CLASSIFICATION AND IDENTIFICATION OF THE AETIOLOGICAL AGENTS OF PRIMARY AMEBIC MENINGO-ENCEPHALITIS TOGETHER WITH PRELIMINARY INVESTIGATIONS OF PUBLIC HEALTH MEASURES

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Raymond Thomas Michael Cursons
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ABSTRACT

The taxonomy of the aetiological agents of Primary Amebic Meningo-encephalitis (PAM) was investigated to determine the reliability of the common features of the three current schemes. It is concluded that the scheme of Singh & Das (1970) is the most suitable and should be generally adopted. The acceptance of one scheme will remove much of the confusion which characterizes the classification of these organisms.

Current identification methods that differentiate between Naegleria gruberi (the non-pathogen) and Naegleria fowleri (the pathogen) were also investigated over a wider range of parameters than previously, to establish their relative usefulness. The conclusions of this investigation are presented in Tables XXII and XXIII. The controversial identification of the 1968 New Zealand cases (isolates BH & BL) as a Myxomycete by Mandal et al. (1970) was re-examined. Evidence is presented to demonstrate that they are N. gruberi.

It was established that there was no general selection for the non-pathogen over the pathogen at 37°C as indicated by their respective Q_{O_2} values at 27°C and 37°C. That there is potential for adaptation to a range of temperatures was shown.

The failure of chlorine as a disinfectant for these soil-amebae was also examined. The ineffectiveness of normal levels of chlorination was confirmed and therefore the use of NaCl and the basic dyes Malachite Green and Brilliant Green investigated. It was found that no amebae could survive a concentration of 1.5% (W/V) of NaCl in axenic culture, of 1.5 $\mu g/cm^3$ of Malachite Green and of 3.0 μg of Brilliant Green.

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PREFACE

Primary Amebic Meningo-encephalitis (PAM) is a normally fatal disease of the central nervous system (CNS) usually involving young, healthy individuals with a recent history of contact with fresh-water. It was first recognised by Fowler in Australia in January 1961 and since then, about 74 diagnosed cases have occurred in various parts of the world (Table I).

On purely histological evidence, all reported cases of PAM, prior to 1968 were attributed to members of the Hartmannella/Acanthamoeba group of amebae (Culbertson et al., 1961; Fowler & Carter, 1965). However, three reports in 1968 (Cerva & Novak; Cerva, Novak & Culbertson; Carter) suggested that the amebae were much smaller in histological sections than for the formerly known pathogenic Hartmannella/Acanthamoeba strains, and probably belonged to the related genus Naegleria. Cultural verification of this suggestion soon followed (Butt, Baro & Knorr, 1968; Culbertson, Ensminger & Overton, 1968; Calicott et al., 1968) and the amaebae were subsequently classified as Naegleria gruberi. Then, in 1970, on the basis of morphological, cultural, and pathogenicity differences, Carter renamed the pathogenic Naegleria, Naegleria fowleri. To date, there are two other synonyms for the pathogenic Naegleria: N. aerobia (Singh & Das, 1970) and N. invades (Chang, 1971).

The first New Zealand cases of PAM occurred in the late autumn of 1968 (Mandal et al., 1970) after the victims had bathed in a Matamata hot-spring. Although the amebae were originally considered to belong to the genus Naegleria, they were later reclassified as a slime mould probably belonging to the genus Echinostelium, Mandal et al. The fifth case occurred in May 1972, after the victim had also swum in a thermal pool, but in this case the aetiological agent was identified as N. fowleri (Nicoll, 1973). Since then the Adelaide Amebic Research Unit has consistently isolated pathogenic Naegleria and Hartmannella/Acanthamoebae from New Zealand sources (Robinson, 1974).

Unfortunately, the classification of these organisms is still a controversial matter (Carter, 1970; Culbertson, 1971). With three different classification schemes existing in the literature (Page, 1967; Singh & Das, 1970; Chang, 1971) all of which overlap to some consider-

able extent creating confusion in the selection of a name, it was considered necessary to review the classification to establish the degree of reliability which could be attached to each of the characters cited. It is essential as a prelude to the diagnosis of PAM that there are reliable characters on which to base an identification, since by its very nature, identification presupposes that classification has already distinguished the species, and that names have been assigned to them. As a follow-up, it was considered essential to review current identification methods over a wider range of experimental parameters than have previously been examined, as well as investigating new methods of identification. Using these methods, it was then decided to reassess the controversial identification by Mandal et al. (1970) of the causative agents of the early New Zealand cases.

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CHAPTER ONE: INTRODUCTION

1. The History of Primary Amebic Meningo-encephalitis

1.1 Discovery

Between the establishment of the families under the order Amoebida, Wenyon (1926) and the systematic treatment on a phylogentic basis by Singh (1952) the small, free-living soil-amebae attracted little research attention perhaps because Schaudin (1903) had convinced most workers that the only real pathogenic ameba of the human intestine was Entamoeba histolytica. This preoccupation with intestinal amebic disease, effectively inhibited the consideration of the role free-living amebae as disease agents to such an extent that when amebae were encountered in bacterial or fungal cultures, the tests for virulence were made by inoculation of the animal only via the intestine. Further support of this preoccupation comes from two retrospectively diagnosed cases of PAM in which the causative agent was thought to be the intestinal ameba Iodamoeba butschlii (Derrick, 1948; Kernohan, 1960).

Interest was revived in 1957 when Jahnes et al., reported that some free-living amebae, loosely classified as Acanthamoebae, were found as contaminants in primary monkey kidney cell culture, (PMK). Then in 1958 medical interest was aroused when Culbertson et al., who observing a similar ameba in cell culture fluid suspected of containing an unknown simian virus, injected this fluid into the brains of both mice and monkeys to determine the virulence of the suspected virus. All the injected animals died and amebae were found in lesions of severe meningo-encephalitis. Direct microscopic examinations of the cell culture fluid used for the inoculation revealed the presence of amebae in the monolayer itself. Thus the effects of the amebae upon the cell culture had been mistaken for viral cytopathogenic effects, (CPE). Removal of the amebae from the cell culture produced no CPE at all. This amebae, designated Hartmannella, strain A-1, was later classified as Hartmannella culbertsoni by Singh and Das (1970).

From this evidence, Culbertson et al. (1961) postulated that a similar disease could occur in man as a result of contact with water which was heavily contaminated by these organisms. Shortly after, the disease was recognised by Fowler in Australia (1961) and Butt in the USA (1962). The first published description of the disease came in 1965 (Fowler & Carter) and this was followed shortly after by reports from the USA, Czechoslovakia, Australia and New Zealand. From one of these reports Butt (1966) coined the term Primary Amebic Meningo-encephalitis (PAM) to distinguish the disease from the rare invasion of the brain by E. histolytica.

Although the early reports suggested that the causative agents of PAM might belong to the Hartmannella/Acanthamoeba group, by 1968 it was generally agreed that the incriminating species belonged to the related genus Naegleria (Butt, Baro, Knorr, 1968; Carter, 1968; Culbertson, Ensminger & Overton, 1968). The pathogens were thus considered to be pathogenic strains of Naegleria gruberi until 1970, when Carter renamed them Naegleria fowleri.

To date there have been about 74 diagnosed cases of PAM reported but this is open to conjecture since retrospective diagnoses indicate that some cases may have occurred unrecognised. The disease has occurred in many countries (Table I) the majority of cases being associated with contact with water.

1.2 Classification and Identification

In 1909, Dangeard stated "rien n'est plus difficle en effet que déterminer une amibe". In 1973, Bovee and Jahn stated, "progress in taxonomy and phylogeny of amebae is slow and use of modern methods even slower". More specifically on the subject of limax-amebae taxonomy Carter (1970) stated that, "their taxonomy is unfortunately a controversial matter; diverse and therefore confusing classification exists in the literature. The chief difficulty is the relative lack of immutable characteristics by which these amebae can be identified. The nucleus is distinctive but common to all,

Table I. Cases of PAM in the Literature (adapted from Carter, 1972)

Country	No. of reported cases	No. of cases with amebae isolated	Identity	Reference
Africa	1	0	-	Grundy & Blowers, 1970
Australia	1*	0	-	Derrick, 1948-49
	4	0	Naegleria	Fowler & Carter, 1965
	2	1	*N.fowleri*	Carter, 1968
	1	1	"	" 1970
	2	2	"	" 1972
	2	2	"	Anderson & Jamieson, 1972b
Belgium	3	3	*N.gruberi*	Jadin *et al.*, 1971
	1	1	*N.fowleri*	Van Den Driessche, 1973
Czecho-slovakia	16	0	-	Cerva & Novak, 1968
	1	1	Naegleria	Cerva *et al.*, 1969
Great Britain	2*	0	-	Symmers, 1969
	1*	0	-	Apley *et al.*, 1970
	1	1	Naegleria	" " 1970
	1	0	Acanthamoeba	Jager & Stamm, 1972
India	2*	0	-	Pan & Gosh, 1971
	1	0	-	Bedi *et al.*, 1972
New Zealand	1*	0	-	Mandal *et al.*, 1970
	3	1	Myxomycetale	" " 1970
	1	1	*N.fowleri*	Nicoll, 1973
USA	1	0	-	Kernohan *et al.*, 1960
	1	0	Hartmannella	Patras & Andujar, 1966
	3	0	-	Butt, 1966
	1	1	Naegleria	Butt, Baro & Knorr, 1968
	1	0	-	Poppiti, R.J., personal comm. to Butt *et al.*, 1968
	1	0	Acanthamoeba	Rorke *et al.*, 1971
	7	0	-	Calicott, 1968
	1	1	*N.gruberi*	Calicott et al., 1968
	3	2	Naegleria	Duma *et al.*, 1971
	1	1	"	" " 1969
	5	0	-	Dos Santos, 1970
	1	0	-	McCroan & Patterson, 1970
	1*	0	-	Kenny, 1971
World Totals	74	19		

* = unconfirmed cases

and total size, locomotive form, pseudopodal type and even cyst morphology are known to vary considerably under different environmental conditions".

Traditionally, taxonomy of sarcodines has followed two distinct trends:

morphological approach - emphasizes the pseudopodial structure and locomotive form

cytological approach - places emphasis on the pattern of nuclear division, i.e. whether promitosis or mitosis.

These early taxonomic schemes, prior to Singh (1952) suffered however from many controversies due to the many inherent technological limitations such as:

a) the limited resolution of the light microscope,

b) the source of staining. Prior to Ralfalko's 1947 use of the Feuglen reaction observers depended upon haematoxylin and other non-specific aniline dyes to examine dividing nuclei which led to the description of many spurious structures,

c) preconceived ideas about what should be present, and

d) the lack of strictly controlled culture conditions which may lead to the production of abnormal forms.

Currently there are three different classification schemes for small, free-living amebae (Table II) all of which tend to overlap to some considerable extent and consequently produce some difficulty in the selection of a name.

The scheme of Singh & Das (1970) is a purely cytological approach based on the type of nuclear division and the presence or absence of certain mitotic structures.

Page (1967a) attempts to combine both the pseudopodal and cytological approaches. He assumes that a correlation exists between the manner of locomotion and the pattern of nuclear division.

Chang's (1971) scheme can perhaps be considered as a hybrid of the other two schemes, in that he follows Singh & Das's scheme for the family Schizopyrenidae, but adopts Page's family Hartmannellidae.

Table II. Current Classification Schemes

Singh & Das (1952, 1970)	Page (1967a)	Chang (1970)
F. Schizopyrenidae: resting nucleus contains 1 or several F-ve nucleoli which during mitosis form polar masses. Nuclear membrane persists throughout division. I.B. may be present. Amebae may have more than one nucleus, & some genera may produce a flagellate stage. G. Naegleria: Polar masses formed. F-ve. I.B. temporary biflagellate; no cytostome. t.s. N. gruberi. F. Hartmannellidae: Resting nucleus has either a single F-ve nucleolus or several nucleoli. During mitosis the nucleolus (i) disappears & a spindle with chromosomes arranged as an equatorial plate develops. The nuclear membrane disappears during mitosis. Amebae may be uni- or multinucleate; no temporary flagellates. G. Hartmannella: Resting nucleus contains a single F-ve nucleolus. During mitosis the nucleolus disappears & a spindle with chromosomes arranged as an equatorial plate is formed. No temporary flagellates. t.s. H. glebae.	F. Vahlkampfiidae: Amebae which are monopodial in locomotion, except temporarily, moving in a markedly eruptive manner, & with the promitotic type of nuclear division. G. Naegleria: Promitosis, temporary biflagellate; no cytostome. F. Hartmannellidae: Amebae which are usually elongated in active locomotion, with only 1 broad lobopodium, except temporarily; usually 75 u in length; vesiculate nucleus, usually single; nuclear division in which the nucleolus & usually the nuclear membrane disintegrate; no flagellate stage. G. Hartmannella: Broadly digitiform or hemispherical lobose pseudopods. Normally uninucleate. Nuclear division with disintegration of nucleolus. Cyst may be present - is smooth-walled & rounded. Locomotion not strongly eruptive. t.s. H. hyalina. G. Acanthamoebae: Broad anterior hyaline lobopodium from which are produced acanthapodia. Cyst: stellate endocyst & wrinkled ectocyst. Normally uninucleate. Nuclear division with disintegration of nucleolus. t.s. A. castellanii.	F. Schizopyrenidae: Active limax form common; transient flagellates may be present; nucleolus origin of polar masses; polar caps & I.B. may be present. G. Naegleria: Double-walled cysts; transient flagellates; polar caps & I.B. in mitosis. t.s. N. gruberi. F. Hartmannellidae: No transient flagellates; motility sluggish; no limax form, nucleolus disappearing, probably forming spindle in mitosis; no polar caps or masses, aster & centrosome not known. G. Hartmannella: Ectoplasm clear or less granular than endoplasm; single walled cyst; single vacuole. t.s. H. glebae. G. Acanthamoebae: Filamentous processes from ecto- or endoplasm; grow axenically in fluid bacteriological media. t.s. A. rhysodes.

KEY: F = family, G = genus, I.B. = Interzonal Body, F-ve = Feulgen negative, ts = type species

From the three classification schemes, it is apparent that Naegleria can be differentiated from Hartmannella/Acanthamoeba in that Naegleria:

1) exhibits promitosis, i.e. ameboid movement and the nuclear membrane both persist until late telophase, and the nucleolus does not disappear but divides into two polar masses, and
2) the possession of a non-dividing flagellate stage with no cytostome.

Recently however, there has been a move towards the use of serological methods as an aid to the rapid identification of pathogens. Suffice it to say at this point that a more critical analysis of the topic will be found in the Discussion.

1.3 Epidemiology

The free-living amebae are ubiquitous in both soil and fresh-water environments throughout the world, depending largely on bacteria or other organisms as a source of nutrients. Growth is also influenced by such physical conditions as moisture and temperature (Fulton, 1970). Although the origin of the pathogenic species remains to be elucidated (Carter, 1972) environmental isolates have been made from a wide variety of sources: sewage (Singh & Das, 1972a; Chang, 1974), air (Kingston & Warhurst, 1969), soil (Anderson & Jamieson, 1972c), and hot-pools (Robinson, 1974). Selective proliferation of the pathogenic species could occur by thermal pollution of surface-waters by industry, etc. (van den Driessche *et al.*, 1973).

The disease, according to Chang (1974) can be divided into two separate clinical entities, a) a non-swimming associated meningo-encephalitis, and b) a swimming associated meningo-encephalitis, both forms being characterized according to:

a) the aetiological agents, both of which are soil-amebae
b) the portal of entry into the body

c) virulence

d) pathology

a) THE NON-SWIMMING ASSOCIATED MENINGO-ENCEPHALITIS is caused by members of the Hartmannella/Acanthamoeba group, the portal of entry thought to be the bloodstream (Chang, 1974). According to Chang, "the meagre number of cases and the lack of a definite mode of transmission make any epidemiological investigation difficult".
The reported cases of the disease (Patras & Andujar, 1966; Rorke & Robert, 1973; Jager & Stamm, 1972) all occurred in defense-weakened patients and, because the single isolation attempt was unsuccessful, the "responsible species" still remains an enigma. Chang (1974) on the basis of a comparison between the cytopathogenicity and pathogenicity of lab strains with the clinical and pathological features reported in the above three cases, considers the most probable species to be Acanthamoeba rhysodes. He also suggests that the species Acanthamoeba castellanii, Hartmannella culbertsoni and Acanthamoeba palestinensis to be synonyms of Acanthamoeba rhysodes. However morphological (Singh & Das, 1970), serological (Adam, 1964; Cerva & Kramar, 1973) and cultural evidence (Stevens & O'Dell, 1973) suggest otherwise.

b) THE SWIMMING-ASSOCIATED MENINGO-ENCEPHALITIS is caused by N. fowleri (Carter, 1970) and is without doubt the more important. Infection is thought to occur in one of two ways:

1) Naegleria-contaminated water may be introduced into the upper nasal passage, or

2) the disease may be precipitated by washing trophpzoites residing in the lower nasal passage of a carrier into the upper nasal passage (Chang, 1974). In this context, it is interesting to note that a pathogenic Naegleria (strain 161A) has been isolated from a normal healthy carrier (Chang, 1974; Lastovica, 1974).

As yet, no pathogenic hartmannellids have been iso-

lated from swimming-associated cases, although they are present together with N. fowleri, in the same natural environment (Singh & Das, 1972a; Chang, 1974). Because of this relatively rare involvement as pathogens, Singh and Das have suggested that the spread of PAM by swimming in fresh-water is mainly due to the entry of the flagellate stage of N. fowleri into the nose and subsequent transformation into the trophozoite stage (Singh & Das, 1972a). Supporting evidence for this comes from:

1) the ease of transformation of the flagellate into the trophozoite stage at 37°C,
2) the greater activity and rigidity of the flagellate stage which allows for greater dispersal,
3) the fact that amebae are usually found on the bottom of a pool attached to some semi-solid substrate, and
4) the result that intra-nasal inoculation of the flagellate stage is more effective in causing PAM and subsequent death than intra-nasal inoculation with the trophozoite stage (Singh & Das, 1972a).

1.4 Pathogenicity

In an experimental study of the pathogenicity of Naegleria meningo-encephalitis in mice, Martinez et al. (1973) found that following inoculation of N. fowleri into mice, the amebae disrupt microvilli, sensory cilia and kinocila of epithelial cells and invasion occurs primarily by direct penetration of sustenacular cells, perhaps through phagocytosis of amebae by these cells (Fig. 1). Following this intracellular invasion, either the host-cell or the amebae are destroyed. It is thought that the host-cell is destroyed by one of three mechanisms. Firstly by secretion of an enzyme or toxin which liquefies the surrounding tissue. Chang (1974 & 1971) has shown that pathogenic Naegleria liberate a cytolytic toxin in PMK cell culture, the cell-free filtrate of which, when again passaged in PMK cell culture, produces CPE. This toxin/extracellular enzyme is also thought responsible for the clear 'halo', i.e. a clear space which surrounds the trophozoite

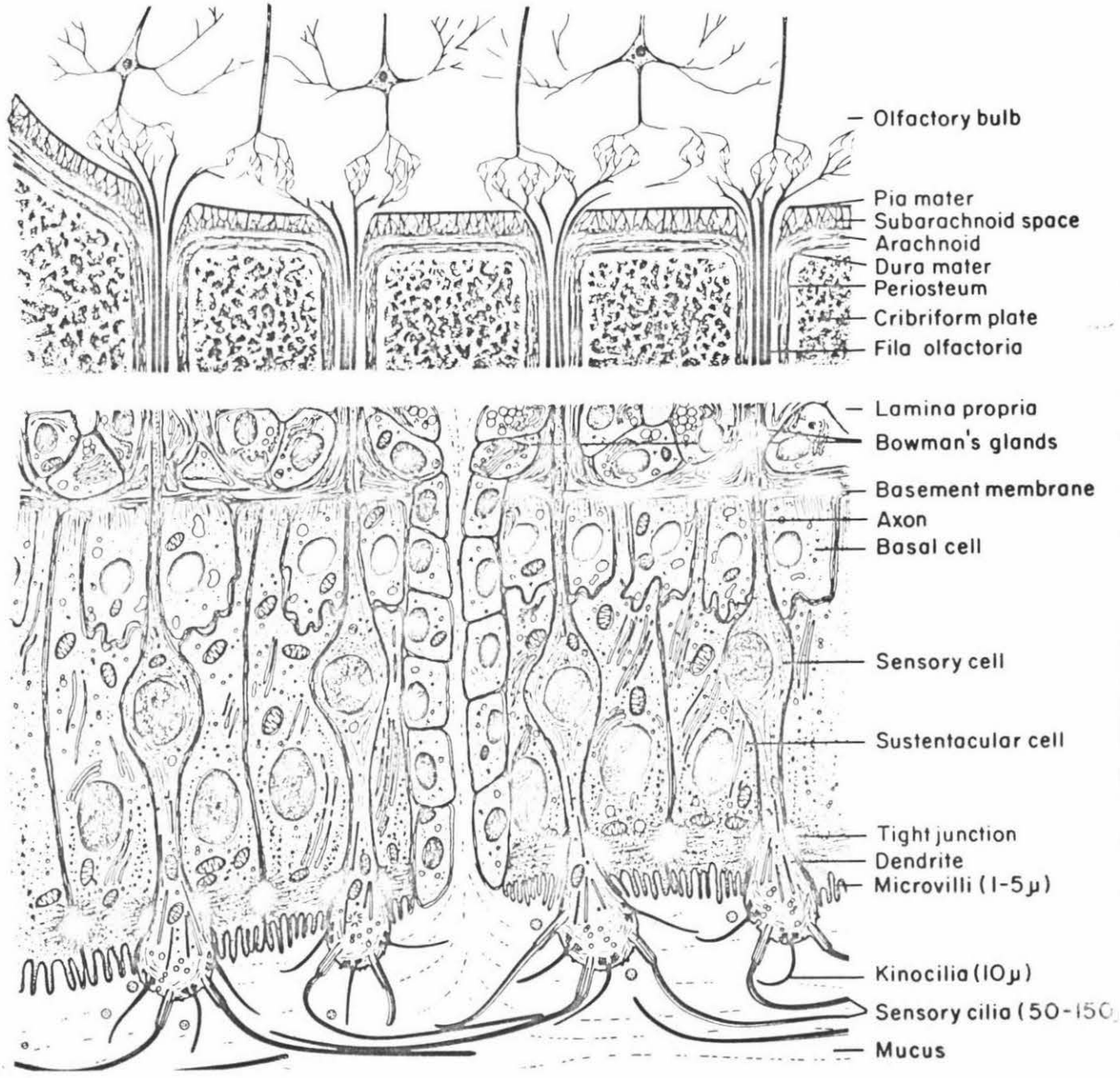


Figure 1. Schematic diagram of olfactory mucosa and adjacent structure:
(From Martinez et al., 1973)

in host-tissue (Vivesvara & Calloway, 1974; Maitra et al., 1974). Secondly by ingestion of the host cytoplasm and erosion of the host-cell, and thirdly by a combination of both these processes.

If the sustentacular cell is destroyed, the amebae then progress inward through the nasal epithelial basement membrane of the lamina propria. The final paths of invasion of the CNS appear to be via the mesaxons of the Schwann cells, which surrounds the filia olfactoria, and along perineural spaces of the olfactory nerves of the submucosal plexus.

In similar studies on pathogenicity, it was demonstrated that the actual production of pseudopodia is used for ingestion of host-cytoplasm (Visvesvara & Calloway, 1974; Maitra et al., 1974). It was further found that in both hosts (i.e. mouse brain and Vero cells) that there was very close contact between the ameba plasma membrane and the cell membranes of host-tissue. From the cell membrane, the amebae formed many micropinocytotic vesicles used for the active transport of nutritive solutes from the brain.

As regards the pathogenicity of Hartmannella/Acanthamoeba for humans and lab animals, it is generally agreed that irrespective of their origin, they possess a relatively low degree of pathogenicity. However they can produce an infection if the resistance of the host is lowered, and the disease is fatal if it is manifested in the brain. Of the two commonly indicted pathogenic species of Hartmannella/Acanthamoeba, H. culbertsoni (strain A-1) produces an acute disease while A. rhysodes (strain HN-3), is responsible for a chronic disease (Culbertson, 1971). Both have been shown to secrete a phospholipase enzyme into Vero cell filtrate, which, when this is passaged into new Vero cells, produces CPE (Visvesvara, 1972; Elson et al., 1970). In the case of A. rhysodes, this extracellular enzyme has been shown to be a lecithinase. Perhaps of significance is the finding of McIntosh and Chang (1971) who found in a comparative study of two different strains of A. rhysodes, H. glebae, and A. castellanii, that all except H. glebae secreted a lipogenic toxin and contained

antigen(s) capable of sensitising erythrocytes. Of the four strains, only *H. glebae* has been shown not to be pathogenic.

1.5 Diagnosis

The Swimming Associated Amebic Meningo-encephalitis

A Naegleria brain infection should be suspected when there is a history of swimming about 7 days prior to the abrupt onset of fever, headache, sore-throat, nausea and vomiting (Carter, 1972; Chang, 1974). Confirmation of the disease may be achieved by the observation of trophozoites in the CSF. This is still the most reliable diagnostic procedure since the standard physical examinations and even detailed neurological examinations do not sufficiently differentiate the disease from acute bacterial meningitis (Culbertson, 1971; Chang, 1974).

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 basilar surface of the cerebrum or cerebellum and over the brain stem (Carter, 1972). The cerebral and cerebellar grey substances show variable sized lesions which tend to be haemorrhagic and quite soft when they are large (Culbertson, 1971). The existence of redness and the destruction of the olfactory nerve occurs solely in amebic meningo-encephalitis and could serve to distinguish it from bacterial meningitis (Carter, 1972).

Elsewhere in the human body, there is generally no evidence of amebic infection or chronic disease as can occur when bacteria are involved. The pathological changes are restricted to pulmonary congestion and oedema, slight bronchopneumonia, acute splenitis and occasionally myocarditis. Carter (1972) thinks that these changes are readily explicable as the non-specific effects of any severe cerebral infection and are not considered specific, with the possible exception of myocarditis. These restricted pathological changes in the human body differ from those in the guinea-pig, where sub-cutaneous, intraperitoneal, or intramuscular injections resulted in a systemic infection and eventually

death (Culbertson, 1971).

The Non-swimming Associated Amebic Meningo-encephalitis

Hartmannella/Acanthamoeba brain infections are difficult to diagnose even in advanced cases due to the lack of specific symptoms and signs, and the absence of amebae in the CSF (Chang, 1974). More informative findings may be revealed by the examination of throat and nasal swabs.

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, 1974). In all the three reported cases, there was lack of evidence of the olfactory bulb area being involved and the absence of inflammatory reactions in the surrounding grey and white matter.

As well as causing meningo-encephalitis, Hartmannella/Acanthamoeba are thought responsible for acute respiratory tract infections, and there is a statistically significant correlation between the presence of amebae in the nose and a previous history of headaches, frequent colds and bleeding from the nose (Carter, 1972). There is also evidence of them causing pulmonary lesions in cattle (Culbertson, 1971), and multifocal necrohaemorrhagic foci in the heart, lungs, liver and pancreas of a dog (Ayers, Billips, & Garner, 1972).

1.6 Treatment

The results of treatment of the early cases where the amebic nature of the disease was not suspected using such traditional antibiotics as sulpha-drugs, penicillin, streptomycin, tetracyclines, and chloramphenicol, were, as could be expected, negative, due to the selective mode of action of these drugs for prokaryotic cells. Even in later cases where the antiprotozoal drugs emetine, chloroquine, and metronidazole were used, the course of the disease was not affected in the slightest (Carter, 1972). *In vitro* studies have since shown that none of the bacterial antibiotics traditionally used are effective against pathogenic *Naegleria* species

(Carter, 1969) and that emetine, though highly effective in vitro, does not protect animals from the disease, probably because it is unable to pass the blood-brain barrier and thus to the source of the infection.

To date, only the drugs, Amphotericin B and Clotrimazole (and possibly 5-Fluorocytosine) appear promising for use as chemotherapeutics (Carter, 1972; Cotter, 1973; Stevens & O'Dell, 1973b; Anderson & Jamieson, 1974). The mode of action of Amphotericin B depends on binding to the membrane sterols and is the only drug so far which has been successful in treatment of the disease (Carter, 1972). However, its clinical usefulness is lessened by the fact its mode of action is directed against eukaryotic cells, and, it is therefore very toxic to any eukaryotic cells at the high doses which are sometimes required in order to achieve recovery. Clinical trials on Clotrimazole have so far been only in vitro, although the required concentrations appear to be well within therapeutic limits (Anderson & Jamieson, 1974). The effect of inoculum size and activity of the two drugs is shown below.

Table III. Comparison of Amphotericin B and Clotrimazole in vitro (Adapted from Anderson and Jamieson, 1974)

Drug	Inoculum size amebae / cm^3	Minimum inhibitory concentration (MIC) μg / cm^3
Clotrimazole	7.3×10^2 2×10^4–10^5	0.15 0.125–0.25
Amphotericin B	1.1×10 1.1×10^2 1.1×10^4	0.06 0.125 0.25

Amphotericin B has been shown to have little effect on hartmannellids (MIC 767 $\mu g/cm^3$). This is thought due to possible differences in the composition of the cell membrane and the drugs of choice so far for hartmannellid infection are sulphadiazine and 5-Fluorocytosine. Sulphadiazine has

been shown to be prophylactic in mice at concentrations ranging between 0.2 - 0.8% of the body weight (Culbertson et al, 1965), while 5-Fluorocytosine has been shown to be prophylactic at 0.15 mg/g body weight (Stevens & O'Dell, 1973b). The possible use of 5-Fluorocytosine as a chemotherapeutic for Naegleria infections and the use of clotrimazole for hartmannellid infection, still remains to be investigated.

It has been suggested that for future treatment of Naegleria infections, a synergistic mixture of Amphotericin B and 5-Fluorocytosine be administered (Cotter, 1973). This would also have the added advantage that should the suspected infection prove to be hartmannellid meningo-encephalitis, proper chemotherapy has already begun. However, in any "suspected amebic meningo-encephalitis", it is strongly recommended that a combination of both anti-Naegleria and anti-hartmannellid drugs be administered as soon as possible.

2. Control Measures

The best prevention of PAM is obviously the abstinence of swimming in lakes, rivers, or swimming-pools where it is thought that the pathogenic amebae are present (Culbertson, 1971). Cerva (1971) states "the constant presence of numerous populations of the limax group cannot be prevented even under the strictest observations of all routine safety measures applied to water systems of swimming-pools. Thus, nowadays systems of bacteriological and chemical inspection of water in swimming-pools, appears to be ineffective". Support of this statement comes from the fact that these amebae are not affected by normally used doses of chlorine (Cerva, 1971; Anderson & Jamieson, 1972b; Brown, 1974) which still remains the most common disinfectant of water (Morris, 1966). Documentary evidence exists showing that 16 cases of the disease were contracted after swimming in an indoor, chlorinated swimming-pool (Cerva, 1971), swimming in a backyard pool filled from the mains supply, and playing 'submarines' in bath-water (Anderson & Jamieson, 1972b).

So far, both 0.75% salination (Anderson & Jamieson, 1972b) and the basic dyes brilliant- and malachite green (Cerva, 1973) have been shown to be amebicidal, but their potential use as 'disinfectants' in field conditions remains to be fully elucidated.

3. Aims of the Investigation

1. To critically review the current classification of these 'soil-amebae' in order to determine reliable common features in the classification schemes.
2. To examine existing and potential identification methods in the hope of providing a wide range of practicable methods for distinguishing between pathogenic and non-pathogenic species of Naegleria.
3. To examine the ability of these amebae to adapt to a range of environmental temperatures.
4. To reassess the controversial identification of the aetiological agent of 1968 N.Z. cases of PAM as Myxomycetes.
5. To examine the potential use of basic dyes and sodium chloride as possible alternative disinfectants to Cl_2.

CHAPTER TWO: MATERIALS

1. Ameba Cultures Used

Table IV. Ameba Cultures Used

Isolate	Origin	Indicted Pathogenicity	Source	Plate No.
Ng-27	Australia	-	NHI	1 + 2
Ng-1518	U.K.	-	NHI	
HB-1	U.S.A.	+	CCAP	
NHI	N.Z.	+	NHI	3
PA14	Australia	+	NHI	
NTH	Australia	+	NHI	
BL	N.Z.	+	Waikato Hospital	4
BH	N.Z.	+	Waikato Hospital	5
PL200f	N.Z.	+	NHI	
H. culbertsoni (A-1)	U.S.A.	+	CCAP	
A. castellani (Neff)	U.S.A.	-	HNI	

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

CCAP = Culture Centre of Algae and Protozoa, Cambridge, U.K.

Note: The hartmannellid strains A-1 and Neff were included in the present study for comparative and control reasons.

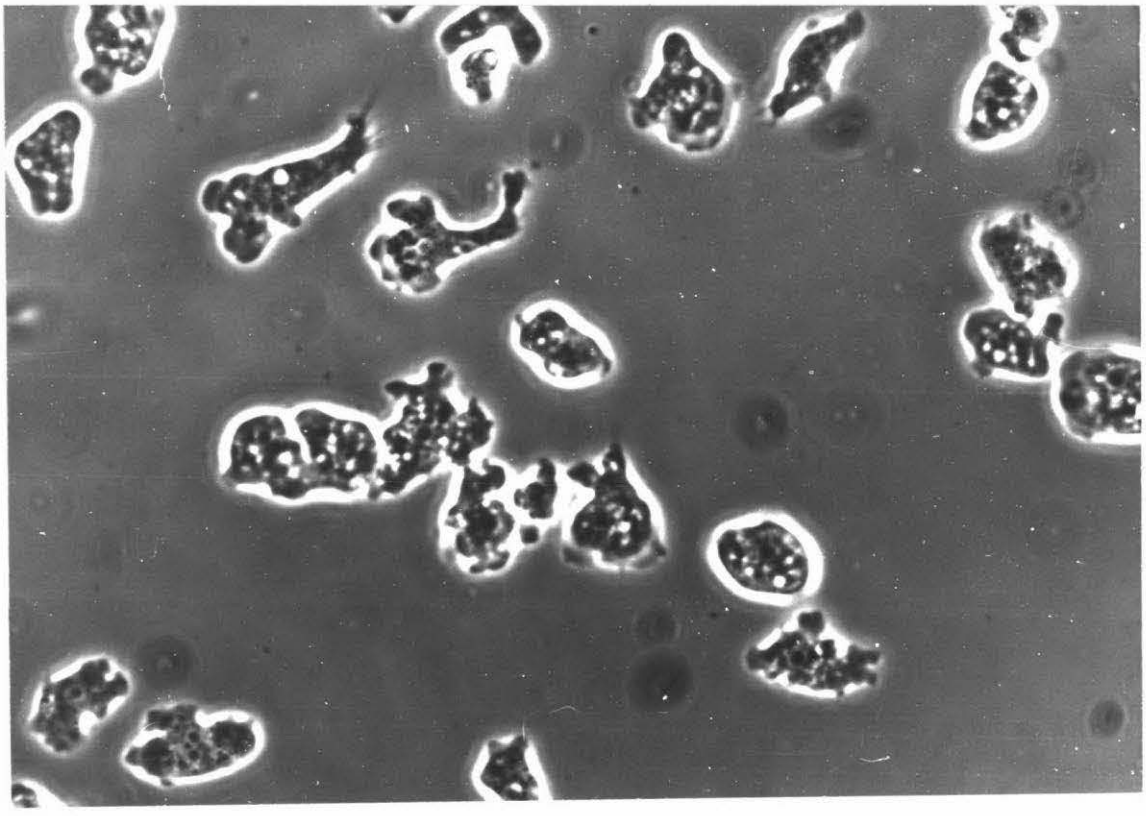


Plate 1. Trophozoite stage of N.gruberi, Ng-27 x 500

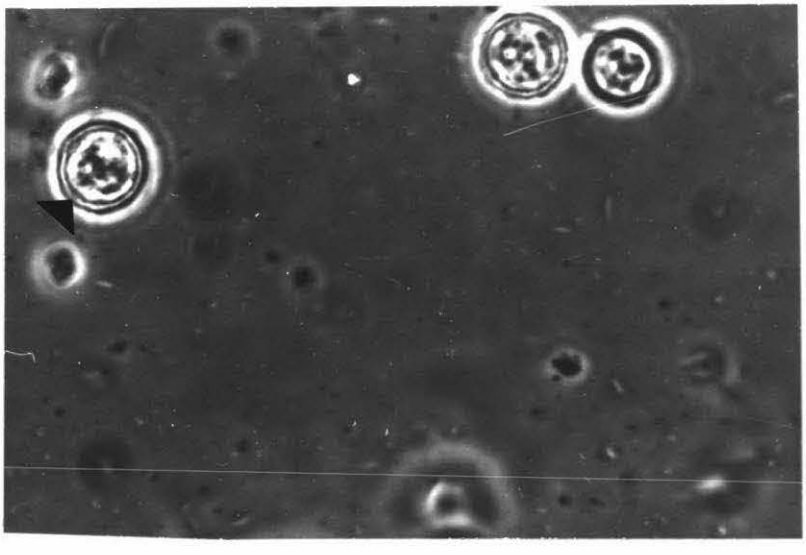


Plate 2. Cyst stage of N.gruberi, Ng-27 x 700.
Arrows indicate pores in the cysts.

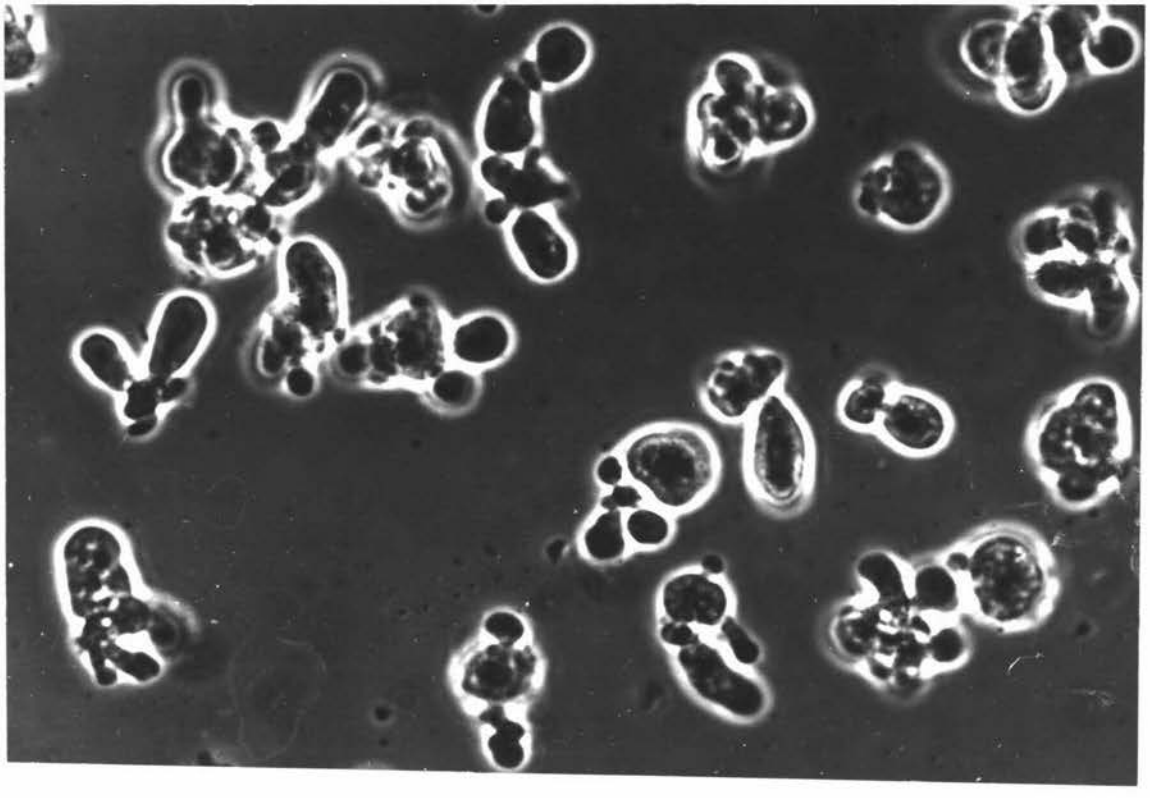


Plate 3. Trophozoite stage of N.fowleri, NHI x 500

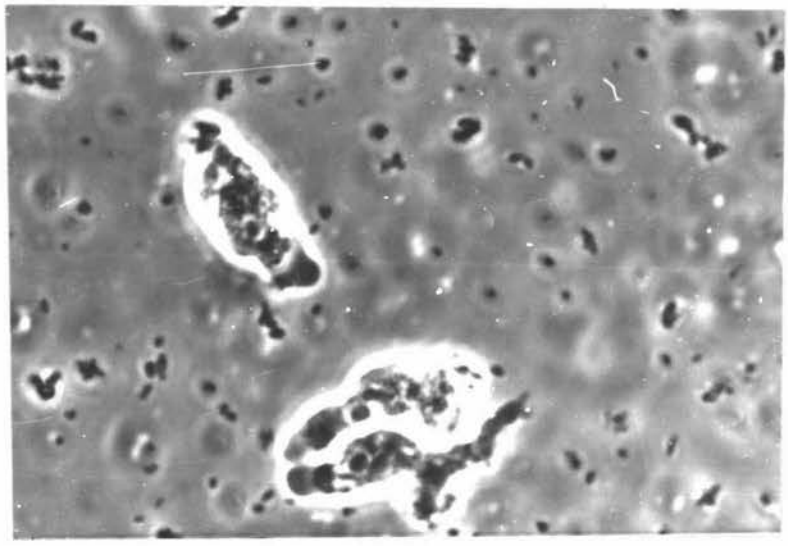


Plate 4. Trophozoite stage of BL x 600

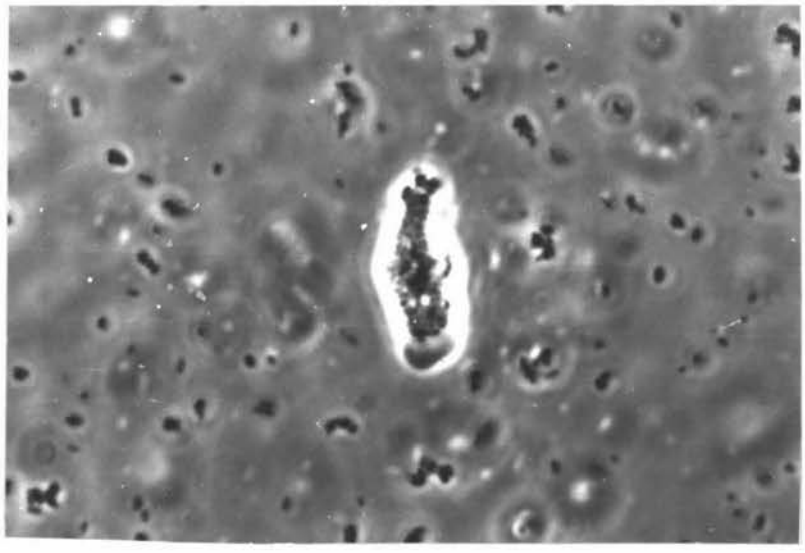


Plate 5. Trophozoite stage of BH x 600

2. Plate Media

NM agar (Fulton, C. 1970)

Difco Bacto-peptone = 2. dextrose = 2 g

K_2HPO_4 = 1.5 g KH_2PO_4 = 1 g

Davis agar = 20 Distilled water = 1 l

Autoclave at 15 psi (121°C) for 15 mins.

PM agar

As for NM agar except that 4 g of peptone is used.

BST agar (Chang, S.L. 1958)

Sucrose = 10 g

tryptose = 2g

PO_4 buffered water pH 7.4 - 7.5 = 1 l

Davis agar = 20 g

Autoclave at 15 psi (121°C) for 15 mins.

GSN agar (Griffin, J. 1972)

Stock = 10 g $CaCl_2$, 6 g KCl, 3 $MgSO_4.7H_2O$, 15 g NaCl

4 g anhyd. $CaHPO_4$

Distilled water to 1 l

Agar, 5 g BBL Trypticase, 5 g Difco yeast extract

1 l stock

Autoclave at 15 psi (121°C) for 15 mins.

Ameba-Saline (AS) agar (Page, 1967a)

NaCl = 0.12 g $MgSO_4.7H_2O$ = 0.004 g

Na_2HPO_40 = 0.142 g KH_2PO_4 = 0.136 g

Agar = 20 g $CaCl_2.2H_2O$ = 0.004 g

Distilled water = 1 l

Autoclave at 15 psi (121°C) for 15 mins

NS Ouchterlony - plates

Noble agar = 15 g

NaCl = 85 g

Trypan Blue (10 cm^3 of a 1% W/V aq. sol^n)

Distilled water = 1 l

Autoclave at 10 psi for 10 mins.

Borate-Saline (BS) Ouchterlony plates

Borate - Buffer (pH 8.4 - 8.5)

Boric acid = 6.184 g

Borax ($Na_2B_4O_7.10H_2O$) = 9.536 g Distilled water to 1 l

NaCl = 4.384 g

Borate-Saline sol^n

5 parts buffer with 95 parts saline

(saline = 8.5 g NaCl/l)

For plates - 10 g Noble Agar

980 cm^3 Borate-saline sol^n

10 cm^3 of a 1% sol^n of Methiolate in saline

Trypan Blue, 10% of a 1% aq. sol^n.

3. Axenic Media

Page's Ameba Saline (PAS) (Page, 1967a)

$NaCl$ = 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 — Distilled water = 1 l

pH 6.8 - 7.0

Autoclave 15 psi (121°C) for 15 mins.

Alsevers solution

Dextrose = 20.5 g — Na citrate.$2H_2O$ = 8 g

Citric acid = 0.55 g — $NaCl$ = 4.20 g

Distilled water = 1 l

Autoclave 10 psi for 15 mins.

Serum (calf, foetal calf, pig, horse)

Allow blood to clot at 37°C for 4 hrs.

Place at 5°C overnight for clot contraction.

Pour off serum from clot and spin down at 3000 rpm for 10 mins at 5°C.

Filter progressively through 5 µm, 0.5 µm & 0.22 µm filters.

Filter through a sterile 0.22 µm filter.

RBC Lysate (sheep or bovine blood)

Make up a 0.3% (W/V) blood/Alsevers sol^n.

Add blood/Alsevers sol^n to twice its vol. of distilled water.

Centrifuge at 36,000 g for 30 mins.

Filter through a 5.0 um, .45 µm & 0.22 µm filter.

Filter through a sterile 0.22 µm filter.

Bacterial Replacement of Serum (Stevens & O'Dell, 1973c)

Grow up an overnight culture of Escherichia coli or Enterobacter cloacae in BHI at 37°C on a reciprocal shaker.

Harvest cells 15,000 g for 10 mins, wash once in distilled water.

Resuspend cells in PAS to make a wet weight of 0.13% (wt/vol).

Autoclave at 15 psi for 10 mins.

CYM Medium (Stevens & O'Dell, 1973c)

Thiamine hydrochloride = 0.001 g d-Biotin = .002 g
Vit. B12 = 0.000001 g Difco casitone = 10 g
Difco yeast extract = 5 g Glucose = 10 g
1 l of PAS Methionine = 0.0895 g
pH 6.8 - 7.0. Autoclave at 15 psi (121°C) for 15 mins.
To this is added asceptically 1 cm^3 of the following sterile cocktail A medium to 9.0 cm^3 of CYM media
40 cm^3 sterile serum (pig, horse, calf)
20 cm^3 sterile RBC lysate
6 cm^3 of a sterile 2,500 ug/cm^3 Panmycin sol^n
34 cm^3 sterile distilled water.

TYG Medium (Stevens & O'Dell, 1973c)

BBL Trypticase = 5 g Difco yeast extract = 5 g
Glucose = 10 g Thiamine hydrochloride = 1.0 mg
d-biotin = 0.2 mg Vit. B12 = 1.0 ug
PAS = 1 l pH 6.8 - 7.0
Autoclave at 15 psi for 15 mins.
To this is added asceptically 0.1 cm^3 of the bacterial lysate (or 1 cm^3 of serum)/8.0 cm^3 of TYG media containing 1.0 cm^3 of antibiotic (150 ug/cm^3 of Panmycin or 2000 ug Penicillin and 1000 units of Streptomycin).

MYAS Medium (De Jonckheere, et al., 1974)

Difco malt extract = 0.1 g Difco yeast extract = 0.1 g
PAS = 1 l pH 6.8 - 7.0
Autoclave at 121°C for 15 mins.
To this is added asceptically 1.0 cm^3 of serum to 8 cm^3 MYAS media containing 1 cm^3 of antibiotic.

Fultons Medium A (Fulton, C., 1970)

HL-5 = Distilled water 650 cm^3 Difco protease-peptone = 10 g
Difco yeast extract = 5 g Autoclave at 15 psi for 15 min.
Dissolve; distribute in 65 cm^3 bottles and when cool add asceptically: 2 cm^3 sterile PO_4; 2 cm^3 sterile DEX.
PO_4 - KH_2PO_4 = 3.4 g 100 cm^3 Distilled water
$Na_2HPO_4.7H_2O$ = 6.7 g Autoclave at 15 psi for 15 mins
DEX - Distilled water = 50 cm^3, Dextrose = 13.5 g
Autoclave at 10 psi for 20 mins.

Mix asceptically 8% (V/V) HL5 foetal calf serum 1 cm^3 .5 cm^3 penicillin (2000 units/cm^3, .5 cm^3 streptomycin (1000 units/cm^3.

Peptone-Neff medium (Stevens & O'Dell, 1973a)

Difco proteose-peptone = 40 g Difco yeast extract = 7.5 g

Glucose = 15 g Vit. B1 hydrochloride = 1.0 mg

Biotin = 0.2 mg Vit. B12 = 1.0 ug

+ 1 l of the following Ac ion sol^n

Ac ion sol^n:

$MgSO_4$ (M.W. 246.50) = 0.2465 g

$CaCl_2$ (M.W. 219.08) = 0.01095 g

KH_2PO_4 (M.W. 136.09) = 0.27218 g

Ferric citrate (M.W. 335.02) = 0.0335 g

Autoclave at 15 psi (121°C) for 10 mins.

To 9.0 cm^3 of peptone-Neff media is added asceptically 1.0 cm^3 of antibiotic.

2% Casitone sol^n

Difco Bacto-Casitone = 20 g PAS = 1 l

Autoclave at 15 psi for 15 mins.

CPN Medium

2% Casitone sol^n = 500 cm^3 peptone-Neff = 500 cm^3

Autoclave at 15 psi for 15 mins.

4. Cell Culture Media

4.1 Vero Cell Culture

4.4% Bicarbonate solution

$NaHCO_3$ = 22 g 12.5 of a 0.4% (W/V) Phenol red sol^n

Distilled water up to 500 cm^3

Autoclave at 15 psi (121°C) for 15 mins.

10 x Trypsin/Versene Mixture

NaCl = 80 g KCl = 4.0 g

2.0 cm^3 of a 1% phenol red sol^n Glucose = 10.0 g

Versene = 2.0 cm^3 Trypsin = 5.0 g

Distilled water up to 1 l

Filter sterilize through an 0.22 μm filter.

Antibiotics

10^6 units of Streptomycin } 20 cm^3 of sterile Distilled

10^6 units of Penicillin } water

Eagles Growth Medium (EGM) (Eagle, 1955)

Eagles = 380 cm^3 20 cm^3 4.4% Bicarbonate

1 cm^3 of antibiotic 40 cm^3 (10%) serum (calf or pig)

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

Eagles Maintenance Medium (EMM)

As above but with only 1% serum added.

Eagles Washing Medium (EWM)

As for growth medium but no added serum.

4.2 Chick Embryo Kidney Cell Culture (Green, 1974)

PO_4-Buffered saline PBS (pH 7.5)

Sol^n A

NaCl = 8 g KCl = 0.2 g $Na_2HPO_4.12H_2O$ = 2.9 g

KH_2PO_4 = 0.2 g Distilled water = 800 cm^3

Sol^n B

$CaCl_2.6H_2O$ = 0.19 g Distilled water = 100 cm^3

Sol^n C

$MgCl_2.6H_2O$ = 0.1 g Distilled water = 100 cm^3

1 l of PBS = 800 cm^3 sol^n A, 100 cm^3 sol^n B, 100 cm^3 sol^n C.

Trypsin Sol^{n} (0.1) %

Trypsin = 1 gm PBS = 1 l

Filter sterilize through 0.22 μ filter.

Earles Working sol^{n} (LaE)

Earles stock sol^{n} = 500 cm^3 Earle, 1943

Distilled water = 4,500 cm^3

To 500 cm^3 of the working sol^{n} is added 25 g of lact-albumin hydrolysate, the remaining 4 l being dispensed in 360 cm^3 amounts. All medium is autoclaved at 15 psi for 15 mins. When cool 40 cm^3 of the lactalbumin hydrolysate sol^{n} is added to each 360 cm^3 amount of Earles working sol^{n}. Final sol^{n} called LaE.

Earles Maintenance Medium EaM

Sterile LaE = 400 cm^3 4.4% bicarb. sol^{n} = 20 cm^3

Foetal calf serum = 10 cm^3

1 cm^3 of penicillin + streptomycin to give a final conc. of 100 units/cm^3 of each.

Earles Based Diluent (SD)

LaE = 400 cm^3 Foetal calf serum = 10 cm^3

Antibiotic (P/S) = 1 cm^3

Earles Based Washing sol^{n}

LaE = 400 cm^3 Antibiotics (P/S) = 1 cm^3

Hanks Working sol^{n} LaH (Hanks, 1948)

Sol^{n} A = 250 cm^3 Sol^{n} B = 250 cm^3

Distilled water = 4,500 cm^3

To 500 cm^3 of the working sol^{n} is added 25 g of lact-albumin hydrolysate, the remainder being dispensed in 360 cm^3 aliquots. Autoclave at 15 psi for 15 mins and add 40 cm^3 of the lactalbumin hydrolysate sol^{n} to each 360 cm^3 aliquot.

1.4% Bicarbonate Buffer

$NaHCO_3$ = 7 g 0.4% phenol red sol^{n} = 12.5 cm^3

Distilled water = 500 cm^3 Autoclave at 15 psi for 15 mins.

Tryptose Phosphate Broth

Tryptose = 20 g Glucose = 2 g NaCl = 5 g

Na_2HPO_4 = 2.5 g Distilled water = 1 l

Autoclave at 15 psi for 15 mins.

Hanks' Growth Medium (HTC)

LaH = 400 cm^3 1.4% Bicarb. = 20 cm^3 Calf serum = 50 cm^3

Tryptose phosphate broth = 50 cm^3

Antibiotics (P/S) = 1 cm^3

All sterile components are added asceptically.

5. Miscellaneous Solutions

0.25% Formal Saline

Formalin = 0.25 cm^3

Physiological saline = 99.75 cm^3

Phosphate Buffered Saline (PBS)

NaCl = 8.5 g Na_2HPO_4 = 1.28 g

$NaH_2PO_4.2H_2O$ = 0.156 g Distilled water to 1 l

pH 7.6

Mounting Fluid

$NaHCO_3$ = 0.0715 g Na_2CO_3 = 0.016 g

Distilled water = 10 cm^3 Glycerol to 100 cm^3

CHAPTER THREE: METHODS

1. Sterilization

All glassware, except that used for analytical work, was sterilized at 121°C at 15 psi for 15 mins.

Analytical glassware was sterilized by dry-heat for a minimum of 160°C for 2 hrs.

2. Culture Techniques

2.1 Cloning. All cultures were cloned 3 times by the plaque method.

1) The inoculum was diluted out to the required cell-conc. using sterile distilled water or PAS.
2) 0.5 cm^3 of the diluted inoculum was then pipetted onto NM agar.
3) 3 drops of an overnight broth of Escherichia coli or Enterobacter cloacae were then added.
4) The mixture was then spread over the plate using a flame-sterilized glass spreader.
5) The plates were then incubated at the required temperature in a moistened incubator.

Note: Before use the agar plates were dried at 37°C overnight to remove excess moisture. They were then stored at 5°C, sealed in plastic bags till required.

2.2 Axenizing

1) Cloned amebae were grown up on AS agar using E. coli as the lawn.
2) The amebic-lawn was then washed off with PAS.
3) The amebic-suspension was then added into TYG medium (if Naegleria) or into CPN medium (if Hartmannella/Acanthamoeba) and incubated at the required temperature for 24 hrs.
4) The culture was then subbed into fresh media and if possible the bacterial lysate was replaced by serum.

2.3 Flagellation

1) Either by the hanging-drop method using PAS at 37°C OR
2) Plates were flooded with PAS and then incubated for 2-4 hrs. at 37°C.

3. Serology

3.1 Preparation of Antigens

1) Axenic cultures were spun down at 2000 rpm for 10 mins.
2) The amebic-pellet was then washed twice in PAS to remove any possible antigens to the media.
3) The washed amebae were then resuspended in PAS to give an approximate concentration of 10^6 cells/cm^3 and injected according to the following schedule.

Immunization schedule (Table V)

1) Rabbits were bled 1 week before the start of immunization to obtain pre-immune serum.
2) 0.5 cm^3 of a 50:50 antigen-complete Freunds adjuvant mixture was then injected intramuscularly into each hind leg, 7 days after the initial bleeding.
3) One week later 1.0 cm^3 of a 1:1 antigen-complete Freunds adjuvant mixture was injected intravenously into the right ear.
4) The rabbits were then bled 30 days after the initial bleeding, and on the 33rd day, another 1.0 cm^3 of antigen was administered intravenously.
5) The rabbits were then bled at weekly intervals and were given a 1.0 cm^3 booster of antigen every 6 weeks.

Table V. Immunization Schedule

Day	Procedure
1	Bleed for preimmune sera. Then inject 0.5 cm^3 of a 1:1 antigen - Freunds Complete Adjuvant (Difco) I.M. into each hind leg.
8	0.5 cm^3 of antigen into each hind leg I.M.
15	1.0 cm^3 of a 1:1 antigen - Freunds Complete Adjuvant I.V. in the ear.
30	Bleed.
33	1.0 cm^3 of antigen I.V. in the ear.
40	Bleed and then bleed weekly.
	Every 6 weeks give 1.0 cm^3 of antigen I.V. as a booster.

I.M. = intramuscularly; I.V. = intravenously

3.2 Preparation of Antisera

1) Approximately 1 universal of blood was collected by exsanguination via the marginal vein.
2) The blood was allowed to clot at 37°C for 4 hrs. and then placed at 5°C overnight for clot contraction.
3) The serum was then poured off and spun down at 3000 rpm for 10 mins. at 5°C and then stored at -20°C.

3.3 Agglutination (Anderson & Jamieson, 1972a)

1) Antigens were prepared as outlined for immunization, but resuspended in 0.25% formal-saline.
2) The antiserum and controls were then diluted in 1:2 aliquots with physiological saline.
3) Next, the same volume of amebic suspension, i.e. antigen, was added to each dilution of antiserum
4) After mixing, the trays were incubated at 30°C for 30 min. and then examined under a stereomicroscope (50% agglutination was shown by clumps of 10 or more amebae).

CONTROLS: 1) preimmune - serum
2) media
3) Hartmannella culbertsoni and Acanthamoeba castellanii.

3.4 Indirect Fluorescent Antibody

1) Antigens were prepared as for immunization.
2) One drop of washed amebae was then placed on acid-washed slides and air-dried.
3) The smears were then fixed in cold acetone for 10 mins.
4) PBS (pH 7.6) diluted antiserum was then pipetted onto the smear and the slides were then placed in a humidity chamber at 37°C for 30 mins.
5) The antisera was then washed off with pH 7.6 PBS and the washing was continued for 30 mins. with slight agitation.
6) Commercial fluorescent anti-rabbit serum (Wellcome) was then added and the slides incubated in humidity chambers for another 30 mins.

7) The slides were then washed with two changes of pH 7.6 PBS for 45 mins. then mounted in pH 8-9 mounting fluid and then observed under darkfield illumination using an Olympic (FLM) Fluorescent microscope.

CONTROLS: 1) preimmune serum
2) media

3.5 Gel-Diffusion (Ouchterlony)

1) Antigens were prepared as for immunization except that they were concentrated in a minimum amount of distilled water.
2) The antigens were then freeze-thawed twice and then lysed again at 12 tons/sq. inch in a chilled French-Press (this was found to be preferable to sonication).
3) The lysate was then centrifuged at 10,000 rpm for 10 mins. at 5°C and the supernatant poured off and frozen at -20°C till required.
4) Before use, 0.25% of a 10% Sodium Dodecyl Sulphate solution (SDS) was added to the antigen and the mixture incubated at 37°C for 30 mins.
5) Samples were then added to precut 5 mm wells of NS agar plates and then placed in a 37°C humidified chamber and observed over a period of 3-5 days for the development of precipitin lines.

CONTROLS: 1) media
2) SDS

Note: It is of the utmost importance to use properly washed antigens when making antiserum, to ensure that no cross-reacting antibodies to the medium occur, as did happen with the gel-precipitin plates. Care must also be taken to exclude cysts which give a non-specific reaction. This may be due to the fact that, for the case of H.culbertsoni anyway, cyst antigens differ from trophozoite antigens (Raizada, Saxene, Murti, 1972).

4. Mouse Pathogenicity

Young Swiss-White mice (weighing 10-16 g) were anaesthetized by injecting intraperitoneally 0.15 cm^3 of a 0.1% (V/V) Nembutal solution, using physiological saline as the diluent, into them.

Next 3-4 drops (0.03-0.05 cm^3), of the amebic suspension were inoculated intranasally into the mice. The mice were then observed over a period of 14 days for subsequent deaths.

for 30 mins. at 37°C.

3) The cells were then centrifuged at 80 g for 5 mins. in graduated conical glass centrifuge tubes and resuspended in 10.0 cm^3 of HTC growth medium.

4) The cells were next recentrifuged at 80 g for 5 mins., the supernatant removed and the packed cell volume (PCV) read off. The cells were then resuspended in 500X's their volume of fresh HTC.

5) The cell-suspension was then dispersed in the following aliquots:

   10.0 cm^3 tubes = 0.7 cm^3 petri dishes = 5.0 cm^3

   250 cm^3 bottles = 15.0 cm^3

6) Tubes were placed in a roller drum and left stationary for 24-48 hrs, then rolled. The monolayers were formed about 2 days after seeding and at this stage, HTC growth medium was replaced by EaM using the following volumes:

   tubes = 1.5 cm^3 petri dishes = 5.0 cm^3

   250 cm^3 bottles = 20.0 cm^3

6. Disinfection

6.1 Disinfection Tests - salt, brilliant green, malachite green

3 day old axenic cultures were asceptically inoculated into axenic CYM containing the disinfectant and incubated for 3 days. 3 different samples were then counted on a modified Fuchs-Rosenthal counting chamber for the total count. A fourth sample was then plaqued out on NM agar for a viable count.

6.2 Effect of Different Cell Numbers on the Chlorination Level

1) 3 day old axenic cultures were centrifuged down at 2000 rpm for 10 mins. and washed 3 X's with Cl-free pH 7.5 buffered glass distilled water (BGDW).
2) The trophozoites were then resuspended in pH 7.5 BGDW to give a final concentration of 2.0×10^5 cells/cm^3.
3) Flasks were then inoculated with the required cell concentration and incubated for the required interval of time before being assayed for the Total Available Chlorine content, and tested for viability by being plaqued out on NM agar.

6.3 Effect of a Maintained Chlorination Level on a Known Concentration of Amebae

Cells were washed as above and then at the required time, were analysed for free available chlorine (FAC), combined available chlorine (CAL) and total available chlorine (TAC), as well as having additional chlorine added to the flasks.

6.4 Analysis of Chlorine

Chlorine was analysed by the DPD method of Palin (1957). The only alterations were that instead of using 1.5 g of p-amino-N:N-diethylaniline sulphate, 3.0 g was used, and instead of using 8.0 cm^3 of 1+3 H_2SO_4, 8.0 cm^3 of 2+3 H_2SO_4 was used.

The amebae were removed from the fluid by filtering through a 5.0 μm millipore filter.

7. Staining

7.1 The Feulgen Reaction : (Page, F.C., 1967a)

7.2 Iron-Haematoxylin : (Singh, B.N., 1952)

7.3 Trichome : (Adam, Paul & Zaman, 1971)

CHAPTER FOUR: RESULTS

1. Classification and Identification of Naegleria and Hartmannella/ Acanthamoeba Isolates

1.1 Nuclear Division

Nuclear division of the isolates was studied by both the Feulgen reaction (Page, 1967a) and iron-haematoxylin staining (Singh, 1952). Some difficulty was experienced in getting the amebae in the right stages of promitosis so that the polar masses and interzonal bodies could be observed. The best preparations were those stained by the iron-haematoxylin method, since the Feulgen-negative polar masses and interzonal bodies were very hard to differentiate when stained by the Feulgen reaction.

Table VI. Comparison of Nuclear Division

Isolate	Possession of a flagellate stage	Type of mitosis exhibited	Division in the flagellate stage	Polar masses + interzonal bodies	Illustration
Ng-27	+	Promitosis	-	+	Fig. 2-14
Ng-1518	+	"	-	+	
BH	+	"	-	+	
BL	+	"	-	+	
HB-1	+	"	-	+	
NTH	+	"	-	+	
NHI	+	"	-	+	
PA14	+	"	-	+	
A-1	-	Normal mitosis	ND	-	Fig. 15-27
Neff	-	"	ND	-	

ND = Not Done

Promitosis: "amoeboid movement and the nuclear membrane persist until late telophase, and the nucleolus does not disappear but divides into two polar masses."

Table VI shows that all Naegleria isolates and strains BH and BL possessed interzonal bodies, a nondividing flagellate stage and exhibited promitosis. Conversely, Hartmannella/Acanthamoeba isolates underwent normal mitosis with the production of an equatorial plate, and did not possess a flagellate stage.

Figures 2 - 14: Promitosis of Naegleria (from Singh & Das, 1970)

Prophase

The amebae do not round off during division. Nuclear division begins with swelling of the nucleus and elongation of the nucleolus. The chromatin granules lying beside the nucleolus begin to fuse and the nucleolus assumes a dumbell shape and divides into two halves, the polar masses (Fig. 3).

Metaphase

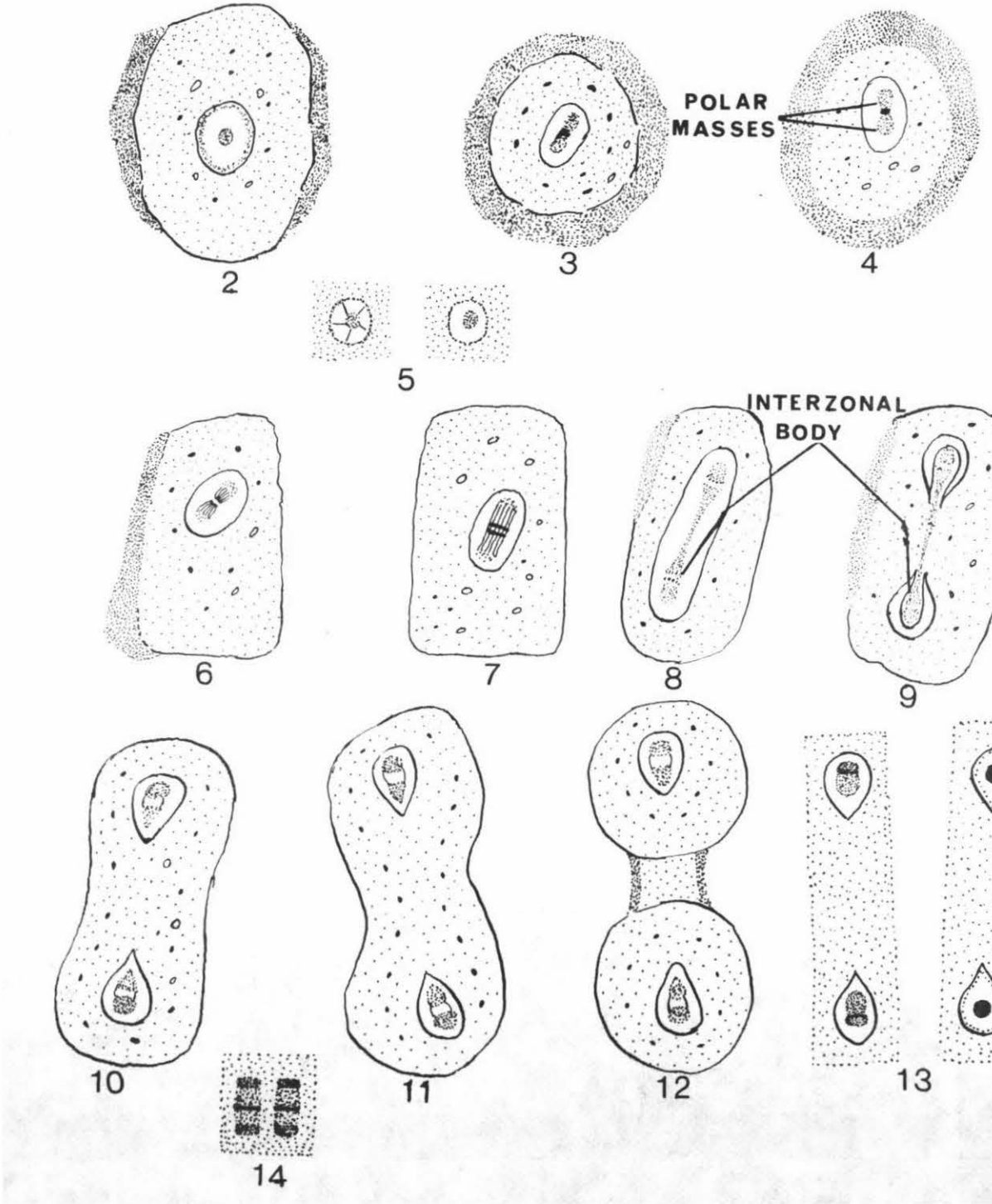
Following formation of the polar masses, a solid mass of chromatic material, without distinguishable chromosomes, occupies the position of the equatorial plate (Fig. 4).

Anaphase

The band of chromatic material divides into two and each half moves towards its pole (Fig. 6). When the chromatic material has reached its pole, the Feulgen negative, granular interzonal body can be seen lying half-way between the polar masses (Figs. 7 & 8). The interzonal body then increases in size and divides in two. The nuclear membrane persists during division becoming elongated and ultimately constricts to give rise to two daughter nuclei (Fig. 9).

Telophase

After the nucleus has divided in two, the ameba elongates and constricts to form two daughter individuals. During this process the thread-like structure joining the two interzonal bodies is ruptured, and each interzonal body fuses with its polar mass forming the nucleolus (Figs. 10 - 13). In a few amebae two nuclei divide at the same time (Fig. 14).


POLAR
MASSES
INTERZONAL
BODY
2
3
4
5
6
7
8
9
10
11
12
13
14

Figures 15 - 27: Mitosis of Hartmannella (from Singh & Das, 1970)

Prophase

The nucleolus fragments forming chromatin granules which fuse, and the nucleolus gradually disappears (Fig. 16).

Metaphase

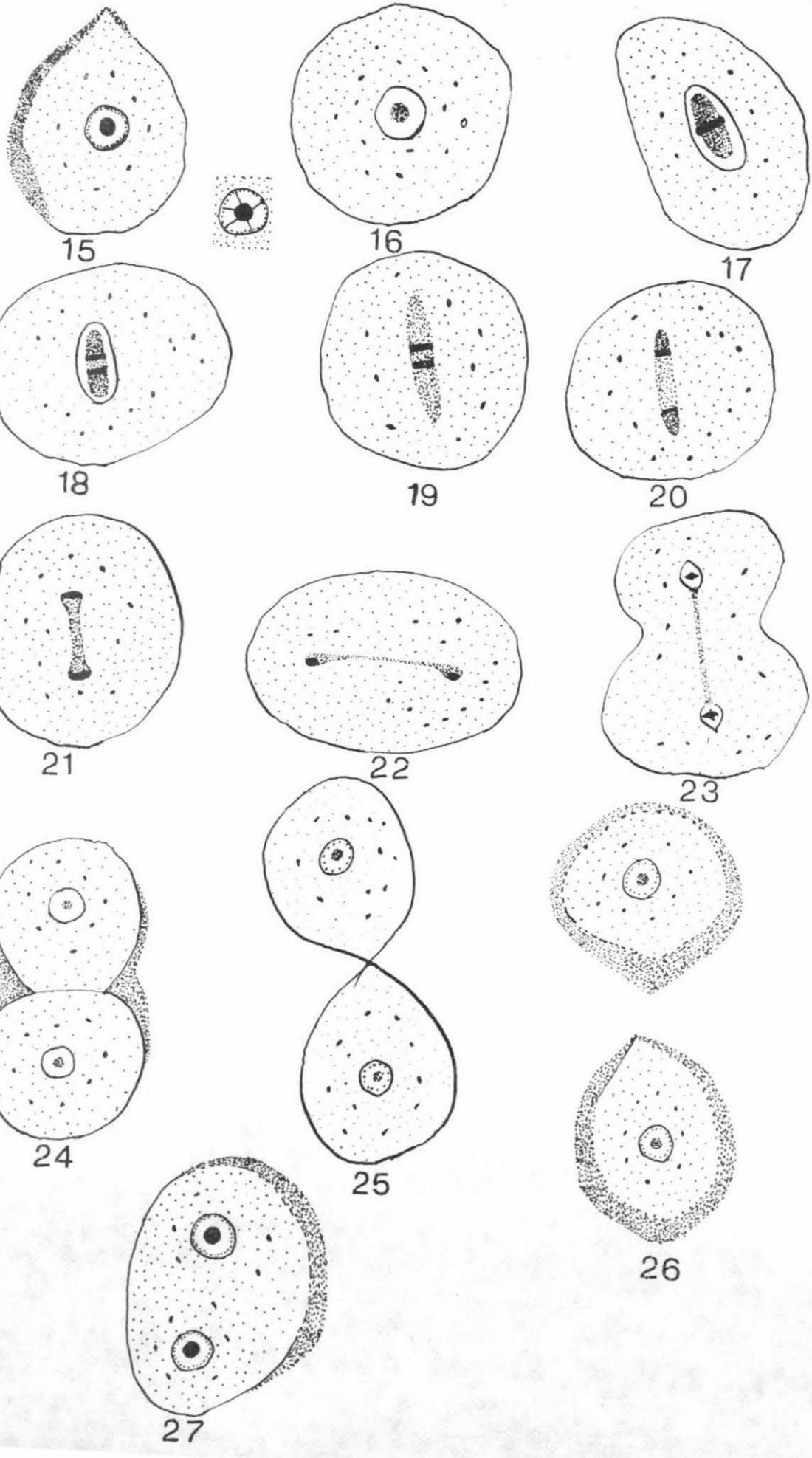
The fused chromatin granules form a solid band at the equatorial plate (Fig. 17) and then divide into two (Fig. 18).

Anaphase

The nuclear membrane disappears and the globular spindle gradually elongates (Figs. 19 & 20). When the chromosome masses reach the poles they are connected by a thread-like structure constricted in the middle (Figs. 21 & 22).

Telophase

The elongated ameba constricts giving rise to two daughter cells (Figs. 23 - 26). Nuclear membranes appear around each chromatin mass (Figs. 23 & 24); these masses fragment into granules, and a nucleolus is gradually formed.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1.2 Plaque Morphology

Plaques were produced on bacterial lawns, as outlined in the Methods, and were used as the basis for clones. Their particular morphologies and plaquing characteristics were recorded as possible differentiation features.

Table VII. The Comparison of Plaque Characteristics

Isolate	Agar Plate	Bacterial lawn	Incubation temperature	Plaquing time	Plaque size diameter	Plate
Ng-27	NM or AS	live *E. coli* or *E. cloacae*	30°C-37°C	36-48 hr	1.5-3.0cm	6
Ng-1518	"	"	30°C	"	"	
BH	"	"	30°C	"	"	
BL	"	"	30°C	"	"	
HB-1	"	"	37°C	48+ hr	up to 1.5cm	7+8 9
NTH	"	"	"	"	"	
NHI	"	"	"	"	"	
PA14	"	"	"	"	"	
A-1	"	"	"	"	"	
Neff	"	"	30°C	"	"	

All isolates formed plaques with either live *E. coli* or *E. cloacae* as the bacterial lawn when incubated at the appropriate temperature. Although strain Ng-27 grew well at 37°C, the remaining non-pathogenic Naegleria strains BH and BL, and the hartmannellid strain Neff, preferred a lower temperature of 30°C. Further, all the non-pathogenic Naegleria, plus strains BH and BL formed relatively larger plaques in a shorter incubation time, than did the other pathogens (Plates 6 - 9).

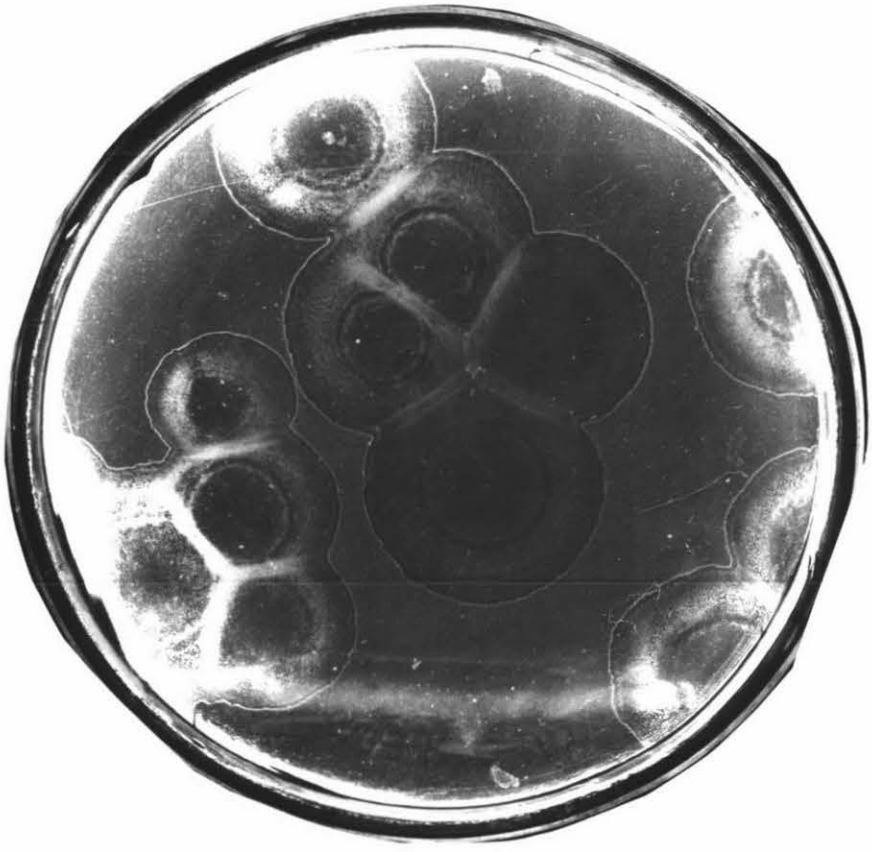


Plate 6. Plaque morphology of N.gruberi, Ng-27 grown on NM agar with E.coli.
Note large size of plaques compared with those on Plates 7 - 9.

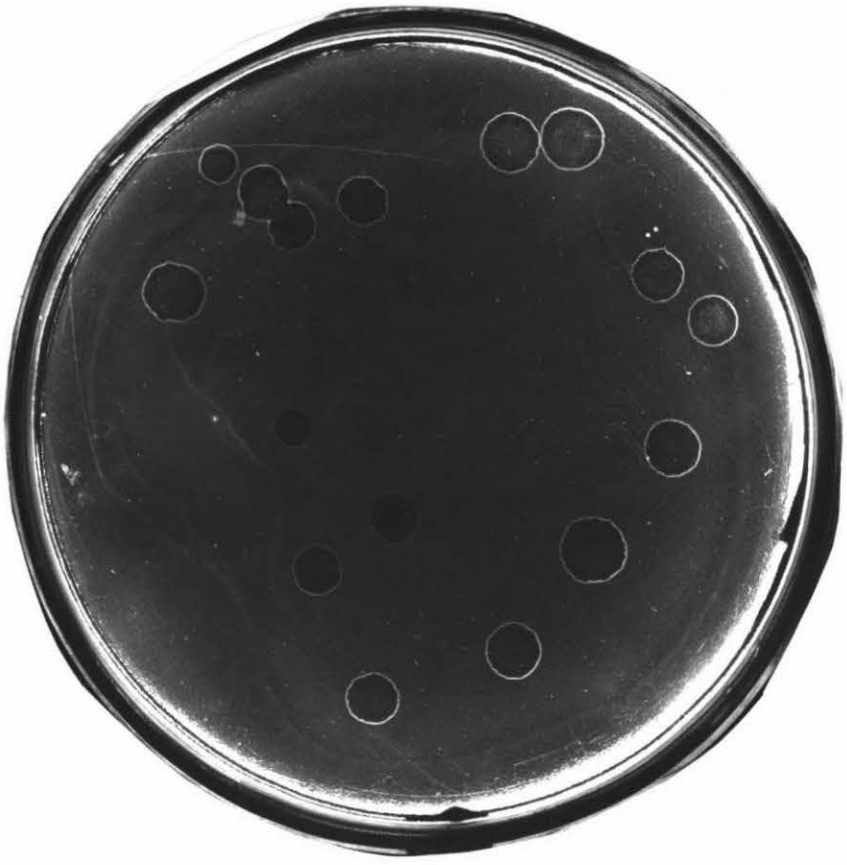


Plate 7. Plaque morphology of N.fowleri, NHI grown on NM agar with E.coli.

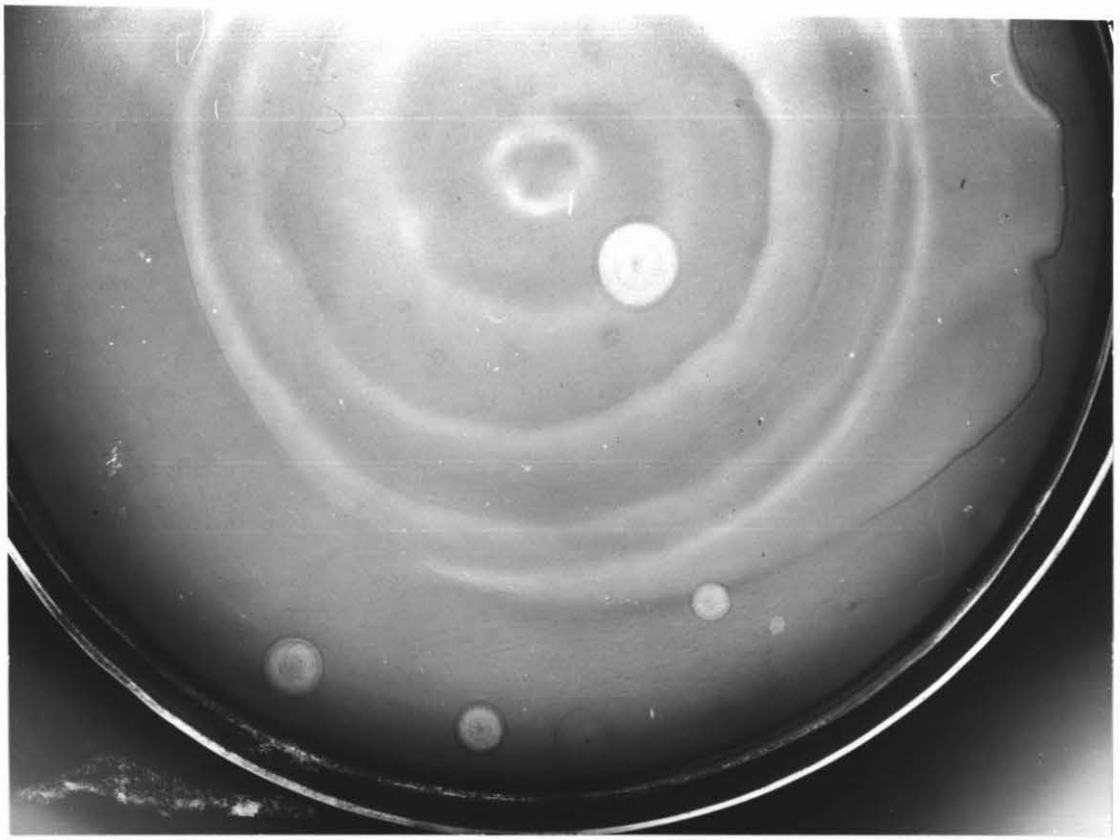


Plate 8. Centred-plaque morphology of N.fowleri, NHI grown on NM agar with E.cloacae.

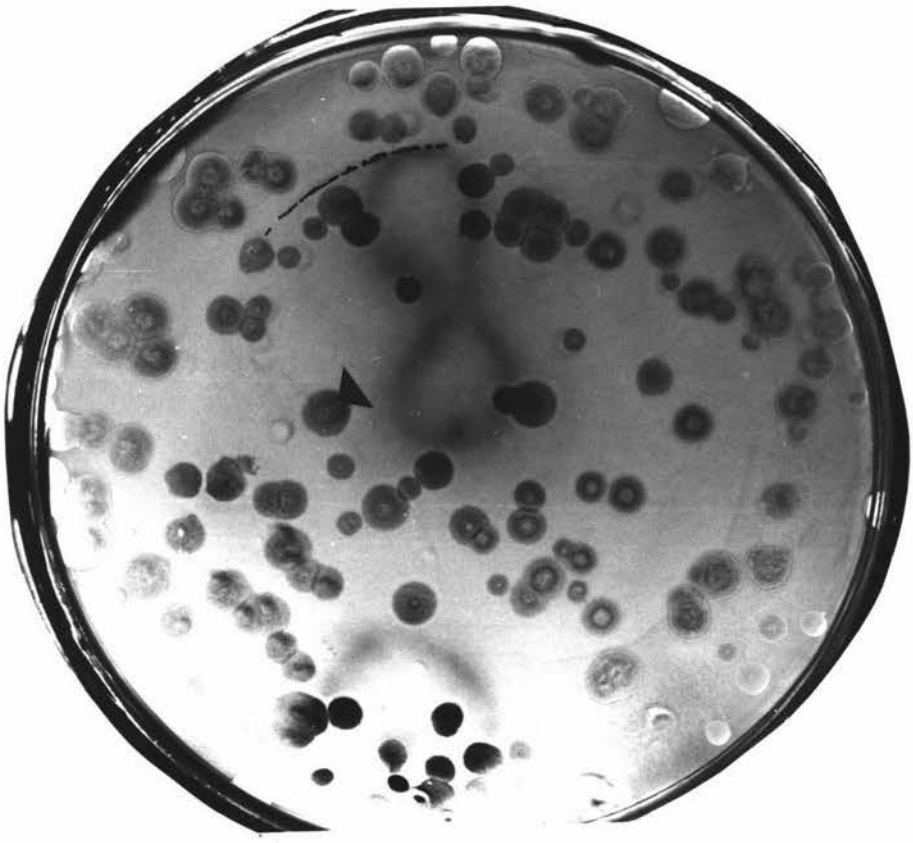


Plate 9. Plaque morphology of N.fowleri, PA14 grown on NM agar with E.cloacae. Note the relatively smaller size as compared to Plate 6 and the centred-plaques.

1.3 Diagnostic Physiological Characters

The possession of unique, simple physiological characters by the pathogenic N. fowleri only is of beneficial use for the differential culturing and preliminary identification of N. fowleri in environmental samples. The following table shows the result of three such tests (tolerance to 1% (wt/vol) NaCl in agar, growth at 45°C and flagellate transformation at 43°C) which can be used to selectively differentiate between N. fowleri and N. gruberi.

Table VIII. Differential Diagnostic Characters of Naegleria

	Isolate	Tolerance of 1% NaCl in agar	Growth at 45° C		Flagellation at 43°C	Controls
Non-pathogens	Ng-27	+	Plates axenic	-	-	Plates 30°C + Flagellation 30°C +
	Ng-1518	+	"	-	-	
	BH	+	"	-	-	
	BL	+	"	-	-	
	Neff	ND	ND		ND	ND
Pathogens	HB-1	-	Plates axenic	+	+	Plates 37°C + Flagellation 37°C +
	NTH	-	"	+	+	
	NHI	-	"	+	+	
	PA14	-	"	+	+	
	A-1	+	"	-	-	Plates 37°C +

ND = Not Done

Only the non-pathogenic Naegleria, strains BH and BL, and the hartmannellid strain A-1 could tolerate 1% (wt/vol) NaCl incorporated into agar. Conversely, the remaining pathogenic Naegleria showed growth at 45°C and flagellation at 43°C. All controls gave a positive result.

1.4 Serology

The use of serological methods as diagnostic tools in the identification of the aetiological agents of PAM has been necessitated from both the clinical and public health viewpoint. The following three serological techniques permit the rapid differentiation of *N. fowleri* from *N. gruberi* as well as indicating to some extent, the taxonomic relationship between the two species.

1.4.1 Agglutination

Table IX. Agglutination Titres

Antisera	Antigen: Ng-27	Ng-1518	BH	BL	HB-1	NTH	NHI	PA14	A-1	Neff
HB-1	1:8	ND	1:8	1:8	1:256-1:512	1:256-1:512	1:256-1:512	1:256-1:512	-	-
Ng-27 Adsorbed HB-1	-	ND	-	-	"	"	"	"	ND	ND
NTH	1:8	ND	1:8	1:8	"	"	"	"	ND	ND
Ng-27 Adsorbed NTH	-	ND	-	-	"	"	"	"	ND	ND
NHI	1:8	1:8	1:8	1:8	"	"	"	"	-	ND
Ng-27 Adsorbed NHI	-	-	-	-	"	"	"	"	-	ND
PA14	1:8	ND	1:8	1:8	"	"	"	"	ND	ND
Ng-27 Adsorbed NHI	-	ND	-	-	"	"	"	"	ND	ND
Pre-immune sera control	-	-	-	-	-	-	-	-	-	ND
Media control	-	-	-	-	-	-	-	-	-	ND

ND = Not Done

As shown in Table IX, all the pathogens, with the exception of the Waikato strains BH and BL, agglutinated to a titre of 1:256 - 1:512 when using antisera prepared against either NHI, HB-1, PA14 or NTH. Using the same antisera, the non-pathogens and strains BH and BL agglutinated to a titre of 1:8. Further, using Ng-27 adsorbed anti-N. fowleri serum, the pathogens still agglutinated to a titre of 1:256 - 1:512. No cross-reaction was noted between the hartmannellid and Naegleria isolates suggesting the absence of common group surface antigens. However, cross-reactions were observed between the anti-N. fowleri sera and the red blood cell lysate of the media, due to incomplete washing of the antigens when preparing the antisera. This cross reaction was removed by precipitation of the antibodies by the addition of RBC to the antisera and then centrifugation at 10,000 rpm for 10 mins. at 5°C. Both controls subsequently then gave a negative result.

1.4.2 Indirect Fluorescent Antibody Titres (IFAB)

The IFAB test was carried out as set out in the Methods, the working dilution of the commercial fluorescein labelled anti-rabbit serum being 1:7.

Table X. IFAB Titres

	Antisera						
Antigen	NHI	Ng-27 adsorbed NHI	HB-1	NTH	PA14	Pre-immune serum	Media
Ng-27	1:64	-				-	-
Ng-1518	ND	ND	ND	ND	ND	ND	ND
BH	1:32					-	-
BL	1:32-1:64					-	-
HB-1	ND		1:1024			-	-
NTH	ND			1:1024		-	-
NHI	ND	1:1024				-	-
PA14	ND				1:1024	-	-
A-1	ND					ND	
Neff	ND					ND	

ND = Not Done

The results show that cross-reactions were observed between anti-*N. fowleri* serum, the free-living *N. gruberi* as well as strains BH and BL at low dilutions, but not at high dilutions. A similar result was also recorded using agglutination. Within experimental limits, the end-point of the IFAB titre closely paralleled that of the agglutination titre. The end titre of the pathogens was not changed using Ng-27 adsorbed anti-NHI serum. Both controls were negative.

1.4.3 Gel Diffusion Analysis (Ouchterlony)

The immunological relationship between N. fowleri and N. gruberi was also assessed by the gel-precipitin technique with regard to complete identity between the two species (as shown by completely confluent lines), to partial identity (as shown by a spur), or to non-identity (as shown by a crossing over of the precipitin lines).

Without exception the antigens of N. gruberi and strains BH and BL exhibited a partial identity reaction using anti-N. fowleri sera. This confirmed the sharing of common group antigens observed by both the agglutination and IFAB reactions. Complete identity was observed between the pathogenic isolates HB-1, NTH, PA14, and NHI, when using anti-N. fowleri sera, but not with the 'pathogenic isolates' BH and BL.

The best separation of antigens was achieved by use of a 0.25% (wt/vol) solution of SDS. However, appropriate controls must be used since cross-reactions between SDS and antiserum were observed at higher concentrations of SDS.

Figures 28 - 33: Comparative gel-diffusion analysis

Figure 28

Using anti-NHI serum. Note the spur against Ng-27. The innermost precipitin line is possibly due to a non-specific cross-reaction with the media because of the proximity of the media control line.

Figure 29

Using anti-NTH serum. Note the unique precipitin line not shared by the non-pathogenic Ng-27 and also the spur against Ng-27.

Figure 30

Using anti-PA14 serum.

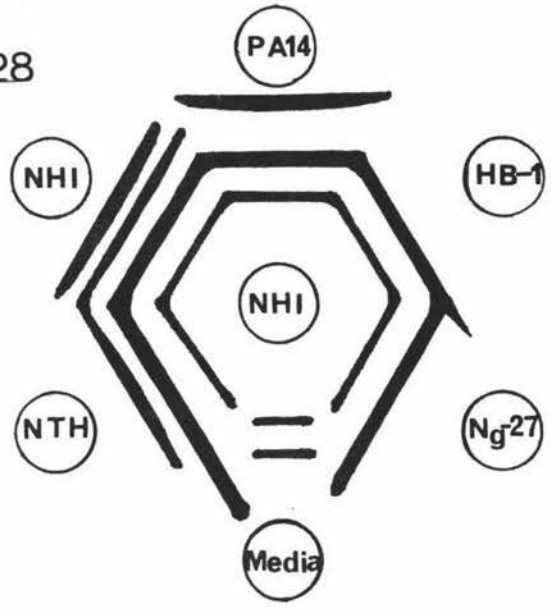
Figures 31 and 32

Using anti-NHI serum. Note the spur against Ng-27 and BL with NHI and the confluent line between BL and BH.

Figure 33

Using anti-NHI serum. Note the unique precipitin line to the pathogenic NHI antigen which is not shared by BH or Ng-27.

28



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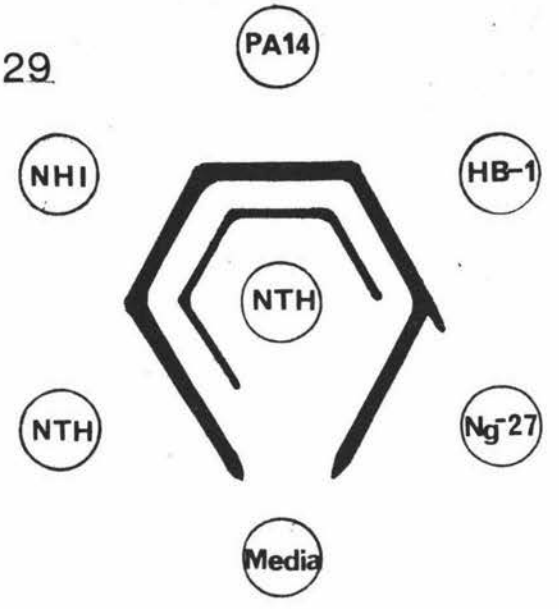

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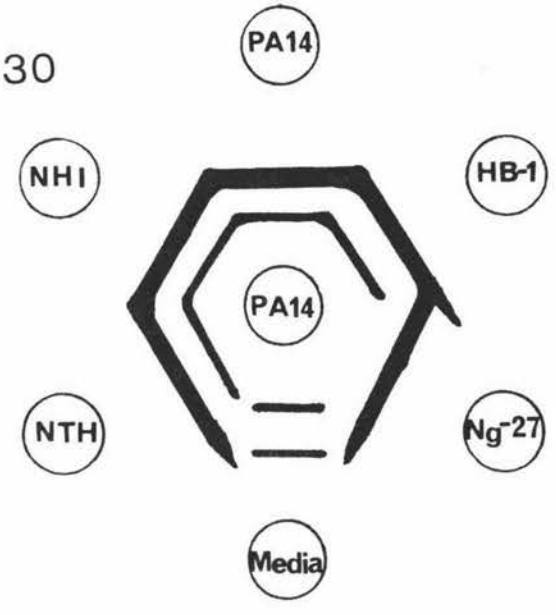

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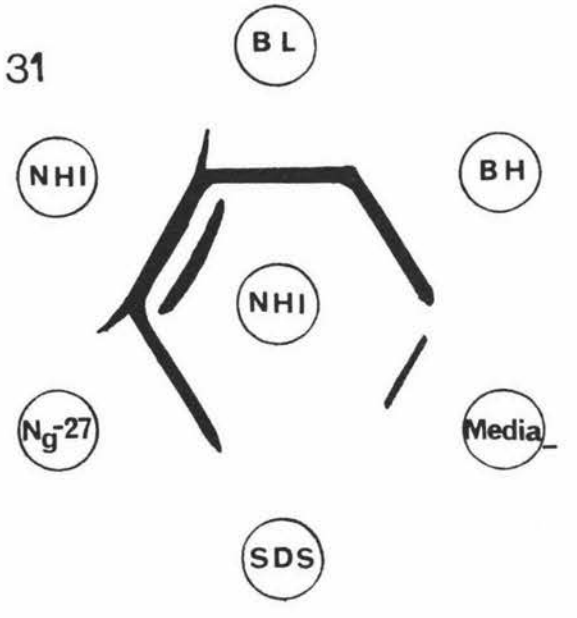

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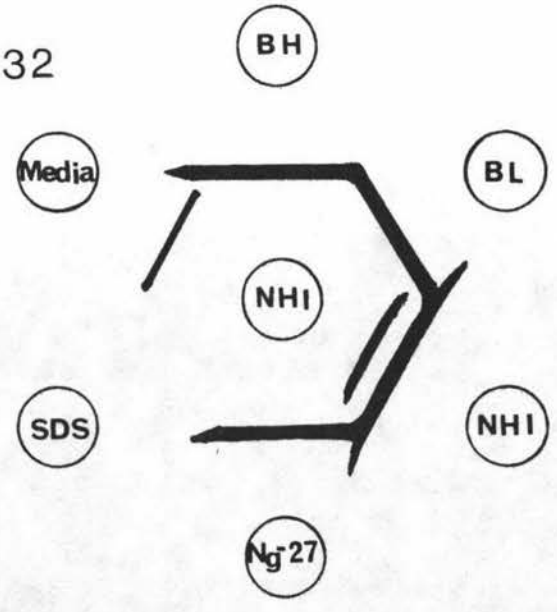

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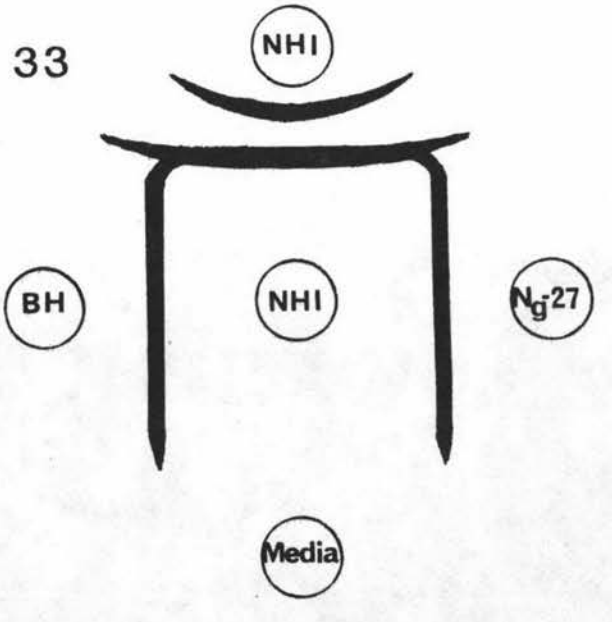


1.5 Mouse Pathogenicity

The virulence of the pathogenic isolates was re-established using traditional intranasal inoculation of trophozoites into mice and then observing for subsequent deaths over a period of 14 days.

Table XI. Mouse-Pathogenicity Results

Isolate	No. of mice used	Size of inoculation cells/cm^3	Number of deaths	Time for deaths
Ng-27	6	10^4	-	-
Ng-1518	ND	ND	ND	ND
BH	6	10^4	-	-
BL	6	"	-	-
HB-1	6	"	5	8-13 days
NTH	6	"	2	10-12 days
NHI	6	"	6	7-8 days
PA14	6	"	6	6-11 days
A-1	6	10^5	6	4-5 days
Neff	ND	ND	ND	-

ND = Not Done

The mice inoculated with H. culbertsoni all died within 4 days, while mice inoculated with N. fowleri died over a period of 6-13 days. The variance in the fatality-time of Naegleria was thought due to differences in the actual volume of inoculum drawn into the nostrils as well as to individual strain virulence. Support for this comes from strain HB-1 which took 8 days to kill 3 mice and 13 days to kill the 4th mouse.

1.6 Vero Cell Culture

Because the role of the mouse as a definitive test of human pathogenicity is not clearly established due to the wide variance in the LD_{50} inoculum reported in the literature, it was decided to investigate the use of cell cultures as an alternative. Initially chick embryo kidney cell cultures were tried but were found to be unsatisfactory due to their low specificity with regard to the CPE formed. Subsequently, Vero cell cultures have been found to give a clear differential result between the pathogenic and non-pathogenic Naegleria on the basis of the cytopathic effects produced.

Table XII. Pathogenicity in Vero Cell Culture

Isolate	Inoculum size	Vessel	Days 1	2	3	4	5	6	Axenic filtrate cell conc.	Plate
Ng-27	10^4 trophozoites	250 cm^3 flask								
Ng-1518	"	"								
BH	"	"								10
BL	"	"								
HB-1	"	"		I	II	III	Dead	Dead		
NTH	"	"			I	III	Dead	Dead		11
NHI	"	"			I	II	III	Dead		12&13
PA14	"	"			I	III	Dead	Dead		
Ng-27	10^4 trophozoites	10.0 cm^3 flaskette								
HB-1	"	"		I	III	Dead	Dead			
NTH	"	"		I	III	Dead	Dead			
NHI	"	"			I	II	Dead			
PA14	"	"		I	III	Dead	Dead			
Ng-27	0.5 cm^3 axenic filtrate	10.0 cm^3 flaskette							$1.23 \times 10^6/cm^3$	
HB-1	"	"			I	II	III		$1.34 \times 10^6/cm^3$	
NTH	"	"					II		$8.1 \times 10^5/cm^3$	
NHI	"	"	II	II	III	Dead	Dead		$25 \times 10^6/cm^3$	
PA14	"	"		I	I	II	III		$1.26 \times 10^6/cm^3$	
Ng-27	0.5 cm^3 cell-culture filtrate	10.0 cm^3 flaskette						I		
HB-1	"	"			I	III	Dead	Dead		
NTH	"	"			I	II	Dead	Dead		
NHI	"	"			I	II	Dead	Dead		
PA14	"	"			I	II	Dead	Dead		

Key: = normal cell monolayer
I = beginning of CPE
II = pronounced CPE
III = very pronounced CPE
CPE = rounding of cells, degeneration accompanied by refractiveness of cells and finally loss of monolayer

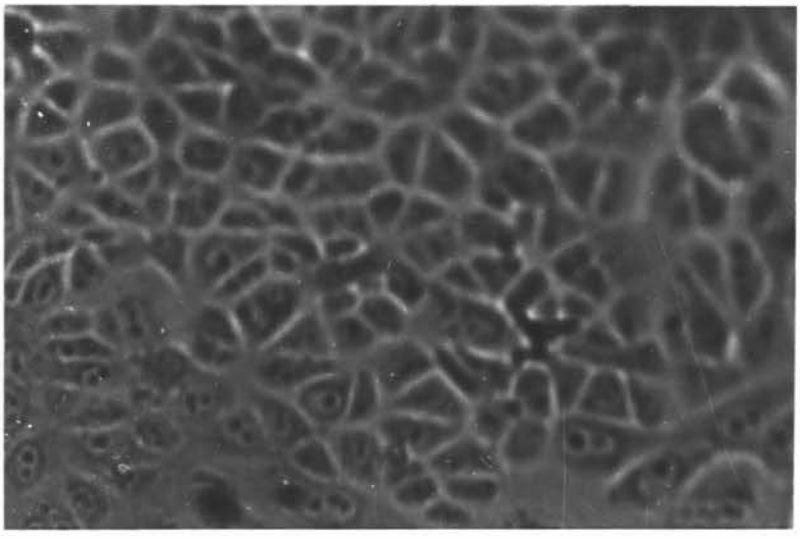


Plate 10. 3 day normal Vero cell culture monolayer x 200.
Note the confluent cell sheet.

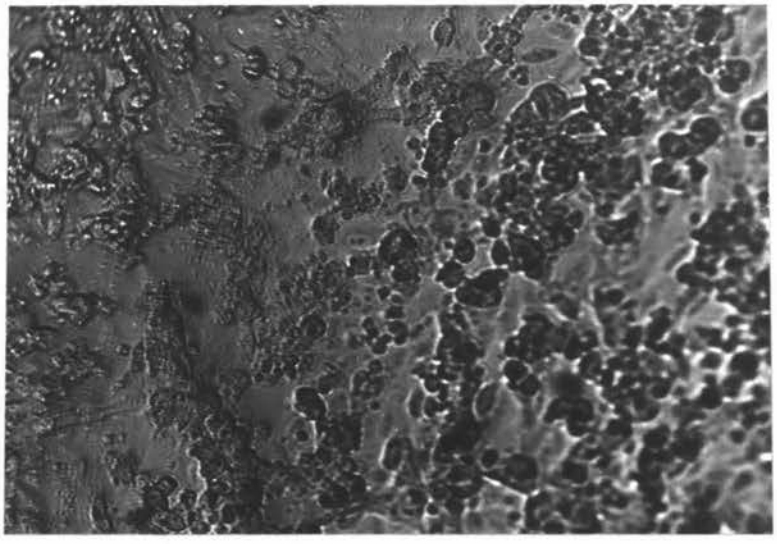
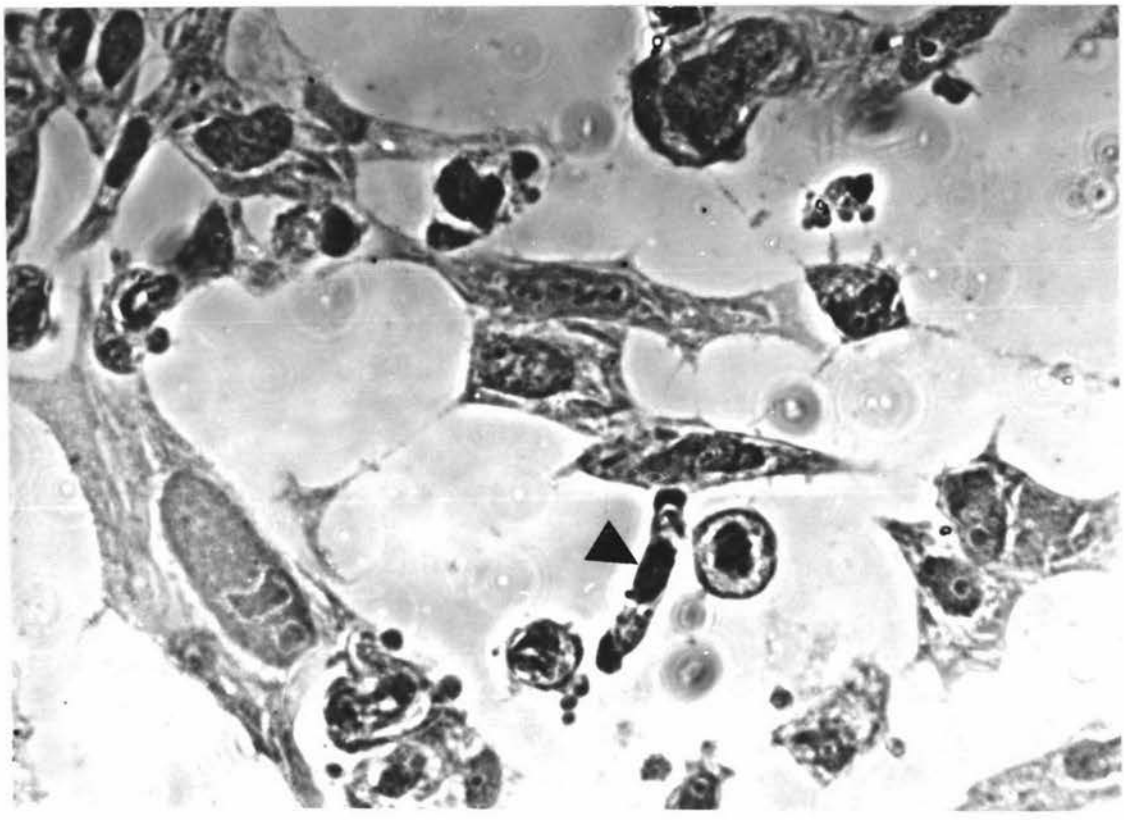
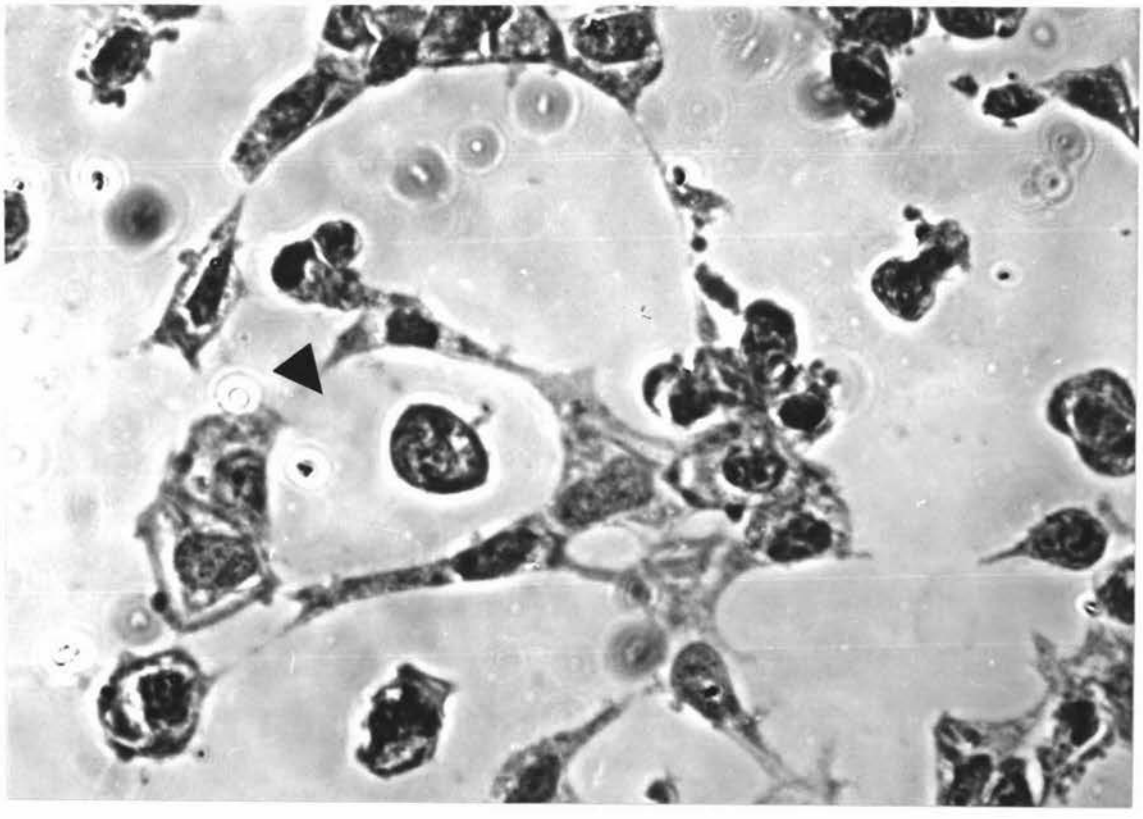


Plate 11. 3 day cytopathic effects of N.fowleri, HB-1 in Vero cell culture x 100.
Note the refractility due to the granulation of the cytoplasm, the rounding up of the cells and the degeneration of the monolayer.





Plates 12 and 13. 3 day cytopathic effects of N.fowleri, NTH in Vero cell culture/stained tichome x 500.
Note the loss of the confluent monolayer in both Plates, the characteristic "limax" form of the trophozoite in Plate 12, and the "halo" around a trophozoite in Plate 13.

The Vero cell culture results complement the mouse pathogenicity results in that only isolates HB-1, NTH, PA14 and NHI formed CPE when using an inoculum of 10^4 cells/cm^3. Further, only the cell-free filtrate of these strains formed CPE when passaged into new Vero cell cultures. The intensity of the CPE of the cell-free filtrate depended on the population size and age of the axenic culture. Typical CPE formed were the rounding of cells accompanied by granulation of the cytoplasm and nuclear pycnosis, refractility and ultimately the loss of the monolayer itself.

2. Respiration Rate ($^{Q}O_2$) and the Temperature Coefficient of Respiration (Q_{10})

The $^{Q}O_2$ and Q_{10} values of the isolates Ng-27, HB-1, PA14, NTH and NHI, were examined at both 27°C and 37°C to examine the possibility of there being any thermal adaptation, and thus selection, by the pathogens in a 10°C rise, and also whether their respective respiration rates could be used in identification.

Table XIII. $^{Q}O_2$ and Q_{10} Values of Naegleria

Isolate	Temp. °C	$Y_2 - Y_1$ (µl)	$X_2 - X_1$ mins.	$^{Q}O_2$ µl/mg/hr	Q_{10}
Ng-27	27	11.0- 5.8 = 5.2	60	5.2	
HB-1	27	9.5- 5.3 4.2	60	4.2	
NTH	27	18.1-11.5 = 6.6	60	6.6	
NHI	27	9.0- 5.3 = 3.7	60	3.7	
PA14	27	14.3- 9.9 = 4.4	60	4.4	
Ng-27	37	17.0- 8.5 = 8.5	60	8.5	1.6
HB-1	37	13.8- 7.0 = 6.8	60	6.8	1.6
NTH	37	44.0-22 = 22	60	22.0	3.3
NHI	37	16.5- 9.5 = 7.0	60	7.0	1.9
PA14	37	29.4-14.8 = 14.6	60	14.6	3.3

Table XIII shows that there is a quantitative relationship between the $^{Q}O_2$ values observed at 27°C and those at 37°C. Further, the Q_{10} values can be divided into two groups.

Gp. 1 NHI, HB-1, Ng-27 Q_{10} of 1.7

Gp. 2 NTH, PA14 Q_{10} of 3.3

The effect of this 10°C from 27 - 37°C on the µl of O_2 consumed, can be seen by comparing Figures 34 and 35.

Apart from the isolates NTH and PA14, the results suggest that at 37°C, there is no selective advantage of the pathogen, as measured by the Q_{10} values, over the non-pathogens.

Figure 34.

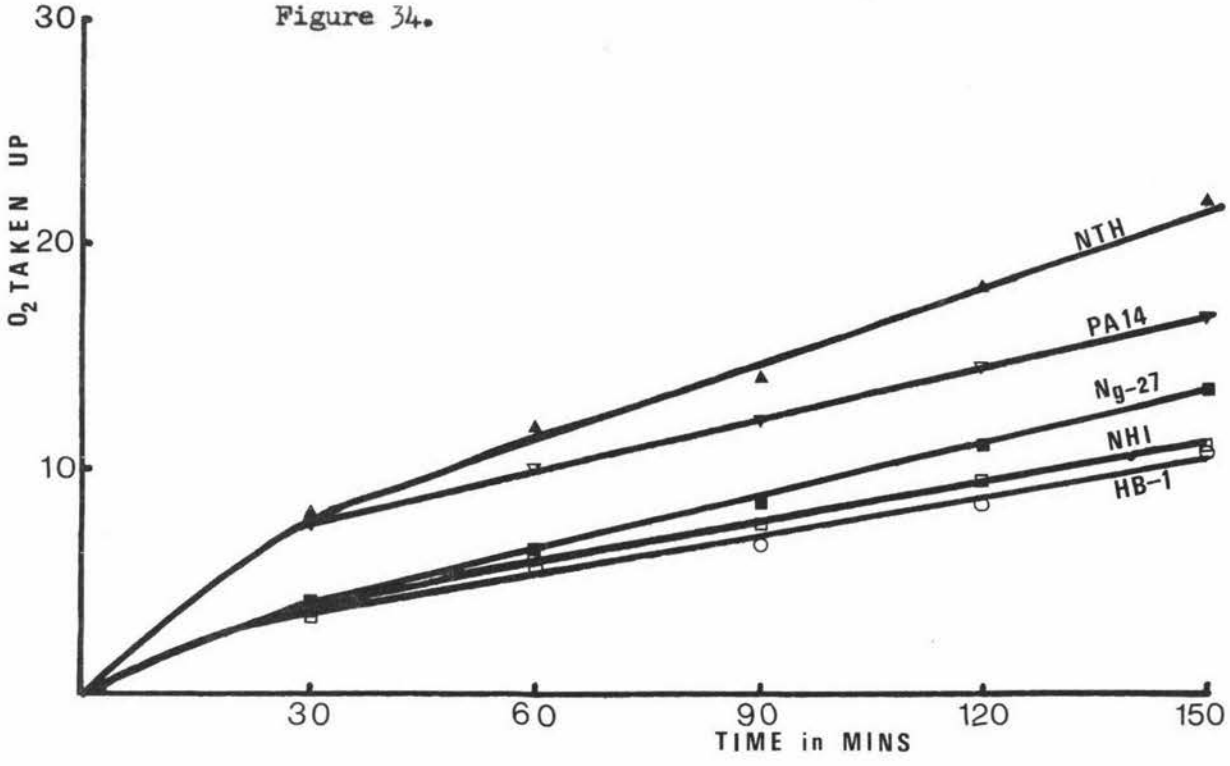
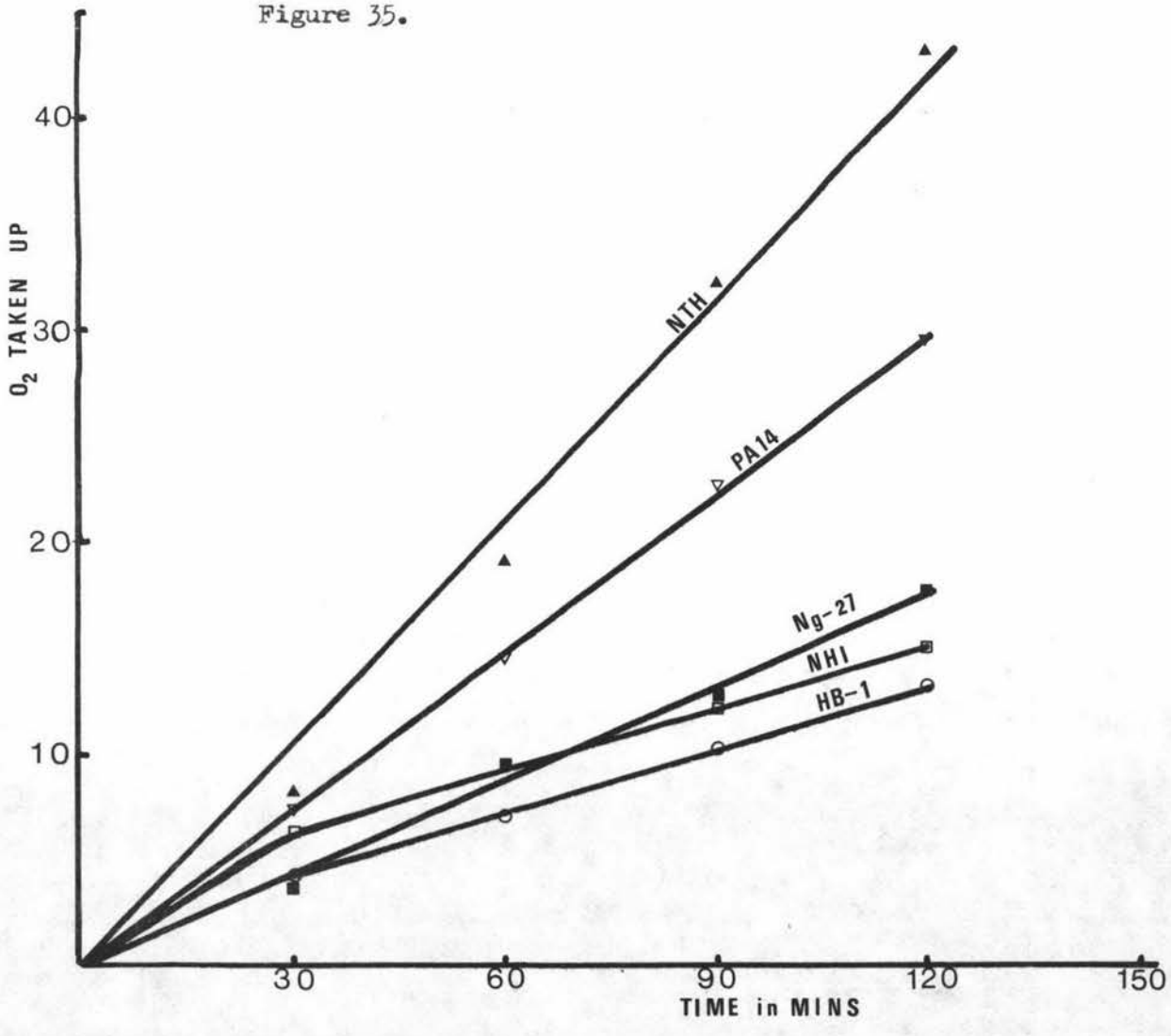


Figure 35.



3. Disinfection

The failure of normal levels of chlorination as an effective disinfectant which has resulted in a number of fatal cases of PAM (Cerva, 1971; Anderson & Jamieson, 1972b) has stimulated the search for alternative disinfectants for use in the field of public health. Currently NaCl (Anderson & Jamieson, 1972b), and the basic dyes malachite green and brilliant green (Cerva, 1973) appear to have the desired amebicidal properties required for use as effective disinfectant against the limax amebae. It was therefore decided to examine their potential use as possible disinfectants.

3.1 Use of Salt

3.1.1 In Monoaxenic Plate Culture

Table XIV. Tolerance of Isolates to NaCl Incorporated into Agar

Isolate	0.5%	0.75%	1.0%
Ng-27	+	+	+
Ng-1518	+	+	+
BH	+	+	+
BL	+	+	+
HB-1	+	+	-
NTH	+	+	-
NHI	+	+	-
PA14	+	+	-
A-1	+	+	+
Neff	ND	ND	ND

ND = Not Done

Table XIV shows that only the non-pathogenic Naegleria and strains BH and BL, could tolerate 1.0% (wt/vol) of NaCl incorporated into agar. The pathogenic isolates HB-1, PA14, NTH and NHI could tolerate up to 0.75% NaCl whilst the hartmannellid A-1 strain could also tolerate 1.0% NaCl.

3.1.2 In Axenic Culture

The axenic salination experiments (Table XV), show that the amebicidal activity of NaCl is a direct effect on the ameba itself, rather than being indirect via the bacterial food-supply. It is thought that the effect of salt on these amebae affects the membranes, due to the malformed, wrinkled appearance of the plasma membranes of the amebae when observed with phase-contrast optics.

The results show that between 0.6%-0.8% (wt/vol) NaCl, a 90% kill (Fig. 36) is achieved. No growth occurs with a concentration of 1.5% (wt/vol) NaCl. Further, the apparent salt tolerance difference observed between pathogen and non-pathogen when using monoxenic plate culture, is not observed in fluid axenic culture. It is thought that this difference could be due to the different feeding mechanisms involved, i.e. phagocytosis on agar culture and pinocytosis in axenic culture.

Figure 36.

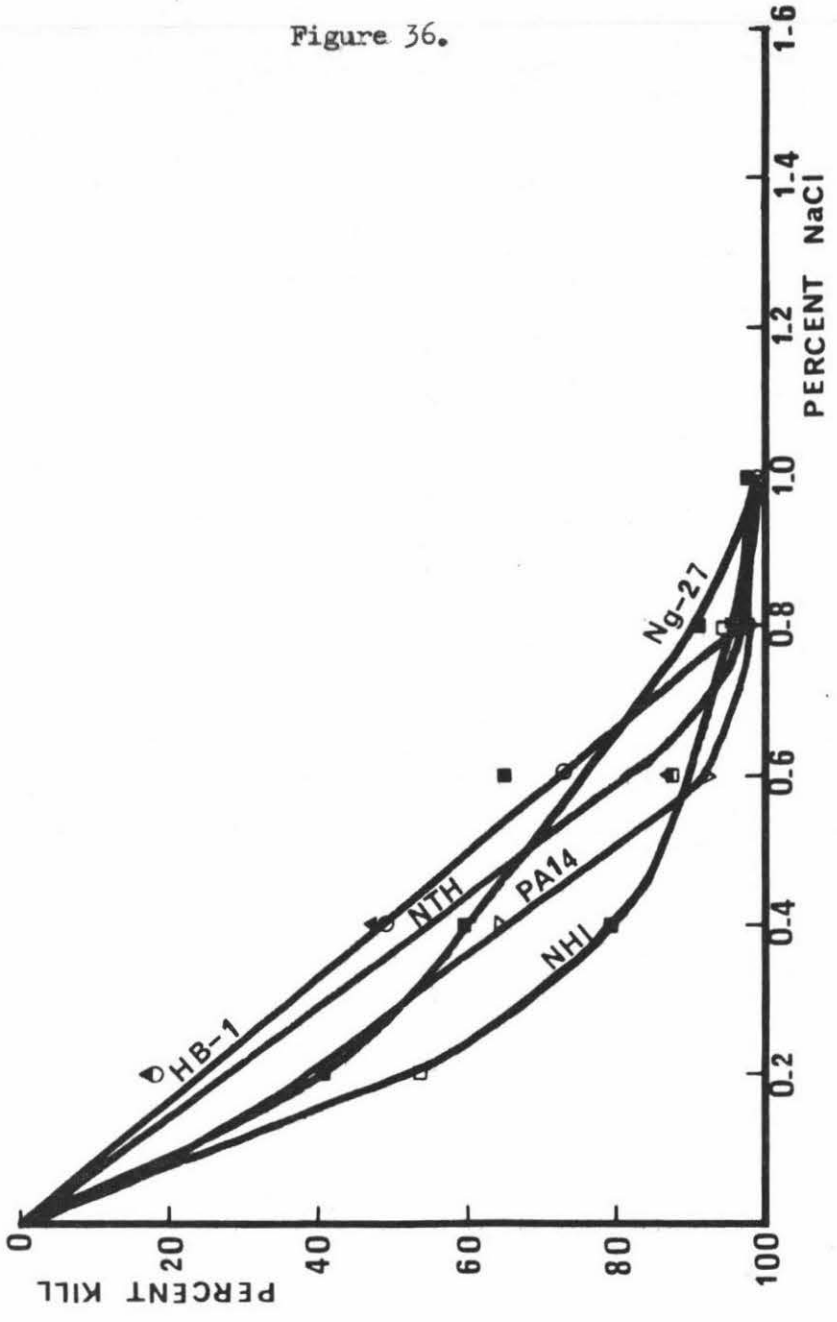

PERCENT KILL
0
20
40
60
80
100
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
PERCENT NaCl
HB-1
NTH
PA14
NHI
Ng-27

Table XV. Tolerance of NaCl in Axenic Culture

Isolate	Inoculum conc. cells/cm^3	Control conc. cells/cm^3	0.2	0.4	0.6	0.8	1.0	1.5	2.5	3.0
						% NaCl				
Ng-27	2.44×10^5	7.6×10^5	4.5×10^5	3.1×10^5	2.7×10^5	6.1×10^4	1.3×10^4	0	0	0
% kill			40.8	59.2	64.5	92	98.3	100	100	100
Viability		+	+	+	+	+	+	−		
HB-1	1.6×10^5	1.28×10^6	1.05×10^6	6.5×10^5	3.4×10^5	2.3×10^4	1×10^3	0	0	0
% kill			18	49.2	73.4	98.2	99.9	100	100	100
Viability		+	+	+	+	+	+	−	−	−
NTH	1.28×10^5	1.24×10^6	1.03×10^6	6.6×10^5	1.6×10^5	1.9×10^4	1×10^3	1×10^3	0	0
% kill			16.9	47	87.1	98.5	99.9	99.9	100	100
Viability		+	+	+	+	+	+	−	−	−
NHI	ND	1.4×10^6	6.47×10^5	2.87×10^5	1.62×10^5	6.73×10^5	2.57×10^4	0	0	0
% kill		0	53.8	79.5	88.4	95.2	98.2	100	100	100
Viability		+	+	+	+	+	+	−	−	−
PA14	ND	1.57×10^6	ND	5.63×10^5	1.18×10^5	8.92×10^4	3.67×10^3	0	0	0
% kill		0		64.1	92.5	94.3	99.8	100	100	100
Viability		+		+	+	+	+	−	−	−

ND = Not Done

3.2 Basic Dyes

3.2.1 Malachite Green

The potential use of Malachite Green as a disinfectant was tested over a wide range of concentrations at 37°C. Total counts were made and subsequent viability assessed by plaquing out on NM agar.

Table XVI. The Effectiveness of Malachite Green as a Disinfectant

Isolate	Inoculum conc. cells/ cm^3	Control conc. cells/ cm^3	0.1 μg	0.25 μg	0.5 μg	1.0 μg	1.5 μg	3.0 μg
Ng-27	2.6×10^5	9.6×10^5	9.6×10^5	1.1×10^5	1×10^3	ND	ND	ND
% kill			0	89%	99.9			
Viability	+	+	+	+	+			
HB-1	5.52×10^5	1.47×10^6	ND	2×10^5	5.7×10^4	3.7×10^4	3.4×10^4	3.4×10^4
% kill				86.4	96.1	97.5	97.7	97.7
Viability	+	+		+	+	+	-	-
NTH	3×10^5	1.01×10^6	1.01×10^6	1.3×10^5	1.2×10^4	1.4×10^4	1.3×10^4	ND
% kill			0	87.2	98.8	98.6	98.7	
Viability	+	+	+	+	+	-	-	
NHI	5.88×10^5	1.41×10^6	1.41×10^6	4.4×10^5	1.2×10^5	ND	ND	ND
% kill			0	69	91.5			
Viability	+	+	+	+	+			
PA14	4.6×10^5	1.02×10^6	ND	1.5×10^5	4.3×10^4	4.5×10^4	4.4×10^4	4.0×10^4
% kill				85.3	95.8	95.6	95.7	96.1
Viability	+	+		+	+	+	-	-

ND = Not Done

Table XVI shows that no Naegleria spp. survived a concentration of 1.5 $\mu g/cm^3$ of Malachite green whilst Fig. 37 illustrates that a 90%+ kill occurred at 0.5 $\mu g/cm^3$ of the dye.

3.2.2 Brilliant Green

Table XVII. The Effectiveness of Brilliant Green as a Disinfectant

Isolate	Inoculum conc. cells/ cm^3	Control conc. cells/ cm^3	0.1 µg	0.25 µg	0.5 µg	1.0 µg	1.5 µg	3.0 µg
Ng-27	2.68×10^5	8.4×10^5	8.4×10^5	8.4×10^5	6×10^4	1.2×10^4	9×10^3	ND
% kill			0	0	92.9	98.6	98.9	
Viability	+	+	+	+	+	+	−	−
HB-1	5.52×10^5	1.47×10^6	ND	1.47×10^6	9.4×10^5	7.1×10^5	3.6×10^4	1.9×10^4
% kill				0	36.1	51.7	97.5	98
Viability	+	+		+	+	+	+	−
NTH	3×10^5	1.01×10^6	1.01×10^6	7.7×10^5	3.7×10^5	2.2×10^4	1.9×10^4	ND
% kill			0	23.8	96.3	97.8	98.9	
Viability	+	+	+	+	+	+	−	
NHI	5.88×10^5	1.41×10^6	1.41×10^6	7×10^5	4.4×10^5	ND	ND	ND
% kill			0	50.3	68.8			
Viability	+	+	+	+	+			
PA14	4.6×10^5	1.02×10^6	ND	1.02×10^6	7.6×10^5	4.4×10^5	3.8×10^4	2.8×10^4
% kill				0	25.5	56.9	96.3	97.2
Viability	+	+		+	+	−	−	−

ND = Not Done

Table XVII shows that a total kill was not achieved till a concentration of 3.0 µg/cm^3 of brilliant green was used. Fig. 38 shows that there appears to be some variation in the response of the different strains to brilliant green as a disinfectant. A 90%+ kill was achieved over a range of 0.5 to 1.5 µg/cm^3 of the dye.

Figure 37.

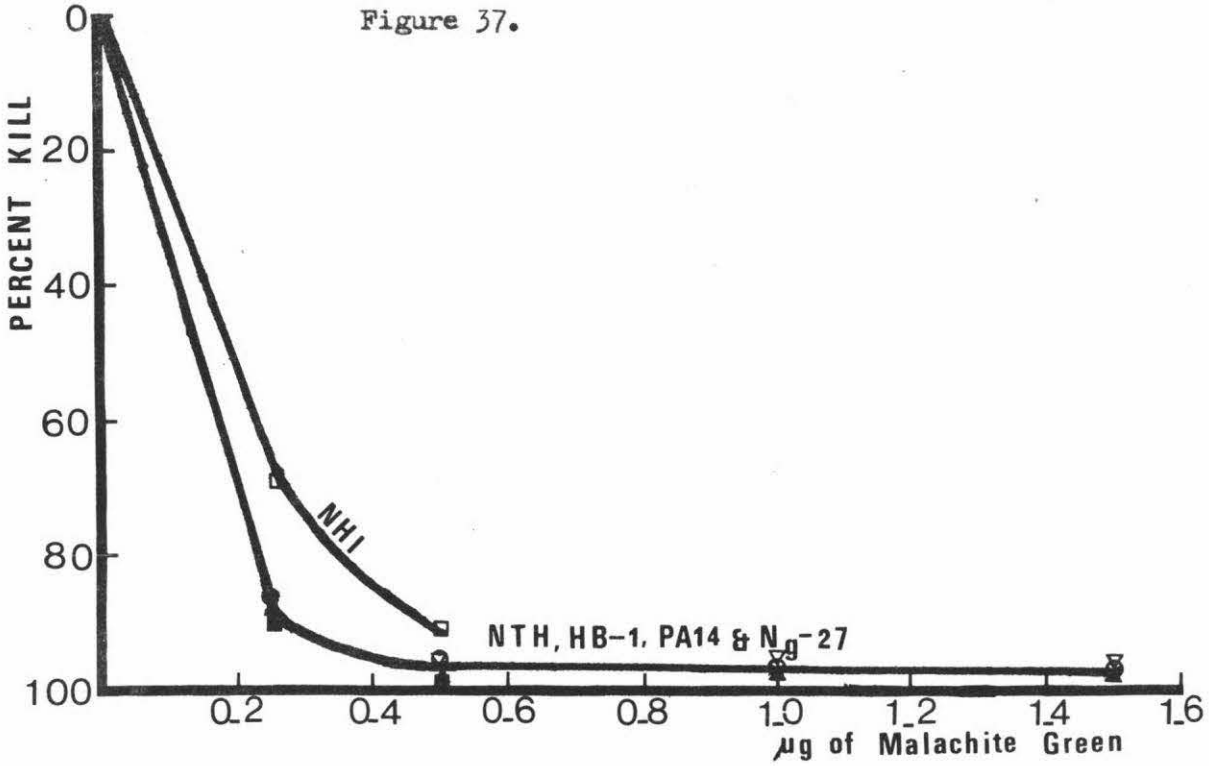


Figure 38.

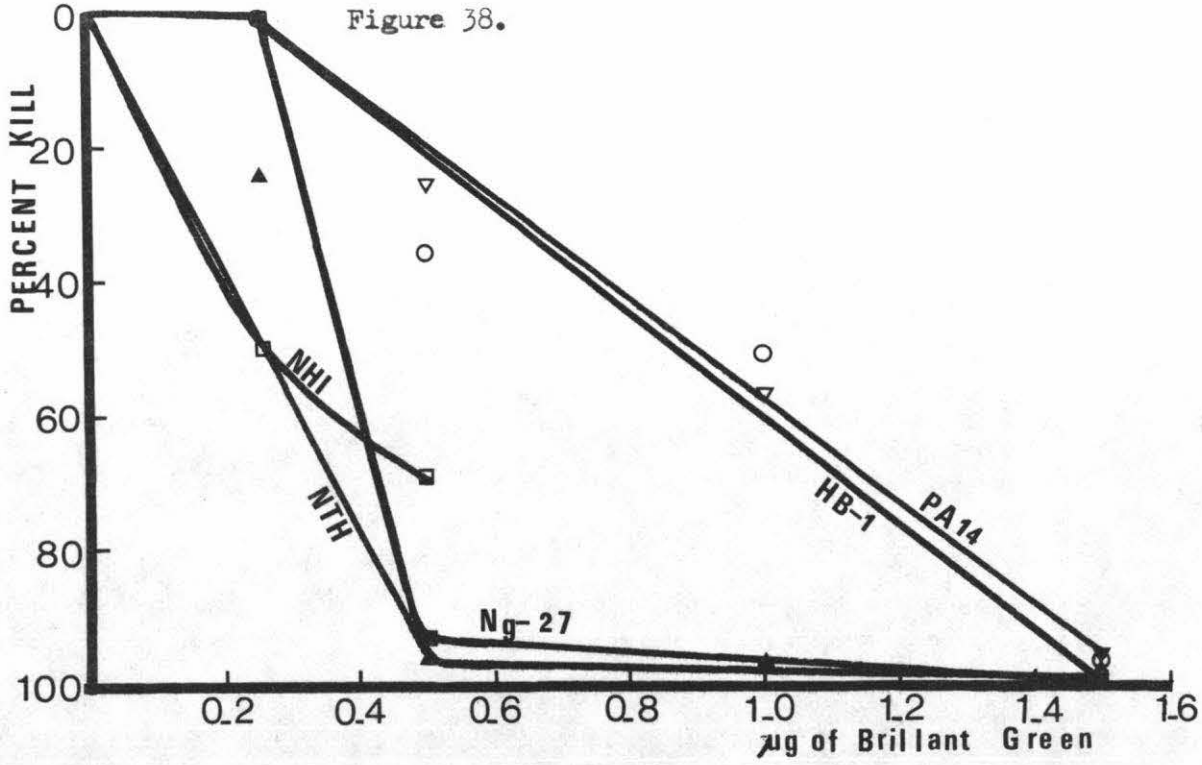
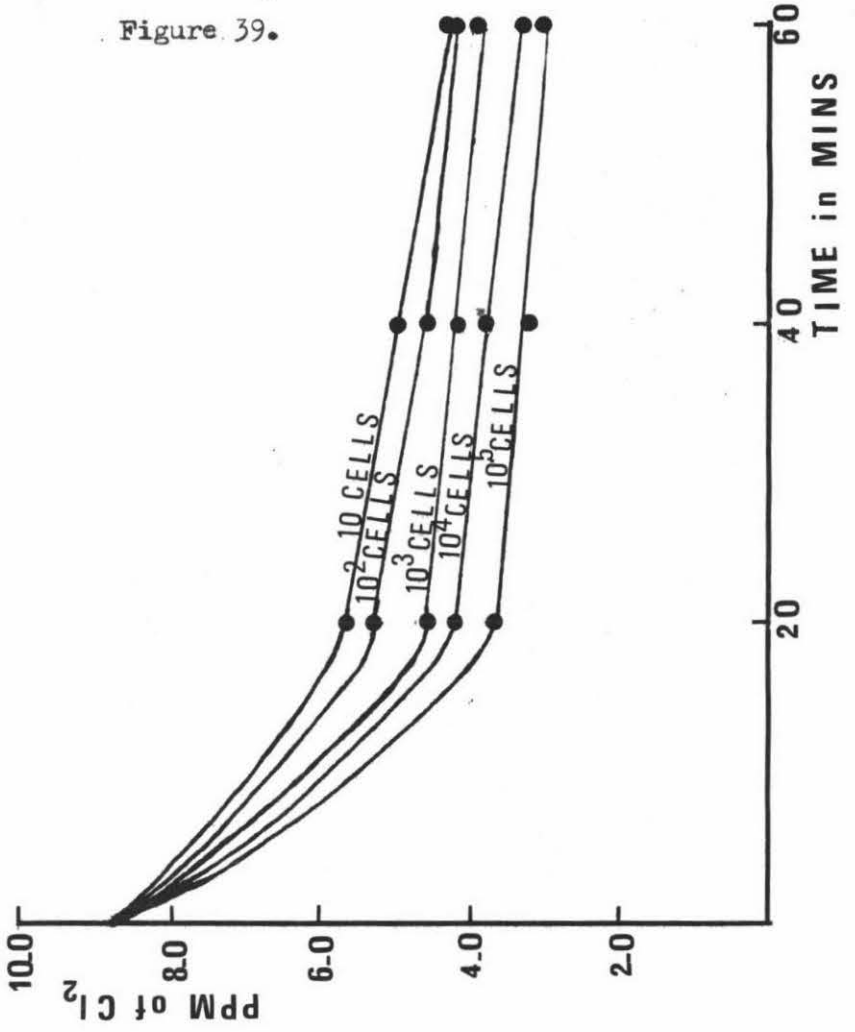


Figure 39.


10.0
8.0
6.0
4.0
2.0
PPM of Cl_2
20
40
60
TIME in MINS
10^2 CELLS
10^2 CELLS
10^3 CELLS
10^4 CELLS
10^5 CELLS

3.3 Chlorine

The effect of different cell numbers on the level of total available chlorine (TAC) was examined, together with the effect of a maintained chlorination level on the viability of these organisms (Cerva, 1971; Anderson & Jamieson, 1972b).

3.3.1 The Effect of Different Cell Numbers on the Chlorination Level

Table XVIII. The TAC Level as a Consequence of Different Cell Numbers

Conc. of cells/cm^3	0 min	20 min	40 min	60 min	
10^5	8.8	3.7	3.2	3.0	
10^4	8.8	4.2	3.8	3.3	Level of Total
10^3	8.8	4.6	4.2	3.9	Available Chlorine
10^2	8.8	5.3	4.6	4.2	(TAC) in ppm
10	8.8	5.7	5.0	4.3	

Fig. 39 illustrates that there is a definite relationship between the level of TAC and the cell population, i.e. the higher the cell population, the lower the TAC level. As can be seen, the greater the "organic mass" present, represented in this case by the cell population, the higher the chlorine demand of the water, since most organic compounds are oxidized by Cl_2 or HOCl due to the high oxidation potential of these molecules.

3.3.2 The Effect of a Maintained Chlorine Level

Figure 40.

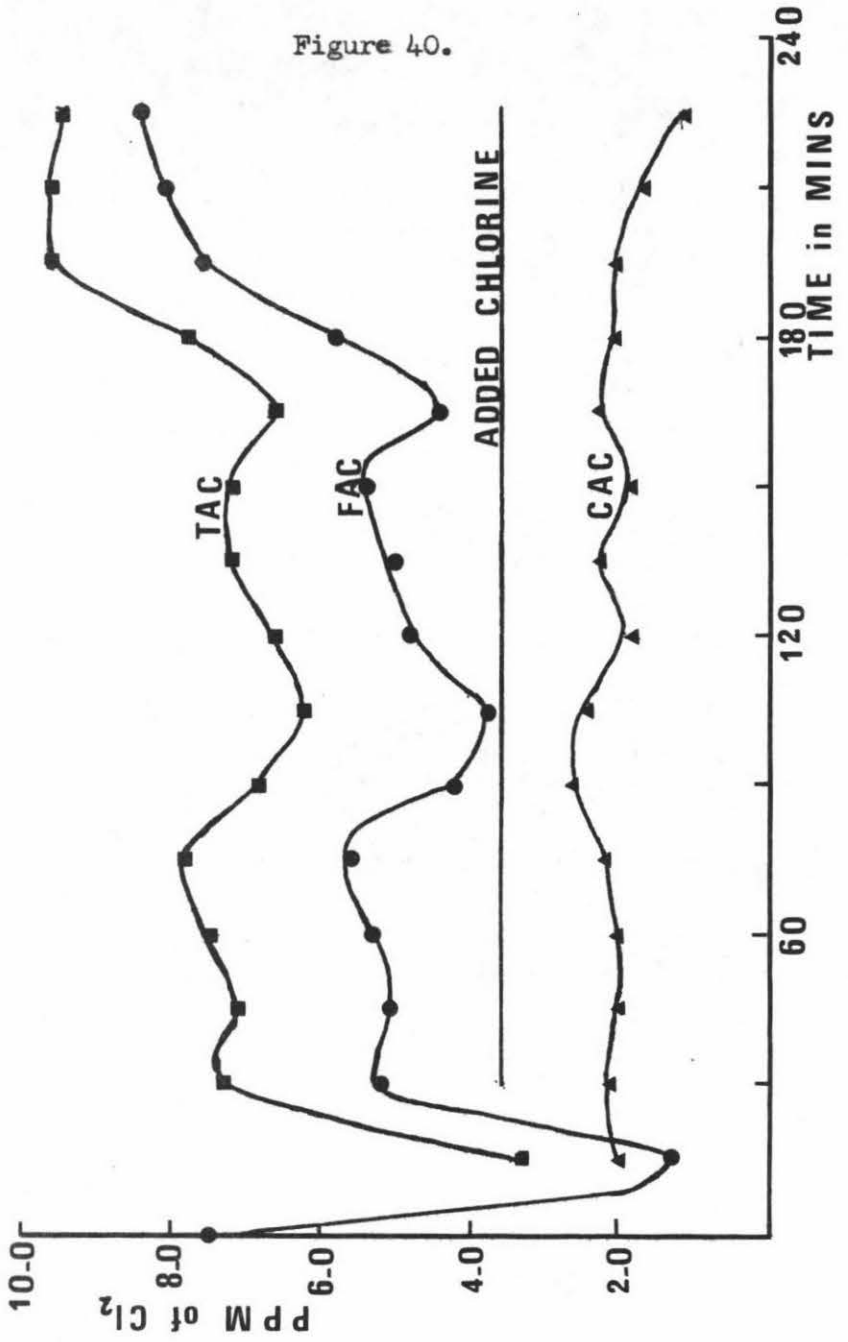

10·0
8·0
6·0
4·0
2·0
PPM of Cl2
TAC
FAC
CAC
ADDED CHLORINE
60
120
180
240
TIME in MINS

Table XIX. Comparative Constituent Chlorine Levels on 10^5 Naegleria/cm^3

Time (mins)	Added Chlorine (mg/l)	Total Available Chlorine (mg/l)	Combined Available Chlorine (mg/l)	Free Available Chlorine (mg/l)	Viability of amebae
0				7.5	+
15		3.3	2.0	1.3	+
30	3.64	7.3	2.1	5.2	+
45	3.64	7.1	2.0	5.1	+
60	3.64	7.4	2.0	5.3	+
75	3.64	7.8	2.2	5.6	+
90	3.64	6.8	2.6	4.2	+
105	3.64	6.2	2.4	3.8	+
120	3.64	6.6	1.8	4.8	+
135	3.64	7.2	2.2	5.0	+
150	3.64	7.2	1.8	5.4	+
165	3.64	6.6	2.2	4.4	+
180	3.64	7.8	2.0	5.8	+
195	3.64	9.6	2.0	7.6	+
210	3.64	9.6	1.6	8.1	+
225	3.64	9.5	1.1	8.4	+

Fig. 40 indicates that there is a drastic drop in the level of the free available chlorine, FAC, level in the first 15 minutes, followed by a subsequent rise in the FAC level and a decrease in the combined available chlorine level as more chlorine is added. The break point occurs after 105 minutes by which time enough chlorine has been added to satisfy the demand of all the reducing agents present.

Table XIX also illustrates that the amebae were still viable after 225 minutes thereby confirming the ineffectiveness of chlorine as a disinfectant for the limax amebae.

CHAPTER FIVE: DISCUSSION

1. Classification of Families of Amebae Associated with Primary Amebic Meningo-encephalitis (PAM)

The taxonomy of the amebae associated with PAM is still a controversial matter. Currently there are three taxonomic schemes (Page, 1967a; Singh & Das, 1970; Chang, 1971) none of which is, as yet, universally accepted. The lack of a universally adopted scheme, has led to much confustion in the literature, resulting from PAM cases, over the nomenclature of these soil-amebae. This has resulted in the frequent use of invalid synonyms for the aetiological agents of PAM so creating some ambiguity concerning which is the correct specific or generic name. At present, there are three different synonyms for the pathogenic species of Naegleria: (N. fowleri, Carter, 1970; N. aerobia, Singh & Das, 1970; N. invades, Chang, 1971), and two different synonyms for the non-swimming associated form of the disease: Hartmannella and Acanthamoeba.

Table II illustrates that the differences between the three classification schemes are due not so much to the individual choice of taxonomic criteria, but from the weight and emphasis placed on what is basically similar data. For instance, Singh & Das use the following criteria as the basis for their taxonomic scheme:

Family Schizopyrenidae - nuclear division, presence or absence of a flagellate stage

Genus Naegleria - nuclear division and the presence of a flagellate stage

Family Hartmannellidae - nuclear division and the absence of a flagellate stage

Genus Hartmannella - nuclear division and the absence of a flagellate stage

Page bases his taxa on:

Family Vahlkampfiidae - nuclear division and movement

Genus Naegleria - nuclear division and a flagellate stage

Family Hartmannellidae - nuclear division, absence of a flagellate stage, movement

Genus Hartmannella - nuclear division, movement and cyst stage

Genus Acanthamoeba - nuclear division, movement and cyst stage

Chang:

Family Schizopyrenidae - nuclear division, movement, presence or absence of a flagellate stage

Genus Naegleria - nuclear division, cyst stage, presence of a flagellate stage

Family Hartmannellidae - nuclear division, movement, absence of a flagellate stage

Genus Hartmannella - trophozoite morphology, cyst morphology, contractile vacuole

Genus Acanthamoeba - trophozoite morphology, nutritional requirements

It can be seen that Singh and Das (1970) using solely cytological features, accordingly place great emphasis on the stages and structures of promitosis, whereas Page (1967a) considers the phenomenon of promitosis in toto. Page further correlates the particular mode of nuclear division with the manner of locomotion thereby emphasizing pseudopodial morphology. Chang (1971) in what is basically a hybridization of the previous two schemes, combines both these approaches.

Although each scheme has its particular merits and demerits, on the whole the scheme of Singh and Das is perhaps the most reliable, and therefore usable, because of the following reasons:

1) Interzonal bodies were found to be a constant feature of nuclear division in this investigation provided the correct promitotic stages could be located. They have also been described by Ralfalko (1947); Singh (1952); Butt, Baro & Knorr (1968); Culbertson, Ensminger, & Overton (1968) and Chang (1971). Because of the difficulty experienced in getting the amebae in the correct stage of promitosis so that the interzonal bodies could be observed using axenic cultures, the writer did feel some sympathy with Page's original criticism that the interzonal bodies were not a consistent enough feature of nuclear division to be used as a taxonomic criterion. However, by using the culture method developed by Singh (1952), appropriate stages of promitosis were finally obtained in sufficient regularity to thus invalidate Page's original criticism.

2) Page places great emphasis on pseudopodial morphology as a taxonomic criterion despite the numerous reports in the literature, Dobell (1914); Minchin (1922); Wenyon (1926); Calkins (1933); Kudo (1946) and Band (1969), which all agree that pseudopodial morphology is too variable a character to be used for classification. Culbertson (1971) states that "the mitotic characteristics have been the most helpful as many of the other morphological factors have been rather inconsistent depending upon differing conditions." The writer's observations also support this view and the variability of pseudopodial morphology is further exemplified by the observations of Ray & Hayes (1954) using Hartmannella astronyxis - "lobopods, filopods and micropseudopodia are all formed and may be present in the same individual at the same time. The kind of pseudopodia produced is conditioned by many factors in the environment .... Bread, flat lobopods are commonly present during active locomotion in individuals which are in a liquid environment and in contact with a solid, smooth surface. In locomotion on agar surface, pseudopods tend to be narrow and tapering and may become extremely long and attenuated. In individuals suspended in a surface film or floating free, relatively short, narrow, radiating filopods are the more common pseudopodial types .... Under conditions of slightly lowered oxygen tension, the limax shape is normally assumed. If the total salt concentration exceeds 0.5%, branched pseudopods are produced. Locomotion, and hence to a large degree the type of pseudopodia formed, depends also upon the nutritional state of the culture and the stage of the life-cycle."

If this statement is accepted there can be no justification for Page's division of the Family Hartmannellidae Alexeiff, emend. Singh (1952), into the two separate genera Hartmannella and Acanthamoeba using pseudopodial morphology as a taxonomic criterion. Furthermore, Hartmannella glebae, Dobell (1914), the type species of the genus Hartmannella as laid down by Singh & Das (1970), also produces acanthapodia, but the cysts are rounded or spherical with a smooth wall

and without pores. This description is also in agreement with the description of H. glebae by Sandon (1927). Thus, "on the basis of cyst character and mode of excystment, H. glebae cannot be included in the genus Acanthamoeba. Only on pseudopodial character, which is of no diagnostic value at all, can H. glebae be placed in the genus Acanthamoeba." - Singh & Das (1970).

3) Page has set up Hartmannella hyalina (Dangeard, 1900) as the type species of the genus Hartmannella despite the fact that Dangeard's original description was based entirely on fixed preparations and does not refer to locomotion. Sandon (1927) describes it as follows, "active form very like N. gruberi, but usually slightly larger, protoplasm rather more fluid and pseudopodia generally formed 'eruptively' ..." The creation of a genus based on such an incompletely studied species is of "little value." - Singh & Das (1970).

4) The criteria used by Singh & Das (1970) in their classification of the small free-living amebae, can be applied to all the other taxa in the order Amoebida and also suggests possible phylogenetic relationships. According to Singh & Das, the Feulgen negative nucleolus or nucleoli in Schizopyrenidae, give rise to polar masses during mitosis. The nuclear membrane persists throughout division. A form such as N. gruberi, which during division has polar masses and interzonal bodies and can readily produce a temporary flagellate stage, may be regarded as a primitive ameba. During the course of evolution it seems that the amebae lose the power to produce interzonal bodies, although they are able to produce temporary flagella, as in the case of Didasculus thorntoni. In a form like Schizopyrenus russelli no temporary flagellate is produced, although the nuclear division of the ameba is indistinguishable from D. thorntoni. Thus the primitive character of temporary flagella production in S. russelli is lost, but it retains the family character in having polar masses. In the Family Entamoebidae Feulgen negative granules or nucleoli do not give rise to polar masses and the nuclear membrane is intact during division.

A spindle with chromosomes arranged as an equatorial plate is formed, as in higher animals and plants.

5) The scheme of Chang (1971) can be questioned on the basis of his use of such doubtful criteria as polar caps (Fulton, 1970) pseudopodial morphology and cyst morphology.

The writer therefore favours the adoption of Singh & Das taxonomic scheme. At the same time it should be realised that taxonomy, by its inherent nature is subject to the changes resulting from the discovery of further scientific knowledge. Perhaps the whole idea of classification can be summed up by Oakley (1962) - "Until an ideal classification that subsumes all the possible information is devised, we shall have to put up with practical classifications that are useful to us. They may be, of course, as ephermeral as the practical requirements they serve, and there is a great deal to be said for not enshrining them in official Latin sets of binomials. The more we know, the better our classifications can be, but we may still find, while we are still so ignorant, that for different purposes different classifications are necessary, and we may even be inclined to relegate to earthly paradises and other non-existent Utopias of classification in which all the properties of an organism are regarded as equally important."

2. Identification of the Aetiological Agents of Primary Amebic Meningo-encephalitis

Efficient rapid means of identification are essential to both treatment and control of the disease because of:

1) the abrupt rapid onset of the disease; to render any chance of survival, drug therapy must be commenced as soon as possible after a positive identification of the aetiological agent, Chang (1974), and
2) the fact that normal levels of chlorination have little effect on these organisms (Cerva, 1971; Anderson & Jamieson, 1972b; Brown, 1974; Table XIX) and therefore currently, there are no effective control measures for PAM. It seems likely in the future that control of the disease will depend, to some extent, on the routine analysis of water to establish the presence or absence of these organisms.

This vital need for rapid identification has perhaps been responsible for the move away from traditional methods of protozoan identification to those of serology over the last few years. This has resulted in the establishment of serology as a powerful diagnostic tool in the field of parasitic protozoology and a resumé of some of these methods together with traditional identification methods for PAM is included with the results of the present study.

2.1 Identification Based on Morphology

2.1.1 The Trophozoite Stage

The comparative studies so far have shown that the pathogenic *N. fowleri* is morphologically identical to the non-pathogenic *N. gruberi* under the compound microscope (Plates 1, 3, 4 and 5) the only slight difference being that the pathogens are slightly smaller in size and form cysts with no opercula, Singh & Das (1970).

Table XX. Morphological Comparison Between *N. gruberi* and *N. fowleri*

	trophozite size µm	nuclear diameter µm	cyst diameter µm	cyst pores
N. gruberi	15–30	4	14	+
N. fowleri	10–15	4	9	-

Electron-microscopic studies have also confirmed this similarity (Carter, 1970; Visvesvara & Calloway, 1974) between the two species. The suggestion by Carter (1970) that the possession of dumbell-shaped mitochondria is a trait of the pathogens only, has been invalidated by Visvesvara & Calloway (1974) who found that *N. gruberi* also possesses these structures. The significance of the possession of dumbell-shaped mitochondria, as opposed to oval-shaped mitochondria, is not yet known. However, it is known that the mitochondria of pathogenic culture forms differ from the mitochondria of mouse-infective forms, in that lead citrate stained sections of amebae in the brain contained only lightly stained mitochondria as opposed to the numerous darkly stained structures of amebae grown in monoxenic cultures. This suggests the possibility of a less active metabolic state when *in vivo* (Maitra *et al.*, 1974). This is perhaps analogous to the situation in Trypanosomes whereby in the culture form the mitochondria undergo extensive proliferation, but in the infective bloodstream form, the mitochondria regress into a single, simple anterior mitochondrion. This structural adaptation in the bloodstream forms is accompanied by the loss of a functional Krebs cycle which is located in the crystal membranes of the mitochondria. Further, the consumption of glucose and O_2 by the bloodstream forms increases to 10 times that of the culture forms (Goodwin, 1964). Thus there is a metabolic adaptation on adoption of the infective form

to meet the demands of a changed environment. In the same way, the mitochondrial differences between the infective and cultural forms of Naegleria, as outlined by Maitra et al. (1974) may also reflect some sort of metabolic adaptation, since the amebae go from a relatively nutritionally poor environment, characterized by a low O_2 content, to a relatively nutritionally rich environment in the brain, characterized by a very high O_2 content. For example, the oxygen content of whole blood is approximately 2.03×10^5 mg/l (20.3 cm^3 of O_2/100 cm^3 of blood), and the oxygen in solution in blood, i.e. not combined with haemoglobin, is 2.85×10^3 cm^3/l (0.285 cm^3 O_2/100 cm^3 blood). The whole blood carries approximately 3×10^4 more oxygen than does pure water at 37°C whilst oxygen in solution in blood has approximately 4×10^2 times more oxygen than pure water at 37°C.

It has also been shown that the serum requirement for the growth of Naegleria in axenic culture is actually a heme requirement, which can also be satisfied by haemoglobin (Band and Balamuth, 1974), and that amebae form many micropinocytotic vesicles from the plasma membrane for the active transport of solutes from the brain (Vivesvara & Calloway, 1974). Therefore, because of the large amounts of oxygen taken in via the carrier haemoglobin molecules and the excess of nutrients, a less active metabolic state, as compared to the free-living state, could constitute an adaptation to the parasitic habitat. Because of the overabundance of oxygen, it seems also highly likely that part of the oxidative metabolic machinery could be missing, as in the Trypanosomes. That oxygen does affect Naegleria spp. can be seen from the findings of Band and Balamuth (1974). They incubated two cultures, identical in every way except that one of the cultures was oxygenated by incubating on a rotary shaker, and found the maximum yield of the rotated

culture to be 5.83 x 10^6 cells/cm^3, and the maximum yield of the other unshaken culture to be 2.44 x 10^6 cells/cm^3.

Thus on adoption of the pathogenic state in which there is a surplus of growth requirements, in the interests of cellular economy a poorly developed mitochondrial system, together with its oxidative metabolic defects, could be of some advantage as a regulatory system. Conversely, in the free-living state where there is not such an over-abundance of growth requirements, a more highly developed mitochondrial system is perhaps a necessity for the maintenance of the level of energy required for the many biosynthetic functions of the cell.

2.1.2 The Flagellate Stage

What little work has been done indicates that the flagellate stage of N. gruberi is morphologically identical to that of N. fowleri (Singh & Das, 1970; Carter, 1970).

2.1.3 The Cyst Stage

There is now reliable evidence to support the view that N. gruberi forms cysts with opercula (Plate 2) via which they excyst. N. fowleri, on the other hand, forms cysts without opercula and excysts via rupture of the cyst wall (Carter, 1970; Singh & Das, 1970; Lastovica, 1974). Singh & Das (1970) further claim that the cysts of pathogenic Naegleria are coated "in a fairly thick gelatinous layer." The cysts of N. gruberi are also said to be larger than those of N. fowleri (Table XXII).

It is tempting to speculate that the loss of specialized structures for excystment may be the result of evolution to a parasitic state. Further, since the cyst stage represents a resistant structure formed normally as a result of an unfavourable environment, the fact that only hartmannellids and not Naegleria

form cysts in the brain, suggests the probability that the former are indeed only pathogenic opportunists. If this is so, then it may explain why these organisms are only rarely involved in cases of PAM.

2.2 Growth and Physiology

2.2.1 Plaque Morphology

Of the 6 pathogenic isolates tested (Table IV) all, with the exception of the Waikato isolates BH and BL, formed smaller plaques on a bacterial lawn (Plates 7-9) in a longer incubation time than did the non-pathogens (Table VII). This could be because the non-pathogens can outgrow the pathogens at 37°C (Chang, 1974; De Jonckhere et al., 1974). Although some of the pathogens occasionally formed centred-plaques (Plates 8 & 9), it was not a consistent enough feature (Plates 7 & 9), to support Chang's claim that centred-plaque formation was indicative of a pathogenic species. However, centred-plaque formation was never observed with any of the non-pathogenic isolates.

The formation of plaques requires the use of live bacterial cultures for the formation of the bacterial lawn. It was further found that the use of viable bacteria was preferable to the use of heat-killed bacteria in general culture maintenance. The use of live over heat-killed bacterial suspensions for the growth of Naegleria spp. is further supported by the work of Anderson & Jamieson (1974b) who found that Naegleria spp. grew profusely on a number of live commonly-isolated Gram-negative bacilli: Proteus vulgaris, Pr. mirabilis, Escherichia coli, Enterobacter aerogenes, Ent. cloacae, Ent. hafniae, Shigella sonnei, Salmonnella typhimurium and Providencia sp., with the exception of Pseudomonas aeroginosa which produced too dense a lawn for the amebae to multiply. A similar observation was observed in the present study with Klebsiella aerogenes which produces an inhibitory

extracellular slime. It thus seems unnecessary to use heat-killed bacteria as a lawn, except perhaps, for axenizing.

2.2.2 Salt Tolerance

The results of experiments to determine the tolerance of amebae to salt (Tables XIV & XV) indicate that support can no longer be given to the claim that 0.5% (wt/vol) of NaCl incorporated into agar, totally inhibits growth of the pathogens (Singh & Das, 1970; Carter, 1970). Using monoxenic agar cultures, growth was not inhibited till 1.0% NaCl, and it was not until 1.5% NaCl that growth was inhibited when using axenic cultures. Additional evidence supporting growth of the pathogens when subjected to a concentration of 0.5% NaCl in agar comes from van den Driessche *et al.* (1973).

The results of the use of axenic cultures also indicate that the amebicidal activity of salt can be considered to be a direct effect on the amebae itself, and not an indirect effect on the bacterial lawn which serves as the food supply. Presumably, the difference in the amebicidal activity of the salt between monoxenic agar cultures and axenic ones is due to the different feeding mechanisms. It is known that amebae in particle-free fluid axenic state, feed exclusively by pinocytosis (Blowers & Olszewski, 1972) as opposed to the normal phagocytotic mechanism in bacterial-monoxenic agar cultures. The use of salt as a disinfectant is discussed in section 4.2.

2.2.3 Temperature

Table VIII illustrates that all pathogens, with the exception of the Waikato isolates, were able to grow at 45°C and underwent flagellate transformation at 43°C. This was also observed by Griffin (1972) who has suggested that the ability to tolerate growth at high temperatures is directly related to virulence, with

nonvirulent strains unable to grow at normal or elevated body temperatures. To be able to cause PAM, an ameba must be able to multiply in the host and to survive fever, as minimal requirements. That protozoa have the ability to adapt to temperature changes is supported by the work of Dallinger (1887) who over a 5 year period increased the temperature tolerance of the amebo-flagellate Tetramitus rostratus from 22°C to 70°C. These results, plus the information that most human infections have occurred during hot weather after swimming in warm water, support the suggestion that the pathogenic Naegleria are indeed thermophillic species.

The adaptive mechanisms and structural differences responsible for this increased temperature tolerance of the pathogens still remains to be elucidated. However, from studies on thermophillic bacteria, it has been shown that their enzymes and other proteins are much more resistant to heat than those of mesophiles. Furthermore, the protein-synthesizing machinery, i.e. ribosomes, etc., of thermophillic bacteria as well as such structures as the cell membrane, are likewise more resistant. Thermophillic bacteria have membrane lipids rich in saturated fatty acids which form much stronger hydrophobic bonds, thus enabling the membranes to remain stable and functional at high temperatures (Brock, 1974). It may be then that such similar changes at both the biosynthetic and structural level may account for the observed temperature tolerance exhibited by the pathogenic Naegleria. Brock suggests that the relatively low temperature of eukaryotes, as compared to that exhibited by prokaryotes, may be due to the inherent thermolability of the organellar membranes of eukaryotes, especially those present in the nucleus and the mitochondria. The mitochondrial membrane seems especially sensitive to heat, and mitochondria disappear when cells are heated to a few degrees above their maximum temperature (Brock, 1974). As yet, nobody has esta-

blished the cause of the pathogens' temperature tolerance.

2.2.4 Respiration Rate ($^{Q}O_2$) and the Temperature Coefficient of Respiration (Q_{10})

There are two important effects of temperature in aquatic habitats, namely:

1) with a rise in temperature there is a decrease in the amount of dissolved oxygen held in the water. The level of dissolved oxygen ranges from 14.63 mg/l at 0°C in pure water down to 6.74 mg/l at 37°C (Montgomery, Thom & Cockburn, 1964), and
2) to increase the metabolic rate of the organisms and thus the rate at which they remove oxygen from the water (Noland & Gojdics, 1967).

Within the biokinetic zone, 10-45°C, the rates of reactions increase between 2 and 4 times for each 10°C rise in temperature (Giese, 1973). Table XIII shows that there is a quantitative relationship between the environmental temperature and the $^{Q}O_2$, i.e. the volume of oxygen consumed/unit of time.

On the basis of the Q_{10} results, the data can be divided into two groups:

1. comprising NHI, HB-1, and Ng-27, with an average Q_{10} of 1.7, and
2. comprising NTH and PA14, with an average Q_{10} of 3.3.

Just why the Australian strains PA14 and NTH should have a Q_{10} which is approximately double that of the other isolates examined is not known. Could it reflect an ecological adaptation to a specific environment? Evidence for this possibility is that NTH was isolated from a fatal case of a child who "played submarines" in bathwater and PA14 was an environmental isolate from the same house. The house had remained unoccupied for a considerable period of time during hot weather (Anderson et al., 1973). The water, which was chlorinated but unfiltered, was delivered in an exposed overland

pipe which became progressively heated during the summer months (Anderson & Jamieson, 1972b). From the results (Table XIII) it can be seen that it is not until a 10°C rise to 37°C occurs, that any marked divergence in their $^{Q}O_2$ values takes place. This could reflect an adaptation to possible periods of low oxygenation, due to high temperatures and stagnation in the water in the overland pipe, in which case the possession of a higher respiration rate is of possible advantage to compensate for the low oxygen level of the environment.

The results also indicate that Naegleria possess the potential to adapt to a wide range of oxygen concentrations, in a number of different environments, e.g. CNS, running and stagnant water. They also have the ability to successfully adapt to changes in their environmental temperature, as can be seen by the doubling and tripling in their Q_{10} values. What is surprising though, is that the pathogens HB-1 and NHI have no selective advantage over the non-pathogen Ng-27 at 37°C with regard to their individual respiration rates. Thus the $^{Q}O_2$ value is of no use as a taxonomic criterion as was previously thought. The inherent ability of the pathogens to adapt to a wide number of differing environments, plus their ability to grow and multiply in both the free-living and parasitic state, illustrates the versatility of these organisms. One wonders whether the retention of their free-living life-cycle may be the result that the adaptation of the parasitic mode of life is evolutionarily a dead end, and the possession of the free-living state, permits their survival in the wild.

2.3 Serology

As mentioned previously, the specificity and relative rapidity of serological methods provides us with relatively quick, efficient means of differentiating between non-pathogens and pathogens, provided that the appropriate antiserum

is available and the necessary controls are used.

Table IX shows that all pathogens, with the exception of the Waikato isolates, agglutinate to a titre of 1:256-1:512 when using anti N. fowleri sera. Using the same antisera, it was found that the non-pathogens agglutinated to a titre of 1:8, thus suggesting the probability of a common group antigen(s) which is (are) shared between both the free-living and pathogenic species of Naegleria. The agglutination titre of the pathogens was not changed when using Ng-27 adsorbed anti N. fowleri serum, tested previously by its failure to agglutinate Ng-27. Similar results have also been observed by Anderson & Jamieson (1972a) and Van Dijck (1974).

The presence of common group antigens was also observed with both the indirect fluorescent antibody test (IFAB), Table X and the gel-precipitin technique, Figs. 28-33. The IFAB results show that fluorescence, when using antiserum prepared against N. fowleri isolates was observed to a titre of 1:1024 in the case of the pathogens, and to a titre of 1:64 for the non-pathogens. Similarly, both Cerva and Kramar (1973), and De Jonckheere et al. (1974) were able to distinguish between N. gruberi isolates and N. fowleri isolates using IFAB titres.

The gel-precipitin results show that:

1) all Naegleria share a common antigen and are therefore serologically related.
2) all pathogens, with the exception of the Waikato isolates, share a unique common antigen between themselves, which is not shared by N. gruberi, and
3) that there is only partial agreement, as shown by the spur, between the pathogens and non-pathogens.

Visvesvara and Healy (1973) also observed a similar difference in the number of precipitin lines between the pathogenic and non-pathogenic Naegleria. They observed 5 precipitin lines between antigens and antisera from all pathogenic strains and 2 lines against N. gruberi antigens using the same antisera. Immunoelectrophoresis (IE) further confirmed these gel-precipitin studies revealing 10-12 bands between antisera against pathogenic antigens, and 4-6 bands for N. gruberi antigens

when using the same antisera. Willaert, Jadin & LeRay (1972) and Willaert & Jadin (1974) have also observed a clear antigenic distinction between a number of different sarcodine genera using IE techniques, as shown in the following table.

Table XXI. Comparative IE Analysis of Different Genera of Amebae

Antigen	Antiserum	Number of Precipitin Lines
N.fowleri	N.fowleri	27
N.jadini	"	14
N.gruberi	"	8
E.histolytica	"	-
A.castellanii (Neff)	"	-
N.jadini	N.jadini	35
N.fowleri	"	14
N.gruberi	"	12
H.culbertsoni	H.culbertsoni	25
A.castellanii (Neff)	"	12
A.polyphaga	"	12
A.rhysodes	"	12
A.castellanii (Neff)	A.castellanii (Neff)	25
A.rhysodes	"	25
H.culbertsoni	"	12
A.polyphaga	"	12
Tetramitus sp.	"	-
Entamoeba sp.	"	-
Naegleria sp.	"	2

From the writer's results (Tables IX & X, Figures 28-33) and these shown in Table XXI it can be deduced that N.fowleri is serologically related, though distinct from, the other two species of Naegleria. Using anti-N. fowleri sera, the results of Willaert et al. (1972), confirm the absence of parent antigens between Naegleria spp. and H. culbertsoni and E. histolytica as has been the case when using IFAB, agglutin-

ation and gel-precipitin techniques. The difference in titres and the number of precipitin lines between the different reports, emphasize the fact that directly comparative serology is only possible where one is dealing with the same batch of antisera.

It can be seen from the above that the use of serological techniques can also be of immense aid in classification as well as identification, due to their high specificity. For example on the basis of IE, it has been shown that the strain 0400 (N. jadini) occupies a position which is serologically intermediate between N. fowleri and N. gruberi (this strain was isolated from a fishpond, can be grown at 30°C but not at 37°C, and is only slightly pathogenic for mice). IE also supports the view that H. culbertsoni, A. castellanii (Neff), A. polyphaga and A. rhysodes are sufficiently related to be included into one genus, Hartmannella, and that A. castellanii and A. rhysodes are probably the same species.

2.4 Pathogenicity

Tables XI & XII show that only the isolates which were pathogenic to mice, also formed CPE in Vero cell culture. Culbertson (1971) also found this relationship saying that the relationship between cell culture and pathogenicity "is pretty close." Chang (1971 & 1974), has also used cell cultures to differentiate between pathogen and non-pathogen. However, when using cell cultures care must be taken to use a suitable concentration of seeding cell culture inoculum, a suitable inoculum concentration of amebae and of course a suitable cell culture source. For example, using an inoculum of 10^5 cells/cm^3, produces CPE with both non-pathogenic and pathogenic Naegleria. Once these variables are standardized, then the use of cell cultures becomes relatively more reproducible.

There thus seems reliable evidence to support the view that the use of cell cultures can differentiate between N. fowleri and N. gruberi, on the basis of the CPE produced by the pathogens. Further, since the role of the mouse as a definitive test of human pathogenicity is not clearly esta-

blished (due to the variance in susceptibility to infection which is often displayed by mice, Anderson & Jamieson, 1972a) the use of cell cultures may in fact prove to be more reliable as a test for pathogenicity because:

1) it is more quantitative as it allows some standardization of some of the variables used, such as the inoculum concentration,
2) it is more reproducible as it eliminates the individual variation of susceptibility to infection of mice,
3) it allows greater ease of reisolation of the amebae without accompanying contamination, and
4) it is relatively quick, easy and cheap.

The CPE exhibited by N. fowleri in Vero cell culture includes the rounding up of Vero cells, refractility due to the granulation of the host cell cytoplasm and nuclear pycnosis, and finally, the loss of the monolayer. These same CPE are also formed when using the cell-free axenic culture filtrate of the pathogens. The intensity of the CPE formed by the cell free filtrate was directly proportional to the population size and/or age of the culture, as has also been found by Chang (1971). It is thought that this cytotoxic substance liberated by the pathogens, corresponds to the "cytolytic toxin" reported by Chang (1971).

3. Classification and Identification of the Waikato Isolates BH and BL of 1968

The original classification of the 1968 Waikato isolates as a slime mould (Myxomycetale) probably belonging to the genus Echinostelium, Mandal et al. (1970), presents an enigma (Table I), since in the original author's words, "no other human cases have been reported as due to a slime mould." There have been many subsequent critics of this identification (Chang, 1974; Culbertson, 1971; Carter, 1972) as from the description of the organism given in the report, it closely resembles that of a species of Naegleria ... "the ameboid form varied between 9-15 μ in diameter and had a clear hyaline ectoplasm and a granular endoplasm containing vacuoles. A vesicular nucleus with a centrally placed nucleolus could be seen now and then. It moved by throwing out one or two broad pseudopodia ..., giving a characteristic creeping motion. Flagellate forms have an elongated or pyriform shape with 2 anterior placed flagella. The cysts measure 12-18 μ d. and were round, with a smooth wall and no pores ..." Mandal et al. (1970).

Initially the amebae were considered by Mandal et al. to belong to the genus Naegleria. However they later reclassified it as a Myxomycete on the basis of the observation that overpopulation of amebae with subsequent lack of food bacteria, and incubation at 55°C, caused "the organisms to adopt both bacillary and spore forms which later returned to the ameboid form under favourable conditions", Mandal et al. (1970). The authors do not make clear what they mean by bacillary forms. Further the writer believes that the hyphal structures as shown by Mandal et al. in Fig. 3 of the report, were in fact mould contamination. As Chang points out, "the ring structure which the authors believed to be a sporangium was located near a mycelium; but sporangia of endosporous mycetozoa are not formed from mycelia but by protruding lobular masses from a plasmodium."

On receipt of the cultures used by Mandal, the isolates BH and BL (Davies, 1974) were cloned three times and subsequently examined using the preceding methods of classification and identification as outlined in the previous sections. Worthy of mention is that on initial re-establishment of the cultures, they failed to be patho-

genic to mice and were thus thought to have lost their pathogenicity (Davies, 1974). However, all attempts at classification and identification have pointed to the isolates being N. gruberi (Tables VI-XII & XIV). Recloning, and selective growth at 45°C and 55°C, failed to produce any pathogenic strains. Their non-pathogenicity was finally confirmed by both mouse-pathogenicity and Vero cell culture, and thus the isolates have been reidentified as N. gruberi. The most likely explanation for the isolates turning out to be N. gruberi, in the light of their original pathogenicity for mice (Mandal et al., 1970), is that since the original cultures were uncloned, they were possibly mixtures containing both N. gruberi and N. fowleri. Evidence for the isolation of N. gruberi from the CSF comes from both Saygi et al. (1972) and Jadin et al. (1971). On re-establishment of the original cultures as used by Mandal, it is possible that the pathogens were "diluted out" by serial subculture leaving the faster growing N. gruberi. One cannot exclude, however, the slight possibility that the pathogenic N. fowleri had reverted back to the free-living N. gruberi.

4. Disinfection

4.1 The Failure of Chlorine as a Disinfectant

Since the introduction of chlorine into water treatment nearly 73 years ago, it has become almost the sole method used for active disinfection of water supplies (Morris, 1970). Its predominant position has been gained because of the potency and range of effectiveness of aqueous chlorine as a germicide; its ease of application, measurement and control; its freedom from toxic or physiological effects; its reasonable persistence in treated waters and the economy of chlorine. No other competitor has such advantages.

When chlorine is added to water, it reacts:

$$Cl_2 + H_2O \rightleftharpoons H^+ + Cl^- + HOCl$$

HOCl has a K_a^o (dissociation constant) of 2.5×10^{-8}, and is therefore affected by the pH of water. $HOCl \rightleftharpoons H^+ + OCl^-$. At the pH values of concern in water supplies, there is essentially no chlorine present as Cl_2.

As far as disinfection with chlorine is concerned, the active species are Cl_2 and HOCl. The ionic form, OCl^-, is relatively ineffective.

Fig. 39 shows the effect of different cell numbers on the level of total available chlorine (TAC) where TAC = free available chlorine (FAC) + combined available chlorine (CAC). It can be seen that as the cell numbers increase, the TAC decreases as expected, since most "organic compounds" are oxidized by Cl_2 or HOCl due to their high oxidation potential. This represents a loss of chlorine for disinfection and is referred to as the chlorine demand of water, i.e. the difference between the amount of chlorine added and the amount that can be determined analytically as residual chlorine. Residual chlorine includes chlorine in all reducible forms, such as Cl_2, HOCl and OCL^-.

Fig. 40 shows the effect of a maintained chlorine level on a constant population of 10^5 cells/cm^3. The Figure also illustrates Break-point chlorination which is, "the addition of enough chlorine to satisfy the demand of all of the reducing agents present, including organic compounds and

ammonia and its derivatives." The break point (which occurred after 105 minutes, Fig. 40) is passed "at the point where the addition of any more chlorine shows up as exactly the same increase in FAC." From the graph, it can be seen that although the FAC level (the part responsible for the disinfection) reaches up to 8.4 ppm, the amebae are still viable after 225 minutes, as shown by growth on NM agar (Table XIX). Additional evidence to show that normal levels of chlorine have little effect on these organisms, comes from Anderson & Jamieson (1972b) who found that superchlorination up to 10 ppm failed to eradicate Naegleria species, and Brown (1974) who found that 6.5 ppm of chlorine had little effect on A. rhysodes. Since normal chlorination levels which vary from 2-3 ppm in drinking water and swimming pools are ineffective a serious public health risk exists (Cerva, 1971).

The reason of the failure of chlorine as a disinfectant for these organisms is not known. Exactly how chlorine kills is still an academic puzzle. Whatever the chemical action, it is generally agreed that the relative efficiency of various disinfecting compounds is a function of the rate of diffusion through the cell wall or plasma membrane. It is assumed that after penetration of the cell membrane is accomplished, the disinfecting compound has the ability to attack the enzyme group whose destruction results in the death of the organism. With this in mind the failure of chlorine as a disinfectant could be due to:

1) the failure of HOCl to penetrate the cell membrane,
2) the presence of insusceptile enzymes to the action of the HOCl, or
3) dissociation of the HOCl into the relatively harmless forms $H^+ + OCl^-$.

It is known however, that normal levels of chlorination do affect active protozoa as well as other eukaryotes such as helminths and flukes (Gainey & Lord, 1961). Therefore most evidence would suggest alternatives 1 and 3.

It is also known that the uptake of solution molecules in particle free axenic culture, occurs exclusively by pino-

cytosis in the case of A. castellanii (Blowers & Olszewski, 1972). Further, the plasma membrane contains relatively few enzymes, there being no evidence of active transport of sugars or amino acids (Korn & Wright, 1973) and is rich in anionic sites. Because of the relatively small size of the HOCl molecule, it seems likely that (apart from the remote possibility that there is active exclusion of the molecule) it will be taken up by the pinocytosis during feeding. The food contained in these membranous pinocytotic channels is then digested by the activity of the enzymes contributed by the lysosomes. Initially the pH is acidic but then becomes progressively alkaline as digestion proceeds. After digestion, the usable products are transferred to the cytoplasm and any undigested residue is lost via defaecation vacuoles (Chapman-Andresen, 1973).

Because of the K_a^o value of HOCl, the molecule is affected by any change in the pH. It is further known that at an alkaline pH, the HOCl molecule is rapidly dissociated into the relatively harmless forms $OCl^- + H^+$. It may be then, that HOCl is taken up by pinocytosis, but then, is dissociated into its relatively harmless ionic forms, and that this accounts for the failure of normal levels of chlorination as a disinfectant against these soil-amebae.

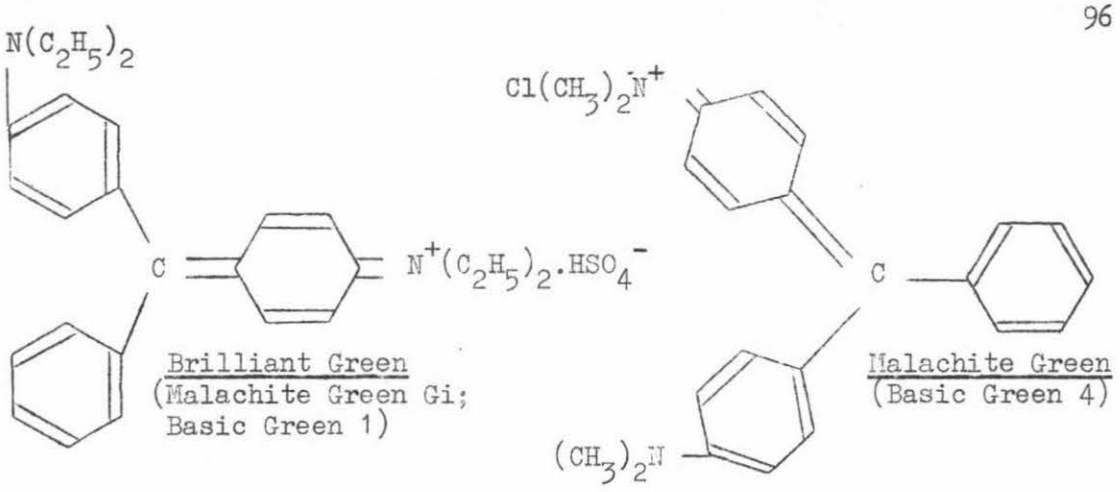
4.2 The Use of NaCl and the Basic Dyes Malachite Green and Brilliant Green as Potential Disinfectants for the Control of Primary Amebic Meningo-encephalitis

The involvement of pinocytosis can also be used to explain the effectiveness of NaCl, malachite green and brilliant green as disinfectants since it is well known that both NaCl and basic dyes are active inducers of pinocytosis (Chapman-Andresen, 1973).

The amebicidal activity of these compounds is possibly due to a "pinocytotic overload" as a result of the induction of a permanent endocytosis. This would result in the rapid internal localization of large amounts of plasmalemma which form the membrane lining the endocytotic vacuoles containing

the disinfectant molecules (Chapman-Andresen, 1972). It is known that such rapid utilization of plasmalemma during intense endocytosis requires a different level of renewal from that of the low plasmalemma renewal rate required in locomotion. Thus the level of surface renewal, which suffices during locomotion, is not adequate for the amount utilized during intense endocytosis (Chapman-Andresen, 1972). This would result in the rapid depletion of membrane precursors, the end result of which would be that the rate of membrane renewal could not keep up with the rate of membrane utilization, thus leaving the ameba with no protective surface membrane. It is further known that the water-expulsion vacuole is also inhibited in pinocytosis, so, creating an osmotic imbalance (Chapman-Andresen, 1961). It is therefore possible, that the ultimate lethality of these compounds as disinfectants, may be a combination of both the phenomena of membrane depletion and osmotic imbalance. That both salt and basic dyes do affect the plasma membrane of amebae can be observed using phase-contrast microscopy, in which locomotion of amebae is inhibited, and the membrane takes on a wrinkled appearance when amebae are incubated in the presence of sublethal doses of these compounds.

The apparent usefulness of salt as a disinfectant is somewhat limited however, when it is taken into account that some fresh-water protozoa can adapt to increasing amounts of salt. Finley (1930) succeeded in adapting 21 fresh-water species, which included members of the 'limax' group to live in undiluted sea-water (3-4% NaCl). The usefulness of the triarylmethane dyes as disinfectants, on the other hand, appears promising. Cerva (1973) found their effectiveness as chemotherapeutics to be approximately equal to that of amphotericin B. The results of the present study confirm this for malachite green at concentrations from 0.5-1.0 $\mu g/cm^3$ (Table XVI), but, in the case of brilliant green, effective disinfection was not obtained till a concentration of 1.5-3.0 $\mu g/cm^3$ was used (Table XVII).



Brilliant Green (Malachite Green Gi; Basic Green 1)

Malachite Green (Basic Green 4)

Cerva further found that with other dyes of the same group, the amebicidal activity was decreased by side chain effects. As yet the potential use of basic dyes as disinfectants remains to be fully investigated in field conditions. Judging from these preliminary results, they should be given full consideration due to their relative high lethality at such low concentrations, and their low cost.

5. Conclusions

1) That Singh & Das' (1970) taxonomic scheme for the classification of the order Amoebida be generally adopted since it appears the most stable and repeatable.

2) That Table XXII gives a compendium of all assayable characters enabling differentiation between the pathogenic N. fowleri and the non-pathogenic N. gruberi.
Table XXIII shows supporting features which are not absolute.

3) That the Waikato isolates BH and BL originally regarded as a myxomycete by Mandal et al. (1970), are strains of N. gruberi.

4) That Naegleria possess the potential to adapt to a wide range of environments and that the $^{Q}O_2$ value cannot be used to differentiate between N. fowleri and N. gruberi.

5) That normal levels of chlorination are ineffective against Naegleria spp. However, 0.8% NaCl, 0.5 $\mu g/cm^3$ of malachite green and 1.5 $\mu g/cm^3$ of brilliant green are useful substitute disinfectants.

Table XXII. Features Used in the Differentiation of *N. gruberi* from *N. fowleri*

Test	*N. gruberi* (non-pathogen)	*N. fowleri* (pathogen)	Reference
Plaque formation over 2-3 days	large	small	Plates 6, 7, 8 & 9
Growth at 45°C	-	+	) Griffin, 1972
Flagellation at 43°C	-	+	) Table VIII
Tolerance of 1% NaCl incorporated into agar	+	-	Table XIV
Agglutination using anti *N. fowleri* serum	1:8 1:16	1:256-1:512 1:256 1:320-1:640	Table IX Anderson & Jamieson, 1972b Van Dijck *et al.*, 1974
IFAB using anti *N. fowleri* serum	1:64 -	1:1024 1:320	Table X Van Dijck *et al.*, 1974
Gel diffusion			Figures 28-33 Visvesvara & Healy, 1973
Immunoelectrophoresis			Visvesvara & Healy, 1973; Willaert *et al.*, 1972, 1974
Cysts with opercula	+	-	Lastovica, 1974; Singh & Das, 1970 Plate 2
Vero cell culture) CPE	-	+	) Table XII
cell-free filtrate) CPE	-	+	) Plates 10,11
Mouse pathogenicity	-	+	Table XI
Growth in Hep-2 cells	+	-	Chang, 1971

Table XXIII. Supporting Features Used in the Differentiation of *N. gruberi* from *N. fowleri*

Test	*N. gruberi* (non-pathogen)	*N. fowleri* (pathogen)	Reference
Formation of Centred Plaques	-	+	Chang, 1971 Plates 8, 9
Opt. growth temperature	30°C	37°C	Table VII

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