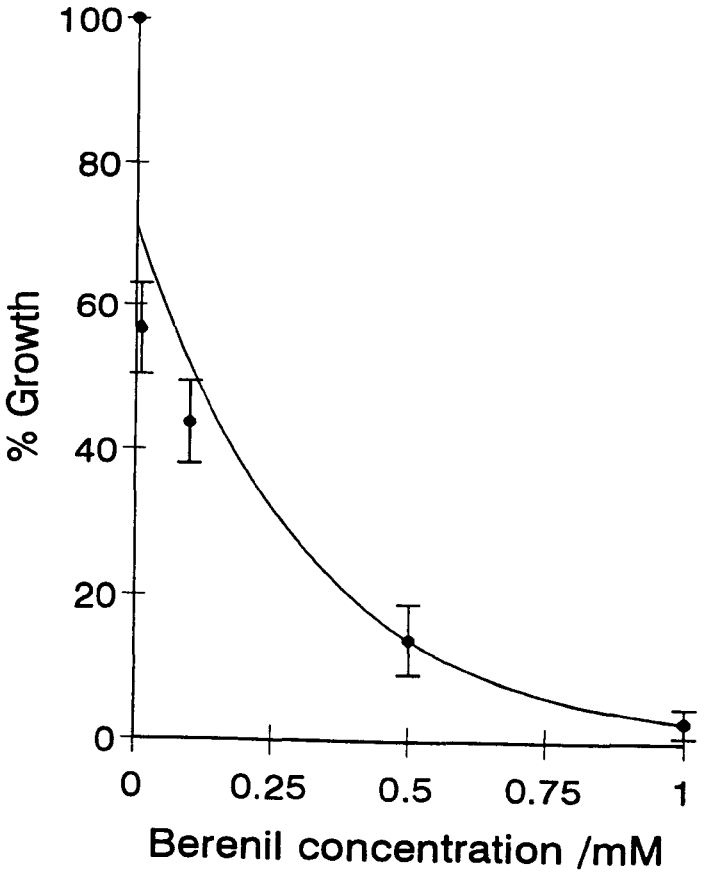


Figure 3.8 The effect of Berenil on the growth of *T. granulosum*. Cells were cultured for 4 days in modified SDM-79 containing a range of concentrations of Berenil. Error bars represent standard deviation where n=3.

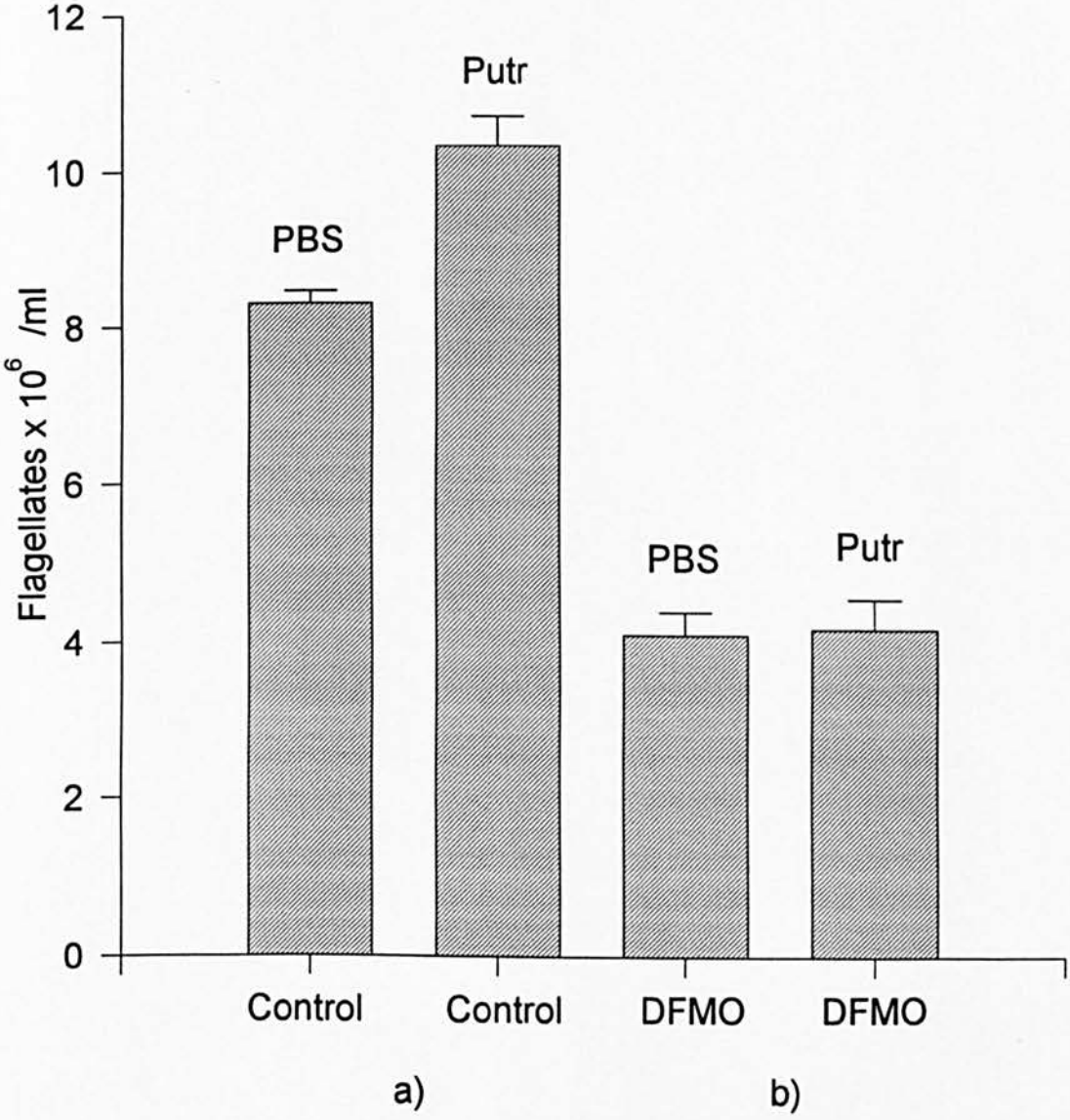


Figure 3.9 The effects of exogenous putrescine on DFMO treated cells. *T. granulosum* was cultured for 4 days in a) modified SDM-79 and b) modified SDM-79 containing 16mM DFMO. After 2 days growth, 100μM putrescine was added to cells in both cultures a) and b). Error bars represent standard deviation where n=3. PBS=Dulbecco's phosphate buffered saline, Putr=putrescine.

without additional spermidine. However, addition of spermine (100μM) to the culture did not cause a significant difference in the numbers of parasites compared to cells cultured in medium with no added spermine (Figure 3.10).

Figure 3.10 Effects of exogenous polyamines on MGBG and Berenil treated cells. *T. granulosum* was cultured for 4 days in a) modified SDM-79, b) modified SDM-79 containing 0.2mM MGBG and c) modified SDM-79 containing 0.2mM Berenil. After two days growth, 100μM spermidine and 100μM spermine were added separately to cultures a), b) and c). Error bars represent standard deviation where n=3. PBS=Dulbecco's phosphate buffered saline.

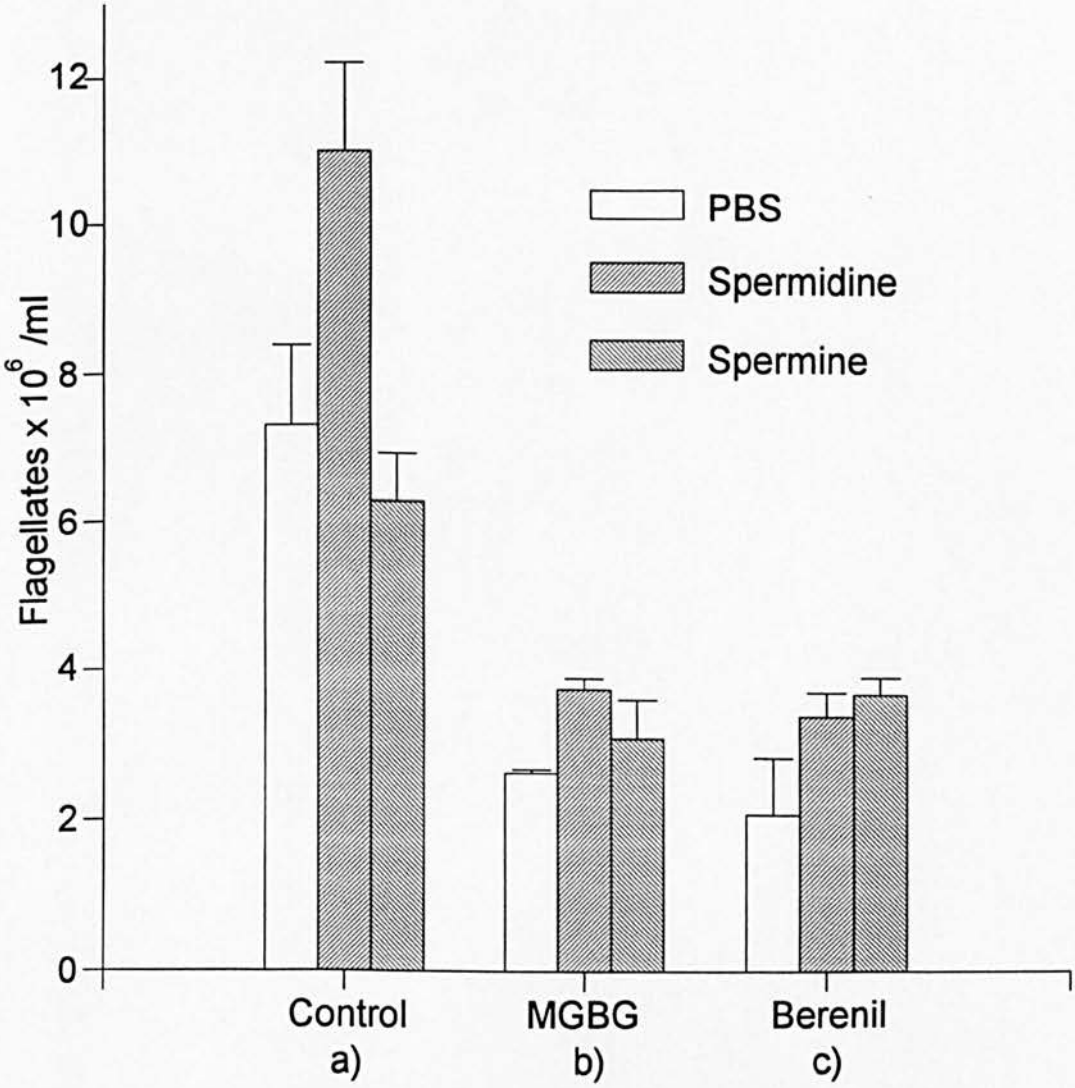
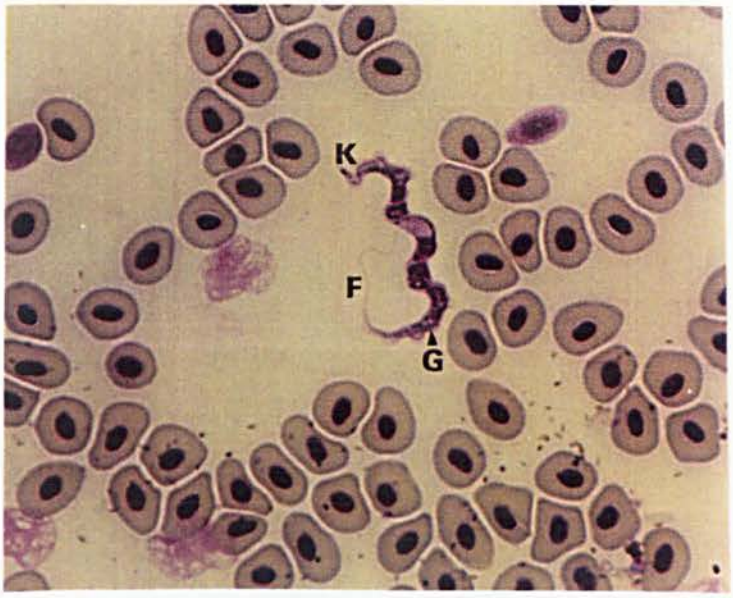
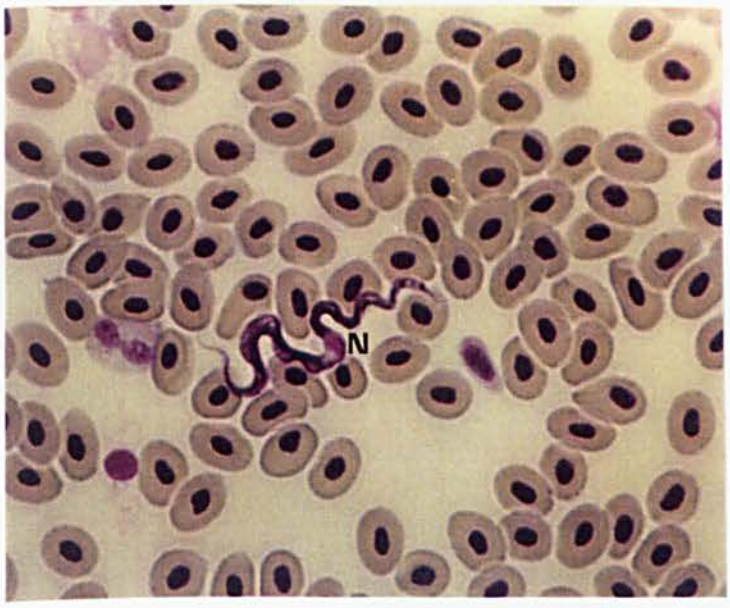

Flagellates x 10^6 /ml
0
2
4
6
8
10
12
PBS
Spermidine
Spermine
Control
a)
MGBG
b)
Berenil
c)

Plate 3.1 *T. granulosum* from the blood of an eel. Note the presence of free flagella (F), granules (G), kinetoplast (K) and nuclei (N). May-Grünwald Giemsa (MGG) (x 818).


K
F
G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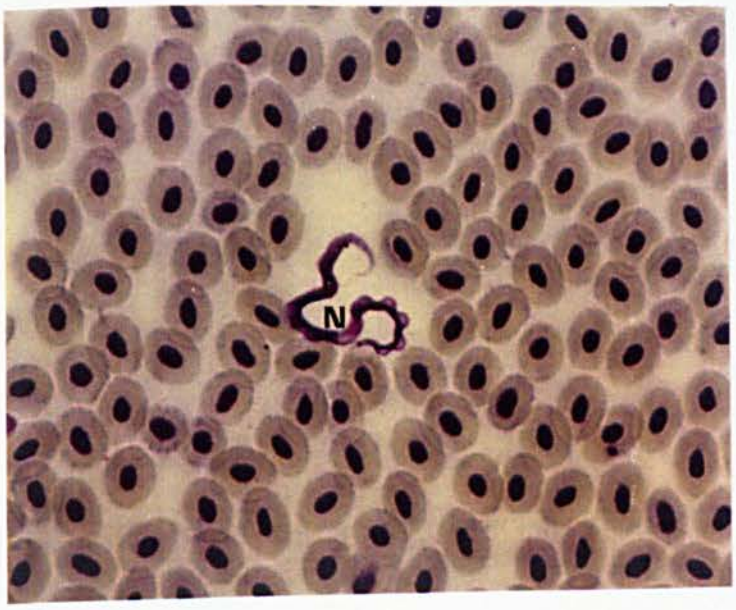
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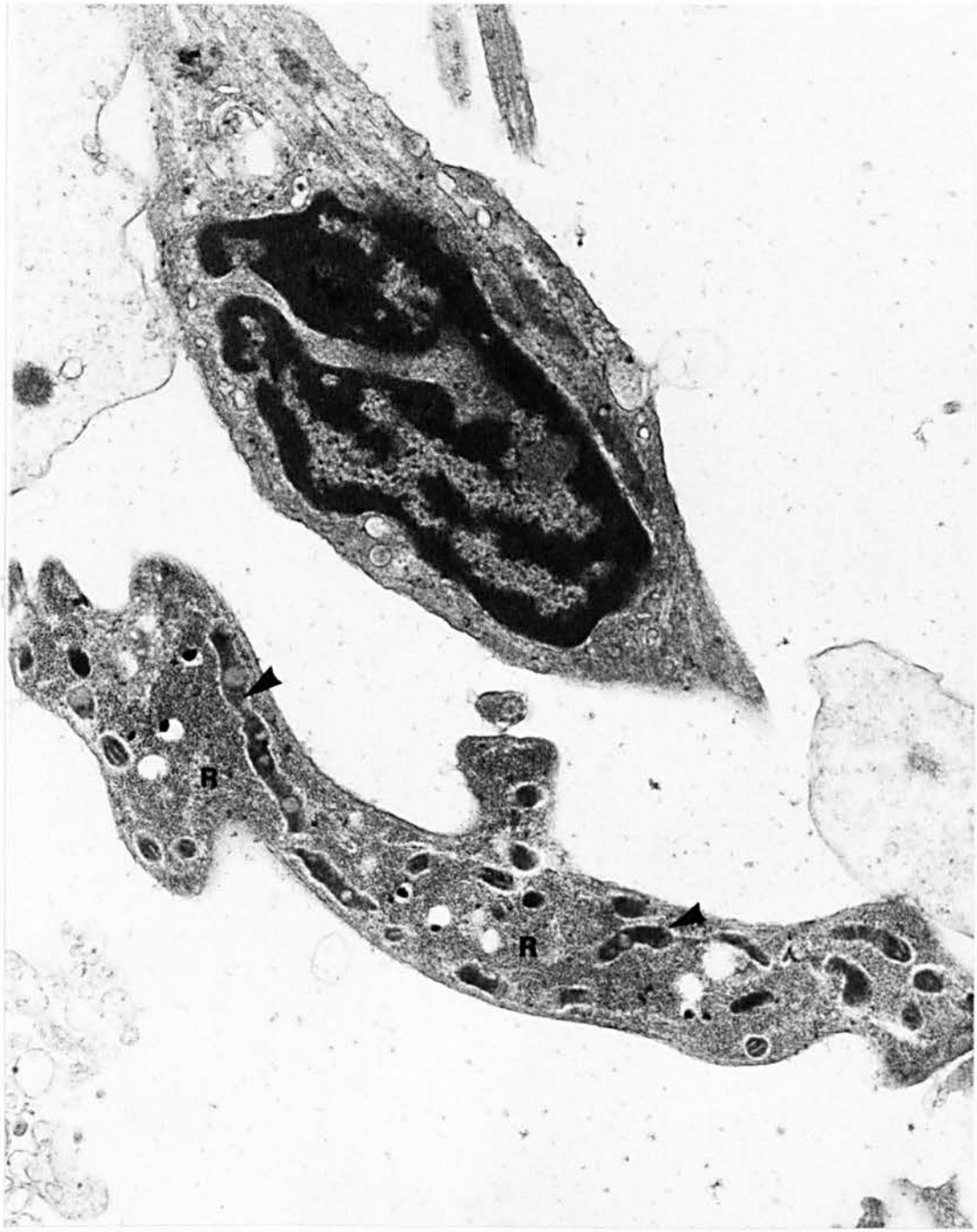


Plate 3.2 Electron micrograph of bloodstream *T. granulosum*. Note the presence of cytoplasmic ribosomes (R). Arrows indicate mitochondrion. Uranyl acetate and lead citrate (UA&LC) (x 25000).

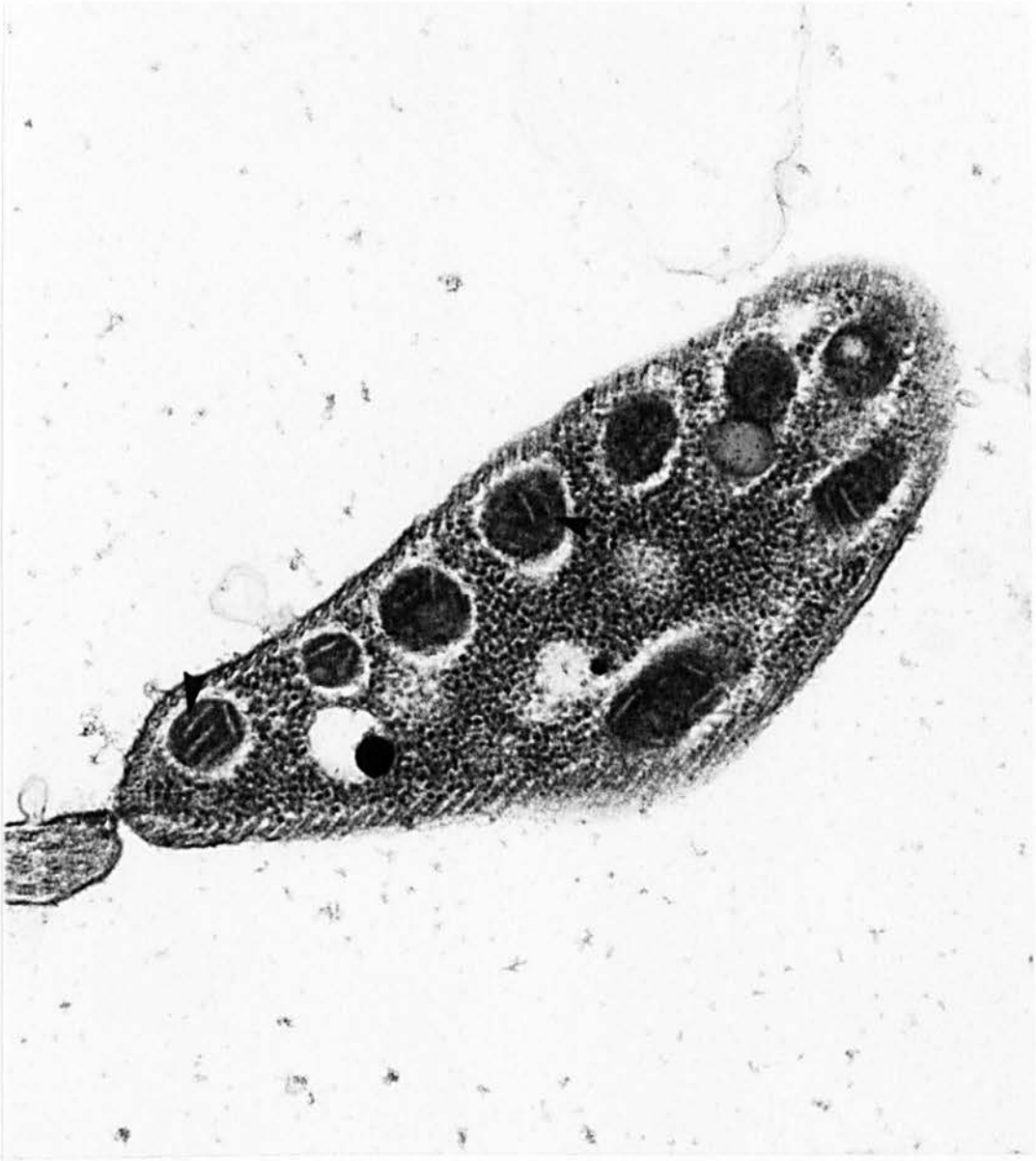


Plate 3.3 Electron micrograph of bloodstream *T. granulosum*. Note the presence of plate-like cristae (arrows). UA&LC (x 58000).

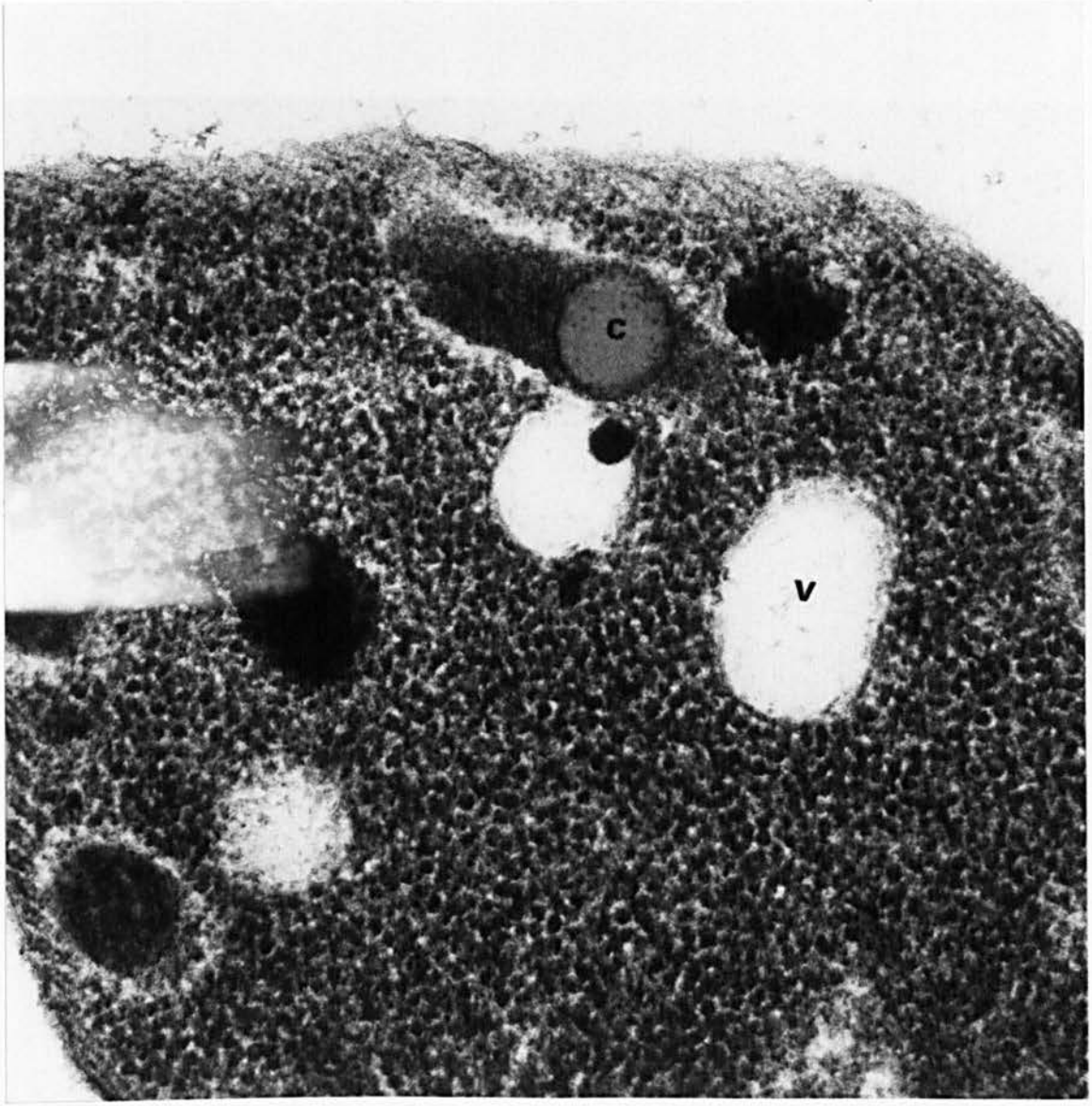


Plate 3.4 Electron micrograph of bloodstream *T. granulosum*. Note the presence of a plate-like crista (C) and empty vacuoles (V). UA&LC (x 171000).

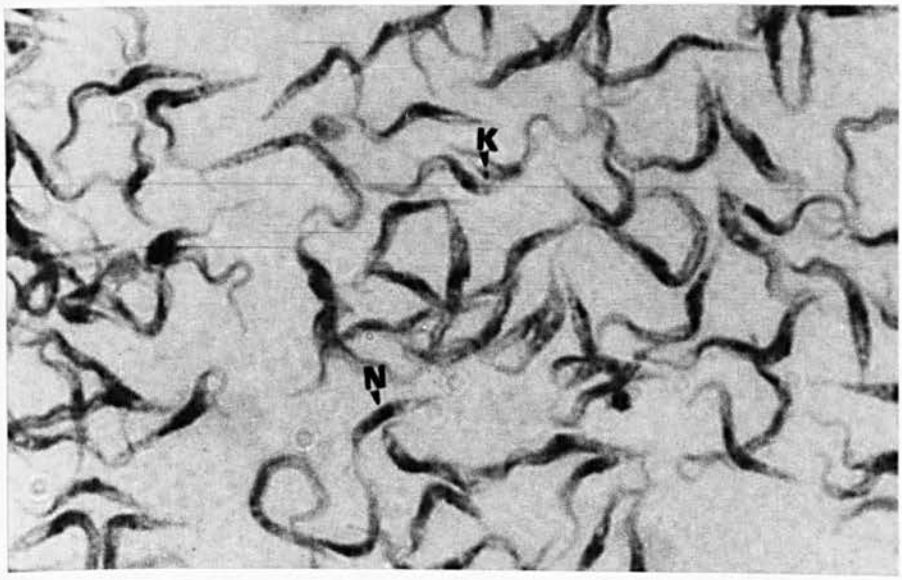


Plate 3.5 *T. granulosum* cultured in modified SDM-79 for 4 days. Note the kinetoplast (K) and nuclei (N). MGG (x 880).

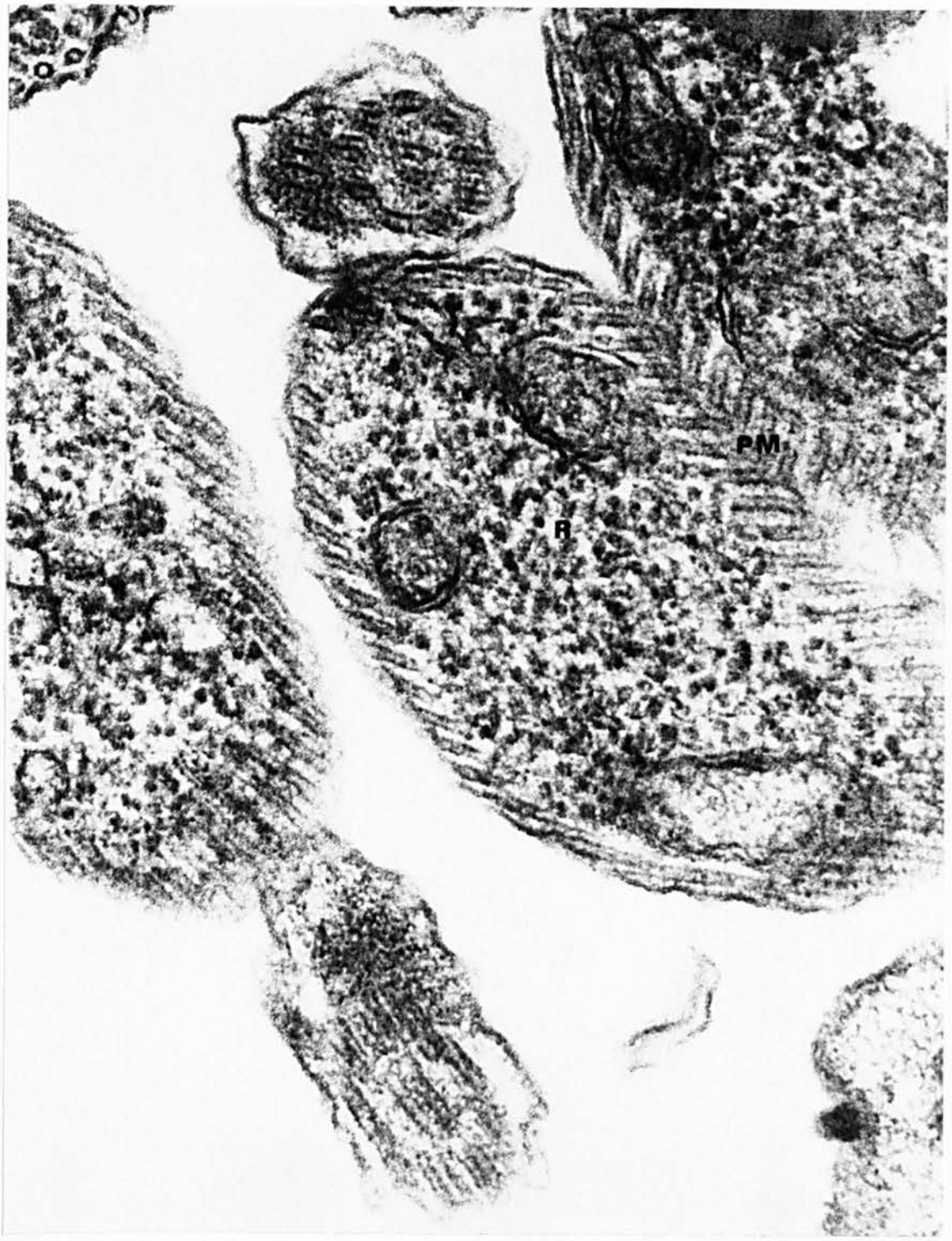


Plate 3.6 Electron micrograph of cultured *T. granulosum*. Note the presence of pellicular microtubules (PM) and ribosomes (R). UA&LC (x 114000).

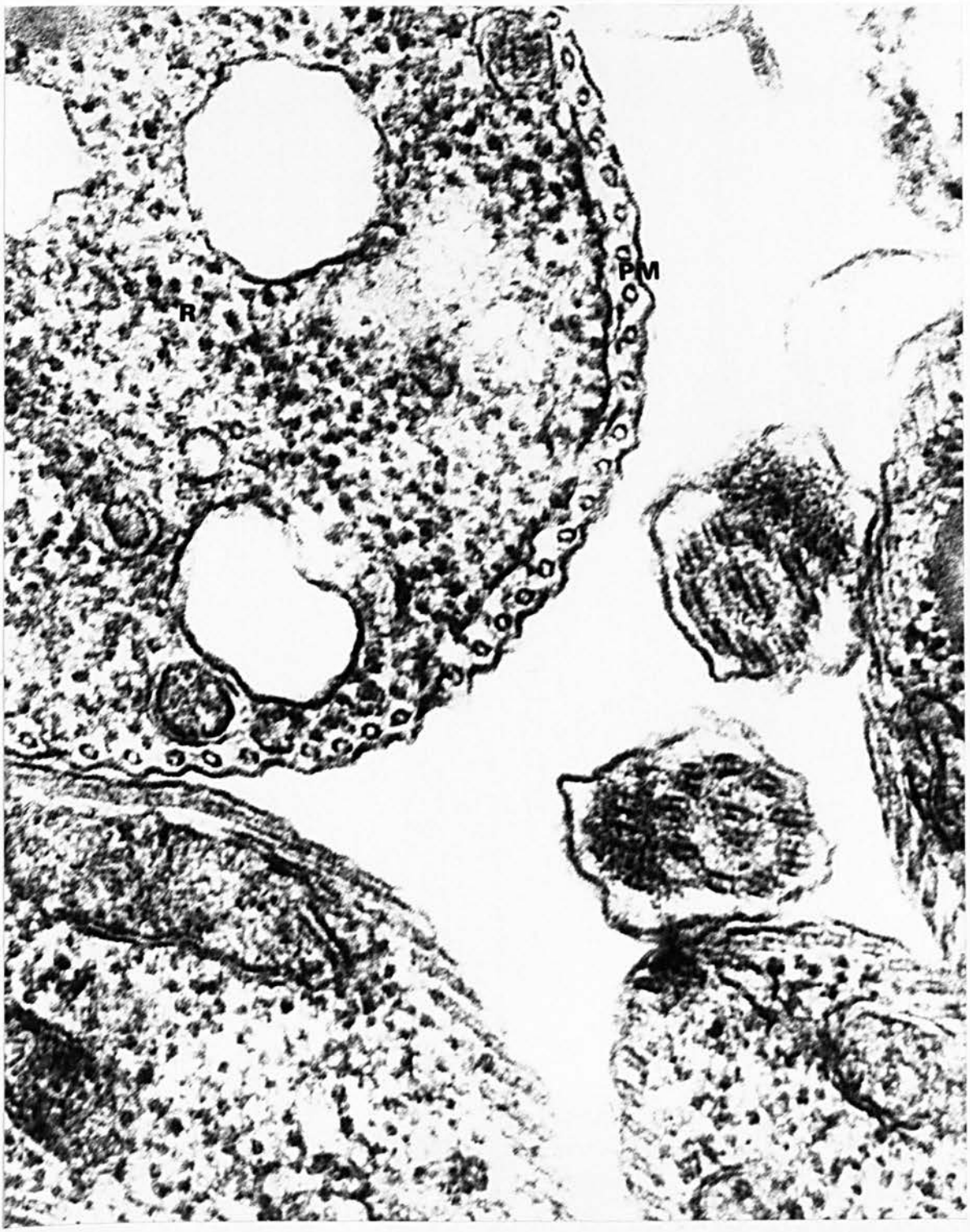


Plate 3.7 Electron micrograph of cultured *T. granulosum*. Note the presence of pellicular microtubules (PM) and ribosomes (R). UA&LC (x 125000).

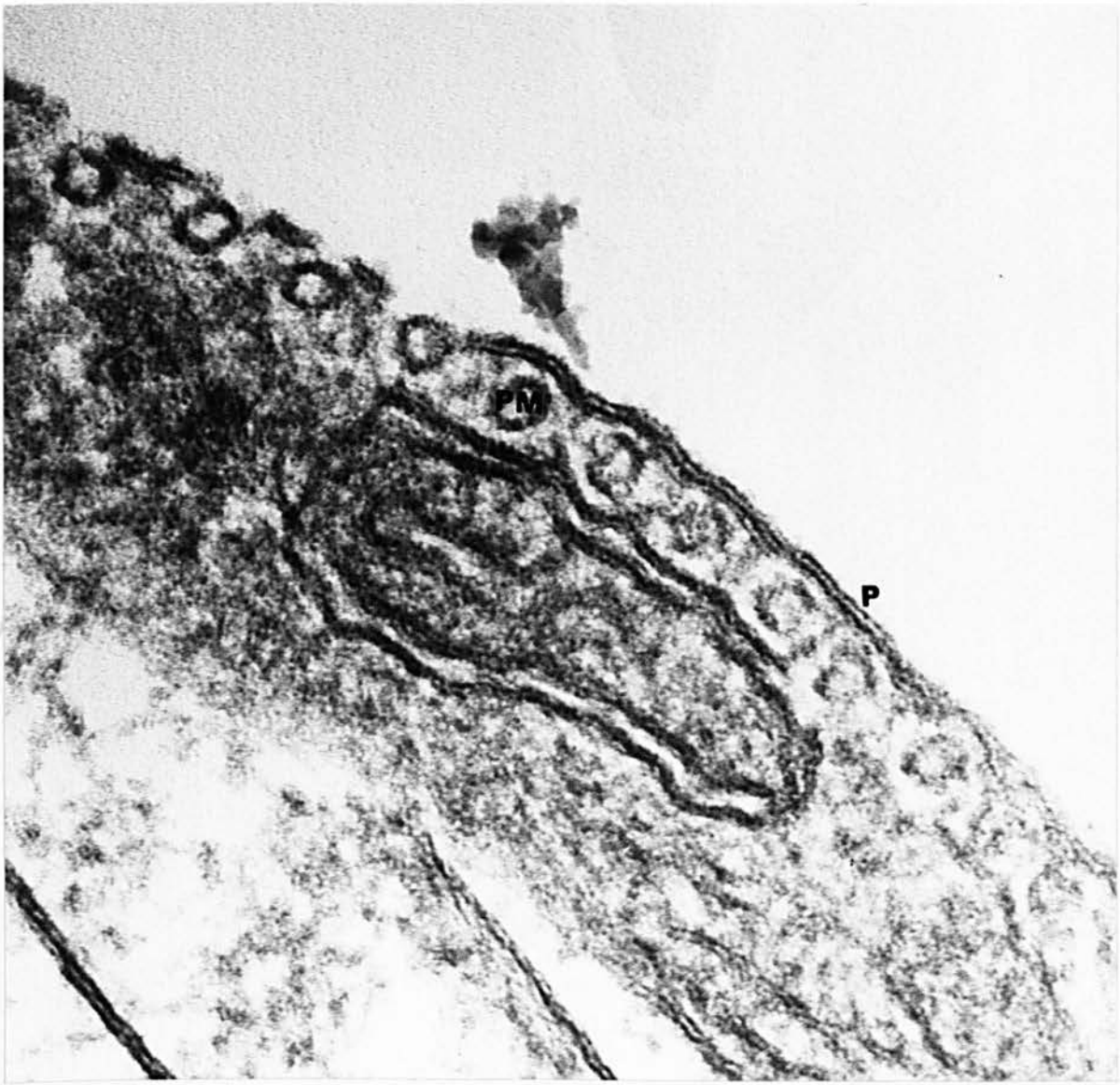
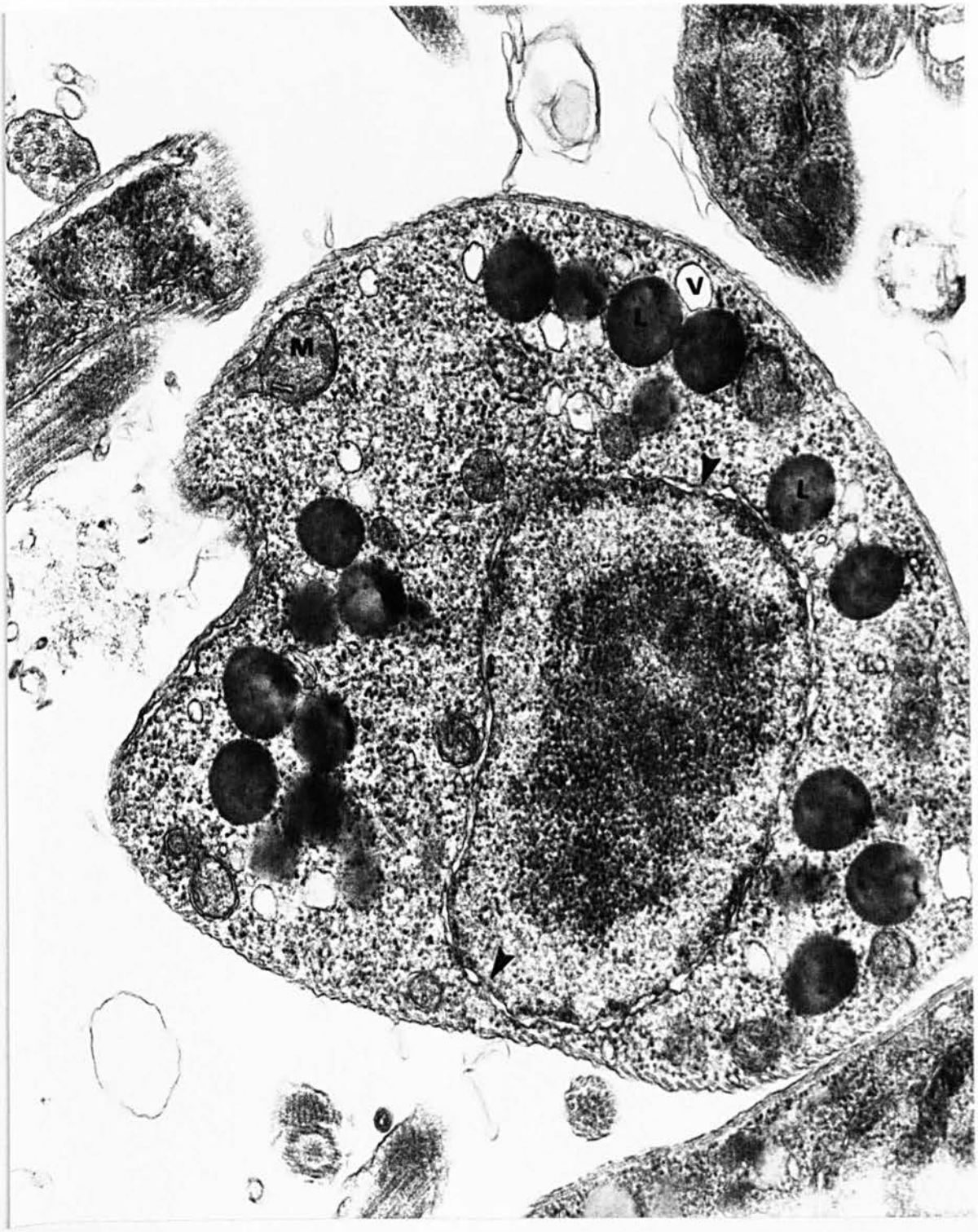


Plate 3.8 Electron micrograph of cultured *T. granulosum*. Note the presence of plasmalemma (P) and pellicular microtubules (PM). UA&LC (x 338000).

Plate 3.9 Electron micrograph of cultured *T. granulosum*. Note the presence of lipid bodies (L), mitochondrion (M) and empty vacuoles (V). Arrows indicate nuclear pores. UA&LC (x 35000).


M
V
L
L

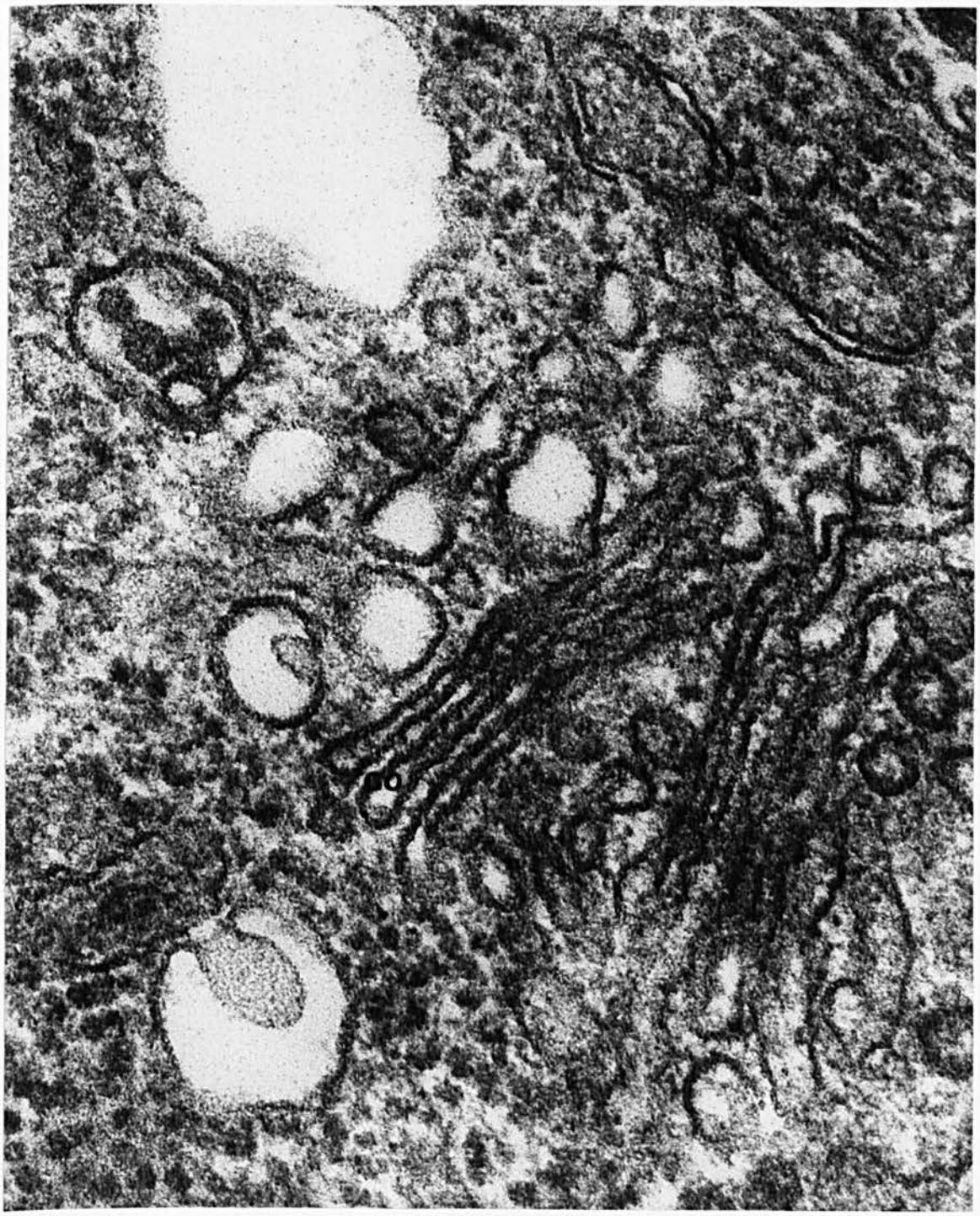


Plate 3.10 Electron micrograph of cultured *T. granulosum*. Note the presence of golgi apparatus (GO). UA&LC (x 189000).

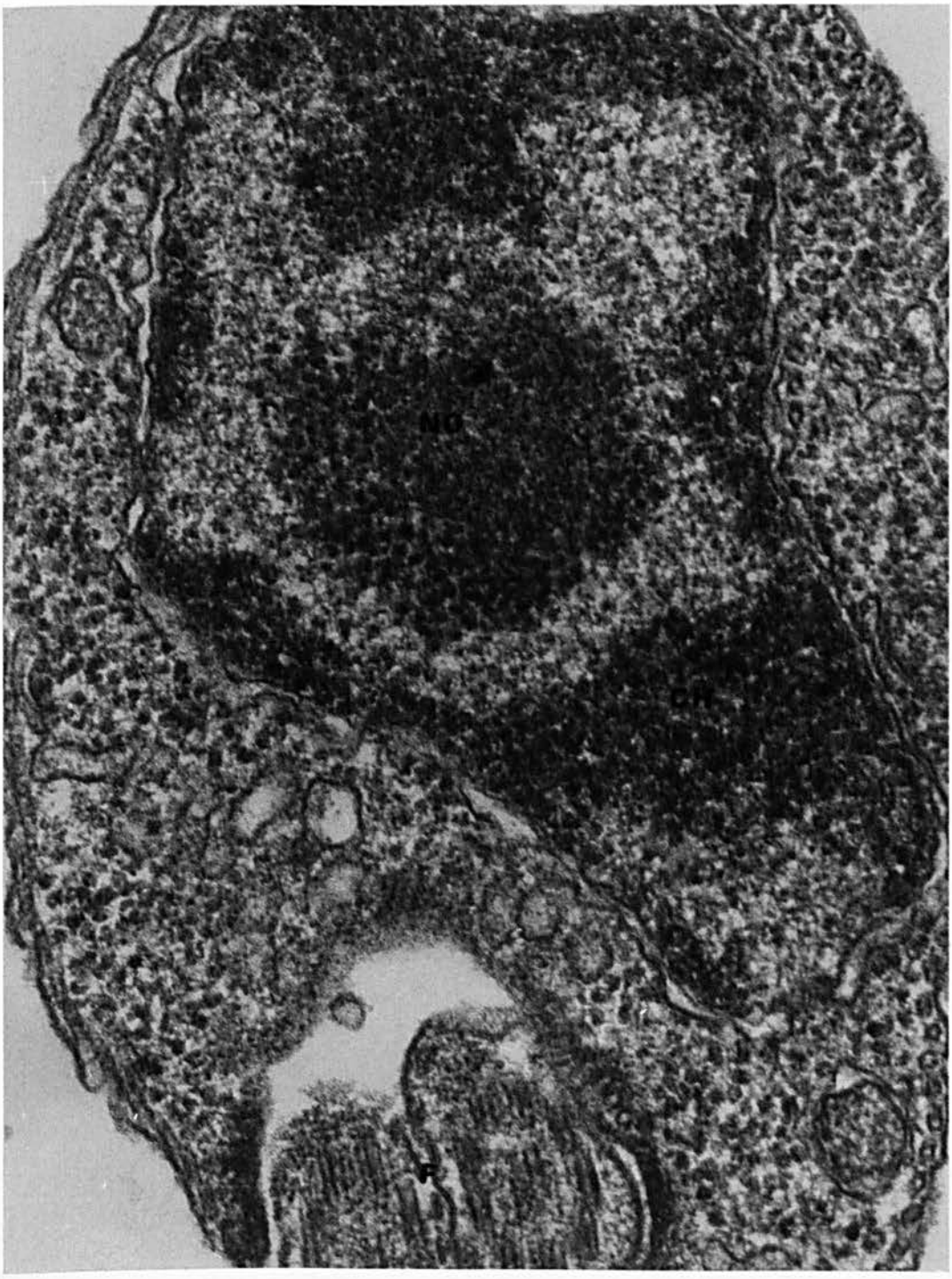


Plate 3.11 Electron micrograph of cultured *T. granulosum*. Note the peripheral chromatin (CH), dividing flagella (F) and nucleolus (NO). UA&LC (x 120000).

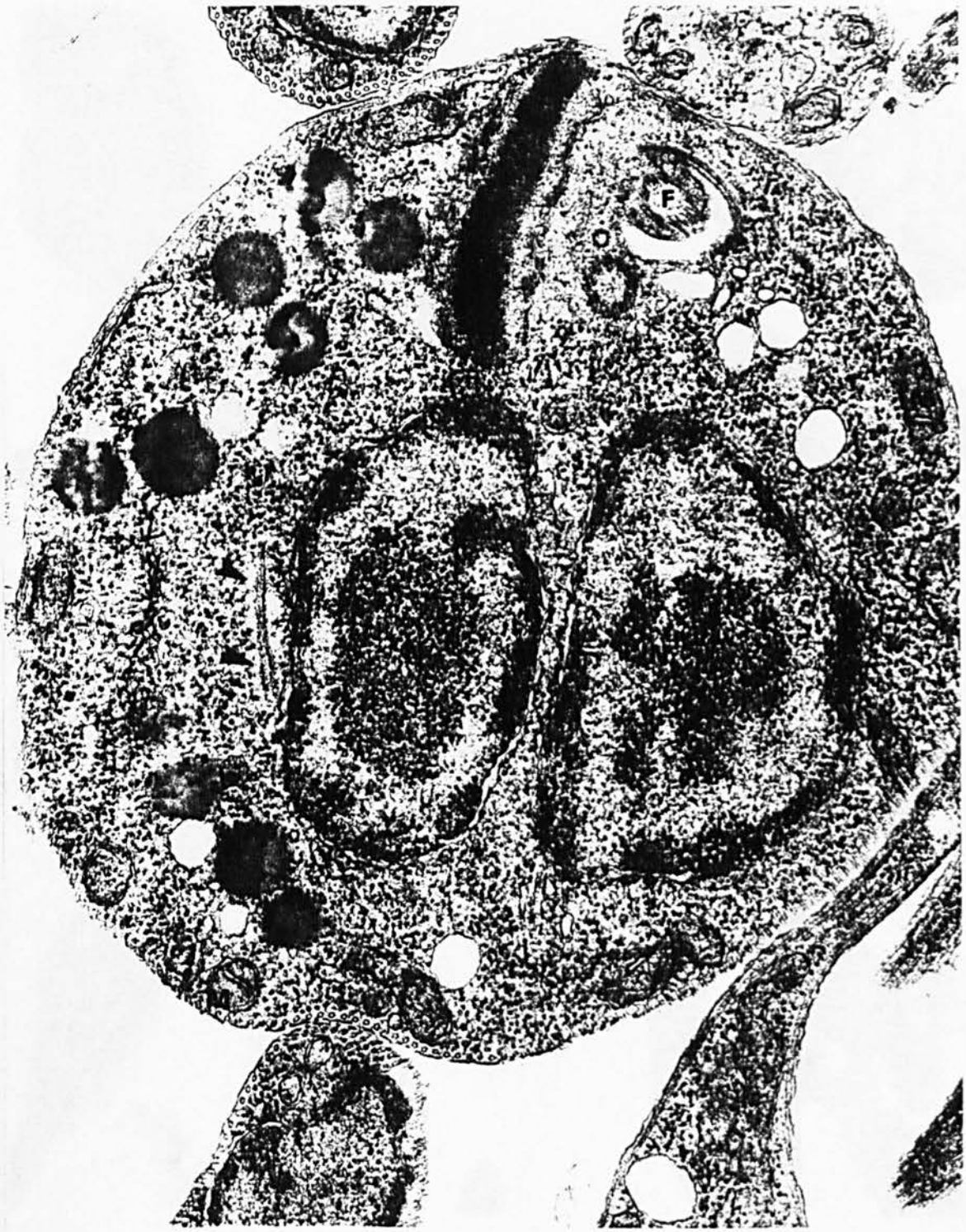


Plate 3.12 Electron micrograph of cultured *T. granulosum*. Note the flagellum (F), kinetoplast (K), mitochondrion (M), divided nuclei (N) and peripheral chromatin (CH). Arrows indicate fibrous structure of unknown origin. UA&LC (x 48000).

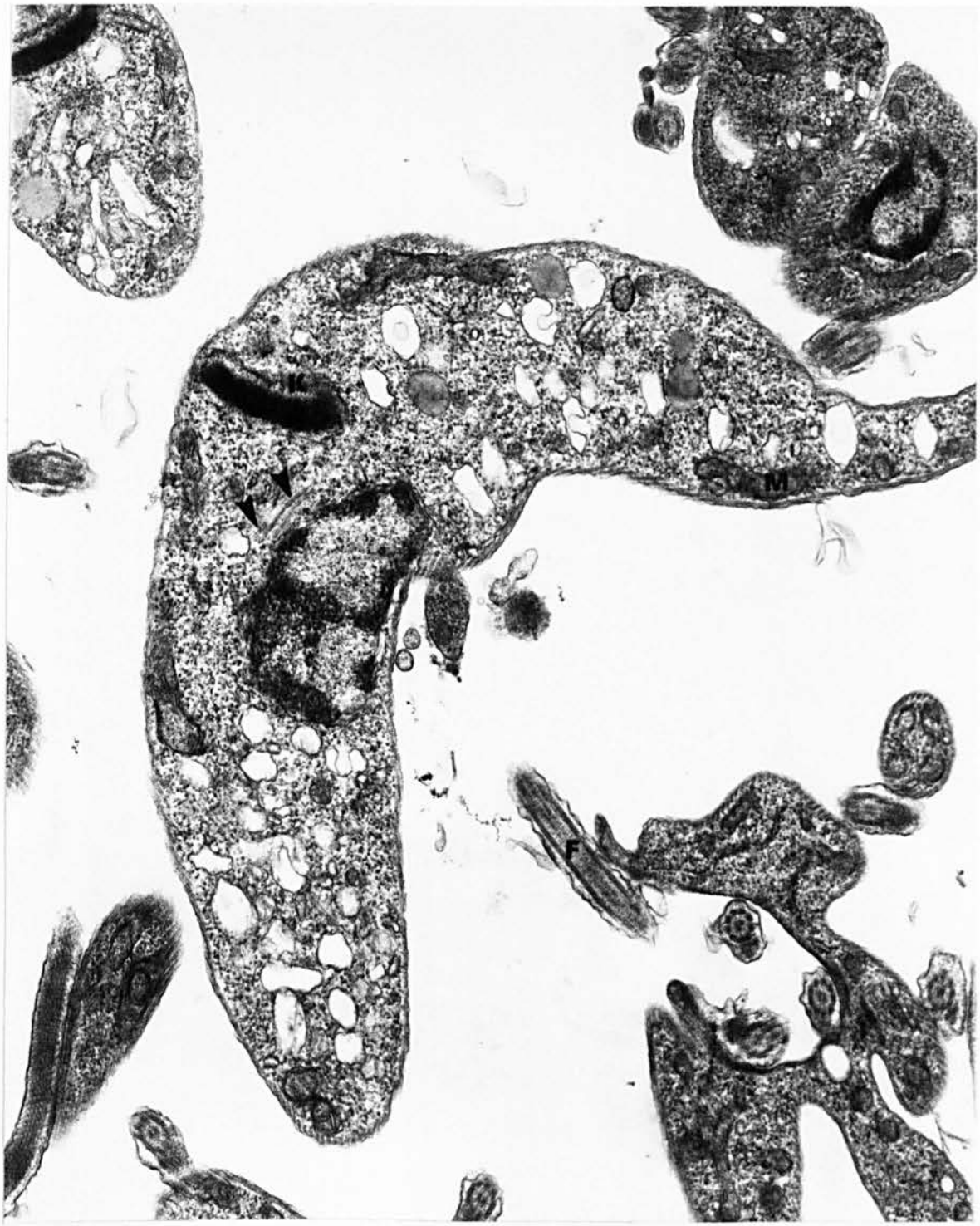


Plate 3.13 Electron micrograph of cultured *T. granulosum*. Note the longitudinal section through the flagellum (F), kinetoplast (K) and mitochondrion (M). Arrows indicate fibrous structure of unknown origin. UA&LC (x 25000).

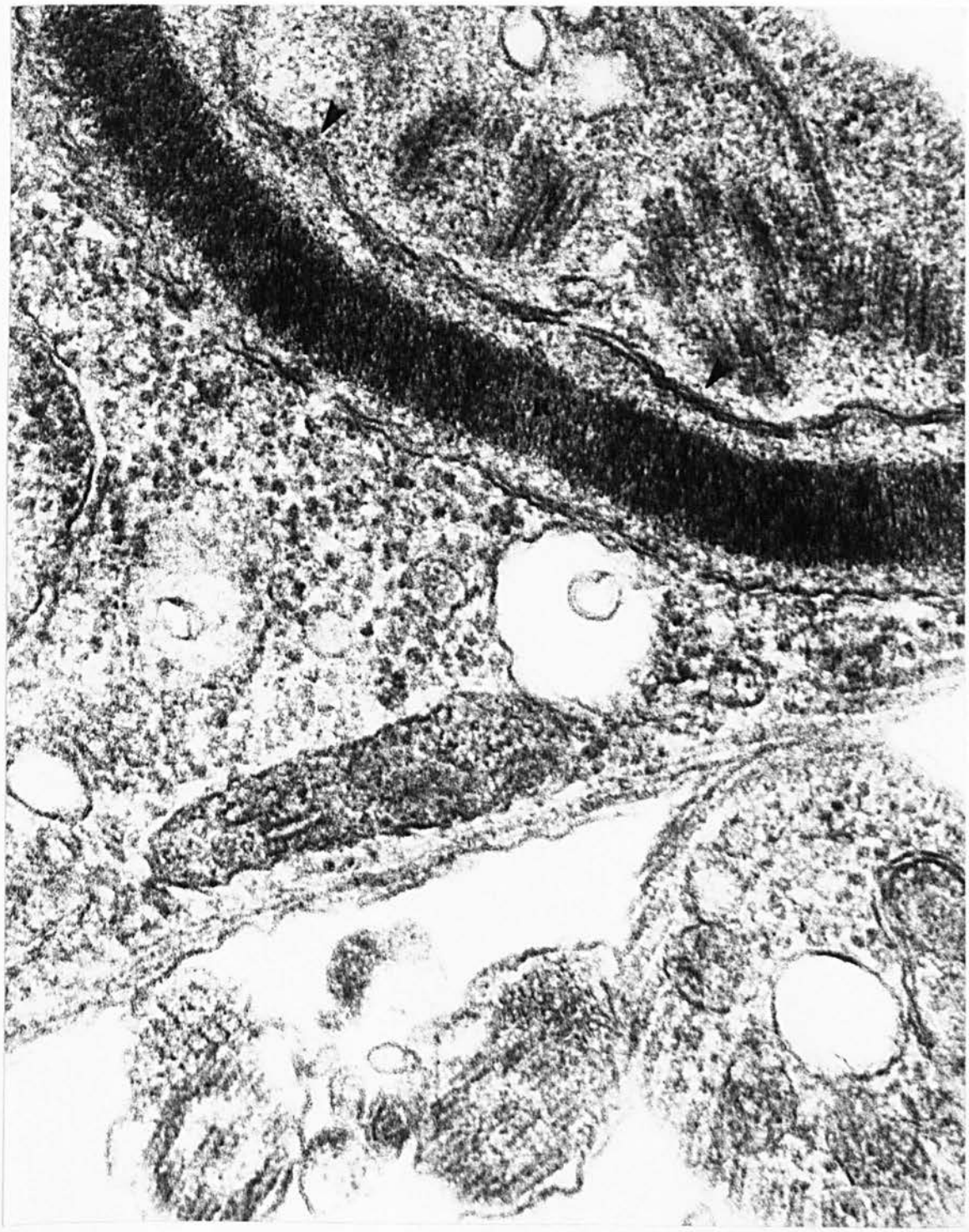


Plate 3.14 Electron micrograph of cultured *T. granulosum*. Note the basal bodies (B) and the dividing kinetoplast (K) housed within the mitochondrial membranes (arrows). UA&LC (x 130000).

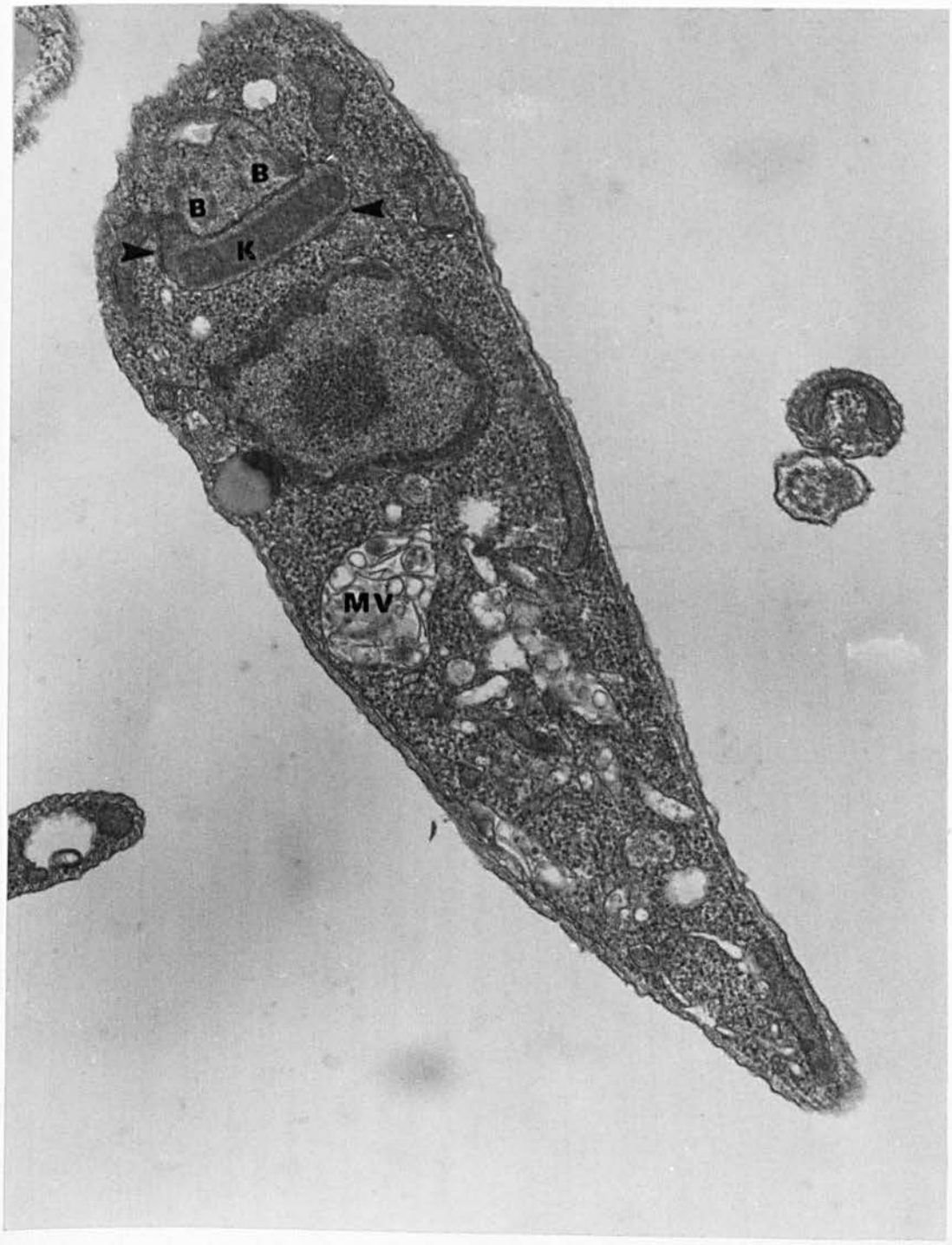


Plate 3.15 Electron micrograph of cultured *T. granulosum*. Note the basal bodies (B), kinetoplast (K) housed within the mitochondrial membranes (arrows), the mitochondrion (M) and a multivesicular body (MV). UA&LC (x 32000).

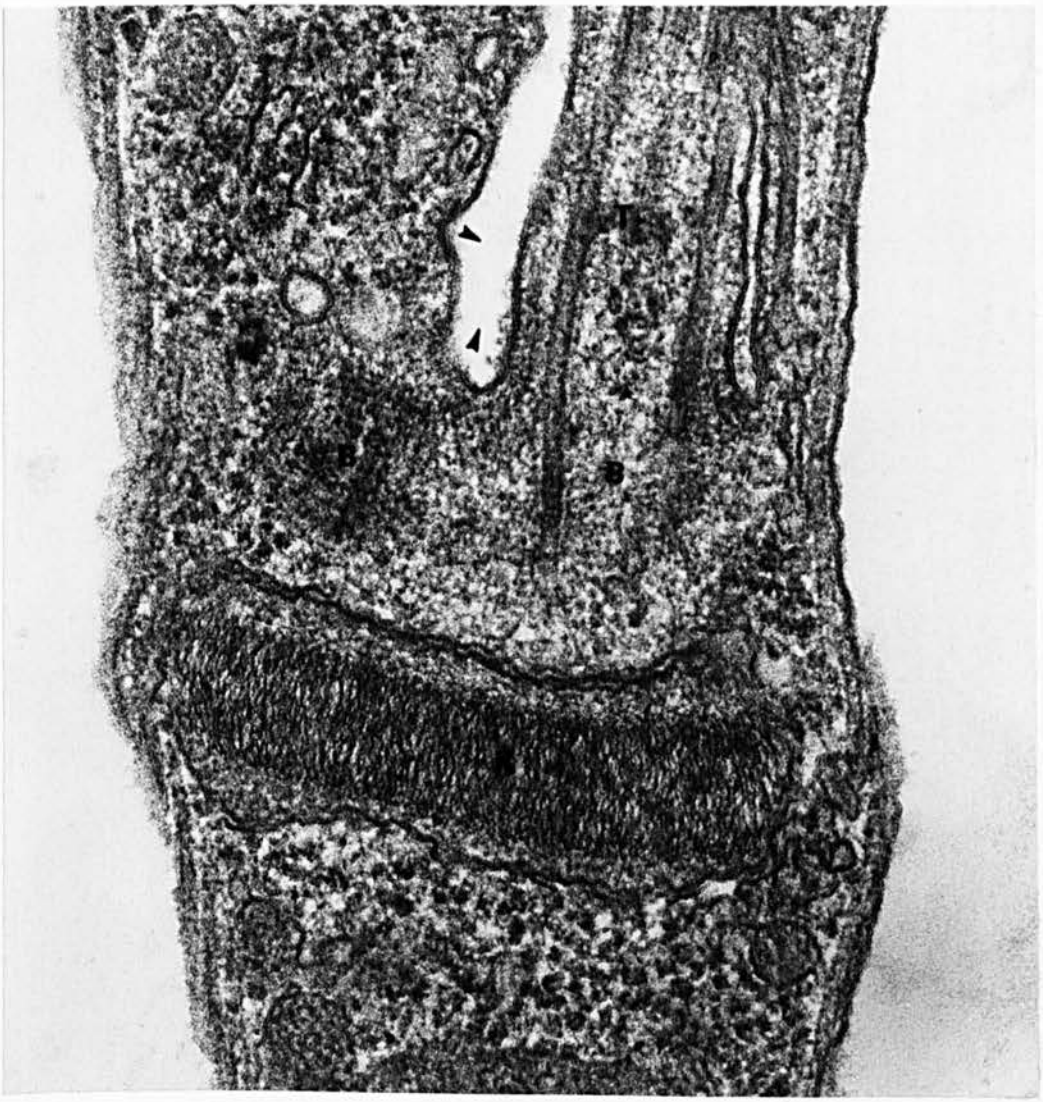


Plate 3.16 Electron micrograph of cultured *T. granulosum*. Note the basal bodies (B), kinetoplast (K) and the terminal plate of the flagellum (T). Arrows indicate flagellar pocket. UA&LC (x 40000).

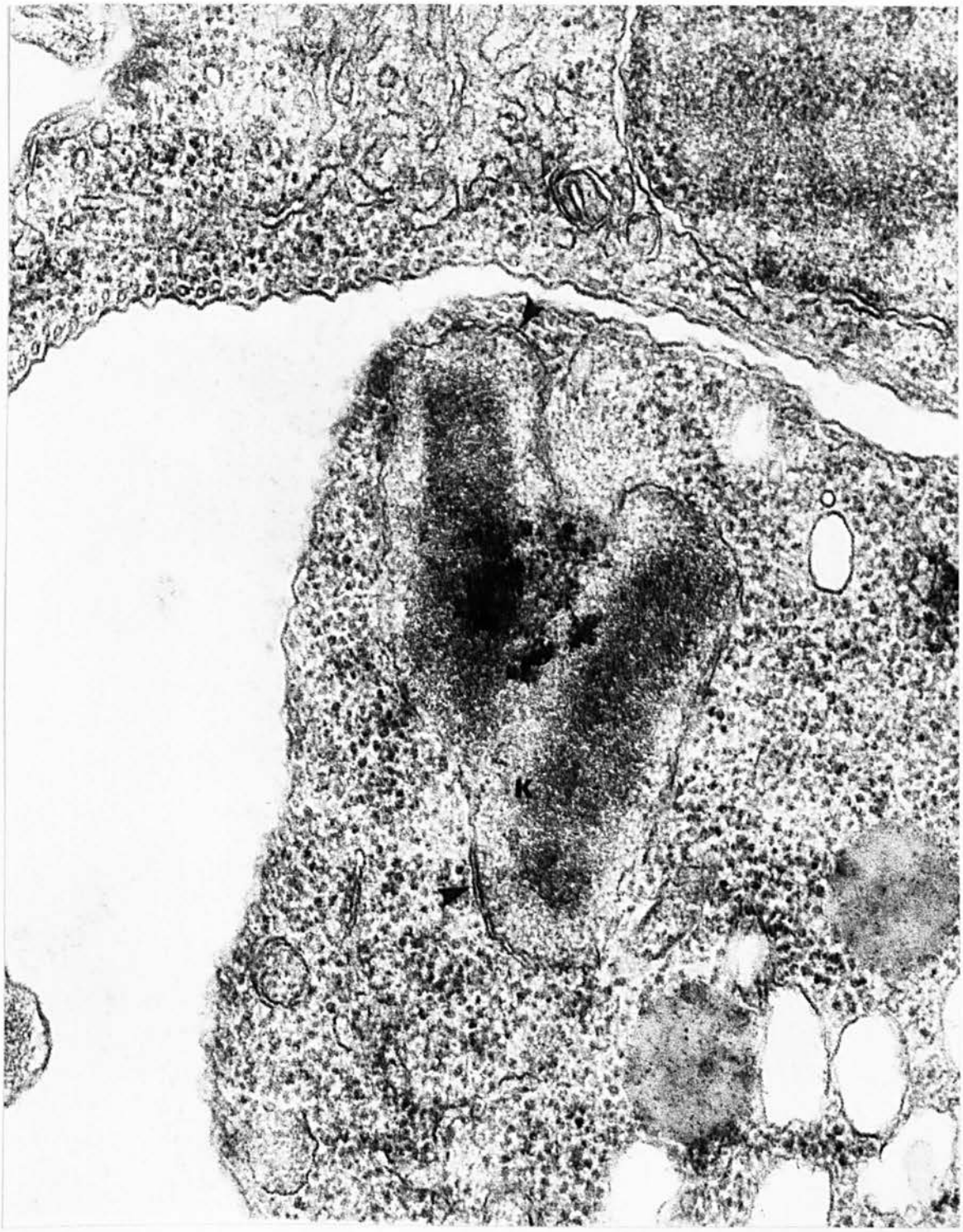


Plate 3.17 Electron micrograph of cultured *T. granulosum*. Note the dividing kinetoplast (K) and the mitochondrial membranes (arrows). UA&LC (x 68000).

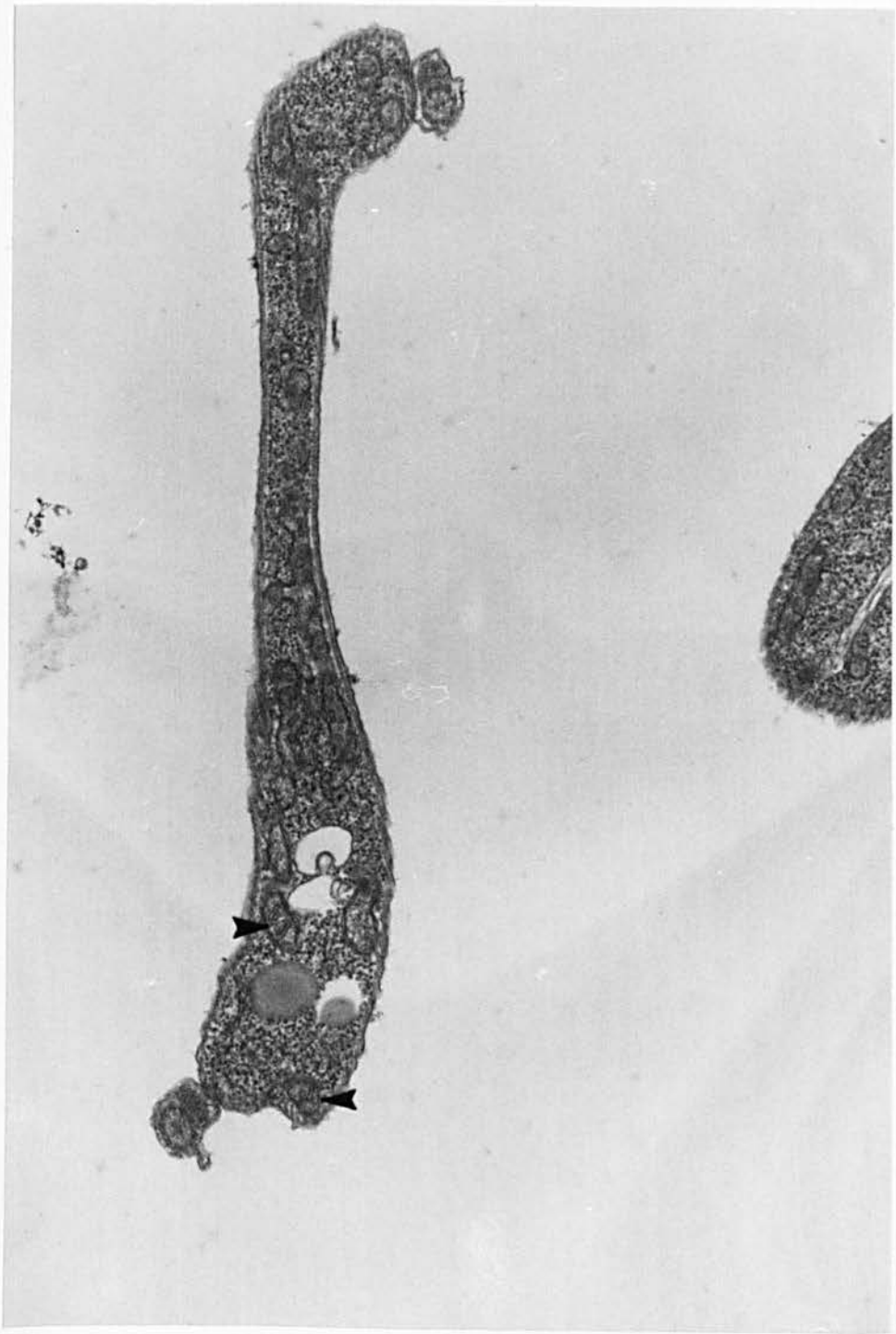


Plate 3.18 Electron micrograph of cultured *T. granulosum*. Note the mitochondrion (arrows) thought to be branching throughout the cell. UA&LC (x 24000).

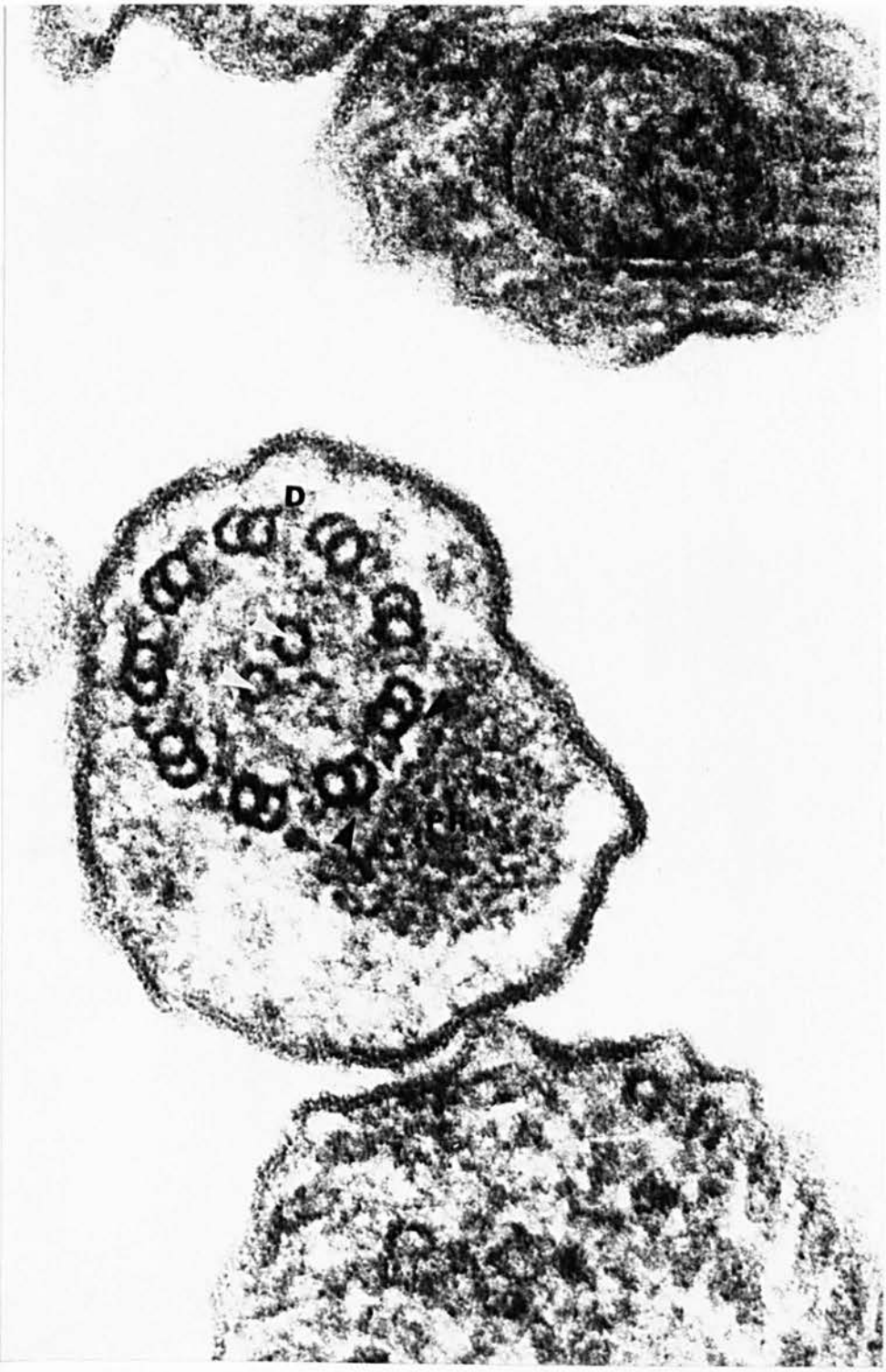


Plate 3.19 Electron micrograph of cultured *T. granulosum*. Note the "9+2" microtubule array, the dynein arms of subtubule A (D), the central microtubules (white arrows) and the paraxial rod (PR) with connections (black arrows) to the microtubule array. UA&LC (x 225000).

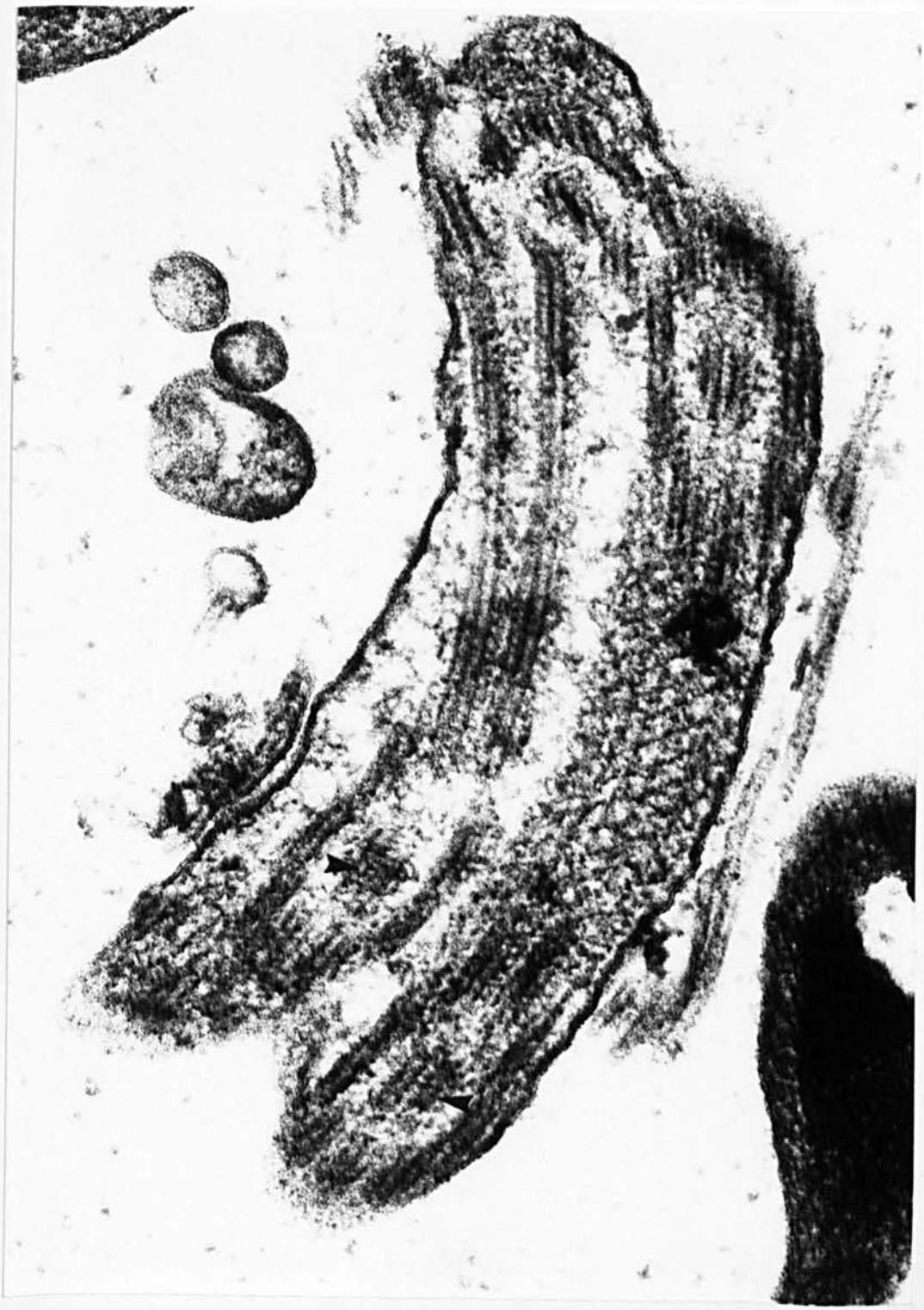


Plate 3.20 Electron micrograph of cultured *T. granulosum* showing a longitudinal cross section through two flagella. Arrows indicate central microtubules. UA&LC (x 166000).

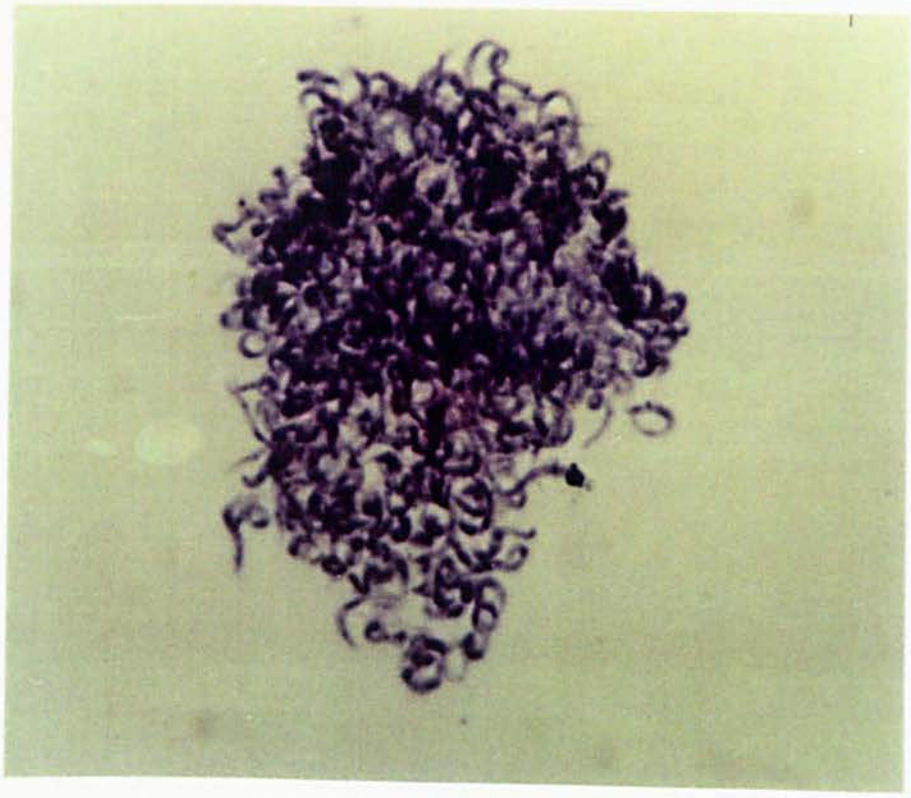
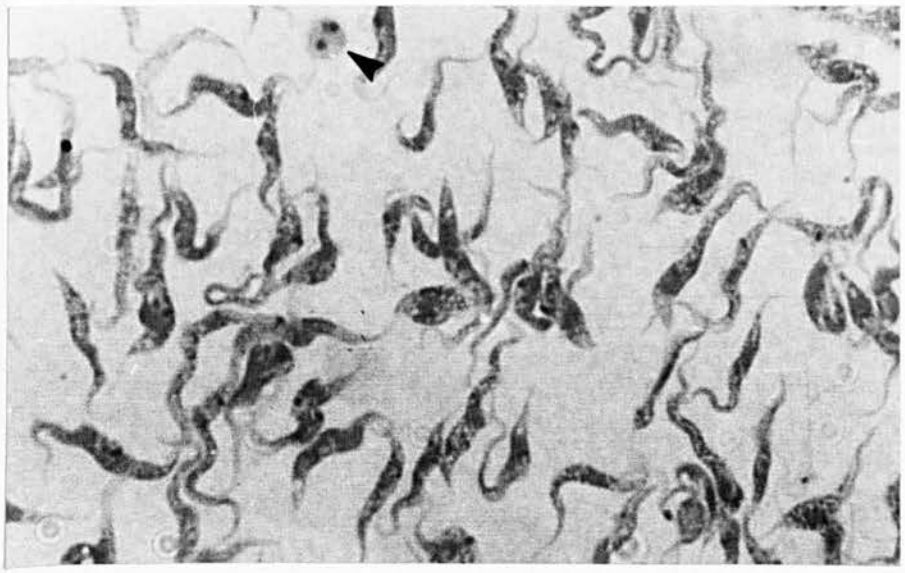
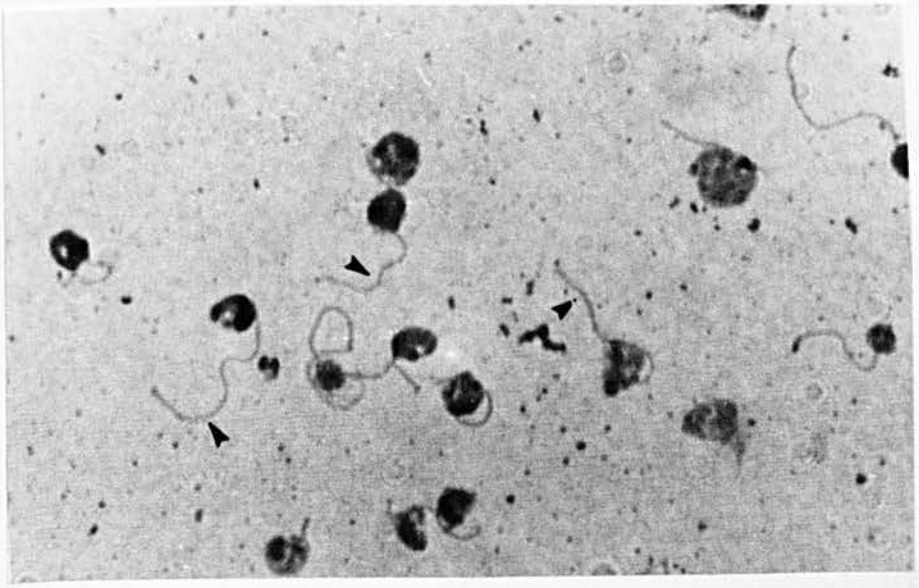


Plate 3.21 *T. granulosum* cultured on blood agar slopes after four days growth. Note the clumping, making quantification difficult. MGG (x 435).

Plate 3.22 *T. granulosum* cultured for 4 days in modified SDM-79 containing 10mM DFMO. Note the shortening and broadening of the parasites compared to cells cultured in drug-free medium (see plate 3.5) and an occasional bizarre shaped trypanosome (arrow). MGG (x 880).

Plate 3.23 *T. granulosum* cultured for 4 days in modified SDM-79 containing 50mM DFMO. Note the altered morphology compared to cells cultured in drug-free medium (see plate 3.5) and to cells cultured in the presence of 10mM DFMO (plate 3.22). Arrows indicate free flagella. MGG (x 880).





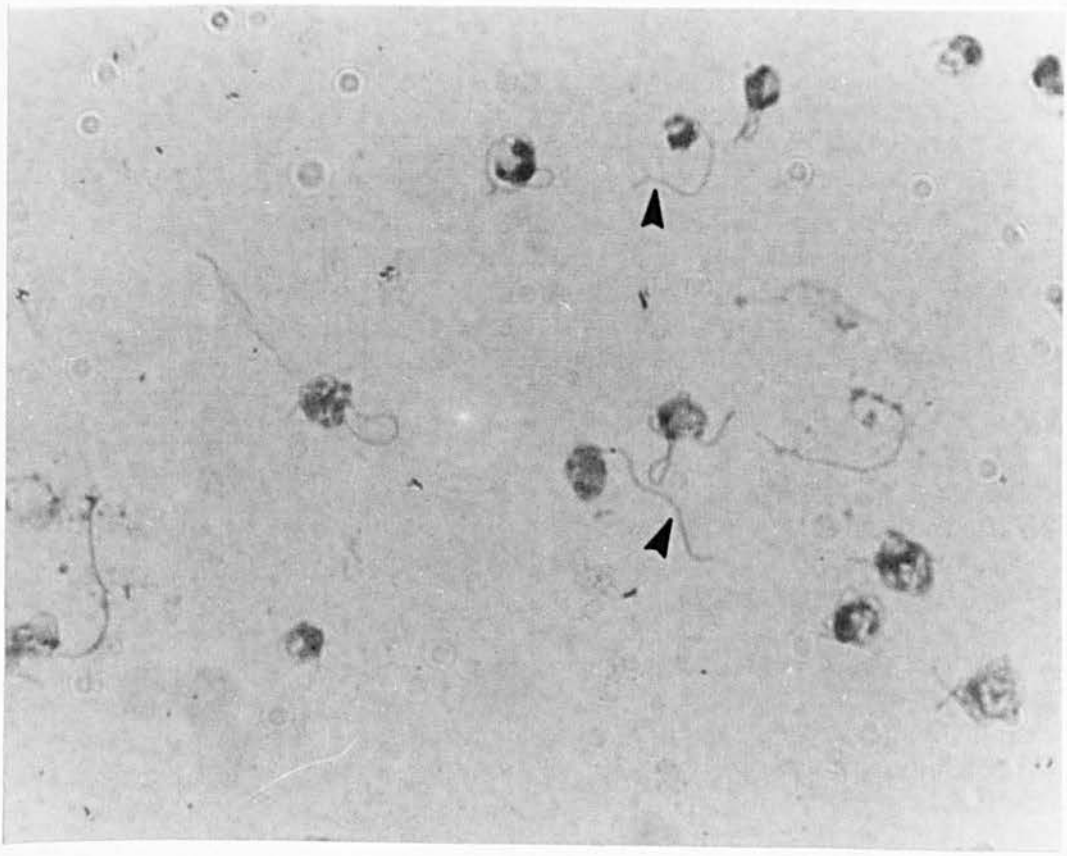


Plate 3.24 *T. granulosum* cultured for 4 days in modified SDM-79 containing 1mM MGBG. Note that cells are similar in morphology to cells cultured 50mM DFMO (see plate 3.23). Arrows indicate free flagella. MGG (x 880).

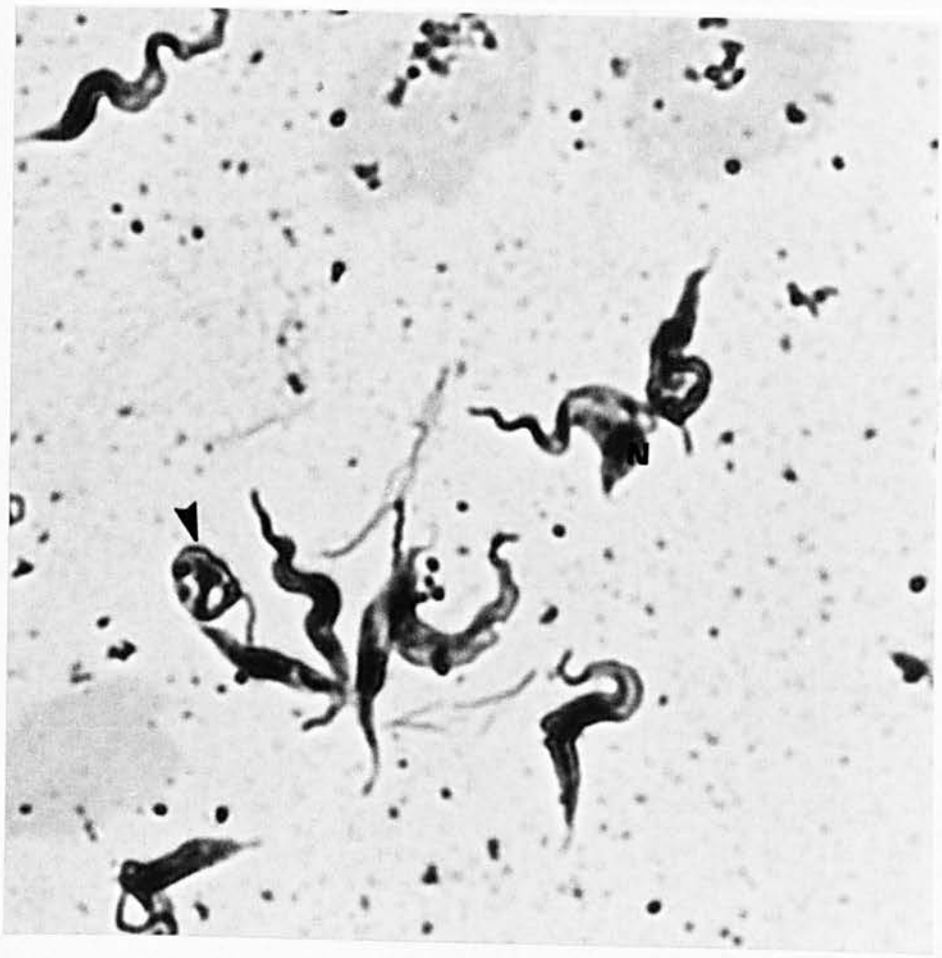


Plate 3.25 *T. granulosum* cultured for 4 days in modified SDM-79 containing 0.05mM Berenil. Note the broadening of cells compared to controls (see plate 3.5), nucleus (N) and the presence of a bizarre cell (arrow). MGG (x 1600).

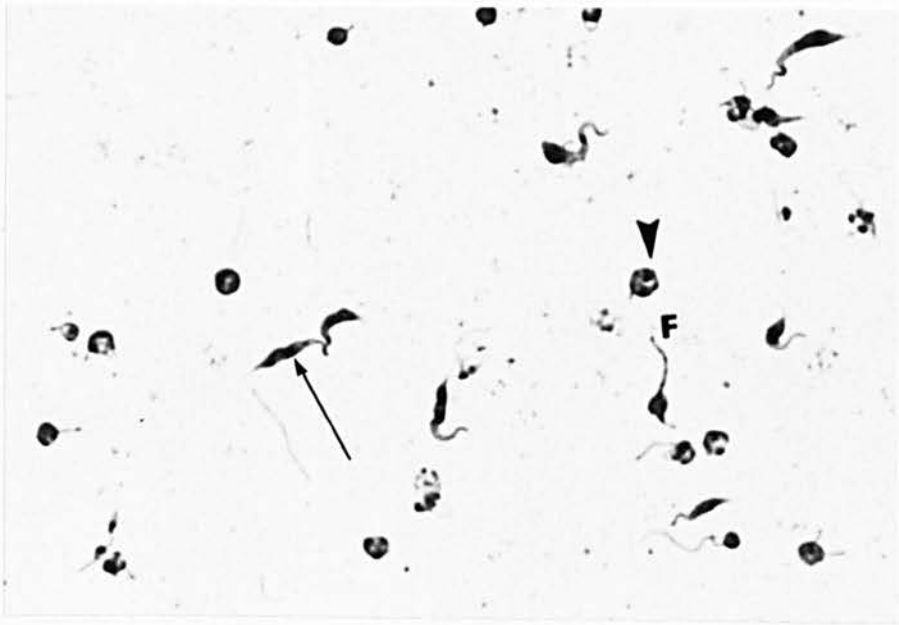


Plate 3.26 *T. granulosum* cultured for 4 days in modified SDM-79 containing 0.2mM Berenil. Note the free flagella (F), nuclei (long arrows) and bizarre forms (arrows). MGG (x 480).

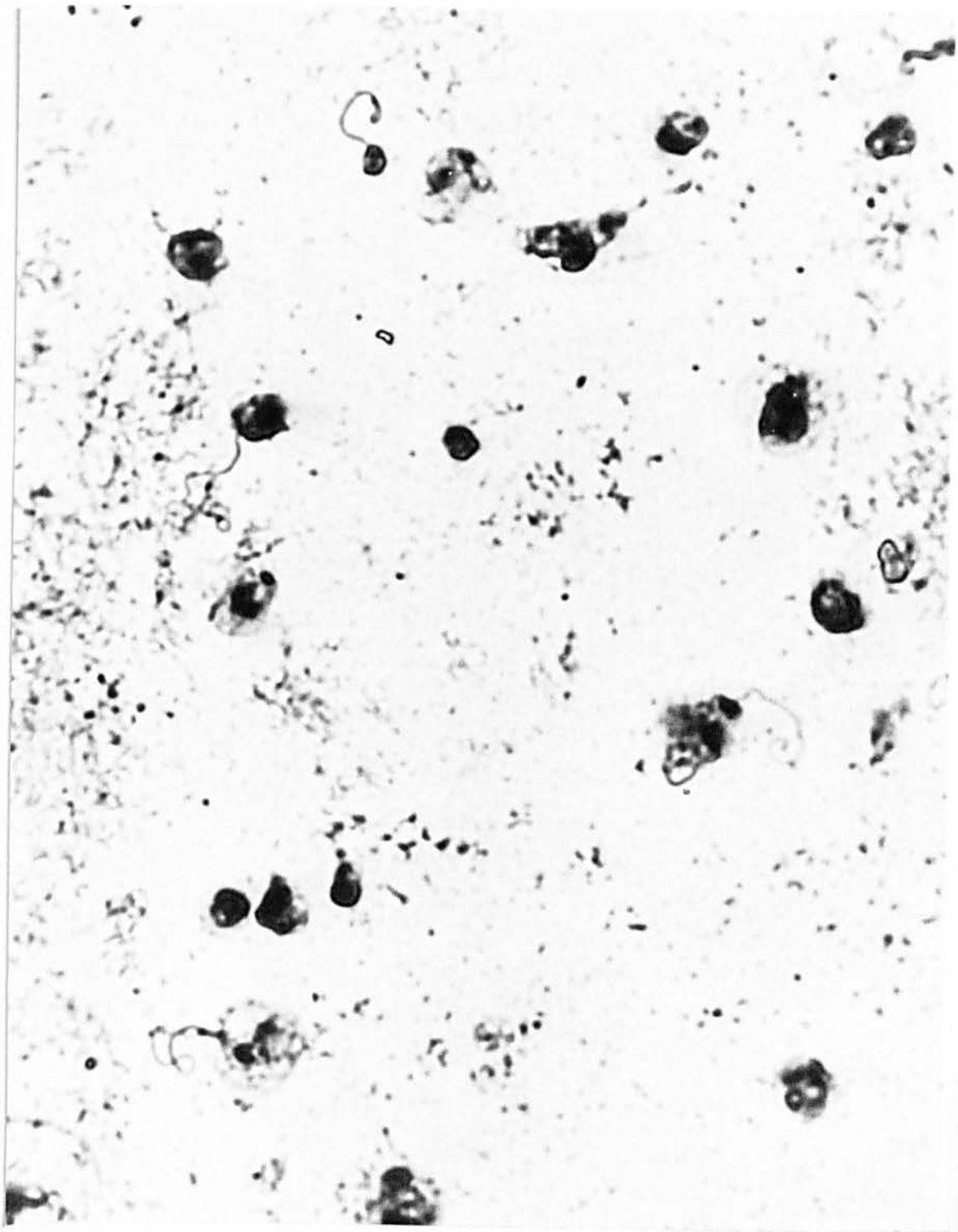


Plate 3.27 *T. granulosum* cultured for 4 days in modified SDM-79 containing 1mM Berenil. MGG (x 1300).

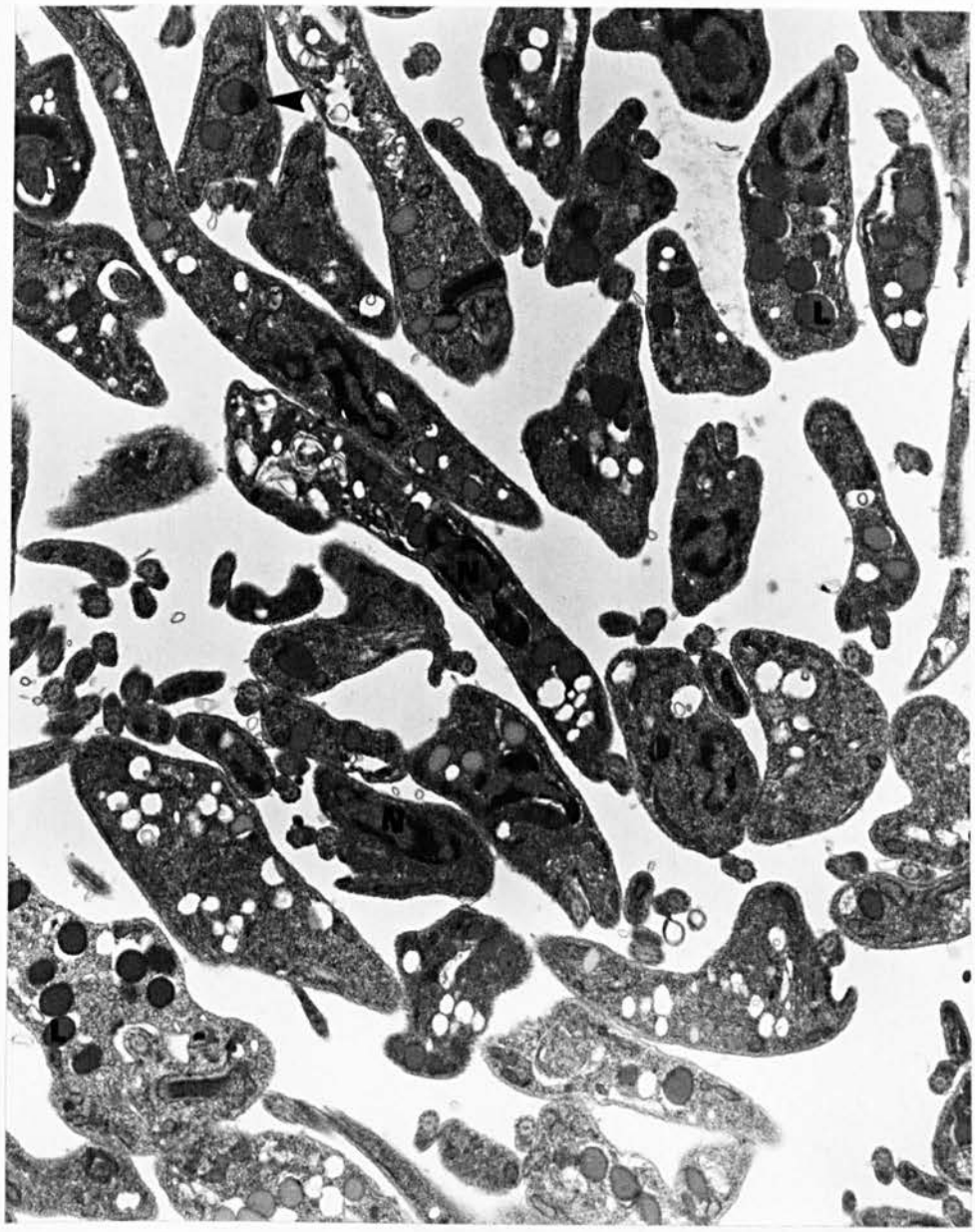


Plate 3.28 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 20mM DFMO. Note the presence of a myelin-like figure (arrow), lipid bodies (L) and nuclei (N). UA&LC (x 12000).

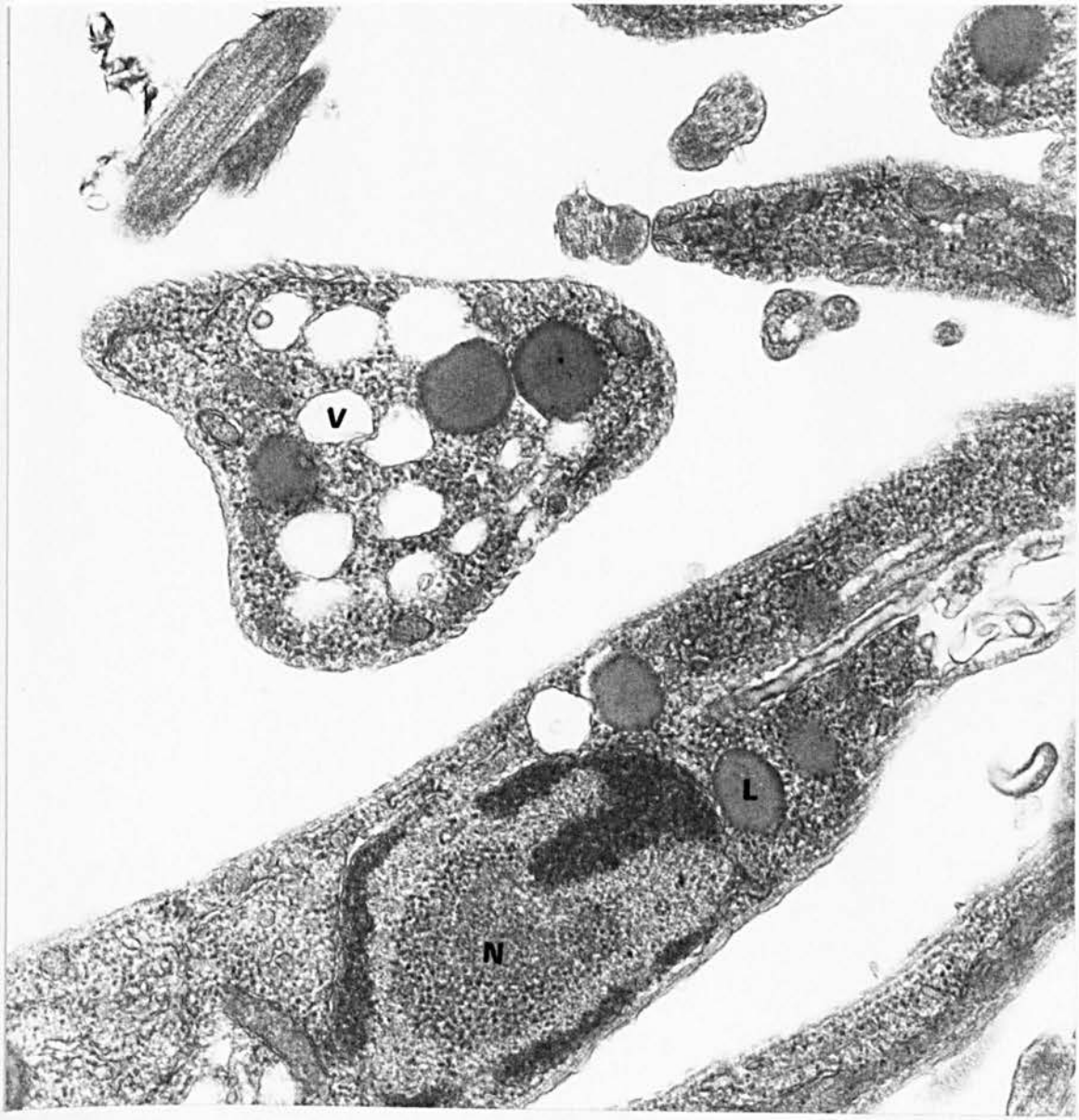


Plate 3.29 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 20mM DFMO. Note the presence of lipid bodies (L), cytoplasmic vacuoles (V) and nucleus (N). UA&LC (x 39000).

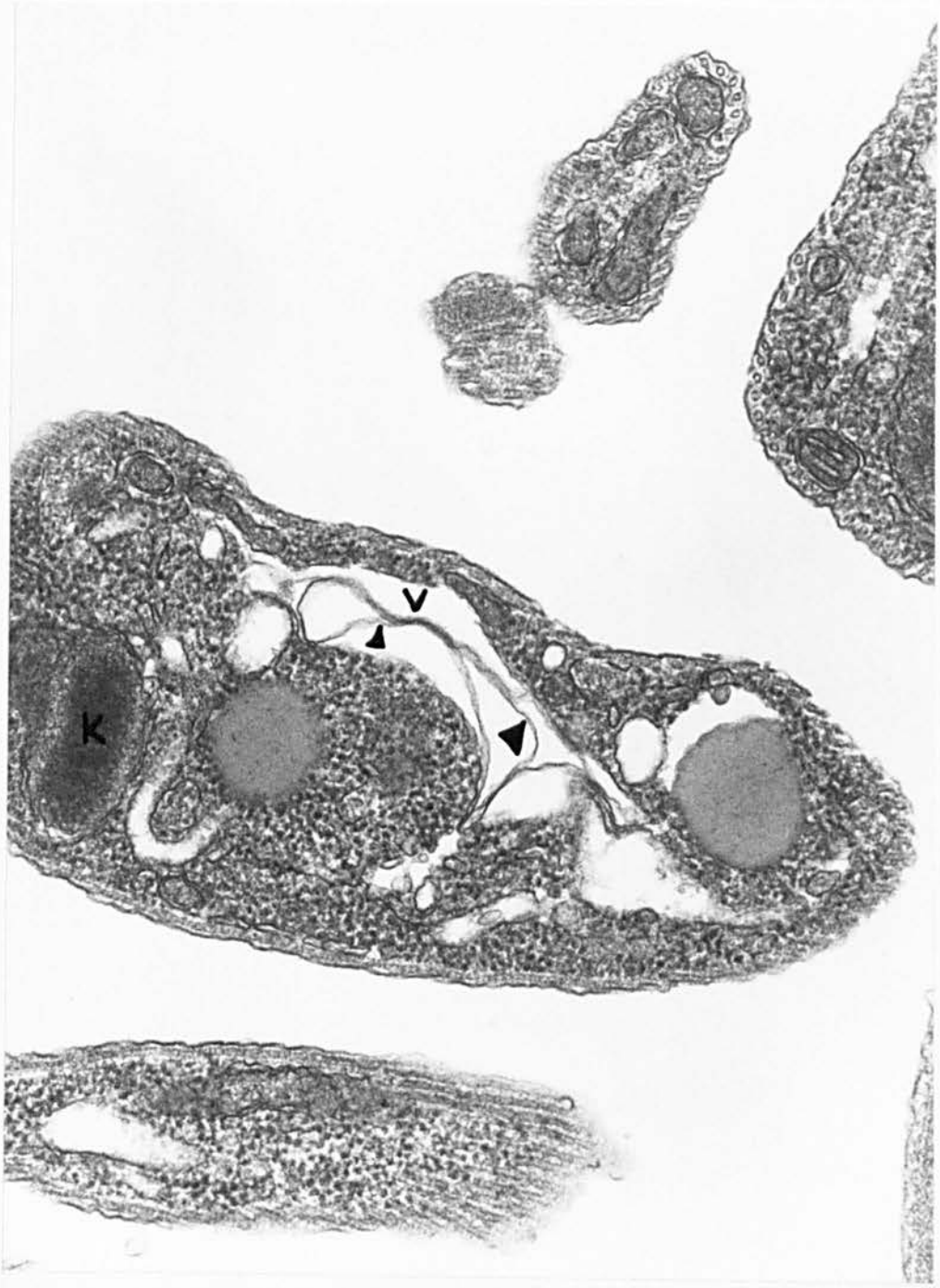


Plate 3.30 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 20mM DFMO. Note the cytoplasmic vacuoles (V) containing membranes (arrows) of unknown origin and the swollen mitochondrion surrounding the kinetoplast (K). UA&LC (x 58000).

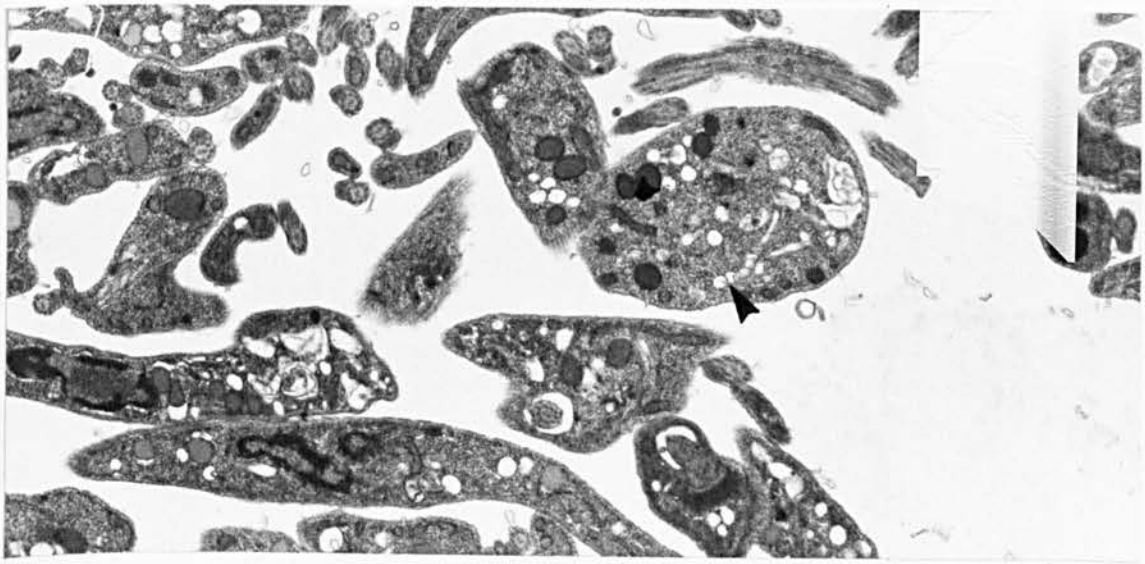


Plate 3.31 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 20mM DFMO. Note the appearance of both electron dense and electron lucent mitochondrion within the same cell (arrows). UA&LC (x 19000).

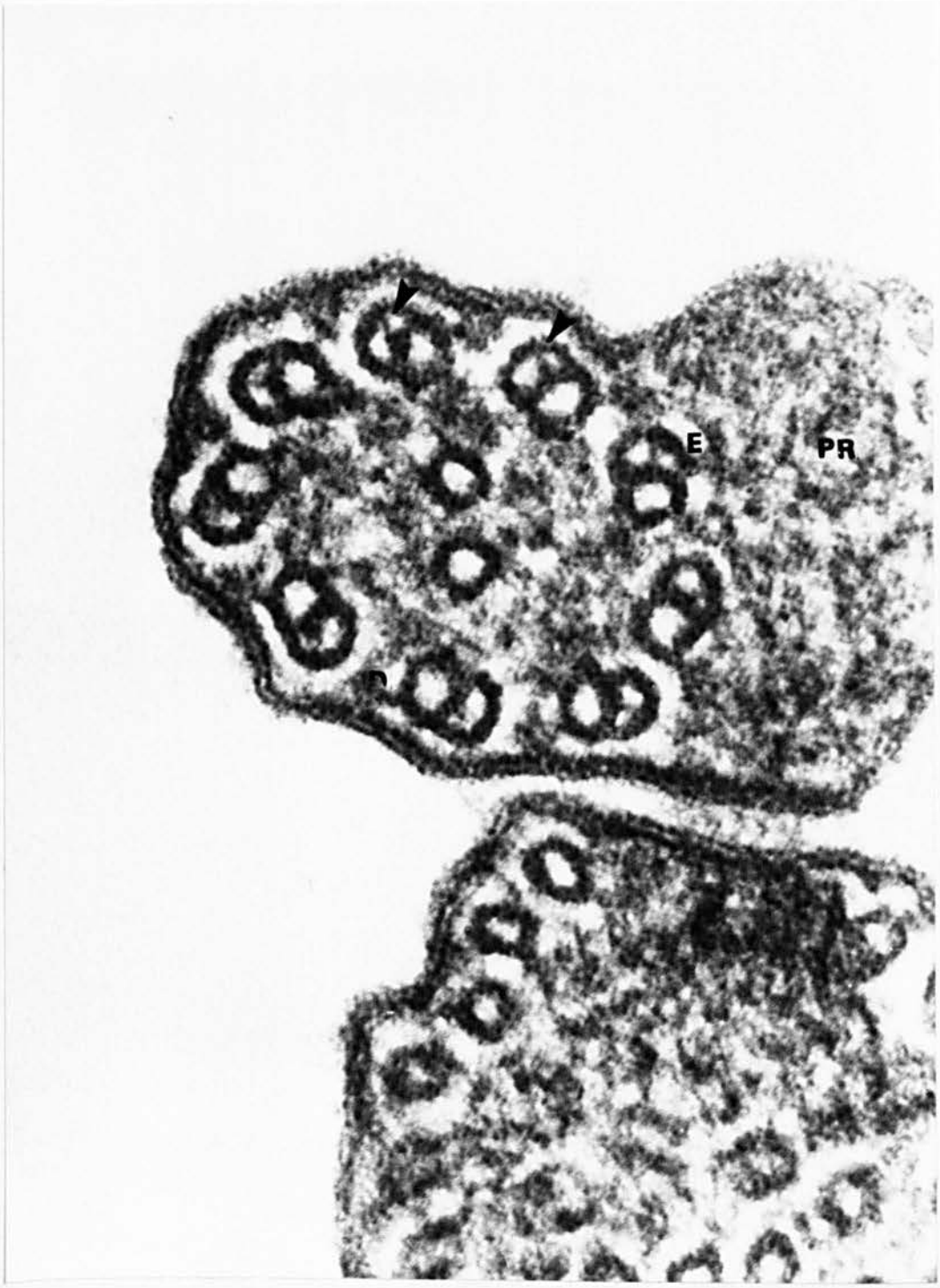


Plate 3.32 Electron micrograph of a single flagellum from *T. granulosum* cultured in modified SDM-79 containing 20mM DFMO. Note the presence of a counter-clockwise arm in the peripheral doublets (arrows), the dynein arms (D) and the extension (E) to the paraxial rod (PR). UA&LC (x 434000).

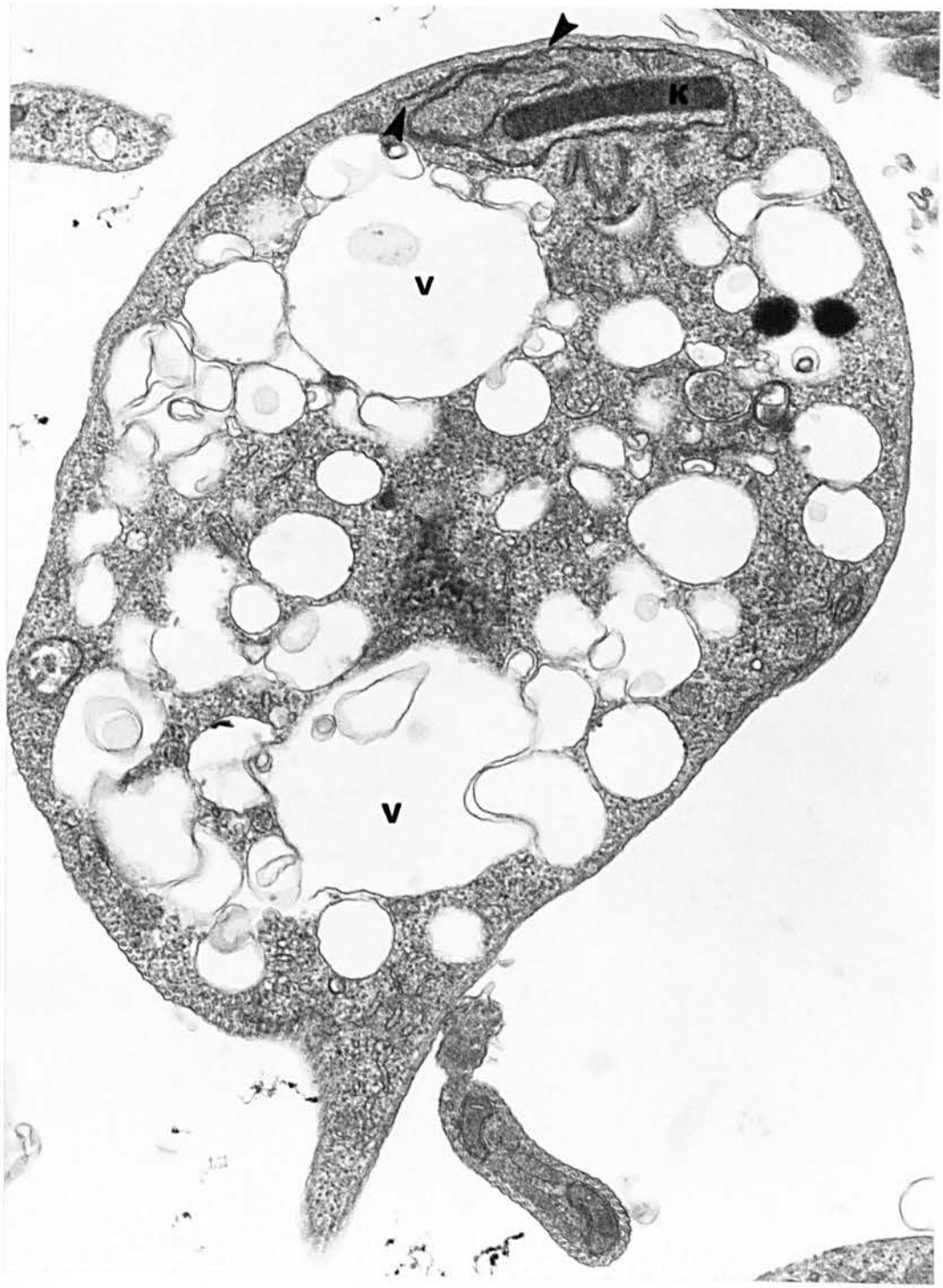


Plate 3.33 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 50mM DFMO. Note the large cytoplasmic vacuoles (V) and the swollen mitochondrial membranes (arrows) surrounding the kinetoplast (K). UA&LC (x 34000).

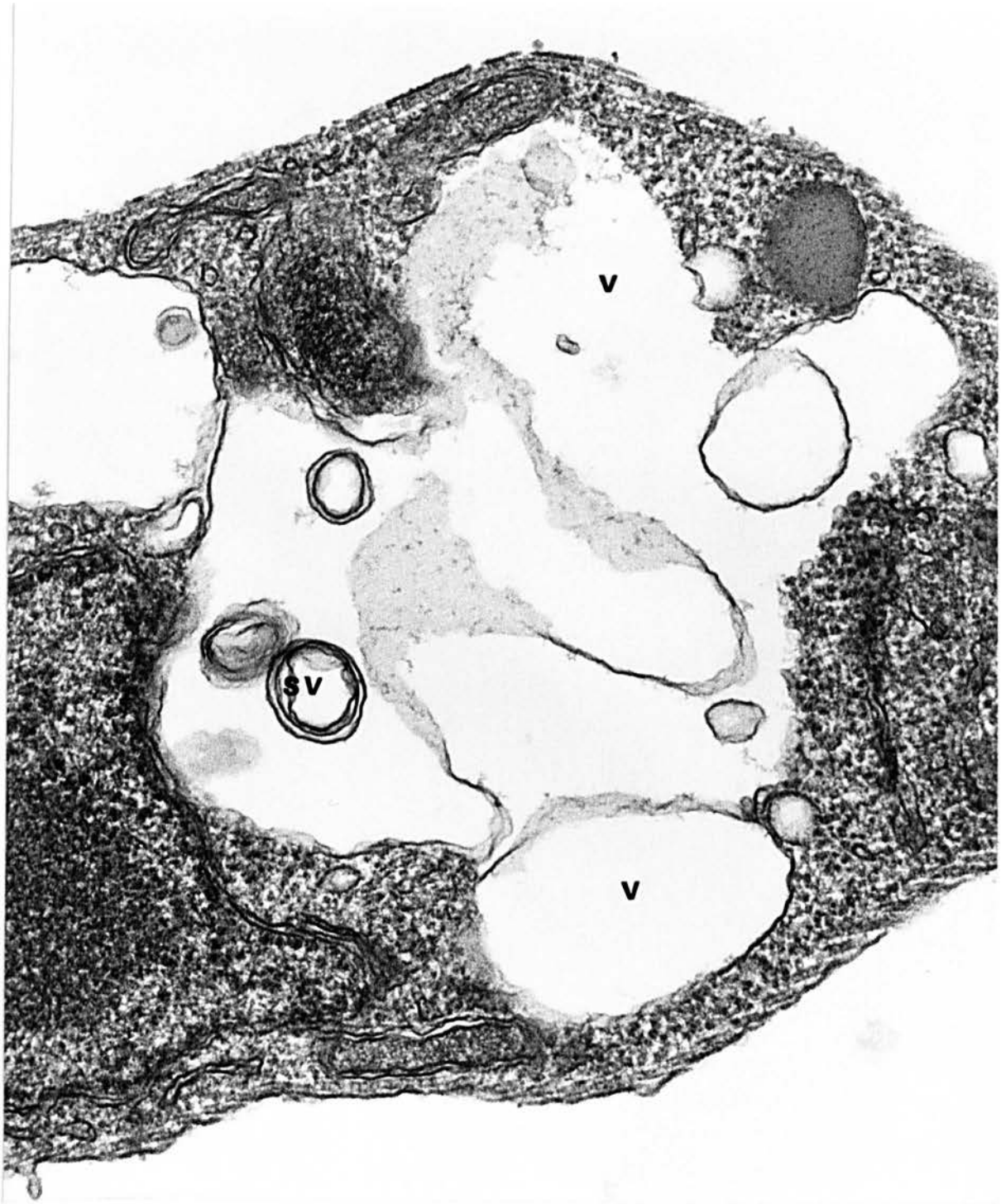


Plate 3.34 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 50mM DFMO. Note the large cytoplasmic vacuoles (V) containing smaller vacuoles of unknown origin (SV). UA&LC (x 83000).

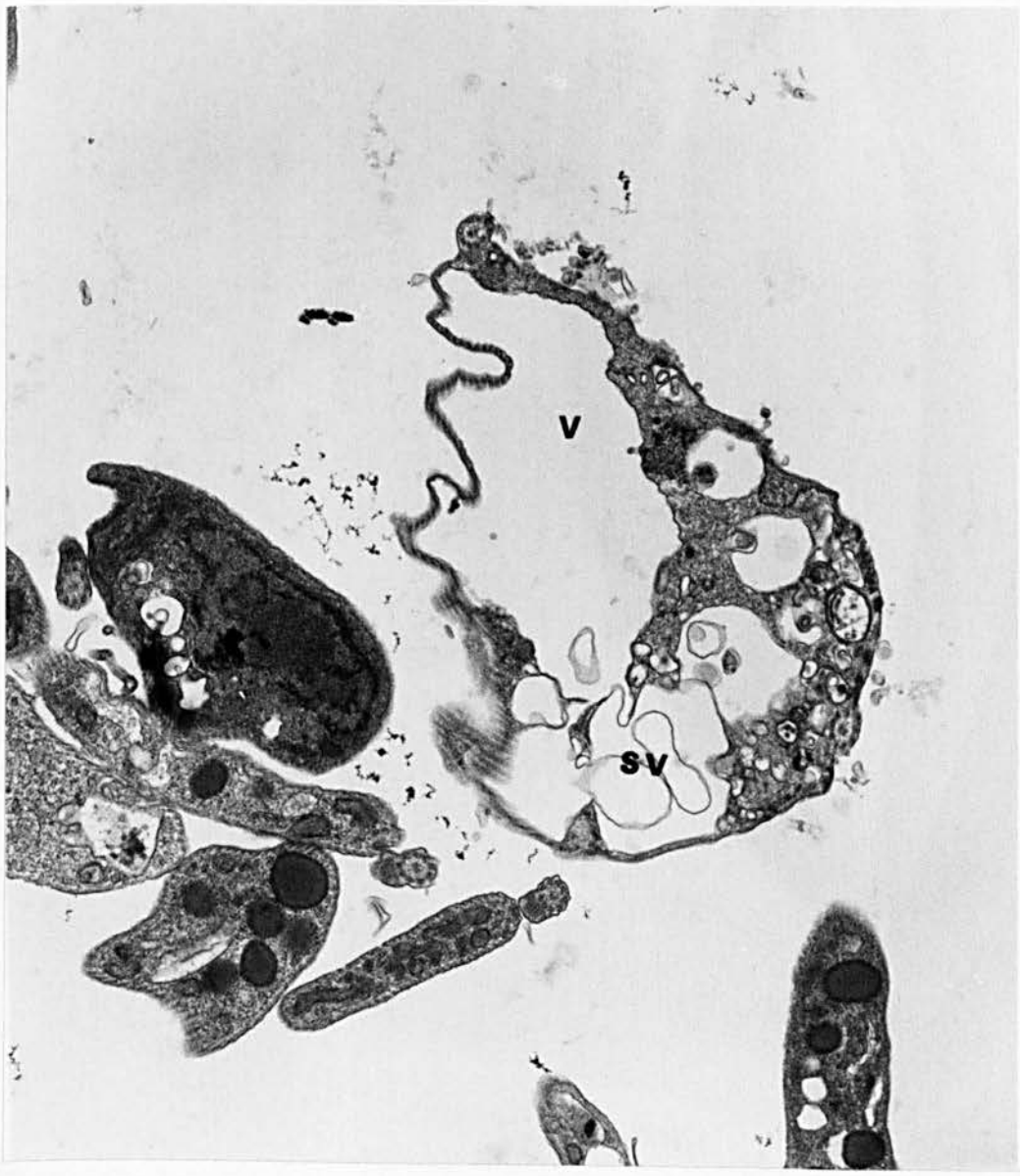


Plate 3.35 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 50mM DFMO. Note the large cytoplasmic vacuoles (V) containing smaller vacuoles of unknown origin (SV). UA&LC (x 20000).

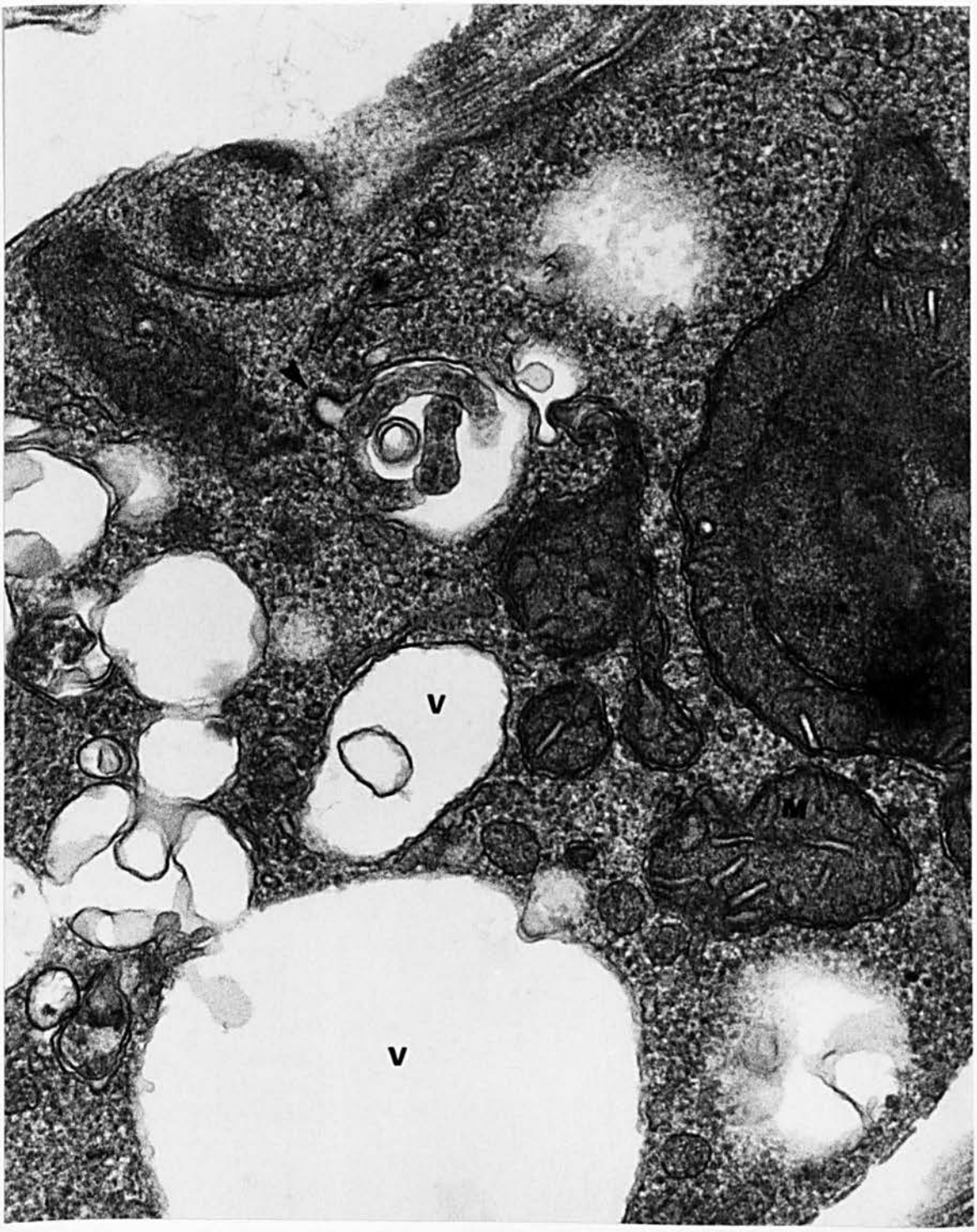


Plate 3.36 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 50mM DFMO. Note the large cytoplasmic vacuoles (V), the shape of cristae within the mitochondrion (M) and the formation of coated vesicles (arrow). UA&LC (x 78000).

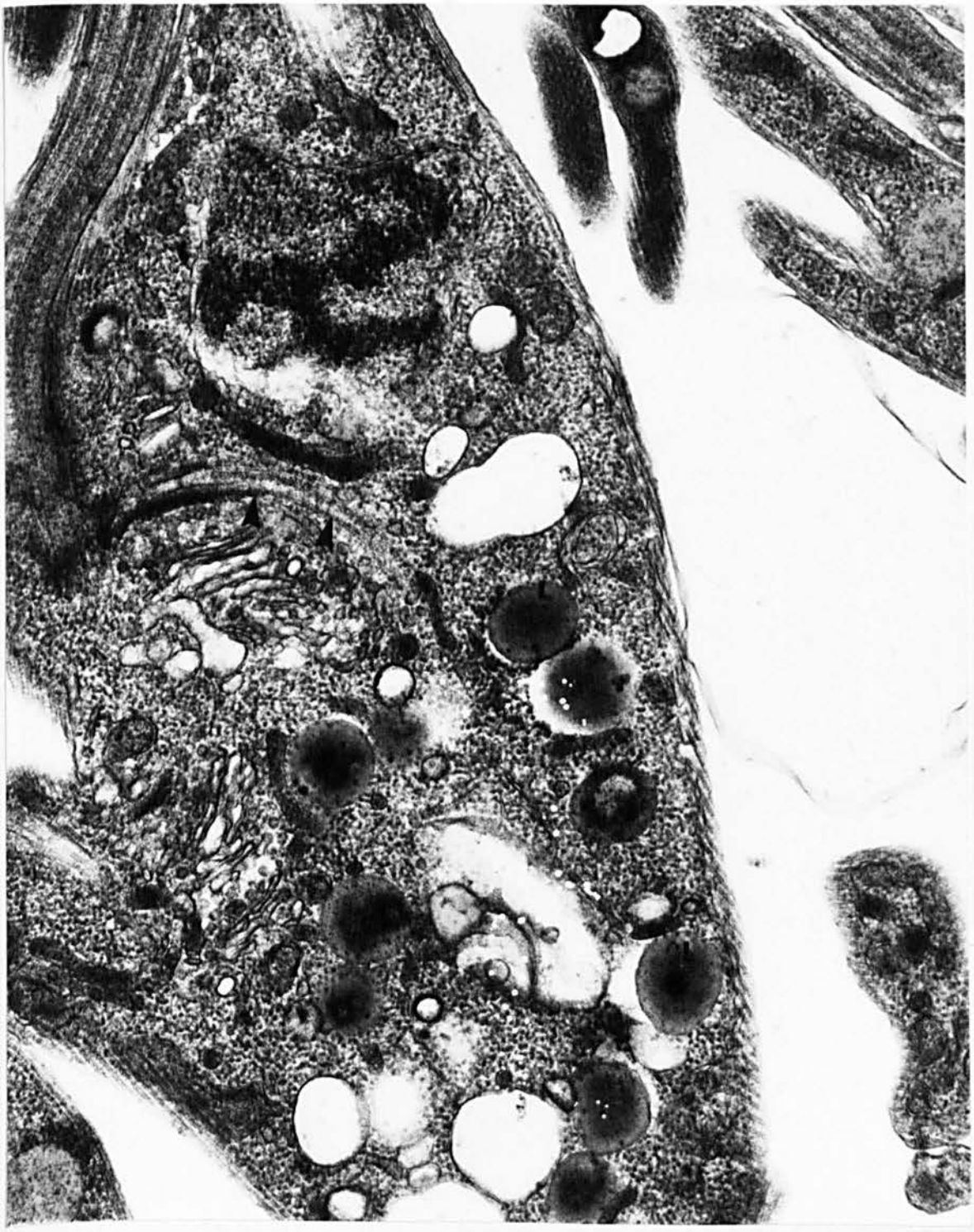


Plate 3.37 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 0.2mM MGBG. Note the lipid bodies (L), cytoplasmic vacuoles and the presence of an unknown fibrous structure (arrows). UA&LC (x 43000).

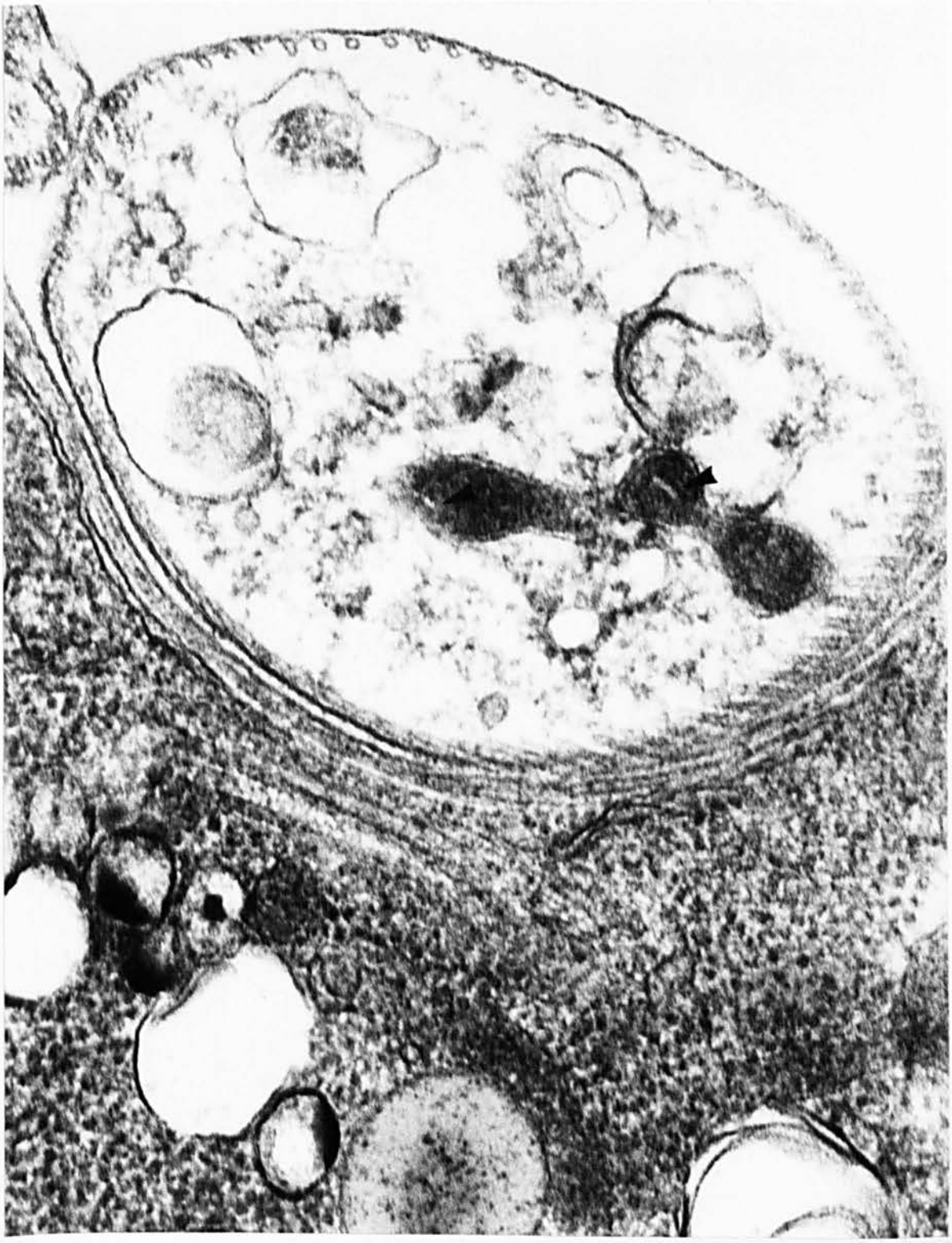


Plate 3.38 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 0.2mM MGBG. Note the appearance of the mitochondrial cristae (arrows) and the lack of cytoplasmic ribosomes. UA&LC (x 122000).

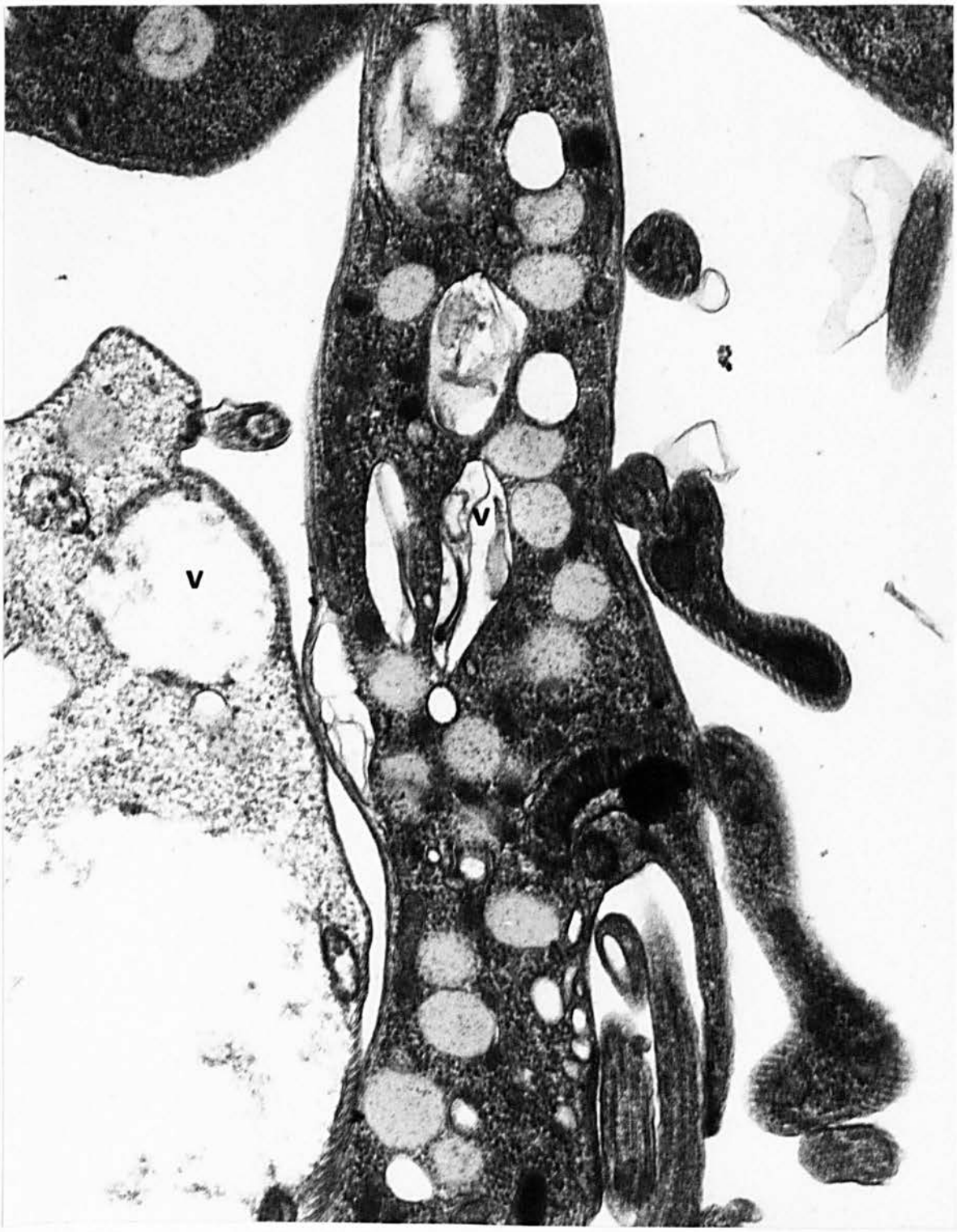


Plate 3.39 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 0.2mM MGBG. Note the presence of cytoplasmic vacuoles (V). UA&LC (x 44000).

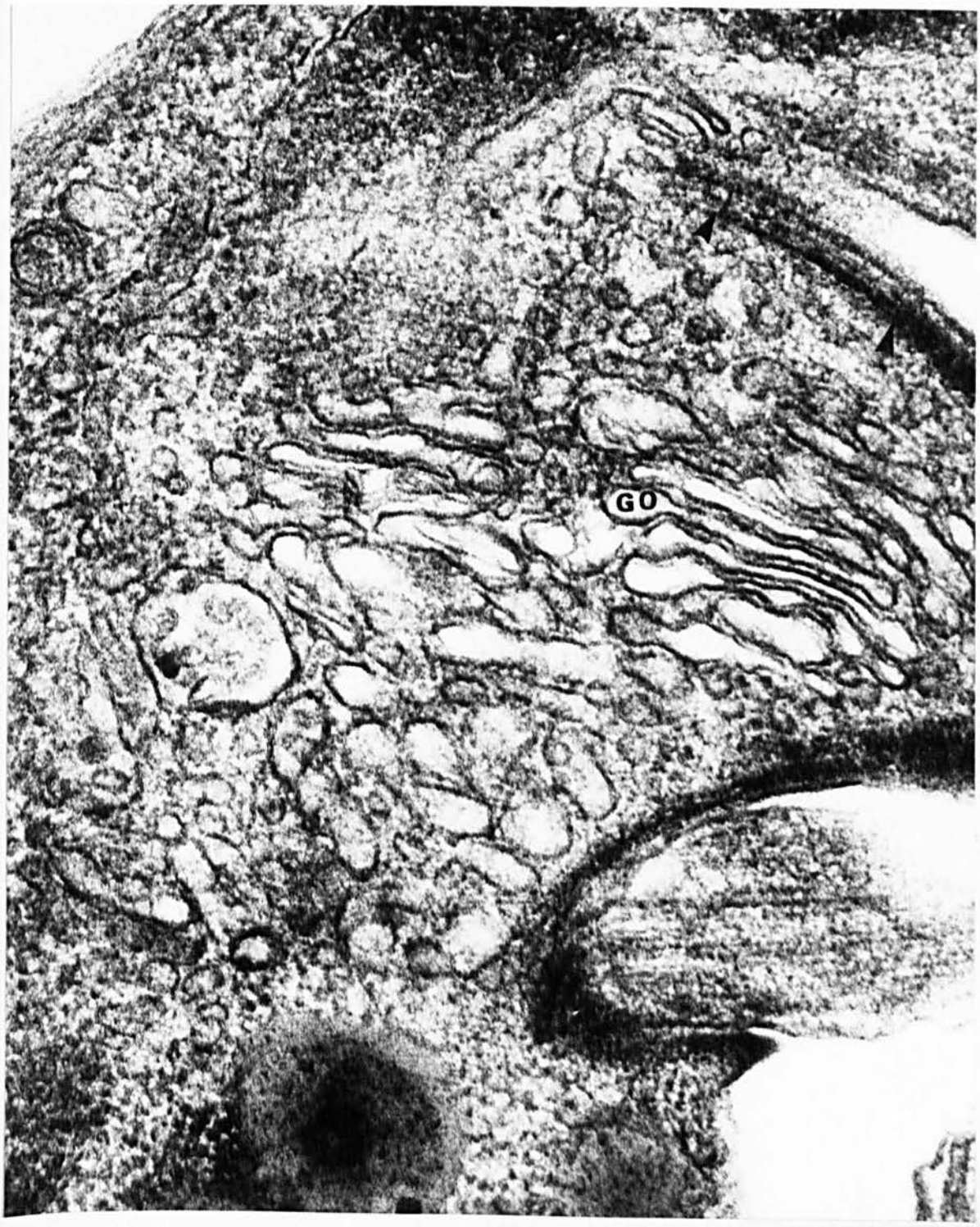


Plate 3.40 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 0.2mM MGBG. Note the presence of golgi (GO) and an unknown fibrous structure (arrows). UA&LC (x 118000).

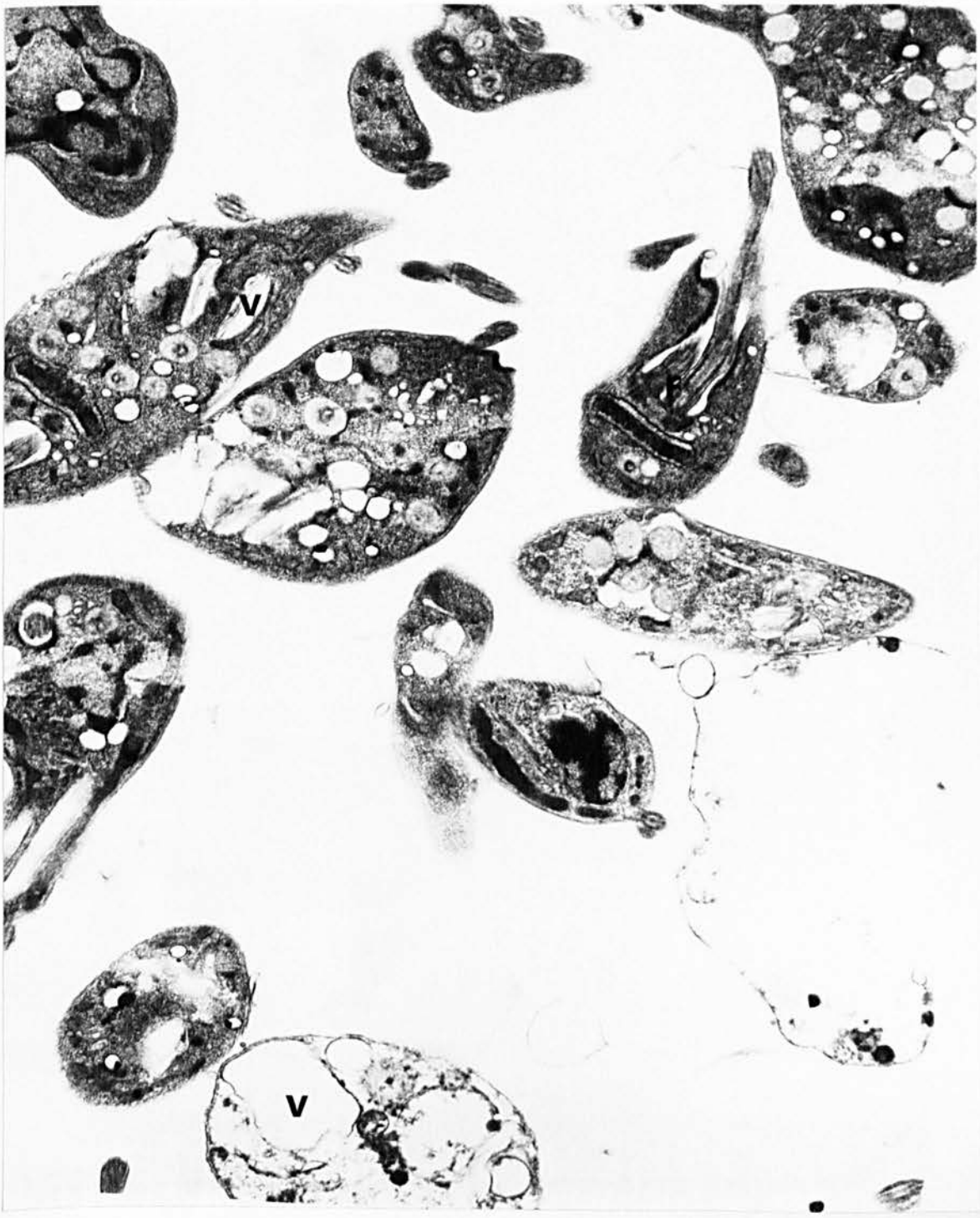


Plate 3.41 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 0.2mM MGBG. Note the presence of cytoplasmic vacuoles (V) and dividing flagella (F). UA&LC (x 15000).

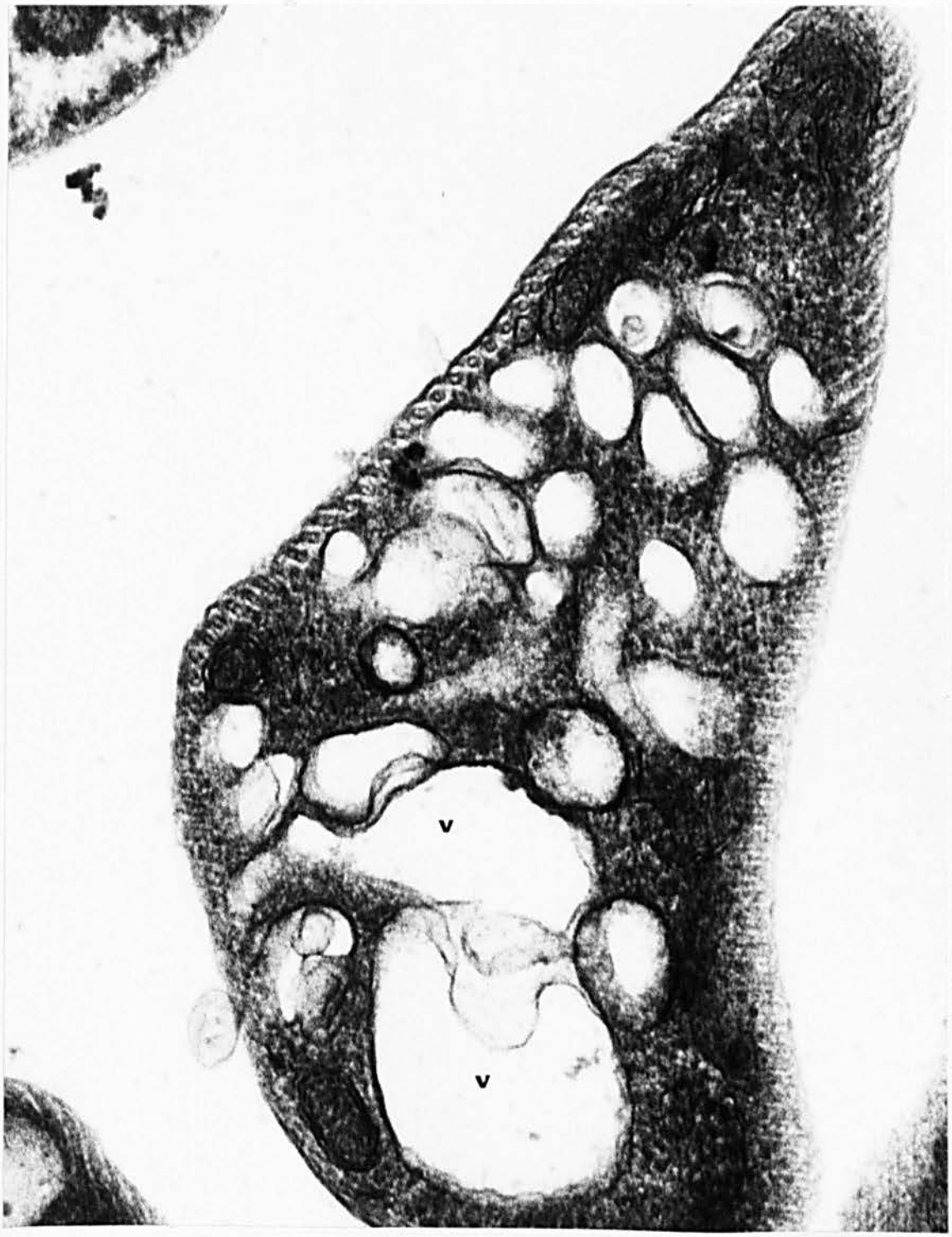


Plate 3.42 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 1mM MGBG. Note the presence of cytoplasmic vacuoles (V). UA&LC (x 101000).

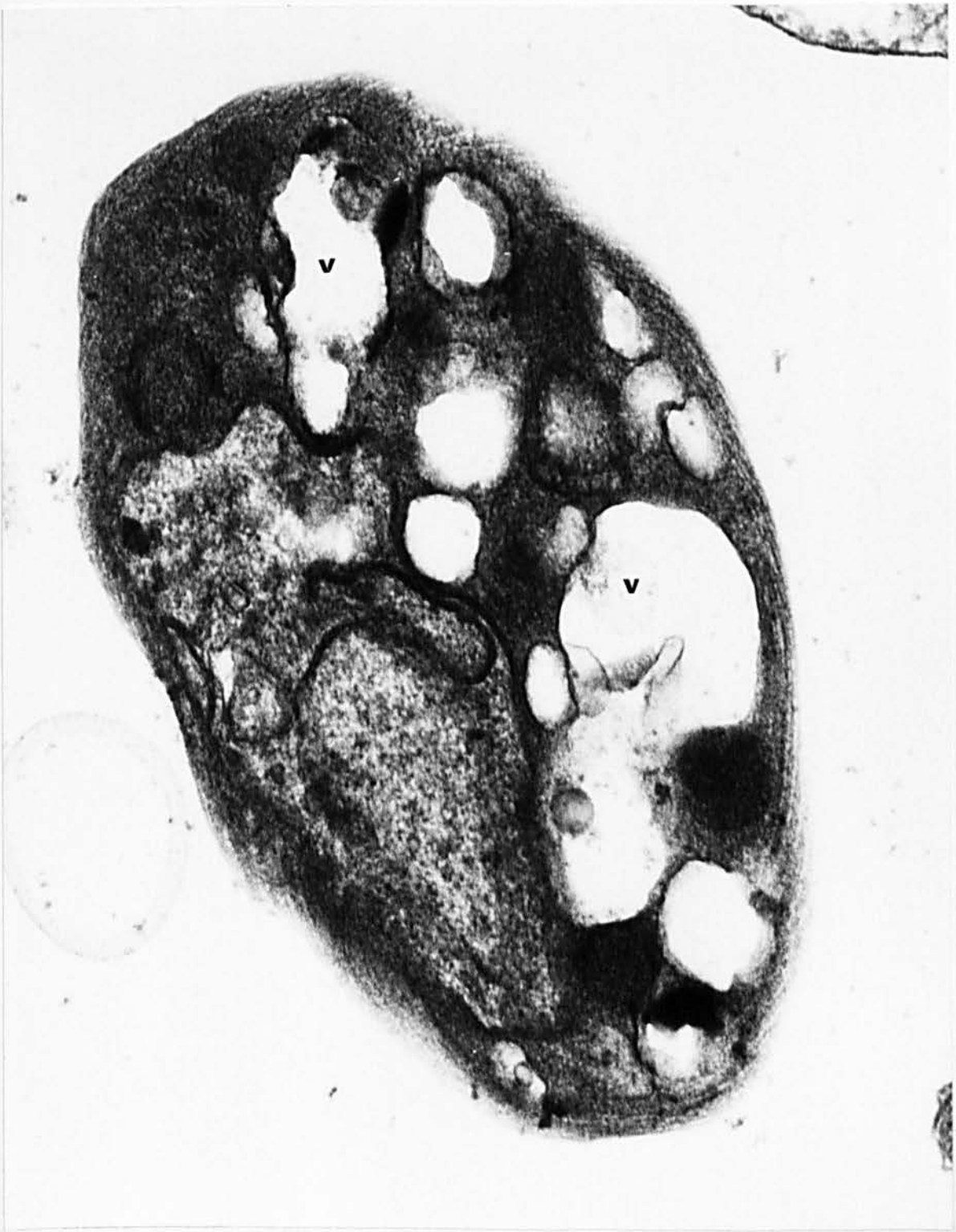


Plate 3.43 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 1mM MGBG. Note the presence of cytoplasmic vacuoles (V). UA&LC (x 76000).

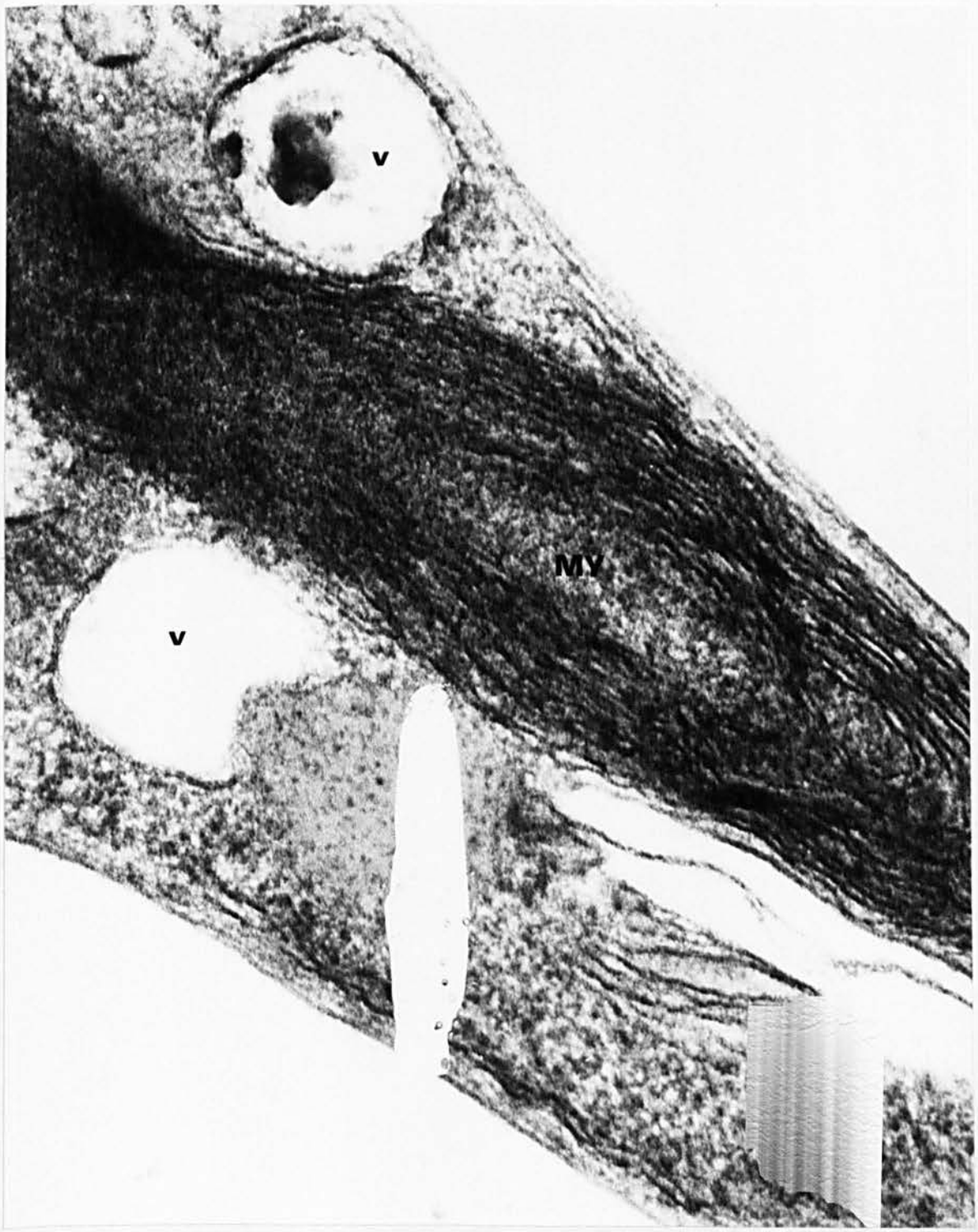


Plate 3.44 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 1mM MGBG. Note the presence of a myelin-like figure (MY) and cytoplasmic vacuoles (V). UA&LC (x 159000).

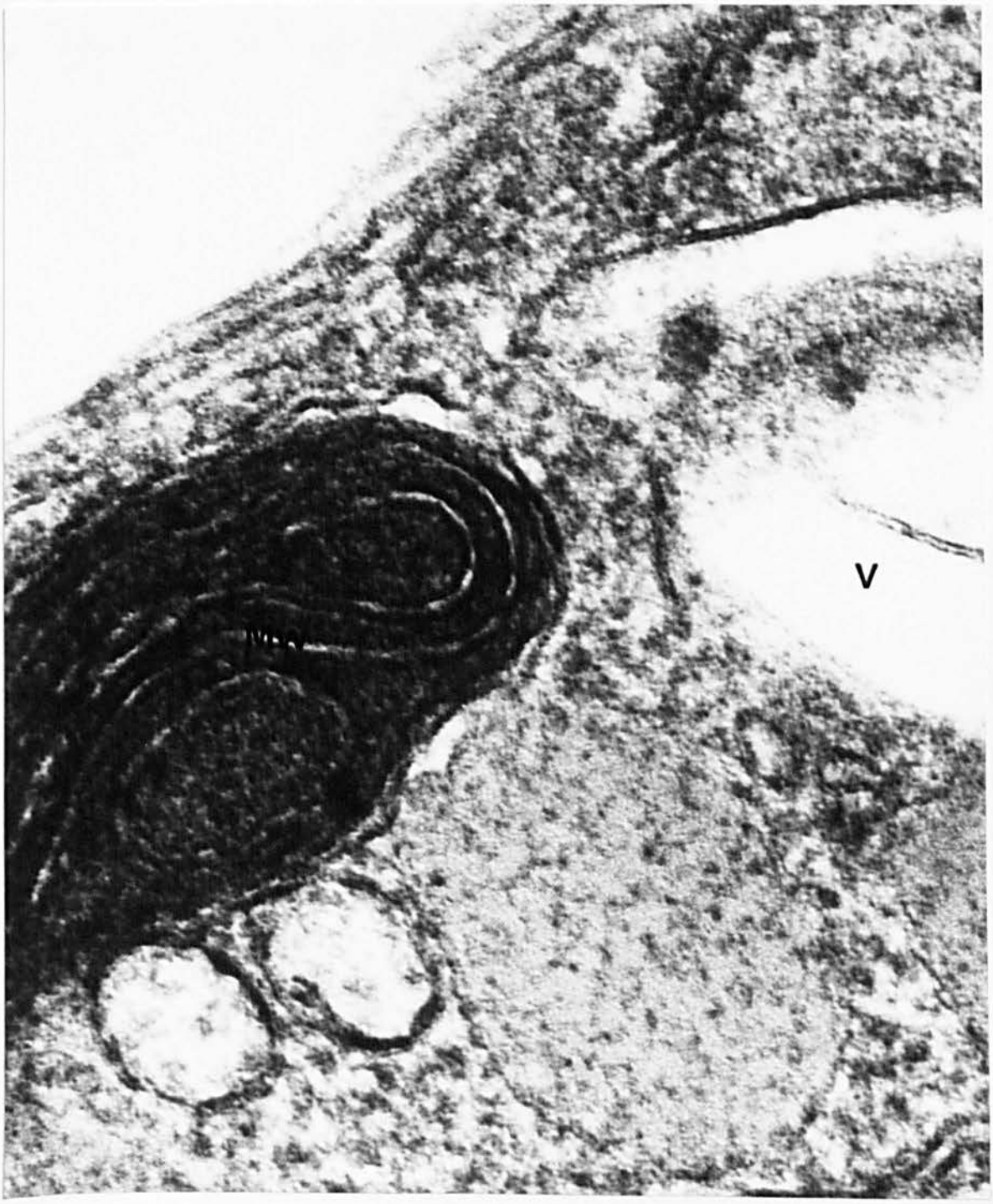


Plate 3.45 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 1mM MGBG. Note the presence of a membranous whirl (MW) and cytoplasmic vacuoles (V). UA&LC (x 227000).

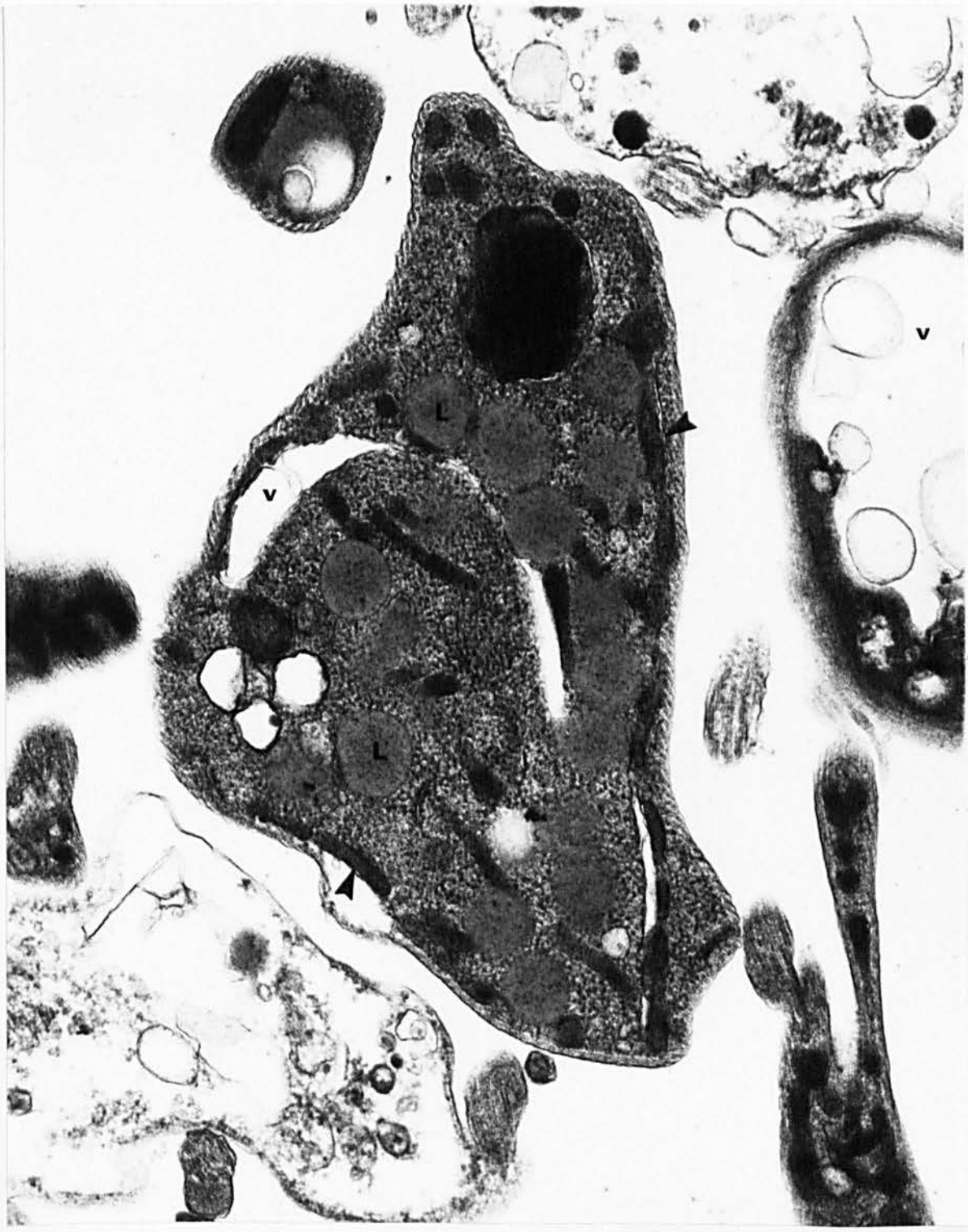


Plate 3.46 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 1mM MGBG. Note the presence of a lipid bodies (L), cytoplasmic vacuoles (V) and what appears to be a highly branched mitochondrion (arrows). UA&LC (x 44000).

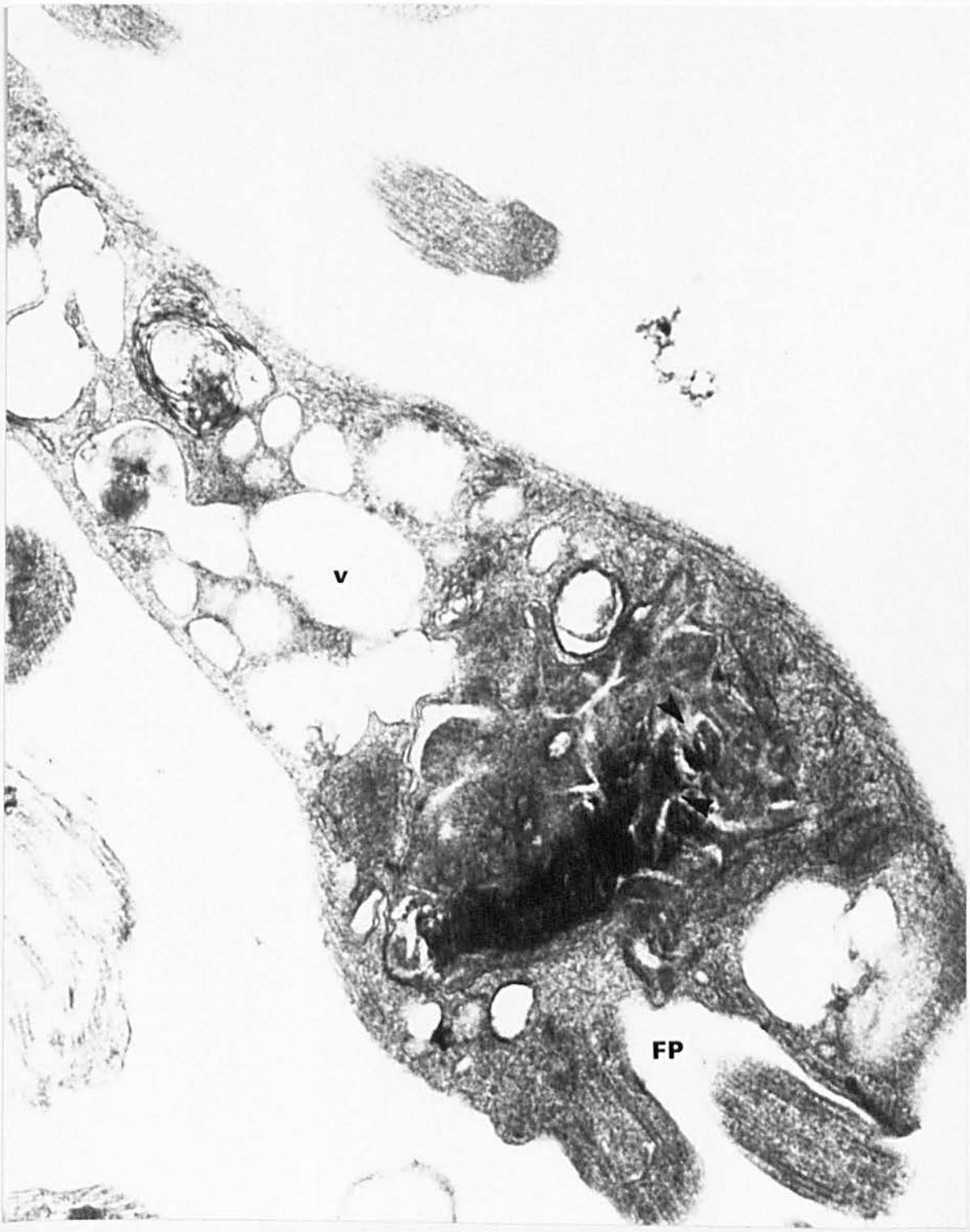


Plate 3.47 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 1mM MGBG. Note the presence of a cytoplasmic vacuoles (V), flagellar pocket (FP) and disruption of the kinetoplast (arrows). UA&LC (x 77000).

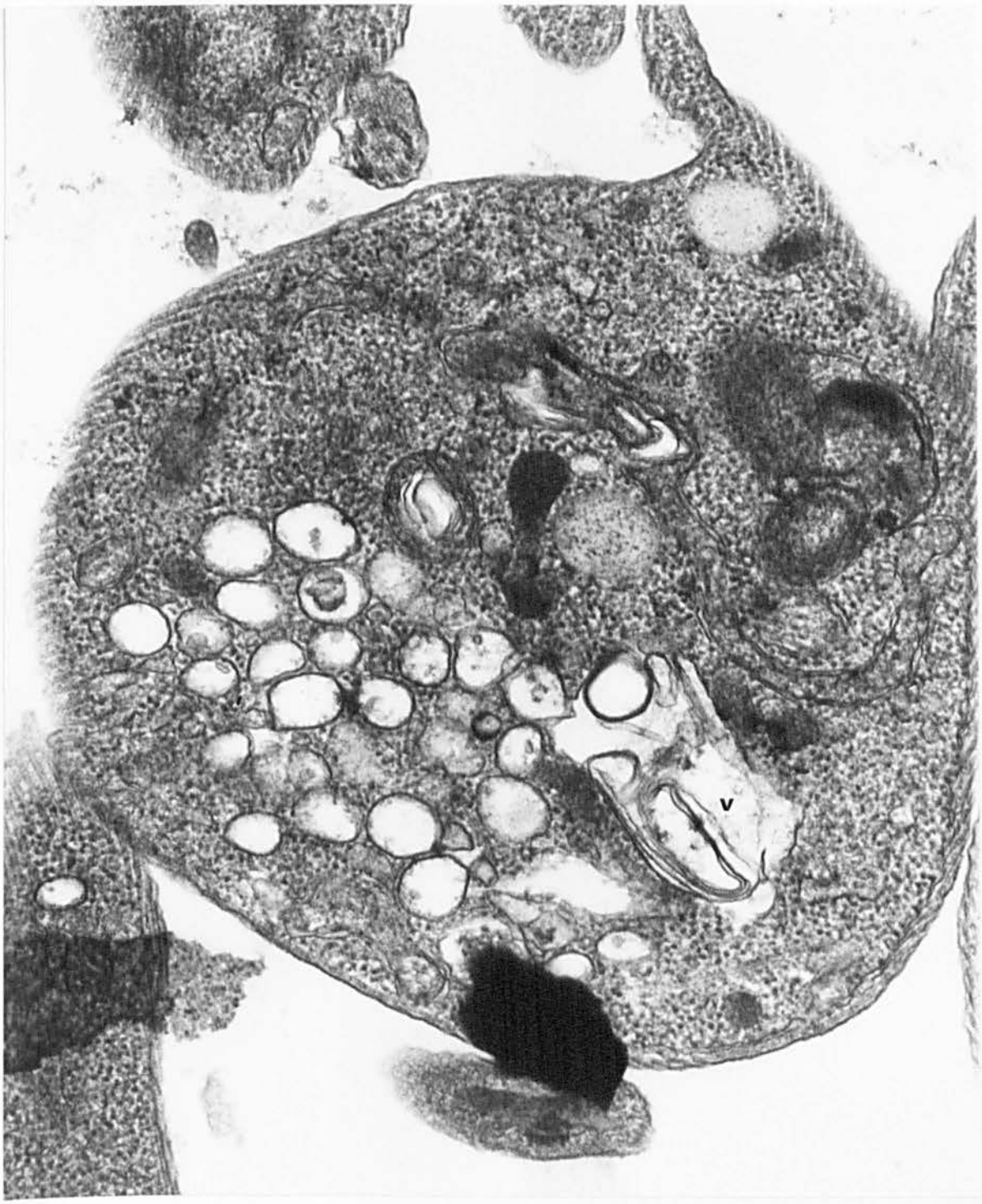


Plate 3.48 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 0.2mM Berenil. Note the presence of a cytoplasmic vacuoles (V). UA&LC (x 78000).

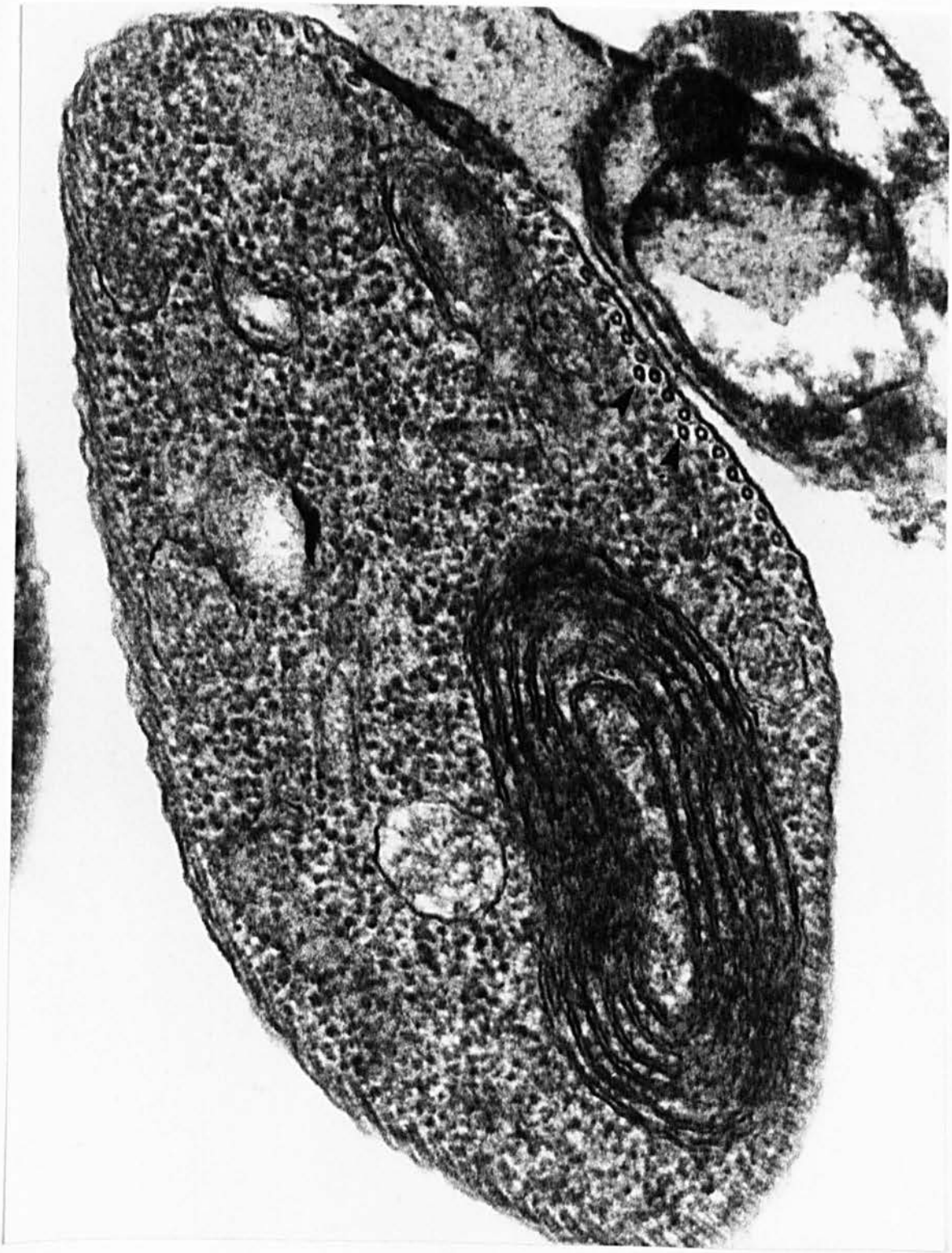


Plate 3.49 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 0.2mM Berenil. Note the presence of additional microtubules (arrows) underlying the usual sub-pellicular microtubules. UA&LC (x 43000).

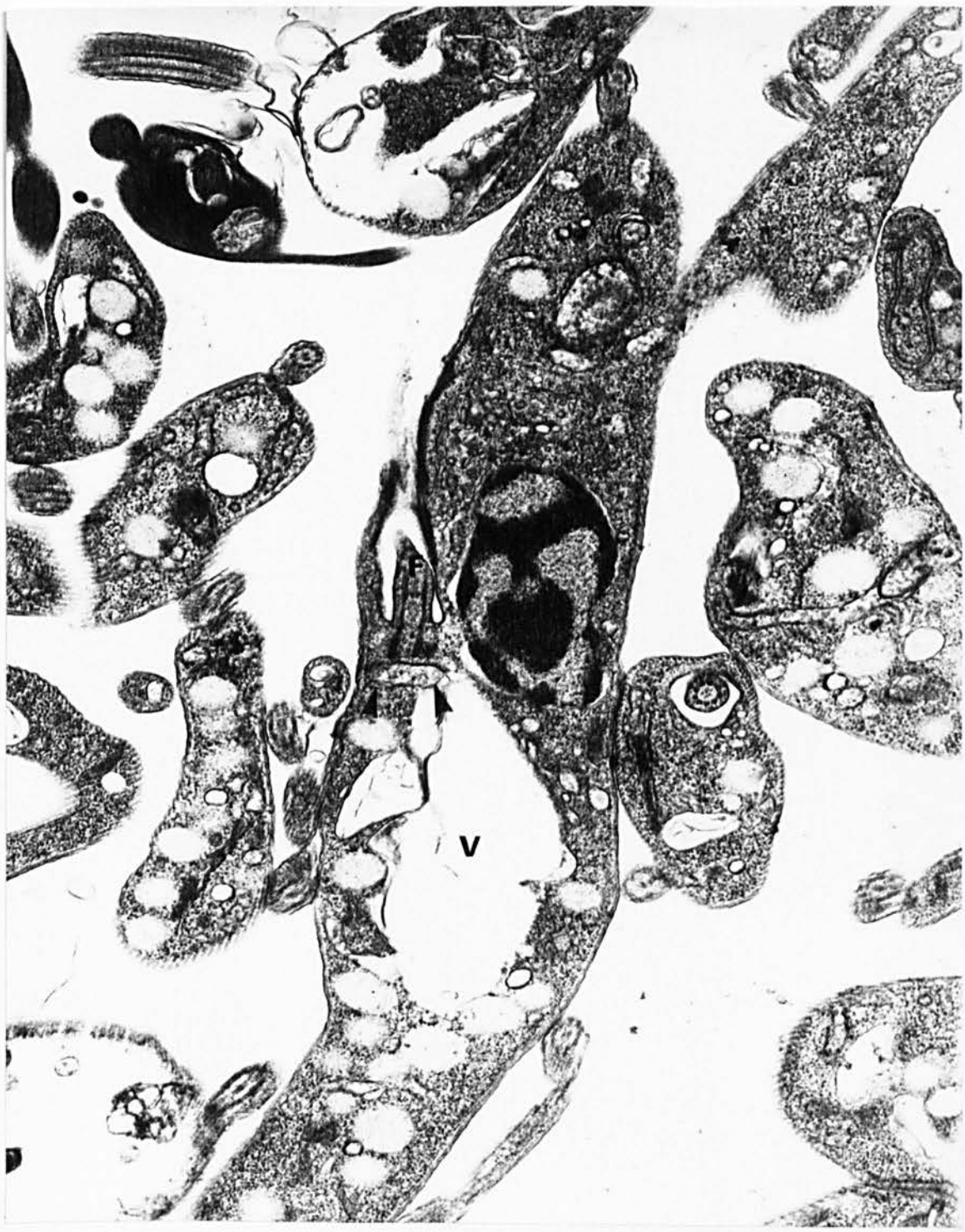


Plate 3.50 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 0.2mM Berenil. Note the presence of cytoplasmic vacuoles (V), flagellum (F) and damage to the kinetoplast (arrows). UA&LC (x 20000).

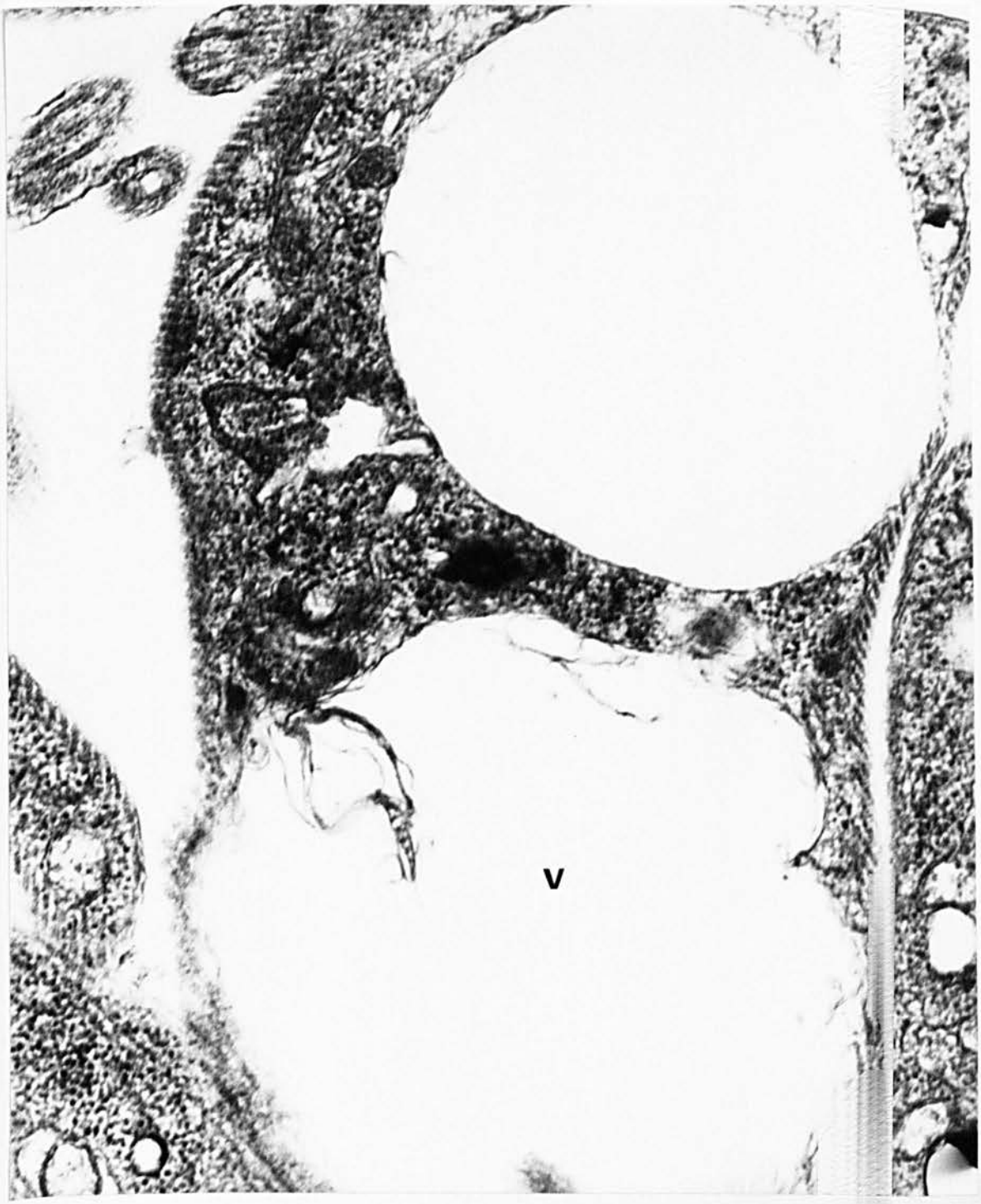


Plate 3.51 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 0.2mM Berenil. Note the presence of cytoplasmic vacuoles (V). UA&LC (x 49000).

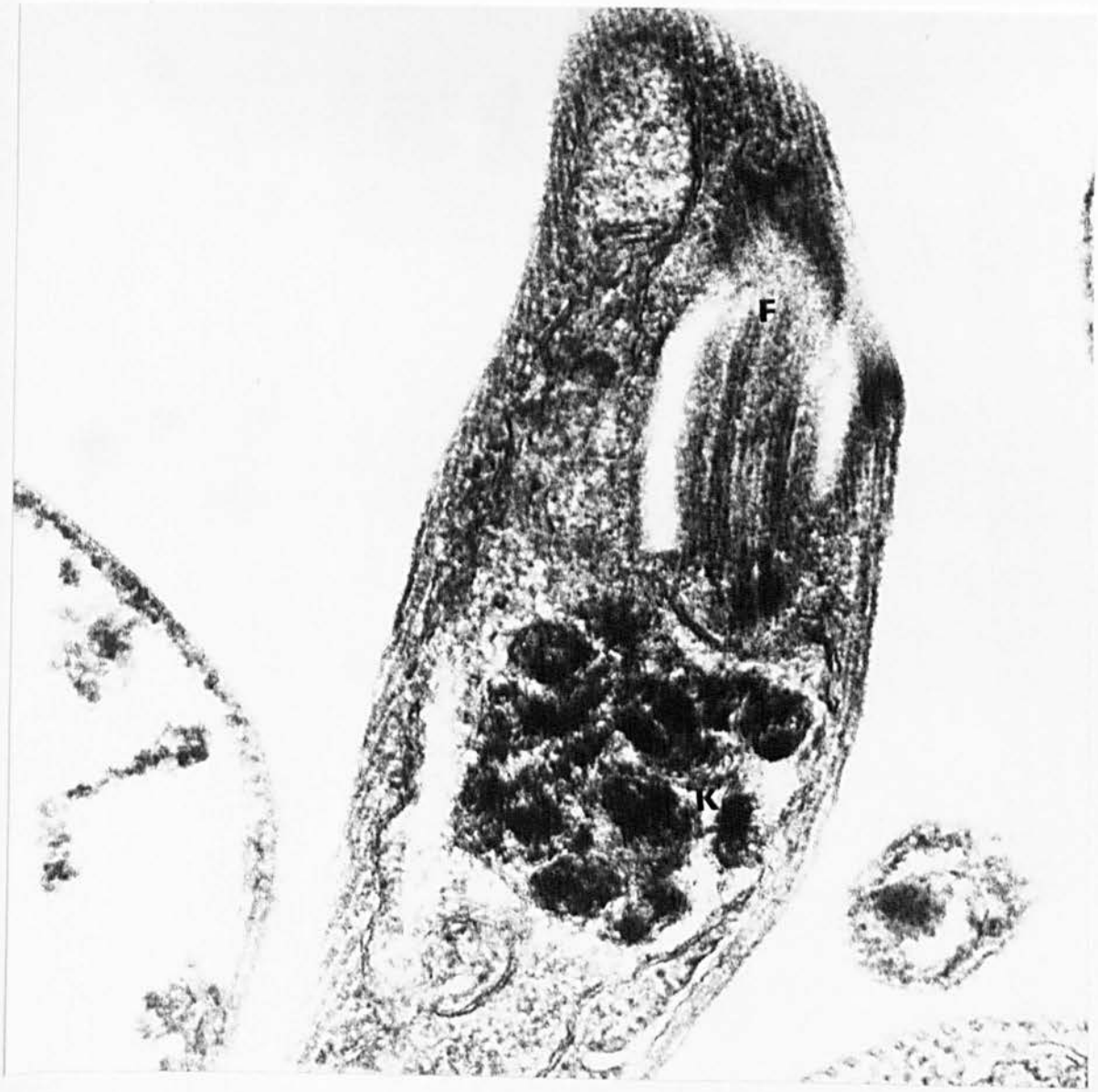


Plate 3.52 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 0.2mM Berenil. Note the presence of a disaggregated kinetoplast (K) and flagellum (F). UA&LC (x 85000).

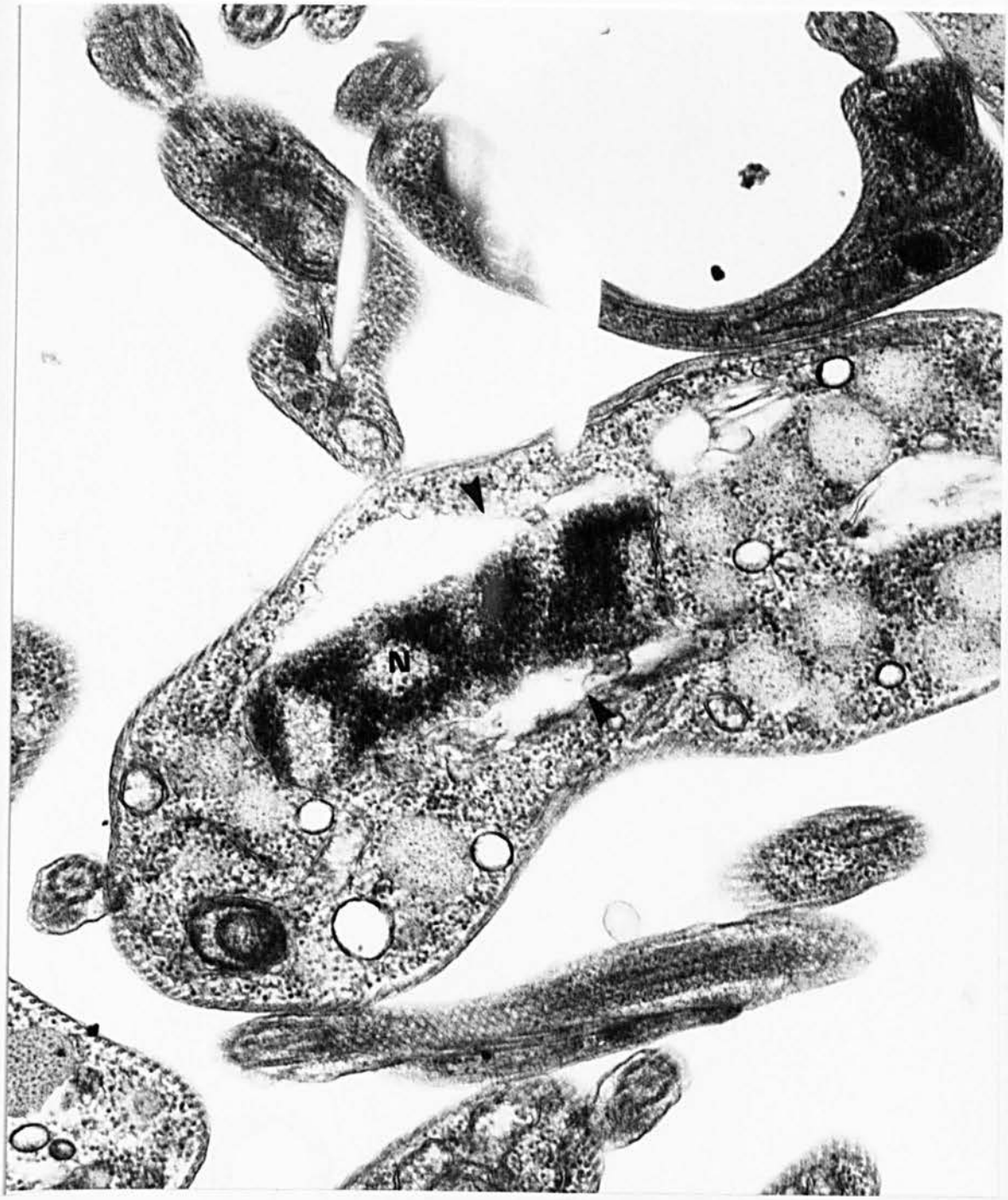


Plate 3.53 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 0.2mM Berenil. Note the unusual appearance of the nucleus (N) and the swollen nuclear membrane (arrows). UA&LC (x 45000).

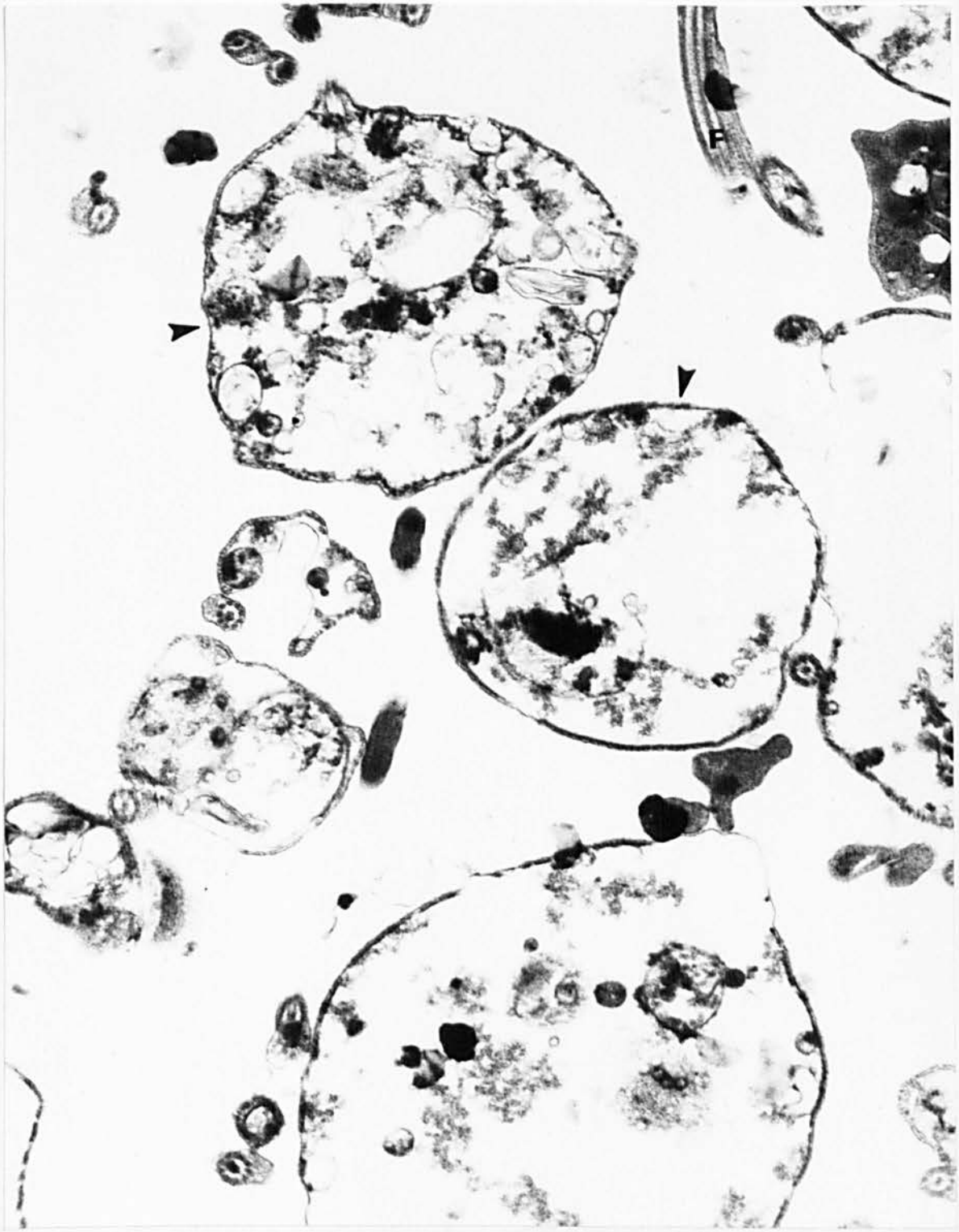


Plate 3.54 Electron micrograph of *T. granulosum* cultured in modified SDM-79 containing 1mM Berenil. Note the flagella (F) and plasmalemma (arrows). UA&LC (x 67000).

3.2 *T. danilewskyi*

3.2.1 Bloodstream *T. danilewskyi*

3.2.1.1 Morphology of bloodstream *T. danilewskyi*

Wet films from the blood of the crucian carp were not examined. Examination of 20 May-Grünwald stained blood smears revealed an intensity of 0.001 / 1000 erythrocytes. As infections were scanty only basic morphometric measurements were made.

Body length (n=6)	32.5±4.9μm
Width at widest point (n=6)	3.1±0.5μm
Free flagellum (n=3)	20.4±0.7μm

All cells observed were trypomastigotes with the kinetoplast located close to the posterior of the cell (Plate 3.55). The nuclei appeared to be oval in shape and located closer to the anterior of the cell compared to the bloodstream forms of *T. granulosum*. The cells were also shorter and slightly wider than *T. granulosum*. The presence of a long (in relation to body length) free flagellum was a distinctive feature in the cells where the flagellum could be clearly observed.

3.2.2 *T. danilewskyi* cultured in drug-free media

3.2.2.1 Morphology of *T. danilewskyi* cultured in modified SDM-79

Once introduced into modified SDM-79 from the horse blood agar slopes, the shape of the parasite had transformed into a cell morphologically indistinguishable from that of *T. granulosum* cultured in modified SDM-79 ($26.7 \pm 4.1 \mu m$ in body length, $1.7 \pm 0.4 \mu m$ wide and $15.2 \pm 1.4 \mu m$).

3.2.2.2 Growth of cultured *T. danilewskyi*

3.2.2.2.1 Growth on nutrient agar slopes

Primary isolates of *T. danilewskyi* from the blood of crucian carp grew well on horse blood nutrient agar slopes reaching similar numbers to *T. granulosum* within 2 weeks.

3.2.2.2.2 Growth in semi-defined and defined media

When introduced into the modified SDM-79, numbers of *T. danilewskyi* present by the stationary phase were similar to those reached by *T. granulosum* under similar conditions. However the approximate doubling time for *T. danilewskyi* was 1 day (Figure 3.11). It was not determined whether *T. danilewskyi* would grow successfully in any of the modifications to modified SDM-79 described previously for *T. granulosum*, but when introduced into the fully defined media TDL-15 and TDL-15 with added haemin, no growth of the parasite was observed.

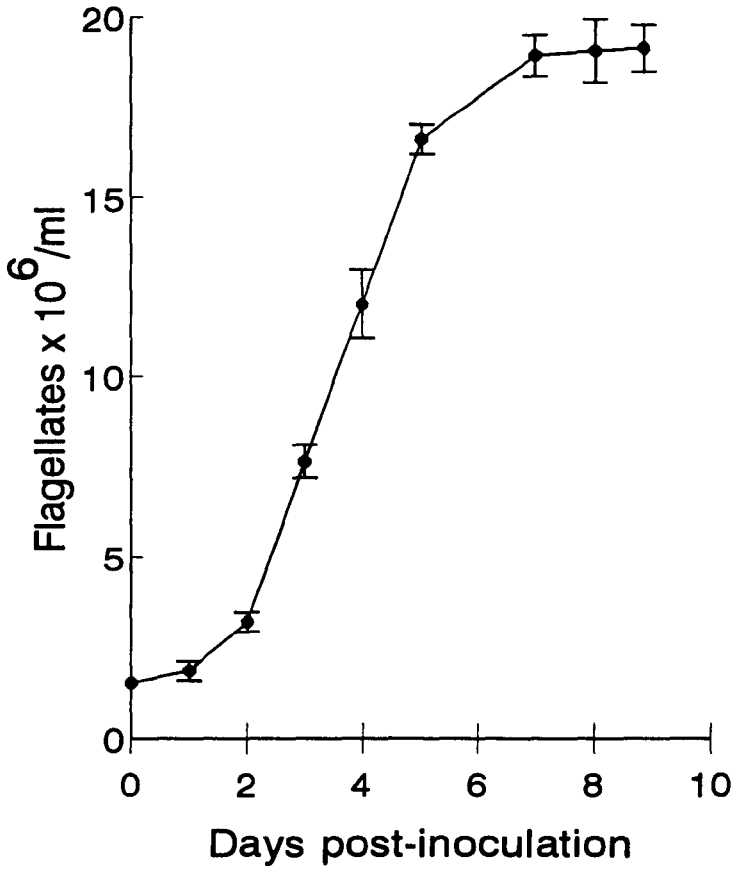


Figure 3.11 Growth of *T. danilewskyi* in modified SDM-79. Error bars represent standard deviation where n=6.

3.2.3 *T. danilewskyi* cultured in medium containing DFMO

3.2.3.1 Effects of DFMO on morphology of cultured *T. danilewskyi*

After 4 days growth in modified SDM-79 containing 10mM DFMO the cells had become shorter and wider although they were not significantly different to *T. granulosum* cultured under similar conditions. As the concentration of DFMO in the medium increased, the cells appeared stumpy but with few bizarre forms observed even at high concentrations (50mM) (Plate 3.56), indicating some resistance to DFMO compared with *T. granulosum*.

3.2.3.2 Effects of DFMO on growth of *T. danilewskyi*

As the concentrations of DFMO present in modified SDM-79 were increased, the numbers of trypanosomes present in the media four days post-inoculation decreased (Figure 3.12), compared to *T. danilewskyi* cultured for four days in drug-free medium. In the presence of high concentrations of DFMO (50mM at day zero), after 4 days culture approximately 75% of the cells had perished compared to greater than than 90% mortality observed in *T. granulosum* cultured under similar conditions. The concentration of DFMO that resulted in 50% mortality after 4 days was 33.5 ± 1.2mM.

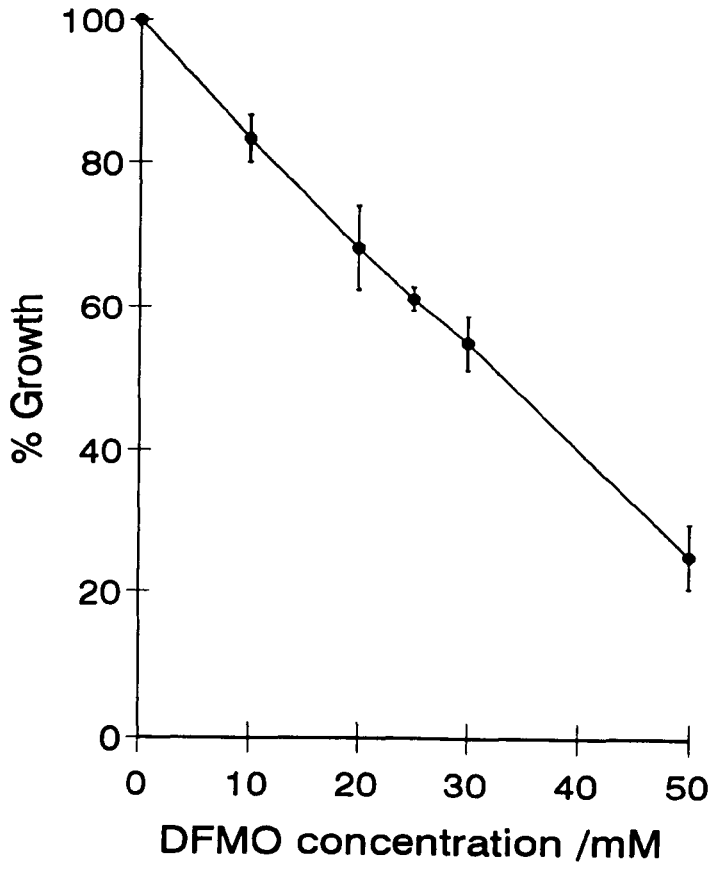
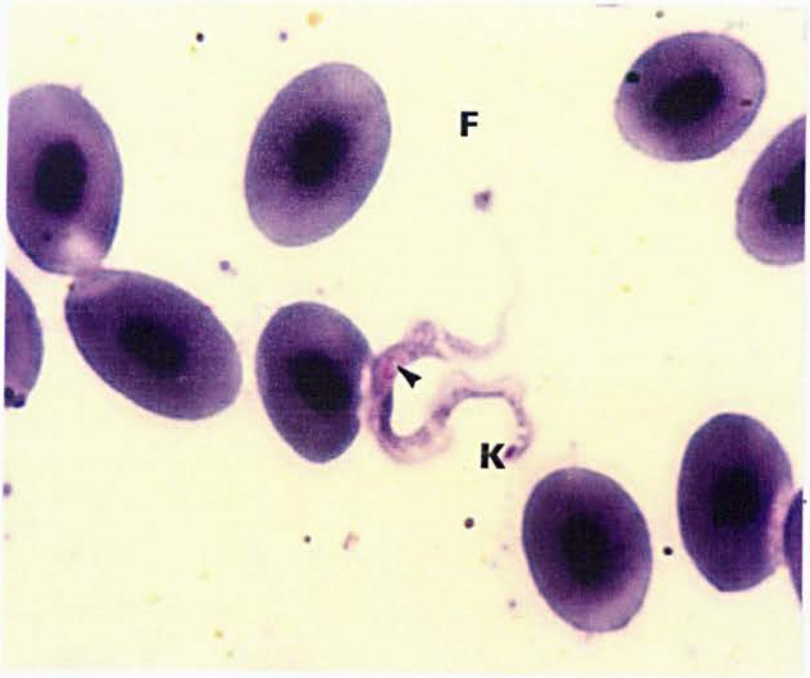


Figure 3.12 The effect of DFMO on the growth of *T. danilewskyi*. Cells were cultured for 4 days in modified SDM-79 containing a range of concentrations of DFMO. Error bars represent standard deviation where n=3.

Plate 3.55 Top: *T. danilewskyi* from the blood of crucian carp. Note the free flagellum (F), kinetoplast (K) and the nucleus (arrow). MGG (x 1700). Bottom: Gill smear from a crucian carp showing bloodstream *T. danilewskyi*. Note the undulating membrane (arrow). MGG (x 1375).


F
K

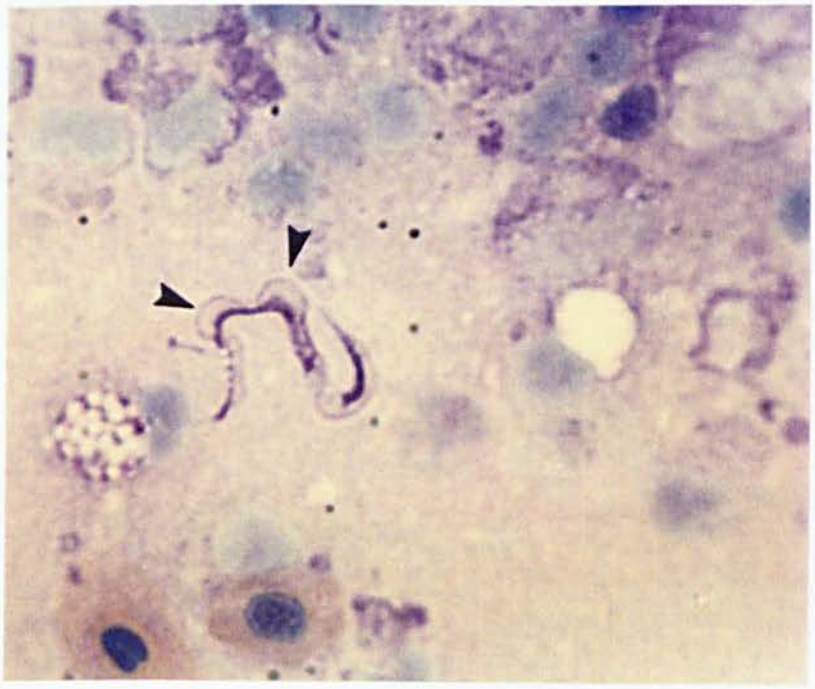
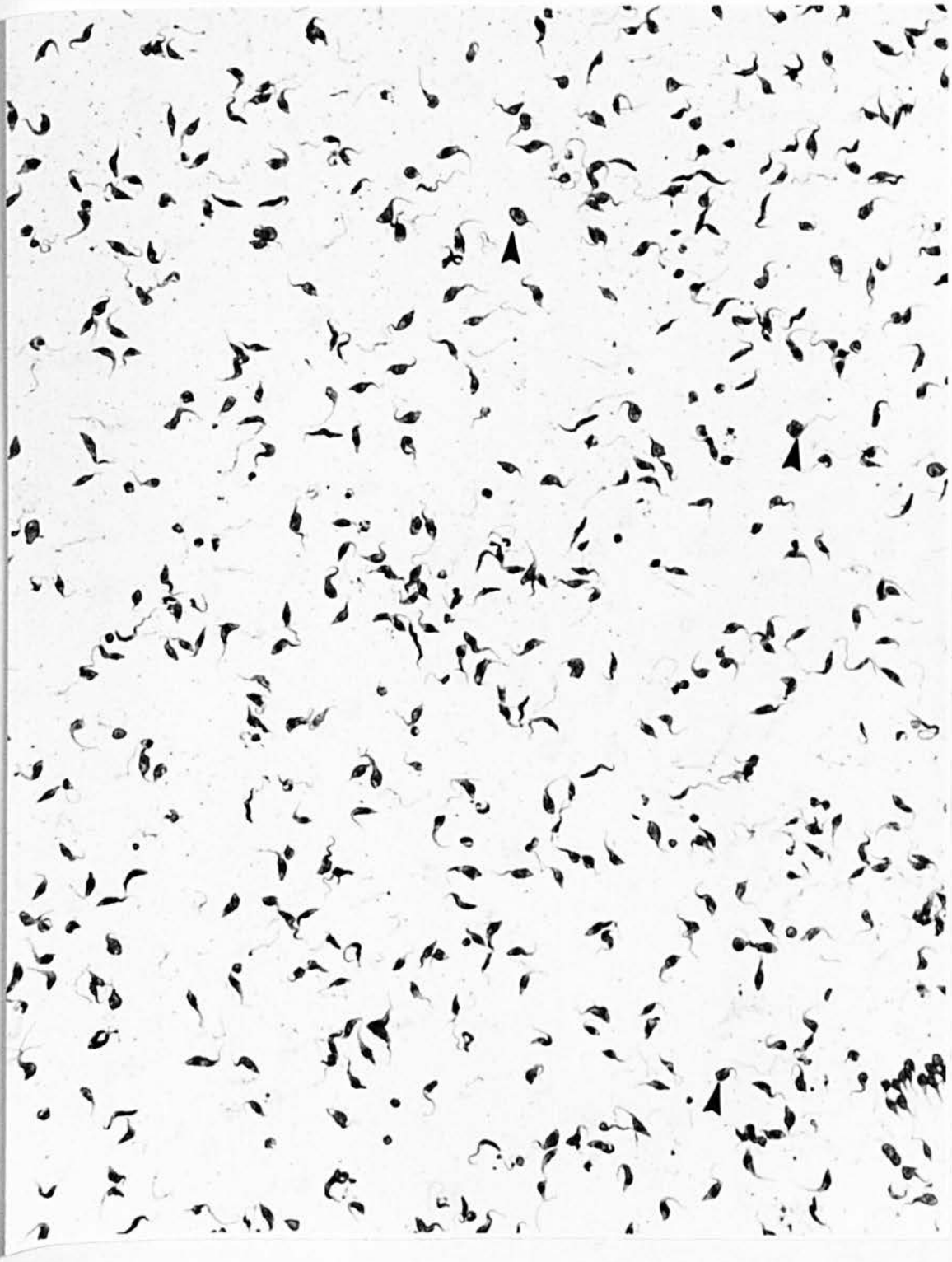


Plate 3.56 *T. danilewskyi* cultured for 4 days in modified SDM-79 containing 50mM DFMO. Note the predominance of stumpy forms and the occasional bizarre form (arrows). MGG (x 350).



CHAPTER 4: DISCUSSION

4.1 Bloodstream forms of piscine trypanosomes

The morphology of bloodstream *T. granulosum* has been reported previously (Laveran and Mesnil 1902a; Bykhovskaya-Pavlovskaya *et al.* 1962; Lom and Dyková 1992; Cruz 1995) and the morphometric measurements described in this study fall within the ranges reported by others, and appear to be similar to the large mature forms described by Needham (1969). However, it is understood that its morphology and the presence of the parasite in the blood of *Anguilla anguilla* should not be the only criteria for species identification, as indicated by Letch (1977), who noted variations in the nuclear indices and body lengths of trypanosomes of the same species. Isoenzyme and polypeptide profiles, combined with characteristics of culture, development in the invertebrate host, host specificity and morphometric analyses should all be considered when defining or identifying a species of fish trypanosome (Woo 1994).

The bloodstream forms of some species of fish trypanosomes divide in the bloodstream of their vertebrate host, for example *Trypanosoma catostomi* (Jones and Woo 1991) and *Trypanosoma cobitis* (Letch 1977). In the current study, no dividing bloodstream forms were seen, although it is known that intensity of *T. granulosum* infection in captive eels increases over long periods, suggesting that division occurs within the eel, and dividing *T. granulosum* has been reported in the blood of eels (Cruz 1995). Needham (1969), on rare occasions saw two kinetoplasts in bloodstream *T. granulosum* and the presence of dividing forms of *T. granulosum* were reported *in vitro* in blood extracted from an eel (Sabrazès and Muratet 1902), suggesting division does take place in the bloodstream.

However, it has also been suggested that division in the blood only takes place in the early part of the infection as dividing forms are not seen during chronic infections (Lom and Dyková 1992).

In this present study, the identification of *T. danilewskyi* from the blood of crucian carp was based on very few measurements as bloodstream forms were scanty. However, the measurements taken were within a range specified previously for *T. danilewskyi* (Laveran and Mesnil 1907).

The ultrastructure of bloodstream forms of *T. granulosum*, have not been reported previously. This may have arisen from difficulty obtaining sufficient numbers of the flagellate, or from the thought that its apparent lack of pathogenicity does not warrant such research effort. However in the present study, elucidation of the fine structure of the species, was considered to merit investigation, especially as it was possible to relate this to the ultrastructure of the culture forms of the same parasite. Ultrastructurally, there appear to be no major differences between the organelles of bloodstream *T. granulosum* and those present in other *Trypanosoma*, confirming the structural unity of the genus. However, the granules, so characteristic of bloodstream *T. granulosum* seen by light microscopy, were not identified ultrastructurally and mitochondria were the most prominent intracytoplasmic organelle. Lipid bodies, so obvious in culture forms (described below), were present in small quantities in the bloodstream form, suggesting perhaps a more efficient utilisation of lipids from the blood and a lack of requirement for storing lipids in large quantities.

Trypanosoma granulosum was not enveloped by a prominent surface coat as in certain

mammalian trypanosomes (Vickerman 1969), but occasionally a fuzzy surface coat was observed, which may be adsorbed foreign material on the plasmalemma, or leached cytoplasmic colloids. It was not the compact coat seen in salivarian trypanosomes, but rather like the diffuse and filamentous coat of *Trypanosoma lewisi*, *Cryptobia keysselitzi* (Vickerman 1970) and *T. danilewskyi* (Lom 1979; Lom *et al.* 1980).

The structure of the mitochondrial cristae of bloodstream *T. granulosum* differ from the structure of mitochondria in bloodstream salivarian trypanosomes. They are plate-like compared with tubular or absent cristae of bloodstream salivarian trypanosomes. It is thought that bloodstream salivarian trypanosomes gain their energy solely from ATP produced during glycolysis, as the Kreb's cycle and oxidative phosphorylation appear inoperative, and NADH is re-oxidised by a non-phosphorylating glycerophosphate oxidase system (Grant and Sargent 1960, 1961; Grant *et al.* 1961). Once salivarian trypanosomes transform to culture forms, cytochrome pigments can be detected, and oxygen uptake is sensitive to cyanide. Accompanied by this switch in respiration, is a change from tubular cristae to many plate-like cristae similar in structure to those observed in *T. granulosum*. The presence of plate-like cristae in bloodstream trypanosomes is not a feature unique to *T. granulosum*. These have been reported in some bloodstream stercorarian trypanosomes (Anderson and Ellis 1965), in bloodstream forms of *T. danilewskyi* (Lom 1979; Paterson and Woo 1984) and the anuran trypanosome *Trypanosoma andersoni* (Reilly and Woo 1982). It is also known that bloodstream *T. cruzi* possesses the enzymes of the tricarboxylic acid cycle (Dusanic 1991), but to suggest that such mitochondrial metabolism exists within *T. granulosum* purely on the appearance of mitochondrial cristae, would be speculative.

4.2 Culture forms of piscine trypanosomes

4.2.1 Cultured in drug-free media

The isolation from fish blood and subsequent growth of both *T. granulosum* and *T. danilewskyi* in diphasic medium, was similar to that described previously for *T. granulosum* (Davies *et al.* 1992; Cruz 1995) and *T. catostomi*, but greater than numbers of *Trypanosoma phaleri* cultured on blood-agar supplemented with various concentrations of NaCl, using Eagle's basal medium with foetal bovine serum as overlay (Jones and Woo 1991). Some of the nutrients present in nutrient agar (peptone 190, beef extract and yeast extract) must be essential for growth of *T. granulosum*, because no growth occurred if this agar was substituted with plain agar in blood-agar cultures. Both trypanosomes were successfully cryopreserved as described previously for *T. granulosum* (Davies *et al.* 1992) for at least 18 months, thereby removing the need to house their vertebrate hosts.

The successful growth of *T. granulosum* and *T. danilewskyi* in a semi-defined medium, modified from the SDM-79 medium used to cultivate several mammalian parasitic protozoa (Brun and Schönenberger 1979; Hunter *et al.* 1991), has been reported in this study and has a three fold advantage over *T. granulosum* and *T. danilewskyi* cultured on blood agar slopes. Firstly, because the medium was more defined, biochemical characterisation was possible, although the serum component of modified SDM-79 obviously remained undefined. Secondly, the use of culture flasks instead of bijou bottles allowed larger harvests and a non-invasive qualitative method of checking the growth of the parasites through the use of an inverted microscope. Finally, the materials needed to make the modified SDM-79 were more economical than those needed for the blood-agar.

Although the maximum cell density of *T. granulosum* cultured in modified SDM-79 was similar to that of *T. danilewskyi*, the growth rate of *T. danilewskyi* was more rapid initially, suggesting a difference in the utilisation of nutrients in the medium between the two cultured parasites. The maximum cell density observed in *T. b. brucei* procyclics cultured in SDM-79 (Brun and Schönenberger 1979) was approximately 4 fold that of *T. granulosum* and *T. danilewskyi* cultured in modified SDM-79 . However *T. b. brucei* procyclics were cultured at 27^{0}C compared to the 20^{0}C of *T. granulosum* and *T. danilewskyi*. An increase in temperature (19^{0}C-28^{0}C) has been shown to increase the growth rate of *T. granulosum* cultured in diphasic medium over 10 days (Cruz 1995), although the effect of temperature on the growth rate in modified SDM-79 has not been determined.

It is known that different batches of serum may provide different growth rates (Brun and Jenni 1987). The absence of growth of *T. granulosum* cultured in medium containing foetal calf serum from Sigma, suggests a difference between the composition of the serum obtained from Sigma and the serum obtained from Gibco which resulted in successful growth. Examination of the certificates of analyses provided by both companies for the relevant batches, revealed that Sigma foetal calf serum contains almost double the gamma globulins and almost quadruple the haemoglobin content of Gibco foetal calf serum. Details of the levels of hormones and growth factors within the sera, so important in the culture of cells, were not available, and mineral content was not available from Gibco for comparison with that provided by Sigma. Most foetal calf serum used for culturing *T. granulosum* and *T. danilewskyi* was from the same batch. However, although different batches of Gibco foetal calf serum were used, only one batch showed a significant decrease in growth of *T. granulosum*. This decrease was seen in figures 3.9 and 3.10 of

the results section and as these experiments were not in conjunction with any biochemical studies and showed good growth, it was deemed appropriate to present the results.

Attempts to replace the missing MEM F14 powder component of SDM-79 with S-MEM and two forms of MEM alpha medium, were unsuccessful possibly as a result of a change in the osmolality of the medium. It is certain that modified SDM-79 was hypotonic compared to SDM-79 or the extra additions to modified SDM-79, and it has been reported that certain fish trypanosomes and trypanoplasms prefer hypo-osmotic media (Jones and Woo 1991; Hamers 1993). Although multiplication was not particularly rapid, more than doubling of the cell number was seen in medium SDM79A. Additional ribo/deoxyribonucleosides in SDM79A compared to the other media investigated, may have had some beneficial effect on cell growth.

Failure to culture *T. granulosum* in the fully defined medium D (Dewey and Kidder 1971), used previously to grow *C. fasciculata* (Hunter *et al.* 1990, 1991), and fully defined TDL-15, used to cultivate *T. danilewskyi* (Wang and Belosovic 1994), suggests there are essential growth factors present in foetal calf serum. To date, the only fish trypanosome successfully cultured in a fully defined medium is *T. danilewskyi* isolated from crucian carp but maintained in goldfish, and cultured in TDL-15 medium (Wang and Belosovic 1994). However, when *T. danilewskyi* isolated in this laboratory was cultured in TDL-15 with/without added haemin, no growth was observed. This suggests a strain difference between our isolate and that of *T. danilewskyi* described by Wang and Belosovic (1994). The inability of different strains of the same parasite to grow in the same medium has been reported previously for *T. danilewskyi* (Islam and Woo 1992).

Insulin, present in the fully defined TDL-15 medium, and known to stimulate the growth of many cells, is also a component of foetal calf serum (Maurer 1987). Preliminary results using *T. granulosum* cultured in modified SDM-79 without additional foetal calf serum but with added insulin, have provided promising results (Mackintosh *et al.* 1994; Davies *et al.* 1995), although insulin preparations may contain polyamines which may add to the growth stimulus (for review of polyamine function see section 5.1.3). The maximum cell density of *T. danilewskyi* cultured in TDL-15 (Wang and Belosovic 1994), was approximately 10 fold lower than *T. granulosum* cultured in modified SDM-79 in the present study. This suggests that refinement is still needed for the cultivation of fish trypanosomes in defined media, which is a desirable goal if fish trypanosomes are to be studied in the absence of complicated physiological and defence systems of both the invertebrate and vertebrate hosts.

The increased growth observed when cells were cultured in the presence of the polyamine oxidase inhibitor, aminoguanidine, is likely due to the increase in polyamines available to the cells as a result of polyamine oxidase inhibition (for review of polyamine oxidase function see section 5.1.2). This increased growth in the presence of aminoguanidine has also been observed in mammalian cells (Ali-Osman and Maurer 1983). The increases in the growth rate of *T. granulosum* stimulated by the addition of putrescine and spermidine to the medium suggests a strong link between certain polyamines and growth of *T. granulosum*, however the addition of spermine did not show a significant change from controls. The addition of putrescine, spermidine and aminoguanidine to the modified SDM-79 may produce substantially higher growth but would probably be of little use for intracellular polyamine analyses.

The inoculation of mammalian bloodstream trypomastigotes into culture medium and subsequent transformation to vector forms, is a common phenomenon amongst trypanosomatids (Gray *et al.* 1987), although culture systems are now available for cultivation of salivarian bloodstream trypomastigotes (Brun *et al.* 1981; Brun and Jenni 1987) and the entire vertebrate section of the life cycle in *T. cruzi* (Baker 1987). Upon transfer into culture medium, trypanosomes from the blood of fishes transform into stages comparable to those present in the leech vector (Lom 1979; Lom and Dyková 1992). It is not known what causes the transformation, but it is assumed that either a lack of stimulus from the fish blood, or an increased stimulus from the culture system is responsible, as demonstrated in *T. b. brucei* (Hirumi *et al.* 1992) and *Trypanosoma congolense* (Ross 1987). The morphology of cultured *T. granulosum* and *T. danilewskyi* appeared different to the bloodstream forms. Culture forms of *T. granulosum* grown in modified SDM-79 were shorter and slimmer with a shorter free flagellum than blood forms. *Trypanosoma danilewskyi* were slimmer with a shorter flagellum than their blood stages, although it was accepted that only a small sample of the latter were used for comparison. The morphological similarities between cultured *T. granulosum* and cultured *T. danilewskyi* makes species identification difficult, a phenomenon observed in other cultured freshwater fish trypanosomes (Zajícek and Pecková 1995).

Although trypomastigote forms of *T. granulosum* dominated the culture from the outset, epimastigotes (not observed in any blood smears) were also present. This abundance of trypomastigotes in cultured fish trypanosomes is unusual (Lom and Dyková 1992) although not unique, as one strain of *T. danilewskyi* was cultured solely in the trypomastigote form (Islam and Woo 1992) and *T. catostomi* cultured on blood-agar medium showed a high (96%) percentage of trypomastigotes, apparently related to NaCl concentration in the

medium (Jones and Woo 1991). However, *T. danilewskyi* in fish tissue culture showed high (63-93%) levels of epimastigotes at the beginning of culture, which declined to lower levels (5-15%) in aging cultures (Smolíková *et al.* 1977) and Cruz (1995) reported epimastigotes of *T. granulosum* cultured on blood-agar slopes, dominant in the exponential phase of growth, being replaced by trypomastigotes at the end of the stationary phase. These differences in the trypomastigote to epimastigote ratios highlight the effects of different culture conditions and strains on the morphology of the parasites.

Examination of the fine structure of cultured *T. granulosum* did not reveal any major differences between *T. granulosum* and other cultured trypanosome species. A cytopharyngeal complex was not obvious, but its presence was not discounted. Prior to cell division, the elongation of the kinetoplast by lateral extension seemed to be similar to that described in another fish trypanosome, *T. cobitis* (Lewis and Ball 1981c). An increase in the presence of lipid bodies suggests the parasite does not sequest and utilise lipids in the same fashion as the bloodstream forms.

4.2.2 Effects of drugs on growth and morphology of cultured piscine trypanosomes

The growth of *T. granulosum* and *T. danilewskyi* cultured in modified SDM-79 was inhibited by high levels of DFMO in the medium (IC_{50}=16mM and 33.5mM respectively), compared to some trypanosomes, for example *T. b. brucei* procyclics (IC_{50}=0.1mM) (Bellofatto *et al.* 1987) (for review of DFMO action see section 5.1.6). Growth of *T. cruzi* trypomastigotes was unaffected by DFMO *in vitro* (Kierszenbaum *et al.* 1987) and a high concentration was needed to affect the growth of *C. fasciculata* (IC_{50}=37mM), although it is known that *C. fasciculata* can efflux DFMO therefore maintaining lower

levels of the drug intracellularly compared to the surrounding medium (Hunter *et al.* 1991). The high levels of DFMO needed to affect growth of *T. granulosum* and *T. danilewskyi* suggests DFMO is not suitable as a chemotherapeutic agent in these species of cultured fish trypanosomes, although the effects of DFMO on the polyamine metabolism of both species will be discussed in section 8.1. The data derived in this section suggests that the inhibition of cell growth by DFMO is polyamine independent, as addition of putrescine to DFMO treated *T. granulosum* did not restore cells to normal growth. One explanation may be that DFMO inhibits the putrescine uptake, so that putrescine added to the medium cannot enter the cells (for review of polyamine uptake see section 5.1.5). However, it has been demonstrated in some cells that DFMO increases putrescine uptake (Balaña Fouce *et al.* 1991a; Walters and Wojcik 1994). *Crithidia fasciculata* (Hunter *et al.* 1990) and *T. b. brucei* (Nathan *et al.* 1981), treated with DFMO show major growth inhibition, but almost normal growth is restored in the presence of putrescine.

Ornithine (structurally similar to DFMO) and sorbitol, at similar concentrations to DFMO (50mM) did not alter the growth rate of *T. granulosum*. This suggests that if ornithine enters the cells, it does not play a significant role in cell growth. Growth inhibition when cells are cultured in medium containing 50mM DFMO was therefore assumed to result from action specific to DFMO.

MGBG has been shown to inhibit the growth of a number of cell types including cultured carrot cells (Kurosaki *et al.* 1992), potato plants (Féray *et al.* 1994), *Acanthamoeba culbertstoni* (Kishore *et al.* 1990) and *T. b. brucei* (Chang *et al.* 1978). The growth of *T. granulosum* was approximately 70 fold more sensitive to the presence of MGBG in the medium than to DFMO. This suggests a more potent action by MGBG. MGBG is a

known inhibitor of S-adenosylmethionine decarboxylase, an important enzyme in the polyamine biosynthetic pathway, and its role in polyamine metabolism of *T. granulosum* will be discussed in section 8.1. The supplement of MGBG treated cells with spermidine (the immediate product of the enzymatic reaction targeted by the drug) demonstrated that inhibition of growth could be partially restored, suggesting one of the antiparasitic actions of MGBG may be related to polyamine metabolism, even though addition of spermine did not significantly alter growth. Polyamines did not reverse the growth inhibitory action of MGBG on the pathogenic amoeba *A. culbertsoni* (Kishore *et al.* 1990) or *T. b. brucei* (Chang *et al.* 1978). In addition to lowering the growth rate of *T. granulosum* (described above), a change in the batch of foetal calf serum also increased the IC_{50} concentrations of both MGBG and Berenil (Figures 3.10).

The effect of Berenil in the medium was more marked on the growth of *T. granulosum* than either DFMO or MGBG. The action of Berenil appears to be particularly related to polyamine metabolism (see section 5.1.6), as addition of both spermidine and spermine significantly increased the growth of Berenil treated cells, although addition of polyamines to the drug-treated cells did not completely reverse the effects of Berenil on growth of *T. granulosum*, possibly because of the relative concentrations of intracellular Berenil and polyamines or the action of Berenil at other sites within the parasite. This reversal of Berenil action by addition of polyamines is also true of *T. b. brucei* (Bitonti *et al.* 1986). However *A. culbertsoni* did not show reversal of Berenil-induced growth inhibition when polyamines were added to the cells (Kishore *et al.* 1990).

The polyamine biosynthesis inhibitor DFMO, caused significant changes to the morphology of both *T. granulosum* and *T. danilewskyi*. Shortening and broadening has

been reported in a number of mammalian trypanosome species (Bacchi *et al.* 1983; Giffin *et al.* 1986), however the balloon-like bizarre shapes of *T. granulosum* appeared to resemble aborted cytokinesis, similar to that described in *T. b. brucei* cultured in medium containing DFMO (Giffin *et al.* 1986). Interestingly, incubation of bloodstream *T. brucei* in the presence of the pyruvate transport inhibitor UK5099, caused a change in morphology similar to that of *T. granulosum* cultured in medium containing high (50mM) levels of DFMO. It was suggested that the change induced by UK5099 resulted from acidification of the cytosol and a change in the osmolarity of the cell (Wiemer *et al.* 1995). It may be that the presence of DFMO causes a change in the osmolarity of the cells leading to changes in morphology. Swelling does not seem to be due directly to an increased concentration of intracellular DFMO, as *T. danilewskyi* did not show similar morphological changes at high concentrations of DFMO, suggesting a difference in the potency of DFMO to the two species of cultured fish trypanosome. The effects of DFMO on the polyamine metabolism of both *T. granulosum* and *T. danilewskyi* will be discussed in section 8.1.

When *T. granulosum* was cultured in the presence of DFMO, the appearance ultrastructurally of cytoplasmic vacuoles, which became larger as the concentration of DFMO in the medium increased, was possibly caused by an osmotic imbalance. Although the mechanism of this effect is unknown, if DFMO is involved in the inhibition of protein synthesis, then the integrity of membrane proteins may be compromised, possibly altering the osmotic balance of the cells. The presence of inner membranes within the expanded vacuoles can not be explained, although similar structures appeared when bloodstream *T. b. rhodesiense* were treated with pentamidine (Macadam and Williamson 1972), and leukaemic cells treated with MGBG, which were reported to be distorted mitochondria,

the smaller vacuoles within the outer vacuoles possibly being the remnants of mitochondrial cristae (Mikles-Robertson *et al.* 1979). Changes in the nucleus and kinetoplast seen in bloodstream *T. b. rhodesiense* treated with DFMO (de Gee *et al.* 1984) were not observed in cultured *T. granulosum.*

The morphological changes seen by light microscopy in *T. granulosum* cultured in medium containing MGBG were similar to the changes described when the parasite was cultured with DFMO, although changes occurred at lower concentrations than with DFMO. Ultrastructurally, a decrease in cytoplasmic ribosomes in some cells suggests MGBG may be involved in inhibition of protein synthesis, but the effect of MGBG on polyamine metabolism will be discussed in a future section. The appearance of large cytoplasmic vacuoles may be explained by distorted mitochondria described earlier when *T. granulosum* was cultured in the presence of DFMO. At higher concentrations (1mM) of MGBG, the partial disruption of the kinetoplast seen in electron micrographs may result from the direct action of MGBG on this structure, as MGBG is known to bind to DNA (Dave *et al.* 1977).

The morphological changes seen by light microscopy described in *T. granulosum* cultured in medium containing Berenil, were similar to those seen in both DFMO and MGBG treated cells. Berenil, when present at similar concentrations to MGBG, appeared to affect the cells to a greater extent than MGBG. Berenil appears to exert an osmotic effect on the cell causing swelling, however the mechanism for this is unknown. The effects of Berenil on the polyamine metabolism of *T. granulosum* will be discussed in a future section. The ultrastructural changes observed in *T. granulosum* cultured in medium containing Berenil were similar to those changes caused by both DFMO and MGBG,

except at low concentrations (0.2mM) the cellular disruption was greater than in cells cultured in medium containing MGBG, suggesting more potent action by Berenil. Berenil also caused disaggregation of the kinetoplast, a phenomenon observed in bloodstream *T. b. rhodesiense* treated with Berenil (Macadam and Williamson 1972). On one occasion elongation of the nucleus and swelling of the nuclear membrane was observed in *T. granulosum* cultured in medium containing Berenil, possibly caused by binding of the drug to DNA. The presence of extra underlying sub-pellicular microtubules in one area of a cell, a phenomenon also observed in cultured *T. rangeli* (Tejero *et al.* 1994) was probably not peculiar to Berenil treated cells, although it has not been observed in *T. granulosum* cultured in drug-free medium. In the presence of 1mM Berenil the lack of intact nuclei, mitochondria or kinetoplasts indicated the beginning of cell death although even at 1mM concentrations the cells appeared as motile as cells cultured in drug-free medium, when viewed under an inverted microscope.

SECTION II

CHAPTER 5: INTRODUCTION

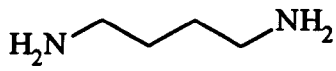
5.1 Introduction to polyamines

Polyamines are aliphatic amines which are ubiquitous in biological material, although their presence and concentration differ markedly between cell types. Of the numerous known polyamines found in nature, putrescine, spermidine and spermine are the three most common (Figure 5.1). Spermine, as its name suggests, was originally discovered as phosphate crystals in semen by Lewenhoeck in 1677. Although the abundance of this and other polyamines in semen is high when compared to other body fluids, such as blood plasma, the function of polyamines within semen is uncertain (Williams-Ashman and Canellakis 1979). Polyamines have been studied in a variety of cell types. These have included mammalian cells in general, protozoa, fungi and bacteria. They occur in high concentrations in actively proliferating tissues such as regenerating liver (Dykstra and Herbst 1965; Russell and McVicker 1971; Hölltä and Jänne 1972), neoplastic cells (Russell and Levy 1971; Russell 1971), and during embryonic development (Raina 1963). Such studies have provided evidence that a rise in polyamine levels is linked to an increase in RNA and protein synthesis.

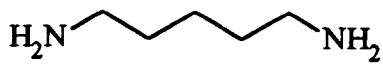
5.1.1 Polyamine biosynthesis

Putrescine (1,4-diaminobutane) is the first polyamine in the polyamine biosynthetic pathway and it is synthesised in two ways. First, putrescine can be formed by the decarboxylation of arginine by arginine decarboxylase (ADC) followed by the hydrolysis of the resultant agmatine (surrounded by a dotted line in Figure 5.2). This pathway is

Diamines

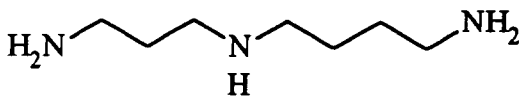


Putrescine
(1,4-diaminobutane)

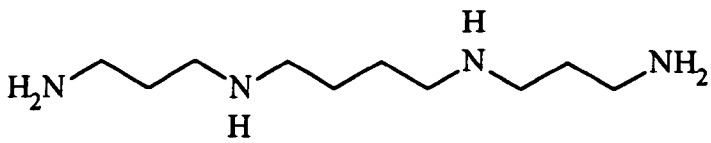


Cadaverine
(1,5-diaminopentane)

Polyamines



Spermidine



Spermine

Figure 5.1 Structures of some naturally occurring diamines and polyamines.

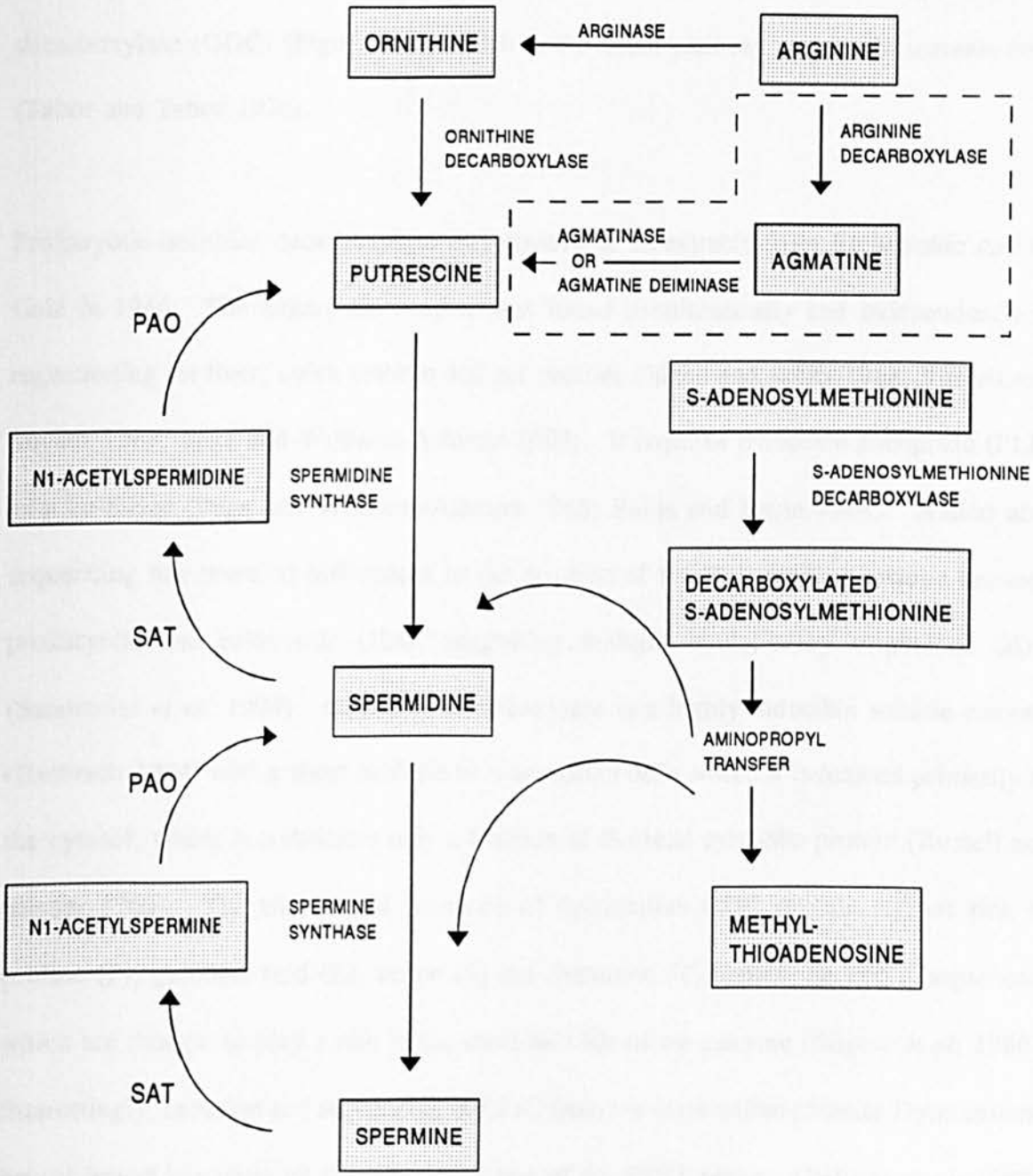


Figure 5.2 A general diagram showing the polyamine biosynthetic pathway. SAT=spermine/spermidine N-acetyltransferase, PAO=polyamine oxidase (adapted from McCann *et al.* 1987).

often found in higher plants, bacteria (Kallio *et al.* 1981) and possibly *Trypanosoma cruzi* (Kiersenbaum *et al.* 1987; Majumder *et al.* 1992; Yakubu *et al.* 1992). Alternatively, putrescine can be synthesised by the decarboxylation of ornithine by ornithine decarboxylase (ODC) (Figure 5.2), which is the usual pathway present in animals cells (Tabor and Tabor 1976).

Prokaryotic ornithine decarboxylase was discovered in extracts from *Escherichia coli* by Gale in 1946. The eukaryotic enzyme was found simultaneously and independently in regenerating rat liver, chick embryo and rat prostate (Jänne and Raina 1968; Russell and Snyder 1968; Pegg and Williams-Ashman 1968). It requires pyridoxal phosphate (PLP) as a co-factor (Pegg and Williams-Ashman 1968; Raina and Jänne 1968). Amino acid sequencing has revealed differences in the position of the PLP binding residue between prokaryotic and eukaryotic ODC, suggesting multiple evolutionary origins of ODC (Sandmeier *et al.* 1994). Ornithine decarboxylase is a highly inducible soluble enzyme (Bachrach 1984) with a short half-life in mammalian cells where it is located primarily in the cytosol, where it constitutes only a fraction of the total cytosolic protein (Russell and Snyder 1969). The amino acid sequence of mammalian ODC reveals regions rich in proline (P), glutamic acid (E), serine (S) and threonine (T), called the PEST sequences, which are thought to play a role in the short half life of the enzyme (Rogers *et al.* 1986). Interestingly, isolation and sequencing of ODC from the mammalian parasite *Trypanosoma brucei brucei* has revealed the absence of one of the PEST regions (Phillips *et al.* 1987), possibly explaining the slower turnover of the parasite ODC compared with its mammalian counterpart.

Putrescine, the product of ornithine decarboxylation, can be converted into spermidine with the addition of an aminopropyl group from decarboxylated S-adenosylmethionine (dcSAM), itself a product of the decarboxylation of S-adenosylmethionine (SAM) by SAM decarboxylase (SAMdc) (Figure 5.2). This enzyme (SAMdc), uses pyridoxal pyruvate as a co-factor in contrast to the pyridoxal phosphate required by ODC (Wickner *et al.* 1970). The reactions involving the additions of one aminopropyl group to putrescine for spermidine and the addition of another aminopropyl group to a spermidine molecule for spermine are catalysed by aminopropyltransferases namely spermidine and spermine synthetase respectively (Tabor and Tabor 1976; Jänne *et al.* 1978). As the aminopropyl group is transferred to putrescine and spermidine molecules, the high energy sulphonium ion of dcSAM is converted to methylthioadenosine (MTA) in equimolar amounts to the polyamine products (Figure 5.2). Adenine is then salvaged by the action of MTA phosphorylase and returned to the purine nucleoside pool (Pegg 1986).

Conversion of ornithine to putrescine in the presence of ODC, and the subsequent addition of one or two aminopropyl groups to form spermidine and spermine is the main route of polyamine biosynthesis in mammals. Amongst trypanosomatids however, polyamine content and biosynthesis mechanisms may be different. Using radiolabelled amino acid precursors in bloodstream *T. b. brucei* (Bacchi *et al.* 1979) and *Leishmania* spp. promastigotes (Bachrach *et al.* 1979a) it has been shown that ornithine is converted to putrescine and spermidine. Spermidine is then converted to spermine in *Leishmania* spp. promastigotes (Bachrach *et al.* 1979a) but not in the bloodstream trypomastigote of *T. b. brucei* (Bacchi *et al.* 1979). It is now accepted that the presence and the concentrations of the three main polyamines putrescine, spermidine and spermine differ among species, culture and bloodstream forms of some trypanosomatids (Bacchi 1981). Polyamines have

been studied in a number of parasitic flagellates including promastigotes of *Leishmania* spp., *L. tropica major*, *L. mexicana* and *L. donovani* (Bachrach *et al.* 1979a, 1979b), *L. infantum* (Balaña-Fouce *et al.* 1991b), *T. b. rhodesiense* (Bacchi *et al.* 1993), *T. b. brucei* (Byers *et al.* 1991), *C. fasciculata* (Hunter *et al.* 1991) and *T. cruzi* (Schwarcz de Tarlovsky *et al.* 1993).

5.1.2 Catabolism of polyamines

The aminopropyltransferase reactions described above are reversible as both spermidine and spermine can be converted back to putrescine via two enzymes, spermine/spermidine-N^1-acetyltransferase (SAT) and flavin adenine dinucleotide (FAD)-dependent polyamine oxidase. The first part of this catabolic pathway involves the conversion of spermine to N^1-acetylspermine by SAT. The second part is the oxidation of N^1-acetylspermine by FAD-dependent polyamine oxidase to yield spermidine and 3-acetamidopropanal. Similarly, N^1-acetylspermidine is the product of spermidine conversion by SAT, which is then converted into putrescine and 3-acetamidopropanal by FAD-dependent polyamine oxidase. Histone acetylase, a predominantly nuclear enzyme also catalyses polyamine acetylation, but virtually all the product of spermidine acetylation by histone acetylase is N^8-acetylspermidine. Conversely N^1-acetylspermidine is the only product of spermidine conversion by SAT, as histones are not substrates of SAT (Della Ragione and Pegg 1983; Della Ragione *et al.* 1983). Since polyamine oxidase activity is usually much greater than SAT activity, levels of intracellular N^1-acetylated polyamines are usually quite low (Pegg 1986).

5.1.3 Functions of polyamines

Two physicochemical attributes of polyamines have helped elucidate their interactions with other cellular constituents. Firstly, at physiological pH, polyamines are largely protonated at their amino groups, exhibiting net positive charges. Secondly, polyamines exhibit considerable conformational flexibility due to the rotations around their carbon-carbon and their carbon-nitrogen bonds. These physicochemical properties allow the polyamines to associate with a variety of negatively charged molecules including nucleic acids, phospholipids and proteins. It is these associations that seem to account for the direct actions of polyamines on cellular events (for reviews see Tabor and Tabor 1972; Bachrach 1973; Tabor and Tabor 1976; Williams-Ashman and Canellakis 1979).

5.1.3.1 Polyamines and cell membranes

Numerous early investigations demonstrated that spermine in low concentrations was able to protect certain gram negative bacteria from osmotic damage. This stabilisation was also seen in erythrocyte membranes, where polyamines reduced the effects of complement and made the membranes more resistant to fragmentation (Bachrach 1973). These results suggested that polyamines maintained membrane structure, possibly by binding to the acidic groups of membrane proteins, and thereby reduce repulsive forces without reducing cohesive forces (Tabor and Tabor 1976; Marton and Morris 1987).

5.1.3.2 Polyamines and the stabilisation of nucleic acids

The interactions of polyamines with nucleic acids have been examined using techniques

such as x-ray diffraction, optical rotatory dispersion, electron microscopy, nuclear magnetic resonance and circular dichroism. These interactions result in the stabilisation of the structures of both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Bachrach 1973, Tabor and Tabor 1972; Tabor and Tabor 1976). However, when evaluating the evidence gained from such studies, possible redistribution of polyamines following cellular disruption must be considered (Tabor and Tabor 1972).

One proposed method of association between polyamines and nucleic acids, involves electrostatic linkages between the positively charged primary or secondary amino groups of the polyamines and the negatively charged highly acidic phosphate groups of the nucleic acid. This results in the neutralisation of those negatively charged groups which usually repel each other therefore increasing the strength of the tertiary structure of the nucleic acid, for example in the double helical structure of DNA (Bachrach 1973). Other associations between polyamines and nucleic acids include hydrogen bonding formed in the narrow groove of DNA between the protonated amino groups of the polyamines and the oxygens of the phosphate groups present on the backbone of the DNA helix (Liquori *et al.* 1967). For example, it has been demonstrated *in vitro* that putrescine can form covalent links with thymine residues in DNA from bacteriophages (Kropinski *et al.* 1973). In addition, using X-ray analyses, intermolecular distances were measured to reveal that a bridge was formed by the polyamines between the two strands of the nucleic acid (Tsoboi 1964). More recently, spermine has been associated with binding to DNA in the major groove further suggesting polyamines play an important role in stabilising the structure of DNA (Feuerstein *et al.* 1986). Further evidence implies that spermidine stabilises isolated supercoiled DNA conformations from *E. coli* even under thermally induced unfolding conditions (Flink and Pettijohn 1975).

The ability of polyamines to influence the structure and activity of tRNA has been studied because changes in the structure of transfer RNA (tRNA) can have adverse effects on protein synthesis (see Tabor and Tabor 1984). It seems that the presence of polyamines facilitates the formation of a more compact, active form of tRNA. Cationic binding to tRNA was thought to be carried out entirely by magnesium cations being tightly bound to tRNA, although it was known that the requirement could be met by other cations for example calcium, cobalt, zinc and manganese (Loftfield *et al.* 1981a). Currently it is considered that polyamines bind to tRNA non-specifically due to electrostatic charges and hydrogen bonding, and *in vitro* experiments on tRNA extracted from *E. coli* have shown that spermine binds more strongly to tRNA than spermidine (Frydman *et al.* 1992).

5.1.3.3 Polyamines and nucleic acid synthesis

Much evidence exists for a relationship between polyamines and synthesis of nucleic acids (see Bachrach 1973; Tabor and Tabor 1976). In a variety of *in vivo* systems a rise in polyamine levels coincides with a rise in DNA and RNA synthesis. This was seen in developing chick embryos (Raina 1963; Russell and Lombardini 1971), in regenerating rat liver after partial hepatectomy (Dykstra and Herbst 1965; Russell and Lombardini 1971) and in rat liver treated with growth hormone (Jänne *et al.* 1968). However, these results only suggested polyamine involvement in nucleic acid synthesis and did not provide evidence for direct causal involvement. More definitive *in vivo* experiments were performed on developing chick embryos providing evidence for a causal relationship between polyamines and nucleic acid synthesis (Caldarera *et al.* 1965). These experiments included injection of spermine into the yolk sac of chick embryos. This resulted in high polyamine oxidase activity which lowered both polyamine and nucleic acid concentrations

within the embryo. However, in the presence of iproniazid (a polyamine oxidase inhibitor), polyamine and subsequent RNA and DNA concentrations in the chick embryo increased. When spermine was injected into the yolk sac in the presence of iproniazid a greater increase in RNA and DNA concentrations was observed compared to the levels of RNA and DNA with just iproniazid present (Caldarera *et al.* 1965). Similar experiments were performed on the same model but polyamine concentrations were altered by injecting an amine oxidase (lowering polyamine levels) or iproniazid (raising polyamine levels) into the yolk sac of the chick embryo. The changes observed in the polyamine levels were accompanied by alterations in the synthesis of DNA and RNA in the developing chick embryo (Moruzzi *et al.* 1968).

The discovery of inhibitors of polyamine biosynthesis has enabled workers to examine the potential regulatory role of polyamines in the synthesis of nucleic acids. In the majority of these experiments, a decrease in polyamine levels was accompanied by a corresponding decrease in DNA synthesis (Canellakis *et al.* 1979). Pegg (1978) demonstrated an inhibition of DNA synthesis in rat liver by intraperitoneal administration of 1,1'-[(methylethanediylidene)-dinitrilo]bis(3-aminoguanidine) (MBAG), an inhibitor of S-adenosylmethionine decarboxylase. Impairment of DNA synthesis in regenerating rat liver was also induced through inhibition of spermidine production *in vivo* by administration of 1,3-diaminopropane (an inhibitor of ODC) together with MBAG (Wiegand and Pegg 1978). However, it may be difficult to dissociate the inhibition of polyamine metabolism and subsequent nucleic acid synthesis from other toxic effects of the drugs. For example, cultured lymphocytes treated with MGBG showed inhibition of DNA synthesis (Knutson and Morris 1978). The inhibition observed may not have been due to decreased polyamine levels as it is known that MGBG has other intracellular actions, including causing severe

mitochondrial damage to a number of cell types (Pegg and Williams-Ashman 1987).

Numerous *in vitro* studies have shown that polyamines stimulate DNA synthesis in both crude and purified DNA synthesising systems (see Tabor and Tabor 1976). For example putrescine, spermidine and spermine can stimulate the activity of the mammalian enzyme DNA polymerase B, with spermine showing the greatest stimulation. Although the mechanism of stimulation is not understood, it is not due to the inhibition of deoxyribonuclease and phosphodiesterase, or the replacement of magnesium ions. It is likely that polyamines stabilise the structure of DNA polymerase B and the order of stimulation is parallelled by the order of molecular size of the polyamines as well as by the number of nitrogen atoms (Chiu and Sung 1972). Studies using *E. coli* polyamine deficient mutants cultured in 2-(*N*-morpholino)-propanesulphonic acid (MOPS) medium in the absence of putrescine, exhibited reduced DNA replication fork movement compared to *E. coli* cultured in the presence of putrescine. Polyamines are therefore thought to act as co-factors in DNA replication (Geiger and Morris 1978). Other *in vitro* studies have shown that polyamines can stimulate DNA-dependent RNA polymerases in numerous models (Tabor and Tabor 1976). This has included stimulation of DNA-dependent RNA polymerase from *E. coli* (Herbst *et al.* 1973) and marked increases in the activities of purified RNA polymerases I and II in the presence of spermidine and spermine (Jänne *et al.* 1975).

Replication proteins for example, primases, helicases and topoisomerases must also be considered as possible sites for interaction with polyamines, as it has been demonstrated *in vitro* that topoisomerases from *E. coli* can be either inhibited or stimulated by polyamines, depending on whether the enzymes act on single or double stranded regions

of the supercoiled DNA. This interaction is of some importance as it is known that topoisomerases are necessary for chromosome segregation at cell division (Srivenugopal and Morris 1985).

5.1.3.4 The effects of polyamines on translation

Many studies have shown that polyamines stimulate several of the intermediate steps of protein synthesis *in vitro*, and a smaller number of experiments have demonstrated this effect *in vivo* (see Tabor and Tabor 1972; Bachrach 1973; Tabor and Tabor 1984).

The activation of amino acids and their subsequent linkage to tRNAs are catalysed by specific aminoacyl-tRNA synthetases which have been shown to vary in their requirements for divalent cations and polyamines (see Tabor and Tabor 1976; Marton and Morris 1987). Detailed kinetic *in vitro* studies have shown that two spermine molecules are bound to purified phenylalanine-tRNA (Phe-tRNA) from yeast and that the resulting compact structure easily forms complexes with Phe-tRNA synthetase, thus enhancing acylation. Phe-tRNA in the absence of spermine but in the presence of physiological concentrations of Mg^{2+}, was more slowly acetylated than Phe-tRNA containing bound spermine suggesting Mg^{2+} could not entirely replace the function of spermine in aminoacylation (Loftfield *et al.* 1981b). However, not all aminoacyl-tRNA synthetases are stimulated by polyamines (see Tabor and Tabor 1984).

Polyamines have a role in maintaining the association between the 30S and 50S subunits of ribosomes from *E. coli*. They can replace Mg^{2+} at approximately 80% of the cationic binding sites of the ribosomes, but if the remaining 20% of cationic binding sites are

replaced by polyamines a loss of activity occurs and extensive structural changes to the ribosome lead to inactivation of the ribosome (Weiss and Morris 1973; Kimes and Morris 1973). However, manganese ions (Mn^{2+}) or calcium ions (Ca^{2+}) are able to replace Mg^{2+} depending on whether the 30S or 50S subunit's cations are replaced (Weiss *et al.* 1973). The above data suggests that the role of maintenance of structural integrity and subunit stability of the ribosomes by polyamines, could be replaced by inorganic divalent cations. Further experiments demonstrated that this does not occur. Polyamine deficient *E. coli* mutants contain ribosomes that are less active in protein biosynthesis than wild type organisms, even under optimum concentrations of Mg^{2+} (Echandi and Algranati 1975b). These *in vitro* studies with *E. coli* mutants have suggested that polyamines are essential for optimal protein synthesis. Other studies demonstrated that the decrease in protein synthesis described resulted from a defect in the 30S subunit of the ribosome (Echandi and Algranati 1975a) possibly at the site of polypeptide initiation (Algranati and Goldemberg 1977).

Equally as important as the effect of polyamines on the rate of protein synthesis, is the frequency with which amino acids are correctly incorporated into a nascent polypeptide chain (translational fidelity). Studies both on cell-free and intact cell systems suggest an important role for polyamines in translational fidelity (Loftfield *et al.* 1981a; see Tabor and Tabor 1984; Marton and Morris 1987). The addition of putrescine, spermidine and spermine to a cell free mammalian translation system permitted more efficient protein synthesis both in quantity and quality of polypeptides, further emphasising a role for polyamines in fidelity of translation. This addition of polyamines was also found to stimulate the synthesis of high-molecular-weight proteins to a greater extent than the stimulation of the synthesis of low-molecular-weight proteins. This difference implied that

an absence of polyamines *in vitro* may be responsible for premature termination of polypeptide elongation, possibly resulting from sluggish elongation or abnormalities in translational fidelity (Atkins *et al.* 1975). In cell-free extracts of some thermophilic bacteria, MS2 phage RNA translation products could not be produced in the absence of polyamines (Uzawa *et al.* 1993).

5.1.4 Polyamines and the cell cycle

Changes in polyamine levels and ODC activity occur throughout the cell cycle reinforcing the notion that polyamines play an important role in DNA replication. Studies on the levels of polyamines at different stages in the cell cycle have provided a greater understanding of their role in cellular proliferation and may provide information useful for controlling such proliferation. In continuously proliferating cell populations it has been shown that polyamine synthesis is initiated in late G1-phase just prior to S-phase (the stage of DNA synthesis) and prior to cell division in the late S/G2 phase. However, in some cells stimulated to proliferate *in vitro* from a quiescent state, an increase in ODC activity and subsequent rise in polyamine levels is detected in early G1 phase, a phenomenon not observed in continuously dividing cells. This suggests that rises in polyamines at different points in the cell cycle may be characteristic of cellular function (Fuller *et al.* 1977; Heby and Andersson 1980). Furthermore, in the algal flagellate *Euglena gracilis* polyamine levels increase in the G1 phase of the cell cycle, in the S/G2 phases prior to cell division, and a third minor rise in polyamine levels occurs in the pre-G1 phase (Adlakha *et al.* 1980).

5.1.5 Uptake of polyamines

Polyamine homeostasis is principally controlled by synthetic and catabolic reactions. However, it is known that uptake and excretion of polyamines from and to the cell's external environment is partially responsible for maintaining this homeostasis. Studies on a variety of mammalian cells (Seiler and Dezeure 1990) including rat enterocytes (Kumagai and Johnson 1988), pig aortic endothelial cells (Bogle *et al.* 1994), human pulmonary artery endothelial cells (Sokol *et al.* 1993), isolated rat pancreatic acini (Alves *et al.* 1992; Stüber *et al.* 1993) and human umbilical cords (Morgan 1992), have shown that polyamine uptake in these cell types appears to be via a saturable energy-dependent process. These conclusions were based upon the following observations: firstly, that polyamine uptake in a number of these studies was shown to be temperature dependent; secondly, that several experiments have shown a reduction in polyamine uptake in the presence of metabolic inhibitors for example potassium cyanide (KCN) and 2,4-dinitrophenol. Studies on carrot protoplasts (Antognoni *et al.* 1994), yeast cells (Kakinuma *et al.* 1992) and a number of parasitic protozoa including *Leishmania infantum* promastigotes (Balaña-Fouce *et al.* 1989), *L. mexicana mexicana* promastigotes, *C. fasciculata* (González *et al.* 1992; González *et al.* 1993), *T. cruzi* epimastigotes (Le Quesne and Fairlamb 1993, 1994) have also revealed a saturable energy-dependent polyamine uptake process.

In parasitic protozoans devoid of polyamine biosynthetic enzymes, polyamine uptake and transport mechanisms may be a route for exploitation by chemotherapy. Studies on the polyamine transport mechanisms of mouse neuroblastoma cells (Rinehart and Chen 1984), rat pancreatic tumour (Nicolet *et al.* 1990) and human leukaemic cells (Walters and

Wojcik 1994; Khan *et al.* 1994) have also revealed saturable, energy dependent mechanism for polyamine uptake which could have important therapeutic implications. Difficulty arises in interpreting the above results as it is not known whether the energy dependence is exerted directly or indirectly on the transporter, and the results are further complicated if metabolism of the polyamines take place. One exception to the rule that polyamines are taken up by energy dependent processes are results from polyamine accumulation in isolated perfused rat lungs which suggested that polyamines enter the lungs via a simple diffusion mechanism rather than an active polyamine uptake process (Rao and Mehendale 1988).

Many polyamine uptake systems appear to require sulphydryl groups for maximal activity, for example in *L. mexicana mexicana* promastigotes (González *et al.* 1992). However, interpretation of polyamine uptake inhibition by use of the sulphydryl reagent N-ethylmaleimide must be treated with caution as the permeant nature of the reagent makes it difficult to distinguish its interaction with the membrane components from its effects on cellular metabolism (Rothstein 1970). The use of the rather impermeable sulphydryl reagent *p*-chloromercuribenzene sulphonate (pCMBS) presumably overcomes this problem. In NB15 neuroblastoma cells, the inhibition of putrescine uptake by pCMBS is decreased by the addition of the reduced sulphydryl compound dithiothreitol (DTT) (Rinehart and Chen 1984). This provides strong evidence that the sulphydryl groups of certain membrane proteins, possibly on the transporter itself, could be involved in polyamine uptake.

5.1.6 Inhibition of polyamine metabolism

Over recent years there has been an increasing awareness that the biosynthesis of polyamines provides a useful target for the design of inhibitors which may be of value as pharmaceutical agents.

As ornithine decarboxylase (ODC) is thought to be the major rate-limiting enzyme in the polyamine biosynthetic pathway, much effort has been focused on the design of specific inhibitors of this enzyme (Koch-Weser *et al.* 1981). The inhibitors of ODC can be placed into two distinct classes, reversible and irreversible. The reversible inhibitors are mainly analogues of ornithine or putrescine. Of fourteen potential inhibitors evaluated, α-methylornithine, an analogue of the amino acid ornithine, has proved to be the most potent reversible inhibitor (Bey *et al.* 1978). Other types of reversible inhibitors, for example L-canaline and α-hydrazinoornithine, are molecules capable of interacting or combining with pyridoxal phosphate (PLP), which acts as a loosely bound cofactor in the decarboxylation of ornithine. The formation of a Schiff's base between the amino group of the substrate and the aldehyde group of the PLP is mandatory in reactions catalysed by ODC (Bey *et al.* 1987) and any blockade of this reaction would result in decreased catalysis of ornithine.

A major disadvantage of competitive reversible inhibitors is that upon metabolic clearance of the inhibitor, there is a supernormal rate of putrescine production due to accumulation of ODC during the inhibitory period (Heby and Jänne 1981) and such shortcomings prompted the search for irreversible inhibitors. One inhibitor of this type is DL-α-difluoromethylornithine (DFMO; Ornidyl®), which is thought to bind to PLP to form a Schiff's base. The ornithine analogue is then decarboxylated resulting in a conjugated

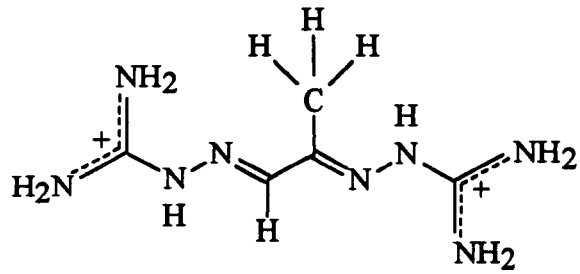
imine (a highly reactive electrophilic intermediate) which inactivates the enzyme through alkylating an hypothetical nucleophilic residue at or near to the active site of the enzyme. Thus the inhibitor is covalently bound to the enzyme resulting in irreversible inhibition (Oredsson *et al.* 1980).

DFMO has demonstrated antiprotozoal properties against several parasites including the human pathogens *T. b. rhodesiense* (Bacchi *et al.* 1993) and *T. cruzi* (Scharcz de Tarlovsky *et al.* 1993), the insect trypanosomatid *C. fasciculata* (Hunter *et al.* 1991), the canine parasite *L. infantum* (Balaña-Fouce *et al.* 1991; Carrera *et al.* 1994) the chicken parasite *Eimeria tenella*, *Plasmodium falciparum* (Bacchi and McCann 1987), *L. mexicana* (González *et al.* 1991) and on short term rapidly fatal *T. b. brucei* infections (Bacchi *et al.* 1980). The basis of these antiprotozoal properties are the biochemical consequences of ODC inhibition and induction of gross morphological transformations (Bacchi *et al.* 1983; Giffin *et al.* 1986). Further evidence suggests that DFMO plays an important role in the methylation index of the cells by changing levels of SAM, dcSAM and S-adenosylhomocysteine (SAH) (Yarlett and Bacchi 1988; Byers *et al.* 1991).

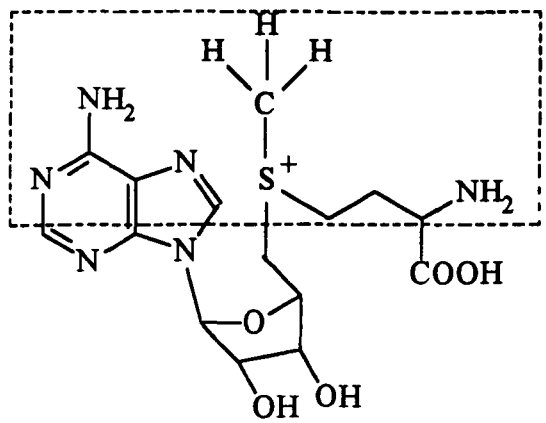
Inhibitors of SAMdc, another rate limiting enzyme in the polyamine biosynthetic pathway have also been developed. The anticancer drug MGBG, which bears a structural resemblance to spermidine and to a lesser degree SAM (Figure 5.3) competitively inhibits the SAMdc's of rat ventral prostate and baker's yeast *in vitro* (Williams-Ashman and Schenone 1972). SAMdc from parasitic protozoa, for example *T. b. brucei* (Bitonti *et al.* 1986) also show decreased spermidine and spermine concentrations but increased putrescine concentrations, when cultured with MGBG. Increase in putrescine is not solely due to the enzyme block but also to the stimulation of ODC and inhibition of diamine

oxidase by MGBG (Jänne *et al.* 1991).

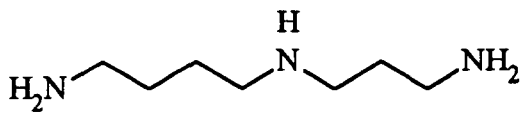
The development of compounds based on the MGBG structure has continued and more potent inhibitors have been designed. EGBG, an ethyl analogue of the parent drug 1,1'-[(methylethanediylidene)dinitrilo]bis(3-aminoguanidine)(MBAG) is an irreversible inhibitor of SAMdc (Pegg 1984). GCP39937 is a hundred times more potent than MGBG (Regenass *et al.* 1992). GCP48664 is a drug with broad antitumour efficacy and an apparent increased therapeutic window compared with the parent compounds MGBG and GCP39937 (Regenass *et al.* 1994; Delworth *et al.* 1995). MDL73811, a structural analogue of dcSAM and an irreversible inhibitor of SAMdc has been found to be a hundred times more potent than DFMO against murine *T. b. brucei* infections and is effective against a strain of *T. b. rhodesiense* that is resistant to melarsoprol and pentamidine and which does not respond completely to DFMO treatment (Bitonti *et al.* 1990). MDL73811 blocks *T. b. brucei* SAMdc leading to a decrease in spermidine (50%) and an increase in putrescine levels (Bitonti *et al.* 1990). After 1 hour exposure *in vivo*, MDL73811 causes a 20-fold increase in SAM levels in *T. b. brucei*, but less than a 2-fold increase in SAM from mammalian cells cultured in the presence of the drug for 6 hours (Byers *et al.* 1991). This can be explained by SAM synthetase from the parasite being less sensitive to inhibition by the product (SAM) than its mammalian counterpart (Yarlett *et al.* 1993). These large increases in SAM, for example in the treatment of *T. b. brucei*, lead to an increase in the parasite's methylation index (SAM:SAH) (Yarlett and Bacchi 1988) and an accompanying rise in protein methylation (Yarlett *et al.* 1991; Bacchi *et al.* 1992), which may lead to abherent methylation reactions within the cell (Ueland 1982). It is therefore possible that the antitrypanosomal effects of MDL73811 are due to major changes in SAM levels rather than a change in polyamine levels. MDL73811 was also



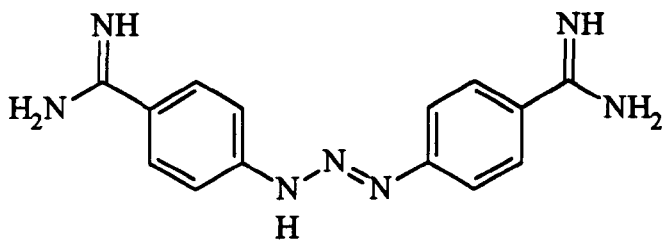
Methylglyoxal Bis(Guanylhydrazone)



S-Adenosyl-L-methionine



Spermidine



Berenil

Figure 5.3 Showing several important structurally related compounds. (adapted from Porter *et al.* 1981).

shown to reduce the capacity of *T. cruzi* to invade and multiply within heart myoblasts, an important process in the pathogenesis of Chagas' disease (Yakubu *et al.* 1993). Finally, Berenil (Figure 5.3), a compound first used in the treatment of animal trypanosomiasis and babesiasis (Hawking 1958) has also been shown to be a potent irreversible inhibitor of a number of SAMdc's from several organisms including from yeast, rat liver (Karvonen *et al.* 1985), *T. b. brucei* (Bitonti *et al.* 1986; Byers *et al.* 1991) and *L. infantum* promastigotes (Balaña-Fouce *et al.* 1991; Carrera *et al.* 1994).

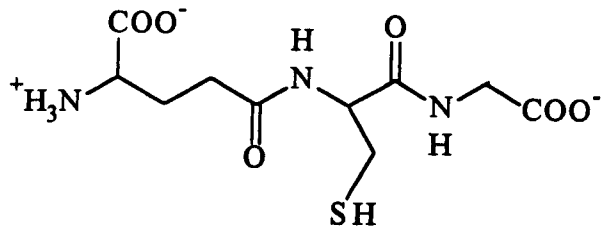
5.1.7 Assay of polyamines

Early separation of polyamines from related metabolites was achieved by crystallization of soluble salts and subsequent gravimetric detection. However, this technique could not detect putrescine or cadaverine, and spermidine salts could not be quantified precisely. More modern techniques utilise the polycationic nature of polyamines as a basis for their separation. Instead of separation of the free amines as in thin layer surface chromatography, electrophoresis and ion exchange chromatography, a pre-column or post-column derivatisation with a fluorescent or coloured agent which binds to the primary or secondary amine group, allows post column detection of polyamines. Interest in high performance liquid chromatography (HPLC) has provided modern separation techniques to be applied to polyamines and their related metabolites. This mainly automated, relatively quick and highly reproducible technique has been exploited in the field of polyamine research (Seiler 1980) and separation by ion-pairing HPLC combined with derivatisation with o-phthalaldehyde has been used to detect polyamines in urine (Seiler and Knödgen 1980; Seiler and Knödgen 1985; Löser *et al.* 1988). Due to the difficulty in measuring polyamines in cerebro-spinal fluid, dansyl chloride was employed as pre-

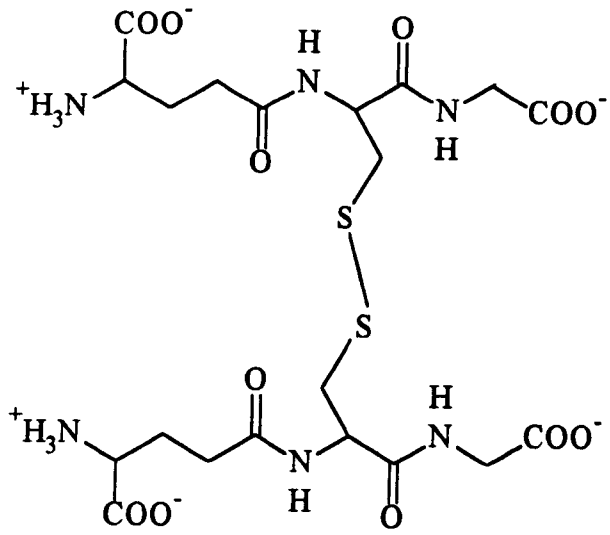
column derivative providing a simple, precise and accurate method for the determination of polyamines (Kabra and Lee 1986). Both dansyl chloride and o-phthalaldehyde derivatisation can be used to detect polyamines in the picomole range, but drawbacks such as poor stability and interference from amino acids have brought difficulties to their applications (Das 1992). Chromophoric labelling of amines with 4-dimethylaminoazobenzene-4'-sulfonyl (dabsyl) chloride (Lin and Chang 1975) and subsequent HPLC separation and detection of fish amines (Lin and Lai 1980) provided the technique used to detect putrescine, spermidine and spermine in rat tissue (Lin and Lai 1982). This technique has allowed development of HPLC methodology with increased sensitivity, simplicity and rapidity of operation (Koski *et al.* 1987; Das 1992). Recently benzoyl chloride, an acid chloride similar to dabsyl chloride has also been employed as a pre-column derivative to detect polyamines in human plasma and unicellular prokaryotes (Mei 1994).

5.2 Introduction to thiols

Most, if not all cells contain at least one low molecular weight thiol. Glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH) (Figure 5.4) is usually the most prevalent of these compounds. GSH is synthesised intracellularly from the amino acids glutamate, cysteine and glycine. This process is catalysed by γ-glutamylcysteine synthetase and GSH synthetase, both of which exhibit negative feedback inhibition in the presence of GSH. The breakdown of GSH and glutathione disulphide (GSSG), is catalysed by γ-glutamyltranspeptidase an enzyme usually found on the external surface of cell membranes (Stryer 1988). Studies on thiol enzymology and metabolism has elucidated some of the varied functions of these compounds.



Glutathione (GSH)



Glutathione disulphide (GSSG)

Figure 5.4 Structures of reduced and oxidised glutathione.

Among their varied functions, thiols are considered to operate as a natural stockpile of reducing power, which can be used as part of a cellular defence mechanism against oxidative stress. If the thiols are not maintained in this reduced state by their specific enzymes then there will be an accumulation of disulphide oxidation products will accumulate and partial loss of protection against cellular damage will occur. The high GSH:GSSG ratio found in cells can be accounted for mainly by the action of the flavoprotein glutathione reductase (GR). This enzyme, in the presence of NADPH, catalyses the conversion of GSSG to GSH (Meister and Anderson 1983).

Work on urinary excretion of toxic compounds in dogs in the late nineteenth century was the stimulus for more recent work which established that GSH could form conjugates with foreign compounds, rendering them more water soluble to aid their excretion (Meister and Anderson 1983). These conjugations may occur spontaneously or may occur in the presence of glutathione S-transferases (GST), which in mammalian cells are enzymes grouped according to their substrate and inhibitor specificities, primary structure and antibody cross-reactivities (Wilce and Parker 1994). Other functions of GSH include enzyme cofactor functions, for example in the glyoxylase conversion of hemimercaptal to S-lactylGSH (Meister and Anderson 1983). Elevated GSH levels have been found in oral cancer cells and squamous cell carcinomas, and are thought to play a role in radiation and alkylating agent resistance in cancer patients (Wong *et al.* 1994).

Although GSH is present in all trypanosomatids studied to date, these organisms differ from mammalian cells in the metabolism of this thiol. In the mid 1980's GR like activity was discovered in the mammalian parasite *T. brucei* (Fairlamb and Cerami 1985), although GR like activity had been detected previously in *T. cruzi* (Boveris *et al.* 1980) the GR

activity discovered in *T. brucei* was found by dialysis to require a low-molecular weight co-factor, that was not needed for the classical GR activity. The co-factor was given the trivial name trypanothione (N^1, N^8-bis(glutathionyl)-spermidine) (Fairlamb *et al.* 1985). It was proposed that the reduction of GSSG was due to a non-enzymatic disulphide exchange reaction involving enzymatically reduced trypanothione. Trypanothione is synthesised via two separate ATP hydrolysis steps from the precursors glutathione and spermidine (Figure 5.5). The enzymes, namely glutathionylspermidine and trypanothione synthetases, catalyse the linkage of two GSH tripeptides covalently joined to a spermidine molecule via their glycine carboxylates (Figure 5.6) (Smith *et al.* 1992). Trypanothione has been found in a number of trypanosomatids including *C. fasciculata* (Fairlamb *et al.* 1986; Shim and Fairlamb 1988; Hunter *et al.* 1990; Steenkamp 1993), *T. b. brucei* (Fairlamb *et al.* 1989, 1992) and *T. cruzi* (Hunter and Fairlamb 1993; Ariyanayagam and Fairlamb 1993, 1994).

The NADPH-dependent flavoprotein responsible for the maintenance of this reduced thiol was named trypanothione reductase (TR) (Shames *et al.* 1986) and it has since been discovered in all trypanosomatids studied to date. It has been purified from the insect trypanosomatid *Crithidia fasciculata* (Shames *et al.* 1986) and the human pathogen *T. cruzi* (Krauth-Siegel *et al.* 1987). TR appears to have striking homology to GR (Shames *et al.* 1986), but it differs in its substrate specificities. TR from *C. fasciculata* will reduce trypanothione disulphide but will not significantly reduce glutathione disulphide. Conversely, GR from human erythrocytes will reduce glutathione disulphide but will not significantly reduce trypanothione disulphide (Shames *et al.* 1986). However, using site-directed mutagenesis to alter certain amino acid residues, it has been possible to produce a mutant *E. coli* glutathione reductase capable of reducing trypanothione at approximately

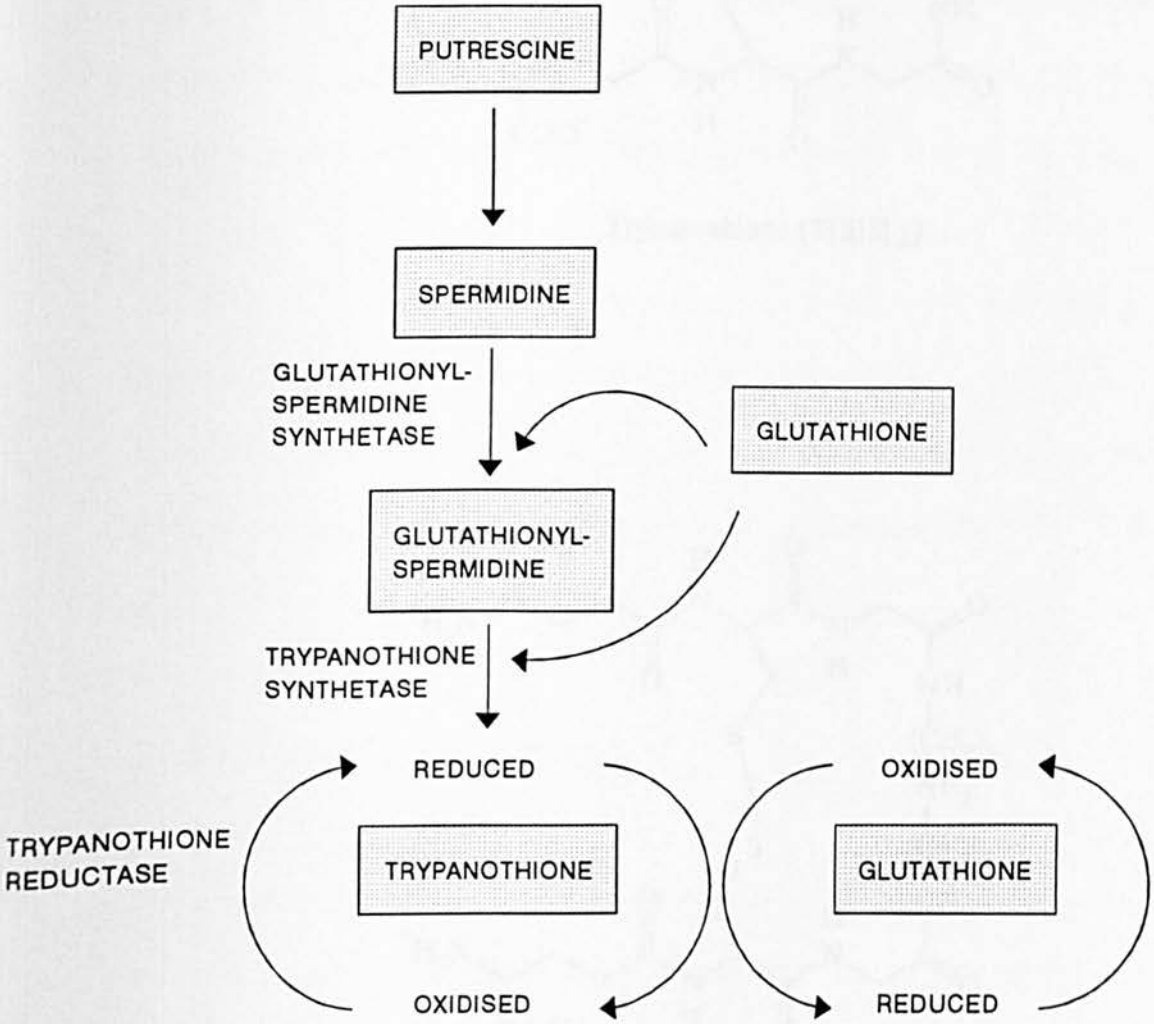
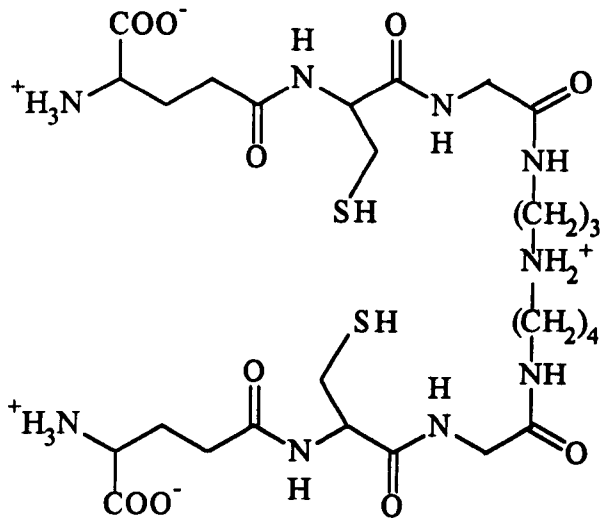
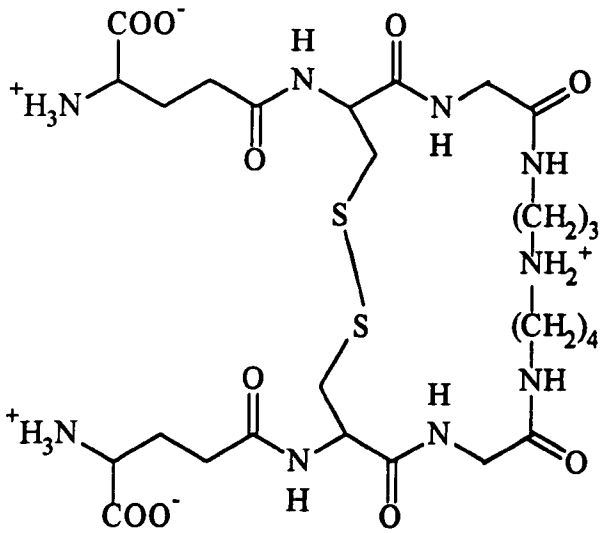


Figure 5.5 A general diagram showing the biosynthesis of trypanothione. (adapted from Fairlamb 1989).



Trypanothione (T[SH] 2)



Trypanothione disulphide (T[S] 2)

Figure 5.6 Structures of reduced and oxidised trypanothione.

10% the rate of natural trypanothione reductases (Henderson *et al.* 1991). In *T. cruzi* epimastigotes, trypanothione disulphide and glutathionylspermidine were found to be substrates of TR whereas glutathione disulphide was neither a substrate nor an inhibitor (Krauth-Siegel *et al.* 1987). It was at the active site of human GR and trypanosomatid TR that four out of five amino acid residues were different. These residues occur where the Gly-I and Gly-II sites, at which the free carboxylates of glutathione disulphide are linked to one molecule of spermidine in trypanothione disulphide, interact with the active site of the flavoprotein disulphide oxidoreductases (Aboagye-Kwarteng *et al.* 1992).

The enzymes normally necessary for destroying hydrogen peroxide namely catalase and glutathione peroxidase are not found in trypanosomatids. Instead a trypanothione dependent peroxidase activity has been detected which accounts for the majority of the hydrogen peroxide metabolism within the parasites (Penketh and Klein 1986; Henderson *et al.* 1987).

5.2.1 Inhibition of thiol metabolism

The advances in the elucidation of trypanothione metabolism have emphasised differences between trypanosomatids and their mammalian host. The importance of the trypanothione system is highlighted by the number of trypanocidal compounds which interfere with this area of trypanosome metabolism (Bacchi and McCann 1987). In *T. brucei* for example, DFMO, by inactivating ODC, decreases intracellular polyamine levels. This indirectly decreases polyamine-glutathione conjugates in the bloodstream (Fairlamb *et al.* 1987) and the procyclic forms of the trypanosome (Bellofatto *et al.* 1987). In another example, pentamidine, by inhibiting SAMdc in *T. brucei*, also decreases levels of spermidine and

indirectly decreases levels of trypanothione (Bitonti *et al.* 1986). Inhibitors of glutathione metabolism, for example buthionine sulphoximine, have demonstrated trypanocidal activity (Arrick *et al.* 1981) due at least in part to a decrease in trypanothione levels.

The most studied aspect of trypanothione metabolism for the development of potential inhibitors is TR. As mentioned above, the enzyme has been purified from certain trypanosome species but inabilities to obtain sufficient quantities of the reductase led to isolation of the TR gene from *T. congolense* followed by cloning, sequencing and overproduction of the enzyme in *E. coli* (Shames *et al.* 1986; Sullivan *et al.* 1989). A comparison of TR from *T. congolense* and human glutathione reductase (GR) revealed that the most important amino acid residues (in terms of their structure and catalytic activity) are almost identical in the two enzymes (Krauth-Siegel *et al.* 1991). A number of drugs have been shown to inhibit TR. These include the cytostatic agent 1,3-bis(2-chloroethyl)-1-nitrosurea (BCNU), a specific inhibitor of TR from *T. cruzi*. However therapeutic use of the drug seems unlikely due to its inhibition of GR from human erythrocytes (Jockers-Scherübl *et al.* 1989). 2,3-Bis(3-(2-amidinohydrazono)-butyl)-1,4-naphthoquinone (GH8693) is also an effective inhibitor of *T. cruzi* TR. It is known as a "turncoat" inhibitor because at low concentrations it is an effective inhibitor of TR, and at higher concentrations it acts as a substrate leading to the generation of toxic oxygen species by transferring electrons to oxygen (Jockers-Scherübl *et al.* 1989). Recently computer-graphically directed ligand design technology predicted a peptide which would specifically inhibit *T. cruzi* TR but not to human GR. The synthesised peptide, which was structurally dissimilar to the substrate trypanothione, inhibited TR and had no effect on GR up to 0.5mM. Although unlikely to serve as a drug due to its high charge, this inhibitor could be used to continue mapping the residues important for ligand binding in TR thereby

assisting rational drug design and targeting (Garforth *et al.* 1994). Chinifur (2-{5'-nitro(furo-2'-yl)-ethene-1-yl}-4(N,N-diethylamino)-1-methyl-but-1-yl-aminocarbonyl-4-quinoline), previously known for its antibacterial activity, has been recently discovered to have antitrypanosomal activity. It is proposed that chinifur binds to TR at the trypanothione binding site, possibly explaining why it inhibits *T. congolense* TR, but only weakly inhibits yeast GR (Cenas *et al.* 1994). The trivalent arsenical drug melarsoprol, a drug still extensively used in the treatment of human African trypanosomiasis, is known to form a stable adduct with trypanothione (Fairlamb *et al.* 1989). This adduct (MelT), is an inhibitor of TR. This mechanism may be partially responsible for the cytotoxicity of the drug (Cunningham *et al.* 1994).

5.3 Aims of the present investigation

The role of polyamines and inhibition of polyamine metabolism has been documented in many parasites, but there have been no recorded studies on the polyamine metabolism of fish trypanosomes (for review of fish trypanosomes see section 1.2). It is the aim of this investigation to elucidate the polyamine metabolism of two fish trypanosomes, concentrating on *Trypanosoma granulosum*, but also including *Trypanosoma danilewskyi*. The intracellular levels of polyamines and their related metabolites are studied in the two fish trypanosomes at different stages of *in vitro* cultivation. The effects of DFMO, MGBG and Berenil on levels of polyamines and related metabolites in both species of trypanosomes are also examined. Using radiolabelled putrescine, the ability of *Trypanosoma granulosum* to synthesise polyamines *in vitro* and the effects of MGBG and Berenil on this synthesis will be determined and discussed. A mechanism of putrescine uptake by cultured *T. granulosum* will also be proposed and the importance of this

mechanism with respect to the polyamine metabolism of the parasite will be examined. Finally, it is proposed to determine the levels of thiols in the two cultured fish trypanosomes and the effects of altering exogenous diamine concentrations on the levels of intracellular thiols in both parasites. The role that these thiols may play in the survival of the two parasites will be discussed.

The implications of this research in relation to fish diseases and other parasitic protozoan infections of humans and animals will be discussed.

CHAPTER 6: METHODS

6.1 Chemicals

The following items were purchased from BDH Ltd:

Acetone (hypersolv), acetonitrile (hypersolv), ethyl acetate (AnalaR), Folin and Ciocalteus phenol reagent, lithium hydroxide (AnalaR), methanesulphonic acid, Iso-octylphenoxypolyethoxyethanol (Scintran Triton X 100), perchloric acid, phosphoric acid, propanol, Scintran toluene and trichloroacetic acid.

The following items were purchased from Sigma Chemical company:

S-adenosyl homocysteine, S-adenosyl methionine (iodide salt), bovine serum albumin (BSA) standard, bromobimane, cadaverine dihydrochloride, camphor sulphonic acid, dansyl chloride, 5 deoxy-5 methyl thioadenosine, dialysis tubing, 1,7-diaminoheptane dihydrochloride, 1,6-diaminohexane dihydrochloride, 1,8-diaminooctane dihydrochloride, 1,9-diaminononane dihydrochloride, diaminopropane dihydrochloride, diethylenetriaminepentaacetic acid (DTPA) diminazene aceturate (Berenil), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), EDTA, fluorescamine, glutathione (reduced form), glutathione reductase (type 4), N-[2-hydroxyethyl]piperazine-N'-[3-propanesulfonicacid](HEPPS),methylglyoxal-bis-(guanylhydrazone) dihydrochloride (MGBG), nicotinamide adenine dinucleotide phosphate (reduced form), octane sulfonic acid, L-proline, putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride and 5-

sulfosalicylic acid.

In addition:

Absolute alcohol was purchased from Hayman Limited.

4-dimethylaminoazobenzene-4-sulfonyl chloride (dabsyl chloride) and silicone oil AR200 (235mPa.s) were purchased from Fluka.

Dulbecco's PBS was purchased from Gibco BRL (Life Technologies Ltd.).

E. coli stock was purchased from Philip Harris.

Putrescine dihydrochloride [2,3-^{3}H(N)]-1.11-2.22TBq/mmol was purchased from Dupont NEN.

Scintimix (2) was purchased from Koch Light.

The following items were generous gifts from Professor A. H. Fairlamb of the London School of Hygiene and Tropical Medicine (LSHTM):

Aminopropylcadaverine, decarboxylated S-adenosylmethionine (synthesised by Dr A. Pegg), glutathionylspermidine trypanothione, homotrypanothione and ovothiol A.

Distilled water used to make any solutions was deionised in a Fisons Fi-stream Cartridge Deioniser. All other reagents used were general purpose reagents.

DFMO was a generous gift from Merrell Dow.

6.2 Analysis of polyamines as their dabsyl derivatives by reverse-phase high-performance liquid chromatography (HPLC)

Approximately 10^8 trypanosomes at different stages of growth, were harvested in an MSE Coolspin centrifuge (1511g, 4^0C, 15 mins). The cells were washed twice in cold Dulbecco's phosphate buffered saline (PBS) and lysed by the addition of 0.25ml filtered deionised water followed by vortexing, and the protein precipitated by the addition of 0.25ml perchloric acid (10%v/v) (containing an internal standard of 1,6-diaminohexane equivalent to 150pmoles when injected onto column), followed by vortexing. The solution was left on ice for 30mins and centrifuged (2960g, 4^0C, 15mins). The supernatant was removed and aliquots were frozen at -20^0C for analysis of S-adenosylmethionine and its related metabolites as described in section 6.2. To the pelleted protein precipitate, 1ml NaOH (1M) was added and incubated at 37^0C overnight. An aliquot of this solubilized sample was used for protein determination as described in section 6.12. The remaining sample was adjusted to pH8.0 with solid potassium carbonate, and after cooling on ice for 30mins, precipitated potassium perchlorate was removed using a Sarstedt MH2 microfuge (12000g, room temperature, 2mins). Aliquots of this supernatant were derivatised as follows. Dabsyl chloride (1.36mg/ml) was dissolved in acetone by heating at 70^0C for 2mins in a shaking water bath. Standard polyamine mixture in water (50μl), or neutralised sample (50μl) was added to 0.1M sodium bicarbonate buffer pH8.0 (200μl), and to the

mixture, dissolved dabsyl chloride solution (400μl) was added and mixed thoroughly. The mixtures were kept in stoppered glass tubes, and placed in a shaking water bath (70^{0}C, 12mins). After cooling for 10mins at room temperature, 1ml of 75%(v/v) ethanol in water was added to the derivatised solution and vortexed. The resulting solution was centrifuged (12000g, room temperature, 5mins) and the supernatant (200μl) used for HPLC analysis (Das 1992). Stock solutions of polyamine standards were made in deionised water and stored at -20^{0}C until needed.

HPLC was accomplished using reverse-phase Spherisorb S5 ODS2(150mm x 46mm) and a guard column containing the same material. Other equipment consisted of a Rheodyne 7125 loop injector, Spectra Physics SP8700XR pump, and the separated samples were detected at 436nm on a Rapiscan SA6508. A 200μl injection of the derivatised sample/standard was applied to the column. Separations of the derivatised polyamines were achieved using a linear gradient from 40% sodium acetate buffer pH4.1/ 60% acetonitrile to 100% acetonitrile in 20 minutes, and the elution was continued for a further 5 minutes at a flow rate of 1.2 ml/min. The data was collected by an RM Nimbus 286 computer and integrated using Apex Chromatography Workstation software, supplied by Autochrome. Standards and samples were quantified according to peak area.

6.3 Analysis of S-adenosyl methionine (SAM) and related metabolites by reversed-phase high-performance liquid chromatography

HPLC was performed using a Beckman Ultrasphere ion pairing 5μm C_{18} (250x4.6mm) and a Waters Bondapak C_{18} pre-column. HPLC equipment consisted of a column heater, heated water bath, Rheodyne 7125 loop injector, Perkin Elmer Binary LC 250 pump, and

detected on a Perkin Elmer LC235 diode array detector at 255nm. Data was collected by an Epson PC AX2E and analysed and integrated by a PE Nelson Omega 2 Analytical Workstation Vs. 2.50.

Aliquots of acidic supernatants extracted as described (section 6.2), were injected directly on to column and a gradient elution system was used to separate the related compounds, consisting of two mobile phases. Mobile phase A was 20ml of acetonitrile to 980ml of 0.1M NaH_2PO_4 and contained $8x10^{-3}$M octane sulfonic acid (OSA) and $1x10^{-4}$M EDTA, adjusted to pH 2.55 with 3M H_3PO_4. Mobile phase B was 70:30 (v/v) mixture of 0.2M NaH_2PO_4 and acetonitrile with $8x10^{-3}$M OSA adjusted to pH 3.1 with 3M H_3PO_4. A linear gradient was used (85%-15% in 30 mins) followed by 15 mins equilibration. Column temperature and mobile phase remained at 40^0C, using a column jacket and water bath. Standards were dissolved in solvent (consisting of perchloric acid (0.01M), $Na_2S_2O_5$ (0.05% w/v) and EDTA (0.01% w/v) dissolved in deionised water) and stored at -20^0C. Standards and samples were quantified according to their peak area (Wagner *et al.* 1982).

6.4 Analysis of thiols by reverse-phase high performance liquid chromatography

Thiol standards were prepared as described previously (Hunter *et al.* 1994). Cells were assayed without washing, as this was found to affect subsequent analyses (Shim and Fairlamb 1988). Approximately 10^8 cells with the addition of cold PBS to a final volume of 50ml were pelleted (1511g, 4^0C, 15mins) and the supernatant carefully aspirated. Pellets were immediately resuspended with vigorous mixing in 0.05ml of HEPPS buffer (40mM N-(2-hydroxyethyl)-piperazine-N'(3-propanesulphonic acid) (HEPPS), 4mM diethylenetriaminepentaacetic acid (DTPA) pH8.0). Thiols were derivatised by the

addition of 0.05ml monobromobimane (2mM in absolute ethanol) to the mixture and placed in a shaking water bath (65^{0}C, 3mins). The samples were then deproteinised with 0.1ml 4M-methanesulphonic acid (pH1.6 with solid LiOH). After standing at 4^{0}C for 15mins, denatured protein was removed by centrifugation (2960g, 4^{0}C, 15mins) and the supernatant stored at -20^{0}C until needed for analysis by HPLC as described below.

Separation of thiols was achieved using two mobile phases. Mobile phase A: *d*-camphor sulphonate in de-ionised water (Li salt, 0.25% (w/v), pH 2.64); mobile phase B: 25% (v/v) *n*-propanol in de-ionised water with *d*-camphor sulphonate (Li salt, 0.25% (w/v)). Following injection the solvent mixture was applied isocratically at 1ml/min for 20mins, followed by a linear gradient of 10-50% B over 40mins, then isocratic at 50% B for 10mins. The column was then equilibrated at 10% B for 15mins. HPLC equipment consisted of either (i) Beckman reverse-phase Analytical Ultrasphere Ion-Pair column (5μm) or (ii) HPLC Technology Limited Ultratechsphere Ion-pair column (5μm), with a Brownlee NewGuard cartridge (7μm), Beckman System Gold 507 Autosampler, Beckman analogue interface 406, Beckman 110B solvent delivery system and a Gilson 121 fluorimeter with filters (ex. 310-410nm, em. 475-650nm). Standards and samples were quantified according to peak area.

6.5 Uptake studies

Trypansomes grown for 4 days in modified SDM-79 were washed twice in either Dulbecco's PBS or Carter's balanced salt solution (CBSS) (Fairlamb *et al.* 1992) and resuspended in the appropriate buffer to determine cell viability over a period of hours (3.5 hours). Viability was determined by counting numbers of live cells using an

improved Neubauer haemocytometer. To determine number of cells lost during centrifugation through the silicone oil, differing quantities of cells were centrifuged (10000g, 1min) and the cells remaining in the supernatant were counted.

Uptake was measured using a rapid sampling technique, involving centrifugation of the cells through silicone oil (Carter and Fairlamb 1993). Approximately 10^9 trypanosomes were washed twice in PBS (1511g, 4^0C, 15mins) and the pellet was resuspended in PBS at $2x10^8$ cells/ml (x2 final concentration). Aliquots of PBS containing 0.1ml unlabelled putrescine (0.5μM-150μM) and [^{3}H]putrescine (0.2μCi/200μl) were overlaid on silicone oil (0.1ml) in 1.5ml eppendorf tubes at 22^0C. To determine the effect of MGBG and Berenil on uptake of putrescine, each drug (0.2mM) was dissolved in the appropriately labelled overlay, prior to the addition of washed cells. Washed cells (0.1ml, $2x10^8$/ml), were prewarmed to 22^0C and pipetted down the side of each tube to ensure adequate mixing. At a fixed time after mixing, the cells were separated from the label by centrifugation (13000g, 1min). The medium above the oil was aspirated, and the region above the silicone oil layer rinsed twice in PBS to remove residual label before aspirating the silicone oil. The pellet was extracted overnight in 1M NaOH (0.1ml, 37^0C) and to the resulting suspension was added 1ml of scintillator (33% Triton X 100, 66% [4g Scintimix (2)/1000ml Scintran toluene]) fluid before counting in a Hewlett-Packard Tri-Carb 2500 TR liquid scintillation counter. The initial rates of uptake were determined by linear regression analysis at 6 time points spaced at 10sec intervals. Km and Vmax were determined using Enzfit software.

6.6 Metabolism of [^{3}H]putrescine label at early time points

To determine whether metabolism of putrescine was involved during uptake studies, the extracts of cells treated with labelled putrescine were separated by HPLC and fractions collected and analysed for radioactivity as described below. The cell contents were analysed by pelleting cells mixed with 1μM putrescine containing [^{3}H]putrescine for 2mins and 30mins, into 50μl TCA (10%) beneath the silicone oil layer. The extract was then analysed for radioactivity following dansylation and separated by HPLC. Dansylation was carried out by extracting the acid supernatant five times with two volumes (1ml) of water saturated ethyl acetate. The remaining ethyl acetate was removed by bubbling gently with nitrogen, the samples were then freeze dried. To this powder 50μl HCl (10mM), 0.2ml saturated sodium carbonate and 0.2ml dansyl chloride (10mg/ml of acetone) were added and the resulting mixture heated at 65^{0}C for 10mins. The solution was cooled to room temperature in the dark, 0.1ml L-proline (25%w/v) added and the mixture centrifuged (13000g, 30secs). An aliquot of this supernatant was analysed for radioactivity following separation by HPLC. HPLC was accomplished using a Perkin Elmer 3000 Fluorescence Spectrometer (ex.340nm, em.515nm), Beckman 506 Autosampler, Beckman 114M solvent delivery module, HPLC Technology Limited UltraTech sphere SP5ODS column, Brownlee NewGuard guard cartridge and separation of polyamines was achieved using two mobile phases. Mobile phase A: acetonitrile; mobile phase B: sodium phosphate buffer (10mM, pH4.4). Following injection the solvent mixture was applied (2ml/min) as a linear gradient 45-80% A over 14mins, then 80-90% A over 1min, then isocratic at 90% A for 7mins. The column was equilibrated at 45% A for 5mins (Kabra and Lee 1986). Integration and reporting were performed using Beckman System Gold v.3.10 and standards were quantified according to peak area. Fractions were collected every 30secs

on a Pharmacia fraction collector Frac 100, and 1ml of scintillator (33% Triton X 100, 66% [4g Scintimix (2)/1000ml Scintran toluene]) fluid was added before counting in a Hewlett-Packard Tri-Carb 2500 TR liquid scintillation counter.

6.7 Effect of MGBG and Berenil on metabolism of putrescine

An aliquot (20μl) of [^{3}H]putrescine (1μCi final concentration) was added to cells cultured for four days in either drug free modified SDM-79, modified SDM-79 containing 0.2mM MGBG or modified SDM-79 containing 0.2mM Berenil. On day five of culture (24hours after the addition of [^{3}H]putrescine) the cells were washed twice in PBS (1511g, 4^{0}C, 15mins), and to the cell pellet was added 500μl TCA (10%) containing 1,7-diaminoheptane as an internal standard. The cells were vortexed, left on ice for 30mins and centrifuged (3500rpm, 4^{0}C, 15mins). The supernatant was extracted five times with water saturated ethyl acetate and samples were freeze dried, dansylated and analysed by HPLC as described in section 6.6.

6.8 Trypanothione reductase assay

Approximately 10^8 cells were washed twice in PBS (1511g, 4^{0}C, 15mins) and the pellet resuspended in 1ml assay buffer (20mM HEPES, 150mM KCl, 1mM EDTA, pH 7.25). The suspension was sonicated in an MSE ultrasonicator (3x15sec, 4^{0}C) and centrifuged (13000g, 5mins, 4^{0}C). To the supernatant (100μl) was added NADPH (1mM, 100μl) and assay buffer (745μl). The solution was mixed and analysed spectrophotometrically at 340nm for several minutes until there was no (non-trypanothione dependent) oxidation of NADPH. The reaction was then initiated by the addition of (55μl) trypanothione ($T(S)_2$)

(1.8mM) and followed at 340nm on an SP8-100 PYE Unicam Spectrophotometer and the activity calculated (E_{340nm}NADPH=6.22mM^{-1}cm^{-1}). A small aliquot of supernatant was used for protein determination as described in section 6.12.

6.9 Glutathione reductase assay

Approximately 10^8 cells were washed twice in PBS at (1511g, 4^{0}C, 15mins) and the pellet resuspended in 1ml assay buffer (20mM HEPES, 150mM KCl, 1mM EDTA, pH 7.25). The suspension was sonicated in an MSE ultrasonicator (3x15sec, 4^{0}C) and centrifuged (13000g, 5mins, 4^{0}C). To eliminate trypanothione dependent oxidation of NADPH, the supernatant was dialysed twice using assay buffer (1000ml, 1hr). An aliquot of the dialysate (100μl) was added to NADPH (1mM, 100μl) and assay buffer (745μl). The solution was mixed analysed spectrophotometrically at 340nm for several minutes until there was no (non-trypanothione dependent) oxidation of NADPH. The reaction was then initiated by the addition of (55μl) glutathione disulphide (GSSG) (18mM) and followed at 340nm on an SP8-100 PYE Unicam Spectrophotometer and the activity calculated (E_{340nm}NADPH=6.22mM^{-1}cm^{-1}). A small aliquot of supernatant was also used for protein determination as described in section 6.12. *Escherichia coli* cultured in nutrient broth for 24 hours were used as positive controls. *E. coli* was extracted and analysed in the same way as *T. granulosum*.

6.10 DFMO determination

Trypanosomes cultured for four days in modified SDM-79 containing 50mM DFMO, were harvested and washed twice in PBS (1511g, 4^{0}C, 15 mins). To the cell pellet was added

(0.5ml) 5%(w/v)TCA in 0.01N HCl. The solution was vortexed, left on ice for 30 mins, and centrifuged (2960g, 4^0C, 15mins). The supernatant was extracted five times with two volumes (1ml) of water saturated ethyl acetate. The remaining ethyl acetate was removed by bubbling gently with nitrogen, then the samples were freeze dried. The samples were then redissolved in HPLC mobile phase A. Mobile phase A consisted of *d*-camphor sulphonate in de-ionised water (Li salt, 0.25% (w/v), pH 2.64); solvent B: 25% (v/v) *n*-propanol in de-ionised water with *d*-camphor sulphonate (Li salt, 0.25% (w/v)). HPLC columns were as in section 6.6. Following injection, linear gradients of 0-15% mobile phase B over 45 mins, followed by 15-0% mobile phase B over 1 min. The system was equilibrated for 8 mins with 0% mobile phase B. Column eluent was derivatised with fluorescamine (0.2g/1000ml) in distilled acetone and detected using a Gilson 121 fluorimeter with filters (ex. 310-410nm, em. 475-650nm). Samples were quantified according to peak area (Fairlamb *et al.* 1987).

6.11 Concentration of polyamines in medium

To 5ml of modified SDM-79 containing 10% foetal calf serum were added 1ml of perchloric acid (60%) and 6μl of 1,6-diaminohexane (2.9mg/ml). The solution was mixed and left an ice for 30mins. The mixture was centrifuged (2960g, 20mins, 4^0C) and the supernatant frozen (-20^0C). Supernatants were applied to an AG-50W H^+ cation exchange column (1ml/min), and polyamines were eluted with 25ml HCl (6N) following elution of amino acids with 20ml HCl (2N). Supernatant were freeze dried and redissolved in 50μl perchloric acid (5%v/v), dabsylated and analysed by HPLC as described in section 6.2.

6.12 Protein determination

Proteins were estimated according to Folin-Lowry method (Lowry *et al.* 1951) on a PYE Unicam SP8-100 uv/vis spectrophotometer. Bovine serum albumin (BSA) was used as a standard and an aliquot of the supernatant was diluted with water so that the protein content of the diluted solution was within the range of concentrations comprising the standard curve (25-150μg/ml). All samples were analysed in duplicate and values were calculated from the standards using linear regression.

6.13 Statistical analyses

All values calculated were the arithmetical mean $\pm$ sample standard deviation. Significance was tested using the Mann-Whitney U test at $p=0.05$. The rank values within each group gave the totals R1 and R2 respectively.

The following equations were used :-

$$U_1 = n_1 \times n_2 + ((n_1(n_1 + 1))/2) - R1$$

$$U_2 = n_1 \times n_2 - U_1$$

Where n_1 and n_2 were the number of data values in each group.

CHAPTER 7: RESULTS

7.1 Biochemical analyses of *T. granulosum*

7.1.1 Detection of polyamines

Dabsylated polyamines were separated over 25mins using reverse-phase high performance liquid chromatography. All polyamines were well separated with no interference from solvent peaks and with approximate retention times: putrescine=13.8mins, 1,6-diaminohexane=14.9mins, spermidine=19.2mins and spermine=22.8mins as shown in Figure 7.1. A linear (r=0.99) relationship of absorbance at 436nm versus amount of polyamine injected was found up to 300 pmoles/0.2ml injection (Figure 7.2) showing good reproducibility after repeated injection. Levels as low as 18.75pmoles/0.2ml injection were detected at 436nm. It was found that underivatised standards stored at -20^{0}C in 1.5ml eppendorf tubes, gave reproducible results upon dabsylation for up to two years.

7.1.1.1 Polyamines in cultured *T. granulosum*

7.1.1.1.1 Cells cultured in drug free medium

Approximately 10^8 cells were needed for detection of polyamines. Preliminary investigations found an occasional unidentified peak with approximately the same retention time as the internal standard 1,7-diaminoheptane. Consequently all subsequent analyses and all results presented in this chapter utilised 1,6-diaminohexane as an internal standard (95%-100% recovery after derivatisation and HPLC) which was well separated from other

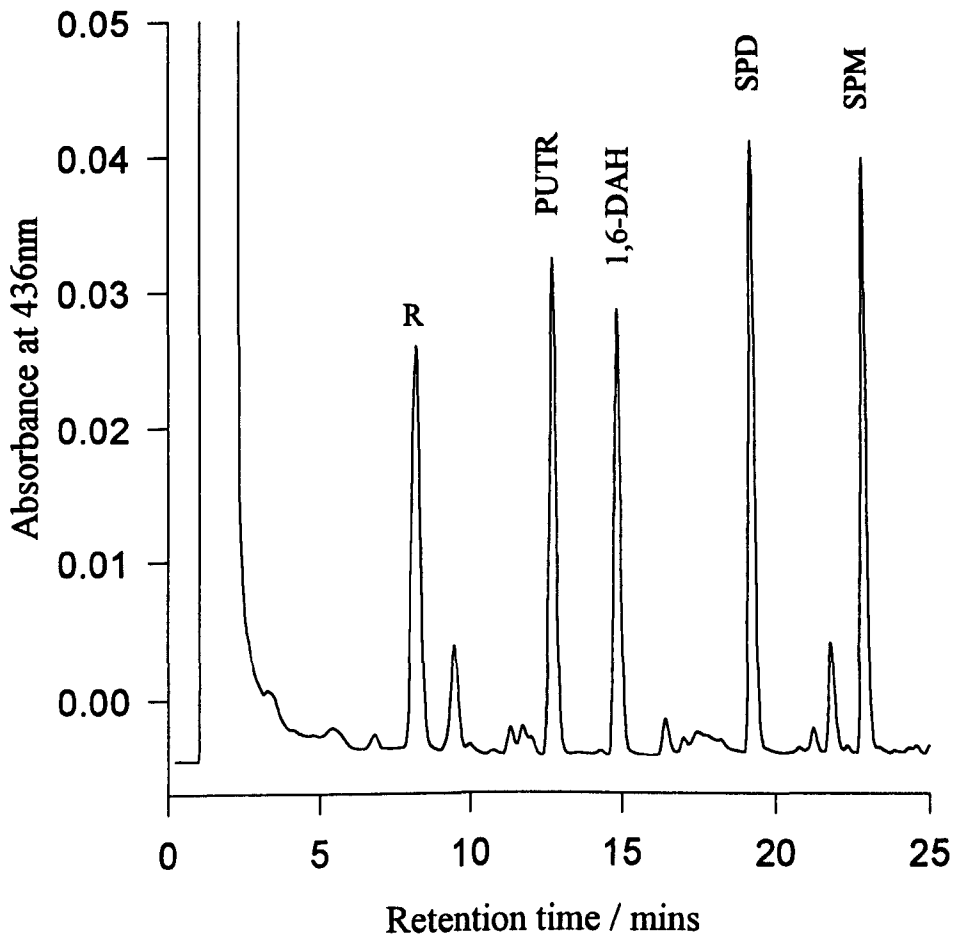


Figure 7.1 Separation of dabsylated polyamine standards by reverse-phase HPLC and subsequent detection at 436nm. Polyamines were derivatised and separated as described in section 6.2 of methods chapter. R=reagent peak, PUTR=putrescine, 1,6-DAH=1,6-diaminohexane, SPD=spermidine and SPM=spermine.

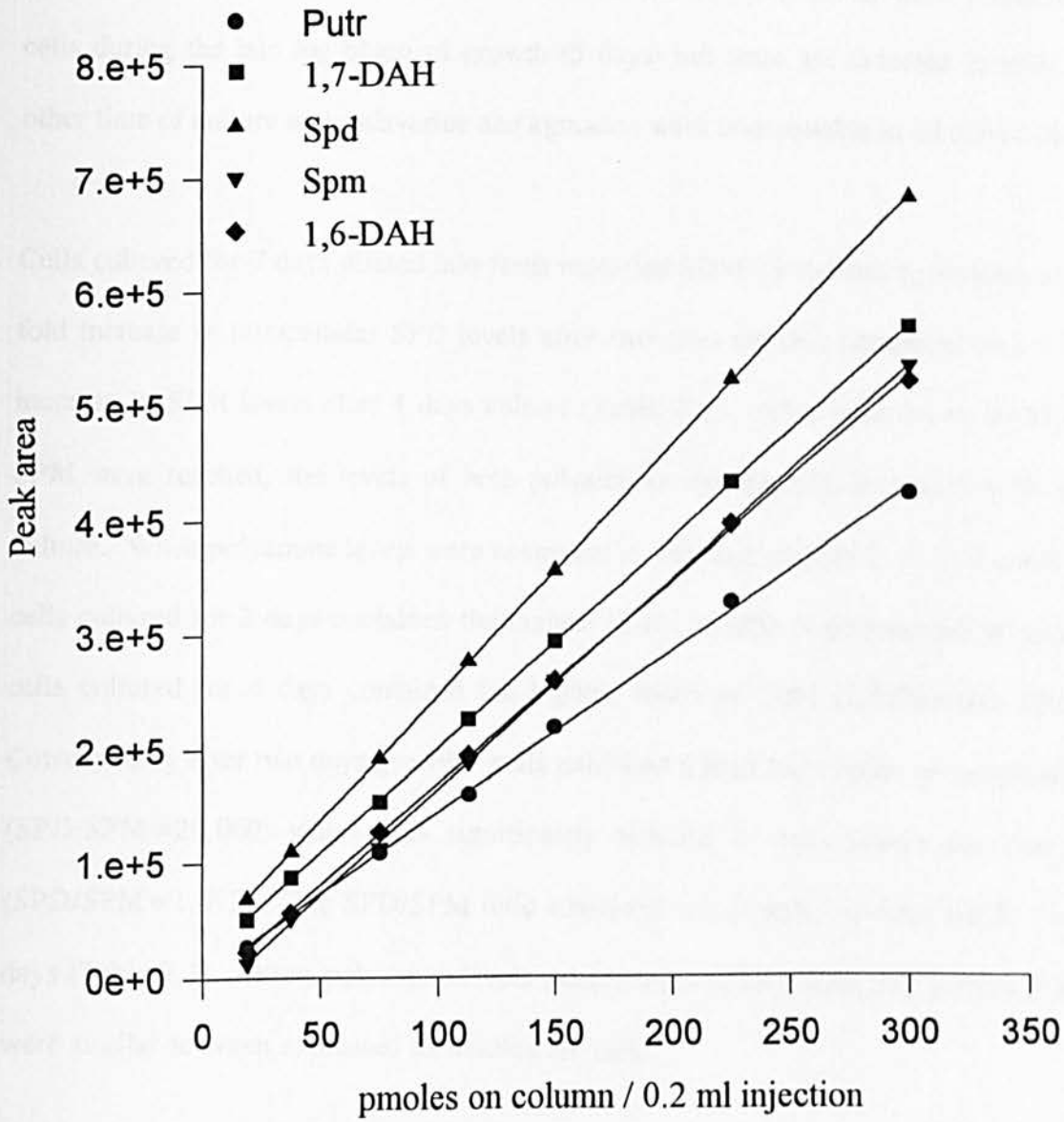


Figure 7.2 Standard curves for the detection of dabsylated polyamines at 436nm. Standards were separated by HPLC as described in 6.2. Values are the mean of three separate determinations. Putr=putrescine, 1,7-DAH=1,7-diaminoheptane, Spd=spermidine, Spm=spermine and 1,6-DAH=1,6-diaminohexane.

peaks (Figure 7.1). It was found upon dabsylation that all extracts of *T. granulosum* cultured in modified SDM-79 contained the polyamines spermidine (SPD) and spermine (SPM) (Table 7.1). Low levels of the diamine putrescine (PUTR) were found in some cells during the late log phase of growth (5 days) but were not detected in cells at any other time of culture and cadaverine and agmatine were undetectable in all cells examined.

Cells cultured for 7 days diluted into fresh modified SDM-79 showed approximately a 13 fold increase in intracellular SPD levels after two days culture, compared to a 3.5 fold increase in SPM levels after 4 days culture (Table 7.1). After peak levels of SPD and SPM were reached, the levels of both polyamines significantly decreased with age of culture. When polyamine levels were examined in cells cultured for 2, 4, 5, 7 and 8 days, cells cultured for 2 days contained the highest levels of SPD (4.654nmoles/10^8cells) and cells cultured for 4 days contained the highest levels of SPM (2.658nmoles/10^8cells). Consequently after two days growth, cells exhibited a high spermidine to spermine ratio (SPD/SPM=20.060) which was significantly reduced in cells grown for four days (SPD/SPM=1.093). The SPD/SPM ratio remained low in cells cultured for 5, 7 and 8 days (Table 7.1). When polyamine levels were expressed as nmoles/mg protein changes were similar to when expressed as nmoles/10^8 cells.

7.1.1.1.2 Cells cultured in media containing polyamine biosynthesis inhibitors

Culturing *T. granulosum* in modified SDM-79 containing a range of concentrations of α-difluoromethylornithine (DFMO) (0, 10, 20, 50mM), significantly altered intracellular concentrations of free polyamines (Table 7.2). The results in table 7.2 were expressed as a percentage of the controls because they are the combination of two experiments, where

Days post-inoculation	nmoles / 10^8 cells				nmoles / mg protein			
	PUTR	SPD	SPM	SPD /SPM	PUTR	SPD	SPM	SPD /SPM
2	nd	4.654 ±0.251	0.232 ±0.084	20.060	nd	6.647 ±0.357	0.335 ±0.124	19.842
4	nd	2.906 * ±0.091	2.658 * ±0.112	1.093	nd	3.719 * ±0.116	3.420 * ±0.144	1.087
5	0.631 ±0.700	1.674 * ±0.331	0.909 * ±0.104	1.842	0.980 ±1.081	2.581 * ±0.511	1.406 * ±0.161	1.836
7 (n=2)	nd	0.363 ±0.117	0.740 ±0.062	0.491	nd	0.532 ±0.173	1.050 ±0.088	0.507
8	nd	0.576 ±0.011	0.485 ±0.052	1.188	nd	0.667 ±0.013	0.563 ±0.062	1.198

Table 7.1 Polyamine levels in cultured *T. granulosum*. Cells were cultured in modified SDM-79. Results are the mean of three separate determinations except where otherwise stated ± standard deviations. nd= not detected. PUTR=putrescine, SPD=spermidine, SPM=spermine and SPD/SPM=spermidine to spermine ratio. * indicates values are significantly different from previous time point, using the Mann-Whitney U test ($p<0.05$).

Conc. of DFMO in medium /mM	% nmoles/10^8 cells		% nmoles/mg protein	
	SPD	SPM	SPD	SPM
0	100.00	100.00	100.00	100.00
10	93.56 * ±1.89	90.30 * ±4.22	84.17 * ±1.70	85.05 * ±3.97
20	156.07 * ±1.52	261.61 * ±21.10	110.84 * ±1.08	186.92 * ±15.08
50	243.91 * ±51.26	219.85 * ±6.64	136.91 * ±28.77	125.19 * ±3.78

Table 7.2 Polyamine levels in *T. granulosum* cultured for four days in medium containing DFMO. All values are expressed as a percentage of the controls. Results are the mean of three separate determinations ± standard deviation. * indicates values are significantly different from control values, using the Mann-Whitney U test ($p<0.05$). SPD=spermidine, SPM=spermine.

although growth rate of the cells in both experiments were similar, thelevels of polyamines in control cells (cells cultured in drug free medium) were significantly different. It was noted that different batches of foetal calf serum were used in making the media in the two experiments. In the presence of 10mM DFMO, *Trypanosoma granulosum* grown for 4 days in modified SDM-79 showed a significant decrease in the levels of both SPD and SPM when these were expressed as nmoles/10^8cells (Table 7.2). In the presence of 20mM DFMO there was approximately a 1.5 fold increase in intracellular SPD levels compared to a larger (2.6 fold) increase in SPM levels. In the presence of 50mM DFMO there was a 2.4 fold increase in intracellular SPD and a 2.2 fold increase in SPM. These changes were found to be statistically significant (see Table 7.2), but when results were expressed as nmoles/mg protein, the changes in levels of SPD and SPM were not as large as when expressed as nmoles/10^8 cells. This was due to a significant increase in protein content in cells grown in the presence of DFMO.

Cells cultured in modified SDM-79 containing MGBG or Berenil at concentrations which caused approximately 50% cell death showed marked changes in both SPD and SPM levels. In the presence of 0.2mM MGBG, SPD levels increased approximately 3 fold whereas SPM levels increased almost 6 fold when expressed as nmoles/10^8 cells (Table 7.3). In the presence of 0.2mM Berenil, SPD levels increased 3.3 fold whereas SPM levels increased 7.7 fold when expressed as nmoles/10^8 cells. When results were expressed as nmoles/mg protein, the increases in levels of SPD and SPM in cells treated with MGBG or Berenil were significant but not as great as the changes observed when the levels of polyamines were expressed as nmoles/mg protein. This was due to the amount of protein per cell being greater in cells cultured for 4 days in the presence of either 0.2mM MGBG or 0.2mM Berenil compared to cells cultured in the absence of either drug.

Conc. of drug in medium / mM	nmoles / 10^8 cells		nmoles / mg protein	
	SPD	SPM	SPD	SPM
Control	0.997 ±0.033	0.897 ±0.030	1.372 ±0.045	1.228 ±0.040
0.2 MGBG	2.973 * ±0.604	5.253 * ±0.585	2.440 * ±0.494	4.303 * ±0.480
0.2 Berenil	3.320 * ±0.596	6.907 * ±1.173	2.497 * ±0.452	5.193 * ±0.884

Table 7.3 Polyamine levels in *T. granulosum* cultured for four days in medium containing MGBG and Berenil. Cells were cultured in modified SDM-79. Results are the mean of three separate determinations ± standard deviation. SPD=spermidine, SPM=spermine. * indicates values are significantly different from control values, using the Mann-Whitney U test ($p<0.05$).

7.1.1.1.3 Cells cultured in medium containin aminoguanidine

When *T. granulosum* was cultured for 5 days in modified SDM-79 containing 10^{-4}M aminoguanidine, polyamine levels did not significantly change from those present in cells cultured in drug-free medium (Table 7.4).

7.1.1.2 Metabolism of putrescine by *T. granulosum*

7.1.1.2.1 Incorporation of [^{3}H]putrescine into spermidine and spermine

When *T. granulosum* was cultured for 5 days in modified SDM-79 with [^{3}H]putrescine added to the cells 24 hours prior to extraction, it was found upon dabsylation and subsequent HPLC and radiometric detection that [^{3}H]putrescine had been incorporated into both SPD and SPM (Figure 7.3) with no [^{3}H]putrescine detected as putrescine.

When cells were cultured for 5 days in modified SDM-79 containing 0.2mM MGBG or 0.2mM Berenil, with [^{3}H]putrescine added 24 hours prior to extraction, [^{3}H]putrescine had been incorporated into both SPD and SPM in much larger quantities (Figure 7.3) than the incorporation observed in the cells cultured in drug free medium.

Cells cultured for 4 days in modified SDM-79 that were exposed to [^{3}H]putrescine for 2 mins showed rapid incorporation of [^{3}H]putrescine into SPD and SPM. When cells were exposed to [^{3}H]putrescine for 30 minutes, [^{3}H]putrescine was taken up by the cells and subsequently incorporated into both SPD and SPM in greater quantities than cells exposed for 2 minutes (Figure 7.4).

Cell treatment	nmoles / 10^8 cells		nmoles / mg protein	
	SPD	SPM	SPD	SPM
Control	1.027 ±0.103	1.457 ±0.041	1.407 ±0.09	1.923 ±0.19
Medium containing 10^{-4}M aminoguanidine	0.857 ±0.084	1.200 n=1	1.271 ±0.134	1.779 n=1

Table 7.4 Polyamine levels in *T. granulosum* cultured for five days in medium containing aminoguanidine. Cells were cultured in SDM-79 for five days. Values are the mean of three separate determinations (except where otherwise stated) ± standard deviation. SPD=spermidine, SPM=spermine.

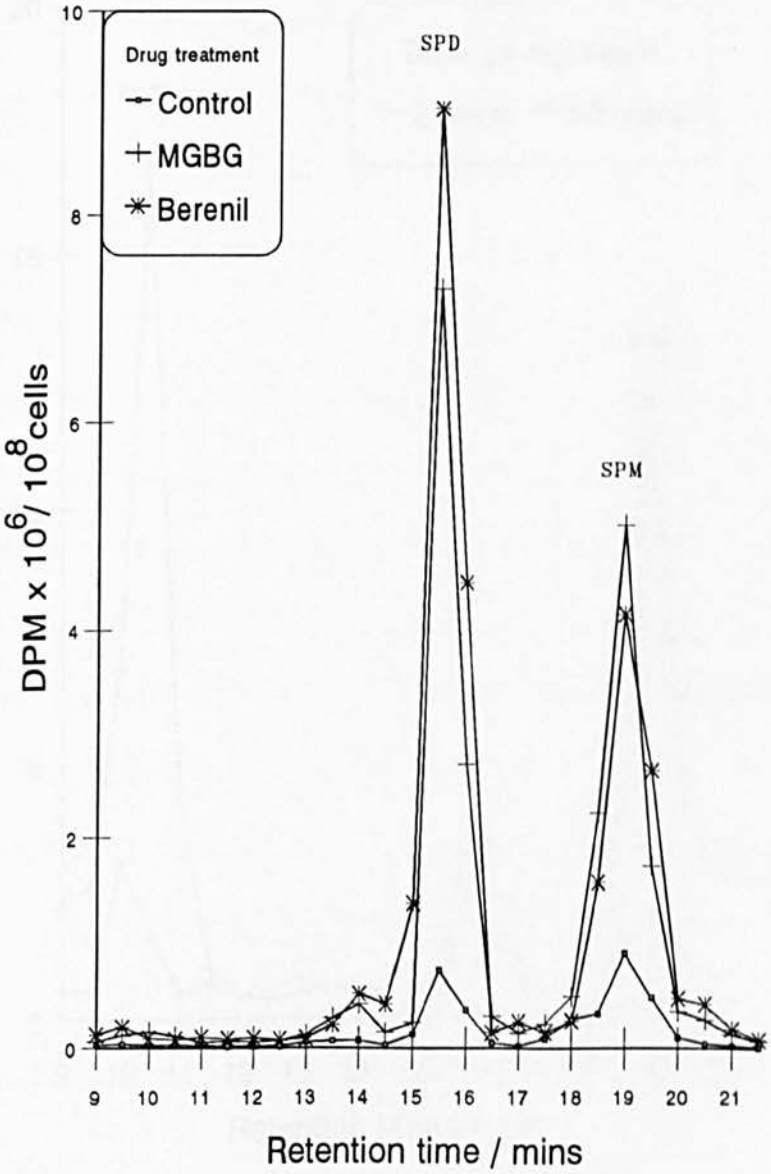


Figure 7.3 The incorporation of [^{3}H]putrescine into spermidine and spermine in *Trypanosoma granulosum* cultured for five days in modified SDM-79 containing 0.2mM MGBG or 0.2mM Berenil. Dansylated polyamines were separated by HPLC and fractions analysed in a scintillation counter as described in section 6.7 of methods chapter. Results are the mean of three separate determinations. SPD=spermidine and SPM=spermine.

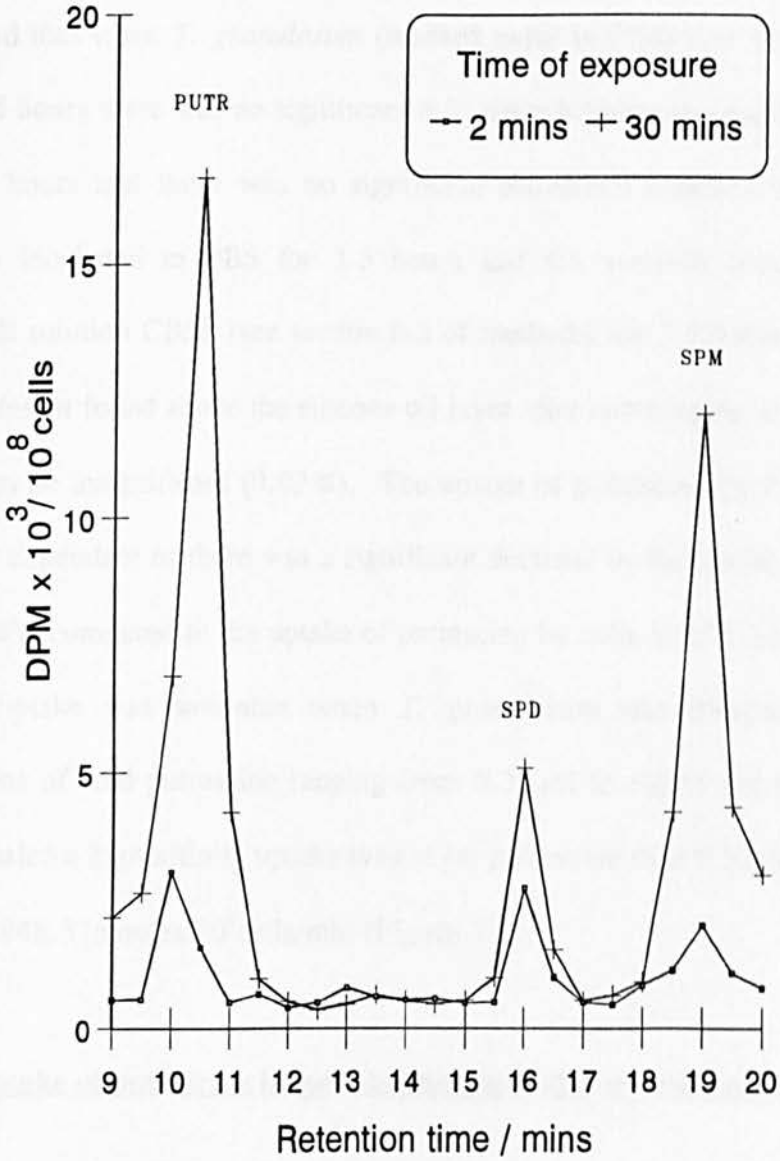


Figure 7.4 The incorporation of [^{3}H]putrescine into spermidine and spermine in *Trypanosoma granulosum* exposed to [^{3}H]putrescine for 2 and 30 minutes. Dansylated polyamines were separated by HPLC and fractions analysed in a scintillation counter as described in section 6.6 of methods chapter. Values are the mean of three separate determinations. Peak PUTR=putrescine, SPD=spermidine and SPM=spermine.

7.1.1.3 Uptake of putrescine by *T. granulosum*

7.1.1.3.1 Uptake of putrescine in the absence of drugs

It was found that when *T. granulosum* (washed twice in PBS) was incubated at 22^{0}C in PBS for 3.5 hours there was no significant difference between the number of cells at t=0 and t=3.5 hours and there was no significant difference between the numbers of *T. granulosum* incubated in PBS for 3.5 hours and the numbers incubated in Carter's balanced salt solution CBSS (see section 6.5 of methods) for 3.5 hours. The percentage of *T. granulosum* found above the silicone oil layer after centrifuging at 10000g for 1 min was found to be insignificant (0.02%). The uptake of putrescine by *T. granulosum* was temperature dependent as there was a significant decrease in the uptake of putrescine into the cells at 4^{0}C compared to the uptake of putrescine by cells at 22^{0}C (results not shown). Putrescine uptake was saturable when *T. granulosum* was incubated with varying concentrations of cold putrescine ranging from 0.25μM to 10μM and Michaelis Menten kinetics revealed a high affinity uptake system for putrescine (Km 0.52μM) and Vmax was found to be 948.37pmoles/10^{8}cells/min (Figure 7.5).

7.1.1.3.2 Uptake of putrescine by cells incubated in the presence of MGBG or Berenil

When *T. granulosum* was incubated with various concentrations of putrescine (0.25-10μM) in the presence of 0.2mM MGBG, Michaelis Menten kinetics revealed an affinity for the uptake of putrescine (Km=0.52μM) (Figure 7.6) similar to the affinity for the uptake of putrescine in cells incubated in a drug free medium, but the rate of putrescine uptake (Vmax=838.53pmoles/10^8 cells/min) was slightly lower in the presence of 0.2 MGBG

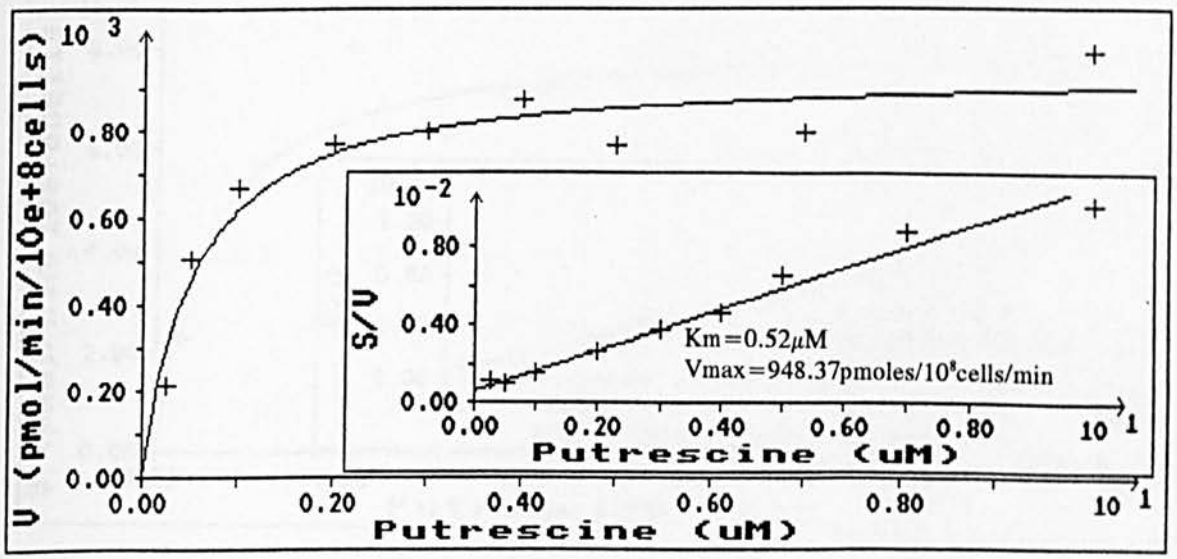


Figure 7.5 Kinetics of putrescine uptake at 22°C in cultured *Trypanosoma granulosum*. Each value represents the initial rate from 6 early time-points (0-60secs) of [^{3}H]Putrescine uptake at a specific concentration of unlabelled putrescine ([S]) (r=0.99). The inset shows S/V vs. S plot.

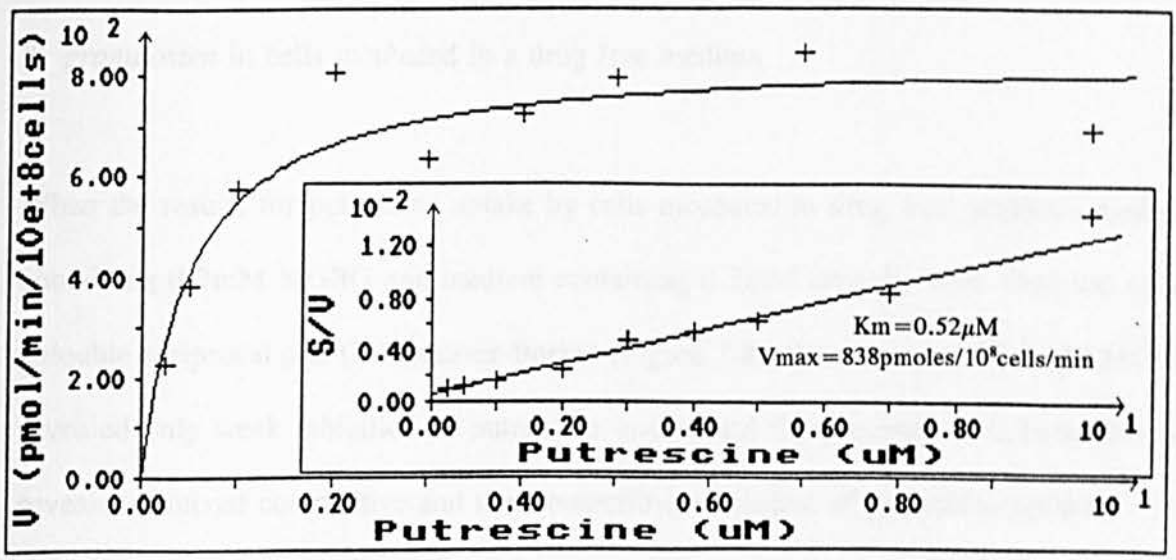


Figure 7.6 Kinetics of putrescine uptake in the presence of 0.2mM MGBG at 22°C in cultured *Trypanosoma granulosum*. Each value represents the initial rate from 6 early time points (0-60secs) of [^{3}H]Putrescine uptake at a specific concentration of unlabelled putrescine ([S]) (r=0.99). All unlabelled concentrations of putrescine contained the same concentration of MGBG. The inset shows S/V vs. S plot.

compared to the rate of putrescine uptake in cells incubated in drug free medium, althoughthe experiment was not repeated therefore it is unknown whether the change was significant.

When *T. granulosum* was incubated with various concentrations of putrescine (0.25-10μM) in the presence of 0.2mM Berenil, Michaelis Menten kinetics revealed an affinity over 3 fold lower (Km=1.86μM) (Figure 7.7) than the affinity for the uptake of putrescine in cells incubated in a drug free medium and the rate of putrescine uptake (Vmax=537.94pmoles/10^8 cells/min) almost half that of the rate of putrescine uptake for *T. granulosum* in cells incubated in a drug free medium.

When the results for putrescine uptake by cells incubated in drug free medium, medium containing 0.2mM MGBG and medium containing 0.2mM Berenil, were analysed using a double reciprocal plot (Lineweaver-Burke) (Figure 7.8), the presence of 0.2mM MGBG revealed only weak inhibition of putrescine uptake and the presence of 0.2mM Berenil revealed a mixed competitive and non-competitive inhibition of putrescine uptake.

7.1.1.4 Polyamines in the medium

Putrescine (0.58$\pm$0.04μM) and spermidine (0.26$\pm$0.03μM) were detected in fresh modified SDM-79 (containing 10% foetal calf serum), but other polyamines were either absent or present at concentrations below the detection limit.

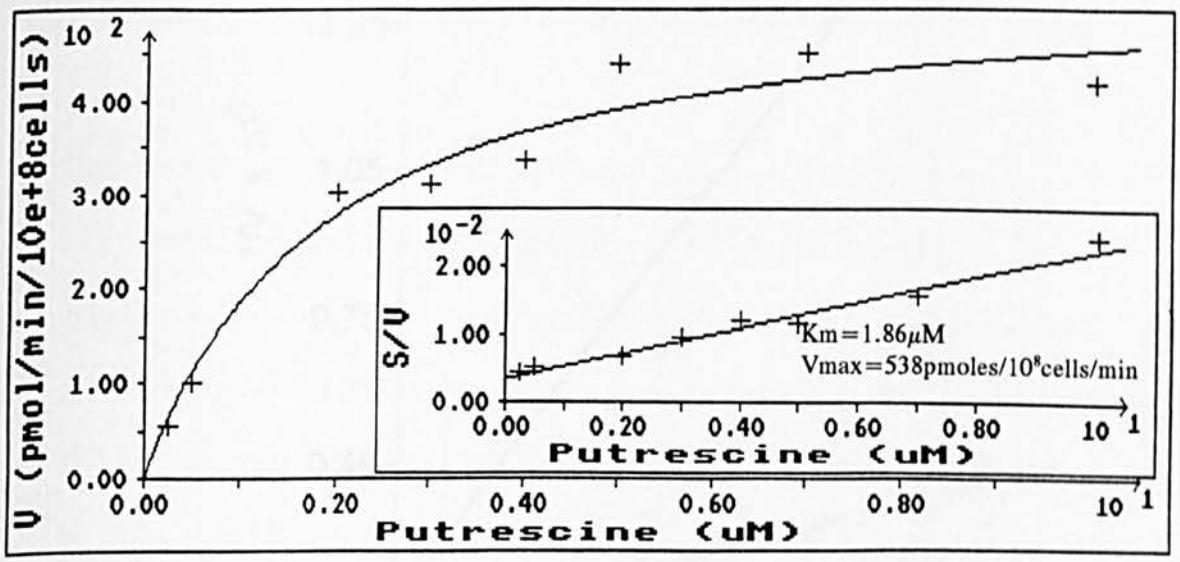


Figure 7.7 Kinetics of putrescine uptake in the presence of 0.2mM Berenil at 22°C in cultured *Trypanosoma granulosum*. Each value represents the initial rate from 6 early time points (0-60secs) of [^{3}H]Putrescine uptake at a specific concentration of unlabelled putrescine ([S]) (r=0.99). All unlabelled concentrations of putrescine contained the same concentration of Berenil. The inset shows S/V vs. S plot.

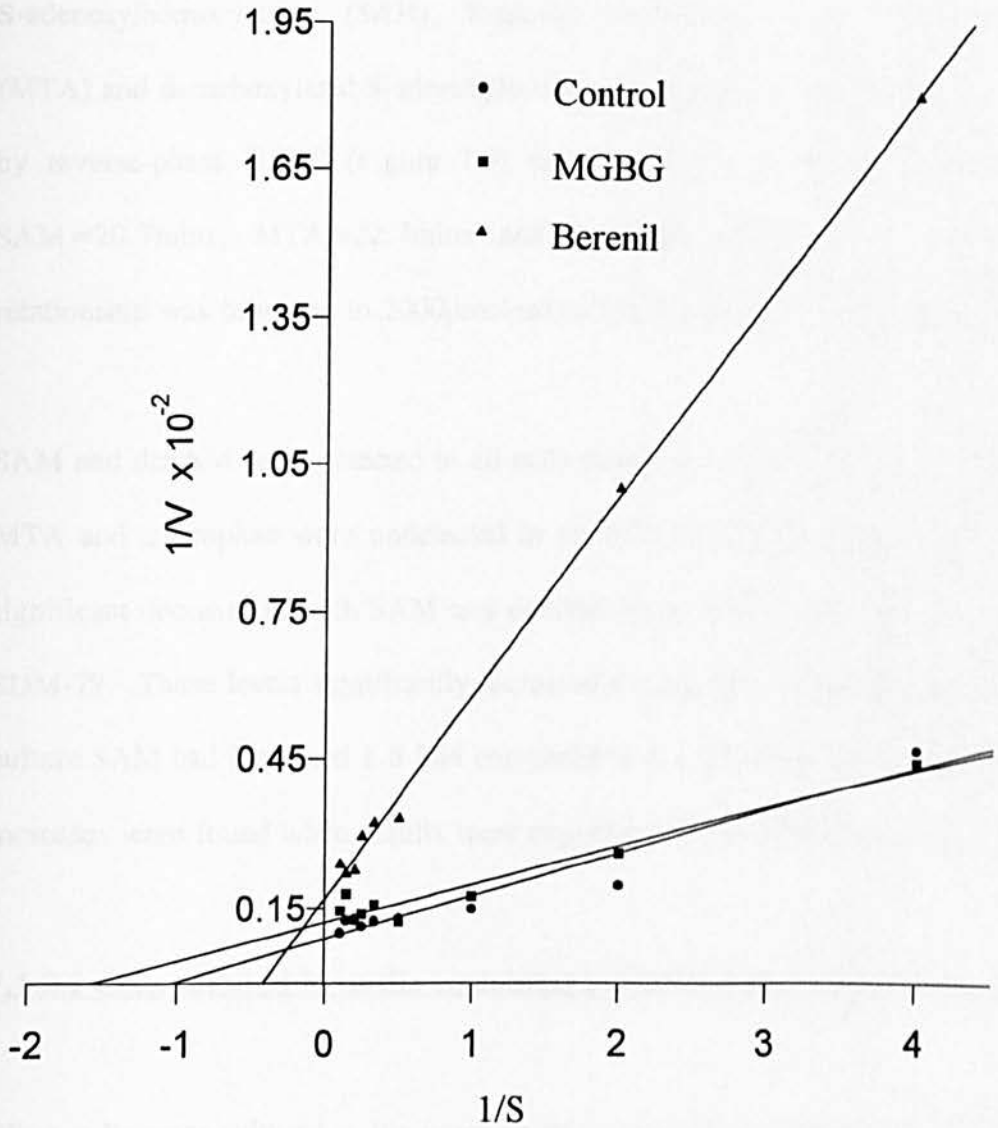


Figure 7.8 Lineweaver-Burke plot of the effect of 0.2mM MGBG and 0.2mM Berenil on uptake of [^{3}H]putrescine into *Trypanosoma granulosum*. Control values represent the uptake of [^{3}H]putrescine into cells in the absence of MGBG or Berenil as described in section 6.7 of methods chapter. Values are the mean of three separate determinations. SPD=spermidine and SPM=spermine.

7.1.2 Detection of S-adenosyl methionine and related metabolites in cultured *T. granulosum*

7.1.2.1 Cells cultured in drug free media

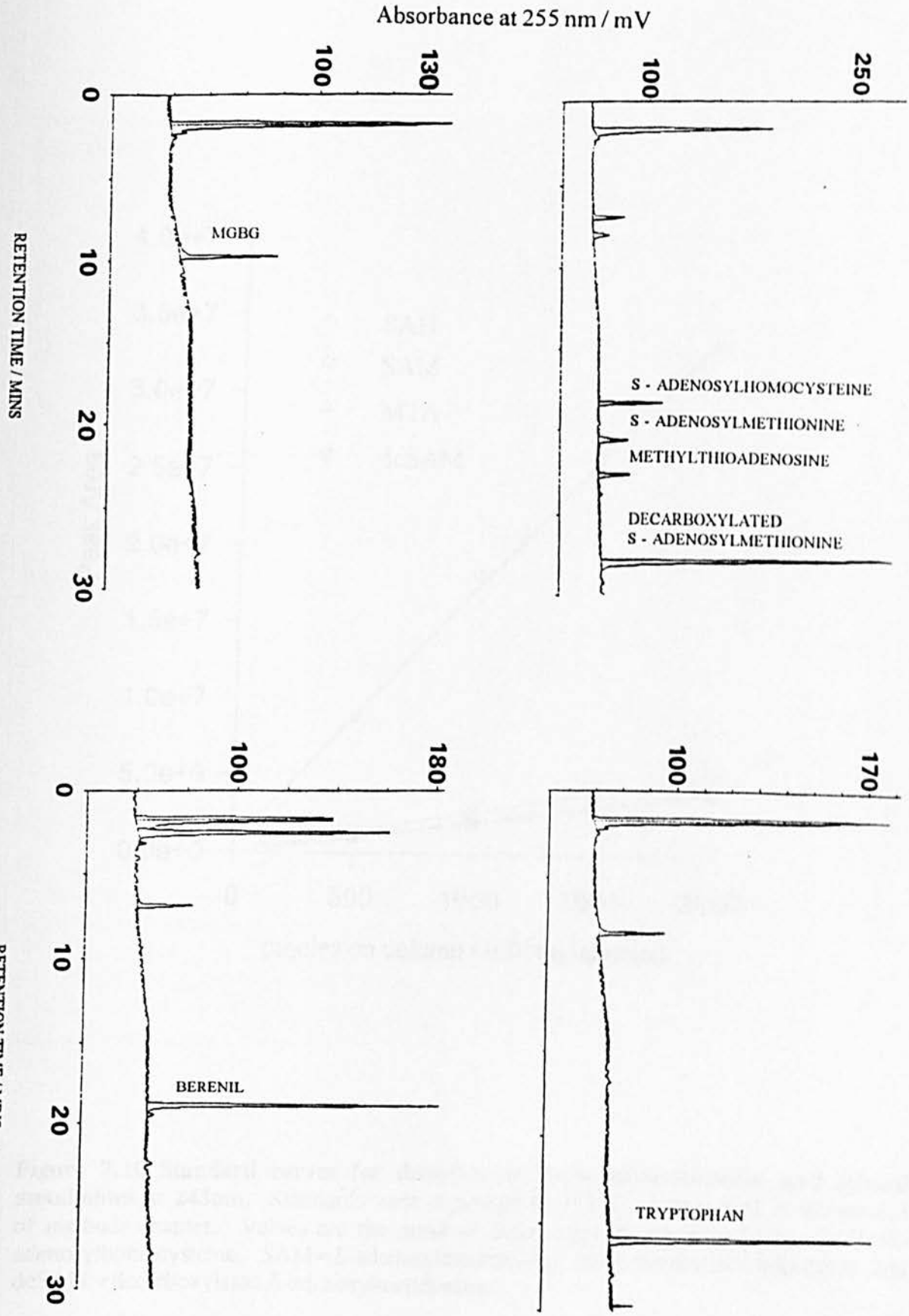
S-adenosylhomocysteine (SAH), S-adenosylmethionine (SAM), methylthioadenosine (MTA) and decarboxylated S-adenosylmethionine (dcSAM) were separated over 35 mins by reverse-phase HPLC (Figure 7.9) (approximate retention times: SAH=18.4mins, SAM=20.7mins, MTA=22.7mins and dcSAM=27.9mins). A linear (r=0.99) relationship was found up to 2000pmoles/0.02ml injected on the column (Figure 7.10).

SAM and dcSAM were detected in all cells grown in modified SDM-79, whereas SAH, MTA and tryptophan were undetected in all cells examined (Table 7.5). There was a significant decrease in both SAM and dcSAM levels after two days growth in modified SDM-79. These levels significantly increased during time of culture and after 8 days of culture SAM had increased 1.8 fold compared to a 3.3 fold increase in dcSAM. Similar increases were found when results were expressed as nmoles/mg protein (Table 7.5).

7.1.2.2 Cells cultured in media containing polyamine biosynthesis inhibitors

When cells were cultured in the presence of a range of concentrations of DFMO (0, 10, 20, 50mM), significant changes were observed in the levels of SAM and dcSAM (Table 7.6). In the presence of 10mM DFMO, cells cultured for 4 days had significantly increased levels of both SAM and dcSAM compared to levels observed in cells cultured in drug free medium. In the presence of 20mM DFMO a more than 2 fold increase in

Figure 7.9 Separation of S-adenosylmethionine and related metabolites by reverse-phase HPLC. Subsequent detection at 254nm as described in section 6.3 of methods chapter.


Absorbance at 255 nm / mV
250
100
S - ADENOSYLHOMOCYSTEINE
S - ADENOSYLMETHIONINE
METHYLTHIOADENOSINE
DECARBOXYLATED
S - ADENOSYLMETHIONINE
130
100
MGBG
0
10
20
30
RETENTION TIME / MINS
170
100
TRYPTOPHAN
180
100
BERENIL
0
10
20
30
RETENTION TIME / MINS

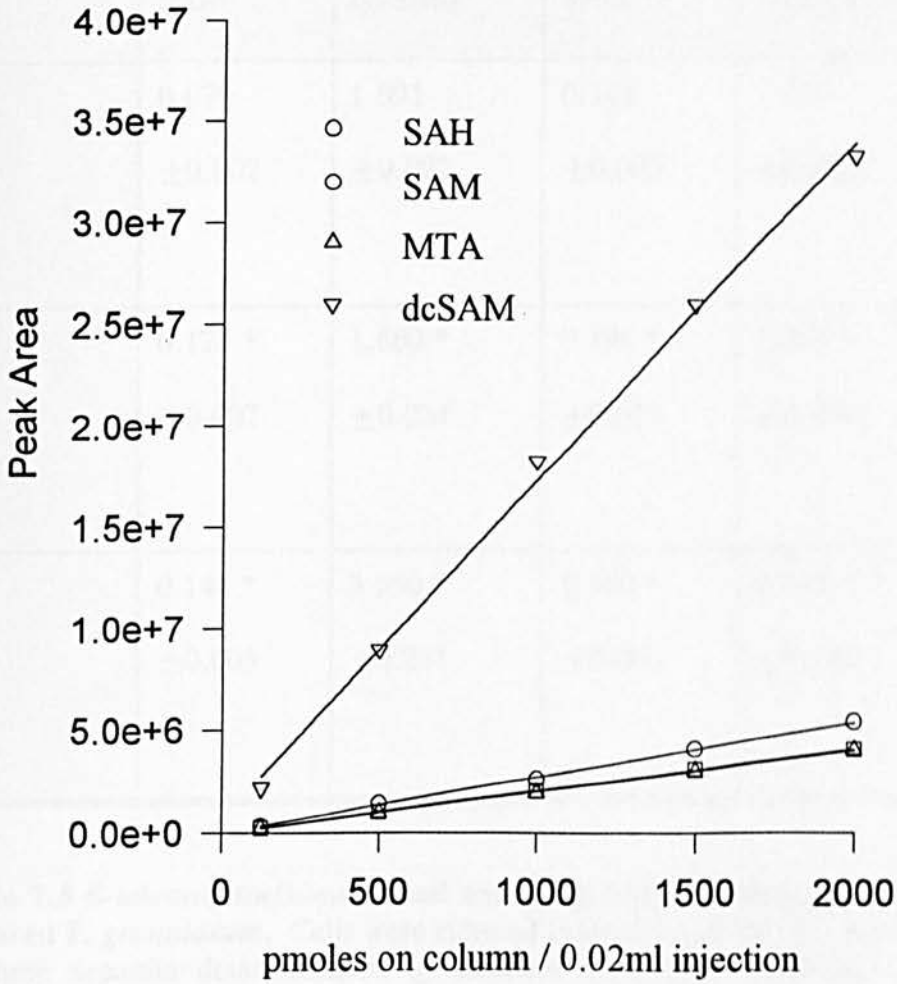


Figure 7.10 Standard curves for detection of S-adenosylmethionine and related metabolites at 245nm. Standards were separated by HPLC as described in section 6.3 of methods chapter. Values are the mean of three separate determinations. SAH=S-adenosylhomocysteine, SAM=S-adenosylmethionine, MTA=methylthioadenosine and dcSAM=decarboxylated S-adenosylmethionine.

Days of culture	nmoles / 10^8 cells		nmoles / mg protein	
	SAM	dc-SAM	SAM	dc-SAM
2	0.079 ±0.002	1.091 ±0.092	0.113 ±0.003	1.557 ±0.132
5	0.126 * ±0.007	1.660 * ±0.034	0.194 * ±0.013	2.559 * ±0.052
8	0.144 * ±0.005	3.550 * ±0.251	0.168 * ±0.006	4.142 * ±0.294

Table 7.5 S-adenosylmethionine and decarboxylated S-adenosylmethionine levels in cultured *T. granulosum*. Cells were cultured in modified SDM-79. Results are the mean of three separate determinations ± standard deviation. * indicates that values are significantly different from values obtained from cells cultured for 2 days using the Mann-Whitney U test ($p<0.05$). SAM=S-adenosylmethionine, dcSAM=decarboxylated S-adenosylmethionine.

Conc. of DFMO in medium / mM	% nmoles / 10^8 cells		% nmoles / mg protein	
	SAM	dc-SAM	SAM	dc-SAM
0	100.00	100.00	100.00	100.00
10	119.99 * ±9.97	199.12 * ±13.34	107.95 ±8.97	187.54 * ±12.56
20	257.62 * ±8.59	252.23 * ±8.55	182.96 * ±6.10	180.22 * ±6.11
50	247.72 * ±59.12	129.21 ±34.56	139.05 * ±33.18	73.58 ±19.68

Table 7.6 S-adenosylmethionine and decarboxylated S-adenosylmethionine levels in *T. granulosum* cultured in medium containing DFMO. Cells were cultured in modified SDM-79. All values are expressed as a percentage of the controls. Results are the mean of three separate determinations ± standard deviation. * indicates a significant difference from control values using the Mann-Whitney U test ($p<0.05$). SAM=S-adenosylmethionine, dcSAM=decarboxylated S-adenosylmethionine.

levels of both SAM and dcSAM was observed compared to the levels in cells cultured in drug-free medium. In the presence of 50mM DFMO there was a similar increase in levels of SAM to the increase observed in cells cultured in medium containing 20mM DFMO but there was no significant change in the levels of dcSAM (Table 7.6). When expressed as nmoles/mg protein the changes observed were not as large as when expressed as nmoles/10^8 cells and indeed when expressed as nmoles/mg protein cells cultured in the presence of 10mM DFMO the levels of SAM did not change significantly.

When *T. granulosum* was grown for 4 days in modified SDM-79 containing MGBG or Berenil at concentrations causing approximately 50% cell death, significant changes were observed in both SAM and dcSAM levels compared to cells grown in drug free medium (Table 7.7). In the presence of 0.2mM MGBG there was a 3.3 fold increase in SAM levels and approximately a 10 fold decrease in dcSAM levels. In the presence of 0.2mM Berenil there was a 77.4 fold increase in levels of SAM but approximately a 10 fold decrease in dcSAM (Table 7.7), similar to the decrease in levels of dcSAM observed in cells cultured in the presence of 0.2mM MGBG.

Conc. of drug in medium / mM	nmoles / 10^8 cells		nmoles / mg protein	
	SAM	dc-SAM	SAM	dc-SAM
Control cells	0.309 ±0.013	4.079 ±0.294	0.425 ±0.018	5.588 ±0.403
0.2 MGBG	1.023 * ±0.005	0.423 * ±0.012	0.840 * ±0.004	0.347 * ±0.009
0.2 Berenil	23.916 * ±3.824	0.497 * ±0.012	17.987 * ±2.876	0.374 * ±0.009

Table 7.7 S-adenosylmethionine and decarboxylated S-adenosylmethionine levels in *T. granulosum* cultured for four days in medium containing MGBG and Berenil. Cells were cultured in modified SDM-79. Results are the mean of three separate determinations ± standard deviation. * indicates a significant difference from control values using the Mann-Whitney U test ($p < 0.05$). SAM=S-adenosylmethionine, dc-SAM=decarboxylated S-adenosylmethionine.

7.1.3 Thiols in cultured *Trypanosoma granulosum*

7.1.3.1 Thiol levels in cells cultured in modified SDM-79

Derivatised thiols were separated over 60mins by reverse-phase HPLC. A linear relationship was found up to 250 pmoles/20μl injection (approximate retention times glutathione=31.3mins, glutathionylspermidine=47.5mins, glutathionylaminopropylcadaverine=48.4mins, approximate retention times: ovothiol A=36.7mins, glutathione=40.2mins, glutathionylspermidine=58.4mins, N^1, N^8-*bis*(glutathionyl)spermidine=51.0mins, N^1, N^9-*bis*(glutathionyl)aminopropylcadaverine=52.7mins glutathionylaminopropylcadaverine=59.4mins (Figure 7.11). When samples were injected onto the HPLC Technology Limited Ultrasphere Ion-pair column (5μm) (6.4 of methods section) retention times of the derivatised thiols increased by approximately 10mins and ovothiol A (OSH-A) was detected for the first time (Figure 7.12). Reduced glutathione (GSH) was the major thiol detected in *T. granulosum* grown in modified SDM-79, and the conjugates glutathionylspermidine (GSPD(SH)) and trypanothione ($T(SH)_2$) were undetectable in all cells grown in modified SDM-79. Of the cells cultured for 2, 5 and 8 days in modified SDM-79, cells cultured for 2 days contained the highest levels of GSH (16.188nmoles/10^8 cells). Upon dilution into fresh medium GSH levels increased approximately 4 fold over the first 2 days and then significantly decreased to 4.415nmoles/10^8 cells by day 8 of culture (Table 7.8).

Glutathione reductase (GR) activity was detectable in twice dialysed *E. coli* cultured in nutrient broth for 24 hours but undetectable in twice dialysed *T. granulosum* extracts,

Figure 7.11 Separation of derivatised thiols by reverse-phase HPLC. Subsequent fluorometric detection as described in section 6.4 of methods chapter. GSH=glutathione, GSPD(SH)=glutathionylspermidine, $T(SH)_2$=trypanothione and $HT(SH)_2$=N^1, N^9-*bis*(glutathionyl)aminopropylcadaverine (homotrypanothione).

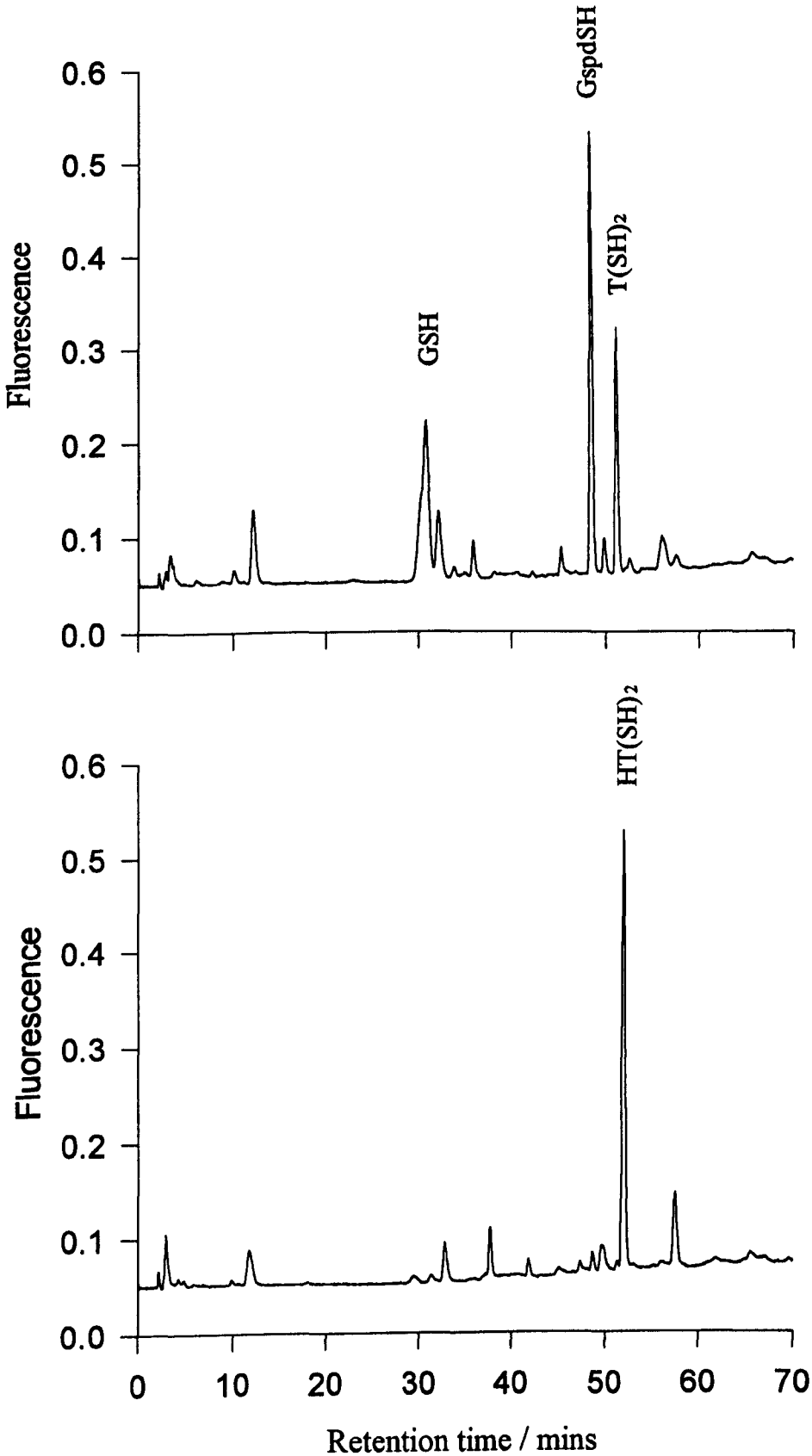
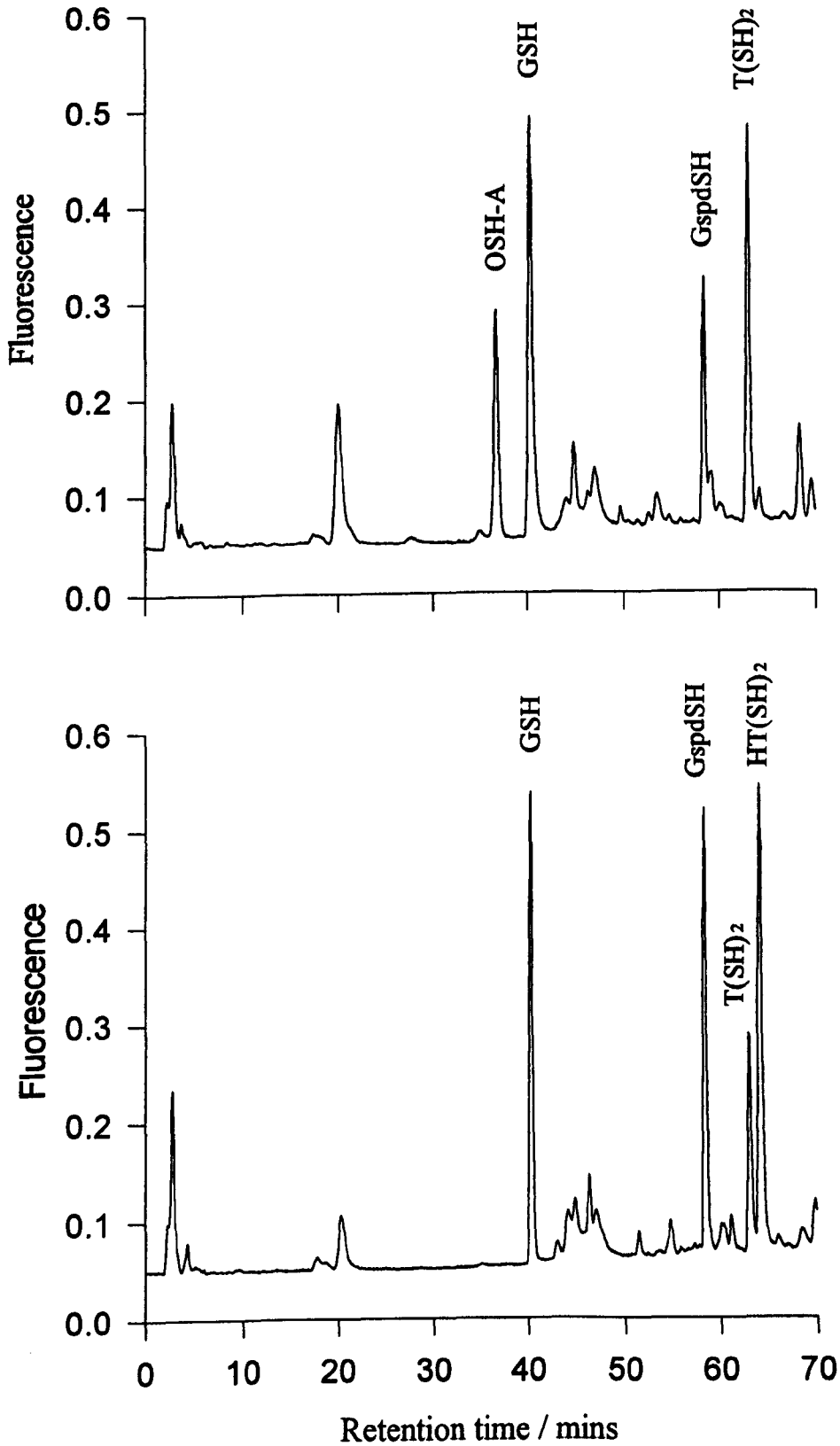

GspdSH
T(SH)2
GSH
HT(SH)2
Fluorescence
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
70
Retention time / mins

Figure 7.12 Separation of derivatised thiols by reverse-phase HPLC. Subsequent fluorometric detection as described in section 6.4 of methods chapter. GSH=glutathione, GSPD(SH)=glutathionylspermidine, $T(SH)_2$=trypanothione, $HT(SH)_2$=N^1, N^9-*bis*(glutathionyl)aminopropylcadaverine (homotrypanothione) and OSH-A=ovothiol A.


Fluorescence
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OSH-A
GSH
GspdSH
T(SH)2
Fluorescence
0.6
0.5
0.4
0.3
0.2
0.1
0.0
GSH
GspdSH
T(SH)2
HT(SH)2
0
10
20
30
40
50
60
70
Retention time / mins

Days of culture	nmoles/10^8 cells			nmoles/mg protein		
	GSH	GSPD(SH)	$T(SH)_2$	GSH	GSPD(SH)	$T(SH)_2$
2	16.188 ±1.671	nd	nd	23.160 ±2.391	nd	nd
5	6.300 * ±0.298	nd	nd	9.707 * ±0.458	nd	nd
8	4.415 * ±0.223	nd	nd	5.146 * ±0.260	nd	nd

Table 7.8 Thiol levels in cultured *T. granulosum*. Cells were cultured in modified SDM-79. Values are the mean of four separate determinations ± standard deviation. nd=not detectable. * indicates values are significantly different from the previous time point using the Mann-Whitney U test ($p<0.05$). GSH=glutathione, GSPD(SH)=glutathionylspermidine and $T(SH)_2$=trypanothione.

however trypanothione reductase (TR) activity was detectable in all *T. granulosum* cell extracts examined (Table 7.9).

7.1.3.2 Thiol levels in cells cultured in media containing additional diamines

The major thiol detected in *T. granulosum* cultured for 5 and 8 days in modified SDM-79 containing putrescine (5μM final concentration) was GSH (Table 7.10) as was found in cells cultured in modified SDM-79 without the addition of exogenous putrescine. In addition $T(SH)_2$ levels were detected whereas GSPD(SH) remained undetected. Cells cultured in the presence of 5μM putrescine showed a significant increase (nmoles/10^8 cells) in GSH levels when cultured for 8 days compared to cells cultured for 5 days, but this increase was not apparent when values were expressed as nmoles/mg protein. These increases in GSH levels between cells cultured in the presence of additional putrescine (5μm) for 5 and 8 days (Table 7.10) were not observed in cells cultured in media lacking additional putrescine (Table 7.8).

Cells cultured for 5 days with 100μl putrescine (100μM final concentration) added 24 hours prior to extraction contained significantly higher (2.557nmoles/10^8 cells) levels of GSH than cells grown in modified SDM-79 with 100μl buffer (control) added 24 hours prior to extraction (1.510nmoles/10^8 cells) (Table 7.11). GSPD(SH) and $T(SH)_2$, both undetected in control cells were detected in cells cultured in modified SDM-79 containing 100μM putrescine. Occasionally a "split peak" appeared on the chromatograms with the same retention time expected of GSPD(SH) and an unidentified peak (still present after further extraction with ethyl acetate) was observed in cells cultured in modified SDM-79 containing 100μM putrescine (Figure 7.13).

	Activity (U/mg protein)
TRYPANOTHIONE REDUCTASE	
T. granulosum	0.037±0.001
GLUTATHIONE REDUCTASE	
T. granulosum	nd
E. coli	0.035±0.022

Table 7.9 Trypanothione reductase and glutathione reductase activities in *T. granulosum*. Results for *T. granulosum* and *E. coli* are the mean of three separate determinations ± standard deviation. nd=not detected.

Days of culture	nmoles/10^8 cells			nmoles/mg protein		
	GSH	GSPD(SH)	$T(SH)_2$	GSH	GSPD(SH) SPD	$T(SH)_2$
5	4.905 ±1.065	nd	0.053 ±0.026 n=3	7.536 ±1.634	nd	0.082 ±0.040 n=3
8	7.165 * ±0.875	nd	0.070 n=1	8.479 ±1.035	nd	0.060 n=1

Table 7.10 Thiol levels in *T. granulosum* cultured in medium containing putrescine. Cells were cultured in modified SDM-79 containing putrescine (5μM) added at day 0. Values are means of four separate determinations (except where otherwise stated) ± standard deviation. nd=not detectable. * indicates values are significantly different from the previous time point using the Mann-Whitney U test ($p<0.05$). GSH=glutathione, GSPD(SH)=glutathionylspermidine and $T(SH)_2$=trypanothione.

Table 7.11 Thiol levels in *T. granulosum* cultured for five days in medium containing the diamines putrescine (100μM) or cadaverine (100μM). Cells were cultured in modified SDM-79. Diamines were added to the culture 24 hours prior to extraction. Results are the mean of six separate determinations $\pm$ standard deviation. * indicates values are significantly different from the control using the Mann-Whitney U test ($p<0.05$). nd=not detected. GSH=glutathione, GSPD(SH)=glutathionylspermidine, GAPC(SH)=glutathionylaminopropylcadaverine, $T(SH)_2=N^1$, N^8-*bis*(glutathionyl)spermidine (trypanothione), $HT(SH)_2=N^1$, N^9-*bis*(glutathionyl)aminopropylcadaverine (homotrypanothione).

Medium	nmoles / 10^8 cells				
	GSH	GSPD(SH)	GAPC(SH)	$T(SH)_2$	$HT(SH)_2$
Control	1.510 ±0.149	nd	nd	nd	nd
containing putrescine	2.557 * ±0.219	0.731 * ±0.202	nd	0.801 * ±0.179	nd
containing cadaverine	2.373 * ±0.161	0.052 * ±0.018	0.069 * ±0.026	<0.050	0.261 * ±0.053

Medium	nmoles / mg protein				
	GSH	GSPD(SH)	GAPC(SH)	$T(SH)_2$	$HT(SH)_2$
Control	2.253 ±0.222	nd	nd	nd	nd
containing putrescine	3.080 * ±0.263	0.881 * ±0.244	nd	0.965 * ±0.215	nd
containing cadaverine	2.728 * ±0.181	0.060 * ±0.021	0.079 * ±0.030	<0.050	0.299 * ±0.060

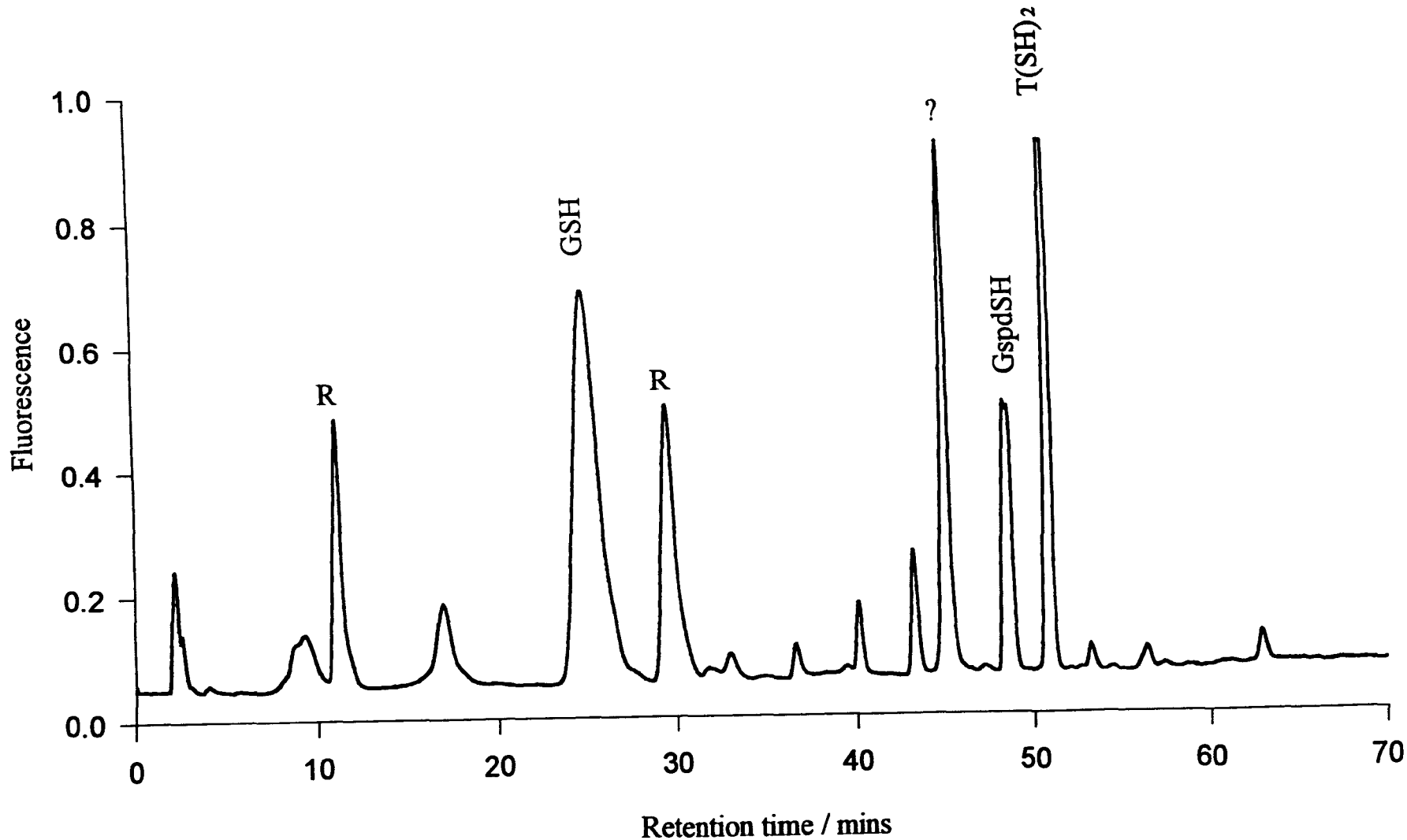


Figure 7.13 Thiols in *Trypanosoma granulosum* cultured in modified SDM-79 with putrescine (100μM) added 24 hours prior to extraction. R=reagent peak, GSH=glutathione, ?=unknown thiol peak, GspdSH=glutathionylspermidine and $T(SH)_2$=trypanothione.

Cells cultured for 5 days with 100μl cadaverine (100μM final concentration) added 24 hours prior to extraction contained significantly higher (2.373nmoles/10^8 cells) levels of GSH than cells grown in modified SDM-79 with 100μl buffer (control) added 24 hours prior to extraction (1.510nmoles/10^8 cells) (Table 7.11). GSPD(SH), $T(SH)_2$, GAPC(SH) and $HT(SH)_2$ were all undetected in control cells but were detected in cells cultured in modified SDM-79 containing 100μM cadaverine.

In addition 0.437$\pm$0.02 nmoles/10^8 cells ovothiol A (OSH-A) was detected in *T. granulosum* cultured in modified SDM-79.

7.2 Biochemical analyses of *T. danilewskyi*

7.2.1 Detection of polyamines in cultured *T. danilewskyi*

7.2.1.1 Cells cultured in drug free medium

Approximately 10^8 cells cultured in modified SDM-79 were needed to detect intracellular free polyamines. Spermidine (SPD) was the only detectable polyamine in *T. danilewskyi* compared to the presence of both SPD and SPM in cultured *T. granulosum*. After 2 days growth in modified SDM-79, SPD levels in *T. danilewskyi* increased approximately 4 fold (Table 7.12) compared to a 13 fold increase in *T. granulosum* and levels of SPD in cells cultured for 4 and 7 days were significantly lower than levels of SPD in cells cultured for 2 days, as found in *T. granulosum*.

7.2.1.2 Cells cultured in medium containing polyamine biosynthesis inhibitors

Culturing *T. danilewskyi* in modified SDM-79 for 4 days in a range of concentrations of DFMO (0, 20 and 50mM), altered intracellular concentrations of free polyamines (Table 7.13). In the presence of 20mM DFMO there was a 2.7 fold increase in free SPD (expressed as nmoles/10^8 cells) compared to cells cultured in the absence of DFMO. Cells cultured in the presence of 50mM DFMO showed a 5.7 fold increase in free SPD levels compared to cells cultured in drug free medium. Expressed as nmoles/mg protein the increases in SPD levels in cells cultured in modified SDM-79 containing DFMO were significantly different from SPD levels in cells cultured in drug free media, but were not as large as the changes expressed as nmoles/ 10^8 cells. All increases in free SPD levels

Days of culture	nmoles / 10^8 cells		nmoles / mg protein	
	SPD	SPM	SPD	SPM
2	0.441 ±0.072	nd	0.631 ±0.110	nd
4	0.284 * ±0.001	nd	0.420 * ±0.001	nd
7	0.118 * ±0.020	nd	0.178 * ±0.031	nd

Table 7.12 Polyamine levels in cultured *T. danilewskyi*. Cells were cultured in modified SDM-79. Results are the mean of three separate determinations ± standard deviation. * indicates values are significantly different from previous time point using the Mann-Whitney U test ($p<0.05$). nd=not detected.

Conc. of DFMO / mM	nmoles / 10^8 cells		nmoles / mg protein	
	SPD	SPM	SPD	SPM
0	0.296 ±0.030	nd	0.438 ±0.044	nd
20	0.801 * ±0.002	nd	0.659 * ±0.002	nd
50	1.685 * ±0.005	nd	1.572 * ±0.005	nd

Table 7.13 Polyamine levels in *T. danilewskyi* cultured for four days medium containing DFMO. Cells were cultured in modified SDM-79. Results are the mean of three separate determinations ± standard deviation. * indicates a significant difference from control values using the Mann-Whitney U test ($p<0.05$). SPD=spermidine, SPM=spermine.

in *T. danilewskyi* cultured in modified SDM-79 containing DFMO were significantly larger than the increases in SPD levels in *T. granulosum* cultured under similar conditions. Interestingly, the IC_{50} for DFMO on *T. danilewskyi* was approximately double that for *T. granulosum* (see section 7.1.3).

When *T. danilewskyi* was cultured for 4 days in modified SDM-79 containing MGBG at a concentration (0.2mM) which caused approximately 50% cell death, there was no significant change in levels of free SPD (Table 7.14), compared to a significant increase in both SPD and SPM in *T. granulosum* cultured under similar conditions. In the presence of Berenil (0.2mM) there was a 3 fold increase in levels of free SPD in *T. danilewskyi* compared to a similar increase in SPD levels in *T. granulosum* cultured under similar conditions. When expressed as nmoles/mg protein the increase in levels of SPD in cells cultured in modified SDM-79 containing Berenil was not as great as when the levels of SPD were expressed as nmoles/10^8 cells. SPM was undetectable in all extracts from *T. danilewsky* is cultured in modified SDM-79 containing any of the polyamine biosynthesis inhibitors.

7.2.2 Thiols in *T. danilewskyi*

7.2.2.1 Cells cultured in modified SDM-79

When *T. danilewskyi* was cultured in modified SDM-79 for 5 days, GSH was the only thiol detected (Table 7.15), as was observed in *T. granulosum* cultured under similar conditions, although *T. danilewskyi* contained more than double the levels of GSH found in *T. granulosum* (Table 7.10).

Conc. of drug in medium / mM	nmoles / 10^8 cells		nmoles / mg protein	
	SPD	SPM	SPD	SPM
Control cells	0.340 ±0.054	nd	0.503 ±0.070	nd
0.2 MGBG	0.450 ±0.110	nd	0.370 ±0.094	nd
0.2 Berenil	0.990 * ±0.091	nd	0.760 * ±0.070	nd

Table 7.14 Polyamine levels in *T. danilewskyi* cultured for four days in medium containing MGBG and Berenil. Cells were grown in modified SDM-79. Results are mean of three separate determinations ± standard deviation. * indicates a significant difference from control values using the Mann-Whitney U test ($p<0.05$). SPD=spermidine, SPM=spermine.

Table 7.15 Thiol levels in *T. danilewskyi* cultured for five days in medium containing the diamines putrescine (100μM) or cadaverine (100μM). Cells were cultured in modified SDM-79. Diamines were added to culture 24 hours prior to extraction. Results are the mean of three separate determinations ± standard deviation. * indicates a significant difference from control values using the Mann-Whitney U test ($p<0.05$). GSH = glutathione, GSPD(SH) = glutathionylspermidine, GAPC(SH) = glutathionylaminopropylcadaverine, $T(SH)_2 = N^1$, N^8-*bis*(glutathionyl)spermidine (trypanothione), $HT(SH)_2 = N^1$, N^9-*bis*(glutathionyl)aminopropylcadaverine (homotrypanothione).

Medium	nmoles / 10^8 cells				
	GSH	GSPD(SH)	GAPC(SH)	$T(SH)_2$	$HT(SH)_2$
Control	4.407 ±1.935	nd	nd	nd	nd
containing putrescine	1.517 * ±0.691	<0.050	nd	0.075 * ±0.015	nd
containing cadaverine	1.726 * ±0.808	nd	0.084 * ±0.119	<0.050	0.076 * ±0.011

Medium	nmoles / mg protein				
	GSH	GSPD(SH)	GAPC(SH)	$T(SH)_2$	$HT(SH)_2$
Control	6.517 ±2.862	nd	nd	nd	nd
containing putrescine	1.667 * ±0.758	<0.050	nd	0.083 * ±0.015	nd
containing cadaverine	1.897 * ±0.886	nd	0.093 * ±0.132	<0.050	0.083 * ±0.012

7.2.2.2 Cells cultured in media containing additional diamines

The addition of 100μM (final concentration) putrescine to the cells 24 hours prior to extraction significantly altered intracellular thiol levels (Table 7.15). GSH levels were decreased compared to cells without additional putrescine. This depletion was the reversal of the situation in *T. granulosum* where GSH levels almost doubled in cells exposed to additional putrescine in their medium. Trypanothione ($T(SH)_2$) and GSPD(SH) were detected in *T. danilewskyi* when the cells were exposed to additional putrescine, these values were approximately 10 fold less than the levels of $T(SH)_2$ and GSPD(SH) found in *T. granulosum* cultured under similar conditions.

When *T. danilewskyi* was cultured in modified SDM-79 with cadaverine (CDV) (100μM final concentration) added 24 hours prior to extraction, the major thiol detected was GSH (Table 7.15), although levels were significantly lower compared to levels of GSH in cells cultured under similar conditions without additional CDV. This decrease was similar to the decrease described above in cells cultured in modified SDM-79 in the presence of additional PUTR. In the presence of additional CDV *T. danilewskyi* contained glutathionylaminopropylcadaverine (GAPC(SH)), homotrypanothione ($HT(SH)_2$) and low levels of $T(SH)_2$. The levels of $HT(SH_2)$ detected in *T. danilewskyi* were approximately 3 fold lower than the levels detected in *T. granulosum* cultured under the same conditions. GSPD(SH) was undetected in *T. danilewskyi* when cultured in the presence of CDV compared to low levels of GSPD(SH) found in *T. granulosum* cultured under the same conditions.

7.3 Detection of intracellular polyamine biosynthesis inhibitors

When *T. granulosum* was incubated in modified SDM-79 containing 50mM DFMO, 0.2mM MGBG or 0.2mM Berenil, it was found that all three drugs were present intracellularly (Table 7.16; Figure 7.9, 7.14). DFMO was also detected in *T. danilewskyi* cultured in modified SDM-79 containing 50mM DFMO (Table 7.16).

Cell type	pmoles / 10^8 cells		
	DFMO	MGBG	Berenil
T. granulosum	334.58 ±4.26	1.03 ±0.06	1.10 ±0.03
T. danilewskyi	300.31 ±16.48	N/A	N/A

Table 7.16 Levels of polyamine biosynthesis inhibitors in *T. granulosum* and *T. danilewskyi* cultured in medium containing 50mM DFMO, 0.2mM MGBG or 0.2mM Berenil. Cells were cultured for 4 days. Results are the mean of three separate determinations ± standard deviation. N/A=not determined.

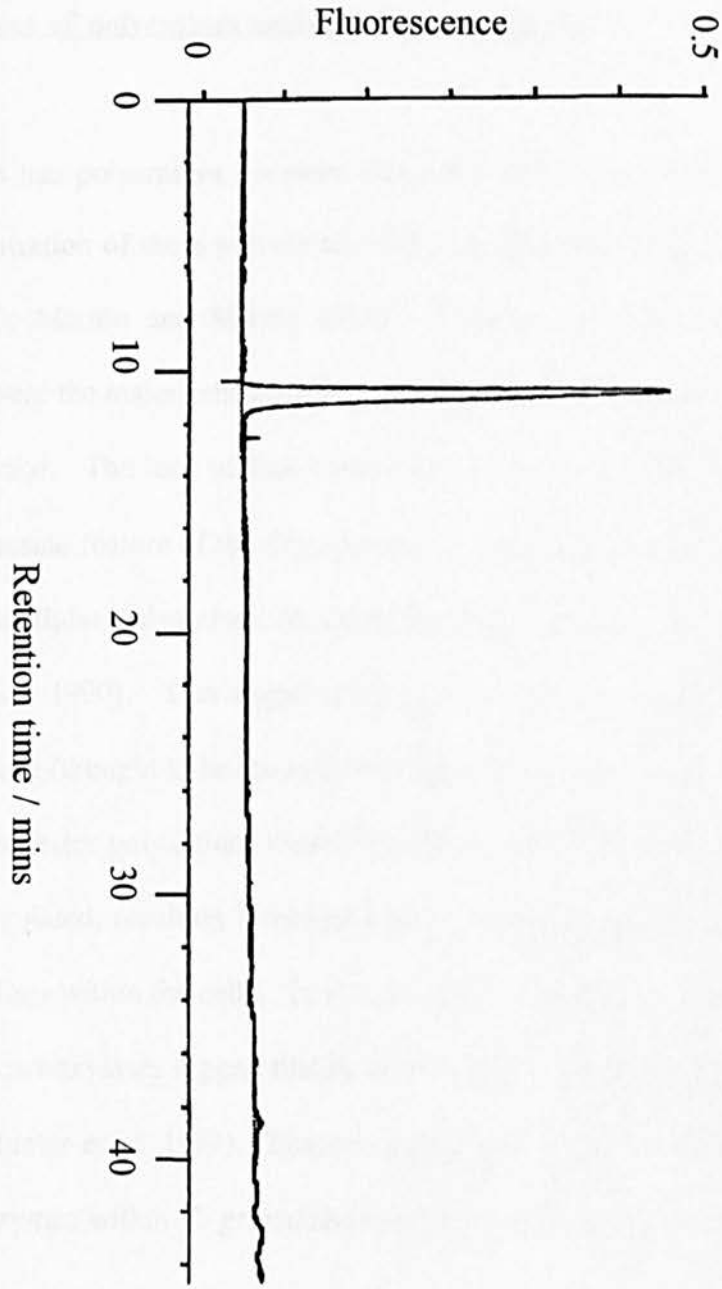


Figure 7.14 Fluorometric detection of post-column derivatised difluoromethylornithine. Separation by reverse phase HPLC as described in section 6.10 of methods chapter.

CHAPTER 8: DISCUSSION

8.1 Analyses of polyamines and related metabolites

It is known that polyamines are required by all cells for normal function and that the type and concentration of these polyamines differ considerably (Tabor and Tabor 1976, 1984; Pegg 1986; Marton and Morris 1987). In cultured *T. granulosum*, spermidine and spermine were the major intracellular free polyamines, compared to spermidine in cultured *T. danilewskyi*. The lack of free putrescine shown by the two fish trypanosomes seems to be an unusual feature of the *Trypanosoma*. Putrescine and spermidine appear to be the major intracellular polyamines found within other members of the genus (Bacchi 1981; Hunter *et al.* 1990). This suggests that in the two fish trypanosomes either ornithine decarboxylase (thought to be the rate limiting enzyme in polyamine biosynthesis) is absent and they sequester polyamines from the medium, or that ornithine decarboxylase activity is highly regulated, resulting in virtually all the putrescine product being used immediately for metabolism within the cells. In *T. cruzi* epimastigotes, the low levels of ornithine and arginine decarboxylases suggest that *de novo* synthesis of putrescine is a minor biosynthetic pathway (Hunter *et al.* 1994). However, the presence, levels and subsequent importance of these enzymes within *T. granulosum* and *T. danilewskyi* have not been determined.

Although the presence of spermine within *T. granulosum* does not confirm spermine synthesis, labelling *T. granulosum* with [^{3}H]putrescine in the present study demonstrated both spermidine and spermine synthesis. *Trypanosoma granulosum* and *T. cruzi* seem to be the only members of the genus *Trypanosoma* known to synthesise spermine, and within which intracellular spermine accounts for a substantial amount of the total intracellular

polyamine content (Schwarcz de Tarlovsky *et al.* 1993; Hunter *et al.* 1994). In bloodstream *T. b. brucei* (Bacchi *et al.* 1983; Bitonti *et al.* 1986; Byers *et al.* 1991) and *Crithidia fasciculata*, a parasite often used as a laboratory model for *T. cruzi* (Fairlamb *et al.* 1986; Le Quesne 1995), free spermine has not been detected. The use of radiolabelled precursors and assay of specific enzyme activities are reliable methods for confirming polyamine biosynthesis in trypanosomatids (Bachrach *et al.* 1979a; Hunter *et al.* 1994). Extended studies using these techniques would help elucidate the polyamine metabolism of *T. granulosum* and *T. danilewskyi*.

The concentrations of polyamines in *T. granulosum* were lower than in most trypanosomatids, with again the exception of *T. cruzi* in which they are similar (Hunter *et al.* 1994) and *T. danilewskyi* which also exhibits these low concentrations of polyamines. It was noted that changes in batches of foetal calf serum used to culture *T. granulosum*, resulted in significant changes in polyamine levels and this is an important point to be considered when comparisons are made to other trypanosomatids and culture systems. Polyamine analyses of *T. granulosum* cultured in modified SDM-79 containing foetal calf serum from Sigma were never undertaken, as the low numbers of cells gained from these cultures, were insufficient to allow polyamine analyses.

Parasites represent a diverse group of organisms, which show equally diverse aspects of polyamine metabolism. For example, cadaverine, a diamine found in certain bacteria where it is synthesised from lysine via a lysine decarboxylase (Fecker *et al.* 1986), is present at higher levels than putrescine in all stages of the epimastigote growth cycle of *T. cruzi* (Algranati *et al.* 1989), although it has not been found in cultured *T. granulosum*, *T. danilewskyi* or other members of the genus. Parasitic helminths responsible for a

number of infections in mammals, exhibit distinct features of polyamine metabolism, some similar to those detected within *T. granulosum*. For example, spermidine and spermine are the only detectable polyamines in *Nippostrongylus brasiliensis* (Sharma *et al.* 1991). The presence of unusual polyamines for example thermospermine, norspermidine and norspermine have been identified in *Euglena gracilis* (Adlakha *et al.* 1980; Villanueva 1981) but not in any of the trypanosomatids studied to date.

Analyses of the polyamine levels during culture of *T. granulosum* and *T. danilewskyi* suggests that the presence of fresh replacement medium either increases polyamine biosynthesis, or increases polyamine uptake from the medium. The term uptake is used throughout the discussion, as transport implies that the mechanism is isolated and does not take into account the metabolism of the label once inside the cell. An increase in spermine levels followed an increase in spermidine levels in *T. granulosum*, possibly attributable to the greater need for spermidine within the cell during early rapid growth of the parasite, giving rise to a change in the spermidine to spermine ratio. A rise in spermidine levels after 2 days culture of *T. granulosum* corresponded with a decrease in both S-adenosyl methionine (SAM) and decarboxylated S-adenosylmethionine (dcSAM) levels. This suggests transfer of aminopropyl groups resulting in synthesis of both spermidine and spermine. Following the initial rise in spermidine levels, a subsequent drop of this polyamine within *T. granulosum* corresponded with increased SAM and a larger increase in dcSAM. These results, combined with the observation of [^{3}H]putrescine incorporation into both spermidine and spermine suggests that *T. granulosum* has the capacity to synthesise both spermidine and spermine with transfer of aminopropyl groups from dcSAM. This incorporation was shown as early as two minutes after exposure of cells to [^{3}H]putrescine, suggesting rapid enzyme activity. The changes described in the levels of

polyamines and related metabolites throughout the period of culture of *T. granulosum* and *T. danilewskyi* are similar to those described in numerous mammalian and plant cells (Dykstra and Herbst 1965; Hölttä and Jänne 1972; Bachrach 1973; Tabor and Tabor 1976, 1984; Adlakha *et al.* 1980). However, although mammalian cells have shown an increase in intracellular polyamines following aminoguanidine (a polyamine oxidase inhibitor) treatment (Brunton *et al.* 1994), in the presence of aminoguanidine, the intracellular polyamine levels of *T. granulosum* remained unchanged, suggesting polyamine oxidase plays a minor role in polyamine homeostasis within *T. granulosum*.

In *T. granulosum* cultured in medium containing relatively low concentrations (10mM) of DFMO, levels of both spermidine and spermine decreased slightly. This might be explained by DFMO inhibiting ornithine decarboxylase activity, a phenomenon observed in numerous cells (Bey *et al.* 1987), including *T. b. brucei* (both *in vivo* and *in vitro*) (Bacchi *et al.* 1980; Giffin *et al.* 1986; Byers *et al.* 1991). However, this reduction may also be due to a dilution of intracellular polyamines caused by an increase in cell volume and protein concentration as was thought to occur in *T. b. brucei* (Giffin *et al.* 1986). In this instance inhibition of ornithine decarboxylase would be of minor importance to the cells, and DFMO would not therefore be a useful chemotherapeutic agent against *T. granulosum*. The increases in intracellular polyamines within *T. granulosum* and *T. danilewskyi* cultured in the presence of high concentrations (50mM) of DFMO are unusual and suggest that *T. granulosum* and *T. danilewskyi* sequestered polyamines from the medium, as described in *Leishmania infantum* promastigotes (Balaña Fouce *et al.* 1991) and leukaemia cells (HL-60) (Walters and Wojcik 1994) cultured in medium containing DFMO.

Interestingly, in both *T. b. brucei* (Bacchi *et al.* 1983) and *Plasmodium falciparum* (Assaraf *et al.* 1987), putrescine and spermidine concentrations decreased dramatically following DFMO treatment, but there was a concomitant rise in intracellular spermine. In some parasites DFMO has little or no effect on growth or ornithine decarboxylase activity, possibly due to the absence of ornithine decarboxylase and uptake of polyamines, as in *T. cruzi* epimastigotes (Hunter *et al.* 1990). When mice infected with the cestode *Echinococcus multilocularis* are fed water containing DFMO, there are no changes in the growth or development of the cysts and only a slight reduction in putrescine levels compared to controls. Therefore it is suggested that the parasite lacks ornithine decarboxylase, although the presence of intracellular DFMO was not reported (Miyaji *et al.* 1993). In addition, certain clones of *L. infantum* promastigotes remain refractory to DFMO and those most refractory to the drug contain the highest putrescine to spermidine ratios although the relevance of these ratios is uncertain (Carrera *et al.* 1994).

The increases in solute concentration within *T. granulosum* and *T. danilewskyi* treated with DFMO may have caused changes in osmolarity resulting in the altered and bizarre shapes described in the previous section. It is also possible that increases in spermidine levels in the presence of high concentrations of DFMO are due to degradation of spermine or polyamine conjugates by catabolic enzymes. It is unlikely that increased *de novo* synthesis of polyamines occurred, although assays of biosynthetic enzyme activities would help elucidate the mechanism.

Increases in the levels of SAM and dcSAM seen in *T. granulosum* cultured in medium containing DFMO have been described previously in trypanosomes (Bacchi *et al.* 1983; Fairlamb *et al.* 1987). In particular, increased levels of SAM and dcSAM, over a 1000

fold in the case of dcSAM, are partially responsible for the antitrypanosomal action of DFMO on *T. b. brucei* (Byers *et al.* 1991). More modest changes were observed in *T. granulosum* however and therefore alterations in the levels of SAM and dcSAM are thought to play a minor role in the antitrypanosomal action of DFMO in this parasite. It has also been suggested that a change in the ratio of SAM to S-adenosylhomocysteine (SAH) is responsible for altering the capacity of the cell to methylate proteins , and this is known to play a role in gene expression (Ueland 1982; Yarlett and Bacchi 1988). As no SAH was detected in *T. granulosum* its significance in the biochemistry of the parasite is unknown.

When *T. granulosum* was cultured in medium containing MGBG (0.2mM), a known inhibitor of SAMdc (Williams-Ashman and Schenone 1972), intracellular levels of both spermidine and spermine increased as did levels of SAM, but levels of dcSAM decreased approximately 10 fold suggesting inhibition of S-adenosylmethionine decarboxylase (SAMdc). Increases in levels of spermidine and spermine with MGBG could result from both polyamines being sequestered from the medium, although it is thought that MGBG inhibits spermidine uptake (Pegg 1988). Alternatively, spermidine and spermine may be displaced from anionic binding sites or polyamine conjugates within the cell. However, it seems that MGBG does not totally inhibit SAMdc, as SAM levels rise approximately 3 fold compared to the 10 fold increase seen in *T. b. brucei* (Byers *et al.* 1991). It is likely that small amounts of synthesised dcSAM are able to transfer aminopropyl groups to putrescine sequestered from the medium. It is known from the uptake experiments in this study that the inhibition of putrescine uptake by MGBG is minimal in *T. granulosum*. This is also true of *T. cruzi* epimastigotes (Le Quesne 1995). Another explanation for the rise in polyamine levels may be that aminopropyl groups are gained elsewhere for

polyamine synthesis. MGBG, is also known to induce spermidine/spermine-N^1-acetyltransferase (SAT) (Pegg and Williams-Ashman 1987). If this induction occurs in *T. granulosum* then increases in spermidine levels may be partially due to SAT activity. Increases in levels of spermidine in cells treated with MGBG are not unusual. For example *Acanthamoeba culbertsoni*, a free-living amoeba, when cultured in a fully defined medium containing MGBG, shows an increase in spermidine levels as well as strong growth inhibition (Kishore *et al.* 1990). MGBG (0.2mM) did not significantly alter polyamine levels in *T. danilewskyi*, suggesting that low (0.2mM) concentrations of MGBG, although able to cause significant cell death, were insufficient to cause an imbalance in polyamine homeostasis. Therefore if biosynthesis is inhibited, then possibly uptake of polyamines by *T. danilewskyi* is regulated differently from uptake in *T. granulosum*. Studies on the enzyme kinetics of SAMdc in *T. granulosum* and *T. danilewskyi* would help elucidate the polyamine metabolism of both parasites.

Growth inhibition observed in mammalian cells cultured in medium containing MGBG is accompanied by decreases in the levels of both spermidine and spermine, but increases in putrescine levels (Linares *et al.* 1994), a phenomenon also observed in cultured carrot cells (Kurosaki *et al.* 1992) and *T. b. brucei* (Bacchi *et al.* 1979) as a result of inhibition of *T. b. brucei* SAMdc (Chang *et al.* 1978; Bitonti *et al.* 1986; Tekwani *et al.* 1992). Paradoxically, in potato leaves treated with MGBG, putrescine levels decrease, possibly due to its transport to another part of the plant (Féray *et al.* 1994).

In several *Leishmania sp.* the incorporation of [^{3}H]putrescine into spermidine is inhibited by MGBG (Bachrach *et al.* 1979b), compared to the apparent greater incorporation of [^{3}H]putrescine into spermidine, when *T. granulosum* is cultured in medium containing

MGBG. Changes in the intracellular levels of SAM and dcSAM (described above) suggested partial inhibition of SAMdc by MGBG. It may be that because cell contents were analysed 24 hours after addition of the label to the trypanosomes, those cultured in the drug free medium rapidly metabolised the label compared with those cells cultured in the presence of MGBG. Further studies on uptake of putrescine by *T. granulosum* using a range of concentrations of MGBG would reveal to what extent SAMdc is inhibited by the drug.

In the presence of Berenil, polyamine levels increased in both *T. granulosum* and *T. danilewskyi*, in contrast to the decrease in spermidine and spermine reported seen in *T. b. brucei* cultured in medium containing Berenil (Bitonti *et al.* 1986). It seems that synthesis of polyamines was somewhat decreased in *T. granulosum* due to the 77 fold increase in SAM and reduced levels of dcSAM, suggesting inhibition of SAMdc. Cells cultured in the presence of Berenil still incorporated [^{3}H]putrescine into both spermidine and spermine however implying some SAMdc activity. The rise in polyamines within *T. granulosum* with Berenil was probably mainly due to increased uptake from the medium although it is known that Berenil inhibits uptake of both spermidine and spermine in the rodent filariid *Acanthocheilonema viteae* (Tekwani *et al.* 1995).

The increased putrescine levels following Berenil treatment, seen in *T. b. brucei* (Bitonti *et al.* 1986) were not observed in *T. granulosum*, suggesting that any putrescine that is either synthesised or sequestered, is rapidly metabolised to spermidine in *T. granulosum*. Spermidine levels also increased in *T. b. brucei* and *A. culbertsoni* exposed to Berenil *in vivo*, possibly due to the huge build up of putrescine combined with an incomplete block of SAMdc (Bitonti *et al.* 1986; Kishore *et al.* 1990). S-adenosylmethionine

decarboxylases from rat liver and yeast are strongly inhibited by Berenil (Karvonen *et al.* 1985) and in cultured rat hepatocytes an increase in putrescine in Berenil treated cells is partially due to the degradation of spermidine by polyamine oxidase (Colombatto and Grillo 1993). In certain clones of *Leishmania infantum* promastigotes a high regression coefficient occurs between the putrescine to spermidine ratio and Berenil growth inhibition, suggesting high putrescine levels may be protective against Berenil toxicity (Carrera *et al.* 1994).

As in the case of MGBG, the presence of Berenil in the culture medium of *T. granulosum* caused more of the tritiated label to be incorporated into spermidine and spermine 24 hours after the addition of the label, than into spermidine and spermine from cells cultured in drug free medium for the same time. This was possibly due to rapid metabolism or even excretion of the label from the cells in the drug-free medium.

Other drugs, for example MDL73811, have shown that inhibition of parasite SAMdc does not necessarily result in a decrease in intracellular polyamine levels. In *T. b. brucei* infected rats treated with MDL73811, the polyamine levels within the parasites remained unchanged even with complete inhibition of SAMdc activity accompanied by a 20 fold increase in SAM levels. This large increase is not observed in mammalian cells (Bitonti *et al.* 1990; Byers *et al.* 1991), suggesting the rise in levels of SAM in *T. granulosum* treated with Berenil may be partially responsible for the antitrypanosomal effect of the drug.

It is possible that *T. granulosum* and *T. danilewskyi* are incapable of *de novo* synthesis of polyamines, as is thought to be the case for *T. cruzi* (Hunter *et al.* 1990) and certain

filarial worms (Walter 1988). Exposing the cells to relevant labelled amino acids followed by subsequent HPLC and radiometric detection would provide experimental means for evidence of the capabilities of *T. granulosum* and *T. danilewskyi* to synthesise putrescine.

8.1.1 Uptake of putrescine

Although *T. granulosum* cultured in a semi-defined medium (containing 10% foetal calf serum) lacks intracellular free putrescine, it possesses a rapid high affinity uptake system for putrescine. This raises a number of questions as to the purpose of such a mechanism and how uptake of polyamines relates to synthesis of polyamines.

The majority of cells are capable of *de novo* synthesis of polyamines from their amino acid precursors, but if cells have a rapid high affinity uptake system for putrescine and the cells are present in a polyamine rich environment then there would be little need for *de novo* synthesis. However, uptake of polyamines from the environment is not the major mechanism controlling polyamine homeostasis amongst most trypanosomatids, as they tend to be capable of *de novo* synthesis. Compared to the rapid uptake described in both *T. granulosum* and *T. cruzi*, the uptake of putrescine appears to be non-saturable and of low velocity in both *T. b. brucei* (procyclic and bloodstream) and *L. donovani* (promastigotes) (Le Quesne 1995). When compared to *L. infantum* promastigotes (K_m=1.1μM, Vmax=0.17 nmole min^{-1} (10^8cells)$^{-1}$) harvested in late log phase (Balaña-Fouce *et al.* 1989), *T. granulosum* had a 5 fold greater rate of uptake and the affinity of the uptake mechanism for putrescine was 2 fold higher (Km halved). However, *T. cruzi* epimastigotes, thought incapable of *de novo* synthesis, when cultured for 5 days (Km=2.1μM, Vmax=0.60nmole min^{-1} (10^8cells)$^{-1}$) the rate of putrescine uptake is similar

to that observed in *T. granulosum* (Km=0.52μm, Vmax=0.95nmole min^{-1} (10^8 cells)$^{-1}$) (Le Quesne 1995), although the affinity is some 4 fold greater in *T. granulosum*. When *T. cruzi* is cultured for 3 days, the rate of transport increases over 5 fold (K_m=2.0μM, Vmax=3.3 nmole min^{-1} (10^8cells)$^{-1}$) without a significant change in Km, compared to 5 day cells (Le Quesne and Fairlamb 1994). This change in the rate of putrescine uptake by *T. cruzi* was accompanied by protein and RNA synthesis (Le Quesne 1995), a phenomenon observed in *L. infantum* promastigotes (Balaña-Fouce *et al.* 1991) and some mammalian cells (Pohjanpelto 1976; Wallace and Keir 1981).

T. granulosum appears to be the only trypanosomatid studied that has a high affinity rapid uptake system for putrescine and yet no known intracellular stage in its vertebrate host. It has been suggested that the uptake of diamines by *T. cruzi* epimastigotes is of physiological significance. Following a blood meal, the excreta of *Rhodnius prolixus* (a *T. cruzi* vector) contain both putrescine and cadaverine in micromolar quantities and as putrescine uptake is enhanced in response to favourable growth conditions, the lack of *de novo* synthesis of polyamines may be part of the parasite's adaptation to its environment within the arthropod host (Le Quesne 1995). In the mammalian host, amastigote forms of *T. cruzi* are bathed in polyamines and if a specific polyamine transporter were present, upon which the parasite depended for maintenance of polyamine homeostasis, then this could possibly be exploited for chemotherapeutic attack. A putrescine uptake system, similar to that of *T. cruzi* epimastigotes is present in *T. granulosum* (in terms of affinity and velocity). This would only be of physiological significance if the parasite was present in a putrescine-rich environment for part of its life cycle. It is known that *T. granulosum* spends part of its life cycle in the intestine of a leech, presumed to contain a high content of fish blood which is thought to be high in polyamines compared to mammalian blood,

due to the presence of nucleated erythrocytes (Bachrach 1973). This suggests that throughout the life cycle of *T. granulosum* the cells would be surrounded by polyamines.

The medium (Dulbecco's PBS) used to wash and incubate the cells, provided the trypanosomes with an environment unlikely to affect the uptake of [^{3}H]putrescine. The presence of KCl, KH_2PO_4, NaCl and Na_2HPO_4 within Dulbecco's PBS, should be remembered when considering the mechanism of putrescine uptake as there are several accounts in a variety of cells suggesting inorganic cations affect polyamine uptake (Davis and Ristow 1988; Khan *et al.* 1989; Alves *et al.* 1992; Antognoni *et al.* 1994; Escribano *et al.* 1994). Uptake was not affected either by extracellular amines present in the modified SDM-79. Extracellular amines are known to stimulate putrescine uptake in neuroblastoma cells (Rinehart and Chen 1984), although these do not affect putrescine uptake in either *T. cruzi* or *L. mexicana* (González *et al.* 1992).

Further studies in *T. granulosum* are needed to elucidate the mechanism of putrescine uptake. Studies using KCN or 2,4 dinitrophenol, known uncouplers of the respiratory chain, or ouabain, to determine whether the Na^+/K^+ pump is involved in putrescine uptake, may provide valuable information. Inhibition of putrescine uptake occurs in *T. cruzi* when cells are incubated in the presence or either *N*-ethylmaleimide, 2,4 dinitrophenol or carbonylcyanide *m*-chlorophenyl hydrazone (CCCP). This suggests that putrescine transport is energy dependent, requires sulphydryl groups on the transporter, or could be linked to a proton gradient (Le Quesne 1995). All of these mechanisms remain to be determined in *T. granulosum*.

The values obtained from the Michaelis Menten equation and the double-reciprocal

(Lineweaver-Burk) plot, show that MGBG does not inhibit putrescine uptake to a great extent. A slight decrease in Vmax suggests that non-competitive inhibition takes place, although further experiments would have to be performed to see if the change was significant. As no competitive inhibition was observed using MGBG, it may be suggested that the uptake mechanism distinguishes between putrescine and compounds of similar shape to spermidine, as is apparent in L1210 leukemia cells where polyamine uptake mechanisms have a definite specificity in terms of aliphatic chain length (Porter *et al.* 1984). The present study has shown that MGBG, structurally similar to spermidine, enters *T. granulosum*, although it remains to be determined whether the mechanism of uptake is through a temperature dependent saturable uptake system separate to polyamine uptake. In some mammalian cells there are both high and low affinity uptake systems for putrescine (Sokol *et al.* 1993) and in others there are two known transporters, one for both spermidine and spermine, and a separate transporter for putrescine, spermidine and spermine (Morgan 1992). The weak inhibition of putrescine uptake by MGBG in *T. granulosum* is also observed in cultured *L. infantum* promastigotes (Balaña-Fouce *et al.* 1989) and the filamentous fungus *Neurospora crassa* (Davis and Ristow 1988), although both mixed (in the nanomolar range) and competitive (in the micromolar range) inhibition of putrescine transport were observed in human endothelial cells exposed to MGBG (Sokol *et al.* 1993).

Mixed inhibition of putrescine uptake was noted in *T. granulosum* cultured in medium containing Berenil. This was a mixture of non-competitive, where the attachment of substrate to available sites is believed to be unimpeded by the presence of inhibitor at all concentrations of substrate and competitive inhibition, where putrescine competes for the available sites for uptake and decreasing the affinity between putrescine and the uptake

mechanism (Neame and Richards 1982; Devés and Krupka 1989). The decrease in Vmax brought about by Berenil may result from a decreasing turnover of carrier sites, by a decreasing amount of carrier available, or both. This makes Berenil a more potent inhibitor of putrescine uptake than MGBG, as demonstrated in *L. infantum* promastigotes, where Berenil and a series of Berenil analogues almost completely inhibited putrescine uptake non-competitively (Balaña-Fouce *et al.* 1991; Reguera *et al.* 1994).

8.2 Analyses of thiols

Glutathione (GSH) was the major intracellular thiol detected throughout the period of culture of both *T. granulosum* and *T. danilewskyi* with similar levels to *T. cruzi* epimastigotes (Hunter *et al.* 1994), *C. fasciculata* (Shim and Fairlamb 1988; Hunter *et al.* 1991) and *T. b. brucei* procyclics (Bellofatto *et al.* 1987). However, as for polyamines, differences were noted in the levels of glutathione in *T. granulosum* cultured in modified SDM-79 containing different batches of foetal calf serum. After inoculation into fresh medium there was approximately a 4 fold increase in the amount of glutathione present in *T. granulosum*, presumably through increased activity of biosynthetic enzymes. The lower concentrations of glutathione detected in cells in the stationary phase, similar to that described in cultured mammalian cells (Post *et al.* 1983; Batist *et al.* 1986), may be due to precursor starvation, for example, cysteine. The elevation of glutathione observed during rapid growth of *T. granulosum*, is also seen in the insect trypanosomatid *C. fasciculata* (Shim and Fairlamb 1988) and in *E.coli* cultured under anaerobic conditions (Smith *et al.* 1995), although the changes in glutathione levels are not as pronounced as those in *T. granulosum*.

Trypanosomatids usually contain 70% of their intracellular glutathione as trypanothione (N^1, N^8-bis(glutathionyl)-spermidine) (Fairlamb *et al.* 1985, 1987; Bellofatto *et al.* 1987; Hunter *et al.* 1991), a phenomenon not observed in *T. granulosum*, *T. danilewskyi* or *T. cruzi* (Ariyanayagam and Fairlamb 1993, 1994) cultured in semi-defined media. This apparent absence of trypanothione from *T. granulosum*, warranted further investigation. It revealed that glutathione was not maintained in its reduced state by glutathione reductase, the mechanism thought to exist in mammalian cells. The absence of classical glutathione reductase activity is a feature of all trypanosomatids studied to date (Fairlamb and Cerami 1992) including *T. granulosum*. The presence of a trypanothione dependent reductase however, suggested that *T. granulosum* had the ability to maintain trypanothione in a reduced form. It seemed that trypanothione was probably present in *T. granulosum* cultured in modified SDM-79 after all, but it was present in levels lower than the detection limit.

When *T. granulosum* was cultured in medium with additional putrescine (5μM), trypanothione was detected, confirming the ability of *T. granulosum* to synthesise trypanothione. This phenomenon also occurs in *T. cruzi* epimastigotes (Hunter *et al.* 1994), further highlighting metabolic similarities between *T. granulosum* and *T. cruzi*. The absence of detectable glutathionylspermidine in extracts of *T. granulosum* containing trypanothione suggested rapid metabolism of glutathionylspermidine, as is thought to be the case for *C. fasciculata* (Henderson *et al.* 1990). When a larger concentration (100μM) of putrescine was added to *T. granulosum* and *T. danilewskyi* 24 hours prior to extraction, both glutathionylspermidine and trypanothione were detected, the latter present in *T. granulosum* at similar concentrations to trypanothione found in bloodstream *T. b. brucei* (Fairlamb *et al.* 1992), *T. cruzi* epimastigotes (Hunter *et al.* 1994), but much lower than

is found in exponentially cultured *C. fasciculata* (Shim and Fairlamb 1988; Fairlamb 1988; Hunter *et al.* 1991). Addition of putrescine to *T. granulosum* also caused an increase in levels of glutathione compared to those cells which did not have the additional putrescine added. It seems that the higher concentration (100μM compared to 5μM) of putrescine was sufficient stimulus for the cell to produce both glutathionylspermidine and trypanothione and stimulate glutathione synthesis. Conversely in *T. danilewskyi*, glutathione levels decreased in the presence of putrescine (100μM). Increases in both glutathionylspermidine and trypanothione were seen in *T. cruzi* epimastigotes cultured in similar conditions, although glutathione levels did not alter significantly (Ariyanayagam and Fairlamb 1993; Hunter *et al.* 1994).

Addition of cadaverine (100μM) to the medium caused *T. granulosum* and *T. danilewskyi* to synthesise the polyamine thiol conjugates glutathionylaminopropylcadaverine and homotrypanothione (N^1, N^9-bis(glutathionyl)aminopropylcadaverine), two thiols thought previously unique to *T. cruzi* epimastigotes (Hunter *et al.* 1994). If homotrypanothione proves to be crucial for parasite survival, then inhibition of homotrypanothione synthesis may lead to a decrease in the survival of the parasite. The enzymes responsible for the catalysis of homotrypanothione synthesis have yet to be characterised in *T. granulosum*, *T. danilewskyi* or *T. cruzi*. The presence of homotrypanothione raises the question of whether a specific thiol reductase is present which may also be another route for chemotherapeutic attack. Interestingly, in the presence of cadaverine there were detectable levels of both glutathionylspermidine and trypanothione, suggesting that syntheses of trypanothione and homotrypanothione are closely related in *T. granulosum*, although the phenomenon was not observed in *T. cruzi* epimastigotes (Hunter *et al.* 1994) or *T. danilewskyi*. From a physiological perspective, it appears that *T. granulosum*, *T.*

danilewskyi and *T. cruzi* have adapted at some stage in their life cycles to metabolising cadaverine, presumably either from the gut of the invertebrate vector or from the blood of the vertebrate host.

The split glutathionylspermidine peak observed in HPLC chromatograms of derivatised extracts of *T. granulosum* cultured in modified SDM-79 containing putrescine (100μM), suggested both N^1-glutathionylspermidine and N^8-glutathionylspermidine were used in equimolar amounts to synthesise trypanothione in *T. granulosum*, a phenomenon also observed in *L. donovani*, but *T. cruzi* only appears to use the N^8isomer (Ariyanayagam personal communication). An unknown thiol peak (29.56mins) was present in some *T. granulosum* samples that had putrescine (100μM) added 24 hours prior to extraction, possibly suggesting other polyamine/thiol conjugates within *T. granulosum*.

The presence of ovothiol A (OSH-A) in *T. granulosum*, *T. cruzi*, *L. donovani* (Ariyanayagam and Fairlamb 1995), *L. major*, *T. b. brucei* (Ariyanayagam, personal communication) and *C. fasciculata* (Steenkamp and Spies 1994) raises a number of interesting questions regarding its possible physiological role. *Leishmania donovani* entering the host macrophage is attacked by an oxidative burst and the presence of high levels of OSH-A may be a factor responsible for *L. donovani* survival within the macrophage. When *T. cruzi* enters the mammalian cell it is possible that the levels of reactive oxygen species are higher than within the bloodstream due to cellular respiration. The relatively high levels of OSH-A in *T. cruzi* compared to the levels in both *T. b. brucei* and *C. fasciculata*, may be an additional mechanism for the parasite to deal with oxidative trauma (Ariyanayagam and Fairlamb 1995; Ariyanayagam, personal communication). However the relatively high levels observed in *T. granulosum* are difficult to explain, due

to the apparent lack of an intracellular stage. The advantages to these parasites of producing high levels of compounds with potential antioxidant and free radical scavenging abilities are not obvious, although ovothiols and the related mercaptohistidines are several tenfold more efficient than glutathione as free radical scavengers (Holler and Hopkins 1990), suggesting an important role for such compounds within any parasite. In the cases of both *T. granulosum* and *T. cruzi* where OSH-A levels are high when compared to *C. fasciculata* and *T. b. brucei*, the importance of this compound remains to be determined (Ariyanayagam and Fairlamb 1995). A trypanothione peroxidase system has not been detected in *T. cruzi* (Henderson *et al.* 1987) and has not been determined in *T. granulosum*. It may be possible that ovothiol A, in the presence of trypanothione and trypanothione reductase could constitute an effective trypanothione peroxidase system, as ovothiol has been shown to replace glutathione peroxidase in sea urchin eggs (Turner *et al.* 1988). The presence of ovothiol A in *T. granulosum* and the lack of pathogenic risk using *T. granulosum* in the laboratory allows an excellent experimental system to study the metabolism and biochemical function of OSH-A in trypanosomes.

GENERAL CONCLUSIONS

This study has been concerned with the isolation, culture and subsequent study of two freshwater fish trypanosomes, namely *T. granulosum* and *T. danilewskyi*, although the former has been examined in more detail. Culture of mostly trypomastigote forms of both trypanosomes in modified SDM-79 has allowed analyses of their polyamine and thiol metabolism and the effects polyamine biosynthesis inhibitors on the growth, morphology, ultrastructure and biochemistry of these parasites.

Perhaps the most remarkable feature of the biochemical analyses performed, was the similarity detected between the two fish trypanosomes, in particular *T. granulosum*, and existing data for *T. cruzi* (Hunter *et al.* 1994; Le Quesne 1995). The low levels of polyamines present in *T. granulosum* and *T. danilewskyi* in this study, and in *T. cruzi* (Hunter *et al.* 1994) are unusual amongst trypanosomes. This may be due to the sequestration of polyamines from their environment. Although polyamine uptake studies for *T. danilewskyi* were not performed, the similarity in the rapid high affinity uptake system for putrescine present in both *T. granulosum* and *T. cruzi* epimastigotes is striking. Such systems seem unusual amongst trypanosomes and this suggests that both *T. granulosum* and *T. cruzi* may have a reduced the need for *de novo* synthesis of putrescine. It is also interesting that *T. granulosum* and *T. cruzi* appear to be the only *Trypanosoma spp.* reported to date, which are able to synthesise spermine.

Analyses of thiols highlighted further similarities between the two fish trypanosomes and the mammalian *T. cruzi* (Hunter *et al.* 1994). Firstly, glutathione appears to be the major intracellular thiol within all three species, while trypanothione, usually present at high

(>70%) concentrations in trypanosomatids (Fairlamb *et al.* 1985, 1987; Bellofatto *et al.* 1987; Hunter *et al.* 1991) is thought to be present at low concentrations in *T. cruzi* (Hunter *et al.* 1994), *T. granulosum* and *T. danilewskyi*. Secondly, in the presence of additional putrescine there is an increased production of trypanothione. This suggests that like *T. cruzi* (Hunter *et al.* 1994), *T. granulosum* and *T. danilewskyi* are able to scavenge putrescine from the medium. A similar response occurs when cadaverine is added to their environment, as *T. granulosum*, *T. danilewskyi*, and *T. cruzi* (Hunter *et al.* 1994) then synthesise homotrypanothione, a compound reportedly absent from African trypanosomes or *Leishmania* (Hunter *et al.* 1994). Thirdly, the importance of ovothiol A (a thiol also found in *T. cruzi*, see Ariyanayagam and Fairlamb 1995), to *T. granulosum* is unclear, but in as much as it offers the parasite extra oxidative defence, it is assumed to be advantageous.

Although it seems unlikely that *T. granulosum* and *T. danilewskyi* would cause extensive mortality to wild fishes, introduction of such infectious agents into an artificial culture system could have devastating micro-economic consequences (see Bauer 1961; Woo 1995). Studies that may lead to the design of an anti-trypanosomal agents for fishes may therefore be of some use in modern aquaculture.

Culturing *T. granulosum* and *T. danilewskyi* in the presence of DFMO (an inhibitor of ornithine decarboxylase), showed that high concentrations of the drug were needed to affect the growth of both parasites. At high (50mM) concentrations, the levels of intracellular polyamines were greater than in controls, and the concomitant changes to the morphology and ultrastructure of the parasites suggested that DFMO caused cell death through osmotic imbalance, rather than from polyamine depletion. Interestingly, in the

presence of DFMO, cultured *T. granulosum* demonstrated ultrastructural variations (Plate 3.32) to the pattern of microtubules ordinarily observed in flagella, although these have been seen in some bloodstream forms of *T. lewisi* (Anderson and Ellis 1965). Curiously, DFMO (50mM) had no effect on the growth of *T. cruzi* trypomastigotes (Kierszenbaum *et al.* 1987) or epimastigotes (Hunter *et al.* 1990), probably due to polyamine uptake from the medium. It is therefore difficult to explain why *T. granulosum*, which has an equally rapid and high affinity uptake system should be affected by DFMO, unless DFMO causes an osmotic imbalance. Addition of exogenous putrescine to *T. granulosum* cultured in medium containing DFMO did not restore growth, suggesting that inhibition of ODC was not responsible for the anti-parasitic action of DFMO. In the event that an anti-parasitic agent might be needed for *T. granulosum* or *T. danilewskyi*, DFMO would not be suitable due to the high concentrations of the drug needed to affect the parasites. *In vivo* studies would however provide valuable data on the effect of DFMO on bloodstream trypomastigotes. Future work on *T. granulosum* and *T. danilewskyi* should include assays of ODC and SAMdc activities and analyses of the incorporation of radiolabelled ornithine into polyamines, to further appreciate the complex mechanisms of polyamine homeostasis within these organisms.

The low concentrations of MGBG and Berenil, needed to affect the growth, morphology and ultrastructure of both *T. granulosum* and *T. danilewskyi* suggest that these drugs might be more suitable than DFMO for chemotherapy of fish flagellate infections. MGBG and Berenil, although known inhibitors of polyamine metabolism, caused an increase in intracellular polyamines within both *T. granulosum* and *T. danilewskyi*, probably due to increased uptake from the medium. It seems likely that the increases in SAM and damage to the kinetoplast, nucleus and possibly the mitochondria, played a significant role in cell

death. Future work should include the effect of these drugs on the bloodstream trypomastigotes of both species, to assess the suitability of these drugs in treating protozoan disease of fishes.

The isolation and culture of both *T. granulosum* and *T. danilewskyi* in modified SDM-79 produced rapid growth and provided cells suitable for biochemical analyses. However, the development of a fully defined medium is a desirable goal for the future, as different batches/brands of foetal calf serum can cause alterations to the growth rate and levels of intracellular polyamines and thiols. Isolating *T. granulosum* in a fully defined medium was beyond the scope of this thesis. In this study, addition of polyamines and aminoguanidine (a polyamine oxidase inhibitor) to modified SDM-79 increased growth of *T. granulosum*. Insulin is also known to stimulate growth of cultured *T. granulosum* (Davies *et al.* 1995). In the future, polyamines and insulin may therefore be useful candidates in the development of a fully defined medium, although it may be difficult to replace the parasite's need for serum (see Brun and Jenni 1987).

The apparent biochemical similarities between *T. granulosum* (and probably *T. danilewskyi*) and *T. cruzi* are surprising considering that their life cycles are so very different. These similarities lead to interesting questions concerning the phylogenetic relationships between the species. Sequencing of small subunit rRNA and comparative studies on RNA editing have provided a means of assessing genetic distances between some flagellates. As a result of these studies *T. b. brucei* appears to be one of the most ancient of kinetoplastid flagellate species whereas the more recent *T. cruzi* apparently shares a clade with an unidentified fish trypanosome (Maslov *et al.* 1994). Interestingly, *C. fasciculata* occupies the crown of the evolutionary tree, where it is far removed from

T. cruzi (Fernandes *et al.* 1993; Vickerman 1994).

Crithidia fasciculata is often used as a laboratory model for *T. cruzi* (Hunter *et al.* 1990, 1991). However, if fish trypanosomes prove to be more closely related to *T. cruzi* than is *C. fasciculata*, and work presented in this thesis clearly supports this similarity, then perhaps in the future fish trypanosomes like *T. granulosum* may be more appropriate models for the hazardous *T. cruzi*. This may be particularly relevant if the future strategy for the chemotherapy of Chagas' disease is to centre on using the polyamine transport system to target the polyamine-glutathione synthetases in the cell (Le Quesne 1995).

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