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WARNING

AMOEBIC MENINGITIS

In all thermal pools

KEEP YOUR HEAD ABOVE WATER

to avoid the possibility of developing the serious illness called **AMOEBIC MENINGITIS.**

This disease can be caught in thermal pools if water enters the nose, while swimming or diving.

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PATHOGENIC FREE-LIVING AMEBAE-
OCCURRENCE IN NEW ZEALAND THERMAL REGIONS
TOGETHER WITH INVESTIGATIONS INTO THEIR
DISINFECTION, IMMUNITY AND VIRULENCE

A thesis presented in partial fulfuilment of
the requirements for the degree of Doctor of
Philosophy in Microbiology at Massey
University, New Zealand

Raymond Thomas Michael Cursons

1978

ABSTRACT

Pathogenic free-living amebae (PFLA), of which *Naegleria fowleri* was the predominant pathogenic species, were isolated from 6 out of 10 pools sampled from the Hamilton, Rotorua and Gisborne Health Districts. The majority of these PFLA contaminated-pools occurred in the Matamata-Taupo region, and this localized geographical distribution appeared to be influenced, in part, by the particular physical and chemical properties of the pool. 'High-risk' pools, which exhibited a high incidence of isolations of PFLA, were shown to be natural pools, i.e. soil enclosures, as opposed to concrete constructed pools. PFLA were also isolated from the soil, and it was thought that soil acted as a reservoir of PFLA. No seasonal distribution in the occurrence of PFLA in thermal pools was noticed.

A comparative study on the disinfecting potential of chlorine, chlorine dioxide, ozone and deciquam 222 for PFLA showed that all 4 disinfectants possessed amebicidal properties, but only at higher levels than those normally used for disinfecting bacteria. Of the 4 disinfectants, deciquam 222 proved to be the most effective amebicide, followed by chlorine, chlorine dioxide and ozone.

An immunological survey of normal human sera for the presence of antibodies to either pathogenic or non-pathogenic *Naegleria* and *Acanthamoeba* spp. established that human sera had a titre ranging between 1/5 - 1/20 for *Naegleria* spp. and between 1/5 - 1/80 for *Acanthamoebae*. No discrimination in titres was observed between blood groups or sexes and fluorescein-labelled class-specific immunoglobulins showed that these antibodies belonged mainly to both the Ig M and Ig G classes. It was also shown that fresh adult human sera, as opposed to cord or specific hyperimmune-rabbit sera, contained a heat-labile neutralizing factor which inhibited the formation of cytopathic effects (CPE) in Vero cell

culture by *Acanthamoeba culbertsoni*, but not by *N. fowleri*. Homologous, as well as heterologous antigens of *Naegleria* spp. were however, shown to cross-react with both the *in vitro*, macrophage inhibition factor assay, and *in vivo*, delayed hypersensitivity, correlates of cell-mediated immunity.

Finally, this study also demonstrated that both pathogenic and non-pathogenic species of *Naegleria* and *Acanthamoeba* secreted both an extracellular phospholipase 2 and lysophospholipase into their axenic cultures. The relative production of phospholipase 2 correlated with the formation of CPE in Vero cell culture by either amebae, or by cell-free filtrates from axenic cultures of amebae. The relative level of production of this enzyme appeared to influence the virulence and hence pathogenic-potential of these micro-organisms.

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PREFACE

Since the first reported cases in New Zealand in 1968 of primary amebic meningo-encephalitis (PAM) (Mandal et al., 1970) the small free-living amebae of the genera Acanthamoeba and Naegleria have been of great interest to the New Zealand Department of Health. Locally it is known as 'Hot-Pool Meningitis' and there have been 7 confirmed cases of the disease all due to Naegleria fowleri and all contracted after swimming in thermal pools in the central North Island (Cursons et al., 1978b). Subsequently, these pathogenic free-living amebae (PFLA) (Adam et al., 1971) have been shown to be responsible for a number of diseases ranging from chronic illnesses such as respiratory infections (Martinez et al., 1975) and humidifier fever (M.R.C. Symposium, 1977) to blindness (Visvesvara & Jones, 1975).

The apparent ubiquity and ease of isolation of these amebae from the environment stimulated a program (initiated by the New Zealand Department of Health and run by the National Health Institute) of isolating amebae from specific thermal pools. Later this program was extended under contract to Massey University to cover 10 thermal pools in 3 different Health Districts, with the aim of sampling the pools for the presence or absence of PFLA and trying to correlate their presence or absence with such parameters as the chemical composition of the waters, pH, temperature, and numbers of bacteria. In addition, comparative disinfection tests were carried out using a variety of compounds to observe their respective amebicidal properties. Because of the repeated isolations of PFLA from some New Zealand thermal pools, an immunological study was also undertaken. It was hoped to explain the enigma of the human population's apparent immunity to these amebae, despite their pathogenicity and distribution. Finally, the virulence of these amebae in relation to their pathogenicity was also studied.

TABLE OF CONTENTS

	Page
ABSTRACT	ii
ACKNOWLEDGEMENTS	iv
PREFACE	vi
LIST OF TABLES	xiii
LIST OF FIGURES	xv
LIST OF PLATES	xvii
CHAPTER 1: INTRODUCTION	1
1.1 The History of Free-living Amebae as Disease Agents	1
1.2 Occurrence and Distribution	4
1.3 Infection	5
1.4 Immunity	6
1.5 Disinfection	7
CHAPTER 2: SAMPLING SITES	9
2.1 Introduction	9
2.2 Hamilton Health District	11
2.2.1 Waignaro Springs	11
2.2.2 Moana-iti Springs	12
2.2.3 Opal Springs	12
2.2.4 Okoroire Springs	13
2.3 Rotorua Health District	16
2.3.1 Soda Springs	16
2.3.2 Butcher's Pool	16
2.3.3 Otumaheke Stream	16
2.4 Gisborne Health District	20
2.4.1 Te Puia	20
2.4.2 Morere	21
CHAPTER 3: MATERIALS	25
3.1 Amebic Cultures used	25
3.2 Plate Media	30
3.2.1 Ameba Saline Agar	30
3.2.2 Ameba 1% Saline Agar	30

... Cont'd

Page

3.2.3 N.M. Agar 30
3.2.4 E.Y. Agar 30
3.2.5 Lysolecithin Agar 31
3.2.6 Haemolytic Agar 31
3.2.7 Proteinase Agar 31
3.2.8 DIFCO Standard Methods Agar 32
3.2.9 DIFCO Violet Red Bile Agar 32
3.3 Axenic Media for Amebae 32
3.3.1 Page's Ameba Saline 32
3.3.2 CYM Medium 32
3.3.3 CYMH Medium 32
3.3.4 4% Neff Medium 32
3.4 Cell Culture Media 32
3.5 Lymphocyte and Macrophage Culture Media 32
3.5.1 Lymphocytes 32
3.5.2 Macrophages 33
3.6 Disinfection Solutions Used 33
3.6.1 Water 33
3.6.2 Chlorine 33
3.6.3 Chlorine dioxide 33
3.6.4 Ozone 33
3.6.5 Deciquam 222 33
3.7 Polyacrylamide Gel Electrophoresis 34
3.7.1 Polyacrylamide Gels 34
3.7.2 Running Buffer 34
3.7.3 Tracking Marker 34
3.7.4 Gel Staining Solution 34
3.8 Staining Solutions 35
3.8.1 Sudan black 35
3.8.2 Ehrlich's haematoxylin 35
3.8.3 Glycerin-jelly 35
3.9 Chemicals and Serological Reagents 35
3.10 Experimental Animals 36

CHAPTER 4: METHODS 37
4.1 Miscellaneous Methods 37
4.1.1 Chemical Analysis of Thermal Pool Water .. 37

... Cont'd

		Page
	4.1.2 Scanning Electron Microscopy	37
4.2	Identification of Amebae and Bacteria	37
	4.2.1 Identification of Amebic Isolates	37
	4.2.2 Total Coliform and Total Bacteria Count ..	39
4.3	Amebic Culture Techniques	40
	4.3.1 Cloning	40
	4.3.2 Axenic Culture	40
4.4	Cell Culture	40
	4.4.1 Neutralization of Amebae in Cell Cultures	41
4.5	Indirect Macrophage Inhibition Assay	41
	4.5.1 Immunization of Guinea-pigs	41
	4.5.2 Lymphocyte Culture and Production of Macrophage Inhibition Factor	41
	4.5.3 Culture of Macrophages	42
	4.5.4 Delayed Hypersensitivity	44
4.6	Disinfection	44
	4.6.1 Production of Disinfectants	44
	4.6.2 Chemical Analysis of Disinfectants	46
	4.6.3 Disinfection Tests	46
4.7	Polyacrylamide Gel Electrophoresis	46
4.8	Cytochemical Staining	47
4.9	Preliminary Isolation and Characterization of Extracellular Enzymes of Pathogenic Free-living Amebae	47
	4.9.1 Detection of Amebic Extracellular Enzymes	47
	4.9.2 Thin Layer Chromotography	47
	4.9.3 Haemolysis	48
	4.9.4 Isolation of Phospholipases from the Supernatants of Naegleria fowleri (MsT)	48
4.10	Protein Determination	50
4.11	Sterilization	50
CHAPTER 5:	RESULTS	52
5.1	Survey of PFLA in New Zealand Thermal Pools ..	52
	5.1.1 The Distribution of PFLA in New Zealand Thermal Pools during the 1976 Survey ..	52
	5.1.2 The Occurrence of PFLA in New Zealand Thermal Pools during the 1977 Survey ..	58

... Cont'd

Page

5.2 The Comparative Use of Disinfectants against Free-living Amebae 62

5.2.1 The Use of Chlorine as a Disinfectant against Free-living Amebae 62

5.2.2 The Use of Alternative Disinfectants to Chlorine against Free-living Amebae .. 63

5.3 Immunity to PFLA 65

5.3.1 The Presence of Antibodies to PFLA in Human Sera 65

5.3.2 The Presence of a Specific Neutralizing Factor against PFLA in Normal Human Sera 76

5.3.3 The Reaction of the Cell-Mediated Immune System to Antigens of *Naegleria* spp. .. 80

5.4 Virulence of PFLA 83

5.4.1 The Presence of Extracellular Enzymes in the Supernatants of Axenic Cultures of Free-living Amebae 83

5.4.2 Production of Phospholipase 2 in Axenic Cultures of Free-living Amebae 91

5.4.3. The Preliminary Isolation of a Phospholipase 2 from Axenic Cultures of *N. fowleri* (MsT) 94

5.4.31 The Isolation of Phospholipase 2 from Serum-supplemented Axenic Cultures 98

5.4.4 Virulence of Amebae and Cell-free Axenic Filtrates in Vero Cell Culture 105

CHAPTER 6: DISCUSSION 118

6.1 Survey of New Zealand Thermal Pools 118

6.1.1 The Distribution of PFLA in New Zealand Thermal Pools during the 1976 Survey .. 118

6.1.2 The Effect of Chemical, Physical and Biological Parameters on the Distribution of PFLA in New Zealand Thermal Pools .. 118

6.1.3 The Incidence of PFLA in New Zealand Thermal Pools 125

... Cont'd

Page

6.2 The Comparative Use of Disinfectants against Free-living Amebae 131
6.2.1 The Use of Chlorine as a Disinfectant against Free-living Amebae 131
6.2.2 The Use of Alternative Disinfectants to Chlorine against Free-living Amebae .. 137
6.2.21 The Use of Chlorine dioxide as a Disinfectant against Free-living Amebae 137
6.2.22 The Use of Ozone as a Disinfectant against Free-living Amebae .. 138
6.2.23 The Use of Deciquam 222 as a Disinfectant against Free-living Amebae 141
6.3 Immunity to PFLA 144
6.3.1 The Presence of Antibodies to PFLA in Human Sera 145
6.3.2 The Presence of a Specific Neutralizing Factor against PFLA in Normal Human Sera 147
6.3.3 The Induction of the Cell-Mediated Immune System 149
6.3.4 Immune Responses in Relation to Infection with PFLA 150
6.4 Virulence of PFLA 156
6.4.1 The Presence of Extracellular Phospholipases in the Supernatants of Axenic Cultures of Free-living Amebae 156
6.4.2 Production of Phospholipase 2 in Axenic Cultures of Free-living Amebae 157
6.4.3 Preliminary Isolation of Phospholipase 2 from Axenic Cultures of N. fowleri (MsT) 160
6.4.4 Virulence of Amebae and Cell-free Axenic Filtrates in Vero Cell Culture 161
6.4.5 The Significance of Phospholipase 2 Production and its Relationship to the Pathogenicity of PFLA 163

... Cont'd

Page

APPENDICES 166

Appendix I Data from the 1976 Survey 166

Appendix II Data from the 1977 Survey 175

Appendix III Papers Published 189

BIBLIOGRAPHY 190

LIST OF TABLES

Page

I. Cases of Primary Amebic Meningo-encephalitis reported after 1974 2

II. Chemical and Physical Characteristics of Thermal Pools from the Waikato Health District 13

III. Chemical and Physical Characteristics of Thermal Pools from the Rotorua-Taupo Health District 20

IV. Chemical and Physical Characteristics of Thermal Pools from the Gisborne Health District 21

V. Ameba Cultures Used 25

VI. Distribution and Incidence of PFLA from New Zealand Thermal Pools during the 1976 Survey 52

VII. Monthly Isolations of PFLA from New Zealand Thermal Pools during the 1976 Survey .. 57

V II. The range of total Coliform and Bacterial Counts from New Zealand Thermal Pools during the 1976 Survey 58

IX. The Incidence of PFLA in New Zealand Thermal Pools during the 1977 Survey 59

X. Monthly Isolations of PFLA from New Zealand Thermal Pools during the 1977 Survey . .. 60

XI. Bacteriological Quality of Thermal Pools and Surrounding Soils during the 1977 Survey .. 61

XII. The Amebicidal Capacity of Chlorine 62

XIII. The Comparative Amebicidal Capacity of Chlorine dioxide, Ozone and Deciquam 222 64

XIV. Presence of Antibodies to PFLA in Human Sera from the Palmerston North Health District . 66

XV. Presence of Antibodies to PFLA in Human Sera from the Hamilton Health District 68

XVI. Presence of Antibodies to PFLA in Human Sera from the Rotorua Health District 70

XVII. Presence of Class-specific Antibodies to PFLA in Human Sera 75

XVIII. Use of Hyperimmune Rabbit and Normal Human Sera in Neutralizing _N. fowleri_ (MsT) and _A. culbertsoni_ (A-1) Vero Cell Culture 77

... Cont'd

Page

XIX. Cross-reactivity of Homologous and Hetero logous Antigens of Naegleria spp. as judged by Delayed Hypersensitivity 80

XX. Cross-reactivity of Homologous and Hetero-logous Antigens of Naegleria spp. as judged by Inhibition of Macrophages 82

XXI. Products of Enzymatic Hydrolysis of Phos-phatidyl choline by Different Phospholipases 84

XXII. Effect of Rosenthal's Inhibitor on Phospholi-pase 2 Activity in EY Agar 88

XXIII. Production of Phospholipase 2 by Naegleria spp. 92

XXIV. Production of Phospholipase 2 by Acanthamoeba spp. 93

XXV. Effect of Protein Concentration of the Reaction of N. fowleri and A. culbertsoni Cell-free filtrates in EY Agar 97

XXVI. Relative formation of Cytopathic Effects in Vero Cell Culture by Naegleria spp. 106

XXVII. Relative formation of Cytopathic Effects in Vero Cell Culture by Acanthamoeba spp. .. 108

XXVIII. Relative formation of Cytopathic Effects in Vero Cell Culture by axenic Cell-free Filtrates 112

XXIX. Addition of N. fowleri (MsT) axenic filtrate to Vero Cell Cultures inoculated with N. fowleri (Ts-1) 113

XXX. Inhibition of the formation of Cytopathic Effects in Vero Cell Cultures inoculated with N. fowleri by anti-phospholipases serum 116

XXXI. Comparison of the properties of Chlorine, Chlorine dioxide, Ozone and Deciquam 222 .. 132

XXXII. Previous Reports of antibodies in Human Sera 146

LIST OF FIGURES

Page

1. Map showing the location of thermal areas of the North Island of New Zealand 10
2. Flow diagram showing the strategy used for the isolation and identification of PFLA 38
3. Method used for the production of chlorine dioxide 45
4. Method used for the production of ozone 45
5. Flow diagram showing the strategy used for the isolation of phospholipases from N. fowleri (MsT) supernatants 45
6. Graph showing average monthly temperature for New Zealand 54
7. Graph showing average monthly rainfall for the Matamata and Taupo regions 55
8. Graph showing average monthly rainfall for the Rotorua and Gisborne regions 56
9. Thin-layer chromatograms of neutral lipids and phospholipids from extracts of EY plates .. 86
10. PAGE profile of the supernatant from serum-supplemented axenic N. fowleri culture 95
11. PAGE profile of 40-60% $(NH_4)_2SO_4$ precipitated fraction of the serum-supplemented axenic N. fowleri culture 95
12. PAGE profile of the XM 50,000 MW ultrafiltration fraction of serum-supplemented axenic N. fowleri culture 96
13. PAGE profile of the SEPHADEX G-100 fraction of serum-supplemented axenic N. fowleri culture .. 96
14. PAGE profile of serum-supplemented axenic culture medium 99
15. PAGE profile of bovine serum 99
16. PAGE profile of serum-free axenic culture medium . 101
17. PAGE profile of the supernatant from serum-free axenic N. fowleri culture 101
18. PAGE profile of SEPHADEX G-100 fraction of serum-free axenic N. fowleri culture 102
19. Gel-diffusion pattern using anti-phospholipases serum serum 103

... Cont'd

Page

20. Graph of predation by Naegleria spp. on the bacterium Enterobacter cloacae 121
21. Graph of predation by Acanthamoeba spp. on the bacterium Enterobacter cloacae 122
22. Distribution of HOCl and OCl^- as a function of pH 134
23. Relative bactericidal efficiency of chlorine containing derivatives 135
24. Effect of nitrogen compounds on the chlorine demand 135
25. Relative effectiveness of chlorine dioxide as a disinfectant 139

LIST OF PLATES

Page

1. Waignaro thermal pool 14
2. Moana-iti thermal pool 14
3. Opal 'Jockey' thermal pool 15
4. Okoroire 'No. 4' thermal pool 15
5. Soda thermal pool 17
6. Butcher's thermal pool 17
7. Upper Otumaheke stream 18
8. Mouth of Otumaheke stream 18
9. Old 'Whey' soil-site of Otumaheke stream .. 19
10. Hot-water source of Te Puia Springs 22
11. Wooden trough used to pipe thermal water to Te Puia pool 22
12. Morere thermal baths pipe supply 23
13. Hut enclosing Morere thermal baths 23
14. Scanning electron micrograph of <u>N. gruberi</u> trophozoite 26
15. Scanning electron micrograph of <u>N. gruberi</u> flagellate 26
16. Scanning electron micrograph of <u>N. gruberi</u> cyst 27
17. Scanning electron micrograph of <u>N. fowleri</u> cyst 27
18. Scanning electron micrograph of <u>A. castellanii</u> trophozoite 28
19. Scanning electron micrograph of <u>A. castellanii</u> cyst 28
20. Scanning electron micrograph of <u>A. culbertsoni</u> cyst 29
21. Photomicrograph of <u>N. fowleri</u> stained by IFAB 72
22. Photomicrograph of <u>N. gruberi</u> stained by IFAB 72
23. Photomicrograph of <u>A. culbertsoni</u> stained by IFAB 73
24. Photomicrograph of <u>A. castellanii</u> stained by IFAB 73
25. Photomicrograph of section through skin of guinea-pig immunized with <u>N. fowleri</u> and skin-tested with saline 81

... Cont'd

Page

26-28. Photomicrographs of sections through skin of guinea-pig immunized with N. fowleri and skin-tested with Naegleria spp. antigens .. 81

29. Photomicrograph of control migration of PEC .. 82

30. Photomicrograph of inhibition of migration of PEC 82

31. Clearing of EY plates by cell-free filtrates from exponential axenic cultures of amebae 85

32. Clearing of EY plates by cell-free filtrates from stationary axenic cultures of amebae 85

33. Clearing of EY plates by cell-free filtrates from 48 hr axenic cultures of amebae 90

34. Inhibition of clearing of EY plates by cell-free filtrates from 48 hr axenic cultures of amebae by Rosenthal's inhibitor 90

35. Clearing and turbidity of egg-yolk after native PAGE of purified phospholipases 104

36. Scanning electronmicrograph of control Vero Cell Culture monolayer 104

37. Photomicrograph of Vero Cell Culture inoculated with N. fowleri showing early cytopathic effects 109

38. Scanning electronmicrograph showing attachment of N. fowleri to Vero cells 109

39. Photomicrograph of Vero Cell Culture inoculated with N. fowleri showing pronounced cytopathic effects 110

40. Scanning electronmicrograph of Vero Cell Culture inoculated with N. fowleri showing pronounced cytopathic effects 110

41. Photomicrograph of control Vero Cell Culture stained with Sudan black 114

42. Photomicrograph of Vero Cell Culture inoculated with A. culbertsoni stained with Sudan black 114

43. Photomicrograph of Vero Cell Culture inoculated with N. fowleri and stained with Sudan black 114

CHAPTER 1: INTRODUCTION

1.1 The History of Free-Living Amebae as Disease Agents

The history of pathogenic free-living amebae (PFLA) of the genera Acanthamoeba and Naegleria has been extensively reviewed elsewhere (Culbertson, 1971; Duma *et al.*, 1971; Chang, 1971, 1974a; Carter, 1972; Cursons, 1974; Cursons & Brown, 1976). In an extensive survey on the worldwide distribution of cases of primary amebic meningo-encephalitis (PAM), Willaert (1974) tabulated 84 cases from all continents with the exception of Antarctica. Prior to 1968, most cases were attributed to *Acanthamoeba* spp. This is possibly a reflection of Culbertson's pioneering work, which demonstrated that an amebic meningo-encephalitis in both mice and primates could be caused by the A-1 strain of Acanthamoeba (Culbertson *et al.*, 1958, 1959, 1965). However in 1968, Butt *et al.*, Carter, and Culbertson *et al.*, showed that the incriminating species of most reported cases belonged not to the genus Acanthamoeba, but to the related genus Naegleria. In 1970 on the basis of morphological, cultural and pathogenicity differences, Carter named the pathogenic species *Naegleria fowleri*.

Subsequent to Willaert's review of 1974, there have been at least 8 other reported cases of PAM (Table I).

Table I. Cases of Primary Amebic Meningo-encephalitis reported after 1974.

Country	Year	Number of cases	Causative Organism	Diagnosis	Reference
New Zealand	1974	1	*N. fowleri* (MsT)	isolation from CSF	Cursons *et al.*, 1976b
U.S.A.	1974	1	*N. fowleri* (Lovell)	isolation from CSF	De Jonckheere, 1977
	1974	1	*Acanthamoeba* sp.	IFAB	Martinez *et al.*, 1977
	1975	1	*Acanthamoeba* sp.	IFAB, post-mortem	Hoffman *et al.*, 1978
Venezuela		1	*A. culbertsoni*	IFAB	Martinez *et al.*, 1977
Peru		1	*A. castellanii*	IFAB	Martinez *et al.*, 1977
Zambia	1972	1	*Acanthamoeba* sp.	post-mortem	Bhagwandeen *et al.*, 1975
Korea	1958	1	*Acanthamoeba* sp.	post-mortem	Ringsted *et al.*, 1975

IFAB = indirect fluorescent antibody

CSF = cerebro-spinal fluid

Conceivably, the actual number of cases may be higher since the symptoms of PAM parallel those of aseptic meningitis. Retrospective studies have disclosed a possible case dating back to 1909 (Symmers, 1969) and fluorescent antibody staining has confirmed that the 1948 case reported by Derrick, originally thought to be due to Iodamoeba butschlii was in fact caused by N. fowleri (McMillan, 1977). Later PAM cases were divided into two types:

i) a swimming-associated acute meningo-encephalitis caused by Naegleria fowleri and

ii) a non-swimming-associated chronic meningo-encephalitis caused by a variety of pathogenic Acanthamoebae, notably Acanthamoeba culbertsoni, Acanthamoeba castellanii and Acanthamoeba polyphaga (Chang, 1974a).

Subsequently, Acanthamoeba spp. have also been indicted in a number of chronic illnesses such as respiratory infections (Martinez et al., 1975), corneal ulceration of the eye (Nagington et al., 1974; Visvesvara & Jones, 1975) and together with Naegleria spp., in humidifier fever (M.R.C. Symposium, 1977). The reidentification of the aetiological agents of the 1968 cases of PAM in New Zealand as N. fowleri (Cursons & Brown, 1975; Cursons et al., 1976a) has dismissed the notion of slime moulds being involved in the aetiology of PAM (Mandal et al., 1970).

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

1.2 Occurrence and Distribution

The free-living amebae appear to be truly ubiquitous organisms. Isolations have been recorded from a variety of environmental sources such as air (Kingston & Warhurst, 1968), humidification systems (M.R.C. Symposium, 1977), freshwater, brackish and ocean sediments (De Jonckheere et al., 1975, De Jonckheere & Van De Voorde, 1977a; Brown & Cursons, 1977; Sawyer et al., 1977; Stevens et al., 1977a; Wellings et al., 1977). They have also been isolated from chlorinated swimming and domestic waters (Cerva, 1971a; Anderson & Jamieson 1972; Cerva & Huldt, 1974), from bottled drinking water (Desmet-Paix, 1974), from a home dialysis unit (Casemore, 1977), from soil (Anderson & Jamieson, 1972; Wellings et al., 1977; Cursons et al., 1978b) and from sewage (Singh & Das, 1972; Chang, 1974a). Isolations have also been recorded from cell cultures (Jahnes et al., 1957; Stevens & O'Dell, 1973a; Willaert et al., 1978), throat and nasal cavities (Elridge & Tobin, 1967; Cerva et al., 1973; Chang et al., 1975), eyes (Nagington et al., 1974, Visvesvara et al., 1975), gastrointestinal washings (Hoeffler & Rubel, 1974), cold blooded vertebrates (Frank, 1974), snails (Kingston & Taylor, 1976), and fish (Taylor, 1977). Variable physical parameters such as temperature and pH are equally tolerated over a wide range with *in vitro* growth reported up to 45°C (Griffin, 1972) and a pH range of 4.6 to 9.5 (Carter, 1970). This apparently ubiquitous distribution is no doubt due to the possession of resistant cysts enabling PFLA not only to withstand unfavourable conditions but also to take advantage of the intermittent occurrence of favourable conditions, e.g. the isolation of pathogenic *Acanthamoebae* from 2°C (Brown & Cursons, 1977).

The distribution of the pathogenic species in relation to non-pathogenic ones is still unknown. In general non-pathogenic species are more prevalent at

ambient temperatures in temperate zones. The repeated isolations of PFLA from waters above ambient temperature, i.e. $\geqslant 30^{\circ}C$ (De Jonckheere et al., 1975; De Jonckheere & Van De Voorde, 1977a; Stevens et al., 1977a; Wellings et al., 1977; Cursons et al., 1978b), combined with their higher optimum temperature of growth (Griffin, 1972) suggests that pathogenic amebae are environmentally selected over non-pathogenic amebae in waters above ambient temperature. The source of pathogenic amebae in these waters is unknown. Cursons et al. (1978b) and Wellings et al. (1977) have succeeded in isolating PFLA from the soil, the preferred habitat of small free-living amebae (Singh, 1975). Thus it is possible that soil acts as a reservoir of pathogens in the same way it does for Cryptococcus, and that contamination occurs via run-off after rain.

1.3 Infection

The invasion of organs and tissues by PFLA is now well documented. The suggestion that invasion by Naegleria occurs primarily via the nasal mucosal epithelium because of the pathological condition of the cribriform plate and subjacent nasal structures (Culbertson, 1971; Carter, 1972) has been verified by Martinez et al. (1973). Using mice they demonstrated that amebic invasion occurs through the disruption of the olfactory mucosa, penetration into the submucosal plexus and ultimate passage of the amebae through the cribriform plate to the central nervous system. A similar route of invasion may also occur via haematogenous spread from the pulmonary, genitourinary and systemic circulation (Culbertson, 1971; Martinez et al., 1975, 1977). Once invasion of the brain has been established, destruction of surrounding brain tissue is thought to be brought about by a combination of phagocytosis and pinocytosis of host tissue by N. fowleri and solely by pinocytosis in the case of

A. culbertsoni (Visvesvara & Callaway, 1974; Maitra *et al.*, 1974, 1976). The possession of appropriate lysosomal hydrolytic enzymes for endocytosis of host tissue in both amebae has also been shown (Bowers & Korn, 1973; Childs, 1973; Ryter & Bowers, 1976; Feldman, 1977). The examination of sections from brains infected with either *N. fowleri* or *A. culbertsoni* reveals areas of extensive demyelination with the trophozoites of the amebae surrounded by a clear halo (Martinez *et al.*, 1975; Chang, 1976; Maitra *et al.*, 1976). Many authors have speculated on the possibility that enzymes secreted by the amebae are responsible for this and indeed, cytotoxic enzymes have been shown to be liberated by both pathogenic *Naegleria* and *Acanthamoeba* spp. (Chang, 1971, 1974a; Chang 1976; Visvesvara & Callaway, 1974; Visvesvara & Balamuth, 1975; Cursons & Brown, 1976, 1978; Maitra *et al.*, 1976). The ability of pathogenic and non-pathogenic free-living amebae to produce phospholipases has also been shown by Elson *et al.* (1970), Chang (1971, 1974a, 1976), Hax *et al.* (1974), Victoria and Korn (1975), Visvesvara and Balamuth (1975) and Cursons *et al.* (1978c). The level of production of such cytopathic enzymes may explain the differences in virulence reported amongst *Acanthamoeba* and *N. fowleri* isolates (Culbertson, 1971; De Jonckheere & Van De Voorde, 1977b).

1.4 Immunity

Because of the frequency and ease of isolation of virulent PFLA from the environment, many authors have been puzzled as regards the low incidence of infection by PFLA in the human population (Anderson & Jamieson, 1972; Cursons *et al.*, 1976b, 1977; John *et al.*, 1977; Wellings *et al.*, 1977; Haggerty & John, 1978). This has led many authors to speculate upon the existence of probable host related susceptibility factors and the demonstration of specific antibodies

to free-living amebae in human sera has been reported (Chang & Owens, 1964; Elridge & Tobin, 1967; Culbertson, 1971; Edwards *et al.*, 1976; Cursons *et al.*, 1977; M.R.C. Symposium, 1977; Tew *et al.*, 1977). Adams *et al.* (1976) reported that mice surviving a primary intravenous infection with *N. fowleri* were subsequently resistant to a further challenge by the same route with a dose of amebae that produced uniformly fatal disease in untreated control mice. This group further demonstrated that mice immunized with live or formalized *N. fowleri*, or live *N. gruberi* subcutaneously (S/C), intraperitoneally (I/P), intravenously (I/V), or intranasally (I/N), were significantly protected against a subsequent challenge with *N. fowleri* (John *et al.*, 1977). The role of cell-mediated immunity (CMI) in resistance to infection by *N. fowleri* was reported by Diffley *et al.* (1976) who demonstrated that guinea-pigs surviving a normally fatal challenge with *N. fowleri*, exhibited a delayed hypersensitivity (DH) response when tested intradermally (I/D) with a soluble fraction derived from *N. fowleri*. Thus support is given for the hypothesis that Man's unwitting exposure to the more ubiquitous non-pathogens may actively immunize him against challenge by PFLA (John *et al.*, 1977). That some innate immunity exists was shown by Wong *et al.* (1975a & b) who demonstrated that primates were apparently immune to I/N or I/V inoculations of *N. fowleri* or *A. culbertsoni* unless on immunosuppressive drugs. However intrathecal (I/T) inoculations were shown to cause an amebic meningo-encephalitis.

1.5 Disinfection

The finding of PFLA in chlorinated domestic and swimming waters (Cerva, 1971a; Anderson & Jamieson, 1972; Cerva & Huldt, 1974; De Jonckheere & Van De Voorde, 1976; Lyons & Kapur, 1977) has led to an expression of concern by public health authorities over

the possible contraction of PAM via these waters. Cerva (1971a), after a review of 16 fatal cases of PAM in an indoor chlorinated swimming pool, stated that, 'the constant presence of numerous populations of the limax group cannot be prevented even under the strictest observations of all routine safety measures applied to water systems of swimming pools.' In 1972 Anderson and Jamieson reported a case of PAM in South Australia the victim having played submarines in domestic bath-water, and, that superchlorination to 10 $mg.1^{-1}$ failed to eradicate Naegleria from a contaminated pool. Subsequently, Derreumaux _et al._, (1974) demonstrated that 0.5 $mg.1^{-1}$ of HOCl, the active disinfecting component of chlorine disinfection, was able to eradicate both _Naegleria_ and _Acanthamoeba_ spp. De Jonckheere and Van De Voorde (1976) found that an initial concentration of chlorine between 0.5 - 1.0 $mg.1^{-1}$ was cysticidal for _Naegleria_ spp. but that _A. culbertsoni_ cysts were not inactivated by 40 $mg.1^{-1}$.

CHAPTER 2: SAMPLING SITES

2.1 Introduction

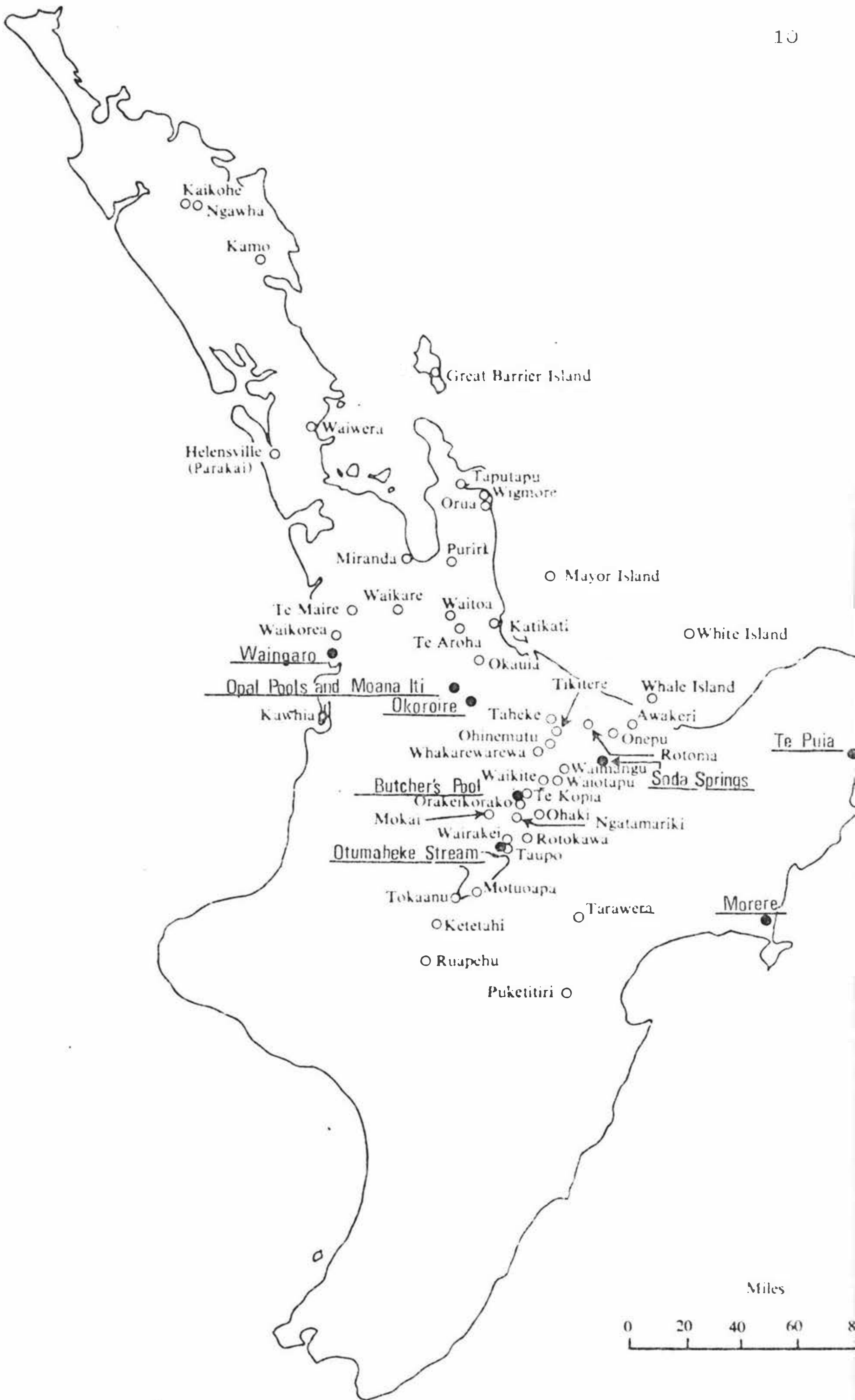
Although thermal springs are situated in both the North and South Islands of New Zealand, the majority lie within the Taupo Volcanic Zone (a belt which extends from the Tongariro National Park to White Island) emphasizing their volcanic association (An Encyclopedia of New Zealand, 1966). There are two main theories as to their origin:

i) that the water is mainly meteoric, that is, ground water which originated as rainfall, but has become heated either by coming into contact with hot rock or by steam rising from magma (molten rock) and

ii) that magma, as it cools and crystallizes, concentrates steam and other gases in the remaining liquid portion. This causes the pressure to rise until the steam forces its way out of the overlying rocks, where it may escape directly to the surface as volcanic steam, or more usually, be condensed in the ground water setting up convection currents.

Whatever the origin of the heat, thermal investigations have shown that the hot-spring areas of the Taupo Volcanic Zone are fed by water at temperatures higher than 200°C. The water at depth is at a pressure corresponding to the weight of ground water above it, but as it rises this gradually decreases. When the pressure becomes less than that at boiling-point, the water boils and gives off steam, eventually reaching the surface as a mixture of steam and boiling-water. It is this steam which supplies the innumerable steam vents in each thermal area, while the hot water flows away as springs (An Encyclopedia of New Zealand, 1966).

Fig. 1 shows the location of thermal springs in the North Island. Although some are commercially developed,i.e.having concrete construction and


Kaikohe
Ngawha
Kamo
Great Barrier Island
Waiwera
Helensville
(Parakai)
Taputapu
Wigmore
Orua
Miranda
Puriri
Mayor Island
Waikare
Te Maire
Waitoa
Waikorea
Katikati
Te Aroha
White Island
Waingaro
Okauia
Opal Pools and Moana Iti
Tikitere
Whale Island
Okoroire
Kawhia
Taheke
Awakeri
Ohinemutu
Onepu
Whakarewarewa
Rotoma
Te Puia
Waikite
Waiotapu
Soda Springs
Butcher's Pool
Te Kopia
Orakeikorako
Mokai
Ohaki
Ngatamariki
Wairakei
Rotokawa
Otumaheke Stream
Taupo
Motuoapa
Tokaanu
Tarawera
Morere
Ketetahi
Ruapehu
Puketitiri
Miles
0
20
40
60

surroundings, with a variable degree of hygiene such as water filtration and disinfection, the majority are natural thermal pools, i.e. with soil banks and surrounded by native flora, having no water treatment or other measures to prevent pollution. Traditionally, thermal pools in New Zealand have become popular with tourists for recreation and relaxation, and because of their high mineral content, have also been considered of some therapeutic benefit in the treatment of arthritic conditions. Maori folklore suggests that, 'one should slowly immerse one's body into a thermal pool,' and it is tempting to speculate on some practical purpose, borne of experience, for this advice. Although no specific diseases have previously been associated directly with prior contact with thermal pools, non-specific diseases of the ear, throat and nose are common (local Health Inspectors, pers. comm.).

Thermal pools to be sampled were chosen after consultation with the National Health Institute and the Health Departments of the Hamilton, Rotorua and Gisborne Health Districts. Previous cases of PAM had been recorded in both the Hamilton and Rotorua districts, while the Gisborne area was sampled because of the high halogen ion content in the thermal waters of that district.

2.2 Hamilton Health District

2.2.1 Waignaro Springs (Plate 1)

Situated north-west of Hamilton, this pool was chosen as a commercially developed 'control' pool because of the concrete and tiled construction of the pool and surroundings, which reduces soil contamination of the water. Overall the hygiene is of a high standard with the pool being emptied and scubbed with HTH (granular $Ca(OCl)_2$) on a weekly basis, and the water being subjected

to a chlorination régime. The source of hot-water is a natural thermal bore from which the water is pumped through PVC piping to the pool. The chemical and physical characteristics are given in Table II. Samples were taken from the pool itself.

2.2.2 Moana-iti Springs (Plate 2)

Situated east of Hamilton and previously called Crystal Springs, this site is where the first New Zealand cases of PAM were recorded. Subsequently, the old natural pool in which the cases were contracted has been closed and replaced by a new concrete pool. Pool hygiene is poor, the only chlorination being intermittent and for the control of algae. Pool surroundings are of grass, and thus soil contamination via bather's feet occurs. Other organic debris (e.g. leaves) were also seen in the water when this site was inspected. The source of hot-water is a natural thermal bore, the chemical and physical characteristics of which are presented in Table II. Sampling in the 1976 survey was from the pool itself, whilst in the 1977 survey, samples were taken from the pool inlet and outlet.

2.2.3 Opal Springs (Plate 3)

Situated adjacent to Moana-iti, these are commercially developed pools of concrete construction. Although no chlorination of the water is practised, pool hygiene is fair with the pool being emptied and scrubbed with HTH on a weekly basis. The source of hot-water is a natural thermal bore, the chemical and physical characteristics of which are given in Table II. The sampling site was usually the Jockey pool which is subject to flooding from the adjacent Waihai River.

2.2.4 Okoroire Springs (Plate 4)

Situated south-west of Hamilton these are concrete thermal pools with gravel bottoms and surrounded by high bush-covered banks, thus soil contamination is high. Pool hygiene is poor, the hot-water source being a thermal spring which percolates up through the bottoms of the pools, the chemical and physical characteristics of which are presented in Table II. Samples were taken from the Number 2 or Number 4 pools.

Table II. Chemical and Physical Characteristics of Thermal Pools from the Waikato Health District

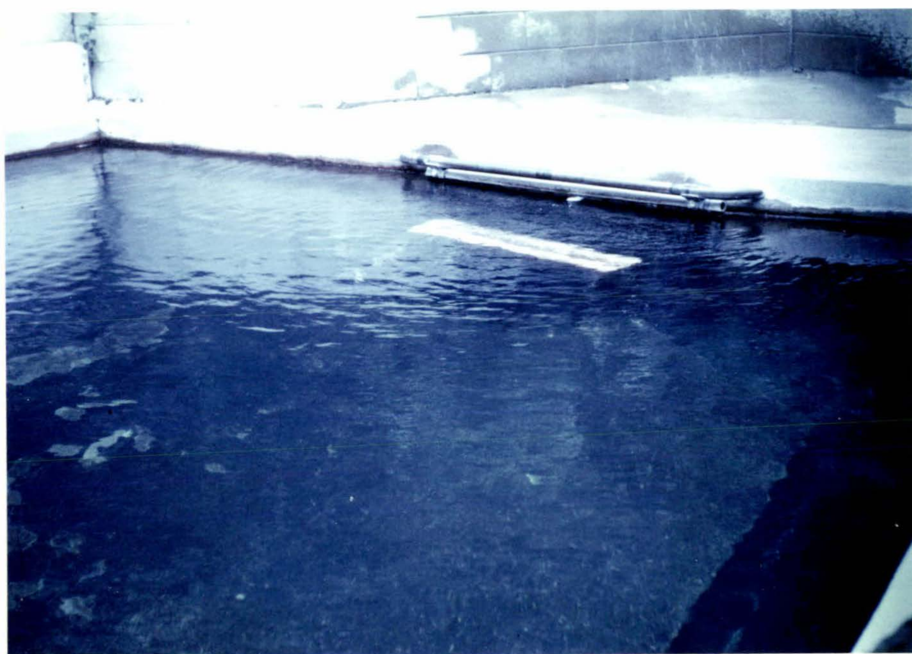
Physical and Chemical Characteristics	Waignaro	Moana-iti	Opal	Okoroire
pH	9.6	7.1	7.1	6.9
Temperature °C	46	39	39	42
Chemicals $g.m^{-3}$				
Lithium	0.09	0.20	0.19	0.20
Sodium	90.0	100.0	97.0	103.0
Potassium	0.07	11.0	10.7	15.4
Magnesium	0.1	12.7	11.5	12.2
Calcium	0.75	11.0	10.7	5.6
Aluminium	0.1	0.1	0.1	0.1
Silicon	22.8	32.4	30.3	59.0
Iron	0.01	0.19	0.31	0.66
Copper	0.01	0.01	0.013	0.013
Lead	0.1	0.1	0.1	0.1
Manganese	0.01	0.093	0.102	0.084
Fluoride	3.50	0.25	0.2	0.3
Chloride	51.0	1.0	1.0	1.0
Bromide	0.1	0.1	0.1	0.45
Iodide	0.04	0.01	0.01	0.01
Carbonate	52.0	nil	nil	nil
Bicarbonate	20.0	384.0	370.0	369.0
Sulphate	11.0	11.0	2.0	2.0
Nitrate	0.2	0.2	0.2	0.49
Total Phosphate	0.09	0.45	0.52	0.58

Plate 1. Waignaro thermal pool.

Plate 2. Moana-iti thermal pool showing inlet bore.
Note surrounding organic debris.

Plate 3. Opal 'Jockey' thermal pool.

Plate 4. Okoroire 'No. 4' thermal pool. Note surrounding vegetation.



2.3 Rotorua Health District

2.3.1 Soda Springs (Plate 5)

Situated south-west of Rotorua, this is a natural thermal pool within a flowing river, the hot-water source percolating up through the bottom. The chemical and physical characteristics of the water-supply are given in Table III. Samples were taken from the pool itself.

2.3.2 Butcher's Pool (Plate 6)

Situated south-west of Rotorua this is a natural thermal pool surrounded by dairy-farm pastures. The hot-water source is a natural thermal spring which percolates up from the pool bed. The chemical and physical characteristics of the water source are shown in Table III. Samples were taken from the pool itself.

2.3.3 Otumaheke Stream (Plates 7,8 & 9)

Situated south of Rotorua in Taupo, this pool was chosen as it was the site of the most recent (1974) fatal case of PAM in New Zealand. This also is a natural thermal pool, the hot-water source being a thermal spring. The chemical and physical characteristics are given in Table III. Samples were taken either from the pool itself or from the origin of the stream.

Plate 5. Soda thermal pool showing overflow.

Plate 6. Butcher's thermal pool.

Plate 7. Upper Otumaheke stream.

Plate 8. Mouth of Otumaheke stream and adjacent Waikato River.

Plate 9. Old Whey soil site of Otumaheke stream.

Table III. Chemical and Physical Characteristics of Thermal Pools from the Rotorua-Taupo Health District

Physical and Chemical Characteristics	Soda Springs	Butcher's Pool	Mouth of Otumaheke Stream	Upper Otumaheke Stream
pH	6.0	7.5	7.7	7.5
Temperature ^{o}C	38	40	35	18
Chemicals g.m^{-3}				
Lithium	1.4	0.62	0.05	0.05
Sodium	206.0	173.0	23.0	44.0
Potassium	18.0	16.0	4.5	4.5
Magnesium	7.0	6.3	2.9	11.3
Calcium	8.6	6.5	4.6	10.2
Aluminium	6.025	0.01	0.01	0.05
Silicon	65.0	65.0	23.0	46.0
Iron	0.82	2.6	0.14	0.07
Copper	0.001	0.002	0.001	0.007
Lead	0.008	0.005	0.005	0.005
Manganese	1.3	0.3	0.32	0.002
Fluoride	0.45	1.15	0.3	0.5
Chloride	249.0	5.0	13.0	5.0
Bromide	0.71	0.10	0.21	0.1
Iodide	0.08	0.01	0.01	0.01
Carbonate	nil	nil	nil	nil
Bicarbonate	180.0	506.0	52.0	167.0
Sulphate	46.0	5.0	29.0	48.0
Nitrate	0.31	0.1	0.27	0.1
Total Phosphate	0.49	1.69	0.28	0.15

2.4 Gisborne Health District

2.4.1 Te Puia (Plates 10 & 11)

Situated north of Gisborne, this is a commercially developed concrete constructed pool with the hot-water source being a natural thermal spring situated about 150m from the pool in unstable terrain, amongst long grass and sparse native bush. The water is 'piped' to the pool via an open wooden trough which is open to considerable contamination from the surrounding vegetation. Overall hygiene is poor with pool maintenance being badly needed. The chemical and physical characteristics of the pool-water are

given in Table IV. sampling was normally from the pool itself.

2.4.2 Morere (Plates 12 & 13)

Situated south of Gisborne, this is a concrete constructed pool, the hot-water source being a natural thermal spring situated some 800m from the pool. Water is piped to the pool through concrete and PVC piping. Overall pool hygiene is fair. The chemical and physical characteristics are given in Table IV. Sampling was generally from the pool itself.

Table IV. Chemical and Physical Characteristics of Thermal Pools from the Gisborne Health District

Physical and Chemical Characteristics	Te Puia	Morere
pH	7.2	6.7
Temperature ^{o}C	28.0	31.0
Chemicals $g.m^{-3}$		
Lithium	12.2	5.4
Sodium	4,400.0	6,100.0
Potassium	38.1	100.0
Magnesium	5.9	137.0
Calcium	550.0	3,900.0
Aluminium		
Silicon	67.0	28.0
Iron	0.9	0.4
Copper	0.15	N.G.
Lead	N.G.	N.G.
Manganese	0.1	N.G.
Fluoride	1.5	0.4
Chloride	8,100.0	15,600.0
Bromide	27.6	80.0
Iodide	18.0	25.0
Carbonate	nil	N.G.
Bicarbonate	14.8	N.G.
Sulphate	71.0	N.G.
Nitrate	N.G.	N.G.
Total Phosphate		

N.G. - not given

Plate 10. Hot-water source of Te Puia springs.

Plate 11. Wooden trough used to 'pipe' thermal water from hot-water source to the Te Puia pool.

SPRINGS

Plate 12. Morere thermal baths pipe supply.

Plate 13. Hut enclosing Morere thermal baths.

2.5 Sample Collection

Duplicate 1.0 l samples of water or a quantity of soil were collected by the local Health Inspectors at each site, in sterile Whirl Pak bags (ROBINSON DESIGNS, Auckland). Wherever possible, samples were collected from near the bottom of the pool. Simultaneously, both the pH and temperature of the water were recorded whilst rainfall and ambient temperature readings were obtained from the nearest meterological station via the New Zealand Meterological Service. The bags containing water were forwarded to either the local hospital bacteriology laboratory, for both a total bacteria and a total coliform count, or to Massey University for the isolation of amebae. Generally, samples collected at the beginning of the week (Monday or Tuesday) were processed on the Friday of that week.

2.6 Frequency of Sampling

In the 1976 survey, monthly water samples only were collected from the sites to constitute a preliminary screening of the thermal pools for the presence of PFLA. Those pools which exhibited a high frequency of PFLA isolations during the 1976 survey, were singled out for sampling on a more regular basis. In 1977, water and soil samples were collected on a weekly basis from the Otumaheke stream site, whereas fortnightly water and soil samples were obtained from the Hamilton sites. Although the local Health Departments endeavoured to cooperate to the fullest, other work commitments occasionally prohibited them from collecting the samples.

CHAPTER 3: MATERIALS

3.1 Ameba Cultures Used

See Table V.

Table V. Ameba Cultures Used

Species	Strain	Pathogenicity	Source	Plates
Naegleria fowleri	NHI	+	NHI	
Naegleria fowleri	MsT	+	NHI	17
Naegleria fowleri	Ts-1	-	SPHKUL	
Naegleria jadini	0400	-	IMTPL	
Naegleria gruberi	P1200f	-	NHI	14, 15, 16
Acanthamoeba culbertsoni	A-1	+	CCAP	20
Acanthamoeba castellanii	1501	-	IMTPL	18, 19
Acanthamoeba rhysodes	1507	-	IMTPL	
Acanthamoeba polyphaga	P23	-	IMTPL	

+ = positive

- = negative

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

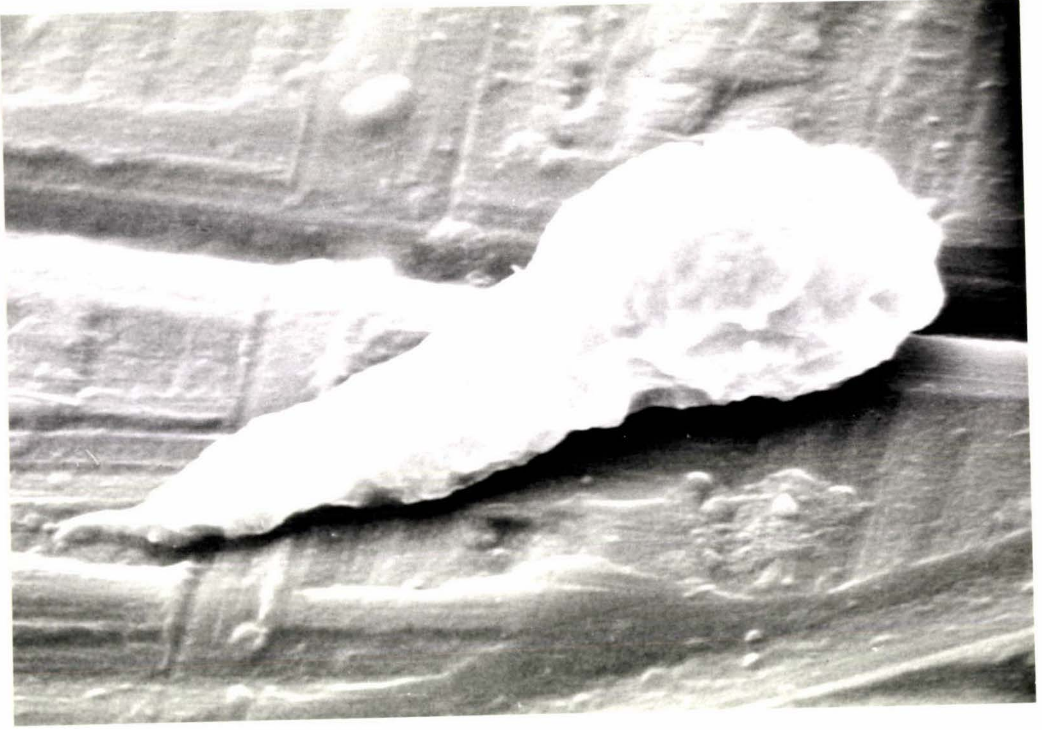
SPHKUL = School of Public Health, Katholieke Universiteit, Leuven, Belgium

IMTPL = Institut de Médecine Tropicale Prince Léopold, Belgium

CCAP = Culture Centre of Algae and Protozoa

Plate 14. Scanning electronmicrograph of *N. gruberi* trophozoite. Mag. *ca* x 8,400. (From Cursons & Brown, 1976)

Plate 15. Scanning electronmicrograph of *N. gruberi* flagellate. Mag. *ca* x 4,200. (From Cursons *et al.*, 1978a).



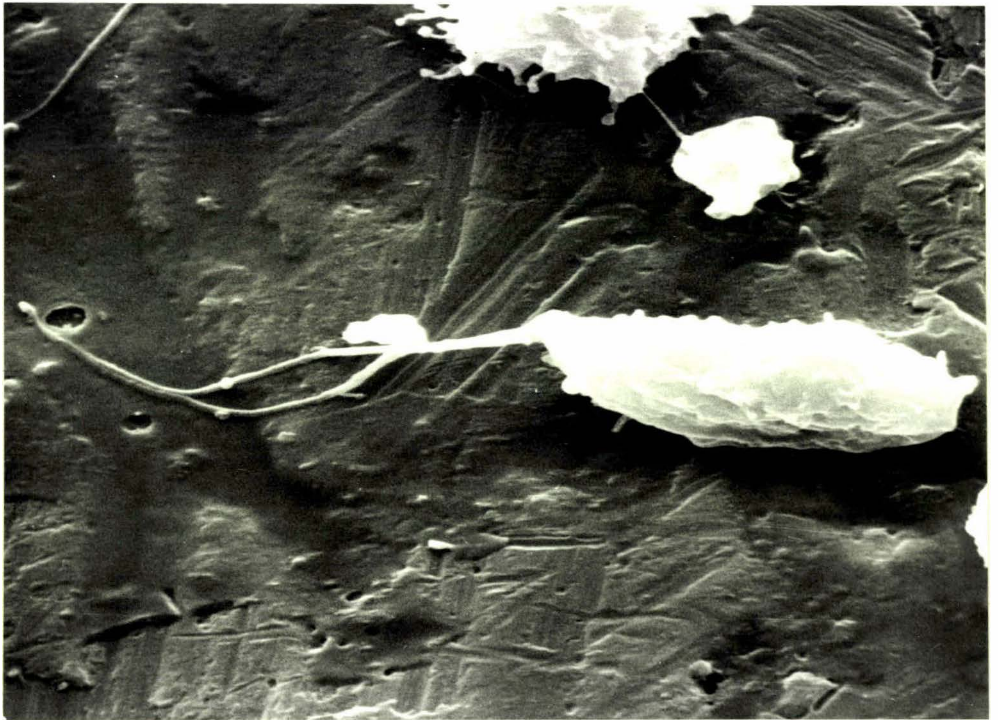
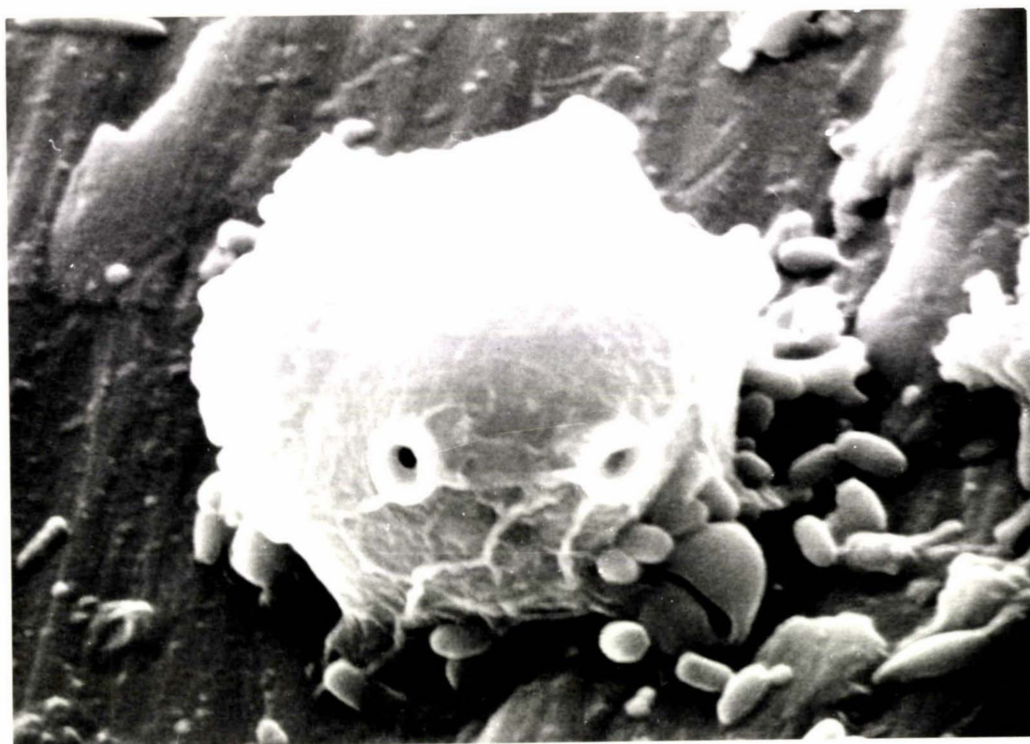


Plate 16. Scanning electronmicrograph of N. gruberi cyst. Mag. *ca* x 10,000. (From Cursons & Brown, 1976).

Plate 17. Scanning electronmicrograph of N. fowleri cyst. Mag. *ca* x 10,600. (From Cursons & Brown, 1976).



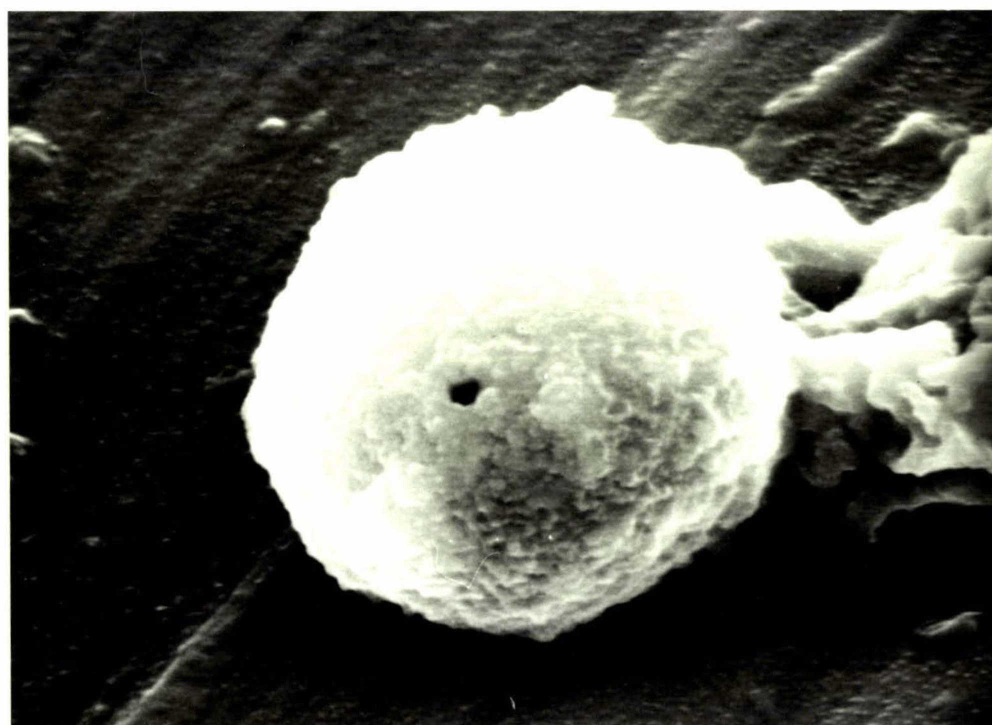
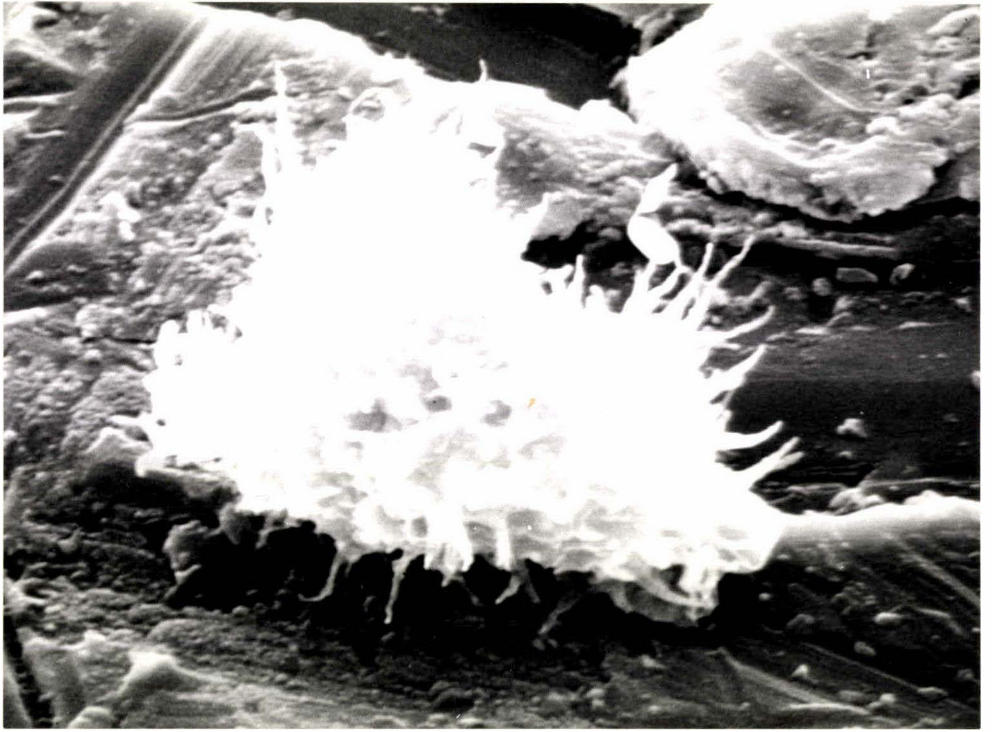


Plate 18. Scanning electronmicrograph of A. castellanii trophozoite. Mag. *ca* x 6,000. (From Brown & Cursons, 1977).

Plate 19. Scanning electronmicrograph of A. castellanii cyst. Mag. *ca* x 8,400. (From Brown & Cursons, 1977).



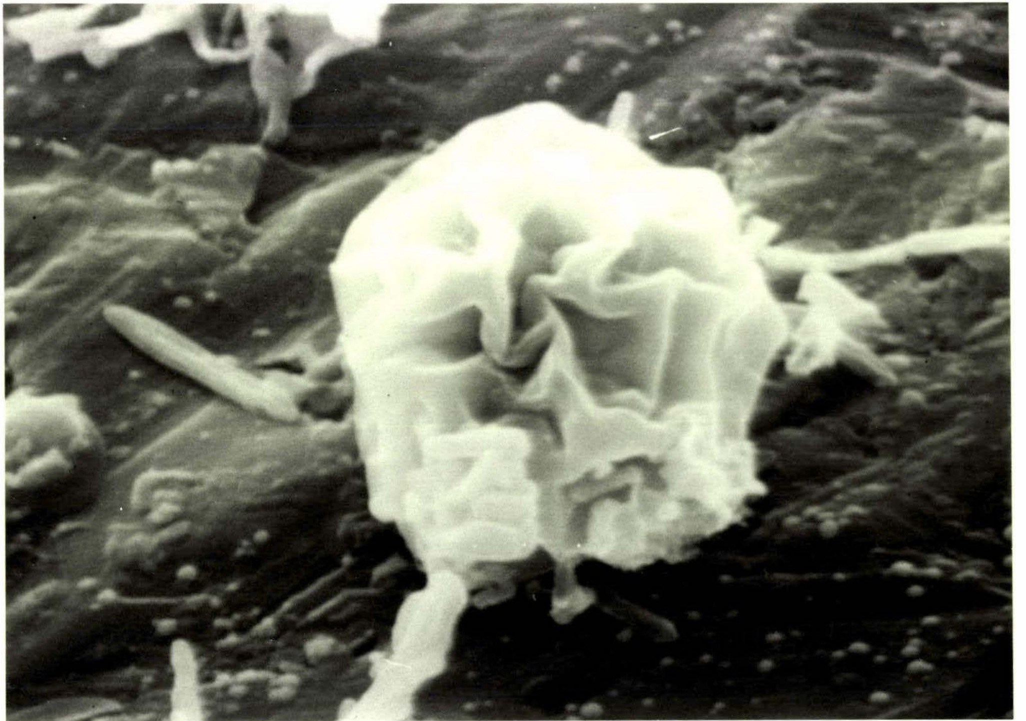
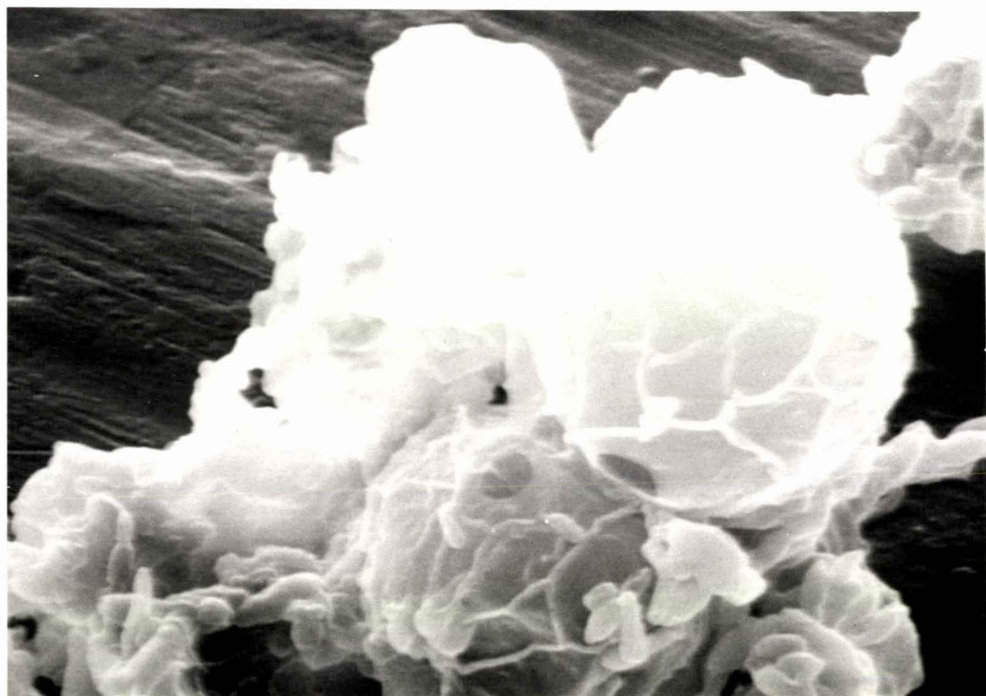


Plate 20. Scanning electronmicrograph of *A. culbertsoni* cyst. Mag. *ca* x 8,600.
(From Cursons *et al.*, 1978a).



3.2 Plate Media

3.2.1 Ameba Saline Agar (Page, 1967; Cursons et al., 1978a) for the isolation of *Naegleria* spp.

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

NaCl	= 10.0 g	Na_2HPO_4	= 0.142 g
$MgSO_4 \cdot 7H_2O$	= 0.004 g	KH_2PO_4	= 0.136 g
$CaCl_2 \cdot 2H_2O$	= 0.004 g	DAVIS agar	= 15.0 g
Distilled water	= 1.0 l		

pH 6.8

Autoclave at 103.4 kPa (121°C) for 15.0 min.

3.2.3 N.M. Agar (Fulton, 1970) for plaquing out either *Naegleria* or *Acanthamoeba* spp.

3.2.4 E.Y. Agar (Cursons et al., 1978c) for the detection of extracellular microbial phospholipases.

Sol. A:

SIGMA hepes buffer	= 0.6 g
$CaCl_2$	= 0.044 g
NaCl	= 9.0 g
$MgCl_2 \cdot 6H_2O$	= 0.081 g
DIFCO Nobel agar	= 10.0 g
Distilled water	= 980.0 cm^3

pH 7.6

Autoclave at 103.4 kPa for 15.0 min. Cool to 45°C, then add 0.3 cm^3 of OXOID egg-yolk emulsion to 20 cm^3 of Sol. A.

The final formulation of this agar was only achieved after extensive testing of barbital, barbital-acetate, phosphate and hepes buffers at different concentrations and pH (Habermann & Hardt, 1972). Of the buffers examined, both hepes and barbital buffers produced the best results but only at relatively low concentrations. The pH optimum for phospholipase 2(A) was found to be pH 7.5 whilst that for

lysophospholipase (B) was more alkaline. Because the buffering capacity of hepes is between pH 6.8 - 8.2, the alkaline limit is 8.2.

The presence and concentrations of particular cations was found also to be of importance. No phospholipase activity was observed without the presence of Ca^{2+} ions being present and the addition of Mg^{2+} ions also had an enhancing effect. In contrast, the presence of Zn^{2+}, Mn^{2+}, Cu^{2+}, Pb^{2+} or excess EDTA had an inhibitory effect on phospholipase activity.

3.2.5 Lysolecithin Agar (Habermann & Hardt, 1972; Cursons et al., 1978c) for the detection of extracellular microbial lysophospholipases. To 20.0 cm^3 of Sol. A (see Materials 3.2.4) at 45°C, add 0.008 g of SIGMA L-α-lysophosphatidyl choline (type I, approximately 98%) instead of the OXOID egg-yolk emulsion.

3.2.6 Haemolytic Agar for the detection of haemolytic activity of extracellular microbial phospholipases.

Phospholipase 2: To 20.0 cm^3 of Sol. A (see Materials 3.2.4) add 0.3 cm^3 of washed erythrocytes and either 0.3 cm^3 of OXOID egg-yolk emulsion, or bovine serum albumin to a final concentration of 4.0% (W/V).

Phospholipase 3: To 20.0 cm^3 of Sol. A (see Materials 3.2.4) add 0.3 cm^3 of washed erythrocytes.

3.2.7 Proteinase Agar for the detection of extracellular microbial proteinases.

Add 0.8 g skim milk powder to 20.0 cm^3 of Sol. A (see Materials 3.2.4), and autoclave at 68.9 kPa for 10.0 min.

3.2.8 DIFCO Standard Methods Agar for total bacterial counts.

3.2.9 DIFCO Violet Red Bile Agar for total coliform counts.

3.3 Axenic Media for Amebae

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

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

3.3.3 CYMH Medium for the serum-free axenic cultivation of *Naegleria* spp. The serum component of CYM medium is omitted.

3.3.4 4.0% Neff Medium (Stevens & O'Dell, 1973b) for the axenic cultivation of *Acanthamoeba* spp.

3.4 Cell Culture Media

All media used for Vero cell culture are described in Cursons (1974) and Cursons & Brown (1976, 1978).

3.5 Lymphocyte and Macrophage Culture Media

3.5.1 Lymphocytes

Washing Medium: WELLCOME medium 199, CO_2-HCO_3^- buffer and 100 units of both penicillin and streptomycin .cm^{-3}.

Growth Medium: WELLCOME medium 199,

CO_2-HCO_3^- buffer, 10% (V/V) complement inactivated normal guinea-pig serum and 100 units of both penicillin and streptomycin $.cm^{-3}$.

3.5.2 Macrophages

Collecting Medium: Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution (HBSS) (Hanks, 1948), 20.0 units of heparin (preservative-free) and 100 units of both penicillin and streptomycin $.cm^{-3}$.

Washing Medium: WELLCOME Eagles MEM, CO_2-HCO_3^- buffer and 100 units of both penicillin and streptomycin $.cm^{-3}$.

Growth Medium: WELLCOME Eagles MEM, CO_2-HCO_3^- buffer, 15.0% (V/V) complement inactivated guinea-pig serum and 100 units of both penicillin and streptomycin $.cm^{-3}$.

3.6 Disinfection Solutions Used

3.6.1 Water

All water used was distilled and deionized.

3.6.2 Chlorine

A solution of sodium hypochlorite (NaOCl) was obtained from BDH.

3.6.3 Chlorine dioxide

Chlorine dioxide was generated in the laboratory (see Method 4.6.1).

3.6.4 Ozone

Ozone was generated in the laboratory (see Method 4.6.1).

3.6.5 Deciquam 222 (Di-decyldimethyl-ammonium bromide)
This was a generous gift from Maui Brothers, Auckland.

3.7 Polyacrylamide Gel Electrophoresis (PAGE)

3.7.1 Polyacrylamide Gels (Kersters & De Ley, 1975)

Sol. A:	28.0%	(W/V)	BDH acrylamide
	0.735%	(W/V)	BDH N, N'-methylenebisacrylamide
Sol. B:	0.23%	(V/V)	SIGMA N,N,N'-tetra-methylethylenediamine
	6.1%	(W/V)	SIGMA 7-9 tris (hydroxy-methyl) aminomethane
Sol. C:	0.14%	(W/V)	BDH ammonium persulphate

2.0 cm^3 of Sol. A was mixed with 1.0 cm^3 of Sol. B. Next 1.0 cm^3 of distilled water was added and mixed followed by 4.0 cm^3 of Sol. C. The gels were then left to polymerise at 22^oC for 2.0 hr after which they were overlaid with 1.0 cm^3 of distilled water.

3.7.2 Running Buffer

64.0 mM SIGMA tris-HCl buffer pH 8.7.

3.7.3 Tracking Marker

0.05% (W/V) Bromophenol blue, containing 5.0% (W/V) sucrose.

3.7.4 Gel Staining Solution (Blakesley & Boezi, 1977)

0.2% (W/V) of SIGMA Brilliant blue dye was dissolved in an equal volume of 2.0 N H_2SO_4 and stood overnight. The precipitate which formed was removed by gravity filtration (WHATMAN No. 1) and to the clear brown filtrate was added 1/9 volume of 10.0 N KOH. To the resulting dark

blue solution was added BDH trichloroacetic acid (100%) to a final concentration of 12% (W/V). Finally the pH was checked to below 1.0.

3.8 Staining Solutions

3.8.1 Sudan black (Culling, 1974) for the demonstration of neutral lipids. 1.0 g of GURR Sudan black was dissolved in 100.0 cm^3 of propylene glycol and heated to 100^oC for 5.0 min. Whilst still hot, the solution was filtered through Whatman No. 2 filter paper, cooled and finally filtered through a scintered glass filter.

3.8.2 Ehrlich's haematoxylin (Culling, 1974) for counterstaining nuclei. 2.0 g of GURR haematoxylin was dissolved in 100.0 cm^3 absolute ethanol. 100.0 cm^3 of distilled water, 100.0 cm^3 of glycerol and 10.0 cm^3 of glacial acetic acid respectively were then added in the order listed. Finally potassium alum was added to excess and the mixture ripened for two months in the light.

3.8.3 Glycerin-jelly for mounting slides. 10.0 g of BBL gelatin was dissolved in 60.0 cm^3 of hot distilled water. To this was added 70.0 cm^3 of glycerol and 0.25 g of phenol.

3.9 Chemicals and Serological Reagents

Unless otherwise stated, all chemicals used were, wherever possible, of an analytical grade and obtained from BDH.

Dimethyl-DL-2,3-distearoyloxypropyl-2-hydroxyethyl-ammonium acetate (Rosenthal's inhibitor) (RH) was obtained from CALBIOCHEM. 0.1 g of this was dissolved in 2.0 cm^3 of hot acetone (70^oC) and then diluted down with hot water (60^oC) to the required concentration.

Gluteraldehyde and osmium tetroxide were obtained from TAAB Laboratories.

Fluorescein isothiocyanate (FITC) conjugated anti-rabbit,-guinea-pig,-human and -human-IgG,-IgM and -IgA were all obtained from WELLCOME Laboratories.

3.10 Experimental Animals

All experimental animals used were obtained from closed inbred colonies from the Small Animal Production Unit, Massey University.

CHAPTER 4: METHODS

4.1 Miscellaneous Methods

4.1.1 Chemical Analysis of Thermal Pool Waters

The chemical analysis of thermal pool waters was performed by courtesy of the Chemistry Division of the Department of Scientific and Industrial Research, Auckland, New Zealand.

4.1.2 Scanning Electron Microscopy (SEM)

The method used for scanning electron microscopy is given in Cursons & Brown (1978).

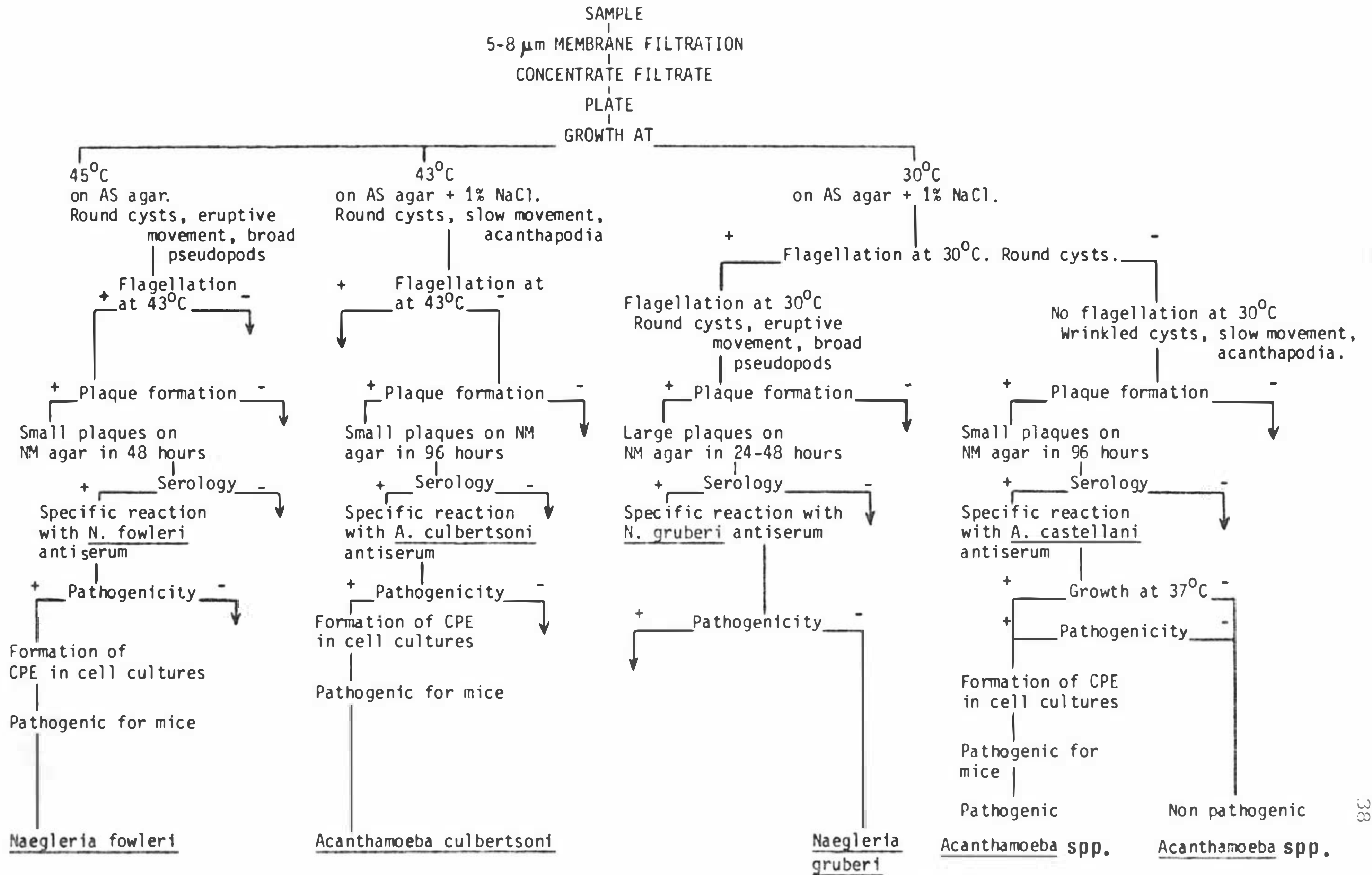
4.2 Identification of Amebae and Bacteria

4.2.1 Identification of Amebic Isolates

Water samples were processed according to Fig. 2, the rational behind each definitive test being described previously (Cursons, 1974; Cursons & Brown, 1976). Briefly this involved the exploitation of cultural, physiological, morphological, serological and pathogenicity differences existing between the different genera and species of free-living amebae. Although this procedure was fairly routine, particular points in the course of the investigation became apparent:

i) Many different species of free-living amebae were isolated which were able to both tolerate and grow at 45°C. In particular, the most common contaminant found in both thermal-waters and soils was that of *Vahlkampfia ustiana* which was shown to be both non-pathogenic in cell cultures and mice. Morphologically, this species resembled *Naegleria* spp. but could be differentiated both on trophozite and cyst dimensions (30-50 μm and 15-22 μm respectively), more conspicuous vacuolation,

Figure 2. Flow diagram showing the strategy used for the isolation and identification of PFLA. (From Cursons & Brown, 1976).


SAMPLE
5-8 µm MEMBRANE FILTRATION
CONCENTRATE FILTRATE
PLATE
GROWTH AT
45°C
on AS agar.
Round cysts, eruptive movement, broad pseudopods
Flagellation at 43°C
+
-
Plaque formation
Small plaques on NM agar in 48 hours
Serology
Specific reaction with N. fowleri antiserum
Pathogenicity
Formation of CPE in cell cultures
Pathogenic for mice
Naegleria fowleri
43°C
on AS agar + 1% NaCl.
Round cysts, slow movement, acanthapodia
Flagellation at 43°C
Plaque formation
Small plaques on NM agar in 96 hours
Serology
Specific reaction with A. culbertsoni antiserum
Pathogenicity
Formation of CPE in cell cultures
Pathogenic for mice
Acanthamoeba culbertsoni
30°C
on AS agar + 1% NaCl.
Flagellation at 30°C. Round cysts.
Flagellation at 30°C
Round cysts, eruptive movement, broad pseudopods
Plaque formation
Large plaques on NM agar in 24-48 hours
Serology
Specific reaction with N. gruberi antiserum
Pathogenicity
Naegleria gruberi
No flagellation at 30°C
Wrinkled cysts, slow movement, acanthapodia.
Plaque formation
Small plaques on NM agar in 96 hours
Serology
Specific reaction with A. castellani antiserum
Growth at 37°C
Pathogenicity
Formation of CPE in cell cultures
Pathogenic for mice
Pathogenic
Non pathogenic
Acanthamoeba spp.
Acanthamoeba spp.

broader pseudopods, a more eruptive movement and the absence of a flagellate stage. It did however, cross react with anti-Naegleria serum but at lower titres. Unless shown to possess some virulence in cell culture, no attempt was made to identify those isolates able to grow at 45°C further than the generic level (Page, 1976).

ii) During isolation, dehydration must be avoided at any of the processing stages because of the extreme sensitivity of N. fowleri. Carter (1970) commented on the relative poor formation of cysts by N. fowleri, and recently Chang (1978) has also reported the extreme sensitivity to dehydration of both the trophozoite and cyst stages. It is felt that a higher incidence of isolation of N. fowleri from thermal pools in the future could be achieved by greater care being taken to avoid dehydration.

iii) Flagellation was usually achieved by flooding ameba-saline agar plates with sterile distilled water. However in a few cases, N. fowleri strains were isolated with a very low rate of flagellation and in some cases, it had to be repeated.

4.2.2 Total Coliform and Total Bacteria Counts

Total coliform, i.e. presumptive, confirmed and faecal counts, and total bacterial counts were performed by the local hospitals' bacteriology laboratories. Total coliform and total bacteria counts on soil were performed in the Massey laboratory using Standard Methods Agar for total bacteria and Violet Red Bile Agar for

coliforms. The method adopted was as follows:

1.0 g of wet soil was added to 99.0 cm^3 sterile distilled water and then shaken. The mixture was then diluted out using sterile distilled water and plated out on the appropriate media. Plates for total bacteria were incubated at 37°C for 24 hr whilst plates for total coliforms were incubated at 37°C for 2 days. All bacterial counts were expressed $.g^{-1}$ dry weight of soil.

4.3 Amebic Culture Techniques

4.3.1 Cloning

All cultures obtained were cloned 3 times by the plaque method (Fulton, 1970; Cursons, 1974) before being used experimentally.

4.3.2 Axenic Culture

Cloned amebae were cultured from ameba-saline agar plates into either 5.0 cm^3 of CYM for *Naegleria* spp. or 5.0 cm^3 of 4.0% Neff for *Acanthamoeba* spp. in sterile universals (8.0 x 2.0 cm) and then placed on gyroshakers (150 rpm) (Cursons *et al.*, 1978d). *N. fowleri* (strains MsT, NHI and Ts-1) and *A. culbertsoni* (strain A-1) were cultured at 37°C and sub-cultured every 24.0 hr.

N. gruberi (strain P1200f), *N. jadini* (strain 0400), *A. castellanii* (strain 1501), *A. rhysodes* (strain 1537) and *A. polyphaga* (strain P23) were cultured at 30°C and sub-cultured every 48.0 hr.

4.4 Cell Culture

All methods used for Vero cell culture are

described in Cursons and Brown (1976 & 1978).

4.4. Neutralization of Amebae in Cell Cultures

i) 1.0 cm^3 of serum was added to 4.0 cm^3 of Eagles maintenance media (EMM).

ii) 1.0 cm^3 of this was added to 1.0 cm^3 of the appropriate dilution of axenically-grown amebae, previously washed and resuspended in EMM in tightly-capped bijou bottles, and then incubated at 37°C for 1 hr on a gyroshaker at 150 rpm.

iii) Eagles growth medium from 24 hr Vero monolayers was tipped off and the monolayer washed in serum-free Eagles medium. Then 1.5 cm^3 of the ameba-serum mixture was added to the monolayers, incubated at 37°C and examined for the formation of cytopathic effects (CPE).

4.5 Indirect Macrophage Inhibition Factor Assay

4.5.1 Immunization of Guinea-pigs

Pre-immune serum was obtained from each guinea-pig by cardiac puncture using a sterile disposable syringe (21 gauge x 4.0 cm). The guinea-pigs were then immunized with 0.5 cm^3 of a 1:1 Complete Freunds Adjuvant (DIFCO): antigen mixture I/M into their hind leg. After 28 days the guinea-pigs were then boosted weekly with 0.1 cm^3 of antigen S/C for the following three weeks.

4.5.2 Culturing of Lymphocytes and Production of Macrophage Inhibition Factor (MIF)

8.0 cm^3 of blood was withdrawn aseptically by cardiac puncture using a sterile disposable syringe (21 gauge x 4.0 cm) containing 160 units

of sterile, preservative-free heparin.

The blood was then centrifuged at 1000 x G for 10 min at 20°C and the buffy coat cells aspirated using a sterile pasteur pipette.

The buffy-coat cells were diluted 1:1 with sterile physiological saline containing 20 units .cm^{-3} of sterile heparin and carefully layered on a sterile Ficoll-Hypaque gradient (Lymphoprep) using 3 volumes of Lymphoprep with 4 volumes of diluted buffy-coat cells. They were then centrifuged in a swing out head at 1250 xG for 20 min at 20°C. The top layer of the diluted plasma was discarded and the lymphocytes collected in 10.0 cm^{3} of medium 199. They were then centrifuged at 1000 xG for 10 min at 20°C and washed again in 10.0 cm^{3} of medium 199.

The cells were then resuspended in growth medium 199 to a concentration of 2.0 x 10^{6} cells .cm^{-3}. To this was added 0.5 mg.cm^{-3} of protein antigen and the mixture cultured at 37°C overnight. After incubation, the cells were centrifuged at 1500 xG for 20 min at 20°C and the supernatant filtered through a sterile 0.22 μm filter and stored at -20°C until required.

4.5.3 Culture of Macrophages

Six days before the isolation of lymphocytes, an unsensitized guinea-pig was injected with 30.0 cm^{3} sterile paraffin oil I/P (16 gauge x 2.0 cm) and the peritoneal cavity massaged.

After 7 days, the guinea-pig was

exsanguinated by cardiac puncture and 150 cm^3 of cold collecting medium introduced I/P. The peritoneal cavity was then agitated and pummelled to resuspend the peritoneal exudate cells (PEC).

A 2-3 cm incision was made just below the diaphragm and a 2.0 x 12.0 cm sterile aluminium centrifuge tube, in which the bottom 2.0 cm had been perforated by smooth small holes, inserted. PEC cells were collected, using a sterile 10.0 cm^3 pipette, from the fluid in the peritoneal cavity, and placed in a sterile separating funnel for 15 min at 5^oC to separate the paraffin oil.

The cells were then collected in sterile 50.0 cm^3 centrifuge tubes and centrifuged at 750 xG for 15 min at 20^oC.

Following centrifugation they were resuspended in sterile 10.0 cm^3 centrifuge tubes, washed twice with Eagles washing medium, adjusted to 10% packed cell volume, and resuspended in Eagles growth medium.

After standing 10 min in an ice bath, sterile capillary tubes were filled to the top and plugged with sterile Cristaseal (HAWKSLEY AND SON, Ltd, Sussex).

They were then wrapped in sterile aluminium foil, placed in a sterile universal, and centrifuged at 200 xG for 2.0 min at 20^oC. The capillary tubes were then cut at the cell-surface with a sterile diamond pencil and placed in sterile cell culture dishes (FALCON) on a sterile blob of silicon grease, making sure that

the orifice of each capillary tube was away from the grease and in contact with the bottom of the dish.

After carefully filling the dish with 2.0 cm^3 of growth medium and 1.0 cm^3 of the lymphocyte cell-free supernatant fluid (see Methods 4.5.2), the mixture was incubated at 37^oC overnight in a desiccator containing 5.0% CO_2, and the inhibition of migration photographed using a LEITZ Ortholux microscope fitted with an Orthomat camera.

4.5.4 Delayed Hypersensitivity

This was tested by inoculating 0.1 cm^3 of antigen containing 5 $mg.cm^{-3}$ of protein intradermally (I/D) into the skin of a sensitized guinea-pig.

4.6 Disinfection

4.6.1 Production of Disinfectants

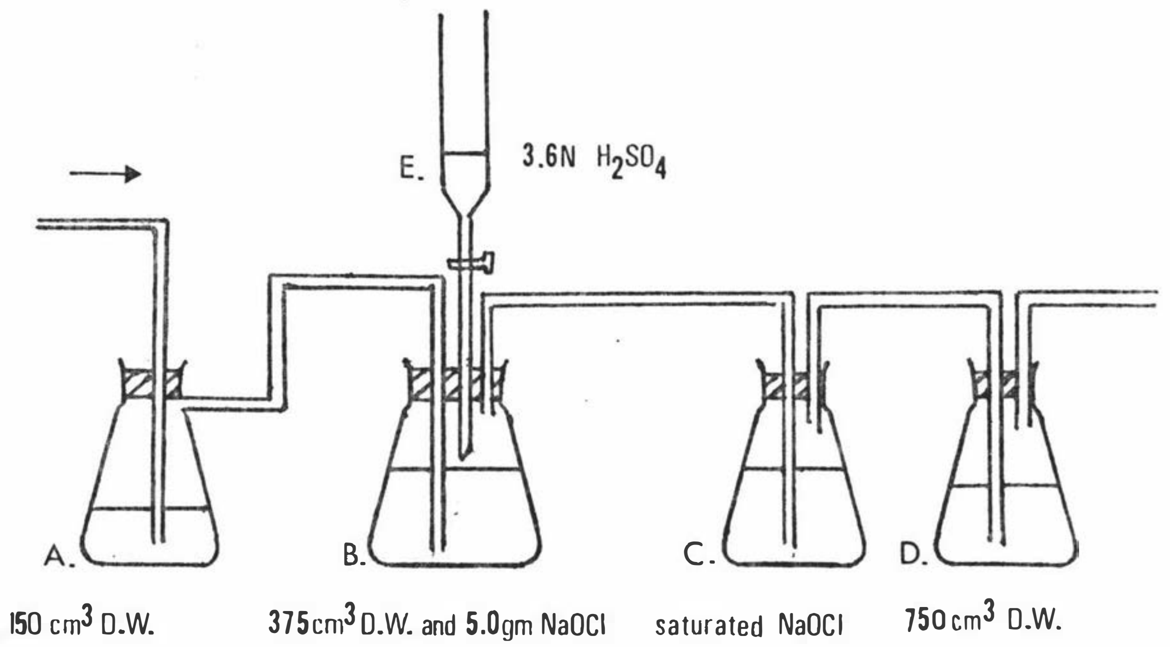
i) Chlorine: a stock solution of BDH sodium hypochlorite was diluted to the appropriate concentration using sterile, chlorine-free, deionized water.

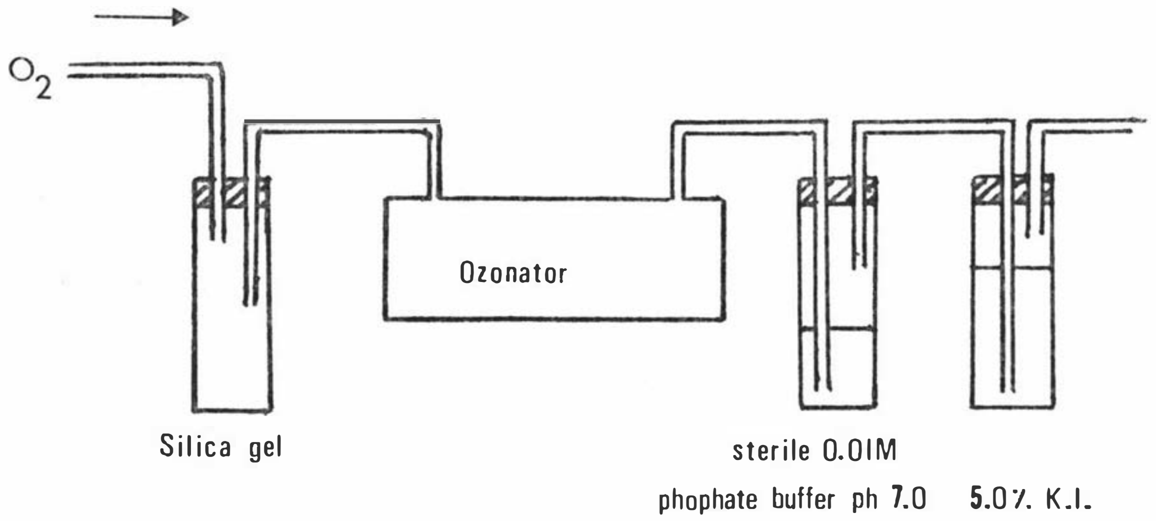
ii) Chlorine dioxide: this was produced as recommended by the American Public Health Association (1971) as shown in Fig. 3:

5.0 g of sodium chlorite was dissolved in 375.0 cm^3 of chlorine-free deionized water and placed in bottle B. 1.0 cm^3 of concentrated H_2SO_4 was added to 9.0 cm^3 of deionized water, mixed and transferred to flask E. A smooth current of air was passed through the system and 5.0 cm^3 increments of H_2SO_4 introduced from E into B at 5.0 min intervals. The airflow was continued

Figure 3. Method used for the production of chlorine dioxide. (Adapted from Standard Methods, 1971)

Figure 4. Method used for the production of ozone.





D.W. = distilled water

for a further 30.0 min and the solution in bottle D stored in a brown bottle at 4°C.

iii) Ozone: this was produced by the electrical discharge of oxygen as shown in Fig. 4. This involves passing dried oxygen through a Mk II ozonizer (BRITISH OXYGEN CRYOPRODUCTS) and collecting the dissolved ozone in sterile 0.01 M phosphate buffer pH 7.0.

4.6.2 Chemical Analysis of Disinfectants

Chlorine, chlorine-dioxide and ozone were analyzed by the DPD method (Palin, 1974). Due to the lack of a specific analytical test, no analysis of Deciquam 222 was performed. The method described by Wang and Langley (1975) was found to yield inconsistent results.

4.6.3 Disinfection Tests

All disinfection tests were performed according to Cursons *et al.* (1978b).

4.7 Polyacrylamide Gel Electrophoresis (PAGE)

Native PAGE was performed according to the method of Kerstens and De Ley (1975) using an Acrylophor (PLEUGER) disc apparatus and a Vokam (SHANDON) Powerpack. Approximately 100 μg of soluble protein in 10 μl, containing 5% (W/V) sucrose, was layered on top of the gels. The two compartments of the Acrylophor apparatus were filled with gel buffer, the gels loaded and pre-electrophoresis was performed with the anode in the lower electrode chamber at a constant current of 1.25 mA.gel^{-1} for 15 min, followed by 3.8 mA.gel^{-1} until the tracking marker had moved through the gel. The gels were then stained overnight at 20°C with Brilliant blue and destained in deionized water in which they were stored. Gels were scanned

using an ISCO gel scanner (Model 1310) at 620 nm using either 30 or 150 $cm.hr^{-1}$.

4.8 Cytochemical Staining for Lipids

A modified Chiffelle and Putts propylene glycol method was used (Culling, 1974).

Coverslips containing cell-cultures infected with amebae were fixed in 10.0% (V/V) formol-saline for 30 min, and then washed 3 times in distilled water.

The coverslips were then dehydrated for 5.0 min in 2 changes of polypropylene glycol and then transferred to the staining solution for 4.0 min.

After staining, the coverslips were differentiated in warm (40^oC) propylene glycol for 3.0 min with some agitation, rinsed in 50% propylene glycol and then washed in distilled water.

The coverslips were then counterstained with 50% Ehrlichs haematoxylin in distilled water for 4.0 min, washed in tap-water to blue the sections and finally mounted in glycerin-jelly.

4.9 Preliminary Isolation and Characterization of Extracellular Enzymes of Pathogenic Free-living Amebae

4.9.1 Detection of Amebic Extracellular Enzymes

Amebic extracellular enzymes were detected by placing 0.1 cm^3 of cell-free axenic culture fluid, that had previously been filtered through an 0.22 μm filter, into either EY or Proteinase Agar plates (Cursons et al., 1978c).

4.9.2 Thin Layer Chromatography

Both reacted and control agar zones were excised from EY agar and were extracted in 8.0 cm^3 of 2:1 chloroform:methanol and 1.0 cm^3

water. The aqueous layer was discarded, and the remainder extracted once more with chloroform: methanol, then the final chloroform layer was evaporated to dryness. The extracted lipid was then dissolved in 0.5 cm^3 chloroform. 10 μl of extracted lipid was spotted onto Silica Gel G plates (MERCK) which had previously been activated at 110°C for 1 hr. Spots were allowed to dry and were placed in hexane: diethyl ether: acetic acid (50:50:1) for neutral lipid analysis, or in chloroform: methanol: acetic acid: water (25:15:4:2) for phospholid analysis. Spots were visualized by spraying with ethanolic 0.1% (W/V) 2,7 dichlorofluorescein (BDH) and observed under a UV light source.

4.9.3 Haemolysis

Haemolysis was performed either in solution or agar using solution A of the EY agar medium. In all haemolytic experiments, NaCl was added to all amebic supernatants to physiological strength. Washed sheep erythrocytes were the target cells, and haemolysis was performed using either egg yolk or 4.0% albumin (Gul & Smith, 1972) incorporated into the agar for phospholipase 2 or erythrocytes alone for phospholipase 3.

4.9.4 Isolation of Phospholipases from the Supernatants of N. fowleri (MsT)

Two 500.0 cm^3 volumes of either CYM or CYMH medium in 2.0 l sterile flasks were inoculated with 0.5 cm^3 or 1.5 cm^3 respectively of N. fowleri (MsT) previously grown up in either CYM or CYMH. After incubation for either 48.0 hr (CYM) or 72.0 hr (CYMH) at 37°C on a gyroshaker (150 rpm) the cells were centrifuged at 3000 xG at 4°C for 15 min. The supernatant was filtered through a 5 μm filter and the protein precipitated out by

Figure 5. Flow diagram showing the strategy used for the isolation of phospholipase 2 and lysophospholipase from the supernatant of axenically grown *N. fowleri* (MsT).

EXPONENTIALLY GROWING CULTURE OF *N. FOWLERI* (MsT)

↓ CENTRIFUGE AT 3,000 G/4^0C/10 MIN

PLACE IN EY PLATE ← COLLECT SUPERNATANT —MEASURE VOLUME→ PROTEIN DETERMINATI

↓

PH 6.8

↓ PRECIPITATE WITH 40-60% $(NH_4)_2SO_4$ AT 4^0C
OR 1:1 CH_3CH_2OH (-70^0C)

CENTRIFUGE 7000 G/4^0C/10 MIN

↓

COLLECT PRECIPITATE & DISSOLVE IN 2.5MM HEPES PH 7.5

↓ DIALYSE OVERNIGHT 4^0 AGAINST 2.4MM HEPES PH 7.5

PLACE IN EY PLATE ← MEASURE VOLUME → PROTEIN DETERMINATION

↓

CONCENTRATE PROTEIN BY FILTERING THROUGH A XM 50,000 ULTRAFILTRATION MEMBRANE

PLACE IN EY PLATE ← MEASURE VOLUME → PROTEIN DETERMINATION

↓

APPLY TO SEPHADEX G-100 EQUILIBRATED IN 2.5MM HEPES PH 7.5

PLACE IN EY PLATE ← COLLECT FRACTIONS → MEASURE PROTEIN

↓

CONCENTRATE PROTEIN FRACTIONS EXHIBITING CLEARING IN EY PLATE BY FILTERING THROUGH A XM 50,000 ULTRAFILTRATION MEMBRANE

↓

PLACE IN EY PLATE ← → MEASURE PROTEIN

↙ PAGE

↘ USE AS ANTIGEN TO MAKE A SPECIFIC ANTISERUM

slowly adding ammonium sulphate $(NH_4)_2SO_4$ to 60% (W/V), stirring continuously with a magnetic stirrer. This mixture was left to settle for 30 min and the precipitated protein collected by centrifugation at 7000 xG at 4^oC for 15 min, then dialyzed overnight against cold 2.5 mM hepes pH 7.5. (Because of the small yield of precipitated protein from CYMH cultures, the $(NH_4)_2SO_4$-precipitated protein was dissolved to a final volume of 2.5 cm^3 and added directly onto the SEPHADEX G-100 for both filtration and desalting). The dialyzed protein was then concentrated by ultrafiltration through a DIAFLO XM 50,000 MW filter to 2.5 cm^3 and then applied to a 2 x 40 cm SEPHADEX G-100 column, eluted using 2.5 mM hepes pH 7.5 and fractions collected using an LKB Ultrorac 7000. $OD_{280\ nm}$ was monitored using an ISCO Optical Unit (Type 6) connected to a UA-5 Absorbance monitor. Those fractions exhibiting activity were pooled together and concentrated to 10.0 $mg.cm^{-3}$ by ultrafiltration using an XM 50,000 MW filter and 10.0 μl applied to native polyacrylamide gels. The remaining protein was used either to make a specific antiserum or stored at -20^oC. A flow diagram depicting the isolation procedure is shown in Fig. 5.

4.10 Protein Determinations

All protein determinations were made either at OD_{280nm} using a PYE UNICAM SP1800 Spectrophotometer or by using the protein-dye binding method of Bradford (1976).

4.11 Sterilization

All glassware, except that used for analytical experiments, was sterilized by steam at 121^oC (103.4 kPa) for 15 min.

All analytical glassware was sterilized by dry-heat for a minimum of 160°C for 2.0 hr.

All heat-labile reagents were sterilized by filtration through a 0.2 μm filter.

CHAPTER 5: RESULTS

5.1 Survey of PFLA in selected New Zealand Thermal Pools

5.1.1 The Distribution of PFLA in New Zealand Thermal Pools during the 1976 Survey

The results in Table VI show the distribution of PFLA amongst the pools sampled from the three Health Districts. Appendix I gives a more detailed summary of results from each sample site. Table VI shows that *N. fowleri* was isolated from 60.0% of the pools sampled whilst pathogenic *Acanthamoeba* spp. were isolated from 40.0% of them. Of the combined total samples taken in 1976, the isolation rate for *N. fowleri* was 23.0% compared to 5.7% for pathogenic *Acanthamoeba* spp.

Table VI. Distribution and Incidence of PFLA from New Zealand Thermal Pools during the 1976 Survey

Health District	Thermal Pool	No. of Samples	Temperature °C	pH	*N. fowleri*	Pathogenic *Acanthamoeba* spp.
Hamilton	Waignaro	7	46	9.6	-	-
	Moana-iti	10	39	7.1	3	-
	Opal	10	39	7.1	3	2
	Okoroire	10	42	6.9	2	1
Rotorua	Soda	9	38	6.0	1	-
	Butcher's	8	40	7.5	-	-
	Mouth of Otumaheke	8	35	7.7	6	-
	Upper Otumaheke	8	18	7.5	5	1
Gisborne	Te Puia	9	28	7.2	-	-
	Morere	9	31	6.7	-	1

- = no isolation

The physical and chemical characteristics of each pool are given in Tables II, III, IV, and Appendix I. PFLA were isolated from a pH range of 6.0 (5.8) - 7.7 (9.2), a water temperature range of 13 (13) - 45 (46) ^{o}C and an ambient temperature range of -1.4 (-2.4) - 23.6 (26.4) ^{o}C. The maxima and minima of both pH and temperature recorded from the samples are given in brackets.

No *N. fowleri* were isolated from Gisborne pools (Te Puia and Morere), Waignaro or Butcher's Pool whilst only 1 isolation was recorded from the Soda Pool. In addition, no or few pathogenic *Acanthamoeba* spp. were isolated from these pools or from Moana-iti. The Gisborne pools were characterized by a very high halogen ion content ($>$ 8000 g. m^{-3}) (Table IV), Waignaro with a high alkaline pH ($>$ pH 9.0) (Table II), and Butcher's with a high iron content (Table III). In general then, most isolations of PFLA came from the Matamata-Taupo region. Fig 6. gives the estimated New Zealand ambient temperature whilst Figs. 7 and 8, the normal monthly rainfalls of the Health Districts sampled. Ambient temperatures of below 10°C were recorded from mid-April to mid-September and this also coincided with the period of highest rainfall. Table VII demonstrates that PFLA can be isolated from New Zealand thermal pools in any calendar month.

Figure 6. Graph showing the average monthly temperature for New Zealand. (From the New Zealand Meterological Office.)

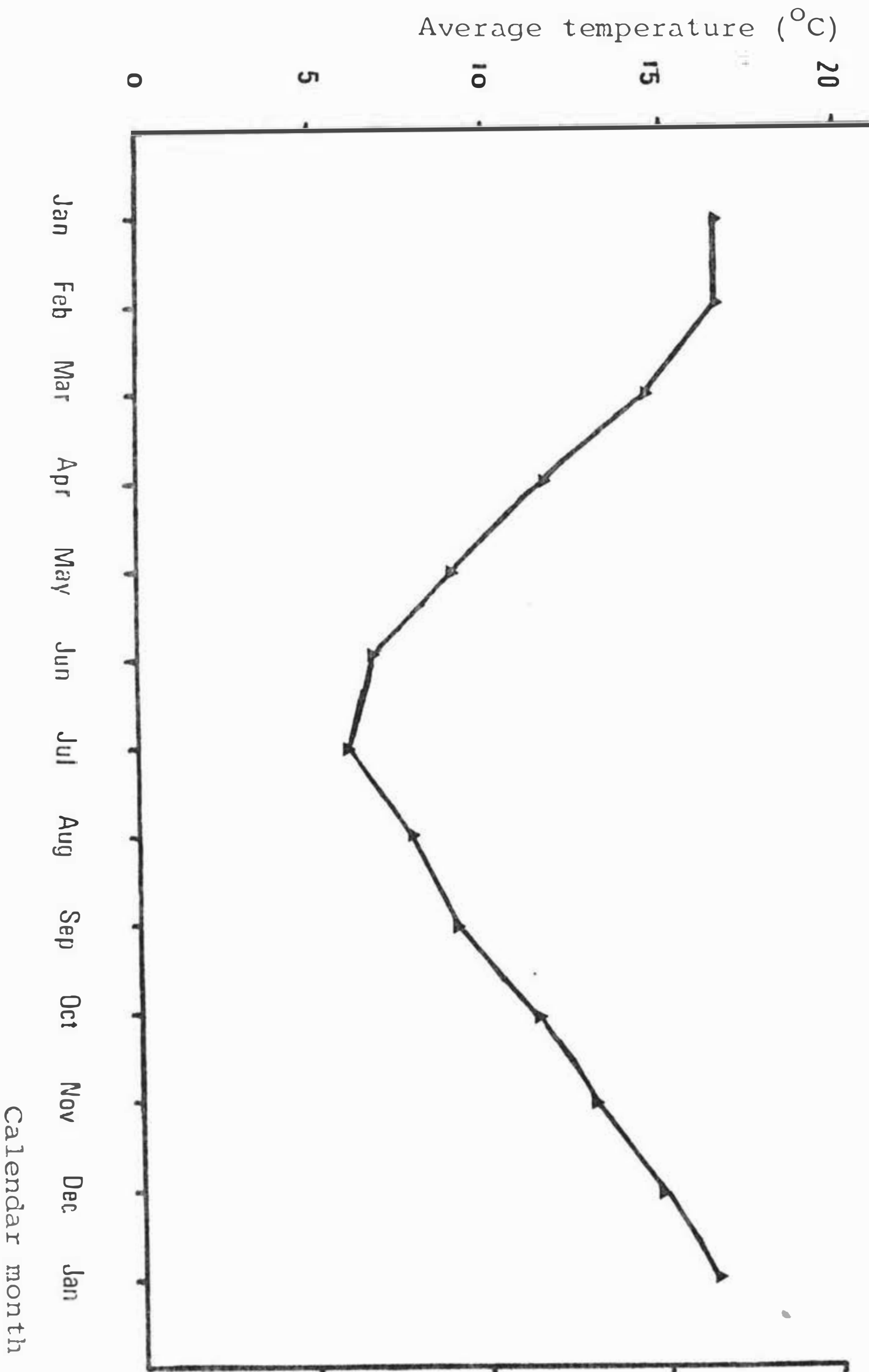

Average temperature (°C)
0
5
10
15
20
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Calendar month

Figure 7. Graph showing the average monthly rainfall for the Matamata and Taupo regions. (From the New Zealand Meterological Office).

▲ = Taupo region

■ = Matamata region

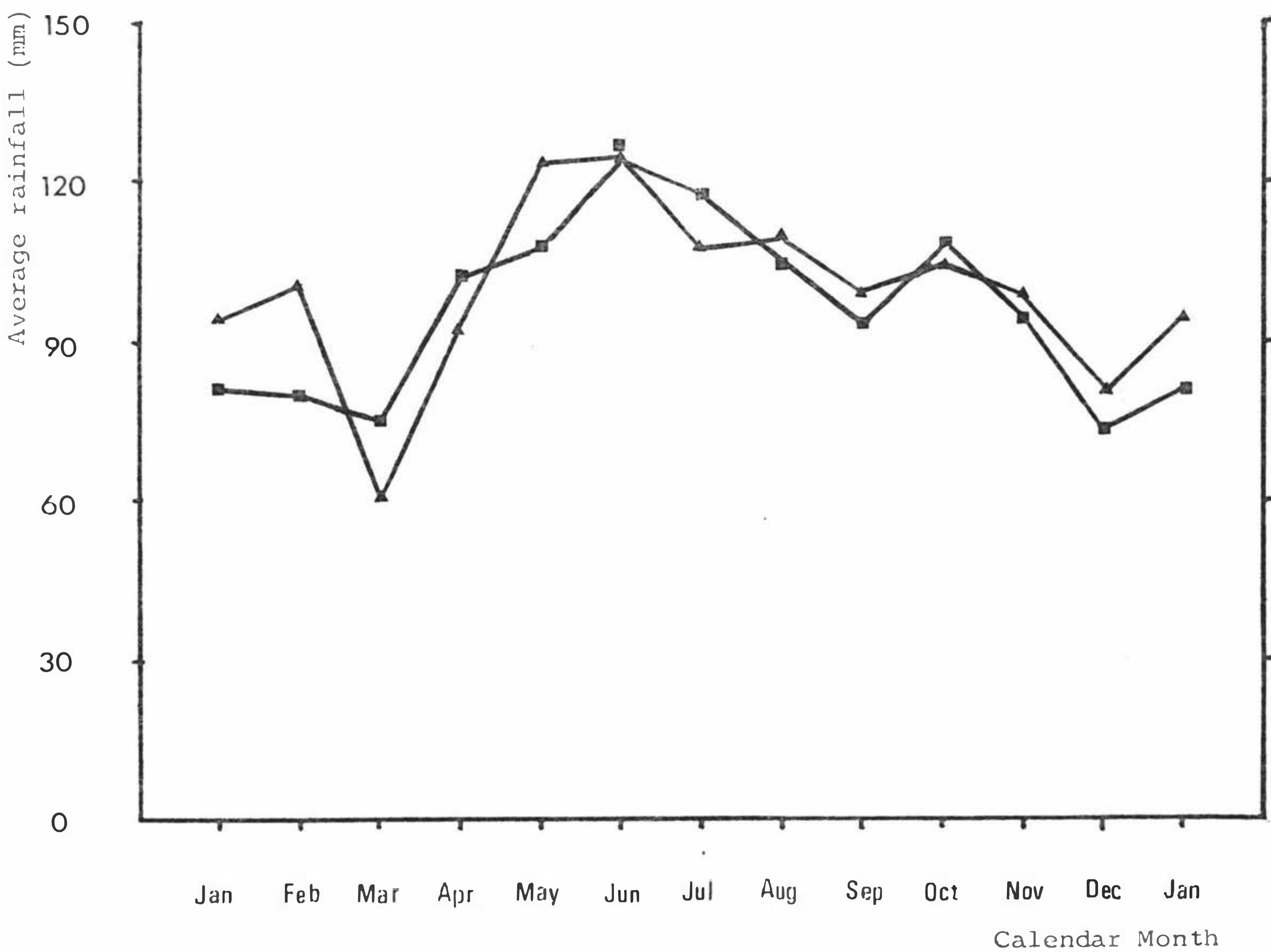

Average rainfall (mm)
150
120
90
60
30
0
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Calendar Month

Figure 8. Graph showing the average monthly rainfall for the Rotorua and Gisborne regions. (From the New Zealand Meterological Office).

▲ = Rotorua region

■ = Gisborne region

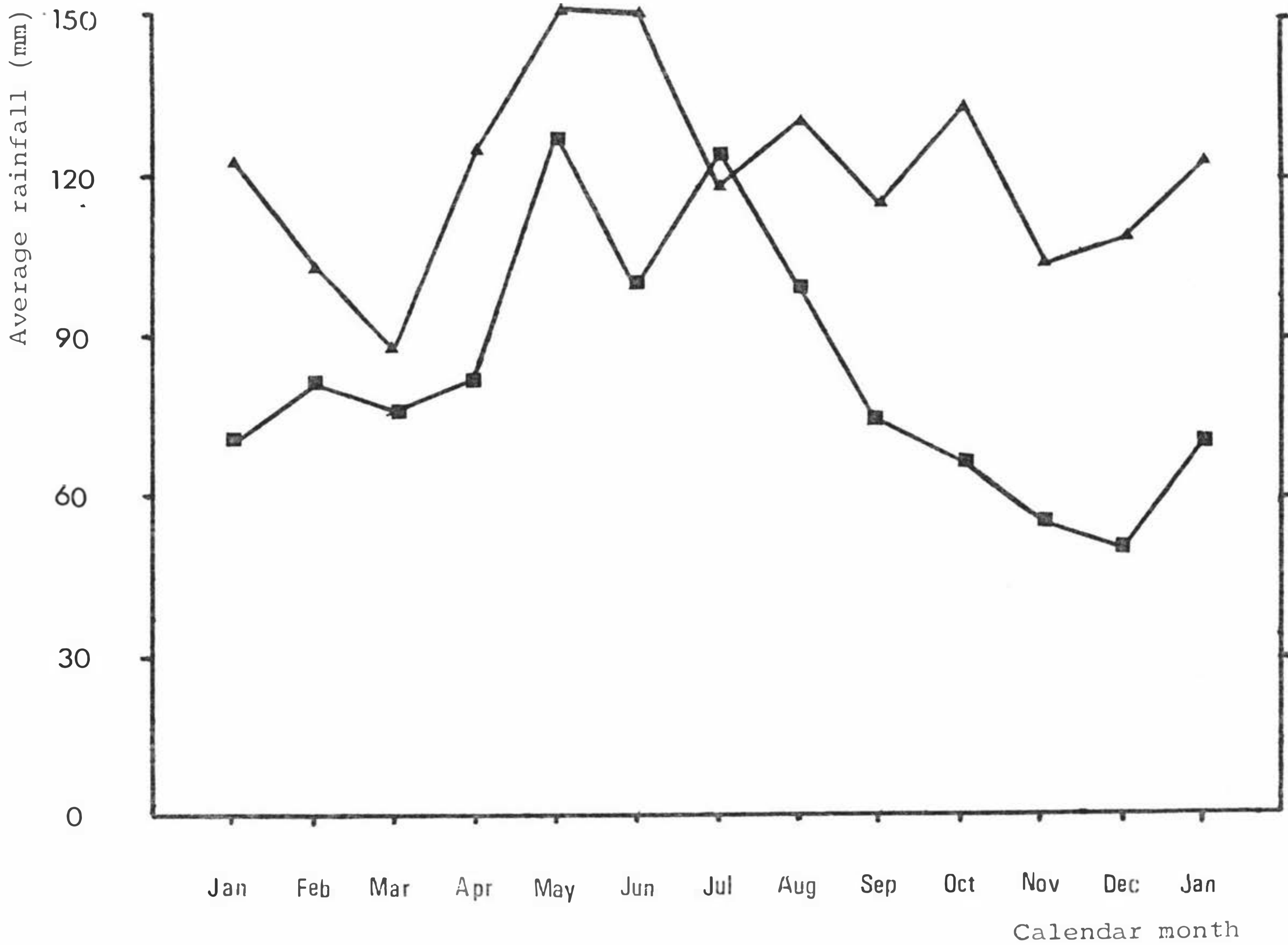

Average rainfall (mm)
150
120
90
60
30
0
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Calendar month

Table VII. Monthly Isolations of PFLA from New Zealand Thermal Pools during the 1976 Survey

Month	No. of Samples	*N. fowleri*	Pathogenic *Acanthamoeba* spp.
Jan	4	2	-
Feb	5	2	1
Mar	4	3	-
Apr	NT	NT	NT
May	6	3	2
June	12	3	-
July	12	3	-
Aug	6	3	-
Sep	12	1	1
Oct	9	3	-
Nov	10	-	1
Dec	8	1	-
Total	88	20	5

\- = no isolation

NT = not taken

However, no correlation between occurrence and the climatic changes could be found (temperature variation in thermal pools is considerably less than that of ambient temperature). Finally Table VIII shows the bacteriological quality of the waters sampled. With the exception of the Butcher's pool, all waters contained bacteria, including coliforms.

Table VIII. The Range of Total Coliform and Bacterial Counts from New Zealand Thermal Pools during the 1976 Survey

Health District	Thermal Pool	No. of Samples	Total Coliforms $.100\ cm^{-3}$	Total Bacteria $.1.0\ cm^{-3}$ $37^{o}C$
Hamilton	Waignaro	1	22	6
	Moana-iti	1	13	80
	Opal	1	35	UC
	Okoroire	1	17	300
Rotorua	Soda	3	35 - 280	NT
	Butcher's	1	nil	NT
	Mouth of Otumaheke	1	870	NT
	Upper Otumaheke	1	12	NT
Gisborne	Te Puia	6	nil - 50	$1x10^{2}$ - $2x10^{4}$
	Morere	5	3 - 7.3	38 - UC

NT = not taken

UC = uncountable($> 10^{7}$ organisms $.cm^{-3}$)

5.1.2 The Occurrence of PFLA in New Zealand Thermal Pools during the 1977 Survey

The results in Table IX and Appendix II show the occurrence of PFLA isolated during the 1977 survey. N. fowleri was isolated from 75% of the pools sampled and 83.3% of soil localities sampled, whereas pathogenic Acanthamoeba spp. were isolated from 50% of the pools and soil localities sampled. Of the combined total samples taken, N. fowleri had an isolation rate of 12.8% from the pools and 16.0% of soils. This compares with 5.3% and 2.3% from the pools and soils respectively for pathogenic Acanthamoeba spp.

Table IX. The Incidence of PFLA in New Zealand Thermal Pools during the 1977 Survey

Health District	Sample Site	No. of Samples	*N. fowleri*	Pathogenic *Acanthamoeba* spp.
Hamilton	Moana-iti Pool	19	-	-
	Moana-iti Soil	17	-	-
	Opal Pools	20	2	-
	Opal Soil	19	5	1
	Okoroire Pools	20	4	2
	Okoroire Soil	20	3	-
Rotorua	Otumaheke Pool (1/3)	35	6	3
	Otumaheke Pool Soil (1/2b)	31	5	-
	Otumaheke Bank Soil (1/2c)	35	4	1
	Otumaheke Whey Site (1/4)	9	4	1

- = no isolation

Again the variability in physical parameters between pools is also apparent (Appendix II) with isolations being made with a pH range of 6.6 - 7.8, a water temperature range of 24.0 - 45.0 °C, an ambient temperature range of -0.9 - 26.5 °C, and a soil temperature range of 8 - 24.4 °C. Table X shows the isolations of PFLA recorded on a monthly basis. Again no obvious seasonal distribution is apparent, with random isolations of PFLA being recorded. However the survey did illustrate that soil can serve as a reservoir of PFLA with a greater number of isolations of

N. fowleri being recorded from the soil than from pool water itself (Table X). The influence of soil on the incidence of PFLA in thermal pools is also reflected in the pools themselves, in that those pools with soil enclosures had a higher incidence of PFLA as compared to the concrete-lined pools.

Table X. Monthly Isolations of PFLA from New Zealand Thermal Pools during the 1977 Survey

Month	No. of Samples		*N. fowleri*		Pathogenic *Acanthamoeba* spp.	
	Pool	Soil	Pool	Soil	Pool	Soil
Jan	NT	NT				
Feb	1	2	-	1	-	-
Mar	10	14	-	7	-	-
Apr	10	14	1	3	-	-
May	14	19	-	2	1	-
June	7	11	2	-	-	-
July	9	13	4	3	-	-
Aug	13	12	2	1	2	-
Sept	10	18	1	2	1	-
Oct	9	13	2	-	1	3
Nov	8	12	-	2	-	-
Dec	3	3	-	-	-	-
Total	94	131	12	21	5	3

NT = not taken
- = no isolation

Table XI shows the bacteriological quality of the waters and soils examined. It can be observed that even the commercially developed concrete-lined pools were contaminated with coliforms, albeit at a lower incidence than the natural pools. All the soils also harboured coliform bacteria. It is interesting to note

that most PFLA were isolated from soils and from those pools with a high coliform count.

Table XI. Bacteriological Quality of Thermal Pools and Surrounding Soils during the 1977 Survey

Health District	Sample Site	Total No. of Samples	No. of Positive Coliform Samples	Range of Total Coliforms $.100\ cm^{-3}$ or $.1.0\ g^{-1}$ soil	Range of Total Bacteria $.1.0\ cm^{-3}$ or $.1.0\ g^{-1}$ soil
Hamilton	Moana-iti Pool	17	6	Nil - $1.61x10^5$	Nil - UC
	Moana-iti Soil	16	16	$4.3x10^3$ - $9.6x10^5$	$1.22x10^4$ - $>10^8$
	Opal Pools	19	5	Nil - $5.4x10^4$	Nil - UC
	Opal Soil	17	17	$1.7x10^3$ - $6.25x10^6$	$5.1x10^5$ - $>10^8$
	Okoroire Pools	19	16	Nil - $1.61x10^5$	$3.06x10^2$ - UC
	Okoroire Soils	18	18	$1.4x10^3$ - $4.63x10^6$	$1.2x10^5$ - $>10^8$
Rotorua	Otumaheke Pool (1/3)	9	9	$2.5x10^2$ - UC	$3.3x10^3$ - UC
	Otumaheke Pool Soil (1/2b)	27	27	$3.92x10^2$ - $1.1x10^6$	$6.02x10^4$ - >108
	Otumaheke Bank Soil (1/2c)	30	30	$3.4x10^3$ - $154x10^6$	$4.6x10^5$ - $>10^8$
	Otumaheke Whey Site (1/4)	9	9	$2.7x10^3$ - $2.1x10^6$	$2.66x10^6$ - $1.8x10^8$

UC = uncountable ($>10^7$ organisms. cm^{-3})

5.2 The Comparative Use of Disinfectants against Free-living Amebae

5.2.1 The Use of Chlorine as a Disinfectant against Free-living Amebae

The amebicidal capacity of chlorine is shown in Table XII. Because initially the deionized water exerts negligible chlorine demand, the initial total available chlorine (TAC) will equal the free available chlorine (FAC). It can be seen that *Naegleria* spp. were more sensitive to chlorine than the *Acanthamoeba* spp. with 0.79 $mg.l^{-1}$ of TAC being amebicidal for *Naegleria* spp. as opposed to 1.25 $mg.l^{-1}$ of TAC for *Acanthamoebae*. The difference, (TAC - FAC), known as combined available chlorine (CAC), represents the chlorine demand which is due to the organic content of the inoculum reacting with the chlorine during the 30 min contact interval.

Table XII. The Amebicidal Capacity of Chlorine

Species (Strain)	pH	Tp °C	Inoculum of Amebae $x10^4$ $.cm^{-3}$	Chlorine Levels $mg.l^{-1}$ TAC	FAC	CAC	Survivors No. of Amebae $. cm^{-3}$	%
N. gruberi (P1200f)	7.0	25	1.92	0.79	0.16	0.630	0	0
				0.675	0.125	0.50	1	0.005
				0.625	0.125	0.50	10	0.052
				0.625	0.125	0.50	15	0.078
				0.55	0.105	0.45	26	0.14
N. fowleri (MsT)	7.0	25	1.92	0.925	0.275	0.650	0	0
				0.750	0.175	0.575	0	0
				0.74	0.19	0.55	0	0
				0.625	0.125	0.50	7	0.036
				0.575	0.125	0.55	18	0.093
A. castellanii (1501)	7.0	25	1.92	1.10	0.25	0.85	0	0
				1.02	0.22	0.80	0	0
				1.0	0.25	0.75	2	0.01
				0.85	0.20	0.65	10	0.052
				0.80	0.175	0.625	17	0.089
A. culbertsoni (A-1)	7.0	25	1.92	1.25	0.25	1.0	0	0
				1.25	0.25	1.0	0	0
				1.09	0.14	0.95	23	0.12
				0.95	0.20	0.75	18	0.093

TAC = total available chlorine
FAC = free available chlorine
CAC = combined available chlorine

5.2.2 The Use of Alternative Disinfectants to Chlorine against Free-living Amebae

Table XIII shows the comparative amebicidal capacity of chlorine dioxide, ozone and deciquam 222. Whereas *Naegleria* spp. were once again more sensitive to the chlorine-containing disinfectant as compared to *Acanthamoebae* spp., no marked difference in the sensitivity amongst the four strains (MsT, P1200f, A-1 and 1501) towards ozone or deciquam 222 could be detected. Of the 3 alternative disinfectants examined, deciquam 222 exhibited the greatest amebicidal capacity followed by chlorine dioxide and ozone.

Table XIII. The Comparative Amebicidal Capacity of Chlorine dioxide, Ozone and Deciquam 222

Species (Strain)	pH	Tp ^oC	Inoculum of amebae $x10^4 . cm^{-3}$	ClO_2 $mg.l^{-1}$			O_3 $mg.l^{-1}$			Deciquam 222 $cm^3.l^{-1}$	
				Initial	Final	Survivors	Initial	Final	Survivors	Initial	Survivors
N. gruberi (P1200f)	7.0	25	1.92	1.1	0.25	40	6.75	0.08	20	0.05	-
				1.1	0.25	30				0.025	4
				1.0	0.25	40					
N. fowleri (MsT)	7.0	25	1.92	2.0	0.5	8	6.75	0.075	20	0.05	-
				1.6	0.35	15				0.025	2
				1.3	0.25	20					
A. castellanii (1501)	7.0	25	1.92	3.4	0.75	-	6.75	0.078	20	0.05	-
				2.9	0.65	1				0.025	6
				2.6	0.6	3					
A. culbertsoni (A-1)	7.0	25	1.92	3.4	0.75	-	6.75	0.08	20	0.05	-
				2.5	0.6	1				0.025	2

5.3 Immunity to PFLA

5.3.1 The Presence of Antibodies to PFLA in Human Sera

Random samples of fresh human sera were obtained from the Palmerston North, Hamilton and Rotorua districts. The samples from the Palmerston North area served as a 'control' batch since no thermal pools are located there. The presence of antibodies to both pathogenic and non-pathogenic free-living amebae was determined by the indirect fluorescent antibody tests (IFAB) the results of which are presented in Tables XIV, XV & XVI and Plates 21-24.

Table XIV. Presence of Antibodies to PFLA in Human Sera from the Palmerston North Health District

Serum No.	Type	Sex	Blood Group	Antigens/Titre of Human Sera			
				N. fowleri (MsT)	*N. gruberi* (P1200f)	*A. culbertsoni* (A-1)	*A. castellanii* (1501)
A_1	Mother		O neg	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = I-II
A_2	Cord		O pos	1/5 = I-II	1/10 = I-II	1/5 = II-III	1/10 = I-II
B_1	Mother		O pos	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = I-II
B_2	Cord		B pos	1/5 = I-II	1/10 = II-III	1/5 = III-IV	1/10 = II-I
C_1	Mother		A neg	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = I-II
C_2	Cord		O pos	1/5 = II-III	1/10 = I-II	1/5 = II-III	1/10 = I-II
D_1	Mother		O neg	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = I-II
D_2	Cord		O neg	1/5 = II-III	1/10 = I-II	1/5 = II-III	1/10 = II-III
E_1	Mother		A pos	1/10 = II-III	1/20 = I-II	1/20 = III-IV	1/40 = II-III
E_2	Cord		A pos	1/5 = I-II	1/10 = I-II	1/5 = II-III	1/10 = I-II
F_1	Mother		A pos	1/10 = I-II	1/20 = II-III	1/20 = III-IV	1/40 = II-III
F_2	Cord		O pos	1/5 = I-II	1/10 = II-III	1/5 = III-IV	1/10 = II-III
G_1	Mother		O neg	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = I-II
G_2	Cord		O pos	1/5 = I-II	1/10 = I-II	1/5 = II-III	1/10 = I-II

... cont'd

H_1 Mother H_2 Cord		A neg A pos	1/10 = I-II 1/5 = I-II	1/20 = I-II 1/10 = I-II	1/20 = II-III 1/5 = II-III	1/40 = I-II 1/10 = I-II
I	M	A pos	1/10 = II-III	1/20 = II-III	1/20 = III-IV	1/40 = II-III
J	M	A pos	1/10 = II-III	1/20 = II-III	1/20 = IV	1/10 = III-IV
K	M	B pos	1/10 = I	1/20 = I	1/20 = II-III	1/40 = I-II
L	F	N.G.	1/10 = II-III	1/20 = II-III	1/20 = II-III	1/40 = II-III
M	M	"	1/10 = II-III	1/20 = II-III	1/20 = II-III	1/40 = II-III
N	F	"	1/10 = I	1/20 = II-III	1/20 = II-III	1/40 = II-III
O	F	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = I-II
P	F	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = I-II
Q	M	AB	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
R	M	N.G.	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
S	M	"	1/10 = I	1/20 = II-III	1/20 = III-IV	1/80 = II-III
T	F	"	1/10 = II-III	1/20 = II-III	1/20 = II-III	1/40 = II-III
U	M	"	1/10 = I	1/20 = I-II	1/20 = II-III	1/40 = II-III
V	F	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III

IV = very high intensity of fluorescence
III = high intensity of fluorescence
II = low intensity of fluorescence
I = very low intensity of fluorescence

N.G. = not given

Table XV. Presence of Antibodies to PFLA in Human Sera from the Hamilton Health District

Serum No.	Type	Sex	Blood Group	Antigens/Titre of Human Sera			
				N. fowleri (MsT)	N. gruberi (P1200f)	A. culbertsoni (A-1)	A. castellanii (1501)
47	N.G.	N.G.	N.G.	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
48	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
49	"	"	"	1/10 = II-III	1/20 = I-II	1/20 = III-IV	1/40 = III-IV
50	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
51	"	"	"	1/10 = I	1/20 = I-II	1/20 = II-III	1/40 = II-III
52	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
53	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
54	"	"	"	1/10 = I	1/20 = I-II	1/20 = II-III	1/40 = II-III
55	"	"	"	1/10 = I	1/20 = I-II	1/20 = II-III	1/40 = II-III
56	"	"	"	1/10 = I	1/20 = I-II	1/20 = II-III	1/40 = II-III
57	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
58	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
59	"	"	"	1/10 = I-II	1/20 = II-III	1/20 = II-III	1/40 = II-III
60	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
61	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
62	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = III-IV	1/40 = III-IV

... cont'd

63	N.G.	N.G.	N.G.	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
64	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
65	"	"	"	1/10 = I	1/20 = I-II	1/20 = II-III	1/40 = II-III
66	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
67	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
68	"	"	"	1/10 = I	1/20 = I-II	1/20 = II-III	1/40 = II-III
69	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
70	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
71	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
72	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
73	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
74	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
75	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = III-IV	1/40 = III-IV
76	"	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III

IV = very high intensity of fluorescence
III = high intensity of fluorescence
II = low intensity of fluorescence
I = very low intensity of fluorescence

N. G. = not given

Table XVI. Presence of Antibodies to PFLA in Human Sera from the Rotorua Health District

Serum No.	Type	Sex	Blood Group	Antigens/Titre of Human Sera			
				N. fowleri (MsT)	*N. gruberi* (P1200f)	*A. culbertsoni* (A-1)	*A. castellanii* (1501)
194		M	N.G.	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = I-II
220		M	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
242		F	"	1/10 = I-II	1/20 = I-II	1/20 = I-II	1/40 = I-II
243		F	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = I-II
261		F	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
272		M	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
274		F	"	1/10 = I	1/20 = I-II	1/20 = I-II	1/40 = I-II
279		F	"	1/10 = I-II	1/20 = II-III	1/20 = I-II	1/40 = II-III
291		M	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
699		M	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
695		M	"	1/10 = I-II	1/20 = I-II	1/20 = I-II	1/40 = II
692		M	"	1/10 = I-II	1/20 = I-II	1/20 = I-II	1/40 = II-III
690		M	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = I-II
729		F	"	1/10 = I-II	1/20 = II-III	1/20 = II-III	1/40 = III-IV
781		F	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
790		M	"	1/10 = I	1/20 = I-II	1/20 = II-III	1/40 = II-III

... cont'd

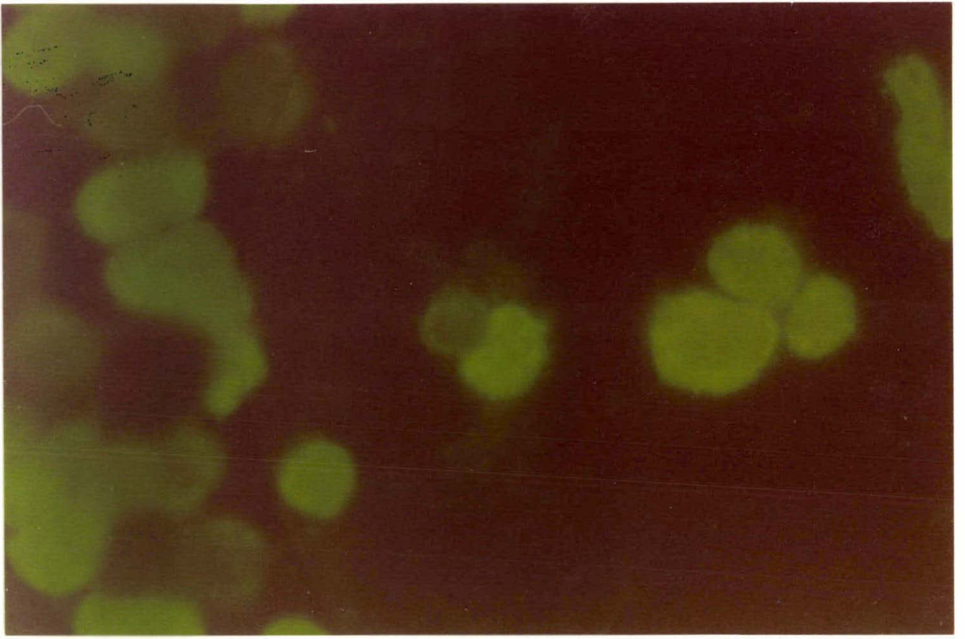
798	M	N.G.	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = I-II
799	M	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
844	F	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = III-IV
848	M	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
862	M	"	1/10 = I-II	1/20 = I-II	1/20 = I-II	1/40 = III-IV
875	M	"	1/10 = I-II	1/20 = I-II	1/20 = III-IV	1/40 = III-IV
878	M	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = I-II
879	F	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
886	N.G.	"	1/10 = II-III	1/20 = I-II	1/20 = II-III	1/40 = I-II
889	"	"	1/10 = I	1/20 = I-II	1/20 = II-III	1/40 = II-III
890	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
897	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III
921	"	"	1/10 = I	1/20 = I	1/20 = II-III	1/40 = III-IV
946	"	"	1/10 = I-II	1/20 = I-II	1/20 = II-III	1/40 = II-III

IV = very high intensity of fluorescence
III = high intensity of fluorescence
II = low intensity of fluroescence
I = very low intensity of fluorescence

N.G. = not given

Plate 21. Photomicrograph of *N. fowleri* trophozoites stained by IFAB using anti-human serum labelled with fluorescein isothiocyanate (FITC). Mag. *ca* x 1000.

Plate 22. Photomicrograph of *N. gruberi* trophozoites stained by IFAB using anti-human serum labelled with FITC. Mag. *ca* x 1200.



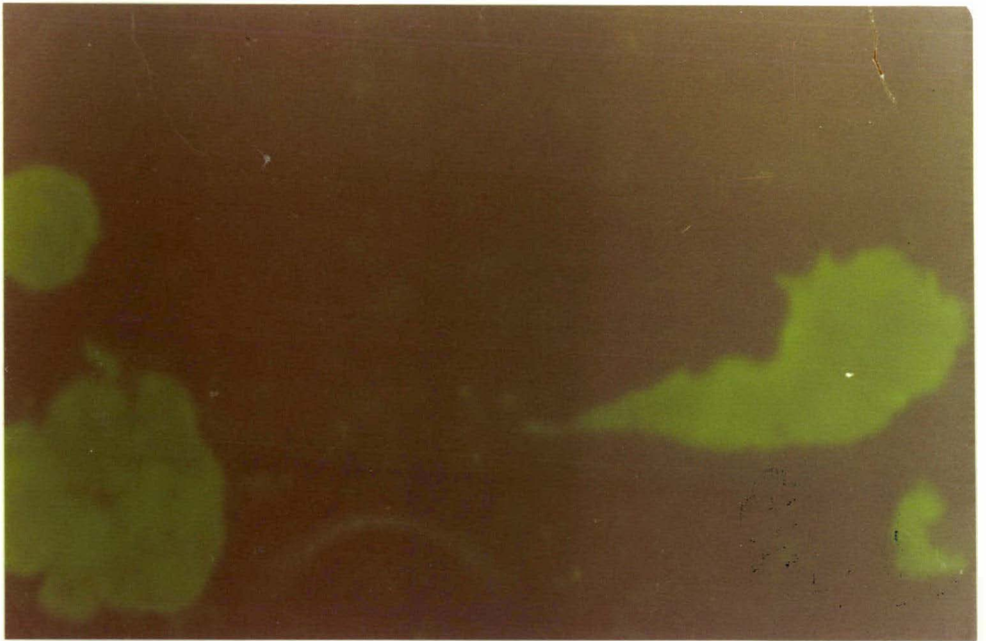
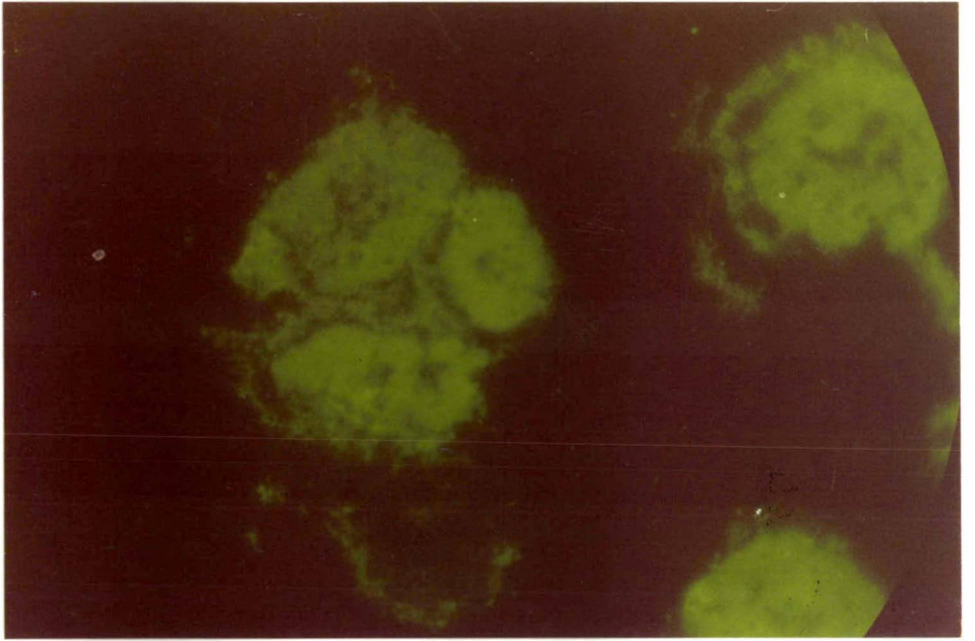
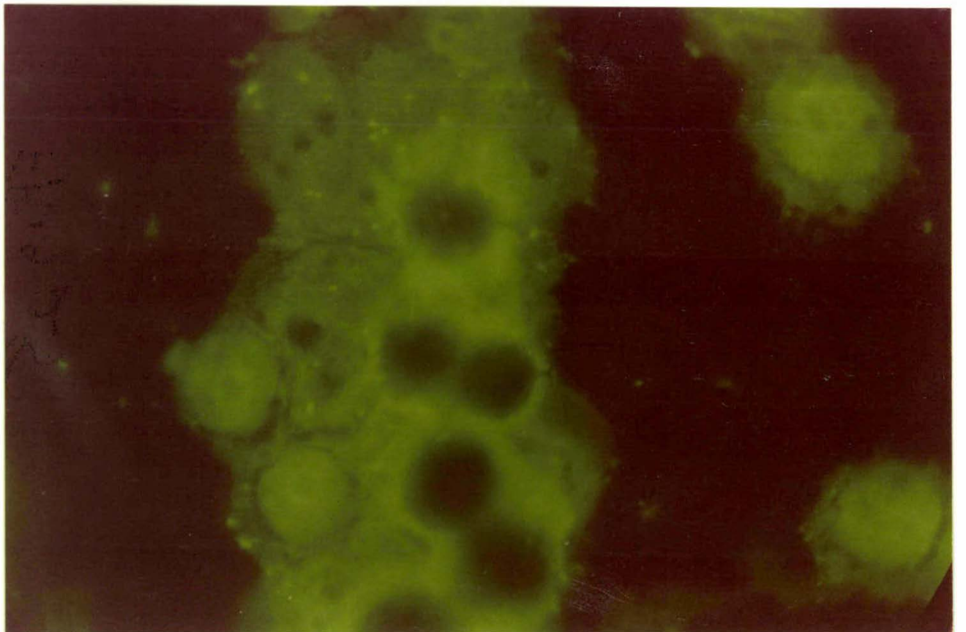


Plate 23. Photomicrograph of *A. culbertsoni* trophozoites stained by IFAB using anti-human serum labelled with FITC. Mag. *ca* x 1200.

Plate 24. Photomicrograph of *A. castellanii* trophozoites stained by IFAB using anti-human serum labelled with FITC. Mag. *ca* x 1000.





Antibodies to PFLA were found in all 93 serum samples tested with titres ranging from 1/5 - 1/20 for *Naegleria* spp. and from 1/20 - 1/80 for *Acanthamoeba* spp. No difference could be detected between different blood groups and sexes, though cord sera displayed a lower titre than maternal sera. Using class-specific labelled anti-immunoglobulins, it was shown (Table XVII) that the antibodies belonged primarily to the immunoglobulin classes IgG and IgM. The observation of antibodies belonging to the IgG class is not surprising in view that it replaces the initially formed IgM antibodies and has the longest half-life of the immunoglobulin classes. However the finding of antibodies to the IgM class with a titre which closely parallels the titre of IgG antibodies, suggests recent contact with PFLA.

Table XVII. Presence of Class-specific Antibodies to PFLA in Human Sera

Adult Human Serum	Titre	Antigen	anti-human IgG, IgM, IgA	anti-human IgG	anti-human IgM	anti-human IgA
952	1/10	*N. fowleri* (MsT)	I	II	I-II	-
	1/20	*N. gruberi* (P1200f)	I-II	II	II	-
	1/40	*A. castellanii* (1501)	II	I-II	II	I
	1/80	*A. culbertsoni* (A-1)	I	I-II	I-II	I
956	1/10	*N. fowleri* (MsT)	I	I-II	I-II	-
	1/20	*N. gruberi* (P1200f)	II	II-III	II	-
	1/40	*A. castellanii* (1501)	I	I	I	I
	1/80	*A. culbertsoni* (A-1)	I	II	II	I
960	1/10	*N. fowleri* (MsT)	II	II	II	-
	1/20	*N. gruberi* (P1200f)	II	II-III	I-II	-
	1/40	*A. castellanii* (1501)	II	II-III	I-II	I
	1/80	*A. culbertsoni* (A-1)	II	II-III	I-II	I

III = high intensity of fluorescence

II = low intensity of fluorescence

I = very low intensity of fluorescence

5.3.2 The Presence of a Specific Neutralizing Factor against PFLA in Normal Human Sera

Table XVIII shows the specificity of the antibodies as judged by *in vitro* neutralization tests using cell cultures as an indicator of pathogenicity. No neutralization was obtained with either unheated, complement inactivated hyperimmune-rabbit, or human sera for *N. fowleri*. On the other hand, fresh unheated adult as opposed to complement inactivated adult, or cord sera, neutralized *A. culbertsoni* at a titre of 1/10 - 1/20. Furthermore, the addition of fresh guinea-pig complement did not affect the results, nor the use of hyperimmune rabbit anti-*A. culbertsoni*-serum with a specific IFAB titre of 1/1000, though some delay in the formation of cytopathic effects (CPE) with the homologous serum was observed (Table XVIII).

Table XVIII. Use of Hyperimmune Rabbit and Normal Human Sera in Neutralizing N. fowleri (MsT) and A. culbertsoni (A-1) in Vero Cell Culture using an inoculum of 1.0×10^4 cells cm^{-3}

Antigen	Antiserum treatment	Titre	IFAB Titre MsT	DAY 1	2	3	4	5	6	7
MsT	Control (no antiserum)			-	II	IV				
	Rabbit-MsT heated 56°C/30 mins	1/10	1/500 = III	-	II	IV				
	Rabbit-MsT heated 56°C/30 mins + G.P.C.'	1/10		-	II	IV				
	Rabbit-MsT unheated	1/10		-	II	IV				
	Human pooled unheated	1/10	1/10 = II	-	II	IV				
A-1			A-1							
	Control (no antiserum)			-	II	IV				
	Rabbit-A-1 heated 56°C/30 mins	1/10	1/500 = III	-	-	I	I	II	II	III
		1/20		-	-	II	II	III	III	IV
		1/40		-	II	IV				
	Rabbit-A-1 heated 56°C/30 mins + G.P.C.'	1/10	1/500 = III	-	-	I	I	II	III	IV
		1/20		-	I	II	II	III	IV	
		1/40		-	II	IV				

... Cont'd

	Rabbit-A-1 unheated	1/10	1/500 = III	- - - I II II III
		1/20		- - I I II III III
		1/40		- I III IV
	Rabbit-1501 heated 56°C/30 mins	1/10	1/500 = I	I II IV
	Rabbit-1501 heated 56°C/30 mins + G.P.C.'	1/10	1/500 = I	I II IV
	Rabbit-1501 unheated	1/10	1/500 = I	I II III IV
		1/20		I II IV
	A_1 Human heated 56°C/30 mins	1/10	1/20 = II	- II IV
	A_2 Human heated 56°C/30 mins	1/10	1/5 = II	- II IV
	A_1 Human unheated	1/10	1/20 = II	- - - - - II IV
A-1		1/20		- - - - II IV
		1/40		- II III IV
	A_2 Human unheated	1/10	1/5 = II	- II IV
	B_1 Human heated 56°C/30 mins	1/10	1/20 = II	- II IV
	B_2 Human heated 56°C/30 mins	1/10	1/5 = III	- II IV
	B_1 Human unheated	1/10	1/20 = II	- - - - - - -
		1/20		- - - - - - II
		1/40		- I III IV
	B_2 Human unheated	1/10	1/5 = II	- - III

... cont'd

	Pooled Human unheated	1/10	. 1/20 = II	-	-	-	-	-	-	-
		1/20		-	-	-	-	-	-	-
		1/40		-	-	-	II	IV		
		1/80		-	I	II	IV			
	Pooled Human heated 56°C/30 mins	1/10	1/20 = II	-	II	IV				
A-1	Pooled Human unheated	1/10	1/20 = III	-	-	-	-	-	-	-
		1/20		-	-	-	-	-	-	-
		1/40		-	-	-	-	-	-	II
		1/80		-	I	II	III	IV		
	Pooled Human heated 56°C/30 mins	1/10	1/20 = III	-	II	IV				
	Pooled Human heated 56°C/30 mins + G.P.C.'	1/10	1/20 = III	-	II	IV				

IV = very high intensity of fluorescence

III = high intensity of fluorescence

II = low intensity of fluorescence

I = very low intensity of fluorescence

I = early CPE; CPE = rounding of Vero cells, degeneration and refractility and finally loss of the monolayer.

II = pronounced CPE

III = very pronounced CPE

IV = total degeneration of monolayer

G.P.C.' = guinea-pig complement

5.3.3 The Reaction of the Cell-Mediated Immune System to Antigens of *Naegleria* spp.

The cross-reactivity of homologous and heterologous antigens of *Naegleria* spp. with components of the cell-mediated immune (CMI) system, as judged by the *in vivo* delayed hyper-sensitivity (DH) response from intradermal (I/D) skin tests and *in vitro* macrophage inhibition factor (MIF) tests, is demonstrated in Plates 25, 26, 27, 28, 29 & 30 and Tables XIX & XX. Table XIX and plates 25, 26, 27 & 28 show that guinea-pigs sensitized against *Naegleria* spp. elicit a typical DH reaction.

Table XIX. Cross-reactivity of Homologous and Heterologous Antigens of Naegleria spp. as judged by Delayed Hypersensitivity

Sensitizing Antigen	Test-Antigen	Diameter of D.H. Nodule after 48 hr
N. fowleri (MsT)	MsT	9.0 ± 0.1 mm
	P1200f	8.0 ± 0.1 mm
	0400	6.7 ± 0.1 mm
	saline	-
N. jadini (0400)	MsT	7.0 ± 0.1 mm
	P1200f	10.5 ± 0.1 mm
	0400	9.0 ± 0.1 mm
	saline	-
N. gruberi (P1200f)	MsT	7.0 ± 0.1 mm
	P1200f	12.0 ± 0.1 mm
	0400	8.3 ± 0.1 mm
	saline	-

- = no reaction

This is judged by the time taken to form the hard-nodule-lesion and infiltration of mono-nuclear

Plate 25. Photomicrograph of section through skin of guinea-pig immunized with *N. fowleri* and skin-tested with saline. Note lack of infiltration by mononuclear cells. Mag. *ca* x 80.

Plates 26-28. Photomicrographs of sections through skin of guinea-pig immunized with *N. fowleri* and skin-tested with *N. fowleri* (plate 26), *N. gruberi* (plate 27) and *N. jadini* (plate 28). Note infiltration by mononuclear cells (dark nuclei) and accompanying necrosis. Mag. *ca* x 80.

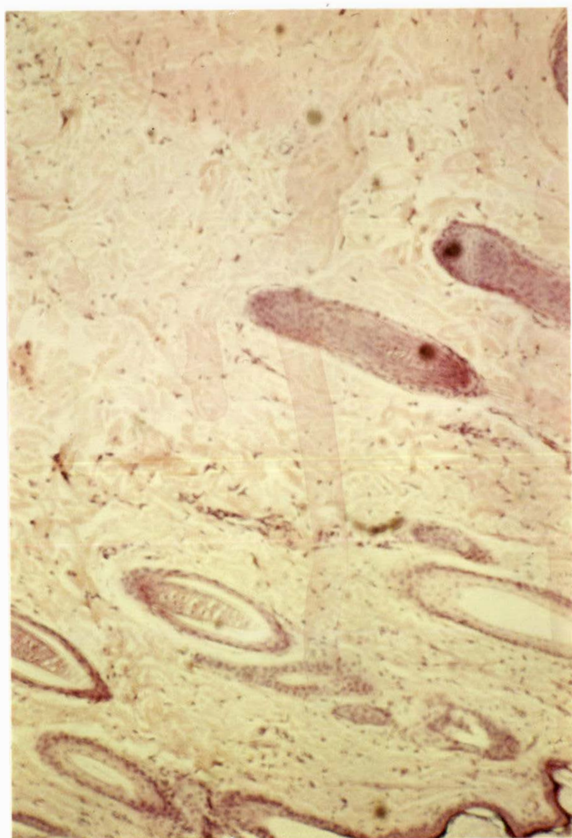


Plate 25

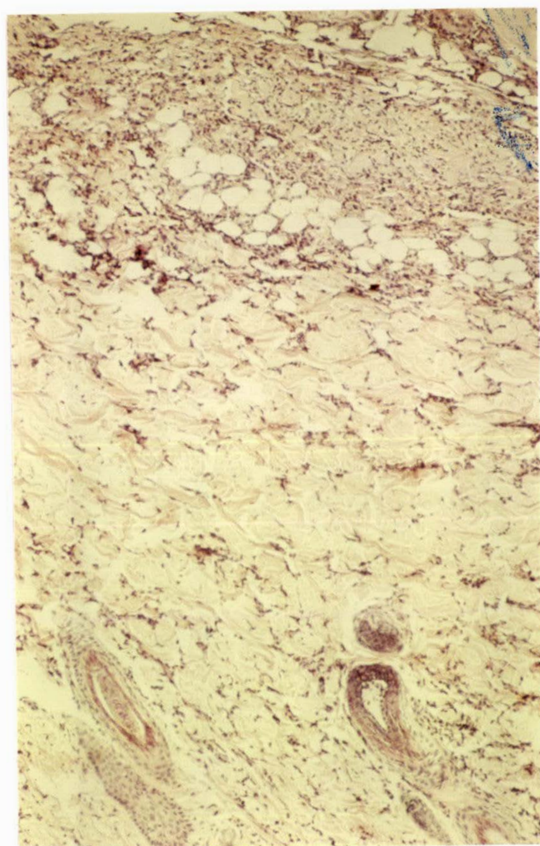


Plate 26

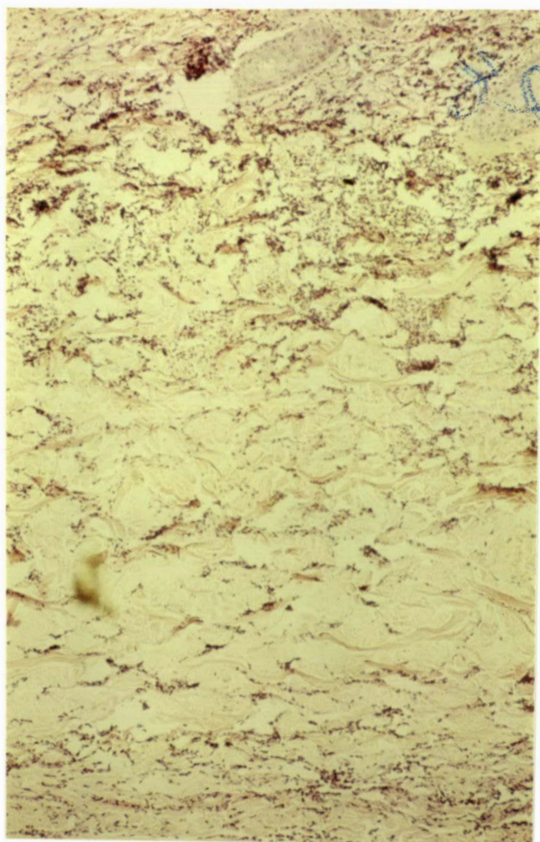


Plate 27

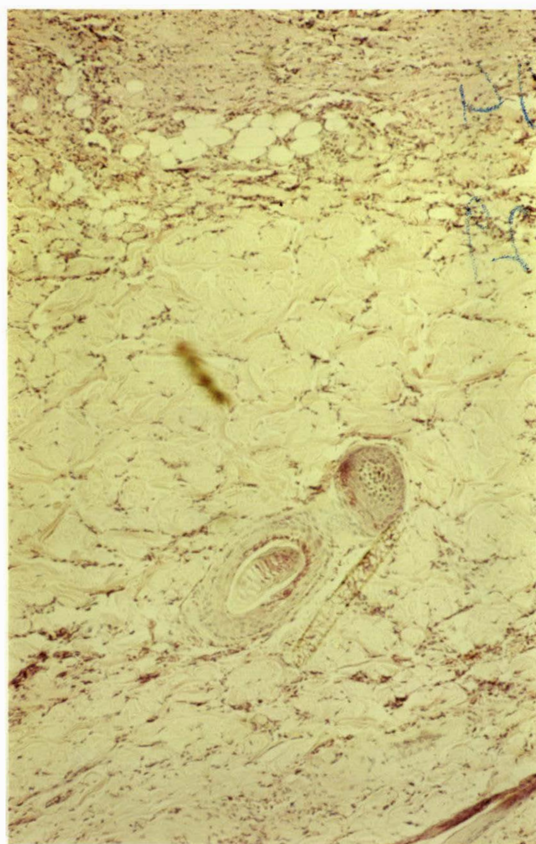
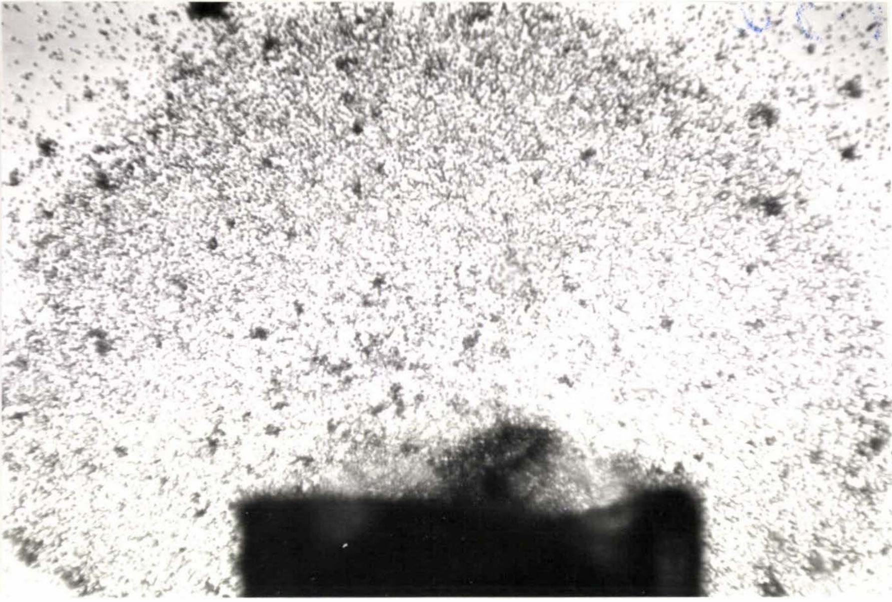
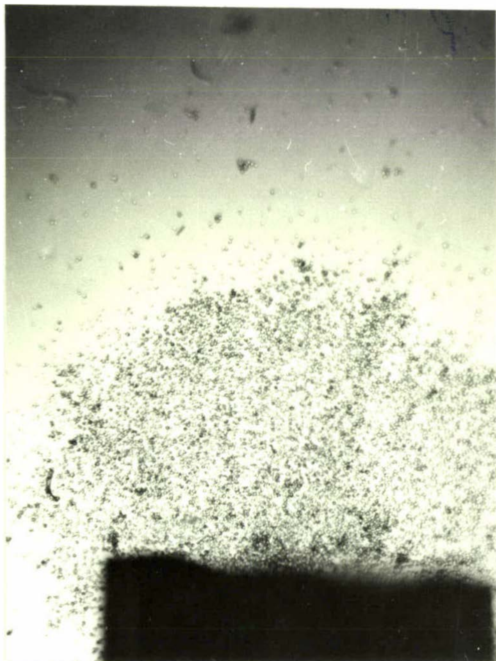


Plate 28

Plate 29. Photomicrograph of migration of peritoneal exudate cells from a capillary tube in which the lymphokine MIF is not present in the culture medium (Control). Mag. _ca_ x 30.

Plate 30. Photomicrograph of the inhibition of migration of peritoneal exudate cells from a capillary tube by the lymphokine MIF which is present in the culture medium. The lymphokine MIF was produced by peripheral lymphocytes that had been isolated from a guinea-pig previously sensitized to _N. gruberi_ (P1200f) when these lymphocytes were cultured in the presence of soluble antigens from _N. gruberi_ (P1200f). Mag. _ca_ x 25.





cells, with either homologous or heterologous antigens. The ability to respond to heterologous antigens is also reflected by the MIF test (plates 29 & 30) though in this case the homologous system is shown to be the most efficient.

Table XX. Cross-reactivity of Homologous and Heterologous Antigens of Naegleria spp. as judged by Inhibition of Macrophages

Sensitizing Antigen	Lymphocyte Stimulating Antigen	% Inhibition of Macrophages
MsT	MsT	52.38%
MsT	P1200f	36.83%
MsT	0400	25.2%
0400	0400	67.62%
0400	MsT	25.64%
P1200f	P1200f	68.36%
Saline	MsT	0
	P1200f	0
	0400	0
Saline	-	0

5.4 Virulence of PFLA

5.4.1 The Presence of Extracellular Enzymes in the Supernatants of Axenic Cultures of Free-living Amebae

By adapting the method of Habermann and Hardt (1972) (see Method 4.91) it was demonstrated that the cell-free filtrates of axenic culture of both pathogenic and non-pathogenic free-living amebae possessed both a phospholipase 2 (phospholipase A) as indicated by the outer clear zone, and a lysophospholipase (phospholipase B) as indicated by an accompanying turbid zone

(Plates 31 & 32). Clearing is due to phospholipase 2 reacting with the phosphatidyl choline (lecithin) present in the egg-yolk to produce the highly lytic intermediate lysolecithin, which exhibits detergent-like activity in solubilizing the lipo-protein mixture, whilst turbidity is due to the production of insoluble fatty acids by lysophospholipase reacting with the lysolecithin (Table XXI).

Table XXI. Products of Enzymatic Hydrolysis of Phosphatidyl Choline by Different Phospholipases

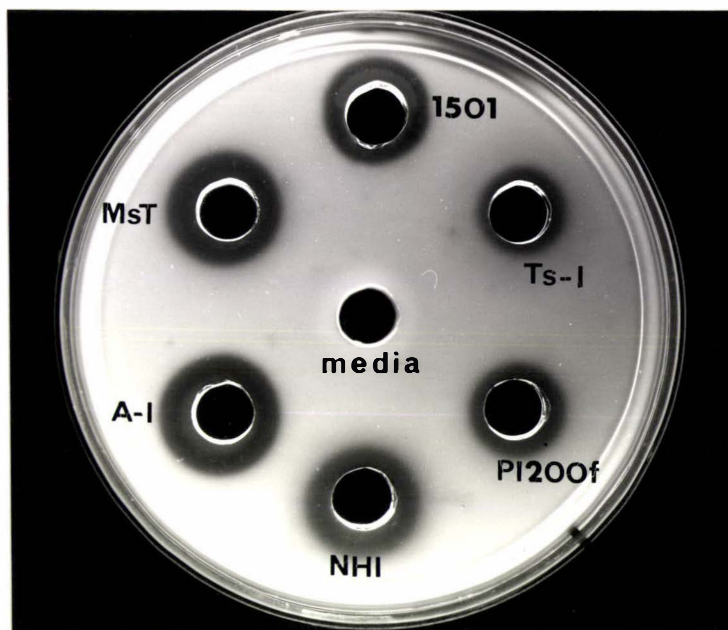
Lipid substrate	Enzyme	Lipid products	Water-soluble-products
Phosphatidyl Choline (lecithin)	Phospholipase 2 (Phospholipase A)	sn-1-Acyl-3-glycerophosphorylcholine + fatty acids	none
	Lysolecithinase (Phospholipase B)	Fatty acids	sn-3-glycerophosphorycholine
	Phospholipase 3 (Phospholipase C)	sn-1, 2-Diglyceride	Phosphorylcholine
	Phospholipase 4 (Phospholipase D)	sn-1, 2-Diacyl-3-glycerophosphate	Choline

Additional evidence comes from extracting the reacted agar zones with a 1:1 mixture of CH_3Cl: CH_3OH for determination of chemical content by thin layer chromotography (TLC) and the requirements for egg yolk (as a source of lecithin) or 4% bovine serum albumin for haemolysis of sheep erythrocytes (Fig.9).

The presence of lysophospholipase was further confirmed by replacing the egg-yolk in the agar plates with 8.0 mg of lysolecithin. No

Plate 31. Clearing of egg-yolk by cell-free filtrates from exponential phase axenic cultures of pathogenic and non-pathogenic strains of *Naegleria* (MsT, NHI, Ts-1, P1200f) and *Acanthamoeba* (A-1, 1501). Note the relative lack of inner ring of turbidity indicating low production of lysophospholipase.

Plate 32. Clearing and turbidity of egg-yolk by cell-free filtrates from stationary phase axenic cultures of pathogenic and non-pathogenic strains of *Acanthamoeba*. Note inner ring of turbidity due to increased production of lysophospholipase. Mag. *ca* x 1.5.


1501
MsT
Ts-1
media
A-1
P1200f
NHI

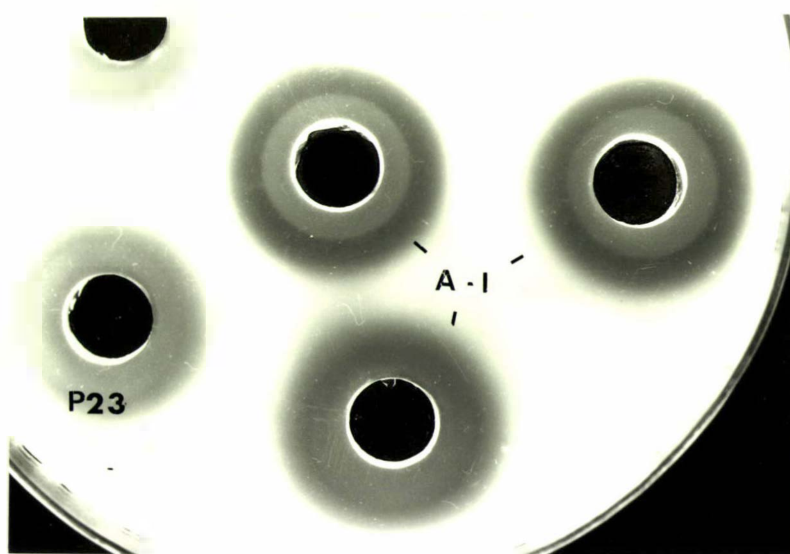
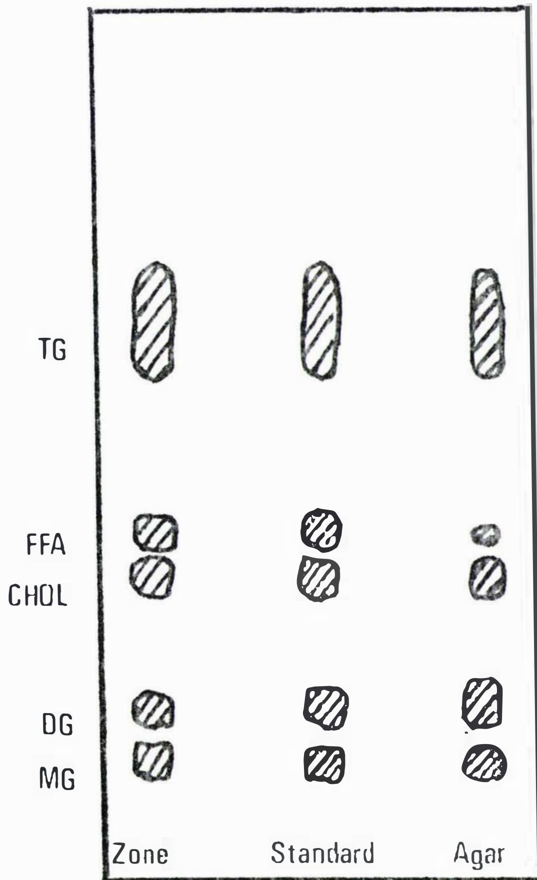
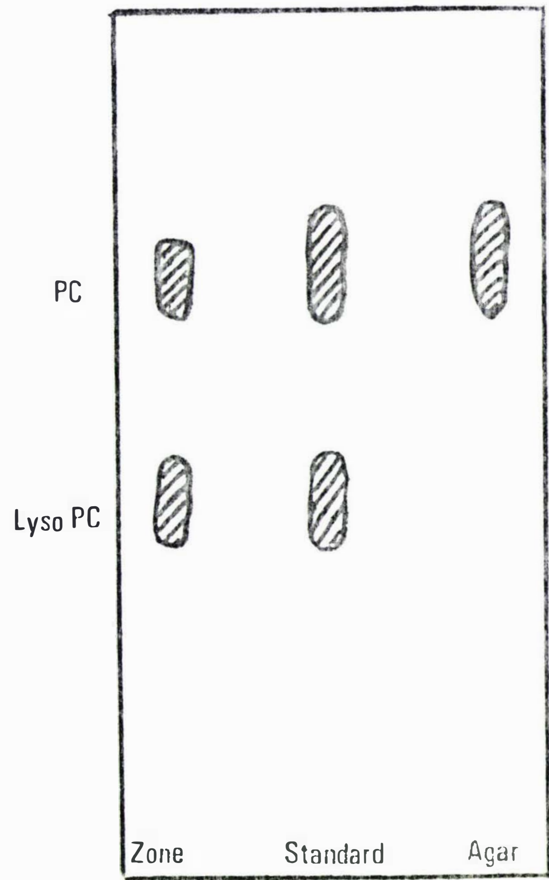

A-1
P23

Figure 9. Thin-layer chromatograms of neutral lipids and phospholipids from extracts of clear zones of EY plates.





Neutral Lipids

Phospholipids

TG = Triglycerides

DG = Diglycerides

MG = Monoglycerides

FFA = Free fatty-acids

CHOL = Cholesterol

PC = Phosphatidyl choline

Lyso PC = Lysophosphatidyl choline

phospholipase C was detected, as evidenced by the lack of turbidity in egg-yolk plates, the lack of diglycerides from TLC and the failure to haemolyse sheep erythrocytes unless an exogeneous source of phosphatidyl choline was present. Replacement of the egg-yolk with casein also failed to reveal any proteinases.

The results in Table XXII and Plates 33 & 34 demonstrate the inhibition of extracellular phospholipase 2 by competitive inhibition using a synthetic analogue of lecithin, Dimethyl-DL-2, 3-distearoyloxypropyldimethyl-2-hydroxymethyl-ammonium acetate, Rosenthal's inhibitor (RH). Furthermore the presence of excess EDTA also inhibited the enzymatic reaction, by complexing with Ca^{2+} and Mg^{2+} which are needed for activity.

Table XXII. Effect of 2.64 & 5.28 x 10^{-2}M Rosenthal's Inhibitor on Phospholipase 2 Activity in EY Agar

Species (Strain)	No. of amebae $.cm^{-3} \times 10^{6}$	Clearing Rx in EY Agar (cm)	Clearing Rx in EY Agar + Acetone (cm)	Clearing Rx in EY Agar + RH (cm)	Clearing Rx in EY Agar (cm)
Naegleria spp.				2.64×10^{-2}M	
MsT	5.1	0.70	0.70	0.34	-
NHI	5.0	0.65	0.65	0.32	-
Ts-1	5.3	0.28	0.28	-	-
P1200f	5.1	0.58	0.58	0.28	-
0400	5.0	0.31	0.31	0.1	-
				5.28×10^{-2}M	
MsT	5.1	0.70	0.70	-	-
NHI	5.0	0.65	0.65	-	-
Ts-1	5.3	0.28	0.28	-	-
P1200f	5.1	0.58	0.58	-	-
0400	5.0	0.31	0.31	-	-

... cont'd

Acanthamoeba spp.				2.64×10^{-2}M	
A-1	5.4	0.76	0.76	0.38	-
1501	5.2	0.60	0.60	0.29	-
1537	3.0	0.41	0.41	0.20	-
P23	5.1	0.65	0.65	0.33	-
				5.28×10^{-2}M	
A-1	5.4	0.76	0.76	-	-
1501	5.2	0.60	0.60	-	-
1537	3.0	0.41	0.41	-	-
P23	5.1	0.65	0.65	-	-

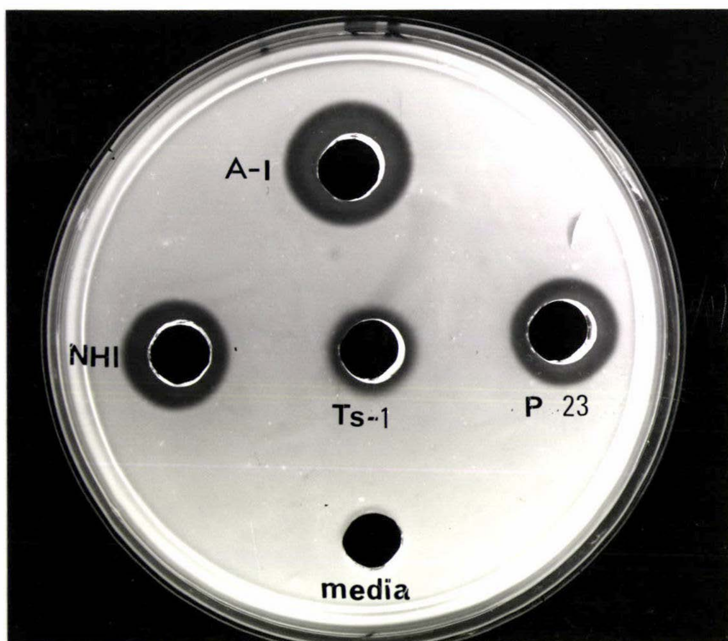
RX = reaction

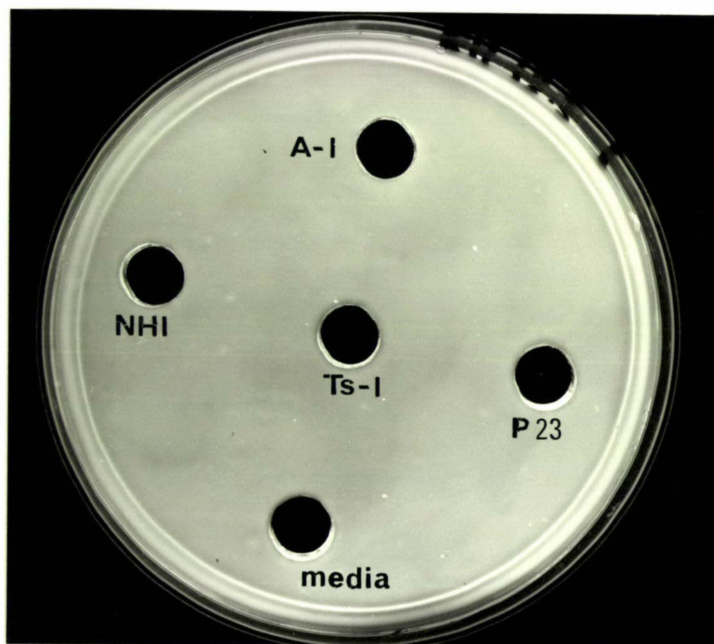
- = no reaction

Rosenthal's Inhibitor (RH) = Dimethyl-DL-2,3-Distearoyloxypropyl-2-hydroxyethyl-ammonium acetate

Plate 33. Clearing of egg-yolk by cell-free filtrates from 48 hr exponential axenic cultures of pathogenic and non-pathogenic strains of *Naegleria* (NHI, Ts-1) and *Acanthamoeba* (A-1, P23).

Plate 34. Inhibition of clearing of egg-yolk by cell-free filtrates from 48 hr axenic cultures of pathogenic and non-pathogenic *Naegleria* (NHI, Ts-1) and Acanthamoeba (A-1, P23) by the incorporation of 5.28×10^{-2}M Rosenthal's inhibitor.


A-I
NHI
Ts-1
P 23
media


A-I
NHI
Ts-I
P 23
media

5.4.2 Production of Phospholipase 2 in Axenic Cultures of Free-living Amebae

Tables XXIII & XXIV show that the maximum activity of phospholipase 2 by both *Naegleria* and *Acanthamoeba* spp. respectively, was obtained from axenic cultures exhibiting an increase in cell numbers. This was especially marked for *Naegleria* spp. and appeared to be linked to exponential growth. (Table XXIII).

Table XXIII. Production of Phospholipase 2 by *Naegleria* spp.

Species (Strain)	cellsx10^6 .cm^{-3} at sample time	Zone diameter of cleared agar (cm) during incubation				
		24 hr	48 hr	72 hr	90 hr	120 hr
N. fowleri (MsT)	4.5	0.74				
	10.0		0.9			
	6.4			0.65		
	2.4				0.25	
	2.5					0.25
N. fowleri (NHI)	3.4	0.65				
	8.9		0.72			
	6.0			0.60		
	2.9				0.22	
	2.5					0.25
N. fowleri (Ts-1)	3.1	0.13				
	8.4		0.31			
	6.9			0.20		
	3.0				-	
	2.8					-
N. gruberi (P1200f)	3.6	0.53				
	8.0		0.61			
	11.0			0.65		
	6.7				0.4	
	6.0					0.3
N. jadini (0400)	2.6	0.25				
	6.0		0.35			
	2.6			0.25		
	2.4				0.10	
	1.0					-
control CYM		-	-	-	-	-

- = no reaction

With *Acanthamoeba* spp. maximum activity was obtained from cell-free supernatants of cultures exhibiting stationary growth (Table XXIV).

Table XXIV. Production of Phospholipase 2 by *Acanthamoeba* spp.

Species (Strain)	cellsx10^6 $.cm^{-3}$ at sample time	Zone diameter of cleared agar (cm) during incubation				
		24 hr	48 hr	72 hr	90 hr	120 hr
A. culbertsoni (A-1)	2.8	0.52				
	6.3		0.78			
	9.8			0.95		
	17.2				1.15	
	8.9					1.4
A. castellanii (1501)	2.2	0.31				
	5.7		0.60			
	7.5			0.68		
	11.1				0.84	
	9.0					0.9
A. rhysodes (1537)	2.2	0.3				
	4.0		0.58			
	6.0			0.64		
	12.1				0.82	
	8.3					0.92
A. polyphaga (P23)	2.5	0.4				
	5.1		0.66			
	13.9			0.70		
	17.7				0.81	
	9.1					0.96
control neff		-	-	-	-	-

- = no reaction

In both genera, stationary-lysis phases were accompanied by an accumulation of lysophospholipase in the culture supernatant, as judged by the increasing ratio of clear:turbid zones in egg-yolk plates. Furthermore, the pathogenic strains

MsT, NHI and A-1 produced comparatively more phospholipase 2 than the non-pathogenic strains. This was especially marked between the pathogenic N. fowleri strains MsT, NHI and the non-pathogenic N. fowleri strain Ts-1.

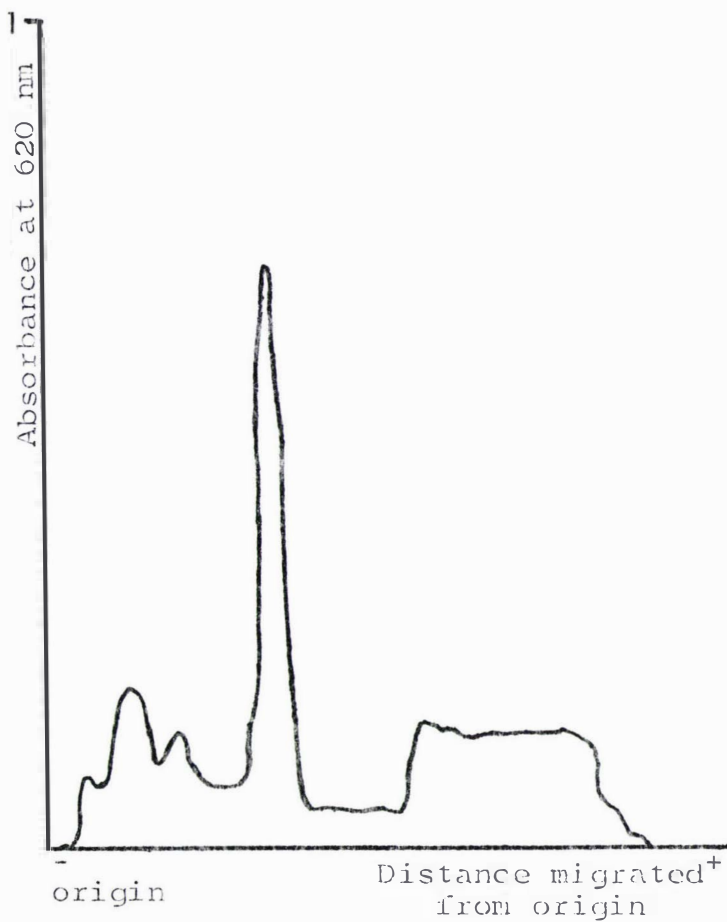
5.4.3 The Preliminary Isolation of Phospholipase 2 from Axenic Cultures of N. fowleri (MsT)

5.4.31 The Isolation of Phospholipase 2 from Serum-supplemented Axenic Cultures

Fig. 5 (see Methods 4.9.4) shows the isolation procedure while Figs 10, 11, 12 & 13 show the relative purification at each stage as judged by polyacrylamide gel electrophoresis (PAGE). The specific activity (i.e. activity $.mg^{-1}$ protein) of each fraction could not be determined due to the inherent variables in agar assay systems e.g. concentration effects on diffusion of proteins through the agar (Table XXV).

Figure 10. PAGE profile of the supernatant from serum-supplemented axenic *N. fowleri* culture.

Figure 11. PAGE profile of the 40-60% $(NH_4)_2SO_4$ precipitated fraction of the serum-supplemented axenic *N. fowleri* culture.


1
Absorbance at 620 nm
-
origin
Distance migrated+
from origin

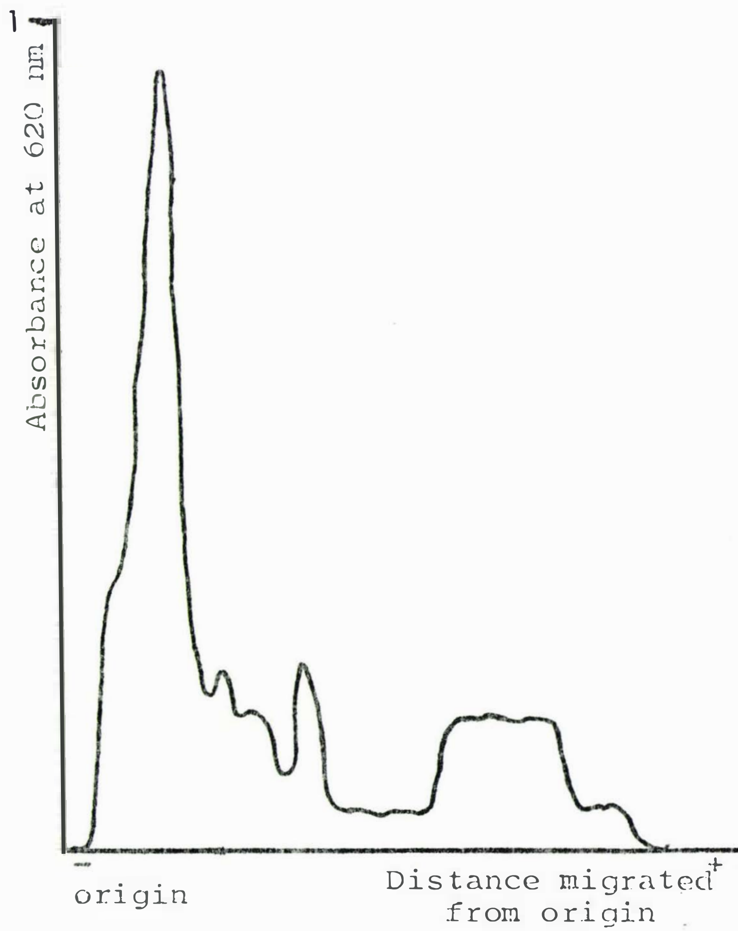

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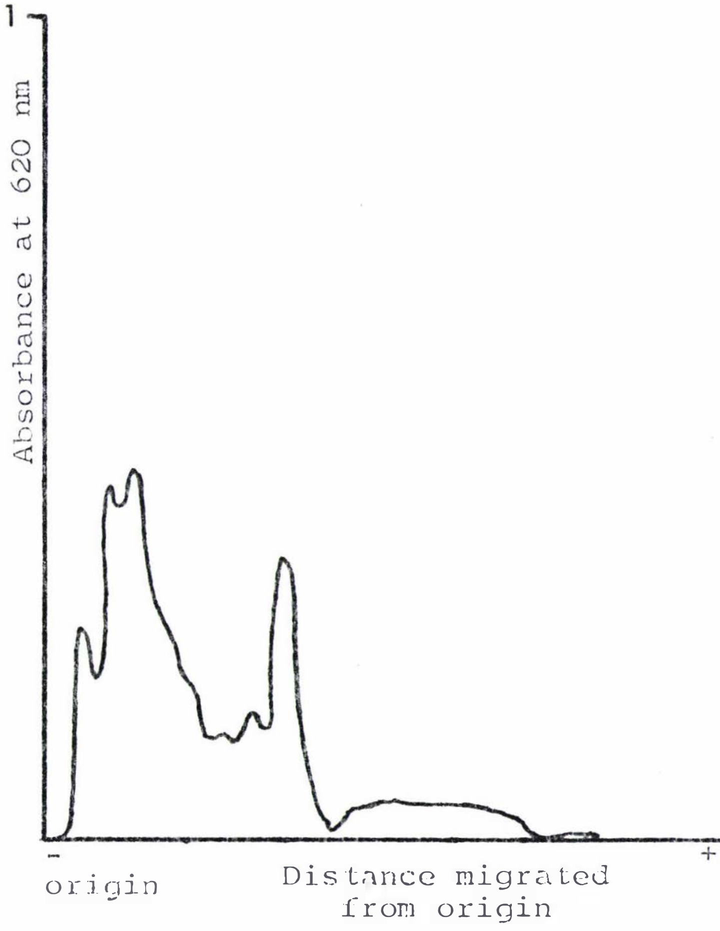
Figure 12. PAGE profile of XM 50,000 MW ultrafiltration fraction of serum-supplemented axenic *N. fowleri* culture.

Figure 13. PAGE profile of the SEPHADEX G-100 fraction of serum-supplemented axenic *N. fowleri* culture.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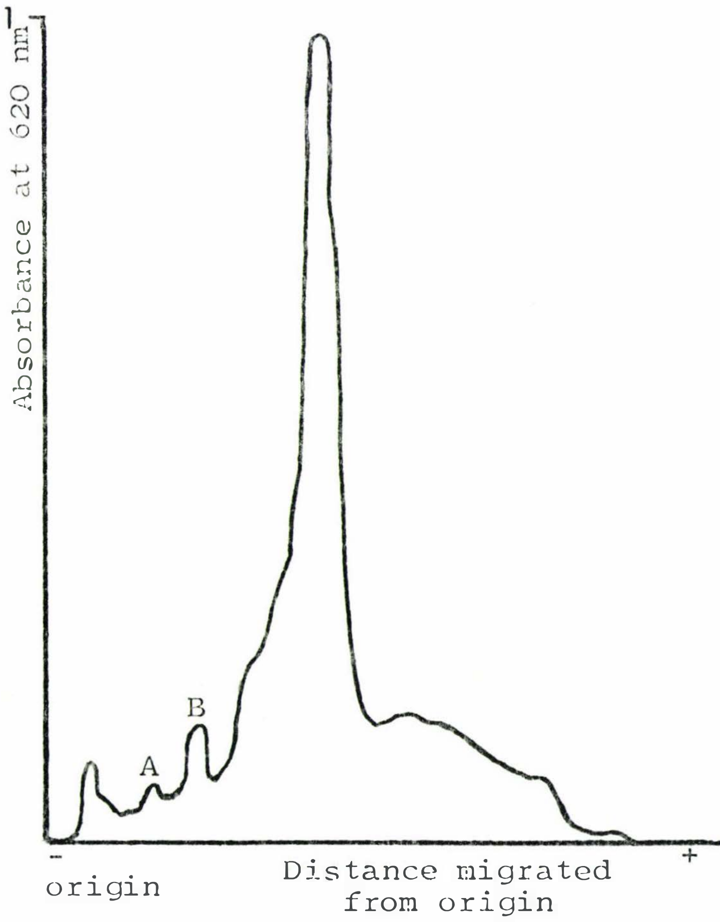
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Absorbance at 620 nm
A
B
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origin
Distance migrated
from origin

Table XXV. Effect of Protein Concentration on the Reaction of *N. fowleri* (MsT) and *A. culbertsoni* (A-1) Cell-free Filtrates in EY agar

Amebic Filtrate	Dilution	Protein Concentration mg. cm^{-3}	Clearing of EY Agar (cm)
N. fowleri (MsT)	Neat	20	0.70
	1/2	10	0.44
	1/4	5	0.32
	1/8	2.5	0.20
Control CYM	Neat	18	-
A. culbertsoni (A-1)	Neat	50	0.80
	1/2	25	0.64
	1/4	12.5	0.43
	1/8	6.25	0.30
Control Neff	Neat	46	-

- = no reaction

Enzyme activity as judged by clearing of the EY agar was retained in the fraction precipitated by either 40-60% $(NH_4)_2SO_4$ or by 1:1 supernatant:cold CH_3CH_2OH. Dialysis against 2.5 mM hepes pH 7.5 at $4^{o}C$ overnight allowed removal of contaminating $(NH_4)_2SO_4$ and proteins, whilst ultrafiltration allowed not only concentration of the enzyme for SEPHADEX gel filtration but also suggested a M.W. range of $<$100,000 & $>$50,000 daltons in that activity was not retained by an XM 100,000 MW filter but was by an XM 50,000 MW filter Further purification was carried out on SEPHADEX G-100.

By following the purification of the

enzyme by PAGE, it can be observed from the protein profiles that the only contaminating media-proteins belonged to the 2% bovine sera added to the media (Figs 14 & 15). No protein bands were obtained from the casitone, yeast-extract or haemin constituents of the media. Although the purification stages removed the contaminating alpha (α), beta (β) and gamma (γ) peaks of bovine sera, the albumin peak was further purified along with both phospholipase 2 and lysophospholipase, being eluted with them in the same fractions. Thus bovine sera albumin also acted indirectly as a MW marker for both enzymes. Its MW of 67,000 daltons agreed with the ultrafiltration result of a M.W. of $<$100,000 but $>$50,000 daltons.

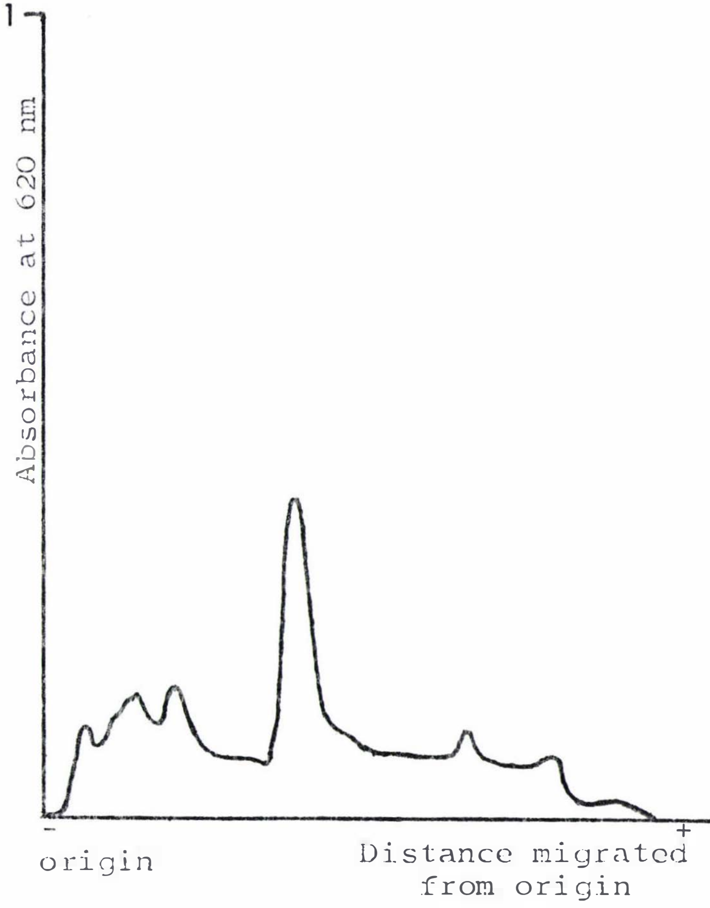
By comparing the PAGE profiles of the SEPHADEX G-100 fraction with media components, 2 extra peaks (labelled A + B) (Fig. 13) representing phospholipase 2 and lysophospholipase were revealed. Evidence for these peaks being both phospholipase 2 and lysophospholipase came from unstained gels where both clearing and turbidity was observed at the appropriate distance from the origin when such gels were placed in egg yolk agar plates (plate 35). Furthermore, albumin must have an excess of negative charges as it migrates substantially towards the anode.

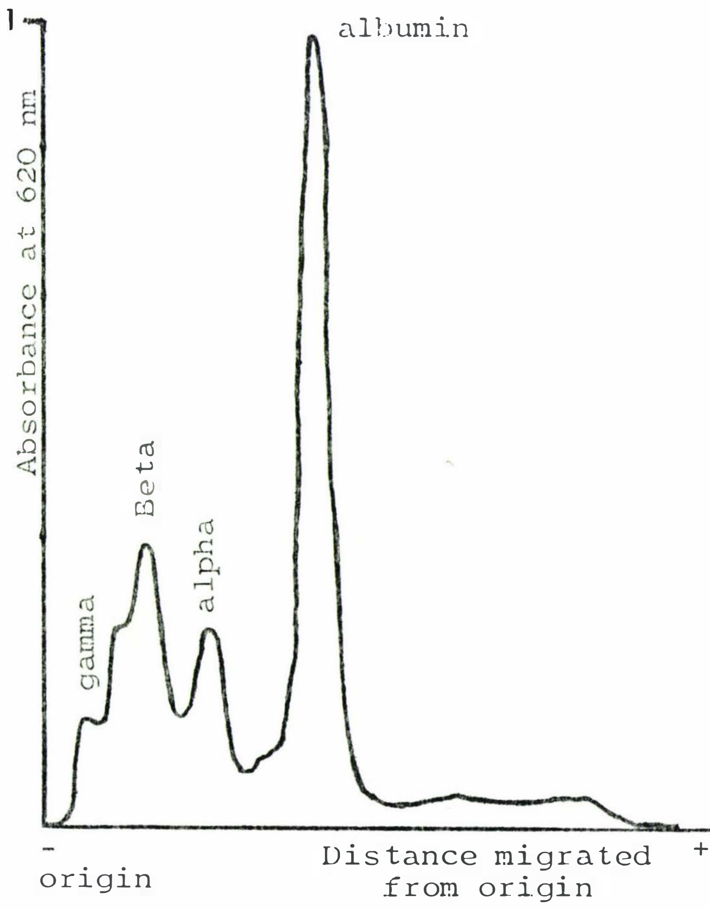
5.4.32 <u>The Isolation of Phospholipase 2 from Serum-free Axenic Cultures</u>

Because bovine serum-free medium PAGE

Figure 14. PAGE profile of serum-supplemented axenic culture medium.

Figure 15. PAGE profile of sterile bovine serum.





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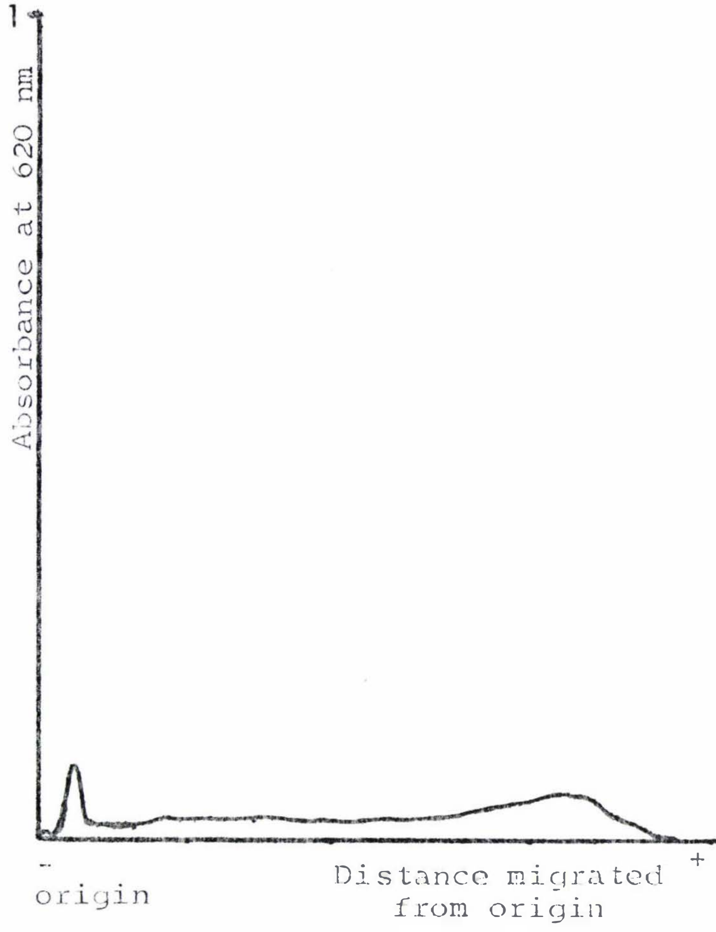
profiles gave no contaminating protein bands (Fig. 16), isolation of phospholipase 2 was attempted using this medium. Protein was again precipitated by either 40-60% $(NH_4)_2SO_4$ of 1:1 supernatant:cold CH_3CH_2OH but because of the comparatively poor yield of total protein, it could be applied directly onto SEPHADEX G-100 for gel-filtration and desalting. Phospholipase active fractions were then pooled together and concentrated by ultrafiltration through an XM 50,000 MW filter and then subjected to electrophoresis on polyacrylamide gels.

By comparing Figs 16, 17 & 18 , it can be seen that two extra protein peaks were observed only on the ultrafiltration fraction, once again close to the origin. Furthermore, unfixed gels reacted on an egg-yolk plate giving both a clear and turbid zone (typical of both a phospholipase 2 and lysophospholipase) at a distance which corresponded to the two peaks observed in the fixed and stained polyacrylamide gel. As well, antiserum raised against the purified fraction exhibiting both phospholipase 2 and lysophospholipase activity yielded only 2 precipitin lines in ge diffusion assays (Fig. 19). This antiserum also completely inhibited the characteristic clearing of the EY agar plate by the cell-free filtrate from an axenic culture of N. fowleri (MsT) when it was incorporated into the axenic culture medium to 10.0% (V/V).

Figure 16. PAGE profile of serum-free axenic culture medium.

Figure 17. PAGE profile of the supernatant from serum-free axenic *N. fowleri* culture.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Absorbance at 620 nm
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origin
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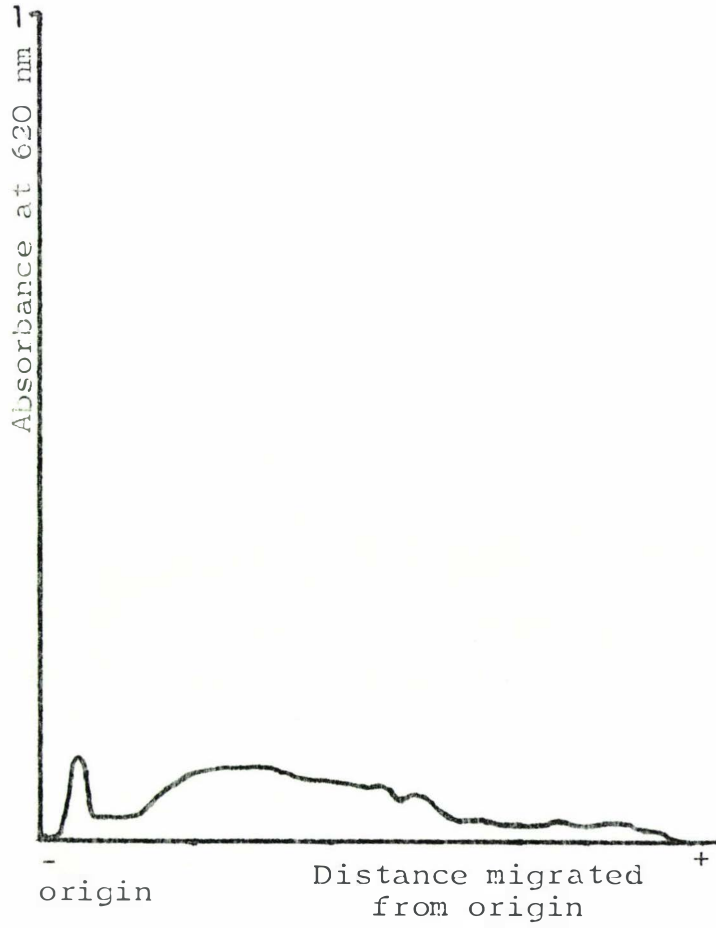
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Absorbance at 620 nm
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origin
Distance migrated
from origin
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Figure 18. PAGE profile of the SEPHADEX G-100 fraction of serum-free axenic *N. fowleri* culture.

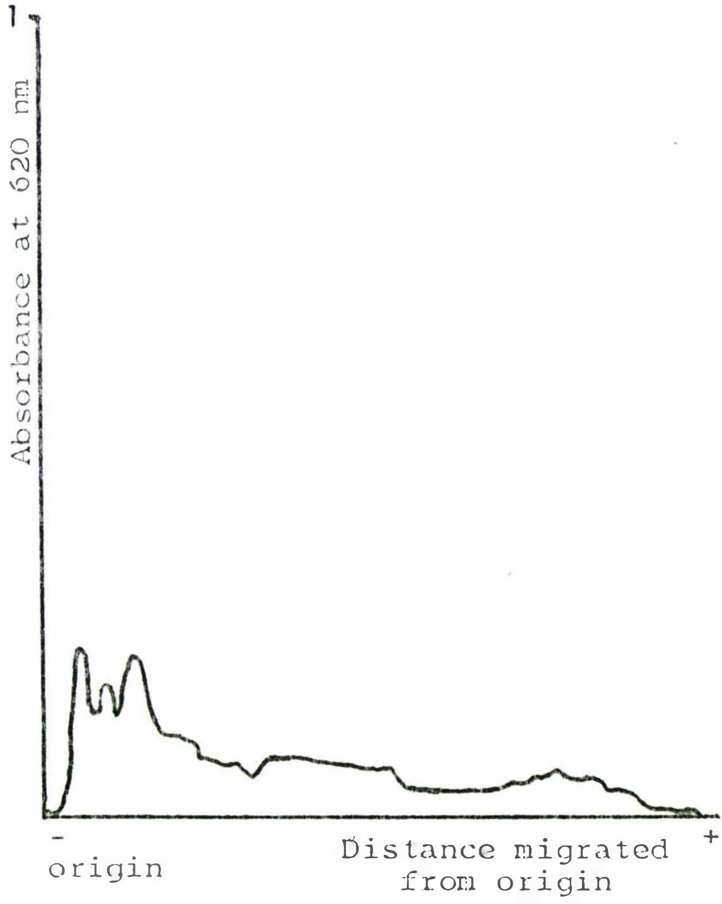

1
Absorbance at 620 nm
-
origin
Distance migrated
from origin
+

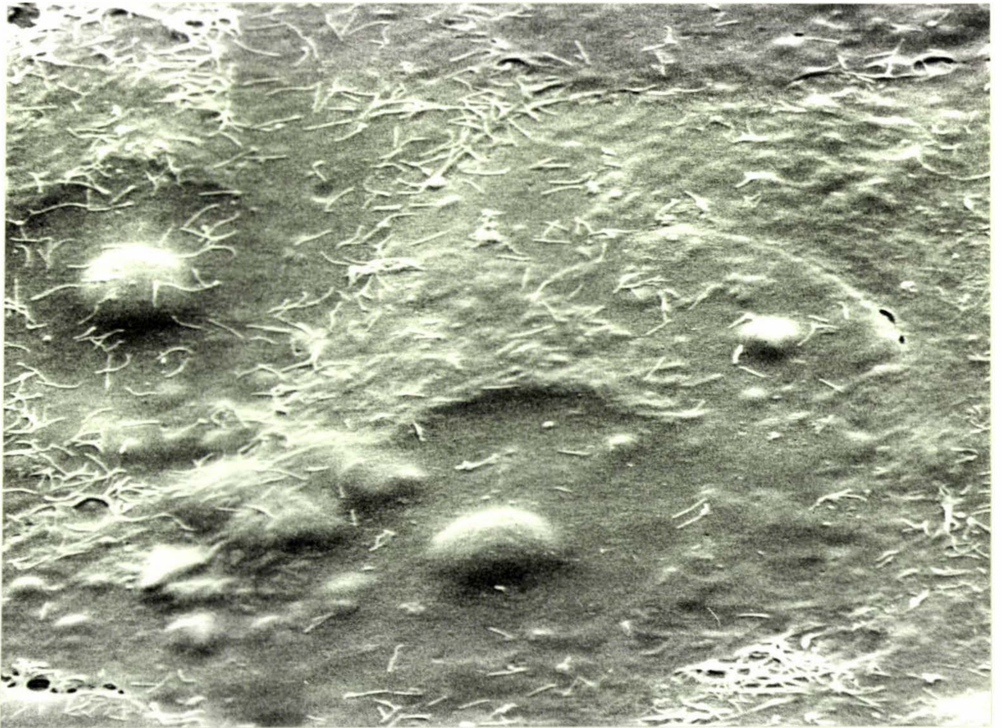
Figure 19. Gel-diffusion pattern using anti-phospholipases serum. Note the two precipitin lines between the anti-serum and phospholipase 2 and lysophospholipase enzymes isolated from the axenic CYMH supernatants of *N. fowleri* (MsT) cultures and the lack of reaction with the media (CYMH).

Phospholipase 2 and Lysophospholipase
Rabbit anti-serum
CYMH media

Plate 35. Clearing and turbidity of egg-yolk (close to the origin) after native PAGE of purified phospholipases.

Plate 36. Scanning electronmicrograph of control Vero cell culture monolayer. Note confluency of of monolayer, and microvilli. Mag. *ca* x 2600.

origin



5.4.4 Virulence of Amebae and Cell-free Axenic Filtrates in Vero Cell Culture

Tables XXVI & XXVII show the relative formation of CPE by both pathogenic and non-pathogenic species of *Naegleria* and *Acanthamoeba* in Vero cell culture. The progressive destruction of the cell monolayer by *N. fowleri* (MsT) is shown in Plates 36, 37, 38, 39 & 40. All pathogenic strains (MsT, NHI, A-1) produced early CPE and at much lower inocula when compared to the non-pathogenic strains (Ts-1, P1200f, 0400, 1501, 1537, and P23).

Table XXVI. The Relative Formation of Cytopathic Effects in Vero Cell Culture by different inocula of *Naegleria* spp.

Species (Strain)	size of inocula cells. cm^{-3}	No. of days for development of CPE						
		1	2	3	4	5	6	7
N. fowleri (MsT)	5×10^5	IV						
	5×10^4	II	IV					
	5×10^3	-	I	III	IV			
	5×10^2	-	-	I	II	III	IV	
N. fowleri (NHI)	5×10^5	IV						
	5×10^4	I	III	IV				
	5×10^3	-	I	II	III	IV		
	5×10^2	-	-	I	I	II	III	IV
N. fowleri (Ts-1)	5×10^5	IV						
	5×10^4	-	-	I	I	II	III	III
N. jadini (0400)	5×10^5	IV						
	5×10^4	-	-	-	-	-	-	-

... cont'd

N. gruberi (P1200f)	5 x 10^5 5 x 10^4	IV -	 -	 -	 -	 -	 -	 -
Control		-	-	-	-	-	-	-

I = beginning of CPE

II = pronounced CPE

III = very pronounced CPE

IV = complete breakdown of monolayer

\- = no CPE

Table XXVII. The Relative Formation of Cytopathic Effects in Vero Cell Culture by different inocula of *Acanthamoeba* spp.

Species (Strain)	size of inocula cells. cm^{-3}	No. of days for development of CPE						
		1	2	3	4	5	6	7
A. culbertsoni (A-1)	5×10^5	IV						
	5×10^4	II	IV					
	5×10^3	-	I	III	IV			
	5×10^2	-	-	-	I	I	III	IV
A. castellanii (1501)	5×10^5	IV						
	5×10^4	-	-	-	-	-	-	-
A. rhysodes (1537)	5×10^5	IV						
	5×10^4	-	-	-	-	-	-	-
A. polyphaga (P23)	5×10^5	IV						
	5×10^4	-	-	-	-	-	-	-
Control		-	-	-	-	-	-	-

I = beginning of CPE

II = pronounced CPE

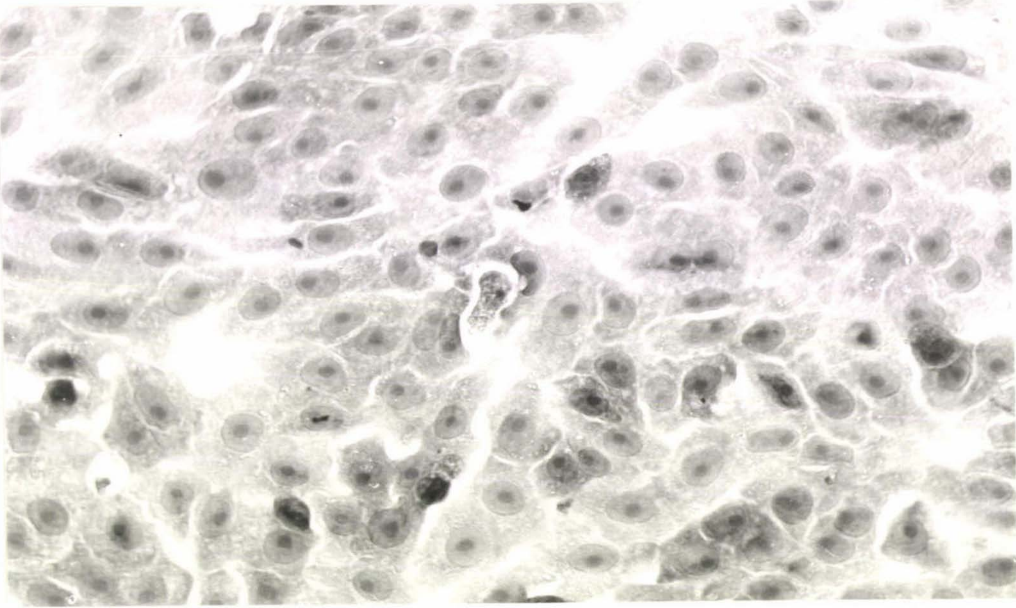
III = very pronounced CPE

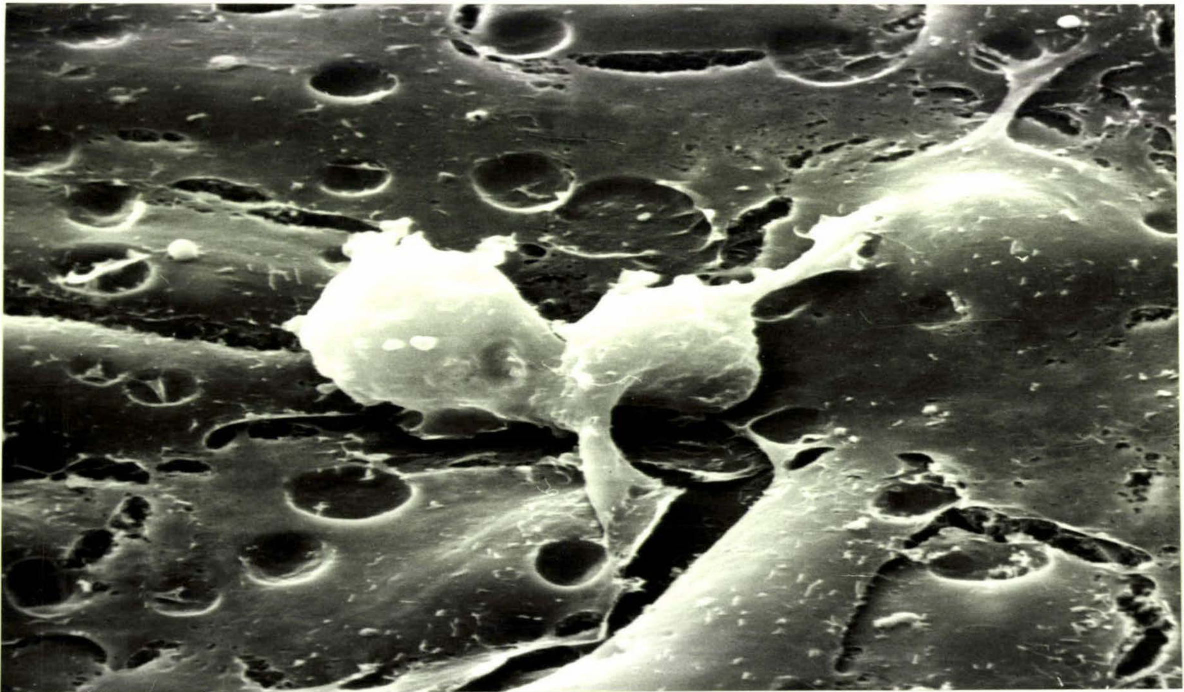
IV = complete breakdown of monolayer

\- = no CPE

Plate 37. Photomicrograph of Vero cell culture monolayer infected with *N. fowleri* showing beginning of CPE. Note the characteristic clear halo surrounding the trophozoite. Mag. *ca* x 300. (From Cursons & Brown, 1978).

Plate 38. Scanning electronmicrograph showing attachment of *N. fowleri* trophozoite onto Vero cell culture monolayer by pseudopods. Note extensive vacuolation of Vero cells' cytoplasm and loss of microvilli. Mag. *ca* x 2000.





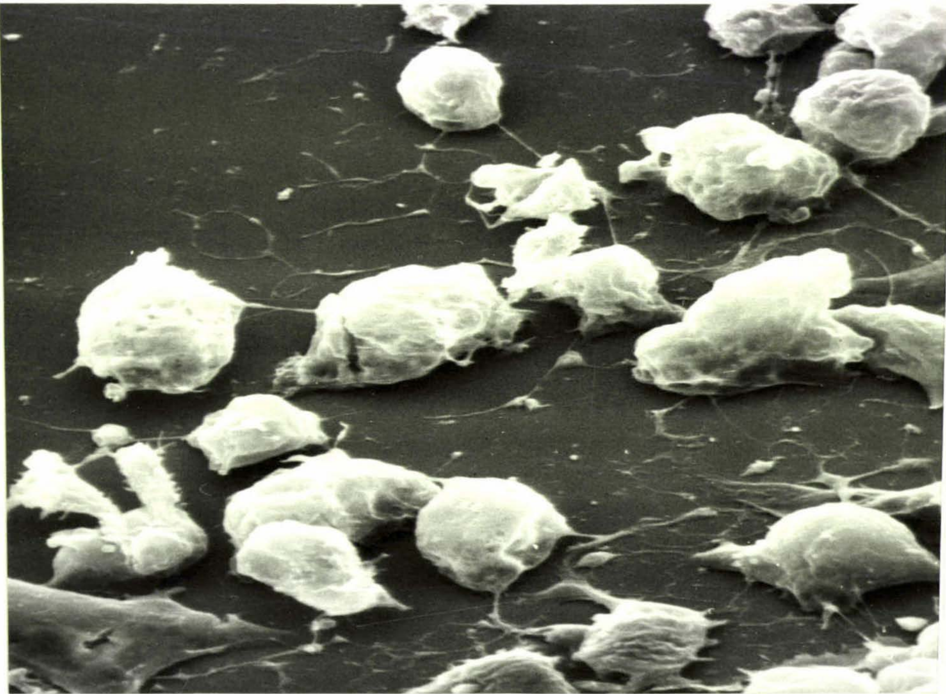


Plate 39. Photomicrograph of Vero cell cultu
layer infected with *N. fowleri* sho
pronounced CPE. Note loss of mono
replacement of Vero cells with *N.*
trophozoites. Mag. *ca* x 150. (Fr
& Brown, 1978).

Plate 40. Scanning electronmicrograph of Ve
ture infected with *N. fowleri*. N
gressive destruction of monolayer
x 1400. (From Cursons & Brown, 1

Table XXVIII shows the formation of CPE in Vero cell culture by phospholipase 2 active cell-free filtrates of axenically grown pathogenic and non-pathogenic amebae. As can be seen, the cytopathogenicity of these cell-free filtrates towards the monolayer depends upon the relative amounts of phospholipase 2 present in the filtrate as assessed by their reaction in EY agar.

Table XXVIII. The Relative Formation of Cytopathic Effects in Vero Cell Culture by Cell-free Filtrates from Axenic Cultures

Species (Strain)	Clearing Reaction in EY agar (cm)	No. of days for development of CPE						
		1	2	3	4	5	6	7
N. fowleri (MsT)	0.72	III	IV					
N. fowleri (NHI)	0.68	III	IV					
N. fowleri (Ts-1)	0.21	I	II	IV				
N. jadini (0400)	0.3	I	III	IV				
N. gruberi (P1200f)	0.60	III	IV					
A. culbertsoni (A-1)	0.90	IV						
A. castellanii (15[illegible]1)	0.70	IV						
A. rhysodes (1537)	0.72	IV						
A. polyphaga (P23)	0.75	IV						
Control CYM	-	I	II	IV				
Control Neff	-	I	II	IV				

I = beginning of CPE

II = pronounced CPE

III = very pronounced CPE

IV = complete breakdown of monolayer

Furthermore, Table XXIX demonstrates that non-cytoxic amounts of crude phospholipase 2 from cell-free filtrates of axenically grown strain MsT, added to Vero cell culture inoculated with

strain Ts-1, resulted in the formation of CPE in a period of time equal to that of pathogenic Naegleria strains.

Table XXIX. Addition of Cell-free Filtrate from the Axenic Culture of N. fowleri (MsT) to Vero Cell Cultures Inoculated with N. fowleri (Ts-1)

Species (Strain)	size of inocula cells . cm^{-3}	% addition of cell-free filtrate	No. of days for development of CPE						
			1	2	3	4	5	6	7
N. fowleri (Ts-1)	5×10^5	0	IV						
	5×10^4	0	-	-	I	I	II	III	IV
	5×10^3	0	-	-	-	-	-	-	-
N. fowleri (Ts-1)	5×10^5	25%	IV						
	5×10^4	25%	I	III	IV				
	5×10^3	25%	-	-	I	II	III	IV	
MsT cell-free filtrate and maintenance media		25%	-	-	-	-	-	-	-
25% Ts-1 cell-free filtrate and maintenance media		25%	-	-	-	-	-	-	-
25% CYM and maintenance media		0	-	-	-	-	-	-	-

I = beginning of CPE

II = pronounced CPE

III = very pronounced CPE

IV = complete breakdown of monolayer

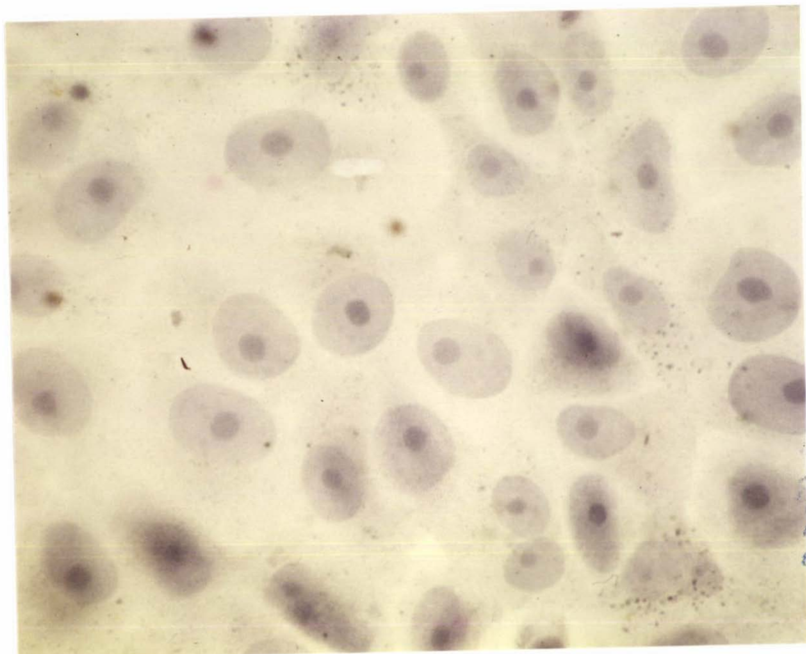
\- = no CPE

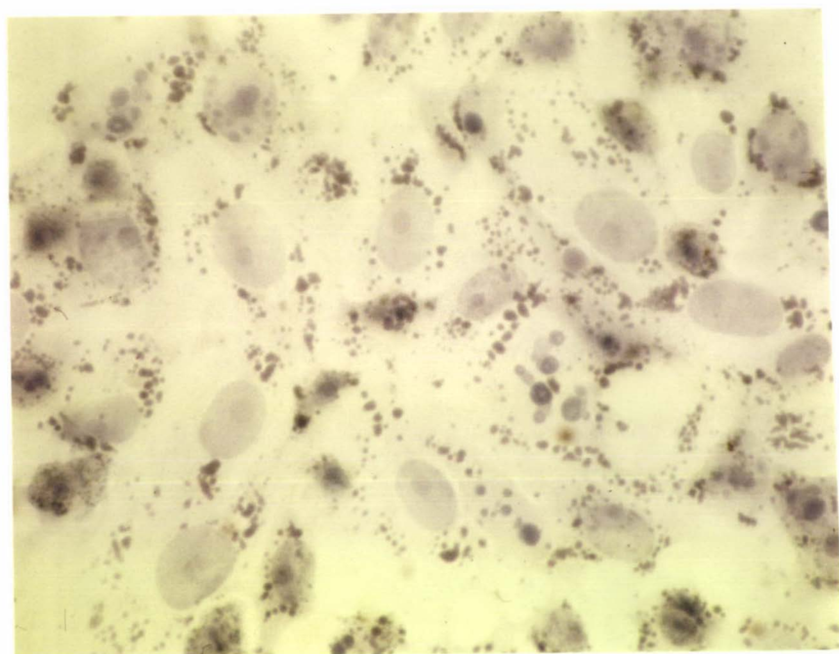
Cytochemical staining with Sudan black of strains MsT or A-1 infected Vero monolayers also revealed extensive lipogenesis (Plates 41, 42 & 43) consistent with monolayer degradation by lipolytic enzymes. Finally Table XXX shows the inhibition and delay in the formation of CPE by N. fowleri strains MsT, NHI and Ts-1 by different concentrations of the specific anti-phospholipase 2

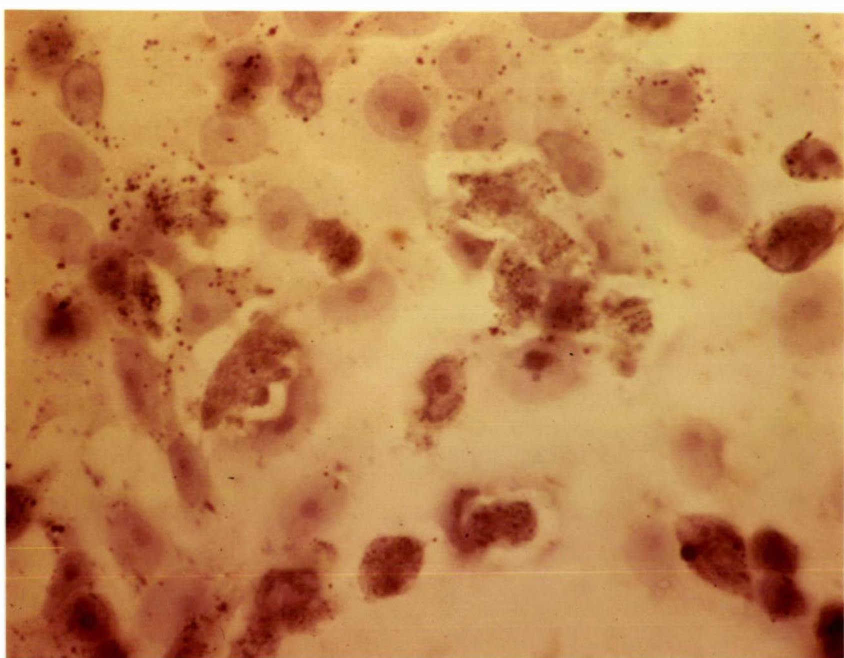
Plate 41. Photomicrograph of control Vero cell culture stained with Sudan black for lipid detection. Note relative lack of lipogenic staining. Mag. <u>ca</u> x 500.

Plate 42. Photomicrograph of Vero cell culture infected with *A. culbertsoni* stained with Sudan black. Note widespread lipogenic staining. Mag. *ca* x 500.

Plate 43. Photomicrograph of Vero cell culture infected with *N. fowleri* stained with Sudan black. Note localized lipogenic staining and trophozoites surrounded by clear halos Mag. *ca* x 500.







and lysophospholipase serum. This was achieved by replacing the 2% (V/V) serum requirement of the maintenance medium, with either 4% (V/V) or 10% (V/V) of sterile rabbit-anti-phospholipase-serum. This anti-serum was specific for N. fowleri strains MsT, NHI and Ts-1, with an IFAB titre of 1/1000, as opposed to the 1/10 IFAB titre for N. gruberi (P1200f).

Table XXX. Inhibition of the Formation of CPE in N. fowleri inoculated Vero Cell Culture by anti-Phospholipase 2 and Lysophospholipase serum

Species (Strain)	Size of inocula cells. cm^{-3}	% addition of anti-phospholipase serum	No. of days for development of CPE						
			1	2	3	4	5	6	7
N. fowleri (MsT)	5×10^5	10%	IV						
	5×10^4		-	I	III	IV			
	5×10^3		-	-	-	-	-	-	-
	5×10^2		-	-	-	-	-	-	-
N. fowleri (MsT)	5×10^5	4%	IV						
	5×10^4		I	III	IV				
	5×10^3		-	-	I	I	II	IV	
	5×10^2		-	-	-	-	-	-	-
N. fowleri (NHI)	5×10^5	4%	IV						
	5×10^4		-	I					
	5×10^3		-	-	I	I	II	III	III
	5×10^2		-	-	-	-	-	-	-
N. fowleri (Ts-1)	5×10^5	4%	IV						
	5×10^4		-	-	-	-	I	I	I

... cont'd

	5×10^4		II	IV					
<u>N. fowleri</u> (MsI)	5×10^3	0%	-	I	III	IV			
	5×10^2		-	-	I	II	III	IV	
	5×10^4		I	III	IV				
<u>N. fowleri</u> (NHI)	5×10^3	0%	-	I	II	III	IV		
	5×10^2		-	-	I	I	II	III	IV
<u>N. fowleri</u> (Ts-1)	5×10^5	0%	IV						
	5×10^4		-	-	I	I	II	III	III
		0%	-	-	-	-	-	-	-

I = beginning of CPE

II = pronounced CPE

III = very pronounced CPE

IV = complete breakdown of monolayer

CHAPTER 6: DISCUSSION

6.1 Survey of New Zealand Thermal Pools

6.1.1 The Distribution of PFLA in New Zealand Thermal Pools during the 1976 Survey

The results presented in Table VI show the geographical distribution of PFLA isolations from the three Health Districts sampled. Whereas no or very few PFLA were recorded from the Gisborne-Rotorua areas, the Matamata-Taupo region had a relatively high incidence of isolation. It is also interesting to note that all recorded fatal cases of PAM have occurred in this region. Furthermore, the findings of a higher incidence of *N. fowleri* than pathogenic *Acanthamoeba* spp. is also in accordance with the reported cases of disease caused by PFLA in New Zealand. No reports of clinical infection due to *Acanthamoeba* spp. have been recorded in New Zealand, though this is possibly a reflection of the physician not considering them as potential pathogens during diagnosis more than their not having occurred. The aforementioned seven cases of PAM have all been due to *N. fowleri* (Nicoll, 1973; Cursons *et al.*, 1976a, 1976b)

6.1.2 The Effect of Chemical, Physical and Biological Parameters on the Distribution of PFLA in New Zealand Thermal Pools

The distribution of PFLA in waters will be affected by both the chemical and physical, as well as the biological characteristics, of the water. For example, many authors (Carter, 1970; Culbertson, 1971; Chang, 1974a; Cursons & Brown, 1976, Cerva, 1978) have reported that *N. fowleri* is extremely sensitive to 0.5 - 1.0% NaCl. Besides the chemical composition of the waters, both temperature and pH will also affect the distribution of these amebae. Stevens *et al.*

(1977a) recorded a temperature range of 35 - 38°C for the isolation of N. fowleri from thermally polluted lakes, whilst Wellings et al. (1977) recorded a range of 16 - 34.5°C. In the latter report, no N. fowleri isolates were obtained from lake waters below 26.5°C but were obtained from the bottom mud at 16°C. Similarly De Jonckheere and Van De Voorde (1977a) succeeded in isolating N. fowleri from waters between 10 - 32°C and Brown and Cursons (1977) isolated pathogenic A. castellanii from frozen lakes at 2°C. Griffin (1972) established that N. fowleri could tolerate 45°C and A. culbertsoni 42°C, whilst Chang (1978) observed that 51°C was lethal for both cysts and trophozoites of N. fowleri.

A temperature of isolation above 42°C has previously been thought important as a selective temperature for PFLA. Griffin (1972) in a study of 12 virulent and 10 non-virulent strains of free-living amebae demonstrated that the ability of strains to grow at high temperatures ($>$ 42°C) was apparently related to virulence. Unpublished observations have shown this to be an oversimplification with some free-living species, particularly Vahlkampfia ustiana (see Method 4.2.1) being able to tolerate 45°C. Subsequently De Jonckheere et al. (1975), and Singh and Hanumaiah (1977) have also established that many non-pathogenic species of free-living amebae are able to tolerate at least 42°C.

The pH range tolerated by PFLA is equally impressive. Carter (1970) demonstrated that N. fowleri could survive a pH range of between 4.6 - 9.5 whilst Kadlec (1975) showed that free-living amebae were able to grow in vitro between a pH of 5.6 - 8.4.

However, physical parameters such as pH and temperature are not the only factors influencing growth in thermal waters. The finding that *N. fowleri* utilizes substantially less oxygen than *N. gruberi* and *A. castellanii* (Buyers *et al.*, 1969; Weik & John, 1977 a & b) is ecologically important particularly in thermal pools since oxygen concentration decreases with an increase in temperature. Furthermore Cerva (1977) established *in vitro* that the optimal temperature for growth of *N. fowleri* was dependent not only on the age and size of inoculum but also on the composition and concentration of the culture medium. He established that if the concentration of soluble organic substances in the media was low, then a lower temperature was more satisfactory for the development of pathogenic strains whilst the reverse, that is a high concentration of organic substances, resulted in a higher temperature for satisfactory growth and development. No such relationship could be confirmed for *N. gruberi*. This may be a reflection on the different nutritional requirements reported between *N. fowleri* and *N. gruberi* (Cerva, 1969, 1977, 1978).

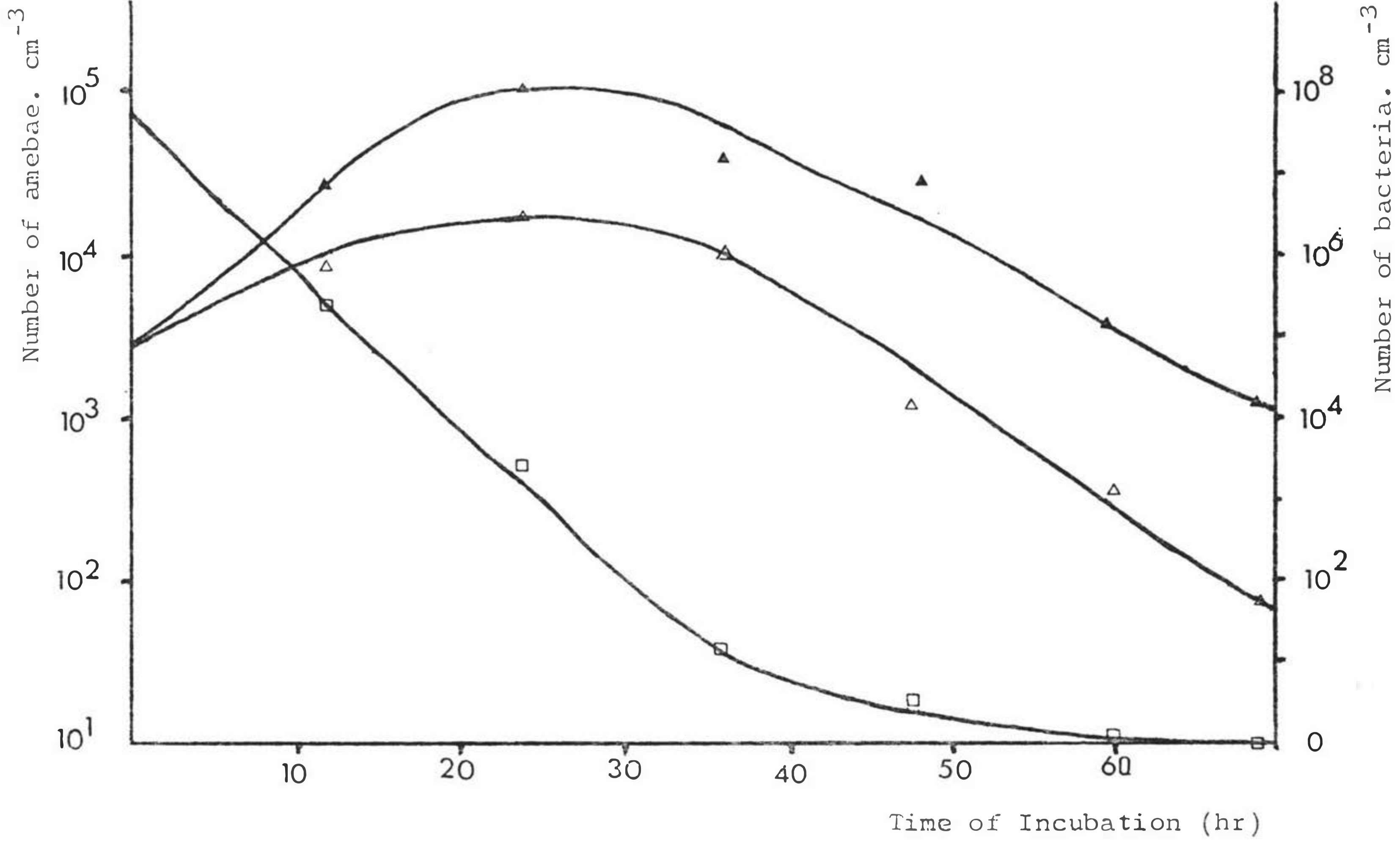
The presence of a suitable food source will also affect the distribution of PFLA in thermal pools. Besides organic debris, PFLA have also been shown to ingest both yeast and bacteria. Preliminary *in vitro* studies using *Enterobacter cloacae* as the bacterial food source have established an inverse correlation between active amebae and numbers (Figs. 20 & 21). Danso and Alexander (1975) and Danso *et al.* (1975) also reported a similar observation. Using *Rhizobium meliloti* as the food source they also demonstrated the inability of both

Figure 20. Graph of predation by pathogenic and non-pathogenic *Naegleria* spp. on the bacterium *Enterobacter cloacae*. (From Brown *et al.*, pers. comm.).

▲ = *N. gruberi* (P1200f)

△ = *N. fowleri* (MsT)

□ = *Enterobacter cloacae*



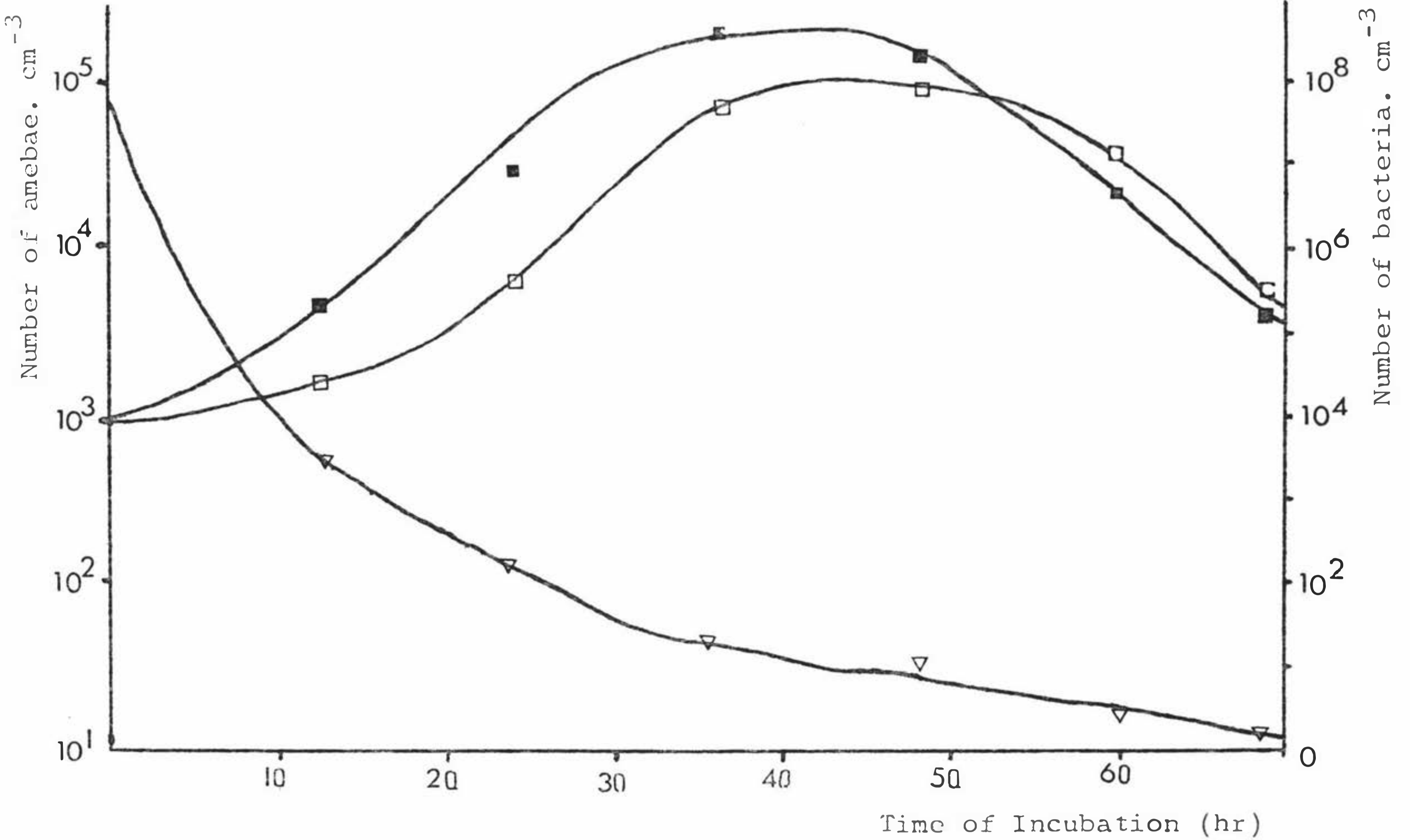
Number of amebae. cm-3
10⁵
10⁴
10³
10²
10¹
Number of bacteria. cm-3
10⁸
10⁶
10⁴
10²
0
10
20
30
40
50
60
Time of Incubation (hr)

Figure 21. Graph of predation by pathogenic and non-pathogenic *Acanthamoeba* spp. on the bacterium *Enterobacter cloacae*. (From Brown *et al.*, pers. comm.).

■ = *A. castellanii* (1501)

□ = *A. culbertsoni* (A-1)

△ = *Enterobacter cloacae*



Number of amebae. cm-3
10⁵
10⁴
10³
10²
10¹
10
20
30
40
50
60
Time of Incubation (hr)
Number of bacteria. cm-3
10⁸
10⁶
10⁴
10²
0

Naegleria and *Acanthamoeba* spp. to totally eliminate their prey. They suggested that the size of the prey population diminished until a density was obtained at which the energy used by the amebae between contacts with surviving food bacteria equalled that derived from feeding. Further, using *Tetramitus rostratus*, *Hartmanella* sp., *Naegleria* sp. and *Vahlkampfia* sp. with *R. meliloti* again as the food source, they demonstrated that amebic multiplication and bacterial decline stopped when the bacteria fell to approximately 10^6 - 10^7 cells. cm^{-3}. At higher bacterial densities, the maximum numbers of *Hartmannella* sp. and *Naegleria* sp. were proportional to the initial concentration of *R. meliloti* provided. Thus it appears that a critical density of bacteria is necessary for amebae to initiate or to maintain feeding. Napolitano *et al.* (1977) using alcohol-killed bacteria in monoxenic culture as the food source for *N. gruberi* reaffirmed the finding. They found that when the initial concentration of bacteria used was 10^9. cm^{-3}, the mean generation time (MGT) was 2.5 hr as opposed to 19.1 hr when 10^7 bacteria. cm^{-3} was added. However in nature, there is probably no simple predator-prey relationship between bacteria and the amebae alone, due to competition for bacteria by other protozoa, and the fact that amebae are not nourished indiscriminately by all species of bacteria (Singh, 1975). Singh (1941, 1945) established that bacteria differ in their edibility by amebae because of their production of exotoxins, pigments and extracellular slime, etc., a conclusion also arrived at by Chang (1971), Anderson and Jamieson (1974) and Menapace *et al.* (1975). Thus, the low incidence of PFLA in the Waignaro, Te Puia, Morere, Butchers and Soda

thermal pools could be due to any one, or a combination of the above characteristics of the particular pool. It is thought that the lack of isolation of PFLA from the Waignaro pools is due to the extremely alkaline pH of 9.6 caused by the pool CO_3-HCO_3^- buffer system. It is not known whether the comparatively high fluoride content also has any detrimental effect on the amebae. Because of the high alkaline pH in this pool, and its detrimental effect on chlorination it is doubtful whether chlorination could be effective in controlling the amebae. It should be emphasized that, the only total coliform and total bacterial counts recorded by the Department of Health to establish the bacterial qualities of the Hamilton pools being surveyed, was unfortunately taken when the Waignaro Pool was flooded, and thus may not represent the true bacteriological quality of the water (Table VIII). The lack of isolation of *N. fowleri* in the Gisborne pools is possibly a consequence of their very high halide ion content (Table IV) which has been shown to be inhibitory to *N. fowleri* (Culbertson, 1971; Cursons & Brown, 1976; Cerva, 1978). On the contrary, the isolation of a pathogenic *Acanthamoeba* sp. is explained by their greater tolerance to NaCl (Culbertson, 1971). These waters also have a comparatively lower coliform and total bacteria incidence, and thus the bacteriological quality of the water may have an indirect effect on the presence or absence of these amebae. Similarly the absence of PFLA in the Butchers Pool may be due to the high concentration of iron (Table III) in the thermal water. Such high concentrations of iron have been shown to have a lethal effect on many micro-organisms (Avakyan, 1974; Cairns *et al.*, 1974; Weinberg, 1978). However the low

incidence of PFLA in the Soda Springs does not correlate with any of the physical parameters such as pH and temperature, nor the chemical composition of the water. It is possible that biological parameters such as predation by carnivorous Protozoa and Metazoa, the presence of toxic bacteria or a high biochemical oxygen demand of the water, is responsible for the low levels of PFLA recorded in this pool.

6.1.3 The Incidence of PFLA in New Zealand Thermal Pools

The results of both the 1976 and 1977 surveys demonstrate a higher incidence of N. fowleri than pathogenic Acanthamoeba spp. in both thermal pools and surrounding soils. Although Acanthamoeba spp. are generally considered to be the most prevalent small free-living amebae in the environment (Singh, 1952; Page, 1967; Menapace et al., 1975; Visvesvara & Balamuth, 1975) it is thought that the reversal of this trend, particularly in thermal pools, can be attributed to the unusual nature of this environment. It has been shown that N. fowleri possesses a higher temperature-tolerance range than most other free-living amebae (Griffin, 1972) and that its oxygen requirements are substantially less than the non-pathogenic N. gruberi or A. castellanii (Buyers et al., 1969; Weik & John, 1977a & b). Thus these two environmental factors may exert a positive selection pressure for N. fowleri over N. gruberi or Acanthamoeba spp. in thermal waters, as has been suggested by De Jonckheere et al. (1975) and Stevens et al. (1977a). Further, Cerva (1977) demonstrated that 20°C was inhibitory to the in vitro multiplication of N. fowleri and Chang (1978) showed that the trophozoites of N. fowleri degenerate at tempera-

tures below $10^{\circ}C$. Thus the detrimental effect of temperatures below $20^{\circ}C$ on *N. fowleri* trophozoites is probably responsible for the close association of swimming-associated PAM infections with waters above ambient temperature i.e. above $30^{\circ}C$.

However, the relatively low incidence of Acanthamoeba from the soil is confusing. This may be a reflection on the soil biomass or even competition between the two genera. Sandon (1927) observed that *Naegleria* and *Acanthamoeba* spp. seldom occurred together in the same soil plot, an observation supported by Menapace *et al.* (1975).

Although the 1977 survey reaffirmed the wide adaptability of these amebae to both pH and temperature, marked differences in the isolation rate of PFLA from thermal waters, as compared to the 1976 survey, were recorded. In 1977 the Moana-iti pool was the only pool which yielded no PFLA whereas in 1976 3 isolations of *N. fowleri* were recorded from it (Tables VI & IX). This could be a reflection of the change of the sample sites (see Sampling Sites 2.2.2) making direct comparisons invalid.

Because of the relatively constant temperature of thermal pool waters, the effect of seasonal change on these pools is very much diminished compared to the effect on non-thermal waters, the temperature of which is directly influenced by climatic change. The ability to form resistant cysts to withstand adverse conditions will also influence seasonal distribution. Soils containing cysts will yield positive isolations on incubation and provide a positive inoculation to thermal pools via runoffs.

This relative lack of seasonality in the incidence of PFLA is reflected in the distribution of cases of PAM due to *N. fowleri* in New Zealand. Most cases of PAM in other countries have occurred during the warm summer months, a finding also reflected in the incidence of isolation of *N. fowleri* (De Jonckheere *et al.*, 1975; Wellings *et al.*, 1977). However in New Zealand, the seven reported cases of PAM occurred randomly in the months of April, May, June, September and December (Mandal *et al.*, 1970; Nicoll, 1973; Cursons *et al.*, 1978b) a reflection of the year round use of thermal pools in contrast to the primarily summer use of non-thermal recreational waters. Nevertheless it is possible that rainfall and temperature could affect the incidence of PFLA in the environment for the following reasons:

i) cold winter temperatures stimulate encystment of the amebae in the soil. Because of the resulting reduced division rate, the population of amebae will be lower than that of the summer months, and

ii) because of this inhibiting effect of cold temperatures on amebic growth, heavy rain at low temperatures will have a diluting effect on the amebic population in the soil and in runoff water. It is acknowledged that biological surveys which have sampling time intervals considerably longer than the cell cycle time of the organisms involved, cannot follow closely responses of the organisms to environmental changes which occur at times other than that just prior to the sampling. This could be, in part, responsible for the recorded differences

between the 1976 - 1977 surveys.

That soil can act as a reservoir for PFLA is not surprising in view that soil is the preferred habitat of small free-living amebae providing food, oxygen, water and a suitable temperature (Singh, 1975). Further the PFLA are adapted to survival in this environment by the possession of resistant cysts, which combined with the ability to encyst rapidly, enables them to exploit alternating adverse and favourable conditions such as desiccation and moisture. Although little has been done on the comparative physiology of non-pathogenic and pathogenic free-living amebic cysts, it is generally agreed that the cysts of N. fowleri are less resistant to environmental extremes with the exception of heat, than N. gruberi (Carter, 1970; Chang, 1978). This may be due to the latter possessing both an outer thin and inner thick cyst wall whereas the former possesses only the inner thick cyst wall (Schuster, 1975). This association of soil with PFLA is further emphasized by the fact that many isolations of PFLA were recorded from soil-contaminated pools (i.e. those with soil enclosures) as compared to commercially developed pools (Table IX). The presence of soil either from runoff or via bathers' feet, is probably the main source of bacterial and amebic contamination of commercially developed pools. Direct sampling from the bore or inlet of both the Moana-iti and Opal pools has demonstrated the comparative sterility of the water with no amebae and few bacteria being isolated.

The effect of the bacterial concentration in the water has been shown by Brown, Keys and

Cursons, (pers. comm., 1978) (Figs. 20 & 21). Further Singh (1941, 1945), Chang (1971), Anderson and Jamieson (1974), Danso and Alexander (1975), Danso *et al.* (1975), Menapace *et al.* (1975) and Napolitano *et al.* (1977) have reported either, discrimination by the amebae as regards edibility or toxicity of bacteria as a food source, or, a dependence of the amebic growth rate on the ratio of bacteria : amebae.

Thus in the New Zealand Health Districts sampled, there appears to be a localised geographical distribution of PFLA contaminated pools in the Matamata-Taupo region, and this appears to be influenced, in part, by the particular physical and chemical properties of the pool. *N. fowleri* is the predominant pathogenic species and its geographical distribution is also in accord with the locality of all recorded cases of PAM in New Zealand. Furthermore, those pools characterized as being 'natural' pools having soil enclosures have been shown to be the 'high-risk pools', the soil providing a reservoir of PFLA. Because of the relatively constant temperature of thermal pools and the ability of PFLA to overwinter as resistant cysts, there is no obvious seasonal distribution of PFLA in thermal pools, though preliminary evidence does suggest that rainfall could in part influence this by contaminating pools via runoff from amebic-containing soils. Finally, the bacteriological quality of the water appears also to affect the incidence of isolation of PFLA, in that those pools frequently contaminated with coliforms had a higher rate of isolation.

Thus this survey has brought to light a number of environmental factors which constitute

the basis of an experimental program being initiated at Massey University and which is designed to elucidate the effect of these factors on the distribution of PFLA.

6.2 The Comparative Use of Disinfectants against Free-living Amebae

Disinfection can be defined as 'the killing of the larger portion (but not necessarily all) of the harmful and objectionable micro-organisms in, or on, a medium by means of chemicals, heat or ultraviolet light etc.' (Wang & Peery, 1975). Thus it is not to be confused with sterilization.

The results demonstrate that all four disinfectants examined (i.e. chlorine, chlorine dioxide, deciquam 222 and ozone) possess amebicidal properties and would be suitable for the disinfection of waters contaminated with PFLA. However the ultimate choice of a particular disinfectant will remain closely tied to the chemical and physical characteristics of the water to be tested and the particular properties of the disinfectant (Table XXXI). Whatever disinfectant is chosen, it is generally accepted that the relative efficiency of any disinfectant will be a function of its rate of diffusion through the cell permeability barrier. After penetration of this barrier, it is believed that the disinfectant destroys either some integral structural component, or attacks the cell's metabolic machinery. Because of the absence of a cell wall, it is believed that the initial site of action will be the plasma membranes of the amebae followed by subsequent interruption of metabolism.

6.2.1 The Use of Chlorine as a Disinfectant against Free-living Amebae

Since the introduction of chlorine into water treatment nearly 80 years ago, it has become almost the only method used for active disinfection of potable water supplies (White, 1972). This predominant position has been gained because of:

Table XXXI. Comparison of the Properties of Chlorine, Chlorine dioxide, Ozone and Deciquam 222

Disinfectant Property	Chlorine Cl_2	Chlorine dioxide ClO_2	Ozone O_3	Quarternary Ammonium Compound QAC Deciquam 222
Disinfecting efficiency	Good	Good $\geqslant Cl_2$	Good $\geqslant Cl_2$	Very good $> Cl_2$
Optimum pH	2 - 7	6.5 - 8.5 +	NK	6.4 - 9.6
tp^{o}C	22 - 25°C	30°C	0 - 4°C	20 - 37°C
reaction with Ca^{2+}, Mg^{2+}	-	-	-	+
reaction with NH_3	+	-	-	-
reaction with organic N	+	+	+	very low
reaction with amino acids	+	-	+	+
reaction with proteins	+	+	+	+
toxicity	high	intermediate	high	low
solubility @ 20 - 30°C	good	very good	very low	very good
reaction with UV	+	+	-	-
stability	low	high	very low	high
residual disinfectant	yes	yes	no	yes
Cost	low	high	low	high

NK = not known

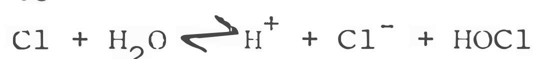
i) its potency and range of effectiveness as a germicide

ii) its ease of application, measurement, control and economy

iii) its relative freedom from toxic or physiological effects and

iv) its reasonable persistence in waters.

When chlorine is added to water it reacts according to



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

HOCl (the active disinfecting species of chlorine) has a dissociation constant(K_a^o) of 2.5×10^{-8} and is therefore affected by the pH of water reacting $HOCl \rightleftharpoons H^+ + OCl^-$. Fig. 22 demonstrates the distribution of HOCl & OCl^- as a function of pH, whilst Fig. 23 compares the germicidal efficiency of chlorine-containing derivatives.

Because chlorine reacts with both organic nitrogenous and ammonia nitrogenous compounds forming organohalides and chloramines respectively (Fig. 24), before active disinfection of such contaminated waters can commence, it is first necessary to satisfy the <u>chlorine demand</u> of the water, i.e. the difference between the amount of chlorine added (TAC) and the amount that can be determined analytically as residual chlorine (FAC). The technique whereby this initial chlorine demand of the water satisfied before active disinfection commences is called '<u>break-point chlorination</u>' the break-point being 'the point where the addition of any more chlorine shows up as an equivalent increase in free available chlorine (FAC). Using a batch system, where only a single dose of chlorine is added, Table XII shows that initial concentrations of 0.74, 0.79, 1.0 and 1.25 $mg.l^{-1}$ Cl_2 had a sterilizing effect on <u>N. fowleri</u>,

Figure 22. The distribution of HOCl and OCl^- as a function of pH. (From Singley, 1971).

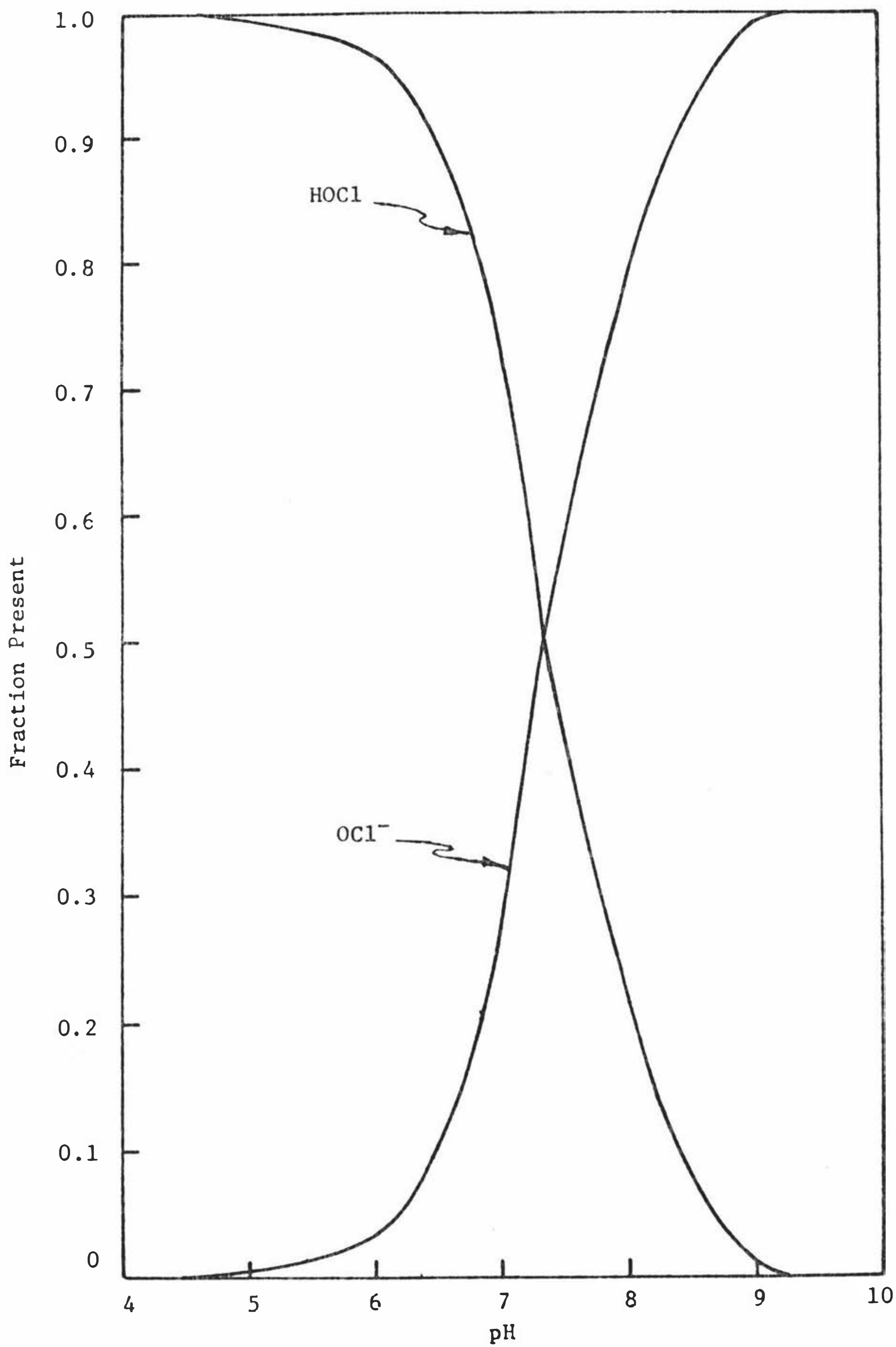
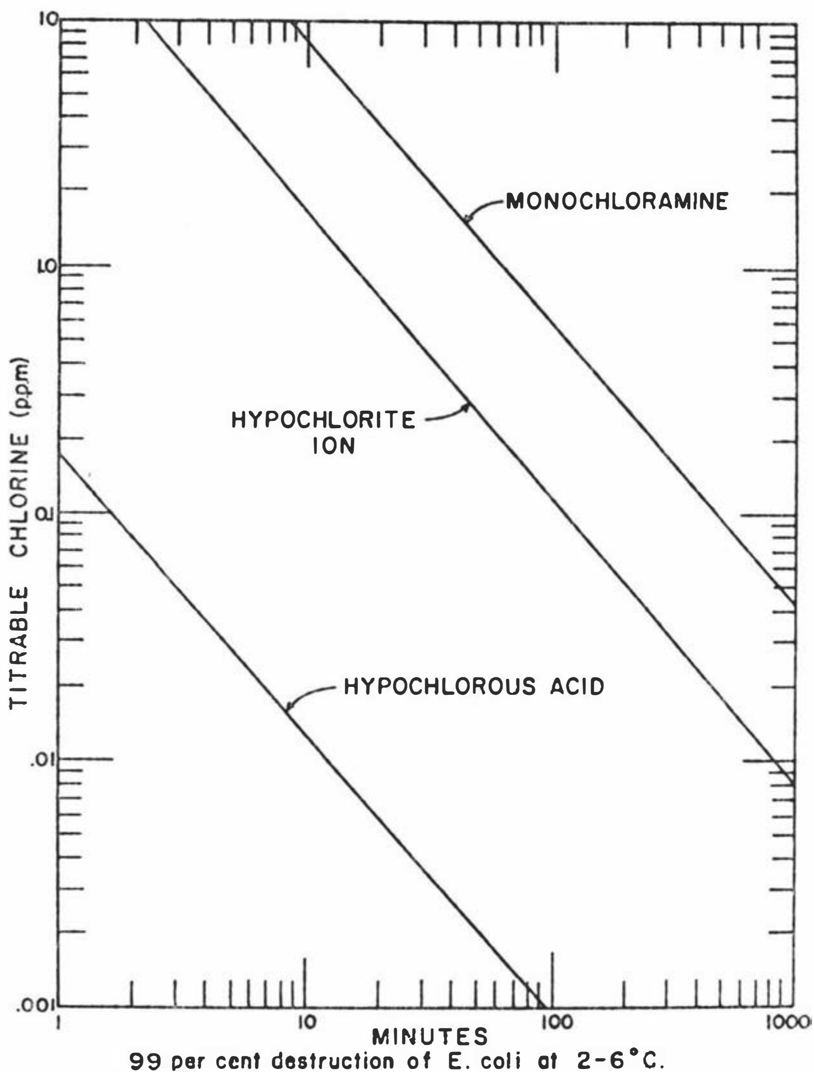
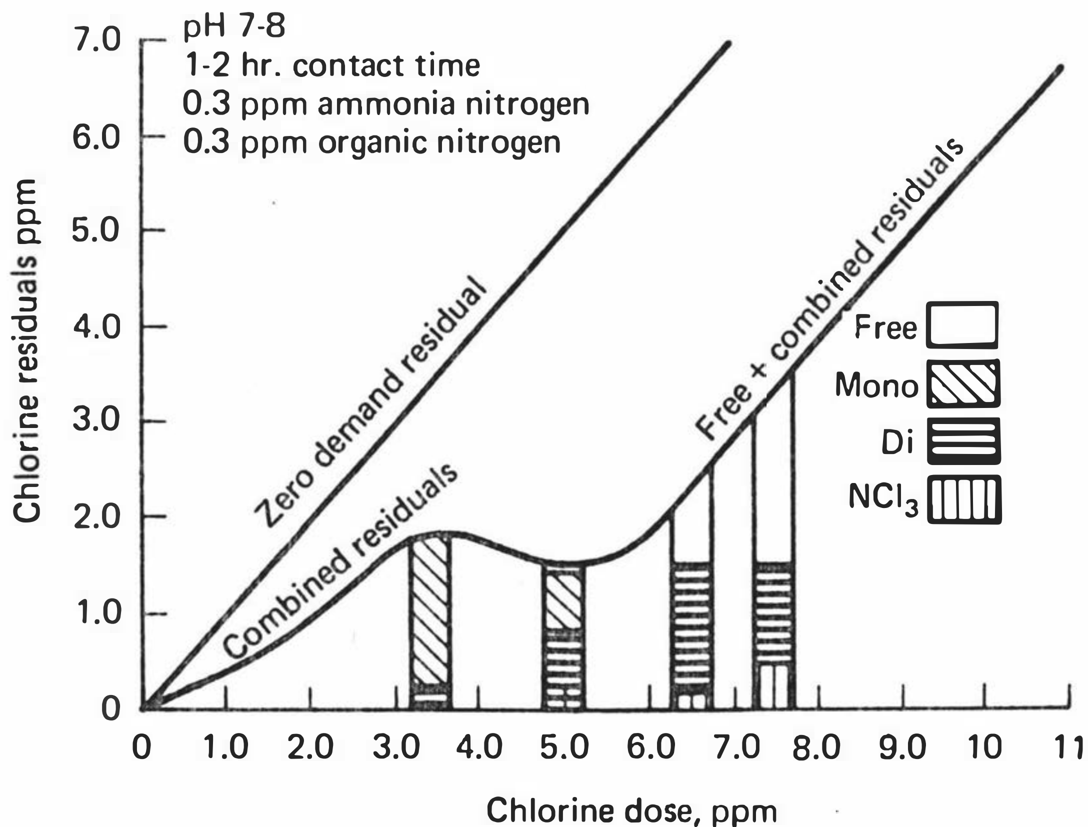

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Fraction Present
HOCl
OCl⁻
4
5
6
7
8
9
10
pH

Figure 23. Relative bactericidal efficiency of chlorine containing derivates. (From White, 1972).

Figure 24. The effect of nitrogen compounds on the chlorine demand. (From White, 1972).



99 per cent destruction of E. coli at 2-6°C.



N. gruberi, A. castellanii and A. culbertsoni respectively. It is thought that the difference in susceptibility to chlorine between the two genera is more likely a consequence of the difference in chemical (especially protein) composition of the cell membranes than differences in metabolism. Experimentally, bactericidal doses of chlorine have been shown to inhibit both glucose oxidation by bacteria (Green & Stumpf, 1946) and sulphydryl enzymes (Knox et al., 1948). More recently, Venkobachar et al. (1977) have demonstrated that chlorine also damages the cell membrane of bacteria resulting in leakage of macromolecules and the complete cessation of oxidative phosphorylation. Thus it is possible that chlorine exerts a double effect on amebae destroying both their cell membrane and metabolism.

The use of chlorine as a disinfectant for PFLA was reported by Anderson and Jamieson (1972) who failed to eliminate N. fowleri which had been superchlorinated with 10.0 $mg.l^{-1}$ of chlorine. However the chlorine demand of the water was not tested and thus there may have been insufficient FAC available for disinfection. Derreumaux et al. (1974) using a batch method, but a different pH and concentration of amebae to those used in this study, demonstrated that water with an initial FAC content of 1.4 $mg.l^{-1}$ of chlorine was able to sterilize 2×10^3 trophozoites $.cm^{-3}$ in 30 minutes. This agrees well with the figure reported in Table XII, i.e. 1.25 $mg.l^{-1}$ chlorine sterilized 1.92×10^4 trophozoites $.cm^{-3}$ in 30 minutes. They also reported Naegleria to be more sensitive to chlorine than Acanthamoeba, as did De Jonckheere

and Van de Voorde (1976) who observed that whereas 10^3 cysts $.cm^{-3}$ of Naegleria were sterilized by 2 $mg.1^{-1}$ of chlorine in 30 minutes, 40 $mg.1^{-1}$ chlorine failed to sterilize Acanthamoebic cysts.

It should be emphasized that all experiments discussed with chlorine have been laboratory batch experiments, which will differ considerably from the conditions found in thermal pools, lakes, rivers and commercial pools. Chlorination in practice involves break-point chlorination to establish, and subsequently maintain, an acceptable residual concentration (FAC) for disinfection. In New Zealand, this varies from 0.1-0.2 $mg.1^{-1}$ for potable water and 0.5 - 0.8 $mg.1^{-1}$ for recreational waters. Thus there is intermittent dosing of the water-supply to meet the fluctuating chlorine-demand of the water. In a swimming-pool situation, the more organic pollutants produced by the bathers, the more FAC will be needed for adequate disinfection. In addition it is important to remember that chlorine as well as the other disinfectants will have an indirect effect on PFLA in potable waters by denying them their bacterial food-source, since most bacteria are destroyed at levels of 0.1 $mg.1^{-1}$ of chlorine (White, 1972), thus starving the amebae and forcing them to encyst. It has been shown that the cyst stage is non-infective (Culbertson, 1971).

6.2.2 The Use of Alternative Disinfectants to Chlorine against Free-living Amebae

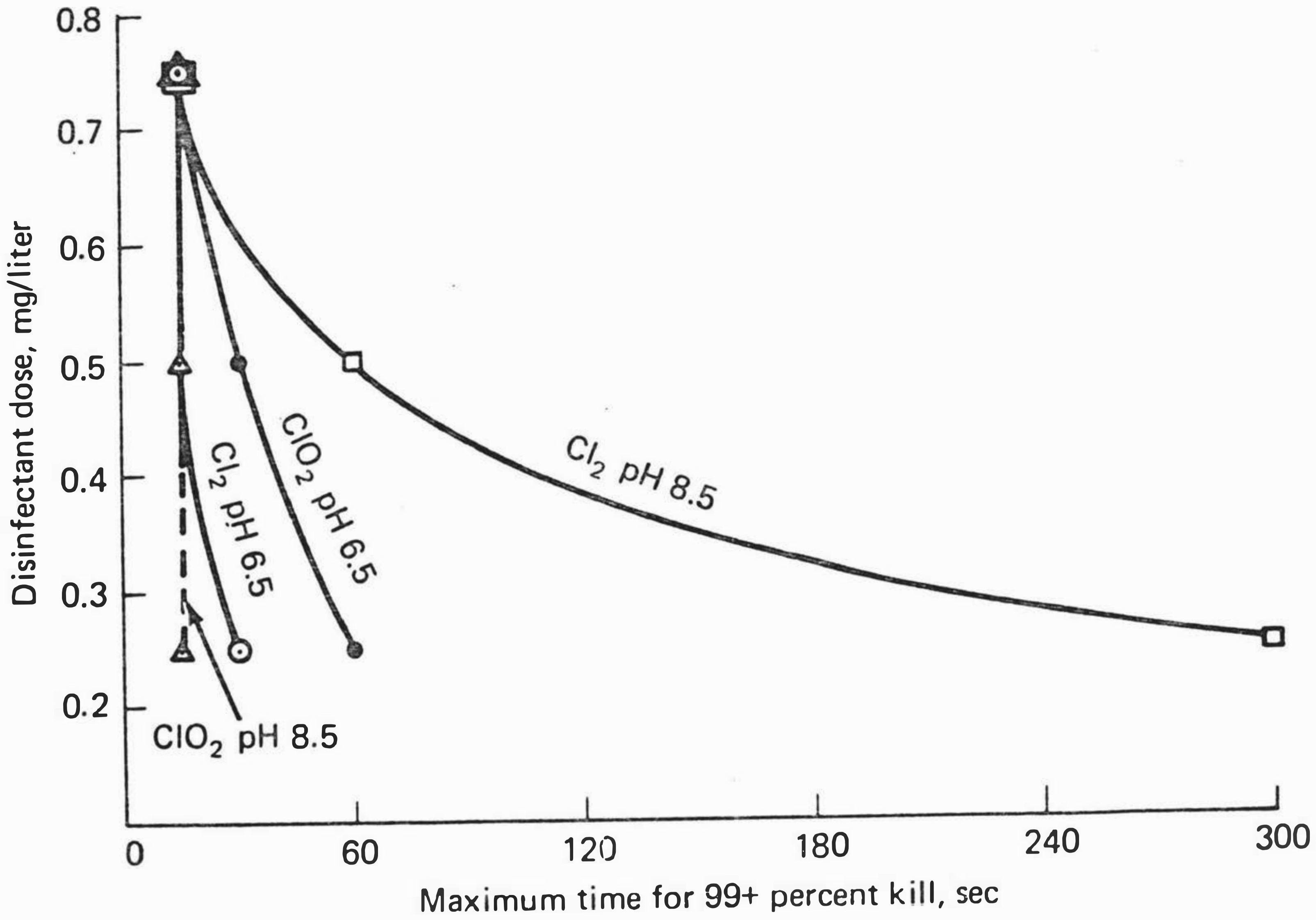
6.2.21 The Use of Chlorine dioxide as a Disinfectant against Free-living Amebae

Although there is relatively little

information on the use of chlorine dioxide as a disinfectant, its mode of action is thought to be the inhibition of protein synthesis (White, 1972). Like chlorine, it is a vigorous and effective oxidizing agent, but is 5 times more soluble in water. Unlike chlorine, it does not react with water or ammonia. It does react with proteins, organic nitrogenous compounds, phenols and aromatics, and thus is useful for the removal of tastes and odours from water. It forms a stable residual and is more active at an alkaline pH. Its main disadvantage is that it is easily expelled from solution and is highly explosive in its gaseous form (Table XXXI). Its relative effectiveness as a germicide as compared to chlorine can be seen from Fig. 25. Table XIII shows that chlorine dioxide is also an effective disinfectant for PFLA. Again Acanthamoeba spp. were more resistant to the disinfectant than Naegleria spp. Because of its particular properties, chlorine dioxide may be a valid alternative to chlorine in those waters with high ammonia contamination and an alkaline pH.

6.2.22 The Use of Ozone as a Disinfectant against Free-living Amebae

The use of ozone as a disinfectant dates back to the 1900's since when it has been extensively used in Europe. Because of the recent association of organohalides, formed in potable water by chlorination, with certain cancers, ozone is receiving more favourable attention


Disinfectant dose, mg/liter
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0
60
120
180
240
300
Maximum time for 99+ percent kill, sec
Cl_2 pH 8.5
ClO_2 pH 6.5
Cl_2 pH 6.5
ClO_2 pH 8.5

because its high germicidal properties and lack of toxic end-products make it a viable alternative to chlorine (Symons & Henderson, 1977). Its main advantages are:

i) it is a very rapid, potent, broad-spectrum germicide active not only against bacteria, but also viruses, parasites and cysts

ii) it does not form any objectionable by-products in water - its end-product is oxygen and thus it lowers both biochemical and chemical oxygen demands (BOD & COD) of water and

iii) it oxidizes taste and odour-causing compounds and removes colour from water.

Compared to these advantages, its disadvantages are:

i) its relatively short half-life of 15-30 minutes does not permit residuals to be maintained

ii) its solubility in water is inversely related to temperature

iii) its high oxidation potential (approximately twice that of chlorine) creates a high ozone demand in most natural waters

iv) unless regular periodic treatment is undertaken, bacterial regrowth occurs very rapidly and

v) it is more expensive than chlorine.

Because of ozone's high oxidation potential, its mode of action is thought to be via chemical oxidation and thus inactivation of essential enzymes (Nebel et al., 1976). Its disinfection activity is often said to be associated with an

'all or nothing' phenomenon and once the ozone demand of the water is satisfied, then disinfection proceeds rapidly. Under the same conditions, it is generally agreed that ozone is a more potent bactericide than chlorine, with concentrations as little as 0.04 $mg.1^{-1}$ of ozone being effective against *Escherichia coli* (Katzenelson *et al.*, 1974).

Table XIII demonstrates that ozone is also amebicidal, but only at much higher concentrations as compared to chlorine or deciquam 222. The relatively higher concentration of ozone needed to constitute amebicidal disinfection is due to both its poor stability, because of its short half-life, and to the ozone demand of the water, probably a function of the greater cytoplasmic mass of amebae as compared to bacteria. This ozone demand is reflected in the difference between the initial and final concentrations of ozone. However no differences in sensitivity to ozone were detected between *Naegleria* and *Acanthamoeba* spp.

6.2.23 The Use of Deciquam 222 as a Disinfectant against Free-living Amebae

Quarternary ammonium compounds (QAC) are synthetic organic disinfectants which are very soluble in water, have no colour and manifest a strong disinfecting action in small quantities while possessing very low toxicity for humans. They are surface-active substances which are easily adsorbed on the surface of solutions, and by virtue of

this, are able to lower the surface tension of solvents. Optimum pH range is usually between 6.4 - 9.6 whilst their biocidal activity is accelerated with increasing temperature (Verbina, 1974). QAC do not react with water and therefore there is no QAC demand, and because of this, possibilities for recycling exist (Wang & Peery, 1975). They are, however, inactivated by hard waters.

Their mode of action is thought to be multiphasic. The cation binds to the acidic groups of the cell membrane neutralizing the membrane potential and thus inhibiting the permeability barrier. Owing to the low surface tension of the QAC, a tighter contact between the solution and microbe is formed, increasing the absorption of the antiseptic at the surface of the microbial cell. This leads to disturbances of the permeability barrier causing leakage of macromolecules as well as denaturation (Verbina, 1974; Wang & Peery, 1975). The relative bactericidal activity of QAC compared to chlorine can be judged from the finding that 3.0 $mg.1^{-1}$ of cetyldimethylbenzylammonium chloride was bactericidal for 2×10^6 coliforms within 15 minutes, whereas 8.0 $mg.1^{-1}$ of chlorine failed to kill 9.8×10^5 coliforms in 30 minutes (Wang & Pek, 1975).

The results in Table XIII show that deciquam 222 is a very potent amebicide being approximately 20 times more

effective than chlorine. Its use has previously been reported by Das and Jadin (1974) who obtained amebicidal concentrations of 0.025 $mg.l^{-1}$. This high amebicidal activity is thought due to the extreme sensitivity of the amebic plasma membrane to surface-active reagents (Carter, 1972). Again no differences in susceptibility between *Naegleria* and *Acanthamoeba* spp. were observed.

In conclusion deciquam 222, chlorine, chlorine dioxide and ozone all possessed potential disinfecting properties for PFLA, but at higher levels than those used for disinfecting bacteria. Of the 4 disinfectants examined, deciquam 222 proved to be the most effective amebicide followed by chlorine, chlorine dioxide and ozone. The final choice of a particular disinfectant, however, must remain tied to the physical and chemical parameters of the water to be disinfected. The necessity for an effective disinfectant can be judged by the increasing number of isolations of free-living amebae from potable and recreational waters (Cerva, 1971a; Chang, 1971; Anderson & Jamieson, 1972; Cerva & Huldt, 1974; Molet *et al.*, 1976; Lyons & Kapur, 1977). The finding that the majority of amebae isolated from these studies belonged to the genus Acanthamoeba, testify to its greater resistance to chlorine than *Naegleria* spp. After a review of 16 fatal cases of PAM from an indoor chlorinated swimming pool, Cerva (1971a) stated that, 'it appears that the constant presence of numerous populations of amebae of the limax group cannot be prevented even under the strictest observations of all routine safety measures applied to potable waters.' However, Lyons and Kapur (1977) in a survey of 30 halogenated public swimming pools concluded that the low amebic densities ($<1 .l^{-1}$) in the majority of

pools illustrated that these organisms could be adequately controlled by proper pool maintenance. The possession of resistant cysts, however, constantly complicates the disinfection process.

6.3 Immunity to PFLA

The ability of a pathogen to develop in its host depends on the balance between the growth-promoting factors, e.g. nutrients required by the organism, and growth-inhibiting factors, e.g. host defence mechanisms, present in the host environment. Successful parasites are considered to be those which can obtain all their requirements from the infected host without causing excessive damage to the latter. Thus well-established parasites, which usually cause chronic infections, have generally reached a homeostatic relationship with the host causing the smallest amount of damage compatible with the need to enter, multiply and be discharged from the host. In this context pathogenic Acanthamoebae may be considered to be more adapted to infecting Man than N. fowleri. This is also reflected by the variety of chronic diseases that Acanthamoeba can cause, e.g. respiratory infections (Culbertson, 1971; Martinez et al., 1975), corneal ulceration (Visvesvara & Jones, 1975), and meningo-encephalitis (Martinez et al., 1977). These conditions are usually associated with some predisposing condition in the host, e.g. undergoing immunosuppressive treatment, in addition to invasion by Acanthamoeba. In this context Acanthamoebae may be regarded as transient commensals except when the host-parasite relationship is upset at which time they show marked virulence. On the contrary, N. fowleri causes an acute meningo-encephalitis (Culbertson, 1971; Carter, 1972). Since both Naegleria and Acanthamoebae are free-living protozoa and the human host is clearly unnecessary for the survival of the amebae, acute infection by N. fowleri may indirectly be a result

of Man's close association with thermal contamination of the environment which selectively favours the more thermophilic _N. fowleri_ (Griffin, 1972) as suggested by De Jonckheere _et al._ (1975).

6.3.1 The Presence of Antibodies to PFLA in Human Sera

In view of the ubiquitous distribution of free-living amebae in the environment (see Introduction 1.2), together with the isolation of _N. fowleri_ and _Acanthamoeba_ spp. from the human nasopharynx (Schumaker _et al._, 1971; Cerva _et al._, 1973; Chang _et al._, 1975), the finding of antibodies to both pathogenic and non-pathogenic free-living amebae in the sera of apparently normal humans was not entirely unexpected. Such intimate contact with Man is highly likely to lead to the induction of an immune response. Previous findings of antibodies to PFLA in human sera are summarised in Table XXXII. More recently, both _Naegleria_ and _Acanthamoeba_ spp. have been implicated in humidifier disease, which is a form of allergic alveolitis, caused by amebae growing in the stagnant water of the humidifier of an air-conditioning unit and being liberated as an aerosol into the atmosphere (M.R.C. Symposium, 1977).

Table XXXII. Previous Reports of Antibodies in Human Sera

Ameba	Serological Test	Titre	Reference
A. castellanii	complement-fixation	1/40 - 1/160	Chang & Owens, 1964
Acanthamoeba spp.	complement-fixation	1/5 - 1/40	Elridge & Tobin, 1967
N. gruberi	gel-diffusion	neat	Edwards *et al.*, 1976
N. fowleri	radio-immunoassay	1/45 - 1/398	Tew *et al.*, 1977
A. castellanii *A. polyphaga*	IFAB gel-diffusion	Neat - 1/100 neat	M.R.C. Symposium, 1977

The results in Tables XIV, XV & XVI show that whereas *Acanthamoebae* had titres ranging from 1/5 - 1/80, *Naegleria* spp. had only a titre of between 1/5 - 1/20. Generally, the intensity of fluorescence and antibody titre of the pathogen, was similar to the non-pathogen and no discrimination was observed between different blood groups or between the two sexes. This serological cross-reactivity between pathogenic and non-pathogenic amebae is probably a result of antigenic similarity (Willaert & Jadin, 1974; Hadas *et al.*, 1977). The results in Table XVII suggest that these antibodies belong to the IgG and IgM classes. Since only IgG crosses the placenta, maternal IgG antibodies are likely to be responsible for the positive reactions with cord sera, as it is unlikely that newborn infants would have had prior contact with PFLA or enough time to synthesise their own antibodies. The finding of specific IgM antibodies in adult sera was surprising in view of its relatively short half-

life and that as the immune response unfolds, IgM antibodies are replaced by IgG antibodies. Since the titre and intensity of the fluorescence of the IgM antibodies closely paralleled that of the IgG antibodies, this may be due to either recent infection or persistent exposure to free-living amebae. In this context, the aforementioned isolation of free-living amebae from the nasopharynx of army recruits (Cerva et al., 1973) could indicate that Acanthamoebae are common transitory human commensals and may explain the higher antibody titre to Acanthamoebae present in normal sera. The observation of a very low IgA titre is not surprising as it has a short half-life and is associated with a localized response.

6.3.2 The Presence of a Specific Neutralizing Factor against PFLA in Normal Human Sera

The detection of a factor present only in fresh adult human sera capable of inhibiting the formation of CPE in Vero cell culture by A. culbertsoni (Table XVIII) suggests that normal human sera contain some 'natural antibody' to Acanthamoebae (Boyden, 1966; Michael, 1969). This view is supported by this factor possessing properties typical of those exhibited by natural antibodies in that:

i) its action was independent of antibody titre (1/10 - 1/20 compared to 1/40 - 1/80)

ii) it was found only in adult but not cord sera

iii) it was specific for A. culbertsoni and not for N. fowleri and

iv) it was heat-labile in that 56°C/30 min inactivated it.

Since the nonspecific term 'natural antibody' is immunologically incorrect however, the factor is best referred to as neutralizing or resistance

factor.

No neutralization was observed with the use of heated or unheated hyperimmune rabbit antisera nor was any neutralization observed against *N. fowleri*. The addition of fresh guinea-pig complement did not affect any of the results. The finding of an amebicidal factor against *Acanthamoebae* in fresh adult sera has previously been reported by Culbertson (1971). He, like Carter (1970), also found that fresh normal human sera were amebicidal for *N. fowleri*. This discrepancy between the neutralization results, reported by Culbertson and Carter to those found in this study are likely to result from differences in assay conditions. Carter reported the highest titre for immobilization and lysis of 10^2 - 10^3 amebae. cm^{-3} to be 1/8. However, the lowest assay titre used in the neutralization of 10^4 amebae. cm^{-3} in Vero cell culture was 1/10. Neutralizing factors in human sera against other protozoans such as *Trichomonas vaginalis* and *Trypanosoma brucei* have also been reported (Honigberg, 1970; Terry, 1976). In the case of *T. brucei* this factor has been shown to be a macroglobulin, possibly IgM (Terry, 1976).

The mode of action of such neutralizing factors remains obscure. Beckerdite-Quagliata *et al.* (1975) reported that normal human serum killing of the bacterium *Serratia marcesens* was due to the activation of the endogenous bacterial phospholipase A and not serum phospholipase A. In this context the lysis of trophozoites by normal human sera (Carter, 1970) may be the result of a similar phenomenon, since both pathogenic and non-pathogenic free-living amebae have also been shown to possess a phospholipase A (Cursons

et al., 1978c). These neutralizing factors may be responsible for the localization of N. fowleri infections to the central nervous system and the need for some predisposing condition before invasion by Acanthamoebae is successful.

6.3.3 The Induction of the Cell-Mediated Immune (CMI) System

Previous work by Diffley et al. (1976) demonstrated that CMI plays an important role in the defence of guinea-pigs infected subcutaneously with N. fowleri. Culbertson et al. (1972) observed granuloma formation, an indicator of CMI (Barrett, 1974), in guinea-pigs infected subcutaneously with N. fowleri, and the finding of granuloma as distinctive pathological lesions in Acanthamoebae infections has been commented upon (Culbertson, 1971; Martinez et al., 1977). Further support of CMI playing a decisive role in resistance to infection by PFLA is demonstrated by the increased susceptibility to infection by PFLA exhibited by cortisone treated animals (Culbertson, 1971; Wong et al.,.1975a & b). This is thought to be due to the suppression of the CMI system, i.e. the T-lymphocytes, by cortisone and related steroids. Although cortisone can suppress the primary IgG response to some extent, the secondary IgG response is largely resistant to steroid hormones (Barrett, 1974).

The results in Tables XIX & XX and Plates 25-30 confirm that both the in vitro MIF and in vivo DH correlates of CMI were able to respond to both heterologous and homologous antigens. As expected, the homologous reaction was greater than the heterologous combination. The similar antigenic identity between pathogenic and non-pathogenic species of free-living amebae has

been commented on by others (Willaert *et al.*, 1972; Visvesvara & Healy, 1975; Willaert & Jadin, 1974; Cursons & Brown, 1976; Hadas *et al.*, 1977) and it is likely that these common group antigens reacting with the T-lymphocyte receptors are responsible for the cross-reactions. Thus *in vitro*, T-lymphocytes obtained from guinea-pigs sensitized to *N. gruberi* antigens responded by producing the lymphokine MIF when presented with *N. fowleri* antigens. John *et al.*, (1977) have shown that mice previously immunized with either live *N. gruberi* or dead *N. fowleri* were significantly protected against a subsequent normally lethal challenge of *N. fowleri*. Further, *N. gruberi* proved to be the more effective immunogen. Thus support is given to their hypothesis that Man's unwitting exposure to the more ubiquitous *N. gruberi*, may immunize him against *N. fowleri*. It is likely that a similar immunization by non-pathogenic *Acanthamoebae* against pathogenic *Acanthamoebae* could occur.

6.3.4 Immune Responses in Relation to Infection with PFLA

Like most microbial infections, infections with PFLA begin at the surface of the mucosal membranes. Infection with *N. fowleri* has been shown to be via disruption of the olfactory mucosa, penetration into the submucosal nervous plexus and passage through the cribriform plate to the subarachanoid space (Carter, 1970; Culbertson, 1971; Martinez *et al.*, 1973). Although intranasal infection by direct extension into the central nervous system (CNS) has also been shown with *A. culbertsoni* (Culbertson, 1971), involvement of the CNS appears to be a secondary phenomenon representing metastatic spread from a

primary focus in the skin, genitourinary or respiratory tracts (Martinez *et al.*, 1977). Following intranasal inoculation, the amebae must resist the cleansing action of the mucociliary blanket and avoid being caught up in the mucus and swallowed. Although no direct attachment to the nasal epithelial cells by *N. fowleri* has been observed, it appears that the mucociliary blanket actively encourages residence of *Naegleria*, with amebae actively destroying it (Carter, 1970; Martinez *et al*., 1973). Chang (1974b) reported that the nasal discharge of allergic rhinitides actually supported a limited multiplication of Naegleria trophozoites with associated CPE in epithelial cells. It is at this stage that secretory IgA may be important in inhibiting the adherence of the amebae to the surface of the mucosal cells thereby preventing entry into the body tissues and thus resistance. In this context, local immune reponse in various mucosal surfaces can be stimulated best by local application of an antigen and it has been demonstrated that intranasal instillation of poliovirus results in the prompt appearance of IgA poliovirus antibody (Ogra *et al.*, 1975). Recent evidence also suggests that local respiratory tract CMI may be important in host defence to a wide spectrum of infectious disease agents and may be relatively independent of systemic CMI (Ballanti *et al.*, 1975). Because IgA has a relatively short half-life of 6 days as compared 25 days for IgG, protective immunity is short-lived and accordingly resistance to respiratory diseases is short-lived. Since it takes a few days for antibodies or immune cells to be formed in appreciable amounts and delivered to the site of infection, previous or continuous exposure in the form of transitory commensals, may play an important part in resistance.

Following destruction of the mucociliary blanket, the amebae are phagocytozed by the sustentacular cells of the olfactory epithelium. This appears to be the most frequent avenue utilized in initial penetration of the mucosal barrier. At this stage the host reaction consists of a slight purulent inflammation with typical associated infiltration by polymorphonuclear leukocytes (PMN) which are observed to be attacking the amebae (Carter, 1970; Martinez et al., 1973).

Deep amebic invasion occurs when the organisms pierce the basal membrane and the lamina propria, reaching the submucosal plexus (Martinez et al., 1973). Once amebae have reached the sub-epithelial tissues, they are exposed to the host defence system comprising:

i) phagocytic cells
ii) antimicrobial substances in body tissues and fluids
iii) inflammation
iv) humoral antibody and
v) CMI

Owing to the delay between induction and a specific effective immune response, the first line of defence consists of the constitutive phagocytes, namely neutrophils, monocytes and macrophages. Experimental evidence supporting this comes from the use of gnotobiotic animals. Although gnotobiotic animals have the same immunological potential as conventional animals, because of their 'sterile' environment, only their constitutive defense system, as opposed to both the constitutive and inducible defence systems of conventional animals, is operating. Thus it has been shown that gnotobiotic guinea-pigs infected

intranasally with N. fowleri exhibit the same symptoms and a similar course of events as those of normal guinea-pigs infected with N. fowleri by the same route (Cerva, 1971b; Phillips, 1974). Histologically, the host's immune response, consisting of inflammation, engulfment of amebae by phagocytes, the formation of multinucleated giant cells together with the close proximity of eosinophils, lymphocytes, plasma cells and phagocytes to the amebae, is seen in pathological lesions of meningo-encephalitis caused by either N. fowleri or Acanthamoeba spp. (Carter, 1970; Culbertson, 1971; Martinez et al., 1973, 1975, 1977; Visvesvara & Callaway, 1974; Sarphie & Allen, 1977; Schuster & Dunnebacke, 1977). Destruction of phagocytes by amebae can also occur (Visvesvara & Callaway, 1974).

To escape the active phagocytosis of PMN and monocytes, amebae invade the unmyelinated axons of the filia olfactoria and are also seen within the perineural spaces with no accompanying inflammatory response. Via this path amebae eventually reach the subarachnoid space, olfactory tracts, olfactory bulbs and contiguous CNS structures (Martinez et al., 1973). The end result shows the nasal mucosa with extensive loss of surface epithelium, severe purulent inflammation and partial necrosis of the submucosa with massive amebic invasion. The nasal cavities are filled with a purulent and haemorrhagic exudate containing numerous amebae. Many amebae are seen in olfactory nerve bundles which are virtually destroyed by purulent inflammation, as are the olfactory lobes. Necrosis stops abruptly at the posterior margin of the olfactory lobe, the adjacent 1 mm or so of cerebral hemisphere showing only moderate, acute inflammation, focal haemorrhage and moderate diffuse amebic invasion. More

posteriorly, tissue reaction is virtually absent, although amebae are freely invading brain tissue. In addition blood vessels are closely packed with amebae within their adventitia and perivascular spaces (Carter, 1970; Martinez *et al.*, 1973).

Because of the acute nature of PAM caused by *N. fowleri* there is little evidence from such infections implicating inducible systems of immunity (namely humoral and CMI) in an active defence role. Although specific complement-fixing antibodies have been observed in guinea-pigs infected with *N. fowleri* (Cerva, 1971b), the results in Table XVIII suggest that antibodies and complement are ineffective directly against the amebae. This may be due to redistribution and endocytosis of surface bound antibody complexes, similar to that seen in *N. gruberi* (Preston *et al.*, 1975) and *Entamoeba histolytica* (Aust-Kettis & Sundqvist, 1978), thus providing the amebae with a powerful mechanism of escape from destruction by host antibodies. Antibodies however, may function indirectly in host defence as opsonins, promoting phagocytosis via the Fc receptors of neutrophils and macrophages. Experimentally, inducible systems of immunity have been shown to play an active role in resistance to infection with *N. fowleri*. Mice previously sensitized to a single dose with either *N. fowleri* or *N. gruberi* via the intraperitoneal or intravenous routes were more resistant to rechallenge with a normally fatal dose of *N. fowleri* by the intraperitoneal, intravenous or intranasal routes (Adams *et al.*, 1976; John *et al.*, 1977; Thong *et al.*, 1978). That this was an inducible response was emphasized by the observation that acquired protective immunity took 3 weeks to develop and was seen to wane 7

weeks after immunization (Thong *et al.*, 1978).

A more active role in defence by the CMI system is suggested by Diffley *et al.* (1976) who found that guinea-pigs which recovered from *N. fowleri* infections exhibited DH when tested with soluble *N. fowleri* antigens. Other evidence implicating CMI in a more active role comes from the increased susceptibility to infection with *N. fowleri* in immunosuppressed animals (Wong *et al.*, 1975a & b). Specifically with Acanthamoeba infections, the formation of granuloma in an attempt to wall off the organism (Culbertson, 1971; Martinez *et al.*, 1977) provides further evidence for an active CMI role in defence. The ability of T-lymphocytes as compared to B-lymphocytes to respond may be a consequence of their prolonged half-life and greater distribution throughout the body. Generally, humoral responses to protozoa develop when the organisms invade the bloodstream as in malaria and trypanosomiasis, whereas CMI is usually developed in the tissues as in cutaneous leishmaniasis (Roit, 1977).

In summary, despite the seemingly high incidence of PFLA in the environment, most primates, and perhaps most domestic animals, appear to be able to resist infection by PFLA. Defence against PFLA is thought to be mediated by both constitutive and inducible immune systems as well as non-specific factors of immunity. Such resistance may occur via contact with the more prevalent antigenically-related non-pathogenic species sensitizing the immune system.

6.4 Virulence of PFLA

6.4.1 The Presence of Extracellular Phospholipases in the Supernatants of Axenic Cultures of Free-living Amebae

In a recent review on lipolytic enzymes, Brockerhoff and Jensen (1974) stated that phospholipase 2 has proven to be of ubiquitous distribution and is found in:

i) the venoms of snakes and bees
ii) as digestive enzymes and
iii) as intracellular enzymes occurring in the tissues of animals or in micro-organisms.

By and large, lysophospholipases are found wherever they are looked for. The appearance of both phospholipase 2 and lysophospholipase in the same cell is common, as lysophospholipase is universally distributed in the cells of animals, plants and micro-organisms, where its activity regularly exceeds that of phospholipase 2 which supplies the highly lytic lysophospholipid substrate (Brockerhoff & Jensen, 1974).

Amongst the protozoa, phospholipase 2 has recently been reported in *Trypanosoma congolense* (Tizard *et al.*, 1977) and possibly *Entamoeba histolytica* (McCaul *et al.*, 1977). Specifically with free-living amebae, phospholipase 2 has previously been demonstrated in both pathogenic and non-pathogenic *Acanthamoeba* spp. (Elson *et al.* 1970; Hax *et al.*, 1974; Victoria & Korn, 1975; Visvesvara & Balamuth, 1975) whilst a filtrate from pathogenic *Naegleria* spp. has been reported by Chang (1971; 1974a & b; 1976) Cursons and Brown (1976, 1978) and Wong *et al.* (1977). Furthermore Dunnebacke and Schuster (1977) have reported an intracellular cytotoxic material from Naegleria lysates termed by these workers Naegleria associated infectious material (NAIM).

The finding, therefore, of both a phospholipase 2 and lysophospholipase in the supernatants of both pathogenic and non-pathogenic free-living amebae, was not altogether unexpected. The possibility of the cytotoxic filtrate from Naegleria being a phospholipase was first suggested by Cursons and Brown (1976, 1978). Chang (1976) identified a phospholipolytic enzyme or factor from the same pathogenic N. fowleri strain MsT, as well as other strains, which hydrolyzed sphingomyelin to choline, sphingosine and fatty acids. The presence of fatty acids as a degradation product suggests a phospholipase 2 (Table XXI), and so may be the same enzyme. As regards NAIM, although this is an intracellular lysate, it shares many properties with the partially purified phospholipase 2 from N. fowleri (MsT), Cursons et al. (1978c). Interestingly enough, freeze-thaw destruction of packed N. fowleri (MsT) also released phospholipase 2 activity. Thus it is possible that the NAIM also may possess phospholipase 2 activity and must warrant further investigation.

In summary, cell-free supernatants of axenically grown free-living amebae, have been shown to possess both phospholipase 2 and lysophospholipase activity.

6.4.2 Production of Phospholipase 2 in Axenic Cultures of Free-living Amebae

In a recent review by Glenn (1976), an extracellular or exoenzyme was described as "one which exists in the medium around the cell, having originated from the cell without any alteration to cell structure greater than the maximum compatible with the cell's normal process of growth and reproduction". The synthesis of

of many exoenzymes is influenced by cultural conditions. Specifically they appear to be subject to control by end-product inhibition, i.e. the repression of exoprotease synthesis by amino acids, or catabolic repression, e.g. synthesis of α-amylase is repressed by glucose. Some exoenzymes follow the growth cycle, and others, more closely, the stationary phase (Glenn, 1976).

It has been hypothesized that in eukaryotes, exoenzymes are synthesised on the ribosomes of the rough endoplasmic reticulum (RER) from whence they are transported to the cisternae of the smooth endoplasmic reticulum (SER), elements of which eventually fuse with the golgi complex. They are further processed in the golgi complex and packaged in secretory vesicles which fuse with the plasma membrane releasing their contents by exocytosis. Further, there is a substantial body of evidence supporting the concept that membrane-bound polysomes in eukaryote cells are engaged in the synthesis of exportable proteins, whereas free polysomes are active in the synthesis of non-exportable proteins (Glenn, 1976). Unlike bacterial exoenzymes, eukaryote ones tend to be large molecules containing disulphide bridges and very frequently have carbohydrate portions. It is thought that the continuing addition of sugar-units, as the protein moves through the various organelles, may often be crucial in rendering the enzyme hydrophilic and releasing it from the secretory vesicle. It appears that the phospholipase 2 produced by *Naegleria* spp. closely follows exponential growth, whereas the phospholipase 2 produced by *Acanthamoeba* spp. is tied to the stationary phase (Tables XXIII & XXIV). Besides phospholipase 2, a full spectrum of lysosomal enzymes have been

identified in N. fowleri (Feldman, 1977) and Acanthamoebae (Muller, 1969; Childs, 1973; Ryter & Bowers, 1976; Stevens et al., 1977b). Many authors have implicated lysosomal enzymes as the extracellular means by which cells effect digestion and/or invasion (Allison, 1967; Glauert et al., 1969). The enhanced invasive capacity of Entamoeba histolytica by the extracellular activity of lysosomal enzymes illustrates this point (Lushbaugh & Miller, 1974). It is highly likely that the phospholipase 2 and lysophospholipase produced by free-living amebae are also lysosomal enzymes; such 'packaging' protecting the cell from the hydrolytic enzymes until released by exocytosis. Although no clear differences have been found in the protein components of the plasma membrane of Acanthamoebae as a function of cell density (Wilkins & Thompson, 1974), an increase in lysolecithin content of the membrane and a decrease in agglutinability of cells by concanavalin A has been reported (Hax et al., 1974; Spies et al., 1975). The difference observed in phospholipase 2 production between Naegleria spp. and Acanthamoeba spp. may be a consequence of a different transport and processing machinery in the former due to its total lack of golgi apparatus which has been shown to be present in the latter (Lastovica, 1975; Ryter & Bowers, 1976).

Feldman (1977) suggests that instead of the classical endoplasmic reticulum (ER), to golgi, to membrane-bound packet scheme, that lysosomal enzymes are synthesized on the system of membrane-associated polysomes and channeled via the ER to a variety of cellular locations.

The production of more phospholipase 2 by

the pathogenic strains as compared to the non-pathogenic strains (Tables XXIII & XXIV) suggests that the relative production of this enzyme may contribute to the virulence of the pathogens. Visvesvara and Balamuth (1975) demonstrated that the pathogenic A. culbertsoni (A-1) produced comparatively more phospholipase 2 than the non-pathogenic A. castellanii (Singh). Chang (1971 & 1974a) established that pathogenic Naegleria strains produced a filtrate cytotoxic for primary monkey kidney (PMK) cells. In a later paper comparing the virulence of pathogenic strains, Chang (1976) correlated the ability of those strains producing early CPE with their ability to produce a phospholipolytic factor. Wong et al. (1977), in comparing high and low virulence strains of N. fowleri also observed a similar phenomenon in that only the highly virulent strains produced a cytolytic filtrate in PMK cells. Similarly Table XXIII demonstrates that the non-pathogenic N. fowleri (Ts-1) produced comparatively less phospholipase 2 as compared with N. fowleri strains MsT and NHI, and that this correlated with its low virulence in Vero cell culture.

6.4.3 Preliminary Isolation of Phospholipase 2 from Axenic Cultures of N. fowleri (MsT)

Because N. fowleri (MsT) produced comparatively more phospholipase 2 than the other Naegleria strains (Table XXIII) this strain was chosen for enzyme production studies. Owing to the inherent variabilities in agar diffusion assays, no quantitation of specific activity could be determined. Table XXV shows the inhibitory effect of different concentrations of protein samples and demonstrates why specific activity was not determined, i.e. dilute protein samples readily diffuse through the agar

producing a relatively large zone whereas more concentrated protein samples were inhibited and consequently produce smaller zones. Habermann and Hardt (1972) upon whose method the assay is based, also found a dose-relationship response, in that reproducibility was only consistent over a limited range of protein concentrations. Although the assay is not quantitative in its present form it is still useful for a comparative assessment of phospholipase activity. PAGE however revealed enzyme purification as judged by visualization of two protein bands which displayed characteristic phospholipase 2 and lysophospholipase activity. Antiserum raised against these proteins displayed only two precipitin lines in gel-diffusion plates as would be expected if only two proteins constituted the antigenic dose. This specific antiserum also completely inhibited the clearing of EY agar by the phospholipase 2 present in the cell-free filtrate from axenic cultures of N. fowleri (MsT). This inhibition was thought to be the result of neutralization of the phospholipase 2 enzyme by the antiserum, by combining with the enzyme's antigenic determinants, and thus prohibiting the enzyme from combining with the active sites of its phospholipid substrates.

6.4.4 Virulence of Amebae and Cell-free Axenic Filtrates in Vero Cell Culture

The formation of CPE in cell culture, as an indicator of pathogenicity of PFLA, depends upon a number of varying factors such as virulence of the amebae, the concentration and age of the inoculum and the susceptibility of the cell type. The results in Tables XXVI & XXVII show that the pathogenic strains (MsT, NHI & A-1) exhibited greater virulence towards Vero cell culture, as

compared to the non-pathogenic strains, by producing not only earlier CPE at an equivalent inocula of amebae, but also CPE at much lower inocula. Examination of monolayers infected with either N. fowleri (MsT) or A. culbertsoni (A-1), has revealed a close association between the Vero cells and amebae with the trophozoites surrounded by a clear halo, as well as extensive lipolysis (Plates 41 - 43).

The virulence of these pathogenic strains was also associated with their ability to produce comparatively more phospholipase 2 in their axenic supernatants, as compared to the non-pathogenic strains in each genus examined (Tables XXIII & XXIV). Furthermore it was shown that cell-free filtrates from either pathogenic or non-pathogenic axenic cultures were cytopathic towards Vero cell culture, depending upon the relative concentration of phospholipase 2 present in the filtrate (Table XXVIII). These results suggest a close relationship between the observed virulence of free-living amebae towards cell cultures and the relative production of phospholipase 2. This close relationship is further supported by:

i) the demonstrations that supplementation of the fluid of N. fowleri (Ts-1) inoculated cell cultures with non-cytotoxic amounts of the cell-free filtrate from axenic cultures of N. fowleri (MsT), resulted in the formation of CPE in a time comparable to the pathogenic N. fowleri strains (Table XXIX) and

ii) that different concentrations of the specific anti-phospholipase 2 and lysophospholipase serum both inhibited and delayed the formation of CPE by N. fowleri (MsT) (Table XXX), presumably by 'neutralizing' the membrane-active phospholipase 2. This 'neutralization' of the formation of CPE by N. fowleri strains parallels their virulence and relative production of phospholipase 2, i.e. those virulent strains producing more phospholipase 2 were the least inhibited by the

anti-serum.

6.4.5 The Significance of Phospholipase 2 Production and its Relationship to the Pathogenicity of PFLA

Virulence, or the capacity of a given pathogen to produce disease, is dependent upon many features of an organism, including its invasiveness, its ability to survive and reproduce in the face of the host's defence mechanisms and its production of toxins antagonistic to the host. The possession of highly lytic enzymes capable of disrupting biological membranes would thus enhance the invasiveness, and hence pathogenic potential, of a micro-organism. Experimentally, phospholipase 2 from snake venom has been shown to hydrolyze myelin *in vitro* (Banik *et al.*, 1976), whilst lysophospholipids, the intermediate product of phospholipase 2 reactions, have been shown to be powerful cytotoxic substances producing intense inflammation (Phillips *et al.*, 1965). Specifically, the free fatty acids produced by the phospholipase 2 of *T. congolense* (Tizard *et al.*, 1977) have been reported to:

i) lyse washed sheep erythrocytes and kill mouse peritoneal macrophages and lymphocytes
ii) suppress T-cell immunity
iii) destroy vascular endothelium and
iv) provoke a myocarditis.

Finally the lethality of many venoms (snake, bee, scorpion) is also due to the presence of phospholipase 2.

Examination of sections of brain infected with PFLA has revealed a close association between the host tissue and amebae. Areas of extensive demyelination with the trophozoites

surrounded by a clear halo are also seen (Martinez et al., 1975; Chang, 1976; Maitra et al., 1976). This has led many authors to speculate on the possibility that either secretion of cytolytic enzymes, phagocytosis of the host cell, or a combination of both are responsible for the pathological lesions (Chang, 1974a, 1976; Visvesvara & Callaway, 1974; Visvesvara & Balamuth, 1975; Cursons & Brown, 1976, 1978; Maitra et al., 1976). Maitra et al. (1976) also reported that, whereas A. culbertsoni had been shown to form only pinocytic channels in brain tissue, both phagocytosis and pinocytosis of host tissue by N. fowleri had been observed.

The close relationship between the relative production of phospholipase 2 with the observed virulence of free-living amebae towards cell cultures suggests that this extracellular enzyme is important in the pathogenicity of these organisms. It is likely that the secretion of such an enzyme constitutes the initial steps whereby host tissue is prepared for endocytosis by these amebae. High levels of a membrane-lytic exoenzyme, such as phospholipase 2, may well be an essential factor for the breakdown of host tissue for pinocytosis by virulent Acanthamoeba spp., and is likely to enhance phagocytosis of host tissue by virulent N. fowleri. Visvesvara and Balamuth (1975) showed that the greater cytopathogenicity of A. culbertsoni, as compared to A. castellanii, was dependent on the former's ability to produce comparatively more phospholipase 2, as was also found with the pathogenic and non-pathogenic Acanthamoebae examined in this study. In a comparative study of different geographical strains of N. fowleri, Chang (1976) correlated the ability of strains which produced early CPE and rapid cell destruction with their ability both to degrade sphingomyelin and to cause severe pathological changes in the white matter of infected brains. Finally Wong et al. (1977)

reported that the pathogenic N. fowleri strains HB-1 and C-66 lost their original virulence and this was associated with a delay in the formation of CPE in cell culture, not only when amebae were added to cell cultures, but also by cell-free filtrates from axenic cultures. Restoration of the original virulence, accomplished by passaging in mice, also resulted in increased formation of CPE not only by amebae, but also by the filtrates.

In addition to the ability to produce a phospholipase 2, other factors must also be involved in pathogenicity, since non-pathogens were also shown to produce the enzyme. Obviously the ability to tolerate and reproduce at 37°C is important. In this context McIntosh and Chang (1971) noted that 3 Acanthamoeba strains which were non-lethal for chick embryos at 37°C were subsequently lethal when the temperature of incubation was dropped to 33°C. Thus because of the multifactorial nature of pathogenicity any lack in the full complementation of pathogenic characteristics will result in considerable attenuation of the strain concerned. In this context the non-pathogenic N. fowleri strains may represent phospholipase-deficient mutants, which, because of this deficit are relatively avirulent.

APPENDICES

Appendix 1. Isolation of Amebae and related Environmental Data from selected North Island Thermal Pools in 1976.

1976 Waingaro Springs

Date	Source	Amebae Non-pathogens	Amebae Pathogens	Total Coliforms .100 cm^{-3}	Total Bacteria .1.0 cm^{-3} at 37 °C	pH	Tp °C	Max. Tp °C	Min. Tp °C	Soil 30 cm. Tp °C	Rain prior to 7 days of sampling
12/1	water	+	-	ND	ND	9.2	46	23.6	20.0	23.7	47.0 mm
24/5	"	-	-	"	"	9.0	39	16.0	-0.7	12.7	7.9 mm
14/6	"	-	-	"	"	8.9	39	10.5	-2.4	11.5	27.5 mm
20/7	"	+	-	"	"	9.0	39	13.4	2.7	11.2	31.1 mm
16/8	"	-	-	"	"	8.8	39	16.5	9.4	13.6	179.8 mm
13/9	"	-	-	22	56	8.8	39	14.9	9.0	13.1	185.7 mm
8/11	"	-	-	ND	ND	ND	ND	17.0	6.9	18.2	58.8 mm

\+ = positive isolation

\- = no isolation

ND = Not Done

1976 Moana-iti Springs

Date	Source	Amebae		Total Coli-forms .100 cm^{-3}	Total Bacteria .1.0 cm^{-3} at 37 °C	pH	Tp °C	Max. Tp °C	Min. Tp °C	Soil 30 cm. Tp °C	Rain prior to 7 days of sampling
		Non-pathogens	Pathogens								
12/1	water	+	*N. fowleri*	ND	ND	6.8	39	23.6	20.0	23.7	47.0 mm
16/2	"	+	-	"	"	7.4	34	23.1	10.1	22.7	2.9 mm
24/5	"	+	-	"	"	7.8	32	16.0	-0.7	12.7	7.9 mm
14/6	"	+	*N. fowleri*	"	"	7.6	30	10.5	-2.4	11.5	27.5 mm
20/7	"	+	-	"	"	7.1	39	13.4	2.7	11.2	31.1 mm
16/8	"	+	-	"	"	7.5	33	16.5	9.4	13.6	179.8 mm
13/9	"	+	-	3	200	7.6	31	14.9	9.0	13.1	185.7 mm
11/10	"	+	*N. fowleri*	"	"	7.6	33	22.1	11.9	17.3	19.9 mm
8/11	"	+	-	"	"	7.2	33	17.0	6.9	18.2	58.8 mm
6/12	"	+	-	"	"	7.4	32	26.4	11.5	22.2	19.6 mm

\+ = positive isolation

\- = no isolation

ND = Not Done

1976 Opal Springs

Date	Source	Amebae Non-pathogens	Amebae pathogens	Total Coliforms .100 cm^{-3}	Total Bacteria .1.0 cm^{-3} at 37 °C	pH	Tp °C	Max. Tp °C	Min. Tp °C	Soil 30 cm. Tp °C	Rain prior to 7 days of sampling
12/1	water	-	-	ND	ND	6.8	39	23.6	20.0	23.7	47.0 mm
16/2	"	+	N. fowleri	"	"	6.8	40	23.1	10.1	22.7	2.9 mm
24/5	"	+	N. fowleri A. polyphaga	"	"	6.8	40	16.0	-0.7	12.7	7.9 mm
14/6	"	-	-	"	"	6.8	39	10.5	-2.4	11.5	27.5 mm
20/7	"	-	-	"	"	7.0	39	13.4	2.7	11.2	31.1 mm
16/8	"	+		"	"	7.0	41	16.5	9.4	13.6	179.8 mm
13/9	"	+	A. polyphaga	35	uncountable	7.6	33	14.9	9.0	13.1	185.7 mm
11/10	"	+	N. fowleri	"	"	7.6	33	22.1	11.9	17.3	19.9 mm
8/11	"	-	-	"	"	6.8	40	17.0	6.9	18.2	58.8 mm
6/12	"	-	-	"	"	7.4	39	26.4	11.5	22.2	19.6 mm

+ = positive isolation

- = no isolation

ND = Not Done

1976 Okoroire Springs

Date	Source	Amebae		Total Coliforms $.100\ cm^{-3}$	Total Bacteria $.1.0\ cm^{-3}$ at 37 °C	pH	Tp °C	Max. Tp °C	Min. Tp °C	Soil 30 cm. Tp °C	Rain prior to 7 days of sampling
		Non-pathogens	pathogens								
12/1	water	+	*N. fowleri*	ND	ND	6.8	42	23.6	20.0	23.7	47.0 mm
16/2	"	+	*N. fowleri*	"	"	6.8	36	23.1	10.1	22.7	2.9 mm
24/5	"	+	*A. polyphaga*	"	"	6.8	34	16.0	-0.7	12.7	7.9 mm
14/6	"	+	-	"	"	7.2	35	10.5	-2.4	11.5	27.5 mm
20/7	"	+	-	"	"	7.2	36	13.4	2.7	11.2	31.1 mm
16/8	"	+	-	"	"	7.0	36	16.5	9.4	13.6	179.8 mm
13/9	"	+	-	11	500	6.8	37	14.9	9.0	13.1	185.7 mm
11/10	"	-	-	ND	ND	7.2	36	22.1	11.9	17.3	19.9 mm
8/11	"	-	-	"	"	7.4	34	17.0	6.9	18.2	58.8 mm
6/12	"	+	-	"	"	6.9	35.5	26.4	11.5	22.2	19.6 mm

+ = positive isolation

- = negative isolation

ND = Not Done

1976 Soda Springs

Date	Source	Amebae Non-pathogens	Amebae pathogens	Total Coliforms .100 cm^{-3}	Total Bacteria .1.0 cm^{-3} at 37 °C	pH	Tp °C	Max. Tp °C	Min. Tp °C	Soil 30 cm. Tp °C	Rain prior to 7 days of sampling
9/3	water	+	*N. fowleri*	ND	ND	6.0	28.3	19.3	15.0	ND	0.7 mm
1/6	"	+	-	"	"	6.0	37	15.5	7.5	"	10.7 mm
29/6	"	-	-	"	"	5.8	36	12.7	7.3	"	0.4 mm
27/7	"	-	-	280	"	6.0	37	10.6	0.9	"	1.4 mm
24/8	"	+	-	90	"	5.8	36	14.0	4.7	"	ND
21/9	"	+	-	ND	"	6.0	36.5	14.5	2.1	"	0.7 mm
19/10	"	+	-	35	"	6.0	37	18.2	9.4	"	65.8 mm
15/11	"	+	-	ND	"	5.8	35	18.1	3.5	"	13.0 mm
16/12	"	+	-	"	"	5.8	37	23.5	12.0	"	11.7 mm

+ = positive isolation

- = no isolation

ND = Not Done

1976 Butcher's Pool

Date	Source	Amebae		Total Coli-forms $.100\ cm^{-3}$	Total Bacteria $.1.0\ cm^{-3}$ at 37^oC	pH	Tp ^oC	Max. Tp ^oC	Min. Tp ^oC	Soil 30 cm. Tp ^oC	Rain prior to 7 days of sampling
		Non-pathogens	Pathogens								
9/3	water	+	-	ND	ND	7.5	35	19.3	15	ND	0.7 mm
1/6	"	-	-	"	"	7.5	41	15.5	7.5	"	10.7 mm
29/6	"	+	-	"	"	7.5	35	12.7	7.3	"	20.3 mm
27/7	"	-	-	-	"	7.5	35	10.6	0.9	"	1.4 mm
24/8	"	+	-	ND	"	7.5	35	14.0	4.7	"	ND
21/9	"	-	-	"	"	8.0	35	14.5	2.1	"	0.7 mm
19/10	"	-	-	"	"	7.5	35	18.2	9.4	"	65.8 mm
15/11	"	+	-	"	"	7.5	35	18.1	3.5	"	13.0 mm

+ = positive isolation

- = no isolation

ND = Not Done

1976 Otumaheke Stream: 1/2 water

Date	Source (Water)	Amebae Non-Pathogens	Amebae Pathogens	Total Coliforms .100 cm^{-3}	Total Bacteria .1.0 cm^{-3} at 37 °C	pH	Tp °C	Max. Tp °C	Min. Tp °C	Soil 30 cm. Tp °C	Rain prior to 7 days of sampling
9/3	Mouth Upper	+ +	N. fowleri N. fowleri	ND "	ND "	ND "	45 18	19.6 ND	15.0 ND	19.4 ND	0.7 mm
31/5	Mouth Upper	+ +	N. fowleri N. fowleri	" "	" "	7.5 7.4	21 15	13.4 ND	9.4 ND	9.9 ND	19.9 mm
29/6	Mouth Upper	+ +	N. fowleri N. fowleri	" "	" "	7.4 7.4	31 15	12.1 ND	3.7 ND	7.1 ND	17.7 mm
27/7	Mouth Upper	+ +	N. fowleri N. fowleri	870 12	" "	ND ND	20 13	11.6 ND	0.6 ND	6.9 ND	0.8 mm
21/9	Mouth Upper	+ +	- N. fowleri	" "	" "	7.7 7.65	20 14	15.4 ND	-1.4 ND	9.9 ND	0.5 mm
18/10	Mouth Upper	+ +	N. fowleri -	" "	" "	7.35 7.32	22 20	16.5 ND	10.4 ND	13.5 ND	59.2 mm
15/11	Mouth Upper	+ +	- A. polyphaga	" "	" "	7.65 7.50	26 17.5	15.7 ND	5.9 ND	13.6 ND	4.3 mm
13/12	Mouth Upper	+ +	N. fowleri -	" "	" "	7.25 7.6	43 21	23.3 ND	9.3 ND	17.6 ND	44.3 mm

+ = positive isolation

- = negative isolation

ND = Not Done

1976 Te Puia Springs

Date	Source	Amebae		Total Coliforms $.100\ cm^{-3}$	Total Bacteria $.1.0\ cm^{-3}$ at 37 °C	pH	Tp °C	Max. Tp °C	Min. Tp °C	Soil 30 cm. Tp °C	Rain prior to 7 days of sampling
		Non-pathogens	pathogens								
2/2	water	-	-	ND	ND	ND	ND	ND	ND	ND	ND
9/6	"	-	-	"	"	7.6	28.9	13.1	6.2	"	5.44 mm
6/7	"	*Acanthamoeba* sp.	-	"	"	7.6	27.8	11.5	-1.3	"	24.9 mm
31/7	"	-	-	nil	$2x10^4$	7.6	27.0	13.4	5.5	"	3.0 mm
1/9	"	-	-	nil	$5.6x10^2$	7.7	26.7	9.5	6.0	"	33.7 mm
27/9	"	-	-	50	$1x10^2$	7.6	28	10.1	8.4	"	143.1 mm
19/10	"	+	-	nil	$1.1x10^4$	7.6	35	18.4	9.7	"	79.9 mm
22/11	"	+	-	nil	$4.8x10^2$	7.6	35	18.0	12.6	"	33.7 mm
21/12	"	-	-	nil	$2.5x10^2$	7.15	35	15.0	12.4	"	30.6 mm

+ = positive isolation

- = negative isolation

ND = Not Done

1976 Morere Springs

Date	Source	Amebae Non-pathogens	Amebae Pathogens	Total Coliforms .100 cm^{-3}	Total Bacteria .1.0 cm^{-3} at 37°C	pH	Tp °C	Max. Tp °C	Min. Tp °C	Soil 30 cm. Tp °C	Rain prior to 7 days of sampling
2/2	water	+	*Acanthamoeba* sp.	ND	ND	ND	ND	ND	ND	ND	ND
9/6	"	+	-	"	"	7.1	36.7	13.1	6.2	"	5.4 mm
6/7	"	*Acanthamoeba* sp.	-	"	"	7.3	36.6	11.5	-1.3	"	24.9 mm
31/7	"	-	-	"	"	7.2	38	13.4	5.5	"	3.0 mm
1/9	"	-	-	3	38	7.2	48.9	9.5	6.0	"	33.7 mm
27/9	"	-	-	-	2.5×10^2	7.1	43	10.1	8.4	"	143.1 mm
26/10	"	-	-	7.3	1.96×10^3	7.1	40	12.8	8.4	"	79.9 mm
22/11	"	+	-	-	2.4×10^3	7.1	42.8	18.0	12.6	"	33.7 mm
21/12	"	-	-	-	uncountable	6.7	41.1	15.0	12.4	"	30.6 mm

+ = positive isolation

- = no isolation

ND = Not Done

Appendix 2. Isolation of Amebae from Water and Soil in 1977.

1977 Moana-iti Springs

Date	Source	Amebae Non-pathogens	Amebae Pathogens	Total Coliforms $.100cm^{-3}$ or 1.0 g^{-1}	Total Bacteria 1.0 cm^{-3} or 1.0 g^{-1} at 37°C	pH	Tp °C	Max. Tp °C	Min. Tp °C	Soil 30 cm. Tp °C	Rain prior to 7 days of sampling
7/3	Water Soil	+ +	- -	nil ND	UC ND	ND ND	ND ND	26.5 ND	14.1 ND	ND 24.4	6.3 mm
23/3	Water Soil	+ +	- -	ND $> 1.61x10^5$	ND ND	ND ND	ND ND	24.6 ND	16.2 ND	ND 21.3	45.2 mm
4/4	Water Soil	+ +	- -	$> 1.61x10^5$ $3.8x10^4$	UC ND	7.0 ND	24 ND	21.7 ND	8.4 ND	ND 21.6	2.1 mm
18/4	Water Soil	- -	- -	nil $5.16x10^4$	8 $> 10^8$	7.4 ND	34 ND	20.2 ND	14.0 ND	ND 19.1	35.7 mm
2/5	Water Soil	+ +	- -	nil $4.7x10^4$	UC $1.11x10^6$	7.5 ND	33 ND	15.0 ND	11.8 ND	ND 16.0	23.1 mm
17/5	Water Soil	- +	- -	1 $4.8x10^4$	UC $1.4x10^6$	7.5 ND	34 ND	13.9 ND	8.3 ND	ND 14.0	16.7 mm
30/5	Water Soil	+ +	- -	ND $5.2x10^3$	ND $1.22x10^4$	ND ND	ND ND	14.6 ND	11.4 ND	ND 13.6	22.3 mm
21/6	Water Soil	- +	- -	nil $2.35x10^5$	nil $2.16x10^6$	6.8 ND	39 ND	14.1 ND	8.1 ND	ND 12.6	64.7 mm

... Cont'd

18/7	Water Soil	- -	- -	nil 9.9×10^{3}	5 1.17×10^{6}	7.0 ND	40 ND	13.3 ND	6.7 ND	ND 12.9	8.1 mm
1/8	Water Soil	+ -	- -	nil 1.7×10^{4}	1.4×10^{4} 2.1×10^{6}	6.8 ND	39 ND	16.0 ND	10.7 ND	ND 11.8	38.2 mm
15/8	Water Soil	- -	- -	3.5×10^{3} 1.7×10^{4}	9.3×10^{3} 1.68×10^{6}	6.7 ND	39 ND	13.7 ND	9.0 ND	ND 12.1	44.7 mm
29/8	Water Soil	- +	- -	nil 2.2×10^{5}	nil 3.22×10^{7}	ND ND	40 ND	13.4 ND	8.7 ND	ND 14.3	30.9 mm
12/9	Water Soil	+ +	- -	6 9.6×10^{5}	1.85×10^{5} 1.0×10^{7}	6.8 ND	39 ND	16.1 ND	1.1 ND	ND 12.4	6.2 mm
26/9	Water Soil	+ -	- -	nil 2.0×10^{5}	nil 1.79×10^{6}	6.8 ND	39 ND	15.9 ND	2.7 ND	ND 13.6	49.1 mm
10/10	Water	-	-	nil	1	ND	39	21.5	7.6	ND	27.0 mm
25/10	Water	+	-	17	4.7×10^{3}	ND	32	16.5	10.8	ND	3.5 mm
7/11	Water Soil	- +	- -	nil 4.3×10^{3}	36 9.4×10^{6}	7.4 ND	33 ND	19.9 ND	10.3 ND	ND 17.7	19.0 mm
21/11	Water Soil	- +	- -	1.61×10^{2} 1.1×10^{4}	1.7×10^{3} 4.4×10^{6}	7.0 ND	36 ND	19.8 ND	17.0 ND	ND 19.6	25.3 mm
6/12	Water Soil	+ +	- -	nil 1.3×10^{4}	2.0×10^{2} 5.9×10^{6}	ND ND	ND ND	ND ND	ND ND	ND ND	ND

+ = positive isolation

- = no isolation

ND = Not Done

UC = uncountable

1977 Opal Springs

Date	Source	Amebae		Total Coli-forms $.100cm^{-3}$ or.1.0 g^{-1}	Total Bacteria 1.0 cm^{-3} or.1.0 g^{-1} at 37°C	pH	Tp °C	Max. Tp °C	Min. Tp °C	Soil 30 cm. Tp °C	Rain prior to 7 days of sampling
		Non-pathogens	Pathogens								
7/3	Water Soil	- +	- N. fowleri	nil ND	9 ND	6.8 ND	40 ND	26.5 ND	14.1 ND	ND 24.4	6.3 mm
23/3	Water Soil	- +	- N. fowleri	nil ND	2.2x10 ND	6.8 ND	39 ND	24.6 ND	16.2 ND	ND 21.3	45.2 mm
4/4	Water Soil	- +	- -	$5.4x10^4$ $6.8x10^5$	UC ND	7.2 ND	39 ND	21.7 ND	8.4 ND	ND 21.6	2.1 mm
18/4	Water Soil	- +	- N. fowleri	265 $2.4x10^5$	$2.65x10^2$ $> 10^8$	6.8 ND	39 ND	20.2 ND	14.0 ND	ND 19.1	35.7 mm
2/5	Water Soil	+ +	- -	nil $1.7x10^3$	UC $1.0x10^6$	7.0 ND	37 ND	15.0 ND	11.8 ND	ND 16.0	23.1 mm
17/5	Water Soil	- +	- -	nil $6.25x10^6$	UC $6.0x10^7$	7.1 ND	38 ND	13.9 ND	8.3 ND	ND 14.0	16.7 mm
30/5	Water Soil	- +	- -	ND $2.4x10^4$	ND $5.1x10^5$	ND ND	ND ND	14.6 ND	11.4 ND	ND 13.6	22.3 mm
21/6	Water Soil	- +	- -	24 $1.97x10^4$	55 $3.9x10^6$	6.8 ND	39 ND	14.1 ND	8.1 ND	ND 12.6	64.7 mm
4/7	Water Soil	+ +	N. fowleri N. fowleri	13 $5.5x10^5$	$4.3x10^4$ $1.2\ 10^6$	7.4 ND	34 ND	13.3 ND	6.7 ND	ND 12.9	53.2 mm

... Cont'd

18/7	Water Soil	- -	- -	nil 2.74×10^{4}	5 1.22×10^{6}	7.2 ND	ND ND	16.0 ND	10.7 ND	ND 11.8	1.9 mm
1/8	Water	+	<u>N. fowleri</u>	nil	10	7.4	31	13.7	9.0	ND	38.2 mm
15/8	Water Soil	- +	- <u>N. fowleri</u>	nil 1.2×10^{4}	10^{2} 1.87×10^{7}	6.9 ND	38 ND	14.5 ND	11.8 ND	ND 12.8	44.7 mm
29/8	Water Soil	+ +	- -	53 1.11×10^{5}	6.1×10^{4} 1.93×10^{7}	ND ND	33 ND	13.4 ND	8.7 ND	ND 14.3	30.9 mm
12/9	Water Soil	- -	- -	nil 9.3×10^{3}	nil 3.54×10^{6}	7.4 ND	32 ND	16.6 ND	1.1 ND	ND 12.4	6.2 mm
26/9	Water Soil	- +	- -	nil 2.73×10^{5}	nil 4.78×10^{6}	7.3 ND	39 ND	15.9 ND	2.7 ND	ND 13.6	49.1 mm
10/10	Water Soil	- +	- -	nil 5.9×10^{4}	nil 1.6×10^{6}	ND ND	39 ND	21.5 ND	7.6 ND	ND 15.8	27.0 mm
25/10	Water Soil	- +	- <u>A. poly- phaga</u>	nil 5.0×10^{4}	nil 8.0×10^{6}	ND ND	39 ND	16.5 ND	10.8 ND	ND 16.3	3.5 mm
7/11	Water Soil	- +	- -	nil 9.2×10^{4}	40 1.6×10^{7}	7.2 ND	40 ND	19.9 ND	10.3 ND	ND 17.7	19.0 mm
21/11	Water Soil	- +	- -	nil 9.4×10^{5}	nil 1.2×10^{7}	7.0 ND	39 ND	19.8 ND	17.0 ND	ND 19.6	25.3 mm
6/12	Water Soil	+ +	- -	nil 1.4×10^{4}	2.4×10^{4} 1.3×10^{6}	7.2 ND	37 ND	ND ND	ND ND	ND ND	ND

+ = positive isolation - = no isolation ND = Not Done UC = uncountable

1977 Okoroire Springs

Date	Source	Amebae: Non-pathogens	Amebae: Pathogens	Total Coliforms $.100\ cm^{-3}$ or $.1.0g^{-1}$	Total Bacteria $.1.0\ cm^{-3}$ or $.1.0g^{-1}$ at 37°C	pH	Tp °C	Max. Tp °C	Min. Tp °C	Soil 30 cm. Tp °C	Rain prior to 7 days of sampling
7/3	Water	+	-	B	UC	7.0	37	26.5	14.1	ND	6.3 mm
	Soil	+	-	ND	ND	ND	ND	ND	ND	24.4	
23/3	Water	-	-	1.6×10^5	UC	7.1	37	24.6	16.2	ND	45.2 mm
	Soil	+	*N. fowleri*	ND	ND	ND	ND	ND	ND	21.3	
4/4	Water	+	-	1.6×10^5	UC	6.8	34	21.7	8.4	ND	2.1 mm
	Soil	+	*N. fowleri*	1.0×10^5	ND	ND	ND	ND	ND	21.6	
18/4	Water	+	-	1.61×10^2	UC	7.0	34	20.2	14.0	ND	35 7
	Soil	+	-	4.63×10^6	$>10^8$	ND	ND	ND	ND	19.1	
2/5	Water	-	-	54	UC	6.8	35	15.0	11.8	ND	23.1 mm
	Soil	+	-	3.7×10^5	3.0×10^6	ND	ND	ND	ND	16.0	
17/5	Water	+	-	2.21×10^5	UC	7.2	34	13.9	8.3	ND	16.7 mm
	Soil	+	-	5.6×10^5	1.04×10^6	ND	ND	ND	ND	14.0	
30/5	Water	+	-	ND	ND	ND	ND	14.6	11.4	ND	22.3 mm
	Soil	+	-	2.5×10^5	ND	ND	ND	ND	ND	13.6	
21/6	Water	+	-	2.4x10	5.4×10^2	6.6	ND	14.1	8.1	ND	64.7 mm
	Soil	+	-	1.03×10^4	2.5×10^5	ND	ND	ND	ND	12.6	
4/7	Water	-	-	5.4×10^1	4.1×10^3	7.0	35	13.3	6.7	ND	53.2 mm
	Soil	+	-	5.7×10^3	6.8×10^5	ND	ND	ND	ND	12.9	

... Cont'd

18/7	Water Soil	+ +	*N. fowleri* *N. fowleri*	nil 3.43×10^{3}	2.4×10^{4} 1.2×10^{5}	7.0 ND	36 ND	16.0 ND	10.7 ND	ND 11.8	8.1 mm
1/8	Water Soil	- -	- -	nil 7.68×10^{3}	1.47×10^{3} 2.17×10^{6}	6.6 ND	35 ND	13.7 ND	9.0 ND	ND 12.1	38.2 mm
15/8	Water Soil	+ +	*N. fowleri* -	5 2.0×10^{4}	3.8×10^{4} 4.43×10^{6}	6.6 ND	32 ND	14.5 ND	11.8 ND	ND 12.8	44.7 mm
29/8	Water Soil	+ +	- -	35 6.0×10^{5}	1.9×10^{3} 1.13×10^{6}	ND ND	ND ND	13.4 ND	8.7 ND	ND 14.3	30.9 mm
12/9	Water Soil	+ +	*A. polyphaga* -	nil 2.12×10^{6}	$> 10^{5}$ 3.0×10^{6}	7.2 ND	30 ND	16.1 ND	1.1 ND	ND 12.4	6.2 mm
26/9	Water Soil	+ +	*N. fowleri* -	2 4.7×10^{5}	6.8×10^{3} 2.1×10^{6}	6.8 ND	35 ND	15.9 ND	2.7 ND	ND 13.6	49.1 mm
10/10	Water Soil	+ +	*N. fowleri* -	2 5.3×10^{3}	UC 5.6×10^{5}	ND ND	39 ND	21.5 ND	7.6 ND	ND 15.8	27.0 mm
25/10	Water Soil	+ +	*A. polyphaga* -	24 1.4×10^{3}	2.9×10^{4} 9.2×10^{5}	ND ND	36 ND	16.5 ND	10.8 ND	ND 16.3	3.5 mm
7/11	Water Soil	- -	- -	12 6.8×10^{4}	3.06×10^{6} 2.3×10^{6}	7.0 ND	36 ND	19.9 ND	10.3 ND	ND 17.7	19.0 mm
21/11	Water Soil	+ -	- -	2 5.7×10^{4}	1.25×10^{4} 5.7×10^{6}	7.2 ND	33 ND	19.8 ND	17.0 ND	ND 19.6	25.3 mm
6/12	Water Soil	+ +	- -	24 3.2×10^{4}	5.0×10^{2} 2.8×10^{6}	ND ND	ND ND	ND ND	ND ND	ND ND	ND

+ = positive isolation   - = no isolation   ND = Not Done   UC = uncountable

1977 Otumaheke Stream: 1/2B; 1/3 water

Date	Source	Amebae: Non-pathogens	Amebae: Pathogens	Total Coliforms $.100\ cm^{-3}$ or $.1.0g^{-1}$	Total Bacteria $.1.0\ cm^{-3}$ or $.1.0g^{-1}$ at 37°C	pH	Tp °C	Max. Tp °C	Min. Tp °C	Soil 30 cm. Tp °C	Rain prior to 7 days of sampling
28/2	Water	+	-	ND	ND	7.8	28	23.6	11.2	ND	34.8 mm
	Soil	+	-	"	"	ND	ND	ND	ND	18.8	
7/3	Water	+	-	"	"	ND	27	22.5	12.6	ND	0.4 mm
	Soil	+	-	"	"	ND	ND	ND	ND	19.8	
14/3	Water	+	-	"	"	7.9	26	21.8	13.0	ND	0.1 mm
	Soil	+	-	"	"	ND	ND	ND	ND	19.2	
21/3	Water	+	-	"	"	ND	25	16.1	7.0	ND	No rain
	Soil	+	_N. fowleri_	$2.1x10^4$	"	ND	ND	ND	ND	18.2	
28/3	Water	+	-	ND	"	ND	25	22.0	5.4	ND	46.8 mm
	Soil	+	-	$6.6x10^4$	"	ND	ND	ND	ND	16.4	
4/4	Water	+	-	ND	"	ND	29	18.2	6.1	ND	2.9 mm
	Soil	+	-	$3.4x10^4$	"	ND	ND	ND	ND	16.4	
12/4	Water	+	-	ND	"	ND	25	18.5	7.4	ND	12.5 mm
	Soil	+	-	$2.4x10^4$	"	ND	ND	ND	ND	15.6	
19/4	Water	+	_N. fowleri_	ND	"	ND	25	16.6	9.8	ND	7.1 mm
	Soil	+	_N. fowleri_	$6.1x10^3$	$> 10^8$	ND	ND	ND	ND	14.7	
27/4	Water	+	-	ND	ND	ND	21.5	12.4	4.6	ND	17.2 mm
	Soil	+	-	$9.1x10^4$	$3.8x10^5$	ND	ND	ND	ND	12.2	

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2/5	Water Soil	+ +	*A. polyphaga* *N. fowleri*	ND 3.8×10^{4}	ND 1.51×10^{6}	ND ND	24 ND	12.4 ND	4.9 ND	ND 10.6	40.1 mm
9/5	Water Soil	+ +	- -	2.5×10^{2} 5.6×10^{4}	ND 4.01×10^{6}	" "	21 ND	12.7 ND	-1.4 ND	ND 9.0	2.2 mm
16/5	Water Soil	+ +	- *N. fowleri*	ND 4.5×10^{4}	ND 5.7×10^{5}	" "	21 ND	13.4 ND	10.5 ND	ND 10.2	12.8 mm
23/5	Water Soil	+ +	- -	UC 3.7×10^{5}	UC 1.95×10^{6}	" "	37 ND	13.4 ND	9.4 ND	ND 10.5	52.2 mm
30/5	Water Soil	+ +	- -	1.2×10^{3} 2.5×10^{3}	3.3×10^{3} 8.6×10^{5}	" "	20 ND	10.1 ND	4.0 ND	8.5 ND	43.4 mm
7/6	Water Soil	+ +	*N. fowleri* -	3.85×10^{2} 6.7×10^{4}	4.25×10^{3} 6.7×10^{5}	" "	ND ND	10.4 ND	1.2 ND	ND 7.7	34.7 mm
13/6	Water Soil	+ +	*N. fowleri* -	3.8×10^{3} 1.17×10^{4}	7.25×10^{3} 4.03×10^{5}	" "	41 ND	12.1 ND	6.4 ND	ND 7.0	20.9 mm
20/6	Water Soil	+ +	- -	8.2×10^{2} 3.92×10^{2}	2.9×10^{4} 1.77×10^{5}	" "	38 ND	8.9 ND	1.1 ND	ND 8.5	39.3 mm
27/6	Water Soil	+ +	- -	6.21×10^{3} 1.65×10^{3}	1.93×10^{4} 1.24×10^{5}	" "	38 ND	13.1 ND	6.1 ND	ND 8.0	26.2 mm
4/7	Water Soil	+ +	*N. fowleri* -	2.6×10^{2} 3.9×10^{4}	3.11×10^{4} 8.15×10^{5}	" "	35 ND	9.8 ND	3.4 ND	ND 8.3	68.4 mm

... Cont'd

12/7	Water Soil	+ +	*N. fowleri* -	5.2×10^3 4.5×10^5	UC UC	ND "	35 ND	10.5 ND	-0.9 ND	ND 6.6	4.8 mm
18/7	Water Soil	+ +	- -	ND 1.88×10^3	ND 6.02×10^4	" "	ND ND	11.0 ND	2.1 ND	ND 6.5	1.0 mm
25/7	Water Soil	+ +	- *N. fowleri*	ND 8.9×10^4	ND 1.39×10^7	" "	39 ND	10.2 ND	1.6 ND	ND 8.0	45.6 mm
8/8	Water	-	*A. polyphaga*	ND	ND	"	ND	12.4	3.1	ND	4.5 mm
18/8	Water	+	-	ND	ND	"	40	12.1	4.1	ND	38.2 mm
22/8	Water	+	*A. polyphaga*	ND	ND	"	45	14.6	3.0	ND	12.4 mm
30/8	Water	+	-	ND	ND	"	36	7.7	4.9	ND	12.1 mm
6/9	Water Soil	+ +	- -	ND 4.4×10^5	ND 5.01×10^7	" "	ND ND	9.3 ND	3.9 ND	ND 8.1	23.8 mm
13/9	Water Soil	+ +	- -	ND ND	ND 4.19×10^5	" "	ND ND	9.9 ND	1.4 ND	ND 8.2	1.7 mm
19/9	Water Soil	+ +	- -	ND 4.63×10^3	ND 3.5×10^6	" "	40 ND	14.2 ND	1.5 ND	ND 9.1	32.9 mm
27/9	Water Soil	+ +	- -	ND 2.9×10^3	ND 7.38×10^6	" "	ND ND	13.4 ND	1.7 ND	ND 9.4	6.2 mm

... Cont'd

4/10	Water Soil	+ +	<u>N. fowleri</u> -	ND 1.8×10^4	ND 1.1×10^7	ND "	ND "	15.6 ND	2.1 ND	ND 11.1	18.9 mm
17/10	Water Soil	+ +	- -	ND 4.0×10^3	ND 1.0×10^7	" "	" "	16.0 ND	6.0 ND	ND 12.0	1.8 mm
31/10	Water Soil	+ +	- -	ND 1.5×10^4	ND 1.5×10^6	" "	" "	16.0 ND	7.6 ND	ND 15.2	8.3 mm
8/11	Water Soil	+ +	- -	ND 1.1×10^6	ND 5.7×10^7	" "	" "	17.9 ND	3.4 ND	ND 14.5	17.8 mm
18/11	Water Soil	+ +	- -	ND 2.5×10^4	ND 2.7×10^6	" "	42 ND	18.3 ND	9.5 ND	ND 15.1	6.5 mm

+ = positive isolation

- = no isolation

ND = Not Done

UC = uncomparable

1977 Otumaheke Stream: 1/2C Soil

Date	Source	Amebae Non-Pathogens	Amebae Pathogens	Total Coliforms $.1.0\ g^{-1}$	Total Bacteria $.1.0\ g^{-1}$ at 37^oC	pH	Tp ^oC	Max. Tp ^oC	Min. Tp ^oC	Soil 30 cm. Tp ^oC	Rain prior to 6 days of sampling and on sample day
28/2	Soil	+	*N. fowleri*	ND	ND	ND	ND	23.6	11.2	18.8	34.8 mm
7/3	Soil	+	*N. fowleri*	ND	ND	"	"	22.5	12.6	19.8	0.4 mm
14/3	Soil	+	*N. fowleri*	ND	ND	"	"	21.8	13.0	19.2	0.1 mm
21/3	Soil	+	-	1.26×10^5	ND	"	"	16.1	7.0	18.2	No rain
28/3	Soil	+	*N. fowleri*	1.56×10^5	ND	"	"	22.0	5.4	16.4	46.8 mm
4/4	Soil	+	-	1.1×10^4	ND	"	"	18.2	6.1	16.4	2.9 mm
12/4	Soil	+	-	3.1×10^5	ND	"	"	18.5	7.4	5.6	12.5 mm
19/4	Soil	+	-	3.5×10^5	$> 10^8$	"	"	16.6	9.8	14.7	7.1 mm
27/4	Soil	+	-	5.8×10^4	4.5×10^7	"	"	12.4	4.6	12.2	19.2 mm
2/5	Soil	+	-	3.6×10^4	4.1×10^7	"	"	12.4	4.9	10.6	40.1 mm
9/5	Soil	+	-	1.92×10^5	1.33×10^7	"	"	12.7	-1.4	9.0	2.2 mm
16/5	Soil	+	-	1.02×10^6	9.5×10^7	"	"	13.4	10.5	10.2	2.8 mm

... Cont'd

23/5	Soil	+	-	6.27×10^{3}	2.87×10^{6}	ND	ND	13.4	9.4	10.5	52.2 mm
30/5	Soil	+	-	4.4×10^{3}	5.02×10^{7}	"	"	10.1	4.0	8.5	43.4 mm
7/6	Soil	+	-	3.05×10^{5}	5.37×10^{7}	"	"	10.4	1.2	7.7	34.7 mm
13/6	Soil	+	-	2.07×10^{4}	3.02×10^{6}	"	"	12.1	6.4	7.0	20.9 mm
20/6	Soil	+	-	3.5×10^{3}	4.6×10^{5}	"	"	8.9	1.1	8.5	39.3 mm
27/6	Soil	+	-	1.02×10^{4}	7.2×10^{6}	"	"	13.1	6.1	8.0	26.2 mm
4/7	Soil	+	-	3.18×10^{5}	1.4×10^{7}	"	"	9.8	3.4	8.3	68.4 mm
12/7	Soil	+	-	1.2×10^{4}	UC	"	"	10.5	-0.9	6.6	4.7 mm
18/7	Soil	+	-	1.16×10^{4}	1.58×10^{6}	"	"	11.0	2.1	6.5	1.0 mm
25/7	Soil	+	-	6.70×10^{4}	1.0×10^{7}	"	"	10.2	1.6	8.0	45.6 mm
8/8	Soil	+	-	2.4×10^{5}	8.13×10^{7}	"	"	12.4	3.1	6.9	4.5 mm
18/8	Soil	+	-	1.54×10^{6}	2.76×10^{7}	"	"	12.1	4.1	9.1	38.2 mm
22/8	Soil	+	-	2.8×10^{5}	2.49×10^{7}	"	"	14.6	3.0	8.5	12.4 mm
30/8	Soil	+	-	5.72×10^{4}	5.20×10^{7}	"	"	7.7	4.9	10.0	12.1 mm
6/9	Soil	+	-	9.64×10^{4}	1.12×10^{7}	"	"	9.3	3.9	8.1	23.8 mm

... Cont'd

13/9	Soil	+	-	ND	1.39×10^6	ND	ND	9.9	1.4	8.2	1.7 mm
19/9	Soil	-	-	ND	ND	"	"	14.2	1.5	9.1	32.9 mm
27/9	Soil	+	-	2.1×10^5	4.15×10^6	"	"	13.4	1.7	9.4	6.2 mm
4/10	Soil	+	-	2.6×10^4	4.15×10^6	"	"	15.6	2.1	11.1	18.9 mm
17/10	Soil	+	<u>A. poly-phaga</u>	3.4×10^3	1.0×10^7	"	"	16.0	6.0	12.0	1.8 mm
31/10	Soil	+	-	1.3×10^5	3.2×10^7	"	"	16.0	7.6	15.2	8.3 mm
8/11	Soil	+	-	3.8×10^5	1.0×10^7	"	"	17.9	3.4	14.5	17.8 mm
18/11	Soil	+	-	2.7×10^5	5.2×10^6	"	"	18.3	9.5	15.1	6.5 mm

+ = positive isolation

- = no isolation

ND = Not Done

UC = uncountable

1977 Otumaheke Stream: 1/4 whey-soil

Date	Source	Amebae		Total Coliforms .1.0 g^{-1}	Total Bacteria .1.0 g^{-1} at 37°C	pH	Tp °C	Max. Tp °C	Min. Tp °C	Soil 30 cm. Tp °C	Rain prior to 7 days of sampling
		Non-pathogens	Pathogens								
6/9	Soil	+	-	$2.47x10^4$	$1.2x10^7$	ND	ND	9.3	3.9	8.1	23.8 mm
13/9	Soil	+	N. fowleri	$1.17x10^4$	$3.0x10^6$	"	"	9.9	1.4	8.2	1.7 mm
19/9	Soil	+	N. fowleri	$4.17x10^3$	$2.66x10^6$	"	"	14.2	1.5	9.1	32.9 mm
27/9	Soil	+	-	$5.0x10^4$	$5.2x10^6$	"	"	13.4	1.7	9.4	6.2 mm
4/10	Soil	+	-	$1.4x10^5$	$9.2x10^6$	"	"	15.6	2.1	11.1	18.9 mm
17/10	Soil	+	A. culbertsoni	$5.0x10^5$	$3.3x10^6$	"	"	16.0	6.0	12.0	1.8 mm
31/10	Soil	+	-	$2.7x10^3$	$5.9x10^6$	"	"	16.0	7.6	15.2	8.3 mm
8/11	Soil	+	N. fowleri	$2.1x10^6$	$1.8x10^8$	"	"	17.9	3.4	14.5	17.8 mm
18/11	Soil	+	N. fowleri	$3.5x10^4$	$8.5x10^6$	"	"	18.3	9.5	15.1	6.5 mm

+ = positive isolation

- = no isolation

ND = Not Done

Appendix 3. Papers Published.

Primary Amebic Meningo-encephalitis (PAM) in New Zealand - aetiological agents, distribution, occurrence and control.
Ray T. Cursons*, Tim J. Brown and Elizabeth A. Keys, Department of Microbiology and Genetics, Massey University.

Traditionally the only pathogenic amebae recognised as occurring in waters has been the aetiological agent of amebic dysentery *Entamoeba histolytica*. Since the accidental discovery of cytopathogenicity in cell culture by pathogenic free-living amebae (PFLA) approximately twenty years ago (Jahnes *et al*.1957), the biomedical interest in these organisms has increased. In man and animals they have been responsible for a whole multitude of diseases, ranging from chronic illnesses such as respiratory infections and blindness, to the very acute disease of Primary Amebic Meningo-encephalitis, (PAM). Taxonomically they belong to the limax and small soil amebae on account of their characteristic slug-like shape and movement. They are ubiquitous in soil and freshwater environments throughout the world where they exist as saprophytic organisms feeding mainly on bacteria, yeast and organic debris present in their microenvironment. Laboratory cultivation may be achieved on non-nutrient agar upon which a lawn of *Enterobacter cloacae* or *Escherichia coli* has been spread or by axenic culture (Cursons & Brown, 1976). The PAM amebae belong to the genus Naegleria within the family *Schizopyrenidae*, and to the genus Acanthamoeba within the family *Acanthamoebidae*. The ease with which these amebae may be isolated from natural habitats has probably resulted in an underestimation of their pathogenic potential. After a review of 16 fatal cases from an indoor chlorinated swimming pool, Cerva (1971) stated that "it appears that the constant presence of numerous populations of amebae of the limax groups cannot be prevented even under the strictest observation of all routine safety measures applied to potable water". The widespread reports of isolation of pathogenic amebae (Brown & Cursons, 1977; Chang, 1974; Wellings *et al* 1977) from soil, water, in thermal pools and even chlorinated waters has led to the expression of real concern by Public Health Authorities, particularly in thermal tourist resorts of New Zealand. In New Zealand

there have been 6 cases of PAM, all of which were fatal, and were contracted while swimming in thermal waters in the North Island. All six cases were caused by the ameba Naegleria fowleri which has caused 88 of the 94 recorded cases in the world to date. Of the 94 cases 84 have been fatal.

Both the pathogenic species N. fowleri and the non-pathogenic one N. gruberi are morphologically identical (Fig. 1) but can be differentiated by serological, physiological, growth and pathogenicity characteristics (Fig. 2). They can further be differentiated from the genus Acanthamoeba on morphological grounds and in that they undergo promitosis, possess a temporary flagellate stage (Fig. 3) and are serologically unrelated as well. The portal of entry has been shown to be the cribriform plate via the nasal mucosa. Typical symptoms, expressed after an incubation period of 3-9 days, are the abrupt onset of headache, sore throat and mild fever. It then progresses rapidly over the next 3-4 days with increasing headache, stiff neck and vomiting. By the end of the third day the patient is normally severely disorientated and in a coma, with death following in about 4-8 days. Conformation of the disease may be achieved by the observation of trophozoites in the CSF. In post-mortem diagnosis, a degree of encephalitis is invariably present. The cerebral and cerebellar grey substances show variable sized lesions which tend to be haemorrhagic and quite soft when they are large. The existence of redness and destruction of the olfactory nerve occurs solely in swimming-associated meningo-encephalitis and could serve to distinguish it from bacterial meningitis which closely resembles PAM. Elsewhere in the body there is generally no evidence of amebic infection, with the possible exception of a myocarditis. Chemotherapy is generally ineffective once symptoms become apparent but the drugs of choice would be Amphotericin B and 5-flurocytosine which act synergistically.

Very much in the minority are cases of PAM caused by species of Acanthamoeba (Fig. 4). With the exception of the pathogenic species A. culbertsoni which produces an acute meningo-encephalitis in both man and animals, the only other known pathogenic species A. castellanii and

A. polyphaga produce systemic diseases which are chronic and thus in line with their lower virulence. The portal of entry is thought to be the bloodstream which they may enter through the alimentary canal or open wounds. Acanthamoebic infections are difficult to diagnose even in advanced cases due to the lack of specific symptoms and the absence of amebae in the CSF. More informative findings may be revealed by the examination of throat and nasal swabs, or by fluorescent antibody staining of sera. Post-mortem diagnosis relies on the presence of confined superficial lesions in the grey matter with a minimum inflammatory reaction, and the finding of double-walled cysts in apparently normal tissue bordering the lesion. Generally there are no lesions in the olfactory bulb region. The chemotherapy again is unreliable but 5-flurocytosine and sulphadiazine are the drugs of choice. To date there has been no reported illness due to Acanthamoeba in New Zealand and the overseas evidence suggests that this is a more "opportunistic pathogen" than Naegleria.

The still poorly understood epidemiology and environmental physiology of the disease has led to the establishment of a survey of six New Zealand thermal areas and a comparative investigation of common disinfectants as a means of control.

Materials and Methods

Culture

All Naegleria spp. were grown axenically in CYM media

All Acanthamoeba spp. were grown axenically in 4.0% Noff.

Disinfection

Cl_2 was produced from either $Ca(OCl)_2$ or NaOCl.

ClO_2 was produced from $NaClO_2$.

O_3 was produced by electrical discharge of dry oxygen.

QAC - Used Deciquam 222 (Di-decydimethyl-ammonium bromide).

All glassware was acid washed and rinsed in distilled water before use.

Only Chlorine-free de-ionized water was used.

FAC, TAC & CAC was measured by the DPD method of Palin (1975) as were ClO_2 & O_3 levels.

The protocol for the test was as follows:

Amebae were centrifuged out at 1500 rpm/10 mins. and resuspended in sterile 0.01M pH7 PO_4 buffer. They were then washed three times in the same buffer, counted using a modified Fuchs Rosenthal haemocytometer and resuspended to a final concentration of 1.2×10^6 cell/cm^3. Then a viable count was done on them using plaque formation on NM agar (Cursons & Brown, 1976).

For the test, a total of 24.6 cm^3 of 0.01M PO_4 buffer pH7 containing the desired concentration of disinfectant was dispersed in 250 cm^3 sterile Erlenmeyer flasks. Then 0.4 cm^3 of amebae were inoculated and the flasks were incubated at 25°C/30 mins. in a Griffin (100 series) mechanical waterbath. After 30 mins., 10 cm^3 was withdrawn for DPD analysis, a crystal of Na thiosulphate added to neutralize the disinfectant (for Cl_2, ClO_2 or O_3) whilst neutral red/$CaCl_2$ was added to neutralize the QAC. Then the remaining 15 cm^3 was filtered through a 5 µm cellulose-acetate filter. The filter was then washed with some sterile PAS and 0.1 cm^3 was diluted out in PAS and plaqued on NM agar, with Enterobacter cloacae serving as the bacterial lawn.

Isolation

Water + soil samples were collected in 11 sterile Whirl Pak bags and processed according to (Fig. 2).

Results and Discussion

Occurrence and Distribution

The results at this stage of the survey indicate that of the pools and soils sampled, the pathogenic N. fowleri is comparatively widespread whilst pathogenic Acanthamoeba spp. are of a relatively low frequency (Table I). The results also confirm that the most probable source of these amebae is the soil (their preferred habitat) and it is interesting to note that of all the pools sampled, the majority of pathogenic isolates

have all occurred from pools with gravel bottoms and soil banks, or surrounded by grassed-in areas. All N.Z. cases of PAM have occurred in soil contaminated pools after periods of unusually warm weather and heavy rainfall. At such times it would be expected that soil runoffs could contaminate the pool indicating that the high risk areas are those pools subject to flooding and soil contamination. On the other hand, pools in which no, or few, amebae have been isolated have been characterized by either very good hygiene standards and management or by a high salt concentration. Previous work has confirmed that 1.0% NaCl is amebicidal (Cursons & Brown, 1976).

Control of PAM

It is clear that control of the disease resides in three main ways:

(i) by abstaining from swimming in those waters where it is thought that FFLA's exist,

(ii) by the use of good water hygiene practice and particularly the exclusion of soil or organic matter from pools, or

(iii) by blocking the life-cycle of the ameba using either physical, chemical or biological means.

Hence it is strongly recommended that all pools be concreted or protected in some way from the entry of soil and flood waters.

Traditionally the presence of pathogens in potable water supplies has been inhibited or prevented by the routine practice of disinfection.

Disinfection

Experimental systems of disinfection were set up using axenic cultures of amebae to ascertain the direct effect of disinfectants on the amebae, indirect effects are also believed to be present in nature. Initially a breakpoint chlorination method was used, but the many variables such as sampling volume, time, pH, produced erratic results. A batch treatment was preferred in that it would at least give some indication of

what initial and residual levels of the disinfectant would be required to be amebicidal. This would also allow the comparative quantitation of chlorine with the other common disinfectants.

Because both pH, temperature and organic content seriously affect the amebicidal action of chlorine, it was decided to use 0.01M PO_4 buffer to give a pH7.0 at 25°C. This agreed with the pH of most hot pools, the temperature was chosen as an average between thermal and temperate pools. The final concentration of 1.92 X 10^4 cells/cm^3 was chosen as this has been shown to be a fatal dosage when administered intra-nasally to either mice or guinea-pigs.

Obviously the choice of a particular disinfectant will be tied closely to the chemical and physical composition of the water to be treated. From the Tables II and III it can be seen that the disinfectants of choice for PFLA are both ClO_2 and Cl_2 as well as the QAC Deciquam 222. Each has its own advantages and disadvantages as listed in Table IV. The apparent inactivity of O_3 may be explained in that these are only preliminary results and that disinfection with this compound has been explained as an all or nothing phenomena, such that the initial ozone demand may have been so great as to leave only a low concentration of residual O_3 for disinfection.

Under experimental conditions the total available disinfectant will be equal to the free available disinfectant prior to inoculation of the amebae (Tables II and III). Table II also indicates that Acanthamoeba spp. generally require a higher amebicidal concentration of chlorine. These results correlate well with those of Cerva (1971) who out of 100 isolations from a swimming pool with a free chlorine level of 0.3 mgl^{-1} (pH7.4 - 7.6) isolated only 4 Naegleria isolates as opposed to the 54 isolates of Acanthamoeba spp. However, from that same pool (from 1962-1965) he reported 16 fatal cases of PAM caused by Naegleria. Other isolations of amebae from chlorinated environments (Cerva & Huldt, 1974; Chang, 1974) also show that Acanthamoeba spp. are potentially more chlorine resistant than Naegleria spp.

The control of these PFLA by the use of disinfectants is further complicated by the presence of resistant cysts in both genera. De Jonckheere & van de Voorde (1976) have shown the cysticidal concentration of free available chlorine to be 2 $\mu g/cm^3$ for 15 mins. for N. fowleri and >40 $\mu g/cm^3$ for 3 hrs. for A. culbertsoni. Notwithstanding the fact that disinfectants are effective, if not totally so, it is uncommon to find any controlled disinfection programme in New Zealand commercial thermal pools. There seem to be three basic reasons for this: a) the problem of controlling dosage in hot organically charged water, b) cost, and c) the potential removal of the "natural" aspect and properties of thermal waters.

The effect of disinfection against amebae is felt indirectly via the food bacteria as well as directly. The survey currently being carried out has not yet shown any quantitative correlation between bacterial numbers and presence or absence of amebae. As other variables may complicate this pattern a controlled experimental programme is being planned to elucidate the problem.

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YEAR	Area	No. of samples		% of samples + for amebae		% of samples + for *N. fowleri*		% of samples + for pathogenic *Acanthamoebae spp*	
		water	soil	water	soil	water	soil	water	soil
73-4	Hamilton and	88	55	50	80	23	0	2.3	4.5
74-5	Rotorua	16	16	37.5	50	33	62.5	0	0
77	Taupo	16	16	75	100	6	25	0	0
77	Hamilton	15	15	46.6	93	0	26.7	0	0

Table I Isolation of amebae from water and soil in New Zealand thermal areas.

Species/ strains	pH	$t^{o}c$	$mg. \ell^{-1}$			inoculum cells $X\ 10^{4}/cm^{3}$	survivors	
			TAC	FAC	CAC		No.	%
N. gruberi (Pl 200f)	7.0	25.0	0.79	0.16	0.63	1.92	0	0
N. fowleri (MsT)	7.0	25.0	0.74	0.19	0.55	1.92	0	0
A. castellanii (1537)	7.0	25.0	1.02	0.22	0.80	1.92	0	0
A. culbertsoni (A-1)	7.0	25.0	1.09	0.14	0.95	1.92	23	0.032

Table II   Effect of chlorine on survival of axenic amebae

Species/ strain	pH	$t^{o}c$	Inoc. cells $X\ 10^4/cm^3$	ClO_2 $mg.l^{-1}$ initial	ClO_2 $mg.l^{-1}$ final	survivors	O_3 $mg.l^{-1}$ initial	O_3 $mg.l^{-1}$ final	survivors	Deciquam 222 $ml.l^{-1}$ initial	survivors
N. gruberi (Pl 200f)	7	25	1.92	1.1	0.25	40	6.26	0.075	+	0.05	0
N. fowleri (MsT)	7	25	1.92	1.6	0.35	15	ND	ND	ND	0.05	0
A. castellanii (1537)	7	25	1.92	2.9	0.65	1	4.25	0.05	+	0.05	0
A. culbertsoni (A-1)	7	25	1.92	2.5	0.60	1	ND	ND	ND	0.05	0

Table III Effect of chlorine dioxide, ozone and Deciquam 222 on axenic amebae

		Cl_2	ClO_2	O_3	QAC
Disinfecting efficiency		Good	Good > Cl_2	Good > Cl_2	V. good > Cl_2
optimum	pH	2 - 7	6.5 - 8.5	NA	7 - 9
	$t^{o}c$	22 - 25	30	0 - 4	20 - 37
reacts with	NH_3	+	-	-	-
	org. N	+	+	+	v. low
reacts with	amino acids	+	-	+	+
	proteins	+	+	+	+
toxicity		high	low	high	low
solubility at 20 - 30°C		good	v. good	v. low	v. good
reacts with UV		+	+	-	-
stability		low	high	low	high
residual disinfectant		+	+	-	+
cost		low	high	low	high

TABLE IV Comparison of the characteristics of chlorine, chlorine dioxide, ozone and Deciquam 222

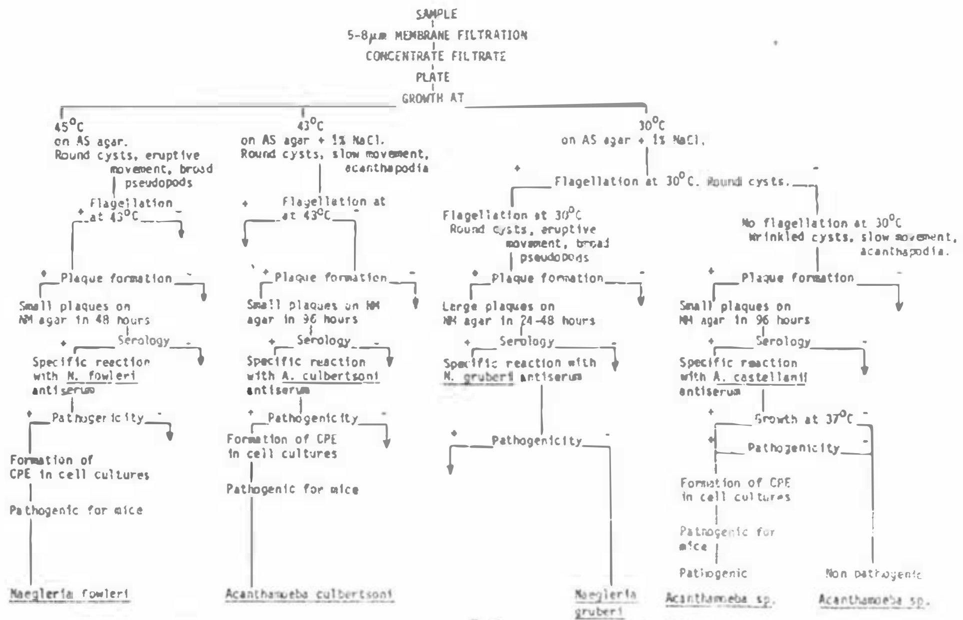

Fig. 2. Flow diagram of test strategy for the isolation and identification of the aeticlogical agents of PAM and closely related species.

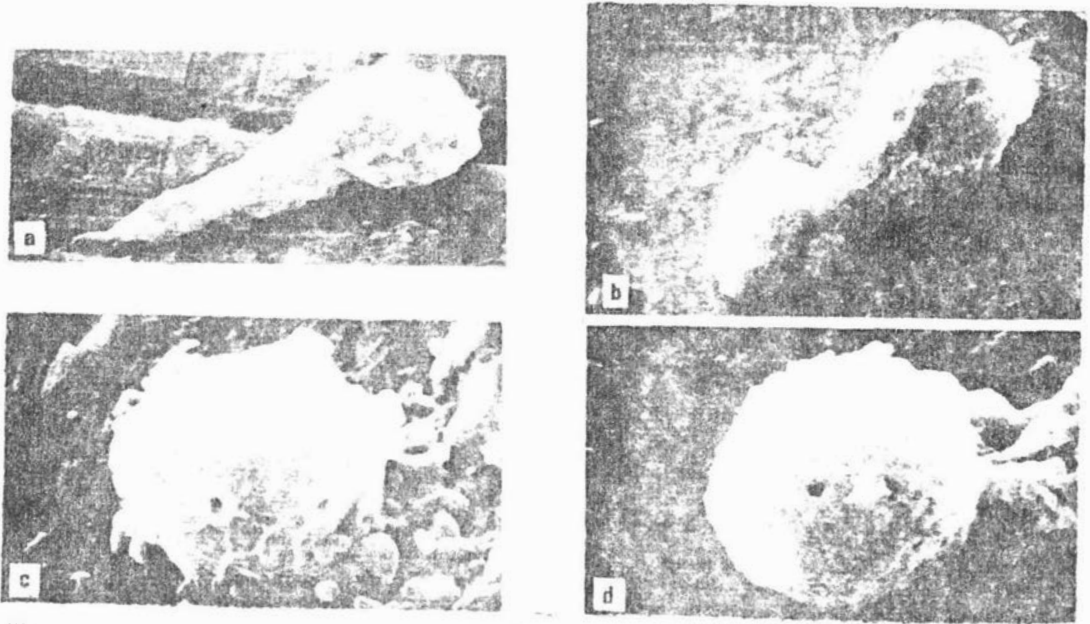

Fig. 1. Scanning electronmicrographs of trophozoites of (a) Naegleria gruberi (X3000), and (b) N. fowleri (X3000), and of cysts of (c) N. gruberi (X4000), and (d) N. fowleri (X4000) showing ostioles; all isolated from New Zealand thermal waters.

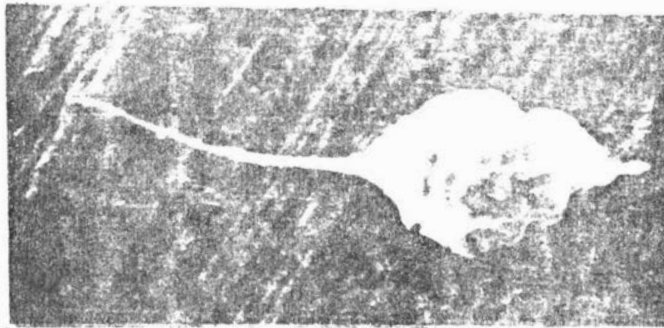

Fig. 3. Scanning electronmicrograph of flagellate stage of N. fowleri strain MsT (X4000)

Fig. 4. Scanning electronmicrograph of trophozoite of Acanthamoeba castellanii (X4500)

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