PRIMARY AMOEBIC MENINGO-ENCEPHALITIS AND ISOLATION OF THE CAUSATIVE *NAEGLERIA* AMOEBAE FROM ONTARIO WATERS

by

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

November 1974

MASTER OF SCIENCE (1974) McMASTER UNIVERSITY
(Biology) Hamilton, Ontario.

TITLE: PRIMARY AMOEBIC MENINGO-ENCEPHALITIS AND ISOLATION OF THE CAUSATIVE *NAEGLERIA* AMOEBAE FROM ONTARIO WATERS.

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NUMBER OF PAGES: x, 135

SCOPE and CONTENT:

Primary amoebic meningo-encephalitis (PAME) is being recognized with increasing frequency throughout the world since its initial discovery by Dr. M. Fowler in 1961. The disease, which has been fatal in all but two reported cases, is mediated by the amoeba-flagellate, *Naegleria*, which enters the host through a nasopharyngeal route. *Naegleria* was considered a free-living saprophyte until the discovery of PAME.

As of 1973, no PAME cases had been reported in Canada and it was not known whether this was due to incorrect diagnosis of meningitis cases, hostile climatic conditions that precluded pathogenic *Naegleria* growth to infective levels, or complete lack of pathogenic strains in Canada.

Naegleria is easily isolatable from soil and fresh water sources and, consequently, a search for a pathogenic strain was undertaken in southern Ontario. Only water accessible to the public was sampled. A grossly polluted canal yielded a pathogenic strain capable of producing PAME when

intranasally introduced into test animals.

A comparative analysis was carried out using the pathogenic strain and three other isolates selected for diversity of natural environment and/or non-pathogenicity. NB-1, a non-pathogenic laboratory *Naegleria* long used as a tool for the study of cell differentiation, was used as a standard.

The cultural needs, optimal temperatures for growth, the doubling rate, temperature tolerance, rate of transformation from amoeba to flagellate, effect of sub-lethal heat shock during this transformation process, cyst survival ability, resistance of amoebae and cysts to desiccation with alcohol and soap solutions, as well as various morphological measurements and taxonomic observations of the amoeba, flagellate and cyst phases were determined for the various strains.

The most distinctive and consistent feature found within the five strains was the thermophilic nature and temperature tolerance of the pathogens. Non-pathogenic strains failed to thrive at temperatures above normal body temperature. In addition, the cyst walls of the most virulent pathogen did not have distinctive pores, a feature normally found in *Naegleria*.

Experimental pathologic testing of the pathogenic strain showed a positive relationship between inoculum size and fatality rate. Tests showed that the rate of

intracranial infestation with *Naegleria* exceeded the number of individuals that succumbed to the disease. Also, some mice that appeared to be near-death from PAME were capable of recovering.

The virulence of the pathogen was not affected by the media used for sub-culturing and the age of the test animals had little effect on the fatality rate.

The pathogenic strain demonstrated a virulence and survival time (i.e., period from time of inoculation until death from PAME) that correlated well with studies conducted by other investigators using pathogenic *Naegleria* isolated directly from PAME victims.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. A. D. Dingle.

I would also like to thank J. J. Darley for his valuable assistance during the electron microscope studies. To Dr. J. Mills-Westermann, Dr. E. L. McCandless, and H. Bacchus, thank you for your advice and efforts on my behalf. And last but not least, I am especially indebted to the Biology office staff and to my children, Lynne and Dale.

To my husband, a truly beautiful person.

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I. INTRODUCTION

The amoebo-flagellate, *Naegleria*, has long been used as a useful tool for the study of cell differentiation. Transformation of amoeboid- to flagellate-form is rapid, synchronous and reproducible with a brief latent period during which all specialized components of the flagellar apparatus are structured *de novo* (Fulton, 1970).

Within the past few years *Naegleria* species have also been identified as facultatively pathogenic organisms (normally free-living but capable of infecting humans) and is the agent responsible for primary amoebic meningo-encephalitis (PAME) in man. Pathogenic strains of *Naegleria* are found in a wide variety of habitats. The pathogen enters the host via a naso-pharyngeal route. Although there have been outbreaks of *Naegleria*l meningo-encephalitis in North America, to date none has been recorded in Canada. This may be due to our climatic conditions which would not normally favour rapid proliferation of pathogenic amoebae or it might possibly be due to lack of knowledge in identifying amoebae infestation as the primary cause of death in meningo-encephalitis cases or, the third possibility, pathogenic strains may not be present in Canada.

General *Naegleria* Description

At present there is still much confusion in the scientific journals over the correct nomenclature to be assigned to *Naegleria* amoebo-flagellates. For the purpose of this study, Page's (1967) classification will be adopted:

Class:	Rhizopodea, von Siebold, 1845
Subclass:	Lobosia, Carpenter, 1861
Order:	Amoebida, Kent, 1880
Family:	Vahlkampfiidae, Jollos, 1917
Genus:	*Naegleria*, Alexieff, 1912; emend. Calkins, 1913

There are now two recognized species; *N. gruberi* (Schardinger, 1899) and *N. fowleri* (Carter, 1970). *N. fowleri* was named for Dr. M. Fowler, the first to diagnose PAME in a human and isolate the causative amoeba. This species is not included in Page's classification.

As indicated, there are now two recognized species, *N. fowleri* and *N. gruberi*. Chang (1971) named his pathogen *N. invadens* while Singh and Das (1972a) use the term *N. amoebida* but it is believed that they are all the same species and, therefore, Carter's nomenclature, being of an earlier date, has priority. Recent findings suggest that there may be intermediate strains between the pathogenic and non-pathogenic strains. These intermediate strains, which have been isolated from the environment, are serologically identical to *N. fowleri* but show greatly reduced or no pathogenicity for laboratory mice (Anderson and Jamieson, 1972a).

Not only is there some confusion over the nomenclature but there is also much debate over the exact characteristics of *Naegleria* species. Some features are common to all strains and these include the following: only the amoebae under-

division and this is characterized by intranuclear karyokinesis, a process whereby the nuclear membrane and nucleolus fail to disappear during division; the amoebae range from 10μm to 40μm in length, possess lobose pseudopodia, a distinctive nucleus with a prominent nucleolus, and one or more contractile vacuoles; the amoebae under adverse conditions form round, double-walled cysts ranging from 6μm to 18μm in diameter and excystment may be brought about by providing a suitable nutrient source; if suspended in an aqueous environment, the amoebae will readily transform into a flagellated form which lacks a cytostome (mouth parts) and usually has two anteriorly placed, equal flagella but may have more; the flagellates are transient and revert to the amoeboid-form (Fulton, 1970; Page, 1967).

Many attempts have been made to definitively characterize the genus. Page (1967) defines *Naegleria* as "all amoebae with the promitotic pattern of nuclear division and with production of a temporary biflagellate phase lacking a cytostome". Fulton (1970) says that 'promitosis' is a misnomer when applied to *Naegleria* division and rather it should be described as "intranuclear mitosis with persistent nucleolus" which he terms intranuclear karyokinesis. *Naegleria* species are not necessarily biflagellate but rather may undergo a change in flagellum number due to ploidy change or adverse conditions that affect the flagellum synthesis stage (Dingle, 1970). Other strains characteristically have some members in the flagellate phase with greater than two flagella. Odd numbers may be due to

loss of one flagellum or an abortive attempt to form two. In any event, flagellum number cannot be used as a strict generic criteria (Fulton, 1970; Fulton & Dingle, 1967).

Fulton (1970) lists the following elements as essentials for classification within the genus *Naegleria*: "(a) intranuclear mitosis with polar masses and an interzonal body, (b) cysts with several preformed pores through which the amebae excyst, (c) temporary flagellates with about 2 anterior and equal flagella". However, Page (1967) reports that interzonal bodies are not invariably present even in one species of *Naegleria* although this characteristic has been used in the past as a diagnostic tool (Chang, 1958; *ibid.* 1970; Singh, 1952). All *Naegleria* form cysts but there is some debate as to whether all pathogenic strains possess pores and/or plugs in their cell walls (Carter, 1970; Culbertson *et al.*, 1968; Sáygi *et al.*, 1973).

Naegleria may be found from the Antarctic to the Arctic and although usually found at the surface of rich soil, it has been found at depths of six meters (reference in Fulton, 1970). Practically any fresh water source will yield *Naegleria* strains though sea water is a hostile environment (Page, 1967; Carter, 1970). Pathogenic strains appear to be more restricted but have been isolated from sewage sludge (Singh & Das, 1972a), tap and super-chlorinated swimming pool waters (Anderson & Jamieson, 1972b), soil samples (Anderson & Jamieson, 1972c),

and lakes (Butt, 1966). The preponderance of this particular amoeba (pathogenic and non-pathogenic) in fresh water habitats was demonstrated by Jamieson and Anderson (1973) who isolated 130 strains of *Naegleria* from 400 water samples and, of these, two were pathogenic to mice.

Most strains may be satisfactorily maintained in the laboratory using gram negative bacteria (e.g., *Escherichia coli*, *Aerobacter aerogenes*, etc.) as the nutrient source. The life cycles of *Naegleria* species in their natural habitats is not known and may well vary from that found under laboratory conditions. Since it is now known that *Naegleria* can invade and proliferate on meningeal tissue and other cell debris, it is conceivable that suitably adapted species may thrive in a number of diverse environments other than the normal soil and/or water habitats.

Comparison of Pathogenic and Non-Pathogenic Characteristics

As with most of the literature concerning *Naegleria* there is a diversity of opinion not only on the similarities but on the differences found between the pathogenic and non-pathogenic strains.

(a) Nutrient source

As mentioned previously, *Naegleria* will proliferate on gram negative bacteria. In the wild, the amoeba probably feed on faecal bacteria found in organic matter, sewage, water or moist soil. *N. fowleri* is more fastidious in diet when grown

under laboratory conditions. The original PAME isolate in Australia proliferated only on plain agar seeded with live *E. coli* (Carter, 1970). Anderson and Jamieson (1972a) have now gotten this same isolate to grow axenically on an agar medium if *E. coli* are heat killed at 60°C for 1 hour. Carter (1970) found that 0.5% NaCl in agar completely inhibited growth and the amoebae disappeared. Van den Driessche (1973), however, grew his isolate from a PAME victim in Belgium on 0.5% salt media. Pathogenic strains have successfully been grown in liquid axenic cultures (Cerva, 1969; Anderson & Jamieson, 1972b).

The general consensus appears to be that the non-pathogenic strains will grow on plain or saline agar (Page, 1967) while the pathogenic strains are much more successful on plain agar (Carter, 1970; *ibid.*, 1972; Culbertson, 1970; Culbertson *et al.*, 1968; Singh & Das, 1972; Jamieson & Anderson, 1973; Butt *et al.*, 1968). The liquid axenic culture method, because of its defined components, would be the ideal medium for growth, biochemical and immunological studies (Cerva, 1969; Fulton, 1974). However, establishing amoebic growth in such a medium is often difficult and time consuming and not suited to a short term study such as this.

(b) Temperature Tolerance and Range

Temperature tolerance is perhaps the most striking and consistent difference found between the pathogenic and non-

pathogenic varieties. All pathogenic strains can tolerate and proliferate at temperatures well above normal body temperature, whereas non-pathogenic *Naegleria* may manage to grow at 37°C but will not thrive. Temperatures several degrees below normal body temperature are usually optimal (Fulton, 1970; Griffin, 1972). Pathogenic varieties survive at room temperature but grow slowly. Carter (1970) found that placing *N. fowleri* amoebae at temperatures of 0°-4°C for periods less than 16 hours rendered them non-viable. Pathogenic amoebae can survive at 46°C, well above the highest human fever (Griffin, 1972).

(c) Cyst Survival

Pathogenic strain cysts are not resistant to freezing or drying but can survive in sea water (4% NaCl) while the amoebae cannot. The cysts can, however, be stored on agar plates and kept in a moist chamber at 21°C for periods up to, and possibly exceeding, 18 months. If stored at -15°, 4°, or 37°C the cysts become non-viable after two months (Carter, 1970). Duma *et al.* (1969) dried and stored PAME isolate cysts for a minimum of 23 months. Non-pathogenic strains have been stored for extensive periods of time on agar plates, or dried. Fulton (1970) reports that NB-1 cysts have survived seven years storage.

(d) Cytology

Pathogenic strains have dumb-bell shaped mitochondria (Martinez *et al.*, 1971). These findings concur with Carter's (1970) who described the mitochondria as "dumb-bell" shaped, "although truly cup-shaped and oval forms are seen occasionally". The endoplasmic reticulum is less conspicuous in the pathogenic strains and not closely applied to the mitochondria (Carter, 1972).

The nucleus diameter of pathogenic and non-pathogenic strains ranges from 2.8μm - 5.5μm (Page, 1967; Carter, 1970) but may be as small as 2.6μm (Cerva *et al.*, 1968). In the cyst stage, the non-pathogenic strains have a nuclear diameter ranging from 3.3μm - 5.2μm (Page, 1967) while the pathogenic *N. fowleri* cyst has a mean nucleus diameter of 1.5μm (Carter, 1970). The cyst nuclear diameter is smaller than either the amoeboid- or flagellate-phase nucleus according to their findings. Carter (1970) reports a mean nucleus diameter of 2μm in the flagellated *N. fowleri* species, which although it is larger than the nucleus diameter found in the cyst is still smaller than that generally found in the amoeboid form.

There are usually two flagella, approximately 12μm - 15μm long, in all species during flagellation.

With regard to cyst wall structure, Carter (1970) claims "pores and associated plugs are not seen" in the pathogenic strains. Other investigators do not insist on the absolute lack of pores but rather have found that pathogenic strains

do possess pores but not in as great a number as found in the non-pathogenic strains. For example, Culbertson *et al.* (1968) found "only a minority of them have the pore-like structure filled with hyaline or 'structureless' substance"; Duma *et al.* (1969) reported that "their walls are smooth and contain several apertures"; and Saygi *et al.* (1973) found "occasional pores in the walls".

There seems to be little disagreement concerning the presence of pores in strains considered at present to be non-pathogenic. The cysts usually possess from two to ten pores containing plugs (Fulton, 1970; Page, 1967) although Chang (1958) reports "one or two but rarely three pores per cyst".

During excystment the amoebae leave via these pores but possibly the cyst wall is ruptured during excystment in the pathogenic strains (Carter, 1970). Carter feels that the most distinctive feature of *N. fowleri* is the "strangely, empty, non-nucleated cysts that appear to be intact and alive, but are non-viable on subculture".

(e) Dimensions

Great variations occur among isolates and even between members of a particular strain. Much of the size difference may be due to the nutrient source and incubation temperature. Chang (1958) found that *N. gruberi* measured directly from river water samples had a diameter of 22μm but after being grown in the laboratory was only 18μm. The measurements of some pathogenic

amoeba isolates are as follows: the Australian isolates have ranged from 15µm-30µm (Carter, 1970); Cĕrva *et al.* (1969) in Czechoslovakia reported amoebae with a mean length of 7.4µm; Saygi *et al.* (1973) in Britain isolated amoebae 14.4µm x 22.8µm from a PAME case; while in the U.S.A., isolates ranging from 15µm to 40µm have been reported (Cotter, 1973; Butt, 1966). Non-pathogenic *N. gruberi* average 14.5µm-33µm when elongated (Page, 1967) and 10µm-20µm in diameter when rounded (Fulton, 1970). All species while in elongated locomotion have a general length:breadth ratio of approximately 3:1.

The flagellates range from 10µm-29µm (average 17.5µm) in length for the non-pathogenic strains. *N. fowleri* has bodily dimensions averaging 8µm x 15µm. Flagellates of all strains are usually fusiform with two anteriorly placed flagella but may be rounded or may retain the amoeboid shape in some cases (Carter, 1970; Page, 1967).

Non-pathogenic strain cysts range from 6.0µm-18.0µm in diameter (average 11.5µm) while *N. fowleri* cysts have a mean diameter of 9.0µm (Page, 1967; Carter, 1970). The isolate from a PAME case in Britain had a mean cyst diameter of 15.6µm (Saygi *et al.*, 1973).

It would appear that pathogenic and non-pathogenic *Naegleria* strains cannot be distinguished by size whether in the amoeboid, flagellate or cystic phase.

TABLE I

Summary of Pathogenic and Non-pathogenic *Naegleria* Characteristics*

Feature	Pathogenic	Non-pathogenic
a) Nutrient requirements	Plain agar + live bacteria** Axenic culture	Plain or saline agar Axenic culture
b) Temperature tolerance	≥46°C	≤37°C
c) Cyst survival time	>23 months (@21°C) ≤2 months (@-15, 4, 37°C)	7 years
d) Cytology		
-mitochondria	"dumb-bell" shaped occasionally ovoid	ovoid
-nuclear diameters		
amoeba	2.6μm-5.5μm (range)	2.8μm-5.5μm (range)
flagellate	2μm (mean)	-***
cyst	1.5μm (mean)	3.3μm-5.2μm (range)
-Number of flagella	=2 (12μm-15μm length)	=2 (12μm-15μm length)
- Cyst wall structure	Few if any pores**	2-10 pores always present
-Excystment	Rupture of cyst wall**	via pores
e) Dimensions		
-amoeba length	5μm-40μm (range)	14.4μm-33μm (mean range)
-flagellate length	15μm (mean)	17.5μm (mean)
-cyst diameter	9.0μm (mean); 15.6μm (mean)	6μm-18μm (range) 11.5μm (mean)

*See preceding text for details and references
**Data in dispute
***Not given in literature

History and Description of Primary Amoebic Meningo-Encephalitis

The term 'primary amoebic meningo-encephalitis' (PAME) was coined by C. G. Butt (1966) and describes infection of the central nervous system in human beings due to free-living amoeba, as distinguished from rare secondary invasion of the brain by *Entamoeba histolytica*. The disease is being recognized with increased frequency and appears to be worldwide.

In 1957, C. G. Culbertson found an agent thought to be a simian virus in a primary monkey tissue culture which caused the deaths of mice and monkeys after intracerebral injection. At autopsy he noted live, motile amoebae. Intranasal inoculation of mice with these amoebae produced meningo-encephalitis and Culbertson at this time suggested that a similar disease might be found in humans (Culbertson, 1958; Culbertson *et al.*, 1959). This research led to the first human diagnosis of PAME in 1961 by Dr. M. Fowler in Australia (Fowler & Carter, 1965), followed shortly by a case in the U.S.A. (Butt, 1966). As of the end of 1972, close to 100 cases of PAME have been reported throughout the world; all but two of these have proved fatal (Cotter, 1973).

Approximately one half of the known PAME cases have been diagnosed in retrospect (Dos Santos, 1970). Prior to 1965, cases had been identified as various types of viral or bacterial meningitis or, as in one case from 1909, 'meningitis carcinomatosa' (Symmers, 1969). PAME resembles fulminating

bacterial meningitis but the purulent cerebrospinal fluid does not contain pathogenic bacteria. The lack of bacteria in the fluid samples does not necessarily alert the physician or pathologist if antibiotics have already been administered. To differentiate between PAME and other meningeal diseases, Carter (1972) recommends microscopic examination of wet mounts of cerebrospinal fluid. The live amoebae are highly motile and the formation of pseudopodia help in identifying them from other host cell types. Others have found the amoebae extremely difficult to discern in the fluid samples (Apley *et al.*, 1970) and recommend culturing the specimen on plain agar plates with live *Escherichia coli* at 37°C. This leads to a minimum 24 hour delay in diagnosis. Although the amoebae have a large nucleus with a distinctively prominent nucleolus, they can easily be mistaken for gitter cells in stained brain tissue preparations. Only if the pathologist is aware that there may be amoebic infestation is there much likelihood that the trophozoites will be noted (Symmers, 1969). Cysts are not found in the diseased tissue.

PAME appears to be worldwide and cases have been reported from Britain (Apley *et al.*, 1970), the U.S.A. (Butt, 1966; Butt *et al.*, 1968; Callicott, 1968; Callicott *et al.*, 1968; Patras & Andujar, 1966), India (Singh & Das, 1972a), Belgium (Van den Driessche, 1973), and Czechoslovakia (Cěrva & Novák, 1968; Cěrva *et al.*, 1968). Epidemics have been

reported in some of these areas. For instance, between 1962 and 1965 sixteen cases were traced to an indoor heated swimming pool in Czechoslovakia (Cĕrva *et al*, 1968), seven persons died in 1950 and 1951 in Richmond, Virginia (Callicott, 1968), and certain areas of Southern Australia may be such that the disease is endemic to the region (Carter, 1972; Jamieson & Anderson, 1973).

Patients often give a history of swimming in fresh or brackish water and the portal of entry in the majority of cases would appear to be the nasal mucosa. In Australia, however, most of the PAME victims had either not been swimming or had done so in salt water which is amoebicidal (Carter, 1972). Some of them had been known to completely submerge themselves in their bathwater or other fresh water sources. After the appearance of the disease, pathogenic *Naegleria* were isolated from the victims' residences, and neighbourhoods; i.e., chlorinated tap water, bath tub drains, watering troughs, etc. (Carter, 1968; Anderson & Jamieson, 1972a; *ibid.*, 1972b). In Britain, two positive and one suspected case of PAME are thought to have resulted from three children playing in a mud puddle. Intranasal inoculation would be possible in all of these cases. There have, however, been cases reported where intranasal infiltration of amoebae seems highly unlikely (Patras & Andujar, 1966; Carter, 1968; *ibid.*, 1972) but no other route of entry has yet been found.

Cases normally occur either in the hottest time of the year or in heated swimming pools or hot springs.

Patients are equally divided between the sexes but usually range from seven to twenty years of age, suggesting swimming or sewage contamination rather than food contamination or air transmission. Singh and Das (1972b) found that the flagellated-phase of pathogenic *Naegleria* is more infective and had greater virulence than the amoeboid-phase. Animal studies using cysts for the intranasal and intracerebral inoculation have failed to cause PAME in all cases and there is no evidence of natural transmission from one animal to another (Carter, 1970; Culbertson, 1970). Carter (1970; *ibid.*, 1972) found that amoeba are normal and mobile in an environmental pH 4.6-9.5 but were immobilized by levels below pH 4.3, by bile salts, or by human serum. These findings would tend to preclude amoebic infestation via an open wound (i.e., the circulatory system) or the gastro-intestinal tract.

The intranasal path of invasion has been studied with light and electron microscopy. These studies demonstrate that the amoebae disrupt the olfactory mucosa and are possibly phagocytosed by the sustentacular cells present within the mucosa. These cells are then destroyed either by amoeboid movement or enzymatic digestion, or possibly both. The submucosal nervous plexus is then invaded and the amoebae pass through the cribriform plate to the central nervous system (Martinez *et al.*, 1971;

ibid., 1973a; *ibid.*, 1973b). Electron microscopic studies by Maitra *et al.* (1974) support the findings concerning entry by way of the olfactory mucosa as well as show clear zones around the amoebae in the brain tissue. The surrounding host cell membranes appear damaged, suggesting that secretions from the amoeba may have damaged the host tissue.

In all cases of PAME investigated to date, the symptoms and temporal aspects are similar. The victim usually enjoys robust good health until beset by an abrupt headache and mild fever three to seven days after the *Naegleria* inoculation (Cerva *et al.*, 1968). Occasionally rhinitis and a sore throat accompany the first symptoms. Over the next three days, the headache intensity and fever increases and the patient suffers neck rigidity and vomiting. By the end of the third day, all patients are severely disoriented, if not actually comatose, and have been diagnosed as suffering from acute pyogenic meningitis. Death from cardio-respiratory failure occurs on the fifth or sixth day of the illness. At autopsy the olfactory bulbs are always markedly reddened and often severely haemorrhagic and necrotic. The olfactory mucosa is severely ulcerated. Amoebae are present in portions of the brain but the sites and degree of encephalon infestation may vary markedly (Carter, 1972).

The similarity of the course of PAME in mice and humans allows the use of mice as suitable models (Carter, 1970;

Martinez *et al.*, 1973a; *ibid.*, 1973b).

The temporal and symptomatic aspects of the disease in experimental pathology testing is as follows; mice after intranasal inoculation appear normal for an initial three day period. On the fourth day they become less mobile, scratch their heads vigorously, and their hair begins to bristle. On the fifth day the mice assume a hunched curled position with circling disoriented movements. Their faces are puffy while the body appears dehydrated. Death usually occurs on the sixth day with the animal shrunken; brain swollen and haemorrhagic necrosis present; lungs and kidney engorged with blood; spleen greatly enlarged and the liver soft and enlarged. Carter (1972) notes that mouse blood lacks the amoebicidal qualities found in human serum and therefore amoebae are found not only in the brain tissue but in other organs of the body. This is in contrast to human PAME where the amoebae infestation is restricted to the brain. It is possible to infect mice intranasally, intracerebrally and intravenously but not via the respiratory or alimentary tracts (Culbertson *et al.*, 1959; *ibid.*, 1968; Carter, 1970).

Serial killing studies have shown that by 36 hours after intranasal inoculation the amoebae have penetrated the nasal mucosal membrane; by 72 hours, they are found in parts of the olfactory lobes; by 84 hours, they have spread throughout the olfactory lobes; and by 96 hours, have spread into

the cerebral hemispheres (Carter, 1970).

The pathogenicity of a particular *Naegleria* strain should not, however, be based solely on mouse pathogenicity studies. There are varying degrees of resistance found within mouse strains which are a determining factor in whether the inoculum proves fatal or not (Griffin, 1972). The amoeba strain may also influence the test results as demonstrated by Saygi *et al.* (1973) who isolated amoebae from a PAME victim in Britain but found that it was non-pathogenic to mice.

All pathogenic strains grow well at temperatures well beyond the highest human fever and *N. fowleri* can proliferate at temperatures up to 46°C (114.8°F), whereas, non-pathogenic strains fail to grow at normal or elevated body temperatures (Griffin, 1972).

Agglutination tests suggest the presence of a common group antigen within pathogenic strains. Cross-agglutination antibody are removed by absorption with a suspension of non-pathogenic strains and yet the serum continues to agglutinate pathogenic strains (Anderson & Jamieson, 1972a). This may possibly prove to be the most accurate classification tool for pathogenic versus non-pathogenic strains.

To date the only drug that appears to have any therapeutic value is a fungicide, Amphotericin B. Massive doses of this drug led to the recovery of a PAME victim in Britain (Apley *et al.*, 1970) and another in Australia (Carter, 1972).

To be effective the drug must be given intravenously in very large dosages. Amphotericin B is highly toxic and treatment is complicated by phlebitis, anemia and nephrotoxicity (Utz *et al.*, 1964). Early diagnosis is imperative as death follows rapidly after the onset of PAME symptoms. Since the amoebae actually devour the tissue involved, even if a fatality were avoided, there is a danger of permanent brain damage if the disease continues for any length of time. Several other antiamoebic agents and antibiotics have been tested but none have proved effective (Culbertson *et al.*, 1968; Carter, 1970; Duma, 1970; Das, 1971).

Ecological Aspects of Pathogens

Since the amoebae may be isolated from soil samples, they are free-living amoebae and can easily contaminate lakes, pools, etc. (Anderson & Jamieson, 1972e). A link between fecal matter and the selective growth of pathogenic free-living amoebae seems apparent (Apley *et al.*, 1970; Singh & Das, 1972a). Industrialized societies via thermal pollution combined with increased fecal discharge from larger populations may result in the selection of the more virulent thermophilic *Naegleria* by decreasing the growth rate of the non-pathogenic, low-temperature tolerant species. Each degree that the water temperature is increased favours the pathogenic strains.

Lamirande in O.U.C. Diving News (1970) points out the possible similarities between Lake Spier, Florida, where PAME cases have occurred, and lakes in Ontario. Lake Spier is a

recreational lake surrounded by vacation cottages. Septic tanks, organic debris, lawn fertilizers and pesticides have increased the nutrient level of the lake and vegetative and bacterial growth is optimal for pathogenic *Naeglerial* growth. The particularly virulent strains of *N. fowleri* can produce death in a mouse with an inoculation of only 39 amoebae while an inoculum of 300 amoebae will consistently prove fatal to 50% of the mice inoculated intranasally (Carter, 1970). From this it is quite apparent that anything that increases the pathogenic population in a fresh water source is potentially dangerous. In the future, along with the increase in Ontario's population and their leisure time, will possibly come greater pollution of the fresh water recreational areas and an increased opportunity for pathogenic *Naegleria*. This may prove particularly hazardous to scuba divers and others who spend considerable amounts of time in the water during the hot months of the year.

Dos Santos (1970) reviewed 16,174 autopsies (1920-1968) from the Richmond, Virginia, area and found five previously unknown and unpublished PAME cases. The Vital Statistics for 1971 (Province of Ontario) reported 12 fatalities from viral encephalitis and 7 from meningococcal infections; in 1972, there were 17 and 28 deaths respectively. This is not to infer that any of these cases were incorrectly diagnosed but rather that the medical profession should be made aware of

the potential danger to ensure a rapid and accurate diagnosis should the disease occur. If pathogenic strains could find a suitable environment and survive the rigors of an Ontario winter then there is a definite possibility that PAME has, or will, occur in Canada.

Since it was not known whether pathogenic strains of *Naegleria* were present in Canada, this research was concentrated on the isolation of a pathogenic strain (if possible) and a comparison of its characteristics with other non-pathogenic strains isolated from fresh water sources in Ontario. To add to the relevance of the study, water samples were taken only from sources which were used by, or were accessible to the public (i.e., canals, recreational ponds and streams, swimming pools, and fish tanks; sewage treatment plants were excluded).

II. METHODS AND MATERIALS

In order to be as brief and as concise as possible, the following materials were used consistently during the course of the research project. Unless otherwise stated, methods and materials are similar to or are modifications from Fulton (1970).

(a) Tris - 2mM Tris (hydroxymethyl) aminomethane-HCl buffer, pH 7.4, prepared with de-ionized water.

(b) Lugol's Iodine - 4 g. I_2 and 6 g. KI dissolved in a few drops of de-ionized water and diluted to 100 ml.

(c) Amoeba saline (Page, 1967) - a dilute salt solution, pH 6.8-7.0, consisting of NaCl, 0.120 g.; $MgSO_4 \cdot 7H_2O$, 0.004 g.; $CaCl_2 \cdot 2H_2O$, 0.004 g.; Na_2HPO_4, 0.142 g.; KH_2PO_4, 0.136 g.; de-ionized water to make 1 litre.

(d) Washing - All washing of cells was done by suspending the cells in Tris in 50 ml. plastic centrifuge tubes, vortexing vigorously (Vortex-genie) and then centrifuging for 45 seconds at a setting of 5 (Swinging Bucket International Clinical Centrifuge, Model CL). A manually operated mechanical brake is built into the lid of the centrifuge and allows rapid stoppage of the rotor by applying manual pressure. Cells are usually washed three times to remove bacteria.

(e) Sterilization - All contaminated glassware was soaked in Liqui-Nox, washed thoroughly and sterilized using dry heat

(180°C for 3 hours). All media and sterile water were autoclaved for 15 minutes at 18 p.s.i. Whenever possible, disposable equipment was used and this included the sampling tubes, petri dishes, syringes, gloves and face masks. These were deposited in heavy polyvinyl bags and incinerated.

Mice that either died of an illness or were sacrificed were treated in the following manner: All counters were scoured with Liqui-Nox solution followed by 70% alcohol. All surgical tools were kept in 70% alcohol and flamed before use. Animals were dipped in 70% alcohol to destroy contaminants that may have been present on their exterior. Surgical gloves and mask were worn at all times. The cranial contents (including the olfactory bulb), the liver, the lungs, and the heart were removed and plated on non-nutrient agar with live *A. aerogenes*. This was then incubated in a humid 37°C incubator for a minimum of 24 hours. All animal parts and incubated tissue were incinerated.

To test for the possibility of cross-contamination of animal tissue cultures, 42 mice healthy three weeks after amoebae inoculation with a non-pathogenic isolate were sacrificed and plated in the above manner. No *Naegleria* growth or contamination was found in any of these test tissue culture dishes after incubating at 37°C for several days.

(f) Cell counts - Cell concentrations were estimated using a Coulter Counter, Model "F" (Coulter Electronics Inc., Hialeah,

Fla.). Sensitivity settings providing suitable accuracy for the various strains were as follows: threshold 20, attenuation 2, and aperture 64.

The cell suspension to be counted was diluted in an electrolyte (0.4% NaCl in de-ionized water) to give counts ranging up to 5000. The diluted cell suspension is vortexed vigorously to prevent clumping of cells. The Coulter Counter absorbs a measured volume (0.5 ml.) and records the total cell count found within that volume. The average of three counts was used to calculate the number of cells/ ml. in the original cell suspension.

(g) Light microscopy - Unless otherwise noted, all light microscopy was done using a Leitz-Wetzlar phase microscope with a Phaco 40/0.65 objective lens (x400) and a blue filter. Measurements were made using a Nikon Filar Micrometer which was calibrated against a stage micrometer with divisions 0.1 and 0.01 mm. (Bausch & Lomb Optical Co.).

(h) Media - Isolates were maintained and grown on either NM (Fulton & Dingle, 1967) or NNA (non-nutrient agar) plates with live *Aerobacter aerogenes* as amoebae nutrient in all cases. The bacteria provide the nutrient source for *Naegleria* and the amoebae derive no nutrition from the media. The various media are as follows:

(i) NM consists of 1.6 g. Difco Bacto-Peptone; 1.6 g. dextrose; 1.2 g. K_2HPO_4; 16.0 g. Difco Bacto-Agar;

0.8 g. KH_2PO_4 and 800 ml. of de-ionized water. The medium is autoclaved at 18 p.s.i. for 15 minutes, cooled to approximately 45°C and poured into sterile petri dishes with approximately 20 ml. per dish.

(ii) NNA contains 2% Difco Bacto-Agar in de-ionized water, prepared in the same manner as NM.

(iii) PM contains the same components as NM, except the Difco Bacto-Peptone is increased from 1.6 g. to 3.2 g.

All media were stored at 12°C until used. NM and PM are both suitable growth media for *Aerobacter aerogenes*.

(iv) *A. aerogenes* was cultured using Penassay Broth (PAB), a medium consisting of 17.5 g. Difco Bacto Antibiotic Medium 3 per litre of de-ionized water. The medium is dispensed at 6 ml. per stoppered culture tube, autoclaved, and stored under refrigeration.

(v) PAB-grown *A. aerogenes* was prepared by inoculating a tube of PAB-medium from an *A. aerogenes* stock slant (PAB-medium containing 20.0 g. Difco Bacto-Agar per litre of PAB-medium) and incubating overnight at 34°C. The resulting stationary phase PAB-culture ($\simeq 10^9$ bacteria/ml.) was then stored at room temperature and used for several days.

(iv) PM-grown *A. aerogenes* was required to provide a a dense bacterial lawn on NNA plates. Approximately 0.1-0.2 ml. of PAB-grown *A. aerogenes* was pipetted onto a PM plate, the bacterial culture spread with a glass rod and incubated at 34°C for a minimum of 18 hours. The glass spreader is stored in 70% ethanol and flamed lightly before use. The bacterial lawn was removed after incubation with the glass rod and sterile distilled water and centrifuged at 7,000 r.p.m. for 15 minutes in a Servall Angle Centrifuge (Ivan Sorvall, Inc., Norfolk). The pellet was resuspended in a minimum amount of sterile de-ionized water, vortexed and 1.0 ml was spread lightly on NNA plates with the glass spreader.

(i) Plates -

(i) Spread plates - a wire loopful of cysts of a particular strain was suspended in 0.5 ml. sterile de-ionized water, vortexed vigorously and approximately 10^5 cysts and 0.1 ml. *A. aerogenes* (PAB-grown) was spread on NM plates. If NNA plates were used, then 10^5 cysts with 1.0 ml. *A. aerogenes* (PM-grown) was spread on NNA plates with a glass rod. The plates were then inverted and incubated at the desired temperature.

(ii) Edge plates - may be either NNA plates spread with PM-

grown bacteria or NM plates spread with 0.1 ml. PAB-grown bacteria. A spot containing cysts or amoebae was then placed on one side of the plate and allowed to grow across the entire diameter of the petri dish.

(iii) Double stripe plates - two parallel stripes of bacteria on an NM or NNA agar plate surface. The cyst or amoeba sample was placed on the end of one of the stripes. Amoebae grow along the stripe and migrate across the sterile, non-nutrient (i.e., bacteria lacking) agar to the other stripe. This method eliminates undesirable organisms and may be repeated several times if a persistent contaminant remains. All isolates were grown on double-stripe plates one or more times before an attempt was made to clone them.

(iv) Stock plates - all strains after cloning were maintained on NNA plates with *A. aerogenes* at 21°-23°C and transferred to fresh edge plates weekly. After satisfactory growth was established on NM plates, stocks were maintained on this medium too.

(v) Clone plates - A few cysts or amoebae (10 - 30) were introduced either into 1.0 ml. PM-grown *A. aerogenes* and spread on NNA plates or into 0.1 ml. PAB-grown bacteria and spread on PM plates. PM clone plates give a denser bacterial lawn which facilitates plaque resolution (Fulton & Dingle, 1967). The plates should be several days old to ensure the rapid absorption of the liquid. If the plates are not sufficiently

dry the amoebae may flagellate and lead to plaques possibly formed by more than one cell.

The cloning technique described above is routinely used by many investigators in the protozoology field (Fulton, 1970). This method, however, does not assure with absolute certainty that the 'cloned' organism is a descendant of one cell. This places limitations on the research data and the correct method would have been the isolation of a single cell. Because of these limitations, the term 'clone' or 'strain' as used throughout the test of this thesis refers to an isolate that may or may not be a descendant of a single cell.

All data reported in the Results section were obtained from the isolated strains after cloning. Isolates were not cloned until their pathogenicity had been tested. At present there is no information as to whether every amoeba within a population is capable of producing PAME. By introducing uncloned, heterogeneous populations of amoebae, directly after isolation, into the nasal chambers of the mice, potentially pathogenic species, or individuals within a species, would not be eliminated (Jamieson & Anderson, 1973). If the amoebae had been isolated, cloned and then the initial pathogenic test carried out, there is a possibility that the particular cell chosen to begin the cloned cell line would not be pathogenic while other members of the heterogeneous isolate might well be pathogenic but eliminated during the cloning process. The pathogenic strains (DC-4 and FT-52) were initially cloned directly from the brain tissue of the

first fatalities and these cell lines were used for all further pathogenicity testing and taxonomic studies.

A. Sampling and Isolating of Strains

Samples of water and solids (i.e., slime, floating debris, soil) were taken from fresh water sources in Southern Ontario using disposable, individually-wrapped sterile plastic #2001 Tubes (17 x 100 mm. style-Falcon, Oxnard). Samples were stored in an upright position at 12°C for various lengths of time.

At the time of isolation, a sterile 10 ml. pipette was gently lowered into the bottom portion of the sampling tube and approximately 0.1 ml.-0.5 ml. of the sediment and water placed in the centre of a previously prepared NNA + live *Aerobacter* edge plate. A minimum of four cultures was prepared for each sample and incubated at 37°C for a minimum of 24 hours. Attempts to isolate *Naegleria* using NM plates proved unsatisfactory as other amoeba, mold, etc. grew at a faster rate and overran *Naegleria* growth. The use of only NNA with live bacteria and the elevated temperature (i.e., 37°C versus normal incubating temperature of 34°C) for isolation purposes, favoured the pathogenic strains of *Naegleria*.

Naegleria amoebae migrate rapidly outward from the central inoculum area and a distinctive pattern is produced during this migration. Upon the formation of the characteristic

migration ridge, amoebae were removed using a wire loop and checked for flagellate transformation by a squash technique. Petroleum jelly was deposited around the edge of an 18 mm. cover slip, a small quantity of sterile distilled water was placed on the cover slip and the amoebae introduced into the aqueous medium. A slide was then inverted over the cover slip, pressed gently into place and the preparation turned over. Three squash plate preparations were made per amoeba sample. The slides were kept at 21°-23°C and microscopic observations commenced approximately one hour after the initial suspension.

Isolates demonstrating flagellate transformation were sub-cultured to fresh plates by removing the cover slip and micro-pipetting the amoebo-flagellate suspension onto NNA and *Aerobacter* edge plates.

B. Pathogenicity Testing of Isolates

As previously noted, isolates were not cloned before their pathogenicity was tested but rather the inoculum consisted of a heterogeneous population of one or more strains of amoebae.

NNA spread plates were prepared with the appropriate isolate and incubated overnight at 37°C. The amoebae were then removed, washed and resuspended in the appropriate amount of sterile de-ionized water to give an approximate cell density of 10^6 amoebae/ml. or 2.0×10^4/20 µl. Cell density was

determined using the Coulter counter. Few bacteria remain after overnight incubation on NNA and the amoebae required only one washing. This is sufficient to remove most of the bacteria while reducing the chance of damaging the trophozoites (Carter, 1972).

All mice (unless otherwise noted) were 15 g. female Swiss/Webster, housed at 21°-23°C and fed freely on standard pellet food. Test animals were anaesthetized using anhydrous ether ('Baker Analyzed' Reagent Grade) and held with their ventral surface uppermost. Using a disposable 1 c.c. tuberculin syringe and a 26-gauge hypodermic needle, 20 µl. of the amoebic suspension was introduced intranasally. The animal must be breathing deeply and evenly to ensure the inhalation of the inoculum which is deposited on the external nares.

Five animals were used as a control for each testing date. These were treated in a like manner to the test animals but instead of an inoculum of amoebae, they received 20 µl. of sterile distilled water containing a small population of *Aerobacter*. No deaths resulted in these animals.

After inoculation all animals were watched for signs of morbidity. All disease fatalities and sacrificed animals were plated as described under Sterilization methods. Ether vapour was used as the killing agent for sacrificed animals. Animals surviving six weeks after inoculation and not required

for further experimentation were dispatched by the animal attendant.

C. Selection of Strains for Taxonomic Studies

Strains were selected for pathogenicity, diversity of environmental habitat and phenotypic differences.

D. Suitable Culture Conditions for Selected Strains

After the selection of strains for taxonomic studies, experiments were performed to select the medium and temperature that produced the greatest cell increase. Fulton and Dingle (1967) used 33°-34°C for preparing NB-1 for experimental work while Page (1967) used room temperature for his strains. The temperature in his laboratory varied from 25° - 30°C depending on the time of the year. It was observed visually that all strains cleared edge plates much more rapidly at 34°C than at room temperature. Consequently temperatures of 30°, 34° and 37°C were selected for incubation purposes.

Nine NNA and nine NM spread plates were prepared for each strain in the following manner: a sterile de-ionized water and cyst suspension was made from each cloned isolate and a count of the cells/ml. made using the Coulter Counter; 0.1 ml of the cyst suspension was mixed with 0.9 ml. PAB-grown *Aerobacter* and NM spread plates prepared; 0.1 ml. of the cyst suspension was also mixed with 9.0 ml. PM-grown bacteria and NNA spread plates prepared; 3 NM plates and 3 NNA plates

of each isolate were then incubated at each of the three temperatures for 20 hours. The plates were then washed, pooled, and a count of the cell density was taken for each treatment. Amoebae were also observed microscopically immediately after removal from incubation to note possible abnormalities. For the purposes of this study, the temperature that resulted in the greatest cell increase is termed the 'optimal' temperature. There may be temperatures other than the three tested that would provide slightly greater cell increase.

E. (a) Temperature Dependent Rates of Advance

(b) Temperature Tolerance

The relative rate of advance at a particular temperature was expressed as the average amount of time required to completely clear an edge plate. Temperature tolerance (i.e., critical temperature) was determined by establishing the highest temperature at which a particular strain of amoebae was capable of prolif erating (i.e., clearing an edge plate).

Two NM edge plates were prepared per strain; DC-4 and FT-52 also had two NNA + bacteria edge plates prepared in addition to the NM plates. The strains were then spotted on all edge plates, the plates were inverted and incubated at the particular temperature being tested. The average amount of time required to completely clear the edge plate was recorded.

As the temperature was elevated it became necessary to seal the dishes in air-tight containers to prevent drying and to help sustain bacterial activity.

F. Generation Time

Generation time for the selected isolates was determined by growing the amoebae in liquid culture, a medium consisting of 7mM MOPS-buffer, 5mM $MgSO_4$, pH 6.8 and bacteria (Lastovica & Dingle, 1971).

The bacteria are first grown in 1 litre batches of PAB in 2.8 litre Fernbach flasks in a gyratory shaker-incubator (Model 2156, Warner-Chilcott Lab., Research Specialties Co., Richmond, Calif.) at 37°C and 100 oscillations per minute, centrifuged in 250 ml. plastic bottles (Sorvall RC-2B Centrifugre with GSA rotor for 10 minutes at 5,000 r.p.m.), and the bacterial products of 2 litres of the PAB culture was then resuspended in 1 litre of MOPS-Mg buffer. The culture was then distributed at 20 ml. per 125 ml. Erlenmeyer flask, stoppered with cotton plugs and placed in the gyratory shaker-incubator to pre-warm (32°C and 37.5°C at 100 oscillations per minute). The 32°C incubating temperature was used as this is the temperature at which the generation time for NB-1 has been previously established (Lastovica & Dingle, 1971).

Pre-warming the buffer before the addition of amoebae

forestalls transformation which would lead to a 2-4 hour delay before meaningful growth readings could be taken.

The amoebae are grown to stationary phase on suitable media and suspended in MOPS-buffer (21° - 23°C); washed once and resuspended in approximately 5 ml. buffer. The suspension density was then determined with the Coulter Counter and a sufficient amount of the amoebic suspension was added to the 20 ml. pre-warmed MOPS-bacterial liquid culture to produce 10^4 to 10^5 amoebae/ml. The amoebae addition should be less than 0.5 ml. to ensure accurate cell density counts at later stages. Samples were removed from the 37.5°C culture at hourly intervals and at 2 hour intervals from the 32°C culture, counted and plotted on semi-logarithmic (3 cycle x 70 divisions) graph paper as cells/ml. versus time (hours) until a definite exponential growth rate had been established. Portions of the slopes were selected in which the growth had doubled (e.g., 2.0×10^5 to 4.0×10^5 cells/ml.) and the time required for these doublings was resolved by reading off the divisions on the abscissa under the slopes. This doubling time is termed the generation time (Fulton & Dingle, 1967).

G. Transformation Kinetics and Heat Shock

Transformation techniques are according to Method C (Fulton, 1970). The heat-shock treatment to achieve multi-flagella per cell was according to methods set forth

by Dingle (1970).

Cells were incubated approximately 18 hours at their optimal temperature on spread plates. Amoebae were then removed from the plates using 10 ml. Tris and a glass spreader and washed as described previously. Tris and the centrifuge tubes were kept chilled in an ice bucket to delay the onset of transformation. After each washing the supernatant was dumped rapidly to prevent excessive loss of amoebae and the cells were resuspended in 20 ml. Tris in a 125 ml. Erlenmeyer flask and incubated in a reciprocating shaker water bath at 25°C and 100 oscillations/minute. Zero time (t=0) was considered to be the time of final suspension in Tris and the commencement of incubation. Routinely, 10^5 amoebae/flask were used to ensure adequate cell densities for counting purposes.

Samples of cell suspensions were removed at predetermined time intervals with a Pasteur capillary pipette and 3 or 4 drops fixed with 1 drop of Lugol's iodine. Care was taken to prevent excessive agitation while pipetting as reversion or de-flagellation may occur. The fixed samples were stored overnight under refrigeration before counting or making cytological observations if the original cell suspension density had been low. This allowed the cells to settle to the bottom of the tube and provided a denser cell population for microscopic purposes.

A drop of the cell-iodine sample was deposited on a

glass slide and an elevated cover slip (clay feet) added. The flagella appear black and distinct while the body and many organelles are orange. For the transformation kinetics, 100 cells were counted per sampling time for the presence or absence of flagella and a graph drawn plotting percent flagellates versus time. This allows the determination of the T_{50} which represents the time at which 50% of the cells are flagellated. A separate count was made of the number of flagella per flagellate.

To determine whether heat-shock would disrupt the metabolism of the isolated strains sufficiently to alter the number of flagella from that found under normal transformation temperatures (i.e., 25°C) to a greater number of flagella per cell as had been found with the NB-1 strain, samples were grown and prepared in the same manner as described for normal transformation. However, at t=0 the flasks were placed in a 38°C reciprocating shaker water bath and incubated for 40 minutes. The flasks were then transferred to the 25°C water bath and allowed to continue transformation a further 2 hours. Samples were then fixed with Lugol's iodine as described previously and a count was made of the number of flagella per cell.

H. Cyst Storage and Viability

The cysts to be tested for duration of viability were prepared in the following manner. A minimum of three 1M

spread plates per strain was incubated at their optimal temperature for 48 hours. This was to ensure complete encystment. DC-4 and FT-52 were grown on NNA spread plates as well as the NM plates. The encysted strains were sealed in air-tight containers to prevent drying and stored at room temperature (21°-23°C). At 14 day intervals, samples of the cysts were removed and tested on NM or NNA edge plates for viability.

I. Resistance of Amoebae and Cysts to Desiccation

To test cyst durability, each strain was incubated to complete encystment as described in the preceding section. The plates were then washed with de-ionized water, centrifuged and cyst samples from the pellet placed in 125 ml. Erlenmeyer flasks containing 10 ml. of one of the following: 1% solution Liqui-Nox; 2% solution Liqui-Nox; or 70% ethanol. The flasks were stoppered lightly and incubated in a water bath at room temperature and 100 oscillations/minute. Samples were taken at 24 hours, washed thoroughly (a minimum of six centrifugations) and a portion of each pellet spotted on an edge plate. Part of each pellet was retained, allowed to dry at room temperature for 24 hours and then spotted on edge plates. A control for each strain was initiated from the original centrifugation pellet, incubated in de-ionized water and edge plated.

To test amoeba durability, amoebae were grown to

stationary phase on NM or NNA spread plates, harvested and suspended in a dense sterile de-ionized water suspension. A small drop of the amoebic suspension was placed on a glass slide and an elevated cover slip placed atop. Using a Pasteur pipette, the desired testing solutions were allowed to diffuse under the cover slip while being observed with phase contrast optics. The solutions were those used for the cyst durability tests and a possible 10% dilution (maximum) may have occurred because of the aqueous solution the amoebae were already suspended in. However, the amoeba suspension was kept to a minimum and the amount of solution that diffused under the cover slips far exceeded it. This was to ensure a minimum amount of dilution. A control slide containing only the original amoeba suspension was maintained.

J. Measurements and Taxonomic Observations

Unless otherwise noted, the materials and methods used in this section are, or are modifications of, those set forth by Page (1967). Strains NB-1, DC-1 and CT-8 were transferred to NNA and re-cultured several times on this new culture medium to ensure consistent growth behaviour. DC-4 and FT-52 were maintained continually on NNA with live bacteria and, therefore, did not require transferring. Plates were incubated at 34°C rather than 23°C as described by Page.

J. (a) Amoebae:

Amoebae were removed directly from the growth plate after approximately 18 hours incubation with a wire loop and suspended in Page's amoeba saline on a cover slip. The cover slip was quickly inverted and placed atop a depressed well slide giving a hanging drop. The slide was left for approximately 15 minutes before observations and measurements were begun to follow amoebae to adhere to the slide and resume normal movement. The hanging drop with amoeba saline produces little cell distortion even after lengthy periods of observations. Length and breadth measurements of 100 amoebae in motion were recorded as well as general observations on their particular type of locomotion and the presence or absence of uropodia.

To measure the nuclear diameters and to observe the general shape of the nucleolus and the types and numbers of vacuoles present in an individual, a sample was prepared as described above and Nissenbaum's fixative (Nissenbaum, 1953) utilized after the waiting period and amoebae adherence to the slide.

Nissenbaum's fixative consists of 10 volumes saturated solution of mercuric chloride; 2 volumes glacial acetic acid; 2 volumes commercial formalin; and 5 volumes tertiary butyl alcohol. Reagents were mixed just prior to use. The fixative was pipetted directly onto the amoebae (or flagellate)

suspension; after 15 seconds the fixative was drained off and the slide flooded with 70% iodine alcohol for 3-4 minutes; washed with 70% alcohol for 3-5 minutes; and was then ready for microscopic observations (x1000) with oil immersion or further staining.

The 70% iodine alcohol consisted of 70% ethanol saturated with iodine until a port wine red colour was obtained. This washing was required to remove the mercuric chloride and prevent black deposits on the specimen.

It is not necessary to invert the slide for 15 minutes before the addition of Nissenbaum's fixative but a greater number of cells are affixed to the slide if the amoebae are allowed to adhere to the glass before the fixative is added. The t-butyl alcohol present in the fixative causes adhesion of amoebae and flagellates to the glass. There is little, if any, distortion of the nucleus, vacuoles or general amoeboid shape using this fixative.

Mitotic events were studied in the manner set forth by Page (1967) with minor modifications. Spread plates were grown for approximately 16 hours at the optimal temperature, a large loopful of amoebae was removed from the plate, suspended in the amoeba saline solution and immediately fixed with Nissenbaum's fixative. The immediate fixation increased the chances of amoebae being present on the slide that were actively undergoing mitosis. The amoebae adhere to the glass

and appear normal morphologically while the bacteria are washed off the slide. After the final alcohol rinse, the cells were stained for five minutes with a modified Kernechtrot (Chroma-Gesellschaft) method.

The Kernechtrot stain is prepared by dissolving 5 g. Michrome Nuclear Fast Red (Herzberg, Michrome No. 1023, Edward Gurr, Ltd., London) in 100 ml. of a 5% (w/v) aqueous $Al_2(SO_4)_3$ solution. The slides were washed with de-ionized water and examined using phase optics (x1000) with oil immersion until an amoeba undergoing mitosis was found. Observations were then made as to whether the nuclear membrane was intact, the appearance of the nucleolus and polar masses, if any. The stain gives clear definition of the nuclear boundary and of other components present within the nuclear membrane. Food vacuoles and other unidentified organelles also appeared quite distinctly with this staining technique.

J. (b) Flagellates:

Flagellates were prepared as previously described under the Transformation section. However, no samples were taken until t=2 hours. At that time, cells were removed using a capillary pipette and fixed with Lugol's iodine. These cells were then used to measure the number of flagella per cell, the flagella length and the flagellate body length and width. Body shape was also noted and recorded.

J. (c) Cysts:

Cysts were washed with de-ionized water and a drop of suspension was placed on a slide with an elevated cover slip. The diameters of 100 individuals were measured and the nuclear diameters of 25 individuals (x1000, with oil immersion). The shapes were recorded and the cyst walls studied for the presence of pores, plugs and other structural aspects.

Several investigators have noted the apparent dearth of pores in the cyst walls of pathogenic strains (Carter, 1970; Culbertson *et al.*, 1968; Saygi *et al.*, 1973). Regular phase contrast light microscopy (x1000) failed to resolve whether pores were or were not present in the strains being categorized and further studies were then done using a scanning electron microscope with methods modified from those of Darley and Lott (1973). Cells were plated on spread plates; incubated at optimal temperature for 72 hours to ensure complete encystment; washed four times using 2mM Tris buffer to remove bacteria and then fixed for 30 minutes in a fixative consisting of 0.2M phosphate buffer (pH 7.2), 4% glutaraldehyde and 20% glycerol. Samples were then rinsed with 20% glycerol, filtered and affixed to aluminum conducting tape (3M Co., St. Paul, Minn.). The samples were rapidly frozen in isopentane cooled by liquid nitrogen; transferred to liquid nitrogen; and finally transferred into 6 inch Pyrex test tubes

standing in a litre plastic beaker which was half filled with liquid nitrogen. The tubes were held firmly in place by a plastic top that fitted over the beaker. The beaker was then placed under the Bell jar of an Edwards Vacuum Coating Unit, Model E12E4, and pumped using the rotary pump only until the nitrogen had sublimed and reached room temperature. This prevents condensation on samples when they are removed from the vacuum. Dried specimen were kept under vacuum until mounting and coating. All samples were pre-coated with carbon followed by paladium-gold 40/60 (E. F. Fullan Inc.) and viewed using an NMR Model 1000 Scanning Electron Microscope. Micrographs were taken using a Groflex RH/10 camera with Ilford FP4 120 black and white panachromatic safety film and a 55 second exposure time. Printing of the micrographs was done on a Laboratory 138S enlarger (Durst) with Latico 140 and Latico 200 lens (f8 setting), No. 2 Ilford paper.

III. RESULTS

A. Sampling and Isolating of Strains

A minimum of 29 strains of *Naegleria* were isolated from the original water samples (Table 1). It is possible that mixed populations existed within these isolates and that more than 29 strains were actually present. Many samples contained several different amoebic forms, none of which proved to be *Naegleria*. *Naegleria* may have been present in these incubated samples but were outgrown by other Protozoan forms. It should also be pointed out that in the majority of the samples, only 1 ml. or less was used for isolation purposes. If the complete sample or an even greater volume had been used, there undoubtedly would have been more than 29 strains isolated.

All samples were taken from the area bordered by Hamilton to the south, Toronto to the east, Guelph to the west, and Midland to the north. The majority of the samples were from public beaches where the water tends to be warm and placid, both features suited to amoebic growth. Samples taken from areas high in organic growth or fecal pollution (i.e., CD-3, DC-4, HP-16, HM-35, and FT-53) contained several types of amoebae.

Only six sampling sites failed to yield any amoeboid forms when 1 ml. of the sample was incubated. At the time of sampling, it was noted that these particular sites possessed

little, if any, vegetative growth. This apparent lack of organic material along the banks and shores was probably due to the chemical content of the factory drainage ditch (WD-6 and WD-7) and the turbulence of the Grand River (GR-40) and Lake Huron (WB-27) at these particular sampling sites. The Royal Botanical Gardens rockgarden sample (RB-47) was taken from a small silt-filled pool that was serving as a drainage reservoir for a nearby road construction operation and appeared to be contaminated with petroleum products.

Of special interest was the isolation of amoebae from the water filters of the McMaster Biochemistry Department (WF-54). The filters are used to de-ionize the water used for viral cancer research and are changed at three month intervals. The presence of amoebae in these filters could be due only to their presence in the hospital water supply. The amoebae were possibly of the *Hartmannella* genus as they looked very much like *Naegleria* in the amoeboid-form but failed to transform into flagellates.

From Table 1 on the following page, it becomes quite apparent that *Naegleria*, and like amoebae, can be isolated from practically all water sources in our environment and are capable of occupying diverse habitats.

TABLE 1

Sample	Sampling Site	Amoebae Isolated	*Naegleria* Isolated	Pathogen
DC-1	Desjardins Canal (Dundas)	+	+	-
CD-2	Cootes Drive (Dundas)	+	-	
CD-3	" " "	+	+	+
DC-4	Desjardins Canal (Dundas)	+	+	+
CD-5	Cootes Drive (Dundas pool)	+	-	
WD-6	Factory drainage (Hamilton)	-		
WD-7	" " "	-		
CT-8	Outdoor swimming pool "	+	+	-
PC-9	Port Credit marina (L. Ont)	+	-	
EB-10	Etobioke Beach (L. Ont.)	+	+	NT*
EB-11	Etobioke R. (Marie Curtis)	+	+	NT
MC-12	Mimico Creek (Toronto)	+	+	-
MB-13	Mimico Beach (L. Ont.)	+	-	
HP-14	Humberside Park (Toronto)	+	+	NT
HP-15	High Park (Toronto)	+	+	NT
HP-16	" " "	+	+	-
DV-17	Don Valley Parkway (Toronto)	+	-	
DR-18	Don River (Toronto)	+	-	
CT-19	Fountain (Hamilton)	-		
MC-20	Mississauga Ck. (Miss.)	+	-	
SC-21	Simcoe Canal (Hwy. 400)	+	+	-
LS-22	Lake Simcoe (Barrie)	+	+	NT
BC-23	Barrie Canal (Hwy. 27)	+	-	
EC-24	Elmvale Canal (Hwy. 27)	+	-	
ML-25	Midland Lake	+	-	
BB-26	Balm Beach (Lake Huron)	+	-	
WB-27	Wymbolwood Beach (L.Huron)	-		
WB-28	Wasaga Beach (L.Huron)	+	+	NT
WB-29	" " "	+	-	
WL-30	" Lagoon (Nancy Is.)	+	+	NT
BR-31	Batteaux R. (Collingwood)	+	+	NT
PR-32	Pretty R. "	+	+	NT
MR-33	Mad River (Hwy. 24)	+	+	NT
HM-34	Hornings Mill Creek Park	+	+	NT
HM-35	Hornings Mill Pond	+	+	-
OR-36	Orangeville Racetrack	+	+	NT
CR-37	Credit River (Hwy. 24)	+	+	NT
EC-38	Eric Creek (Hwy. 24)	+	-	
ER-39	Eramos River (Hwy. 24)	+	-	
GR-40	Grand River (Guelph)	-		
WB-41	Hwy. 6 Creek (Wentworth)	+	+	NT
CD-42	Cootes Drive Canal (Dundas)	+	-	
OH-43	Ontario Hydro site (Dundas)	+	-	
YR-44	York Road (Dundas)	+	+	NT
YR-45	" " "	+	+	-

(Continued)

TABLE 1 continued-

Sample	Sampling Site	Amoebae Isolated	*Naegleria* Isolated	Pathogen
RB-46	R.B.Gardens (Hwy. 2)	+	+	NT
RB-47	" " rockgarden	-		
HH-48	Hamilton Harbour (Hwy. 6)	+	+	NT
OP-49	Pond (Hwy. 2 overpass)	+	+	NT
CP-50	Cootes Paradise-High Level	+	+	NT
CP-51	" " canal-Hwy. 8	+	-	
FT-52	Fish tank-McMaster Biol.	+	+	+
FT-53	" " " "	+	-	
WF-54	Filters-McMaster Biochem.	+	-	
NB-1	Courtesy A.D.Dingle			-

*NT- not tested

B. Pathogenicity Testing of Isolates

Since this research project was aimed primarily at trying to locate possible pathogens in the area, the pathogenicity of randomly selected isolates was tested first before a decision was made as to which isolates were to be compared and characterized. Only 10 of the 29 isolates and NB-1, the control, were tested for pathogenicity (Table 1). NB-1 failed to prove pathogenic during this testing confirming earlier tests by Fulton (1970).

To test for pathogenicity, the appropriate isolate was incubated overnight and an inoculum of approximately 2.0×10^4 amoebae per animal was prepared. Ten or more 15 g mice were lightly anaesthetized and intranasally inoculated with the amoeba isolate. All animals were observed for the following six weeks for signs of disease. All fatalities were tested for amoebic infestation by incubating the brain and other organs on NNA plates with live *A. aerogenes*. Of the isolates tested, three showed varying degrees of pathogenicity.

(a) Strain CD-3: One fatality occurred three days after an inoculum of 2.0×10^4 was tested on 10 animals. The *Naegleria* that emerged from the incubated brain tissue were extremely small, approximately 10um or less. The strain failed to thrive and was lost.

(b) Strain DC-4: Three fatalities resulted from the initial pathogenicity test, one at day 6 and two at day 7. An inoculum

size of 2.7×10^4 was used with ten test animals. The cloned cell line proved pathogenic and was used for all experimental pathology experiments and pathogenic versus non-pathogenic comparative tests.

(c) Strain FT-52: One fatality resulted four days after an inoculum of 2.7×10^4 was used with eleven test animals. The amoeba membrane appeared ruffled and amoebae were slow to emerge from the incubated brain tissue. Further testing of the cloned strain failed to produce fatalities.

Mice succumbing to PAME displayed the classic symptoms of the disease; that is, bristling hair, extreme dehydration of the body with puffy face parts, ataxic movements and continuous circling, head scratching, followed by loss of mobility and death. Opening of the cadaver disclosed brain tissue that was usually hemorrhagic and/or necrotic and olfactory bulbs that were soft and pulpy. The liver, and often the spleen, were usually extensively enlarged with necrotic patches. The lungs and heart occasionally appeared hemorrhagic but necrosis was not observed. The intestinal tract appeared black as a result of the darkly-coloured fecal material.

Although not only the cranial contents, but the liver, lungs and heart were plated from PAME fatalities, amoebae emerged only from the brain tissue. Microscopic examination of wet squash mounts from these organs immediately following death failed to reveal any amoebic forms. However, even if

amoebae were present they would be extremely difficult to discern in these particular organs and failure to observe them microscopically could not be used as a definitive test for the presence or absence of amoebic infestation. Mouse blood lacks the amoebicidal factor present in human serum and other investigators have found trophozoites in these organs.

Amoebae were seen microscopically in brain tissue. They appeared very active in fresh mounts. Vacuoles containing a golden-coloured substance, possibly lipid, were present in amoebae from brain tissue; these vacuoles are not seen in amoebae grown on agar plates with bacteria.

C. Selection of Strains for Taxonomic Studies

Strains were selected for pathogenicity, diversity of natural environment and comparative qualities. NB-1 was selected to act as a control as it has been studied for many years and is among the better taxonomically described strains. NB-1 was derived by four single plaque isolations of the Cambridge 1518/1a strain (Fulton & Dingle, 1967).

Strain DC-4 was the first isolate chosen because of its pathogenicity in mouse studies. It was isolated from the Desjardins Canal, Dundas, Ontario; a sluggish fresh water canal containing much sewage material and other organic matter. The sample was taken on September 26, 1973, after a long hot spell in the region. The temperature of the water was not

taken at this particular site until August 22, 1974, when it registered 34°C.

Strain DC-1 was then selected as a comparative strain to DC-4 as it was sampled at the same time and from the same site. It, however, was non-pathogenic and appeared to have dietary, thermic and phenotypic differences.

Strain CT-8 was isolated from an outdoor swimming pool. The pool temperature ranged from 15.5° - 21.0°C; pH from 7.2 - 7.8; chlorination averaged 1.0 p.p.m. (ranged from 0.6 - 2.0 p.p.m.); the pool walls were washed weekly, net cleaning was done daily; and the water was under constant filtration. CT-8 was among the most rapidly growing strains isolated and the most easily cultivated of the five selected for further study. It was non-pathogenic.

Strain FT-52 was isolated from fish tanks used for zebra fish studies in the Biology Department, McMaster University, Hamilton. The tanks were kept at elevated temperatures (26°-28°C). Various fungicides and therapeutic agents for fish survival were used when required. FT-52 was smaller in size than other strains isolated, grew very rapidly when conditions were suitable but was initially difficult to culture and maintain. Mouse pathogenicity tests suggest that it is pathogenic but not to the same extent as DC-4.

D. Suitable Culture Conditions for Selected Strains

(a) Medium - Only NNA and NM plates spread with live *Aerobacter* were tested for culturing suitability. There may be other media that would provide greater growth but these are easily prepared, store well and provide satisfactory growth for most laboratory work. It was found that CT-8 and DC-1 had the greatest growth when grown on NM while DC-4 and FT-52 grew well only on NNA. DC-4 at this time had not completely adapted to NM and grew poorly while FT-52 actually decreased on this particular medium. NB-1 was not tested but was grown on NM for most experiments. All isolates were maintained on the medium that appeared to be most suitable for them. DC-4 and FT-52 gradually adapted to NM and NM-stock plates were then instituted for these two isolates.

(b) Temperature - Isolates were tested to try to determine a temperature that would lead to the greatest cell increase. As may be noted in Table 2, 30°, 34° and 37°C were selected and it was found that CT-8 and FT-52 grew optimally at 34°C; whereas DC-1 and DC-4 had the greatest increase at 37°C. NB-1 was not tested as other investigators have shown 33°-34°C to produce the most satisfactory growth (Fulton & Dingle, 1967).

Growth in all cases was slowed when isolates were incubated at 30°C, regardless of the medium used. It may be noted that DC-1 and CT-8 grew exceptionally well on NM medium. The NM medium provided a denser bacterial lawn and allowed a greater amoebae density for the DC-1 and CT-8

TABLE 2

Growth of Isolates on NNA & NM at Various Temperatures

The Table shows the temperatures and media tested to determine suitable culture conditions for the isolates. Three plates per treatment were prepared with an inoculum for each strain as shown in the column "t=0". Plates were incubated at the various temperatures for 20 hours, washed, pooled and the average cells per plate determined. Cell counts were established with the Coulter counter. The medium and temperature showing the greatest increase in cell number was selected as the most suitable culture condition. The amoebae tend to grow well at temperatures within a given range (plateau) and any isolate that showed a greater cell increase at 34°C would probably grow as efficiently at 33°C or 35°C (Dingle, personal communication).

TABLE 2

Growth of Isolates on NNA & NM at Various Temperatures

Isolate	t=0*	T(°C)	NM**	NNA**
DC-1	1.6×10^5	30	1.75×10^7	1.85×10^5
		34	1.99×10^7	2.1×10^6
		37 ✓	2.44×10^7	4.5×10^6
CT-8	3.3×10^5	30	1.77×10^7	2.3×10^6
		34 ✓	3.38×10^7	6.5×10^6
		37	1.3×10^7	2.1×10^6
DC-4	2.1×10^4	30	1.5×10^4	2.5×10^5
		34	2.15×10^4	2.5×10^6
		37	8.0×10^4	6.5×10^6 ✓
FT-52	7.1×10^5	30	2.5×10^5	3.6×10^6
		34	3.7×10^5	5.0×10^6 ✓
		37	4.6×10^5	4.2×10^6

* Figures represent cells inoculated per plate at t=0.
** Cells/plate at t=20 hours.
✓ Temperature and medium producing greatest cell increase.
DC-1 = NM at 37°C
CT-8 = NM at 34°C
DC-4 = NNA at 37°C
FT-52= NNA at 34°C

isolates than the more sparse NNA plates. The strain DC-4 decreased in cell density when incubated on NM at 30°C. FT-52 decreased at all temperatures when grown on NM and proved extremely fragile even after NM stock plates were possible.

E. (a) Temperature Dependent Rates of Advance

To test the relative rate of advance of the isolates, amoebae were inoculated onto edge plates and the length of time that they required to completely clear the edge plate was recorded. These figures are given in Table 3. Strains CT-8, FT-52 and NB-1 cleared the plates in the shortest period of time when incubated at 34°C. Particularly rapid growth was apparent in the strains NB-1, CT-8 and DC-4 at the appropriate temperature for they required only 35, 31 and 36 hours respectively to completely clear the edge plates.

Both FT-52 and DC-4 had adapted to NM medium and little, if any, differences could be discerned in the growth rates of these strains on the NM and NNA media at suitable temperatures (i.e., sub-lethal).

At room temperature, the most rapid growth was achieved by CT-8 and an average of only 80 hours was required to clear a plate. By far the slowest growth at this temperature was that of DC-4 which required seven days to cover an agar plate.

E. (b) Temperature Tolerance

Table 3 gives the critical temperature beyond which the isolate cannot survive for any length of time. All of

the strains tested were able to survive and migrate for at least 24 hours at temperatures beyond the uppermost temperature at which they were capable of proliferating (i.e., clearing an edge plate). Only DC-4 and FT-52, the pathogens, were able to grow successfully at temperatures well beyond normal body temperature. In all cases, the temperature one degree past the critical temperature led eventually to disintegrated and crenated amoeboid forms. There was no evidence of cyst formation at the elevated temperatures.

Of special interest was strain DC-4's ability to proliferate at 43°C (109.5°F), well beyond the range that a human can survive. The NNA plates provided a slightly more suitable nutrient source for DC-4 until 42°C was reached. It may have been that beyond this point the bacteria were unable to survive on the NNA plates in a suitable state to provide the needed nutritional source for amoebic growth. The NM plates, by providing adequate nutrition for the bacteria, may have allowed them to remain viable and available for the amoebae.

TABLE 3

Temperature Dependent Rates of Advance and Temperature Tolerance

Table 3 gives the average amount of time required to completely clear an edge plate at a particular temperature and the critical temperature.

The amount of time required to completely clear an edge plate was determined by inoculating the edge of two edge plates/strain, incubating at the desired temperature until complete clearage of the plates, and the average amount of time needed was then recorded.

The critical temperature, for the temperature tolerance results, was deemed to be the highest temperature at which an isolate was capable of clearing an edge plate.

TABLE 3

Temperature Dependent Rates of Advance and Temperature Tolerance

Strain	Temperatures °C											
	23	34	36	37	38	39	40	41	42	43	44	
DC-1 *	144	56		60	80	-	-	-	-	-	-	(hr)
CT	80	31		65	-	-	-	-	-	-	-	(hr)
NB-1	100	35	70	-	-	-	-	-	-	-	-	(hr)
DC-4 (NNA)	168	48		36	40	55	48	60	72	-	-	(hr)
(NM)**	168	48		36	48	60	60	60	72	162	-	(hr)
FT-52 (NNA)	100	60		96	96	96	168	-	-	-	-	(hr)
(NM)**	100	60		96	96	120	-	-	-	-	-	(hr)

* Average amount of time (hours) required to clear an edge plate
** DC-4 and FT-52 were tested on NNA and NM edge and stripe plates

F. Generation Time

The generation time, or doubling rate, was determined using liquid culture rather than the agar plates used in all other aspects of this research project. Using liquid medium facilitates sampling at the predetermined time intervals, limits the accummulation of metabolic wastes that might slow amoebae growth as well as other difficulties inherent in using agar plates. The generation time for NB-1 had previously been determined using 32°C as the incubation temperature; consequently, the same temperature was used for one of the incubation temperatures (Lastovica & Dingle, 1971). The 37.5°C incubation temperature was used to determine any differences in growth rate at higher temperatures.

TABLE 4

Summary* of Generation Time at 32°C and 37.5°C

T(°C)	DC-1	DC-4	CT-8	FT-52	NB-1
32.0	1.7 hr	2.4 hr	1.65 hr	1.5 hr	1.8 hr
37.5	2.4 hr	1.6 hr	1.75 hr	1.5 hr	**

* See pages 63 and 65 (Table 5 & Figure 1) for details
** NB-1 tested at 32°C only

Strains PT-52's generation time was unaffected by the differences in temperature and was extremely rapid at at both temperatures. The same appears to be relatively true for Strain CT-8. Both strains in their natural environment would be subjected to varying and elevated

temperatures over fairly short periods of time.

Under liquid culture conditions DC-1 had a relatively long generation time at 37.5°C and a much shorter one at 32°C. This was consistent with Table 2 and 3 as well as later findings. It would appear that DC-1 grows well up to, but not exceeding, 37°C. This heat-sensitive quality could quite concaivably be the determining factor in its non-pathogenicity. Strain DC-4, although isolated from the same source, showed a marked decrease in generation time with increased temperature. DC-4 had a generation time of 1.6 hours at 37.5°C, only surpassed by FT-52. This is consistent with Table 2 and 3 where it was noted that the rate of growth increased dramatically at, or slightly beyond, normal body temperature. This is to be expected in a pathogenic organism.

TABLE 5

Generation Times at 32° and 37.5°C

Amoebae were grown to stationary phase on agar plates, washed in MOPS-buffer, cell densities established using the Coulter counter, and 0.1 ml. of the amoeba-buffer suspension added to 20 ml. MOPS-bacteria liquid culture (pre-warmed to the desired temperatures). T=0 shown on the table is the cell density introduced into the liquid cultures. The flasks were stoppered tightly and incubated at 32° or 37.5°C. Samples were taken from the 37.5°C incubating samples at hourly intervals and from the 32.0°C cultures at 2 hour intervals. Cell densities were determined and plotted on semi-logarithmic graph paper as cells/ml. of suspension versus time (see Figure 1).

The generation time for NB-1 had previously been determined (Lastovica & Dingle, 1971), consequently the same temperature was used during this experiment. The higher 37.5°C was used to determine any differences in growth rate at higher temperatures.

TABLE 5

Generation Times at 32°C and 37.5°C

Time (hrs)	Cells/ml of suspension								
	DC-1		DC-4		FT-52		CT-8		NB-1
0	2.25×10^5		1.5×10^5		1.5×10^5		2.17×10^5		3.5×10^5
	32°	37.5°	32°	37.5°	32°	37.5°	32°	37.5°	32° *
1		8.1×10^4		3.5×10^4		6.6×10^4		5.75×10^4	
2	1.25×10^5	9.0×10^4	7.75×10^4	4.35×10^4	1.02×10^5	1.02×10^5	9.6×10^4	8.75×10^4	7.5×10^4
3		8.6×10^4		7.35×10^4		1.48×10^5		1.16×10^5	
4	2.19×10^5	1.4×10^5	8.9×10^4	9.9×10^4	2.6×10^5	2.5×10^5	2.16×10^5	2.15×10^5	1.5×10^5
5		1.9×10^5		1.78×10^5		4.15×10^5		3.55×10^5	
6	2.6×10^5	2.45×10^5	1.5×10^5	2.2×10^5	6.58×10^5	6.06×10^5	5.9×10^5	5.07×10^5	3.3×10^5
7	4.1×10^5		2.2×10^5	3.76×10^5	1.0×10^6	9.7×10^6	7.8×10^5	6.06×10^5	4.75×10^5
12	3.1×10^6	1.25×10^5	1.25×10^5	2.15×10^5	5.6×10^6	4.4×10^6	4.8×10^6	4.0×10^6	2.06×10^6
Gen. Time	1.7 hr	2.4 hr	2.4 hr	1.6 hr	1.5 hr	1.5 hr	1.65 hr	1.75 hr	1.8 hr

* Only 32°C used for NB-1 generation time

FIGURE 1

Minimum Generation Time

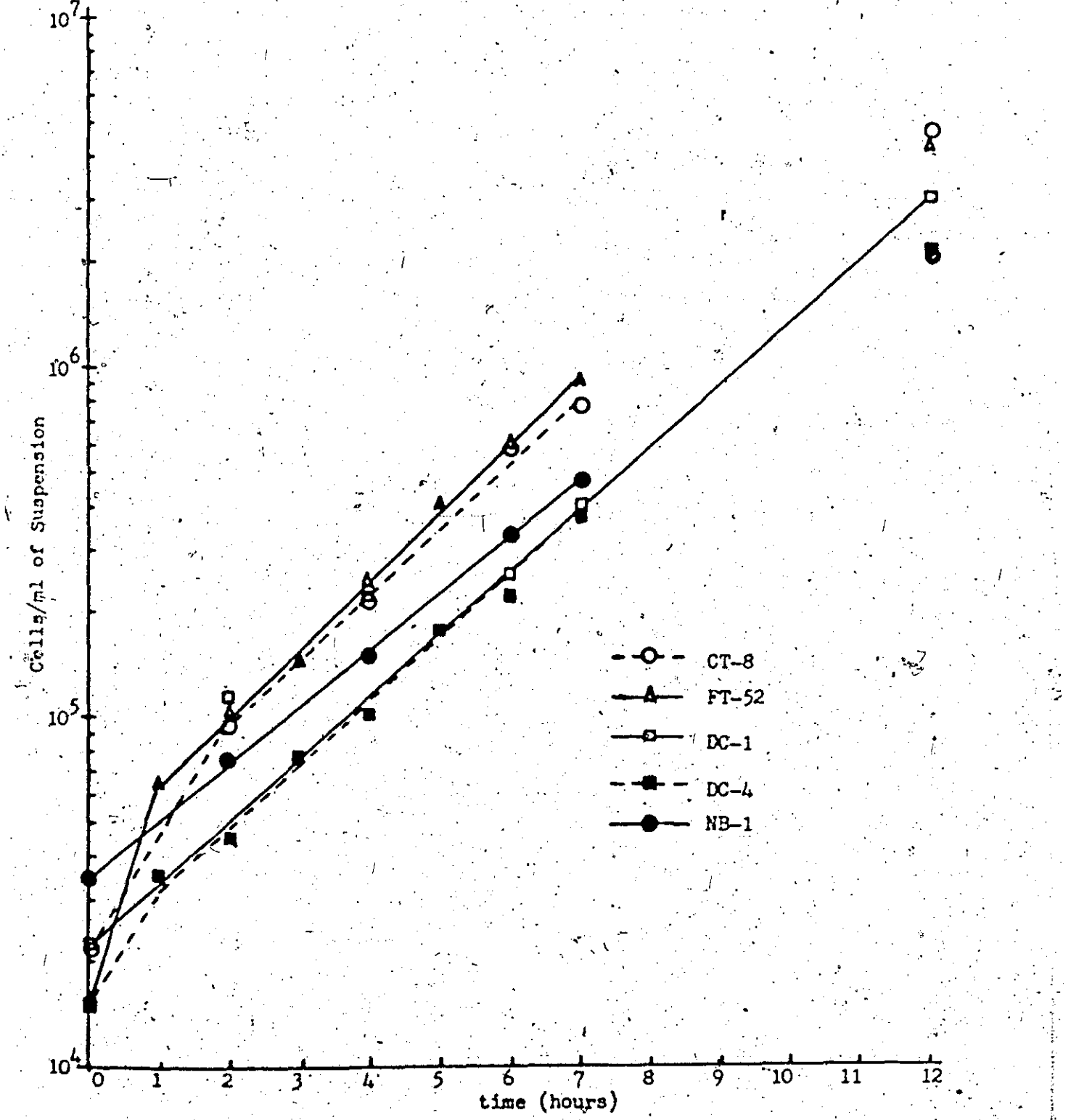
The slopes shown in Figure 1 are those that gave the shortest generation time. All strains, except DC-4, had the fastest doubling rate at 32°C. The points shown on the graph are derived from Table 5, the results of one experiment. To ascertain the generation time, portions of the slopes were selected in which the growth had doubled (e.g., 2.0×10^5 to 4.0×10^5 cells/ml.) and the time required for these doublings was resolved by reading off the divisions on the abscissa under the slopes.

Strain DC-1 failed to produce a linear growth rate at t=2 hrs and t=4 hrs. This may have been due to clumping of bacteria in the liquid culture which would give a greater cell density than was actually present.

It may also be noted that (with the exception of DC-1) generation times were not calculated from incubation periods beyond t=7 hours. The cell densities at t=12 hours for these four samples fails to correspond to the linear relationship established during the initial seven hour period. This may be due to the amoebae reaching stationary phase or to the depletion of the bacterial food source which would lead to reduced mitotic activity.

FIGURE 1

Minimum Generation Time



G. Transformation Kinetics and Heat Shock

The rate at which a particular strain of *Naegleria* transforms from the amoeboid to the flagellate phase is reproducible and an innate characteristic of the organism being studied. To a limited extent, it is also indicative of the metabolic rate of the strain. By heat shocking the cells with sub-lethal temperatures during the incubation period, the metabolic processes can be disrupted leading to over-production of flagellar components and a state of 'hairiness' (i.e., multi-flagella). The T_{50} of a given strain is the amount of time required for 50% of the population to become flagellated.

TABLE 6

Summary of Transformation Kinetics and Heat Shock*

	DC-1	DC-4	CT-8	FT-52	NB-1
T_{50} (min.)	73.0	70.0	61.0	58.5	69.5
Mean f/F** (25°C)	2.2	2.0	2.2	2.0	2.3
Mean f/F** (38°C)	3.2	2.3	3.9	2.2	4.2

*See Table 7 and Figure 2 for more detail
**Mean number of flagella/flagellate at 25°C (normal transformation conditions) and at 38°C (heat shock treatment).

It may be noted in Table 6 that FT-52 and CT-8 have a T_{50} much lower than the other isolates. This would suggest a faster metabolic rate was in operation within these two

strains for flagellar components (i.e., flagella, rhizoplasts and associated structures of the flagellar apparatus) arise *de novo* during the initial incubation period. As seen in Table 4, the generation times for these strains are particularly fast adding further support to a faster metabolic rate.

Of particular interest was the change in the number of flagella per cell with an elevated temperature treatment (Table 7). Strains DC-4 and FT-52, both pathogenic in varying degrees, were little affected by increasing the temperature during the initial 40 minute incubation period. This would be expected in an organism capable of growing at, or well above, normal body temperature as the pathogenic strains must. The increased temperature, although of relatively short duration, was sufficient to alter the metabolic processes of the non-pathogenic strains as was demonstrated by the increased flagellum number.

DC-1 was not affected to the same degree as NB-1 or CT-8. Its optimal growth temperature was 37°C and a shift of only one degree is very slight, yet a definite disruption of the flagellum synthesis had occurred.

Multi-flagella may be a trait of a particular strain but under normal transformation temperatures none of these isolates exhibited this characteristic. Only sub-lethal temperatures induced a mean number of flagella per cell substantially greater than the two normally found.

FIGURE 2

Transformation Curve of Amoebae-to-Flagellates

This figure shows the transformation of the 5 *Naegleria* strains under normal transformation conditions (i.e., 25°C, Tris buffer, 100 oscillations per minute). Amoebae were incubated overnight on agar plates, washed and suspended in Tris buffer. The buffer and centrifuge tubes were kept in an ice bucket during this preparation period to prevent the onset of transformation. Zero time was designated as the time at which incubation at 25°C commenced.

Samples of the suspensions were removed at predetermined intervals, fixed in Lugol's iodine and examined microscopically for the presence or absence of flagella.

It may be noted that all strains, except for DC-1, attained a 90+ percent flagellation stage before the 120 minute point was reached. DC-1, however, normally ranged in the 80 - 90 percent flagellate range and rarely exceeded this range. Another of DC-1's characteristics was the presence within the flagellate population of large numbers of amoeboid-shapes possessing two or more flagella. Although there were many amoeboid-flagellates, the majority did display the typical flagellate fusiform shape.

FIGURE 2

Transformation Curve of Amoebae-to-Flagellate

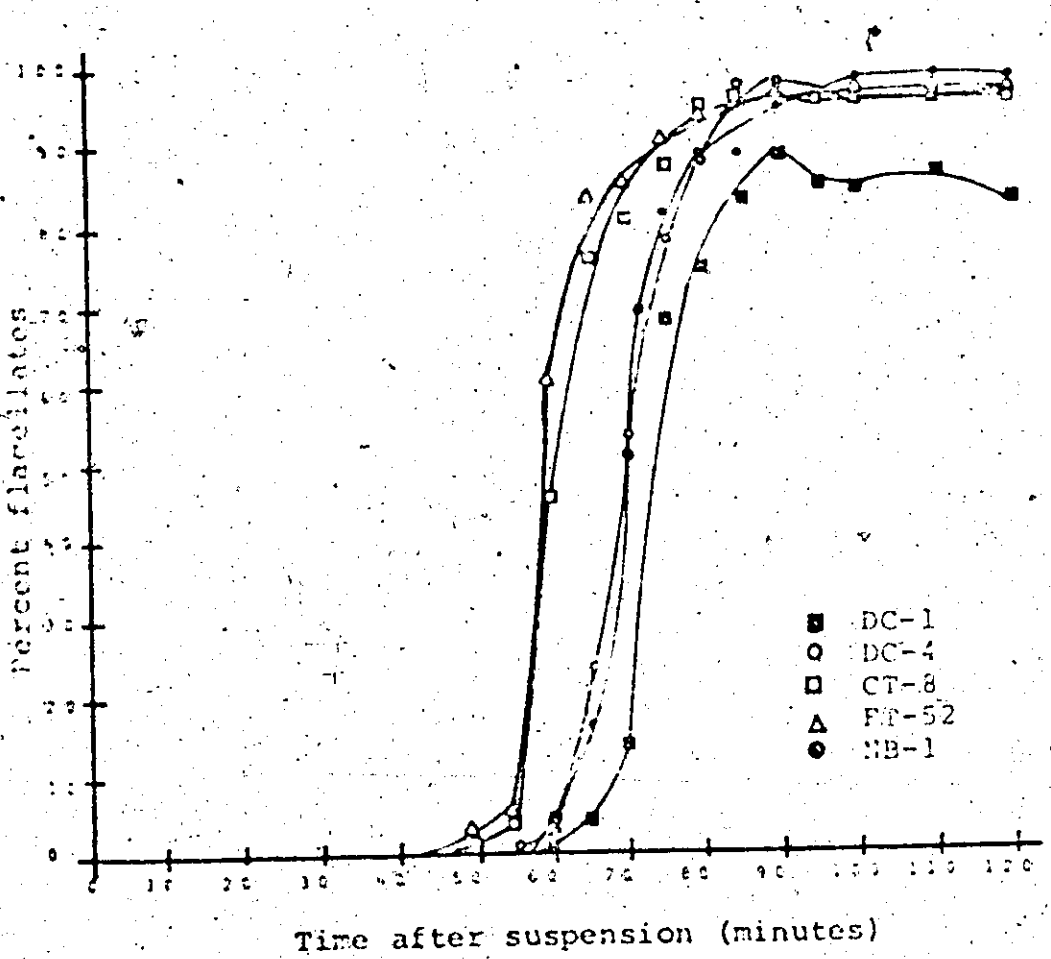


TABLE 7

Frequency of Flagella Distribution

Table 7 shows the number of flagella per flagellate when the *Naegleria* strains were incubated at the normal transformation temperature (25°C) and when the cells were incubated at sub-lethal temperatures (38°C). For the 25°C frequency of distribution readings, the amoebae were suspended as for normal transformation kinetic studies and samples were taken at t = 2 hours. For the heat-shock treatment, the cells were suspended in the regular manner but incubated initially for 40 minutes at 38°C, then transferred to the 25°C water bath and allowed to incubate for a further 2 hours before samples were removed. All samples were fixed in Lugol's iodine.

Each frequency distribution represents 100 individuals.

TABLE 7

Frequency of Flagella Distribution

Strain	T(°C)	Number of flagella							Mean
		1	2	3	4	5	6	7	
DC-1	25	5	77	6	12	0	0	0	2.2
	38	0	40	24	14	20	2	0	3.2
DC-4	25	7	88	3	3	0	0	0	2.0
	38	6	69	15	10	0	0	0	2.3
CT-8	25	6	76	14	4	0	0	0	2.2
	38	5	13	20	24	25	12	1	3.9
FT-52	25	6	91	4	0	0	0	0	2.0
	38	15	62	15	8	0	0	0	2.2
NB-1	25	4	78	6	11	1	0	0	2.3
	38	2	10	14	36	22	14	2	4.2

H. Cyst Storage and Viability

When stored on NNA and NM agar plates at room temperatures and in sealed containers the cysts of all strains retained their viability after 19 weeks storage. Excystment occurred within 24 hours of culturing onto fresh edge plates. Strain FT-52, although it retained its viability on NNA plates, failed to excyst after only 14 weeks storage on NM plates. FT-52 cysts were sparse on all plates.

I. Resistance of Amoebae and Cysts to Desiccation

The possible ability of amoebae and cysts to survive solutions of Liqui-Nox soap and alcohol was of great importance when handling the strains and preventing possible contamination of laboratory equipment and research material. Both amoebae and cysts were tested in 70% ethanol as well as 1% and 2% solutions of Liqui-Nox.

The addition of either of the soap solutions or the alcohol to the amoebae suspensions resulted in immediate lysis and degeneration of the organism.

The cysts retained their viability and were not desiccated by the soap solutions or drying periods after the soap immersion. The 70% alcohol, however, appeared to damage the cyst walls (i.e., they appeared rather mucoid) and the cysts failed to excyst after plating onto fresh agar plates.

The results of this experiment emphasize the durability

of the cyst under adverse conditions and the lack of resistance to desiccation of the amoeboid form.

As described under Sterilization in Methods and Materials, to prevent possible contamination of research animal material, all surgical tables, tools, etc. were scoured with Liqui-Nox followed by 70% ethanol. Cysts are not found in animal tissue and as demonstrated during this particular area of the research project, the amoebae are highly susceptible to desiccation by soaps and alcohol. All diseased animals, petri dishes containing diseased tissue or used for culturing purposes, and surgical gloves were incinerated to destroy the cysts.

J. Measurements and Taxonomic Observations

Qualitative microscopic observations of the isolates suggested a difference in size between the various strains. Direct measurements using an ocular micrometer were made to determine if actual quantitative differences in dimensions were present in the amoeboid, flagellate and cyst forms. Observations were also made concerning cyst structure and shape, flagellate shape, patterns of locomotion, mitotic processes, and the shape and number of various organelles (e.g., nucleolus, contractile vacuoles, etc.) within the trophozoite's protoplasm.

All strains were transferred to NNA with live *Aerobacter* before the taxonomic observations and measurements were

made. This common culture was used to ensure that any deviations found among the strains could not be attributed to a particular medium but rather were definite differences. Non-nutrient agar precluded components within the medium affecting the metabolic processes and/or growth patterns of the selected strains and allowed comparisons relatively free from extraneous factors. Axenic media would have been the most suitable but finding the optimal axenic culture for each strain is a time consuming and very demanding undertaking, and not suited to such a short-term project.

The measurements given in Table 8 are to be used for comparative purposes only as measurements would differ if the medium, incubating temperature or sampling time were altered.

(a) Size- The amoebae ranged from 10µm - 34µm when in locomotion (Figure 3). The length:breadth ratio gives an approximation of the over-all body size when compared with the mean length. Strain CT-8 was found to be considerably larger than the other strains not only during the actual quantitative measurements but during more qualitative measurements at different temperatures and on different media. The amoebic, flagellate and cyst forms were all larger than other strains. Strain FT-52 was consistently smaller than the other strains while DC-1, DC-4 and NB-1 were relatively similar in size.

TABLE 8

Measurement of *Naegleria* Strains

Each parameter was measured for 25 individuals except for the amoebae lengths, cyst diameters, flagellate body lengths and flagella/cell which are the measurements for 100 individuals.

All measurements were made using phase contrast optics with a Nikon Filar Micrometer calibrated against a stage micrometer with divisions of 0.1 and 0.01 mm. A Phaco 40 objective (x400) lens was used for all measurements except nuclear diameters (x1000, with oil immersion).

TABLE 8

Measurements of *Naegleria* Strains

	DC-1	DC-4	CT-8	FT-52	NB-1
Amebae:					
Length in locomotion; range	10.0-25.0	14.5-33.0	19.0-34.0	11.5-26.0	11.0-26.5
Mean	18.3	19.8	26.4	15.7	18.4
Body length:breadth ratio	2.8:1	2.8:1	2.7:1	3:1	2.7:1
Nuclear diameter; range	3.6-5.3	3.0-4.8	3.8-6.6	3.8-5.1	3.1-5.4
Mean	4.1	4.0	4.8	4.4	4.2
Contractile vacuoles/cell	1-4	1-3	1-2	1-3	1-3
Cysts:					
Cyst diameter; range	6.5-14.0	8.0-16.0	10.0-18.5	6.0-11.5	9.0-17.0
Mean	9.3	11.8	13.4	8.9	12.7
Nuclear diameter; range	2.8-5.0	2.8-5.0	3.6-6.2	2.6-4.8	3.0-4.9
Mean	3.9	3.9	4.7	3.7	4.0
Flagellates:					
Body length; range	12.5-19.0	14.5-21.0	14.5-29.5	12.0-17.5	11.5-21.0
Mean	15.9	17.6	21.5	14.6	17.1
Body length:breadth	2.0:1	2.0:1	1.8:1	2.0:1	2.1:1
Flagella length; range	10.5-18.5	11.0-19.0	12.0-19.5	10.5-17.5	11.0-19.5
Mean	12.2	15.5	15.0	14.9	15.0
Flagella/cell**	2.2	2.0	2.2	2.0	2.3

** See Table 6

FIGURE 3

Frequency Distribution of Amoebae Lengths

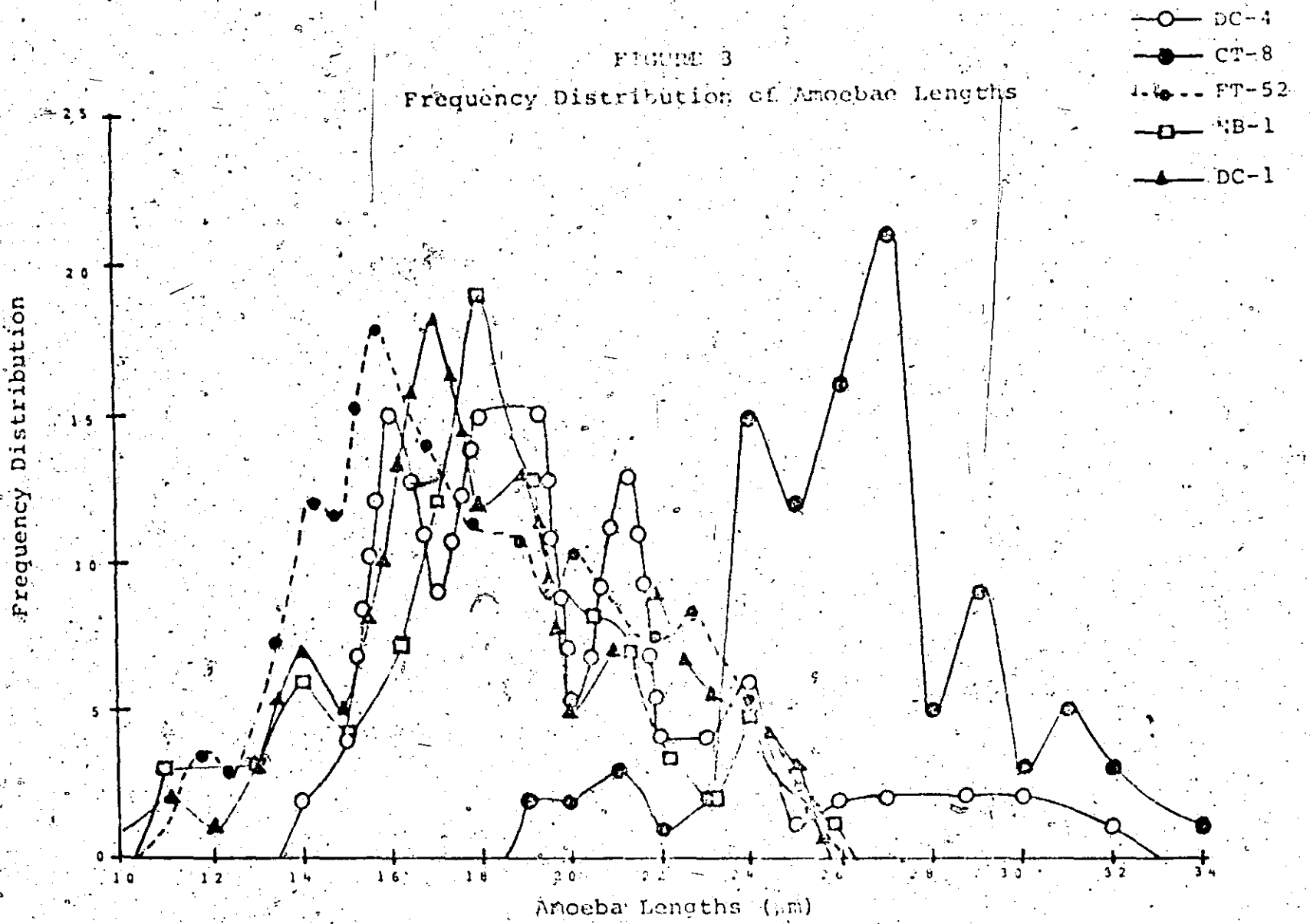
Figure 3 gives the frequency distribution of amoebae lengths for the five strains under study.

Amoebae were incubated on growth plates for approximately 18 hours, removed directly from the plates and suspended in a hanging drop preparation with Page's amoeba saline. After the amoebae had adhered to the glass slide and resumed normal movements, the lengths of 100 individuals in locomotion was measured using (x400) phase contrast optics and a Nikon Filar micrometer.

It is apparent in Figure 3 that the CT-8 population lies in the upper range of dimensions measured. Strain FT-52 was slightly smaller than DC-1, DC-4 and NB-1.

FIGURE 3

Frequency Distribution of Amoebae Lengths



The flagellates ranged from a mean length of 14.6μm (FT-52) to 21.5μm (CT-8). As in the amoeboid form the flagellates of NB-1, DC-1 and DC-4 were relatively similar in length. The mean flagellum length in all strains was within the range 12.2μm (DC-1) and 15.5μm (DC-4). The flagellum length of CT-8 was considerably shorter than the flagellate body length (i.e., 15μm versus 21.5μm respectively) and only in strain FT-52 did the flagella length exceed the body length.

The cysts ranged from a mean diameter of 8.9μm (FT-52) to 13.4μm (CT-8). Although the mean diameters of strains DC-1, DC-4 and NB-1, as well as the other two just cited, appeared to be dissimilar, the diameter measurements fell over a very large range and cyst size could not be used as a strict guide to strain identification. All strains had some very large cysts and these were occasionally binucleate.

It may be noted that the amoeboid form was larger in general size than the other forms and that the flagellate appears to be larger than the cystic form. Microscopic examination of live, motile amoebae show many contractile vacuoles coalescing and dispersing quite freely while other organelles move in varying degrees within the cytoplasm. In general, the amoebae appear fluid. This is not true of the flagellate form where the cell appears much more compact and organelles are found in approximately the same position within all fixed flagellates. The cystic forms appear even more

condensed and this includes the nuclei which, according to the measurements given in Table 8, have a smaller diameter than that found in the amoebic form.

(b) Locomotion- Strains CT-8, FT-52 and NB-1 proceeded primarily with monopodial extensions, whereas DC-1 and DC-4 often produced multipodial structures. Strains DC-4, CT-8 and NB-1 moved in a relatively smooth manner while DC-1 and FT-52 were often sinuous while in motion. During increased activity, all strains produced violent hyaline eruptions but this was more restrained, or absent, at low temperatures or under low-activity conditions (e.g., onset of karyokinesis).

Flagellates of all strains moved with violent thrashing of the flagella and no strain differences in locomotion could be discerned.

(c) Nucleus and nucleolus - The nuclear diameter in the cystic form of all strains was smaller than that found in the corresponding amoeboid-form. In the amoebae, the mean nuclear diameter ranged from 4.m (DC-4) to 4.7.m (CT-8); the cyst nuclear diameter ranged from 3.7.m (FT-52) to 4.7.m (CT-8). Whether these are significant differences and the nucleus actually more condensed in the cyst or not is not clear. Studies on more than 25 individuals would have to be undertaken to arrive at meaningful statistical differences, if any. The nuclear diameter would be extremely dependent on the mitotic state of the particular individual i ng

measured.

The nuclear diameter appeared to bear little relationship to the mean amoeba length possibly because the amoeba tends to shorten and round-up and the nucleus enlarges as the amoeba enters mitosis; therefore, although the amoeba area and nuclear diameter increased, the length would tend to decrease. It was observed in earlier microscopic work that at temperatures above the optimal temperature for a particular strain, the nuclei and amoebae were often enlarged possibly because of an inability to complete karyokinesis. In addition, occasionally more than one nucleus was seen in enlarged amoebae at these increased temperatures suggesting an inability to complete cytokinesis. In any event, the nuclear diameter is dependent to a large extent on the incubation temperature and the stage of division of the amoeba.

The nucleolus was usually rounded, but occasionally ovoid, and tended to be centrally located within the nucleus. Except during active mitosis and extreme locomotory activity, there appeared to be little variation in shape.

All strains underwent intranuclear karyokinesis or 'promitosis'. The nuclear envelope remained intact throughout the division process and the nucleolus did not disappear.

(d) Contractile vacuoles, food vacuoles - From one to four contractile vacuoles were seen in the 25 individuals of each strain during this study. However, this was observed

to vary constantly as various sized vacuoles coalesced and dispersed within any particular amoeba. After staining, other much smaller vacuoles (possibly food vacuoles) could be seen and these appeared as rolled membranous structures. As mentioned earlier, DC-4 amoebae growing in brain tissue contained large vacuoles filled with a golden, waxy substance that moved freely within the cytoplasm but was not dispersed in the normal manner of a contractile vacuole and may have been enlarged food vacuoles containing lipids or other brain material.

(e) Body Form and Structures- Amoebae in all strains were slug-like while in rapid locomotion but rounded with or without multipodial-type structures when inactive. The uropodium (or uroid process) is found on the posterior portion of an amoeba in motion and appears as a pinched-off nub trailing one or more fine short filaments. Uropodial structures were occasionally seen on members of all selected strains during locomotion.

Flagellates were normally fusiform in shape after the initial rounding up period (a stage observed just prior to, and at the initiation of, flagellum outgrowth). Strain DC-1 invariably contained many members (11% or greater) that retained the amoeboid-shape yet possessed flagella. This phenomenon was observed in the other strains but not to the same degree and never exceeded 4% of the population being counted.

Cysts were usually spherical but occasionally ovoid; the walls appeared smooth and double. Cysts were usually uninucleate but large cysts occasionally were binucleate. From one to four pores with plugs can be seen in Figures 4, 5, 6 and 7. More pores are probably present but with electron micrographs it is impossible to establish the exact number. Figures 8 and 9 (DC-4, the pathogenic strain) lack definitive apertures although indentations are apparent on the cyst in Figure 9.

Cysts of all strains generally appeared intact after excystment, but ruptured (Figure 7) and collapsed cysts were also present.

In summary, direct measurements confirmed the greater size of CT-8 and lesser size of FT-52 but failed to provide any definite differences in the other measurements. Strain CT-8 was in the top range of sizes when compared with other published *Naegleria* measurements (Fulton, 1970; Page, 1967).

Only strain DC-1 displayed any distinct characteristics while in the flagellate-form. The amoeboid-shaped flagellate was conspicuous as was the less than 90 percent flagellation rate invariably observed in this particular isolate.

In the cystic form, CT-8 characteristically had several plugs and pores, whereas fewer were noted in the other strains. Only DC-4 lacked distinctive pore structures but conceivably

FIGURES 4 - 9

Scanning Electron Microscope Studies of *Naegleria* Cysts

Micrographs of cysts of the various *Naegleria* strains were made to determine the presence or absence of pores and plugs in the cyst cell walls.

Cysts were incubated for 72 hours to ensure complete encystment, washed and fixed for 30 minutes in 0.2M phosphate buffer (pH 7.2), 4% glutaraldehyde and 20% glycerol. Samples were then rinsed with 20% glycerol, filtered and affixed to aluminum conducting tape, rapidly frozen in isopentane cooled by liquid nitrogen, transferred to liquid nitrogen and finally transferred to containers nested in liquid nitrogen. The container was then placed in an Edwards Vacuum Coating Unit and pumped using a rotary pump unit until the nitrogen had sublimed and reached room temperature. This prevents condensation on the samples when they are removed from the vacuum. All samples were pre-coated with carbon followed by paladium-gold 40/60 and viewed using a NMR Model 1000 Scanning Electron Microscope.

FIGURE 4: Strain NB-1

Note the prominent pore structure in the non-pathogenic NB-1 cyst.

(Magnification, 12,000x)

FIGURE 5: Strain CT-8

A preponderance of pores was noted in this particular non-pathogenic strain.

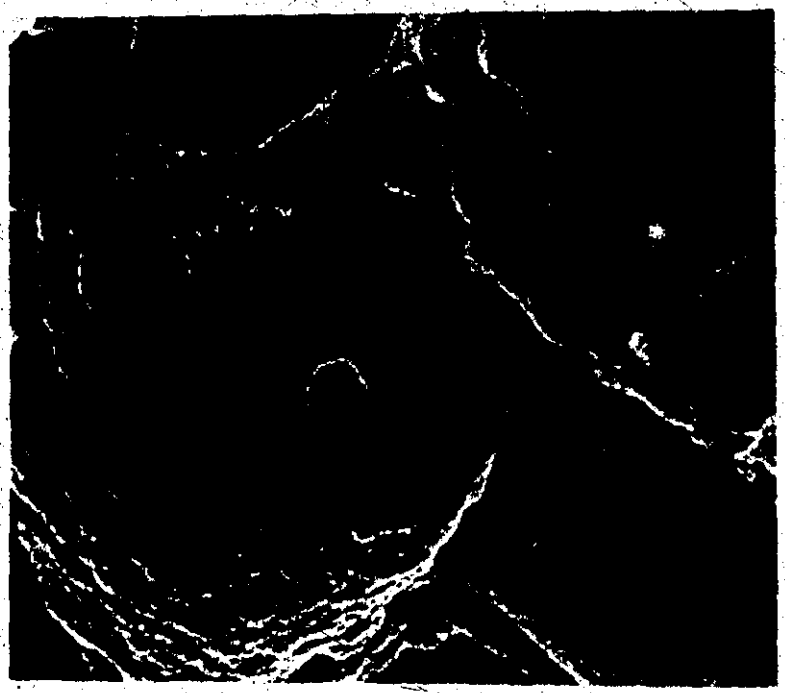
(Magnification, 12,000x)

FIGURE 6: Strain DC-1

A non-pathogenic isolate sampled from the same site as the pathogenic DC-4 strain. Only one pore is apparent in this micrograph but it is distinct with prominent features. (Magnification, 12,000x)

FIGURE 7: Strain FT-52

A prominent pore is seen in the adjacent micrograph with a possible plug. The cyst also appears to be ruptured but this may be an artifact. Strain FT-52 was pathogenic but only to a limited extent.
(Magnification, 12,500x)



Scanning Electron Microscope Studies of *Naegleria* Cysts

FIGURE 8: Strain DC-4

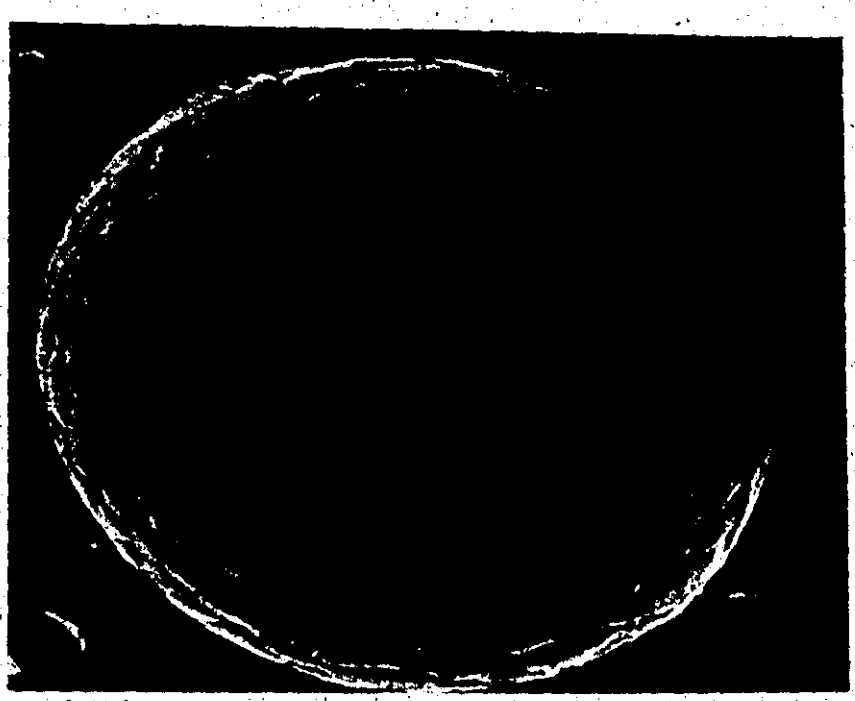
No pore structure can be discerned in the pathogen cyst wall structure. The outer surface is lightly ridged with only minor indentations.

(Magnification, 12,000x)

FIGURE 9: Strain DC-4

A possible pore or aperture (note arrow) in the cyst wall of the pathogenic DC-4 strain.

(Magnification, 12,000x)



the occasional member within the population may have possessed discrete pores that were not observed during the scanning electron microscope study. It is quite apparent, however, that if pores are present in DC-4 cyst walls, they are not in the same abundance as that found in the other strains.

Measuring substantially more than 25 - 100 individuals might provide distinct strain differences and further electron microscope work would undoubtedly confirm the presence or absence of cyst pores in the DC-4 strain. However, the prime purpose of this study was to locate, if possible, a pathogenic strain and compare its virulence to other known pathogenic *Naegleria*. Consequently, the number of cells and organelles measured were restricted to the same number as used by Page (1967) and there is probably no significant statistical data to be found in such a small sampling.

K. Experimental Pathology (DC-4)

Test #1: The original test for pathogenicity of the heterogeneous DC-4 isolate. The inoculation techniques are as described in the section Pathogenicity Testing of Isolates. Ten test animals were used and an inoculum size of 2.7×10^4 amoebae/animal. Three out of the ten animals succumbed to PAME.

Test #2: DC-4 cell line was cloned from brain tissue of the first fatality resulting from Test #1. Test #2 was conducted to ensure that the cloned line retained the pathogenic aspects of the original isolate. Two animals died of PAME.
(Inoculum size = 2.7×10^4 and 10 test animals)

Test #3: The inoculum size was increased to see if a relationship existed between size and the number of fatalities. Carter (1970) found that inoculum size corresponded positively with the mortality rate and inversely with the length of the disease. General observations were also made concerning disorientation of mice and their subsequent recovery.
(Inoculum size - 4.0×10^4 and 12 test animals)
Five of the 12 animals succumbed to PAME while 3 displayed signs of disorientation but recovered. Table 10 shows the relationship of inoculum size versus fatalities at the various inoculum sizes tested. A positive relationship appears with a definite correlation between inoculation dosage and the number of resulting fatalities.

TABLE 9

Experimental Pathology Test Results (DC-4)

NNA spread plates (NM plates for Tests #7 and 8) were prepared and incubated overnight at 37°C. The amoebae were then removed, washed and resuspended in sterile de-ionized water at a density of $\approx 10^6$ amoebae/ml. as established using the Coulter counter.

All mice were female Swiss/Webster, 15 g. (except in Tests #4 and 9). Test animals were lightly anaesthetized using anhydrous ether and held with the ventral surface uppermost. A disposable 1 c.c. syringe and 26-gauge hypodermic needle were used to intranasally introduce 20µl.-40µl. (depending on the desired inoculum size) into their nostrils. The animals must be breathing deeply and evenly to ensure complete inhalation of the inoculum.

Five animals were inoculated with sterile de-ionized water and a small *Aerobacter* population and used as controls for each test date.

Animals were then observed for the following six weeks for signs of disorientation and morbidity. Those succumbing were checked for PAME by plating their cranial contents, liver, heart, spleen and lungs onto NNA plates with live bacteria and incubating at 37°C for one to several days. All animals surviving six weeks and in good health were destroyed.

TABLE 9

Experimental Pathology Test Results (DC-4)

Test No.	Inoculum Size	Animal Weight	Number & Day of Death	Fatalities PAME	Disoriented* Animals
1**	2.7×10^4	15 g.	1 @ day 6 2 @ day 7	3/10	not observed
2	2.7×10^4	15 g.	1 @ day 2 1 @ day 7	2/10	not observed
3	4.0×10^4	15 g.	3 @ day 2 1 @ day 11 1 @ day 20	5/12	1 @ day 7 3 @ day 8
4	4.0×10^4	20 g.	2 @ day 13	1/6	4 @ day 13
5	4.0×10^4	15 g.	1 @ day 2 1 @ day 7 1 @ day 8	3/10	4 @ day 7
6	4.0×10^3	15 g.	1 @ day 11	1/10	3 @ day 11
7***	4.0×10^4	15 g.	1 @ day 2 1 @ day 4 1 @ day 9 1 @ day 13 1 @ day 15	5/12	1 @ day 2 2 @ day 3
8***	2.0×10^4	15 g.	1 @ day 2 1 @ day 3 1 @ day 13	3/11	not observed
9	2.0×10^4	10 g.	1 @ day 3	1/6	not observed

* Animals severely disoriented, hunched, circling and hair bristling but recovered

** Initial pathogenicity test consisting of a mixed population of amoebae

*** Amoebae inoculum grown on NM rather than regular NNA plates

Test #4: The general observations made during Test #3 concerning apparent recovery of severely disoriented and ill mice, were tested in a more quantitative manner. Also included in this test was a comparison of age versus possible resistance. (Inoculum size = 4.0×10^4 with 24 test animals; 20 g. each)

Half of the inoculated mice were maintained in the usual manner and any fatalities tested for PAME and recorded. The remaining half were sacrificed at Day 3 and the brain and body parts plated in the usual manner, incubated and observed for possible amoebic infestation. A comparison was then made between the number of mice that succumbed to PAME and the number of sacrificed mice that had positive amoeba results. These results were also later compared for possible age resistivity with the results of Tests #5 and #8.

Table 11 shows only a 16.6% fatality rate for the 20 g. mice although 33% of the sacrificed animals were infested. One point that was noted was the length of survival time of the two fatalities that both occurred on Day 13 (see Table 9), approximately 6 days after the onset of symptoms. This might possibly show a greater resistance to the disease.

Test #5: Test #4 had suggested that amoebic infestation was not necessarily fatal in all cases. To further test for spontaneous recovery and possible age resistivity, 20 animals, 15 g. each, were inoculated. Ten were watched for signs of PAME

TABLE 10

Inoculum Size versus Fatalities

Inoculum Size	Fatalities	% Fatalities
4.0×10^1	0/10	0%
4.0×10^2	0/10	0%
4.0×10^3	1/10	10%
2.0×10^4	3/11, 1/6	23.5%
2.7×10^4	3/10, 2/10	25%
4.0×10^4	5/12, 2/12, 3/10, 5/12	32.6%

Legend: Table 10 includes all fatalities over the entire experimental research period. This includes Test #1 which consisted of a mixed, heterogeneous amoebic population: Test #4 which used larger animals and Test #9 which tested younger animals. These differences do influence the data; for example, if only Tests #3, 5 and 7 results (where only uniformly matched animals were used) were tabulated with an inoculum size of 4.0×10^4, the fatality rate for this particular inoculum size would average 38.5% rather than the 32.6% shown on Table 10.

while the remaining ten were sacrificed at Day 3 and tested as described in Test #4. Results were later compared with those of Tests #4 and #8.

(Inoculum size = 4.0×10^4 with 20 animals, 15 g. each)

Table 11 shows that the 15 g. animals had approximately double the mortality rate of the larger (viz., older) animals, 30% fatalities versus 16.6% as well as 100% infestation versus 33% infestation 3 days after inoculation. Deaths ranged from t[illegible] to eight days after inoculation, a much shorter period than that seen in Test #4 (Table 9).

Test #6: Carter (1970), using *N. fowleri* isolated from a PAME victim, found that only 39 amoebae could produce a PAME fatality in a mouse and that the LD_{50} for this particular strain was 300 amoebae. The LD_{50} is the number of amoebae required to kill 50% of the inoculated mice. An attempt was made to establish the minimum dosage and LD_{50} for DC-4. Serial dilutions of Test#5 inoculum were used resulting in intranasal inoculation sizes of 4.0×10^3, 4.0×10^2 and 40 amoebae/inoculation. Ten test animals were inoculated per dilution.

Table 10 shows the deaths resulting from the dilutions. The lowest dosage leading to a fatality was 4.0×10^3 and an LD_{50} was not attained. Several animals in the 4.0×10^3 group became severely disoriented during the study but only one death occurred. In the 4.0×10^2 inoculum size, animals became disoriented but none to any extreme degree. The animals receiving

TABLE 11

Spontaneous Recovery Rate versus Animal Weight

Test #	Weight	Inoculum Size	Fatalities*	Infestation**
4	20 g.	4.0×10^4	16.6%	33%
5	15 g.	4.0×10^4	30%	100%
8	15 g.***	2.0×10^4	27%	91%
9	10 g.****	2.0×10^4	16.6%	100%

* Animals succumbing to PAME
** Animals sacrificed and exhibited amoebic infestation after incubation
*** Amoebae inoculum grown on NM medium as compared to the other inoculations which were all grown on NNA
**** All animals were from the same litter and mixed males and females

Legend: Animals in Tests #4, 5, 8 and 9 were treated in the following manner. Half of each inoculated group was maintained under normal conditions while the remaining half were sacrificed at Day 3 and their cranial contents (including the olfactory bulbs), heart, lungs and liver were incubated on NNA plates with live *Aerobacter* at 37°C.

The number of mice that succumbed to PAME was then compared to the number of sacrificed animals that demonstrated amoebic infestation. As can be seen in Table 11, in all cases the rate of infestation was significantly higher than the fatality rate.

approximately 40 amoebae intranasally displayed no adverse effects.

Test #7: It has been noted that pathogenic *Naegleria* isolates grow poorly, if at all, on salts-containing media (Carter, 1970; Culbertson *et al.*, 1968). NM is a dilute salt medium (0.25%) but it was noted that DC-4 originally grew very poorly on NM plates. After the cell line was successfully established on NM medium, the pathogenicity of the clone was tested to see if NM medium might have reduced or enhanced its pathogenicity for mice, or its virulence. Results were compared with Test #3 (NNA-grown amoebae) and Test#8 for dosage versus fatalities. (Inoculation size = 4.0×10^4 with 12 test animals).

Table 10 shows that NM-grown amoebae proved just as virulent as NNA-grown DC-4 in Test #3 (i.e., 42%). Fatalities ranged over a period from 2 to 15 days with the two fatalities on Day 13 and 15 displaying severe PAME symptoms for over a week before finally succumbing. The animals in this group were especially prone to head scratching and mutilated their faces, particularly in the eye and region directly above the cranial cavity.

Test #8: The spontaneous recovery rate of NM-grown inoculum was tested as well as the possible correlation of dosage to number of fatalities.

(Inoculation size = 2.0×10^4 with 22 test animals)

Test #8 correlates with Test #7 (see Tables 9 and 10).

An inoculum of 2.0×10^4 led to 27% fatalities while 4.0×10^4 led to 42% fatalities respectively. It is consistent with the direct relationship between inoculum size and resulting fatalities with NM-grown amoebae that had already been demonstrated with NNA-grown DC-4.

The NM-grown amoebae had a similar rate of infestation as that found in Test #5, NNA-grown DC-4 (i.e., 27% fatalities and 91% infestation versus 30% fatalities and 100% infestation respectively). However, in Table 11 it can be seen that Test #5 had double the inoculum size of Test #8. Whether the NM-grown inoculum had greater pathogenicity or whether the animals in the various groups had different degrees of resistance is unknown.

Test #9: The possible greater susceptibility of younger mice was also tested. It was not possible to purchase 10 g. mice but one of the experimental animals produced a litter and these were raised to the 10 g. size. The litter consisted of a mixture of males and females. Half of the animals were sacrificed at Day 3 while the remainder were observed for PAME fatalities. Results were compared with Tests #4, 5 and 7.
(Inoculum size = 2.0×10^4 with 12 test animals, 10 g. each)

Using the 10 g. animals, the fatality rate was similar to that found with the 20 g. animals (Test #4; 16.6%) but the infestation was 100% as compared to only 33% in the older mice (see Table 11). Again, note should be made of the differences

in inoculum size between the two tests as well as the short period between inoculation and death in the smaller animal (Table 9).

As mentioned, all the 10 g. animals in this particular study came from one litter and were of both sexes. There were, therefore, three variables in the particular group; namely, a possible inherent resistance to the disease; possible differences in male versus female resistivity; and finally, the difference in age (viz., weight) as compared to the animals in the other tests. Because of these shortcomings, it is difficult to interpret the results for the younger animals. All animal-based pathology studies have innate and unknown variables unique to each animal being studied but in the other tests these were held to a minimum by using the same sex and randomly selected members from different litters.

IV. DISCUSSION

Culbertson *et al.* (1968) and Butt *et al.* (1968) found that their isolate, HB-1, from a PAME victim did not grow, encyst or transform as readily as non-pathogenic *N. gruberi* strains. Fulton (1970) comments on these supposed differences using NEG, his particular strain of *N. gruberi*, as an example: "After prolonged cultivation in particulate-free axenic media, *N. gruberi* NEG amebae initially do not grow well when returned to cultures with bacteria, and sometimes many generations are required before the amebae will grow rapidly, if they ever do at all. Whether this change is adaptation or selection is not known.". It may well be that the difficulties encountered with HB-1 are caused by a failure of the organism to adapt to a change in diet (i.e., from brain tissue to bacteria) or the investigators may be using techniques unsuited for their particular strain. Difficulty was experienced in stabilizing FT-52 and DC-4 growth on NM but after growth was established, the amoebae grew extremely well. Of the five strains studied, only FT-52 failed to encyst well on NM plates. However, in all other respects the pathogenic strains proved comparable, or even superior, to the other non-pathogenic strains studied. All strains encysted well (with the exception of FT-52), grew

profusely under suitable conditions, transformed rapidly and synchronously, and excysted readily on spread or edge plates.

Several minor differences in rates were found with NB-1, the control, as compared to the findings of others but these differences were well within the range of experimental variability. For instance, the generation time given by Fulton and Dingle (1967) is 1.7 hours as compared to 1.8 hours found during this research; the T_{50} varied insignificantly from 69 minutes (Dingle, 1970) to 69.5 minutes. The average number of flagella after heat shock was 4.2 rather than 4.5 as reported by Dingle (1970). The NB-1 rate variations were insignificant and it can be used as a standard, or control, for comparative purposes with the other strains. Since the NB-1 results are comparable to previous reported findings, the data on the other isolates should also represent each strain's rate of transformation, generation time, flagellation percent under normal and heat-shock treatment, and other characteristics with a fair degree of accuracy. It may well be that the other strains could attain a faster doubling time or T_{50} as well as develop a greater number of flagella during heat-shock treatment but one would not expect any findings dramatically different from those found during this research.

Dingle (1970) found that by heat-shocking cells of strain NB-1 during the amoeba-flagellate transformtion, the

number of flagella could be increased to as many as 18 flagella per cell. In general, he found that the sub-lethal temperatures doubled the flagella per individual and the average moved from 2.2 under normal transformation conditions to 4.5 flagella under heat-shock treatment. The critical period for flagellum synthesis is the initial 40 minute incubation period and by heat-shocking during this period, the synthesizing process is altered leading to multi-flagellation. NB-1, the control strain, was affected by the heat-shock treatment as were the other non-pathogens, DC-1 and CT-8. However, the metabolic processes of FT-52 and DC-4, the pathogens, were relatively unaffected by incubation at 38°C.

The non-pathogenicity of NB-1 was confirmed and CT-8 and DC-1 were also found to be non-pathogenic. Only DC-4 appeared consistently pathogenic while FT-52's pathogenicity was not conclusively demonstrated.

When testing the temperature tolerance of the various strains it was found that only FT-52 and DC-4 could successfully multiply and clear an agar edge plate at temperatures beyond 37°C. Griffin (1972) reported that *N. fowleri* could grow at 45°C and tolerate 46°C. Fulton (1970) reported that NB-1 will grow "....from below 20° to 40-41°". True growth, as compared to the migration of amoebae, was not observed in NB-1 beyond 36°C although the amoebae could survive and migrate for at least 24 hours when incubated at 40°C. Proliferation, such

as required to clear an edge plate, did not occur.

Carter (1970) found that *N. fowleri* required 4-7 days at 37°C to cover an edge plate. This is significantly slower than pathogenic DC-4 which required only 36 hours when incubated at this temperature. Griffin (1972) found that at room temperature the pathogenic strains of *N. fowleri* required one week to two months to cover an agar slant. At 23°C, DC-4 consistently cleared an edge plate in one week, but this rate was much slower than that of the other four strains studied. Strain CT-8, the most prolific at room temperature, required only 80 hours to completely clear a plate, whereas NB-1 and FT-52 required 100 hours. Strain DC-4's growth rate, while being well below the other isolates at room temperature, was far superior in the range from 37°-42°C (98.6°-107.6°F) which is the range from normal body temperature to death from fever in humans.

Carter also found that although decreased temperatures (i.e., 21° or 30°C as compared to 37°C) retarded *N. fowleri* growth, infectivity remained unchanged. Less than 16 hours at 0°-4°C destroyed amoebae viability. In its natural environment, however, the amoebae would probably encyst as the temperature decreased over a period of several weeks. Carter found that cysts could survive storage at -15°C for up to two months. The length of time that a cyst will remain viable when stored under relatively optimal conditions is of interest not

only to the protozoologist who may be maintaining the protozoan for experimental purposes but to health officials who may be attempting to reduce or eradicate pathogenic strains within a given area. It is known that some encysted pathogenic strains when stored on plain agar in a moist environment and at room temperature can survive for two years and possibly longer (Carter, 1970) while others can survive this length of time in a dried state (Duma *et al.*, 1969). Fulton (1970) reports that NB-1 cysts are viable up to seven years. The strains used in this research had remained viable in the sampling tubes at 12°C for at least five months before isolation and testing for pathogenicity was undertaken, suggesting that the pathogenic *Naegleria* can survive lowered temperatures for considerable lengths of time. However, whether the *Naegleria* strains were in the amoeboid, flagellate, cyst or intermittent stages is not known.

Cyst and trophozoite resistance to soap solutions and 70% ethanol was studied to obtain qualitative information concerning the durability of *Naegleria* under adverse conditions. This aspect of the investigation was of particular relevance when dealing with the pathogenic strains and in preventing possible contamination of test animal material, surgical areas and laboratory utensils. Carter (1970) reports loss of viability of amoebae and cysts after 24 hours immersion in solutions of 70% alcohol, 4% formalin, and 1% hospital soap. The

strains used throughout this project showed varying degrees of resistance to chemical desiccation. The amoebae were highly susceptible while the cysts were only affected by immersion in 70% ethanol for a 24 hour period. Since the cysts can survive soap solutions and chlorination (Jamieson & Anderson, 1972b), one can readily see why amoebae can be isolated from swimming pools and fish tanks with relative ease (e.g., PT-52 and CT-8).

However, why pathogenic strains would proliferate to infective levels in heated swimming pools remains unanswered. Investigators have found the pools to be more than adequately maintained with a low bacterial population; yet cases of *Naegleria* meningoencephalitis related to swimming pools have occurred in Belgium (Van den Driessche, 1973), Czechoslovakia (Cerva & Novak, 1968), and Australia (Anderson & Jamieson, 1972b). Although attempts to isolate pathogenic amoebae from the pool in Czechoslovakia have not been successful, there appears to be little doubt as to the source of the infection. In Australia, super-chlorination up to 10 p.p.m. failed to destroy *Naegleria* present in the pool but salination to 0.7% (w/v) eradicated all species (Anderson & Jamieson, 1972b). Chlorination should destroy, or at least limit, the *Naegleria* food source and yet the number of PAME cases directly attributed to pools and the ease with which CT-8 was isolated during this research project, suggests that in its natural habitat there

may be many nutrient sources (i.e., cell debris, organic litter) utilized by *Naegleria* strains. Under laboratory conditions, few strains (if any) would survive or proliferate on as sparse a bacterial population as that found in well-maintained chlorinated pools. The amoebae should encyst if the nutrients become depleted or are sparse, and cysts are, according to all research to date, non-infectious. *Naegleria* was considered to be a harmless saprophyte, coprozoic and free-living, until Culbertson in 1958 first noted the ability of the amoebae to grow on cell debris and brain tissue. There may be many as yet unrecognized sources of food and many environments suitable for specially adapted strains.

Samples were incubated at 37°C during this research to attempt to isolate pathogenic strains. According to Griffin (1972) even 37°C allows non-pathogenic amoebae to outgrow the pathogenic *Naegleria* strains; therefore 43°C would be preferable for isolation purposes. The overgrowth at 37°C by the non-pathogens would result in an underestimation of the number of potentially pathogenic species present in mixed inocula. Since it was not known at the beginning of this research project whether pathogenic srains were to be found in this locale, the lower incubation temperature was used and possibly pathogenic strains were lost during the isolation process.

Other investigators have found that pathogenic strains are more fastidious in their diet and will grow only on NNA with live *A. aerogenes* or *E. coli* (Butt *et al.*, 1968; Carter, 1970; Culbertson *et al.*, 1968). The results of this research would tend to support their findings. Strains DC-4 and FT-52 grew poorly, if at all, on the low salt NM medium initially while proliferating well on NNA with live bacteria. They did adapt to the NM and grew vigorously on this medium but using NNA for the initial isolation is recommended to ensure growth of any pathogens that may be in the sample.

Measurements:

As mentioned earlier, all measurements and general morphological observations are primarily for comparative purposes and should not be considered as set dimensions or strict criteria for any particular strain. The nuclear diameter, amoeba size and shape, the cyst diameter, and to a certain extent the flagellate dimensions, are all dependent on factors present during a given experiment which may be altered inadvertently, or deliberately, and lead to different findings in the next set of tests. Some of the more important factors are the mitotic or growth phase of the trophozoite, the incubating temperature and the type of medium used for culturing purposes.

Fulton (1970) and Chang (1971) note the differences that occur by varying the medium. NNA medium was used for

the culture medium during the measurements study and was the medium used by Page (1967) for his taxonomic studies. Fulton (1970) disagrees with the design of Page's study and claims that results are less reproducible with NNA and live bacteria due to uneven distribution of the bacterial lawn. However, practically all other investigators working with pathogenic varieties use only NNA with bacteria. For comparative purposes, a medium that has the least effect on the metabolic processes of any of the strains was deemed the most suitable. Consequently, NNA with live bacteria was used throughout the measurements and taxonomic observations to produce as uniform comparative results as possible.

It was noted during the course of this study that increasing the temperature, particularly at or approaching the lethal range for a given strain, resulted in extremely enlarged amoebae and nuclei as well as multi-nucleate enlarged amoebae suggesting that the amoebae were no longer capable of undergoing karyokinesis or cytokinesis.

All the isolates closely approximated the normal measured ranges for *Naegleria*. The eight strains measured by Page (1967) fell within the range of 14.5µm-41µm while in active translational locomotion. The medians for the various strains are as follows: 21.4µm, 24.8µm, 21.4µm, 24.2µm, 26.2µm, 22.1µm, 22.1µm, and 24.2µm. Unfortunately, Page fails to supply the mean values. Cerva (1969) reported a mean length of 7.4µm for his pathogenic strain isolated from a PAME

victim. The five strains studied during this project ranged from 10.0um-34.0um when in active motion. The means for the isolates are as follows: 18.3um, 19.8um, 26.4um, 15.7um, and 18.4um. Only FT-52 (mean 15.7um) and CT-8 (mean 26.4um) appeared distinctly different in size but these dimensions lie within the ranges normally observed.

Mean flagellate body length ranged from 14.8um - 20.8um (i.e., 18.7um, 15.9um, 14.8um, 17.8um, 20.8um, 16.7um, 14.8um, and 19.4um) in Page's study and from 14.6um-21.5um (i.e., 15.9um, 17.6um, 21.5um, 14.6um, and 17.1um) during this study. Once again there appeared to be little, if any, significant difference between the pathogens and non-pathogens.

Page's presumably non-pathogenic cyst diameters ranged from 8.6um-16.6um (mean 11.5um) as compared to 6.5um - 18.5um (mean 11.8um) for the non-pathogen DC-1, NB-1 and CT-8. The pathogenic DC-4 and FT-52 cyst diameters ranged from 6.0um-16.0um (mean 10.4um); whereas *N. fowleri* (Carter, 1970) averaged 9.0um and Saygi *et al.* (1973) reported a mean diameter of 15.6um. There appears to be a wide variation in cyst diameters but pathogenic strains could not be identified on the basis of cyst dimensions.

The cysts of non-pathogenic strains contained one or more pores, while DC-4, the one clear pathogen isolate in this study, showed no definitive pore structure. FT-52 did possess distinct pores and plugs, but its pathogenicity is still somewhat in question.

Rate of Locomotion:

Most investigators include a rate of locomotion chart in their measurements of *Naegleria* strains. Page (1967) measured ".....the rate of locomotion: for 1 min or 100 micrometer units (whichever came first) in 10 amoebae of each strain." and found the rate ranged from 46μm-122μm per minute. This was at room temperature which varied from 25°-30°C. As he says, "The values obtained for locomotive rates are only an indication of the potential rate for each strain".

Carter (1970) measured his over a period of one minute in a similar manner and found the *N. fowleri* isolate's rate of locomotion ranged from 30μm-66μm per minute, with an average of 45μm per minute. Page failed to give an average rate but comparison of Page's and Carter's locomotory rates shows that *N. fowleri* averaged lower than the lowest rate recorded by Page. This may have been due to the laboratory temperatures or may be a significant difference.

Since *Naegleria* activity is influenced by the temperature of the environment, the composition of the liquid suspension, the length of the observation time, the mitotic state, and other variables, this particular measurement appears to have little validity even under controlled conditions. Consequently, since the rate of locomotion can be changed so drastically by altering any of the environmental conditions, this measurement was not included in the comparative analysis of the strains.

Experimental Pathology:

The survival time (average 7.4 days) of mice inoculated with DC-4 corresponds with that found by other investigators using strains isolated directly from PAME victims. Nicoll (1973) found death resulted in 6-7 days after inoculation; Martinez *et al.* (1973b), 5-7 days after; Carter (1970), 3-8 days after; while Van den Driessche (1973) found his isolate caused death in 2-3 days. As noted previously, the older (viz., larger) animals survived 13 days before succumbing - much beyond the normal survival time.

The DC-4 inoculum size bore a definite relationship to the fatality rate but no direct relationship between inoculum size and duration of disease could be discerned. Carter (1970) reports that the dosage influenced the length of the disease: for example, 4,000 amoebae resulted in death in 5-6 days; 300 amoebae, 8 days; fewer than 300 amoebae, 11 days or more. Singh and Das (1972b) used the flagellated form of *N. aerobia* (considered here to be *N. fowleri*) and found not only similar correlations of inoculum size to duration of illness but an increase in virulence. An inoculum of 2,500 flagellates produced the same or a shorter incubation period than 20,000 amoebae. They found that 1,000 flagellates led to 75% death rates; 500, 66%; 100, 57%; and 50, to a 25% death rate. This is not a linear relationship and suggests that only so many individuals within a population are capable

of producing PAME. Deaths with the various inoculum sizes of flagellates occurred in 4-5 days. Singh and Das postulate that in the wild, *N. aerobia* is constantly changing from amoeba on the bottom where it feeds to a flagellate which then swims about and is more pathogenic than the amoeba. Possibly it could penetrate further into the inner nares but would then revert to the amoeba.

Although a near linear relationship was established with the DC-4 inoculum size versus fatality rate (Table 10), there is some doubt as to whether the relationship would continue beyond the 40,000 amoebae per inoculum (the maximum dosage used). It was noted at the time of inoculation that the nasal chambers of the experimental animals were completely filled by the 20µl used and increasing the inoculum size possibly would not increase the fatality rate appreciably.

The maximum mortality rate achieved with strain DC-4 was 42% with an inoculum of 40,000 amoebae. Martinez *et al.* (1973b) reports a 45% fatality rate with 10^3-10^4 amoebae; Nicoll (1973), 100% fatalities with 3,000 amoebae; Carter (1970), a 90% fatality rate over a three year period. It should, however, be pointed out that many of these investigators (e.g., Culbertson, Carter, Singh and Das, etc.) report killing the animals when they appear moribund. This practice was not followed in the DC-4 pathology studies; if moribund-appearing animals had been destroyed, the mortality rate would have been

increased substantially.

The selection of animal strains also enters into the resulting fatality rate. This may be illustrated by the fact that two of Culbertson's pathogenic strains killed the mice (Albino, 10-12 g.) used by Singh and Das; whereas, only one of these strains would kill the mice (S.P.F. (Lilly) of ND-4 lineage, 9-11 g.) routinely used by Culbertson (Griffin, 1972). Only Swiss/Webster mice, supplied from one source, were used throughout the DC-4 testing and different results might have been obtained if different strains of mice had been tested for susceptibility.

There are many puzzling aspects concerning *Naegleria* meningo-encephalitis. One of these is the likelihood of another portal of entry other than the nasopharyngeal route but where this entry point may be is unknown. The gastro-intestinal tract, the circulatory system and the respiratory tract have virtually been excluded either through animal testing or elimination (i.e., the inability of amoebae to survive human serum, pH levels below 4.3 and bile salts). In Australia several PAME victims had either not been swimming or had done so in salt water. Of these, some had an opportunity to be in contact with waste water and septic tank effluent and, thereby, possible intranasal contamination. Others, however, appear to have developed the disease without ever being in a situation where they could have been infected via the nasopharyngeal route. A

case reported by Patras and Andujar (1966) concerns an older man admitted to the hospital with peripheral edema. On the seventeenth day of his hospital stay, the patient developed a severe headache and died on the nineteenth day of PAME. Cerva's (1969) findings show that the average survival time from inoculation until death in a human is approximately ten days or less. There is no explanation as to the source of the amoebae or their route of entry.

This brings one to the point of considering possible carrier states and sites of amoebic infestation other than the brain. It has been emphasized several times in this thesis that proliferation at temperatures well above 37°C is a prime requisite for any potential pathogen of man. There is, however, always the insidious possibility that amoebae now considered non-pathogenic because of their temperature-intolerance could adapt sufficiently to invade brain tissue. At the onset of fever, the population might be depleted or possibly encyst, although *Naegleria* cysts have never been found in tissue. Other amoebae (i.e., *Hartmannella*) are known to encyst in animal tissue and the present data on *Naegleria* cysts may reflect limited testing. In any event, as the fever subsided the parasite could commence growing again. Less temperature-tolerant strains would probably not invade the brain but possibly other cooler areas of the body. There are several diseases of unknown etiology, multiple sclerosis to name but

one, that are typified by slow degeneration of the affected organs with sporadic remissions. This is all supposition, of course, but the possible parasitic role played by *Naegleria* has just begun to be investigated.

Carter (1972) mentions the statistically significant correlation found between the presence of *Hartmannella* amoebae (an amoeba often mistaken for *Naegleria*) in the nasal passages and a previous history of headaches, frequent colds and nose bleeding. Also amoebae, again of the *Hartmannella* variety, have repeatedly been isolated from the same subjects suggesting a possible carrier state. One final case concerns a patient suffering from Hodgkin's Disease for ten years prior to his death. After his death, an abscess containing amoebae (of unknown classification) was found in brain sections. Whether this abscess was related to his disease is unknown.

Since PAME has been overlooked for all these years, there could be many other low-profile diseases attributable to amoebic infestation. At present, *Naegleria* is the only agent known to cause PAME but its role may not be limited to this one tissue or disease.

During the experimental pathological studies with mice using strain DC-4, amoebae emerged only from incubated brain tissue. Other investigators (Carter, Culbertson, Duma, etc.) have found amoebae infestation of the liver, spleen, lungs, and surrounding various blood vessels in mouse studies. However,

incubating these organs and tissue from animals succumbing to DC-4 produced meningo-encephalitis failed to produce any signs of amoebic infestation. Whether these findings were due to the particular pathogenic strain DC-4 or to the strain of mice (Swiss/Webster) is unknown but does point to possible serum factor differences in mouse populations.

It was also noted that a large number of mice inoculated intranasally with DC-4 developed PAME symptoms (i.e., extreme disorientation, bristling hair, and hunched positions) but recovered. This has not been reported by other investigators, again possibly because of the mouse strain used or the pathogenic *Naegleria* strain used but more probably because of the general practice of killing moribund-appearing animals. If mice are capable of recovering from severe *Naegleria* invasion then there is always the possibility that humans can, and have, recovered from PAME. The disease, being only recently recognized, is still only in the initial investigative stages and may prove to be less virulent than believed at present.

Naegleria Speciation:

Investigators have attempted to classify *Naegleria* for many years and at present several taxonomic classifications are in use. The possible species of a *Naegleria* isolate is a particularly difficult problem to solve. Whether there is only one species (*N. gruberi*), two species (*N. gruberi* and *N. fowleri*), or numerous species is not definitively

established at present.

As to whether the strain DC-4 is *N. gruberi* or *N. fowleri*, or possibly an intermediate strain (Anderson and Jamieson, 1972a), is unresolved at present and possibly only immunological testing can determine its true species.

In common with *N. fowleri* is DC-4's virtual absence of pores within the cyst wall structure but it appears to lack the virulence ascribed to *N. fowleri*. The cultural fastidiousness and other attributes assigned to *N. fowleri* are gradually being found to be untrue; for example, Cérva's axenic medium using fresh horse serum produces good pathogen growth (Cerva, 1969) and Anderson and Jamieson (1972a) are now growing *N. fowleri* on heat-killed *E. coli* and on an axenic medium described by Fulton (1970).

Carter (1970) considers the cyst of *N. fowleri* to be its most distinguishing feature. He claims that the commonest type of cyst formed by *N. fowleri* is such that, "The wall is intact and apparently the same as in the mature cyst....but the interior appears almost empty." This type of cyst is seen at times with all the strains tested during this research and cannot be considered a definite characteristic for any particular strain. They are particular prevalent on NNA plates. Possibly the amoeba has excysted or degenerated within the cyst and the movement noted within the empty-appearing cyst wall is due to bacteria entering the cyst.

Pathogens in Canadian Waters:

The DC-4 sample was taken during the latter part of September, 1973, following a prolonged heat spell in the area. Whether the pathogenic strain would be in sufficent numbers to be easily isolatable at other times of the year or following cooler summers is unknown.

The organic and faecal pollution, the possible thermal pollution from the Dundas Hydro plant, and the shallowness of much of the canal allows extensive warming during the summer months and would contribute to increasing the pathogenic population while slowing the growth rate of the non-pathogenic *Naegleria*.

When one considers the temperature of the Desjardins Canal (i.e., 34°C and possibly higher after extensive hot weather) it becomes readily apparent that the canal provides an optimal environment for pathogenic DC-4 growth and that in this natural habitat, DC-4 would grow at the same rate, or possibly faster, than the non-pathogenic DC-1 strain. As the temperature surpassed the 34°C measured figure of August 22, 1974, DC-4 would increase its growth rate dramatically while DC-1 would decrease leading to waters seriously polluted with the pathogenic strain. If one compares the generation time of DC-1 and DC-4 at 32°C and 37.5°C, a complete reversal of doubling time is found.

With the isolation of DC-4 from the Desjardins Canal

in Dundas, Ontario, there is no doubt that pathogenic strains of *Naegleria* exist in Canada. The canal is grossly polluted and because of the organic and fecal material, as well as the shallowness of the waters, temperatures are elevated beyond that found in surrounding less polluted waters. Ecology investigators have found that the winter temperature of the Desjardins Canal corresponds directly with the effluent material in the water. High biological activity is found in the waste material and leads to temperatures in the canal during the winter months that are 4° - 6°C higher than those found in the nearby West Pond. The canal rarely, if ever, freezes over during the winter and the water has a mean temperature of 7°C during this time. This is the surface temperature and the bottom waters, because of the accumulation of effluent, are warmer than the surface waters.

Pathogenic *Naegleria* have been found in soil samples and are free-living. Whether the pathogenic strain found in the Desjardins Canal is a mutation or a selectively adapted strain caused by increased water temperatures and pollution is not known. However, the Desjardins Canal and other such polluted fresh waters could act as ideal breeding sites and lead to the dispersion of cysts via wind, animals, etc. to other sites or via the flagellates to other waterways in the

vicinity.

There appears to be little doubt that the pathogenic strains are capable of growing over a wide range of temperatures but that elevated temperatures favor these strains while slowing or completely curtailing non-pathogenic growth.

Since approximately one-half of the known PAME cases have been diagnosed in retrospect it would appear that this is an old disease that has gone unrecognized. Although many points in the ecology and epidemiology of potentially pathogenic free-living amoebae may remain obscure, it seems highly probable that thermal and faecal pollution of surface waters by increased industry or population could cause selective proliferation to a dangerous level of the thermophile *Naegleria* and an increase in the number of PAME victims.

Although the effects of pollution have been stressed, it should be pointed out that waters need not be grossly polluted to contain sufficient numbers of pathogenic amoebae to cause PAME in susceptible individuals.

The one common factor found in all water sources containing pathogenic *Naegleria* is the elevated water temperatures. Most PAME cases occur during hot spells or in heated waters. It has been suggested that this is because more people are swimming and for longer periods of time but this does not apply when heated swimming pools or hot springs are considered. Rather it would appear that the water temperature is the

triggering factor that allows the amoebae to proliferate to a sufficient number to become infectious.

Since the disease is so virulent and the therapy extremely toxic, prophylaxis remains the best answer at present. Prolonged swimming and diving in stagnant, warm fresh water should be avoided, particulary by persons with histories of nose, ear or sinus disturbances or disease. Reducing the number of heavily polluted water sources, such as the Desjardins Canal, that could serve as reservoirs for pathogenic strains would also reduce the incidence of pathogenic *Naegleria* being transferred to other less polluted waters.

Since PAME has been reported in Britain, Belgium, New Zealand and Czechoslovakia, countries all with climatic conditions similar to those found in southern Canada, there was a high probability that a pathogenic *Naegleria* strain would be found. With the isolation of the DC-4 strain there appears little doubt that conditions are suitable for pathogens and that PAME will eventually be diagnosed in our country.

Summary

To conclude this discussion, the results and conclusions drawn from this investigation will be summarized.

Primary amoebic meningo-encephalitis (PAME) is being recognized with increasing frequency throughout the world since its initial discovery in 1961 in Australia. As of 1973, no cases had been reported in Canada and it was not known whether this was due to incorrect diagnosis of meningitis cases, hostile climatic conditions that precluded pathogenic Naegleria growth to infective levels, or complete lack of such pathogenic strains in Canada. Consequently, a search was undertaken in diverse fresh water environments in the southern Ontario area. Water samples were collected, Naegleria strains isolated and intranasally introduced into mice to test for possible pathogenicity. Of the samples tested, two displayed possible pathogenic qualities (i.e., CD-3 and FT-52) and one (DC-4) proved virulent to a degree comparable with isolates from PAME victims.

A comparative analysis was carried out using the pathogenic DC-4 strain and three other isolates selected for diversity of natural environment and non-pathogenicity (i.e., CT-8, a swimming pool isolate; FT-52, a fish tank isolate; and DC-1, a non-pathogen sampled from the same site as the pathogen, DC-4. NB-1, a laboratory Naegleria strain long used as a tool for the study of cell differentiation, was used as a standard, or control.

The following studies were conducted:

1) The cultural needs of the various isolates were established and it was found that the pathogenic strains initially required non-nutrient agar with live bacteria to thrive. This is in agreement with the findings of other investigators. All other isolates grew well on NM medium (a dilute salt medium) with Aerobacter and the pathogens adapted rapidly to this medium too.

2) The temperature that produced the best growth under laboratory conditions was established and the generation time, or doubling rate, of each of the isolates was ascertained at 32°C and 37.5°C. The pathogen (DC-4) was found to have an extremely rapid generation time at 37.5°C but a rate well below the other four strains when grown at 32°C. FT-52, a low virulence pathogen, had a similar doubling rate at both temperatures suggesting a wide temperature range for growth. DC-1, CT-8 and NB-1 grew much slower at the elevated temperature than at the lower temperature.

The highest temperature at which a particular strain would proliferate was also established. Both pathogenic isolates grew at temperatures well above normal body temperature (i.e., FT-52 grew at 40°C; DC-4 at 43°C) while the non-pathogens were unable to sustain growth at temperatures above 38°C. The most distinctive and consistent feature found within the five strains studied was the thermophilic nature and temperature tolerance of the pathogens. This

finding concurs with studies conducted by Griffin (1972).

3) Naegleria amoebae when immersed in an aqueous environment transform from the amoeboid form to a flagellate form possessing usually 2 flagella. This transformation is rapid, synchronous and reproducible using standard transformation techniques and the T_{50} (time at which 50% of a given population of amoebae have transformed) can be considered a constant for a particular strain. The rate of amoeba-to-flagellate transformation for NB-1 is well documented (T_{50}=69 minutes). The isolates all transformed readily and had T_{50}'s as follows: DC-1, 73 minutes; DC-4, 70 minutes; CT-8, 61 minutes; FT-52, 58.5 minutes; and NB-1, 69.5 minutes. Therefore, it seems apparent that the rate of transformation cannot be used to differentiate between pathogenic and non-pathogenic strains.

Heat shock with sub-lethal temperatures during the transformation incubation period has been found to disrupt the metabolic processes and dramatically increase the number of flagella per cell (Dingle, 1970). Amoebae of the various strains were incubated at 38°C for 40 minutes and then transferred to normal transformation temperatures. The non-pathogenic strains were affected by the elevated temperature and developed significantly more flagella than under normal transformation conditions, whereas the pathogenic strains were little affected by the 38°C incubation and their flagella count was only altered slightly. The results of the transformation and heat shock studies cannot be compared with those of others in the field for none, at least to the best of my knowledge,

have conducted these tests on pathogenic strains under similar controlled conditions.

4) The cyst survival ability and viability after storage was tested and all strains were found capable of surviving on NNA plates at room temperatures and in a moist chamber for at least 19 weeks (the length of the cyst survival study). One strain, FT-52, although capable of surviving on NNA plates, remained viable for only 14 weeks when stored on NM plates. This agrees with cyst survival studies conducted on pathogenic and non-pathogenic strains by other investigators.

Cysts and amoebae of the various strains were also tested for ability to withstand alcohol and soap solutions. The amoebae were extremely susceptible to desiccation by 1% and 2% soap solutions and by 70% ethanol. The cysts were hardy and survived 24 hours immersion in soap solutions followed by 24 hours drying at room temperature but were not viable after 24 hours immersion in 70% ethanol. Although the amoebae can easily be destroyed by normal laboratory cleansers, the cysts are capable of surviving adverse conditions and possibly extensive storage periods. Therefore, extreme care should be exercised when handling cyst cultures to prevent contamintion of the surroundings.

The survival of cysts in soap solutions is contradictory to results reported by Carter (1970). Extensive wa shing of the cyst suspension after removal from the test solution is essential to remove residual incubation material

from the cyst walls. Retention of soap solutions or alcohol within the cyst wall would likely lead to lysis of the amoeba at excystment.

5) Measurements and morphological observations were made on the various strains and comparisons were made between the strains and with pathogenic and non-pathogenic studies done by other investigators. The pathogenic DC-4 does not have definitive pores in the cyst walls; this is considered by some investigators to be a characteristic of pathogenic strains. In all other respects, the strains fell well within the dimensions reported by others and their morphology, mitotic events, locomotion, protoplasmic organelles, etc. could not be used for differentiation or diagnostic purposes.

6) The experimental pathology studies (DC-4) included tests for a correlation between inoculum size and number of fatalities as well as a possible relationship between inoculum size and survival time of those succumbing to PAME. There was a positive relationship between dosage size and resulting fatalities but no correlation could be found between the dosage and the survival time of fatalities.

An observation made during the preliminary tests for pathogenicity led to studies concerning the possible recovery of animals obviously infested with amoebae and possible in some stage of PAME. This phenomenon had not been reported by other investigators and is perhaps the most important finding to come out of this study. Clinical observations of severely

disoriented animals showed that many of these same animals eventually recovered completely. In further studies, half of the animals in each test group were sacrificed at Day 3 and the other half observed for PAME symptoms and fatalities. It was found in all cases that the number of sacrificed animals with amoebic infestation exceeded by a minimum factor of 2, the number of animals actually succumbing to the disease. This applied to animals weighing 10 g., 15., and 20 g. as well as when different inoculum sizes were tested. These findings suggest that PAME may not be as fatal a disease as previously supposed or that Strain DC-4 differs from other pathogenic isolates in virulence.

The resistance or susceptibility of animals in different weight groups was tested. The results were inconclusive but suggested that although larger animals might succumb at the same rate, they had a longer survival time than the smallest animals tested.

Since there had been much discussion in scientific papers concerning the most suitable media to use for pathogenic growth and it had been found that initially DC-4 grew well only on NNA with live bacteria, experiments were conducted to test the effects of NM on amoebae growth and virulence. Possible enhancement or reduction of virulence was not noted with the NM-grown amoebae and, indeed, NM- and NNA-grown amoebae produced the same fatality rate (i.e., 42%) when tested under similar experimental conditions.

Strain DC-4, the only strain used for the experimental pathology studies, demonstrated a virulence and produced fatalities within a survival time that correlated well with studies conducted using pathogenic Naegleria isolated from PAME victims. The species of DC-4 was not determined and further studies should be conducted on this particular strain. Serological tests would help establish whether DC-4 was N. gruberi, N. fowleri or an intermediate strain. Also, further electron microscope studies would determine positively the presence or absence of discrete pores in the cyst wall structure and also quite likely would show the means of excystment, whether through such pores, if found, or by rupture of the cyst wall.

As far as the southern Ontario scene is concerned, the possibility of a PAME occurrence cannot be negated simply because the disease has never been reported in Canada. Pathogenic Naegleria has now been demonstrated to be present in our waterways and probably capable of causing disease if, and when, a susceptible person comes in contact with the pathogen.

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