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THE *IN VITRO* AND *IN VIVO* TESTING OF CHEMOTHERAPEUTIC AGENTS AGAINST PATHOGENIC FREE-LIVING AMEBAE

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Science in Microbiology

at

Massey University, Palmerston North

New Zealand

Christopher John Elmsly

1980

ABSTRACT

During the last ten years, there has been an increasing awareness of sporadic cases of Primary Amoebic Meningo-encephalitis (PAM) affecting primarily younger age groups and appearing in an acute fulminant form. The earliest positive case (Willaert, 1974) may have been in England in 1909 which shows that the disease has been with us for a long time.

The pathogenic free-living amebae (PFLA), which comprises the genus _Naegleria_ and the genus _Acanthamoeba_, are the causative organisms of PAM and AM*respectively. PAM is a rapidly fatal disease affecting the central nervous system (CNS), the treatment of which to date has been successful in only a small number of cases, and therefore the continual screening of suitable chemotherapeutic agents against amebae of the _Naegleria_ spp. and _Acanthamoeba_ spp., is of great importance.

AM is also essentially confined to the CNS although it may take the form of chronic granulomata in the liver, spleen, uterus and kidneys (Martinez _et al_., 1977).

Six chemotherapeutic agents: Amphotericin B, 5-Fluorocytosine, Kanamycin, Oxytetracycline, Tylosine and Levamisole were tested for activity against a non-pathogenic and a pathogenic species of _Naegleria_ and a non-pathogenic and a pathogenic species of _Acanthamoeba_ in axenic culture.

For the _Naegleria_ spp., Amphotericin B and Oxytetracycline were found to be active and the _Acanthamoeba_ spp. were found to be only susceptible to Levamisole.

The synergistic combinations of drugs against the amebae were also investigated in axenic culture. In preliminary trials Kanamycin together with Oxytetracycline showed promise against _Naegleria fowleri_ (MsM) but this was later shown not to be the case. Amphotericin B in combination with 5-Fluorocytosine was also shown not to be synergistic, however Amphotericin B in combination with Oxytetracycline proved to be effective against _N. fowleri_.

Amphotericin B was combined with 5-Fluorocytosine against _A. culbertsoni_ (A-1) but was not found to be synergistically active.

* Amebic meningitis caused by Acanthamoeba infections.

Levamisole was also tested against N. gruberi (P1200f) and A. castellanii (0.1) at various stages in growth of the amebae (i.e. 24, 48 and 72 hour stock cultures) to determine the effect of using aged amebae. It was found that the age of the stock culture bore no relation to the activity of the drug.

After axenic culture testing, the susceptibility of the pathogenic N. fowleri (MsM) and A. culbertsoni (A-1) to the agents which showed activity, was investigated in a vero cell culture system. For N. fowleri (MsM) the results of axenic culture testing were confirmed,with Amphotericin B and Oxytetracycline protecting the monolayer from the destructive effects of the amebae,both when used singly and at a greater efficiency when added together as a synergistic combination.

Levamisole, although effective to some extent against Acanthamoeba spp. in axenic culture,failed to show any activity against the amebae in vero cell culture testing.

In vivo animal protection studies were then performed using drugs that had been shown either in this or other studies to be effective against either Naegleria or Acanthamoeba spp. Chemotherapeutic agents tested on N. fowleri (MsM) included two imidazoles; Miconazole nitrate and Ketoconazole (previously known as R41,400), as well as Amphotericin B. The synergistic combination of Amphotericin B with either Tetracycline or Oxytetracycline was also investigated.

For A. culbertsoni (A-1), 5-Fluorocytosine, and Polymyxin B were tried both singly and in combination.

These drugs were injected by intraperitoneal (I.P.) and intraventricular (I.vent.) routes. The results were not promising, with none of the drugs offering significant protection even whilst using Amphotericin B which is considered the drug of choice.

The question of adequate drug levels reaching the brain was tested out with two imidazoles, Ketoconazole and Miconazole. Serum samples were assayed against Candida pirapsilosis and C. pseudotropicalis respectively at various time intervals after innoculation with the drug, and a gradual increase and breakdown of the drug in the animal system could then be shown. These results showed that based on in vitro results, the levels of the imidazoles obtained in the serum after the first eight hours after injection, should have been sufficiently high to prevent amebic multiplication.

ACKNOWLEDGMENTS

I would like to personally thank the following:

The Department of Microbiology and Genetics, Massey University for providing the opportunity and facilities for this investigation.

My supervisor, Dr Tim Brown for his supervision and guidance; and Dr Ray Cursons for his many suggestions.

Professor D.F. Bacon and other academic and technical staff of the Department.

Mrs Elizabeth Keys for her technical assistance and friendship throughout this study.

Ethnor Pty. Ltd., and Roche Products Pty. Ltd., for their financial support and supply of chemotherapeutic agents.

Mrs Penny van Doorn for the excellent typing.

Massey University Library staff for the numerous interloan requests.

The Central Photographic Unit, Massey University.

The Small Animal Production Unit for the seemingly endless supply of mice, and all those others, without whom this study would not have been possible.

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CHAPTER ONE: INTRODUCTION

1.1 The History of the Free-living Amebae as Disease Agents

The history of Pathogenic Free-living Amebae (PFLA) of the genera Acanthamoeba and Naegleria has been extensively reviewed elsewhere (Culbertson, 1971; Duma et al., 1971; Chang, 1971, 1974a; Carter, 1972; Cursons, 1974; Cursons and Brown, 1976).

The commonest disease caused by PFLA is Primary Amebic Meningo-encephalitis (PAM) caused by ameba quite different to those traditionally regarded as parasitic in man,and are not ordinarily parasitic in lower animals. They are ubiquitous in the environment and are free-living in water, sewage, soil and other decaying organic matter(Carter, 1972).

Willaert in 1974, tabulated 84 cases from all the continents with the exception of Antarctica. Since then at least 10 additional cases have been reported (Table 1).

Acanthamoeba spp. were the first agents implicated in this disease, but PAM is now known to be caused by a free-living amebae of the genus Naegleria. This was due mainly to the first case prototype of this illness which was described by Fowler and Carter in 1965. In 1968 Carter, Culbertson et al., and Butt et al., showed that the incriminating species of most reported cases belonged to the genus Naegleria, and in 1970 on the basis of morphological, cultural and pathogenicity differences Carter renamed the pathogenic species Naegleria fowleri distinguishing it from the non-pathogenic Naegleria gruberi.

Prior to 1968, all cases of PAM were attributed to the Acanthamoeba spp. which was probably due to the pioneering work of Culbertson et al., (1958, 1959, 1965) who, whilst working on the production of polio vaccine found an ameba which contaminated the cultures of monkey kidney cells. These cultures, when innoculated intracerebrally into mice, produced a necroting, hemorrhagic meningo-encephalitis that killed mice in 4-7 days. The responsible amebae was identified as an Acanthamoeba and they predicted on the basis of the finding, that this amebae could be capable of producing disease in humans. This ameba was previously considered to be a harmless free-living organism.

The disease caused by PFLA can be divided into two entities (Chang, 1974 (a)):

a) a swimming-associated acute meningo-encephalitis known as Primary Amebic Meningo-encephalitis (PAM) (Martinez et al., 1977). This is the most important of the two and is caused by Naegleria fowleri (non-pathogen - Naegleria gruberi). Infection is thought to occur in two ways:
   i) Naegleria contaminated water may be introduced into the upper nasal passages;
   ii) it may be due to the washing of trophozoites residing in the lower nasal passages of a carrier into the upper nasal area. A pathogenic strain of Naegleria was isolated from a normal healthy carrier (Visvesvara et al., 1974).

b) a non-swimming-associated chronic meningo-encephalitis (Amebic Meningo-encephalitis (AM) caused by amebae of the Acanthamoeba/Hartmanella group. It is considered that the most probable species is A. rhysodes along with another pathogenic species A. culbertsoni. Amebic Meningo-encephalitis due to the involvement of the Central Nervous System (CNS), appears to be a secondary phenomenon representing metatastic spread from a primary focus in the skin, genitourinary tract or respiratory tract (Martinez et al., 1977). Cutaneous ulceration is a possible point of entry with hematogenous spread to the CNS and lower respiratory tract. Infections in experimental animals have been reported (Martinez et al.,) due to this spread.

Subsequently Acanthamoeba spp. have been indicated in a number of chronic illnesses such as respiratory infections (Martinez et al., 1975), corneal ulceration of the eye leading to blindness (Nadington et al., 1974; Visvesvara et al., 1974) and together with Naegleria spp. in humidifier disease (M.R.C. Symposium, 1977). The reidentification of the etiological agents of the 1968 cases of PAM in New Zealand as N. fowleri species (Cursons and Brown, 1975, 1976) has dismissed the notion of slime moulds in the etiology of PAM (Mandel et al., 1970).

Henceforth, in the text, the nomenclature of Martinez et al., 1977, of PAM for Naegleria infections and AM for Acanthamoeba meningo-encephalitis will be adopted.

1.2 Classification

Study of the basic classification of the small free-living amebae has been stimulated by the discovery of their role in human disease. Carter (1970) accepted the decision of Page to retain the family designation of Vahlkampfiidae (for *Naegleria* spp.) in preference to the revision by Singh and Das (1970). Recent studies by Fulton (1970) and more recently by Schuster (1975) on the mitosis of *Naegleria* and related amebae displaying promitosis,have affirmed the lack of validity of details such as "interzonal body" and "polar caps" for use in establishing a higher taxa such as Schizopyrenidae. These points and others are extensively reviewed elsewhere by others (Page, 1974; Chang, 1971; Schuster, 1975; Visvesvara *et al*., 1975).

In reviewing *Acanthamoeba* spp. it was clear that they should be moved from the Hartmanellidae and related limax amebae. Page (1967) for one, proposed the replacement of the Acanthamoebae in the Mayorellidae.

In a recent review by Cursons and Brown (1976) the controversy regarding the classification of the PFLA appeared to be settled with the majority preferring Chang's 1971 classification scheme. The identification of isolates involves the exploitation of specific cytological, morphological, physiological, immunological, growth and pathogenicity characteristics in an ordered sequence readily usable by hospital and public health laboratory staff (Cursons and Brown, 1976).

1.3 Occurrence and Distribution

The ability of PFLA to form resistant cysts undoubtedly enables them not only to withstand unfavourable conditions, e.g. the isolation of *Acanthamoeba* spp. from 2°C (Brown and Cursons, 1977) and from Antarctic soils (Brown *et al*., pers. comm.), but also to take advantage of the intermittent occurrence of favourable conditions. The PFLA appear to be truly ubiquitous organisms, as isolations have been recorded from a variety of environmental sources such as air (Kingston and Warhurst, 1968), humidifier systems (M.R.C. Symposium, 1977), freshwater, brackish and ocean systems (De Jonckheere *et al*., 1975; Brown and Cursons, 1977; Stevens *et al*., 1977a; Wellings *et al*., 1977), chlorinated swimming and domestic waters (Cerva, 1971a; Anderson and Jamieson, 1977; Cerva and Huldt, 1974), from bottled drinking water (Desmet-Paix, 1974), from a home dialysis unit (Casemore, 1977), from soil (Anderson and Jamieson, 1972; Cursons *et al*., 1978b), and from sewage (Singh and Das, 1972; Chang, 1974a)

Isolations have also been recorded from cell cultures (Jahnes et al., 1957; Stevens and O'Dell, 1973a; Willaert et al., 1978) throat and nasal cavities (Elridge and Tobin, 1967; Cerva et al., 1973; Chang et al., 1975), eye infections (Nagington et al., 1974; Visvesvara et al., 1975), gastrointestinal washings (Hoeffler and Rubel, 1974), cold-blooded vertebrates (Frank, 1974), snails (Kingston and Taylor, 1976), and fish (Taylor, 1977).

Temperature and pH are equally tolerated over a wide range with in vitro growth reported up to 45°C (Griffin, 1972) and a pH range of 4.6 to 9.5 (Carter, 1970).

The distribution of the pathogenic species in relation to non-pathogenic ones is still unknown (Cursons, 1978) though in general, non-pathogenic species are more prevalent at ambient temperature in temperate zones. The repeated isolations of PFLA from waters above ambient temperature, i.e. greater or equal to 30°C (De Jonckheere et al., 1975; Stevens et al., 1977a; Wellings et al., 1977; Cursons et al., 1978b), combined with their higher optimum temperature of growth (Griffith, 1972) suggests that the pathogenic amebae are environmentally selected over non-pathogenic amebae in waters above ambient temperature. The source of the pathogenic amebae in these waters is unknown though the fact that Cursons et al. (1978b), and Wellings et al. (1977) have isolated PFLA from the soil, which is the preferred habitat of small free-living amebae (Singh, 1978), makes it possible that the soil acts as a reservoir of pathogens in the same way as it does for Cryptococcus, and that contamination occurs via run off after rain (Cursons, 1978).

1.4 Pathogenicity

The invasion of organs and tissues by PFLA is now well documented (Culbertson et al., 1959, 1968, 1972; Carter, 1968, 1970, 1972; Callicot et al., 1968; Chang, 1971, 1974a & b, 1976; Culbertson, 1971; Martinez et al., 1973, 1975, 1977; Visvesvara and Balamuth, 1975; Wong et al., 1975 a & b). The CNS invasion by Naegleria spp. occurs primarily via the nasal mucosal epithelium, mainly due to the pathological condition of the cribriform plate and subjacent nasal passages (Culbertson, 1971; Carter, 1972) and this has been verified experimentally by Martinez et al. (1973). Using mice they showed that amebic invasion occurs through the disruption of the olfactory mucosa, penetration into the submucosal plexus, probably by phagocytosis by the amebae of the sustentacular cells of the olfactory

neuroepithelium,and finally through the cribriform plate to the CNS.

In cases of Acanthamoeba meningo-encephalitis,the involvement of the CNS appears to be a secondary phenomenon representing metatastic spread from a primary focus in the skin, genitourinary or respiratory tract (Martinez et al., 1975, 1977; Culbertson, 1971). Martinez et al. (1975) reported lower respiratory tract infections in experimental animals.

AM due to Acanthamoeba spp., appears to be due to an opportunistic infection of the CNS. AM occurs in patients who are chronically ill, debilitated or in those whose cell mediated immune responses have been impaired as a result of either underlying systemic disease or its treatment by immunosuppressive methods (Kernohan et al., 1960; Jager and Stamm, 1972; Robert and Rorke, 1973; Bhagwandeen et al., 1975).

Acanthamoeba infections of sites with reduced accessibility to the immune system (e.g. the eye) also demonstrates the opportunistic nature of these infections. Isolations of Acanthamoeba from the cornea of the eye (Nagington et al., 1974) were shown to be of low virulence and infection only resulted after damage to the cornea (Visvesvara et al., 1975).

Once CNS invasion has occurred, destruction of the surrounding tissue is thought to be brought about by a combination of phagocytosis and pinocytosis of host tissue by N. fowleri,and solely by pinocytosis in the case of A. culbertsoni (Visvesvara and Callaway, 1974; Maitra et al., 1974, 1976). The extensive reports of the possession of lysosomal and hydrolytic enzymes is reported elsewhere (Bowers and Korn, 1973; Martinez et al., 1975; Chang, 1976; Maitra et al., 1976; Cursons and Brown, 1976), and it is also speculated that the levels of the cytopathic enzymes produced may explain the degrees of virulence among the Acanthamoeba and the N. fowleri isolates (Cursons, 1978; Culbertson, 1971; De Jonckheere and van de Voorde, 1977b).

1.5 Immunity

Many authors have pondered over the low incidence of PAM and AM cases with regards the ease and frequency of isolation of pathogenic PFLA from the environment (Anderson and Jamieson, 1971; Cursons et al., 1977b, 1977; John et al., 1977; Wellings et al., 1977; Haggerty and John, 1978). This has led many to speculate on the existence of probable host related susceptibility factors and the demonstration of specific

antibodies to free-living amebae in human sera has been reported (Chang and Owens, 1964; Edwards et al., 1976; Cursons et al., 1977; MRC Symposium, 1977).

Adams et al., (1976) reported that mice surviving a primary intravenous injection of N. fowleri were subsequently resistant to further challenge by the same route with a dose of amebae that produced a uniformly fatal disease in untreated control mice. It was further demonstrated by this group that mice immunized with live or formalized N. fowleri or live N. gruberi either subcutaneously, intraperitoneally, intravenously or intramuscularly were significantly protected against a subsequent challenge with N. fowleri (John et al., 1977). The role of cell mediated immunity (CMI) in resistance to infection by N. fowleri was reported by Diffley et al. (1976), who demonstrated that guinea-pigs surviving a normally fatal challenge with N. fowleri, exhibited a delayed hypersensitivity when tested intradermally with a soluble fraction derived from N. fowleri (Cursons et al., 1977). Thong (1978) stated that protective immunity to PAM could be transferred to syngeneic mice by immune sera but not by immune spleen cells. The immunity may be related to agglutinating antibodies demonstrated in immune sera or antitoxic antibodies in immune sera may be the active principle. These all tend to support the hypothesis that unwitting exposure to the more ubiquitous non-pathogenic N. gruberi may immunize against N. fowleri and the same may also occur with Acanthamoeba spp. The fact that some underlying immunity exists was demonstrated by Wong et al. (1975 a & b) who demonstrated that primates were apparently immune to intranasal or intravenous innoculations of N. fowleri or A. culbertsoni unless on immunosuppressive drugs. However intrathecal innoculations were shown to cause amebic meningo-encephalitis. Culbertson has shown that mice immunized with Acanthamoeba spp. are resistant to intranasal challenge with A. culbertsoni but was unable to show the same with Naegleria spp.

1.6 Control Measures

Free-living amebae are widely dispersed in the environment and the fact that they can be isolated from chlorinated domestic and swimming waters (Cerva, 1971a; Anderson and Jamieson, 1972; Cerva and Huldt, 1974; De Jonckheere and van de Voorde, 1976), as well as untreated recreational waters has led to an expression of concern by public health authorities over the possible contraction of PAM or AM via these sources.

Cerva (1971a), after reviewing 16 fatal cases of PAM from an indoor chlorinated swimming pool stated that, there will always be the constant presence of limax amebae even under the strict observations of

all routine safety measures applied to swimming pools and water systems. This was supported by a reported case of PAM in South Australia by Anderson and Jamieson (1972), in which the victim contracted the disease from domestic bath water, and that super chlorination to $10mg.l^{-1}$ failed to eradicate Naegleria from the contaminated pool. However, Lyons and Kapur (1977) in a survey of 30 halogenated public swimming pools concluded that the low amebic densities (less than one per litre), in the majority of pools illustrated that these amebae could be adequately controlled by proper pool maintenance. The possession of resistant cysts however complicates the disinfection process.

Derreumaux et al. (1974) demonstrated that $0.5mg.l^{-1}$ of HOCl, the active disinfecting component of chlorine disinfection was able to eradicate both Naegleria and Acanthamoeba spp. De Jonckheere and van de Voorde (1976), showed that an initial concentration of chlorine between 0.5 - $1.0mg.l^{-1}$ was cysticidal for Naegleria spp. but that Acanthamoeba culbertsoni cysts were not inactivated by levels up to $40mg.l^{-1}$.

In a study of alternative disinfectants by Cursons et al., (1978b) it was shown that deciquam 222, chlorine, chlorine dioxide and ozone all possessed potential disinfecting properties for PFLA, but at higher levels than those for disinfecting bacteria. Deciquam 222 was found to be the most effective followed by chlorine, chlorine dioxide and ozone, but the final choice of disinfectant must depend on the physical and chemical properties of the water to be treated.

1.7 Diagnosis

Early diagnosis and treatment along with careful intensive care treatment therapy is extremely important in the treatment of infections due to PFLA; more in those caused by Naegleria spp. The survival of a nine year old female in Torrance, California (Siedel et al., pers. comm. 1978) and that of a fourteen year old male in Australia (Anderson and Jamieson, 1972) could be attributed to this. Fluid restriction, management of cerebral edema and other complications of amebic meningo-encephalitis are all important in the care of these patients (Siedel et al., pers. comm. 1978).

Infections due to Naegleria spp. are usually characterized by a previous history of swimming in freshwater some 7-14 days before expressing typical meningitis symptoms (Cursons et al.,1977; Carter, 1972; Chang, 1974a). The symptoms include severe headache (usually frontal), sore throat, nausea, vomiting, fever (39-41°C) accompanied

by a stiff neck. Clinical isolation of amebae can be routinely done by cultivation of Cerebral Spinal Fluid (CSF), brain tissue or nasal discharge on Page's Ameba Saline Agar spread with live E. coli or E. cloacae; by axenic CYM culture; or by passaging of suspected material through cell culture, at 37-45°C (Cursons et al., 1978). The examination of CSF is still probably the most routine method of diagnosing general meningitis. The differences between amebic and bacterial meningitis are slight and although in positive amebic cases there tend to be a predominance of neutrophils in the CSF, a high protein concentration and low sugar levels, complete diagnosis relies on finding amebae in the fluid and the further cultivation of these for complete diagnosis. Species identification can then be achieved by a method outlined by Cursons and Brown (1976).

In post-mortem diagnosis, a degree of encephalitis is invariably present. Severe brain swelling and redness, combined with purulent and haemorrhagic exudate containing numerous amebae is more extensive on the ventral surface of the cerebrum or cerebellum and over the brain stem. Amebae are also numerous in the olfactory nerve bundles which are virtually destroyed by purulent inflammation (Carter, 1969, 1972). The grey matter of the cerebral hemispheres and cerebellum shows variable sized lesions which tend to be haemorrhagic and quite soft when they are large (Culbertson, 1971). Puralent meningitis is usually inconspicuous and confined to the antero-basal aspects of the brain, and it is only rarely that one can find inflammation or amebic invasion in the posterior cerebral hemispheres, brain stem or cerebellum, and never in the spinal cord (Carter, 1969, 1972).

The Indirect Immuno Fluorescent Antibody (IFAB) technique applied to hydrosoluble protein extracts of either Naegleria or Acanthamoeba spp. is a valuable tool in the identification of species. It can also be applied to identify amebae in brain sections of suspected or proven patients, though is a time consuming process and is not recommended for routine laboratory practice. Antisera can be produced in rabbits and can be made species specific by suitable absorption methods. IFAB methods can also be used to provide rapid screening methods for detection of PFLA in swimming pools, tap and other domestic and recreational water supplies.

Immunoperoxidase methods have been used to demonstrate both Naegleria and Acanthamoeba spp. in brain sections of patients who have died from PAM and AM respectively by Culbertson (1975) and Cursons et al., (1976). This is a method that may be shown to be more valuable

in the future than immunofluorescence techniques. It has certain advantages over IFAB in that permanent preparations can be made, no specialized equipment is necessary and clear definitive staining of tissue elements results (Culbertson, 1975).

Acanthamoeba meningitis infections are difficult to diagnose even in advanced cases due to the lack of specific symptoms and the apparent lack of amebae in the CSF (Chang, 1974a). There is usually a history of poor health and immunological incompetence with few patients giving a past history of swimming. The onset is slow (>10 days) and insidious, with the lung, brain and kidneys being infected (Martinez et al., 1976). Acanthamoeba infections may initially produce a severe bronchopneumonia, the organisms then dessiminating and reaching the CNS via the bloodstream (Marino, 1975).

Post-mortem diagnosis relies on the presence of superficial lesions in the grey matter with granulomatosis inflammation, and the presence of trophozoites and double walled wrinkled cysts in apparently normal tissue bordering the lesion (Chang, 1974a; Carter, 1972; Culbertson, 1971; Hoffmann et al., 1978). Many authors regard this as diagnostic of Acanthamoeba infections.

In the case of eye infections reported by Nagington et al. (1974) and Jones et al. (1975), positive diagnosis was possible by taking corneal scrapings, with subsequent isolation and identification of *Acanthamoeba* spp.

1.8 PAM Cases and Their Treatment

Since Willaert published the extensive review of world-wide cases due to PAM in 1974, there have been at least ten additional cases reported (Table I). Symmers (1969) reports a possible earliest case dating back to 1909. A later case reported by Derrick et al. (1948) was originally thought to be due to *Iodamoeba butschlii*, but was later proven by fluorescent antibody staining to have been caused by *N. fowleri* (McMillan, 1977). The confusion in this case arose through the patient having widespread alimentary and systemic invasion as well as the typical pattern of cerebral invasion by morphologically identical amebae, thought to be caused by starvation of the patient, perhaps by reducing his gastric activity, bile secretion and amebicidal serum factor (Carter, 1970). The reidentification of the etiological agents of the 1968 cases of PAM in New Zealand as *N. fowleri* (Cursons and Brown, 1975; Cursons et al., 1967a) has dismissed the notion of slime moulds being involved in

COUNTRY	YEAR	NUMBER OF CASES	CAUSATIVE ORGANISM	DIAGNOSIS	TREATMENT	OUTCOME	REFERENCE
NEW ZEALAND	1974	1	*N. fowleri* (MsT)	isolation from CSF	Penicillin Ampicillin Amphotericin B	died	Cursons *et al.*, 1976b
	1978	1	*N. fowleri* (MsM)	isolation from CSF	Amphotericin B	died	Cursons *et al.*, pers. comm., 1978
U.S.A.	1974	1	*N. fowleri* (Lovell)	isolation from CSF	Unknown	died	De Jonckheere, 1977
	1974	1	*Acanthamoeba* sp.	IFAB	Steroids Penicillin	died	Martinez *et al.*, 1977
	1975	1	*Acanthamoeba* sp.	IFAB post-mortem	Unknown	died	Hoffman *et al.*, 1978
	1978	1	*Naegleria* sp.	isolation from CSF	Amphotericin B Miconazole Rifampin	survived	Seidel *et al.*, pers. comm., 1978
VENEZUELA		1	*A. culbertsoni*	IFAB	Steroids	died	Martinez *et al.*, 1977
PERU		1	*A. castellanii*	IFAB	Steroids Antibiotics	died	Martinez *et al.*, 1977
ZAMBIA	1972	1	*Acanthamoeba* sp.	post-mortem	Antibiotics Amphotericin B	died	Bhagwandeen *et al.*, 1975
KOREA	1958	1	*Acanthamoeba* sp.	post-mortem	Penicillin Streptomycin Chloramphenicol	died	Ringsted *et al.*, 1975

Table I: Cases of Primary Amebic Meningo-encephalitis Reported After 1974 (modified from Cursons, 1978)

COUNTRY	YEAR	NUMBER OF CASES	CAUSATIVE ORGANISM	TREATMENT	REFERENCE
UGANDA	1968	1	*Naegleria*	Metronidazole - Emetine - Penicillin Chloroquine.	Grundy & Blowers, 1970
U.S.A.	1967	1	*A. astronyxis*	Ampicillin Penicillin - G	Callicott *et al.*, 1968
	1978	1	*Naegleria*	Amphotericin B Miconazole Rifampin	Seidel *et al.*, pers. comm. 1978
INDIA	1970	2	*Naegleria*	Streptomycin Isonicoteinhydrosine Sulphadexanathosone Amphotericin B	Pan & Ghosh, 1971
	1973	3	*N. fowleri*	Unknown	S.R. Das, pers. comm. to Willaert(1974)
ENGLAND	1969	2	*Naegleria*	Antibiotics Sulphadiazine Amphotericin B	Apley *et al.*, 1970
AUSTRALIA	1971	1	*N. fowleri*	Amphotericin B Sulphadiazine	Anderson & Jamieson, 1972

Table II: Probable and Definite Survivors of Primary Amebic Meningo-encephalitis

the etiology of PAM (Mandel et al., 1970).

The drug treatment of PAM has been very discouraging with Willaert's summary (1974) providing information of only ten possible survivors of the disease. The Californian case of Seidel et al. (1978) brings the world total to eleven cases (Table II). In the earlier cases, where the amebic nature of the disease had not been suspected, treatment consisted only of antibacterial agents such as sulpha-drugs, penicillin, streptomycin, tetracyclines and chloramphenicol (Fowler and Carter, 1965; Butt et al., 1968; Cerva and Novak, 1968; Dos Santos, 1970; Van den Driessche et al., 1973). However, even in later cases where the antiprotozoal drugs emetine, chloroquine and metronidazole were often used, the course of the disease was not affected in the slightest (Carter, 1968, 1970, 1972; Duma et al., 1971) except in the unproven case of Grundy and Blowers (1970) in which survival was attributed to chloroquine. Naegleria were supposedly isolated from the CSF but failed to survive for any length of time in culture and subsequently were not positively identified. The patient also presented atypical symptoms and treatment consisted of metronidazole, emetine, penicillin sulphane and chloroquine.

The in vitro activity of antibacterial agents against pathogenic Naegleria has been extensively reviewed by many (Carter, 1969; Mandel et al., 1970; Prasad, 1972; Thong et al., 1977; Lee et al., 1979, Donald et al., 1979). Of the antiprotozoal drugs, emetine hydrochloride was shown to be effective in vitro against N. fowleri (Carter, 1969; Prasad, 1972; Das, 1975) although it does not protect animals from the disease (Culbertson et al., 1968), probably due to its inability to pass the blood-brain barrier (Parmer and Cottrill, 1949). Chloroquine and metronidazole have also been shown to be ineffective both in in vitro and in vivo studies (Carter, 1969; Mandel et al., 1970; Duma et al., 1971).

Amphotericin B was the only drug to appear promising in the early 70's, and as can be seen in Table II, it was used in the treatment of all survivors except the unproven case of Grundy and Blowers (1976) and Callicott et al;, (1968). Amphotericin B is an antifungal polyene antibiotic and in vitro tests have shown it to be very effective against Naegleria spp. (Carter, 1969; Mandel et al., 1970; Duma et al., 1971; Schuster and Rechthand, 1975; Visvesvara and Balamuth, 1975; Duma and Finley, 1976; De Jonckheere and van de Voorde, 1977; Donald et al., 1979) and to show in vivo promise (Culbertson et al., 1968; Carter, 1969; Das 1971; Thong, 1978, 1979).

Carter (1969) suggested that amphotericin B be tried in the treatment of PAM by simultaneous intravenous and intraventricular administration. The doses recommended were: 0.25mg.kg^{-1} IV and 1.0mg into the cerebral ventricles (I.vent.) in the first 24 hours which were as high as he dared propose due to the highly toxic nature of the drug. Carter (1972) also suggested using sulphadiazine as well as amphotericin B initially, in case the amebae should prove to be Acanthamoeba. These amebae have shown to be resistant to both these drugs in vitro (Casemore, 1970; Chang, 1971; Visvesvara and Balamuth, 1975; Duma and Finley, 1976; Nagington and Richards, 1976; Donald et al., 1979), but there is evidence to show that they are affected by sulphadiazine in vivo (Culbertson et al., 1965). Subsequently the treatment was tried on two patients in the U.S.A. (Duma et al., 1971) who were in the early stages of the disease and should have responded. The first patient (patient 3, Duma et al., 1971) was given 1.5mg of amphotericin B through a ventricular tap which was repeated 16 hours later. 10mg amphotericin B was also administered I.vent. together with 10mg dexamethasone. The patient was also given 400mg metronidazole (orally); 200mg chloroquine base and 4mg dexamethazone intramuscularly (IM) every 6 hours. However, 72 hours after admission he became shocklike, respiration ceased and he died. The second patient (patient 4, Duma et al., 1971) received similar treatment though he died 66 hours after admission.

Carter (1972) reported similar findings to Duma et al (1971) in two patients that had been treated in the same way (seventh and ninth patients Table III, Carter, 1972). There was also the added difficulty in getting the drug into the swollen brains by the intraventricular method.

Apley et al (1970) described three cases of PAM in Britain, two of which were diagnosed presumptively because of association with the fatal proven case. They had the same early symptoms, however, neither actually developed the convincing signs of meningitis. N. gruberi was cultured from the CSF of the child who died and from only one of the others. Amphotericin B was administered to the fatal case after finding amebae in the CSF. It was given IV in one daily dose of 0.25mg.kg^{-1} given over three to four hours. This was increased to 1mg.kg^{-1} over one week, but the patient died on the sixteenth day after admission. It is interesting to note that on the seventh day after admission amebae were seen in the CSF though many appeared to be dead. By the eleventh day after admission, the CSF contained no amebae. It is also interesting that the drug was given by the intravenous route only, and yet produced

high levels in the CSF, apparently destroying most of the amebae in the CNS. The patient's survival had been notably prolonged and Carter (1972) postulated that maybe IV treatment on its own, but at a higher dosage rate, may be successful in further cases. This was in fact proved in a later case (Anderson and Jamieson, 1972).

The second British case, a brother of case one, was admitted to hospital two days after case one, complaining of a headache and sore throat with neck pains. In view of patient one, amphotericin B and sulphadiazine treatment was begun although the CSF was clear. By day seven he was symptom clear though on day eight they returned and although the CSF was clear, some amebae were cultured which appeared to be similar to those from case one. Amphotericin B was given, $0.25 mg.kg^{-1}$ IV over four hours increasing to $0.75 mg.kg^{-1}$ after four days for a total of 10 days after which the CSF was clear and no amebae were cultured. He was discharged symptom free.

The third case was admitted to hospital six days after case one. He complained of sore throat, headache, vomiting and abdominal pain although CSF appeared normal. He was given amphotericin B and sulphadiazine though signs of drug toxicity were noted after four days and the treatment was stopped. It was on day eight that the growth of amebae from case two was reported and although the patient was well, amphotericin B treatment was recommended at $0.25 mg.kg^{-1}$. He was discharged after fourteen days, symptom free with no amebae having been isolated at any time (Apley *et al.*, 1970). "Case three must be considered to be only doubtfully infected with Amebae" (Apley *et al.*, 1970). Griffin (1976) has disputed the diagnosis of *Naegleria* meningo-encephalitis in cases one and two and believes that *Acanthamoeba* were in fact the ameba involved, and that sulphadiazine was responsible for the treatment of case two and the prolonged survival in case one. He also considers that the level of sulphadiazine in the CSF prevented the growth of amebae in culture.

Pan and Ghosh (1971) reported the survival of two children (aged 6 months and 3 years) with CNS infections of slow onset (3-5 months). CSF samples showed "motile amebae with thin pseudopods" and, although no strains were isolated, treatment was with amphotericin B, sulphadiazine and intrathecal steroids. These two cases are considered inconclusive in the nature of the etiological agent involved and the effective agent in their treatment (Donald, 1979).

The first successful treatment of N. fowleri PAM was that reported by Anderson and Jamieson (1972). A fourteen year old boy from Queensland was in the fourth day of illness and comatose by the time treatment was begun. N. fowleri was cultured from the CSF, in which they could be plainly seen. Amphotericin B was given at a dose of $1mg.kg^{-1}$ per day IV, as well as penicillin,-ampicillin and sulphadiazine which he had been having for three days previously. He was afebrile and talking rationally within two days. After five days the CSF white cell count had dropped but amebae were still seen, therefore amphotericin B was given intrathecally (IT) and later I.vent. in small doses (0.1mg on alternate days). The fluid gradually cleared and he was discharged from hospital without any neurological defects.

The second successful treatment of a N. fowleri PAM case is that of a nine year old female from Torrance, California who showed typical symptoms of meningo-encephalitis three days before admission to hospital (Seidel et al., pers. comm.). Routine CSF cell count procedures revealed organisms with ameboid movements and the following medications were given: amphotericin B - 1.5mg IT and $1mg.kg^{-1}$ IV; sulphadiazine $50mg.kg^{-1}$ IV; chloramphenicol $25mg.kg^{-1}$ IV and penicillin 3.4×10^5 units IV. The patient was then transferred to Harbor General Hospital where she was in a coma on admission but responsive to pain and tactile stimulation. The following treatment was administered:

(i) Amphotericin B was given IV at a dose of $1.5mg.kg^{-1}.day^{-1}$ given in two doses daily for three days after which it was decreased to $1mg.kg^{-1}.day^{-1}$ given in a single daily dose for six days.

(ii) Amphotericin B was also given IT at $1.5mg.day^{-1}$ for two days after which it was decreased to 1.0mg every other day for eight days. This was administered through a lumbar intrathecal catheter.

(iii) Miconazole was given IV at a dose of $350mg.m^{-2}.day^{-1}$ given thrice daily for nine days.

(iv) Miconazole IT at $10mg.day^{-1}$ for two days then 10mg every other day for eight days.

(v) Rifampin was given orally at a dose of $10mg.kg^{-1}.day^{-1}$ thrice daily for nine days.

Sulphadiazine (IV - $4gms.day^{-1}$) was continued for three days until studies confirmed the diagnosis of Naegleria meningo-encephalitis. Penicillin and chloramphenicol were continued for three days until CSF

cultures were shown to be negative for bacteria. Dexamethasone and diphenylhydantoin were given for increased intracranial pressure and seizure activity respectively. The patient stabilized clinically over the first forty eight hours. Gradually over the next month of hospitalization her mental status improved and no significent neurological deficits were noted at discharge (Seidel et al.,-pers. comm).

In other reported cases of PAM where there was proof of N. fowleri infection, and where amphotericin B was given as a treatment, the course of the disease was often too advanced to see any effect (Van Den Driessche et al., 1973; Donald, 1979).

Amebic meningitis due to Acanthamoeba are a lot less common than those of Naegleria probably due to the need for some predisposing factor (Martinez et al., 1977; Kernohan et al., 1960; Jager and Stamm, 1972; Bhagwandeen et al.,1975). Callicott et al. (1968) reported a survival due to A. astronyxis which was isolated from the CSF although the authors were unable to provide evidence as to whether the disease was in fact due to Acanthamoeba.

Several cases of Acanthamoeba infection have been reported though only after post-mortem examination where the brain sections were stained by indirect immunofluorescent antibody techniques (Ringsted et al., 1976; Martinez et al., 1977; Hoffmann et al., 1978; Willaert, 1978).

A possible case was reported by Kenney (1971) in a patient hospitalized for acute gastritis of unknown origin. Compliment fixation tests revealed no antibodies to Entamoeba histolytica though did reveal some to A. culbertsoni which rose over the next two months. Clinical examination failed to reveal any symptoms of cerebral involvement and the patient refused a spinal tap. The patient was put onto antiamebic treatment consisting of dehydro-emetine and chloroquine (IM). Compliment fixation tests two months later showed that the serum titre had decreased.

The only human Acanthamoeba infections positively diagnosed during life were those in the eye. Nagington et al. (1974) repeatedly isolated Acanthamoeba from two English patients with corneal ulcers. Warhurst and Thomas (1975) identified the amebae as A. castellanii and A. polyphaga. In one case, chloramphenicol, iodoxuridine, 3-fluor-thymidine, methicillin, gentamicin and later sulphadiazine were tried without any effect. After six months, because of corneal ulceration, pain and loss of vision, a corneal graft was performed which was rejected.

The other infection was in a 59 year old farmer with an identical condition which required enucleation of the eye after one year. Treatment was in this case, chloramphenicol, acetylcysteine, 3-fluorothymidine and clotrimazole.

At the same time as the above eye infections, Jones *et al*. (1975) cultivated *A. polyphaga* from corneal ulcers of two patients in Houston, Texas. They reported suppression of the amebae with paromomycin. It seems that these infections may not be so rare, and in cases of chronic corneal ulceration, amebic infection should always be considered (Nagington *et al*., 1974).

CHAPTER TWO: MATERIALS

2.1 Ameba Cultures Used

Table : Ameba Cultures Used

SPECIES	STRAIN	PATHOGENICITY	SOURCE	PLATE
Naegleria fowleri	MsM	+	MU	2, 4, 6
Naegleria fowleri	MsT	+	MU	-
Naegleria gruberi	P1200f	-	NHI	1, 3, 5
Acanthamoeba culbertsoni	A-1	+	CCAP	8, 10
Acanthamoeba castellanii	1501	-	IMTPL	7, 9

+ = positive

- = negative

NHI = National Health Institute, Wellington, N.Z.

CCAP = Culture Centre of Algae and Protozoa

IMTPL = Institute de Médecine Tropicale Prins Léopold, Belgium

MU = Massey University, Palmerston North, N.Z.

2.2 Plate Media

2.2.1 Ameba Saline Agar (Page, 1967; Cursons et al., 1978a)

for the isolation of *Naegleria* spp.

NaCl	=	0.12g
$MgSO_4.7H_2O$	=	0.004g
$CaCl_2.2H_2O$	=	0.004g
Na_2HPO_4	=	0.142g
KH_2PO_4	=	0.136g
Agar	=	15.0g
Distilled water	=	1.0 litre

pH 6.8

Autoclave at 103.4 KPa (121°C) for 15 minutes

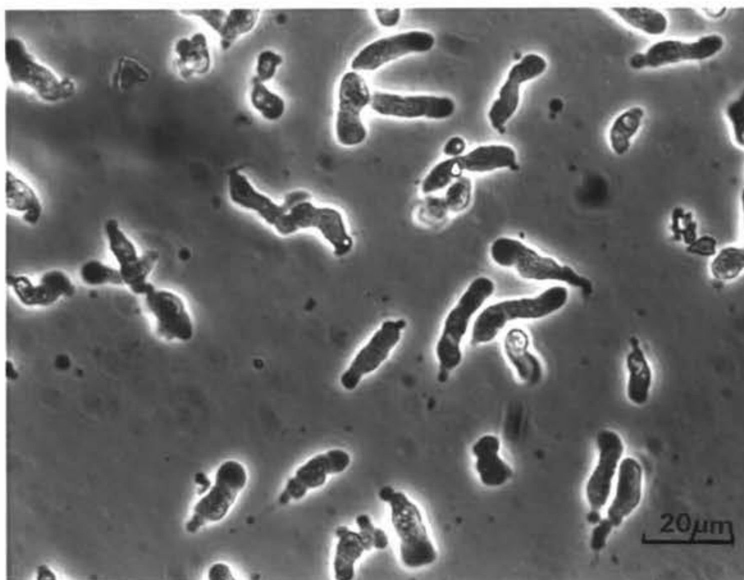


Plate 1 Trophozoite stage of Naegleria gruberi (P1200f) (Note: limax shape, with broad anterior and narrow posterior extremities, also the single pseudopod of clear hyaline ectoplasm.)

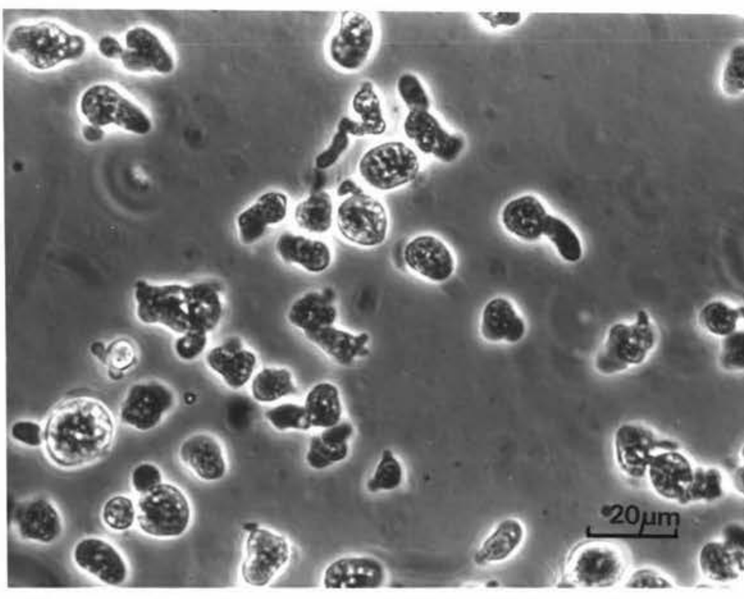


Plate 2 Trophozoite stage of Naegleria fowleri (MsM)

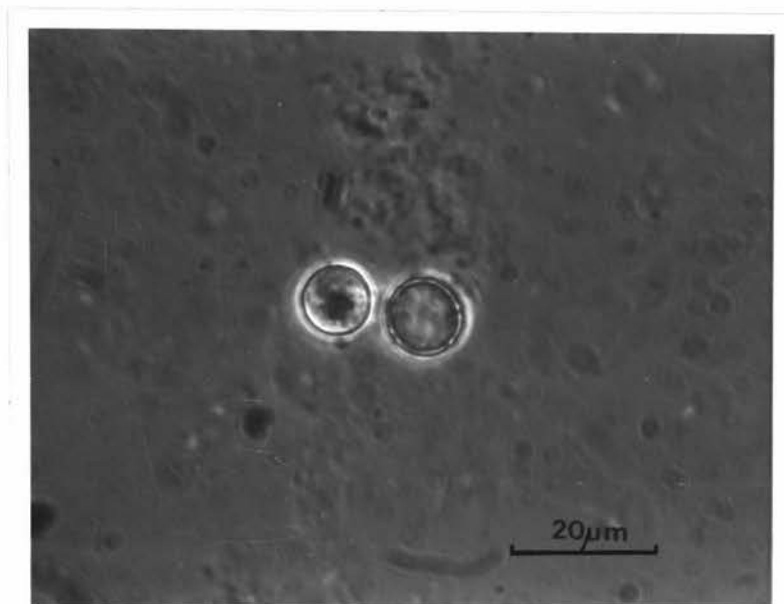


Plate 3 Cyst stage of Naegleria gruberi (P1200f)

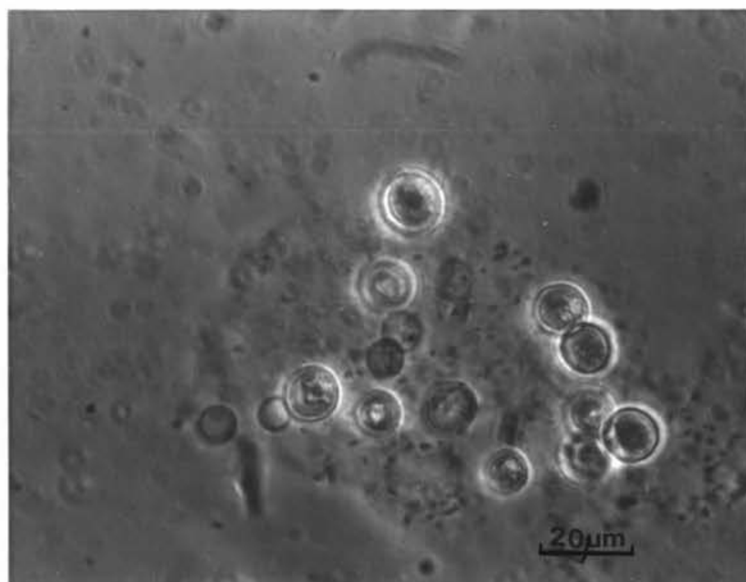


Plate 4 Cyst stage of Naegleria fowleri (MsM)

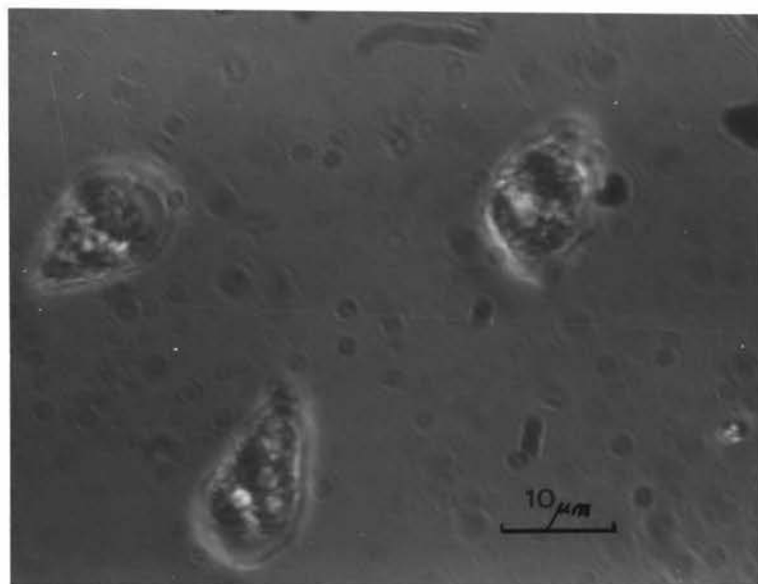


Plate 5 Flagellate stage of <u>Naegleria gruberi</u> (P1200f)

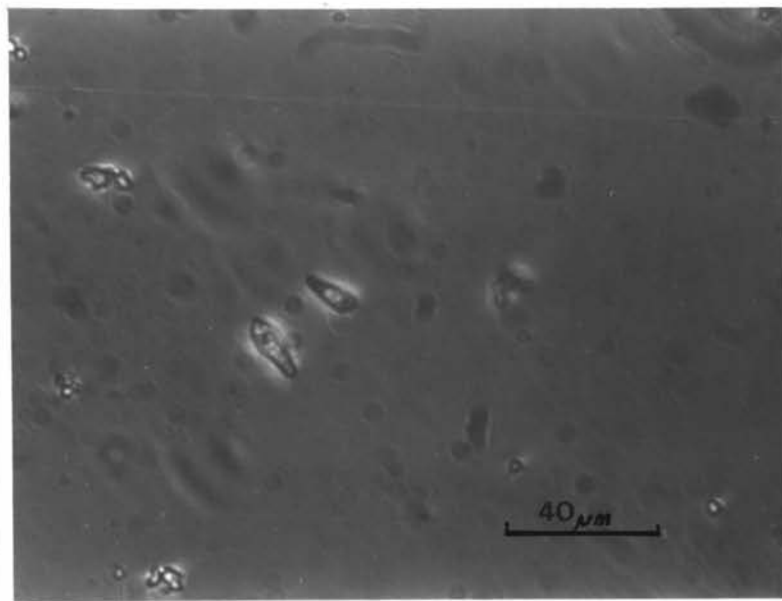


Plate 6 Flagellate stage of <u>Naegleria fowleri</u> (MsM)

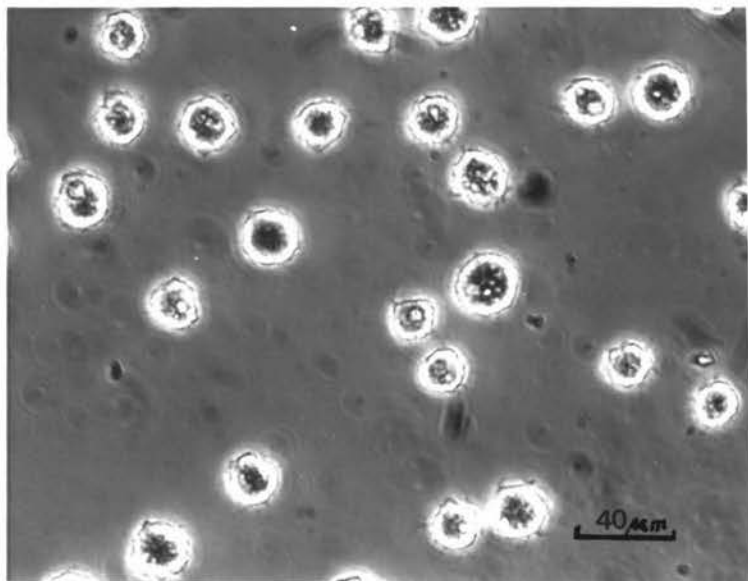


Plate 7 Trophozoite stage of Acanthamoeba castellanii (1501)
(Note the fine Acanthapodia)

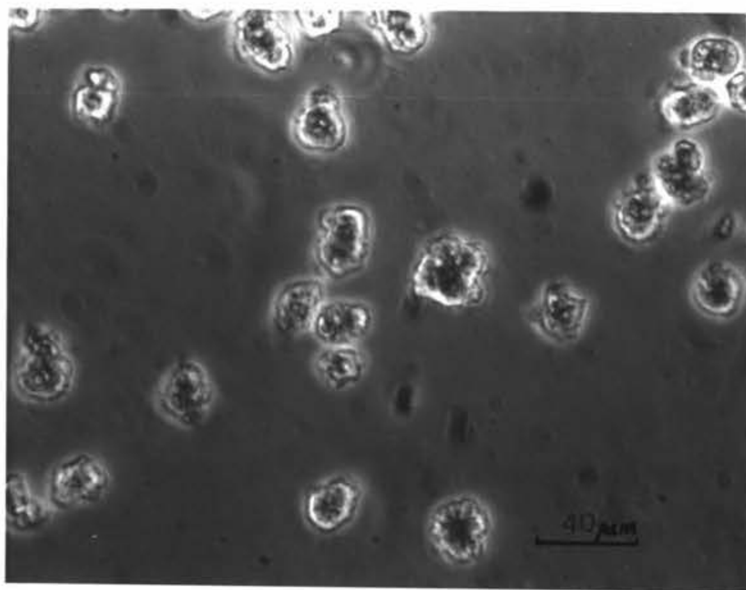


Plate 8 Trophozoite stage of Acanthamoeba culbertsoni (A-1)

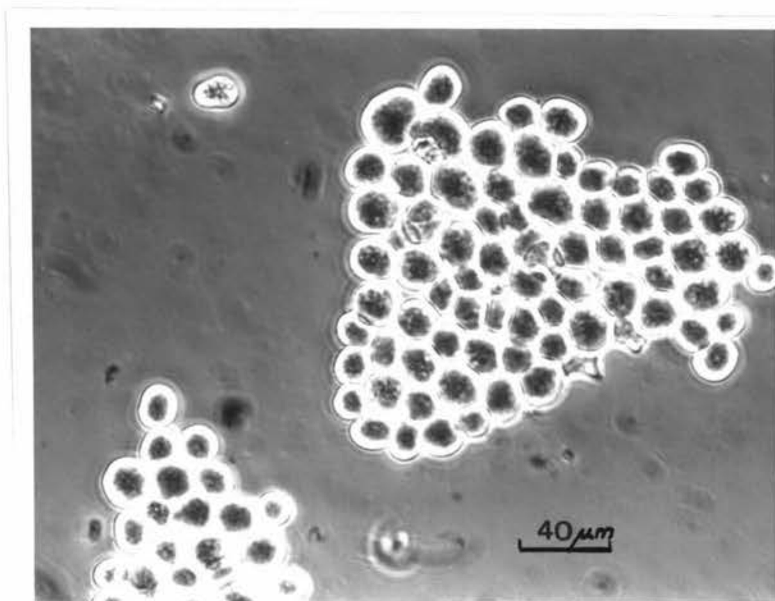


Plate 9 Cyst stage of Acanthamoeba castellanii (1501)
(Note: double-walled structures with a wrinkled ectocyst, stellate to round endocysts)

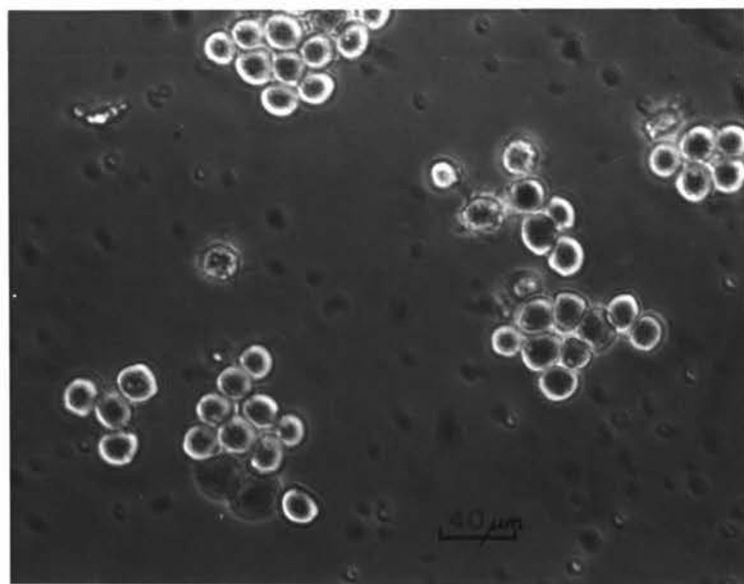


Plate 10 Cyst stage of Acanthamoeba culbertsoni (A-1)

2.2.2 Ameba 1% Saline Agar (Cursons, 1978, modified from Page, 1967) for the isolation of *Acanthamoeba* spp.

NaCl	=	10.0g
$MgSO_4.7H_2O$	=	0.004g
$CaCl_2.2H_2O$	=	0.004g
Na_2HPO_4	=	0.142g
KH_2HPO_4	=	0.136g
Agar	=	15.0g
Distilled water	=	1.0 litre

pH 6.8

Autoclave at 103.4 KPa (121^oC) for 15 minutes

2.2.3 Sabouraud Dextrose Agar (Difco) for the growth of *Candida* spp. in the assay of the imidazoles R41,400 and Miconazole nitrate.

2.3 Axenic Media for Amebae

2.3.1 Page's Ameba Saline (PAS) (Page, 1967; Cursons *et al.*, 1978d) for diluting out either *Acanthamoeba* or *Naegleria* spp. and as a base for CYM medium.

NaCl	=	0.12g
$CaCl_2.2H_2O$	=	0.004g
$MgSO_4.7H_2O$	=	0.004g
Na_2HPO_4	=	0.142g
KH_2PO_4	=	0.136g
Distilled water	=	1.0 litre

pH 6.8

Autoclave at 103.4 KPa (121^oC) for 15 minutes

2.3.2 CYM Medium (Cursons et al., 1978; modified from Stevens and O'Dell, 1973b) for the axenic cultivation of *Naegleria* spp.

Glucose	= 10.0g
Difco Yeast Extract	= 5.0g
Difco Casitone	= 10.0g
L-methionine	= 0.08g
d-Biotin	= 0.002g
Thiamine HCl	= 0.001g
Vitamin B_{12}	= 0.000001g
Page's Ameba Saline	= 1.0 litre

pH 6.8

Autoclave at 103.4 KPa (121°C) for 15 minutes

To $4.5cm^3$ of CYM, add aseptically $0.5cm^3$ of the following cocktail:

Sterile serum	= $50.0cm^3$
Sterile hemin (0.1% W/V)	= $10.0cm^3$
Sterile distilled water	= $40.0cm^3$
Penicillin/Streptomycin	200,000 units.cm^{-3}

2.3.3 4.0% Neff Medium (Stevens and O'Dell, 1973b) for the axenic cultivation of *Acanthamoeba* spp.

Difco proteose-peptone	= 40.0g
Glucose	= 15.0g
Difco Yeast Extract	= 7.5g
d-Biotin	= 0.002g
Thiamine HCl	= 0.001g
Vitamin B_{12}	= 0.000001g

plus 1.0 litre of the following Ac ion solution:

Ac´Ion Solution

$MgSO_4.7H_2O$	= 0.2465g
$CaCl_2.2H_2O$	= 0.01095g
KH_2PO_4	= 0.27218g
Ferric citrate	= 0.0335g

Autoclave at 103.4 KPa (121°C) for 10 minutes

To $9.0cm^3$ of 4% Neff medium is added $1cm^3$ of Penicillin/Streptomycin solution (2000,000 units.cm^{-3})

2.3.4 CGHV and CGHVS (Cursons *et al.*, 1979)

Semi-defined media for the axenic testing of drugs against *Naegleria* (CGHV) and *Acanthamoeba* (CGHVS) spp.

Medium A

Casitone	= 40.0g
Glucose	= 2.5g
Rutin	= 0.0075g
L-methionine	= 0.27g
Folic acid	= 0.0025g
HEPES	= 0.6g
d-Biotin	= 0.002g
Thiamine HCl	= 0.001g
Vitamin B_{12}	= 0.000001g
$CaCl_2.6H_2O$	= 0.006g
$MgSO_4.7H_2O$	= 0.004g
NaCl	= 0.12g
Distilled water	= 952.2cm^3 giving a final volume

of 996cm^3

pH 6.8

Autoclave at 34.5 KPa (108°C) for 15 minutes

Medium B

Hemin	= 0.2g
L-Histidine	= 0.2g
Triethanolamine	= 4.0cm^3
Distilled water	= 196.0cm^3

Sterilize by heating to 60°C for 30 minutes

CGHV: Mix 4.0cm^3 of Medium B with Medium A to give a 0.4% solution of B in A

Add Penicillin/Streptomycin to a final concentration of 200 units each

CGHVS: Add 5.0g NaCl to Medium A before autoclaving and then mix as for CGHV

2.4 Cell Culture Media

2.4.1 Vero Cell Culture

2.4.2 4.4% Bicarbonate Solution

$NaHCO_3$ = 22g
12.5cm^3 of a 0.4% (W/V) Phenol red solution
Distilled water up to 500cm^3
Autoclave at 103.4 KPa (121°C) for 15 minutes

2.4.3. 10x Trypsin/Versene Mixture

NaCl = 80g
2.0cm^3 of a 1% Phenol red solution
Versene = 2.0cm^3
KCl = 4.0g
Glucose = 10.0g
Trypsin = 5.0g
Distilled water up to 1 litre
Filter sterilize through a 0.22μm filter

2.4.4. Antibiotics

10^6 units of Streptomycin } 20cm^3 of sterile
10^6 units of Penicillin } distilled water

2.4.5 Eagle's Growth Medium (EGM) (Eagle, 1955)

Eagles = 380cm^3
1cm^3 of antibiotic solution
20cm^3 of 4.4% bicarbonate solution
40cm^3 (10%) serum (calf or pig)
The Eagles is filter sterilized through a 0.22μm filter and bottled in 380cm^3 with the other ingredients being asceptically added when required. The medium is then gassed with CO_2 till orange

2.4.6 Eagle's Maintenance Medium (EMM)

As for Eagle's Growth Medium but with only 1% serum added.

2.5 Antibiotic Solutions

2.5.1 Amphotericin B (E.R. Squibb & Sons Pty. Ltd.)

Fungizone Intravenous is supplied as a sterile lyophilized powder in vials providing 50mg Amphotericin B.P. and 41mg sodium desoxycholate with 25.2mg sodium phosphates as a buffer.

The preparation of solutions was as follows: An initial concentration of 5mg amphotericin.cm^{-3} was first obtained by adding 10cm^3 of sterile distilled water to the vial of drug powder and shaking until the solution was clear. Further dilutions for *in vitro* drug testing were made using sterile distilled water to give the appropriate working solutions. In *in vivo* drug trials all further dilutions were made using sterile physiological saline solution.

2.5.2 Tetracycline Hydrochloride (Upjohn Pty. Ltd.)

Supplied as Panmycin capsules, each containing 250mg tetracycline hydrochloride (Batch No. A7500).

The actual weight of powder in each capsule is 437.0mg.

The preparation of the stock solution was as follows: To the contents of a capsule was added 25cm^3 of sterile physiological saline to give a solution of 10mg.cm^{-3} which may be used as is, or at further dilutions for *in vivo* experimentation.

2.5.3 Polymyxin B Sulphate (Sigma Chemical Co.)

Supplied as a pure powder containing 8000 USP units.cm^{-3} (Lot 107C-0352)

The preparation of the stock solution for *in vivo* trials was as follows: 0.021g was dissolved in 20cm^3 of sterile distilled water to give a stock of 1.05mg.cm^{-3}. Further dilutions for *in vivo* work were done in sterile physiological saline.

2.5.4 5-Fluorocytosine (Roche Products Pty. Ltd.)

Supplied as 500mg lots of pure substance (Ancotil - Lot A. 410/774). For Intrathecal injection route trials, the stock solution was prepared by dissolving 50.0mg in $10cm^3$ of sterile physiological saline. Further dilutions were made also in sterile physiological saline. For Intraperitoneal injections the drug was prepared after the average weight of the mice had been worked out as there was usually no further dilutions made to get the highest dose.

2.5.5 Miconazole (Ethnor Pty. Ltd.)

Supplied as I.V. solution containing 10mg Miconazole nitrate.cm^{-3} (Lot No. 77K03/430).
Dilutions of miconazole were made directly from the above stock as required, either in sterile physiological saline for *in vivo* trials or in sterile distilled water for *in vitro* trials.

2.5.6 R41,400 (Ethnor Pty. Ltd.)

Supplied as R41,400 base (renamed Ketoconazole).
Preparation of the stock solutions was as follows:
0.02g of R41,400 dry powder was dissolved in 0.8 cm^3 1N HCl and then made up to $2cm^3$ with sterile distilled water to give a stock solution of 10mg.cm^{-3}. Further dilutions were either made in sterile physiological saline (*in vivo* trials) or in sterile distilled water (*in vitro* trials).

2.5.7 Levamisole HCl (Janssen Pharmaceuticia Ltd. Belgium)

Batch No. F29/1.
Preparation of stock solution: 0.05g was dissolved in $10cm^3$ of sterile distilled water to give a stock solution of 5000μg.cm^{-3} Levamisole. Further dilutions were made in sterile distilled water.

2.5.8 Kanamycin (Sigma Chemical Company)

Supplied as Kanamycin sulphate.
Preparation of stock solution: 0.02g was dissolved in $5cm^3$ of sterile distilled water to give a stock solution of $4,000\mu g.cm^{-3}$. Further dilutions were made in distilled water.

2.5.9 Oxytetracycline (Pfizer Ltd.)

Supplied as Oxytetracycline HCl - Lot No. 303,51721 potency 900μ/mg.
Preparation of stock solution: 0.05g was dissolved in $10cm^3$ of distilled water to give a final concentration Of $5,000\mu g.cm^{-3}$. Further dilutions were also made in distilled water.

2.5.10 Tylosine

Supplied as tylosine base of potency = 1040 $units.mg^{-1}$
Preparation of the stock solution was by dissolving 0.05g in $10cm^3$ of distilled water to give a concentration of $5,000\mu g.cm^{-3}$. Further dilutions were also made in distilled water.

2.6 Miscellaneous Solutions

2.6.1 Phosphate Buffered Saline (PBS)

NaCl	= 8.5g
Na_2HPO_4	= 1.28g
NaH_2PO_4	= 0.156g
Distilled water	= 1 litre

pH 7.6
Autoclave at 103.4 KPa (121°C) for 15 minutes

2.6.2 Physiological Saline (as a diluent for in vivo drug trials)

NaCl	= 0.8g
Distilled water	= 1 litre

Autoclave at 103.4 KPa (121°C) for 15 minutes

2.6.3 Hanks Solution (Hanks J.H.) (Used as a buffer in conjunction with Erythrosine B dye for viability staining.)

Solution A:

NaCl	=	160g
KCl	=	8g
$MgSO_4.7H_2O$	=	2g
$MgCl_2.6H_2O$	=	2g
$CaCl_2.6H_2O$	=	5.5g
Distilled water	=	1 litre

Autoclave at 10lbs for 5 minutes

Solution B:

$Na_2HPO_4.2H_2O$	=	1.2g
KH_2PO_4	=	1.2g
Glucose	=	20g
Distilled water	=	1 litre

pH 7.0

Autoclave at 10lbs for 5 minutes

Hanks working solution is made by adding 250cm^3 of Solution A to 250cm^3 of Solution B to 4500cm^3 of sterile distilled water in equivalent ratios.

2.6.4 Erythrosine B (H.J. Phillips *et al.*)

Erythrosine B	=	0.4g
NaCl	=	0.81g
KPO_4	=	0.06g
methyl p-hydroxybenzoate	=	0.05g
Distilled water	=	95cm^3

The solution is heated to boiling to dissolve all materials, cooled and the pH adjusted to 7.2 - 7.3 with 1N NaOH.

The final volume is adjusted with distilled water to 100cm^3.

CHAPTER THREE: METHODS

3.1 Sterilization

All glassware, except that used for analytical experiments, was sterilized by steam at 103.4 KPa (121°C) for 15 minutes. All analytical glassware was sterilized by dry heat at a minimum of 160°C for 2 hours. All heat-labile reagents were sterilized by filtration through a 0.22µm millipore filter.

3.2 Axenic Culture Techniques

3.2.1 Maintenance of stock axenic cultures

Stock cultures of *Naegleria fowleri* (MsM and MsT) and *Naegleria gruberi* (P1200f) grown in CYM medium and *Acanthamoeba culbertsoni* (A-1) and *Acanthamoeba castellanii* (1501) grown in 4.0% Neff medium were obtained from the culture collection at Massey University, Palmerston North.

The amebae strains were further subcultured into $4.0cm^3$ of either CGHV medium (for *Naegleria*) or CGHVS medium (for *Acanthamoeba*) in sterile universals (8.0cm x 2.0cm) and placed on gyroshakers (150rpm) (Cursons *et al*., 1978d). The pathogenic *N. fowleri* (MsM) and *A. culbertsoni* (A-1) were cultured at 37°C and subcultured every 24 hours. The non-pathogenic *N. gruberi* (P1200f) and *A. castellanii* (1501) were cultured at 30°C and subcultured every 48 hours. All amebae strains were subcultured at least ten times in the semi-defined media (CGHV and CGHVS) before drug testing was begun to avoid carryover of any substances in the complex media (CYM and Neff) antagonistic to the drugs (Cursons *et al*., 1979).

3.2.2 Axenic drug testing

All tests were carried out in $6cm^3$ Bijou bottles in a total volume of $2.0cm^3$. Stock cultures of amebae were counted on a Fuchs Rosenthal bright line hemocytometer. The cultures were diluted so that $0.2cm^3$ gave a final concentration of $2\text{-}3 \times 10^6$ $amebae.cm^{-3}$. The drug solutions, prepared as outlined in the Materials, were diluted so that $0.2cm^3$ gave the appropriate final concentration. Media, either CGHV for *Naegleria* spp. or CGHVS for *Acanthamoeba* spp., was added to give a final volume of $2.0cm^3$ (i.e. $1.6cm^3$ of media in single drug experiments or $1.4cm^3$ of media in synergy experiments. All experiments were done in duplicate at least three times. Controls were included in all

experiments with distilled water or the appropriate diluent at the highest concentration used, replacing the drug solution.

Samples were withdrawn at 24, 48, 72 and 96 hours and surviving amebae counted on a hemocytometer. Where the samples were needed to be diluted before counting, $0.1cm^3$ was diluted in $0.8cm^3$ of Hanks solution to which had been added $0.1cm^3$ of Erythrosine B dye. When viewed under the phase contrast microscope, non viable amebae were stained red while viable amebae were not affected by the dye. Where amebae numbers were too few to use dilution techniques; viability was defined by the appearance of the amebae and motility. Viable *Naegleria* spp. show an extended limax form and are grey in colour. Non viable amebae are round, lacked pseudopodia and were darker grey due to increased granulation. Non viable *Acanthamoeba* spp. appear yellow by phase contrast, with no internal structures being discernible and lacking acanthapodia. Where numbers appeared to be below 10^4 amebae.cm^{-3}, viability plating was done. A neat or diluted aliquot of the test culture was plated out on Ameba Saline Agar (for *Naegleria*) or Ameba 1% Saline (for *Acanthamoeba*) seeded with *Enterobacter cloacae*. These plates were incubated at the appropriate temperature for 48 hours and then examined for viable amebae.

3.3 Cell Culture Techniques

3.3.1 Maintenance of stock Vero cell cultures and preparation of KIMAX Tubes for drug testing

Vero cells were maintained in $250cm^3$ KIMAX cell culture bottles. The following procedure was carried out every three to four days to maintain the Vero cells in the bottles and also to prepare monolayers in $10cm^3$ KIMAX tubes:

1) $4.0cm^3$ of a 10x Trypsin-Versene (T/V) stock was added to $36.0cm^3$ of sterile distilled water and heated to 37°C in a water bath.
2) The cell culture fluid was decanted off and the monolayer gently washed with $5.0cm^3$ of the T/V mixture.
3) After a 1 minute wash, the T/V mixture was discarded and a further $8.0cm^3$ of T/V mixture added. The bottle was then placed at 37°C for 1 minute then drained again except for the last few drops.
4) The bottle was left at 37°C until the cells were detached from the glass (approximately 3 minutes). Next, $10.0cm^3$ of EGM was added and the cells counted and dispersed in the following aliquots:
   $250cm^3$ bottles = $3x10^5$ cells.cm^{-3}($3.0cm^3$ of a three day old monolayer)

$10cm^3$ tubes = 1.5×10^5 cells.cm^{-3} ($1.5cm^3$ of a three day old monolayer)

5) Tubes were left stationary overnight at 37°C and then placed on a roller drum until a complete monolayer was formed.

3.3.2 Cell Culture drug testing

Once a monolayer had formed, the tubes were treated as follows:

1) The EGM fluid was discarded and the monolayer washed with $1.0cm^3$ sterile PBS (pH 7.6). EMM was then added to give a final volume of $2.0cm^3$ (i.e. $1.6cm^3$ of EMM in a single drug experiment and $1.4cm^3$ in synergy experiments).
2) The seeding inocula of ameba was obtained from 24 hour exponential axenic cultures of N. fowleri (MsM) and A. culbertsoni (A-1) routinely passaged through mice, cell cultures and maintained in CYM and 4.0% Neff respectively. The amebae were counted and diluted with EMM and $0.2cm^3$ added to the cell culture tubes to give the appropriate concentrations - $9 \times 10^3 ml^{-1}$ for N. fowleri (MsM) and $1 \times 10^3 ml^{-1}$ for A. culbertsoni (A-1).
3) The stock drug solutions, prepared as outlined in the Materials section, were diluted so that $0.2cm^3$ gave the appropriate final concentration.

Control tubes were included in all experiments, with distilled water or the appropriate diluent at the highest concentration used, replacing the drug solution. Controls to determine the cytotoxic effect (CTE) of the drugs were also included.

The tubes were examined every 24 hours for six days and the presence or absence of cytopathic effects (CPE) and CTE noted. After 6 days, the tubes were centrifuged at 1500 rpm for 10 minutes in a bench centrifuge, the supernatant discarded and the deposit resuspended in $1.0cm^3$ PAS and plated on Ameba Saline Agar (for Naegleria) or Ameba 1% Saline Agar (for Acanthamoeba) seeded with Enterobacter cloacae. The plates were incubated at 37°C for 48 hours and examined for viable amebae.

3.4 In Vivo Testing of drugs

Young male white mice (Massey strain) were first weighed and then anaethetized by injecting $0.15cm^3$ of a 0.1% (V/V) Nembutal Solution intraperitoneally (IP), using physiological saline as the diluent. Amebae cultures of either N. fowleri (MsM) or A. culbertsoni (A-1) grown

axenically in CYM or 4.0% Neff respectively, were counted and diluted to 2×10^6 amebae.cm^{-3}. Mice were then inoculated intranasally with $0.05cm^3$ of the amebic suspension, and laid in the cage on their back till recovered. This was to aid penetration of the amebae into the nasal area.

Drug solutions for in vivo testing were prepared daily maintaining the W/V proportions of powder to diluent outlined in the Materials; physiological saline being used as the diluent. The concentration of drug used was adjusted to give the correct dose in proportion to the weight of the mice. The volume of drug given was wherever possible kept to a maximum of $0.1cm^3$ for intraperitoneal injections and $0.02cm^3$ for intraventricular injections (see below).

The drugs were given immediately after intranasal inoculation of amebae and continued daily or at times depending on the trial. For all further drug injections the mice were conscious and were sufficiently immobilized by correct handling procedures. Control mice received either physiological saline or the necessary diluent replacing the drug solutions.

Death due to amebae was verified by removing the brains of dead mice, and plating these on Ameba Saline Agar (for *Naegleria*) or Ameba 1% Saline Agar (for *Acanthamoeba*) seeded with *Enterobacter cloacae*. Amebae could be seen after 24 hours incubation at 37°C.

3.4.1 Intraperitoneal injection method

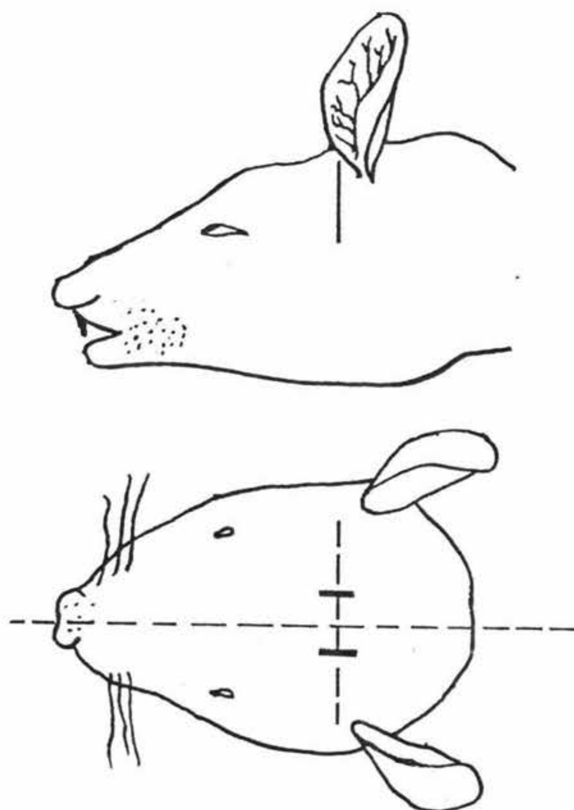
The peritoneum presents a large absorptive area and as long as the needle is placed in the lower quadrant and directed at approximately 45° cephalad, there is little danger of piercing the abdominal viscera. The neeles used were 27 guage 0.5 inch insulin syringes of $1cm^3$ volume and the depth of penetration was approximately 1cm. The technique can easily be performed by one person.

3.4.2 Intraventricular injection method (modified from T.J. Haley et al., 1957).

The method of injection is as follows: the animal is grasped firmly by the loose skin behind the head. The skin was pulled tight with the rest of the hand resting on the back and tail of the mouse to prevent spasmodic moving during penetration. A $1cm^3$ insulin syringe (27 guage 0.5 inch needle) was inserted perpendicularly through the skull into the brain and the volume of drug or diluent injected. The site of injection was 2mm from either side of the midline on a line

drawn through the anterior base of the ears (Figure 1). The site of injection is critical as penetration into the ventricles is not accomplished by injecting further rostrally and damage to the vital centres is produced by injecting more caudally. The size and length of needle is also critical for the same reasons and in addition, an increase in length resulted in extensive brain damage and sometimes death from movement during injection. Therefore a hollow tube was placed over the needle with approximately 2mm of the needle projecting which ensured that the needle did not penetrate too far.

Figure 1.



3.5. Bioassay of Imidazole levels in mouse serum

The assay method used was based on that adopted by Jamieson for the assay in mice of another Imidazole, Clotrimazole (Jamieson, 1975).

3.5.1 R41,400

Candida pirapsilosis was used as the test organism as it required a minimum inhibitory concentration of $1.14\mu g.cm^{-3}$ (Dixon et al., 1978). Assay plates were prepared using Sabouraud dextrose agar (Difco). Large glass petri dishes (14cm diameter) were used,

into which was poured 70cm^3 of agar. 0.5cm^3 of an overnight culture of C. pirapsilosis was spread over the agar to produce a lawn.

R41,400 was administered to 15 mice at a concentration of 60mg/kg^{-1} intraperitoneally and after 0, 2, 4, 6 and 8 hour periods, 3 mice were sacrificed and their blood pooled. The serum was obtained by incubating the blood at 37°C for 1 hour, then at 5°C for 1 hour and finally centrifuging at 3000 rpm for 10 minutes.

Dilution standards were prepared in sterile horse serum and 0.1ml of either these standards or the pooled mouse sera was placed onto antibiotic assay discs (Watman A.A. 13mm) which were then placed onto the surface of Sabouraud dextrose agar plates.

The plates were incubated overnight at 37°C, and the zones of inhibition were measured with calipers fitted with a Vernier scale. A standard curve was drawn up from which was read the concentrations of drug present in each of the pooled mouse sera. From these results, graphs were constructed showing the gradual breakdown of the drugs in the blood stream of the mouse relative to time (Elmsly et al., 1980).

3.5.2 Miconazole nitrate

The assay test was identical to the one for R41,400 except that Candida pseudotropicalis was used as the test organism, because it had a MIC of less than or equal to 1μg.cm^{-3} (Elmsly et al., 1980).

CHAPTER FOUR RESULTS

4.1 In Vitro Axenic Drug Testing of Naegleria spp.

Six drugs were screened for activity against N. gruberi (P1200f) and N. fowleri (MsM). A minimum inhibitory concentration (MIC) and a minimum amebicidal concentration (MAC) were noted with those drugs that showed activity. The MIC is defined as the lowest concentration of the drug which produced obvious reduction in the number of trophozoites over 96 hours. The MAC is defined as the lowest concentration of the drug at which no living trophozoites could be found after 96 hours.

4.1.1 Kanamycin and Tylosine

Figures 2 and 3 show the results obtained with Kanamycin. Neither N. gruberi (P1200f) nor N. fowleri (MsM) showed any effects from the action of the drug. They were all active, motile limax amebae, and numbers closely followed those of the control.

Figures 4 and 5 show the effects of Tylosine on the Naegleria spp. N. fowleri (MsM) showed slightly more susceptibility to the drug than did the non-pathogen, N. gruberi (P1200f). The amebae of both species showed signs of drug damage at $250\mu g.cm^{-3}$. They were smaller, more vacuolated and tended to round up after 48 hours.

Figure 2 The Effect of Kanamycin on Naegleria gruberi (P1200f)
(Drug concentrations shown figures 1-24 are in $\mu g.cm^{-3}$)

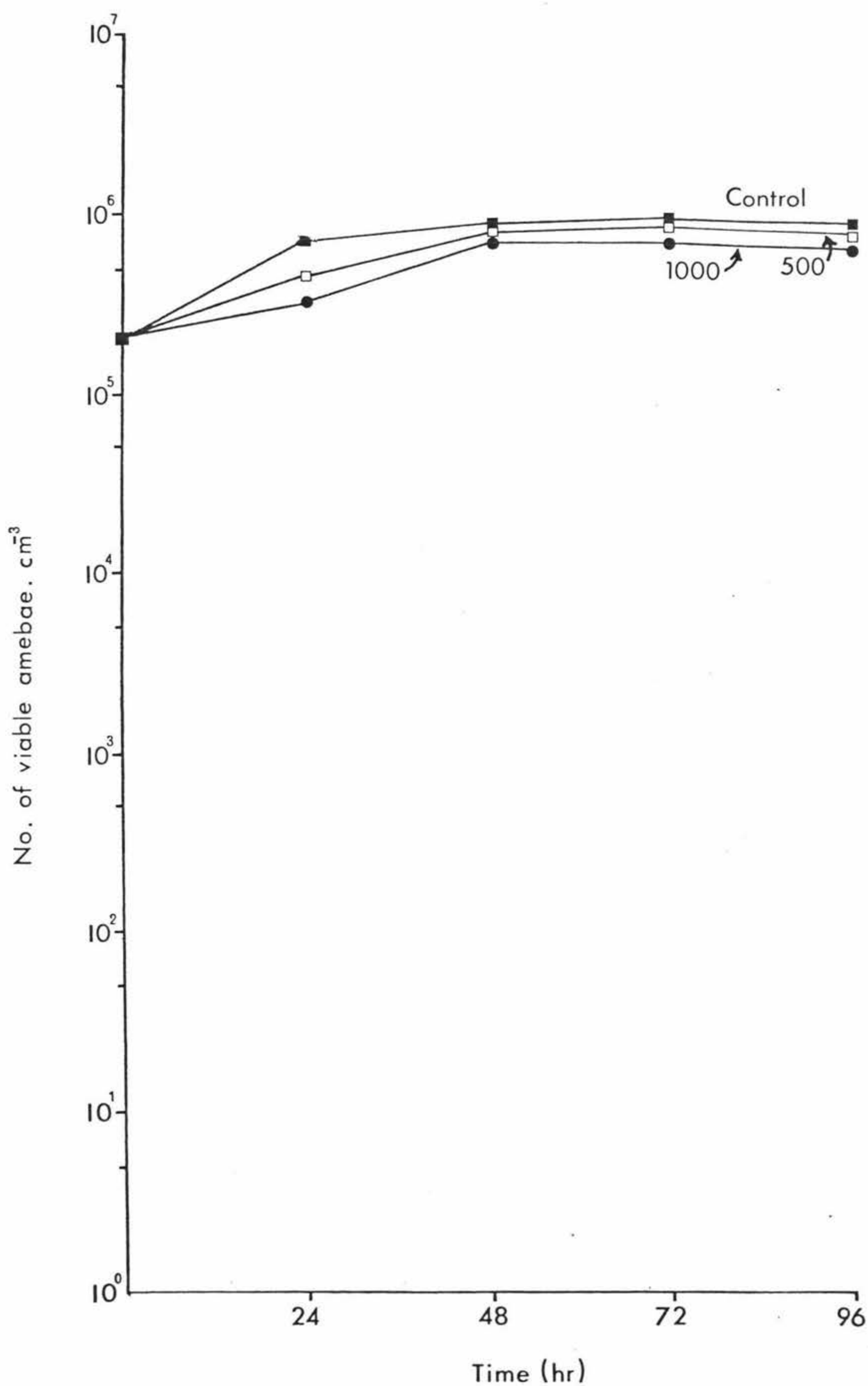


Figure 3 The Effect of Kanamycin on Naegleria fowleri (MsM).

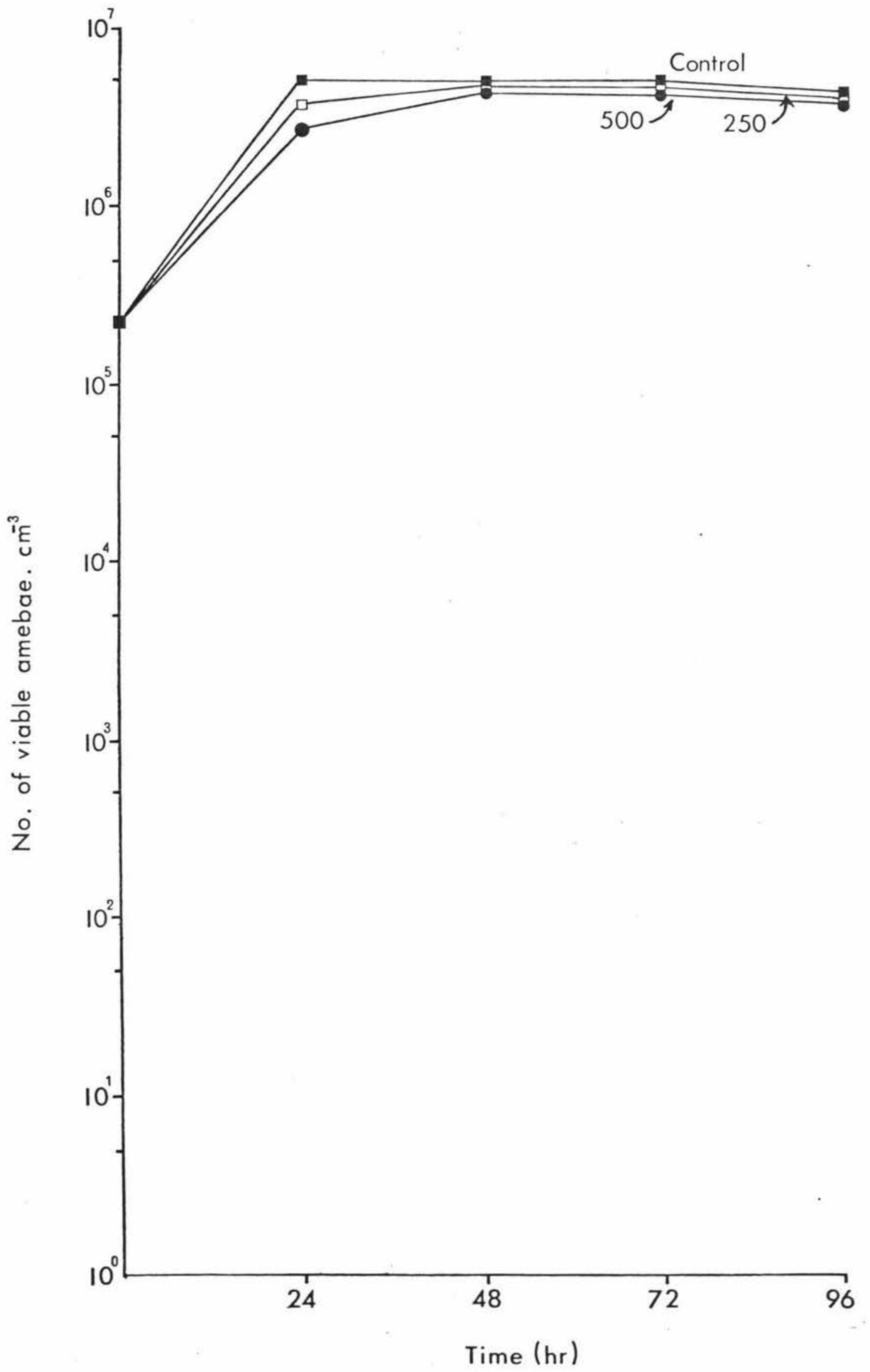


Figure 4 The Effect of Tylosine on Naegleria gruberi (P1200f)

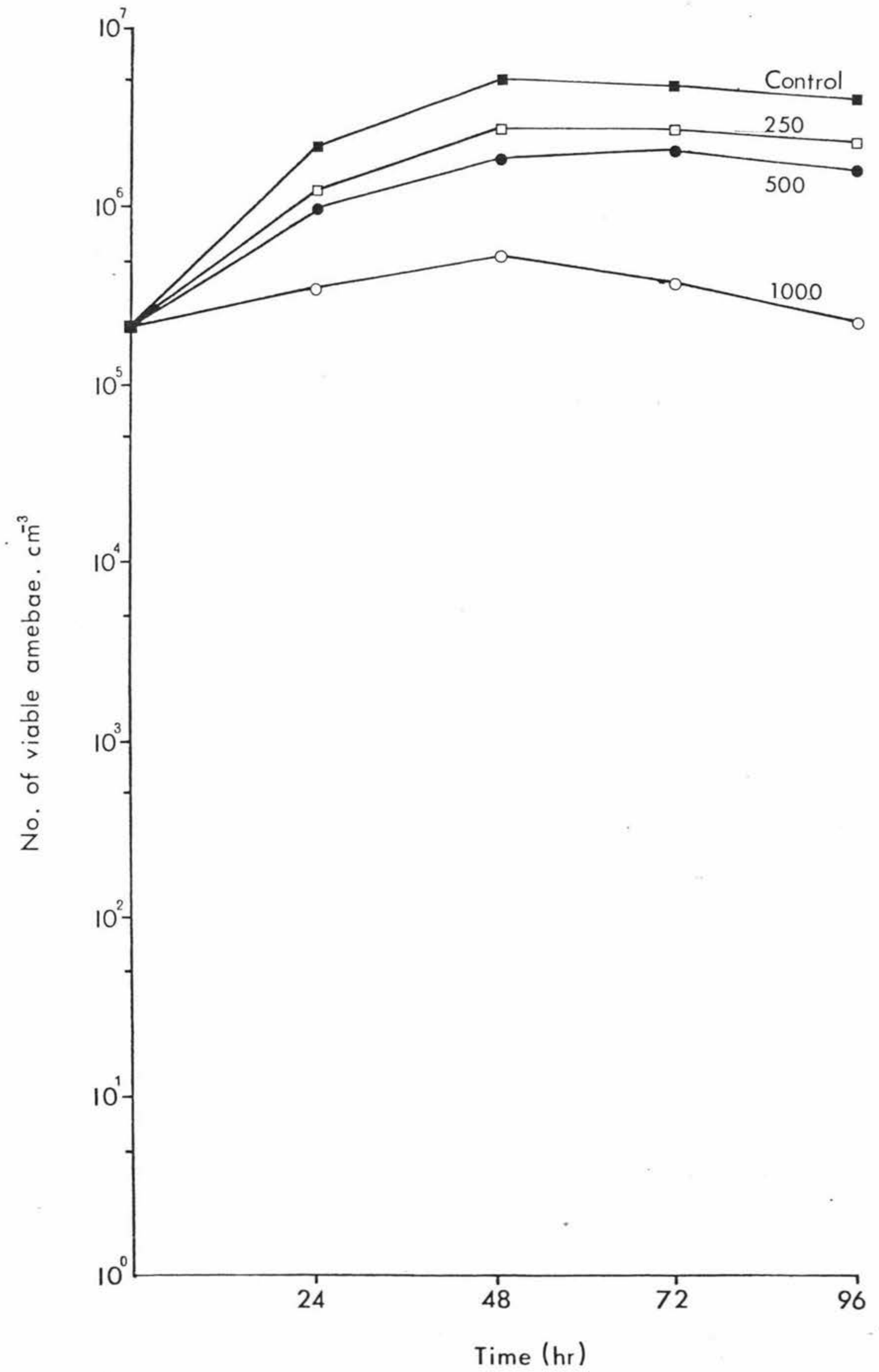
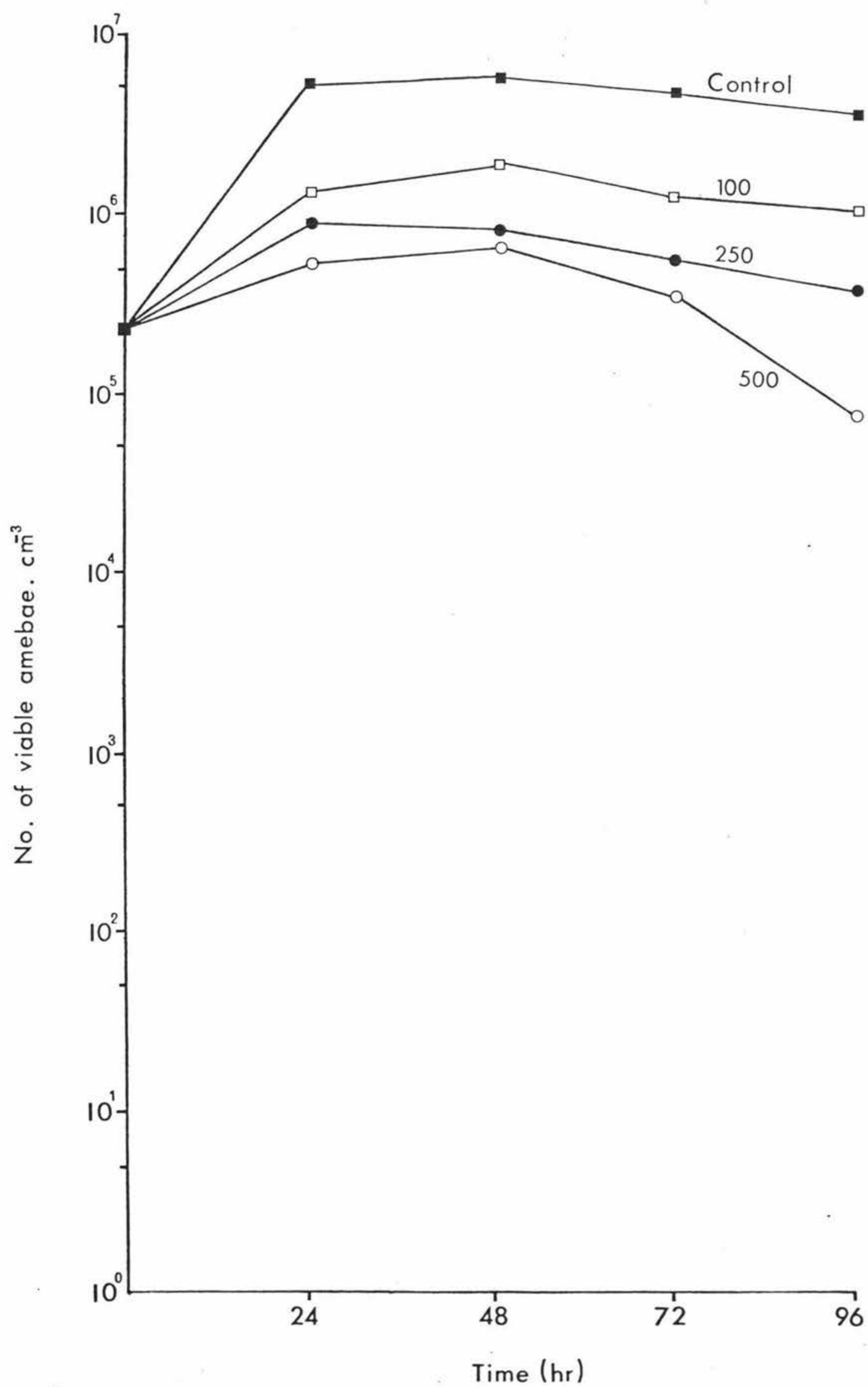


Figure 5 The Effect of Tylosine on Naegleria fowleri (MsM)



4.1.2 Oxytetracycline

Figures 6 and 7 show the results obtained with oxytetracycline. The MAC for both N. gruberi (P1200f) and N. fowleri (MsM) was 500μg.cm^{-3}. The sensitivity to this concentration was greater with the pathogenic N. fowleri (MsM) species, with media sterilization occurring after 72 hours compared to 96 hours with N. gruberi (P1200f). The MIC for both N. gruberi (P1200f) and N. fowleri (MsM) was 100μg.cm^{-3}. At concentrations $\geq$ 100μg.cm^{-3} all amebae appeared small, round and non-motile.

4.1.3 Levamisole

Levamisole was slightly active against N. gruberi (P1200f) at a concentration of 1000μg.cm^{-3}. The amebae, however, showed no obvious signs of drug damage and were limax and motile (Figure 8). Figure 9 shows the effect of levamisole on N. fowleri (MsM). The amebae appeared to be more susceptible to the drug than did the non-pathogen but again, except for the slight drop in numbers, the amebae were all limax and motile.

Figure 6 The Effect of Oxytetracycline on Naegleria gruberi (P1200f)

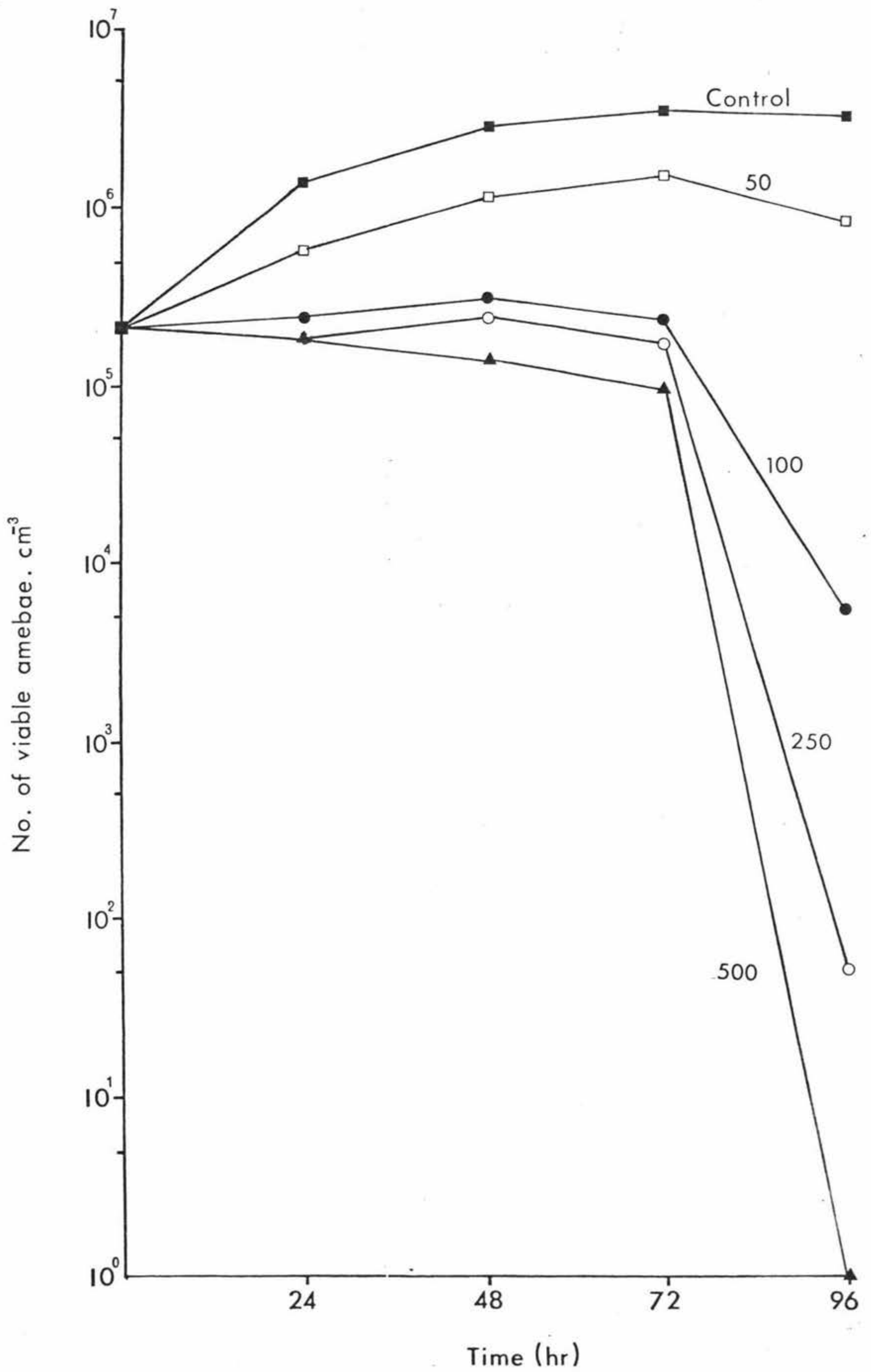


Figure 7 The Effect of Oxytetracycline on Naegleria fowleri (MsM)

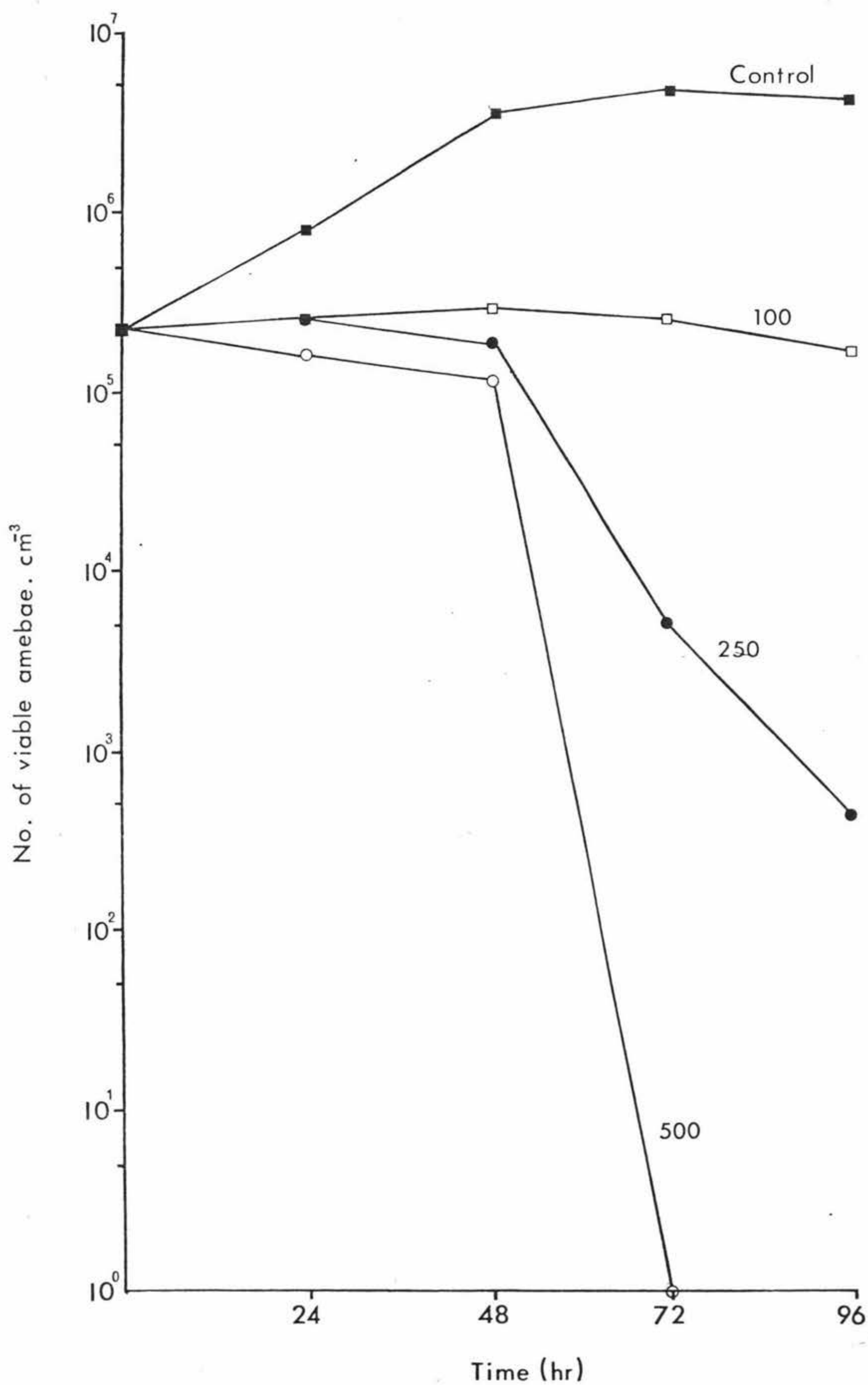


Figure 8 The Effect of Levamisole on <u>Naegleria gruberi</u> (P1200f)

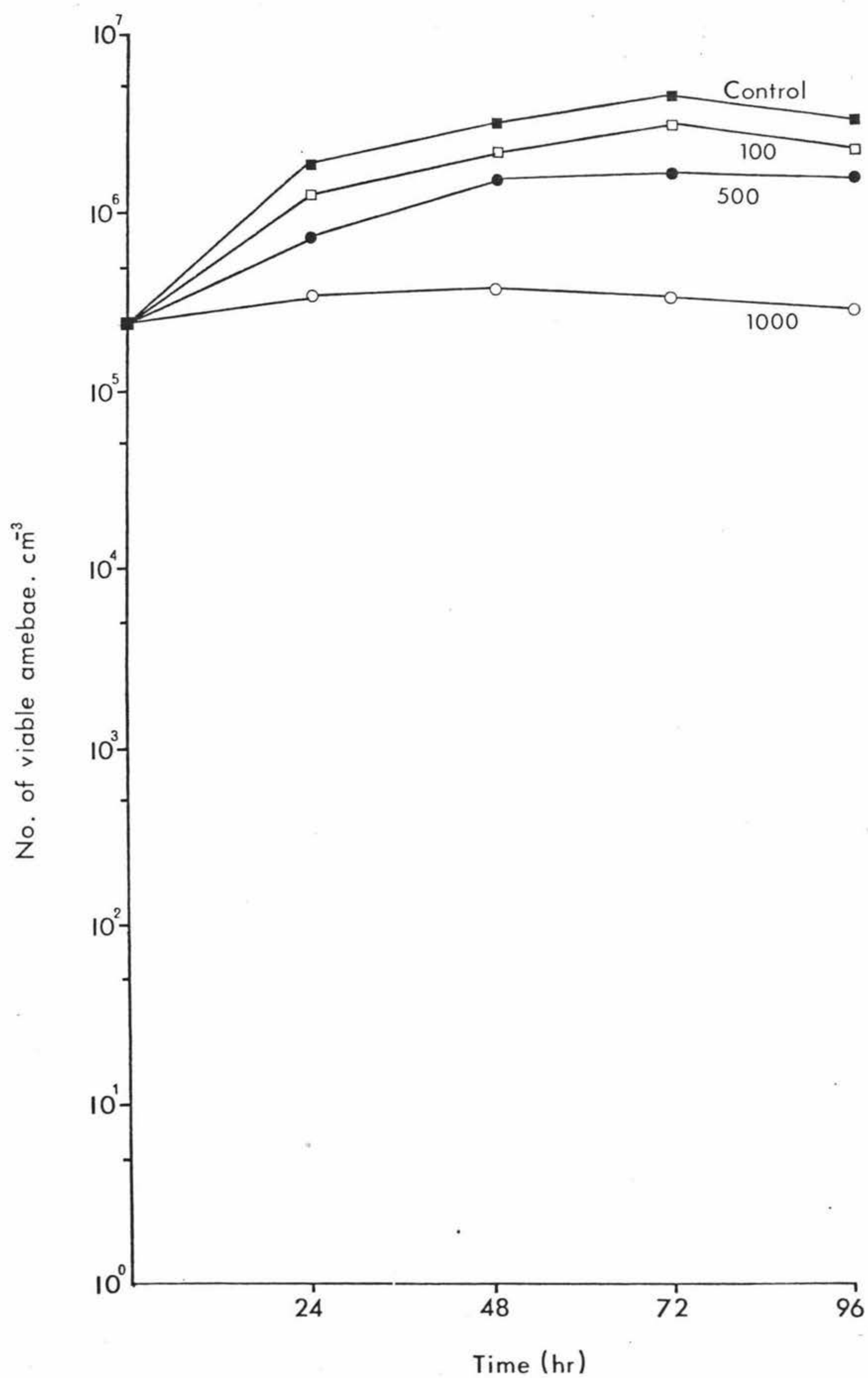
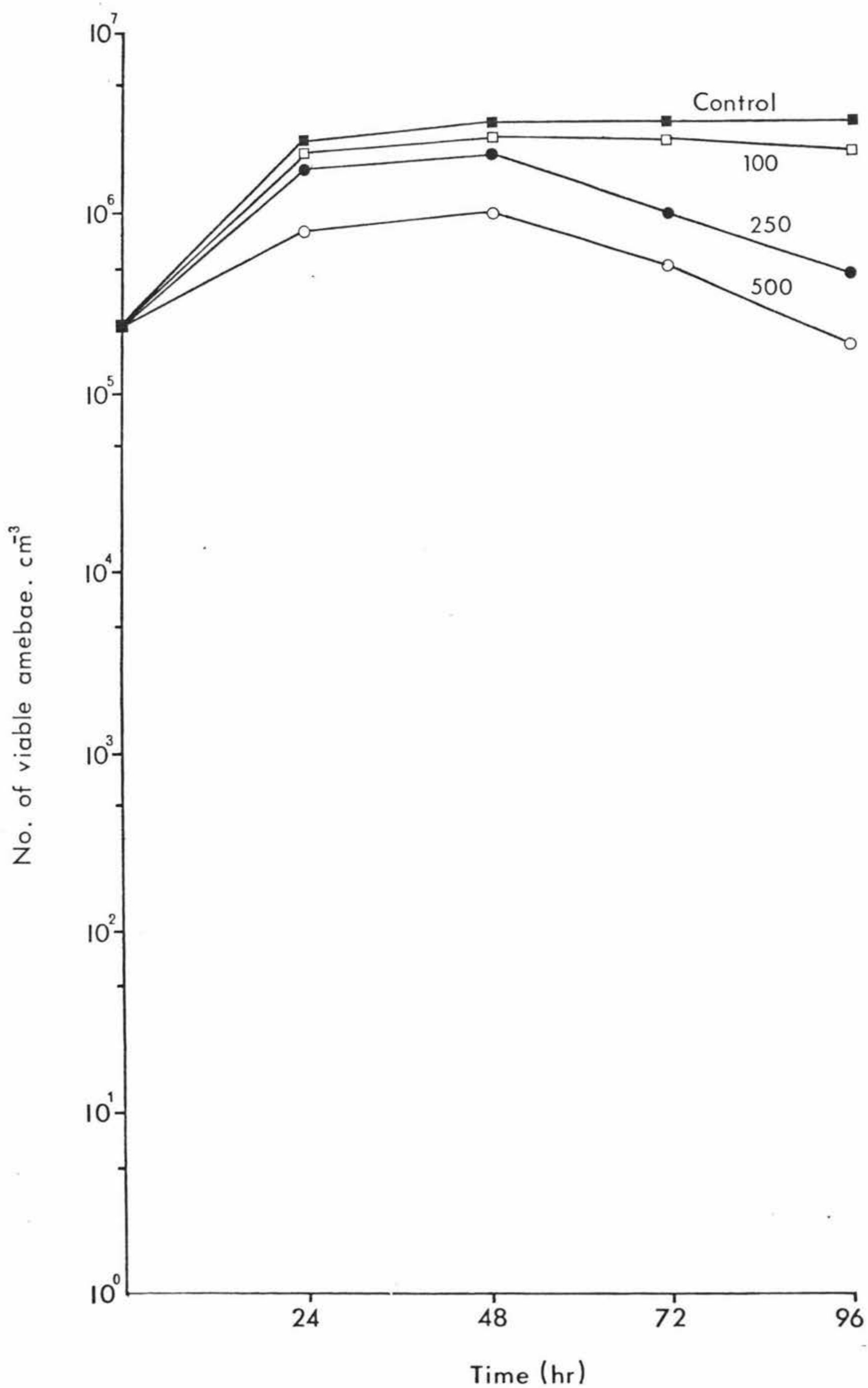


Figure 9 The Effect of Levamisole on Naegleria fowleri (MsM)



4.1.4 5-Fluorocytosine

5-Fluorocytosine was tested for activity against the pathogen, N. fowleri (MsM). The amebae were not affected by the drug at the highest concentration tested, $500\mu g,cm^{-3}$, and were all limax and motile (Figure 10).

4.1.5 Amphotericin B

Figure 11 shows the activity of Amphotericin B. against N. fowleri (MsM). $0.5\mu g.cm^{-3}$ completely sterilized the media in 48 hours and of the concentrations tested, $0.25\mu g.cm^{-3}$ was the MIC. At drug concentrations $\geqslant 0.125\mu g.cm^{-3}$, which was the lowest tested, all amebae appeared rounded, vacuolated and non-motile after 24 hours in the test medium.

Figure 10 The Effect of 5-fluorocytosine on <u>Naegleria fowleri</u> (MsM)

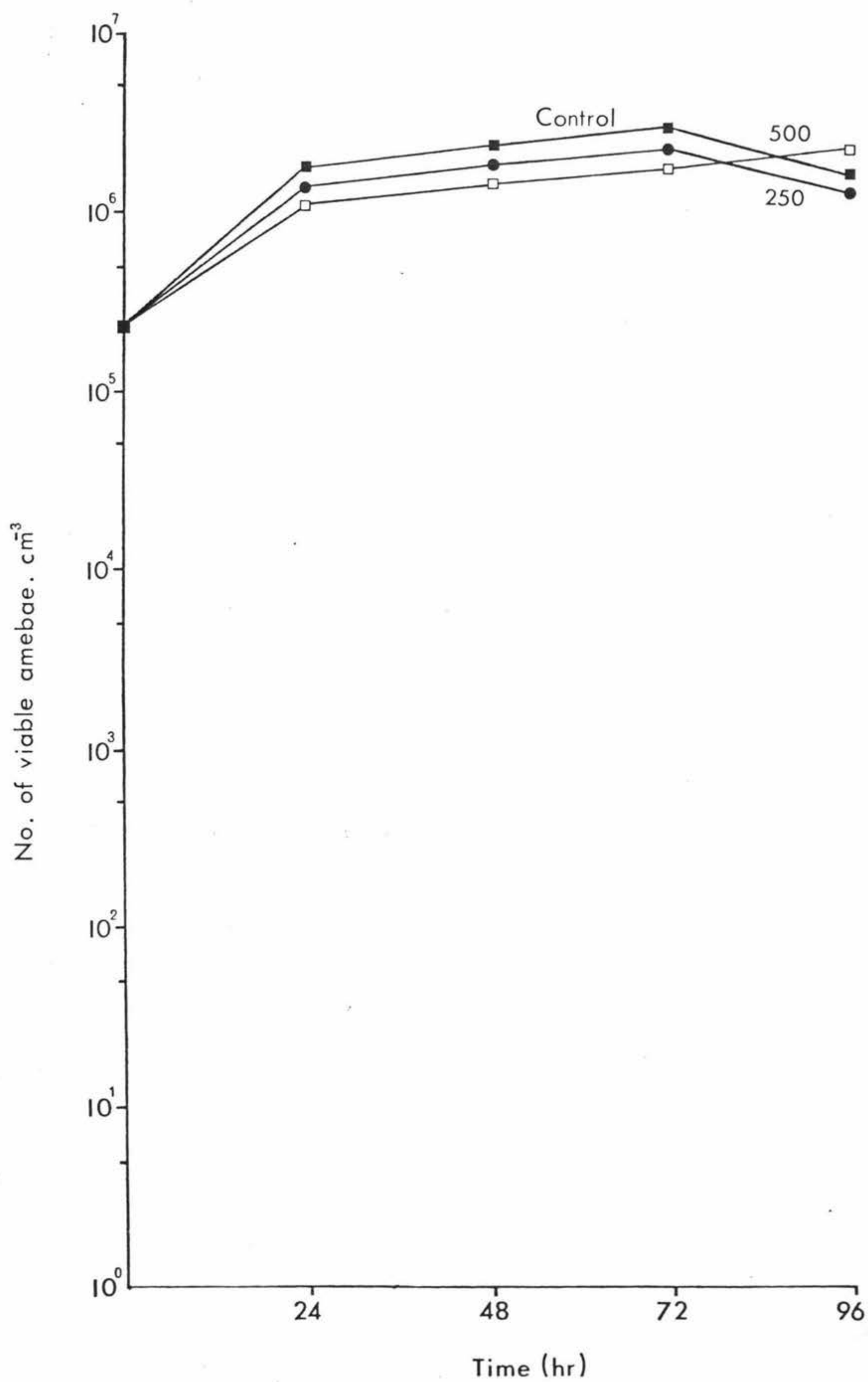
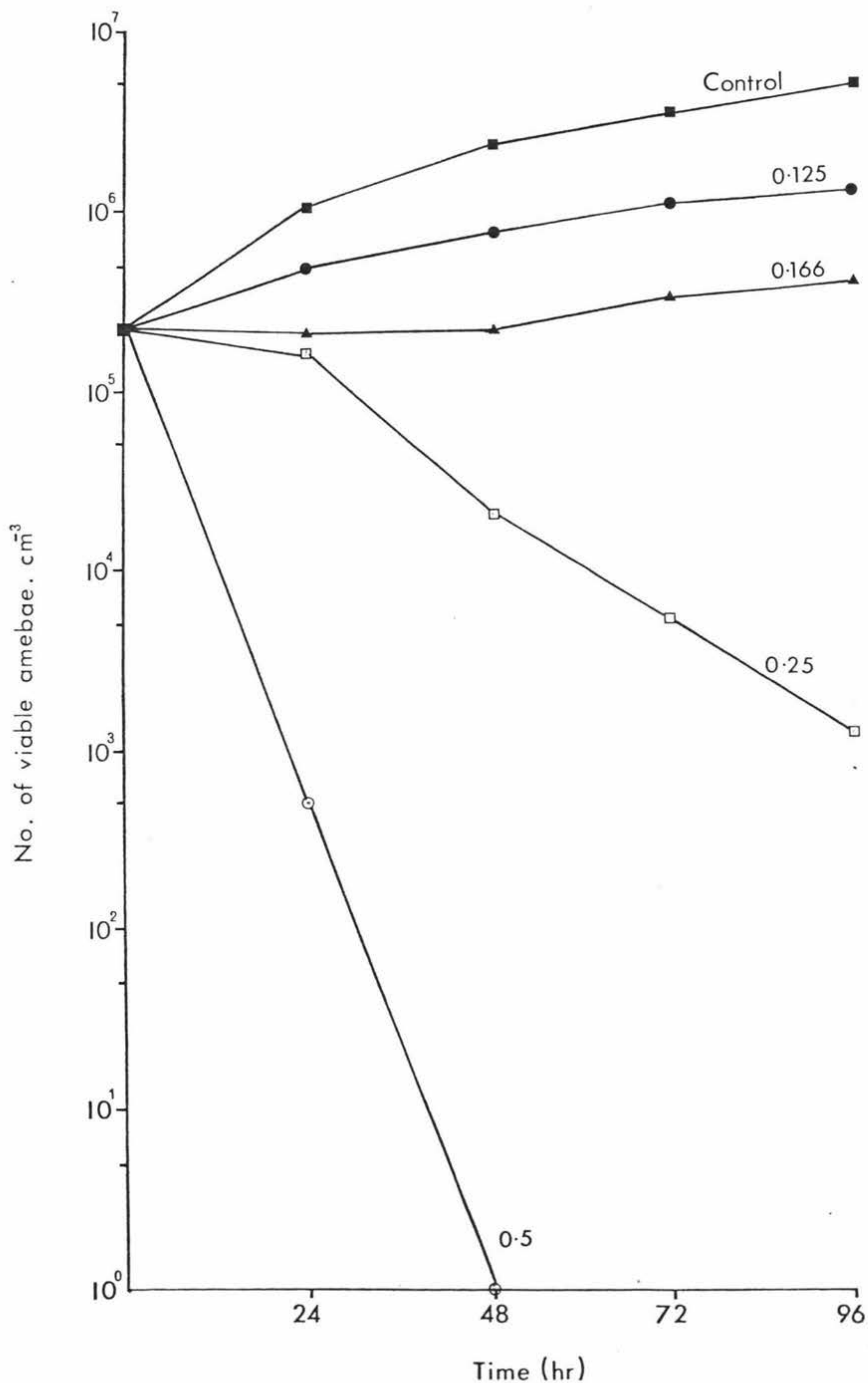


Figure 11 The Effect of Amphotericin B on Naegleria fowleri (MsM)



4.2 In Vitro Axenic Drug Testing of Acanthamoeba spp.

Five drugs were screened for activity against A. castellanii (1501) and A. culbertsoni (A-1). The results are shown in Figures 12-20 with the MIC and MAC defined as in section 4.1.

4.2.1 Kanamycin, Tylosine, Oxytetracycline

Figures 12 and 13 show the activity of kanamycin sulphate on A. castellanii (1501) and A. culbertsoni (A-1). The numbers of amebae of both species were not affected by the drug although at concentrations $\geq$ 500μg.cm^{-3}, both A. castellanii (1501) and A. culbertsoni (A-1), showed slight drug damage becoming rounded and more vacuolated.

Tylosine showed a slight inhibitory effect on both A. castellanii (1501) and A. culbertsoni (A-1)(Figures 14 and 15). The non-pathogenic A. castellanii (1501) showed no effects of drug damage other than a drop in viable amebae at 1000μg.cm^{-3}, whereas the pathogenic A. culbertsoni (A-1) became rounded, vacuolated and somewhat smaller in size at concentrations $\geq$ 500μg.cm^{-3}.

Figures 16 and 17 show the effects of oxytetracycline on the amebae. A. castellanii (1501) appeared to be more sensitive to the drug with a MIC of 1000μg.cm^{-3}. Other than this drop in numbers, the amebae did not show any obvious signs of drug action. A. culbertsoni (A-1) showed only a slight decrease in numbers and at concentrations $\geq$ 250μg.cm^{-3} the amebae became more vacuolated and rounded, with an absence of acanthapodia although their size remained normal.

Figure 12 The Effect of Kanamycin on Acanthamoeba castellanii (1501)

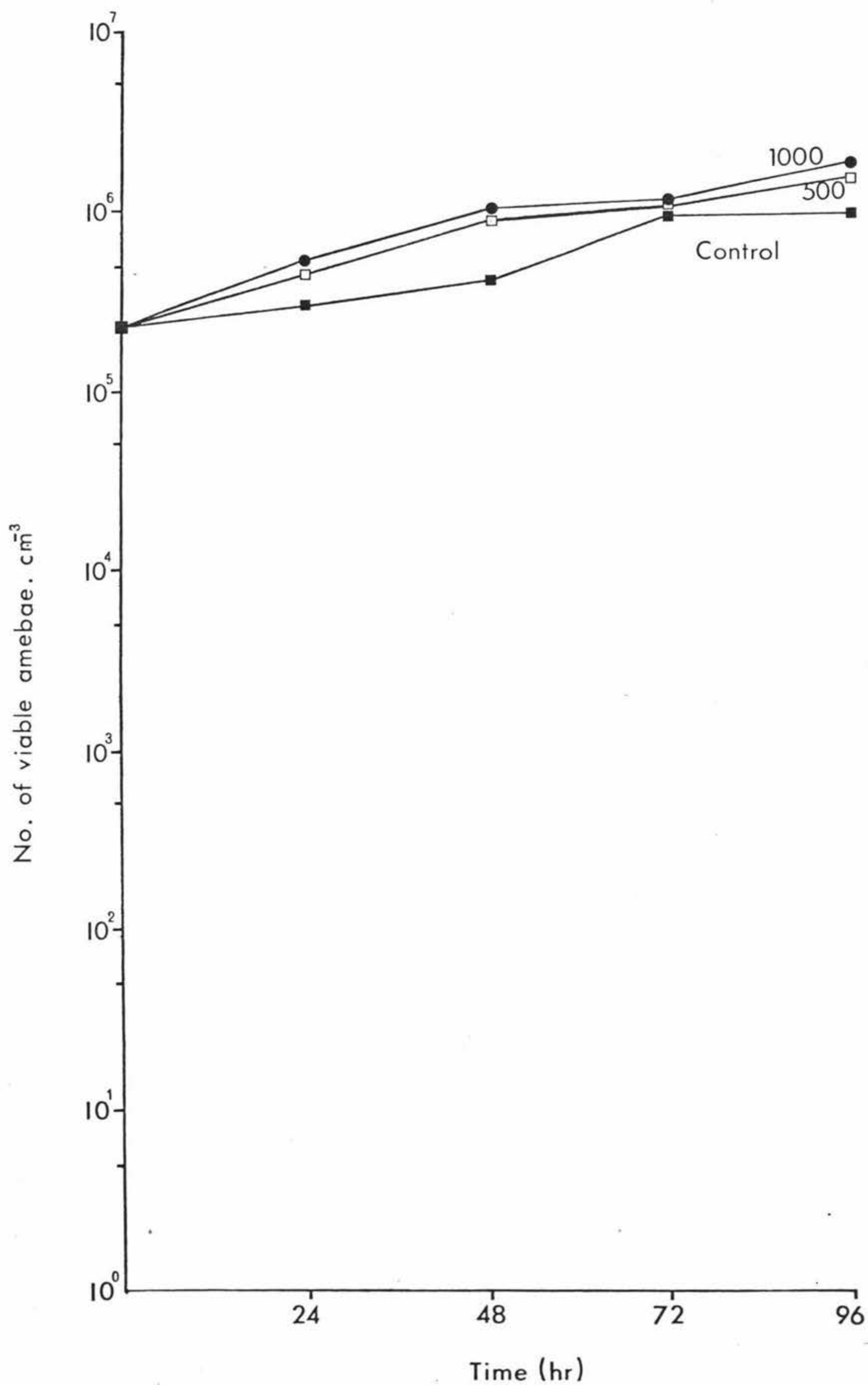


Figure 13 The Effect of Kanamycin on Acanthamoeba culbertsoni (A-1)

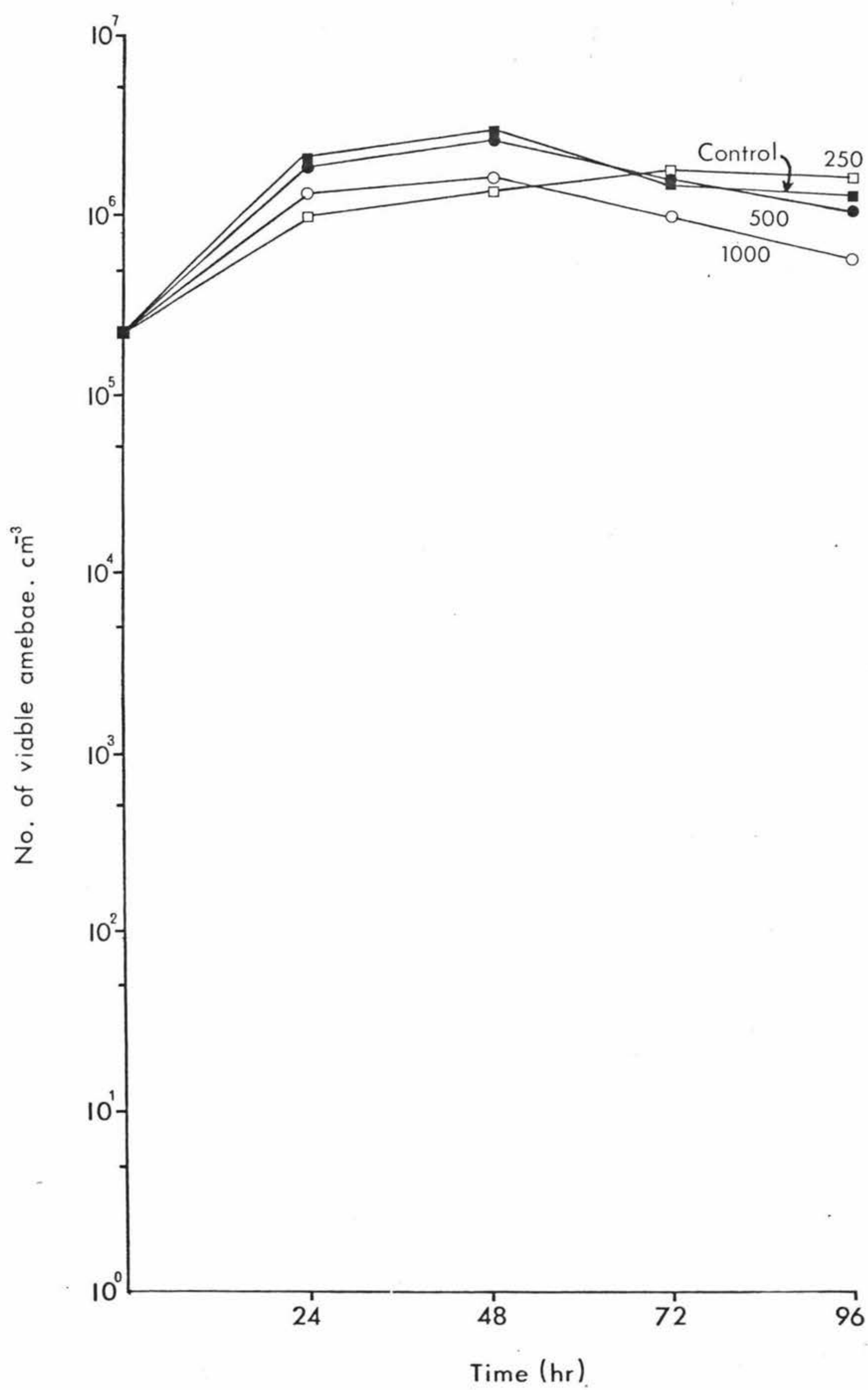


Figure 14 The Effect of Tylosine on Acanthamoeba castellanii (1501)

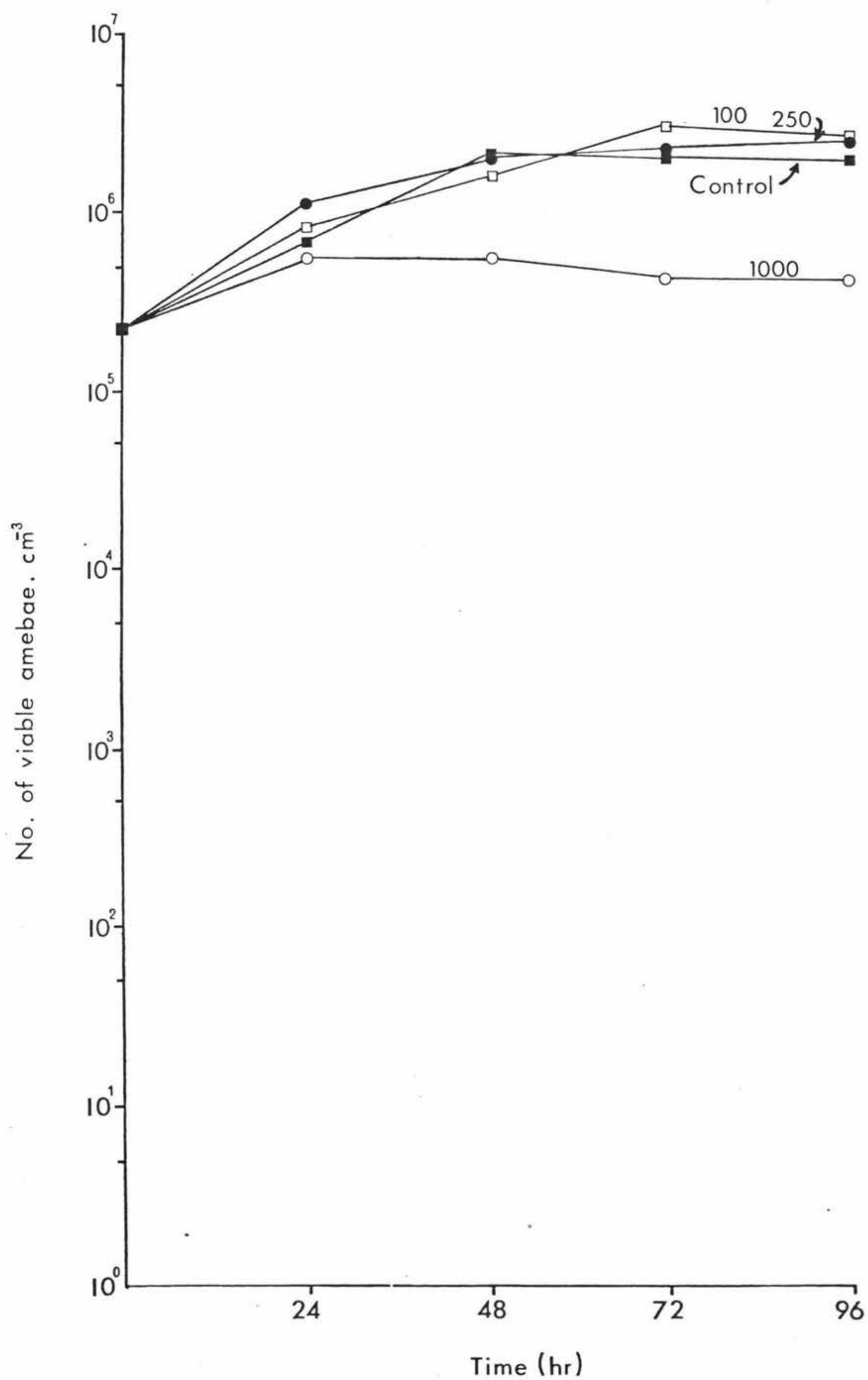


Figure 15 The Effect of Tylosine on Acanthamoeba culbertsoni (A-1)

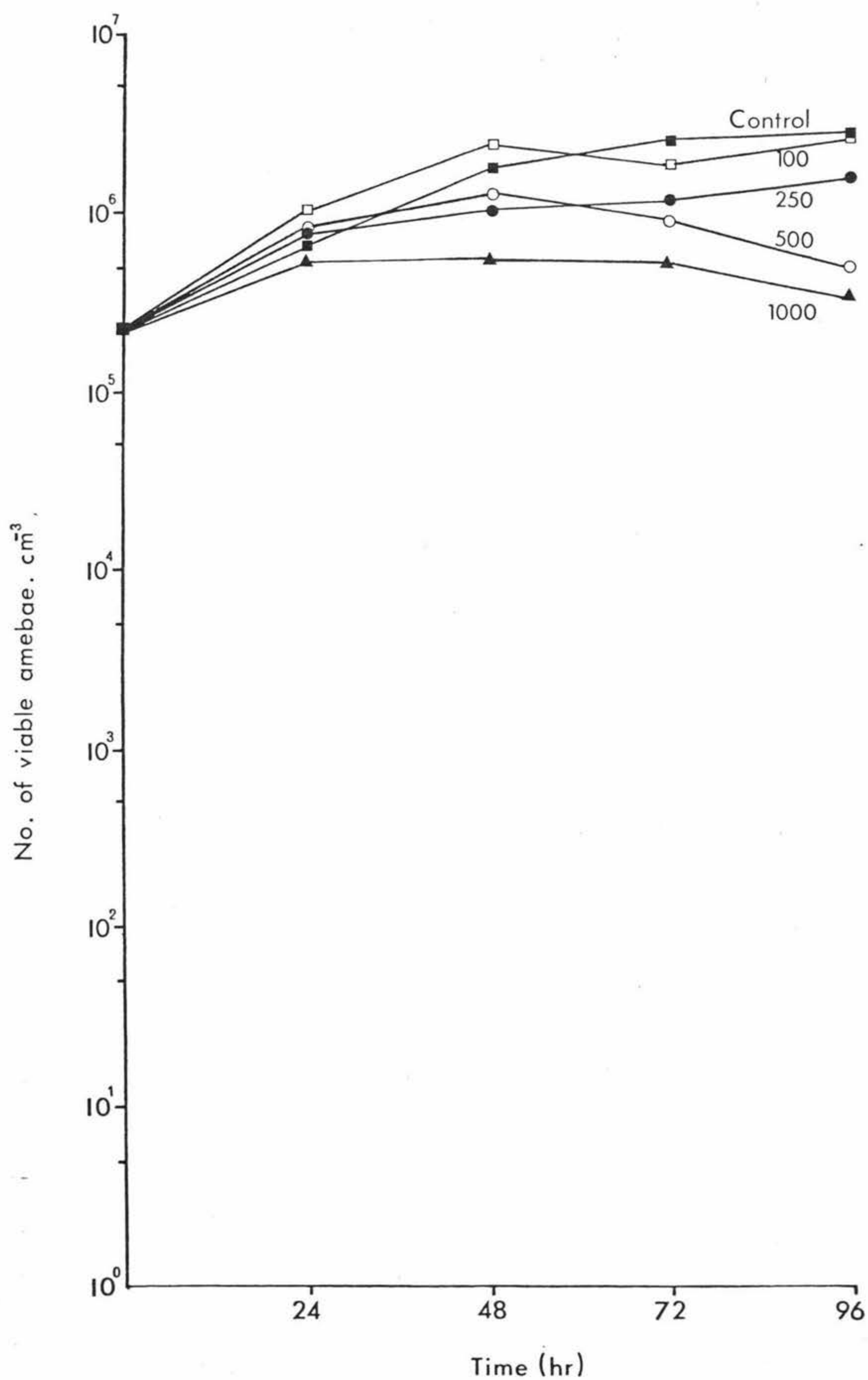


Figure 16 The Effect of Oxytetracycline on Acanthamoeba castellanii (1501

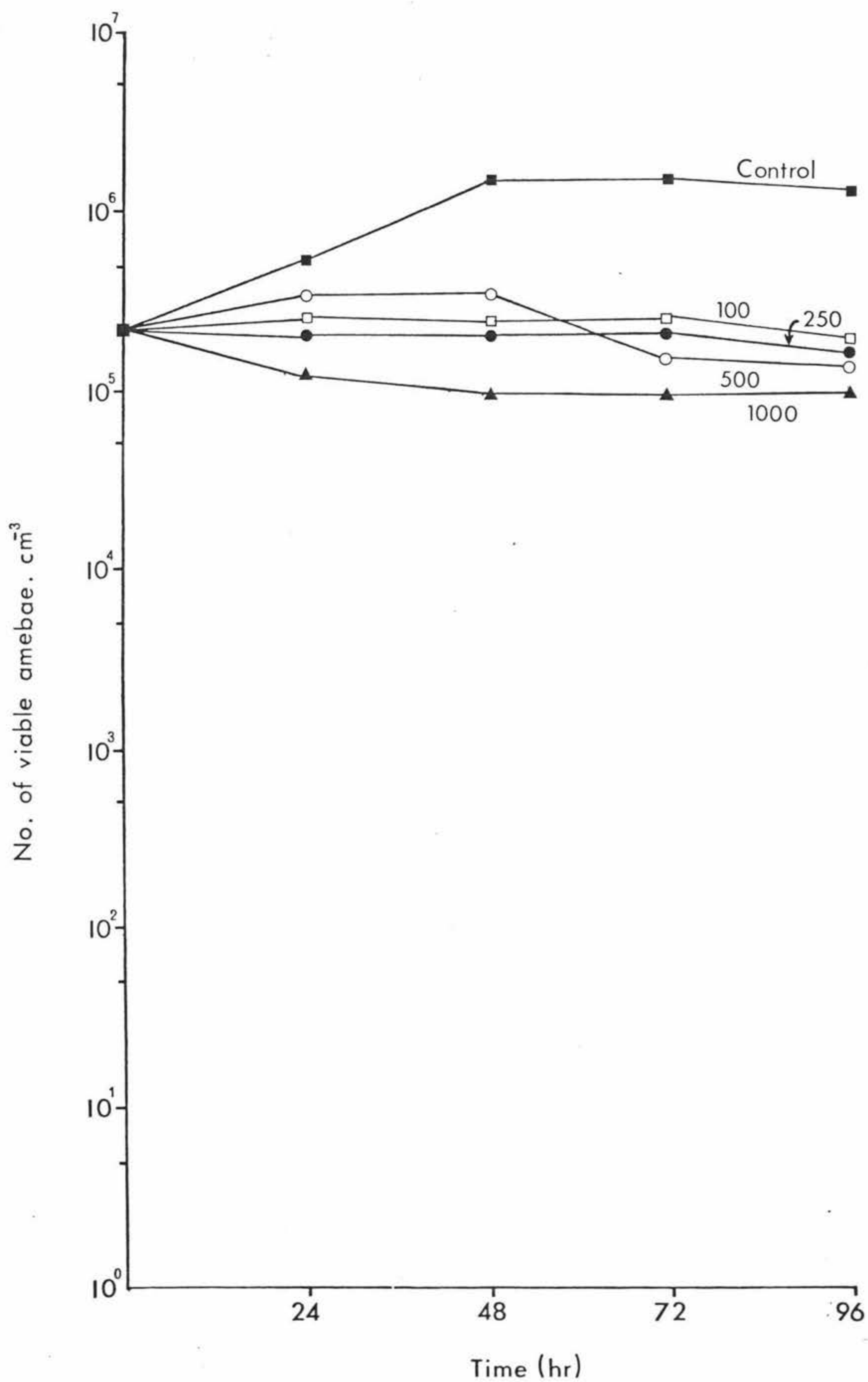
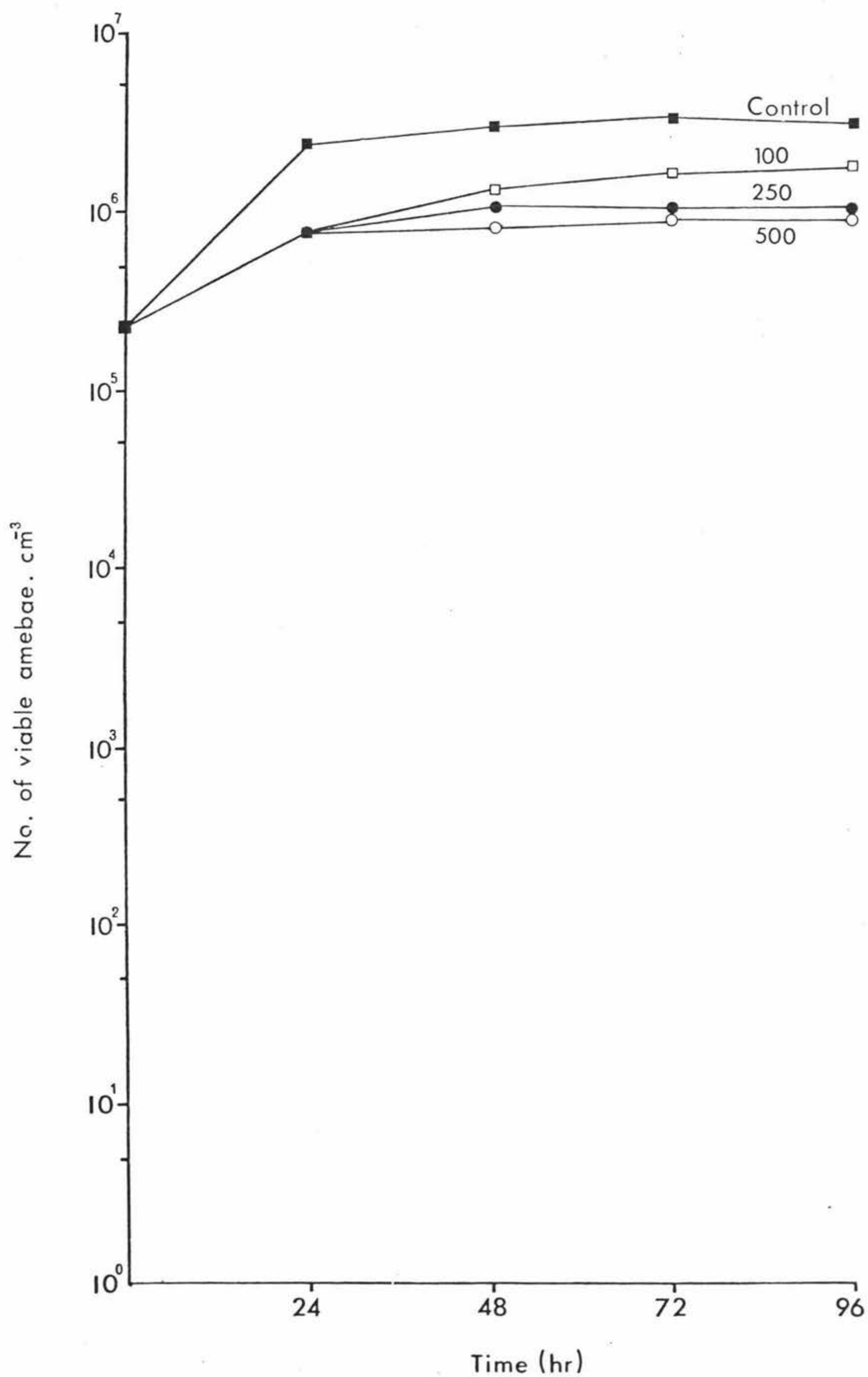


Figure 17 The Effect of Oxytetracycline on Acanthamoeba culbertsoni (A-1)



4.2.2 Levamisole

Figure 18 shows the results of screening A. castellanii (1501) against levamisole. An inhibitory effect was seen at all concentrations tested and at concentrations $\geqslant 250\mu g.cm^{-3}$, the amebae showed signs of drug toxicity. They became rounder, smaller and more vacuolated than normal. The drug has a MIC of $250\mu g.cm^{-3}$.

However, levamizole failed to show the same inhibitory effect on A. culbertsoni (A-1) (Figure 19). The amebae appeared normal even at the highest concentration tested ($500\mu g.cm^{-3}$).

4.2.3 5-Fluorocytosine

5-Fluorocytosine was tested at three concentrations against A. culbertsoni (A-1). Figure 20 shows that the drug had an inhibitory effect on the amebae at all concentrations tested.

Figure 18 The Effect of Levamisole on Acanthamoeba castellanii (1501)

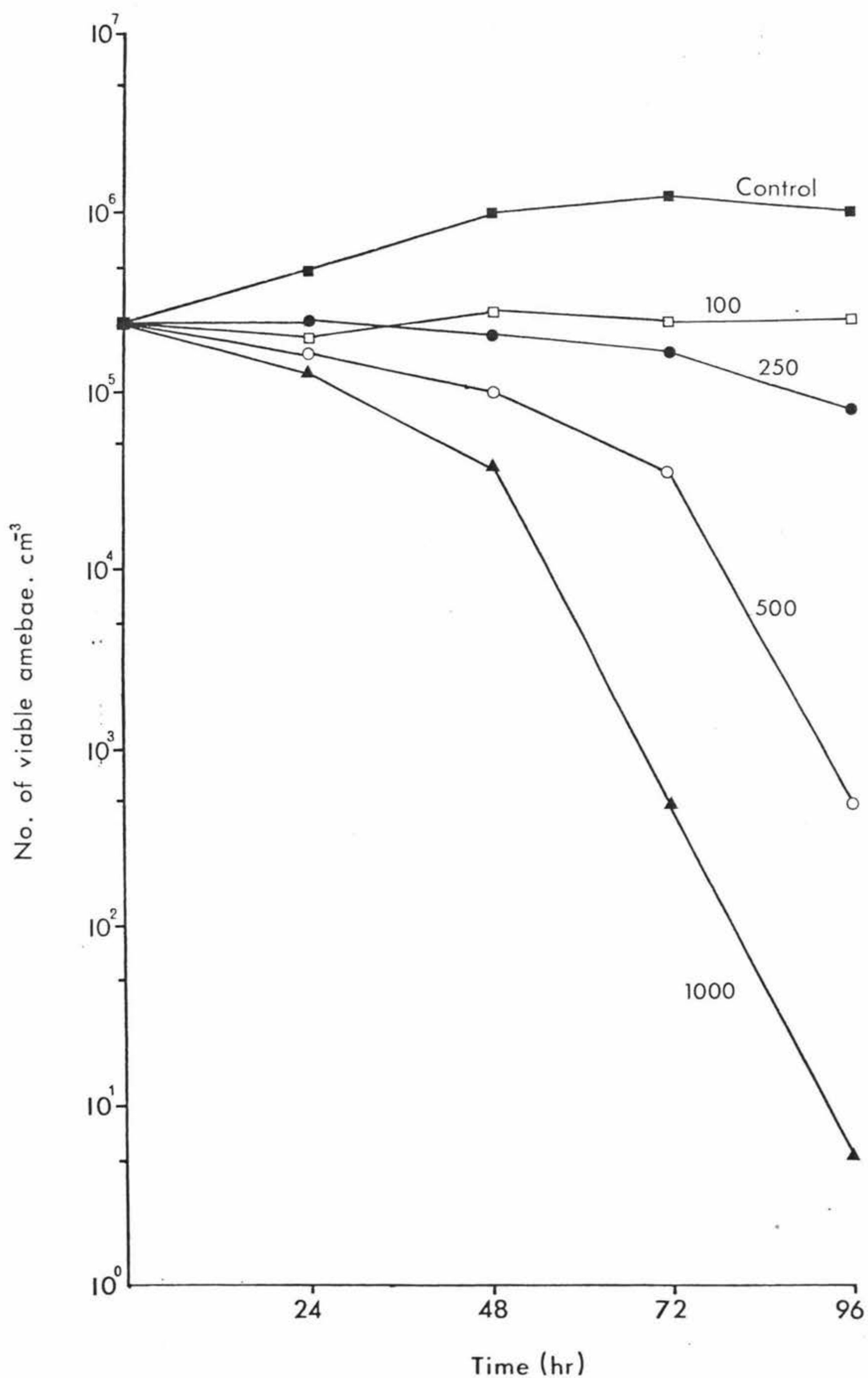


Figure 19 The Effect of Levamisole on Acanthamoeba culbertsoni (A-1)

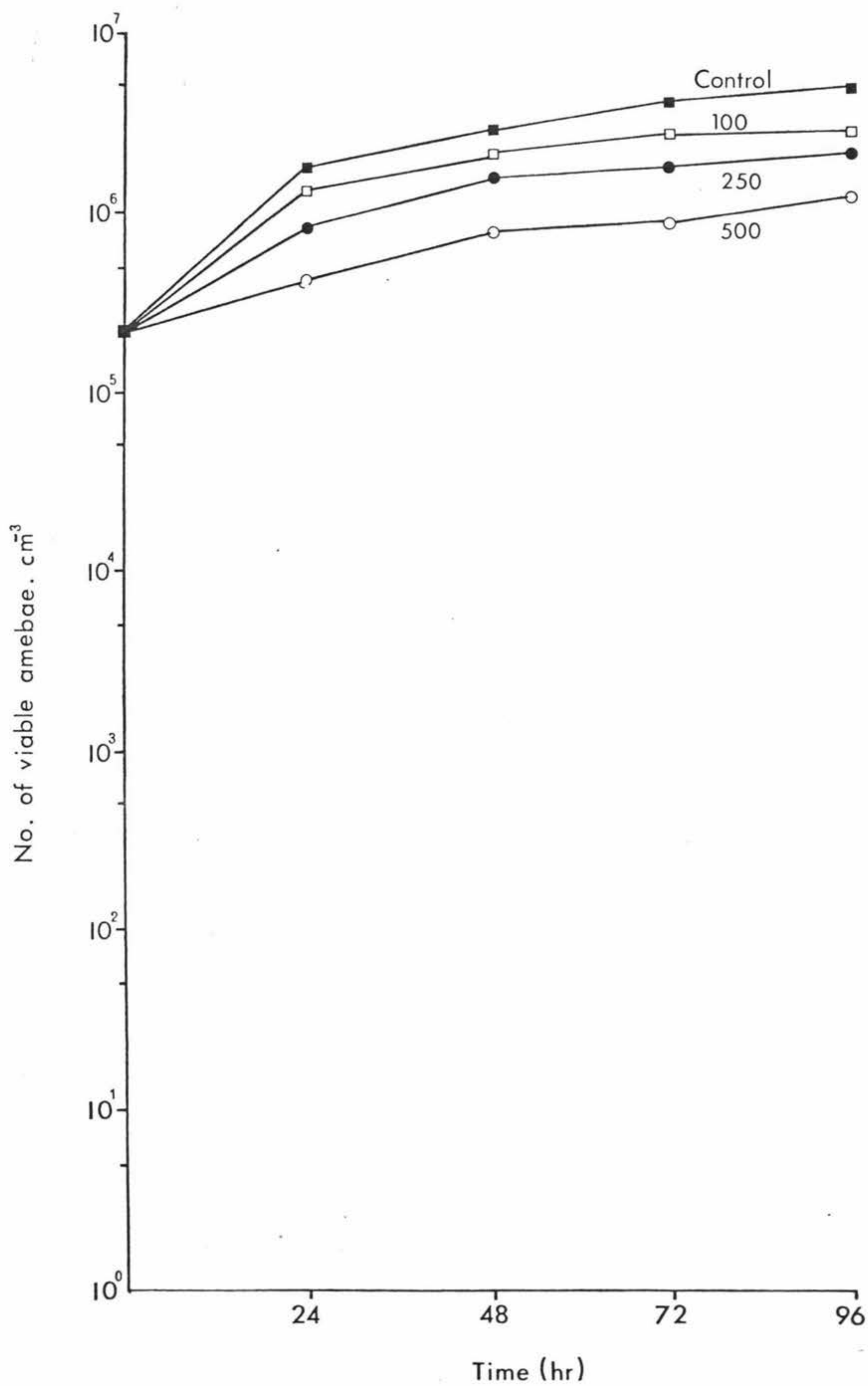
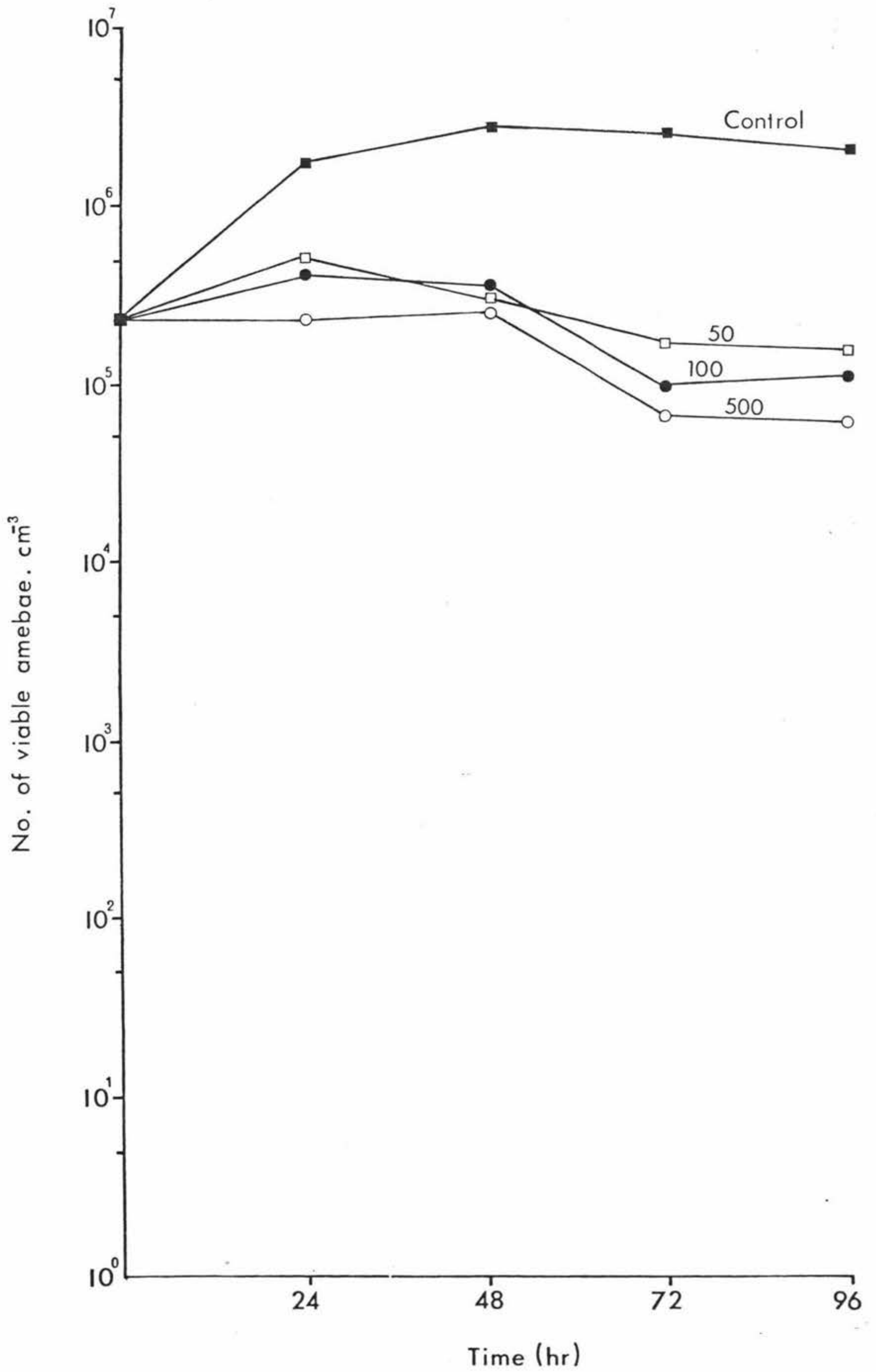


Figure 20 The Effect of 5-Fluorocytosine on Acanthamoeba culbertsoni (A-1)



4.3 In Vitro Axenic Testing of Various Aged Cultures of N. gruberi (P1200f) and A. castellanii (1501) against Levamisole

4.3.1 N. gruberi (P1200f)

Figures 21 and 22 along with Figure 8 give the results of testing various aged cultures of N. gruberi (P1200f) against levamisole. The results are all consistent with each other and it appears as if the age of the parent culture does not affect the activity of the drug in any way.

4.3.2 A. castellanii (1501)

Figures 23 and 24 along with Figure 18 show the results of testing various aged cultures of A. castellanii (1501) against levamisole. The results show (as in the case of N. gruberi (P1200f) that the age of the parent culture does not greatly affect the activity of the drug.

Figure 21 The Effect of Levamisole on a 48 Hour Culture of *Naegleria gruberi* (P1200f)

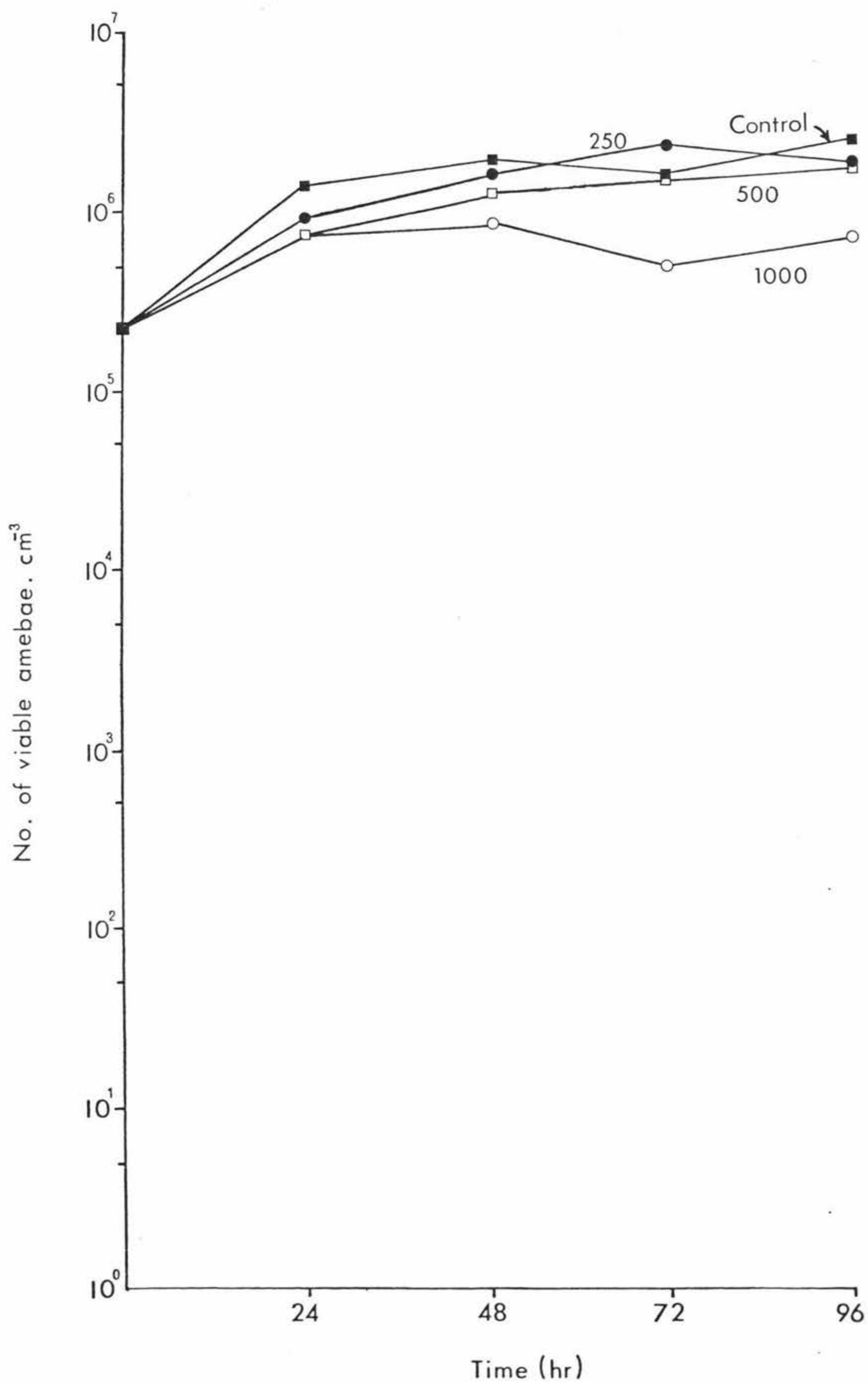


Figure 22 The Effect of Levamisole on a 96 Hour Culture of *Naegleria gruberi* (P1200f)

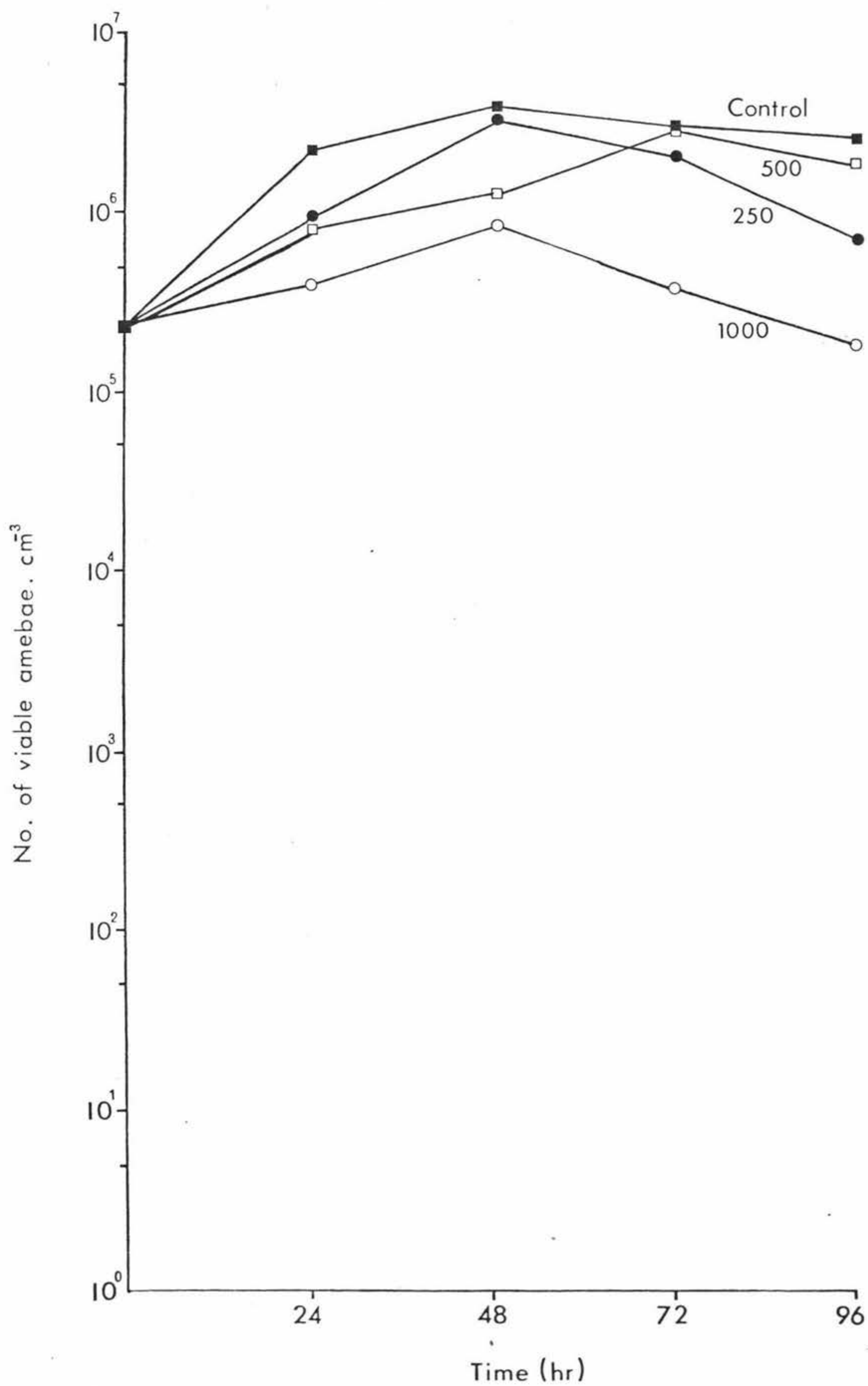


Figure 23 The Effect of Levamisole on a 48 Hour Culture of *Acanthamoeba castellani* (1501)

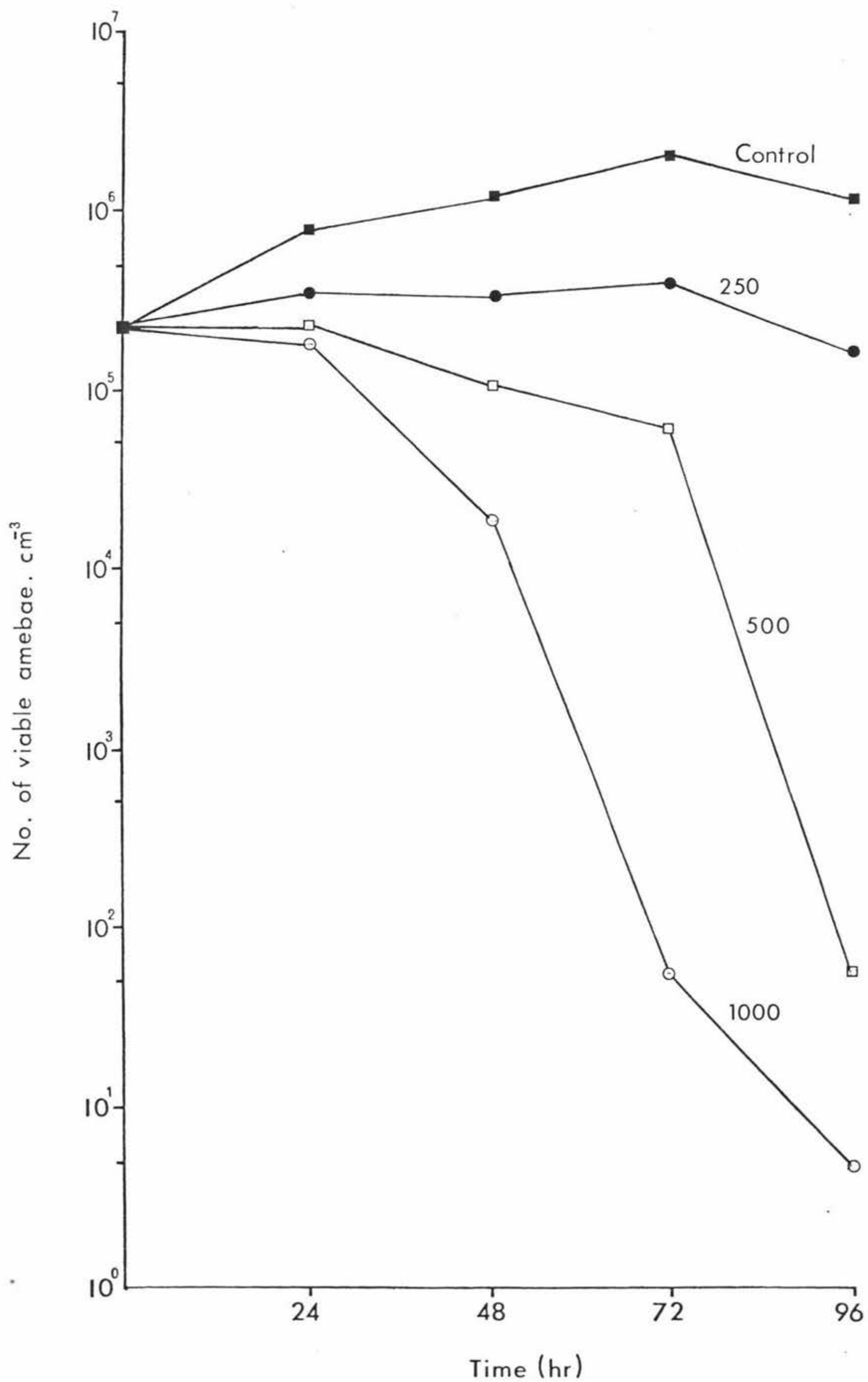
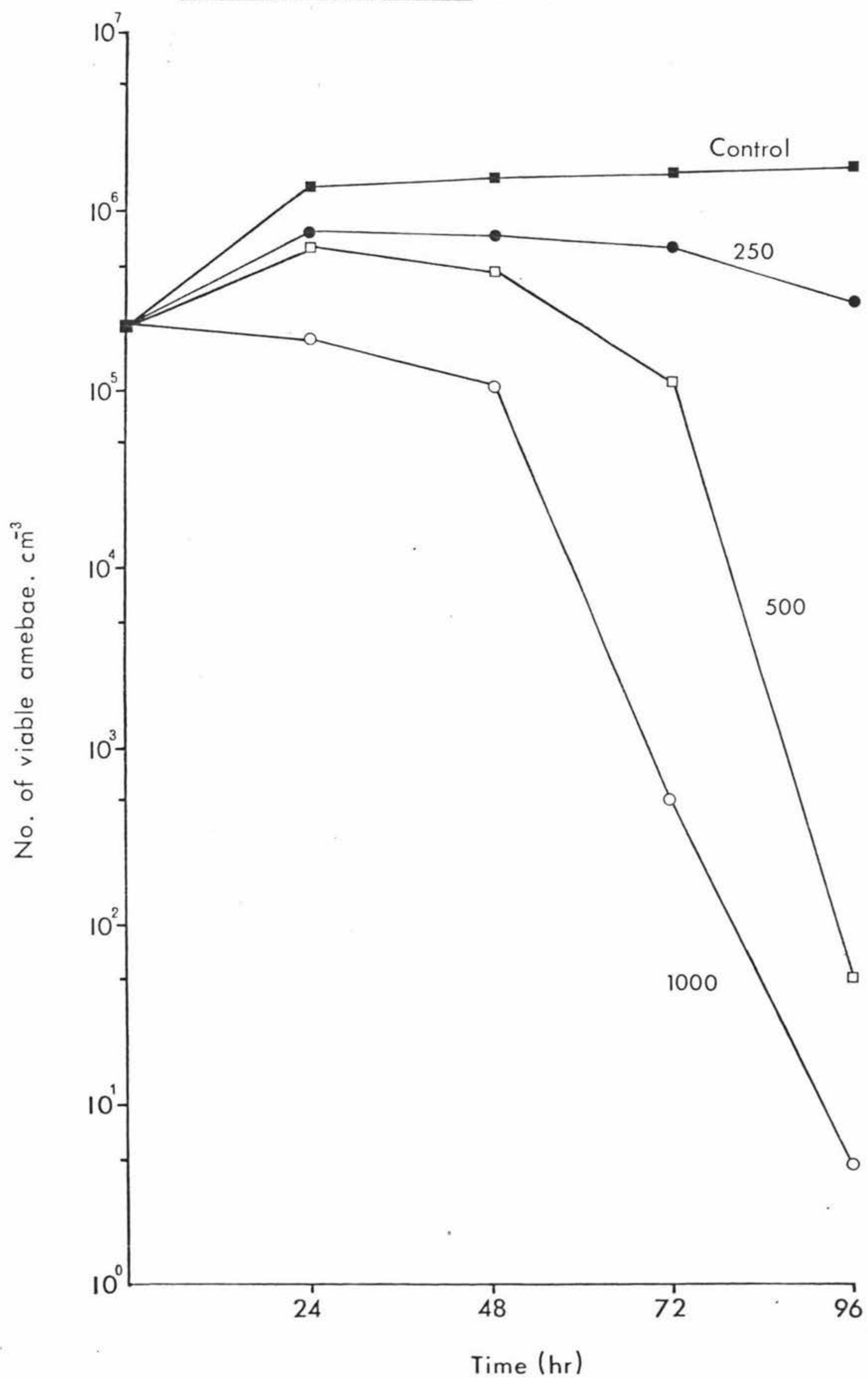


Figure 24 The Effect of Levamisole on a 96 Hour Culture of *Acanthamoeba castellani* (1501)



4.4 The Testing of Drug Combinations against *Naegleria fowleri* (MsM) in Axenic Culture

4.4.1 Amphotericin B and 5-Fluorocytosine

0.5μg.cm^{-3} Amphotericin B completely sterilized the medium in 48 hours (Figure 11) when tested alone in axenic culture with *N. fowleri* (MsM). 5-Fluorocytosine had no activity against *N. fowleri* (MsM).

When added together in combination there was no significant difference to the results when amphotericin B is used alone. 0.25μg.cm^{-3} amphotericin B when used together with 250μg.cm^{-3} 5-fluorocytosine showed the same effect on the decrease in amebae numbers as 0.25μg.cm^{-3} amphotericin did alone (Figure 25).

Figure 25 The Effect of Amphotericin B and 5-Fluorocytosine Alone and in Combination on *Naegleria fowleri* (MsM)

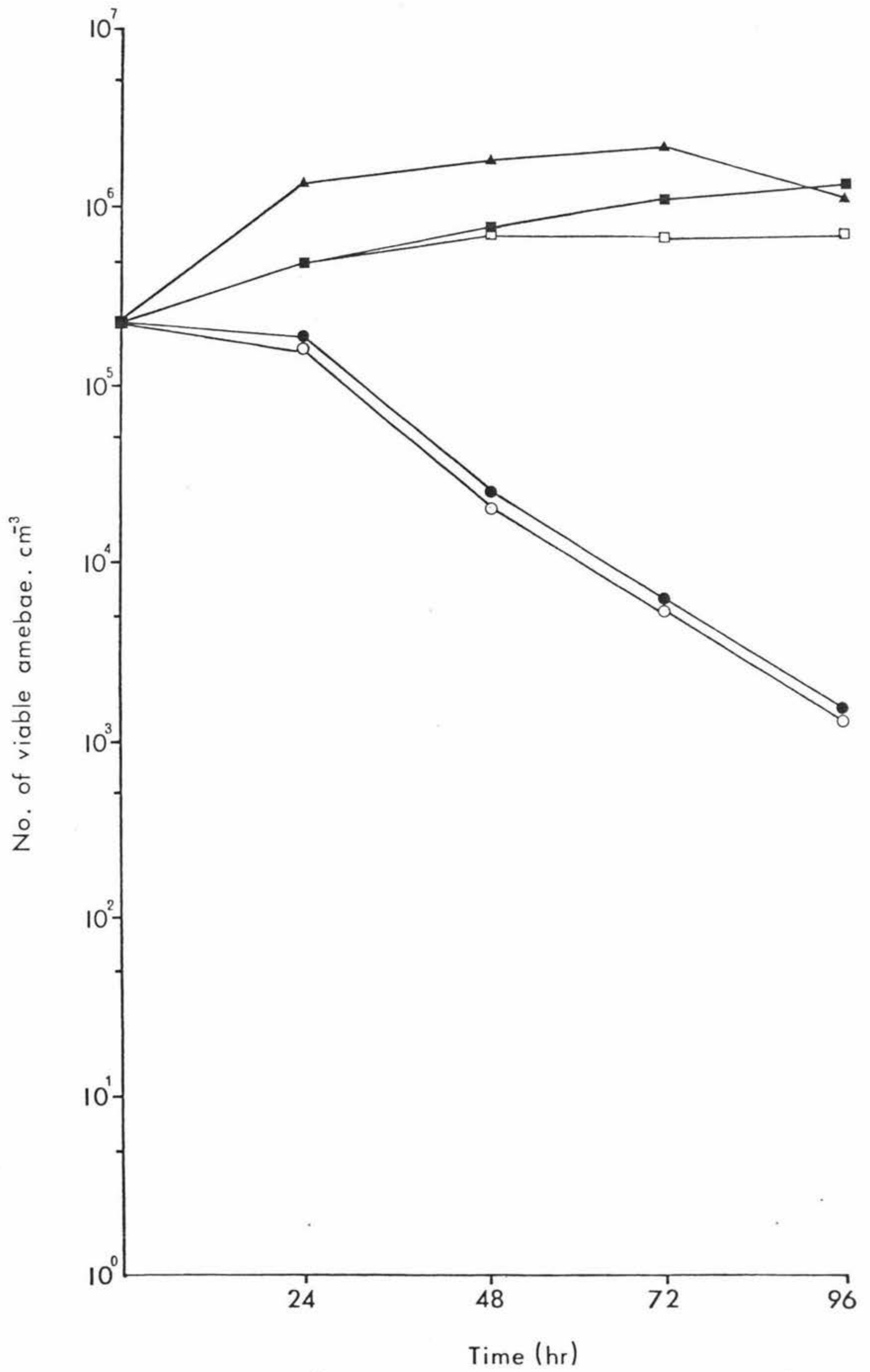
■ = $0.125\mu g.cm^{-3}$ Amphotericin B

○ = $0.25\mu g.cm^{-3}$ Amphotericin B

▲ = $250\mu g.cm^{-3}$ 5-Fluorocytosine

□ = $0.125\mu g.cm^{-3}$ Amphotericin B + $250\mu g.cm^{-3}$ 5-Fluorocytosine

● = $0.25\mu g.cm^{-3}$ Amphotericin B + $250\mu g.cm^{-3}$ 5-Fluorocytosine



10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
No. of viable amebae . cm^{-3}
24
48
72
96
Time (hr)

4.4.2 Amphotericin B and Oxytetracycline

Amphotericin B at a concentration of $0.5\mu g.cm^{-3}$ sterilizes the medium in 48 hours (Figure 11). Oxytetracycline showed activity against N. fowleri (MsM) at concentrations $\geqslant 100\mu g.cm^{-3}$ and at $500\mu g.cm^{-3}$ sterilized the medium in 72 hours. The results of experiments using these two drugs together are shown in Figures 26 and 27.

$0.125\mu g.cm^{-3}$ amphotericin B is ineffective in reducing amebae numbers on its own, but combined with $200\mu g.cm^{-3}$ oxytetracycline, it becomes amebicidal in 72 hours (Figure 26).

Figure 27 shows the results when $0.25\mu g.cm^{-3}$ amphotericin B is combined with 50 and $100\mu g.cm^{-3}$ oxytetracycline. When combined with $50\mu g.cm^{-3}$ oxytetracycline, the combination will sterilize the medium in 96 hours. When combined with $100\mu g.cm^{-3}$, the result is complete sterilization of the medium in 48 hours.

Figure 26 The Effect of Amphotericin B and Oxytetracycline alone and in Combination on *Naegleria fowleri* (MsM)

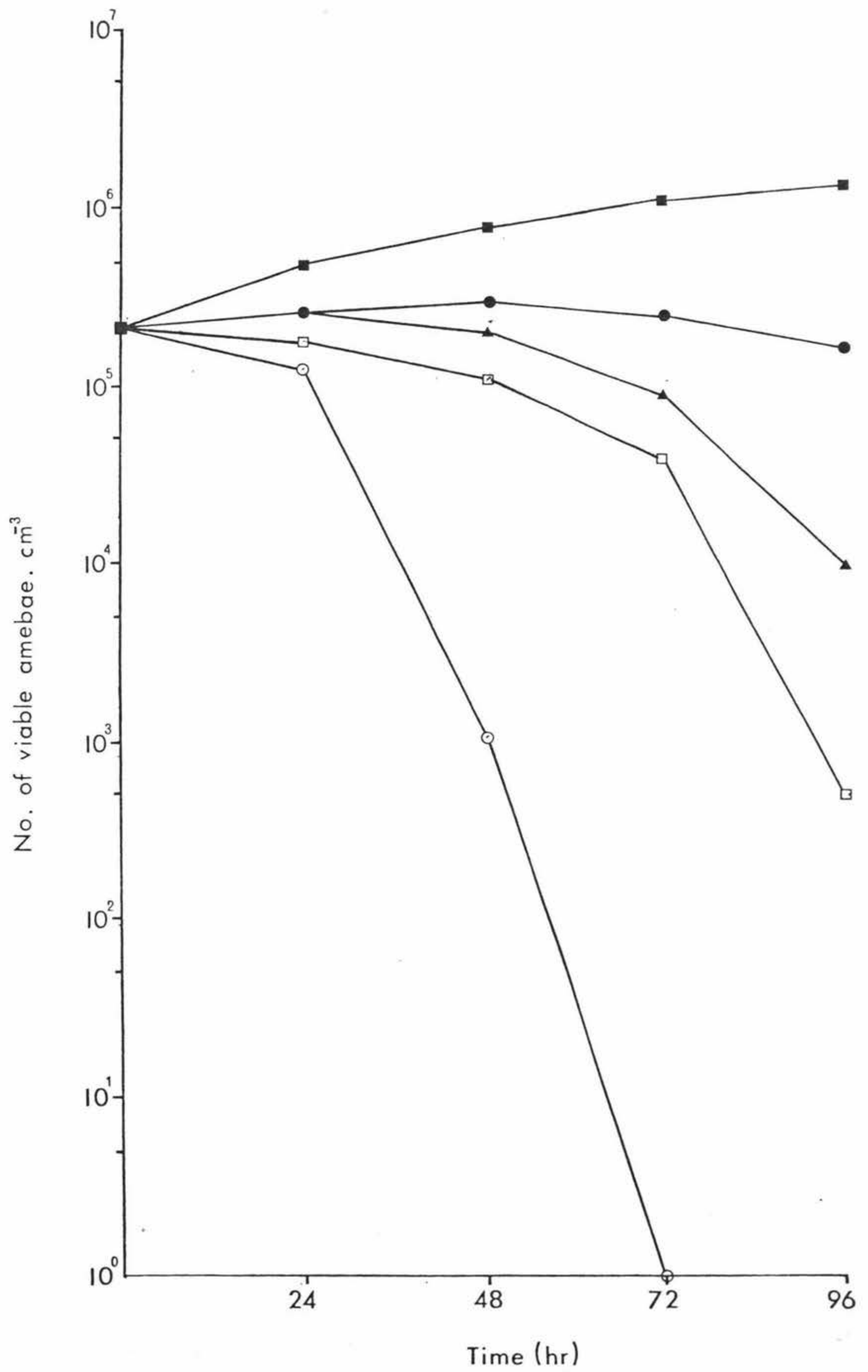
■ = 0.125$\mu g.cm^{-3}$ Amphotericin B

● = 100$\mu g.cm^{-3}$ Oxytetracycline

▲ = 200$\mu g.cm^{-3}$ Oxytetracycline

□ = 0.125$\mu g.cm^{-3}$ Amphotericin + 100$\mu g.cm^{-3}$ Oxytetracycline

○ = 0.125$\mu g.cm^{-3}$ Amphotericin B + 200$\mu g.cm^{-3}$ Oxytetracycline



10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
No. of viable amebae . cm^{-3}
24
48
72
96
Time (hr)

Figure 27 The Effect of Amphotericin B and Oxytetracycline alone and in Combination on *Naegleria fowleri* (MsM)

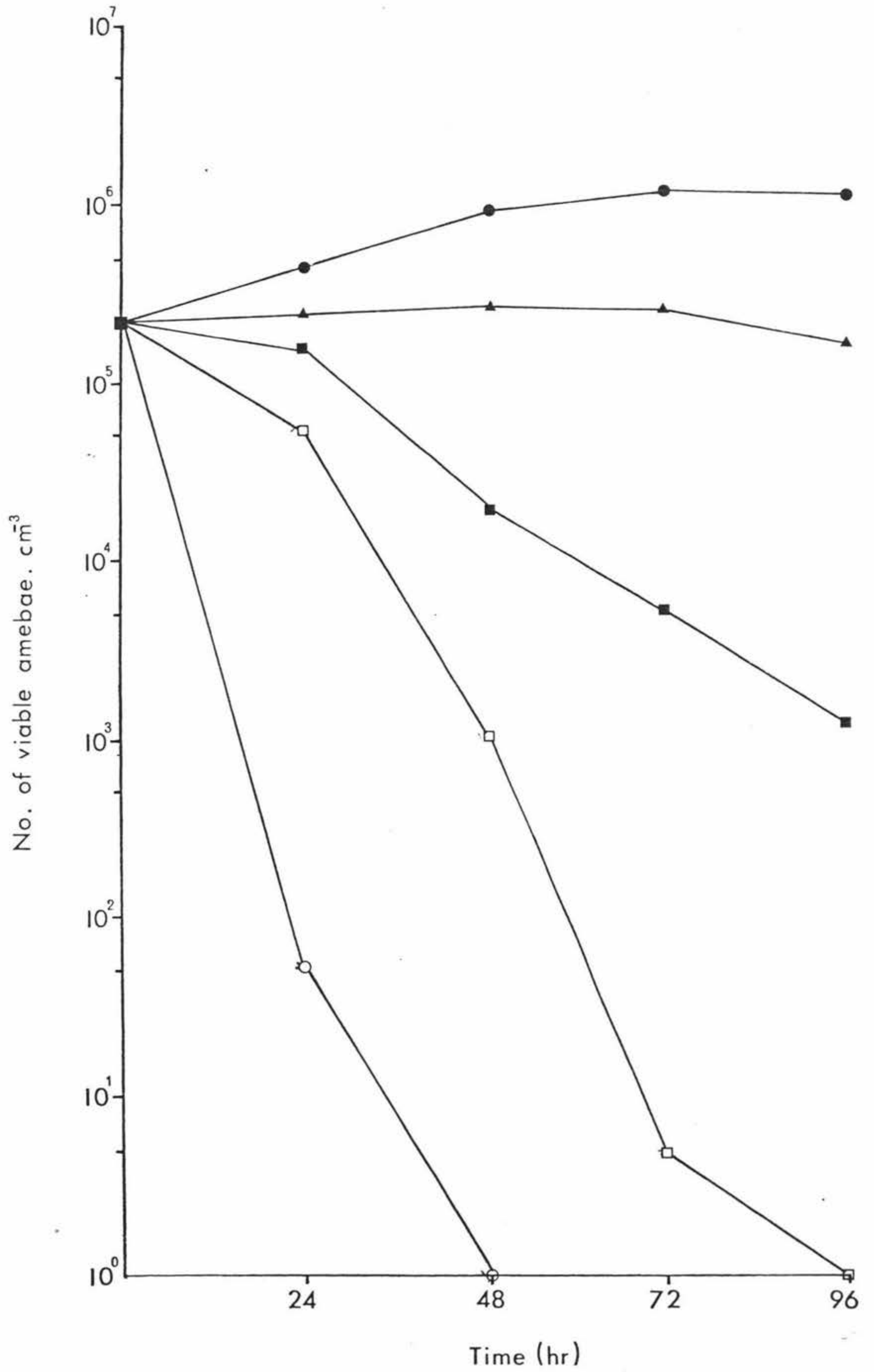
■ = 0.25$\mu g.cm^{-3}$ Amphotericin B

● = 50$\mu g.cm^{-3}$ Oxytetracycline

▲ = 100$\mu g.cm^{-3}$ Oxytetracycline

□ = 0.25$\mu g.cm^{-3}$ Amphotericin B + 50$\mu g.cm^{-3}$ Oxytetracycline

○ = 0.25$\mu g.cm^{-3}$ Amphotericin B + 100$\mu g.cm^{-3}$ Oxytetracycline



10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
No. of viable amebae. cm^{-3}
24
48
72
96
Time (hr)

4.4.3 Oxytetracycline and Tylosine

Figure 28 shows the results of combined oxytetracycline and tylosine on N. fowleri (MsM). There appears to be no synergy, and when 250µg.cm^{-3} oxytetracycline is combined with 250µg.cm^{-3} tylosine, the decrease in amebae numbers is no different to using 250µg.cm^{-3} oxytetracycline alone. The lower concentrations of 250µg.cm^{-3} tylosine combined with 125µg.cm^{-3} oxytetracycline, however, showed a slightly higher decrease in numbers of amebae which was greater than using either drug alone.

Figure 28 The Effect of Oxytetracycline and Tylosine Alone and in Combination on Naegleria fowleri (MsM)

■ = Control

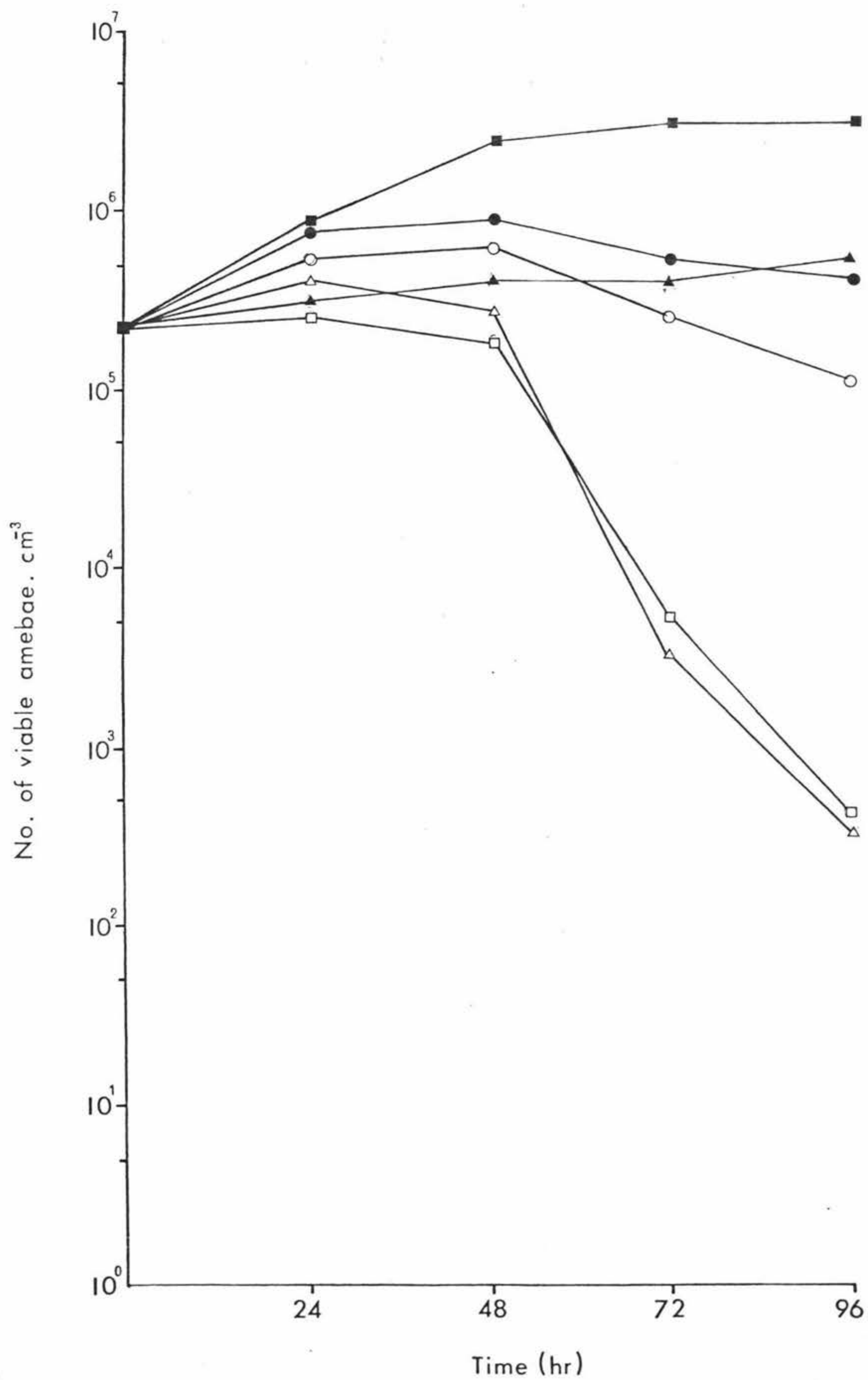
● = $250\mu g.cm^{-3}$ Tylosine

▲ = $125\mu g.cm^{-3}$ Oxytetracycline

□ = $250\mu g.cm^{-3}$ Oxytetracycline

○ = $125\mu g.cm^{-3}$ Oxytetracycline + $250\mu g.cm^{-3}$ Tylosin

△ = $250\mu g.cm^{-3}$ Oxytetracycline + $250\mu g.cm^{-3}$ Tylosin



10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
No. of viable amebae. cm^{-3}
24
48
72
96
Time (hr)

4.5 The Testing of Drug Combinations against *Acanthamoeba culbertsoni* (A-1) in Axenic Culture

4.5.1 Amphotericin B and 5-Fluorocytosine

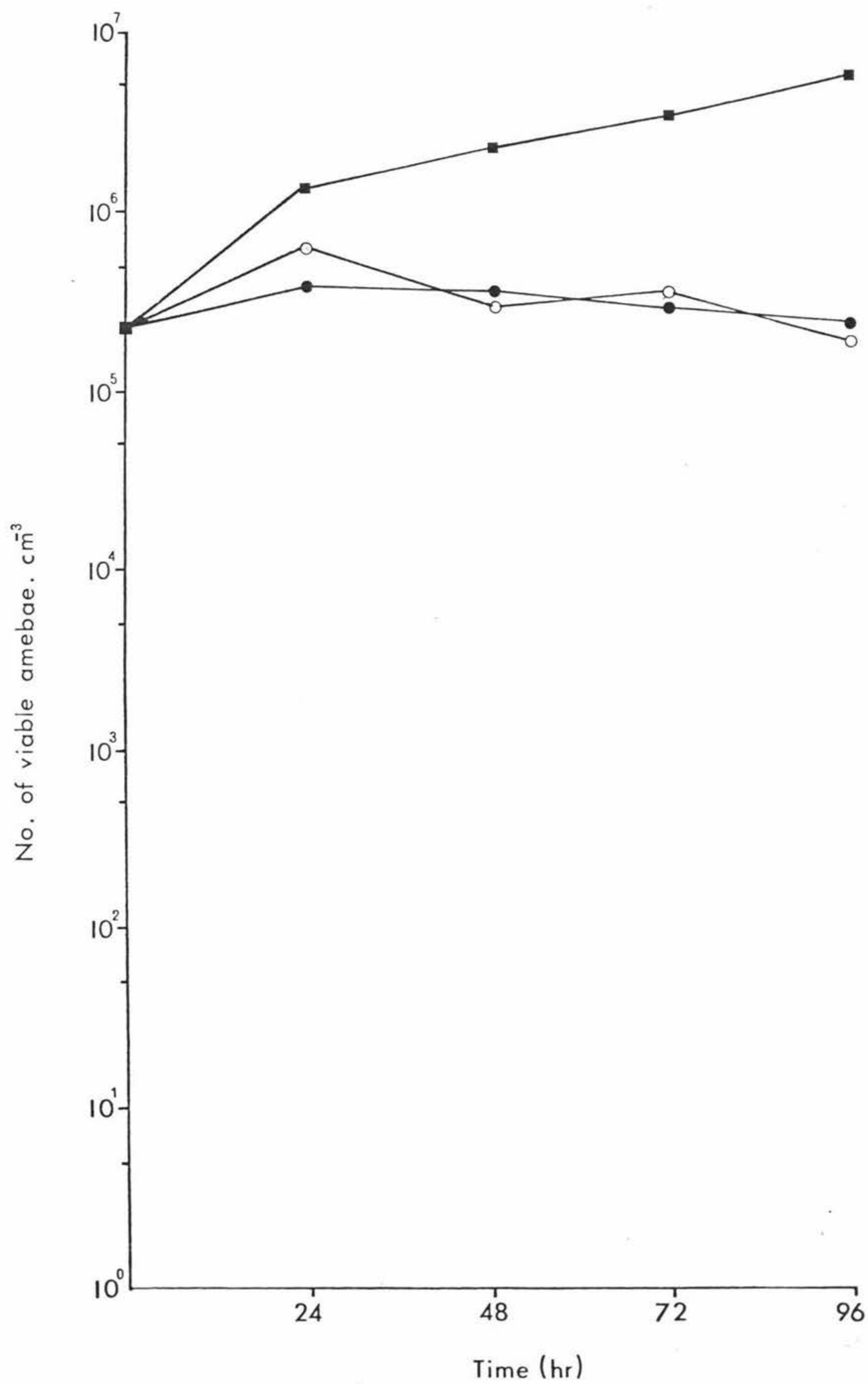
Amphotericin B has a slight effect on *A. culbertsoni* (A-1) at a concentration of 50μg.cm^{-3} (Donald, 1979). 5-Fluorocytosine has an inhibiotry effect on *A. culbertsoni* (A-1) (Figure 19). When 0.25μg.cm^{-3} amphotericin B was combined with 250μg.cm^{-3} 5-fluorocytosine, the effect on the amebae was the same as if 250μg.cm^{-3} 5-fluorocytosine was used alone (Figure 29).

Figure 29 The Effect of Amphotericin B and 5-Fluorocytosine Alone and in Combination on _Acanthamoeba culbertsoni_ (A-1)

■ = 0.25μg.cm^{-3} Amphotericin B

● = 250μg.cm^{-3} 5-Fluorocytosine

○ = 0.25μg.cm^{-3} Amphotericin B + 250μg.cm^{-3} 5-Fluorocytosine



10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
No. of viable amebae. cm^{-3}
24
48
72
96
Time (hr)

4.6 Cell Culture Drug Testing of Naegleria fowleri (MsM)

Vero cell cultures can be used as indicators of pathogenicity of PFLA (Cursons and Brown, 1978). Drugs which showed activity against amebae in axenic cultures were tested in a cell culture system. The ability of the drug to prevent the formation of a CPE and/or to kill the amebae were determined in each case. The cytotoxic effect (CTE) of the drug alone was also determined. In all tables, I through to IV, shows the progress of CPE with IV denoting that the cells had become rounded and detached from the glass. CTE was denoted by I through to III where III showed advanced CTE. A CTE could be differentiated from a CPE visually, in that the cells appeared darker, had a wrinkled outline and did not detach from the glass. In the case of a CPE, the cells were rounded with a smooth outline, and became smaller and detached from the glass as the CPE advanced to IV.

A preliminary experiment was carried out in order to determine the size of inocula of N. fowleri (MsM) on the time needed for the development of a CPE. The results are shown in Table IV. From these, an inoculum size of $9x10^3$ amebae.cm^{-3} was selected and used in all subsequent experiments with N. fowleri (MsM).

Table IV The Effect of Size of Inocula of N. fowleri (MsM) on Time Needed for Development of CPE

Inoculum size (amebae.cm^{-3})	Time (Days) 1	2	3	4	5	6	7	8
9×10^5	III	IV						
9×10^4	I	I	III	IV				
9×10^3	-	-	I	II	III	IV		
9×10^2	-	-	-	-	I	II	II	III
9×10^1	-	-	-	-	-	-	-	-

4.6.1 Amphotericin B

In cell culture amphotericin B prevented the formation of a CPE at a concentration of $0.05\mu g.cm^{-3}$ and no viable amebae were recovered when the drug was added at the same time as the amebae (Table V). In Table VII where $0.02\mu g.cm^{-3}$ was used, it failed to protect the monolayer. Once a CPE had begun to form (after three days), it took $0.25\mu g.cm^{-3}$ to prevent amebae growth and cytopathic effect did not progress past stage I. $0.1\mu g.cm^{-3}$ although failing to kill the amebae did prevent the CPE from progressing past stage II. In the cytotoxicity tests, all the concentrations tested (0.05-$0.5\mu g.cm^{-3}$) had no effect on the Vero cell monolayer.

4.6.2 Oxytetracycline

Table VI shows the results of testing oxytetracycline in cell culture against N. fowleri (MsM). When the drug was added to the cells at the same time as the amebae, $25\mu g.cm^{-3}$ protected the monolayer from CPE and was amebicidal. If a CPE was allowed to develop before the drug was added, $200\mu g.cm^{-3}$ was needed to kill the amebae. 50 and $100\mu g.cm^{-3}$, although not amebicidal, protected the monolayer from total destruction. $200\mu g.cm^{-3}$ was found to be cytotoxic to the monolayer, which began to develop after day 4.

Table V The Effect of Amphotericin B on *Naegleria fowleri* (MsM) in Cell Culture

	Amphotericin B µg.cm^{-3}	TIME (days) 1	2	3	4	5	6	Ameba viability
AMEBAE ADDED TIME = 0; DRUG ADDED TIME = 0	0	–	–	I	II	III	IV	+
	0·05	–	–	–	–	–	–	–
	0·1	–	–	–	–	–	–	–
	0·25	–	–	–	–	–	–	–
	0·5	–	–	–	–	–	–	–
AMEBAE ADDED TIME = 0; DRUG ADDED TIME = 3 DAYS	0·05	–	–	I	II	II	III	+
	0·1	–	–	I	II	II	II	+
	0·25	–	–	I	I	I	I	–
	0·5	–	–	I	I	I	I	–
NO AMEBAE ADDED; DRUG ADDED TIME = 0	0·05	–	–	–	–	–	–	
	0·1	–	–	–	–	–	–	
	0·25	–	–	–	–	–	–	
	0·5	–	–	–	–	–	–	

Table VI The Effect of Oxytetracycline on *Naegleria fowleri* (MsM) in Cell Culture

	Oxytetracycline $\mu g.cm^{-3}$	TIME (days) 1	2	3	4	5	6	Ameba viability
AMEBAE ADDED TIME = 0 DRUG ADDED TIME = 0	0	–	–	I-II	II	III	IV	+
	25	–	–	–	–	–	–	–
	50	–	–	–	–	–	–	–
	100	–	–	–	–	–	–	–
	200	–	–	–	–	–	–	–
AMEBAE ADDED TIME = 0 DRUG ADDED TIME = 3 DAYS	25	–	–	I	II	II	III	+
	50	–	–	–	I	II	II	+
	100	–	–	–	I	I	II	+
	200	–	–	–	–	I	I	–
NO AMEBAE ADDED DRUG ADDED TIME = 0	25	–	–	–	–	–	–	
	50	–	–	–	–	–	–	
	100	–	–	–	–	–	–	
	200	–	–	–	<u>I</u>	<u>I</u>	<u>II</u>	

4.6.3 Amphotericin B in Combination with Oxytetracycline

Amphotericin B when added to the monolayer with oxytetracycline displayed synergism. When the drugs were added at the same time as the amebae, $0.01 \mu g.cm^{-3}$ amphotericin B was not effective against either the amebae or the CPE, although when added together with $10 \mu g.cm^{-3}$ oxytetracycline, CPE was prevented but amebae were still isolated from the medium. $50 \mu g.cm^{-3}$ oxytetracycline added to the Vero cell culture with $0.01 \mu g.cm^{-3}$ amphotericin B however, was amebicidal. The same results were seen if $0.02 \mu g.cm^{-3}$ amphotericin B was used with 10 or $50 \mu g.cm^{-3}$ oxytetracycline (Table VII).

Once a CPE had developed (3 days after addition of amebae) $0.01 \mu g.cm^{-3}$ amphotericin B combined with $100 \mu g.cm^{-3}$ oxytetracycline halted progress of CPE and was amebicidal. The same effect was seen with $0.02 \mu g.cm^{-3}$ amphotericin B combined with either 50 or $100 \mu g.cm^{-3}$ oxytetracycline.

Table VII The Effect of Amphotericin B and Oxytetracycline Together on Naegleria fowleri (MsM) in Cell Culture

	Am. B $\mu g.cm^{-3}$	OT $\mu g.cm^{-3}$	TIME (days) 1	2	3	4	5	6	Ameba viability
AMEBAE ADDED AT TIME = 0; DRUGS ADDED AT TIME = 0	0	0	−	−	I	II	III	IV	+
	0·01	0	−	−	I	II	III	III	+
	0·01	10	−	−	−	−	−	−	+
	0·01	50	−	−	−	−	−	−	−
	0·01	100	−	−	−	−	−	−	−
	0·02	0	−	−	−	II	III	IV	+
	0·02	10	−	−	−	−	−	−	+
	0·02	50	−	−	−	−	−	−	−
	0·02	100	−	−	−	−	−	−	−
AMEBAE ADDED AT TIME = 0; DRUGS ADDED AT TIME = 3 DAYS	0·01	0	−	−	I	II	III	IV	+
	0·01	10	−	−	I	I	II	II	+
	0·01	50	−	−	I	I	I	I	+
	0·01	100	−	−	I	I	I	I	−
	0·02	0	−	−	I	I	II	III	+
	0·02	10	−	−	I	I	I	II	+
	0·02	50	−	−	I	I	I	I	−
	0·02	100	−	−	I	I	I	I	−

4.7 Cell Culture Drug Testing of Acanthamoeba culbertsoni (A-1)

As for N. fowleri (MsM) cell culture testing, a preliminary experiment was carried out in order to determine the size of inocula of A. culbertsoni (A-1) on the time needed to develop CPE. The results are shown in Table VIII and from these an inoculum size of $1x10^3$ amebae.cm^{-3} was selected and used in the following experiment with levamisole which showed promise in in vitro tests against Acanthamoeba spp.

Table VIII The Effect of Size of Inocula of Acanthamoeba culbertsoni (A-1) on Time Needed for Development of CPE

Inoculum Size (amebae.cm^{-3})	Time (Days)							
	1	2	3	4	5	6	7	8
1×10^5	III	IV						
1×10^4	-	I	II	III	IV			
1×10^3	-	I	II	II	III	III	IV	
1×10^2	-	-	-	-	II	II	II	II
1×10^1	-	-	-	-	-	I	I	I

4.7.1 Levamisole

In axenic culture, levamisole was active against the non-pathogenic A. castellanii (1501), however was only mildly active against the pathogenic A. culbertsoni (A-1). This was reinforced in the Vero cell culture tests with all the concentrations tested ($50\text{-}500\mu g.cm^{-3}$) failing to kill the amebae in 6 days. When the drug was added at the same time as the amebae, however, it prevented the total destruction of the monolayer in concentrations $\geqslant 100\mu g.cm^{-3}$. When added to the cell culture system after a CPE had begun, none of the concentrations tested affected the progress of CPE. No CTE was seen, even at the highest concentration tested ($500\mu g.cm^{-3}$) (Table IX).

Table IX The Effect of Levamisole on Acanthamoeba culbertsoni (A-1) in Cell Culture

	Levamisole $\mu g.cm^{-3}$	TIME (days) 1	2	3	4	5	6	Ameba viability
AMEBAE ADDED TIME = 0 DRUG ADDED TIME = 0	0	-	I	I	II	III	IV	+
	50	-	I	I	II	III	IV	+
	100	-	-	-	I	II	III	+
	250	-	-	-	-	II	II	+
	500	-	-	-	-	I	II	+
AMEBAE ADDED TIME = 0 DRUG ADDED TIME = 3 DAYS	50	-	-	I	II	III	IV	+
	100	-	-	I	II	III	IV	+
	250	-	-	I	II	III	IV	+
	500	-	-	I	II	III	IV	+
NO AMEBAE ADDED DRUG ADDED TIME = 0	50	-	-	-	-	-	-	
	100	-	-	-	-	-	-	
	250	-	-	-	-	-	-	
	500	-	-	-	-	-	-	

4.8 In Vivo Drug Testing of Naegleria fowleri (MsM) and (MsT)

Drugs that have previously been shown to exhibit in vitro promise against Naegleria spp. were tested in an in vivo system using mice. Tables X to XX show the results of testing five drugs either alone or in synergistic combinations against the pathogens N. fowleri (MsM) and (MsT).

4.8.1 Ketoconazole

Ketoconazole (R41,400) has previously been shown to be highly effective against Naegleria spp. both in axenic culture and in Vero cell culture (Donald, 1979). In Table X, mice were inoculated with N. fowleri (MsM) and treatment consisted of ketoconazole administered IP ($0.2cm^3$) every day until death. As can be seen in the table there are no deviations in the time of death of either the controls or higher concentrations.

In Table XI, the drug was given twice daily (at 12 hours) and again, the results show no deviations from the time of death of the controls.

Intraventricular (I.Vent) injection of the drug was tried in Table XII and the results tend to show the ineffectiveness of R41,400 in treating mice. Five mice were given $2.5mg.kg^{-1}$ ketoconazole I.Vent without being additionally inoculated with N. fowleri (MsM), and survived thereby showing the drug was not responsible for the deaths. Amebae were also isolated from those mice in which post mortem brain isolations were performed showing again, the inability of the drug to be active against the amebae in vivo.

Table X The Treatment of mice (25.0 ± 3.0gms) Innoculated with *N. fowleri* (MsM) Using Ketoconazole (R41,400) Given I.P. every 24 hrs

Total Daily Dose of Drug ($mg.kg^{-1}$)	Day of Death	No. Deaths/ No. Used	Average Time of Death (days)
0	5*, 6*, 6, 6, 6, 6	6/6	5.8
5	4*, 4*, 4, 5, 5, 5	6/6	4.5
10	5*, 5*, 5, 6, 6, 7	6/6	5.7
20	4*, 5, 5, 5, 5, 5	6/6	4.8
60	4*, 4*, 4, 5, 5, 5	6/6	4.5
80	3*, 4*, 4, 4, 5, 5	6/6	4.2

* Amebae isolated from the brain

Table XI The Treatment of mice (22.0 ± 2.0gms) Innoculated with *N. fowleri* (MsM) Using Ketoconazole given I.P. every 12 hrs

Total Daily Dose of Drug ($mg.kg^{-1}$)	Day of Death	No. Deaths/ No. Used	Average Time of Death (days)
0	4*, 4*, 5, 5, 7, 7	6/6	5.3
20	4*, 5, 5, 5, 6, 6	6/6	5.2
40	4*, 4*, 5, 5, 6, 7	6/6	5.2
80	4*, 4*, 5, 5, 6, 6	6/6	5.0

* Amebae isolated from the brain

Table XII The Treatment of mice (22.0 ± 3.0gms) Intranasally Innoculated with N. fowleri (MsM) using Ketoconazole (R41,400) Administered by I.Vent. Injection

Total Daily Dose of Drug ($mg.kg^{-1}$)	Day of Death	No. Deaths / No. Used	Average Time of Death (days)
0	5*,6 6 6 6 6 6 6 7 7	10/10	6.1
0·1	5* 5* 5 6 6 6 6 6 7 7	10/10	5.9
1·0	5* 5* 5 6 6 6 6 6 7 7	10/10	5.9
2·5	4* 5 5 7 7	5/5	5.6
2·5 No Amebae	-	5/5	-

* Amebae isolated from the brain

4.8.2 Miconazole

Table XIII shows the results of treating mice infected with N. fowleri (MsM) using IP injection of miconazole. $120mg.kg^{-1}$ per day resulted in one prolonged survival and one survival. All mice that died were shown to have been infected with N. fowleri (MsM) by post mortem brain culture.

Miconazole nitrate given by I.Vent injection also failed to cure any mice, even when used at $2.5mg.kg^{-1}$. The average time of death for all the concentrations tested ($0.1 - 2.5mg.kg^{-1}$) were not significantly different to that of the controls (Table XIV).

Table XIII The Treatment of mice (25.5 ± 2.0gms) Innoculated with N. fowleri (MsM) Using Miconazole Nitrate Administered by I.P. Injection

Total Daily Dose of Drug ($mg.kg^{-1}$)	Day of Death	No. Deaths/ No. Used	Average Time of Death (days)
0	5*, 5*, 6, 7, S S	4/6	5.75
15	5*, 5*, 6, 6, 7, 8	6/6	6.2
30	4*, 5*, 5*, 6, 6, 6	6/6	5.3
60	5*, 6*, 6, 7, 7, 7	6/6	6.3
120	4*, 6*, 6, 6, 13, S	5/6	7.0

* Amebae isolated from the brain

Table XIV The Treatment of mice (25.5 ± 2.0gms) Innoculated with N. fowleri (MsM) Using Miconazole Nitrate Given by I. Vent. Injection

Total Daily Dose of Drug ($mg.kg^{-1}$)	Day of Death	No. Deaths/ No. Used	Average Time of Death (days)
0	4*,4*, 5,5,5,5,5,6,6, 6, 7,7,7,7, 7	16/16	5.75
0·1	4*, 5,5,5,5,5, 5,5,6, 6,6, 6,6,6,6,7	16/16	5.5
1·0	3*, 5,5,5,5,5, 6,6, 6,6, 7,7, 7,7.	14/14	5.7
2·5	5*, 6, 6, 6, 6	5/5	5.8
2·5 No Amebae	-	5/5	-

* Amebae isolated from the brain

4.8.3 Amphotericin B and Tetracycline Alone and in Combination

Tables XV to XVIII show the results of treating mice intranasally inoculated with N. fowleri (MsM) with varying doses and methods of injection of amphotericin B and tetracycline, both alone and in synergistic combination.

Table XV shows the results of treating mice with amphotericin B given I.Vent and tetracycline given IP. When uninfected control mice were used, amphotericin B at a concentration of $0.2mg.kg^{-1}$ did not adversely affect the mice alone, but when tetracycline was added IP in combination, the resultant drug toxicity was fatal for two mice. However, tetracycline alone and in combination with amphotericin B resulted in a longer life span of infected mice.

Amphotericin B was administered both I.Vent and IP along with tetracycline given IP in Table XVI. The concentration of amphotericin B was increased from $0.125mg.kg^{-1}$ I.Vent plus $o.25mg.kg^{-1}$ IP to $0.25mg.mg^{-1}$ I.Vent and $0.25mg.kg^{-1}$ IP after 4 days. This treatment although failing to completely treat mice, resulted in a lack of viable amebae being isolated from the brain.

Table XVII shows the results of a treatment regime where mice were treated with amphotericin B (I.Vent) and tetracycline (given both I.Vent and IP). Amphotericin B was too toxic at the doses tested ($0.3-1.5mg.kg^{-1}$) and resulted in the rapid death of mice. A concentration of $0.3mg.kg^{-1}$ amphotericin B alone was not fatal to mice until combined with tetracycline,when the death rate was very rapid indeed with the average time of death decreasing to 2.4 days.

The experiment shown in Table XVIII was based on one published by Thong et al (1979) in which the same experiment was performed using amphotericin B ($2.5mg.kg^{-1}.day^{-1}$) combined with tetracycline ($150mg.kg.day^{-1}$), both given IP. The authors claimed 87.5% survival. Using N. fowleri strain MsM, there were two survivors, whilst the others (marked *) showed very few limax amebae in post mortem brain cultures. The brain contained many cysts though most, if not all, appeared to be dead. The drug regime, used against N. fowleri (MsT) resulted in one survival. The dead mice subjected to post mortem brain culture failed to show any viable amebae, however, the brain specimens contained many cysts, again all appeared to be dead (■).

The drugs were given for a period of 10days, in order to duplicate as close as possible the experiment of Thong et al. (1979). In the case of MsM, two mice subsequently died; these had previously been healthy and both developed typical PAM symptoms before death.

Table XV Treatment of mice (wgt = 23.0 ± 3.0gms) using Amphotericin B (given I.vent) and Tetracycline (given I.P.)

Total Daily Dose of Drug ($mg.kg^{-1}$)		Day of Death	No. Deaths/ No. Used	Average Time of Death (days)
Amphotericin B	Tetracycline			
0.1 ▲	0	-	0/6	-
0.2 ▲	0	-	0/6	
0	0	5, 7, 7	3/3	6.3
0.1	0	5 ✱, 6 ✱, 6, 7, 8	5/5	6.4
0	145	9 ✱, 9 ✱, 10, 10	4/4	9.5
0.1	145	5 ✱, 9 ✱, 10, 10, 10	5/5	8.8
0.2	145	3 ■, 7 ✱, 7, 10, 10	5/5	7.4
0.2 ▲	145 ▲	6, 7, S, S, S	2/5	-

▲ - Drug only - mice not infected with N. fowleri ✱ - Amebae isolated from the brain

■ - No amebae isolated from the brain

Table XVI Treatment of mice (wgt = 26.0 ± 2.0gms) with Amphotericin B and Tetracycline

Amphotericin B was administered both I.vent. and I.P. with Tetracycline given I.P.

Total Daily Dose of Drug ($mg.kg^{-1}$)		Day of Death	No. Deaths/ No. Used	Average Time of Death (days)
Amphotericin B	Tetracycline			
0	0	4, 5, 7, 8	4/4	6.0
0	190	5, 7, 9, 10	4/4	7.75
0.375▲	0	4*, 4*, 6, 6, 6	5/5	5.2
0.375▲	190	4■, 4■, 5■, 7, 8	5/5	5.6

▲ - Given as $0.125mg.kg^{-1}$ I.vent. + $0.25mg.kg^{-1}$ I.P. This was increased to $0.25mg.kg^{-1}$ I.vent. + $0.25mg.kg^{-1}$ I.P. after 4 days.

■ - No amebae isolated from brains on death.

Table VII Treatment of mice (23.0 ± 3.0gms) with Amphotericin B and Tetracycline alone and in combination

Amphotericin B was administered I.vent.and Tetracycline given both I.vent. and I.P.

Total Daily Dose of Drug ($mg.kg^{-1}$)		Day of Death	No. Deaths/ No. Used	Average Time of Death (days)
Amphotericin B	Tetracycline			
0	0	6 ○, 6, 6, 7, 7	5/5	6.4
0	4.4 (I.vent.)	3 ○, 4 ○, 6, 6, 6	5/5	5.0
0	134.4 ▲	6 ○, 6, 7, 7, 7	5/5	6.6
0.3	0	4, 5, 5, 5, 6	5/5	5.0
0.3	134.4 ▲	1 ●, 1 ●, 2 ●, 3,5	5/5	2.4
1.5	0	1 ●, 1 ●, 3 ●, 4,5	5/5	2.8
1.5	134.4 ▲	1 ●, 1 ●, 3 ●, 3,4	5/5	2.4

▲ - Administered as $4.4mg.kg^{-1}$ I.vent. and $130mg.kg^{-1}$ I.P.

● - No amebae isolated from the brain

○ - Amebae isolated from the brain.

Table XVIII The Treatment of mice (wgt = 29.0 ± 4.0gms) Infected with *N. fowleri* (MsM) or (MsT) Using Amphotericin B and Tetracycline Given IP

	Total Daily Dose of Drug ($mg.kg^{-1}$)		Day of Death	No. Deaths/ No. Used	Average Time of Death (days)
	Amphotericin B	Tetracycline			
MsM	0	0	5, 7, 8	3/3	6.7
MsM	2.5	150	3, 8, 11*, 18* S S	4/6	10
MsT	0	0	6, 7, 8	3/3	7.0
MsT	2.5	150	4, 4, 7■, 8■, 8■, S	5/6	6.2

*, ■ - see text for explanation

4.8.4 Amphotericin B and Oxytetracycline

Table XIX shows the results of treating mice infected with N. fowleri (MsM) using amphotericin B (I.Vent) and oxytetracycline (given IP). Uninfected mice were not affected by the use of amphotericin B and oxytetracycline and the treatment of infected mice resulted in a lengthened average time of death from 4.5 days (control) to 8.0 (using $0.1mg.kg^{-1}$ amphotericin B and $150mg.kg^{-1}$ oxytetracycline.

Amphotericin B alone at $0.1mg.kg^{-1}$ increased the average time of death from 4.5 days to 6.75 days.

The results in Table XX are those of an experiment in which $2.5mg.kg^{-1}$ amphotericin B and $150mg.kg^{-1}$ oxytetracycline was given IP to mice infected with either N. fowleri (MsM) or N. fowleri (MsT). There was one surviving mouse in treating those infected with N. fowleri (MsM) and post mortem brain culture showed limax amebae in the others (marked *). There were also a large number of cysts in brain smears which appeared to be non viable. There were no survivors in the mice infected with N. fowleri (MsT), and post mortem brain cultures revealed no viable amebae although there were numerous cysts (marked ■), again all appeared to be non viable.

The drug dosages were continued for a period of 10 days only, to resemble those experiments done using amphotericin B and tetracycline at the same concentrations. Subsequently two mice developed typical PAM symptoms, though on brain culture, no viable amebae were found, only cysts, again many of which appeared non viable.

Table XIX The Treatment of mice (wgt - 21.0 ± 4.0gm) Infected with *N. fowleri* (MsM) using Amphotericin B (I.vent.) and Oxytetracycline (I.P.)

Total Daily Dose of Drug ($mg.kg^{-1}$)		Day of Death	No. Deaths/ No. Used	Average Time of Death (days)
Amphotericin B	Oxytetracycline			
0	0	4 ■ , 4 ■ , 5, 5	4/5	4.5
-	150	4 ■ , 5 ■ , 8, 9	4/4	6.5
0.1	-	6 ■ 6, 7, 8	4/4	6.75
0.1	150	6 ■ , 6, 9, 11	4/4	8.0
0.1 ▲	150 ▲	————	0/4	-

▲ - Drugs only - mice not infected with *N. fowleri* (MsM)

■ - Amebae isolated from brain

Table XX The Treatment of mice (wgt = 29.0 ± 4.0 gms) Infected with *N. fowleri* (MsM) or MsT) Using Amphotericin B and Oxytetracycline Given IP

	Total Daily Dose of Drug ($mg.kg^{-1}$)		Day of Death	No. Deaths/ No. Used	Average Time of Death (days)
	Amphotericin B	Oxytetracycline			
MsM	0	0	5, 7, 8	3/3	6.67
MsM	2.5	150	3, 4, 5, 7*, 12*, S	5/6	6.2
MsT	0	0	6, 7, 8	3/3	7.0
MsT	2.5	150	4, 4, 4, 7■, 8■, 12■	6/6	6.5

* ■ - see text for explanation

4.9 In Vivo Drug Testing of Acanthamoeba culbertsoni (A-1)

4.9.1 5-Fluorocytosine

5-Fluorocytosine given at a dose of $250mg.kg^{-1}.day^{-1}$ IP significantly increased the average time of death (8.0 days) over that of the control mice (4.8 days) (Table XXI). A dosage of $125mg.kg^{-1}.day^{-1}$ resulted in an average time of death parallel to the control mice, but at $250mg.kg^{-1}.day^{-1}$, one mouse had an increased survival time and one other survived the infection.

Table XXII shows the results of treating mice that had been infected with A. culbertsoni (A-1) using I.Vent dosing of 5-fluorocytosine. The doses used were $1.0 - 5.0mg.kg^{-1}.day^{-1}$ and none of the results were significantly deviated from those of the controls.

4.9.2 Polymyxin B Sulphate

Table XXIII shows the results of treating mice with polymyxin B sulphate (I.Vent) at doses of $0.044mg.kg^{-1}.day^{-1}$ and $0.087mg.kg^{-1}.day^{-1}$. The dosage of $0.087mg.kg^{-1}$ was not visually toxic to the mice, and there was no significant change in the death rate as compared to the controls. Deaths due to A. culbertsoni (A-1) were verified by isolating amebae from post mortem brain studies.

Table XXI The Treatment of mice (29.5 ± 4.0gms) Intranasally Innoculated with A. culbertsoni (A-1) Using 5-Fluorocytosine Injected I.P.

Total Daily Dose of Drug (mg.kg^{-1})	Day of Death	No. Deaths/ No. Used	Average Time of Death (days)
0	4*, 4*, 5, 5, 5, 6	6/6	4.8
125	3*, 4*, 4, 4, 5, 7	6/6	4.5
250	5*, 7, 7, 8, 13, S	5/6	8.0

* Amebae isolated from the brain

Table XXII The Treatment of mice (23.5 ± 3.0 gms) Intranasally Infected with A. culbertsoni (A-1) Using 5-Fluorocytosine Injected I. vent.

Total Daily Dose of Drug (mg.kg^{-1})	Day of Death	No. Deaths/ No. Used	Average Time of Death (days)
0 ▲	-	0/7	-
0	2*, 3*, 3*, 4,4,5,5	7/7	3.75
1.0	3*, 3*, 4, 4, 4, 4,5	7/7	3.86
2.5	2*, 3*, 3, 3, 3, 4,5	7/7	3.29
5.0	3*, 3*, 3*, 3, 4, 4,5	7/7	3.57

* Amebae isolated from the brain

▲ Drugs only - not innoculated with amebae

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Table XXIII The Treatment of mice (24.0 ± 3.0 gms) Infected with *A. culbertsoni* (A-1) Using Polymyxin B administered I. Vent.

Total Daily Dose of Drug ($mg.kg^{-1}$)	Day of Death	No. Deaths / No. Used	Average Time of Death (days)
0	4*, 4*, 4, 4, 4	5/5	4.0
0.044	4*, 4*, 4, 4, 4	5/5	4.0
0.087	4*, 4*, 5, 5, 5	5/5	4.6
0.087▲	-	0/5	-

* Amebae isolated from the brain

▲ Drug only - not innoculated with amebae

4.9.3 5-Fluorocytosine and Polymyxin B Sulphate in Combination

Table XXIV shows the results of treating mice infected with A. culbertsoni (A-1) with a combination of polymyxin B and 5-fluorocytosine given I.Vent. The results are consistent with the control mice and shows the drug had little or no effect. One of the control mice died when testing $1mg.kg^{-1}$ 5-fluorocytosine combined with $0.083mg.kg^{-1}$ polymyxin B. These mice were not infected with amebae and this death is attributed to technique rather than drug toxicity as a higher dose of 5-fluorocytosine tested ($5mg.kg^{-1}$) was not toxic.

Table XXIV The Treatment of mice (wgt = 25.0 $\pm$ 2.0 gms) Infected with *A. culbertsoni* (A-1) using the Synergistic Combination of Polymyxin B and 5-Fluorocytosine given I.vent.

	Total Daily Dose of Drug ($mg.kg^{-1}$)		Day of Death	No. Deaths/ No. Used	Average Time of Death (days)
	5-Fluorocytosine	Polymyxin B			
	0	0	4*, 4*, 4, 5	4/4	4.25
(1)	1	0.083	4, S, S, S	1/4	-
	1	0.083	4*, 4, 4, 5	4/4	4.25
(1)	5	0.083	-	0/4	-
	5	0.083	5*, 5, 6, 6	4/4	5.5

(1) Not innoculated with *A. Culbertsoni* (A-1)

* Amebae isolated from the brain

4.10 In Vivo Determination of Serum Drug Levels

In order to evaluate the effectiveness of drugs tested *in vivo*, we must be able to show that adequate levels of drug were reached in the serum. Two drugs tested were the imidazoles - miconazole nitrate and ketoconazole (R41,400).

4.10.1 Miconazole Nitrate

Serum was obtained at various time intervals after injection of $60 mg.kg^{-1}$ miconazole, and assayed against *Candida pseudotropicalis* (see Methods). The resulting zone diameters (Plates 11, 12; Table XXV) were then transformed to miconazole concentrations using the standard curve (Figure 30).

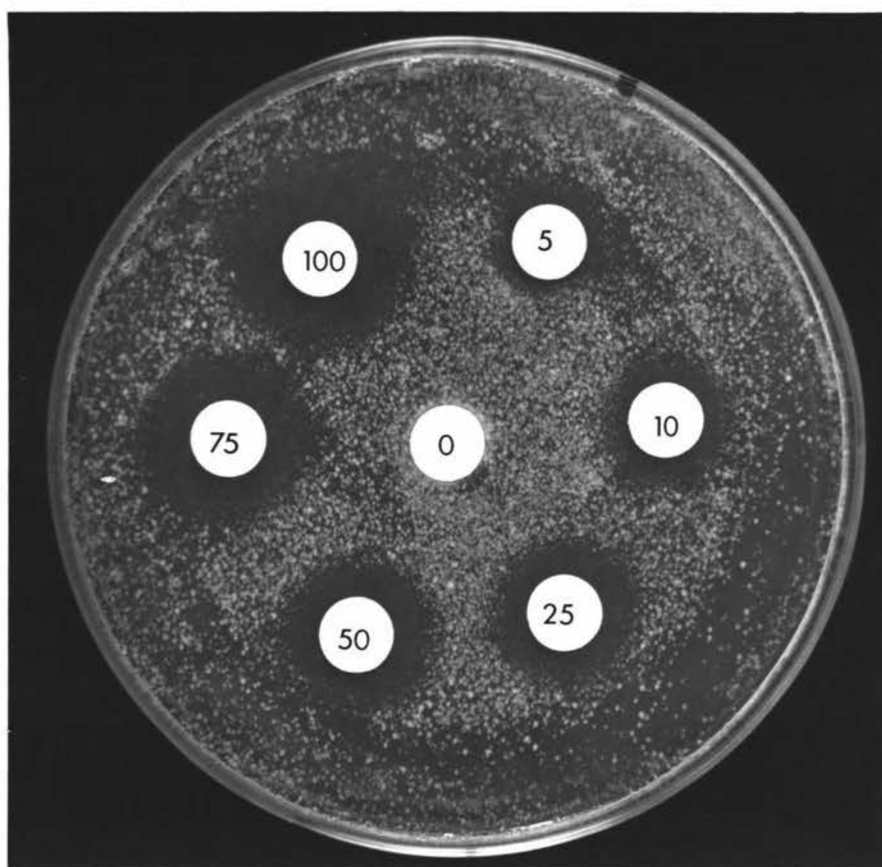
Figure 31 shows the resultant graph which maps the gradual build-up and breakdown of the drug in the bloodstream of the mouse. A maximum level of $45 \mu g.cm^{-3}$ was found after 4 hours which decreased to $1.2 \mu g.cm^{-3}$ after 8 hours.

Table XXV The Results of the Assay of Serum Samples Against *C. pseudotropicalis* for Miconazole

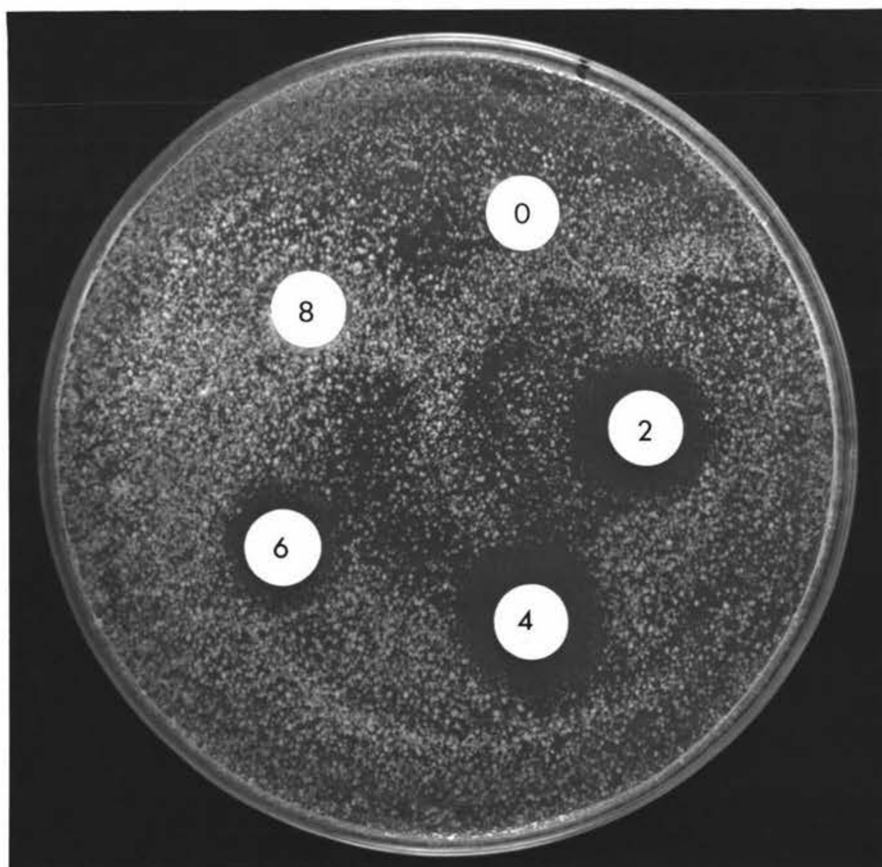
Sample (hr)	Zone Diameter (cm)
0	1.3
2	2.4
4	2.6
6	1.6
8	1.4

Plate 11 Inhibition of a background lawn of *Candida pseudotropicalis* by Miconazole nitrate standards (concentrations in $\mu g.cm^{-3}$)

Plate 12 Inhibition of a background lawn of *Candida pseudotropicalis* by serum samples taken at various time intervals, containing unknown concentrations of Miconazole nitrate (time in hours)



1cm



4.10.2 Ketoconazole (R41,400)

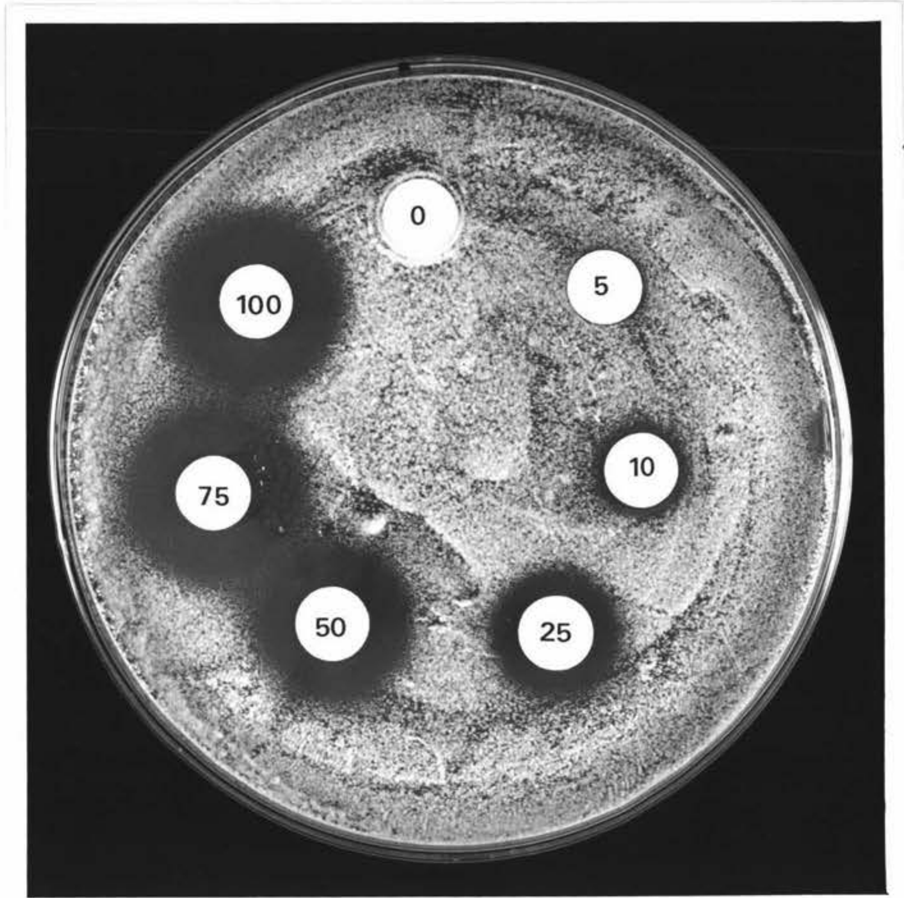
Mouse serum, taken at various time intervals, was assayed for ketoconazole using C. piropsilosis The zone diameter relating to serum samples can be seen in Table XXVI and Plates 13 and 14, and the resulting build-up and degradation of the drug over the eight hours can be seen in Figure 33.

Table XXVI The Results of the Assay of Serum Samples Against C. piropsilosis for Ketoconazole

Sample (hr)	Zone Diameter (cm)
0	1.3
2	2.7
4	2.9
6	2.2
8	1.9

Plate 13 Inhibition of a background lawn of *Candida pirapsilosis* by R41,400 standards (concentrations in $\mu g.cm^{-3}$)

Plate 14 Inhibition of a background lawn of *Candida pirapsilosis* by serum samples taken at various time intervals, containing unknown concentrations of R41,400 (time in hours)



1cm

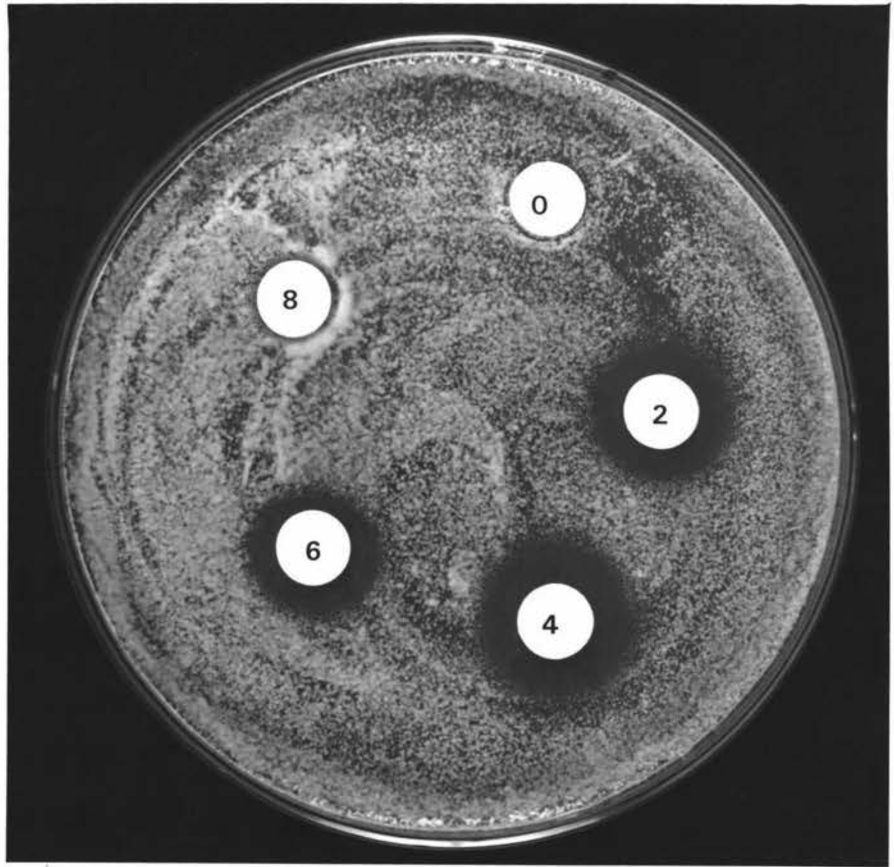


Figure 30 Standard Curve showing Zone Diameter Plotted Against known Miconazole Concentrations

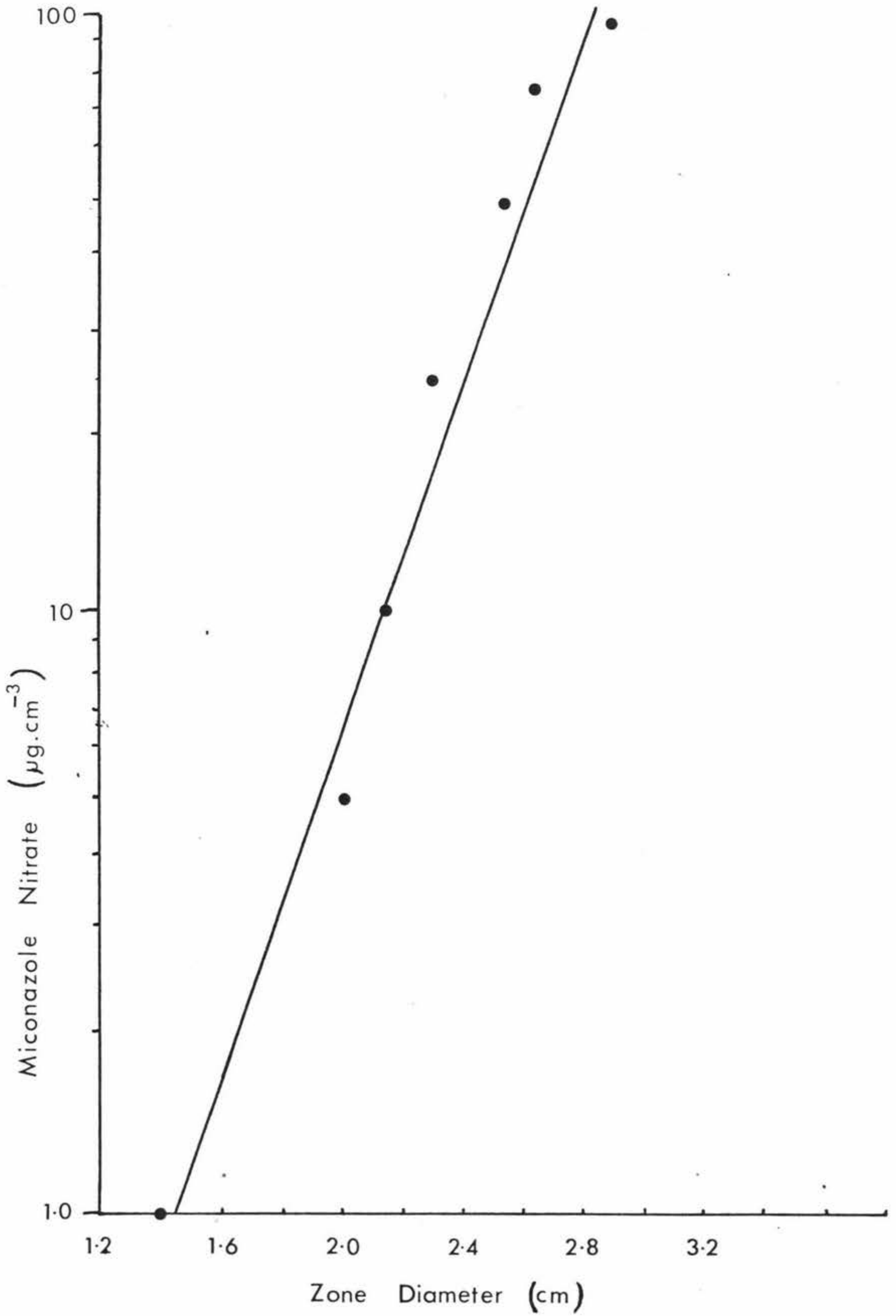


Figure 32 Standard Curve Showing Zone Diameter Plotted Against known R41,400 Concentrations

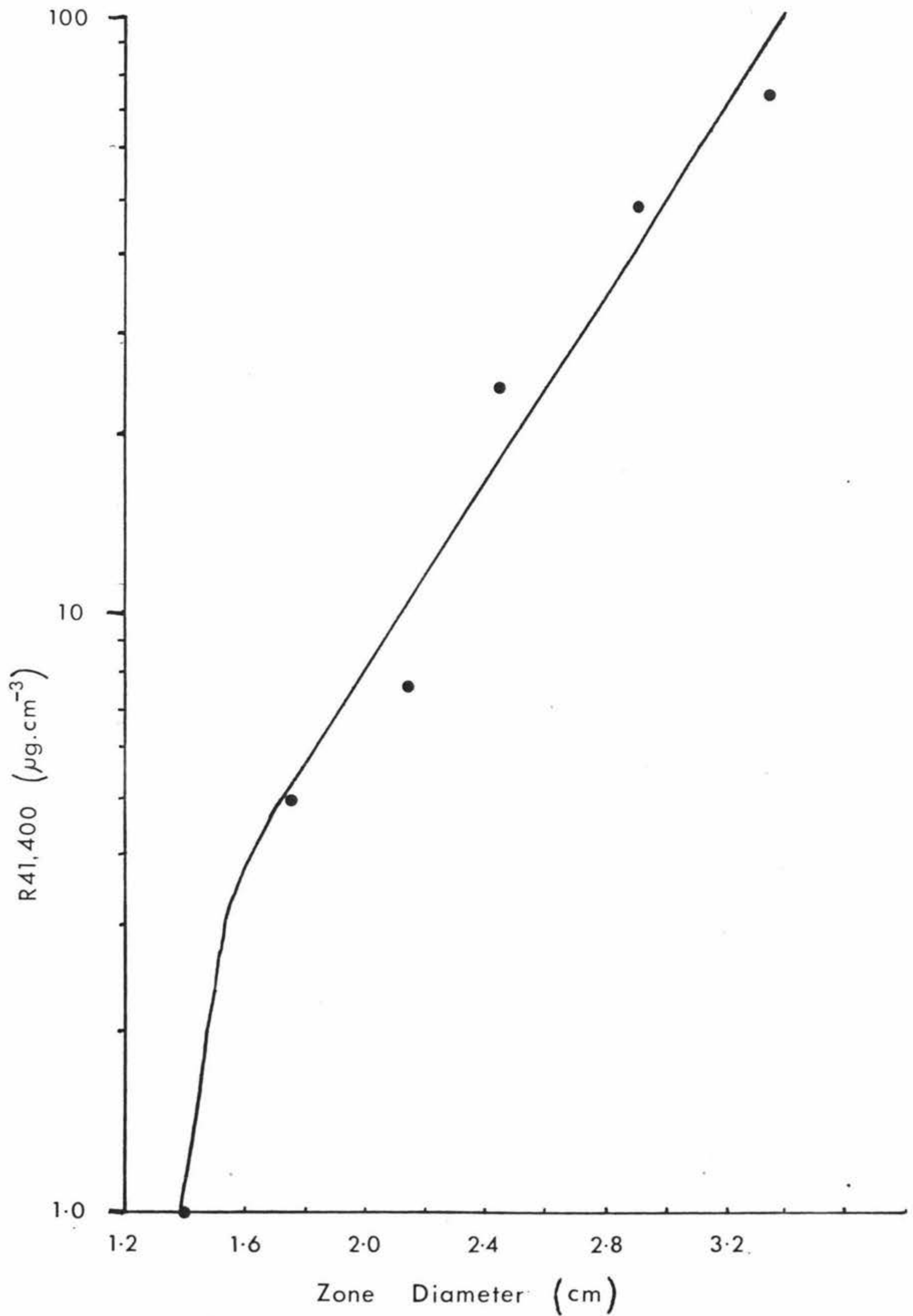


Figure 31 Concentrations of Miconazole Nitrate in the Serum of Mice after an Initial Injection of 60mg.kg^{-1} I.P.

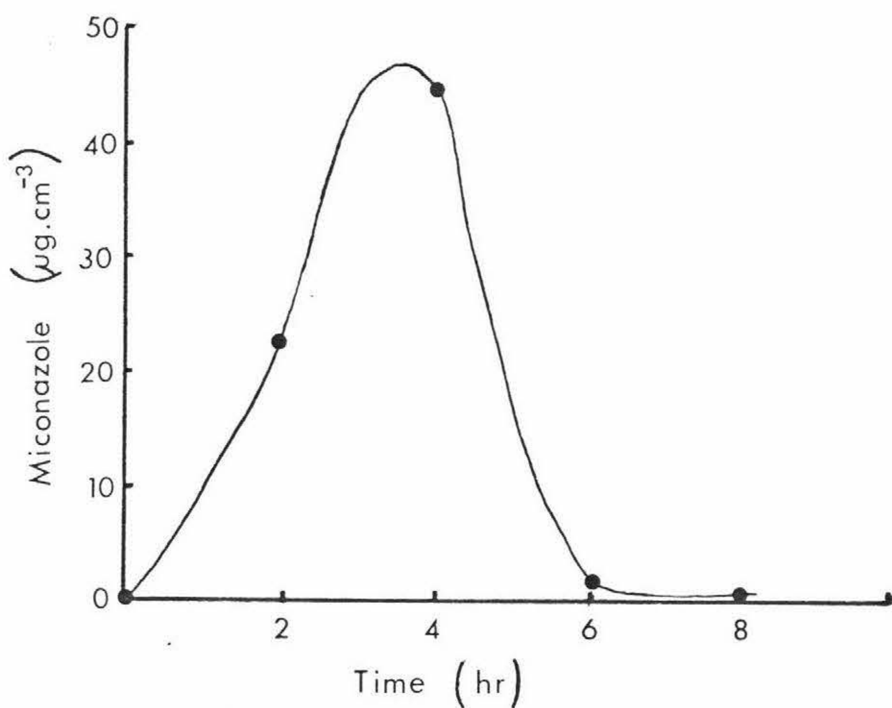
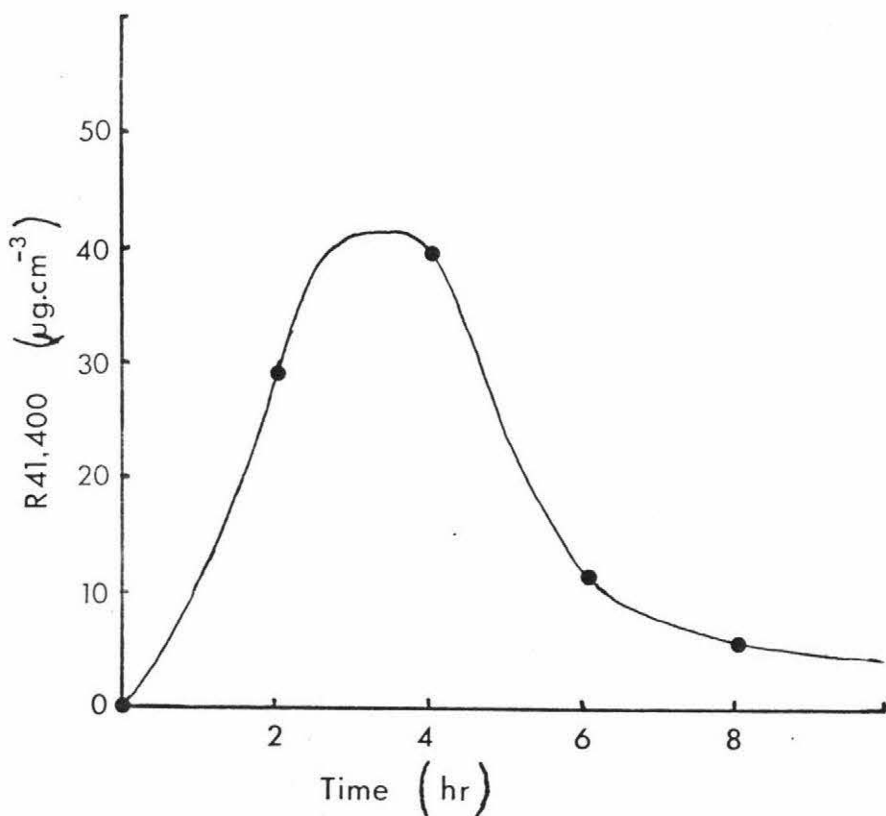


Figure 33 Concentrations of R41,400 in the Serum of Mice after an Initial Injection of 60mg.kg^{-1} I.P.



CHAPTER FIVE DISCUSSION

5.1. Treatment of Naegleria Infections

Treating earlier cases where the amebic nature of the disease was not realized with traditional antibiotics such as sulpha drugs, streptomycin and penicillin produced negative results because of the selectivity of these drugs for prokaryotes. Later when antiprotozoal and in particular anti-amebic drugs were tried, success was again negative (Carter, 1972). It has since been shown in in vitro studies that none of the traditional antibiotics were effective against Naegleria spp. (Martinez et al., 1973; Donald, 1979).

The combination of kanamycin, tylosine and oxytetracycline is used successfully against Mycoplasma spp. (Turner, 1960; Taubeneck, 1962; Hudson, 1965; Ford, 1969; Shepard, 1969) and it was suggested that the trio be tested against Naegleria spp. and Acanthamoeba spp. for a possible synergistic action (Cursons, pers. comm.).

Kanamycin is a polybasic, water soluble antibiotic resembling neomycin. It has a broad range of activity against gram positive and gram negative microorganisms (Goodman and Gilman, 1975) but has no effect against Naegleria spp. at the concentrations tested. $1000\mu g.cm^{-3}$ for N. gruberi (P1200f) and $500\mu g.cm^{-3}$ for N. fowleri (MsM) failed to bring about any difference in the growth rate or activity of the amebae. The amebae were active, motile and demonstrated limax movement even after 96 hours in the test medium (Figures 2 and 3).

Tylosine is an antibiotic of the macrolide group of drugs and is not considered to be a broad spectrum antibiotic (Gingerich et al., 1977). It inhibits beta-hemolytic streptococci at $<1.0\mu g.cm^{-3}$ with Mycoplasma spp. being susceptible at concentrations $<0.09\mu g.cm^{-3}$. For both N. gruberi (P1200f) and N. fowleri (MsM) there was a slight reduction in the numbers of amebae after the 96 hour incubation period with N. fowleri appearing to be more sensitive to the drug (Figures 4 and 5). N. gruberi (P1200f) was tested at a highest concentration of $1000\mu g.cm^{-3}$ and although the numbers of amebae did not drop below the original inoculum size, they did show a deliterious response to the drug action. At drug concentrations as low as $250\mu g.cm^{-3}$, they were rounded up, highly vacuolated, and smaller in size after 48 hours in the test media. N. fowleri (MsM) was more sensitive to tylosine (Figure 5) with levels of $100\mu g.cm^{-3}$ showing the same toxic effects on the amebae as did $250\mu g.cm^{-3}$ on N. gruberi. $500\mu g.cm^{-3}$ resulted in a drop in amebae

numbers after 96 hours that was below the original inoculation number. However, this drug would, on its own, be unlikely to have any in vivo effect as it is bound by protein (40%) and has a very short half-life (1.62 hours) (Gingerich et al., 1977). Nevertheless, synergistic combinations may show promise in the future.

Oxytetracycline was also investigated in axenic culture and in the cell culture system. This drug is a broad spectrum antibiotic similar to tetracycline, which has proven activity against the protozoan diseases (Goodman and Gilman, 1975), and also against Naegleria spp. (Donald, 1979; Thong et al., 1978a, 1979). N. gruberi (P1200f) was shown to be sensitive to oxytetracycline (Figure 6). $500\mu g.cm^{-3}$ was amebicidal resulting in no viable amebae after 96 hours in the culture medium. At $50\mu g.cm^{-3}$, the lowest concentration tested, the amebae, although showing a decrease in numbers, were active and motile, and it was only in concentrations $\geqslant 100\mu g.cm^{-3}$ that they showed drug toxicity effects, appearing smaller, rounded and non-motile. N. fowleri (MsM) appeared more sensitive to oxytetracycline with $500\mu g.cm^{-3}$ sterilizing the media after 72 hours compared to 96 hours with N. gruberi (P1200f). $100\mu g.cm^{-3}$ could be considered the MIC. In cell culture testing, $25\mu g.cm^{-3}$ completely protected the monolayer from CPE and was amebicidal after the six day incubation period, when added at the same time as the amebae. If a CPE was allowed to develop before addition of oxytetracycline, $200\mu g.cm^{-3}$ was needed to halt progression of CPE and to kill the amebae, however, this concentration was found to be cytotoxic to the monolayer if added alone. $50\mu g.cm^{-3}$ and $100\mu g.cm^{-3}$ although failing to kill the amebae, protected the monolayer from total destruction (Table VI).

Amphotericin B and oxytetracycline were investigated for a synergistic effect against N. fowleri (MsM) in both axenic and Vero cell culture. In axenic culture, $0.125\mu g.cm^{-3}$ amphotericin B when used with $100\mu g.cm^{-3}$ oxytetracycline was only inhibitory, however when combined with $200\mu g.cm^{-3}$ oxytetracycline the effect was media sterilization after 72 hours (Figure 26). Increasing the amphotericin B concentration to $0.25\mu g.cm^{-3}$ resulted in media sterilization in 96 hours when combined with $50\mu g.cm^{-3}$ oxytetracycline and in 48 hours when combined with $100\mu g.cm^{-3}$ oxytetracycline (Figure 27).

This synergistic activity was also seen in cell culture (Table VII). $0.01\mu g.cm^{-3}$ amphotericin B alone had no effect on the monolayer and amebae were isolated after the six day incubation period, however in combination with $10\mu g.cm^{-3}$ oxytetracycline, CPE was

completely halted although amebae were isolated after the incubation period, and it was only after adding 50μg.cm^{-3} oxytetracycline in combination with 0.01μg.cm^{-3} amphotericin B that no amebae were isolated. The same effect can be seen with 0.02μg.cm^{-3} amphotericin B in that 50μg.cm^{-3} oxytetracycline was needed to both halt CPE and kill all the amebae. Once a CPE had been allowed to develop before adding the drugs, 0.01μg.cm^{-3} amphotericin B combined with 100μg.cm^{-3} oxytetracycline or 0.02μg.cm^{-3} amphotericin B combined with 50μg.cm^{-3} oxytetracycline was needed to halt progression of the CPE and kill the amebae (Table VII). Lower concentrations in each case of oxytetracycline although failing to kill the amebae, did halt progression of CPE.

Preliminary experiments into the synergistic combinations of oxytetracycline, tylosine and kanamycin found that the combination of oxytetracycline and tylosine had potential activity against N. fowleri (MsM). However, further in vitro experimentation showed this not to be the case (Figure 28) and in fact the activity of 250μg.cm^{-3} oxytetracycline combined with 250μg.cm^{-3} tylosine was no more active than using 250μg.cm^{-3} oxytetracycline alone.

Tetracycline has also been shown to be effective against Naegleria spp. Thong et al. (1977) reported inhibition of N. fowleri using 10μg.cm^{-3} tetracycline in axenic culture although this probably reflects the initial inoculum size. Donald (1979) showed a slight reduction in numbers of N. fowleri and N. gruberi at concentrations of 50 and 100μg.cm^{-3} in axenic testing, while in cell culture testing 50μg.cm^{-3} completely inhibited the formation of a CPE after a six day incubation period, although viable amebae were still recoverable. At 100μg.cm^{-3} no viable amebae were found.

Levamisole is a newly synthesized broad spectrum animal and human anthelmintic, which is very soluble in water, and is fast and short acting in the in vivo situation. In terms of its anthelmintic activity, it is thought to act on the fumerate reductase system by interaction with one or more -SH groups in the active portion of the enzyme, forming stable -S-S bonds (Levamisole - an agent with multiple agents. Preclinical reports provided by Janssen Pharmaceutica). It has also been shown to be effective both in vitro and in vivo on the immune response system, acting by restoring T cell activity; by ressembling the action of the thymus hormone (van de Velde et al. 1978), and by restoring functions of effector cells involved in cell mediated immunity. The anthelmintic group of drugs has been shown to be

effective against protozoa (Cursons pers. comm.), which prompted the study on PFLA. Levamisole was slightly active against N. gruberi (P1200f) at a concentration of 1000μg.cm^{-3} (Figure 8) and in the case of N. fowleri (MsM) which showed more sensitivity to the drug, levels as low as 250μg.cm^{-3} brought about a decrease in numbers as compared to the control. In both cases, however, the amebae were normal, showing no signs of drug toxicity.

5-Fluorocytosine is primarily an antifungal agent, acting by conversion within sensitive fungal cells to 5-flououracil which is a well known metabolic antagonist. It is a drug of low toxicity (Block and Bennett, 1972) and substitutes itself into RNA leading to abnormal protein synthesis. It may also block DNA synthesis by blocking thymidylate synthetase. Donald (1979) used 5-fluorocytosine against N. gruberi (P1200f) and N. fowleri (MsT) and showed that when tested at concentrations $\leqslant$100μg.cm^{-3}, there was no activity. Figure 10 shows the activity of 5-fluorocytosine on N. fowleri (MsM) with the highest levels tested being 500μg.cm^{-3}. This level of drug was not effective against N. fowleri in the least and the amebae were all normal in appearance. Duma and Finley (1976) tested 5-fluorocytosine against six strains of N. fowleri isolated from human cases and no effect was seen at 1000μg.cm^{-3}. However, Das in 1975 reports an amebicidal effect of 200μg.cm^{-3} in axenic culture against pathogenic Naegleria spp. The original inoculum size may have had an effect as Das used 5 x 10^3 amebae.cm^{-3} compared to 2.5 x 10^5 amebae.cm^{-3} in this study.

Amphotericin B although used against protozoa in a few instances (Horvath and Zierdt, 1974; Kinsky, 1967) has had its major application in systemic fungal infections of humans (Hamilton-Miller, 1973; Kinsky, 1967). Amphotericin B is a polyene antibiotic closely related chemically to nystatin and is believed to initiate its action at the plasma membrane of the cell where it binds to sterols (mainly ergosterol) creating "holes" in the membrane, increasing membrane permeability (Andreoli, 1974), and causing leakage of important cellular contents leading to lysis and death (Ghosh and Ghosh, 1963; Gale, 1974).

Carter (1969) reported a minimum immobilizing level of 0.6μg.cm^{-3} and a MIC of 0.075μg.cm^{-3} in vitro. This confirmed the 1968 in vivo results of Culbertson et al who showed that large doses of Amphotericin B in combination with large doses of sulphadiazine in the diet, had activity against N. fowleri (HB-1) in mice. When 1000 amebae

were inoculated intranasally into mice, the mice all died within seven days, however treatment with 5.0 or 7.5mg.kg^{-1} amphotericin B given subcutaneously twice daily, and combined with 0.5% sulphadiazine in the diet, resulted in death of only four out of ten (60% survival). At a lower 2.5mg.kg^{-1} amphotericin B, only two mice survived although the average time of death was increased to as long as 24 days. Sulphadiazine treatment was begun three days before intranasal infection and amphotericin B one day before, with the infecting number of amebae being only 240.

Carter (1969) showed that sulphadiazine had no effect on Naegleria spp. in vitro at levels in excess of those likely to be found in the brain at therapeutic levels. Attainable CSF levels are reported to be 90μg.cm^{-3} (Goodman and Gilman, 1975) and Carter used 1280μg.cm^{-3} with no obvious effects on the Naegleria spp.

Carter in 1969 administered 7.5mg.kg^{-1} amphotericin B once daily IP to mice which resulted in a 60% survival rate. The serum level of amphotericin B was shown to be 1.2μg.cm^{-3}.

Culbertson et al; (1971) found that a single intracerebral injection of amphotericin B prevented PAM in most mice given intranasal lethal doses of N. fowleri (HB-1). At a dose of 37.5mg, five out of eleven mice died with only three showing any signs of amebic invasion and at a lower dose of 18.75mg given intracerebrally, three out of nine died, with four showing signs of amebic lesions.

Das (1971) gave 30mg.kg^{-1} amphotericin B every 24 hours to mice subcutaneously, resulting in complete protection against amebic infection.

In vitro results support the effectiveness of the drug against Naegleria spp. Duma and Finley (1976) reported that 0.62μg.cm^{-3} was the MIC for six pathogenic N. fowleri strains tested. Mandel et al (1970) reported a MIC of $5 \times 10^{-4} - 1 \times 10^{-3}$μg.cm^{-3} for a N. fowleri strain isolated from a human case. De Jonckheere and van de Voorde (1977b) studied the effect of amphotericin B on three pathogenic strains of N. fowleri, reporting a MIC of 0.1 - 0.15μg.cm^{-3} and a MIC of 1.0μg. cm^{-3} for two strains of N. fowleri which were non-pathogenic to mice. Chang (1971) also reports a difference in the susceptibility of pathogenic and non-pathogenic species to amphotericin B. 1μg.cm^{-3} was the MAC for pathogenic Naegleria compared to 2μg.cm^{-3} for the non-pathogens.

Donald (1979) used two strains of Naegleria - a pathogenic N. fowleri (MsT) and a non-pathogenic N. gruberi (P1200f) both of which

had an MAC of 0.5μg.cm^{-3}. Figure 11 shows the effect of the drug on N. fowleri (MsM). 0.5μg.cm^{-3} completely sterilized the medium in 48 hours. In cell culture testing, 0.05μg.cm^{-3}, the lowest concentration tested, both killed all amebae and protected the monolayer from CPE. Once a CPE had been allowed to develop, 0.25μg.cm^{-3} was required to halt it and be amebicidal. 0.1μg.cm^{-3}, although not amebicidal, protected the monolayer from destruction, with a CPE developing to a stage II after the six day incubation period (Table V). These results are comparable to those obtained by Donald (1979).

Differences in the susceptibility of non-pathogenic and pathogenic species and in particular between strains in in vitro testing appear in the literature. Some of these differences may be a result of different screening techniques, whether the amebae are grown axenically, or with bacteria as their food source. It is also necessary to consider different inoculum sizes and different media. In this study, CGHV and CGHVS for the axenic growth of Naegleria and Acanthamoeba spp. respectively was developed by Cursons et al (1979) specifically to minimize the antagonistic effects of media on the drugs. Serum, a common component of medium used to culture these amebae, contains sterols which may preferentially bind with drugs (e.g. amphotericin B). In this semi-defined medium, the serum component is replaced with haemin while yeast extract was replaced with vitamins to eliminate any antagonistic effects that may affect the results of, say, 5-fluorocytosine (Cursons et al., 1979). This does not, however, explain the results obtained using two strains of N. fowleri - MsM and MsT and this media. N. fowleri (MsM) is the most recently isolated strain, obtained from a human case in 1978 (Cursons et al., 1979) and has been maintained in CYM, and for the purposes of drug assays, in CGHV. Virulence of both MsM and MsT is maintained periodically by Vero cell passage and animal passage in mice. The results of testing amphotericin B against MsM in this study and MsT (Donald, 1979) using identical media and inoculum, could possibly be attributed to a loss of virulence in the MsT. N. fowleri (MsT) was isolated from another New Zealand case in 1974 (Cursons et al., 1976) and although virulence has been maintained as for N. fowleri (MsM) it is possible that virulence has been selected out by routine passage. Chang (1971) mentioned that cases of reduction in virulence are varied, and in axenic cultures of pathogenic amebae, there may exist both "high" and "low" virulence amebae, which may be selected for routine passage. Low virulence amebae, if genetically selected for, can be boosted to a higher pathogenicity

by animal or tissue culture passage although not to as high a virulence level as the original as they take longer to kill mice, and grow more slowly on MKC cells. This could be attributed to the fact that lower virulence strains are less deficient in enzyme systems such as phospholipases, which are partially responsible for the degree of pathogenicity (Cursons, 1979).

Duma (1971) found that with regard to Naegleria spp. inoculum size, temperature, time of incubation and whether or not they were grown in the presence of bacteria played a role in virulence. This was also found by Chang (1971) who reported a loss of CPE caused by N. fowleri (HB-1) in cell culture. Visvesvara and Calloway (1974) observed a decrease in virulence for mice of the HB-1 and E66 strains of N. fowleri after intranasal inoculation.

Reviewing the experiments of Culbertson et al. (1968) N. fowleri (HB-1) was used, and also at an inoculum of 240 amebae. Although the controls all died, the amebae may have been lacking in virulence to the degree that they were affected by amphotericin B. Mandel et al. (1970) reported a MIC of $5 \times 10^{-4} - 1 \times 10^{-3} \mu g.cm^{-3}$ against a recently isolated strain of N. fowleri which may reflect the higher virulence compared to the in vitro results of Duma and Finley (1976) who reported an MIC of $0.62 \mu g.cm^{-3}$, and those of Chang (1971) who reported $1 \mu g.cm^{-3}$ against pathogenic N. fowleri. De Jonckheere and van de Voorde (1977) reported two strains of non-pathogenic N. fowleri having an MIC of $>1.0 \mu g.cm^{-3}$ amphotericin B whereas three pathogenic N. fowleri had a far lower MIC of $0.1 - 0.15 \mu g.cm^{-3}$. This again reflects the virulence factor which may reflect a sensitivity loss to amphotericin B or for that matter any other drug.

Tables XV to XX show the in vivo results of amphotericin B given IP and I.Vent. Table XVI shows amphotericin B given at a dosage of $0.125 mg.kg^{-1}$ I.Vent as well as $0.25 mg.kg^{-1}$ IP. This was increased to $0.25 mg.kg^{-1}$ I.Vent as well as $0.25 mg.kg^{-1}$ IP after 4 days when the mice had become accustomed to the dosage. All mice showed typical symptoms of PAM before death, and viable amebae were isolated from brain samples. Increasing the I.Vent dosage to $0.3 mg.kg^{-1}$ resulted in 100% death of mice due to N. fowleri (Table XVII). A further increase to $1.5 mg.kg^{-1}$ I.Vent was tried in order to elevate CSF drug levels, however this concentration was toxic to the mice and although amebae could not be isolated from the brains of mice, they all died with an average time of death of 2.8 days as compared to 6.4 days for the controls.

Deaths in this case were attributed to the high concentration of amphotericin B which proved toxic.

These results however, do not rate with previous published data, and it is believed that virulence factors could be responsible. Culbertson et al. (1968) used a total daily dose of 7.5mg.kg amphotericin Bsubcutaneously claiming a 60% survival rate. Goodman and Gilman (1975) state that $5mg.day^{-1}$ $(0.65mg.kg^{-1})$ given IV, yields plasma levels of 0.5 - $3.5\mu g.cm^{-3}$ persisting for 6-8 hours. CSF levels are 1/40th of the plasma levels which by extrapolating would be approximately $0.0125\text{-}0.09\mu g.cm^{-3}$. These CSF levels would be comparable to infecting an I.Vent dosage of 0.1 - $0.3mg.kg^{-1}$ and although CSF levels may have been reached, the survival rate in this study was 0%, maybe reflecting the virulence and inoculum number.

In vitro testing by Schuster and Rechthard (1975) showed that the age of the amebae culture had a bearing on the action of amphotericin B. The drug was amebicidal when added at 0.25, 0.5, and $1.0\mu g.cm^{-3}$ to the lag phase but was mainly inhibitory when added to the log phase. Ultrastructural modifications could be seen at all concentrations tested ranging from distortion of nuclear shape, absence of pseudopod formation, to an increase in autophagic vacuoles. These changes became more pronounced with increase in exposure time and concentration of amphotericin B.

A similar trial was performed using levamisole against N. gruberi (P1200f) in this study (Figures 8, 21 and 22). It was thought that the age of the parent culture may have a bearing on the effect of drugs in axenic culture. A 24 hour culture of N. gruberi (P1200f), considered to be in the log phase, was tested along with N. gruberi cultures from the lag phase of growth (48 and 96 hour growth). Levamisole was inactive against N. gruberi (P1200f), and the experiments showed that use of parent cultures of different ages had no effect on the growth of the amebae whether treated with drug or not. Repeating the experiment with A. castellanii (1501) which is sensitive to levamisole, reinforced the conclusion that the age of the parent inoculum did not affect the activity of the drug (Figures 18, 23 and 24).

In 1969, Carter suggested that the treatment of PAM with amphotericin B be tried by simultaneous IV and I.Vent administration in the doses - $0.25mg.kg^{-1}$ IV and 1.0mg into the cerebral ventricles in the first 24 hours after diagnosis. Subsequently, this treatment

was tried on two patients in the USA (Duma et al., 1971) and on two patients in Australia (Carter, 1972). Although all four patients failed to respond to treatment, Duma et al. (1971) could not isolate amebae from fresh necropsy tissue in the second case (see Section 1) and in the first case, samples of amebae were confined to one small area, the cisterna cerebromedullaris. Wet mount preparations of CSF from both patients before death, showed poorly motile amebae that were rounded up, highly vacuolated, granulated and ruptured.

In the fatal case of Apley et al. (1970), amphotericin B was administered only by the IV route at a concentration of $0.25 mg.kg^{-1}$ increasing to $1 mg.kg^{-1}$ over one week. On the seventh day after admission 650 $amebae.mm^{-3}$ were seen in the CSF which contained amphotericin B at a concentration of $0.184 \mu g.cm^{-3}$ although many appeared to be dead. By day eleven the CSF concentration was $0.224 \mu g.cm^{-3}$ and no amebae could be seen. This shows that although amphotericin B levels were high enough, the patient died. Autopsy found severe cerebral changes that suggested anoxic damage as well as continued amebic invasion, and again amebae were not found in all areas of the meninges but were located in the small area of the brain stem.

The second and third cases reported by Apley et al. (1970) were also treated in the same way as case one. Although amebae were not seen in the CSF of either patient, they were cultured from the CSF of case two. Case three is considered only a doubtful case of PAM while case two is considered to be due to Naegleria and survival is attributed to the use of amphotericin B. It is also considered that although treatment levels of amphotericin B were low, treatment having started so early in the course of the disease was responsible for the cure. There is controversy over this case as the Naegleria spp isolated was N. gruberi and was subsequently found to be non-pathogenic in mice (Warhurst et al., 1970; Saygi et al., 1973), and also died at febril temperatures (Griffin, 1972). Griffin (1976) feels that the amebae were in fact Acanthamoeba, and that sulphadiazine given on admission was responsible for the prolonged survival in the first case and survival in the second. It is argued that although no Acanthamoeba spp. were isolated from the CSF of patient one taken two days after admission, the presence of sulphadiazine may have prevented the in vitro growth of Acanthamoeba. Finally Stamm (1974) confirmed cultural evidence that N. gruberi was present by visualizing amebae in histological brain sections with anti-N. gruberi immunofluorescence. He did not obtain any labelling with Acanthamoeba antisera.

The first survival where there is definite proof that N. fowleri was involved and survival can be attributed to amphotericin B was that reported by Anderson and Jamieson (1972). Amphotericin B was given IV at a dose of $1mg.kg^{-1}$ immediately the diagnosis was confirmed. The patient although comatose at the time of initiating treatment, became afebrile and was talking rationally within two days. Five days later the CSF white cell count had fallen from an initial count of $12,000.mm^{-3}$ to $15.mm^{-3}$ but amebae were still seen. As a result amphotericin B was given I.Vent in small doses (0.1mg on alternate days) and the CSF gradually cleared.

The most important point seems to be to administer amphotericin B at the maximum possible dose at the earliest possible time after PAM is suspected. This was confirmed in the case report by Siedel et al. (pers. comm. 1978). Amphotericin B although initially given at a dose of $1.0mg.kg^{-1}$ IV combined with 1.5mg intrathecally (IT), was increased to $1.5mg.kg^{-1}$ IV per day divided into two doses for a period of three days, then reduced to $1.0mg.kg^{-1}$ for six days. IT administration of $1.5mg.day^{-1}$ was continued for two days, then reduced to 1.0mg every other day for eight days.

There is the added problem in PAM of raised intracranial pressure, and the resultant difficulty in getting drugs into swollen brains (Carter, 1972) often means corticosteroids (e.g. dexamethazone) are often used which raises the question of reduced efficiency of amphotericin B. The drug binds to sterols and the similar lipid molecular structure of corticosteroids may induce preferential binding with these compounds as opposed to the amebae. Mandel et al (1970) found that 50 times the concentration of amphotericin B was needed to inhibit the growth of N. fowleri in the presence of dexamethazone at concentrations between 0.0025 and $0.25\mu g.cm^{-3}$. Thus, in considering the cases of Duma et al. (1971), the presence of dexamethazone may have lowered the available amphotericin B thereby not achieving effective levels. Siedel et al (pers. comm. 1978) used dexamethazone for increased intracranial pressure and diphenylhydantoin (Dilatin) for seizure activity, although the higher doses of amphotericin B administered may have overcome the antagonistic effect of the corticosteroids while still maintaining effective levels of amphotericin B.

The toxicity of amphotericin B is the most limiting factor to its effectiveness (Utz et al., 1964; Goodman and Gilman, 1975).

Adverse effects of IV treatment include chills, fever, nausea, vomiting, anemia, jaundice, phlebitis, hypokalaemia and nephrotoxicity. Heparin can be added to the infusion to combat phlebitis and alkali therapy may prevent nephrotoxicity during prolonged treatment (Goodman and Gilman, 1975). Intrathecal administration may result in pain along lumbar nerves, headache, nerve palsies, chemical memingitis and impaired vision (Avery, (edited)1976). Siedel et al. (pers. comm. 1978) reported several complications to therapy including raised serum creatine levels which may reflect amphotericin B toxicity, and severe anemia with low reticulocyte counts that were resolving at discharge.

Synthetic derivatives of amphotericin B may solve some of the toxicity problems. Amphotericin B methyl ester is a water soluble derivative of amphotericin B reported to be less toxic than the parent compound (Bonner et al., 1972; Fisher et al., 1975). Mechlinski and Schaffner (1972) reported that the ester exhibits the full antifungal activity of the parent compound. In a separate study on 465 clinical isolates of C. albicans, both amphotericin B methyl ester and amphotericin B possessed comparable activity to approximately half the strains, whereas for the remainder, the activity of the ester was slightly lower than that of the parent compound. Rarely did the ester show superior activity to amphotericin B (Gadebush et al., 1976). A dose of $5mg.kg^{-1}$ amphotericin B methyl ester given IV in primates, serum levels were 7.2 - 12.2 times higher than those resulting from $1.0mg.kg^{-1}$ amphotericin B. Visvesvara et al. (1975b) report that the ester form of amphotericin B was amebicidal for two strains of N. fowleri at a concentration of $10\mu g.cm^{-3}$.

The problems of toxicity, and the high concentrations of amphotericin B required may be circumvented by the use of drug combinations. Amphotericin B has been shown to potentiate the action of otherwise ineffective antibiotics against fungal species (Kwan et al., 1972). Huppert et al. (1974) reported effective treatment of experimental coccidiodomycosis in mice with a combination of amphotericin B and tetracycline. The effective treatment was achieved with amphotericin B at a quarter the concentration required when the drug was used alone.

Amphotericin B in combination with Rifampicin has been found to be effective at concentrations less than either drug used alone against C. albicans (Medoff et al., 1972) and Aspergillis (Kitahara et al., 1976b) in vitro, and against Histoplasma capsulatum and

Blastomyces dermatitides both in vitro and in vivo (Kitahara et al., 1976a). Rifampicin combined with amphotericin B was found to be synergistic in combination against Naegleria spp. Rifampicin at $50\mu g.cm^{-3}$ combined with $0.5\mu g.cm^{-3}$ amphotericin B reduced the time required to sterilize the media from 96 hours to 48 hours compared to amphotericin B alone (Donald, 1979). In cell culture testing, rifampicin was found to antagonize the action of amphotericin B however, due probably in part to the CTE effect of rifampicin which also increased in combination (Donald, 1979). Thong et al. (1979) investigated the effect of amphotericin B and rifampicin against N. fowleri, in vivo, in mice. Rifamycin (the parent compound of rifampicin) when injected at $150mg.kg^{-1}$ IP had no effect on the survival rate, although when combined with $2.5mg.kg^{-1}$ amphotericin B, the survival rate increased to 40%. $2.5mg.kg^{-1}$ amphotericin B given IP, only had a 10% survival rate.

A greater synergistic effect has been shown to occur between amphotericin B and tetracycline. Donald (1979) showed that $50\mu g.cm^{-3}$ tetracycline is combined with up to $0.5\mu g.cm^{-3}$ amphotericin B, no significant difference is seen in either in vitro or cell culture testing to that obtained with amphotericin B alone. However, $0.25\mu g.cm^{-3}$ amphotericin B and $100\mu gmcm^{-3}$ tetracycline was amebicidal in 96 hours and increasing the concentration of amphotericin B to $0.5\mu g.cm^{-3}$, the amebicidal time dropped to 72 hours for media sterilization. The synergistic effect was also observed in cell culture testing where $0.05\mu g.cm^{-3}$ amphotericin B combined with $50\mu g.cm^{-3}$ tetracycline completely protected the monolayer and no viable amebae were recovered. When used alone, amphotericin B was needed at a tenfold concentration ($0.25\mu g.cm^{-3}$) to do the same job (Donald, 1979).

In vivo experiments tend to support this synergistic combination. Thong et al. (1978a) tested tetracycline alone at dosages of 50, 100 and $150mg.kg^{-1}$ given IP once daily and although mortality at the highest dose was 100% the mean survival time was increased to 13.4 days compared to 7.4 days in untreated mice. 28.6% of the mice survived with amphotericin B alone at a dose of $2.5mg.kg^{-1}$, and when administered in combination with $100mg.kg^{-1}$ tetracycline or $150mg.kg^{-1}$ tetracycline with $2.5mg.kg^{-1}$ amphotericin B, the survival rates were 66.7% and 84.6% respectively. Thong et al (1979) used the combination in treating delayed PAM in mice, and initiated the treatment of $150mg.kg^{-1}.day^{-1}$ tetracycline and $2.5mg.kg^{-1}.day^{-1}$ amphotericin B (both IP) 72 hours after intranasal inoculation with

N. fowleri (Northcott strain). The survival rate was 87.5% compared to that of 37.5% when using 2.5mg.kg^{-1} amphotericin B IP alone.

Tables XV to XVIII show the results of testing the combination of tetracycline and amphotericin B against N. fowleri (MsT) and MsM) in vivo. Carter (1969) suggested a regime of 0.25mg.kg^{-1} IV and 1.0mg of amphotericin B into the cerebral ventricles within the first 24 hours after positive diagnosis. Table XVI was designed along this dosage schedule and amphotericin B was administered at 0.125mg.kg^{-1} I.Vent and 0.25mg.kg^{-1} IP which was increased to 0.25mg.kg^{-1} I.Vent and 0.25mg.kg^{-1} after 4 days. Tetracycline given IP at a concentration of 190mg.kg^{-1} increased the survival rate over the controls by 7.75 days compared to 6.0. However, when amphotericin B was combined with tetracycline, there was a 100% death rate although viable amebae were not able to be isolated from the brain.

Table XVII shows the results of testing amphotericin B given I.Vent only, along with tetracycline given both I.Vent and IP. Again results are very disappointing with no survivors and in fact the amphotericin B appears too toxic. These results show preliminary experiments with amphotericin B and, 1.5mg.kg^{-1} amphotericin B as can be seen is very toxic given I.Vent, with effects being relatively quick. The mice displayed signs of drug toxicity including trembling and diarrhoea. The average time of death was $\leq$ 5 days from initiating the treatment and no viable amebae could be found in the brain. 0.3mg.kg^{-1} amphotericin B administered I.Vent was not toxic on its own until tetracycline was administered also, after which mice died with an average time of death being 2.4 days. Tetracycline given alone did not affect the death rate compared to the controls.

It was found by experimentation that 0.2mg.kg^{-1} amphotericin B given I.Vent daily for any length of time was the highest tolerated dose. Control mice showed signs of drug toxicity in varying degrees which on cessation of the treatment gradually returned to normal over a period of 10-14 days. Table XV shows the results of administering amphotericin B (I.Vent) at doses of 0.1 and 0.2mg.kg^{-1} along with tetracycline given IP at a dosage of 145mg.kg^{-1}. Tetracycline alone, as in previous experiments, increased the survival time which however decreased from 9.5 - 8.8 days in combination with 0.1mg.kg^{-1} amphotericin B treatment. Viable amebae were isolated from post mortem brain tissue. 0.2mg.kg^{-1} amphotericin B (I.Vent) when combined with 145mg.kg^{-1} tetracycline appeared to be toxic to the mice and in a control

batch of mice (not inoculated with N. fowleri (MsM) resulted in the death of two mice, and although this regime was used to treat infected mice, viable amebae were still isolated from the brains of dead mice.

The principal reason for the lack of survivals was thought to be one of ameba virulence, and to test this theory we undertook to repeat the experiments of Thong et al. (1979) except that treatment was begun from the day of inoculation with amebae rather than 72 hours post infection as the authors had done (Table XVIII). The amebae used were N. fowleri (MsM) which is the most recent isolate, and N. fowleri (MsT) which was isolated from a human case in 1974. The drug regime - 2.5mg.kg^{-1} amphotericin B and 150mg.kg^{-1} tetracycline both given IP resulted in two survivals against N. fowleri (MsM) and an increased survival period of 10 days compared to 6.7 for that of the controls. Post mortem brain cultures showed very few limax amebae although there were a number of cysts. These cysts appeared to be non-viable and culture at 37°C for 48 hours failed to show any change. The treatment of N. fowleri (MsT) was disappointing in that the average time of death decreased to that of the control mice (6.2 and 7.0 days respectively). Post mortem brain culture, however, failed to show any amebae, only numerous cysts all of which appeared dead and again culture at 37°C failed to show any viable amebae after 48 hours. Thong et al. (1979) treated their mice for a period of seven days while in this study, the treatment went for 10 days. The mouse that died after 18 days post infection (Table XVIII) developed typical PAM symptoms on day 16 and post mortem brain culture was positive for amebae. This tends to support the idea that the drug levels were high enough only to be inhibitory in this case. N. fowleri (MsM) was not completely eliminated from the brains of mice as post mortem culture technique demonstrated whereas they were in the case of N. fowleri (MsT). This supports the belief that the concentration of drug needed to completely protect mice is directly related to virulence, which may in turn be related to time elapsed since isolation and/or passage in vivo.

The virulence of both strains were constantly kept up through cell culture and animal passage, although loss of virulence has been found by others (Chang, 1971; Duma, 1971).

Oxytetracycline was also tested for in vivo synergy with amphotericin B (Tables XIX and XX). Tablé XIX shows the results of treating mice infected with N. fowleri (MsM) using 0.1mg.kg^{-1} amphotericin B given I.Vent combined with 150mg.kg^{-1} oxytetracycline

given IP. Uninfected mice were not affected by the drug dosages and the treatment of infected mice resulted in a lengthened average time of death from 4.5 days (control) to 8.0 days. The average time of death for oxytetracycline only was also increased over that of the control mice (6.5 days). The results in Table XX are those for an experiment conducted along the lines of that performed by Thong _et al._ (1979), and parallels the _in vivo_ animal protection studies outlined by the results in Table XVIII. $2.5mg.kg^{-1}$ amphotericin B was combined with $150mg.kg^{-1}$ oxytetracycline and administered IP to mice infected with either _N. fowleri_ (MsT) or (MsM). In both classes, the treatment of the infected mice resulted in average times of death that were lower than those of the controls. Treating mice infected with _N. fowleri_ (MsM) resulted in one survival, and post mortem brain samples contained large numbers of non viable cysts and a few viable limax amebae that appeared to be suffering from drug damage. The treatment of _N. fowleri_ (MsT) however, resulted in a 100% death rate and brain cultures showed only cysts, again appearing non viable. This parallels the experiments using amphotericin B and tetracycline given IP, and again virulence of amebae is the probable cause of the low survival rate and subsequent differences in results to those of Thong _et al._ (1979). Oxytetracycline like tetracycline is adequately absorbed into the CSF, and CSF levels can be expected to be ¼ that of the plasma level. These levels rise in cases of meningitis. Tetracycline attains higher levels in the plasma than does oxytetracycline, therefore it would be expected to be able to obtain higher CSF levels with tetracycline given in the same concentration. Toxicity effects are similar with both drugs (Garrod _et al._, 1973).

In all _in vivo_ experiments, the inoculation number was 1×10^5 $amebae.cm^{-3}$ which it was hoped would ensure high brain population numbers typical of those found in advanced cases of PAM, and it is probable that this high inoculation number is a factor that must be taken into consideration when evaluating results.

5-Fluorocytosine was tested in combination with amphotericin B against _N. fowleri_ (MsM) _in vitro_ to test for possible synergistic combination. Figure 29 shows the results of these tests and it was found that a combination of amphotericin B ($0.25\mu g.cm^{-3}$) and 5-fluorocytosine ($250\mu g.cm^{-3}$) was no more active against the amebae than $0.25\mu g.cm^{-3}$ amphotericin B alone.

The imidazole group of drugs which includes drugs such as

clotrimazole, R41,400 and miconazole has been shown to be effective in the in vitro situation, against a number of pathogenic and non-pathogenic strains of Naegleria, by a number of different research groups.

Miconazole nitrate is a substituted 1-phenyl-imidazole synthesized by Janssen Pharmaceutica in Belgium and inhibits the growth of most pathogenic fungi and gram positive bacteria, but has no effect on gram negative bacteria. Its activity is due to interference with the permeability of the cell wall and to interference with mitochondrial and peroxisomal enzyme systems resulting in a build-up of peroxide (Miconazole - Systemic Use. Preclinical reports provided by Ethnor Pty. Ltd.). R41,400 is another imidazole, recently renamed ketoconazole, which is a newly synthesized member of the imidazole group of drugs which appears to share many of the properties of other imidazoles. However, it differs from other imidazoles in that it is water soluble which means there are no problems regarding the solubility of the agent when testing in vitro and also may have important clinical applications in that solvents as vehicles required to obtain stable pharmaceutical preparations are not needed (Dixon et al., 1978).

In vitro experiments with miconazole prove it to be very effective, however its effective use in vivo seems doubtful (Elmsly et al., 1980). Nagington and Richards (1976) reported a 48 hour MIC of $10\mu g.cm^{-3}$ when miconazole was tested on N. gruberi. Thong et al. (1977) showed slight inhibition of N. fowleri at 10 and $100\mu g.cm^{-3}$, while Duma and Finley (1976) reported mean MIC's for six strains of N. fowleri at 24 hours of 0.78 - $25\mu g.cm^{-3}$ and at 48 hours of 0.98 - $1.97\mu g.cm^{-3}$. Donald (1979) showed a MAC of $10\mu g.cm^{-3}$ for the non-pathogenic N. gruberi (P1200f) and a MAC of $50\mu g.cm^{-3}$ for N. fowleri (MsT). In cell culture, $5\mu g.cm^{-3}$ was needed to be amebicidal when added at the same time as the amebae, although this level was also found to be cytotoxic to the monolayer. Once a CPE had begin, $10\mu g.cm^{-3}$ was needed to halt progression of the CPE and kill all the amebae (Donald, 1979).

R41,400 reported by Janssen Pharmaceutica to be effective against superficial and systemic fungal infections, was also shown to be effective against Naegleria spp. In vitro trials showed that N. gruberi has a MIC of $50\mu g.cm^{-3}$ whereas N. fowleri (MsT) has a MIC of $10\mu g.cm^{-3}$. In cell culture, $10\mu g.cm^{-3}$ was effective in eliminating the amebae both when added at the same time as the amebae, and once a CPE had been allowed to begin. It was, however, cytotoxic to the monolayer at $10\mu g.cm^{-3}$. Donald (1979) also carried out preliminary in vivo tests against N. fowleri (MsT) in mice using R41,400 as a

treatment. Using 40mg.kg^{-1} IP, the survival time of the mice was reduced from 9.0 days in the control batch to 4.6 days in treated mice, and viable amebae were isolated from all mice subjected to autopsy. When twice daily dosing was tried (at 12 hours), the effect was the same with mice dying at 8.4 days compared to 12.5 days with the controls. This treatment consisted of 40mg.kg^{-1} per day given in two doses each 12 hours. These trials were only preliminary tests and the results of in vitro work warranted further in vivo trials in this study.

Initial experiments were performed using N. fowleri (MsM) and the drugs were given in doses 5, 10, 20, 60 and 80mg.kg^{-1} IP every day. The survival rates were zero, and the average time of death in all dosage schedules were approximately the same as for the controls (Table X). Preclinical data on R41,400 shows that serum levels in experimental animals fall rapidly within eight hours of administration (R41,400 - A New Orally-Active Antifungal Agent - Preclinical report by Janssen Pharmaceutica Pty. Ltd.), therefore twice daily dosing was tried in order to maintain effective serum levels. Table XI shows these results again the death rate being 100% with no significant deviation from that of the controls.

The same results can be seen for the IP treatment of mice with miconazole nitrate (Table XIII). Miconazole was administered IP at doses ranging from 15-120mg.kg^{-1}. 120mg.kg^{-1} resulted in the prolonged survival in one mouse of 13 days (as compared to 5.75 days in the controls) and the survival of another. However, in the control batch we also got two survivals and it seems likely that the drug was not responsible for the survival in the 120mg.kg^{-1} regime batch. Natural resistance to the infection and the added fact that mice have a tendency to cough and sneeze while intranasal inoculation is being carried out are most likely to be the cause of these survivals (Elmsly et al., 1980).

The problem of getting sufficient concentrations of either miconazole or R41,400 into the CSF appeared to be the most logical problem. At most only about 10% of miconazole serum levels are expected in the CSF (Fisher, 1978; Thong et al., 1979; Elmsly et al., 1980), which prompted the I.Vent trials. Tables XII and XIV show the results of I.Vent injection of the imidazoles into mice and in the case of miconazole, virtually no deviation from the average time of death for either the controls or the highest dosage (2.5mg.kg^{-1}) is

seen, being 5.75 and 5.8 days respectively, and viable amebae were isolated from the brains of all mice subjected to brain autopsies.

Jamieson (1975) used another imidazole - clotrimazole, in mice against N. fowleri and this also failed to protect them although it was shown to be effective against 18 strains of N. fowleri in the in vitro situation. She found that after giving her mice twice daily doses of $50mg.kg^{-1}$ orally, she had serum levels that should have been sufficiently high enough to inhibit the amebic multiplication. In order to determine the amount of drug available in the serum, we modified the method of Jamieson (1975), and the subsequent graphs showing the gradual build-up and degradation of both miconazole and R41,400 were built up (Figures 31 and 33), which tend to support present information. For miconazole nitrate, a maximum level of $45\mu g.cm^{-3}$ was found after 4 hours of injecting $60mg.kg^{-1}$ IP. This decreased to approximately $2.0\mu g.cm^{-3}$ after 6 hours. R41,400 peaked at $40\mu g.cm^{-3}$ after 4 hours and was after 6 hours still relatively high at $13\mu g.cm^{-3}$. Therefore, twice daily IP dosing would have kept the serum levels elevated, and the serum levels achieved in mice in the first 8 hours should have been sufficiently high to inhibit amebic multiplication. In the well vascularized area of the nasal mucosa, where amebae are found three days after intranasal inoculation, there would have been ample exposure to amebicidal levels of either R41,400 or miconazole, especially so when twice daily doses are given (Jamieson, 1975; Elmsly et al., 1980). However, in the human situation patients would be unlikely to receive any treatment until at least 72 hours after infection, by which time the amebae would have reached the meninges and the brain. For miconazole, the highest dosage for human infections recommended by the manufacturers is $3600mg.day^{-1}$ ($54mg.kg^{-1}$) given in three IV infusions. In cases of fungal meningitis, IT therapy is recommended at a dose of 20mg. In the case reported by Siedel et al (pers. comm.) miconazole was one of the drugs used. The doses employed were $350mg.m^{-2}.day^{-1}$ given IV three times daily for nine days and 10mg IT for two days decreasing to 10mg every other day for eight days. The role miconazole played in the survival of this case is therefore doubtful, although latest reports describe a possible synergistic activity in combination with amphotericin B (Thong et al., 1979).

Therefore to date, the only drug effective in the treatment of PAM is amphotericin B and although the results of this study tend not

to support this, no doubt it should be administered immediately the diagnosis is made, and at the highest dose possible. The simultaneous administration of tetracycline would also be recommended. The problem of host susceptibility, amebae virulence and related factors should be remembered, and could be the reasons why amphotericin B although administered in the correct doses at an early enough stage has usually failed to bring about a cure.

5.2 Treatment of Acanthamoeba Infections

The treatment of Acanthamoeba infections has to date been far from successful (Kerrohan *et al.*, 1960; Jager and Stamm, 1972; Robert and Rorke, 1973; Nagington *et al.*, 1974; Bhagwandeen *et al.*, 1975; Martinez *et al.*, 1977). Except in the case of eye infections (Nagington *et al.*, 1974, 1976) the main problem in Acanthamoeba infections is the difficulty of diagnosis and in the recorded cases of Acanthamoeba Meningitis (AM) all have been diagnosed post mortem. Even in advanced cases, there is often a lack of specific signs and symptoms indicative of the disease and no amebae are seen in the CSF (Chang, 1974a).

Culbertson *et al.* (1965) showed that sulphadiazine was effective in protecting mice against *A. culbertsoni*. When 10 mice were inoculated intranasally with 500 amebae, all were dead within seven days. However, sulphadiazine incorporated into the diet at dosages of 4, 8 and $12mg.day^{-1}$ resulted in 100% survival, the treatment beginning at the day of infection. When treatment was begun 72 hours after infection $15mg.kg^{-1}$ resulted in 90% survival, which however dropped to 70% survival when treatment was begun 96 hours post infection.

In 1972, Carter suggested that sulphadiazine be used in addition to amphotericin B in the treatment of PAM in case the causative agent should occasionally prove to be *Acanthamoeba* sp. This could have possibly been the causative agent in the cases reported by Apley *et al.* (1970) in which the infection was assumed to be due to *Naegleria* sp. and

amphotericin B as well as sulphadiazine was administered. It was argued by many (Stamm, 1974; Griffin, 1976; Warhurst et al., 1970) that the amebae were Acanthamoeba and survival was attributed to sulphadiazine therapy. To date, however, the only clinical experience with sulphadiazine in the treatment of humans is that reported by Nagington et al. (1974) where it was used to treat an eye infection (case 1). Sulphadiazine was given at a dose of 500mg every 6 hours, but the infection did not respond to treatment. However, 6 months had passed before the first symptoms were noted and treatment begun, therefore, the infection was in an advanced stage.

Amphotericin B has been shown to be ineffective against Acanthamoeba sp. at levels attainable in humans (Duma and Finley, 1976; Visvesvara and Balamuth, 1975; Casemore, 1970). However, a report by Nagington and Richards (1976) gives a MIC of $1\mu g.cm^{-3}$ for A. polyphaga and A. castellanii isolated from cases of eye infections, using an inoculum size of 5×10^3 $amebae.cm^{-3}$. Donald (1979) found that although A. castellanii (1501) was not susceptible to levels $\leqslant 100\mu g.cm^{-3}$, $100\mu g.cm^{-3}$ was amebicidal for A. culbertsoni (A-1).

5-Fluorocytosine, which has no activity against Naegleria sp. has been shown to be active against Acanthamoeba sp. Casemore (1970) reported an amebicidal concentration of $100\mu g.cm^{-3}$ and inhibition in the range of $12.5 - 50\mu g.cm^{-3}$ against six strains of A. castellanii. Das found that $200\mu g.cm^{-3}$ was needed to be amebicidal for A. culbertsoni. Jones et al (1975) studied the effect of 5-fluorocytosine on A. polyphaga isolated from a human eye infection and found a MIC of $25\mu g.cm^{-3}$ and a MAC of $100\mu g.cm^{-3}$ for an inoculum size of 5×10^4 $amebae.cm^{-3}$. Donald (1979) found both A. castellanii (1501) and A. culbertsoni (A-1) susceptible to 5-fluorocytosine, and for the levels tested, $10\text{-}250\mu g.cm^{-3}$, there was inhibition although all concentrations produced the same decrease in numbers. In this study, the results were found to be essentially the same although the decrease in amebae numbers were not as great after the 96 hour incubation period, and in all concentrations tested ($50 - 500\mu g.cm^{-3}$) there was an initial period of growth followed by a decrease in amebae numbers (Figure 20).

In cell culture testing, Donald (1979) showed that $250\mu g.cm^{-3}$ added at the same time as the amebae was amebicidal and $50\mu g.cm^{-3}$ although not amebicidal, inhibited the formation of a CPE by A. culbertsoni (A-1). However, once a CPE had been allowed to develop before adding the drug, no concentration tested was able to halt its progression. This has been confirmed by in vivo testing by Stevens and

O'Dell (1974). Mice inoculated with A. culbertsoni (A-1) and receiving 3mg (200mg.kg^{-1}) from the day of infection, showed a significant increase in survival over the controls. However, when drug treatment was begun 24 hours post infection, no significant difference in the time or rate of death was obtained. From all dead infected mice, the authors were able to isolate numerous viable amebae and they believe that the ineffectiveness of the drug was due to the amebae being able to develop a resistance, probably by way of a change in the structure of the cell membrane. This phenomenon has also been found in fungi which have developed a resistance to 5-fluorocytosine (Shadomy, 1970; Holt and Newman, 1973).

5-Fluorocytosine has low toxicity and is well absorbed from the gastrointestinal tract. The recommended dosage is 150mg.kg^{-1}.day^{-1} given 6 hourly, with CSF levels obtained through IV or oral administration being good at levels up to 50% of those found in the serum. At a daily dose of 140mg.kg^{-1} given orally, serum levels two hours after administration ranged from 65-82μg.cm^{-3} with CSF levels ranging from 40-62μg.cm^{-3} (Block and Bennett, 1972; Shadomy et al., 1969). The results of in vivo experimentation can be seen in Tables XXI and XXII. Given IP, a dose of 125mg.kg^{-1} failed to change the rate and course of the disease, but at a dose of 250mg.kg^{-1} one mouse had a prolonged survival time of 13 days and one completely survived the treatment. However, given I.Vent, the results were not as expected, and all the dosages tried (1.0, 2.5 and 5.0mg.kg^{-1}) failed to alter the course of the disease as compared to the controls. In all mice subjected to post mortem studies, viable amebae could be isolated from the brain. Stevens and O'Dell (1974) used 5-fluorocytosine in vivo against two strains of A. culbertsoni, A-1 and A-3 and the results are comparable to the ones obtained through this study. The A-3 strain had been subjected to animal passage before trials began and as a result of this increased virulence, all the control mice died as well as those given either 100mg.kg^{-1} or 200mg.kg^{-1} 5-fluorocytosine, with no deviation in death rate from those of the control. However, in using strain A-1 which had not been subjected to animal passage, 100μg.cm^{-3} resulted in seven out of eleven survivors. A-3 was obtained by animal passage of the A-1 strain, therefore, they were essentially the same species except for the virulence factor; and, therefore, the survival rate was attributed to this virulence and the fact that A-3 could develop a higher resistance to the drug.

These experiments tend to point to the fact that 5-fluorocytosine may be of limited use in human Acanthamoeba infections, especially with the added problem that treatment would not be initiated until some time after infection (Stevens and O'Dell, 1974).

Synergistic combinations, found valuable in treating Naegleria infections have also been tested against Acanthamoeba sp. Polymyxin B sulphate was found by Nagington and Richards (1976) to be inhibitory against A. polyphaga and A. castellanii at concentrations of $10\mu g.cm^{-3}$ when an inoculum of 5×10^3 amebae was used. The effectiveness of polymyxin B sulphate was also shown by Duma and Finley (1976), who found that two strains of A. castellanii and three strains of A. polyphaga were inhibited by concentrations ranging from 50 to $100\mu g.cm^{-3}$. Donald (1979) showed both A. castellanii (1501) and A. culbertsoni (A-1) to be only slightly inhibited by concentrations up to $63.5\mu g.cm^{-3}$ in axenic culture. In cell culture tests, polymyxin B sulphate had no effect on the amebae or on the formation of a CPE when used at a concentration of 500 $units.cm^{-3}$ ($63.5\mu g.cm^{-3}$). However, in synergistic combination with 5-fluorocytosine, there was increased activity in both axenic and cell culture against A. culbertsoni (A-1) Polymyxin B at a concentration of 500 $units.cm^{-3}$ with 5-fluorocytosine at either 50 or $100\mu g.cm^{-3}$ resulted in axenic culture media sterilization within 72 hours, and in cell culture testing, 5-fluorocytosine at a concentration of $100\mu g.cm^{-3}$ although not amebicidal on its own, was when combined with 100 $units.cm^{-3}$ polymyxin B (Donald, 1979).

This showed the need for further in vivo experimentation which was performed in this study. Polymyxin B is nephrotoxic and neurotoxic (Newton, 1956) with peak serum levels obtained after an IV dose of 60mg being $1.5\mu g.cm^{-3}$. Penetration into the CSF, however, is poor (Garrod et al., 1973) and IT administration is recommended at dosages of 10,000 -50,000 units (1.25-6.25mg) given once daily. Table XXIII shows the results of treating mice intranasally infected with A. culbertsoni (A-1) with polymyxin B given I.Vent. The dosage of $0.087mg.kg^{-1}$ corresponds to the recommended dose in adults of 6mg given IT. The average time of death was unaltered in all the dosages tested from that of the controls, and viable amebae were isolated from brain tissue of all dead mice. This result was not surprising since polymyxin B in cell culture tests failed to either kill the amebae or prevent CPE (Donald, 1979).

The synergistic combination of polymyxin B and 5-fluorocytosine also failed to protect mice from the effects of A. culbertsoni (A-1)

(Table XXIV). Both polymyxin B and 5-fluorocytosine were administered I.Vent and although the highest dosages of $5mg.kg^{-1}$ 5-fluorocytosine and 0.083 $mg.kg^{-1}$ polymyxin B increased the average time of death from 4.25 days (controls) to 5.5 days, the result was far from encouraging. Again, viable amebae were isolated from the brains of dead mice. The combination of these drugs may, however, show promise in Acanthamoeba eye infections. 5-fluorocytosine has been used in treating mycotic surface infections of the eye (Garrod et al., 1973) applied topically at 1-1.5% concentration. Polymyxin B, as an ophthalmic solution containing 20,000 $units.cm^{-3}$ instilled 2-10 times per hour are recommended for bacterial eye infections (Goodman and Gilman, 1975) and can also be given by subconjunctivial injection. This combination has been used in the treatment of a case of A. polyphaga keratitis (Jones et al., 1975) and although polymyxin B at 20,000 $units.cm^{-3}$ was used with 1% 5-fluorocytosine (topically), there was no curative result. 5-fluorocytosine was also given orally at a dose of $150mg.kg^{-1}.day^{-1}$. However, the treatment was not initiated until one year after the infection had begun and was therefore at a very advanced stage.

The combination of 5-fluorocytosine and amphotericin B has been shown to be synergistically active against fungi (Medoff et al., 1972; Arroyo et al., 1977), so was tested against A. culbertsoni (A-1) in vitro in order to see if amphotericin B would reinforce the already proven 5-fluorocytosine (Figure 29). The results show no signs of synergism, and $0.25\mu g.cm^{-3}$ amphotericin B combined with $250\mu g.cm^{-3}$ 5-fluorocytosine showed a decrease in numbers that was no different to using $250\mu g.cm^{-3}$ 5-fluorocytosine alone.

The antibiotics kanamycin, tylosine and oxytetracycline were also tested against A. castellanii (1501) and A. culbertsoni (A-1) in axenic culture (Figures 12-17). Kanamycin although tested in concentrations as high as $1000\mu g.cm^{-3}$ failed to reduce the numbers of amebae, although at concentrations $\geq 500\mu g.cm^{-3}$, the amebae showed signs of drug damage. Tylosine showed the same effect with the non-pathogenic A. castellanii (1501) appearing to be more sensitive to tylosine. A. culbertsoni (A-1) was the only species to show signs of drug damage and took the form of becoming highly vacuolated, smaller, and rounder at concentrations $\geq 500\mu g.cm^{-3}$. Tables 16 and 17 show the effect of oxytetracycline on A. castellanii (1501) and A. culbertsoni (A-1). The non-pathogenic A. castellanii appeared more sensitive to the drug although none of the amebae showed signs of drug damage. A. culbertsoni (A-1) although not as sensitive to the drug showed signs of drug damage

at $\geq$250µg.cm^{-3}. They became rounded, with an absence of acanthapodia although their size remained normal.

In human cases, infections due to Acanthamoeba sp. have occasionally appeared to occur in the development of impaired host immune responses, specifically in patients with alcoholism problems (Patras et al., 1966), diabetes mellitis (Duma et al., 1978) and Hodgkins disease (Jager et al., 1972) and it was also shown by Markowitz et al. (1978) that treatment with drugs such as methyl-prednistone (a corticosteroid) or tetracycline increases the infection rate in mice to A. castellanii. Tetracycline is known to interfere with leucocyte migration (Martin et al., 1974) and also the capacity of neutrophils to phagocytose yeast and bacteria (Forsgren et al., 1974). Therefore, it seems possible that the treatment of Acanthamoeba infections with drugs that restore immune deficiencies may show promise. Levamisole, a widely used anthelmintic is known to have an immuno-modulating effect, especially on the depressed cell mediated immune response (Verhaegen et al., 1973; Fischer et al., 1975), and several authors have described clinical applications of this effect to conditions known to be due to an imbalance or suppression of the cell mediated immune response (Perk et al., 1975; Fegies et al., 1977). Toxoplasma gardii infections in mice affect both the anatomy and function of the thymus with additional interference on the migration and differentiation of thymocytes. Levamisole, either alone or in combination with sulpha-methoxazole-trimetoprin treatment, induced a great increase and normalization of the percentages of T-lymphocytes and a consequent enhancement of the cellular immune responses with depression of the antitoxoplasma antibody titres (Fegies et al., 1977). Figures 18 and 19 show the effect of levamisole on A. castellanii (1501) and A. culbertsoni (A-1). A. castellanii (1501) was sensitive to the drug and at concentrations $\geq$250µg.cm^{-3} the amebae displayed signs of drug damage. They were more rounded and highly vacuolated with loss of acanthapodia. However, the effect on A. culbertsoni (A-1) was unexpected and 500µg.cm^{-3} although showing a decrease in numbers when used against A. castellanii (1501) was not active against the pathogen. The amebae showed no signs of drug damage and all appeared normal even at the highest concentration tested (500µg.cm^{-3}).

In cell culture, levamisole failed to be amebicidal although when added at the same time as the amebae, 250µg.cm^{-3} protected the monolayer and prevented the destruction of the Vero cells (Table IX). When added after a CPE had developed, the highest concentration tested

($500 \mu g.cm^{-3}$) had no effect either on the CPE or the amebae.

This variation in activity of drugs against A. culbertsoni (A-1) and A. castellanii (1501) was also reported by Donald (1979). Amphotericin B was shown to be amebicidal for the pathogen A. culbertsoni (A-1) at $100 \mu g.cm^{-3}$, whereas the non-pathogenic A. castellanii (1501) was not affected by $\leqslant 100 \mu g.cm^{-3}$. The variation in results found in the various studies and between different species and strains of amebae is puzzling and probably reflects a different sterol concentration and makeup in the cell membrane. Although levamisole is not active against A. culbertsoni (A-1) it is worth further investigation in the in vivo situation in order to test the possibility of a depressed immune response.

Levamisole was also used to test the problem of using aged parent cultures as the inoculum in axenic culture drug tests, where it was thought the age of the culture used may reflect the activity of the drug. Figures 18, 23 and 24 show the results of testing levamisole against 24, 48 and 96 hour parent cultures of A. castellanii (1501), and as shown, the results are all comparable, thereby concluding that the age of the parent culture whether in lag or log phase does not affect the results of the drug.

5.3 Drug dosage and Animal Size

When administering a drug it is necessary to ensure that the doses necessary for the control of the infecting agent will not produce toxic effects worse than those of the disease. One of the major problems in the present in vivo studies was conversion of drug dosages from those used in human cases to those for use in mice for both Naegleria and Acanthamoeba infections. Here, the drug dose was expressed as $mg.kg^{-1}$ body weight and adjusted in direct proportion to the respective weights of the mice. However, as long ago as 1830, Hufeland suggested that a better adjustment for man would be to give doses that are proportional to surface area and in 1909, Moore arguing from results of experiments with anti-tryparosomal drugs in mice and donkeys made the same recommendations. He pointed out that

relative surface area is important with regards to maintaining an even body temperature, and in order to maintain the relative greater loss of heat, small animals have higher metabolic respiration and pulse rates and eat relatively more food than the larger animals. Butler and Richie (1960) stated that this is also supported by clinical experience where an adult dose is adjusted for children on a weight basis and it happens that the children are underdosed, and vice versa where a child dose is adjusted from an adult, the adult is overdosed. One must also consider when injecting a drug into small animals, such as mice, the relatively large volumes they must endure.

Pelikan (1960) stated in summary to his work that although the surface-area relationship is probably preferable to that of weight for making an intra-species adjustment of dose for the differences in sizes, other relationships will often apply so that the final adjustments for any particular drug can only be made by direct observation.

Another problem was that of actual drug administration. I.Vent injection of drugs was tried firstly because the infection was localized in the brain area and secondly because the doses required to produce an effect by this mode of administration are in general much less than those required by other routes (Feldberg and Sherwood 1953a, b, 1954a, b, 1955; Haley and McCormich, 1957; Brittain, 1966). However, it was found that there are drug losses into the immediate area surrounding the infection site and into the peripheral brain tissue. Numerous authors have previously reported that absorption into the blood stream takes place after I.Vent injection of histamine or adrenaline and has been attributed to the circulation of the injected material into the subarachroid space and its absorption by blood vessels or the arachnoid villi (Bedford, 1953; Bhawe, 1958; Draskoci et al., 1960). Cairns (1950) has shown that following the injection of 0.03cm^3 of fluid into the subcortical tissue, only 2-8% is recovered in the brain, and it was suggested that the high injection pressure, associated with injections made into the tissue, results in the rupture of the arachnoid villi allowing passage of material into the sagittal sinus (Mims, 1960). Therefore it would seem likely that losses of material injected directly into the cerebral ventricles of mice (I.Vent) could be explained in the same way, since the injection volume traditionally used (0.02cm^3) is large in relation to the volume of fluid likely to be present in the ventricular system of such a small

animal. Cairns (1950) found that reducing the volume to 0.005cm^3 did not significantly reduce the overspill into the bloodstream. It would seem reasonable to assume that with direct I.Vent injection, there would be a greater opportunity for the injected material to leak back along the needle track, than there would be, if say a canula was implanted a week or so before injection. Shaw et al (1974) found that leakages were shown up by the presence of injected material in the subcutaneous tissue overlying the skull and is probably the route by which most of the misplaced material travels, since it is the shortest route to the subarachnoid space.

An obvious implication of these findings is that caution is needed in interpreting the effects of direct I.Vent injections since in some circumstances, entry of the injected material into the periphery could be a complicating factor and may explain the poor results obtained. This is especially so when using the synergistic compounds amphotericin B and, tetracycline or oxytetracycline against Naegleria sp. or, polymyxin B against Acanthamoeba sp. Atkinson et al. (1969) showed that amphotericin B given intrathecally to an adult volunteer was removed from theCSF by bulk flow through the arachnoid villi. Patients treated by intrathecal injection of amphotericin B have not fared significantly better than those who have received intravenous amphotericin B treatment alone (Butler, et al., 1964), therefore, it seems that in the treatment of Naegleria infections, at least, amphotericin B therapy should be confined mainly to IV injections. Results of IP inoculation of amphotericin B and either tetracycline or oxytetracycline were significantly improved over direct I.Vent injection methods and it seems possible that with I.Vent injection methods, the drug levels of the CSF were not as high as expected. In the light of this evidence, further work is needed in the field of drug dosage and in increasing the concentration of drugs in the CSF; direct I.Vent injection having not proved as effective as expected.

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