THE SUSCEPTIBILITY OF PATHOGENIC FREE-LIVING AMEBAE TO CHEMOTHERAPEUTIC AGENTS

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Jennifer Jane Donald

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ABSTRACT

The treatment of infections caused by pathogenic free-living amebae (PFLA) has, until only recently, been far from successful. The continued screening of chemotherapeutic agents against amebae of the genera Naegleria and Acanthamoeba is therefore of the utmost importance.

Seven chemotherapeutic agents, amphotericin B, rifampicin, tetracycline, polymyxin B sulphate, 5-fluorocytosine, miconazole and R41,400 were screened for activity against a non-pathogenic and pathogenic species of Naegleria and a non-pathogenic and pathogenic species of Acanthamoeba in axenic culture. For the Naegleria spp. amphotericin B, miconazole and R41,400 were found to be active. Acanthamoebae spp. were found to be susceptible only to 5-fluorocytosine and R41,400.

The possible use of combinations of drugs against the amebae was also investigated in axenic culture. For Naegleria fowleri (MsT) amphotericin B with either tetracycline or rifampicin showed a synergistic effect. Polymyxin B sulphate and 5-fluorocytosine showed synergistic activity against Acanthamoeba culbertsoni (A-1) but when polymyxin B was combined with tetracycline or rifampicin no significant additive effect was seen.

After axenic culture testing the susceptibility of the pathogenic species, N. fowleri (MsT) and A. culbertsoni (A-1), to the agents which showed activity, was investigated in a Vero cell culture system. For N. fowleri (MsT) the results of axenic testing were confirmed with amphotericin B, miconazole and R41,400 protecting the monolayer from the destructive effects of the amebae. 5-Fluorocytosine inhibited the formation of cytopathic effect (CPE) when the cell cultures were inoculated with A. culbertsoni (A-1) but viable amebae were still present. R41,400 had no effect on A. culbertsoni (A-1) at concentrations at or above those which were cytotoxic to the Vero cells.

The use of combinations of drugs was also investigated in Vero cell culture. Amphotericin B and rifampicin showed an antagonistic rather than a synergistic effect when used against N. fowleri (MsT) in cell culture but amphotericin B and tetracycline showed synergistic activity.

For A. culbertsoni (A-1) the synergistic activity of polymyxin B and 5-fluorocytosine was confirmed. The lack of an additive effect

between polymyxin B and either tetracycline or rifampicin was also shown in cell culture.

The new imidazole derivative R41,400, which showed promise against N. fowleri (MsT) in in vitro tests was then tested in the in vivo situation. Mice experimentally infected with N. fowleri (MsT) were treated once or twice daily intraperitoneally with different doses of R41,400. At the higher dosage levels tested the drug appeared to have a deleterious effect, the average time for death being less than that for the controls.

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CHAPTER ONE: INTRODUCTION

1.1. The History of 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 & Brown, 1976).

The commonest disease caused by PFLA is known as Primary Amebic Meningo-encephalitis (PAM) and in an extensive worldwide survey, Willaert (1974) tabulated 84 cases from all continents with the exception of Antarctica. Since then at least ten additional cases have been reported.

Prior to 1968, most cases were attributed to Acanthamoeba spp.. This is probably a reflection of the pioneering work of Culbertson et al. (1958, 1959, 1965) whom, whilst working on the production of polio vaccine, found an ameba which contaminated the cultures of monkey kidney cells. When the cultures were inoculated intracerebrally into mice and primates, a necrotizing, hemorrhagic meningo-encephalitis was produced that killed the animals in four to seven days. The responsible ameba was identified as an Acanthamoeba and they predicted, on the basis of this finding, that this ameba could be capable of producing disease in humans. This ameba was previously considered to be a harmless, free-living ameba.

However, in 1968, Butt et al., Carter and Culbertson et al., showed that the incriminating species of most reported human cases belonged to the related genus Naegleria. In 1970, on the basis of morphological, cultural and pathogenicity differences, Carter named the pathogenic species Naegleria fowleri distinguishing it from the non-pathogenic Naegleria gruberi.

The disease caused by PFLA can be divided into two types:

i) a swimming - associated acute meningo-encephalitis (Primary Amebic Meningo-encephalitis (PAM) - Martinez et al.(1977) caused by N. fowleri.

and ii) a non-swimming - associated chronic meningo-encephalitis (Amebic Meningo-encephalitis (AM) - being regarded as a secondary invasion of the central nervous system (CNS) having spread

from other foci of infection (Martinez et al., 1977)) caused by a variety of pathogenic Acanthamoebae, notably Acanthamoeba culbertsoni, Acanthamoeba castellanii and Acanthamoeba polyphaga (Chang, 1974a).

Subsequently, Acanthamoeba spp. have also been indicated in a number of chronic illnesses such as respiratory infections (Martinez et al., 1975), corneal ulceration of the eye resulting in blindness (Nagington et al., 1974; Visvesvara et al., 1975) and together with Naegleria spp. in humidifier fever (M.R.C. Symposium, 1977).

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

The controversy regarding the classification of PFLA (Cursons & Brown, 1976) appears to be settled with the majority of authors 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 & Brown, 1976).

1.2 Occurence and Distribution

The summary of recorded isolations from a wide variety of environmental sources provided by Cursons (1978) emphasizes the truly ubiquitous distribution of PFLA. The ability of PFLA to form resistant cysts undoubtedly enables them not only to withstand unfavourable conditions, e.g., the isolation of pathogenic Acanthamoebae from $2^{o}C$ (Brown & Cursons, 1977), but also to take advantage of the intermittent occurrence of favourable conditions.

The distribution of the pathogenic species in relation to non-pathogenic ones is still unknown (Cursons, 1978). In general, non-pathogenic species are more prevalent at ambient temperatures in temperate zones. The repeated isolations of PFLA from water above ambient temperature, i.e., $\geqslant 30^{o}C$ (De Jonckheere et al., 1977; De Jonckheere & Van De Voorde, 1977a; Stevens et al., 1977; Wellings et al., 1977; Cursons et al., 1978b), combined with their higher optimum temperature of growth (Griffin, 1972) suggests that pathogenic amebae are environmentally selected over non-pathogenic amebae in

waters above ambient temperature. The source of pathogenic amebae in these waters is unknown but as Cursons et al. (1978b) and Wellings et al. (1977) have succeeded in isolating PFLA from soil it is possible that soil acts as a reservoir of pathogens and contamination occurs via run-off after rain (Cursons, 1978).

1.3. Pathogenicity

The invasion of organs and tissues by PFLA is now well documented (Culbertson et al., 1959, 1968, 1972; Carter, 1968, 1970, 1972; Callicott et al., 1968; Chang, 1971, 1974a & b, 1976; Culbertson, 1971; Martinez et al., 1973, 1975, 1977; Visvesvara & Balamuth, 1975; Wong et al., 1975a & b; Hoffman et al., 1978). It has been established experimentally that the portal of entry into the CNS in N. fowleri infection is via disruption of the olfactory mucosa, penetration of the organisms into the submucosal nervous plexus, probably by phagocytosis of the amebas by the sustentacular cells of the olfactory neuroepithelium and passage through the cribiform plate to the subarachnoid space (Martinez et al., 1973).

However, in cases of Acanthamoeba meningo-encephalitis the involvement of the CNS appears to be a secondary phenomenon representing metastatic spread from a primary focus in the skin, genitourinary or respiratory tract (Martinez et al., 1977). Cutaneous ulceration as a possible point of entrance with hematogenous spread to the CNS was reported by Bhagwandeen et al. (1975) and Martinez et al. (1977) report involvement of the genitourinary tract. Lower respiratory tract infection in experimental animals have been reported (Martinez et al., 1975).

AM due to Acanthamoeba spp. appears to be an opportunistic infection of the CNS. AM occurs in patients who are chronically ill, debilitated or those whose cell-mediated immune responses have been impaired as a result of either underlying systemic disease or its treatment by immunosuppresive methods (Kernohan et al., 1960; Jager & Stamm, 1972; Robert & Rorke, 1973; Sotelo-Avila et al., 1974; Bhagwandeen et al., 1975).

Acanthamoeba infections of sites with reduced accessibility to the immune system e.g., the eye also demonstrate the opportunistic nature of these infections. Isolates from the cases reported by Nagington et al. (1974) were shown by Visvesvara et al. (1975) to be

of low virulence and infection resulted after damage to the cornea.

Once invasion of the brain has been established in PAM and AM, destruction of surrounding brain 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 & Callaway, 1974; Maitra et al., 1974, 1976).

The examination of sections from brains infected with either N. fowleri or A. culbertsoni reveals areas of extensive demyelination leaving the trophozoites surrounded by a clear halo (Martinez et al., 1975; Chang, 1976; Maitra et al., 1976). Many authors have speculated on the possibility that enzymes secreted by the amebae are responsible for this. Experiments have shown that both cytotoxic enzymes and phospholipases are produced by PFLA (Elson et al., 1970; Chang, 1971, 1974a, 1976; Hax et al., 1974; Visvesvara & Callaway, 1974; Victoria & Korn, 1975; Visvesvara & Ballamuth, 1975; Cursons & Brown, 1976, 1978; Cursons et al., 1978c; Maitra et al., 1976). The level of production of such cytopathic enzymes may explain the differences in virulence reported amongst Acanthamoeba and N. fowleri isolates (Culbertson, 1971; De Jonckheere & Van De Voorde, 1977b).

1.4. Immunity

The low incidence of PAM and AM in the human population has puzzled many authors in view of the ease and frequency of isolation of virulent PFLA from the environment (Anderson & Jamieson, 1972; Cursons et al., 1976b, 1977; John et al., 1977; Wellings et al., 1977; Haggerty & John, 1978). This has led to speculation on the existence of host related susceptibility factors and the demonstration of specific antibodies to free-living amebae in human sera has been reported (Chang & Owens, 1964; Edwards et al., 1976; Cursons et al., 1977; M.R.C. Symposium, 1977).

Observations that previous exposure of mice to live N. gruberi significantly protected them against a subsequent lethal challenge with N. fowleri (John et al., 1977) supports the idea that unwitting exposure to the ubiquitous N. gruberi may immunize against N. fowleri. A similar immunization may also occur with Acanthamoeba. Cell-mediated immunity (CMI) also appears to play an important part in protection against PFLA (Diffley et al., 1976; Cursons et al., 1977).

1.5. Diagnosis

Successful treatment of this rapidly progressive disease is wholly dependent on prompt and definitive diagnosis. The survival of a nine year old female in Torrance, California (Seidel et al., pers. comm., 1978) and a fourteen year old male in Australia (Anderson & Jamieson, 1972) is attributed to early diagnosis and prompt treatment.

A Naegleria brain infection should be suspected when there is a history of swimming about seven days prior to the abrupt onset of fever, headache, sore-throat, nausea and vomiting (Carter, 1972; Chang, 1974a). The most important laboratory procedure for the diagnosis of PAM is the microscopic examination of cerebro-spinal fluid (CSF). Overall, the CSF is indistinguishable from that obtained from patients with bacterial meningitis and diagnosis relies upon amebae being seen in the fluid and the culture of these for complete diagnosis. Species identification can then be achieved by the method outlined by Cursons & Brown (1976).

In post-mortem diagnosis, a degree of encephalitis is invariably present. The brain shows swelling and redness with the purulent exudate more extensive on the ventral surface of the cerebrum or cerebellum and over the brain stem (Carter, 1972). The grey matter of the cerebral hemispheres and cerebellum shows variable sized lesions which tend to be hemorrhagic and quite soft when they are large (Culbertson, 1971). The existence of redness and destruction of the olfactory nerve occurs only in PAM and could serve to distinguish it from bacterial meningitis (Carter, 1972).

The immunofluorescent antibody (IFAB) technique applied to histologic brain sections taken post-mortem, is a valuable tool in identification of amebae in brains of patients who died from meningo-encephalitis. Antisera can be produced in rabbits and can be made species-specific by suitable absorption. In addition to their value in clinical diagnosis IFAB provide rapid screening methods for the detection of PFLA in swimming pool, tap and other domestic and recreational water supplies.

Immunoperoxidase methods have been used by Culbertson (1975) and Cursons et al. (1976) to demonstrate both Naegleria and Acanthamoeba in the brain sections of patients who died from PAM and AM respectively. This method may be shown in the future to be more valuable than the immunofluorescent technique. It has certain advantages over IFAB, e.g.,

permanent preparations can be made, no specialized equipment is necessary and clear, definitive staining of the tissue elements results (Culbertson, 1975).

Acanthamoeba brain infections are difficult to diagnose even in advanced cases due to lack of specific symptoms and signs and the absence of amebae in the CSF (Chang, 1974a). Nasal and throat swabs may provide more information.

Post-mortem diagnosis relies on the presence of confined, superficial lesions in the grey matter with a minimum inflammatory reaction, and the finding of double-walled wrinkled cysts in apparently normal tissue bordering the lesion (Chang, 1974a). In all the reported cases, except that reported by Bhagwandeen et al. (1975), there was lack of evidence of the involvement of the olfactory bulb and the absence of inflammatory reactions in the surrounding grey and white matter. These observations may help in distinguishing between PAM and AM.

Positive diagnosis was possible in the cases of eye infections reported by Nagington et al. (1974) and Jones et al. (1975) by isolation, and subsequent identification of Acanthamoebae spp. taken from corneal scrapings.

1.6. Control Measures

The necessity for an effective disinfectant can be judged by the increasing number of isolations of free-living amebae from potable and treated and untreated recreational waters (Cerva, 1971a; Chang, 1971; Anderson & Jamieson, 1972; Cerva & Huldt, 1974; Molet et al., 1976; Lyons & Kapur, 1977). The majority of amebae isolated in these studies belonged to the genus Acanthamoeba indicating its greater resistance to chlorine than Naegleria spp..

In reviewing sixteen fatal cases of PAM from an indoor chlorinated swimming pool Cerva (1971a) stated that, "it appears that the constant presence of numerous populations of amebae of the limax group cannot be prevented even under the strictest observations of all routine safety measures applied to potable waters." However, Lyons & Kapur (1977) in a survey of 30 halogenated public swimming pools concluded that the low amebic densities ($< 1.1^{-1}$) in the majority of the pools illustrated that these organisms could be adequately controlled by proper pool maintainence. The possession of resistant cysts, however, constantly compli-

cates the disinfection process.

In a study of alternative disinfectants, Cursons et al. (1978b) found that deciquam 222, chlorine, chlorine dioxide and ozone all possessed potential disinfecting properties for PFLA, but at higher levels than those for disinfecting bacteria. Of the four disinfectants examined, deciquam 222 proved to be the most effective amebicide followed by chlorine, chlorine dioxide and ozone. The final choice of a particular disinfectant must however, remain tied to the physical and chemical properties of the water to be disinfected.

1.7. PAM Cases and Their Treatment

In 1974, Willaert provided an extensive worldwide survey of cases of PAM. Since then at least ten additional cases have been reported (Table I). Conceivably, the actual number of cases may be higher since the symptoms of PAM parallel those of aseptic meningitis. Retrospective studies have disclosed a possible case dating back to 1909 (Symmers, 1969) and fluorescent antibody staining has confirmed that the 1948 case reported by Derrick, originally thought to be due to Iodamoeba butschlii was in fact caused by N. fowleri (McMillan, 1977). The reidentification of the etiological agents of the 1968 cases of PAM in New Zealand as N. fowleri (Cursons & Brown, 1975; Cursons et al., 1976a) has dismissed the notion of slime moulds being involved in the etiology of PAM (Mandal et al., 1970).

The results of treatment of PAM have been far from encouraging. Willaert's summary (1974) provides information on ten possible survivors of PAM and the Californian case of Seidel et al. (pers. comm., 1978) makes the world total eleven survivors (Table II). Such a result is hardly surprising in the earlier cases, where the amebic nature of the disease had not been suspected, and treatment consisted only of antibacterial agents such as sulpha-drugs, penicillin, streptomycin, tetracyclines and chloromphenicol (Fowler & Carter, 1965; Butt et al., 1968; Cerva & 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 unproved case of Grundy and Blowers (1970) in which survival was attributed to chloroquine. In this case, amebae, believed to be Naegleria, were

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

seen in the CSF and survived for awhile in culture but were not positively identified. The patient also presented atypical clinical features. Treatment consisted of metronidazole, emetine, penicillin, sulphane and chloroquine.

The ineffectiveness of antibacterial agents against pathogenic Naegleria has been confirmed in vitro by Carter (1969) and Mandal et al. (1970). Of the antiprotozoal agents, emetine HCl is effective against N. fowleri in vitro. Carter (1969) reported a minimum immobilizing level of 12.5 $\mu g.cm^{-3}$ and Krishna Prasad (1972) and Das (1975) report minimum amebicidal concentrations of 16 and 15 $\mu g.cm^{-3}$ respectively, but, it does not protect animals from the disease (Culbertson et al., 1968) probably because it is unable to pass the blood-brain barrier (Parmer & Cottrill, 1949). The ineffectiveness of chloroquine and metronidazole has also been confirmed by in vitro tests and animal protection studies (Carter, 1969; Mandal et al., 1970; Duma et al., 1971).

The only drug to appear promising in the early 1970's was the antifungal agent amphotericin B and as can be seen from Table II it was used in the treatment of all survivors (except the unproved case of Grundy & Blowers, 1970 and Callicott et al., 1968). Amphotericin B is a polyene antibiotic and has been shown to be highly amebicidal to pathogenic Naegleria in vitro (Carter, 1969; Mandal et al., 1970; Duma et al., 1971; Schuster & Rechthand, 1975; Visvesvara & Balamuth, 1975; Duma & Finley, 1976; De Jonckheere & Van De Voorde, 1977; Donald et al., 1979) and to protect mice from the disease (Culbertson et al., 1968; Carter, 1969; Das, 1971).

In 1969, Carter suggested that amphotericin B be tried in the treatment of PAM by simultaneous intravenous (IV) and intraventricular (IVent) administration; the doses recommended were - 0.25 $mg.kg^{-1}$ IV and 1.0 mg into the cerebral ventricles in the first 24 hours. Carter (1972) also suggested sulphadiazine should always be used as well as amphotericin B in case the amebae should occasionally prove to be Acanthamoebae. These amebae have been shown to be resistant to both drugs in vitro (Casemore, 1970; Chang, 1971; Visvesvara & Balamuth, 1975; Duma & Finley, 1976; Nagington & Richards, 1976; Donald et al., 1979), but there is good evidence that they are affected by sulphadiazine in vivo (Culbertson et al., 1965).

Subsequently, such 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. In the first of these, (Patient 3, Duma et al., 1971) a right ventricular tap was performed and after removal of fluid (containing many motile amebae) 1.5 mg of amphotericin B dissolved in 5 cm^3 of 5% dextrose injection solution (D_5W) was slowly injected into the ventricle. 10 mg of amphotericin B and 10 mg dexamethasone were administered IV. A nasogastric tube was inserted, through which 400 mg of metronidazole was given four times daily. The patient also received chloroquine base, 200 mg and dexamethasone, 4 mg, intramuscularly (IM) every six hours. Sixteen hours after admission the ventricular tap was repeated and 1.5 mg of amphotericin B was again injected into the ventricle. Seventy-two hours after admission he became shock-like, respirations ceased and he died.

The second case (Patient 4, Duma et al., 1971) received, within two hours of admission, 1.5 mg amphotericin B diluted in 5 cm^3 D_5W intracisternally (ICist) and 10 mg amphotericin B and 50 mg hydrocortisone IV over the next four hours. In addition, chloroquine sulphate, 200 mg IM; metronidazole, 500 mg by nasogastric tube every six hours; and diphenylhydantoin (Dilantin), 100 mg IM every eight hours, was given. Eighteen hours after admission, the patient again received 1.5 mg amphotericin B ICist and the IV amphotericin B increased to 20 mg. The patient died 66 hours after admission.

Carter (1972) reports similar findings to Duma et al.(1971) in two patients (7th and 9th, Table III, Carter, 1972) who had been treated in the same way.

Apley et al.(1970) described three cases of PAM in Great Britain, two of which were diagnosed presumptively because of association with the fatal proven case. They had the same early symptoms but neither actually developed convincing signs of meningitis. Naegleria was cultured from the CSF of the child who died and from one of the others but the amebae proved to be N. gruberi.

In the fatal infection, amphotericin B treatment was begun two days after admission, when amebae had been seen in the CSF. Amphotericin B, 0.25 $mg.kg^{-1}$ in one daily dose over three to four hours IV increasing over a week to 1 $mg.kg^{-1}$, was administered till the patient died on the sixteenth day after admission. Sulphadiazine, 160 mg IV every six hours was also given. On the seventh day after admission, 650 $amebae.mm^{-3}$ were seen in the CSF, but many appeared to be dead. The concentration of amphotericin B in the CSF was 0.184 $\mu g.cm^{-3}$. On the eleventh day after admission, the CSF contained no amebae and the

amphotericin B concentration was 0.224 $\mu g.cm^{-3}$. Although the diagnosis in this case was made relatively early and treatment with amphotericin B started promptly the patient died after being treated for thirteen days.

The second case reported by Apley et al. (1970) was the brother of case one and was admitted to hospital two days later. On the morning of admission he complained of headache and in the evening developed a sore throat and neck pains. A CSF sample was taken and it was clear and no amebae were seen. The clinical picture was that of an upper respiratory tract infection but in view of case one treatment with amphotericin B and sulphadiazine was begun. By the seventh day he was symptom-free. On the eighth day he again complained of sore throat with head and neck pains. No amebae were seen in the CSF but some were grown and appeared similar in morphology to those isolated from the CSF of case one. By the twelfth day he was afebrile and had no signs of meningitis but in view of growth of amebae from the CSF taken four days earlier, amphotericin B treatment was started again. 0.25 mg amphotericin $B.kg^{-1}$ IV daily over four hours increasing to 0.75 $mg.kg^{-1}$ after four days for a total of ten days was given. CSF taken on the twelfth and eighteenth days appeared normal and no amebae were grown. The patient was discharged, symptom-free. Ten days later a CSF sample was taken and again no amebae were grown.

The third case was admitted to hospital six days after case one. On the morning of admission he complained of sore throat and headache, vomiting and abdominal pain. The CSF was normal and no treatment was given. On the third day, the temperature had become normal but the headache continued and there was slight neck stiffness. No amebae were seen in the CSF but in view of slight lymphocytosis treatment was started with sulphadiazine and amphotericin B (0.25 $mg.kg^{-1}$ daily in one dose over four hours IV). On the fourth day signs of drug toxicity were noted and treatment was stopped. On the eighth day, growth of amebae from case two was reported and although the patient was well, daily amphotericin B, 0.25 $mg.kg^{-1}$ increasing to 0.75 $mg.kg^{-1}$ was given IV for ten days. CSF specimens on the 8th, 14th and 24th days were normal. No amebae were isolated at any time from this case. He was discharged on the fourteenth day, symptom-free (Apley et al., 1970).

Apley et al. (1970) do not believe that isolation of amebae from the CSF of case two was due to laboratory cross-infection "but case three must be considered to have been only doubtfully infected with

amebae." Griffin (1976), has disputed the diagnosis of Naegleria meningo-encephalitis in cases one and two. He contends that an Acanthamoeba was involved and that sulphadiazine, rather than amphotericin B, which was given on admission, was responsible for the prolonged survival in the first case and survival in the second.

The two cases reported by Pan and Ghosh (1971) were similarly inconclusive in the nature of the etiological agent involved and the effective agent in treatment. Their report deals with two Indian children, aged six months and three years, with CNS infections of slow onset (3-5 months). CSF samples showed "motile amebae with thin pseudopods". No strains were isolated and both patients survived. They were treated with amphotericin B, sulphadiazine and intrathecal steroids.

Anderson and Jamieson (1972) reported the case of a fourteen year old boy from Queensland who had typical acute symptoms and was already in the fourth day of illness and comatose by the time amphotericin B treatment was begun. The diagnosis was confirmed by finding 12,000 white cells.mm^{-3} and numerous amebae in the CSF; the amebae were cultured and shown to be N. fowleri. Amphotericin B was given in a dose of 1 $mg.kg^{-1}$ per day IV and penicillin, ampicillin and sulphadiazine, he had been having for three days previously, were continued. Within two days he became afebrile and was talking rationally. After five days the CSF white cell count had fallen to 15.mm^{-3} but many atypical amebae were still present. Amphotericin B was therefore given IT and later IVent in small doses (0.1 mg on alternate days) and the fluid gradually cleared. He was discharged from hospital without any neurological deficit. This case represents the first survival where there is definite proof that N. fowleri was involved and survival can be attributed to amphotericin B.

Seidel et al. (pers. comm., 1978) report a more recent case in Torrance, California. The patient, a nine year old female, presented with typical symptoms of meningo-encephalitis three days before admission to hospital. Lumbar puncture revealed a purulent CSF and motile amebae were seen. Amphotericin B (1 $mg.kg^{-1}$), sulphadiazine (50 $mg.kg^{-1}$), chloramphenicol (25 $mg.kg^{-1}$) and penicillin G (3.4×10^5 units) were all administered IV immediately on admission. 1.5 mg amphotericin B was also given IT.

The patient was then transferred to Harbour General Hospital and was in a coma on admission but responsive to pain and tactile

stimulation. On arrival the treatment outlined in Table III was instituted.

Table III: Treatment Protocol Used in a Case of PAM
(Seidel et al., pers. comm., 1978)

DRUG	ROUTE	DOSAGE
Amphotericin B	IV	1.5 $mg.kg^{-1}$ per day ÷ bid x 3 days → 1.0 $mg.kg^{-1}$ per day qd x 6 days
Amphotericin B	IT	1.5 mg per day x 2 days → 1mg QOD x 8 days
Miconazole	IV	350 $mg.m^{-2}$ per day ÷ tid x 9 days
Miconazole	IT	10 mg x 2 days → 10 mg QOD x 8 days
Rifampin	Oral	10 $mg.kg^{-1}$ per day ÷ tid x 9 days

bid = twice daily
tid = three times daily
qd = every day
QOD = every other day

Sulphadiazine (4 g per day IV) was continued for three days until studies confirmed the diagnosis of Naegleria meningo-encephalitis. Penicillin and chloramphenicol were continued for three days until bacterial CSF cultures were negative. Decadron (dexamethasone) and Dilantin (diphenylhydantoin) were given for increased intracranial pressure and seizure activity, respectively.

The patient stabilized clinically over the first 48 hours. Gradually over the next month of hospitalization her mental status improved. No significant neurological deficits were noted at discharge.

In a few of the other cases of PAM, where proof that N. fowleri was the etiological agent involved and amphotericin B given at effective doses the course of the disease was often too advanced to see any effect (Van Den Driessche et al., 1973; Cursons et al., 1976, pers. comm. 1979).

Callicott et al. (1968), isolated an ameba identified as A. astronyxis from a spinal fluid sample from a patient with a purulent

meningitis that remitted spontaneously. The authors were unable to provide evidence that the organism caused the illness and was not just a cultural contaminant.

Kenney (1971), reported the case of a patient hospitalized for acute gastritis of unknown origin. Complement fixation tests revealed no antibodies to Entamoeba histolytica but did reveal antibodies to A. culbertsoni. Over the next two months a rising titer to A. culbertsoni antigen was reported. Clinical investigation by a physician did not reveal any symptomatology suggestive of cerebromeningeal involvement. The patient refused a spinal tap.

The gastro-intestinal symptoms continued and a stool examination revealed amebae which were called Iodamoeba butschlii. Because of the titre to Acanthamoeba antigen, the patient was put on antiamebic therapy consisting of Dehydro-Emetine (IM) and chloroquine. Complement fixation tests two months later demonstrated that the serum titer had decreased. This case appears to demonstrate a form of disease between the symptomless carrier state and fulminating meningo-encephalitis which may be found to be more common than at present believed.

The only human Acanthamoeba infections positively diagnosed during life under circumstances where chemotherapy could have been tried were those in the eye. Nagington et al. (1974) repeatedly isolated Acanthamoeba from two English patients with corneal ulcers. Warhust and Thomas (1975) identified the amebae as A. castellanii and A. polyphaga. One of the infections was in a 32 year old woman who had a mild unilateral keratoconjunctivitis and uveitis which did not respond to treatment which included chloramphenicol, idoxuridine, 3-fluorothymidine, gentamicin, methicillin and later on sulphadiazine (500 mg, six hourly). Six months after treatment, because of corneal ulceration, pain and loss of vision, a corneal graft was performed but the graft was rejected. The other infection described was in a 59 year old farmer with an identical clinical condition which required enucleation of the eye after one year. Treatment in this case included chloramphenicol, acetylcysteine, 3-fluorothymidine and clotrimazole eye drops.

Jones et al. (1975) cultivated A. polyphaga from corneal ulcers of two patients in Houston, Texas. They reported suppression of the amebae with paromomycin. Griffin (1978) reported seeing similar material at the Armed Forces Institute of Pathology, Washington D.C. and it seems likely that Acanthamoeba in the eye will not prove to be strikingly rare or unusual.

CHAPTER TWO: MATERIALS

2.1. Ameba Cultures Used

Table IV: Ameba Cultures Used

SPECIES	STRAIN	PATHO-GENICITY	ORIGINAL SOURCE	PLATE
Naegleria fowleri	MsT	+	NHI	2
Naegleria gruberi	P1200f	-	NHI	1
Acanthamoeba culbertsoni	A-1	+	CCAP	5&6
Acanthamoeba castellanii	1501	-	IMTPL	3&4

+ = positive

- = negative

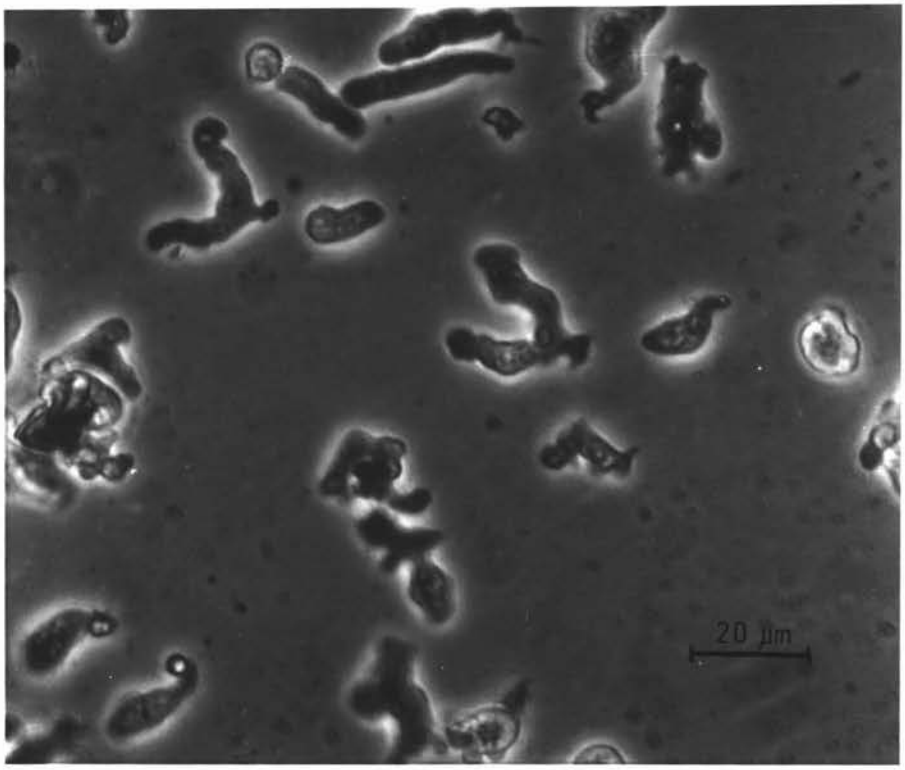
NHI = National Health Institute, Wellington, N.Z.

CCAP = Culture Centre of Algae and Protozoa.

IMTPL = Insitut de Médecine Tropicale Prince Léopold, Belgium.

Plate 1. Trophozoite stage of Naegleria gruberi (P1200f)

Plate 2. Trophozoite stage of Naegleria fowleri (MsT)


20 µm

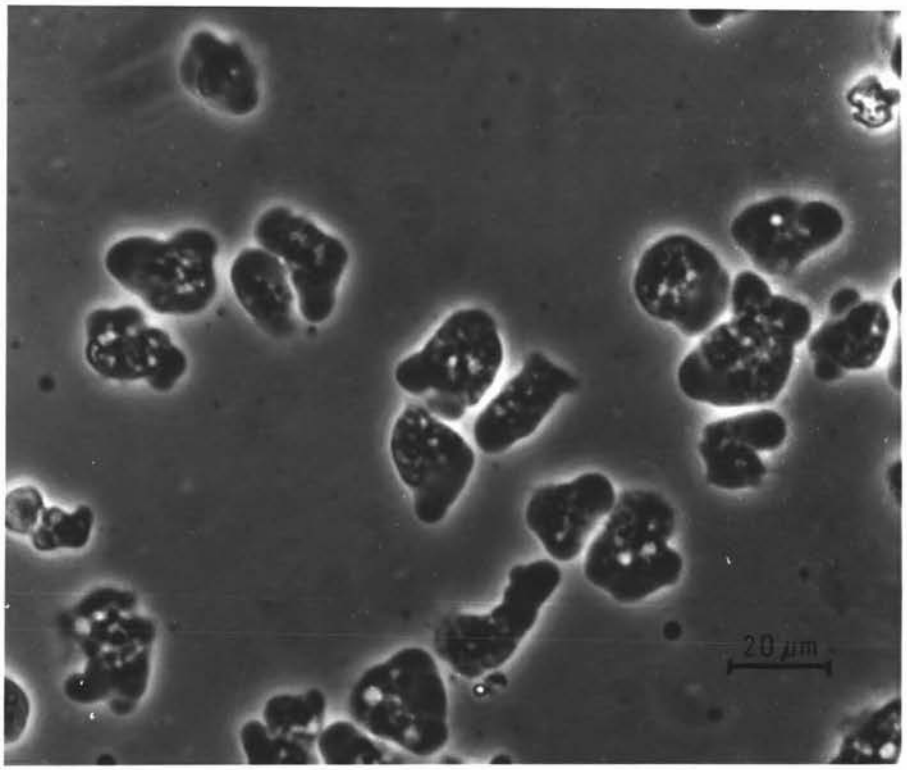
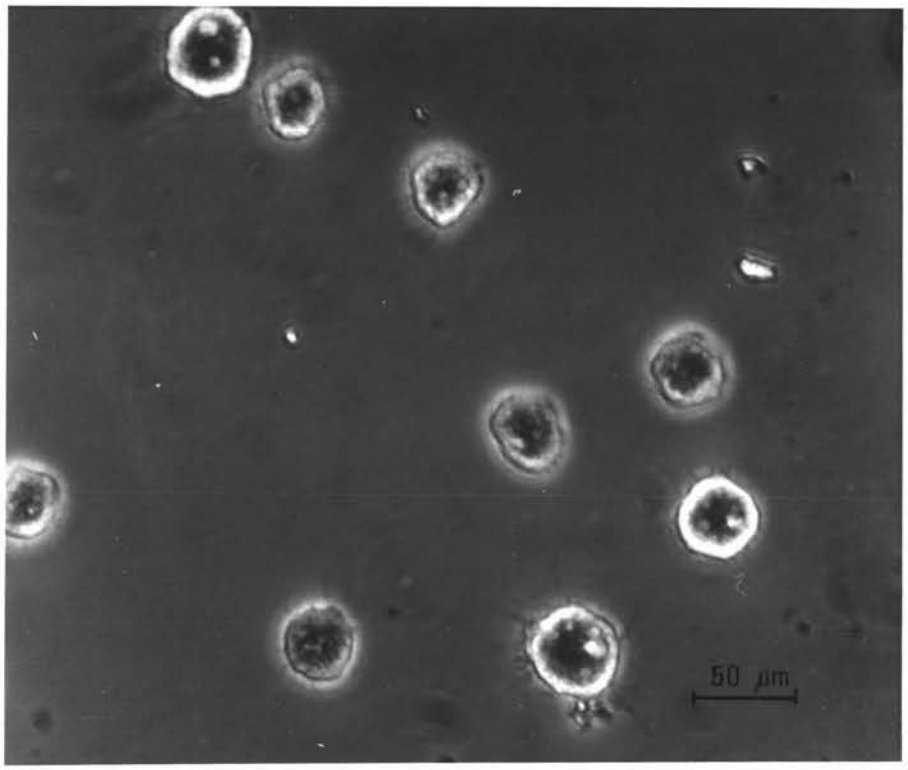

20 µm

Plate 3. Trophozoite stage of Acanthamoeba castellanii (1501)
Note fine acanthapodia.

Plate 4. Cyst stage of Acanthamoeba castellanii (1501)
Note double-walled structure, with stellate endocyst.


50 µm

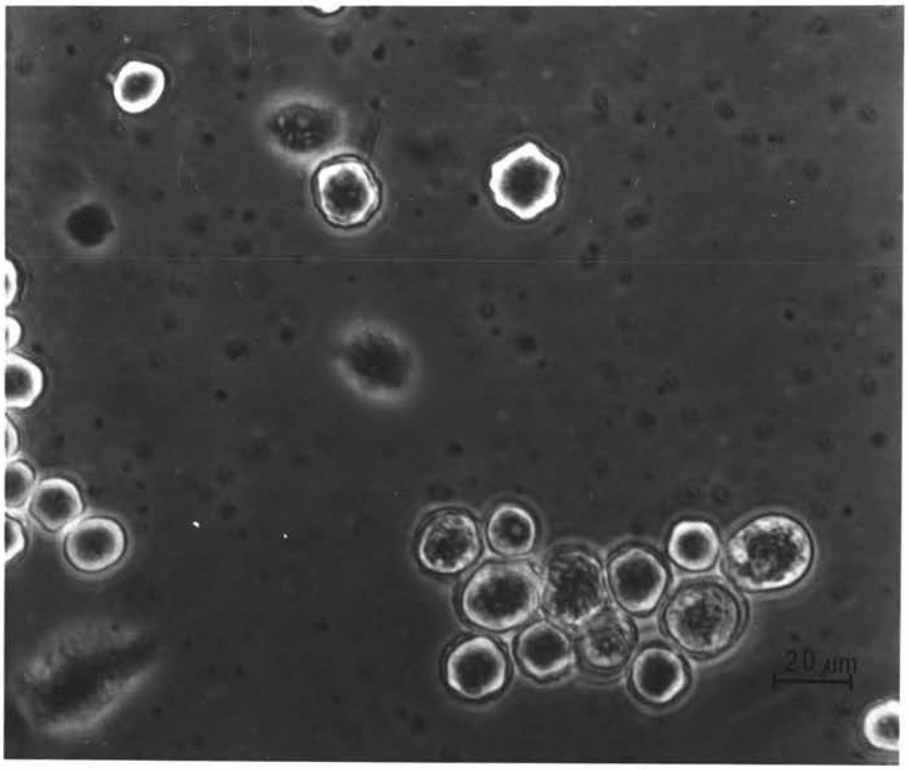
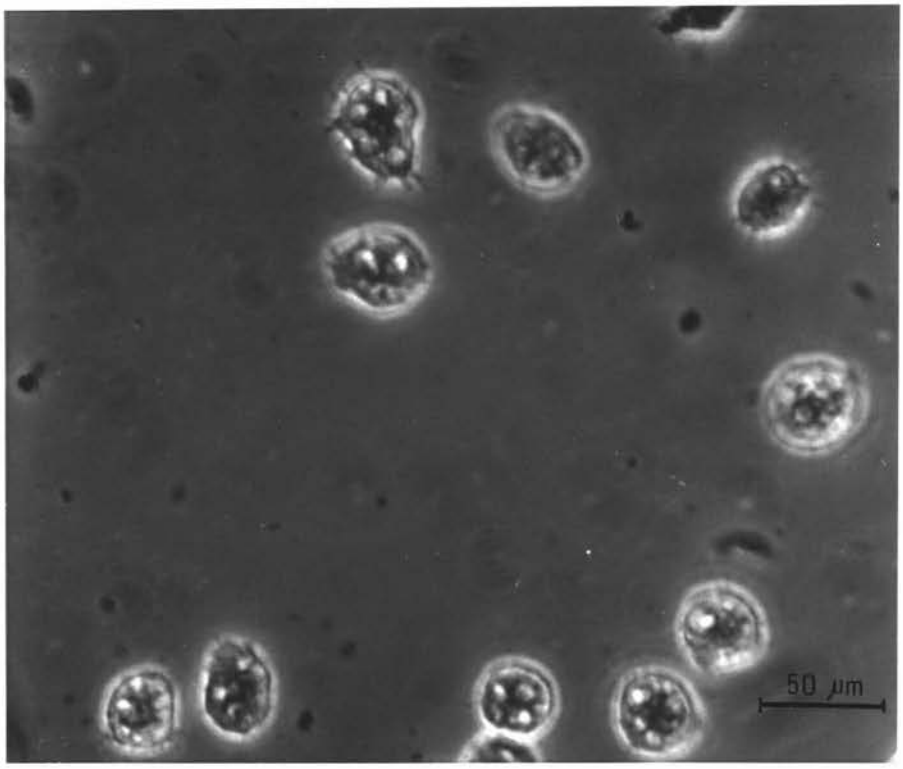
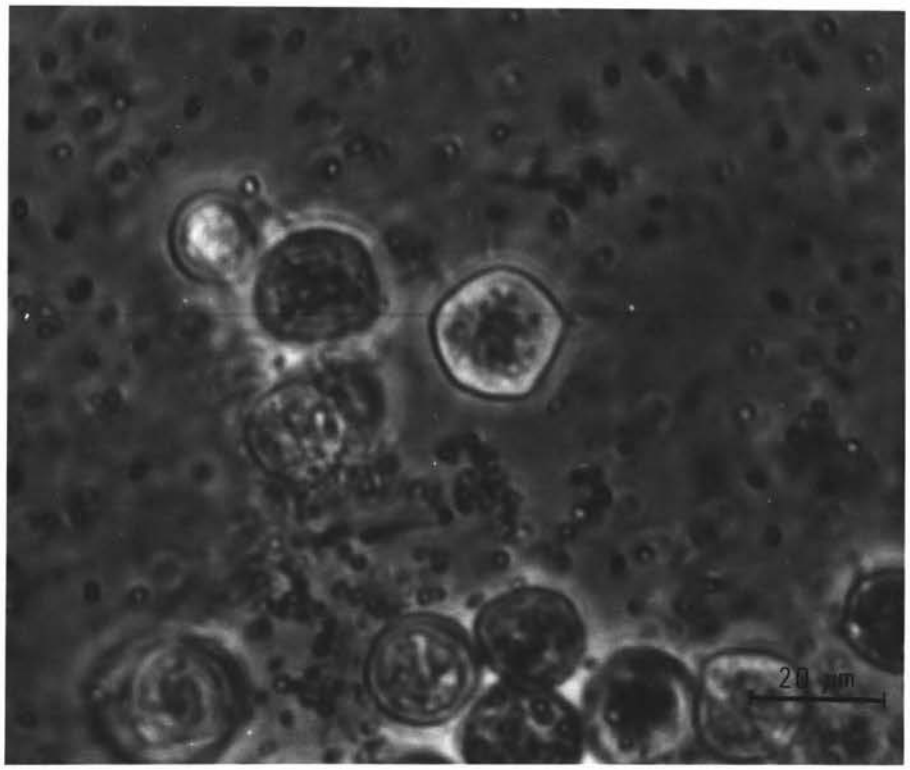

20 µm

Plate 5. Trophozoite stage of Acanthamoeba culbertsoni (A-1)

Plate 6. Cyst stage of Acanthamoeba culbertsoni (A-1)
Note round double-walled structure.


50 µm


20 µm

2.2. Plate Media

2.2.1. Ameba Saline Agar (Page, 1967)

for the isolation of *Naegleria* spp.

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

pH 6.8

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

2.2.2. Ameba 1% Saline Agar (Cursons, 1978, modified from Page, 1967)

for the isolation of *Acanthamoeba* spp.

NaCl	= 10.0 g
$MgSO_4.7H_2O$	= 0.004 g
$CaCl_2.2H_2O$	= 0.004 g
Na_2HPO_4	= 0.142 g
KH_2PO_4	= 0.136 g
Agar	= 15.0 g
Distilled water	= 1.0 litre

pH 6.8

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

2.3. Axenic Media for Amebae

2.3.1. Page's Ameba Saline (PAS) (Page, 1967)

for diluting out either Naegleria or Acanthamoeba spp. and as a base for medium CYM.

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

pH 6.8

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

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

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

pH 6.8

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

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

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

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

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

plus 1.0 litre of the following Ac ion solution:

Ac Ion Solution

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

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

To 9.0 cm^3 of 4% Neff medium is added 1 cm^3 of Penicillin/Streptomycin solution (200,000 units.cm^{-3}).

2.3.4. CGHV and CGHVS (Cursons et al., 1979). Semi-defined media for the axenic testing of antibiotics against Naegleria (CGHV) and Acanthamoeba (CGHVS) spp.

Medium A:

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

Giving a final volume of 996 cm^3

pH 6.8

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

Medium B:

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

Sterilize by heating to 60°C for 30 minutes

CGHV:

Mix 4.0 cm^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 5.0 g NaCl to Medium A before autoclaving and then mix as for CGHV.

2.4. Cell Culture Media

Vero Cell Culture

4.4% Bicarbonate solution

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

10x Trypsin/Versene Mixture

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

Antibiotics

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

Eagles Growth Medium (EGM) (Eagle, 1955)

Eagles = 380 cm^3
1 cm^3 of antibiotic
20 cm^3 of 4.4% bicarbonate
40 cm^3 (10%) serum (calf or pig)

The Eagles is filter sterilized through a 0.22 μm filter and bottled in 380 cm^3 amounts with the other ingredients being aseptically added when required. The medium is then gassed with CO_2 till orange.

Eagles Maintainence Medium (EMM)

As above but with only 1% serum added.

2.5. Antibiotic Solutions

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

Supplied as Fungizone intravenous, a sterile lyophilized powder in vials providing 50 mg amphotericin B and 41 mg sodium desoxycholate with 25.2 mg sodium phosphates as a buffer.

Preparation of Solutions: The dry powder was reconstituted as follows: An initial concentration of 5 mg amphotericin.cm^{-3} is first prepared by adding 10 cm^3 sterile distilled water to the vial of dry powder and shaking the vial until the solution is clear. Further dilutions were made with sterile distilled water to give the appropriate working solutions.

2.5.2. Rifampicin (Lepetit Pharmaceuticals Ltd.)

Supplied as Rifadin capsules containing 150 mg rifampicin. Actual weight of powder in capsule = 193.5 mg.

Preparation of stock solution: 103.3 mg of dry powder was dissolved in 20 cm^3 $^N/_{100}$ HCl giving a stock solution containing 4000 μg Rifampicin.cm^3. Further dilutions were made with sterile distilled water to give the appropriate working solutions.

2.5.3. Tetracycline hydrochloride (Upjohn Pty. Ltd.)

Supplied as Panmycin capsules, each containing 250 mg tetracycline hydrochloride (Batch No. A7500). Actual weight of powder in each capsule = 437.0 mg.

Preparation of stock solution: 55.94 mg of dry powder was dissolved in 20 cm^3 of distilled water giving a stock solution containing 1600 μg tetracycline HCl.cm^{-3}. Further dilutions were made with sterile distilled water to give the appropriate working solutions.

2.5.4. Polymyxin B sulphate (Sigma Chemical Co.)

Supplied as pure powder containing 8000 USP units.mg^{-1} (Lot 107C-0352)

Preparation of solutions: 5.0 mg of dry powder was dissolved in 20 cm^3 of distilled water giving a stock solution of 2000 units.cm^{-3}. Further dilutions were made with sterile distilled water.

2.5.5. 5-Fluorocytosine (Roche Products Pty. Ltd.)

Supplied as 500 mg lots of pure substance (Lot A 375214).

Preparation of solutions: 80 mg of pure 5-fluorocytosine was dissolved in 20 cm^3 of distilled water giving a stock solution of 4,000μg.cm^{-3} 5-fluorocytosine. Further dilutions were made in sterile distilled water.

2.5.6. Miconazole (Ethnor Pty. Ltd.)

Supplied as IV solution containing 10 mg Miconazole.cm^{-3} (Lot No. 77K03/430).

Preparation of stock solution: A stock solution of 400μg.cm^{-3} was prepared by diluting the IV solution in sterile distilled water. Further dilutions were also made in sterile distilled water.

2.5.7. R41,400 (Ethnor Pty. Ltd.)

Supplied as R41,400 base.

Preparation of solution: 0.01 g of R41,400 dry powder was dissolved in 0.1 cm^3 1N HCl then made up to 5 cm^3 with distilled water giving a stock solution of 2000μg.cm^{-3}. Further dilutions were made with sterile distilled water.

All antibiotic stock solutions were prepared fresh when required and filter sterilized through a 0.22μm Millipore filter before use.

2.6. Miscellaneous Solutions

Phosphate Buffered Saline (PBS)

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

pH 7.6

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

2.7. Experimental Animals

Young Swiss-White male mice were obtained from closed, inbred colonies from the Small Animal Production Unit, Massey University.

CHAPTER THREE: METHODS

3.1. Sterilization

All glassware, except that used for analytical work, was sterilized at 103.4 kPa (121°C) for 15 minutes. Analytical glassware was sterilized by dry heat at a minimum of 160°C for two hours.

3.2. Axenic Culture Techniques

3.2.1. Maintainence of stock axenic cultures

Axenic cultures of Naegleria fowleri (MsT) and Naegleria gruberi (P1200f) in CYM medium and Acanthamoeba culbertsoni (A-1) and Acanthamoeba castellanii (1501) in 4.0% Neff medium were provided by Dr. R.T.M. Cursons, Massey University, Palmerston North. The amebae were then added to CGHV (if Naegleria) and CGHVS (if Acanthamoeba). Pathogenic species were incubated at 37°C and subsequently sub-cultured every 24 hours and non-pathogenic species were incubated at 30°C and sub-cultured every 48 hours. All stock cultures were maintained in Universal bottles and incubated on rotary gyroshakers (150 r.p.m.).

All amebae were sub-cultured 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 antagonistic to the drugs (Cursons et al., 1979).

3.2.2. Axenic drug testing

All tests were carried out in 6 cm^3 Bijou bottles in a total volume of 2.0 cm^3. Stock cultures of amebae were counted on a Fuchs-Rosenthal bright-line hemocytometer. The cultures were diluted so that 0.2 cm^3 gave a final concentration of 2-3 x 10^5 amebae.cm^{-3}. The stock drug solutions, prepared as outlined in Materials, were diluted so that 0.5 cm^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 2.0 cm^3, i.e., 1.3 cm^3 of media in single drug experiments and 0.8 cm^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 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 agar (for Acanthamoeba) seeded with Enterobacter cloacae. These plates were incubated at the appropriate temperature for 48 hours and then examined for viable amebae, viability being defined by the appearance of the organism and motility. Viable Naegleria show an extended limax form and are grey in colour. Non-viable cells are rounded, darker grey due to increased granulation and lack pseudopdia. Non-viable Acanthamoeba appear yellow by phase contrast, no internal structure is discernible and they lack acanthapodia.

3.3. Cell Culture Techniques

3.3.1. Maintainence of stock Vero cell cultures and preparation of KIMAX tubes for drug testing

Vero cells were maintained in 250 cm^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 10 cm^3 KIMAX tubes:

1) 4.0 cm^3 of a 10x Trypsin-Versene (T/V) stock was added to 36.0 cm^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 washed gently with 5.0 cm^3 of the T/V mixture.

3) After a 1 minute wash, the T/V mixture was discarded and a further 8.0 cm^3 of T/V mixture added. The bottle was then placed at 37°C for 1 minute and then drained again except for the last few drops.

4) The bottle was left at 37° until the cells were detached from the glass (approximately 3 minutes). Next, 10.0 cm^3 of EGM was added and the cells counted and dispensed in the following aliquots:

250 cm^3 bottles = 3×10^5 cells.cm^{-3} (3.0 cm^3 of a three day old monolayer)

10 cm^3 tubes = 1.5×10^5 cells.cm^{-3} (1.5 cm^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 1.0 cm^3 sterile PBS (pH 7.6). EMM was then added to give a final volume of 2.0 cm^3 i.e., 1.3 cm^3 of EMM in single drug experiments and 0.8 cm^3 in synergy experiments.

2) The seeding inocula of ameba was obtained from 24 hour exponential axenic cultures of N. fowleri (MsT) and A. culbertsoni (A-1) routinely passaged through cell cultures. The amebae were counted and diluted with EMM and 0.2 cm^3 added to the cell culture tubes to give the appropriate final concentrations, 3×10^2 and 3×10^3 amebae.cm^{-3} for A. culbertsoni (A-1) and N. fowleri (MsT) respectively.

3) The stock drug solutions, prepared as outlined in the Materials section, were diluted so that 0.5 cm^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 six days the tubes were centrifuged at 1500 r.p.m. for ten minutes in a bench centrifuge, the supernatant discarded and the deposit resuspended in 1.0 cm^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 R41,400

Young Swiss White male mice were first weighed and then anaesthetized by injecting 0.15 cm^3 of a 0.1% (V/V) Nembutal solution, using physiological saline as diluent, intraperitoneally (IP). Amebae cultures (N. fowleri, MsT) were counted and diluted to 1 x 10^6 amebae.cm^{-3}. Mice were then inoculated intranasally with 0.1 cm^3 of the amebic suspension.

R41,400 solutions for in vivo testing were prepared daily maintaining the W/V proportions of powder to diluent outlined in Materials. Physiological saline was used in place of distilled water. The amount of R41,400 powder used was adjusted to give the correct dose, in proportion to the weight of the mice, in 0.1 cm^3. The drug was given IP immediately after intranasal inoculation of amebae and continued every 12 or 24 hours thereafter depending on the experiment. Control mice received 0.1 cm^3 of a 10% 0.1 N HCl (V/V) in physiological saline at the same times.

CHAPTER FOUR: RESULTS

4.1. In Vitro Axenic Drug Testing of Naegleria spp.

Seven drugs were screened for activity against N. gruberi (P1200f) and N. fowleri (MsT). The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the test compound which produced obvious reduction in the number of trophozoites over 96 hours. Minimum amebicidal concentrations (MAC) were defined as the lowest concentration at which no living trophozoites could be found after 96 hours.

4.1.1. Amphotericin B

Figures 1 and 2 show the results obtained with four different concentrations of amphotericin B. The MAC for both N. gruberi (P1200f) and N. fowleri (MsT) was 0.5 $\mu g.cm^{-3}$. 0.75 $\mu g.cm^{-3}$ completely sterilized the media within 48 hours. At drug concentrations $\geq$ 0.25 $\mu g.cm^{-3}$ all the amebae appeared rounded and vacuolated.

Figure 1. The Effect of Amphotericin B on Naegleria gruberi (PI 200f).
(Drug concentrations shown in Figs.1–8 are in $\mu g.cm^{-3}$)

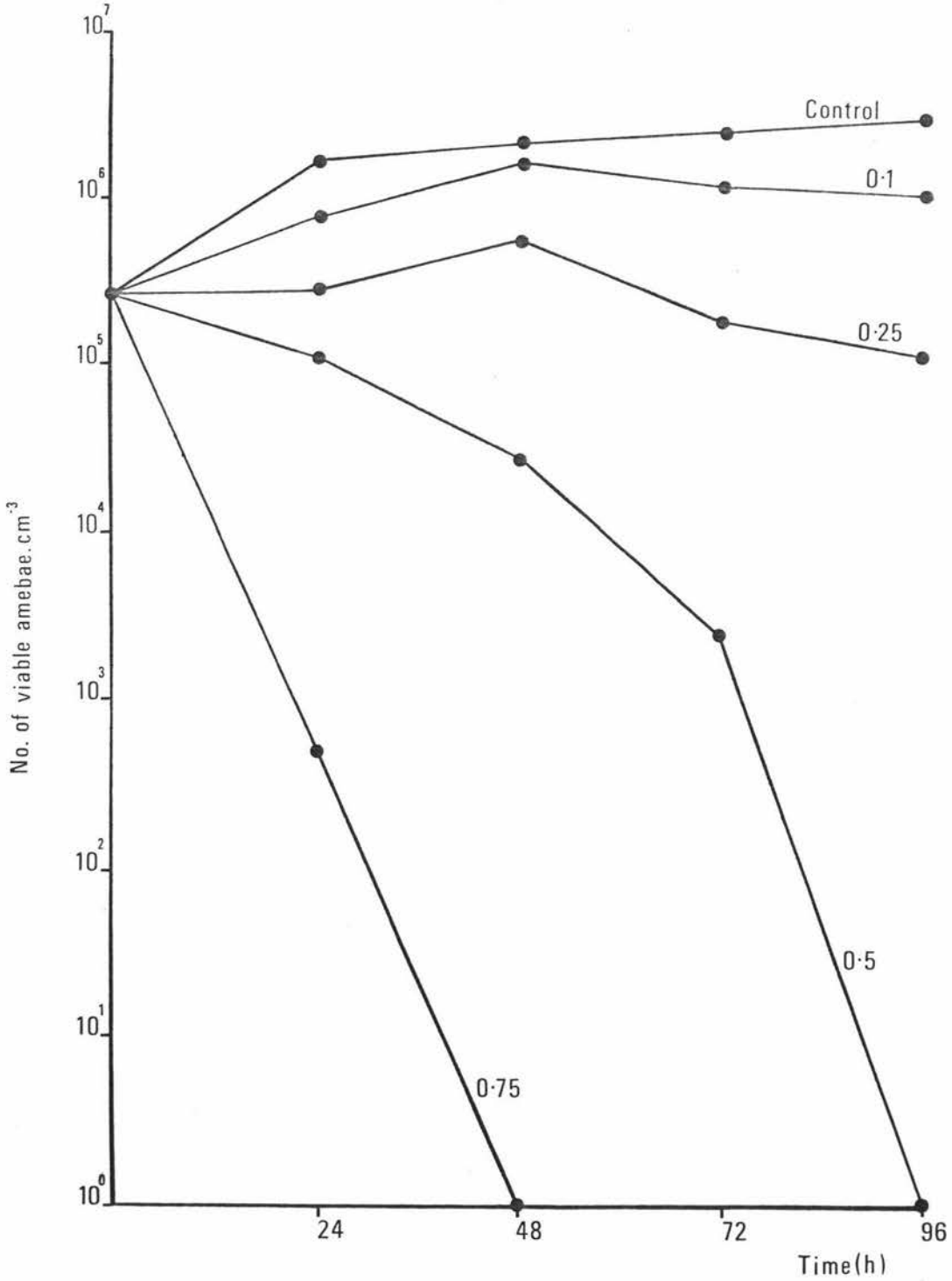
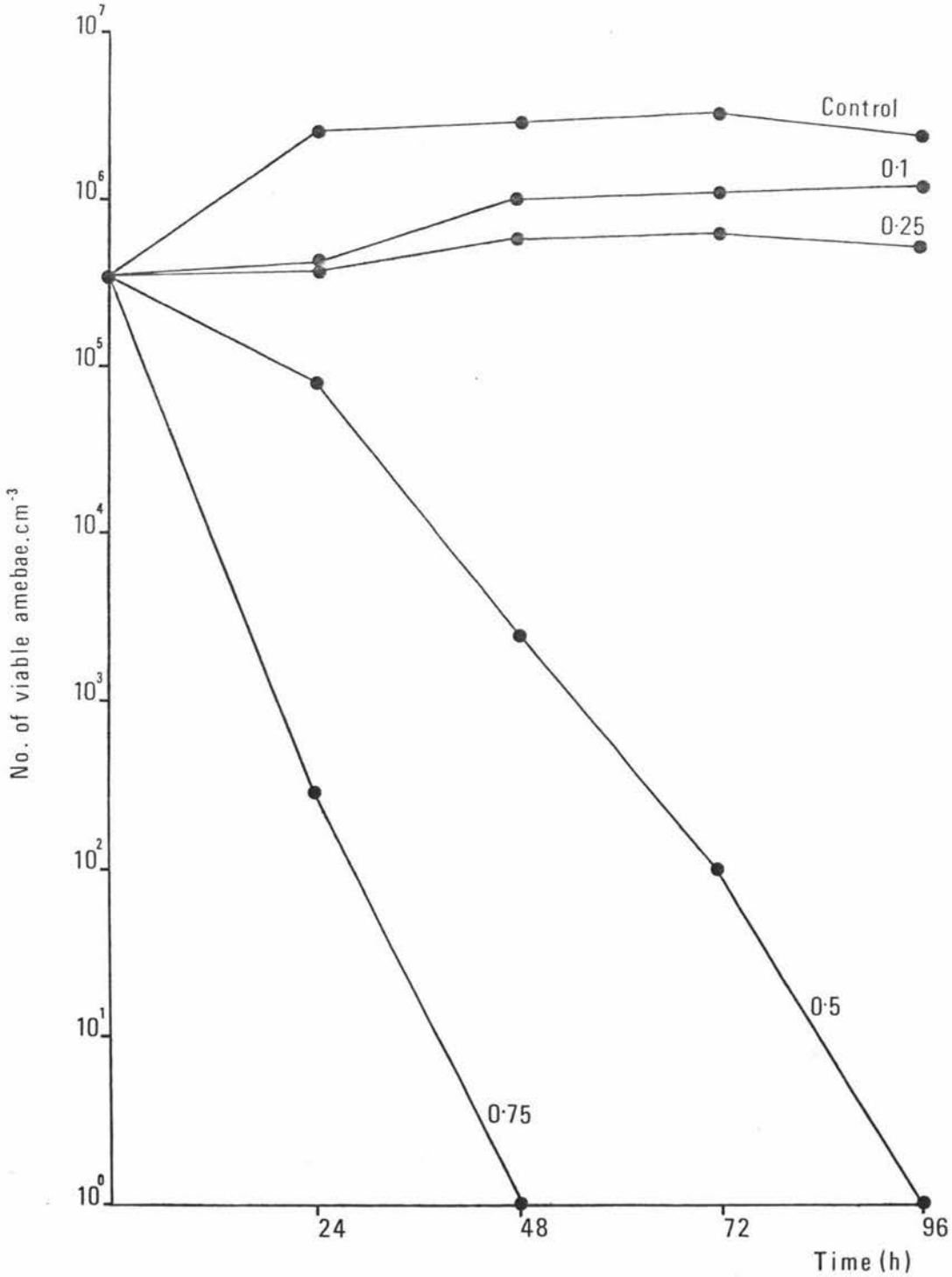


Figure 2. The Effect of Amphotericin B on Naegleria fowleri (MsT).



4.1.2. Tetracycline, Rifampicin and 5-Fluorocytosine

Figures 3 and 4 show the results obtained with three concentrations of tetracycline. For both N. gruberi (P1200f) and N. fowleri (MsT) a slight reduction in numbers was recorded with concentrations $\geq 50\,\mu g.cm^{-3}$. The amebae showed no signs of drug damage and all were active, motile limax amebae.

No inhibitory effect was seen with 100 $\mu g.cm^{-3}$ rifampicin against either N. gruberi (P1200f) (Figure 5) or N. fowleri (MsT) (Figure 6). Only at 500 $\mu g.cm^{-3}$ did the amebae show any signs of drug damage. The number of viable amebae was the same as with 100 $\mu g.cm^{-3}$ but there was evidence of increased vacuolation.

5-Fluorocytosine did not effect the growth of N. gruberi (P1200f) even at a concentration of 500 $\mu g.cm^{-3}$ (Figure 7). At the same concentration N. fowleri (MsT) showed a slight reduction in the number of viable amebae. The remaining viable amebae were active and limax in form (Figure 8).

Figure 3. The Effect of Tetracycline on Naegleria gruberi (P1200f).

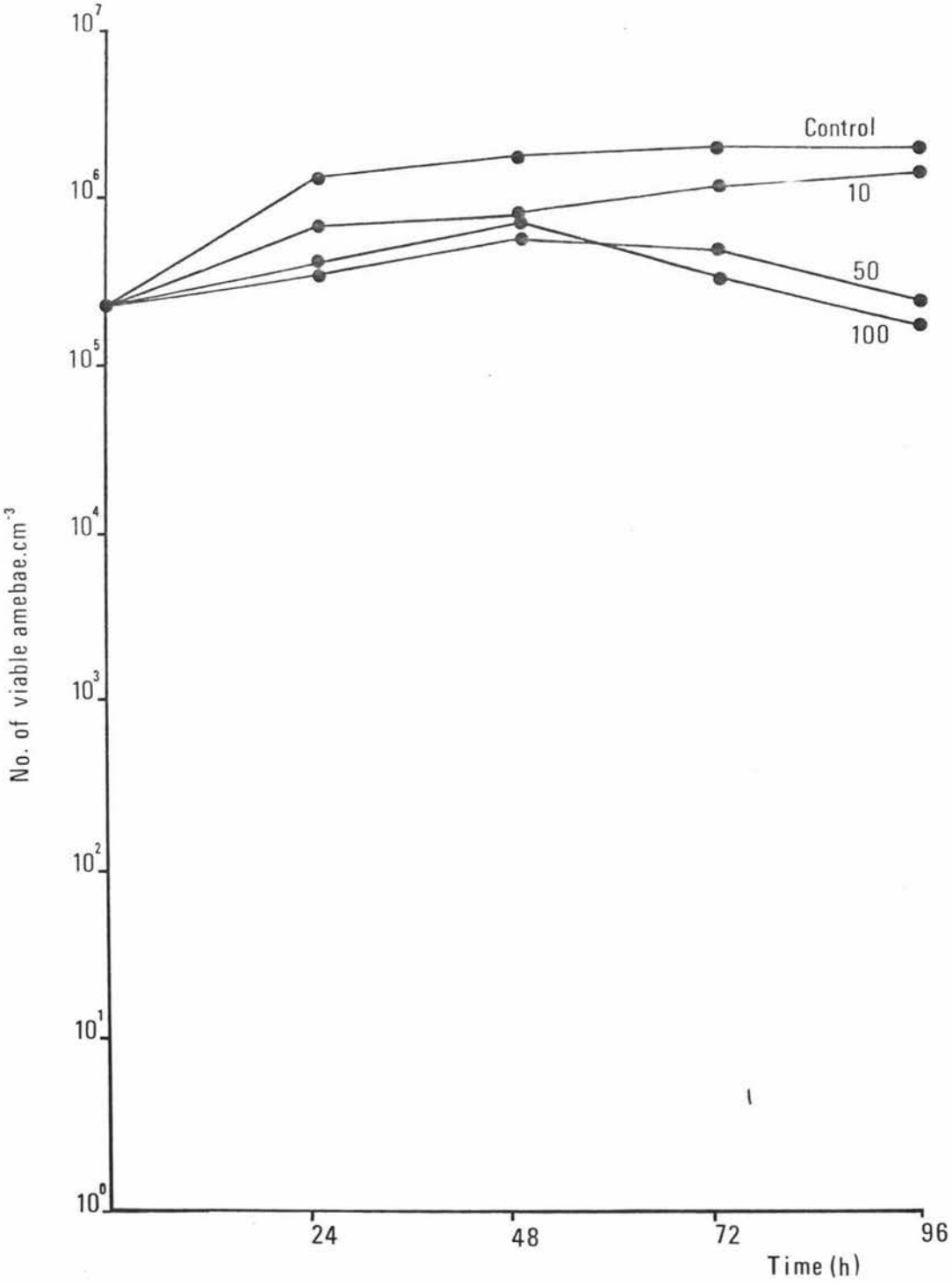


Figure 4. The Effect of Tetracycline on Naegleria fowleri (MsT).

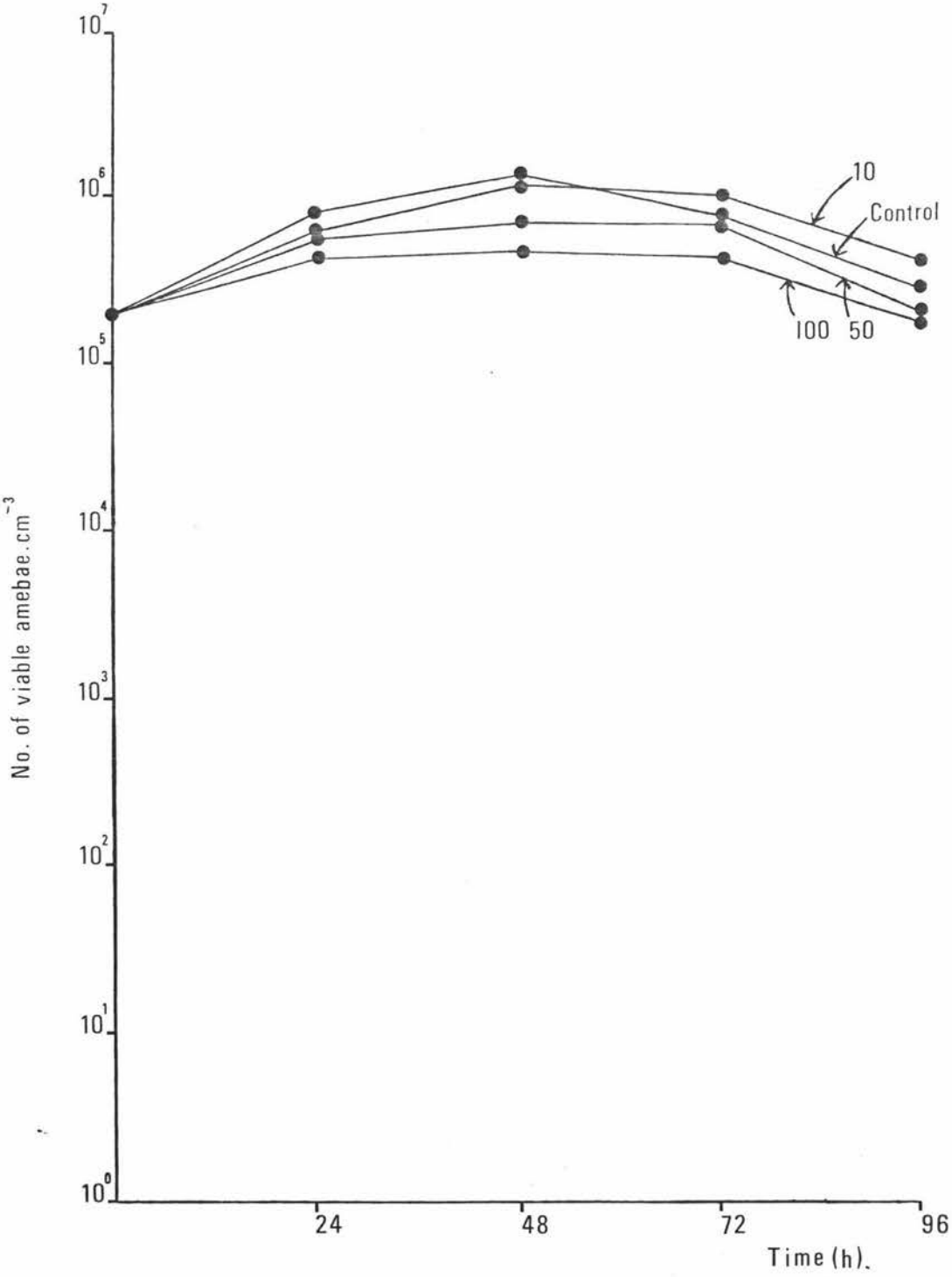


Figure 5. The Effect of Rifampicin on Naegleria gruberi (P1200f).

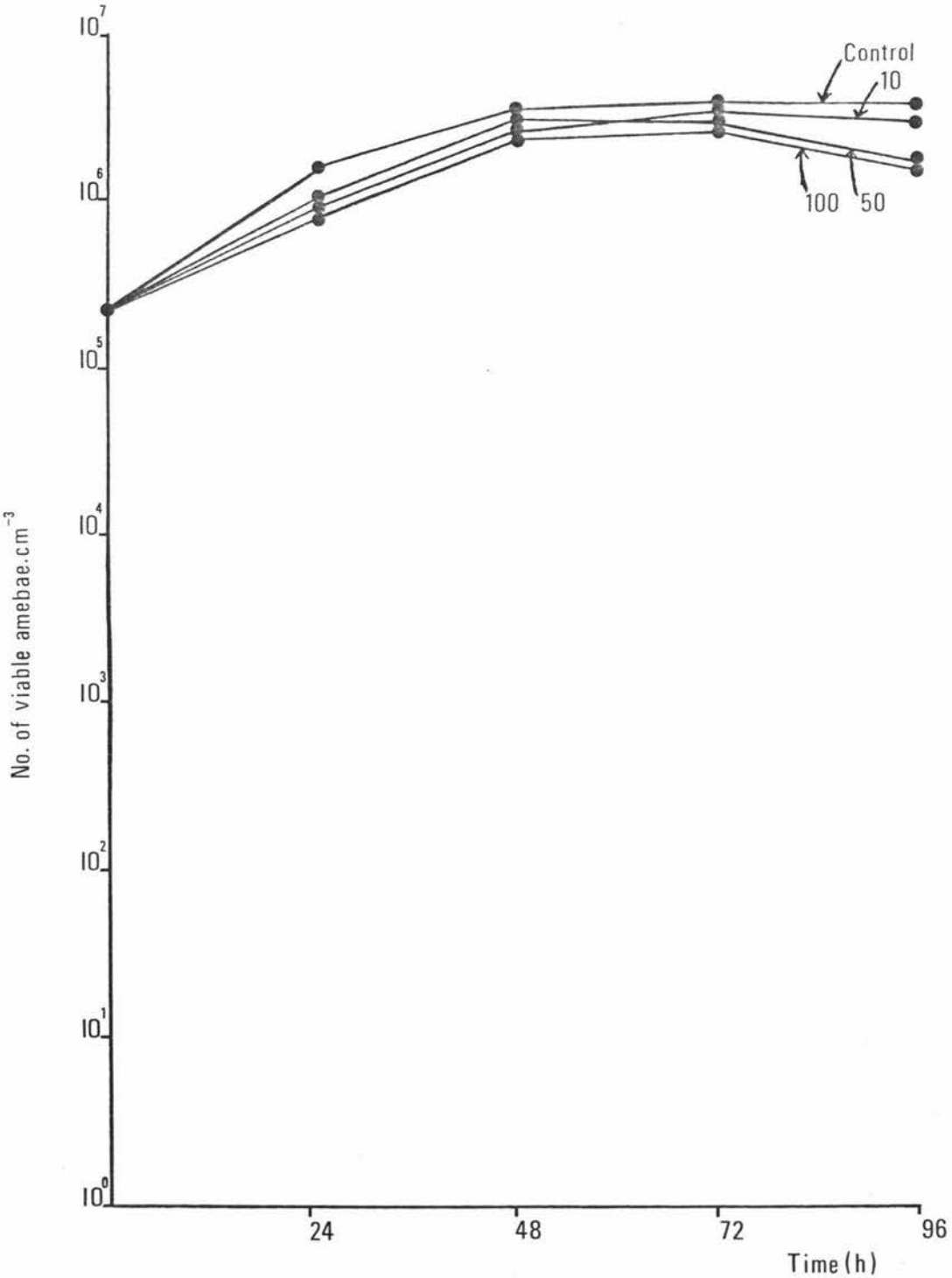


Figure 6. The Effect of Rifampicin on Naegleria fowleri (MsT).

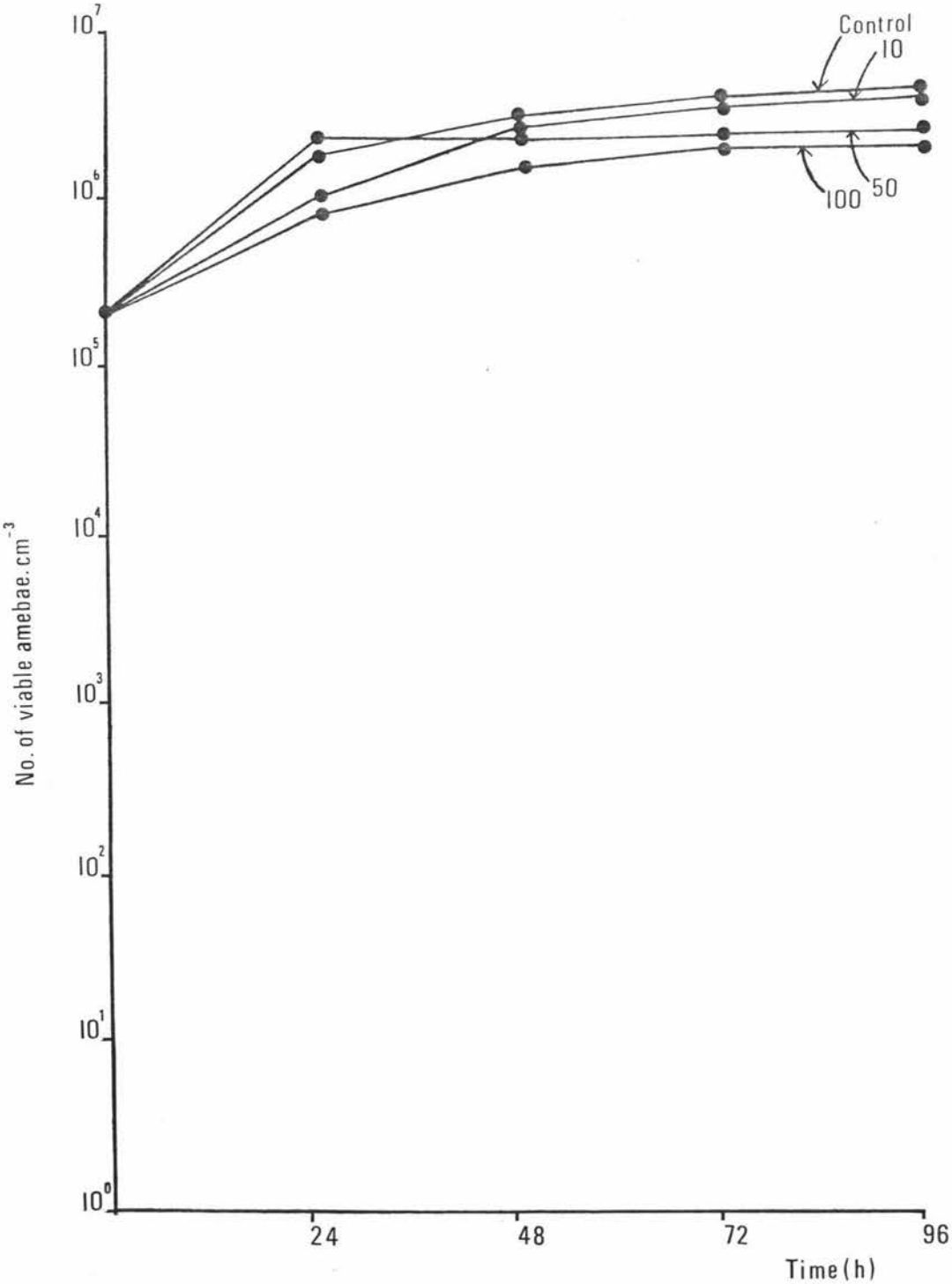


Figure 7. The Effect of 5-Fluorocytosine on Naegleria gruberi (P1200f).

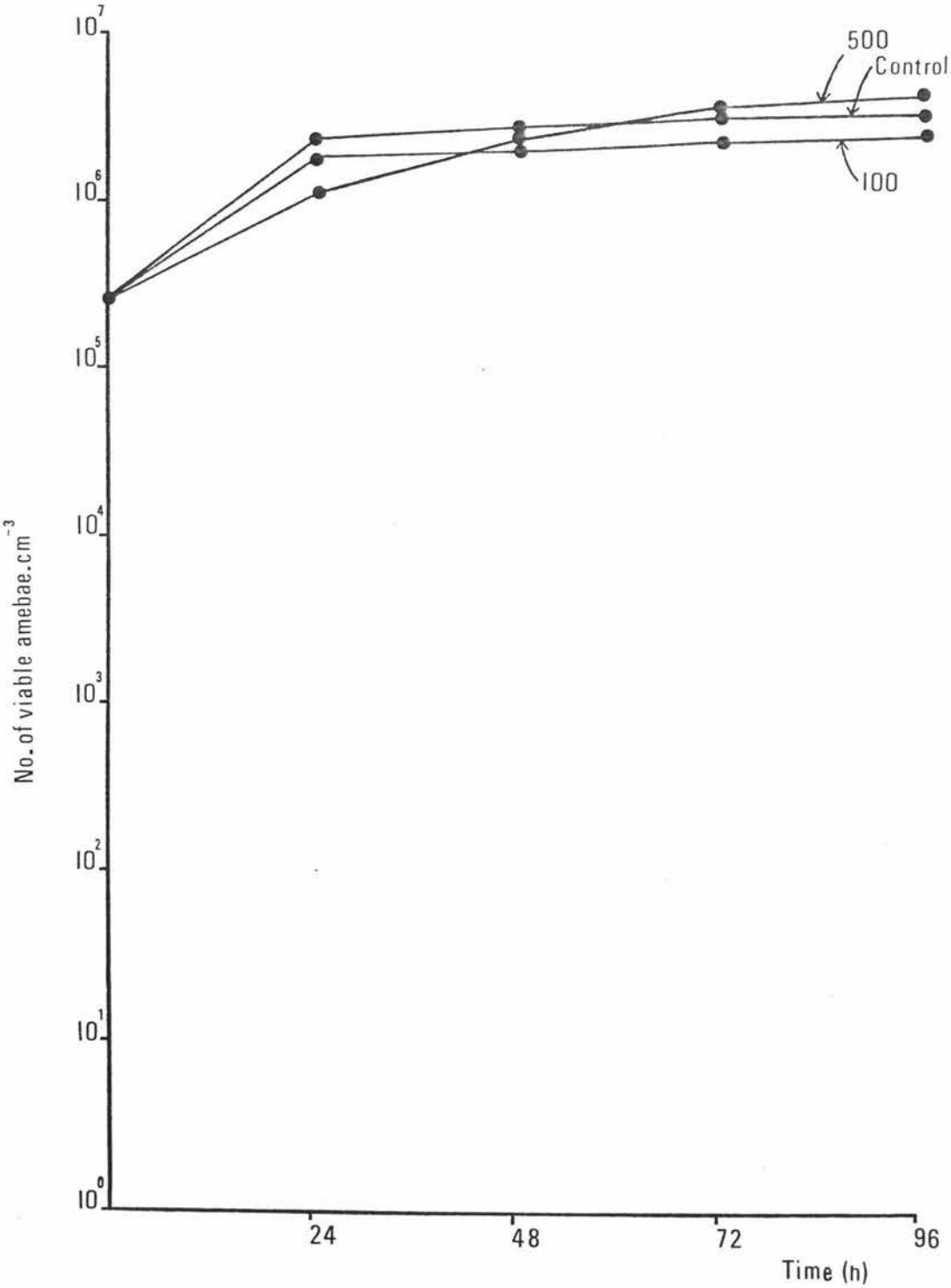
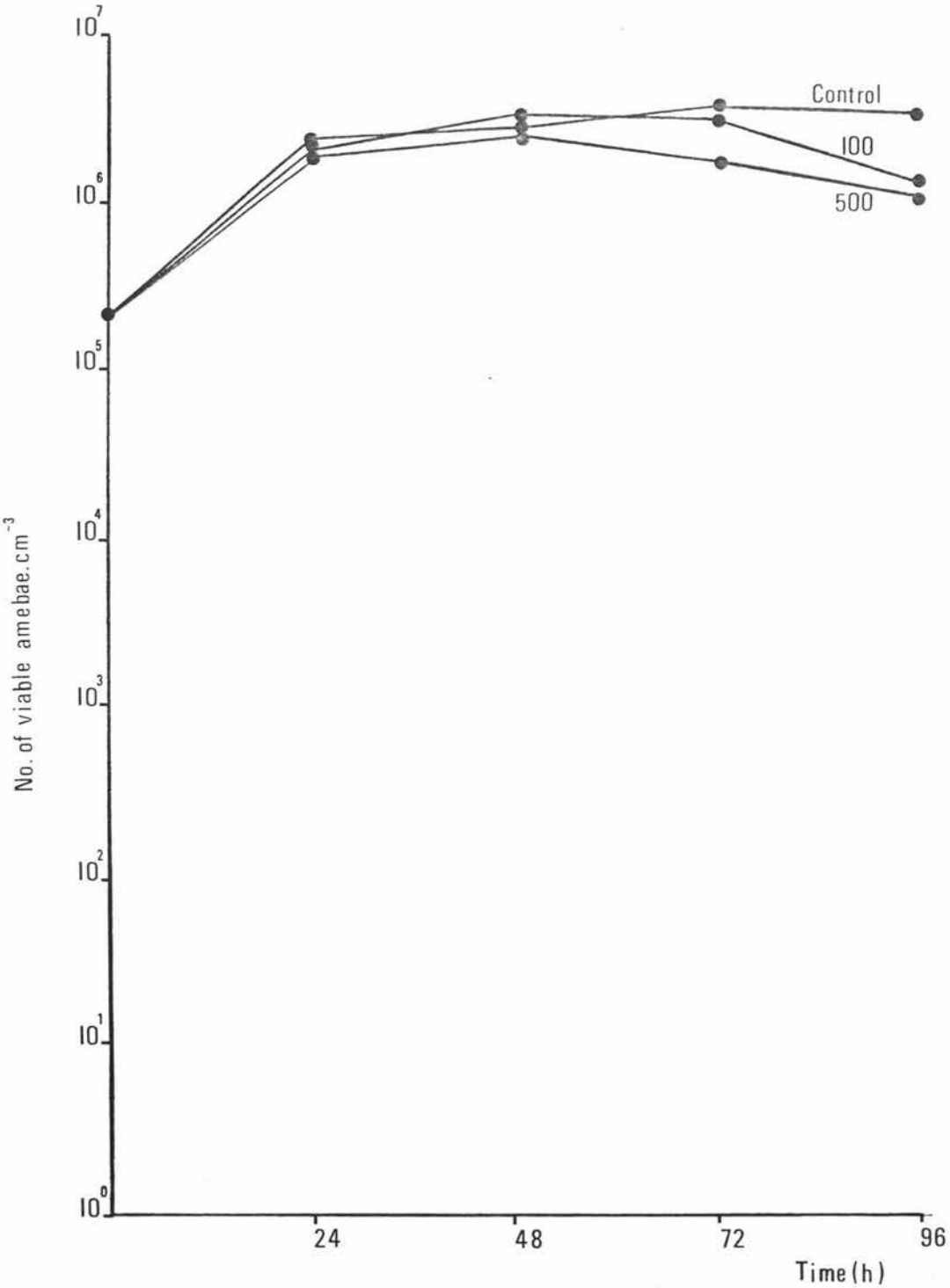


Figure 8. The Effect of 5-Fluorocytosine on Naegleria fowleri (MsT).



4.1.3. Polymyxin B sulphate

Polymyxin B sulphate has no inhibitory activity against Naegleria spp. at concentrations $\leqslant$ 500 units.cm^{-3} (Figures 9 & 10). No signs of drug damage were evident throughout the experiment.

4.1.4. Miconazole

Figures 11 and 12 show the results obtained with four different concentrations of the imidazole derivative miconazole. The two Naegleria spp. showed different responses, with the non-pathogenic N. gruberi (P1200f) being more susceptible. The MIC was 5 μg.cm^{-3} and MAC, 10 μg.cm^{-3}. For N. fowleri (MsT), an initial period of growth, followed by inhibition, resulted with 5 μg.cm^{-3}. The MIC was 10 μg.cm^{-3} and MAC 50 μg.cm^{3}. 100 μg.cm^{-3} miconazole sterilized the media of both species in 48 hours.

Figure 9. The Effect of Polymyxin B on Naegleria gruberi (P1200f)
(Drug concentrations shown in Figs.9&10 are in units.cm^{-3})

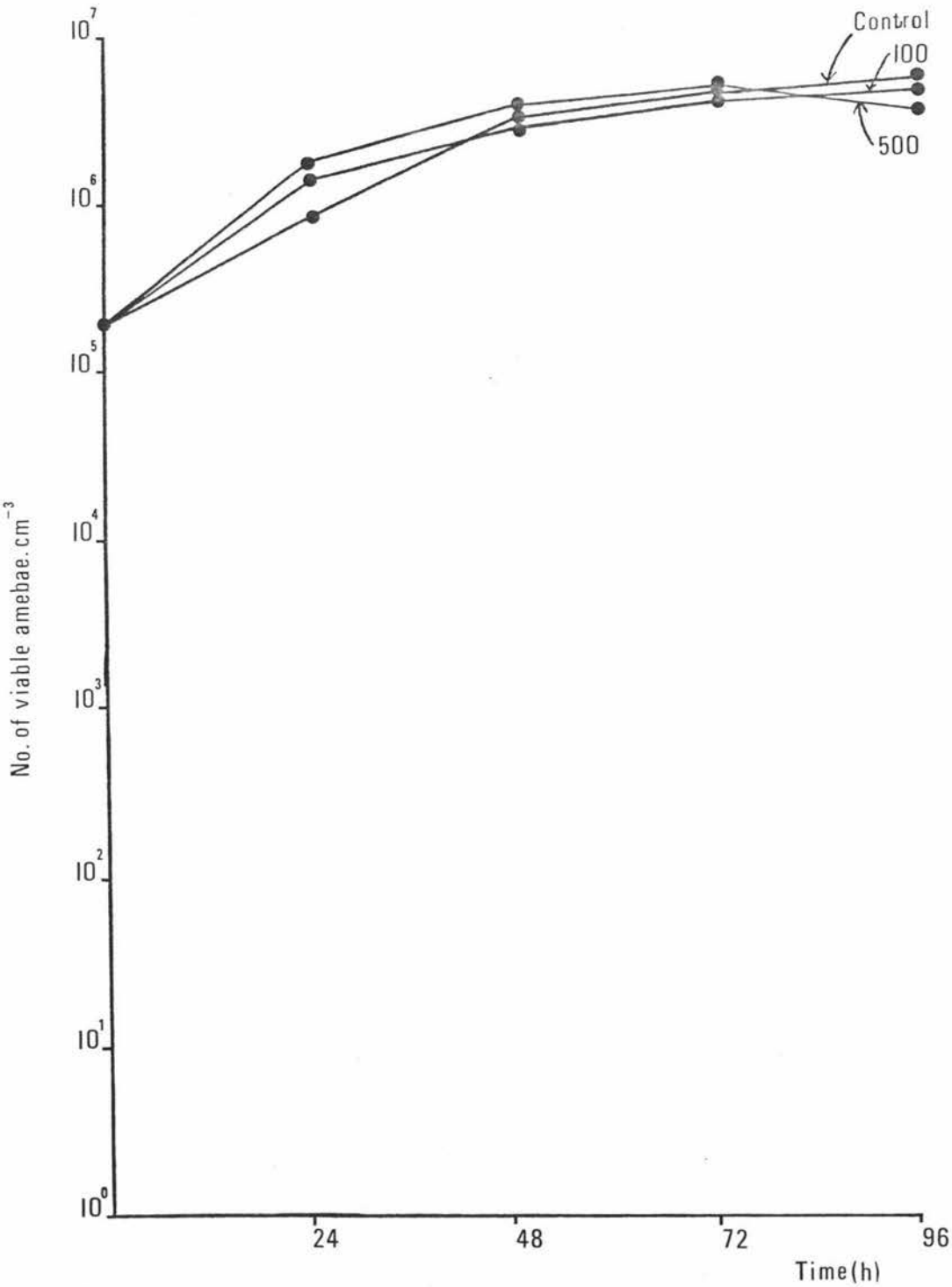


Figure 10. The Effect of Polymyxin B on Naegleria fowleri (MsT).

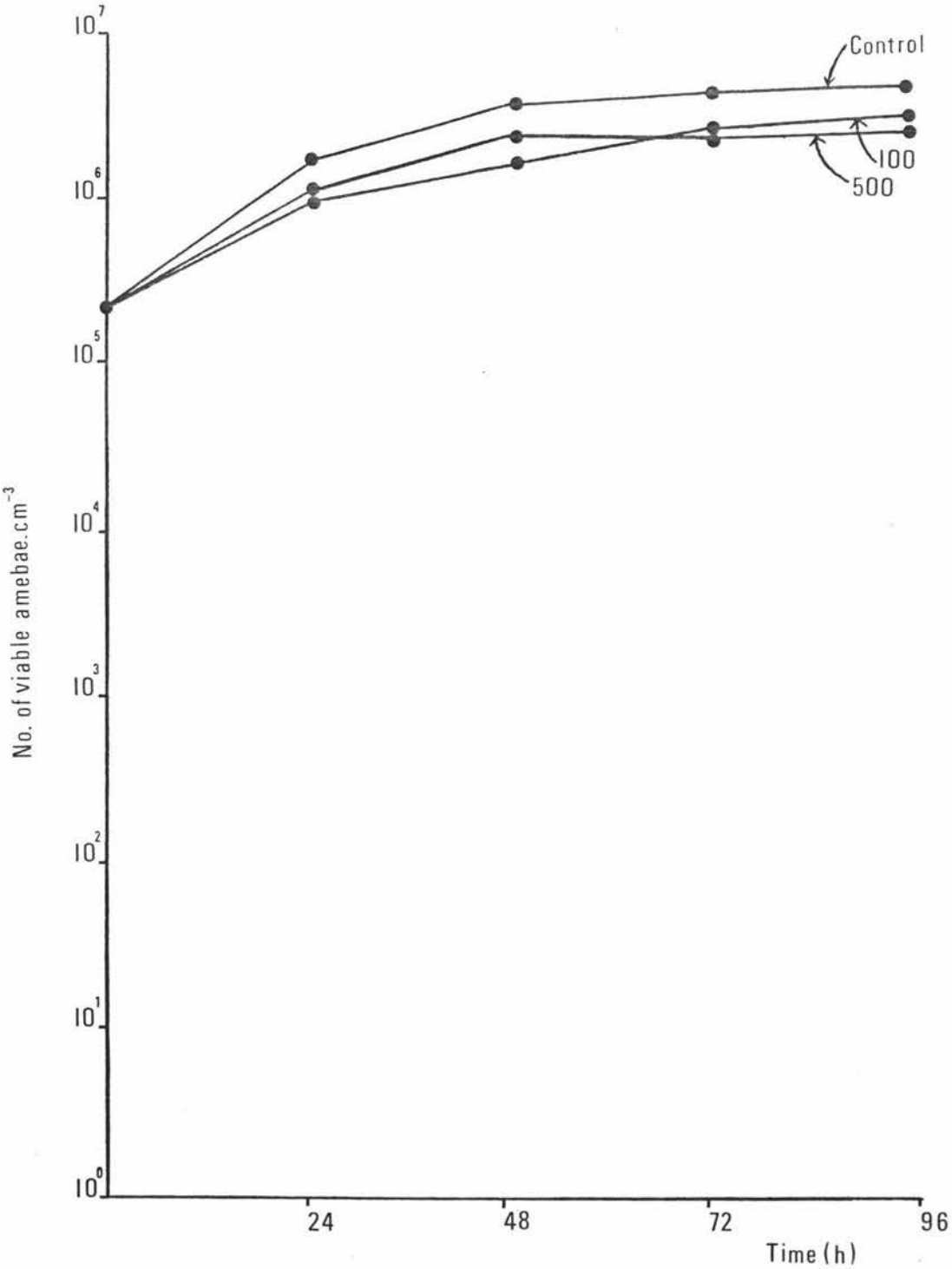


Figure 11. The Effect of Miconazole on Naegleria gruberi (P1200f).
(Drug concentrations shown in Figs.11–14 are in $\mu g.cm^{-3}$)

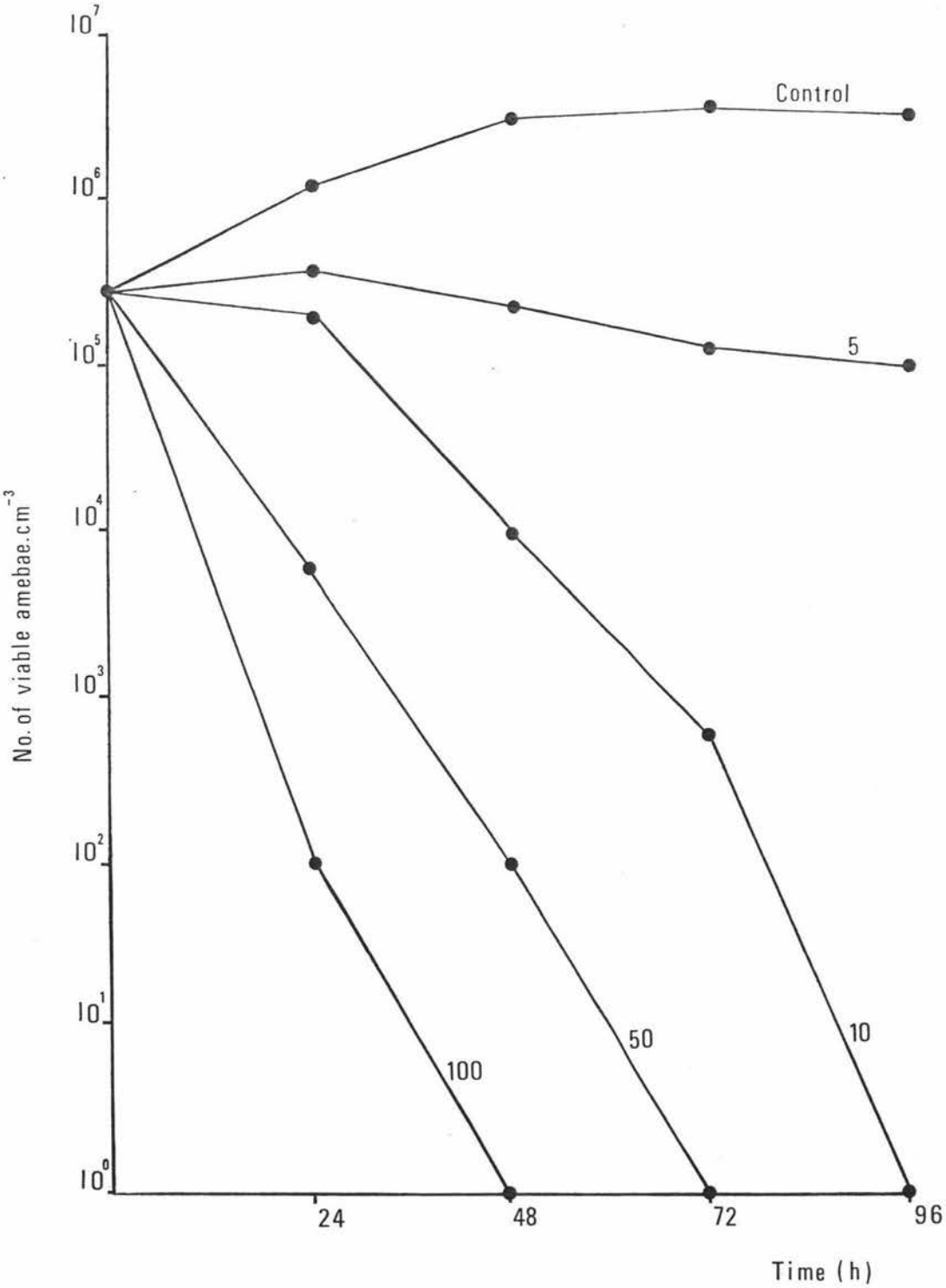
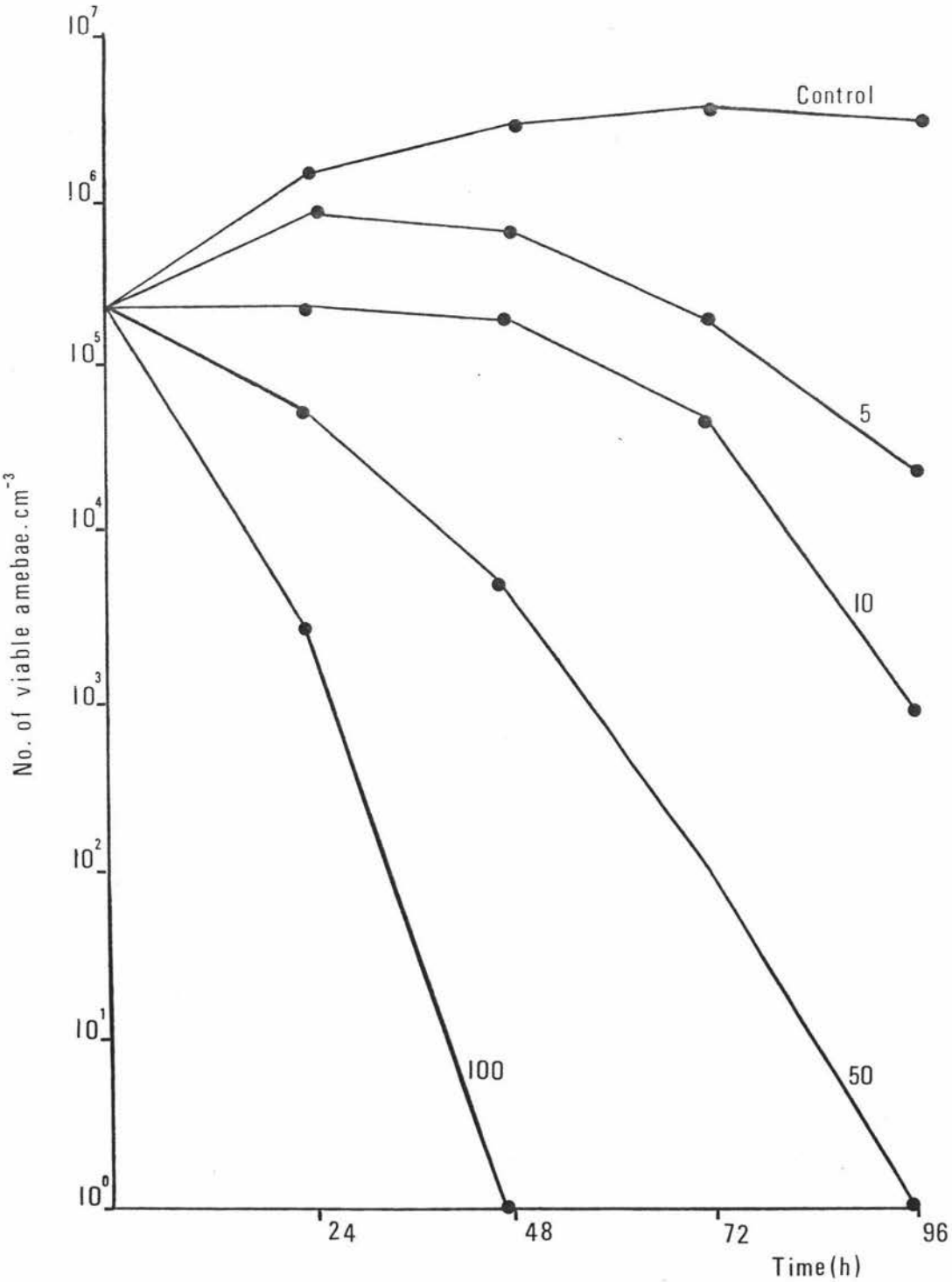


Figure 12. The Effect of Miconazole on Naegleria fowleri (MsT).



4.1.5. R41,400

The two Naegleria spp. also showed a variable response to the imidazole derivative, R41,400, with the pathogenic sp. appearing more susceptible than the non-pathogenic sp. For N. gruberi (P1200f) 10 $\mu g.cm^{-3}$ was only inhibitory (Figure 13) whereas it was amebicidal for N. fowleri (MsT) (Figure 14). 50 $\mu g.cm^{-3}$ was amebicidal within 48 hours for both species.

Figure 13. The Effect of R41,400 on Naegleria gruberi (PI200f).

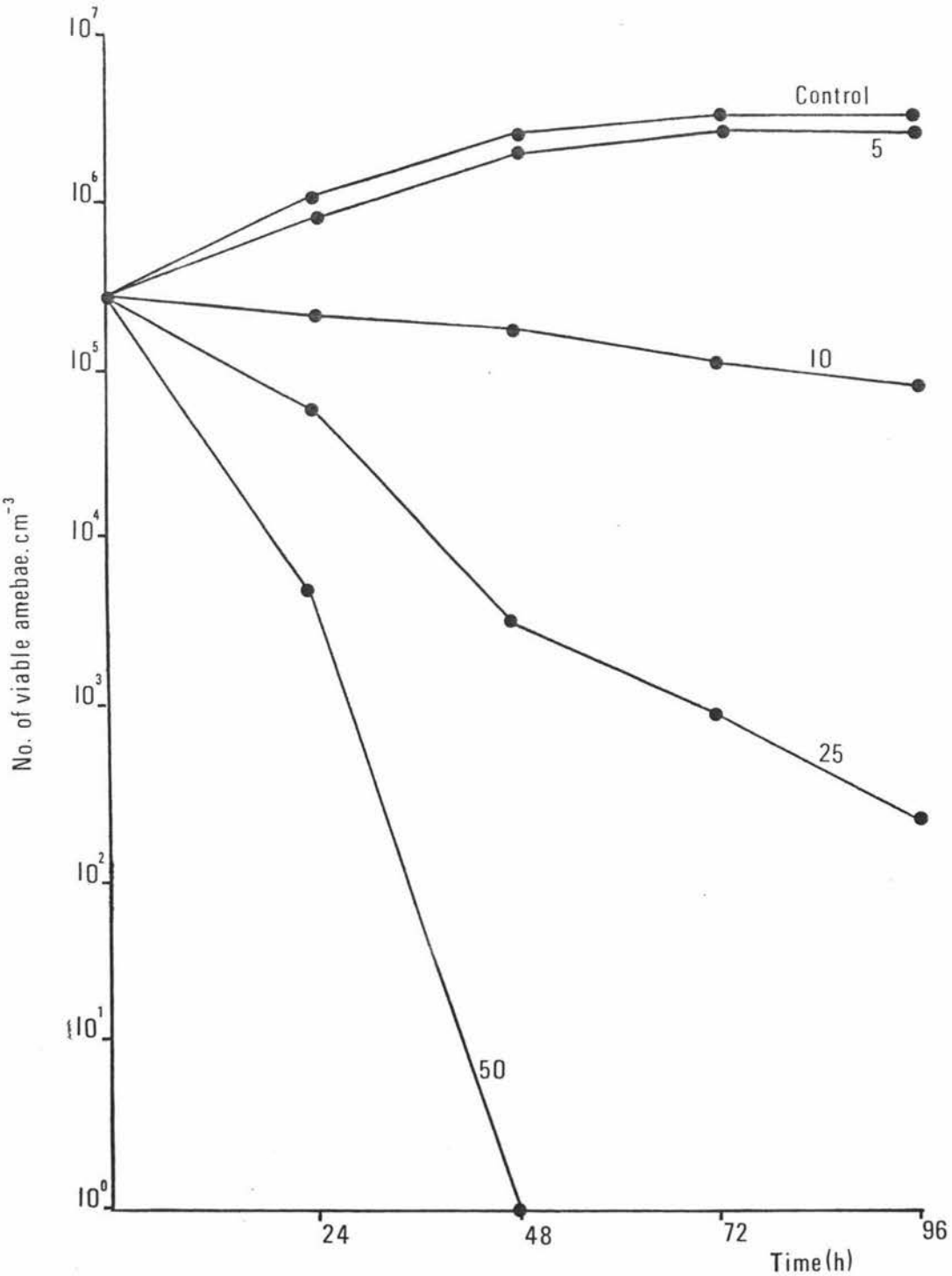
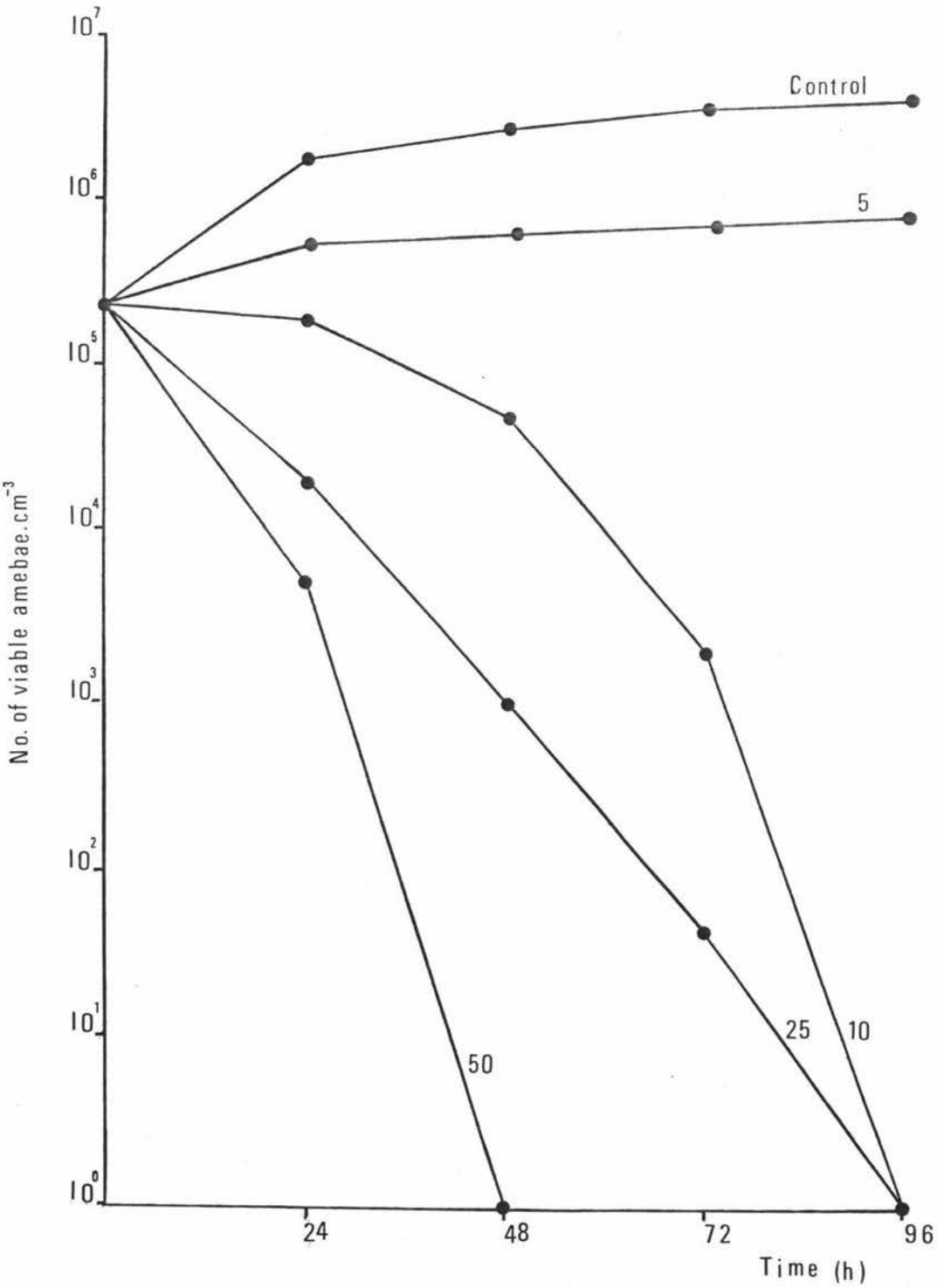


Figure 14. The Effect of R41,400 on Naegleria fowleri (MsT).



4.2. In Vitro Axenic Drug Testing of Acanthamoeba spp.

Seven drugs were screened for activity against A. castellanii (1501) and A. culbertsoni (A-1). The results are shown in Figures 15 to 28. MIC and MAC are defined in section 4.1.

4.2.1. Amphotericin B

The results obtained with four concentrations of Amphotericin B are shown in Figures 15 and 16. The non-pathogenic A. castellanii (1501) and pathogenic A. culbertsoni (A-1) showed a different response to the highest concentration tested. The MAC for A. culbertsoni (A-1) was 100 $\mu g.cm^{-3}$ but this concentration was only slightly inhibitory for A. castellanii (1501).

Figure 15. The Effect of Amphotericin B on Acanthamoeba castellanii (1501).
(Drug concentrations shown in Figs. 15–22 are in $\mu g.cm^{-3}$)

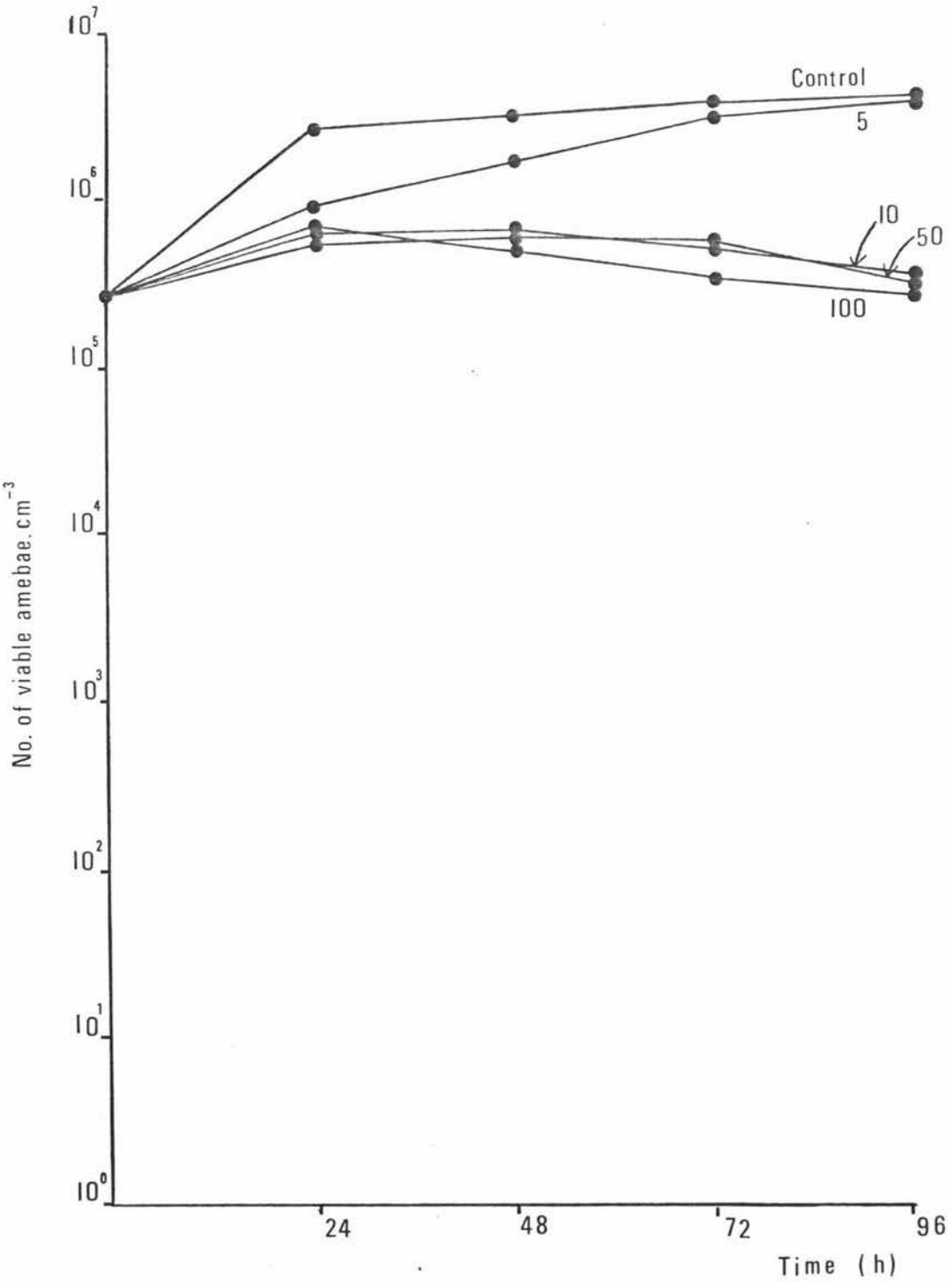
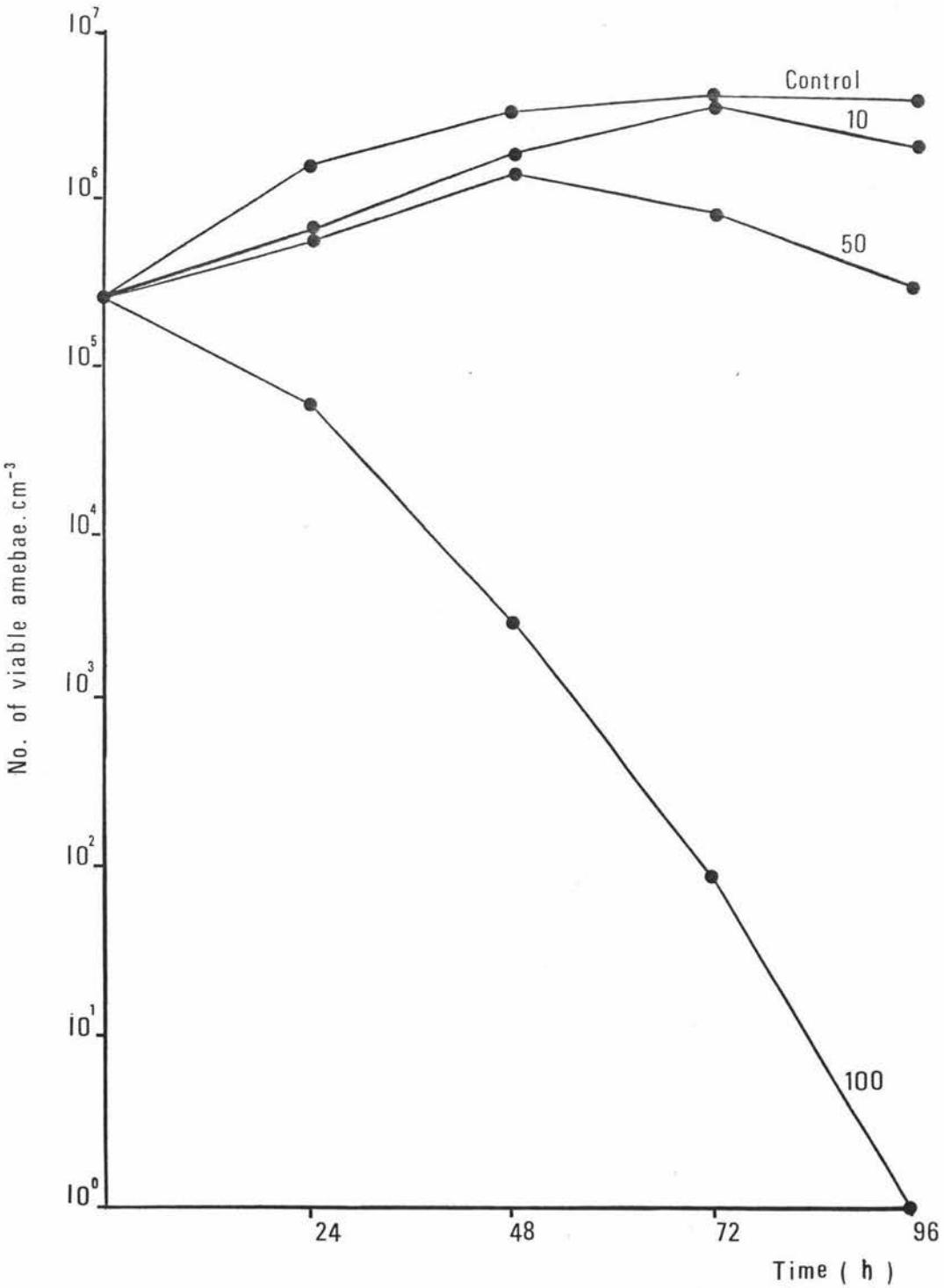


Figure 16. The Effect of Amphotericin B on Acanthamoeba culbertsoni (A-1).



4.2.2. Tetracycline and Rifampicin

The results of screening of tetracycline and rifampicin are shown in Figures 17 to 20.

Tetracycline showed only a slight inhibitory effect on both Acanthamoebae sp. at a concentration of 400 $\mu g.cm^{-3}$ (Figures 17 and 18). No visual signs of drug damage could be detected on the organisms.

Figures 19 and 20 show the results obtained with rifampicin. Compared with the control culture the 500 $\mu g.cm^{-3}$ test cultures contained a higher proportion of cysts.

Figure 17. The Effect of Tetracycline on Acanthamoeba castellanii(1501).

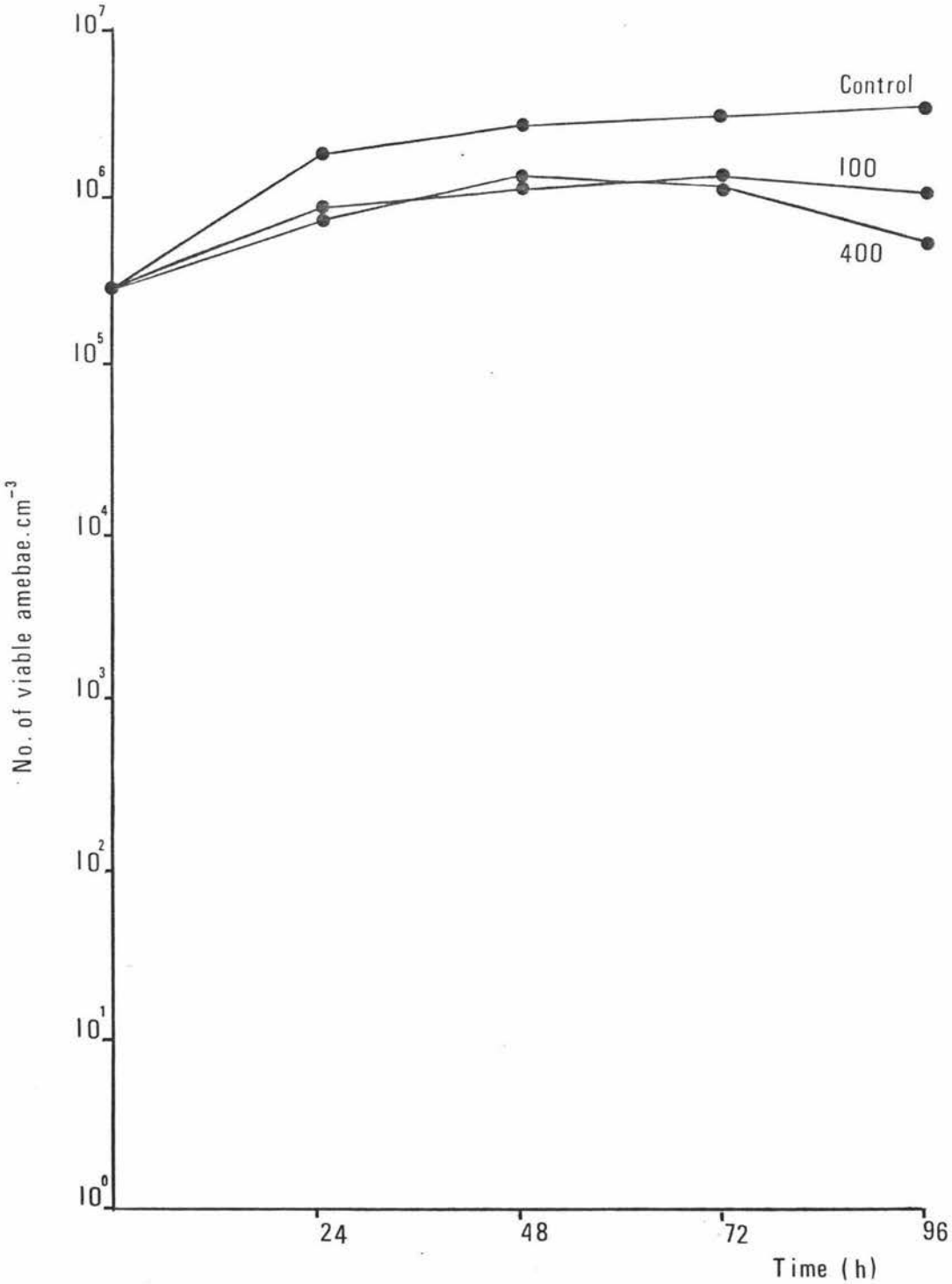


Figure 18. The Effect of Tetracycline on Acanthamoeba culbertsoni(A-1).

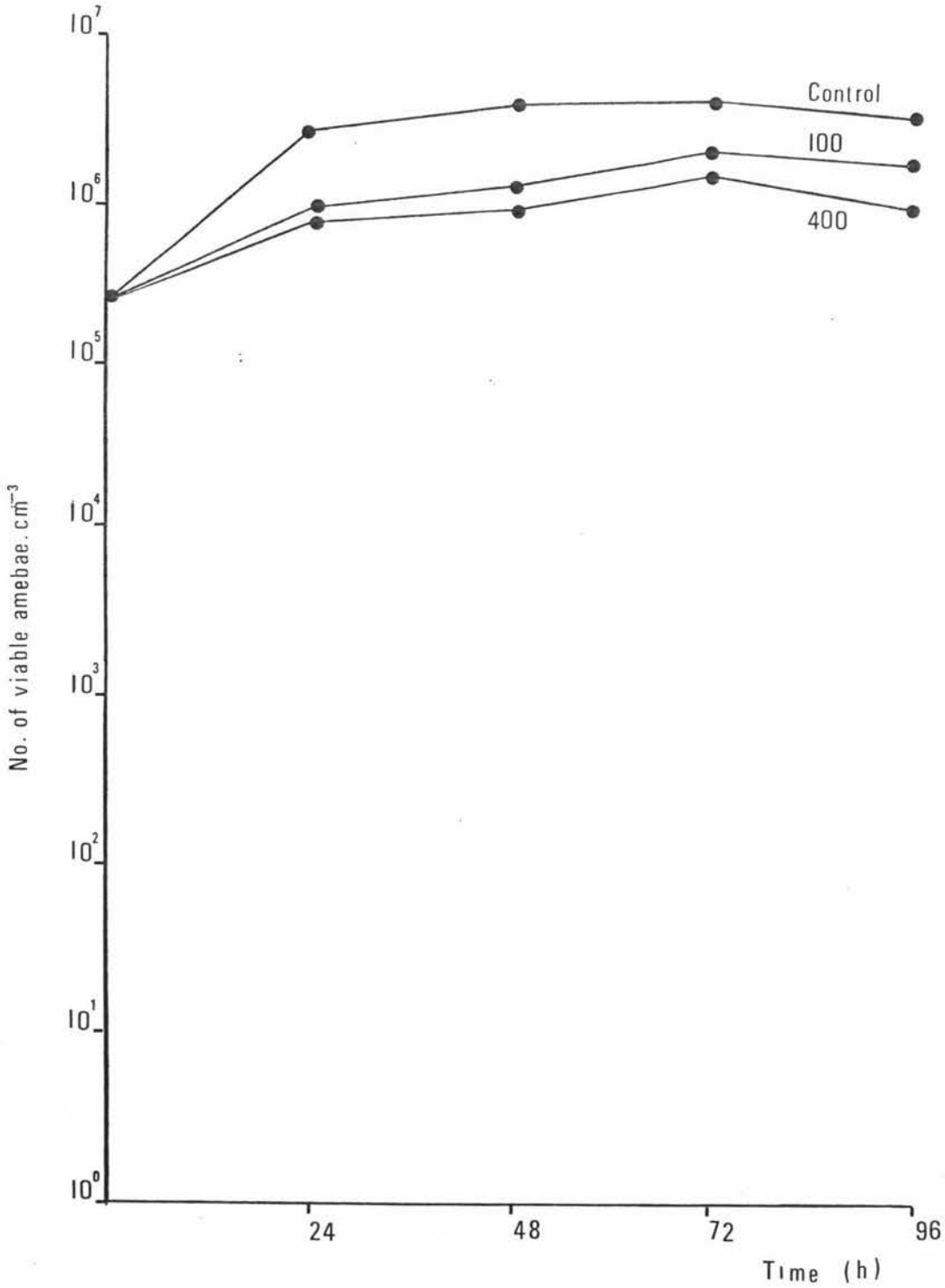


Figure 19. The Effect of Rifampicin on Acanthamoeba castellanii (1501).

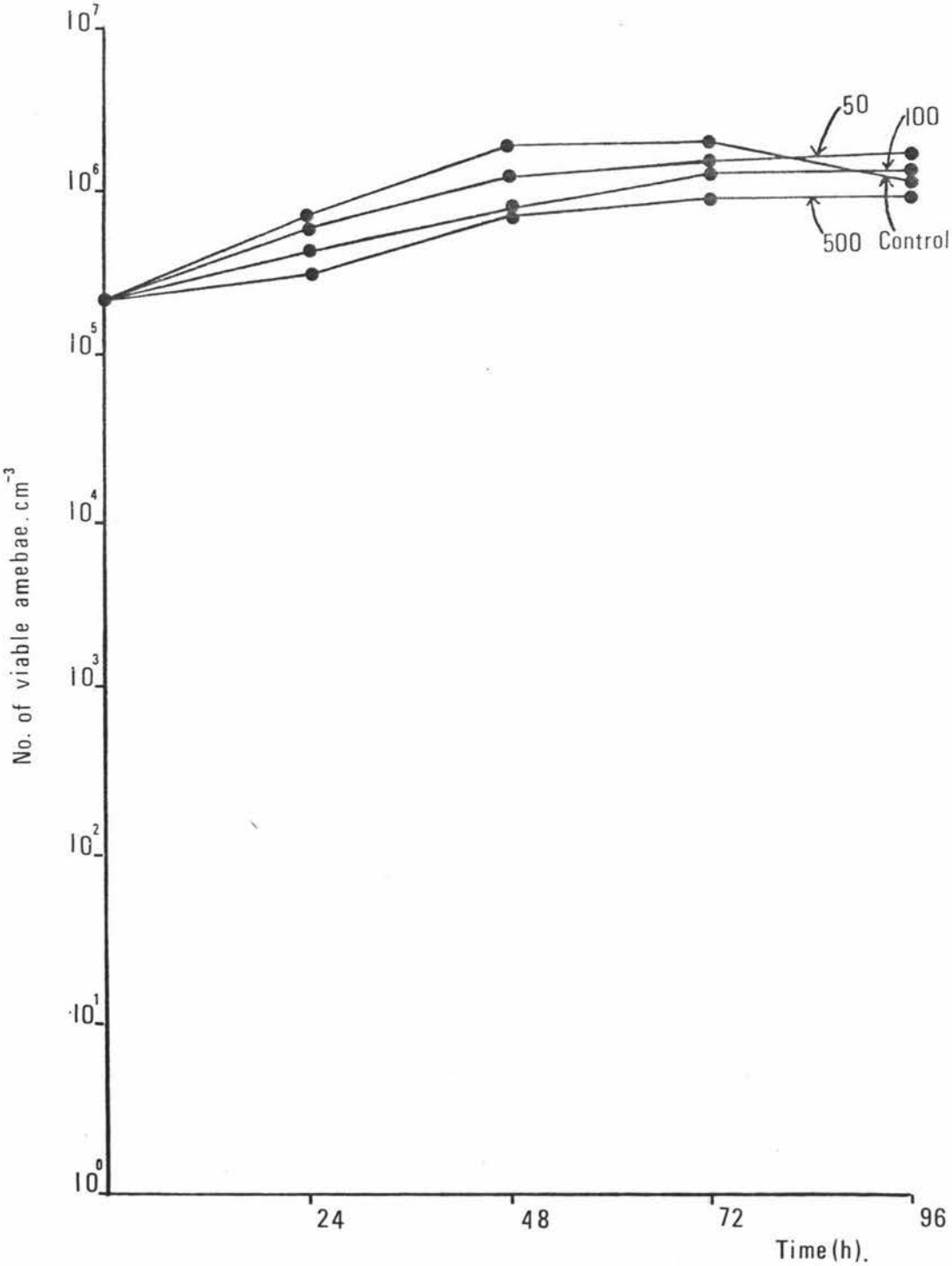
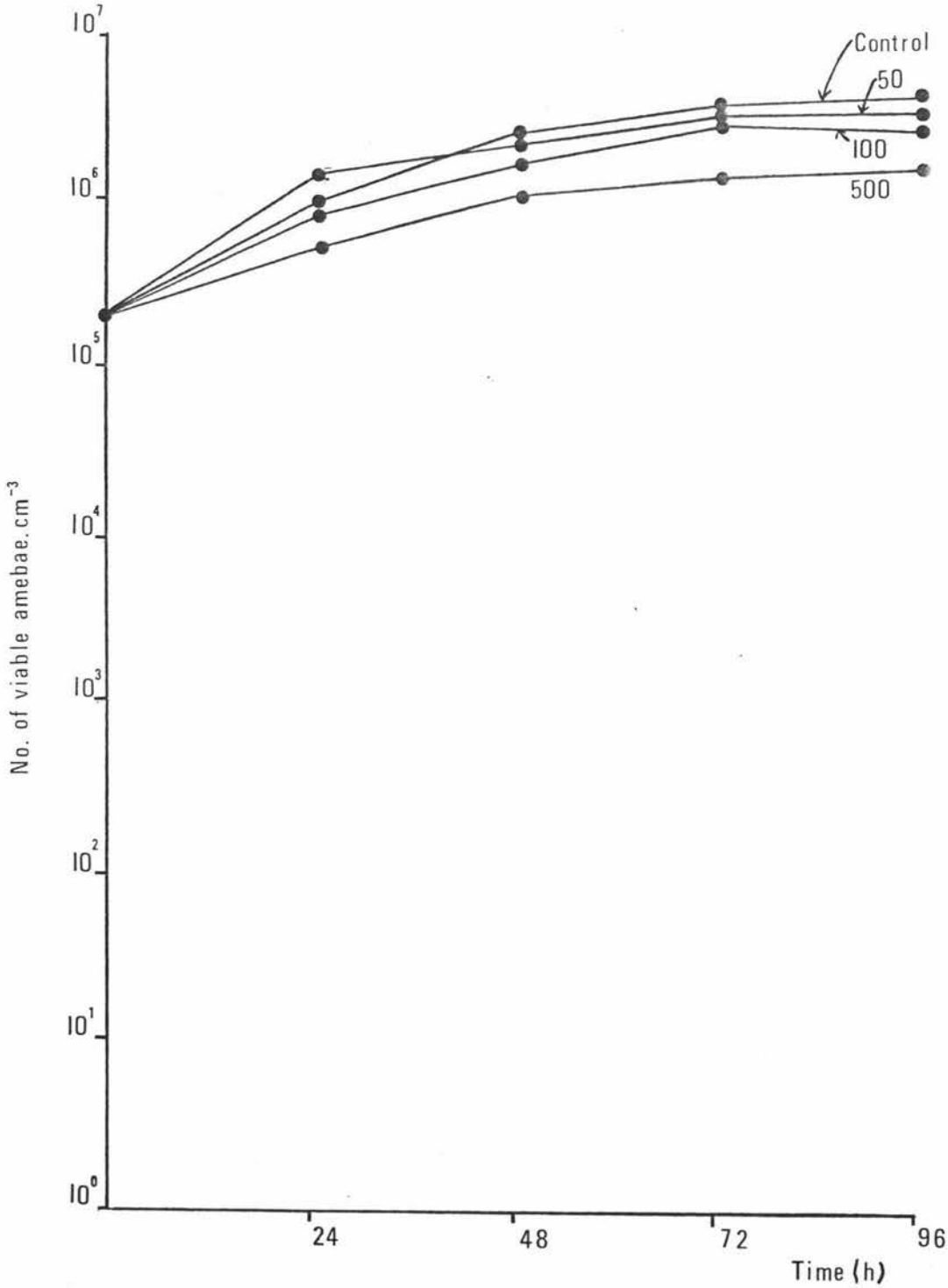


Figure 20. The Effect of Rifampicin on Acanthamoeba culbertsoni(A-1).



4.2.3. 5-Fluorocytosine

Figure 21 shows the results of screening of 5-fluorocytosine at four concentrations against A. castellanii (1501). A similar inhibitory effect was observed at all concentrations tested.

Figure 22 shows the results for A. culbertsoni (A-1). A greater inhibitory effect was seen to that with A. castellanii (1501) with the number of surviving amebae after 96 hours being lower.

Figure 21. The Effect of 5-Fluorocytosine on Acanthamoeba castellanii (1501).

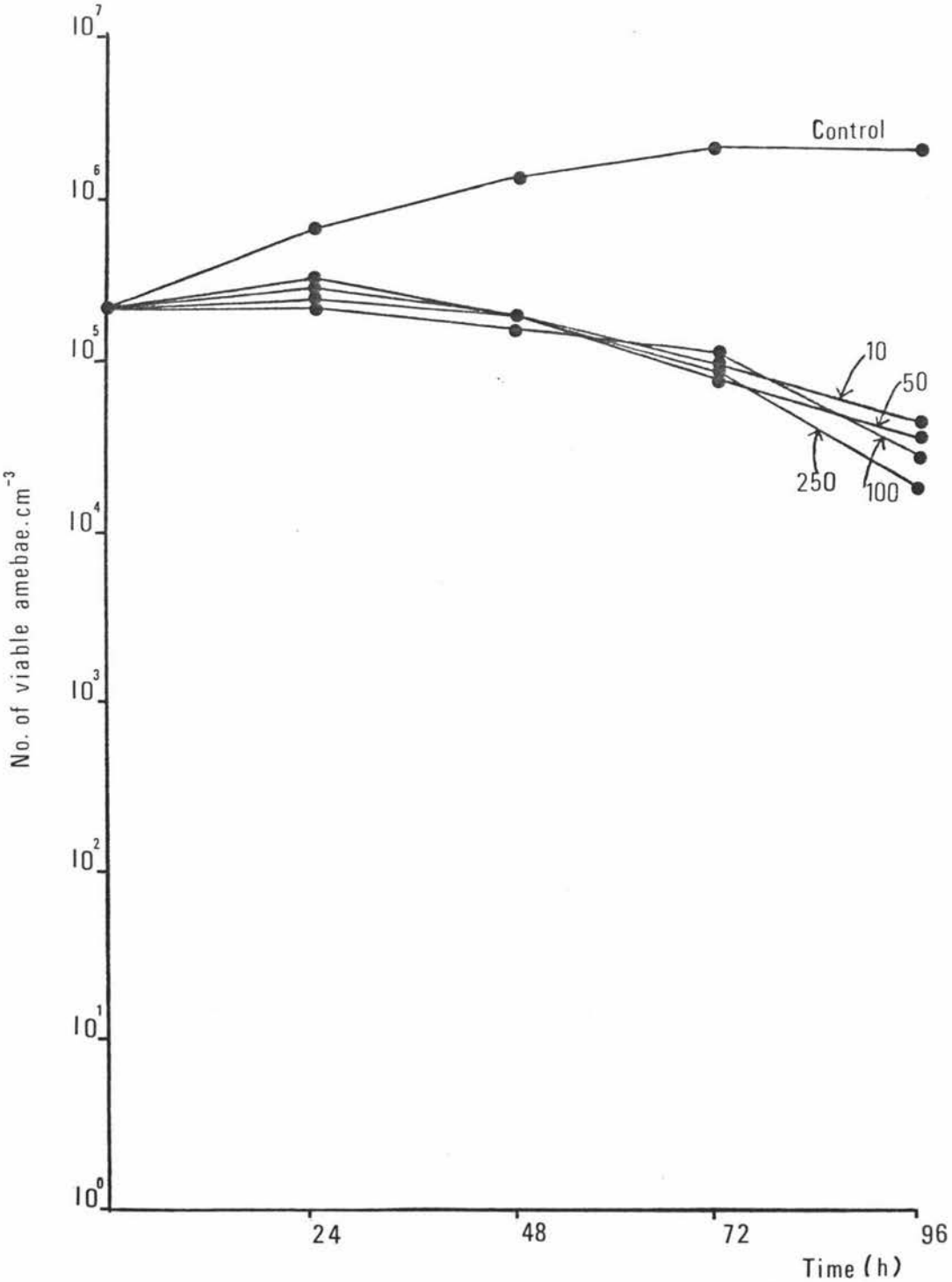
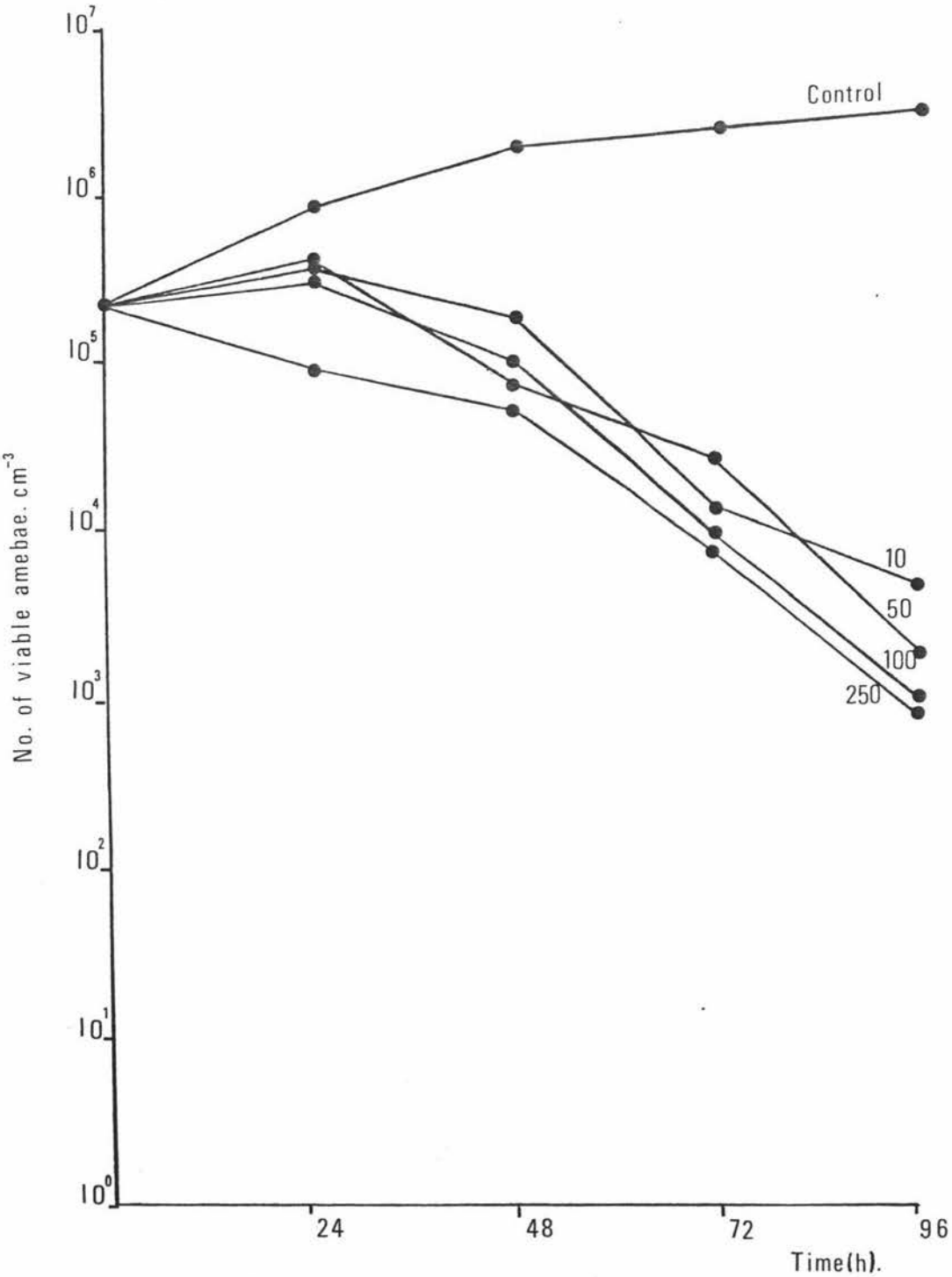


Figure 22. The Effect of 5-Fluorocytosine on Acanthamoeba culbertsoni (A-1).



4.2.4. Polymyxin B sulphate

Figures 23 and 24 show the results of screening of polymyxin B sulphate against Acanthamoebae. Concentrations of $\leq$ 500 units.cm^{-3} had only a slight inhibitory effect. Only at the highest concentration (500 units.cm^{-3}) were increased vacuolation and a lack of acanthapodia obvious with both species.

4.2.5. Miconazole

Miconazole at concentrations $\leq$ 100 μg.cm^{-3} had no effect on either pathogenic or non-pathogenic Acanthamoebae (Figures 25 and 26). None of the amebae in the test cultures showed any variation in appearance from the control organisms.

Figure 23. The Effect of Polymyxin B on Acanthamoeba castellanii (1501).
(Drug concentrations shown in Figs. 23 & 24 are in units.cm^{-3})

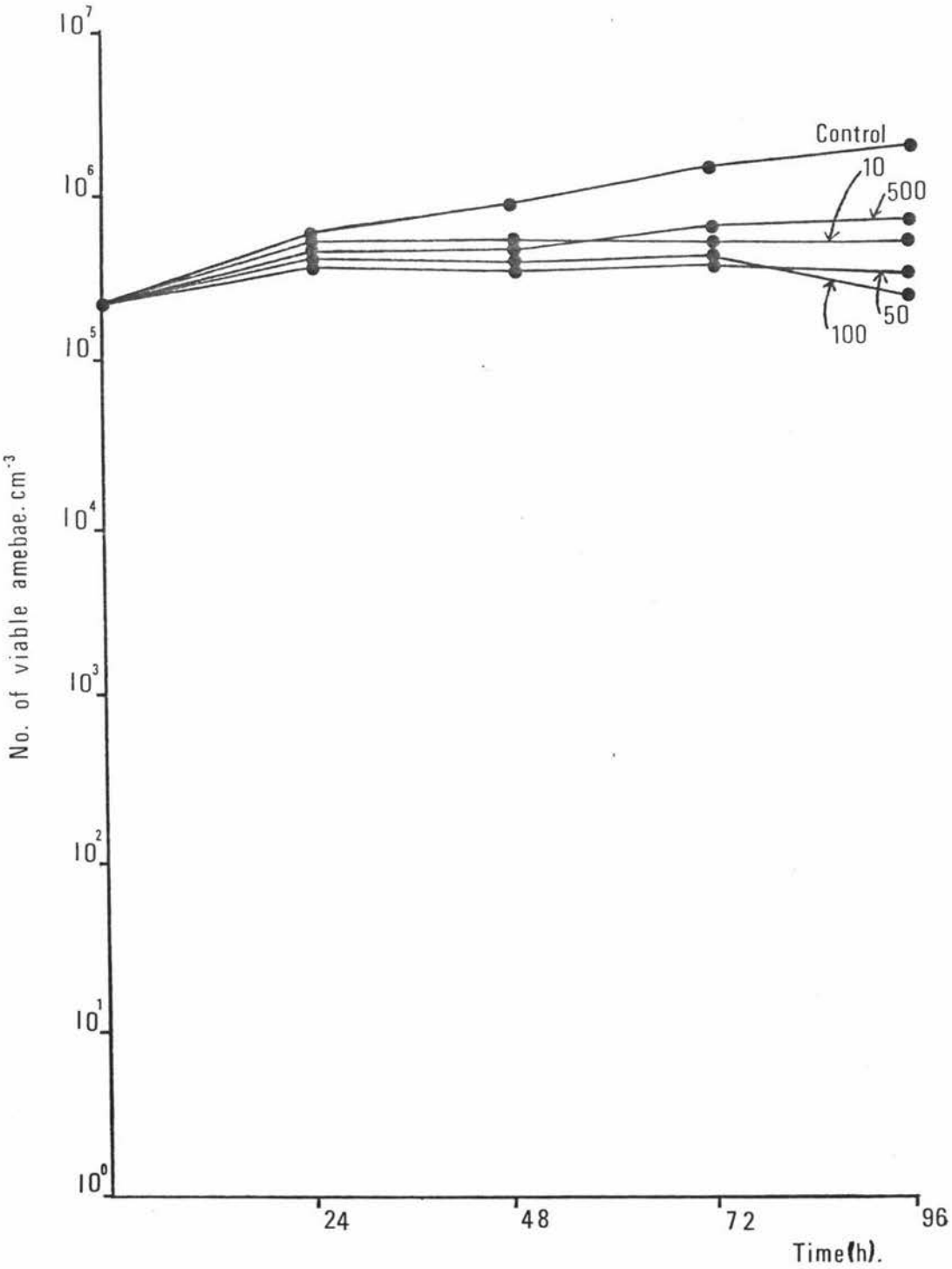


Figure 24. The Effect of Polymyxin B on Acanthamoeba culbertsoni (A-1).

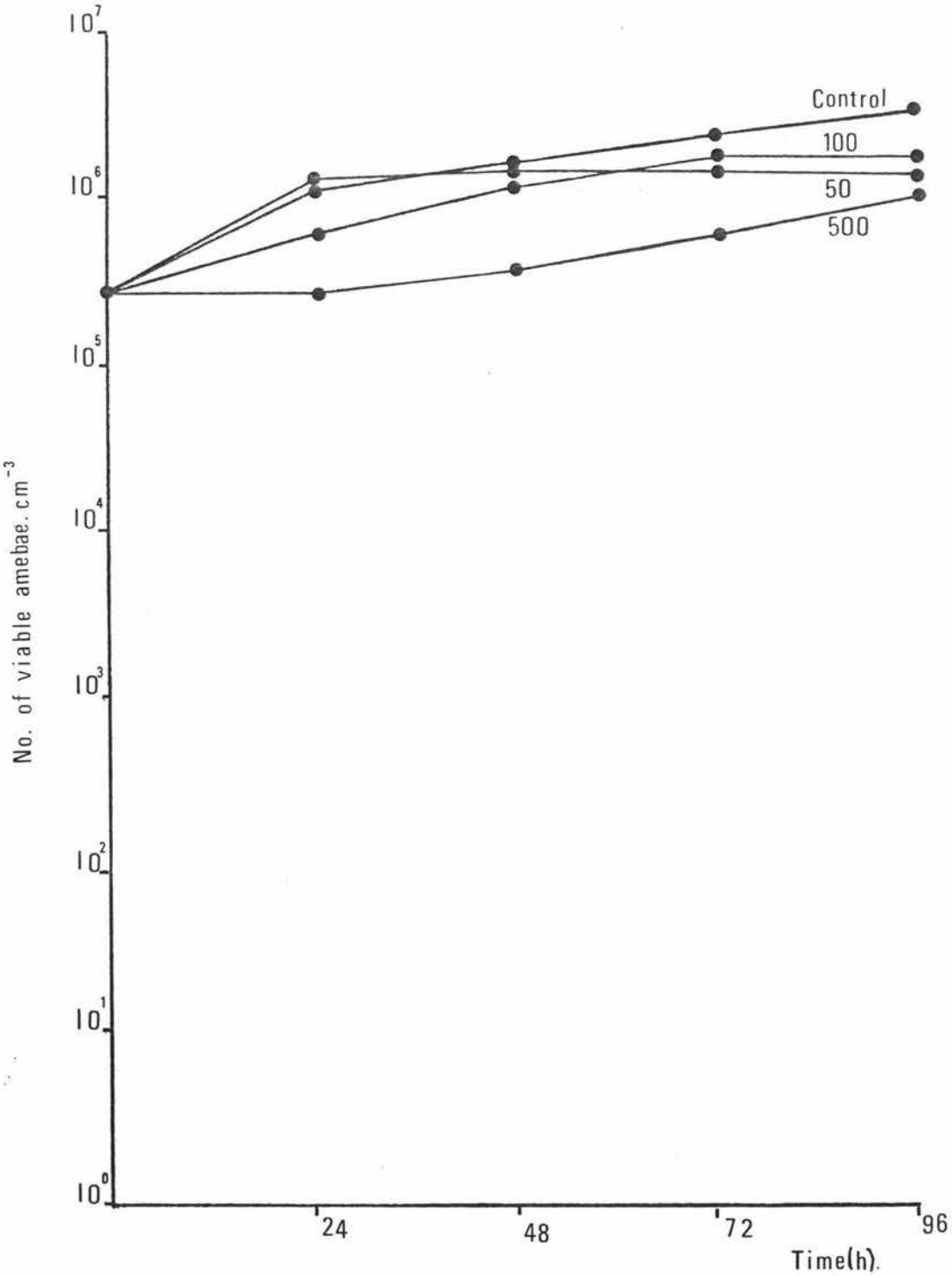


Figure 25. The Effect of Miconazole on Acanthamoeba castellanii (1501).
(Drug concentrations shown in Figs. 25-28 are in $\mu g.cm^{-3}$)

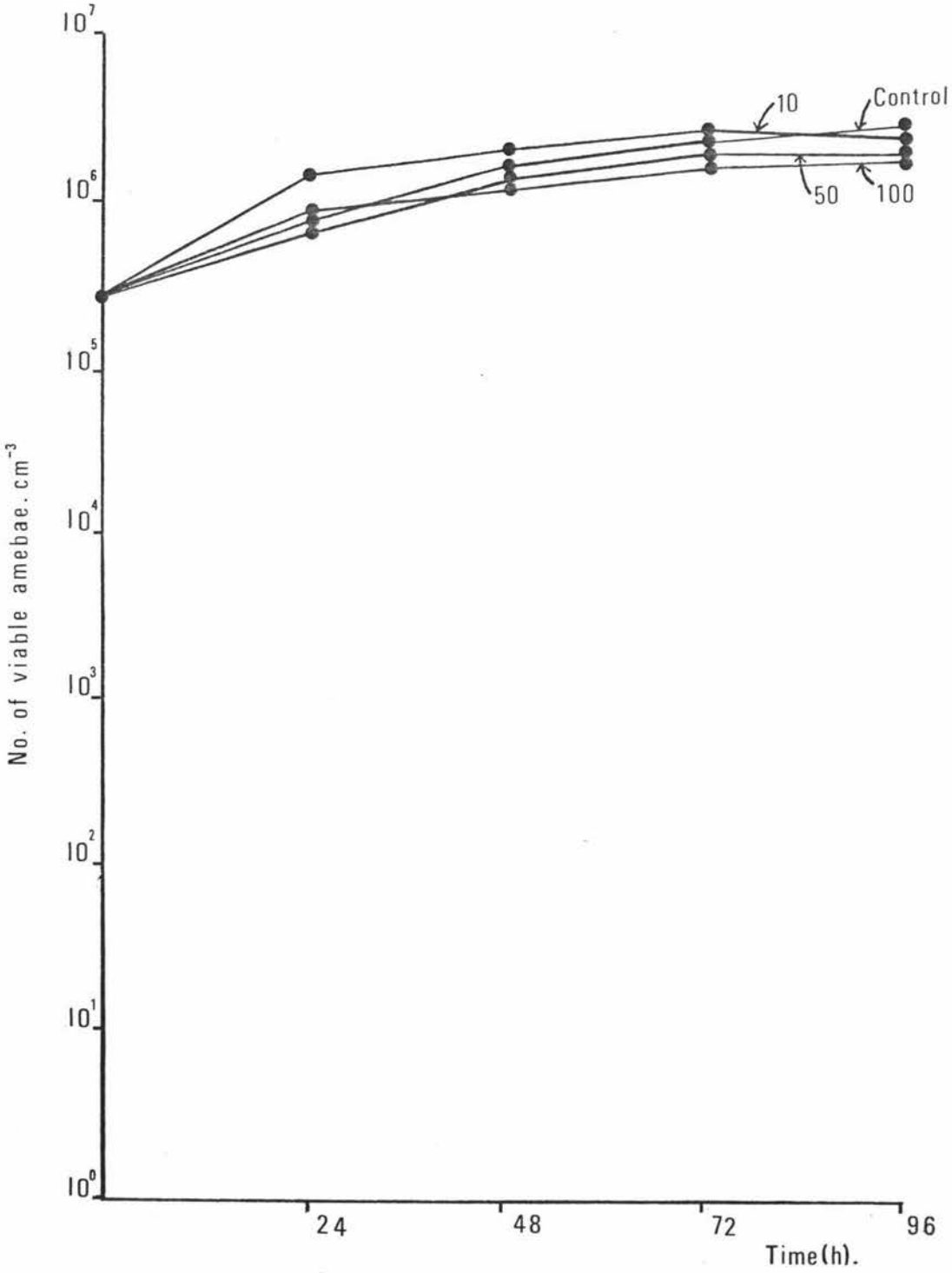
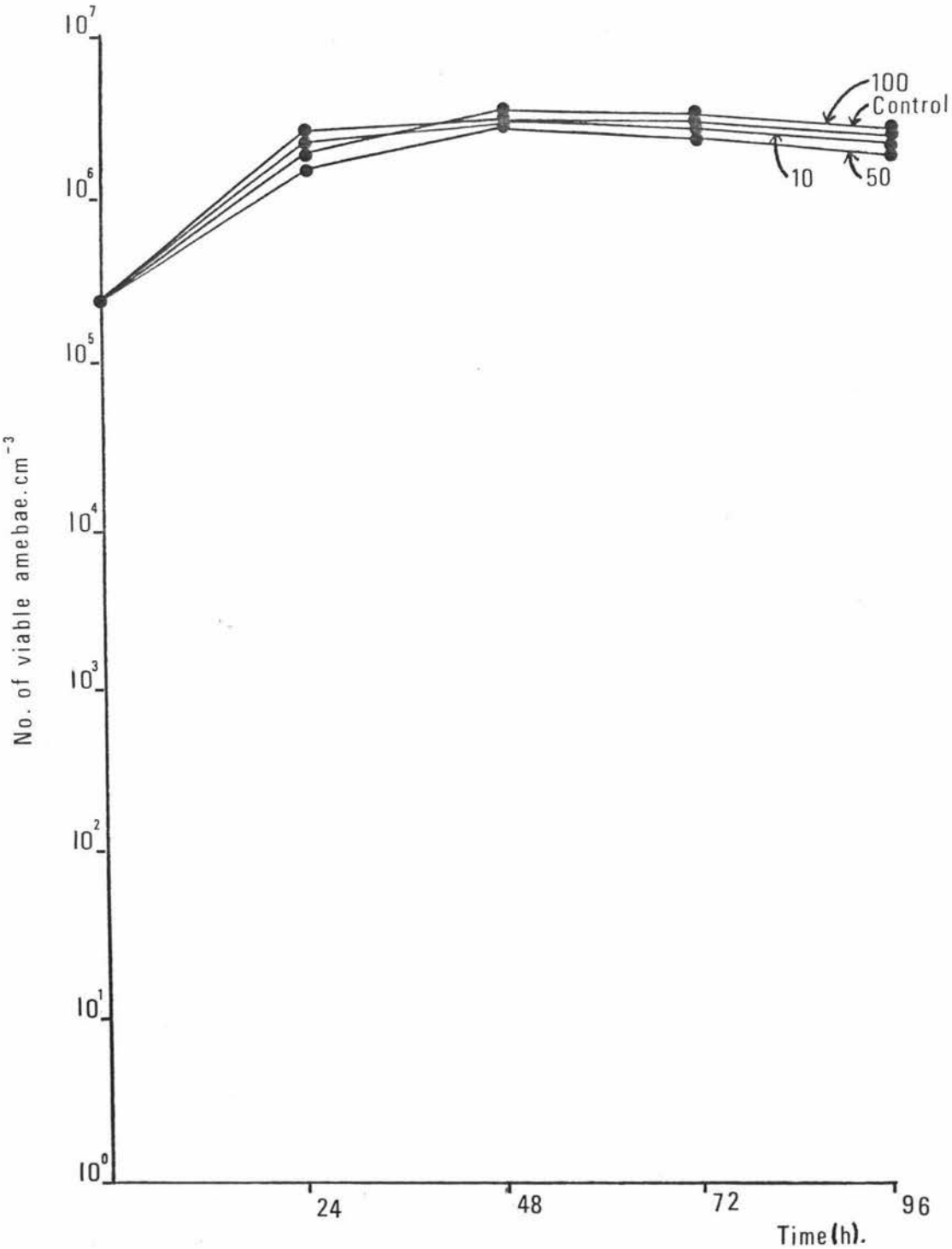


Figure 26. The Effect of Miconazole on Acanthamoeba culbertsoni (A-1).



4.2.6. R41,400

Figures 27 and 28 show the results obtained with four different concentrations of the imidazole derivative, R41,400, against the two Acanthamoebae spp.. Both species showed similar trends but A. culbertsoni appeared more susceptible and after 96 hours there were lower numbers of surviving amebae. At all the drug concentrations tested (25-100 $\mu g.cm^{-3}$) the amebae showed signs of drug damage with increased vacuolation and no visible acanthapodia.

Figure 27. The Effect of R41,400 on Acanthamoeba castellanii (1501).

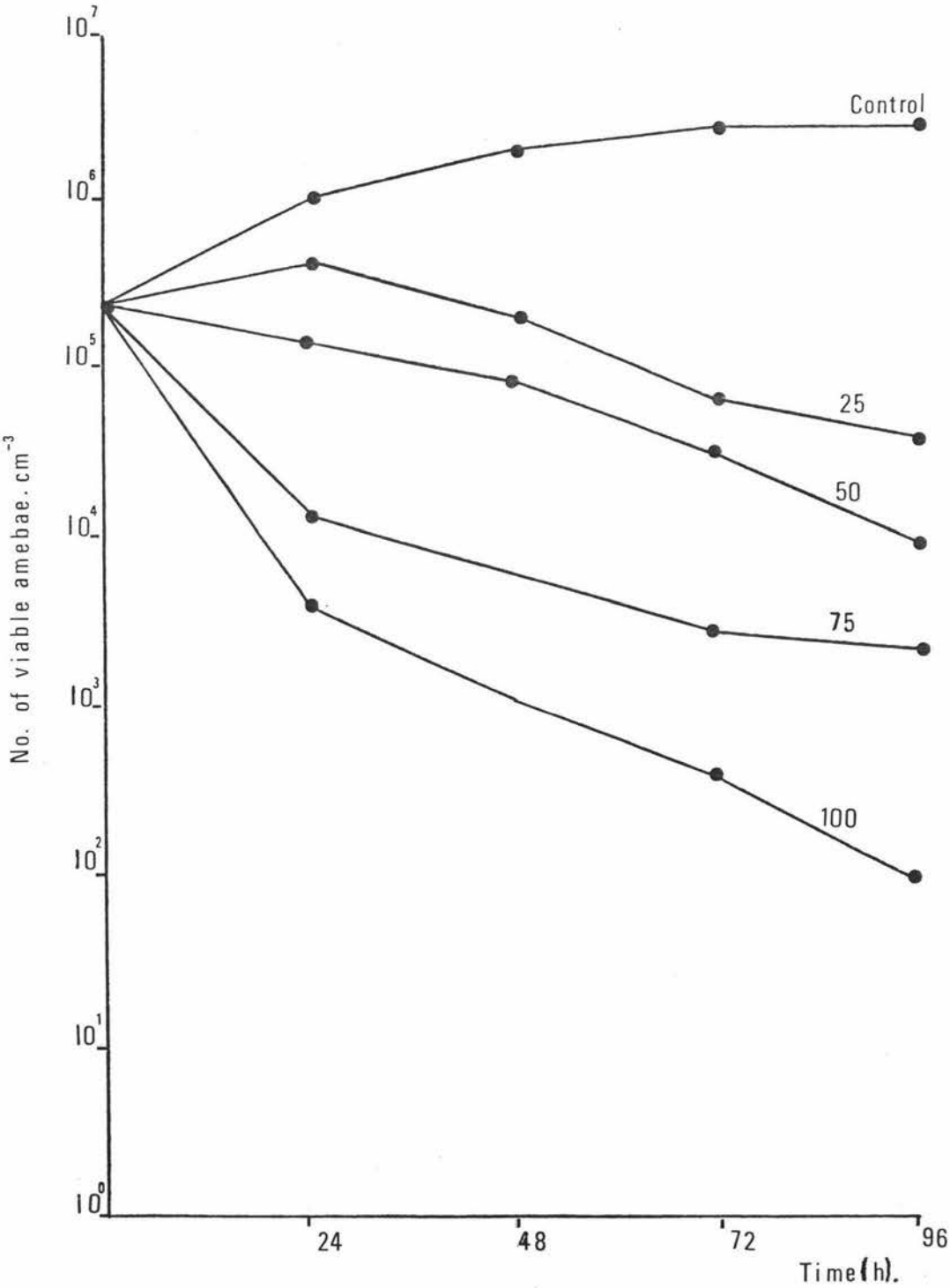
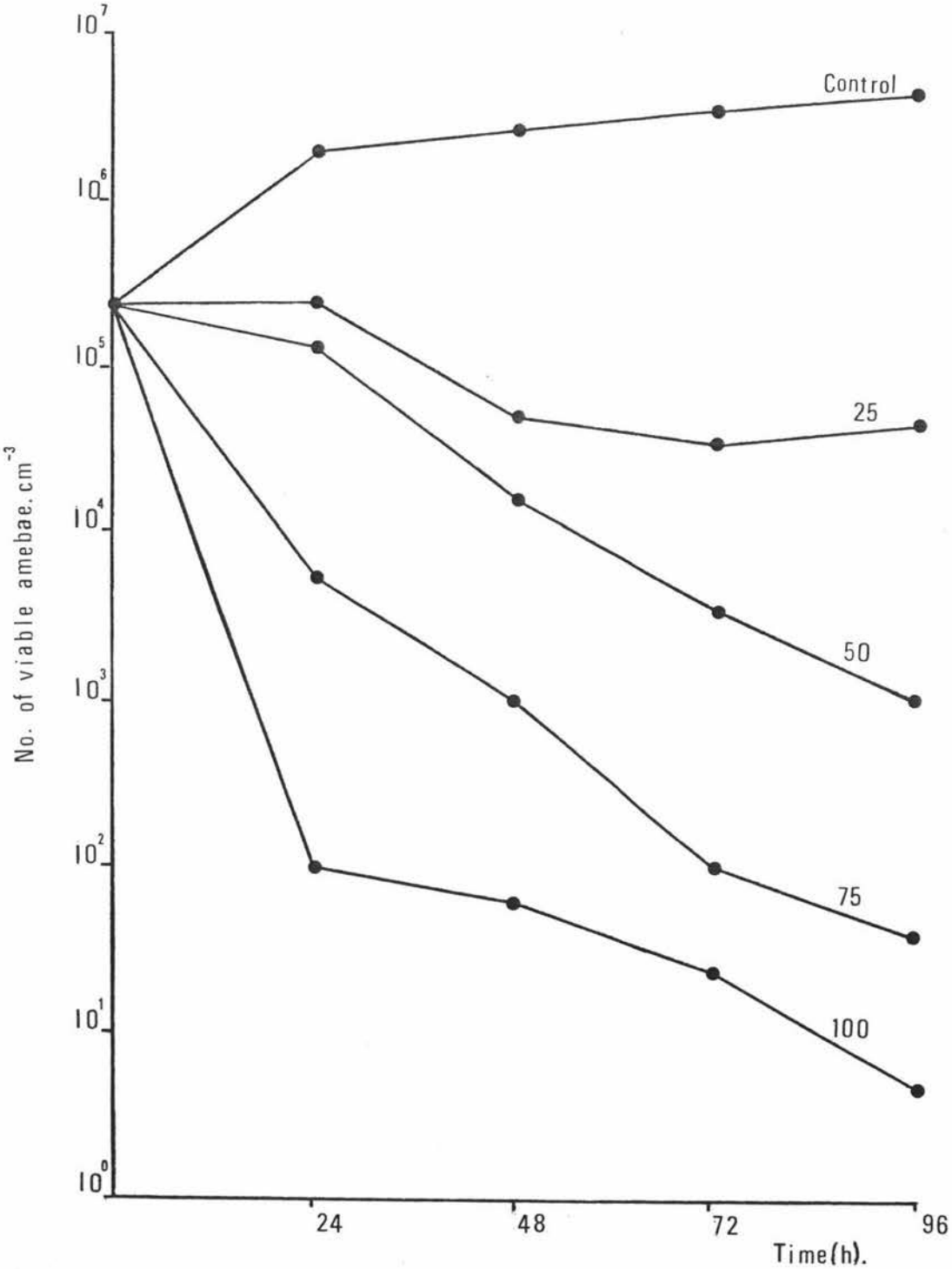


Figure 28. The Effect of R41,400 on Acanthamoeba culbertsoni (A-1).



4.3. The Testing of Drug Combinations against Naegleria fowleri (MsT) in Axenic Culture

4.3.1. Amphotericin B and Tetracycline

The MAC for amphotericin B alone in axenic culture for N. fowleri (MsT) is 0.5 $\mu g.cm^{-3}$ (Figure 2). Tetracycline alone has only a slight inhibitory effect on N. fowleri (MsT) (Figure 4). The results of experiments using these two drugs together are shown in Figures 29 and 30. When 50 $\mu g.cm^{-3}$ tetracycline is used in combination with two different concentrations of amphotericin B no significant difference is seen from that obtained with amphotericin B alone. Only when tetracycline was present at a concentration of 100 $\mu g.cm^{-3}$ was any synergistic effect seen. 0.25 $\mu g.cm^{-3}$ amphotericin B and 100 $\mu g.cm^{-3}$ tetracycline was amebicidal in 96 hours. With 0.5 $\mu g.cm^{-3}$ amphotericin B and 100 $\mu g.cm^{-3}$ tetracycline the time required to completely sterilize the medium was reduced from 96 to 72 hours.

Figure 29: The Effect of Amphotericin B and Tetracycline Alone and in Combination on Naegleria fowleri (MsT)

● = 0.25 μg.cm^{-3} Amphotericin B

■ = 50 μg.cm^{-3} Tetracycline

▲ = 100 μg.cm^{-3} Tetracycline

□ = 0.25 μg.cm^{-3} Amphotericin B + 50 μg.cm^{-3} Tetracycline

△ = 0.25 μg.cm^{-3} Amphotericin B + 100 μg.cm^{-3} Tetracycline

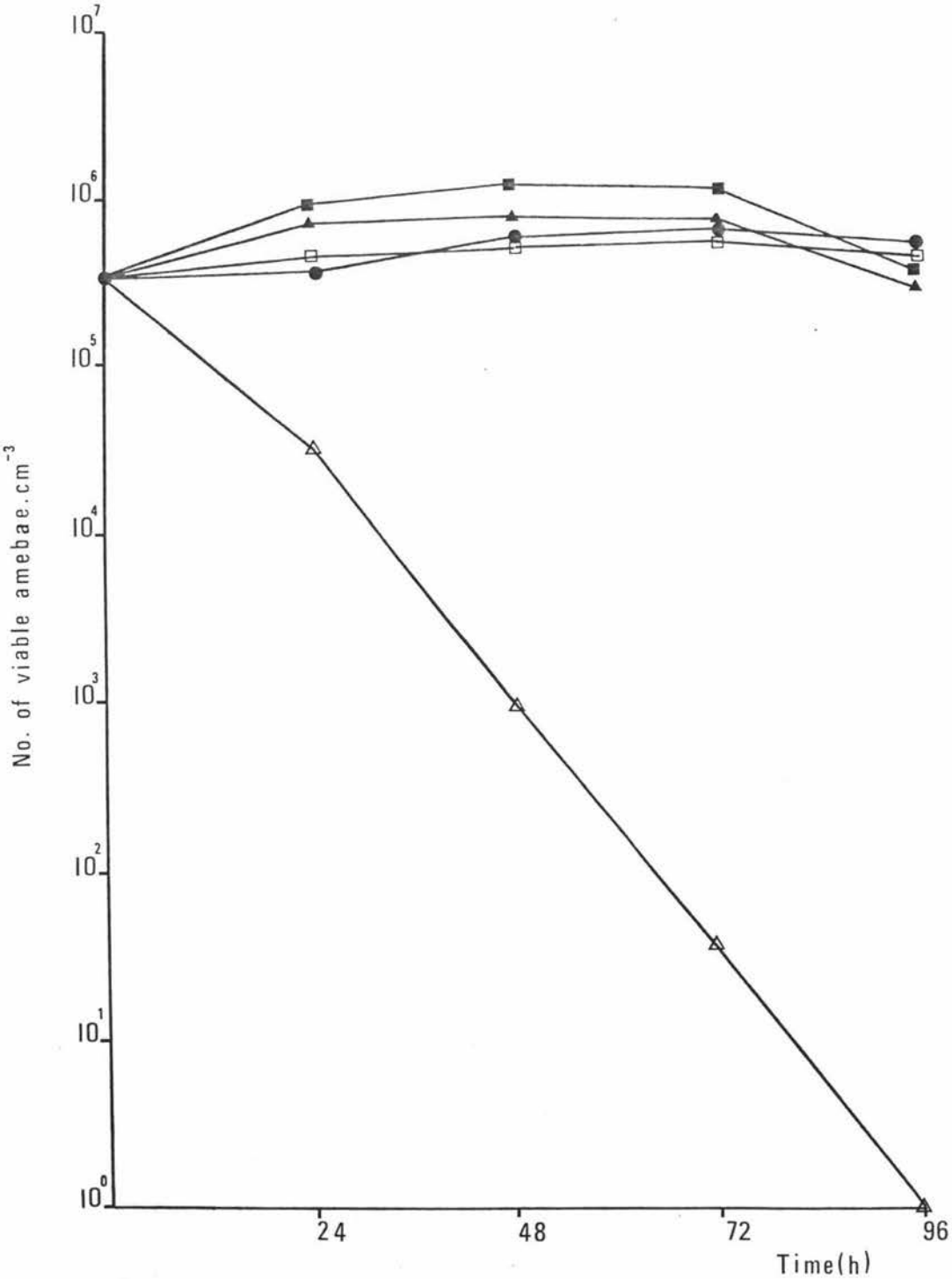

10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
No. of viable amebae.cm^{-3}
24
48
72
96
Time(h)

Figure 30: The Effect of Amphotericin B and Tetracycline Alone and in Combination on Naegleria fowleri (MsT)

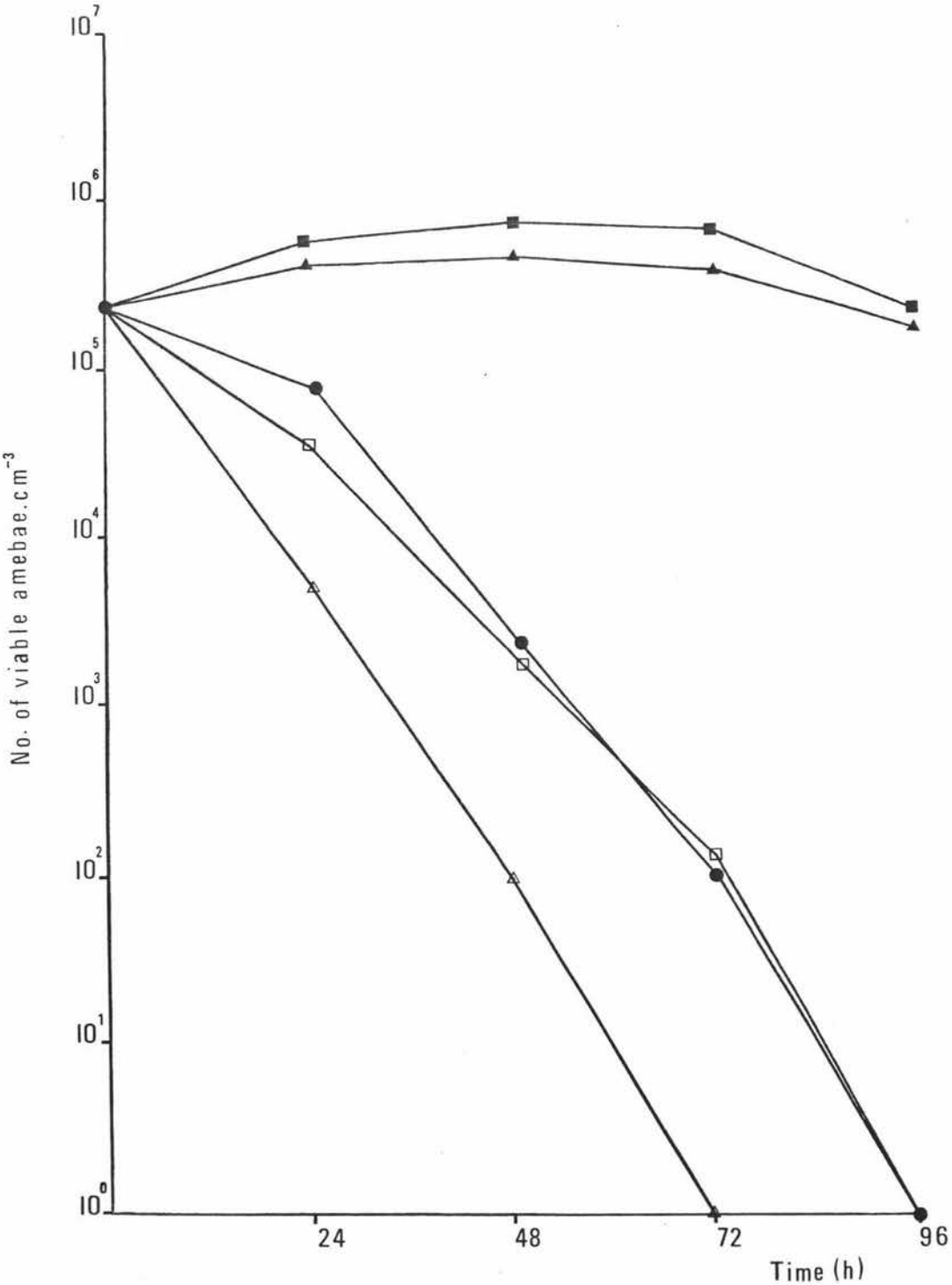
● = 0.5 μg.cm^{-3} Amphotericin B

■ = 50 μg.cm^{-3} Tetracycline

▲ = 100 μg.cm^{-3} Tetracycline

□ = 0.5 μg.cm^{-3} Amphotericin B + 50 μg.cm^{-3} Tetracycline

△ = 0.5 μg.cm^{-3} Amphotericin B + 100 μg.cm^{-3} Tetracycline


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

4.3.2. Amphotericin B and Rifampicin

Figures 31 and 32 show the results obtained with amphotericin B and rifampicin alone and in combination. Rifampicin alone has no inhibitory effect on N. fowleri (MsT) at concentrations $\leq 100\,\mu g.cm^{-3}$ (Figure 6). Figure 31 shows the results obtained with $0.25\,\mu g.cm^{-3}$ amphotericin B in combination with two different concentrations of rifampicin. A synergistic effect is seen with both 10 and $50\,\mu g.cm^{-3}$ rifampicin, the inhibitory effect being greater with the higher concentration. When rifampicin, at 10 and $50\,\mu g.cm^{-3}$, is combined with $0.5\,\mu g.cm^{-3}$ amphotericin B the time required to completely sterilize the media is reduced from 96 hours to 72 and 48 hours respectively (Figure 32).

Figure 31: The Effect of Amphotericin B and Rifampicin Alone and in Combination on Naegleria fowleri (MsT)

● = 0.25 μg.cm^{-3} Amphotericin B

■ = 10 μg.cm^{-3} Rifampicin

▲ = 50 μg.cm^{-3} Rifampicin

□ = 0.25 μg.cm^{-3} Amphotericin B + 10 μg.cm^{-3} Rifampicin

△ = 0.25 μg.cm^{-3} Amphotericin B + 50 μg.cm^{-3} Rifampicin

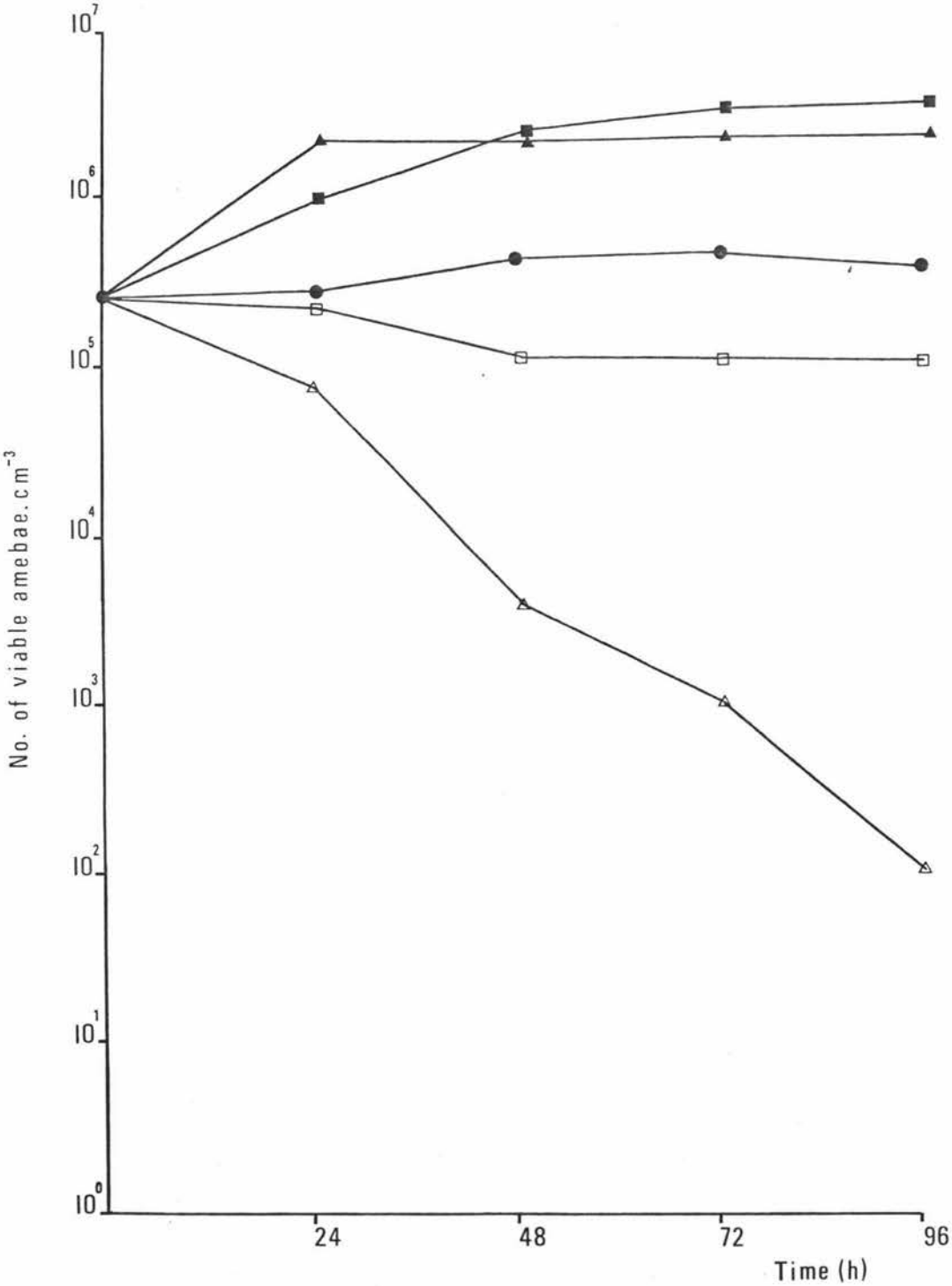

No. of viable amebae. cm^{-3}
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
24
48
72
96
Time (h)

Figure 32: The Effect of Amphotericin B and Rifampicin Alone and in Combination on Naegleria fowleri (MsT)

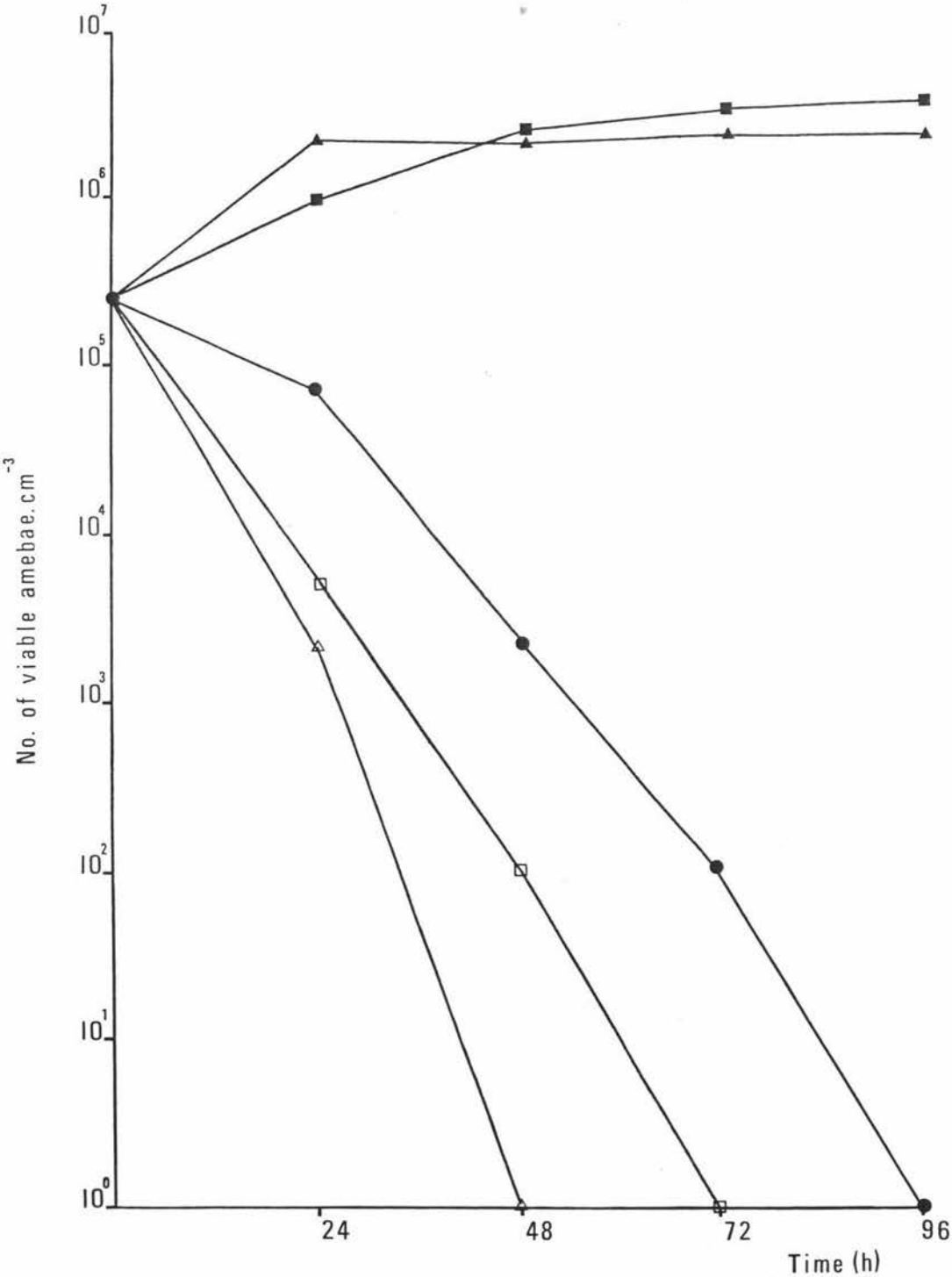
● = 0.5 μg.cm^{-3} Amphotericin B

■ = 10 μg.cm^{-3} Rifampicin

▲ = 50 μg.cm^{-3} Rifampicin

□ = 0.5 μg.cm^{-3} Amphotericin B + 10 μg.cm^{-3} Rifampicin

△ = 0.5 μg.cm^{-3} Amphotericin B + 50 μg.cm^{-3} Rifampicin


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

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

4.4.1. Polymyxin B and 5-Fluorocytosine

Polymyxin B alone has only a slight initial inhibitory effect at a concentration of 500 units.cm^{-3} on A. culbertsoni (A-1) (Figure 24). 5-Fluorocytosine is amebastatic at concentrations $\leqslant 250\,\mu g.cm^{-3}$ (Figure 22). Figure 33 shows the results obtained when 100 units.cm^{-3} polymyxin B is combined with both 50 and 100 $\mu g.cm^{-3}$ 5-fluorocytosine. No synergistic effect is seen. When the concentration of polymyxin B is increased to 500 units.cm^{-3} complete kill results with both 50 and 100 $\mu g.cm^{-3}$ 5-fluorocytosine (Figure 34).

Figure 33: The Effect of Polymyxin B and 5-Fluorocytosine Alone and in Combination on Acanthamoeba culbertsoni (A-1)

●= 100 units.cm^{-3} Polymyxin B

■= 50 μg.cm^{-3} 5-Fluorocytosine

▲= 100 μg.cm^{-3} 5-Fluorocytosine

□= 100 units.cm^{-3} Polymyxin B + 50 μg.cm^{-3} 5-Fluorocytosine

△= 100 units.cm^{-3} Polymyxin B + 100 μg.cm^{-3} 5-Fluorocytosine

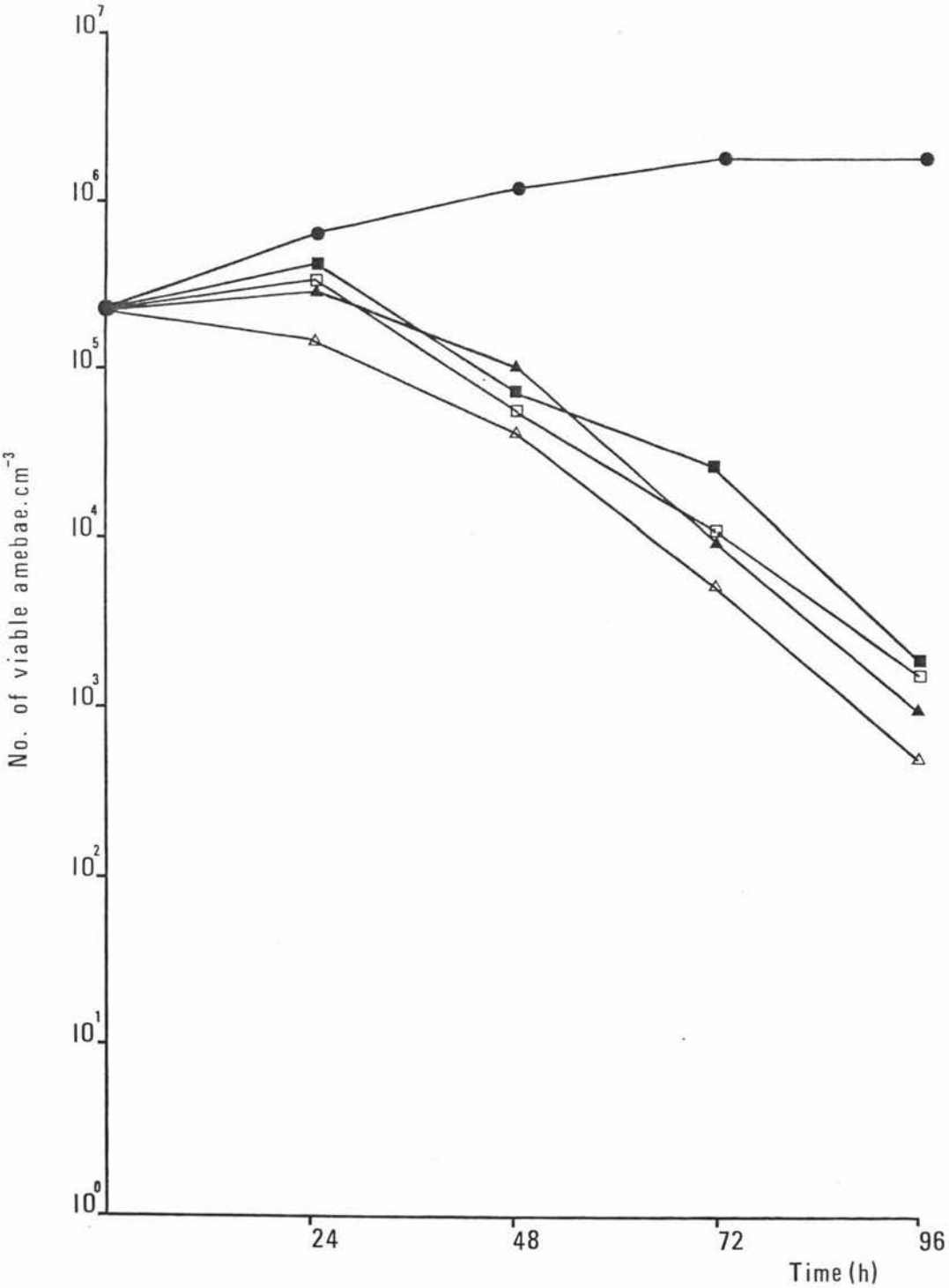

No. of viable amebae.cm^{-3}
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
24
48
72
96
Time (h)

Figure 34: The Effect of Polymyxin B and 5-Fluorocytosine Alone and in Combination on Acanthamoeba culbertsoni (A-1)

● = 500 units.cm^{-3} Polymyxin B

◮ = 10 μg.cm^{-3} 5-Fluorocytosine

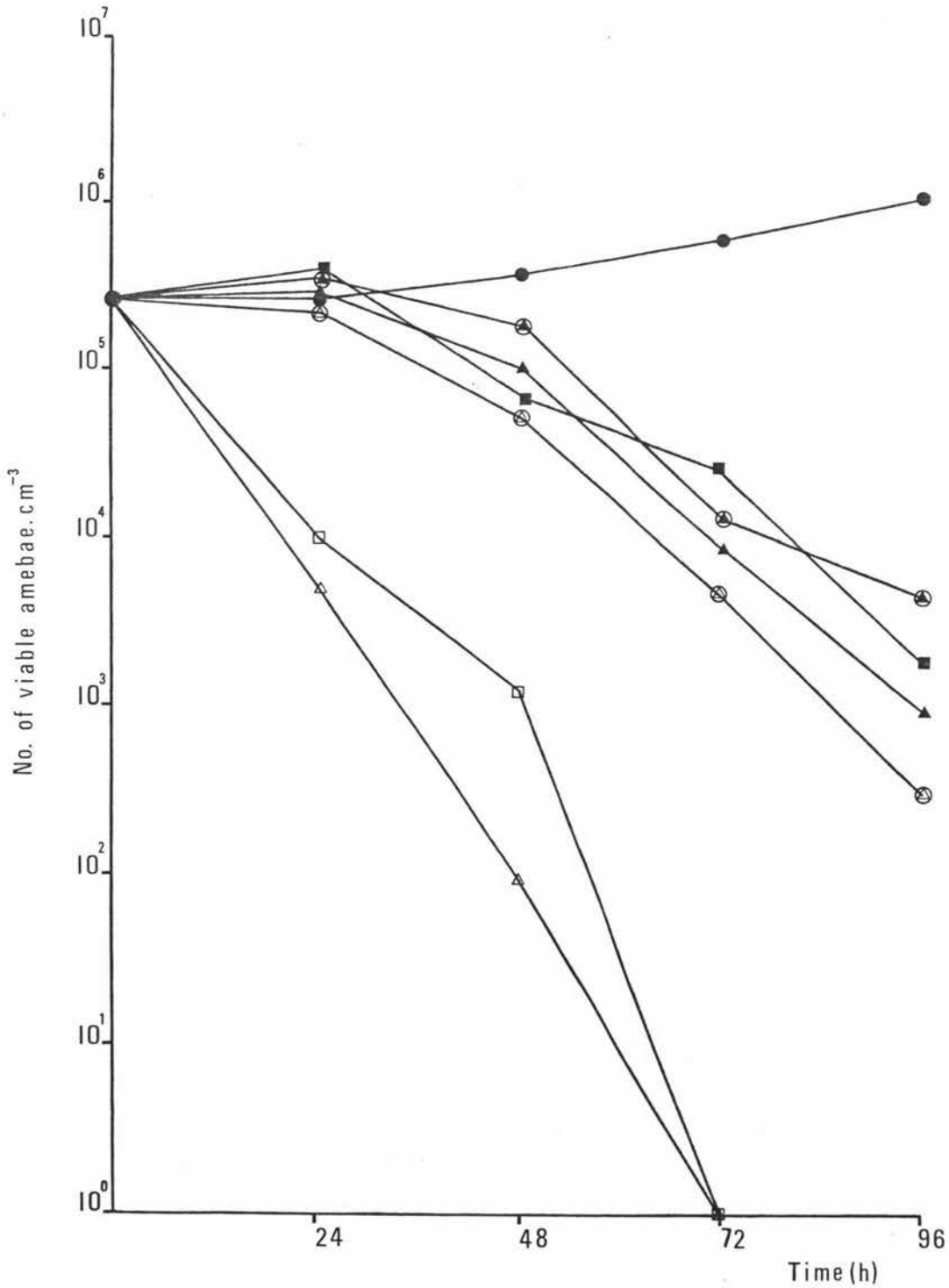
■ = 50 μg.cm^{-3} 5-Fluorocytosine

▲ = 100 μg.cm^{-3} 5-Fluorocytosine

◭ = 500 units.cm^{-3} Polymyxin B + 10 μg.cm^{-3} 5-Fluorocytosine

□ = 500 units.cm^{-3} Polymyxin B + 50 μg.cm^{-3} 5-Fluorocytosine

△ = 500 units.cm^{-3} Polymyxin B + 100 μg.cm^{-3} 5-Fluorocytosine


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

4.4.2. Polymyxin B and Tetracycline

Tetracycline has no effect on Acanthamoebae spp. even at a concentration of 400 $\mu g.cm^{-3}$ (Figures 17 and 18). When combined with 100 and 500 units.cm^{-3} polymyxin B a slight synergistic effect is seen (Figures 35 and 36).

4.4.3. Polymyxin B and Rifampicin

Rifampicin at concentrations $\leq 500\ \mu g.cm^{-3}$ have no effect on Acanthamoebae spp. (Figures 19 and 20). Figures 37 and 38 show the results of using polymyxin B and rifampicin together. Although an initial inhibitory effect was seen with all four combinations, growth of the test cultures was evident. The amebae showed no signs of drug damage except when combinations of the highest concentrations of both agents were used.

Figure 35: The Effect of Polymyxin B and Tetracycline Alone and in Combination on Acanthamoeba culbertsoni (A-1)

● = 100 units.cm^{-3} Polymyxin B

■ = 100 μg.cm^{-3} Tetracycline

▲ = 400 μg.cm^{-3} Tetracycline

□ = 100 units.cm^{-3} Polymyxin B + 100 μg.cm^{-3} Tetracycline

△ = 100 units.cm^{-3} Polymyxin B + 400 μg.cm^{-3} Tetracycline

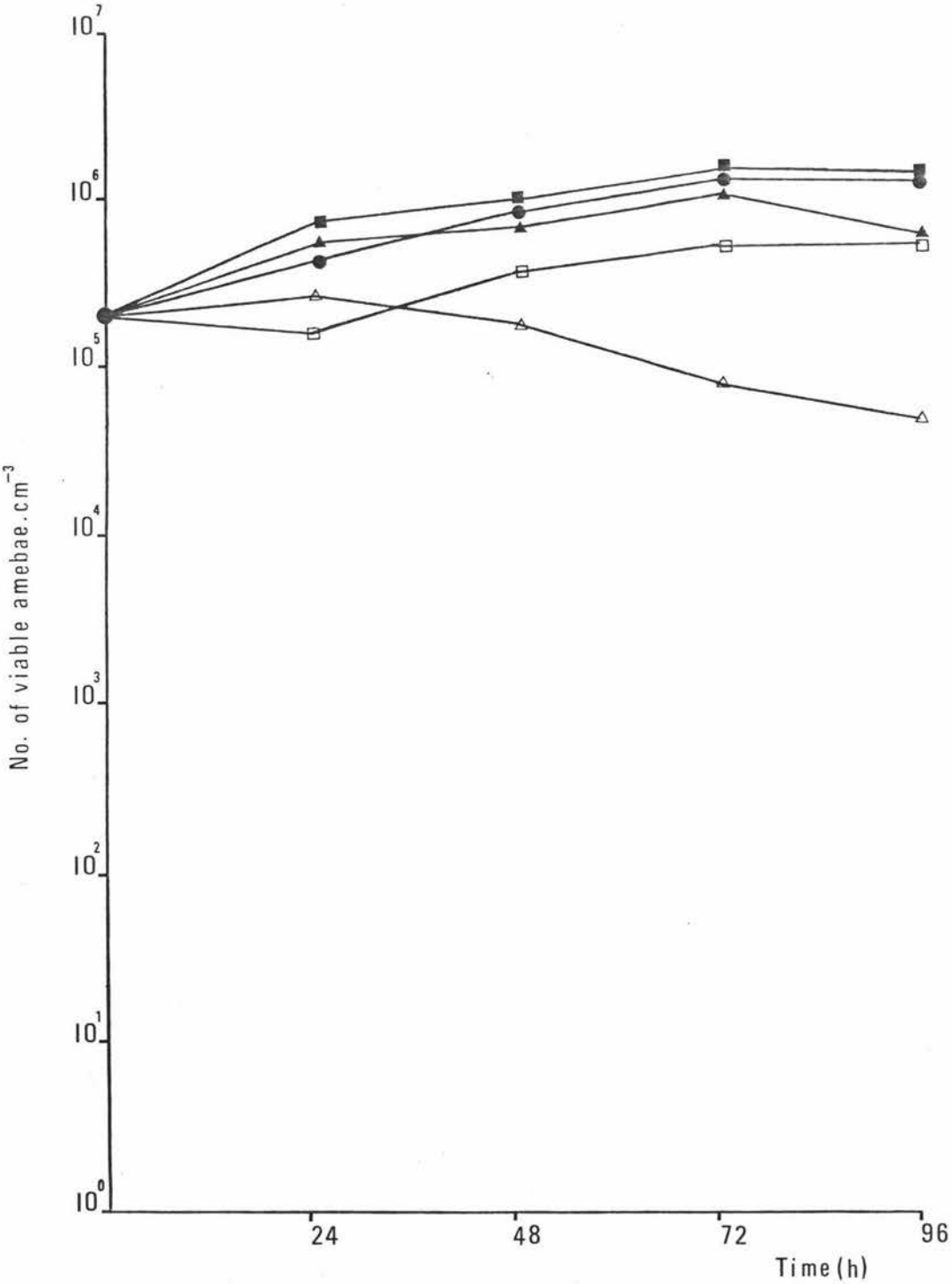

No. of viable amebae.cm^{-3}
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
24
48
72
96
Time (h)

Figure 36: The Effect of Polymyxin B and Tetracycline Alone and in Combination on Acanthamoeba culbertsoni (A-1)

● = 500 units.cm^{-3} Polymyxin B

■ = 100 μg.cm^{-3} Tetracycline

▲ = 400 μg.cm^{-3} Tetracycline

□ = 500 units.cm^{-3} Polymyxin B + 100 μg.cm^{-3} Tetracycline

△ = 500 units.cm^{-3} Polymyxin B + 400 μg.cm^{-3} Tetracycline

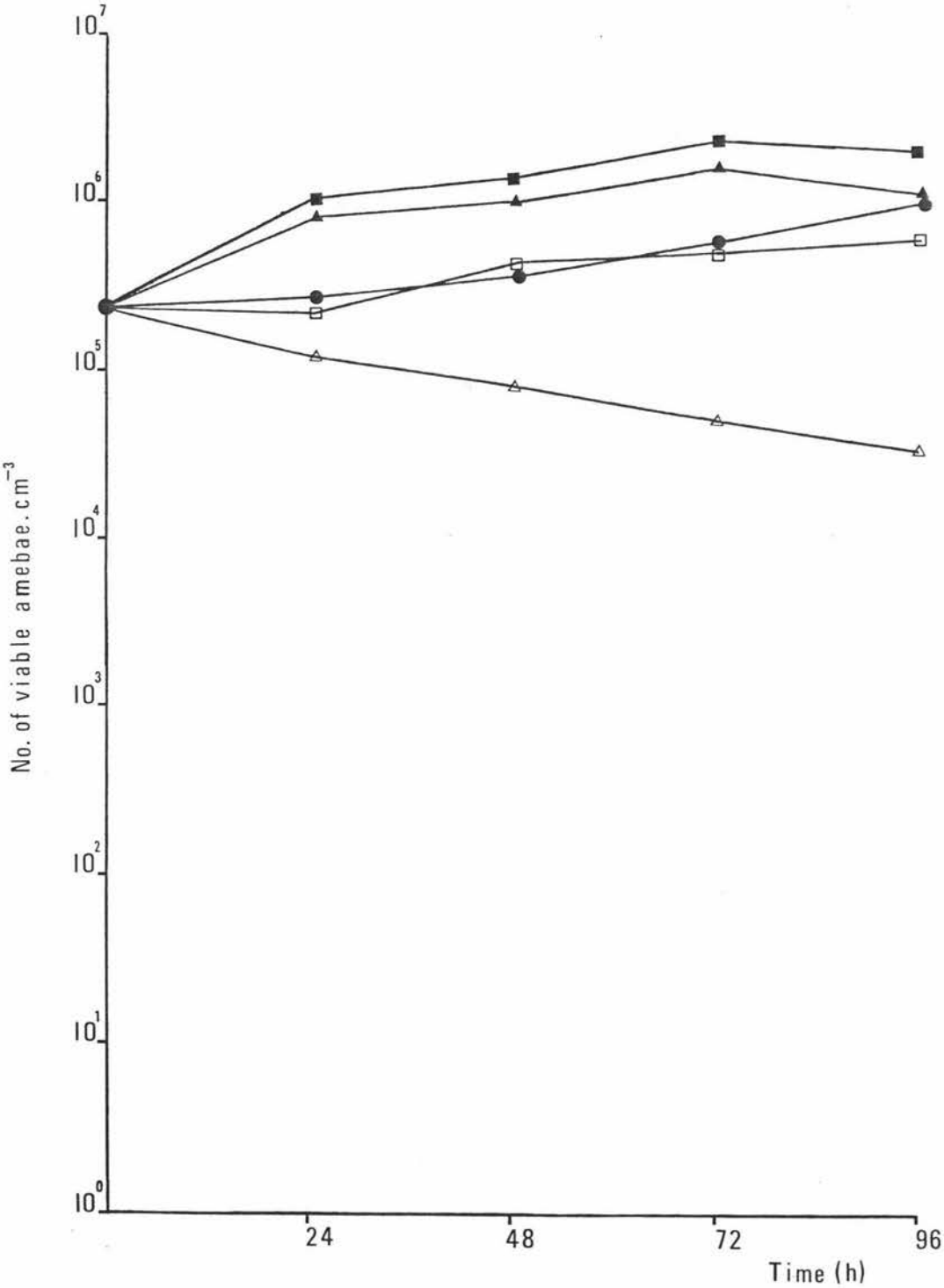

10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
No. of viable amebae. cm^{-3}
24
48
72
96
Time (h)

Figure 37: The Effect of Polymyxin B and Rifampicin Alone and in Combination on Acanthamoeba culbertsoni (A-1)

● = 100 units.cm^{-3} Polymyxin B

■ = 100 μg.cm^{-3} Rifampicin

▲ = 500 μg.cm^{-3} Rifampicin

□ = 100 units.cm^{-3} Polymyxin B + 100 μg.cm^{-3} Rifampicin

△ = 100 units.cm^{-3} Polymyxin B + 500 μg.cm^{-3} Rifampicin

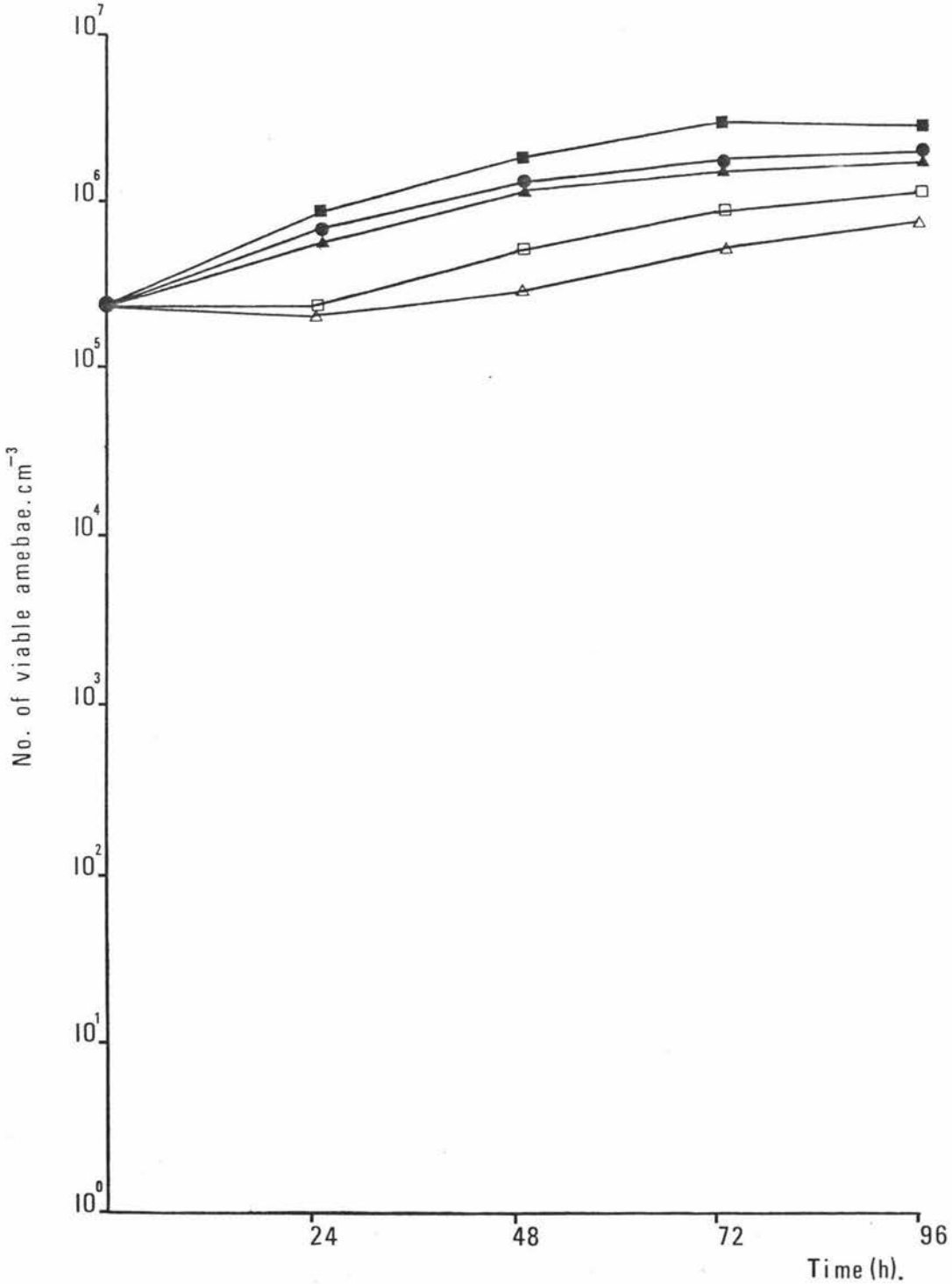

10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
No. of viable amebae.cm^{-3}
24
48
72
96
Time (h).

Figure 38: The Effect of Polymyxin B and Rifampicin Alone and in Combination on Acanthamoeba culbertsoni (A-1)

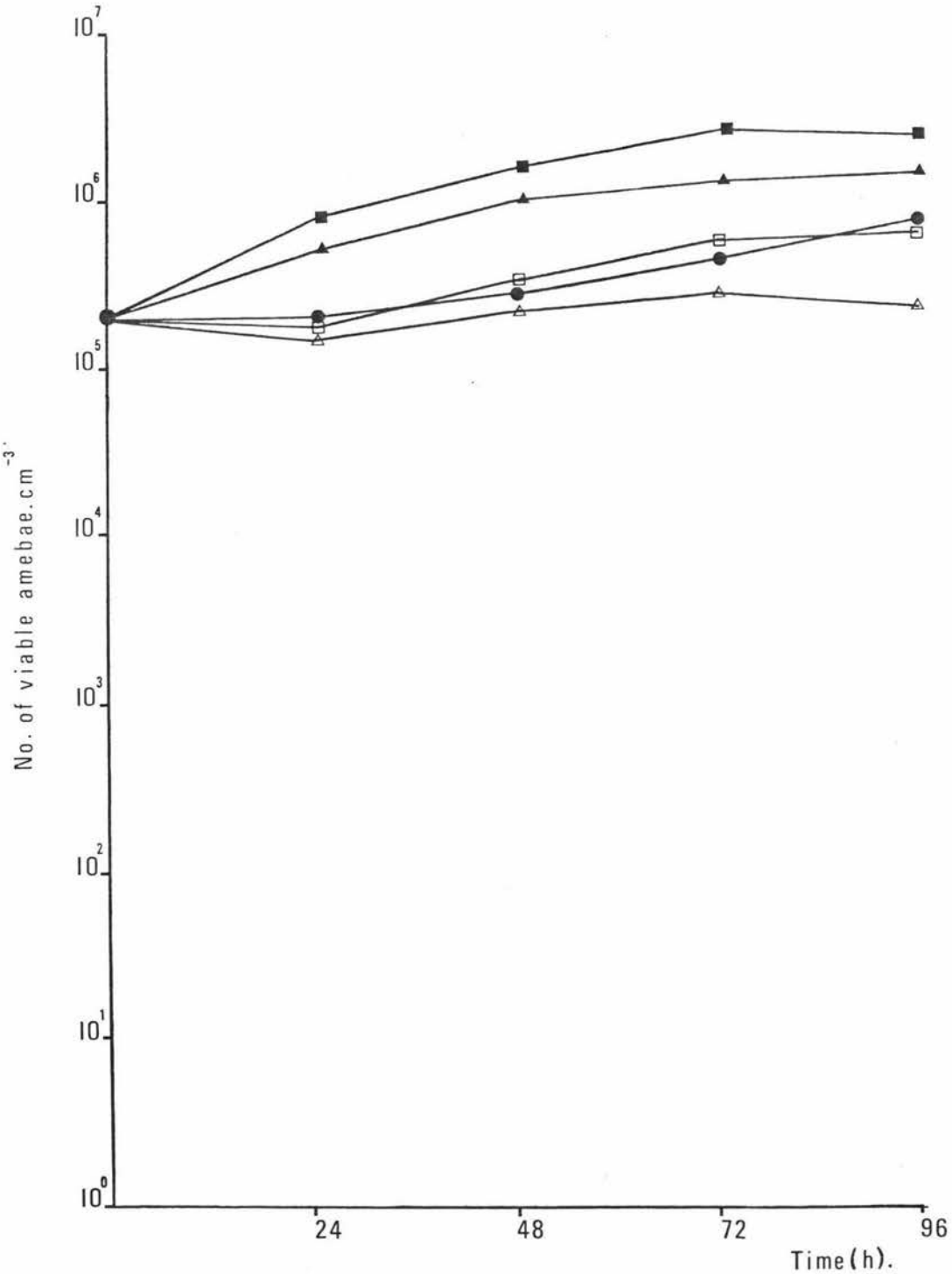
● = 500 units.cm^{-3} Polymyxin B

■ = 100 μg.cm^{-3} Rifampicin

▲ = 500 μg.cm^{-3} Rifampicin

□ = 500 units.cm^{-3} Polymyxin B + 100 μg.cm^{-3} Rifampicin

△ = 500 units.cm^{-3} Polymyxin B + 500 μg.cm^{-3} Rifampicin


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(h).

4.5. Cell Culture Drug Testing of Naegleria fowleri (MsT)

Vero cell cultures can be used as an indicator of pathogenicity of PFLA (Cursons & Brown, 1978). After testing in axenic cultures, drugs which showed activity against the amebae 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. To denote a CTE the same notation as for a cytopathic effect (CPE) is used except the numerals are underlined, i.e., I = beginning of CTE; II = pronounced CTE; III = advanced CTE. A CTE could be differentiated visually from a CPE as the Vero cells appeared darker, had a wrinkled outline and did not detach from the glass. For a CPE the cells were rounded with a smooth outline and became detached from the glass as the CPE advanced to stage IV.

A preliminary experiment was carried out to determine the effect of the size of inocula of N. fowleri (MsT) on the time needed for the development of a CPE. The results are shown in Table V. From these an inoculum size of 3.0×10^3 amebae.cm^{-3} was selected and used in all subsequent experiments with N. fowleri (MsT).

Table V: Effect of Size of Inocula of Naegleria fowleri (MsT) on Time Needed for Development of CPE

Inoculum size (cells.cm^{-3})	TIME (days)							
	1	2	3	4	5	6	7	8
3.0×10^5	II	IV						
3.0×10^4	-	I	III	IV				
3.0×10^3	-	-	I	II	III	IV		
3.0×10^2	-	-	-	I	II	III	IV	
3.0×10^1	-	-	-	-	I	II	III	IV

- = normal cell monolayer
I = begining of CPE
II = pronounced CPE
III = advanced CPE
IV = complete breakdown of monolayer
CPE = cytopathic effect, i.e., rounding of cells, degeneration accompanied by refractiveness of cells and finally loss of monolayer

4.5.1. Amphotericin B.

Table VI shows the results obtained with amphotericin B in cell culture. The MAC against N. fowleri (MsT) in axenic culture was found to be 0.5 $\mu g.cm^{-3}$ (Figure 2). In the cell culture tests 0.1 $\mu g.cm^{-3}$ completely inhibited the formation of a CPE and no viable amebae were recovered when the drug was added at the same time as the amebae. Once the CPE had begun a concentration of 0.25 $\mu g.cm^{-3}$ was amebicidal although the CPE did develop to 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.

4.5.2. Miconazole

Table VII shows the results of testing of miconazole in cell culture against N. fowleri (MsT). In axenic culture 5 $\mu g.cm^{-3}$ caused inhibition after an initial period of growth (Figure 12). The MAC was 50 $\mu g.cm^{-3}$. In cell culture no viable amebae were recovered from the test culture containing 5 $\mu g.cm^{-3}$ miconazole. But 5 $\mu g.cm^{-3}$ miconazole had a CTE on the monolayer which developed at Day 4. When the drug was added after the amebae had had time to begin the CPE, viable amebae were recoverable from the cultures containing 5 $\mu g.cm^{-3}$ miconazole but not from 10 $\mu g.cm^{-3}$ cultures.

Table VI: The Effect of Amphotericin B on Naegleria fowleri (MsT) in Cell Culture. (In Tables VI - XXI, I = beginning of CTE; II = pronounced CTE; III = advanced CTE).

		TIME (days)						
	AmB $\mu g.cm^{-3}$	1	2	3	4	5	6	AMEBA VIABILITY
AMEBAE ADDED AT TIME = 0 DRUG ADDED AT TIME = 0	0	-	-	I	II	III	IV	+
	0.05	-	-	I	II	III	IV	+
	0.1	-	-	-	-	-	-	-
	0.25	-	-	-	-	-	-	-
	0.5	-	-	-	-	-	-	-
AMEBAE ADDED AT TIME = 0 DRUG ADDED AT TIME = 3 days	0.05	-	-	I	II	III	IV	+
	0.1	-	-	I	II	II	III	+
	0.25	-	-	I	I	II	II	-
	0.5	-	-	I	I	I	I	-
NO AMEBAE ADDED DRUG ADDED AT TIME = 0	0.05	-	-	-	-	-	-	
	0.1	-	-	-	-	-	-	
	0.25	-	-	-	-	-	-	
	0.5	-	-	-	-	-	-	

Table VII: The Effect of Miconazole Nitrate on Naegleria fowleri (MsT) in Cell Culture

		TIME (days)						
	Miconazole $\mu g.cm^{-3}$	1	2	3	4	5	6	AMEBAE VIABILITY
AMEBAE ADDED AT TIME = 0 DRUG ADDED AT TIME = 0	0	-	-	I	II	III	IV	+
	1	-	-	I	I-II	II	III	+
	5	-	-	-	I	I-II	II-III	-
	10	-	-	II	III	III	III	-
	25	II	III	III	III	III	III	-
AMEBAE ADDED AT TIME = 0 DRUG ADDED AT TIME = 3 DAYS	1	-	-	I	I-II	III	III	+
	5	-	-	I	I-II	II	II-III	+
	10	-	-	I	I	II	III	-
	25	-	-	I	II-III	III	III	-
NO AMEBAE ADDED DRUG ADDED AT TIME = 0	1	-	-	-	-	-	-	
	5	-	-	-	I	I-II	II-III	
	10	-	-	II	III	III	III	
	25	II	III	III	III	III	III	

4.5.3. R41,400

Table VIII shows the results obtained for R41,400 testing of N. fowleri (MsT) in cell culture. 1 $\mu g.cm^{-3}$ R41,400 slightly inhibited the formation of the CPE as it took one day longer to establish. 5 $\mu g.cm^{-3}$ almost completely inhibited the formation of a CPE although viable amebae were still recoverable. Only at 10 $\mu g.cm^{-3}$ were no amebae recovered after the six day incubation period. But 10 $\mu g.cm^{-3}$ has a CTE on the monolayer. Even when the drug is added three days after the amebae a similar effect is seen to when they are added together.

Table VIII: The Effect of R41,400 on Naegleria fowleri (MsT) in Cell Culture

		TIME (days)						
	R41,400 $\mu g.cm^{-3}$	1	2	3	4	5	6	AMEBAE VIABILITY
AMEBAE ADDED AT TIME = 0 DRUG ADDED AT TIME = 0	0	-	-	I	II	III	IV	+
	1	-	-	-	I	II	III	+
	5	-	-	-	-	-	I	+
	10	-	-	-	I	III	III	-
	25	I	II	III	III	III	III	-
AMEBAE ADDED AT TIME = 0 DRUG ADDED AT TIME = 3 DAYS	1	-	-	I	II	III	IV	+
	5	-	-	I	I	I	II	+
	10	-	-	I	I	II	III	-
	25	-	-	I	II	III	III	-
NO AMEBAE ADDED DRUG ADDED AT TIME = 0	1	-	-	-	-	-	-	
	5	-	-	-	-	-	-	
	10	-	-	-	I	III	III	
	25	I	II	III	III	III	III	

4.5.4. Tetracycline and Rifampicin

The effect of tetracycline on N. fowleri (MsT) in cell culture is shown in Table IX. At a concentration of 50 $\mu g.cm^{-3}$ complete inhibition of the CPE results but viable amebae were recovered. Only at 100 $\mu g.cm^{-3}$ were there no viable amebae. No CTE is seen at any of the concentrations tested.

In axenic culture rifampicin at concentrations $\leq$ 100 $\mu g.cm^{-3}$ had no effect on the growth of N. fowleri (MsT) (Figure 6). A similar effect was seen in cell culture (Table X). The formation of a CPE and the recovery of viable amebae occurred in all test cultures. Rifampicin was cytotoxic to the Vero cells at a concentration of 50 $\mu g.cm^{-3}$.

Table IX: The Effect of Tetracycline on Naegleria fowleri (MsT) in Cell Culture

		TIME (days)						
	Tetracycline $\mu g.cm^{-3}$	1	2	3	4	5	6	AMEBA VIABILITY
AMEBAE ADDED AT TIME = 0 DRUG ADDED AT TIME = 0	0	-	-	I	II	III	IV	+
	10	-	-	I	II	III	IV	+
	50	-	-	-	-	-	-	+
	100	-	-	-	-	-	-	-
NO AMEBAE ADDED DRUG ADDED AT TIME = 0	10	-	-	-	-	-	-	
	50	-	-	-	-	-	-	
	100	-	-	-	-	-	-	

Table X: The Effect of Rifampicin on Naegleria fowleri (MsT) in Cell Culture

		TIME (days)						
	Rifampicin $\mu g.cm^{-3}$	1	2	3	4	5	6	AMEBA VIABILITY
AMEBAE ADDED AT TIME = 0 DRUG ADDED AT TIME = 0	0	-	-	I	II	III	IV	+
	10	-	-	I	II	III	IV	+
	50	-	-	I	II	III	IV	+
	100	-	I	II	III	IV	IV	+
NO AMEBAE ADDED DRUG ADDED AT TIME = 0	10	-	-	-	-	-	-	
	50	-	-	-	I	II	III	
	100	-	I	III	III	III	III	

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

A preliminary experiment was carried out to determine the effect of the size of inocula of A. culbertsoni (A-1) on the time needed to develop a CPE. The results are shown in Table XI. From these, an inoculum size of 3.0×10^2 amebae.cm^{-3} was selected and used in all subsequent experiments with A. culbertsoni (A-1).

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

Inoculum size (cells.cm^{-3})	TIME (days)							
	1	2	3	4	5	6	7	8
3.0×10^5	IV							
3.0×10^4	II	IV						
3.0×10^3	-	-	II	IV				
3.0×10^2	-	-	I	III	IV			
3.0×10^1	-	-	-	I	II	III	IV	

4.6.1. 5-Fluorocytosine

In axenic culture 5-fluorocytosine inhibited the growth of A. culbertsoni (A-1) at concentrations $\geqslant 10\,\mu g.cm^{-3}$ (Figure 22). A similar effect was seen in cell culture (Table XII). No CPE was formed at concentrations $\geqslant 50\,\mu g.cm^{-3}$. Only at $250\,\mu g.cm^{-3}$ 5-fluorocytosine were no viable amebae recovered. When the drug was added three days after the amebae no inhibitory effect was evident and the CPE progressed as for the control. No CTE was seen even at the highest concentration tested ($250\,\mu g.cm^{-3}$).

Table XII: The Effect of 5-Fluorocytosine on Acanthamoeba culbertsoni (A-1) in Cell Culture

		TIME (days)						
	5-FC $\mu g.cm^{-3}$	1	2	3	4	5	6	AMEBA VIABILITY
AMEBAE ADDED AT TIME = 0 DRUG ADDED AT TIME = 0	0	-	-	I	III	IV	IV	+
	10	-	-	-	-	I	II	+
	50	-	-	-	-	-	-	+
	100	-	-	-	-	-	-	+
	250	-	-	-	-	-	-	-
AMEBAE ADDED AT TIME = 0 DRUG ADDED AT TIME = 3 days	10	-	-	I	III	IV	IV	+
	50	-	-	I	III	IV	IV	+
	100	-	-	I	III	III	IV	+
	250	-	-	I	III	III	IV	+
NO AMEBAE ADDED DRUG ADDED AT TIME = 0	10	-	-	-	-	-	-	
	50	-	-	-	-	-	-	
	100	-	-	-	-	-	-	
	250	-	-	-	-	-	-	

4.6.2. Polymyxin B

A similar result to that found in axenic culture for the effect of polymyxin B on A. culbertsoni (A-1) was seen in cell culture. The results are shown in Table XIII. Concentrations $\leq$ 500 units.cm^{-3} polymyxin B were not cytotoxic to the Vero cells but had no effect on the formation of a CPE.

4.6.3. R41,400

R41,400 showed some inhibitory effect on the growth of A. culbertsoni (A-1) in axenic culture (Figure 28). No inhibitory effect was seen in cell culture at or above the cytotoxic level (Table XIV).

Table XIII: The Effect of Polymyxin B Sulphate on Acanthamoeba culbertsoni (A-1) in Cell Culture

		TIME (days)						
	PB Units.cm^{-3}	1	2	3	4	5	6	AMEBA VIABILITY
AMEBAE ADDED AT TIME = 0 DRUG ADDED AT TIME = 0	0	-	-	I	III	IV	IV	+
	50	-	-	I	III	IV	IV	+
	100	-	-	I	III	IV	IV	+
	500	-	-	I	II	III	IV	+
NO AMEBAE ADDED DRUG ADDED AT TIME = 0	50	-	-	-	-	-	-	
	100	-	-	-	-	-	-	
	500	-	-	-	-	-	-	

Table XIV: The Effect of R41,400 on Acanthamoeba culbertsoni (A-1) in Cell Culture

		TIME (days)						
	R41,400 $\mu g.cm^{-3}$	1	2	3	4	5	6	AMEBA VIABILITY
AMEBAE ADDED AT TIME = 0 DRUG ADDED AT TIME = 0	0	-	-	I	III	IV	IV	+
	5	-	-	I	III	IV	IV	+
	10	-	-	I	III	IV	IV	+
	50	I	II	III	IV	IV	IV	+
NO AMEBAE ADDED DRUG ADDED AT TIME = 0	5	-	-	-	-	-	I	
	10	-	-	-	I	II	III	
	50	-	I	II	III	IV	IV	

4.6.4. Tetracycline and Rifampicin

The effect of tetracycline on A. culbertsoni (A-1) in cell culture is shown in Table XV. No inhibitory effect on the formation of a CPE was seen at all the concentrations tested (10-100 $\mu g.cm^{-3}$).

The ineffectiveness of rifampicin against A. culbertsoni (A-1) seen in axenic culture (Figure 20) was also shown in cell culture (Table XVI). No inhibition of the growth of the amebae was seen at or above the cytotoxic concentration.

Table XV: The Effect of Tetracycline on Acanthamoeba culbertsoni (A-1) in Cell Culture

		TIME (days)						
	Tetracycline $\mu g.cm^{-3}$	1	2	3	4	5	6	AMEBA VIABILITY
AMEBAE ADDED AT TIME = 0 DRUG ADDED AT TIME = 0	0	-	-	I	III	IV	IV	+
	10	-	-	I	III	IV	IV	+
	50	-	-	II	III	IV	IV	+
	100	-	-	II	III	IV	IV	+
NO AMEBAE ADDED DRUG ADDED AT TIME = 0	10	-	-	-	-	-	-	
	50	-	-	-	-	-	-	
	100	-	-	-	-	-	-	

Table XVI: The Effect of Rifampicin on Acanthamoeba culbertsoni (A-1) in Cell Culture

		TIME (days)						
	Rifampicin $\mu g.cm^{-3}$	1	2	3	4	5	6	AMEBA VIABILITY
AMEBAE ADDED AT TIME = 0 DRUG ADDED AT TIME = 0	0	-	-	I	III	IV	IV	+
	10	-	-	I	III	IV	IV	+
	50	-	-	II	IV	IV	IV	+
	100	II	III	IV	IV	IV	IV	+
NO AMEBAE ADDED DRUG ADDED AT TIME = 0	10	-	-	-	-	-	-	
	50	-	-	-	I	II	III	
	100	-	I	III	III	III	III	

4.7. The Testing of Drug Combinations against Naegleria fowleri (MsT) in Cell Culture

4.7.1. Amphotericin B and Tetracycline

Table XVII shows the results obtained when amphotericin B and tetracycline were used together against N. fowleri (MsT). When used alone amphotericin B was amebicidal at a concentration of 0.1 $\mu g.cm^{-3}$ (Table VI). Tetracycline inhibited the growth of the amebae at 50 $\mu g.cm^{-3}$ but viable amebae were recovered. Only at 100 $\mu g.cm^{-3}$ were no amebae found (Table IX). When used together 0.05 $\mu g.cm^{-3}$ amphotericin B and 50 $\mu g.cm^{-3}$ tetracycline was amebicidal when the drugs were added at the same time as the amebae. A similar synergistic effect was seen when the drugs were added three days after the amebae. When amphotericin B alone is added three days after the amebae 0.25 $\mu g.cm^{-3}$ is required to kill the amebae (Table VI). By using a combination of amphotericin B and tetracycline the concentrations required are 0.1 $\mu g.cm^{-3}$ amphotericin B and 100 $\mu g.cm^{-3}$ tetracycline.

4.7.2. Amphotericin B and Rifampicin

Amphotericin B and rifampicin showed a synergistic effect when tested in axenic culture (Figures 31 and 32). In cell culture the use of these two drugs together appeared to be antagonistic (Table XVIII). The amebicidal concentration of amphotericin B alone in cell culture is 0.1 $\mu g.cm^{-3}$ (Table VI). When rifampicin is present at either 10 or 50 $\mu g.cm^{-3}$, 0.25 $\mu g.cm^{-3}$ amphotericin B was required to kill all the amebae. The toxic nature of rifampicin is obvious in this table with a CTE developing after three days with 50 $\mu g.cm^{-3}$.

Table XVII: The Effect of Amphotericin B and Tetracycline together on Naegleria fowleri (MsT) in Cell Culture

	AmB_{-3} $\mu g.cm$	Tet_{-3} $\mu g.cm$	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	+
	0.05	10	-	-	-	I	II	III	+
	0.05	50	-	-	-	-	-	-	-
	0.05	100	-	-	-	-	-	-	-
	0.1	10	-	-	-	-	-	-	-
	0.1	50	-	-	-	-	-	-	-
	0.1	100	-	-	-	-	-	-	-
AMEBAE ADDED AT TIME = 0 DRUGS ADDED AT TIME = 0	0.05	10	-	-	I	II	III	IV	+
	0.05	50	-	-	I	II	III	IV	+
	0.05	100	-	-	I	II	III	IV	+
	0.1	10	-	-	I	II	II	III	+
	0.1	50	-	-	I	I	I	II	+
	0.1	100	-	-	I	I	I	I	-

Table XVIII: The Effect of Amphotericin B and Rifampicin together on Naegleria fowleri (MsT) in Cell Culture

			TIME (days)						
	AmB $\mu g.cm^{-3}$	Rif $\mu g.cm^{-3}$	1	2	3	4	5	6	AMEBA VIABILITY
AMEBAE ADDED AT TIME = 0 DRUGS ADDED AT TIME = 0	0	0	-	-	I	II	III	IV	+
	0.05	10	-	-	I	II	III	IV	+
	0.05	50	-	-	II	IV	IV	IV	+
	0.1	10	-	-	-	I	II	III	+
	0.1	50	-	-	II	III	IV	IV	+
	0.25	10	-	-	-	-	-	-	-
	0.25	50	-	-	-	II	II	III	-

4.8. The Testing of Drug Combinations against Acanthamoeba culbertsoni (A-1) in Cell Culture

4.8.1. Polymyxin B and 5-Fluorocytosine

Table XIX shows the results obtained when polymyxin B and 5-fluorocytosine were used together on A. culbertsoni (A-1). When used alone 5-fluorocytosine at a concentration of 50 $\mu g.cm^{-3}$ completely inhibited the formation of a CPE but viable amebae were still recovered at a concentration of 100 $\mu g.cm^{-3}$ (Table XII). When 5-fluorocytosine was added three days after the amebae no inhibition of the formation of a CPE resulted even at a concentration of 250 $\mu g.cm^{-3}$. Polymyxin B alone did not inhibit the growth of amebae in cell culture at concentrations $\leq$ 500 units.cm^{-3} (Table XIII).

When used together no CPE was formed when the drugs were added at time = 0. With 100 units.cm^{-3} polymyxin B and 100 $\mu g.cm^{-3}$ 5-fluorocytosine no viable amebae were recovered after the six day incubation period. When the drug combinations were added to the cell monolayer three days after the amebae the formation of the CPE was halted to a greater or lesser extent. With 100 units.cm^{-3} polymyxin B the inhibitory effect increased with the concentration of 5-fluorocytosine. With 500 units.cm^{-3} polymyxin B and all three concentrations of 5-fluorocytosine tested the CPE did not develop any further once the drugs were added, but only with 250 $\mu g.cm^{-3}$ 5-fluorocytosine were no viable amebae recovered.

Table XIX: The Effect of Polymyxin B sulphate and 5-Fluorocytosine together on Acanthamoeba culbertsoni (A-1) in Cell Culture

	PB Units. cm^{-3}	5-FC $\mu g. cm^{-3}$	TIME (days) 1	2	3	4	5	6	AMEBA VIABILITY
AMEBAE ADDED AT TIME = 0 DRUGS ADDED AT TIME = 0	0	0	-	-	I	III	IV	IV	+
	100	50	-	-	-	-	-	-	+
	100	100	-	-	-	-	-	-	-
	100	250	-	-	-	-	-	-	-
	500	50	-	-	-	-	-	-	-
	500	100	-	-	-	-	-	-	-
	500	250	-	-	-	-	-	-	-
AMEBAE ADDED AT TIME = 0 DRUGS ADDED AT TIME = 3 DAYS	100	50	-	-	I	II	III	III	+
	100	100	-	-	I	I	II	II	+
	100	250	-	-	I	I	I	I	+
	500	50	-	-	I	I	I	I	+
	500	100	-	-	I	I	I	I	+
	500	250	-	-	I	I	I	I	-

4.8.2. Polymyxin B and Tetracycline

Table XX shows the results obtained when polymyxin B and tetracycline are used in combination against A. culbertsoni (A-1) in cell culture. A slight synergistic effective was seen when these two drugs were used in combination in axenic culture (Figures 35 and 36). No inhibition of the formation of a CPE was seen at any of the concentrations tested in cell culture.

4.8.3. Polymyxin B and Rifampicin

The lack of a synergistic effect between polymyxin B and rifampicin seen in axenic culture (Figures 37 and 38) was confirmed in cell culture (Table XXI).

Table XX: The Effect of Polymyxin B Sulphate and Tetracycline together on Acanthamoeba culbertsoni (A-1) in Cell Culture

			TIME (days)						
	PB units.cm^{-3}	Tet μg.cm^{-3}	1	2	3	4	5	6	AMEBA VIABILITY
AMEBAE ADDED AT TIME = 0 DRUGS ADDED AT TIME = 0	0	0	-	-	I	III	IV	IV	+
	100	10	-	-	I	III	IV	IV	+
	100	50	-	-	II	III	IV	IV	+
	100	100	-	-	II	III	IV	IV	+
	500	10	-	-	II	III	IV	IV	+
	500	50	-	-	II	III	IV	IV	+
	500	100	-	-	II	III	IV	IV	+

Table XXI: The Effect of Polymyxin B sulphate and Rifampicin together on Acanthamoeba culbertsoni (A-1) in Cell Culture

			TIME (days)						
	PB units. cm^{-3}	Rif $\mu g.cm^{-3}$	1	2	3	4	5	6	AMEBA VIABILITY
AMEBAE ADDED AT TIME = 0 DRUGS ADDED AT TIME = 0	0	0	-	-	I	III	IV	IV	+
	100	10	-	-	II	III	IV	IV	+
	100	50	-	-	II	IV	IV	IV	+
	100	100	II	III	IV	IV	IV	IV	+
	500	10	-	I	I	III	IV	IV	+
	500	50	-	I	II	III	IV	IV	+
	500	100	II	III	IV	IV	IV	IV	+

4.9. In Vivo Testing of R41,400

Tables XXII, XXIII, and XXIV show the results obtained in the in vivo testing of the imidazole derivative, R41,400. In the first experiment, R41,400 was administered IP once daily. Tables XII and XXIV, represent duplicate experiments with the drug administered every twelve hours.

Table XXII: Intranasal Infection of 25.0 ± 3.2g Swiss-White Male Mice with Naegleria fowleri (MsT). R41,400 given in the doses indicated.

Total Daily Dose of R41,400	Day of Death	No. Deaths /No. Used	Average Time of Death(days)
0	5*,5*,11,15*,S,S	4/6	9.0
10mg.kg^{-1} (0.25mg)	6*,9*,10,18*,21,S	5/6	12.8
20 mg.kg^{-1} (0.5mg)	4*,7,12*,S,S,S	3/6	7.7
40 mg.kg^{-1} (1.0mg)	3*,4*,4*,6*,6*,S	5/6	4.6

* = Amebae isolated from the brain

S = Survivors

Table XXIII: Intranasal Infection of 22.0 ± 4.0g Swiss-White Male Mice with Naegleria fowleri (MsT). R41,400 given in the doses indicated.

Total Daily Dose of R41,400	Day of Death	No. Deaths /No. Used	Average Time of Death (days)
0	6*,8*,17,18*,20*	5/5	13.8
10mg.kg^{-1} (0.11mg bid)	7,7,8*,11,S,S	4/6	8.3
20mg.kg^{-1} (0.22mg bid)	7,8*,8*,8*,9,11*	6/6	8.5
40mg.kg^{-1} (0.44mg bid)	7,8*,8*,8*,11*,19*	6/6	10.2

Table XXIV: Intranasal Infection of 27.0 ± 4.0g Swiss-White Male Mice with Naegleria fowleri (MsT). R41,400 given in the doses indicated.

Total Daily Dose of R41,400	Day of Death	No. Deaths /No. Used	Average Time of Death(days)
0	10*,11,14*,15,S,S	4/6	12.5
10mg.kg^{-1} (0.14mg bid)	6*, 12*, 12*, 12*,13*,14*	6/6	11.5
20mg.kg^{-1} (0.27mg bid)	10*,12,12,13,S,S	4/6	11.8
40mg.kg^{-1} (0.54mg bid)	7*,7*,8,9*,11*,S	5/6	8.4

Although R41,400 was effective *in vitro* against *N. fowleri* (MsT) both in axenic culture (Figure 12) and in cell culture (Table VII) it was not curative *in vivo* and appeared to have an adverse effect on the mice (Tables XXII, XXIII, XXIV).

At the highest daily dose used (40 $mg.kg^{-1}$) the survival time of the mice was reduced. This effect was most marked when the drug was administered in one daily dose where the average time of death decreased from 9.0 days, in control mice, to 4.6 days (Table XXII). At the lowest daily dose (10 $mg.kg^{-1}$) an increased survival time resulted when the drug was administered only once daily but when divided into daily doses the average time of death was less than that of the controls.

CHAPTER FIVE: DISCUSSION

5.1. Treatment of Naegleria Infections

With the awareness of the nature of the etiological agent of PAM and the finding that the traditional anti-protozoal durgs were ineffective the search for effective agents to treat this disease began. Although used in some instances against protozoa (Horvath & Zierdt, 1974; Kinsky, 1967), amphotericin B has had its major application in systemic fungal infections of humans (Hamilton-Miller, 1973; Kinsky, 1967). Evidence supports the belief that the site of action of amphotericin B is at the plasma membrane of the cell, where the lipophilic drug combines with sterols, possibly creating pores in the membrane (Andreoli, 1974; Holz, 1974) and causing leakage of cellular constituents across the membrane (Ghosh & Ghosh, 1963; Gale, 1974).

In 1968, Culbertson *et al.*, showed that relatively large doses of amphotericin B in combination with large doses of sulphadiazine in the diet had significant activity against *N. fowleri* (HB-1) in mice. When 1000 amebae were inoculated intranasally into ten mice, all the mice died within seven days. At a total daily dose of 5.0 or 7.5 $mg.kg^{-1}$ amphotericin B, administered subcutaneously twice daily, and 0.5% sulphadiazine in the diet only four out of ten mice died. When the amphotericin B dose was 2.5 $mg.kg^{-1}$ only two mice survived but the average time of death was 24 days. The sulphadiazine treatment was begun three days before intranasal infection and the amphotericin B one day before. Sulphadiazine alone was shown to be ineffective even at a concentration of 1% in the diet and the infecting number of amebae being only 240.

Carter (1969) showed that sulphadiazine had no effect on the organism *in vitro* at levels in excess of those likely to be attained therapeutically in the brain. The attainable CSF level of sulphadiazine is reported to be 90 $\mu g.cm^{-3}$ (Goodman & Gilman, 1975) and a concentration of 1280 $\mu g.cm^{-3}$ had no effect on the *Naegleria* spp. used by Carter.

Carter (1969) confirmed the *in vivo* result of Culbertson *et al.* (1968) for amphotericin B. He reports a minimum immobilizing level of 0.6 $\mu g.cm^{-3}$ and a minimum inhibitory concentration (MIC) of 0.075 $\mu g.cm^{-3}$ *in vitro*. Disintegration of the amebae in the immob-

ilization test was advanced within two hours at 0.6 $\mu g.cm^{-3}$. In the *in vivo* situation 7.5 $mg.kg^{-1}$ amphotericin B administered once daily IP gave a 60% survival rate. A bioassay of the mouse serum showed the level of drug to be 1.2 $\mu g.cm^{-3}$.

Culbertson *et al.*(1971) found that an intracerebral injection of a single dose of amphotericin B prevents PAM in most mice given intranasal lethal doses of *N. fowleri* (HB-1). When a dose of 37.5 mg was given five out of eleven mice died and only three of the mice showed any signs of amebae invasion. With a dose of 18.75 mg given intracerebrally three out of nine mice died but four mice showed signs of amebic lesions.

Das (1971) employed the subcutaneous route of dosing and found that 30 $mg.kg^{-1}$ amphotericin B given every 24 hours completely protected the mice from amebic infection. Das also tested sulphadiazine and found that 250 $mg.kg^{-1}$ given orally had no effect on the course of the disease.

Differences in the susceptibility of non-pathogenic and pathogenic species and also between strains *in vitro* appear in the literature. Many of these differences may be a result of the different screening techniques, whether the amebae are grown with bacteria or in axenic culture and if in axenic culture the differences in the media employed and different inocula sizes. The semi-defined media, CGHV and CGHVS developed by Cursons *et al.*(1979) were used in this study to minimize the antagonistic effects of media components on the drugs. Serum is a common component of axenic media for these amebae and it contains many sterols which will bind amphotericin B. In the semi-defined media the serum component was replaced with hemin and yeast extract was replaced with vitamins to eliminate any antagonistic effect on 5-fluorocytosine (Cursons *et al.*, 1979).

A number of workers have tested amphotericin B *in vitro*. Duma and Finley (1976) report a mean minimum motility inhibitory concentration against six pathogenic *N. fowleri* strains of 1.39 $\mu g.cm^{-3}$ amphotericin B. The MIC was 0.62 $\mu g.cm^{-3}$. Mandal *et al.*(1970) reported an MIC of 0.0005-0.001 $\mu g.cm^{-3}$ against a *N. fowleri* strain isolated from a human case.

De Joncheere and Van De Voorde (1977) studied the effect of amphotericin B on three pathogenic strains of *N. fowleri* and report an MIC of 0.1-0.15 $\mu g.cm^{-3}$. But two strains of *N. fowleri* which were non-pathogenic to mice had an MIC greater than 1.0 $\mu g.cm^{-3}$.

Chang (1971) also reports a difference in the susceptibility of pathogenic and non-pathogenic species. 1 $\mu g.cm^{-3}$ amphotericin B had an antiamebic effect on pathogenic Naegleria but 2 $\mu g.cm^{-3}$ was required to affect the non-pathogenic species.

The results presented in Figures 1 and 2 were obtained in in vitro axenic testing of amphotericin B against N. gruberi (P1200f) and N. fowleri (MsT). The range at which Naegleria spp. are susceptible are in agreement with most published reports although the absolute values differ. An amebicidal value of 0.5 $\mu g.cm^{-3}$ was found after 96 hours exposure. The difference is thought to be due to the use of a different media for the growth of the amebae and also the different inoculum size. The size of the inoculum used (2-3 x 10^5 amebae.cm^{-3}), chosen to reflect the number of amebae that may be present in the cerebrospinal fluid (CSF) of infected patients, was higher than that used in most other studies (Duma & Finley, 1976; Visvesvara & Balamuth, 1975). Duma et al.(1971) report that the number of amebae present in the subarachnoid space alone may be in excess of 150,000 trophozoites.

When tested in cell culture a concentration of 0.1 $\mu g.cm^{-3}$ was amebicidal (Table VI). No amebae were recovered after the six day incubation period and the cell monolayer remained unchanged. When the drug was added three days after the amebae a concentration of 0.25 $\mu g.cm^{-3}$ was required to completely inhibit the formation of any further CPE. The differences in the MAC in cell culture between when the drug is added at the beginning and at time = three days is probably a reflection of the inoculum size. Only 3 x 10^3 amebae.cm^{-3} are added at the beginning but once the CPE have started to appear the number of amebae present will approach that used in axenic culture. Amphotericin B had no CTE on the Vero cells at the highest concentration tested (0.5 $\mu g.cm^{-3}$). Chang (1971) used a cell culture system and found that a concentration of 2 $\mu g.cm^{-3}$ had a slight CTE on Monkey kidney cells.

Schuster and Rechthand (1975) carried out an extensive study on the in vitro effects of amphotericin B on growth and ultrastructure of both N. gruberi and N. fowleri. Amphotericin B was amebicidal at all the concentrations tested (0.25, 0.5 & 1.0 $\mu g.cm^{-3}$) when the drug was added to cultures in lag phase but was mainly inhibitory when added to log phase cultures. The drug produced ultrastructural modifications at all concentrations (0.05-1.0 $\mu g.cm^{-3}$). These changes

included distortion of nuclear shape, increase in cytoplasmic membranes, decrease in the number of food vacuoles, absence of pseudopod formation, mitochondrial abnormalities and increase in autophagic vacuoles. These alterations became more pronounced with increased exposure time and concentration of amphotericin B.

In 1969, Carter suggested that amphotericin B be tried in the treatment of PAM by simultaneous IV and IVent administration; the doses recommended were - 0.25 $mg.kg^{-1}$ IV and 1.0 mg into the cerebral ventricles in the first 24 hours. Subsequently such treatment was tried on two patients in the U.S.A. (Duma et al., 1971) and on two patients in Australia (Carter, 1972). A description of the treatment regimen for these cases can be found in section 1.7.

Although all four of these patients died autopsy findings are of interest. In the second case of Duma et al. (1971) viable organisms could not be isolated from fresh necropsy tissue and in the first case organisms were cultured from only one small, localized area, the cisterna cerebromedullaris. Wet preparations of CSF from both patients just before death showed poorly motile organisms that were rounded up, vacuolated, granulated and ruptured.

In the fatal case reported by Apley et al. (1970) (described in section 1.7 along with two other cases) 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 but many appeared to be dead. The concentration of amphotericin B in the CSF was 0.184 $\mu g.cm^{-3}$. On the eleventh day after admission the CSF contained no amebae and the amphotericin B concentration was 0.224 $\mu g.cm^{-3}$.

Although adequate levels of amphotericin B were present in the CSF the patient died. However, soon after admission he stopped breathing and thereafter was kept alive by a respirator. The severe cerebral changes found at necropsy suggested anoxic damage as well as continued amebic invasion. As in the case of Duma et al. (1971; Patient 3) amebae were not seen in all areas of meningitis but found only in a localized area, in this case the brain stem.

The second and third cases reported by Apley et al. (1970) were treated in the same way as case one. Amebae were not seen in the CSF of either patient but were cultured from the CSF of case two. The authors believe that the third case was only a doubtful case of PAM but case two was a case of Naegleria meningo-encephalitis and survival

can be attributed to amphotericin B. The success in treatment at relatively low levels of amphotericin B being due to treatment beginning early in the course of the disease.

Griffin (1976) has disputed the diagnosis of Naegleria meningo-encephalitis in the cases described by Apley et al. (1970). He states that "Naegleria, by phase contrast does not look like the amoeba from the CSF illustrated by Apley et al., while Acanthamoeba does. Also the description 'they had three or four spiky pseudopodia and moved very slowly' describes Acanthamoeba in CSF but not N. fowleri or N. gruberi which are monopodial in locomotion." The Naegleria spp. isolated from these cases proved to be N. gruberi and careful experiments showed it to be nonpathogenic in mice (Warhurst et al., 1970; Saygi et al., 1973) and as do other N. gruberi it dies at febrile temperatures (Griffin, 1972).

Griffin (1976) contends that an Acanthamoeba was involved in these cases and sulphadiazine, which was given on admission, was responsible for the prolonged survival in the first case and survival in the second. Culbertson et al. (1965) showed that Acanthamoebae were effected by sulphadiazine in vivo. The N. gruberi isolated, Griffin believes was a secondary invader. No Acanthamoebae were cultured from the CSF of patient 1 taken two days after admission but the presence of sulphadiazine may have prevented Acanthamoeba growth.

In reply to Griffin (1976), Warhurst et al. (1976) believe "that there is no evidence for Dr. Griffin's theory that the N. gruberi was not the primary pathogen in this case". Acanthamoebae were specifically looked for and throat and nasal swabs and CSF samples were treated appropriately but no Acanthamoebae were found. The contention that the sulphadiazine would have eliminated any Acanthamoebae is disputed as amebae were seen to be still moving, although very sluggishly, in a specimen of CSF taken four days after the patient received both sulphadiazine and amphotericin B. Finally, Stamm (1974) confirmed the cultural evidence that N. gruberi was present by visualizing amebae in histologic brain sections with anti - N. gruberi immunofluorescence. He did not obtain any labelling with Acanthamoeba antisera.

The first survival were there is definite proof that N. fowleri was involved and survival can be attributed to amphotericin B is the case reported by Anderson and Jamieson (1972). In this case ampho-

tericin B was given IV at a dose of 1 $mg.kg^{-1}$ immediately the diagnosis was confirmed. The patient was comatose when treatment began but within two days he became afebrile and was talking rationally. After five days the CSF white cell count had fallen from an initial count of 12,000.mm^{-3} to 15.mm^{-3} but many atypical amebae were still present. Amphotericin B was therefore given IT and later IVent in small doses (0.1 mg on alternate days) and the fluid gradually cleared. This case illustrates the need to administer amphotericin B at the maximum possible dose immediately PAM is suspected. This has been confirmed in the case reported by Siedel et al. (pers. comm. 1978). Initially amphotericin B was given IV at a dosage of 1.0 $mg.kg^{-1}$ plus 1.5 mg IT. On transfer to Harbour General Hospital the dosage of amphotericin B was increased to 1.5 $mg.kg^{-1}$ per day divided bid for three days and then reduced to 1.0 $mg.kg^{-1}$ per day for six days. IT administration of 1.5 mg per day was continued for two days and then reduced to 1.0 mg every other day for eight days (Table III).

The problem of raised intracranial pressure in PAM and the difficulty of getting drugs into swollen brains (Carter, 1972) often means corticosteroids e.g., dexamethasone,are used but this raises problems of reduced efficacy of amphotericin B. As a polyene antibiotic amphotericin B binds to sterols and the similar lipid molecular structure of corticosteroids and the lipid constituents of the cell membrane of the ameba may cause the amphotericin B to combine with the corticosteroids. Mandal et al. (1970) found that 50 times the concentration of amphotericin B was required to inhibit growth of N. fowleri isolates in the presence of dexamethasone at concentrations between 0.00025 and 0.25 $\mu g.cm^{-3}$. In the cases of Duma et al. (1971) dexamethasone may have lowered the available amphotericin B so effective levels were not achieved. Seidel et al. (pers. comm. 1978) used dexamethasone and also diphenylhydantoin (Dilantin) for increased intracranial pressure and seizure activity respectively. Amphotericin B was given at much higher doses than in the case of Duma et al. (1971) so the antagonistic effect of dexamethasone was probably overcome and effective levels of amphotericin B reached.

Although effective, amphotericin B therapy is limited by its extreme toxicity (Utz et al., 1964; Goodman & Gilman, 1975). IV therapy is complicated by phlebitis, hypokalaemia, anemia and nephrotoxicity. Heparin can be added to the infusion to combat phlebitis and alkali therapy may prevent nephrotoxicity during prolonged treatment (Goodman

& Gilman, 1975). Seidel et al. (pers. comm. 1978) reported several complications to therapy which included, raised serum creatinine levels believed to be secondary to amphotericin B toxicity and severe anemia with low reticulocyte count that was resolving at discharge.

The toxicity of amphotericin B has prompted the investigation of synthetic derivatives of amphotericin B. Amphotericin B methyl ester which is a water soluble derivative of amphotericin B is reported to be considerably less toxic than the parent compound (Bonner et al., 1972; Keim et al., 1973; Fisher et al., 1975). In addition it exhibits the full antifungal activity of the parent compound (Mechlinski & Schaffner, 1972). In primates Jagdis et al. (1977) showed that a dose of 5 mg amphotericin B methyl ester.kg^{-1} yielded concentrations in serum that were 7.2 to 12.2 times higher than those resulting from 1.0 mg amphotericin B.kg^{-1} during the eight hour post-infusion period. Maximal concentrations of the methyl ester in serum after 10 and 15 mg.kg^{-1} doses were in the same range as those obtained with a 5 mg.kg^{-1} dose.

Visvesvara et al. (1975b) found that amphotericin B methyl ester was amebicidal for two strains of N. fowleri at a concentration of 10 μl.cm^{-3}. In vivo trials were reported to be in progress. The results of these will determine future use of the methyl ester in treatment of PAM.

In the hope of being able to reduce the concentration of amphotericin B required, the use of drug combinations was investigated (Figures 29-32; Tables XVII & XVIII). 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. Effective treatment was achieved with a quarter the dosage of amphotericin B. Amphotericin B used together with rifampicin has been found to be more effective than either drug alone when tested against Candida albicans (Medoff et al., 1972) and Aspergillus (Kitahara et al., 1976b) in vitro and against Histoplasma capsulatum and Blastomyces dermatitidis in vitro (Kobayashi et al., 1972, 1974) and in mice (Kitahara et al., 1976a). Also suboptimal doses of amphotericin B in combination with either rifampicin or 5-fluorocytosine were better than single-drug therapy in the treatment of disseminated Aspergillus fumigatus infection in mice (Arroyo et al., 1977).

Rifampicin is a broad spectrum antimicrobial agent with activity against bacteria, mycobacteria and fungi (Goodman & Gilman, 1975; Garrod et al., 1973). It is believed to inhibit DNA-dependent RNA polymerase activity. Rifampicin was tested against two Naegleria spp. in vitro. Figures 5 and 6 show that Naegleria spp. are not susceptible to levels $\leq 100 \mu g.cm^{-3}$ rifampicin. Only at $500 \mu g.cm^{-3}$ did the amebae show any signs of drug damage. A similar effect was seen in cell culture (Table X). A CPE developed and viable amebae where recovered at the highest concentration tested ($100 \mu g.cm^{-3}$). Rifampicin was toxic to the Vero cells at a concentration of $50 \mu g.cm^{-3}$. Thong et al. (1977) tested rifampicin in axenic culture against N. fowleri and found an inhibitory effect at a concentration of $100 \mu g.cm^{-3}$. This difference is probably due to the lower inoculum size used by Thong et al..

Tetracycline was also investigated in axenic culture and the cell culture system. This drug is a broad spectrum antibiotic with proven activity against other protozoan diseases (Goodman & Gilman, 1975). For both N. gruberi (P1200f) and N. fowleri (MsT) a slight reduction in numbers was recorded with concentrations of 50 and 100 $\mu g.cm^{-3}$ (Figures 3 & 4) in axenic culture. Thong et al. (1977) report inhibition of N. fowleri with $10 \mu g.cm^{-3}$. In cell culture $50 \mu g.cm^{-3}$ completely inhibited the formation of a CPE although viable amebae were present after the six day exposure. At $100 \mu g.cm^{-3}$ no viable amebae were recovered (Table IX). The difference in the effectiveness of tetracycline seen in axenic and cell culture is probably a reflection of inoculum size. Tetracycline had no toxic effect on the Vero cells at all the concentrations tested (10-100 $\mu g.cm^{-3}$).

Amphotericin B and tetracycline were investigated for a synergistic effect against Naegleria spp. in both axenic and cell culture. In axenic culture when $50 \mu g.cm^{-3}$ tetracycline is used in combination with two different concentrations of amphotericin B, $0.25 \mu g.cm^{-3}$ and $0.5 \mu g.cm^{-3}$ no significant difference is seen from that obtained with amphotericin B alone. Only when tetracycline was present at a concentration of $100 \mu g.cm^{-3}$ was any synergistic effect seen (Figures 29 & 30). $0.25 \mu g.cm^{-3}$ amphotericin B and $100 \mu g.cm^{-3}$ tetracycline was amebicidal in 96 hours. With $0.5 \mu g.cm^{-3}$ amphotericin B and $100 \mu g.cm^{-3}$ tetracycline the time required to completely sterilize the medium was reduced from 96 to 72 hours.

Synergistic activity between amphotericin B and tetracycline was also seen in cell culture (Table XVII). Concentrations of

0.05 $\mu g.cm^{-3}$ amphotericin B and 50 $\mu g.cm^{-3}$ tetracycline completely protected the Vero cell monolayer and no amebae were recovered. These concentrations are much lower than those found in axenic culture. If the drugs are added once the amebae have established a CPE a situation closer to the clinical picture is obtained. In this case 0.1 $\mu g.cm^{-3}$ amphotericin B and 100 $\mu g.cm^{-3}$ was amebicidal. When used alone a concentration of 0.25 $\mu g.cm^{-3}$ amphotericin is required for the same result.

A synergistic effect has been reported *in vivo* between amphotericin B and tetracycline by Thong *et al* (1978a). They tested tetracycline alone at dosages of 50, 100 and 150 $mg.kg^{-1}$ given once daily IP. At the highest dose although the mortality was 100% there was prolongation of the mean survival time to 13.4 days compared with 7.4 days in untreated mice. With amphotericin B alone at a dose of 2.5 $mg.kg^{-1}$ per day 28.6% of the mice survived. The survival rate was increased to 66.7% and 84.6% in mice who received the combined schedules of 2.5 $mg.kg^{-1}$ amphotericin B plus 100 $mg.kg^{-1}$ tetracycline, respectively.

Amphotericin B and rifampicin were also investigated for possible synergistic activity (Figures 31 & 32; Table XVIII). In axenic culture a marked synergistic effect is seen, greater than that seen between tetracycline and amphotericin B. A synergistic effect is seen when either 10 or 50 $\mu g.cm^{-3}$ rifampicin is combined with 0.25 $\mu g.cm^{-3}$ amphotericin B, the inhibitory effect being greater with the higher concentration of rifampicin (Figure 31). When rifampicin, at 10 and 50 $\mu g.cm^{-3}$ is combined with 0.5 $\mu g.cm^{-3}$ amphotericin B the time required to completely sterilize the media is reduced from 96 to 72 and 48 hours respectively (Figure 32).

However in cell culture rifampicin appears to antagonize the action of amphotericin B. Alone amphotericin B is amebicidal at 0.1 $\mu g.cm^{-3}$ but viable amebae can be recovered when 10 and 50 $\mu g.cm^{-3}$ rifampicin is also present with this concentration of amphotericin B (Table XVIII). This effect is thought to be due to the cytotoxic nature of rifampicin which appeared to make the cells more susceptible to the amebae. Rifampicin is cytotoxic at a concentration of 50 $\mu g.cm^{-3}$ (Table X) and when used at this concentration together with either 0.05 or 0.1 $\mu g.cm^{-3}$ amphotericin B a combined CTE and CPE develops which is greater than the CTE caused by rifampicin alone.

Thong et al. (1979) investigated the effect of amphotericin B and rifampicin against N. fowleri infection in mice. They found that rifamycin (the parent compound of which rifampicin is a semi-synthetic derivative), injected IP at 50, 100 and 150 $mg.kg^{-1}$ per day had no effect on the course of the disease. A dose of 2.5 $mg.kg^{-1}$ amphotericin B per day resulted in a 10% survival rate. A similar survival rate occurred when 100 $mg.kg^{-1}$ rifamycin per day was combined with the same dose of amphotericin B but was increased to 40% in the group receiving amphotericin B and 150 $mg.kg^{-1}$ per day rifamycin.

In Man, brain and CSF levels of rifampicin have been shown to be appreciably lower than blood levels. D'Oliveira (1972) found a CSF: plasma ratio of 20% in early tuberculous meningitis. This figure results from the high degree of plasma protein binding of rifampicin, so that only 20% of total drug is freely diffusable and able to penetrate into the CSF. Rifampicin was given orally by D'Oliveira (1972) in divided doses totalling 600 mg daily in ten cases of probable tuberculous meningitis. The highest CSF concentration occurred on the second day of treatment and ranged from 0.12 to 1.37 $\mu g.cm^{-3}$. Levels tended to fall by the fourth day of treatment. Detectable CSF rifampicin levels were only found by Sippel et al. (1974) in patients with meningeal inflammation.

Rifampicin was administered orally at a dosage of 10 $mg.kg^{-1}$ per day divided tid for nine days in the case of Siedel et al. (pers. comm. 1978) (Table III). It's effect in this case is difficult to evaluate in view of the high dose of amphotericin B given but as a synergistic effect has been shown both in vitro and in vivo its use in the treatment protocol can only be regarded as advantageous.

A greater synergistic effect was shown to occur between amphotericin B and tetracycline (Figures 29 & 30, Table XVII). This has been confirmed by Thong et al. (1978a) in vivo. In view of the clinical experience with tetracycline in other infections (Goodman & Gilman, 1975), its use in combination with amphotericin B in the treatment of PAM is recommended. For IV injection a dose of 500 mg tetracycline administered by slow infusion is recommended for adults. CSF levels of 10% that of the plasma levels have been found for tetracycline (Garrod et al., 1973). Wood and Kipnis (1954) studied the CSF levels achieved in patients in the convalescent stage of poliomyelitis and found that six hours after a single IV dose these ranged from 0.16-0.62 $\mu g.cm^{-3}$. Six hours after the last of three

doses given at six-hourly intervals, the tetracycline levels ranged from 1.25-5.0 $\mu g.cm^{-3}$.

5-Fluorocytosine, like amphotericin B is primarily an antifungal agent but is of low toxicity (Block & Bennett, 1972). 5-Fluorocytosine exerts its chemotherapeutic effect by conversion within sensitive fungal cells to 5-fluorouracil, a known metabolic antagonist. 5-Fluorocytosine had no activity against Naegleria spp. when tested in vitro in axenic culture at concentrations $\leqslant$ 100 $g.cm^{-3}$ (Figures 7 & 8). Duma and Finley (1976) tested 5-fluorocytosine against six strains of N. fowleri isolated from human cases. No effect was seen at a concentration of 100 $\mu g.cm^{-3}$. Das (1975) reports an amebicidal effect on pathogenic Naegleria at a concentration of 200 $\mu g.cm^{-3}$ in axenic culture. The number of amebae in this test was only 5×10^3.

Polymyxin B sulphate is a surface-active bactericidal antibiotic which alters the permeability of the bacterial cell envelope by binding to the negatively charged phospholipid component and causing cell lysis (Newton, 1956). Eukaryotic cells also contain phospholipids in their cell membranes and it was reasonable to expect polymyxin B to disrupt permeability barriers in them as well. Two studies have shown this to be true in the yeasts Candida tropicalis (Nicholls, 1970), Saccharomyces cerevisiae and Candida albicans (Schwartz et al., 1972).

Mandal et al. (1970) reported that a N. fowleri sp. isolated from a case of PAM was not susceptible to levels of polymyxin B in excess of those attainable therapeutically in humans. Duma and Finley (1976) tested polymyxin B at a concentration of 100 $\mu g.cm^{-3}$ and found it had no effect on six N. fowleri strains. Polymyxin B was tested at 100 and 500 units.cm^{-3} (12.7 and 63.5 $g.cm^{-3}$; Garrod et al., 1973) and was found to have no effect on either N. gruberi (P1200f) or N. fowleri (MsT) (Figures 9 & 10).

Miconazole is a substituted 1-phenyl-imidazole synthesized at Janssen Pharmaceutica in Belgium, which inhibits the growth of most pathogenic fungi and of gram positive bacteria but has no effect on gram negative bacteria. Antifungal and antibacterial activity is due to interference with the permeability of the cell wall and plasmalemma and to interference with mitochondrial and peroxisomal enzyme systems resulting in a drug-induced peroxide accumulation (Miconazole - Systemic Use. Preclinical reports provided by Ethnor Pty. Ltd.).

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 on N. fowleri at 10 and 100 $\mu g.cm^{-3}$. Duma and Finley (1976) report mean minimum motility immobilizing concentrations above 39.69 $\mu g.cm^{-3}$ for six strains of N. fowleri. Mean MIC's at 24 hours ranged from 0.78 to 25 $\mu g.cm^{-3}$ and at 48 hours were reported between 0.98 and 1.97 $\mu g.cm^{-3}$.

The susceptibility of Naegleria spp. to miconazole was confirmed in this study (Figures 11 & 12). The non-pathogenic N. gruberi (P1200f) appeared more susceptible with an MAC of 10 $\mu g.cm^{-3}$. For the pathogenic species N. fowleri (MsT), 50 $\mu g.cm^{-3}$ was required to kill all the amebae in 96 hours. In cell culture, N. fowleri (MsT) was susceptible at 5 $\mu g.cm^{-3}$ when the drug was added at the same time as the amebae. Once a CPE had been established 10 $\mu g.cm^{-3}$ was required to halt the progression of the CPE. No viable amebae were recovered. But miconazole was found to be toxic to the Vero cells at a concentration of 5 $\mu g.cm^{-3}$ (Table VII).

R41,400 is a newly synthesized member of the imidazole group of drugs which appears to share many of the properties of the other imidazoles such as miconazole and clotrimazole. R41,400 differs however from most other imidazoles in one important aspect, it is water-soluble. This means that there are no problems with regards the solubility of agents when testing in vitro and also may have important clinical implications in that solvents or vehicles required to obtain stable pharmaceutical preparations are not needed (Dixon et al., 1978).

In vitro activity of R41,400 compared favourably with miconazole when tested against the filamentous stages of the etiologic agents of systemic and subcutaneous mycoses (Dixon et al., 1978). Preclinical tests by Janssen Pharmaceutica report in vivo efficacy in the treatment of superficial and systemic fungal infections.

In axenic culture R41,400 showed activity against both pathogenic and non-pathogenic Naegleria spp.. N. fowleri (MsT) appears more susceptible than N. gruberi (P1200f) and at a concentration of 10 $\mu g.cm^{-3}$ no viable amebae were recovered after 96 hours exposure (Figure 14). Even at a concentration of 25 $\mu g.cm^{-3}$ 96 hours was still needed to give a complete amebicidal effect. For N. gruberi (P1200f) 10 $\mu g.cm^{-3}$ was only inhibitory and a concentration of 50 $\mu g.cm^{-3}$ was needed before an amebicidal effect was seen (Figure 13). R41,400 was also effective in cell culture against N. fowleri (MsT) (Table VIII). A concentration of 5 $\mu g.cm^{-3}$ was inhibitory when the drug was added at time = 0 and time = three days but viable amebae were still

recovered. Only at 10 $\mu g.cm^{-3}$ were no viable amebae found after the six day exposure period. R41,400 was cytotoxic at a concentration of 10 $\mu g.cm^{-3}$.

A comparative summary of results of miconazole and R41,400 activity against N. fowleri (MsT) is provided in Table XXV. In axenic culture R41,400 appears more effective than miconazole. In cell culture the concentration of miconazole required for inhibitory and amebicidal activity is dependent on the number of amebae present whereas R41,400 does not show this effect. But these drugs show a two-fold difference in cytotoxicity with R41,400 being less toxic.

In view of its efficacy in vitro against N. fowleri (MsT) preliminary experiments were carried out with R41,400 in vivo. The results of these experiments are shown in Tables XXII, XXIII and XXIV. A number of points should be noted regarding these experiments:

1) The virulence of N. fowleri (MsT) appeared to have diminished since it was first isolated from a case of PAM in 1976. Cursons et al. (1978) reported that an inoculum of 5×10^4 amebae caused death of all mice in five to eighteen days when inoculated intranasally.

2) No dosage schedules for IP administration are available for R41,400. All preclinical data involves oral administration of the drug.

3) No assays of mouse serum were carried out to determine if effective levels were reached.

From the results of in vivo testing R41,400 appeared to exert a deleterious rather than curative effect on N. fowleri infection. At the highest daily dose used (40 $mg.kg^{-1}$) the survival time of the mice was reduced. This effect was most marked when the drug was administered in one daily dose where the average time of death decreased from 9.0 days, in control mice to 4.6 days (Table XXII). At the lowest daily dose (10 $mg.kg^{-1}$) an increased survival time resulted when the drug was administered only once daily but when divided into twice daily doses the average time of death was less than that of the controls. Preclinical data on R41,400 shows that serum concentrations in experimental animals fall rapidly within eight hours of administration and this is the reason twice daily dosing was tried. But at the

Table XXV: The Effect of Miconazole and R41,400 on N. fowleri (MsT)

METHOD OF TESTING		INHIBITORY OR AMEBICIDAL CONCENTRATION OF	
		MICONAZOLE $\mu g.cm^{-3}$	R41,400 $\mu g.cm^{-3}$
1. In vitro axenic drug testing	- MAC	50	10
2. Cell culture drug testing			
Drug added at time = 0	- MIC	1-5	5
	MAC	5	10
Drug added at time = 3 days	- MIC	5	5
	MAC	10	10
3. Cytotoxic dose		5	10

lowest dosage schedule this appeared to have a deleterious effect. The maintainence of high serum levels may be affecting the immune system of the mice.

These experiments were only preliminary and obviously more work is necessary. The dosages chosen appear too high and may have affected the immune system thereby making the mice more susceptible to N. fowleri infection. Miconazole, which appeared more toxic in cell culture tests, has been administered twice daily to mice at a dose of 70 $mg.kg^{-1}$ in the treatment of coccidiodomycosis (Preclinical data).

No in vivo tests have been carried out with miconazole as yet but its in vitro efficacy warrants such trials. More information is available about miconazole in clinical trials and this may guide future testing of R41,400. The highest dosage for human infections recommended by the manufacturers is 3600 $mg.day^{-1}$ given in three IV infusions, each administered over a period of 30 minutes to one hour. This dose is equivalent to approximately 54 $mg.kg^{-1}$. For fungal meningitis IT therapy is also recommended at a dose of 20 mg.

Miconazole was included by Siedel et al. (pers. comm. 1978) in the treatment of a human PAM case. The doses employed were IV 350 $mg.m^{-2}$ per day divided tid for nine days and 10 mg IT for two days decreasing to 10 mg every other day for eight days. The role miconazole played in the survival of this case cannot be evaluated as in vivo activity is unknown.

Another imidazole derivative, clotrimazole has been shown to be effective in vitro against 18 strains of N. fowleri. The MIC ranged from 0.03 to 0.215 $\mu g.cm^{-3}$ and the MAC, determined after five days exposure, from 0.125 to 0.25 $\mu g.cm^{-3}$ (Jamieson, 1975). Jamieson and Anderson (1974) showed the amebicidal effect varied with inoculum size. Clotrimazole was amebicidal at a concentration of 0.15 $\mu g.cm^{-3}$ with an inoculum of 730 amebae but failed to prevent the growth of 7,300 and 73,000 amebae at this concentration.

In in vivo studies clotrimazole failed to prevent N. fowleri infection in mice, with doses twice daily of 50 $mg.kg^{-1}$ given orally, when treatment was begun immediately after intranasal inoculation of the amebae (Jamieson, 1975). The serum levels achieved in mice in the first 32 hours after infection should have been sufficiently high to inhibit amebic multiplication. A peak level of 6 $\mu g.cm^{-3}$ was found three hours after a single dose of 50 $mg.kg^{-1}$.

Although clotrimazole appeared effective *in vitro* this finding was not substantiated *in vivo*. A similar result appears to be true for R41,400 although further experimentation is necessary. To date therefore the only drug effective in the treatment of PAM is amphotericin B. It is essential that the maximum possible dose is administered immediately the diagnosis is confirmed. The simultaneous administration of tetracycline would also be recommended.

5.2. Treatment of Acanthamoeba Infections

Treatment of infections due to *Acanthamoeba* spp. has to date been totally unsuccessful (Kernohan *et al.*, 1960; Jager & Stamm, 1972; Robert & Rorke, 1973; Nagington *et al.*, 1974; Sotelo-Avila *et al.*, 1974; Bhagwandeen *et al.*, 1975; Jones *et al.*, 1975; Ringsted *et al.*, 1975; Martinez *et al.*, 1977). The main problem encountered with AM is the difficulty in diagnosis of the disease and all cases have been diagnosed post-morten. Even in advanced cases there is a lack of specific symptoms and signs indicative of the disease and no amebae are seen in the CSF (Chang, 1974a). Nasal and throat swabs may provide more information.

In 1972, Carter suggested that sulphadiazine should always be used as well as amphotericin B in the treatment of PAM in case the amebae should occasionally prove to be *Acanthamoebae*. In 1965 Culbertson *et al.* had showed that sulphadiazine was effective in protecting mice from infection with *A. castellanii*. When ten control mice were inoculated intranasally with 500 *Acanthamoeba* all were dead within seven days. When sulphadiazine, incorporated into the diet, was given at dosages of 4, 8 and 12 mg daily all the mice survived. Treatment was begun on the day of infection. When the sulphadiazine, at a dose of 15 mg daily, was begun 72 hours after infection a 90% survival rate resulted but was reduced to 70% when the treatment was begun 96 hours post-infection.

To date the only clinical experience of the effectiveness of sulphadiazine in humans is that reported by Nagington *et al.* (1974) (Case 1) where it was used to treat an eye infection. Sulphadiazine was given at a dose of 500 mg six-hourly but the infection did not respond to treatment. Unfortunately this treatment was begun six months after the first symptoms when the infection was advanced.

Although effective against Naegleria spp. amphotericin B has been shown to be ineffective in vitro against a number of Acanthamoebae spp. at levels attainable therapeutically in humans. Duma and Finley (1976) and Visvesvara and Balamuth (1975) reported that amphotericin B at a concentration of 50 $\mu g.cm^{-3}$ had no effect on Acanthamoeba spp.. Casemore (1970) reports marked inhibition by 100 $\mu g.cm^{-3}$ amphotericin B against six strains of A. castellanii. A report by Nagington and Richards (1976) on the susceptibility of A. polyphaga and A. castellanii isolated from eye infections gives an MIC of 1 $\mu g.cm^{-3}$. The inoculum size used by Nagington and Richards (1976) was 5 x 10^3 amebae.cm^{-3}.

A marked difference in the susceptibility of the two species used in this study was found with amphotericin B (Figures 15 & 16). The non-pathogenic A. castellanii (1501) was not effected at levels $\leqslant$ 100 $\mu g.cm^{-3}$ whereas for A. culbertsoni (A-1) 100 $\mu g.cm^{-3}$ was amebicidal. The variation in results found in the different studies and between different species and strains is puzzling. Differences between studies may result from the different media used, screening techniques, and the size of the inoculum and this emphasizes the need to standardize testing. Differences between species and strains may reflect different sterol concentrations in the cell membrane.

The antifungal agent, 5-fluorocytosine, which has no activity against Naegleria spp. is active against Acanthamoebae. Casemore (1970) reported an amebicidal concentration of 100 $\mu g.cm^{-3}$ and inhibition in the range of 12.5 to 50 $\mu g.cm^{-3}$ against six strains of A. castellanii. Das (1975) found that 200 $\mu g.cm^{-3}$ was needed for an amebicidal effect against A. culbertsoni in axenic culture with an inoculum of 5 x 10^3 amebae.cm^{-3}. Jones et al. (1975) studied the effect of 5-fluorocytosine on an A. polyphaga strain isolated from a human eye infection. In axenic culture with an inoculum of 5 x 10^4 amebae.cm^{-3} the MIC was 25 $\mu g.cm^{-3}$ and the MAC was 100 $\mu g.cm^{-3}$.

Duma and Finley (1976) found in their study that two strains of A. castellanii and three strains of A. polyphaga were not inhibited by concentrations of 5-fluorcytosine up to 100 $\mu g.cm^{-3}$. The inoculum size used was only 2 x $10^3.cm^{-3}$ but the amebae were grown in an enriched media, containing pyrimidines which probably had an antagonistic effect on the 5-fluorocytosine.

In this study both A. castellanii (1501) and A. culbertsoni (A-1) were found to be susceptible to levels of 5-fluorocytosine ranging from 10 to 250 $\mu g.cm^{-3}$ (Figures 21 & 22). In contrast to

Stevens and O'Dell (1974), who found that concentrations $\geqslant 10 \mu g.cm^{-3}$ 5-fluorocytosine were amebicidal to the avirulent strain but the virulent strain was capable of growing in the presence of $40 \mu g.cm^{-3}$, the pathogenic A. culbertsoni (A-1) appeared more susceptible than the non-pathogenic A. castellanii (1501). For A. castellanii (1501) there was little difference in the pattern of susceptibility at concentrations between 10 and $250 \mu g.cm^{-3}$. For A. culbertsoni (A-1) an initial period of growth followed by inhibition occurred at concentrations between 10 and $100 \mu g.cm^{-3}$. Only at $250 \mu g.cm^{-3}$ was the inhibitory effect seen throughout the 96 hours exposure.

In cell culture (Table XII) $50 \mu g.cm^{-3}$ inhibited the formation of a CPE by A. culbertsoni (A-1) when the drug was added at the same time as the amebae. Only at $250 \mu g.cm^{-3}$ was the drug amebicidal. Once the CPE had begun to form even a concentration of $250 \mu g.cm^{-3}$ did not halt the progression of the CPE. This effect has been confirmed in in vivo studies by Stevens and O'Dell (1974). Mice infected with A. culbertsoni (A-1) and receiving a single daily 3 mg dose of 5-fluorocytosine ($200 mg.kg^{-1}$) from the onset of infection showed a significant increase in survival over the controls. But when the drug treatment was initiated 24 hours post-infection or later, no significant difference was obtained. The authors found numerous amebae in the brain tissue of infected dead mice that had received intensive treatment with 5-fluorocytosine and believe that the ineffectiveness of the drug was due to the organisms capacity to develop resistance, thought to be due to a change in the cell membrane. The use of 5-fluorocytosine as an antifungal agent has also been limited by the ability of fungi to develop resistance (Shadomy, 1970; Holt & Newman, 1973). Even though 5-fluorocytosine promoted survival of A-1 infected mice when treatment was started at the onset of amebic infection, the failure of the drug to protect animals when treatment was begun 24 hours post-infection indicates that it may be of limited use in human Acanthamoeba infections (Stevens & O'Dell, 1974).

Synergistic combinations of drugs have been found to be of value against Naegleria spp. (Figures 29-32; Tables XVII & XVIII; Thong et al., 1978a, 1979) and the possibility of the use of drug combinations was also investigated for Acanthamoebae. For Naegleria a membrane-active agent, amphotericin B was found to potentiate the action of the otherwise ineffective agents tetracycline and rifampicin. For Acanth-

amoebae the membrane-active polymyxin B sulphate was investigated in combination with 5-fluorocytosine, tetracycline and rifampicin.

Nagington and Richards (1976) found that polymyxin B sulphate was inhibitory to A. polyphaga and A. castellanii, isolated from eye infections, at a concentration of 10 μg.cm^{-3} when 5 x 10^3 amebae.cm^{-3} were present. Duma and Finley (1976) reported a 24 hour MIC ranging from 50 to $\geqslant$ 100 μg.cm^{-3} and a 48 hour MIC of 79.37 to $\geqslant$ 100 μg.cm^{-3} against two strains of A. castellanii and three strains of A. polyphaga. The two ocular pathogens were the most resistant to polymyxin B.

Polymyxin B showed a slight inhibitory effect on A. castellani (1501) at concentrations ranging from 10 to 500 units.cm^{-3} (1.27-63.5 μg.cm^{-3}) (Figure 23). A. culbertsoni (A-1) was inhibited only at a concentration of 500 units.cm^{-3} (Figure 24). In cell culture, polymyxin B had no effect on the amebae or the formation of a CPE at a concentration of 500 units.cm^{-3} (Table XIII).

When used together polymyxin B and 5-fluorocytosine showed synergistic activity both in axenic and cell culture against A. culbertsoni (A-1). In axenic culture when 100 units.cm^{-3} polymyxin B is combined with either 50 or 100 μg.cm^{-3} 5-fluorocytosine the result is very similar to that obtained with 5-fluorocytosine alone (Figure 33). But when the polymyxin B concentration is increased to 500 units.cm^{-3} an amebicidal effect is seen with both 50 and 100 μg.cm^{-3} 5-fluorocytosine within 72 hours (Figure 34). Even with the combination of 500 units.cm^{-3} polymyxin B and 10 μg.cm^{-3} 5-fluorocytosine there was a ten-fold difference in the number of surviving amebae over that obtained with 10 μg.cm^{-3} 5-fluorocytosine alone.

Synergistic activity of these two agents was also seen in cell culture (Table XIX). All the combinations tested inhibited the formation of a CPE when the drugs were added at the same time as the amebae. Viable amebae were recovered with a concentration of 100 μg.cm^{-3} 5-fluorocytosine when used alone but the combination of 100 units.cm^{-3} polymyxin B plus 100 μg.cm^{-3} 5-fluorocytosine was amebicidal.

When 5-fluorocytosine was added to the cell culture once CPE had begun to form no inhibitory effect was seen (Table XII). The combined use of polymyxin B and 5-fluorocytosine inhibited the progression of the CPE at concentrations of 100 units.cm^{-3} and 250 μg.cm^{-3}, 500 units.cm^{-3} and 50 μg.cm^{-3} and 500 units.cm^{-3} and 100 μg.cm^{-3}, respectively. An amebicidal effect was seen with 500 units.cm^{-3} polymyxin B and 250 μg.cm^{-3} 5-fluorocytosine.

Like other members of the family but to a lesser extent polymyxin B is nephrotoxic and neurotoxic (Newton, 1956). The doses recommended for IV administration are 1.5 to 2.5 $mg.kg^{-1}$ (12,000 - 20,000 $units.kg^{-1}$) per day as a single dose or divided into two doses (Garrod et al., 1973). Peak serum levels of 1.5 $\mu g.cm^{-3}$ were obtained one hour after a dose of 60 mg. But polymyxin B penetration into the CSF is reported to be low (Garrod et al., 1973) and IT administration is recommended. The recommended dose is 10,000 - 50,000 units given once daily.

5-Fluorocytosine is a synthetic antifungal agent of low toxicity that is well absorbed from the gastrointestinal tract. The currently recommended dose is 150 $mg.kg^{-1}$ per day divided into six-hourly doses. Block and Bennett (1972) studied the serum and CSF levels of 5-fluorocytosine in five patients. At a daily dose of 100 $mg.kg^{-1}$, one hour after administration serum levels ranged from 80 to 101 $\mu g.cm^{-3}$ and CSF levels from 27 to 62 $\mu g.cm^{-3}$. At a daily dose of 140 $mg.kg^{-1}$, two hours after administration, serum levels ranged from 65 to 82 $\mu g.cm^{-3}$ and CSF levels from 40 to 62 $\mu g.cm^{-3}$. Shadomy et al. (1969) reported serum levels from 18 to 40 $\mu g.cm^{-3}$ in four patients three to four hours after a dose of 150 $mg.kg^{-1}$. On average CSF levels are about 50% those found in the serum.

The combined use of polymyxin B and 5-fluorocytosine in Acanthamoeba experimental infections warrants further investigation. The concentrations required may be prohibitive for systemic use but should be effective in Acanthamoeba eye infections. Ophthalmic solutions containing 20,000 $units.cm^{-3}$ instilled two to ten times per hour are recommended for bacterial eye infections (Goodman & Gilman, 1975). The drug can also be given once or twice daily by subconjunctival injection, 5 to 10 mg (40,000 to 80,000 units) in 0.5 cm^3 of isotonic NaCl injection solution. 5-Fluorocytosine, applied topically at 1% to 1.5% concentration, has been used in mycotic surface infections of the eye (Garrod et al., 1973) but the agent penetrates the eye poorly when applied topically. 5-Fluorocytosine penetrates the CSF to levels 50% of those in the serum and also shows good penetration into the aqueous humor (Richards & Jones, 1970) and so oral therapy together with topical application is indicated. Polymyxin B (20,000 $units.cm^{-3}$) and 1% 5-fluorocytosine were used topically in a case of A. polyphaga keratitis (Jones et al., 1975), with no curative result. 5-Fluorocytosine was

also given orally at a dose of 150 $mg.kg^{-1}$ per day. However this treatment was not begun until one year after the infection started and was at a very advanced stage.

Polymyxin B has been shown to potentiate the effect of tetracycline on Saccharomyces cerevisiae and Candida albicans in vitro (Schwartz et al., 1973). This combination and also polymyxin B and rifampicin was investigated for synergistic activity against A. culbertsoni (A-1). Tetracycline alone showed only a slight inhibitory effect on both Acanthamoeba spp. at a concentration of 400 $\mu g.cm^{-3}$ (Figures 17 & 18). No visual signs of drug damage could be detected on the amebae. This was confirmed in cell culture as no inhibitory effect on the formation of a CPE by A. culbertsoni (A-1) was seen at all the concentrations tested (10-100 $\mu g.cm^{-3}$) (Table XV).

A similar result was found when rifampicin was used alone. No inhibitory effect was seen at concentrations of 50 and 100 $\mu g.cm^{-3}$ on both Acanthamoeba spp.. At 500 $\mu g.cm^{-3}$ a higher proportion of cysts were present in the test cultures (Figures 19 & 20). In cell culture, no inhibitory effect was seen at concentrations of rifampicin of 10 to 100 $\mu g.cm^{-3}$ (Table XVI). At concentrations at and above the cytotoxic concentration (50 $\mu g.cm^{-3}$) the Vero cells appeared more susceptible to the amebae with the effect being greater than simply the additive effect of CPE and CTE.

When used in combination, 400 $\mu g.cm^{-3}$ tetracycline and either 100 or 500 $units.cm^{-3}$ polymyxin B showed a greater inhibitory effect than either agent alone (Figures 35 & 36). With 100 $units.cm^{-3}$ polymyxin B and 400 $\mu g.cm^{-3}$ tetracycline there was an initial period where the numbers did not change followed by a decrease in numbers of surviving amebae to $8 \times 10^4.cm^{-3}$ after 96 hours. With 500 $units.cm^{-3}$ polymyxin B and 400 $\mu g.cm^{-3}$ tetracycline the number of surviving amebae decreased to $5 \times 10^4.cm^{-3}$ after 96 hours. However in cell culture, no synergistic activity was found between polymyxin B and tetracycline (Table XX).

For polymyxin B and rifampicin no synergistic activity was seen either in axenic culture (Figures 37 & 38) or in cell culture (Table XXI). In axenic culture at all the drug combinations tested, an initial inhibitory effect was seen in the first 24 hours but growth occurred in all the test cultures after that. In cell culture, an antagonistic rather than synergistic effect was seen. This was

probably due to the cytotoxic nature of rifampicin, as was found when rifampicin was used alone (Table XVI).

The imidazole derivatives, miconazole and R41,400 were also tested for activity against the two Acanthamoeba spp.. Miconazole in axenic culture had no activity against either A. castellanii (1501) or A. culbertsoni (A-1) at concentrations $\leqslant 100 \mu g.cm^{-3}$. None of the amebae in the test cultures showed any variation in appearance from the control organisms (Figures 25 & 26).

Nagington and Ricards (1976) reported a MIC of $10 \mu g.cm^{-3}$ miconazole for a strain of A. polyphaga and $100 \mu g.cm^{-3}$ for a strain of A. castellanii. These two strains were isolated from cases of human eye infection. The inhibitory effect could be due to the low inoculum used, 5×10^3 amebae.cm^{-3}. Duma and Finley (1976) showed that miconazole had no effect on two strains of A. castellanii and three strains of A. polyphaga at a concentration of $100 \mu g.cm^{-3}$. The inoculum used in this study was 2×10^3 amebae.cm^{-3}. From these results it is obvious that there are marked differences in strain susceptibilities to miconazole.

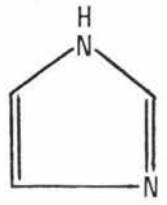
In contrast to miconazole, R41,400 showed inhibitory activity against both Acanthamoebae spp. with the pathogenic A. culbertsoni (A-1) appearing more susceptible (Figures 27 & 28). At all the concentrations tested (25-100 $\mu g.cm^{-3}$) the amebae showed signs of drug damage with increased vacuolation and no visible acanthapodia. The MIC for A. culbertsoni (A-1) was $25 \mu g.cm^{-3}$. After an initial period of growth this concentration was also inhibitory for A. castellanii (1501).

Although R41,400 had an inhibitory effect on Acanthamoebae spp. in axenic culture the concentrations required would be prohibitive for systemic use. In cell culture, the formation of a CPE by A. culbertsoni (A-1) was not inhibited even at a concentration of 50 $\mu g.cm^{-3}$ when the drug was added at the same time as the amebae (Table XIV). At this concentration a marked CTE occurred.

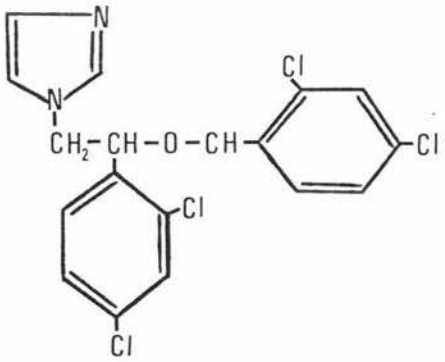
Reports on the activity of another imidazole derivative, clotrimazole, against Acanthamoebae are contradictory. Nagington and Richards (1976) report an MIC of $0.1 \mu g.cm^{-3}$ for A. polyphaga and $1.0 \mu g.cm^{-3}$ for A. castellanii. Duma and Finley (1976) found that two strains of A. castellanii and three strains of A. polyphaga were not affected by a concentration of $100 \mu g.cm^{-3}$ clotrimazole.

Acanthamoebae have been shown to be susceptible to the parent compound, imidazole base (Verma & Kreshna Murti, 1975). After 72 hours exposure to a concentration of 40mM (272.32μg.cm^{-3}) no viable amebae were recovered. The imidazole derivatives differ in the side chains attached to the parent 1,3- diazole ring. Figure 39 compares the structural formulae of imidazole, miconazole and clotrimazole.

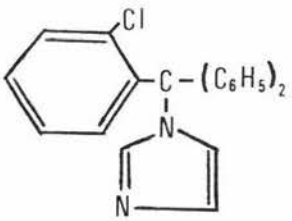
Figure 39: Structural Formulae of Imidazole base, Miconazole and Clotrimazole



Imidazole
(1,3-diazole)



Miconazole



Clotrimazole

Unfortunately the structural formula of R41,400 has not been determined but this may show which of the side chains is necessary for activity against Acanthamoebae and provide direction for the synthesis of a new derivative.

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