COMPARATIVE STUDIES ON RELATED FREE-LIVING AND PATHOGENIC LIMAX AMOEBAE WITH PARTICULAR REFERENCE TO NAEGLERIA

by

ALBERT JOSEPH LASTOVICA

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DECLARATION

This thesis is the result of the candidate's own original work except where acknowledgements are made in the text.

The work has not been previously submitted, and is not being concurrently submitted in candidature for any other degree.

Candidate..Albert Joseph Lastovica.....

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ABSTRACT

Over two hundred and forty strains of limax amoebae including eight potentially pathogenic strains of Naegleria and Acanthamoeba were isolated from a variety of habitats in South Africa.

The amoeboid and flagellate stages of all Naegleria strains examined by light microscopy were found to be similar in morphology. Differences were noted in the excystment of Naegleria fowleri, Naegleria gruberi and other limax amoebae.

Time-lapse microcinematography has been utilised to study cytokinesis and motility in the amoeboid and flagellate stages of Naegleria fowleri and Naegleria gruberi. A new phenomenon was observed in Naegleria where amoebae attempted cytokinesis after brief exposure to a temperature of $9^{\circ}C$.

The amoeboid and flagellate stages of virulent and avirulent Naegleria were found to be similar in surface topology when examined under the scanning electron microscope. Differences were noted in the excystment patterns of various limax amoebae. No structure suggestive of a surface active lysosome was found in any of the cells examined.

A comparative transmission electron microscope study of virulent and avirulent Naegleria and other limax amoebae has revealed striking similarities as well as sharply defined ultrastructural differences. Particles suggestive of a virus were detected in several of the pathogenic Naegleria. Surface-active lysosomes were not found in any of the amoebae examined.

Distinct feeding preferences were evident in Naegleria fowleri and Naegleria gruberi when they were tested on a variety of gram-negative and gram-positive bacteria. Differences were noted on the growth of these amoebae at various temperatures. Cell fractions of Pseudomonas aeruginosa affected excystment, encystment, cell division and the amoeba-to-flagellate transformation in Naegleria.

Differences were observed in the transformation kinetics of Naegleria fowleri and Naegleria gruberi at $25^{\circ}C$ and $43^{\circ}C$. A variety of inhibitors affected motility and transformation in Naegleria. For the first time, La^{3+} ion, a competitive inhibitor of Ca^{2+} ion, was shown to inhibit amoeboid motion, transformation and flagellar beating in Naegleria. Electron microscope observations suggested that La^{3+} ion acts on the plasmalemma.

Material from two suspected cases of primary amoebic meningoencephalitis was examined and cultured but no limax amoebae were isolated.

New criteria for the classification of virulent and avirulent Naegleria have been advanced.

CHAPTER 1

INTRODUCTION

Limax ("slug-shaped") amoebae are ubiquitous in soil, freshwater and other habitats (169,300). While the majority of these amoebae are free-living, some may become parasitic. Certain species of Acanthamoeba and Naegleria are pathogenic to man and other animals. They may invade the brain and meninges and cause fatal primary amoebic meningoencephalitis (46,74,133). To date, nearly 100 cases of primary amoebic meningoencephalitis have been reported from such diverse areas as Australia, Belgium, Britain, Czechoslovakia, India, New Zealand, the United States, Venezuela and Zambia. Primary amoebic meningoencephalitis poses a serious human health hazard because not only are the responsible organisms extremely common and exceptionally virulent, but effective therapy is very limited. Pathogenic limax amoebae are capable of maintaining their virulence for a considerable time, as Culbertson found that Acanthamoeba cultured in the laboratory for over a decade were still pathogenic to mice (90,91).

Differentiation of pathogenic and non-pathogenic limax amoebae is of critical importance. To date, most of the reports on pathogenic Acanthamoeba or Naegleria have been concerned with clinical details, studies of virulence or effective chemotherapy. Biological information, if included at all, forms but a small portion of these reports. Little in-depth comparison of the biology of virulent and avirulent limax amoebae is available in the literature. The primary purpose of this thesis is to compare and contrast selected aspects of the biology of a number of strains of pathogenic and non-pathogenic limax amoebae, particularly those of the genus Naegleria. The light microscope, the transmission electron microscope and the scanning electron microscope were used to study and compare these organisms. Consideration has been given to the occurrence, isolation, growth, temperature tolerance and ecology of limax amoebae. A qualitative and quantitative comparative study of the amoeba-to-flagellate transformation in pathogenic and non-pathogenic Naegleria has been undertaken.

A growing body of evidence indicates that actomyosin-like protein complexes function in non-muscle contractile phenomena, particularly in the cytoplasmic streaming of amoebae and in flagellar beating (204,216). Naegleria exists in three distinct states in its life cycle - as an amoeba, as a flagellate and as a cyst. Environmental factors are capable of causing the amoeba to cease vigorous cytoplasmic streaming and to transform into a rigid, fusiform cell with functional flagella.

A secondary aspect of this thesis is to investigate selected aspects of motility in Naegleria. The phase-contrast microscope, the transmission electron microscope and time-lapse microcinematography were utilised to study motility. In order to obtain information on divalent cation and other requirements, specific metabolic or motility inhibitors such as caffeine, colchicine, cytochalasin B, EDTA, EGTA, lanthanum nitrate and PCMB were used.

CHAPTER 2

DISTRIBUTION, ECOLOGY, ISOLATION AND CULTIVATION OF LIMAX AMOEBAE

A. ABSTRACT

During the period January 1971 to July 1976, over 240 strains of Naegleria, Acanthamoeba and other limax amoebae were isolated from a variety of habitats in South Africa. Seven strains of Naegleria fowleri and a strain of Acanthamoeba have been isolated from a heavily polluted section of the Eerste River, Cape Province, South Africa. These isolates have been identified as being potentially pathogenic, the first such report in Africa.

Limax amoebae have been found in several new habitats. Both Naegleria and Acanthamoeba have been found in the alimentary tract of the clawed toad, Xenopus laevis, and in the faeces of the Black duck, Anas sparsa. These animals may act as vectors for the transmission of limax amoebae from one site to another. Naegleria gruberi has been found in marine mud, and strains of Acanthamoeba have been found in desert sand and sea water.

B. INTRODUCTION

B.1 Distribution

B.1.i Naegleria

Limax amoebae are extremely common in a variety of terrestrial and aquatic environments. Viable cysts of Naegleria and other limax amoebae have been isolated from the air (238).

Naegleria has a worldwide distribution and has been isolated from soil samples collected in polar, temperate and tropical environments (169,361). Fellers & Allison (161) in their study of the protozoa in the soils of New Jersey, described Naegleria as being "very abundant". In 1924, Allison (4) reported that Naegleria occurred in 19 of 23 diverse surface soil samples collected in the United States. The only published reports of limax amoebae in South Africa are those of Fantham et al.(155-159) for the period from 1922 to 1925, and, nearly 50 years later, those of Lastovica (249,255).

Although most common in surface soil, Sandon (361) found encysted Naegleria at depths of 2,4 metres. Kofoid (241) asserted that Naegleria cysts could occur at depths in excess of 6 metres.

Chang (74,75), Fulton (169) and Page (312,313) have described Naegleria as being common in freshwater habitats such as lakes, rivers, swamps, ponds and sewage effluents. A number of workers (58,64,71,74)

have isolated Naegleria and other limax amoebae from swimming pools. Both Naegleria fowleri (8,10) and Naegleria gruberi (450) have been isolated from domestic tap water supplies. In 1972 Anderson & Jamieson (220) isolated, by membrane-filtration, 130 strains of Naegleria from 400 samples taken from swimming pools, rain water tanks, thermal springs, piped water supplies, lakes and dams. Two of these isolates were pathogenic to mice. Singh & Das (393) isolated pathogenic Naegleria and Hartmannella from sewage-sludge samples in Lucknow, India. Chang (personal communication, March, 1976) has reported the isolation of pathogenic Naegleria from a lake in Florida, U.S.A. Other strains of pathogenic Naegleria have been isolated from water, mud and sewage sludge in Belgium (117), Canada (114), Poland (232) and the United States (37,187). The majority of limax amoebae isolated from soil and water are non-pathogenic (76,108,169).

The alimentary tract and faeces of man and a variety of animals provide another habitat for limax amoebae. Naegleria is a coprozoic amoeba - a free-living amoeba that can live in faeces. Naegleria gruberi was first isolated by Schardinger (365) in 1899 from a diarrhoeic human stool. In 1912, Chatton & Lalung-Bonnaire (80) found Naegleria in human intestine in a case of intermittent diarrhoea. Recently Jadin et al.(214) isolated a strain of Naegleria, three strains of Vahlkampfia and six strains of Acanthamoeba (one of which was pathogenic to mice) from 348 human faecal samples. Various workers (25,29, 166,214,316) have isolated many Naegleria and hartmannellid strains from the alimentary tracts and faeces of an assortment of animals, including reptiles.

In 1971, Shumaker et al.(380) cultivated Naegleria amoebae pathogenic to mice from the nasal swab of a healthy seven-year old boy. Initially these amoebae were classified as Naegleria gruberi but subsequent investigations in two different laboratories (252,431) have identified the strain as Naegleria fowleri. Dreizin et al.(129) isolated, from the nasopharynx of young children with respiratory diseases, two strains of limax amoebae pathogenic to tissue culture cells.

Pathogenic Naegleria have been isolated on numerous occasions from the brain and cerebrospinal fluid of humans infected with primary amoebic meningoencephalitis (12,22,32,36,46,63,66,67,69,95,128,137,197, 198,210-212,265,268,305,320,360,405,417,420,436,444).

B.1.ii Acanthamoeba and Hartmannella

Acanthamoeba species are as widely distributed as Naegleria but to date have posed far less of a human health problem. Richards (350-352) reported viable Hartmannella amoebae and encysted Naegleria from the intestinal contents of freshwater molluscs. Cheng (81) found Hartmannella tahitiensis associated with mass mortalities of the oyster, Crassostrea commercialis. Getz (182) unsuccessfully attempted to infect molluscs with Acanthamoeba. Pathogenic hartmannellid amoebae have been isolated from a bull (283) and a beaver, Castor canadensis (293). Acanthamoeba pathogenic to mice have been isolated from a lake in Rumania (338), and a number of lakes in Poland (230,231,233).

Cerva et al.(68) examined 3082 human nasal smears and isolated Acanthamoeba castellanii, Acanthamoeba polyphaga and Hartmannella vermiformis from 7 percent of the samples. Skocil et al.(397-402) found limax amoebae in up to 26 percent of nasal smears taken at various times in civilian and military communities in Czechoslovakia. These authors (402) believed that a correlation exists between the presence of limax amoebae in the nasal passage and a history of headache, rhinitis or epistaxis. They also concluded that there is a seasonal fluctuation in infectivity, as the majority of amoebae were found in late spring and early summer. Limax amoebae were isolated from pillows, blankets, floors and floor polishers, and the authors state that transmission of limax amoebae by air is of primary importance.

Wang & Feldman (441-442) isolated 73 strains of limax amoebae from 18367 human pharyngeal swabs. They also isolated hartmannellid amoebae from human throat swabs. Armstrong et al.(13,443) isolated, from the upper respiratory tract of patients, Hartmannella amoebae that were cytopathogenic to tissue cultures.

Hartmannellid amoebae have also been isolated from the gastro-intestinal tract (199), the ear (217,259) and the eye (297,434,445) of humans. Pathogenic Acanthamoeba have been isolated from the cerebro-spinal fluid of humans infected with primary amoebic meningoencephalitis (24,41,188,190,355).

B.2 Ecology

Little information is available on how limax amoebae exist in nature, their life cycles and interactions with other organisms. Certain limax amoebae may be mutually exclusive, as Sandon (361) reported that the widely distributed amoebae, Naegleria gruberi and Hartmannella hyalina generally do not co-exist. Chang (75) stated that Acanthamoeba was

much more common than Naegleria in sewage effluents and polluted river water, while the reverse was the case in freshwater lakes. Bovee (27), in a detailed study of a small stream in Florida, U.S.A., found that limax amoebae were extremely common and co-existed with many other species of amoebae. Presumably Naegleria lives on bacteria and other micro-organisms, and in turn is preyed on by other organisms such as Thecamoeba (313). The overlay plaque technique of Menopace et al.(287) could provide useful information about the basic ecology of limax amoebae in future studies.

Naegleria and Acanthamoeba are a human health problem, their distribution is widespread and they have been isolated from a variety of animals. Frank (165) has suggested that cold-blooded vertebrates are possibly important epidemiological vectors in the spread of amoebae from one site to another. Any potentially pathogenic amoeba would have to survive not only the habitat of a poikilotherm, but also a temperature change and other physiological stress when infecting homoiotherms. Jadin (206,213) has suggested that humans may act as carriers of pathogenic limax amoebae. Page (316) has proposed the term amphizoic to describe protozoa which not only occur, but thrive both in the free-living (exozoic) state and in the alimentary tract of animals (endozoic). There may well be many more free-living amoebae which can colonise the vertebrate intestine. Marine invertebrates may acquire amoebae from the constant stream of sea water which passes through their bodies.

It is still unknown why some strains of limax amoebae are virulent while others are not. The possibility exists that some normally free-living amoebae on entering a mammal may acquire the ability to phagocytose host cells instead of bacteria. Observations by various investigators tend to refute this concept. Butt (37) isolated, from lake water, a pathogenic Naegleria species that remained pathogenic after growth on bacteria. Cerva et al.(67) reported a series of fatal cases of primary amoebic meningoencephalitis originating from a heated swimming pool over a four-year period - implying a continuous source of infection. Griffin (186) showed that non-pathogenic limax amoebae did not grow at 37°C or higher, while pathogenic strains of Naegleria and Acanthamoeba grew at temperatures of up to 46°C. Pathogenic Naegleria have been isolated from a thermally polluted canal in Belgium (117). Jamieson (personal communication, Dec.1973) reported finding pathogenic Acanthamoeba and Naegleria in several New Zealand hot springs. These

observations support the concept that there are distinct virulent and avirulent limax amoebae.

B.3 Cultivation

Early workers (349,466) grew Naegleria in a variety of ill-defined media with a mixture of micro-organisms present. Today, the usual methods of cultivation are: (i) on the surface of nutrient and non-nutrient agar with a bacterial associate as a food source (74,169,333, 365); (ii) with a suspension of bacteria in non-nutrient buffer (177, 265); and (iii) in axenic liquid media (15,54,74,169,309,366,451).

Naegleria is most conveniently cultivated on dilute agar with a bacterial food source. These amoebae will not grow on concentrated media (71,169). Chang (71,73,74) has studied the growth of pathogenic and non-pathogenic Naegleria on a variety of nutrient agars with different bacteria. Non-nutrient agar seeded with live bacteria has been used to grow pathogenic Naegleria (47,312) as the virulent amoebae grow poorly or not at all on nutrient agar (47, Chapter 7 of this thesis). Fulton (169) reported that Naegleria gruberi could grow on agar between 20-40°C with an optimum of 33°C. Griffin (186) found that pathogenic limax amoebae could grow at temperatures of up to 46°C. Naegleria amoebae have been grown in non-nutrient buffer with a dense suspension of Klebsiella (177,256). At 30°C these amoebae grew with a doubling time of about two hours to a stationary phase yield of 2×10^6 amoebae/ml.

Fulton (169) described an axenic medium for the cultivation of Naegleria. He later reported (173) a simplification of his medium A that replaced one of the undefined fractions, proteose-peptone, with a single amino-acid, L-methionine. Band & Balamuth (18) stated that they could replace the serum requirements of axenic media with hemin for prolific growth of Naegleria. Stevens & O'Dell (408) grew pathogenic Acanthamoeba to a stationary phase yield of 3×10^6 amoebae/ml in axenic aerated suspension cultures of modified Neff medium. Cerva (56) grew pathogenic Acanthamoeba castellanii in chick-embryo culture.

B.4 Effects of physical and chemical agents

Freezing at -20°C or lower temperatures will kill Naegleria trophozoites (46,72,91). Carter (46) reported that Naegleria fowleri amoebae were viable for up to a month at 21°C, but were rendered non-motile at 4°C. Fresh human serum, sea water and bile salts will destroy Naegleria amoebae (46). Drying will kill the trophozoites of Naegleria, but the cysts will survive for lengthy periods (91,169,312). Naegleria gruberi cysts can survive brief exposure to a temperature of

45°C which kills the amoebae (71). Investigators (115) have found that Naegleria fowleri cysts are more sensitive to chlorine than are Naegleria gruberi cysts. Under the same test conditions pathogenic and non-pathogenic Acanthamoeba cysts were insensitive to a chlorine concentration of 4 µg/ml.

Long-term preservation of limax amoebae cultures presents a problem. Fulton (169) reported limited success in the freeze-drying of Naegleria cysts. Ensminger & Culbertson (154) had some success in preserving Acanthamoeba by freezing in 10% glycerol. Investigators (23,91,211,382, 473) have had poor to moderate success in the preservation of Hartmannella and Naegleria amoebae using low temperatures and dimethylsulphoxide as a cryoprotectant.

B.5 Isolation

Isolation of limax amoebae from the environment can readily be accomplished by plating a soil or water sample on to sterile nutrient or non-nutrient agar seeded with a food bacterium. Investigators (74,75, 117,169,249,312,313,390) have used this basic procedure to isolate limax amoebae from various habitats. Anderson & Jamieson (220) and Chang (74) have described methods for isolation of limax amoebae from large volumes of water. Growth of amoebae along a bacterial streak (169) on sterile agar allows the purification of amoebae contaminated with other organisms. Bacteria-free environmental isolates have been obtained by the use of antibiotics (74).

B.6 Isolation of limax amoebae from South African habitats

During the period January 1971 to July 1976, an investigation into the isolation and potential pathogenicity of limax amoebae from a variety of South African habitats was undertaken. The morphology of these isolates was studied under the light microscope and they were compared and contrasted with reference cultures of virulent and avirulent limax amoebae. Some of the results presented in this chapter have been published (249,255).

C. MATERIALS

Organisms Sources are recorded on p.146-147.
Media Described on p.148-149.
Chemicals Described on p.149-150.

D. METHODS

D.1 Isolation and cultivation

Two hundred and thirty four 2-3ml samples of soil, freshwater, sea water, mud, sewage sludge, intestinal contents and faeces of animals, and sand were collected in sterile 10 ml screw-top bottles. An additional 56 samples were collected from the Eerste River in the Cape Province, South Africa where Station A was established three metres upstream from a main sewage outlet on a sunny portion of the bank, and Station B was established three metres below the outlet in a shaded section of the river.

Six Xenopus laevis specimens from the University of Cape Town, Zoology Department aquarium were examined for the presence of limax amoebae. These animals, 5cm in body length and apparently healthy, were killed by blows on the head. They were soaked in 90% alcohol for five minutes to destroy any surface protozoa. Their skins were removed with sterilised dissecting instruments and discarded. A new sterile set of dissecting instruments was used to remove the brain, lungs and liver. The stomach was cut open in the cardiac region and with the aid of forceps the gut contents were squeezed out onto PA plates seeded with Klebsiella aerogenes and incubated at 34°C. Portions of the brain, liver and lungs as well as the contents of the intestine were treated in a similar manner.

All samples were kept at 22-23°C and processed as soon as possible, usually within three days of collection. About 0,2ml of each sample was inoculated and spread over the surface of a NM or PA agar plate seeded with viable Klebsiella aerogenes or Escherichia coli as a food source. The plates were kept at 25°C, 34°C or 43°C and examined frequently for the appearance of plaques of amoebae in the bacterial lawn.

D.2 Purification and identification

Purification of amoeba isolates was accomplished by inoculating one end of a bacterial streak on NM or PA agar plates with a loopful of the amoeba isolate. These plates were grown at 25°C, 34°C or 43°C and were examined frequently. Amoebae would outgrow contaminating micro-organisms on the bacterial streaks. When the amoebae reached the end of the bacterial streak, some were removed with a sterile loop and then inoculated onto a freshly prepared bacterial stock plate. If contamination persisted, the purification step was repeated with variations in temperature and medium.

For identification of amoeba isolates the following criteria were used:

D.2.i Morphological examination

Viable and iodine-stained amoebae, cysts and flagellates were examined by phase-contrast microscopy and compared with established descriptions (Chapter 3 of this thesis, 74,312,313)

D.2.ii Flagellation tests

All potential Naegleria isolates were tested for the ability to transform to flagellates at least three times, by being placed in glass-distilled water at 25°C, 34°C or 43°C.

D.2.iii Ability to grow at 43°C

According to Griffin (186), only pathogenic Naegleria and Acanthamoeba strains are able to grow at 43°C.

D.2.iv Mouse inoculation

A limited number of mouse pathogenicity studies were carried out on some of the isolates. Healthy laboratory mice weighing 20-30gm were anaesthetised, held ventral surface upwards and then inoculated intranasally with 5000-10000 amoebae in 0,4ml doses. Four to six mice were inoculated with each amoeba isolate and were then kept in separate cages. They were examined daily for any abnormal behaviour and at the end of 14 days, some of the mice were killed, dissected and examined for amoebae in the brain and spinal fluid.

E. RESULTS AND DISCUSSION

The fact emerges that limax amoebae are widespread in their distribution in South Africa, as is shown in Table 2:1. Naegleria is very common, being found in 41,5% of the samples examined. Marine mud samples yielded two isolates. This is a new environment for Naegleria and has not been previously reported in the literature, although E.Willaert of the Institute for Tropical Medicine in Antwerp, Belgium, has also found similar Naegleria strains in marine mud (personal communication, January, 1975). Six Naegleria isolates were tested for mouse pathogenicity by intranasal inoculation but proved to be avirulent.

Acanthamoeba was widely distributed and was found in 34,2% of the samples examined. This genus can inhabit environments that Naegleria does not appear to be able to colonise. Six strains of Acanthamoeba were isolated from various marine aquaria at the University of Cape Town. Apparently lifeless desert sand revealed the presence of 15 strain of Acanthamoeba which, to my knowledge, is the

first time this protozoan has been isolated from such an environment. It would appear that *Acanthamoeba* is hardier than *Naegleria*, at least in so far as it is found in more diverse environments.

TABLE 2:1

ISOLATION OF LIMAX AMOEBAE CAPABLE OF GROWTH AT 34°C FROM VARIOUS SOUTH AFRICAN LOCALITIES

SOURCES	NUMBER OF SAMPLES	TOTAL AMOEBA ISOLATES	*NAEGLERIA* ISOLATES	OTHER ISOLATES
1. Fresh water				
Lakes	31	29	19	7 *Acanthamoeba*, 3 unknown sp.
Streams	42	34	18	9 *Acanthamoeba*, 7 unknown sp.
Ponds	34	28	20	7 *Acanthamoeba*, 1 *Hartmannella*
2. Sea water	4	4	0	2 *Acanthamoeba*, 2 *Paramoeba*
3. Marine aquaria	10	9	0	6 *Acanthamoeba*, 3 *Paramoeba*
4. Marine mud	2	2	2	
5. Soil	43	31	15	10 *Acanthamoeba*, 1 *Hartmannella*, 5 unknown sp.
6. Sand	20	17	0	15 *Acanthamoeba*, 2 unknown sp.
7. Sewage sludge	14	12	5	5 *Acanthamoeba*, 2 unknown sp.
8. Mud	24	18	10	6 *Acanthamoeba*, 2 unknown sp.
9. *Xenopus laevis* Alimentary tract	6	14	6	6 *Acanthamoeba*, 2 unknown sp.
10. Black duck faeces *Anas sparsa*	4	5	2	2 *Acanthamoeba*, 1 unknown sp.
	234	203	97	75 31

Origin of sources:

1,5,7 & 8. Various localities. Collected in South Africa and South West Africa from January 1971-July, 1976

2. Collected from Oudekraal, Cape Town on the Atlantic Coast, January, 1975

3. Aquaria of the Zoology and Oceanography Departments, University of Cape Town, May, 1974

4. Marine mud of Lake St. Lucia, Natal. Collected July, 1971

6. Namib Desert, South West Africa. Collected October, 1971
"Red Desert", Natal South Coast. Collected July, 1971
Kalahari Desert, Cape Province. Collected July, 1976

9. *Xenopus laevis* from Zoology Department, University of Cape Town, concrete aquarium, August, 1974

10. Duck faeces from the Percy Fitzpatrick Institute for African Ornithology, University of Cape Town, August, 1975

Microscopic examination of Xenopus laevis brain, lung and liver samples and the PA agar plate cultures of these samples did not reveal any amoebae. The stomach and intestine of these frogs had trophozoites and cysts of Acanthamoeba sp., Naegleria gruberi amoebae and numerous other micro-organisms. Two strains of Naegleria, two strains of Acanthamoeba and an unidentified limax amoeba were isolated from four faecal samples from the Black duck (Anas sparsa). This duck was chosen for study as it feeds in shallow, slow-moving or stagnant waters - a likely habitat for limax amoebae.

Xenopus and Anas are widespread in South Africa and the possibility exists that they could act as vectors or reservoirs of potentially pathogenic limax amoebae.

TABLE 2:2

LIMAX AMOEBAE CAPABLE OF GROWTH AT 43°C WHICH WERE ISOLATED FROM A HEAVILY POLLUTED SECTION OF THE EERSTE RIVER, CAPE PROVINCE

DATE	STATION	TEMPERATURE OF RIVER WATER °C	TOTAL AMOEBAE ISOLATES GROWN AT 34°C	IDENTIFIED AMOEBAE ISOLATES GROWN AT 43°C
Feb. 21	A	22,4	4	Acanthamoeba sp.
	B	24,0	4	Naegleria fowleri
Mar. 19	A	24,5	3	Naegleria fowleri
	B	24,0	4	Naegleria fowleri
May 5	A	15,5	3	Naegleria fowleri
	B	15,5	4	Naegleria fowleri
June 2	A	12,5	3	
	B	13,0	2	
July 4	A	11,1	3	
	B	10,0	2	
July 30	A	13,0	1	
	B	13,0	3	
Aug. 24	A	12,0	2	
	B	13,0	1	
Sept. 23	A	16,0	2	Naegleria fowleri
	B	16,0	3	Naegleria fowleri
			44	

Station A: Located 3m above sewage outlet. 3 samples collected per date from sunny, slow-moving sewage polluted water

Station B: Located 3m below sewage outlet. 4 samples collected per date from slow-moving, sewage polluted water in the shade

The results presented in Table 2:2 on the preceding page tabulate the first report of potentially pathogenic limax amoebae isolated in Africa. Seven strains of Naegleria fowleri and a strain of Acanthamoeba were isolated from a heavily polluted section of the Eerste River, Cape Province. These isolates were capable of growth and transformation at 43°C. They were not tested for mouse pathogenicity, and thus are designated potentially pathogenic. De Jonckheere et al.(117) isolated 24 strains of Naegleria fowleri from a thermally polluted canal in Belgium, but only one of the isolates was pathogenic to mice. The authors concluded that the majority of Naegleria fowleri isolates may have become avirulent from lack of contact with a suitable host, and that mouse pathogenicity tests should not be relied on exclusively for the determination of the pathogenicity of Naegleria.

In my study, none of the isolates capable of growth at 43°C were isolated during the period 2nd June - 24th August, 1975, the coldest months of the South African year with resulting low water temperatures, (Table 2:2). Other workers have found that limax amoebae are more prevalent during the hottest months of the year (47,402).

My isolation experiments, although short-term, indicate that limax amoebae which are a potential human health hazard are widespread in South Africa.

CHAPTER 3

LIGHT-MICROSCOPY OF LIMAX AMOEBAE

A. ABSTRACT

Bright-field and phase-contrast microscopy were used to examine the morphology of six strains of non-pathogenic Naegleria gruberi, five strains of pathogenic Naegleria fowleri, a strain of Naegleria jadini, and two strains of Acanthamoeba.

Both amoeboid and flagellate stages of all the Naegleria strains examined were similar in morphology. Light-microscopy revealed that excystment in Naegleria gruberi was by preformed exit pores piercing the cyst wall. No such structures were seen in the cysts of Naegleria fowleri or Naegleria jadini in which excystment was by rupture of the cyst wall.

Like Naegleria, each Acanthamoeba trophozoite possessed a conspicuous nucleus and nucleolus and, in addition, had numerous fine acanthopodia. Excystment from the wrinkled, double-walled cysts of Acanthamoeba was via prominent ostioles covered by opercula.

B. INTRODUCTION

Naegleria gruberi was first described by Schardinger (365) in 1899. In the ensuing years various investigators (3,14,71,72,74,127,169,267,298, 312,313,319,349,364,392,449,465) have published detailed accounts of the morphology of Naegleria gruberi. The first account of the morphology of Naegleria fowleri was that of Fowler & Carter (164) in 1965. Other morphological descriptions of this pathogenic species have subsequently been published (37,46,95), or have been included in clinical and other studies (44,55,74,78,133,136,205,210,305,392,405,420,447). Naegleria jadini, a third species of Naegleria, has recently been described by Willaert & Le Ray (459) and Das et al.(113). Observations by light-microscopy on the morphology of Acanthamoeba and other limax amoeba have been published by various investigators (1,24,40,41,74,88-91,98,188,280,285, 313,315,317,323,392,411,426,434).

This chapter compares and contrasts the morphology of virulent and avirulent Naegleria and other selected limax amoebae. Some of the results presented here have been published (249,255).

C. MATERIALS

Organisms	Sources are recorded on p.146-147.
Media	Described on p.148-149.
Chemicals	Described on p.149-150.
Apparatus	A Wild model M20 binocular microscope and a Zeiss Photomicroscope III with bright-field and phase-contrast optics. An optical and a stage micrometer.

D. METHODS

Naegleria gruberi, strains 1815 and 27 were grown at 25°C while all Naegleria fowleri strains and the APG strain of Acanthamoeba were grown at 37°C in Fulton's A medium (169). For convenience and reproducibility of the amoeba-to-flagellate transformation process, the Naegleria strains were grown at 25°C or 37°C on PA agar with Klebsiella aerogenes as a food source. For the production of cysts and flagellates, the procedures outlined in Chapter 5 of this thesis were followed. During transformation and reversion, cells were monitored by phase-contrast microscopy.

Amoebae, cysts and flagellates of the strains examined were observed and photographed live, or stained with Lugol's iodine in both hanging-drop and wet mount preparations under phase-contrast. For examination of mitosis in Naegleria gruberi the haematoxylin and eosin procedure of Jamieson & Anderson (219) was used. At least 50 individuals of each strain were measured for length, breadth, nuclear and nucleolar diameter of the amoebae, length and breadth of flagellate and cyst diameter. Rates of locomotion were determined on six to ten rapidly moving amoebae of each strain.

E. RESULTS

Under phase-contrast, the morphological details of the amoebae were seen in vivid detail. A comparative study of six strains of Naegleria gruberi, five strains of Naegleria fowleri and a strain of Naegleria jadini revealed that the morphology of both amoeboid and flagellate stages of these protozoans is identical. Differences were noted in the cyst morphology and the excystment patterns of the Naegleria species. A study of pathogenic Acanthamoeba revealed similarities and differences when compared to the Naegleria amoebae examined. Over 300 micrographs were taken and a representative selection is shown in Figs 3.1 - 3.33, p.18-24.

E.1 Naegleria species

E.1.i Amoebae

The typical limax shape with a single broad pseudopod and a cylindrical tapering body is shown by the 1815 strain of Naegleria gruberi (Fig. 3.5), the RL strain of Naegleria fowleri (Fig. 3.8) and the 0400 strain of Naegleria jadini (Fig. 3.10). When free floating, amoebae assume a more compact, spherical shape with no obvious polarity and several pseudopodia (Figs 3.7, 3.11). Under phase-contrast the clear ectoplasm is easily differentiated from the granular endoplasm (Figs 3.1-3.6, 3.9, 3.11). A terminal uroid process was seen on occasion (Fig. 3.1). The limax shape found in rapidly advancing amoebae had a length/breadth ratio of about 3/1. For Naegleria gruberi the mean length was 22,4 µm (range 15,1 - 30,7 µm) and the mean breadth was 6,9 µm (range 4,9 - 8,9 µm). The mean length of Naegleria fowleri was 14,2 µm (range 8,0 - 31,9 µm), while the mean breadth was 5,9 µm (range 4,1 - 11,7 µm). The mean length of Naegleria jadini was 29,3 µm (range 21,1 - 40,5 µm) and the mean breadth was 9,8 µm (range 7,1 - 13,4 µm).

Some of the strains, especially those of Naegleria fowleri, were variable in size, particularly if cultivated axenically. The amoebae move by means of broad, eruptive, often single pseudopodia usually formed near, or to one side of the last pseudopod. This leads to a somewhat sinuous progression, although pseudopodia may erupt at any point and cause the amoebae to change direction. Rapidly moving amoebae travelled between 30 - 90 µm/min. at 25°C.

In all the Naegleria amoebae examined the nuclei and nucleoli were conspicuous features (Figs 3.1 - 3.11, 3.13 - 3.16). The nucleus was usually single and found in the anterior of the amoeba. The nucleolus occasionally had vesicles present, and both nucleus and nucleolus were deformed when the amoeba moved. For the Naegleria gruberi strains the mean nuclear diameter was 2,5 µm (range 1,5 - 4,0 µm) and for Naegleria jadini the mean nuclear diameter was 3,6 µm (range 2,9 - 3,9 µm). Figs 3.13 - 3.16 illustrate mitosis in four strains of Naegleria gruberi. At telophase the interzonal body and the polar caps were occasionally seen.

Trophozoites of Naegleria gruberi had oval or cylindrical mitochondria up to 1,5 µm long by 0,5 µm wide. Naegleria fowleri amoebae had, in addition to the oval or cylindrical mitochondrial phenotypes, cup-shaped and dumbbell-shaped mitochondria 1 - 3 µm long by 0,5 µm wide.

A contractile vacuole up to 2,5 µm in diameter was usually found toward the posterior of a motile amoeba (Fig. 3.8) and was formed by the coalescence of smaller vacuoles (Figs 3.1 - 3.3). The contents of the vacuole are expelled by fusion of the vacuole and cell membranes. This appears to serve an osmoregulatory function. Amoebae freshly subcultured in Fulton's A medium often had large numbers of empty food or contractile vacuoles. Under the light-microscope, food vacuoles were inconspicuous in the amoebae.

Pale yellow or yellow-green inclusions up to 1 µm in diameter were seen under phase-contrast in all three Naegleria species. Possibly the inclusions are lipid bodies.

E.1.ii Naegleria flagellates

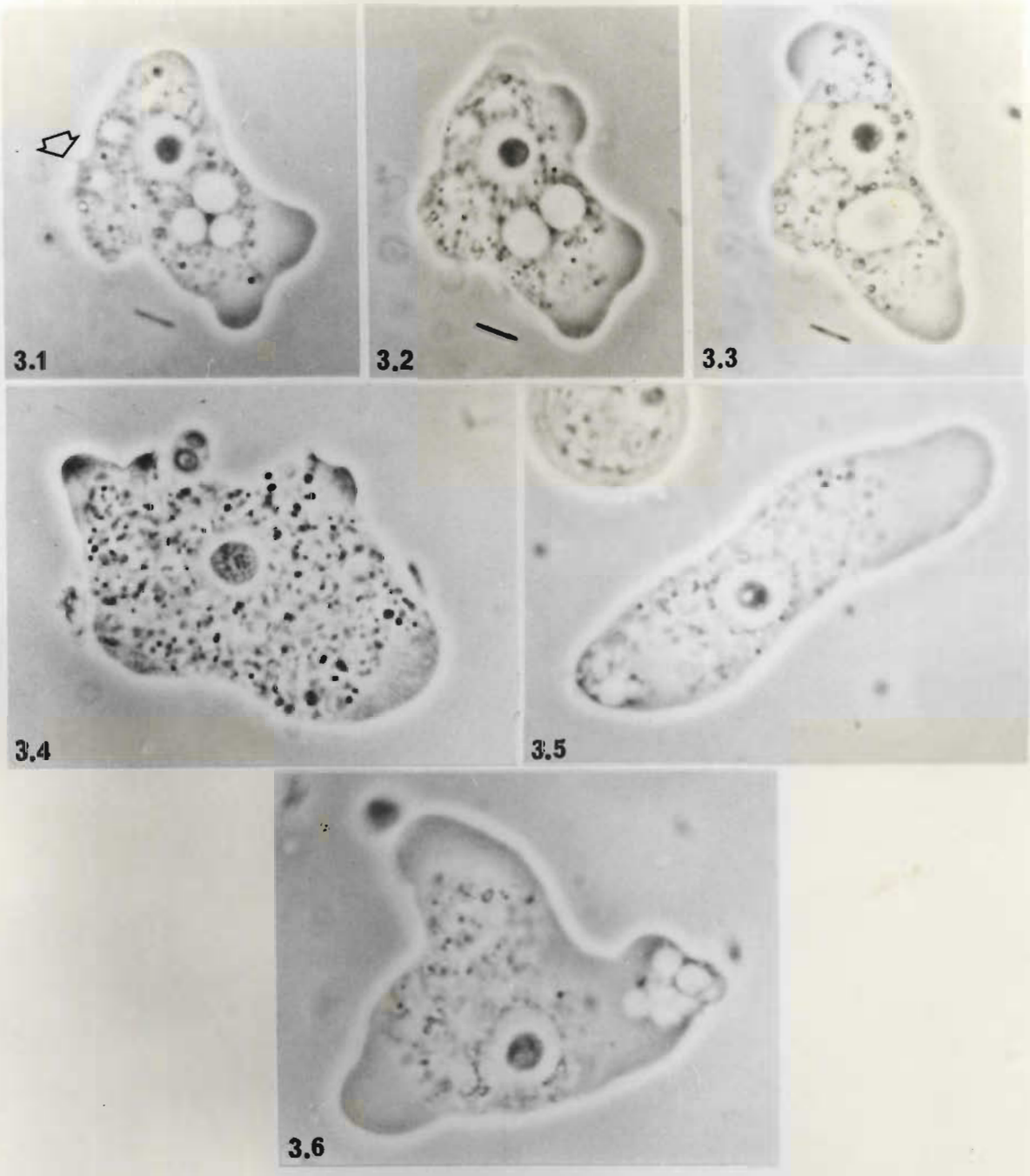
The Naegleria flagellates had a smooth surface and a rigid shape which was usually fusiform (Figs 3.17, 3,21, 3,22) and sometimes oval (Figs 3.23, 3.24) or other shapes (Figs 3.18, 3.19).

The mean measurement of Naegleria gruberi flagellates was 15,3 by 8,1 µm. The mean dimensions of Naegleria fowleri flagellates were 13,0 by 6,2 µm, while Naegleria jadini flagellate's mean was 16,8 by 9,6 µm. Cytoplasmic inclusions of the flagellates were similar to those of the amoebae, except that there was usually only one large contractile vacuole present, and, of course, the components of the flagellar apparatus. The number of flagella was variable, with two being the most common, but up to eight flagella per flagellated cell were observed. The sequence of the steps of transformation and reversion was identical in all three species of Naegleria as seen under the phase-contrast microscope. When flagellates reverted back to amoebae the flagella were non-motile and still attached to the actively streaming amoeba (Fig. 3.20).

E.1.iii Naegleria cysts

Cysts of Naegleria gruberi were smooth-walled, spherical or oval in shape with a mean diameter of 12,5 µm. The nuclei were conspicuous in Naegleria cysts, while food vacuoles were not. The cyst wall had a thick inner layer and a thinner outer layer with up to five exit pores about 0,6 µm in diameter, which were also visible under phase-contrast. Usually one nucleus was present in each cyst, and the nuclear membranes were surrounded by a layer of granules. Under the light-microscope it was obvious that excystment was via an exit pore in the cyst wall (Figs 3.25 - 3.28).

Occasionally, doubly encysted forms - one cyst inside another - were encountered, particularly in old cultures with bacteria. A small number



Figs. 3.1 – 3.3 An amoeba of the NEG strain of Naegleria gruberi with a prominent nucleus, nucleolus, endoplasm, ectoplasm and uroid (arrow). Phase contrast micrographs were taken at 2 second intervals at 25°C and illustrate pseudopod extension and the fusing of 3 contractile vacuoles into 1 large vacuole. The slight blurring is due to cytoplasmic streaming while the micrographs were being taken. X5 000.

Fig. 3.4 A Naegleria gruberi, strain 27 amoeba. X5 300.

Fig. 3.5 The typical elongated limax form is demonstrated by an amoeba of the 1815 strain of Naegleria gruberi. X4 900.

Fig. 3.6 A Naegleria gruberi, strain SA-1 amoeba. X5 000.

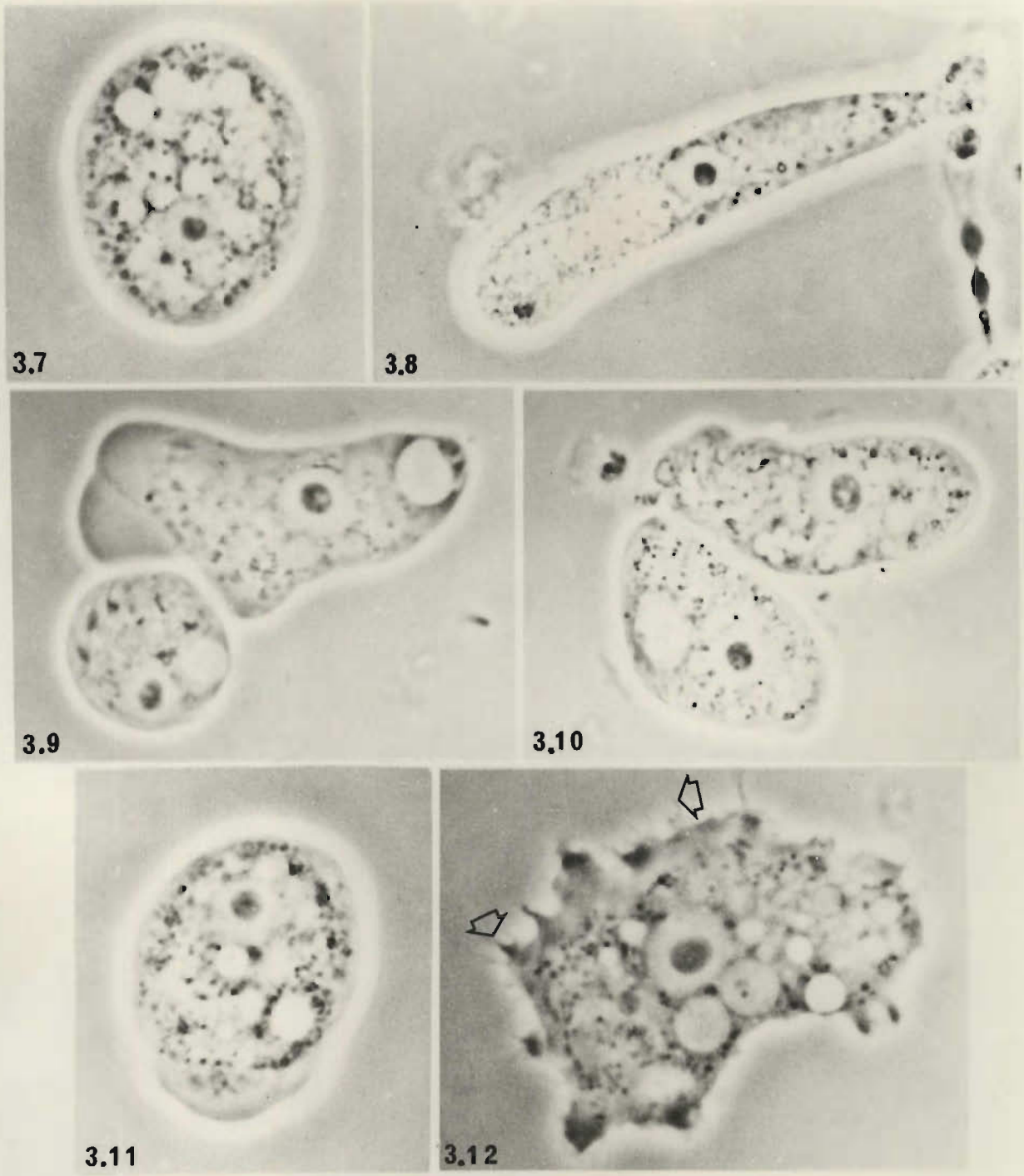


Fig. 3.7 A non-motile amoeba of Naegleria fowleri, strain PA-14. X6 200.

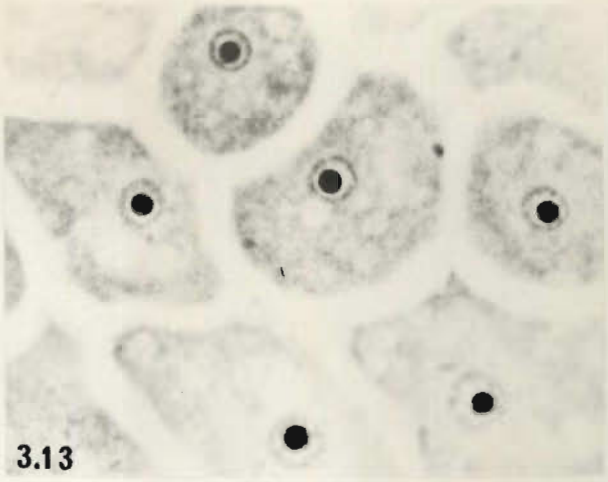
Fig. 3.8 A Naegleria fowleri, strain RL amoeba. X4 850.

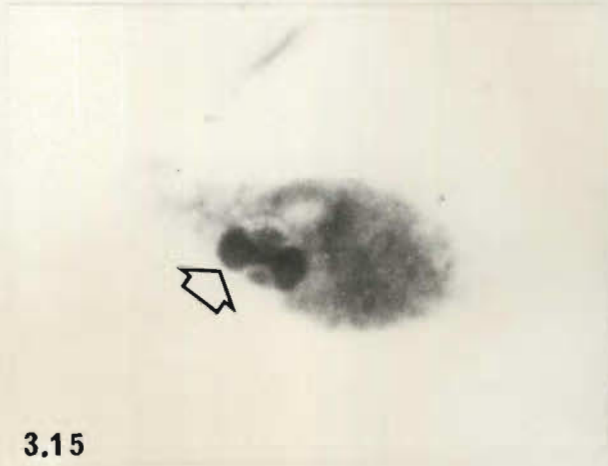
Fig. 3.9 Amoebae of the Oram strain of Naegleria fowleri with conspicuous contractile vacuoles. X4 950.

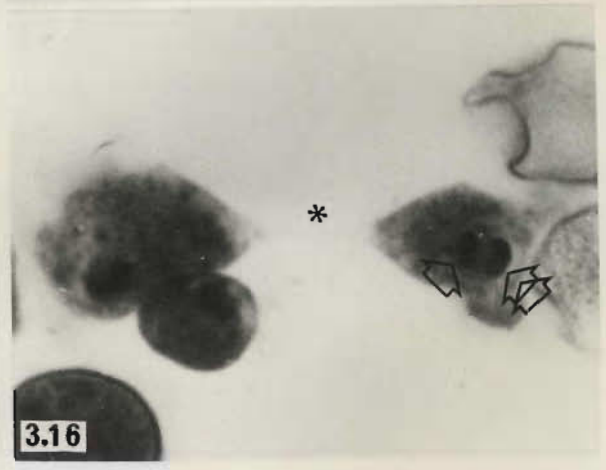
Fig. 3.10 Naegleria jadini, strain 0400 amoebae. X4 850.

Fig. 3.11 A Naegleria fowleri, strain Northcott amoeba. X5 950.

Fig. 3.12 An Acanthamoeba, strain APG amoeba with conspicuous acanthopodia (arrows), nucleus, food and contractile vacuoles. X6 000.







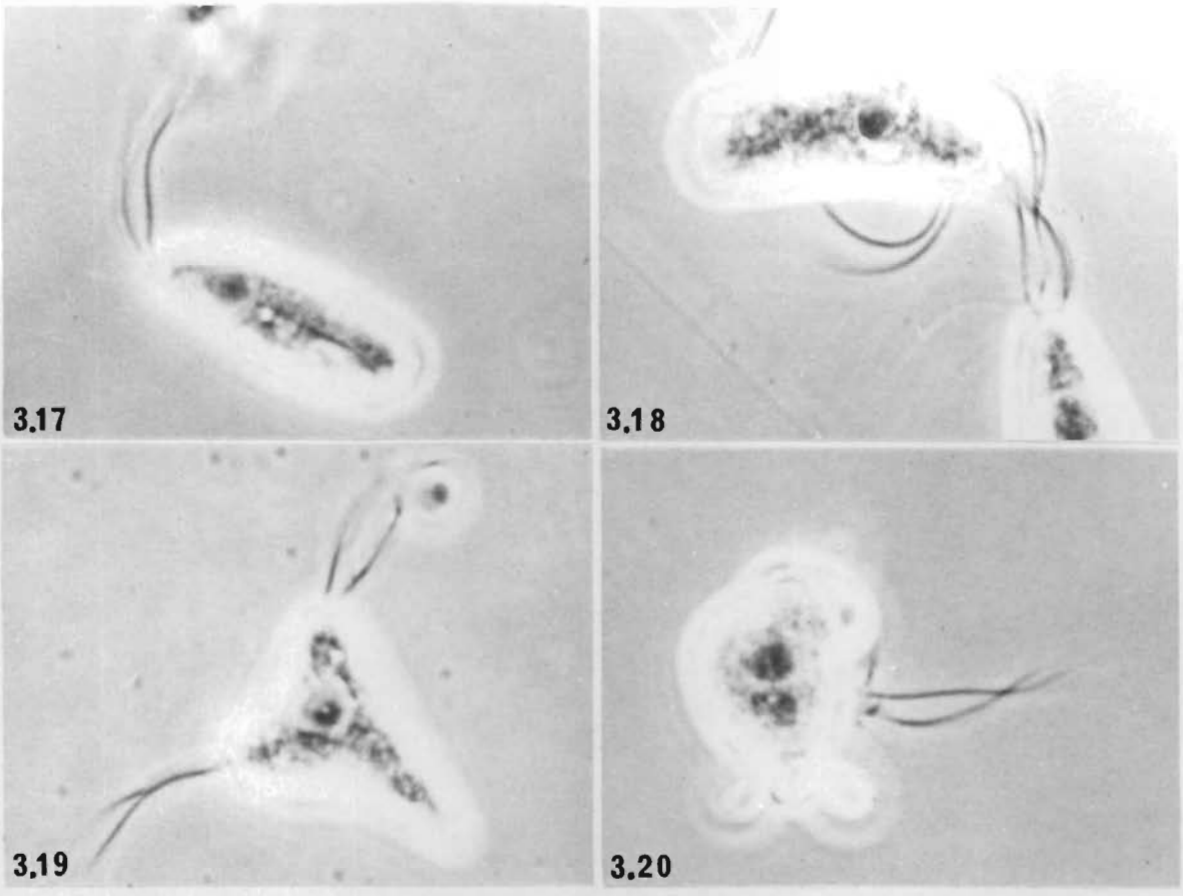
Mitosis in 4 strains of Naegleria gruberi amoebae.

Fig. 3.13 Interphase nuclei of strain NEG amoebae. X3 200.

Fig. 3.14 Thickening and compaction of the nucleolus occurs in late prophase in a strain 27 amoeba. X5 000.

Fig. 3.15 At late anaphase, a strain SA-1 amoeba displays a dumbbell shaped nucleolus (arrow) within the nuclear membrane. X4 800.

Fig. 3.16 A strain NB-1 amoeba in telophase. The 2 daughter cells are still joined by a thin connecting strand of cytoplasm (*). The interzonal body (single arrow) and the polar cap (double arrow) are seen within the nuclear membrane. X4 950.



Flagellates of 4 strains of Naegleria gruberi.

Fig. 3.17 A flagellate of the SA-2 strain with 2 flagella. X5 400.

Fig. 3.18 An NEG strain flagellate. X5 100.

Fig. 3.19 A triangular flagellate of the SA-1 strain. X5 600.

Fig. 3.20 An NB-1 strain flagellate reverting back to an amoeba. The "paralysed" flagella are attached to an actively motile amoeba. X5 300.

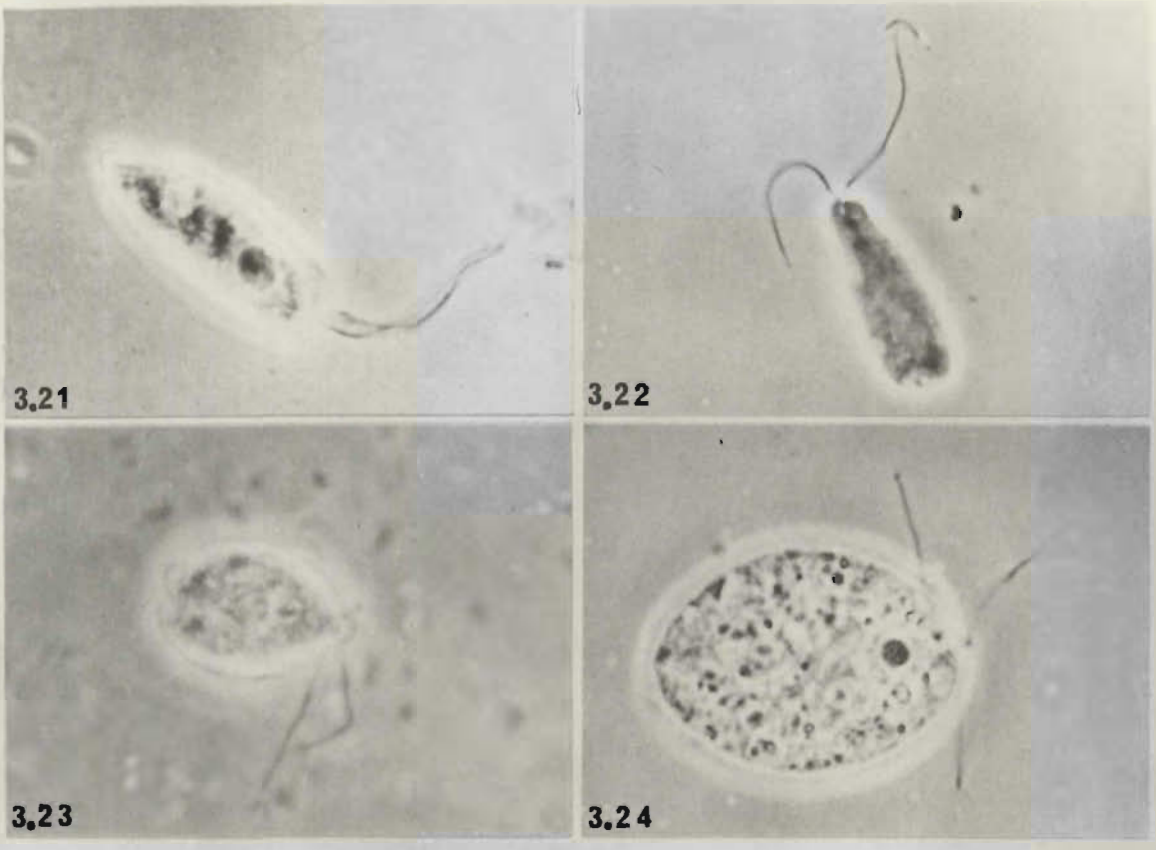
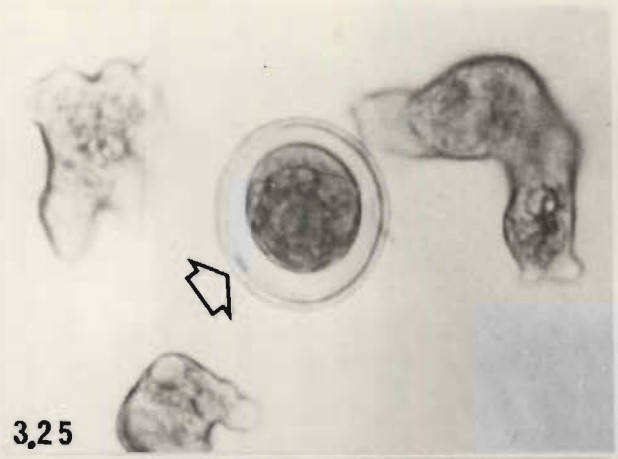
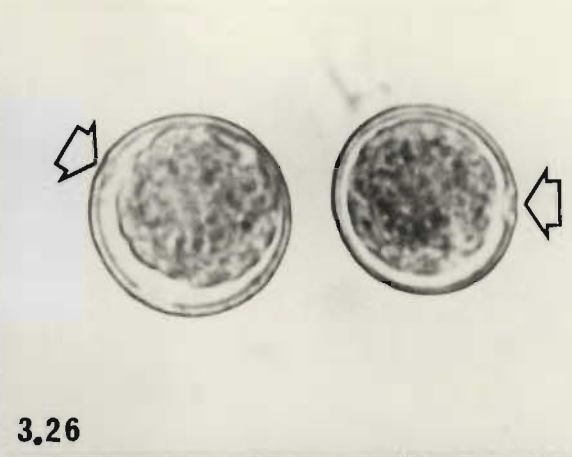
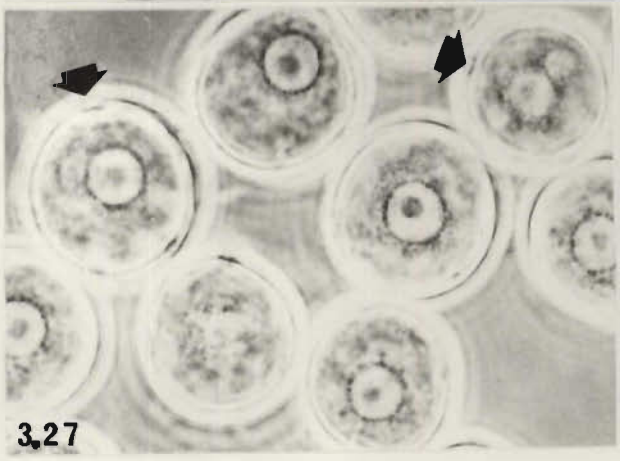


Fig. 3.21 A Naegleria fowleri, Oram strain flagellate. X5 500.
Fig. 3.22 A flagellate of the Naegleria fowleri, RL strain. X4 600.
Fig. 3.23 A Naegleria fowleri, Northcott strain flagellate. X5 300.
Fig. 3.24 A flagellate of the Naegleria jadini, 0400 strain. X5 150.







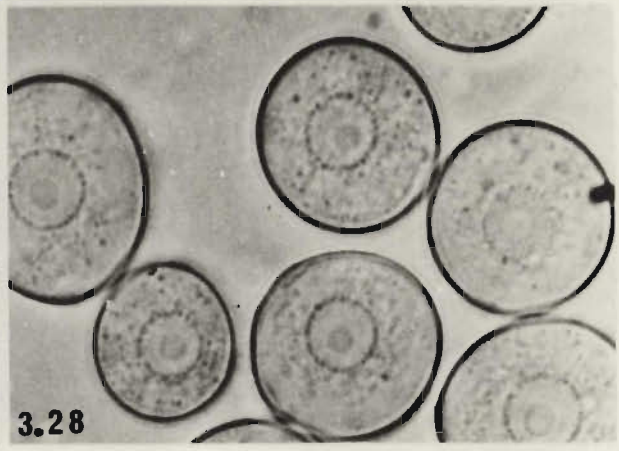


Fig. 3.25 A cyst of the SA-1 strain of Naegleria gruberi surrounded by several amoebae. An exit pore (arrow) is present. X5 750.

Fig. 3.26 Cysts of the Naegleria gruberi, NEG strain, with conspicuous exit pores (arrows). X5 750.

Fig. 3.27 A group of Naegleria gruberi, strain NB-1 cysts with prominent exit pores(arrows), nucleoli, nuclear membranes and vacuoles. X4 800.

Fig. 3.28 Naegleria gruberi, strain SA-2 cysts. X5 000.

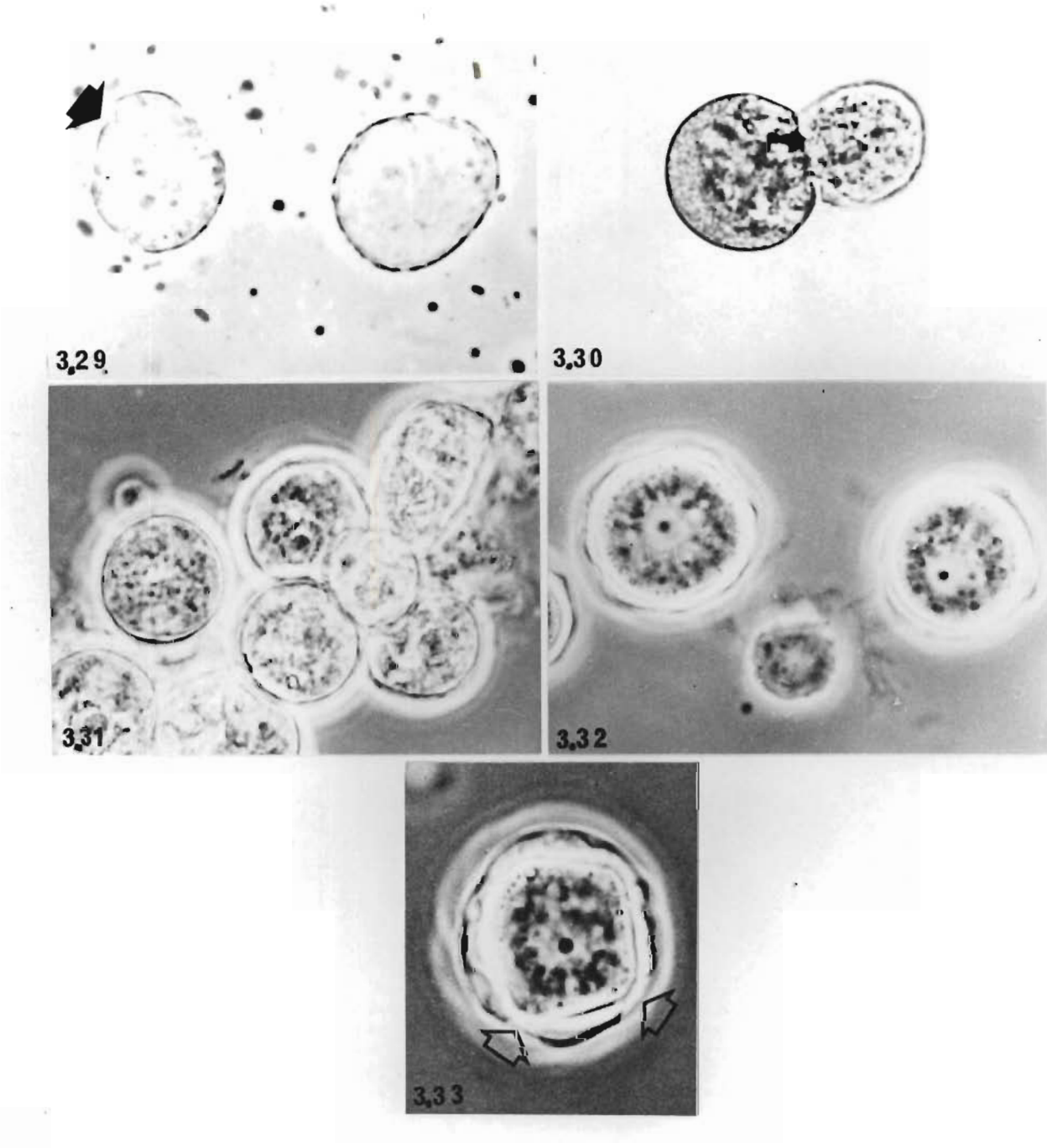


Fig. 3.29 Empty Naegleria fowleri, Oram strain cysts. The arrow indicates a rupture in the cyst wall. X5 500.

Fig. 3.30 An amoeba of the K-71 strain of Naegleria fowleri excysting from a single walled cyst via a prominent rupture. X5 500.

Fig. 3.31 A group of Naegleria jadini, strain 0400 cysts. X5 300.

Fig. 3.32 Acanthamoeba castellanii cysts. X5 600.

Fig. 3.33 A cyst of Acanthamoeba, strain APG with conspicuous ostioles (arrows). X6 500.

of deformed cysts were encountered - possibly the result of mechanical damage.

Naegleria fowleri cysts were somewhat smaller, with a mean diameter of 9,8 µm for the round to oval smooth-walled cysts (Figs 3.29, 3.30). The cyst wall was ruptured for the excystment of the amoeba, and no structures suggestive of exit pores were seen (Figs 3.29, 3.30).

A centrally placed nucleus and granules up to 1 µm in diameter were seen in these cysts. Up to 25% of the cysts appeared to be non-viable.

Naegleria jadini cysts were single-walled with no obvious exit pores or plugs and had a mean diameter of 11,8 µm (Fig. 3.31).

E.2 Acanthamoeba

E.2.i Amoebae

An amoeba of the Acanthamoeba strain APG is shown in Fig. 3.11. These amoebae when rounded were up to 22,9 µm in diameter, and like the Naegleria strains had a conspicuous nucleus with a mean diameter of 4,4 µm with a prominent, centrally placed nucleolus measuring 3,5 µm in diameter. Unlike Naegleria, these amoebae had prominent, fine pseudopodia (acanthopodia). Food, and contractile vacuoles up to 2,2 µm in diameter, cylindrical mitochondria measuring up to 1,5 by 0,5 µm, as well as inclusion bodies (lipid?) up to 1,5 µm in diameter were present in these amoebae. The amoebic phase of Acanthamoeba castellanii displayed the same morphological features.

E.2.ii Cysts

The cysts of both Acanthamoeba strains (Figs 3.22, 3.33) examined were quite different from those of Naegleria. The cysts of the APG strain with a mean diameter of 13,6 µm and the Acanthamoeba castellanii strain with a mean diameter of 12,9 µm. In both these strains excystment was via an ostiole up to 2,1 µm across, plugged by an operculum (Fig. 3.33). The cyst walls were smooth when young, but gradually as they aged, they became more wrinkled (Chapter 5 of this thesis). The centrally placed nucleus was not always easy to see. The cysts were spherical and double-walled - the outer exocyst covered the entire surface while an inner endocyst was discontinuous in the area of the ostioles.

F. DISCUSSION

The results presented on the morphology of Naegleria gruberi, Naegleria fowleri, Naegleria jadini and Acanthamoeba are in good agreement with the findings of other investigators using the light microscope (46,83, 84,113,312), and with the transmission electron microscope studies in this thesis (Chapter 6).

The morphology of the amoeboid and flagellate stages of all three species of Naegleria was similar, but distinct from other amoebo-flagellates (301,416,417,448). For differentiation under the light microscope, emphasis must be placed on the cyst morphology. Naegleria gruberi cysts were found to be smooth in surface texture, but Page (319) reported that certain strains may also be rough or angular.

A number of investigators (169,312,391,392) have used the exit pores in the cyst walls of Naegleria gruberi as a valuable taxonomic criterion. Excystment occurs via cyst wall rupture in Naegleria fowleri cysts which are reported to have no exit pores (44,46,47,252). Willaert & Le Ray (459) could not find any pores in the cyst wall of Naegleria jadini, and concluded that excystment took place by rupture of the cyst wall. Page (319, and personal communication Nov.1974) could not distinguish pores or plugs in Naegleria fowleri and Naegleria jadini by optical or cytochemical methods. Using a scanning electron microscope, I (Chapter 5 of this thesis, 252) did not observe any structure suggestive of cyst wall pores in Naegleria fowleri. Jadin (205) and Schuster (371) though, using scanning and transmission electron microscopes, have presented evidence of pores in the cyst wall of some strains of Naegleria fowleri and Naegleria jadini. The possibility of contaminated cultures in this instance must be considered, or it may be that there are different cyst phenotypes, those with, and those without pores.

Clearly the issue is not settled, but under the light microscope Naegleria gruberi does display obvious exit pores in the cyst wall, while Naegleria fowleri and Naegleria jadini cysts do not.

The morphological observations of Acanthamoeba reported in this chapter agree with published descriptions. The rougher surface morphology and the presence of ostioles in the Acanthamoeba cysts are quite distinct features which differentiate them from other amoebae. Visvesvara & Balamuth (426) stated that several species of Acanthamoeba could be differentiated by cyst morphology. The trophozoites of Acanthamoeba are quite distinct from those of Naegleria and the two amoebae are therefore easily distinguished.

The results presented in this chapter indicate that it is possible with light-microscopy to differentiate pathogenic and non-pathogenic Naegleria and other limax amoeba, provided that attention is paid to the details of cyst morphology.

CHAPTER 4

A TIME-LAPSE MICROCINEMATOGRAPHIC ANALYSIS OF MOTILITY AND CYTOKINESIS IN NAEGLERIA FOWLERI AND NAEGLERIA GRUBERI

A. ABSTRACT

Time-lapse microcinematography was employed to study motility and cytokinesis in the amoeboid and flagellate stages of Naegleria fowleri and Naegleria gruberi. Frame by frame analysis of the resulting cinematographic film has indicated that the processes of amoeboid motion, amoeba-to-flagellate transformation and the reversion of flagellate-to-amoeba were identical in the four strains of Naegleria studied. A new phenomenon of temperature shock-induced cytokinesis was also recorded in Naegleria amoeba. In this phenomenon, cells with one or more nuclei would attempt cytokinesis after a brief exposure to a temperature of 9°C. Usually the cytokinesis was abortive, and the daughter cells would coalesce along a connecting strand of cytoplasm to form a single amoeba. Occasionally, the thin connecting strand of cytoplasm would break and the daughter cells, either nucleated or anucleated, would separate.

B. INTRODUCTION

The technique of time-lapse microcinematography has been extremely useful in the analysis of motility and other biological phenomena in a variety of amoebae (191,193,194,203,280). As there was no published report of this technique having been used to study Naegleria, it was decided to undertake a time-lapse microcinematographic study of the amoeboid and flagellate stages of Naegleria fowleri and Naegleria gruberi. Particular emphasis was paid to the phenomena of amoeba-to-flagellate transformation and subsequent reversion of flagellate-to-amoeba. A portion of the results of this chapter has formed the first comparative time-lapse microcinematographic study of transformation and reversion in pathogenic and non-pathogenic Naegleria (258).

C. MATERIALS

Organisms — Sources are recorded on p.146-147.

Media — Described on p.148-149.

Chemicals — Described on p.149-150.

Apparatus — A modified 16mm Bolex H 16 reflex cinematographic camera was mounted on a Zeiss microscope inside a temperature-controlled chamber.

Illumination of the specimen and advance of the film was done automatically by ancillary electronic equipment. Kodak tri-X reversal film type 7278, or Agfa Gevaert type 25 film was used. Film was exposed at two frames/second.

D. METHODS

D.1 Production, harvesting and preparation of amoebae and flagellates

The NB-1 and NEG strains of Naegleria gruberi were grown at 25°C and the N.f. 1969 and Oram strains of Naegleria fowleri were grown at 37°C in Fulton's A medium (169). These axenic cultures were used for studies of motility and cytokinesis in the various amoebae. For convenience and reproducibility of the amoeba-to-flagellate transformation process, the Naegleria strains were grown at 25°C or 37°C on PA agar with Klebsiella aerogenes as a food source. For the production of flagellates, the procedure outlined on p.39 was followed.

A rubber O ring was quickly dipped in molten wax and then placed on a clean microscope slide. A few minutes later, when the wax had hardened, about three quarters the volume enclosed by the rubber O ring was filled with mineral oil and allowed to temperature equilibrate. Next, a small amount of Naegleria amoebae in buffer was floated on the mineral oil layer. Initially, the entire O ring was filled with amoebae in suspension, but this was unsatisfactory, as the volume was too large and the cells invariably slipped out of focus. As the transformation process is aerobic, care was taken to leave an air space under the coverslip when it was laid on the O ring. The prepared slide was then immediately placed on the microscope stage in the temperature-controlled chamber set at 25°C for Naegleria gruberi, or at 37°C for Naegleria fowleri. The initiation of transformation was calculated from the moment when the Tris-HCl buffer first came into contact with the amoebae.

For studies of motility in the amoebae, the axenic, liquid-grown cultures were placed directly on the mineral oil layer in the O ring. This procedure was modified for cytokinetic studies, as the prepared microscope slide was warmed to the appropriate temperature for the amoeba strain, and rapidly chilled in a 9°C refrigerator for 1-3 minutes, and then quickly returned to the initial temperature. The cells were monitored and filming commenced once the initiation of cytokinesis had started several hours later.

D.2 Time-lapse microcinematography

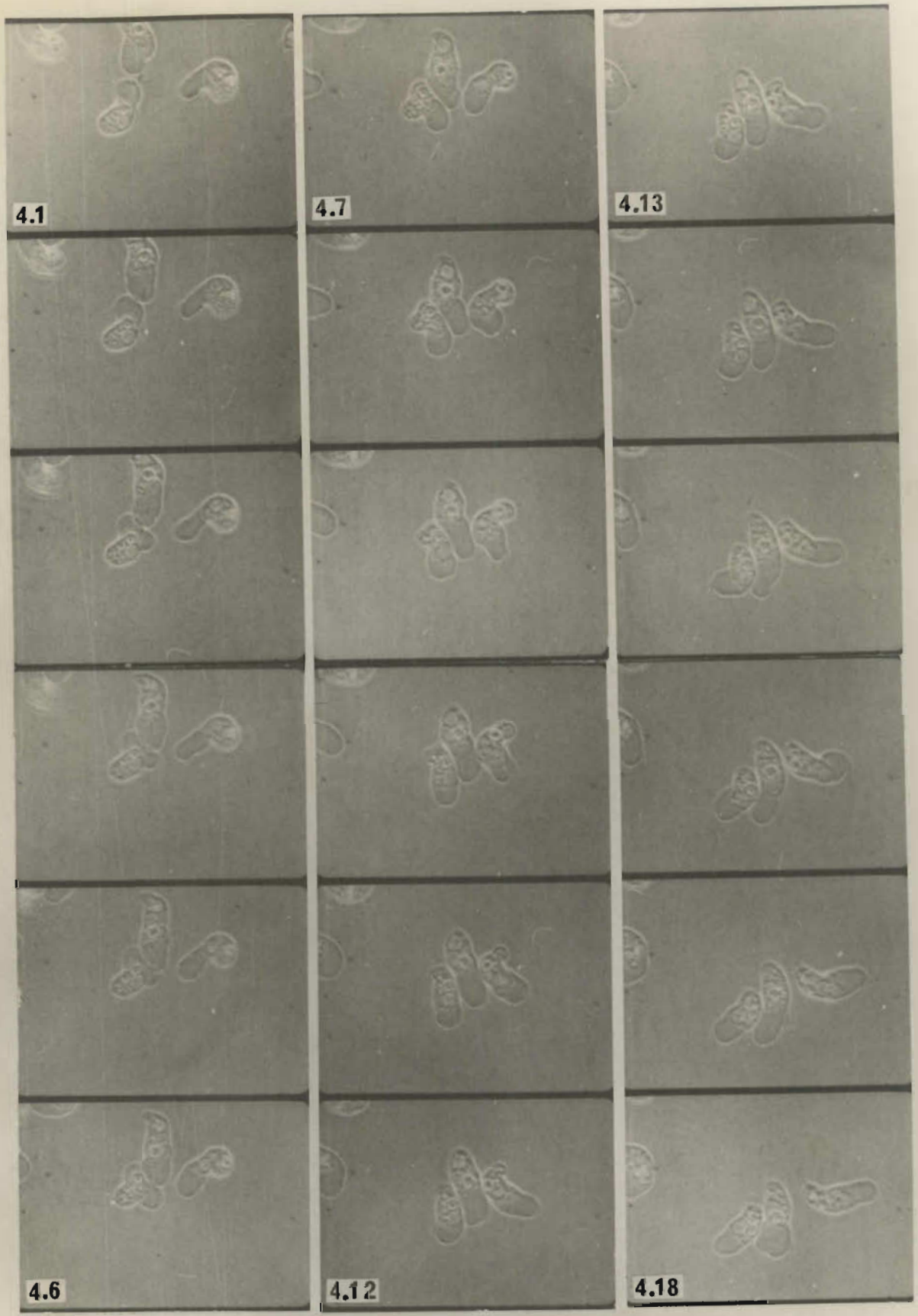
All stages of transformation, reversion, amoeboid motion and cytokinesis were monitored visually through the microscope. Filming was done mainly under manual control as the organisms would often move out of the frame or out of focus, so that constant visual maintenance was essential. As a single sample would occupy the microscope for most of a day, and as the experiments were repeated several times for each strain, the preparation of 250 metres of exposed film took 8 months. This film was critically examined and edited to a length of 50 metres. The resulting composite film was viewed many times and specific sections were analysed frame by frame.

E. RESULTS

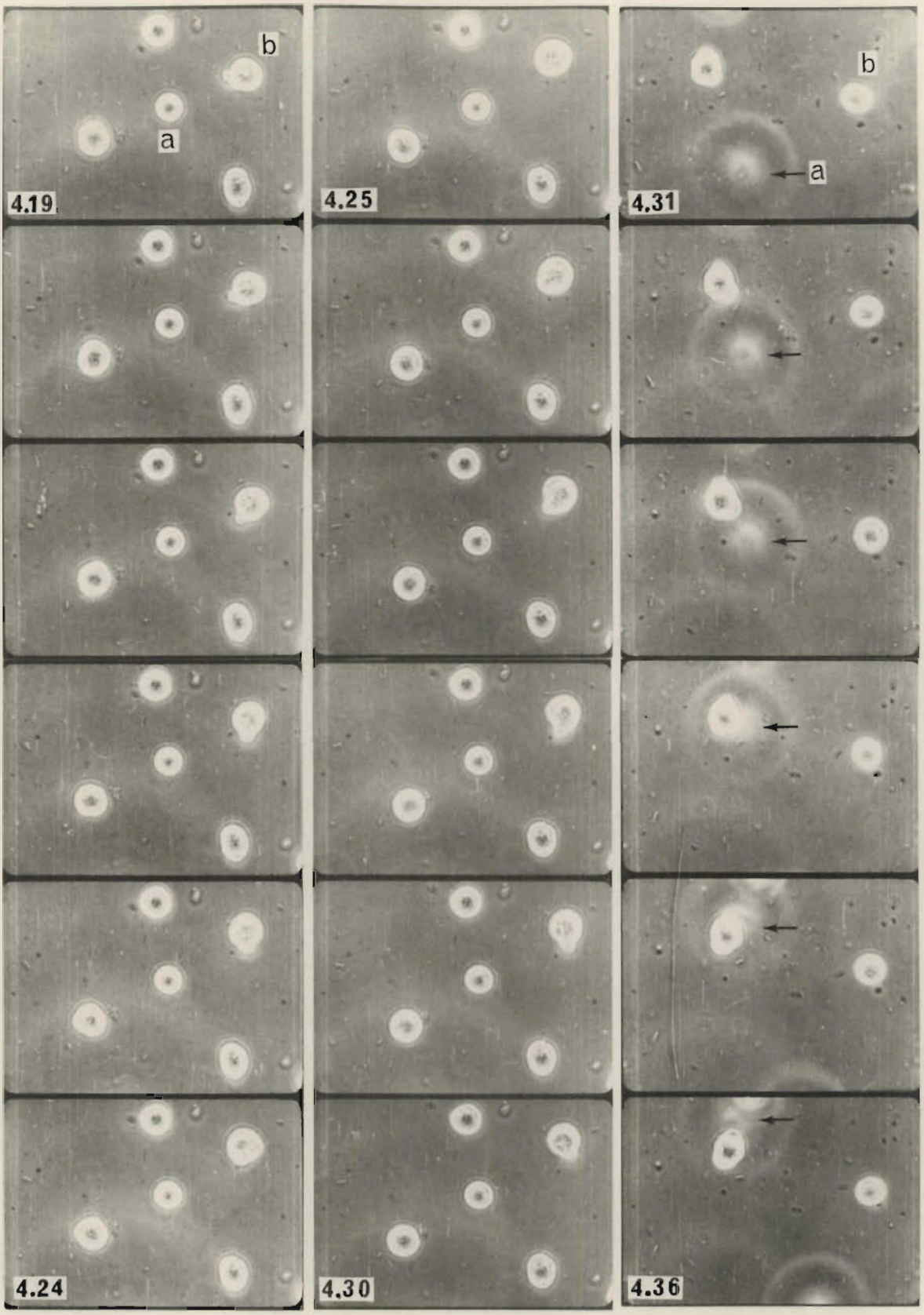
A representative selection of the time-lapse microcinematographic studies is shown in Figs 4.1-4.90, p.30-34.

Amoebae of the Naegleria gruberi, NEG strain illustrate the characteristic, aesthetically pleasing movement of limax amoebae in Figs 4.1-4.18. Typically, the amoebae moved by means of broad, eruptive, often single pseudopodia usually formed near or to one side of the last pseudopod. This led to a somewhat sinuous progression, although amoebae can expel pseudopodia at any point on the surface of the cell and change direction. The anterior end of the amoebae was usually seen to be broader than the posterior end, and uroid filaments at the posterior end were seen on occasion. The uroid filaments of Naegleria were capable of rotational movement. Deformation of nuclei and other organelles occurred while the amoebae moved and was quite evident when the film was projected.

For the first time, it was possible by means of frame by frame analysis of the cinematographic film to examine the sequence of stages in amoeba-to-flagellate transformation and flagellate-to-amoeba reversion, as is shown in Figs 4.19-4.36 for the Oram strain of Naegleria fowleri, and in Figs 4.37-4.54 for the 1969 strain of Naegleria fowleri. Detailed time course studies of transformation are reported in Chapter 8. Depending on the amoeba strain, temperature, and other conditions, 60-90 minutes after the cells had been suspended in Tris-HCl buffer, pH 7,2, the amoebae rounded up and spun rapidly while the flagellar apparatus was being elaborated. Concomitant with or prior to spinning the transforming amoeba attempted to stabilize its cell shape - often a spinning cell hesitated, stopped, threw out pseudopodia and continued spinning again. Stabilisation of the flagellate shape is most

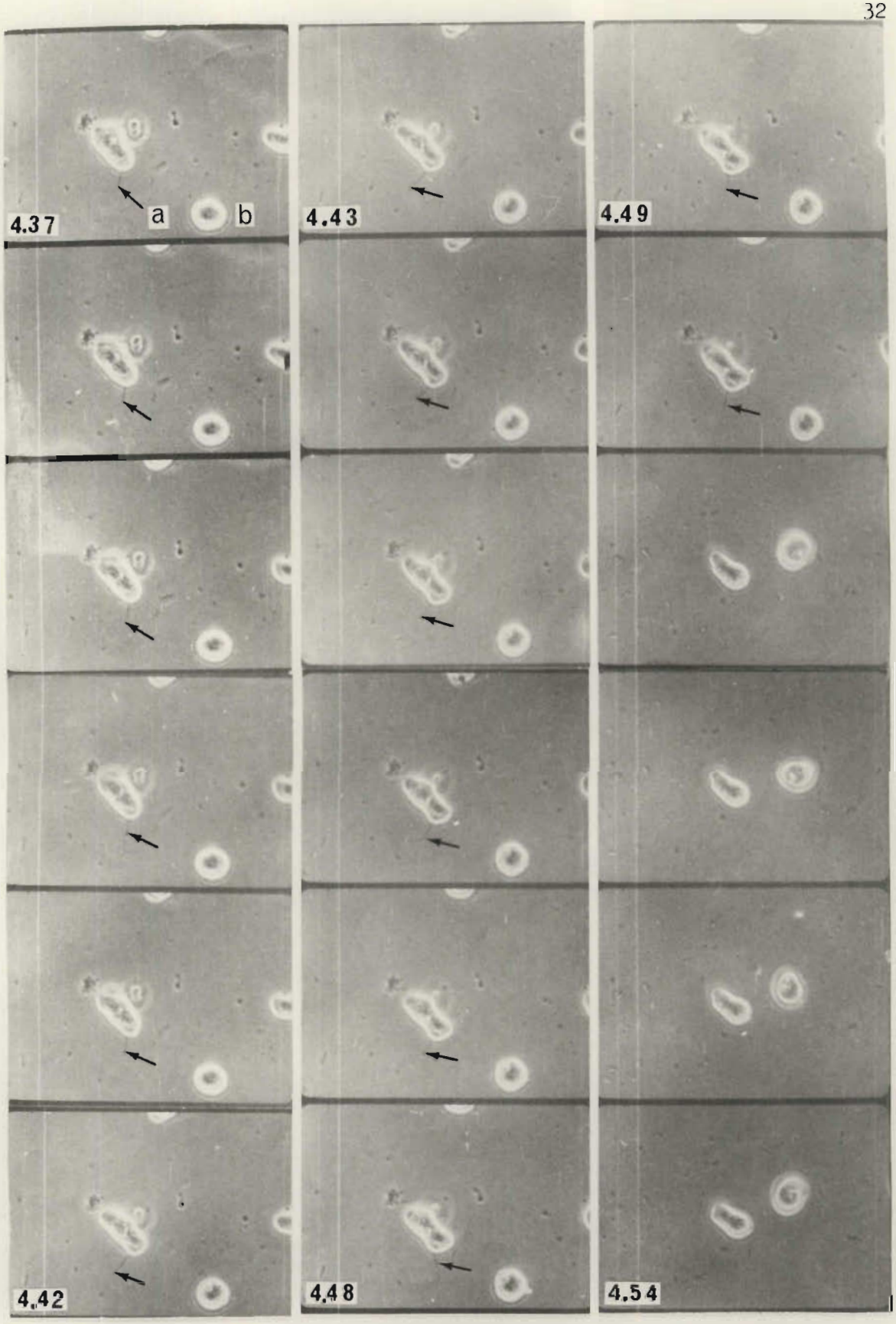


Figs. 4.1 – 4.18 Three limax amoebae of Naegleria gruberi, strain NEG execute a graceful "pirouette". Phase contrast microscopy clearly shows endoplasm, ectoplasm, nuclear details and contractile vacuoles in these actively motile amoebae.
Temperature = 25°C, film speed = 2 frames/second. X1 200.

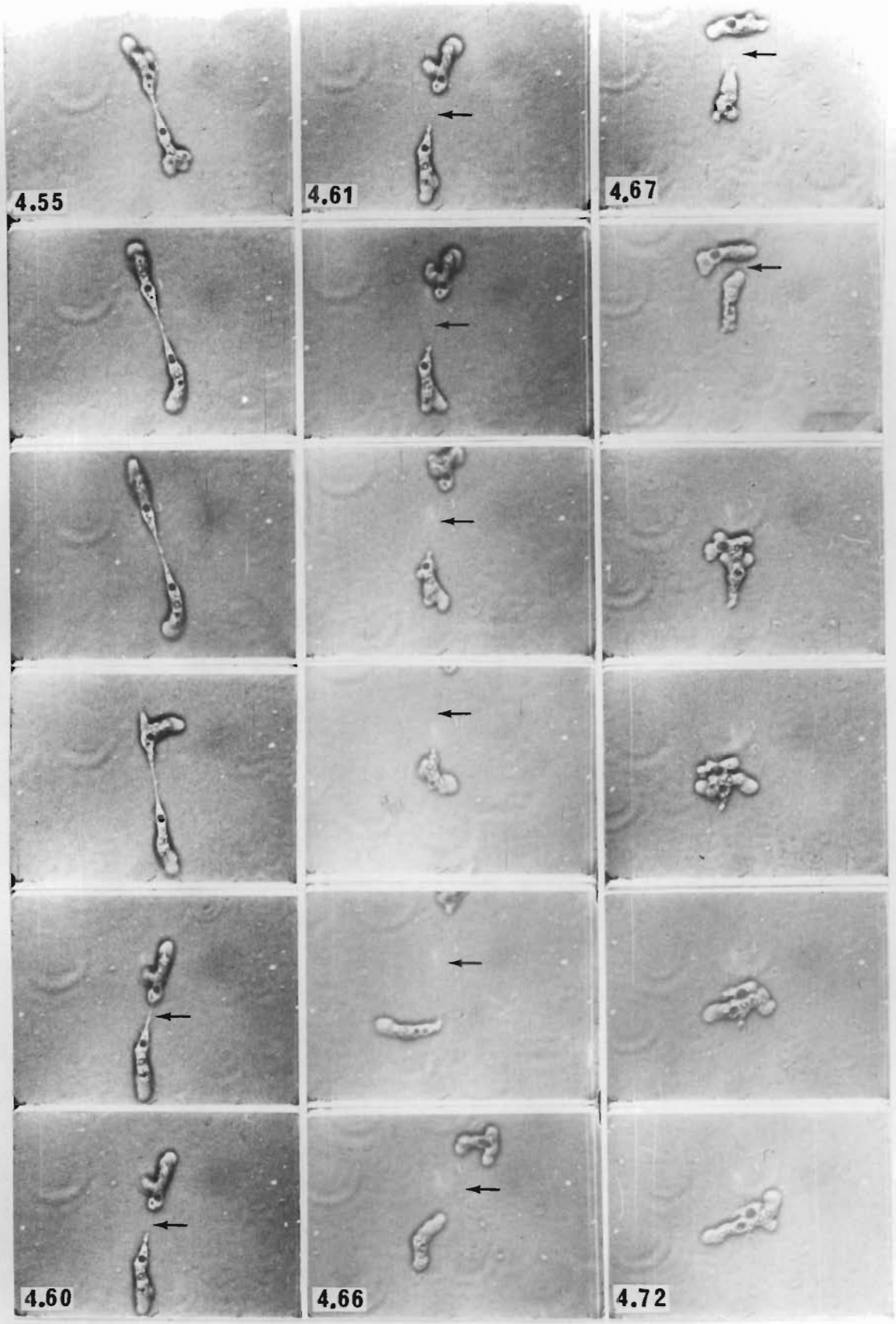


Figs. 4.19 - 4.30 A field of transforming Naegleria fowleri, Oram strain amoebae. Cell a is spherical and spinning rapidly, while cell b is a flagellate that is undergoing stabilization of cell shape. X1 200.

Figs. 4.31 - 4.36 Detail of above field. Cell a (now out of focus) is a fully stabilized flagellate and swims away, while cell b has stabilized into a sphere and is spinning rapidly. X1 640. Temperature = 37°C, film speed = 2 frames/second.



Figs. 4.37 - 4.54 Reversion in the Naegleria fowleri, 1969 strain. Cell a is a motile amoeba with attached, non-motile flagella (arrows) which are visible in frames 4.37 - 4.50. In frames 4.51 - 4.54, cell a has absorbed the flagellar axonemes. Cell b is a flagellate undergoing shape changes prior to reversion to an amoeba.
Temperature = 37°C, film speed = 2 frames/ second. X1 200.



Figs. 4.55 - 4.72 Figures 4.55 - 4.90 represent a selection of frames in sequence of a film lasting 2 minutes and 38 seconds. The film shows an amoeba of the NB-1 strain of Naegleria gruberi attempting a 9°C temperature induced cytokinesis. Both daughter cells are nucleated and after almost complete cytokinesis has occurred, these cells are still joined by a very fine strand of cytoplasm (arrows) in Figs. 4.59-4.68. Gradually, the daughter cells come together along the cytoplasmic strand, and finally fuse (Figure 4.69).

Figs. 4.73 - 4.90 In this sequence the bi-nucleated amoeba unsuccessfully attempts cytokinesis. Endoplasm, ectoplasm, nuclei and contractile vacuoles are clearly visible.
Temperature = 25°C, film speed = 2 frames/second. X1 200

probably due to a supporting microtubular cytoskeleton. Finally, the fully stabilised, and usually fusiform shaped flagellates swam off. Flagella on motile flagellates could not be resolved when the film was shown at two frames per second. After two hours most flagellates had reverted back to amoebae. Reversion was characterised by a loss of cell shape and the commencement of amoeboid motion. Concurrent with, or prior to this process, the flagella beat more and more slowly and finally became entirely paralysed. For a short time, the actively streaming amoebae dragged the now useless flagella behind them. The last step in reversion was the absorption of the flagella into the amoeboid cytoplasm.

Figs 4.55-4.90 illustrate a remarkable sequence of cinematographic frames taken over a two minute and 38 second period, of an amoeba of the NB-1 strain of Naegleria gruberi attempting a cold shock-induced cytokinesis. An abortive cytokinesis in which both nucleated daughter cells were still connected by a thin, long strand of cytoplasm is illustrated in Figs 4.55-4.68. This strand is barely visible in the figures but is quite obvious when the film is projected on a screen. The two daughter cells gradually moved together along the connecting cytoplasmic strand and fused (Fig. 4.69). The resulting binucleated amoeba attempted cytokinesis again, but without success (Figs 4.69-4.90). Amitotic cytokinesis (one daughter cell with a nucleus, the other without a nucleus) was observed on occasion with Naegleria amoebae after subjecting them to the 9°C cold-shock treatment. At times, the cytoplasmic strand would break and the nucleated and anucleated cells would separate. The eventual fate of these cells is not known.

F. DISCUSSION

The time-lapse microcinematographic studies presented in this chapter have yielded valuable information about the basic biology of Naegleria fowleri and Naegleria gruberi, and have confirmed and extended similar observations by convertional light microscopy in this thesis (Chapter 3), and by other research workers (47,55,72,169,222,312,333,349,392,460).

It was found that brief exposure to a temperature of 9°C induced cytokinesis in Naegleria trophozoites. Although cytokinesis induced by this treatment was usually abortive, on occasion the thin, connecting, cytoplasmic strand broke, producing separated daughter amoebae. Band et al.(19-21) have observed a similar cytokinetic phenomenon in Acanthamoeba. Trophozoites of Acanthamoeba were starved and then placed in media which induced cell division without mitotic nuclear division (amitosis) and

nuclear division without cell division. The amitotic division was characterised by separation of the daughter cells with each cell having a portion of the original nucleus, and connected by a long, thin, cytoplasmic strand. Cytokinesis induced by a 9°C temperature shock will provide a most useful tool for future studies of cell division in Naegleria, the more so as Cytochalasin B will block cytokinesis but not karyokinesis (Chapter 8 of this thesis).

Other manipulations of temperature can profoundly affect the biology of Naegleria. Fulton & Guerrini (117) found that they could induce mitotic synchrony in 60-70% of a population of Naegleria gruberi amoebae by shifting the temperature from 30°C to 38°C and back to 30°C. An increase in temperature while the amoebae are transforming into flagellates will induce additional flagella (126).

Naegleria is a valuable experimental organism as the various stages in the life cycle are hardy in laboratory manipulation and the organism is easily grown. Rational use of temperature and other parameters will provide fundamental information on the basic processes of mitosis and phenotypic change in Naegleria, the results of which are directly applicable to other cell types.

CHAPTER 5

SCANNING ELECTRON MICROSCOPY OF LIMAX AMOEBAE

A. ABSTRACT

The scanning electron microscope was used to examine six strains of non-pathogenic Naegleria gruberi, nine strains of pathogenic Naegleria fowleri and six other strains of limax amoebae. Excystment of Naegleria gruberi amoebae occurred via preformed exit pores in the cyst wall. Exit pores were not found in the strains of Naegleria fowleri examined where excystment occurred by rupture of the cyst wall. Excystment in Acanthamoeba sp. took place via a prominent ostiole plugged with an operculum. Schizopyrenus erythaenusa and Singhella leptocnemus excysted by dissolution of the cyst wall. Amoebae and flagellates of Naegleria gruberi and Naegleria fowleri were similar in surface topology, and no structure suggestive of a surface active lysosome was found in any of the cells examined.

B. INTRODUCTION

The scanning electron microscope (S.E.M.) is a most powerful tool for the high resolution study of the surface topology of cells. To date, published S.E.M. studies of limax amoebae include those by Pasternak et al. (322) and Chambers & Thompson (70) dealing with, respectively the encystment and excystment of Acanthamoeba castellanii. J.B.Jadin (205) and J.M.Jadin et al.(209) have reported on the cyst morphology of Naegleria and Acanthamoeba. Various investigators have reported on the surface morphology of the trophozoites of Acanthamoeba culbertsoni and Hartmannella agricola (362) and Naegleria fowleri (271,276).

Scanning electron microscopy was used to examine the various morphological stages of the life cycle of Naegleria and to compare and contrast virulent and avirulent Naegleria with each other, and with other selected limax amoebae.

An examination of the cysts is an obvious starting place for comparative studies, as cysts are easily recognisable, readily available, and hardy in laboratory manipulation. In addition, differences in cyst morphology detected under the S.E.M. may be important taxonomically, as has been found for the shelled amoeba, Difflugia (146). The amoeboid and flagellate stages of Naegleria were examined for the presence of any unusual surface features such as structures similar to the surface-active lysosomes of Entamoeba histolytica (143,144,344,345,347,356,357). A portion of the results presented in this chapter has been published (252).

C. MATERIALS

Organisms	Sources are recorded on p.146-147.
Media	Described on p.148-149.
Chemicals	Described on p.149-150.
Apparatus	Hitachi Scanscope SSM 2, an A.M.R. model 1000, or a Cambridge Stereoscope Mark IIA scanning electron microscopes and ancillary equipment.

D. METHODS

D.1 Production, harvesting and preparation of cysts

Strains of Naegleria gruberi, Acanthamoeba, Schizopyrenus and Singhella were grown at 34°C on NM agar in association with Klebsiella aerogenes. Strains of Naegleria fowleri were grown at 37°C on PA agar in association with the same bacterium. Approximately 10^5 cysts and 0,1ml of a liquid Difco Penassay broth culture of Klebsiella aerogenes was spread over the surface of the agar in petri dishes, and incubated at 34°C or 37°C. The Naegleria gruberi excysted, grew to stationary phase, and encysted within 72 hours. The Naegleria fowleri strains took up to 11 days for complete encystment to occur. Strains of Acanthamoeba, Schizopyrenus and Singhella took up to 17 days for complete encystment at 34°C. Encysted Naegleria fowleri were stored at 37°C and used 20-24 days after preparation, while all other amoeba cysts were stored at 34°C and used 8-16 days after preparation. Usually one petri dish of Naegleria gruberi cysts was adequate for a preparation for the scanning electron microscope. As Naegleria fowleri and other amoeba cysts were sparser, three petri dishes of each strain were used for the preparation of samples.

For harvesting, 15ml of Tris-HCl buffer pH 7,2 was added to the petri dishes and the cysts removed with a bent glass rod. Cyst suspensions of Naegleria fowleri, G.J., Oram, McMahon and of Naegleria gruberi, 27 and 1815, had 2mg/ml of digitonin added to the first buffer wash in order to lyse any amoebae present while leaving the cysts unaffected. These samples stood for 15 minutes at 25°C to allow lysis to occur and were then processed in the same way as the other cyst preparations. The Tris-HCl buffer solution and all other subsequent solutions were used at 25°C. Cyst suspensions were centrifuged for 115 seconds at 500g in a swinging-bucket rotor. The supernatant fluid was discarded and the cysts washed twice more with buffer. Cysts were prepared for the S.E.M. as outlined in section D.3 of this chapter.

D.2 Production, harvesting and preparation of amoebae and flagellates

Strains of Naegleria fowleri were grown at 37°C and the 27 and 1815 strains of Naegleria gruberi were grown at 25°C in Fulton's A medium (169). Naegleria gruberi strains NB-1, NEG, SA-1, SA-2 were grown on NM agar at 34°C with Klebsiella aerogenes while all other amoebae were grown on PA agar at 34°C with the same bacterium as a food source. Liquid-grown amoebae were briefly centrifuged twice at 500g for 60 seconds in a swinging-bucket rotor and washed twice with Tris-HCl buffer at 25°C. Amoeba grown on agar were harvested in the same way as were the cysts. Amoebae suspensions were prepared for the S.E.M. as outlined in section D.3 of this chapter.

For the production of flagellates, 4 to 6 petri dishes of each strain of Naegleria fowleri were grown at 37°C for 36-48 hours. When the amoebae had cleared 50-75% of the bacterial lawn, 15ml of Tris-HCl buffer was added to each petri dish. A bent glass rod was used to harvest the amoebae and the resulting suspensions were centrifuged and washed twice more with buffer to remove most of the bacteria. Each cell suspension was poured into a separate 50ml flask and the volume brought up to 15ml with buffer. Amoebae were allowed to transform by being placed in a shaking water bath at 37°C. Naegleria gruberi was treated in a similar manner except that transformation was accomplished at 25°C. During transformation, the cells were monitored by phase-contrast microscopy. When the maximum proportion of cells was flagellated, a volume of 6% glutaraldehyde equal to the volume of the cell suspension was added to the flask, which was quickly swirled and then allowed to stand for 60 minutes at 25°C. From this point, the cells were treated for the S.E.M. as outlined in section D.3 of this chapter.

D.3 Scanning electron microscopy

Cells were fixed with 3% glutaraldehyde in buffer for 60 minutes. After centrifugation, the cells were washed twice more with buffer. Next, they were taken through a graded ethanol series to absolute ethanol taking 15-20 minutes per step. The absolute ethanol was changed twice before the cell suspension was pipetted directly onto the specimen stubs. These were air-dried overnight in a desiccator and surface-coated by vacuum evaporation of a gold palladium alloy. As a potential technical improvement, some of the suspensions were freeze-dried and coated by the procedure of Darley & Lott (106) after reaching the absolute alcohol stage.

Before and during preparation cysts, amoebae and flagellates were routinely examined under phase-contrast microscopy for any evidence of

artifact formation or distortion. Several preparations of each strain were examined under the scanning electron microscope. At each viewing at least 200 individual cells of each preparation were examined critically for shape, surface texture and distinctive morphological features. Magnifications were measured from contact prints of the original S.E.M. negatives.

E. RESULTS

A selection from over 400 scanning electron micrographs illustrating the fine structure of all the amoeba strains examined is shown in Figures 5.1 - 5.32, p.42-46.

Naegleria gruberi cysts measured before preparation for the scanning electron microscope were between 8 and 19 µm in diameter. The cysts of all 6 strains of Naegleria gruberi were spherical to slightly oval. Under the light microscope the cyst surface texture was reticulated. Under the S.E.M., all strains of Naegleria gruberi showed up to five definite preformed exit pores piercing the cyst wall. In strain NEG (Fig. 5.1), the excysted amoebae have left three empty cyst casings behind with clearly indicated perforations in the cyst walls. A distinct collar or circular ridge circumscribes the exit pore, as seen in strain 27 (Fig. 5.16). Cysts of strain NB-1 (Fig. 5.2) exhibit plugged exit pores, while in Fig. 5.11, both the plugged and empty pores of strain 1815 are illustrated. These plugs are probably dissolved by the excysting amoebae before emergence. Two South African strains of Naegleria gruberi, SA-1 and SA-2 had features similar to the other Naegleria gruberi strains examined.

The cyst size of the pathogenic Naegleria fowleri strains, measured before S.E.M. preparation, varied from 4 to 11 µm in diameter. These eight strains exhibited a more varied cyst morphology with respect to shape and surface texture than did the strains of Naegleria gruberi examined. Usually the cysts were spherical, but oval cysts were occasionally seen. Surface textures varied from smooth or finely reticulate for strain 161A (Figs 5.8 - 5.10), to ridged or coarsely reticulate for the other strains (Figs 5.3 - 5.7). In no case was any structure resembling an exit pore or associated plug seen in pathogenic cysts. Cysts of all Naegleria fowleri strains examined displayed ruptures of the cyst wall. Examples of this may be seen for Naegleria fowleri strains 161A (Fig. 5.10), G.J. (Fig. 5.13), N.f. 1966 (Fig. 5.4), Oram (Fig. 5.5), and PA-90 (Fig. 5.15). The ruptures are prominent and usually two or more µm across. The sequence of cyst rupture is illustrated for strain 161A in Figs 5.8 - 5.10. The smooth,

structureless cysts (Fig. 5.8) become invaginated (Fig. 5.9) and develop large ruptures in the cyst walls (Fig. 5.10).

Cyst preparations of Naegleria fowleri strains G.J. (Fig. 5.12), McMahon (Fig. 5.17), PA-90 (Fig. 5.14) and Naegleria gruberi strains 27 (Fig. 5.16) and 1815 (Fig. 5.11) which had been treated with digitonin to lyse any amoebae present, exhibited a rougher, more reticulated surface topology than did cysts not so treated. The action of digitonin may have been to remove material adhering to the cyst surface.

The cyst size of Acanthamoeba castellanii varied in diameter from 21 to 35 µm, for Acanthamoeba culbertsoni it was 11 to 25 µm, and for Acanthamoeba rhysodes, 12 to 30 µm. The surface texture of the cysts of the Acanthamoeba sp. examined is much more sculpted than is that of Naegleria. Young cysts of Acanthamoeba have numerous fine interconnecting folds or ridges outlining the circular areas of the ostioles, and forming irregular asymmetric patterns over the remainder of the cyst. With age, these ridges become more accentuated, and the mature cysts have a pronounced wrinkled appearance (Figs 5.20, 5.21). Acanthamoeba rhysodes was found to have an extremely fluted surface texture (Fig. 5.22). The three strains of Acanthamoeba examined excyst via an ostiole plugged by a distinct circular plug (operculum) which is at least 1 µm in diameter. One or more ostioles are present on the cyst surface, and the ostioles are delimited by a circular ridge. Figure 5.22 shows an ostiole with the covering operculum in place. Two breached Acanthamoeba culbertsoni cysts are depicted in Fig. 5.20 with the ostioles being especially prominent. Figure 5.21 illustrates a young Acanthamoeba castellanii cyst with an ostiole.

Schizopyrenus erythaenusa cysts (Figs 5.18, 5.19) are spherical and range from 10 to 22 µm in diameter. The smooth, structureless surface is well displayed in Fig. 5.19. Singhella leptocnemius cysts are 9 to 20 µm in diameter and have a finely reticulated surface (Fig. 5.23). Both these limax amoebae appear to excyst by partial or complete dissolution of the cyst wall.

The amoeboid phase of pathogenic and non-pathogenic Naegleria appears identical under the S.E.M. (Figs 5.24 - 5.28). The typical limax form was seen, usually with a single blunt pseudopod. Naegleria fowleri amoebae tended to develop multiple pseudopodia and elongate more than did the amoebae of Naegleria gruberi. Although not depicted, the amoebae of the Acanthamoeba sp. examined had numerous filamentous projections (acanthapodia) as well as lobopodia.

There was no difference between the flagellates of Naegleria gruberi

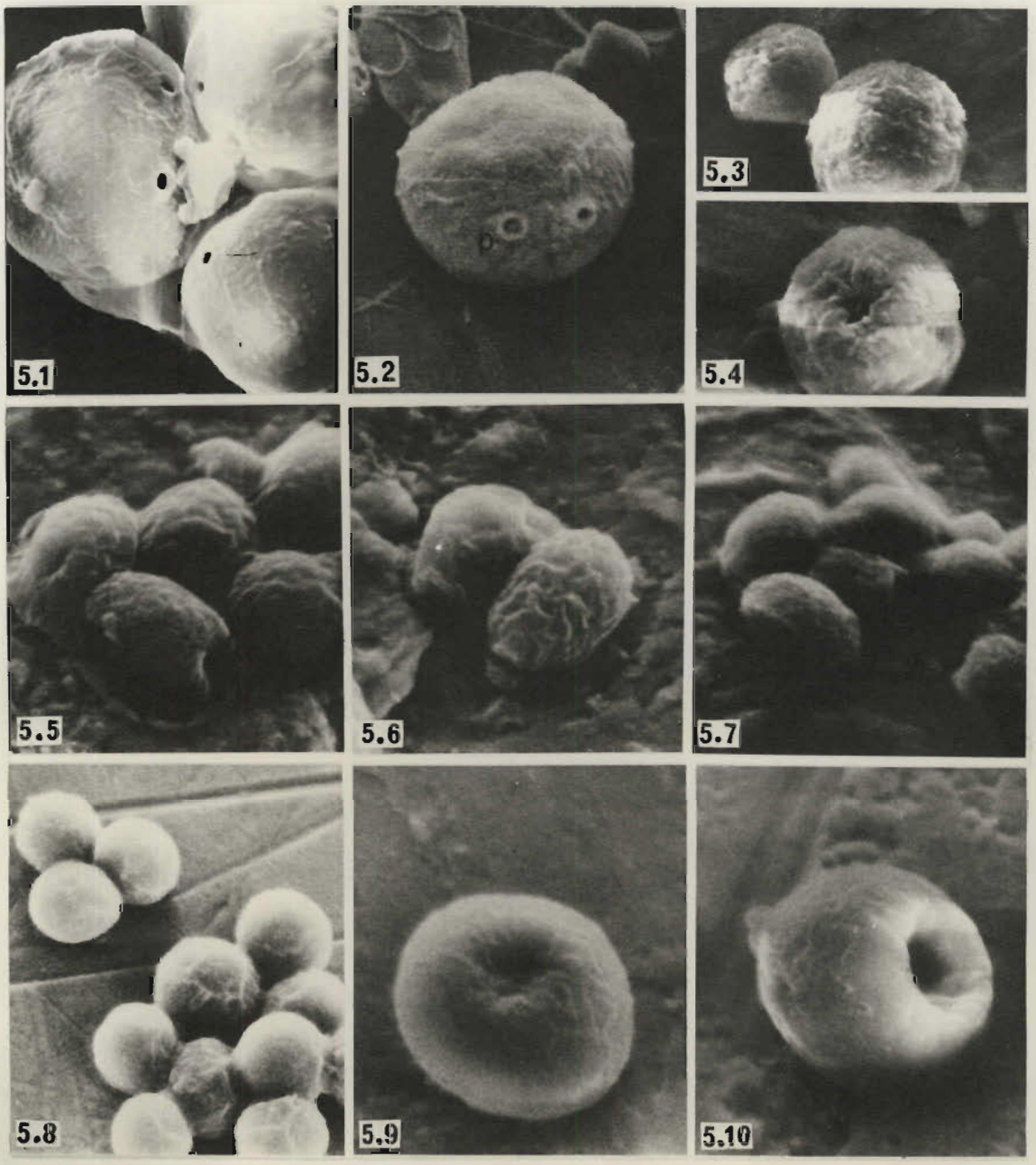


Fig. 5.1 Cysts of Naegleria gruberi, NEG, after excystment. Exit pores are especially prominent in the empty cyst walls. X3 000.

Fig. 5.2 A plugged exit pore (p) in a Naegleria gruberi, NB-1 cyst. X2 275.

Fig. 5.3 Two cysts of Naegleria fowleri, 1966. X2 500.

Fig. 5.4 Wall rupture in a Naegleria fowleri, 1966 cyst. X3 350.

Fig. 5.5 A group of Naegleria fowleri, Oram cysts. The two cysts in the foreground exhibit wall ruptures. X4 800.

Fig. 5.6 Ridged surface of Naegleria fowleri, Northcott cysts. X5 000.

Fig. 5.7 Cysts of Naegleria fowleri, 1969. X4 350.

Figs. 5.8 - 5.10 Excystment sequence in Naegleria fowleri, 161A.

Fig. 5.8 A group of poreless cysts. X2 875
Fig. 5.9 Invaginating cyst. X4 700.
Fig. 5.10 A fully ruptured cyst. X4 950.

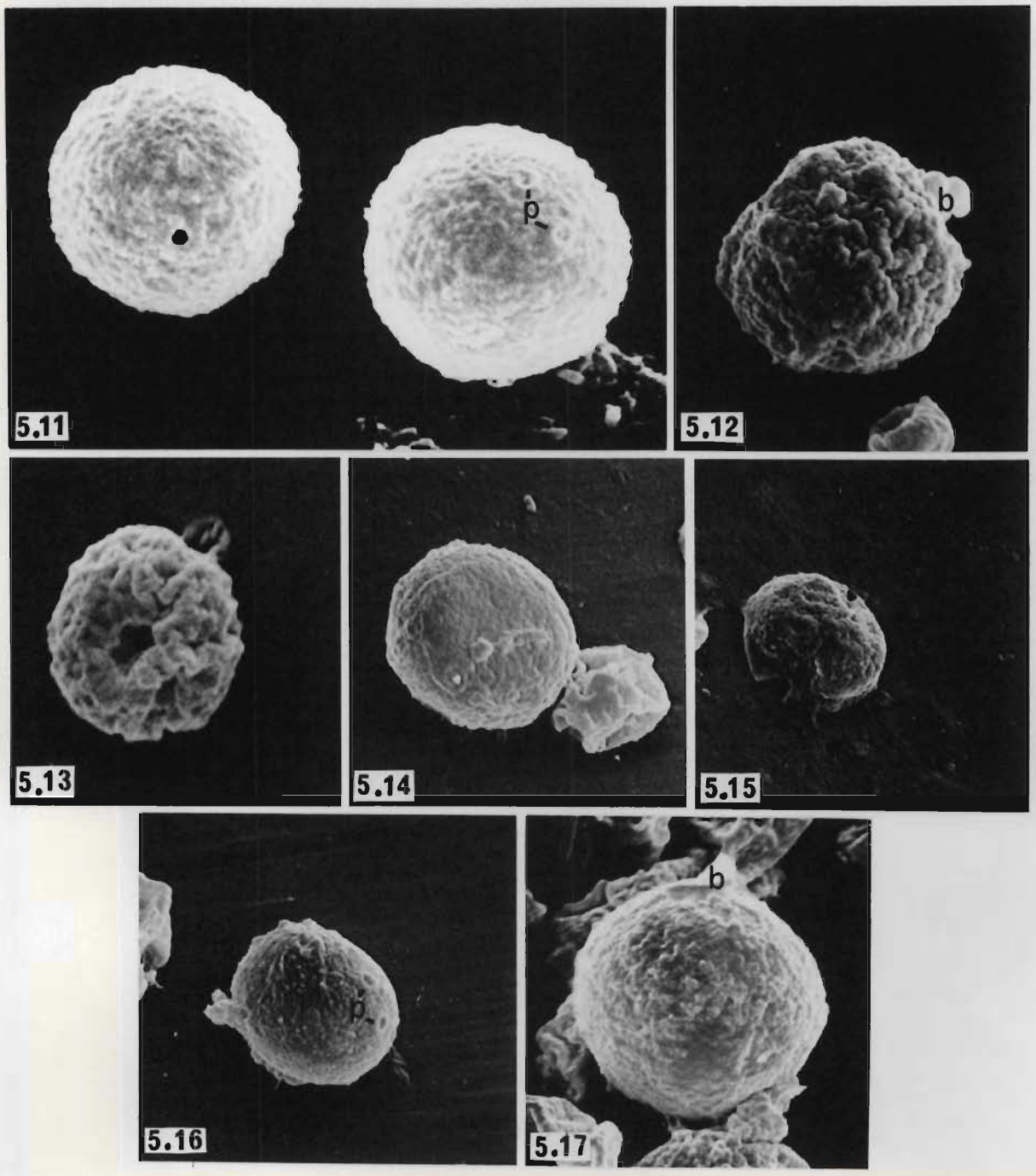


Fig. 5.11 Naegleria gruberi, strain 1815. The left hand cyst displays a prominent breached exit pore, while the right hand cyst shows two plugged exit pores (p). X5 800.

Fig. 5.12 Poreless Naegleria fowleri G.J. cyst with adhering bacteria (b). X7 750.

Fig. 5.13 Ruptured cyst of Naegleria fowleri, G.J. strain. X5 500.

Fig. 5.14 Poreless cyst of Naegleria fowleri, PA-90 strain. X2 250.

Fig. 5.15 Naegleria fowleri, PA-90, with prominent ruptures. X2 100.

Fig. 5.16 Naegleria gruberi, strain 27 cyst with plugged exit pores (p). X2 250.

Fig. 5.17 Poreless cyst of Naegleria fowleri, McMahon strain, with an adhering bacterium (b). X5 000.

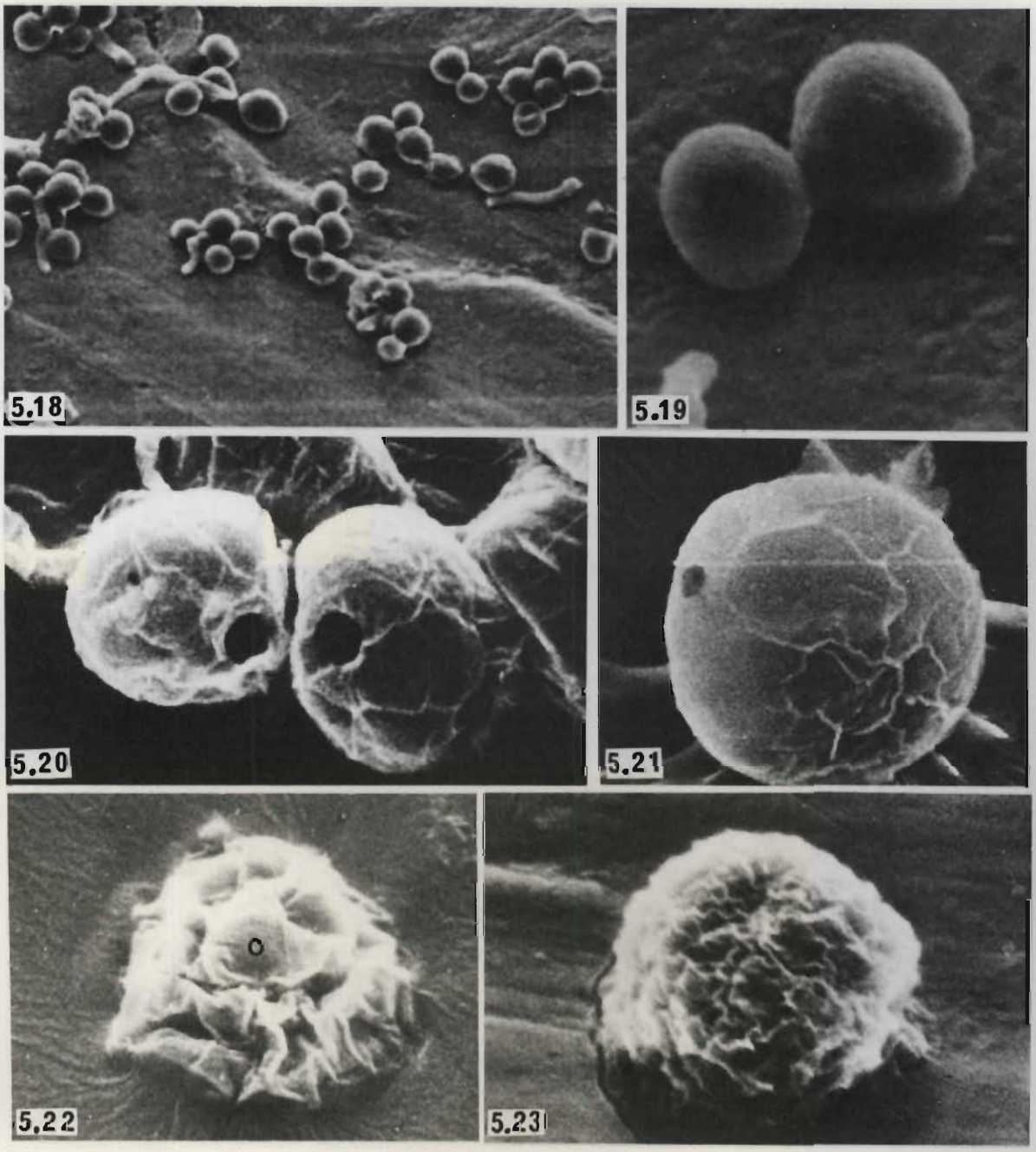


Fig. 5.18 Spherical cysts and elongated amoebae of Schizopyrenus erythaenusa. X900.

Fig. 5.19 Two smooth textured, poreless cysts of Schizopyrenus erythaenusa. X4 600.

Fig. 5.20 Acanthamoeba culbertsoni cysts with ridged surfaces and prominent ostioles. X5 800.

Fig. 5.21 A breached Acanthamoeba castellanii cyst with an ostiole and ridged surface. X6 450.

Fig. 5.22 Acanthamoeba rhysodes cyst displaying a deeply fluted surface texture with an ostiole capped by an operculum (o). X6 000.

Fig. 5.23 Singhella leptocnemius cyst with a finely fluted surface. X4 600.

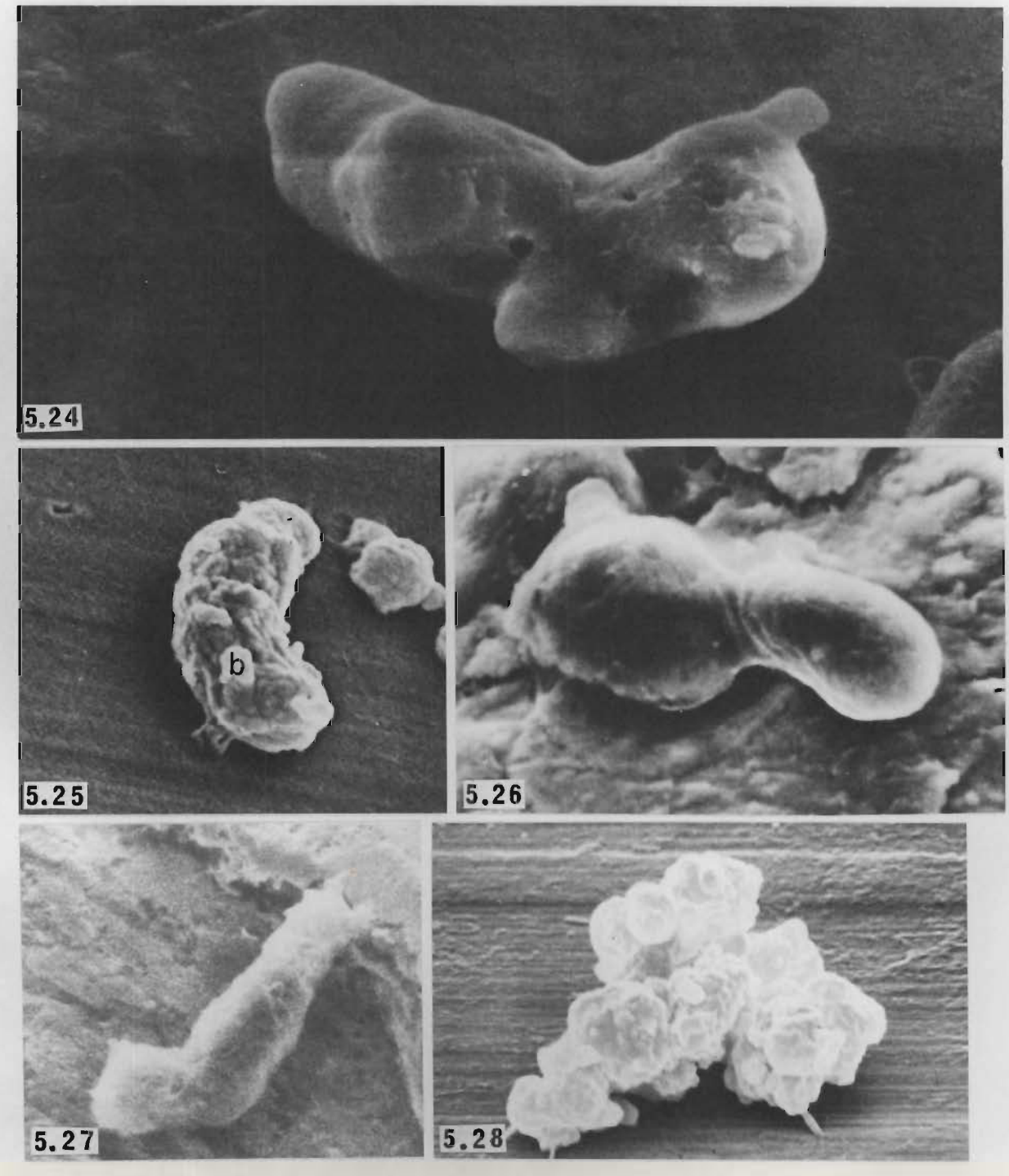


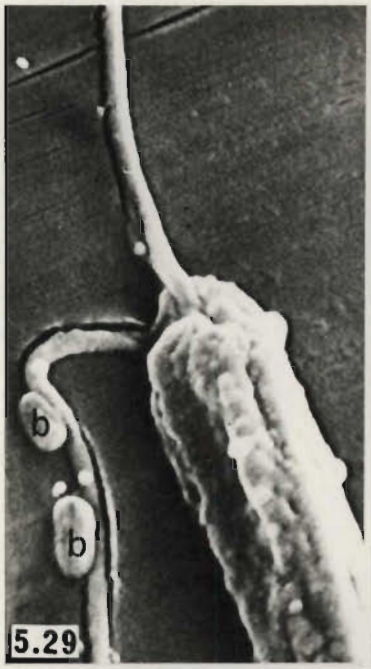
Fig. 5.24 A Naegleria gruberi, NB-1 amoeba exhibiting typical limax shape, with a blunt advancing pseudopod. Pores visible in the surface of the amoeba are probably openings of contractile vacuoles in systole. X4 375.

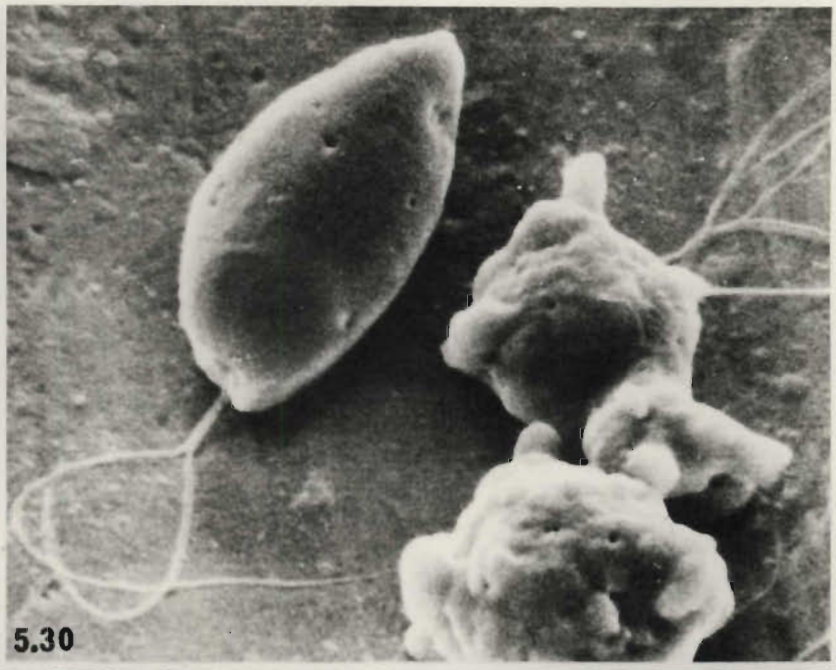
Fig. 5.25 Limax amoeba of Naegleria fowleri, G.J. strain with adhering bacteria (b). X4 900.

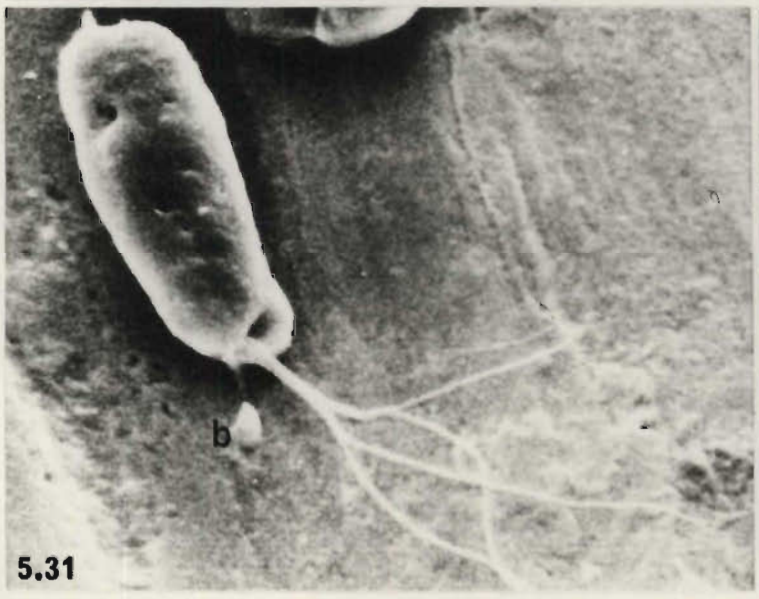
Fig. 5.26 An amoeba of Naegleria gruberi, Bristol strain. X4 200.

Fig. 5.27 A Naegleria fowleri, 1966 strain amoeba. X3 300.

Fig. 5.28 A group of Naegleria fowleri, Oram strain amoebae. X5 000.







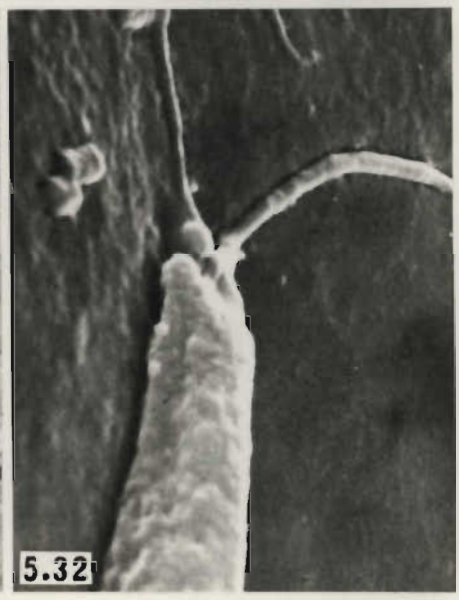


Fig. 5.29 Naegleria fowleri, G.J. flagellate with adhering bacteria (b). X6 800.

Fig. 5.30 A Naegleria gruberi, NB-1 flagellate exhibits a rigid, tapered shape and two flagella. Cells on the right are reverting. They have lost the stable flagellate form and the now motile amoebae possess non-functional flagella. X3 075.

Fig. 5.31 A Naegleria gruberi, NEG flagellate with four flagella. A cytoplasmic bleb (b) is present. X2 700.

Fig. 5.32 Naegleria fowleri, Oram flagellate with two flagella. X5 000.

and Naegleria fowleri under the S.E.M. (Figs 5.29 - 5.32). Figure 5.30 shows a flagellate of Naegleria gruberi, strain NB-1 exhibiting the typical flagellate form of the genus - rigid, fusiform and with two flagella equal in length to the cell, emerging from a definite pore or pit in the cell surface. Next to the flagellate are reverting cells, with typical amoeboid form, but the flagella have not yet been drawn into the cell cytoplasm. Figure 5.31 illustrates a Naegleria gruberi NEG flagellate with four flagella and a cytoplasmic bleb attached by a thin strand of cytoplasm. The two Naegleria fowleri flagellates shown (Figs 5.29, 5.32) have somewhat thicker flagella but this is a direct result of using the procedure of Darley & Lott (106). Other methods of preparing Naegleria fowleri flagellates produced cells that were morphologically similar to those of Naegleria gruberi.

No structure suggestive of a surface active lysosome was seen on any of the amoeboid or flagellated cells examined

F. DISCUSSION

The scanning electron microscope studies presented in this chapter are in good agreement with reports by other workers (70,209,322).

The presence or absence of preformed cyst exit pores has been considered a valuable taxonomic feature for the differentiation of Naegleria species, as these pores are an established diagnostic feature of Naegleria gruberi and are visible under the light microscope (Chapter 3 of this thesis, 169, 391) and the transmission electron microscope (368,371).

J.B.Jadin (205) depicted a Naegleria fowleri cyst with exit pores. J.M.Jadin et al.(209) in their S.E.M. study of Naegleria cysts reported that some Naegleria fowleri cysts had preformed exit pores, while other pathogenic strains of Naegleria did not, or at least pores could not be detected under the S.E.M. Recently Schuster (371) in a transmission electron microscope study of a strain of Naegleria gruberi, a strain of Naegleria jadini and two strains of Naegleria fowleri presented evidence that cysts of all the Naegleria strains possess preformed exit pores. Schuster's transmission electron micrographs show that in Naegleria gruberi a prominent collar surrounds the exit pore making observations of it in the light and electron microscopes extremely easy. In Naegleria fowleri the cyst pores are much less obvious. Schuster (371) also says "Lastovica, however, found no pores in Carter's 1966 isolate of Naegleria fowleri and several other strains he examined with the scanning electron microscope. It is conceivable that the pores may easily be concealed by debris adherent to

the cyst wall, or by partial deformation of the cyst wall during preparative drying process. The difficulty encountered by various investigators in observing cyst pores in Naegleria fowleri at the light microscope level was due to the pores being flush with the surface of the cyst wall itself". Schuster presents transmission electron microscope data on two strains of Naegleria fowleri, Carter's 1966 strain and strain MB-41, identical to the HB-1 strain.

When I examined the 1966 strain of Naegleria fowleri every precaution was taken to ensure an unbiased observation of cyst topology, but still no pores or plugs were detected. While J.M.Jadin et al.(209) report cyst pores in Naegleria fowleri, no micrographs are presented in their report on comparative S.E.M. studies of amoeba cysts. They also report being unable to find any cyst pores in pathogenic Naegleria aerobia.

In agreement with the results presented in this chapter, Carter (46) stated that excystment of Naegleria fowleri occurred by rupture of the cyst wall and that pores and associated plugs were not seen. Singh (390) and Singh & Das (391) also reported a lack of preformed pores in the cyst wall of Naegleria fowleri, and they suggested that some digestion of the cyst wall took place to allow excystment of the amoebae. In this chapter careful S.E.M. investigation of nine strains of Naegleria fowleri has failed to reveal any preformed exit pores or associated plugs.

At this point, the issue is not settled for all strains of Naegleria fowleri, because contaminated cultures could have originated in some (or possibly all) the laboratories concerned. Another possibility is that there could be several morphological forms of the cysts of each strain of Naegleria fowleri, as has been suggested by Carter (46). Allowing a cloned culture of known pedigree of Naegleria to excyst, treating with digitonin to lyse the amoebae present while the cysts are unaffected, and examining the empty cyst structures to determine the method of encystment should be a way of settling the question.

The surface texture of Naegleria fowleri cysts under the S.E.M. varies from smooth to coarsely reticulate. Singh & Das (391) have observed that the cysts of Naegleria fowleri are coated in a "fairly thick gelatinous layer". Under phase-contrast microscopy some of the pathogenic cysts were observed to have a similar coating. Possibly the rough surface texture of the cysts under the scanning electron microscope may be due to varying amounts of this material adhering to the cyst walls.

Encystment is quite different in Acanthamoeba sp., as the amoebae emerge via an ostiole after a covering operculum has been removed.

Chambers & Thompson (70) speculate that most likely enzymatic action brings about the removal of the operculum in Acanthamoeba, leaving the rest of the cyst unaffected. Encystment in other species of limax amoebae such as Schizopyrenusa involves dissolution of the cyst wall.

A study of the surface texture of the cyst is taxonomically useful, as Naegleria cyst morphology is quite different from that of the cysts of the other limax amoebae studied.

There is no published S.E.M. information on the flagellate stage of Naegleria, and only brief reports (271,276) on the S.E.M. of the amoeboid state of Naegleria. Martinez et al.(271) claim to have observed structures comparable to surface active lysosomes in the trophozoites of one strain of Naegleria fowleri. Their evidence is not convincing, and to resolve the question a combination of scanning and transmission electron microscopy must be utilised as Proctor & Gregory (344,347) have done for Entamoeba histolytica.

In the extensive transmission and scanning electron microscope studies of Naegleria fowleri presented in this thesis, no structure suggestive of a surface active lysosome was ever found. Failure to find structures similar to the surface active lysosomes of Entamoeba histolytica (143,144,344,345,347,356,357) means that the actual mechanism of pathogenesis in Naegleria does not depend on such an organelle.

CHAPTER 6

TRANSMISSION ELECTRON MICROSCOPY OF NAEGLERIA FOWLERI, NAEGLERIA GRUBERI AND A PATHOGENIC STRAIN OF ACANTHAMOEBA

A. ABSTRACT

Comparative ultrastructural examination of six strains of non-pathogenic Naegleria gruberi, 13 strains of pathogenic Naegleria fowleri and a pathogenic strain of Acanthamoeba was undertaken. Striking similarities but also well-defined ultrastructural differences were observed in the Naegleria gruberi and Naegleria fowleri examined.

In Naegleria gruberi only cylindrical or oval mitochondria were observed, while all Naegleria fowleri examined possessed prominent cup-shaped or dumbbell-shaped mitochondria in addition to some oval or cylindrical mitochondria. Frequently, cristae of Naegleria fowleri amoebae display a honeycomb pattern when seen in transverse section, unlike those of Naegleria gruberi. Electron-opaque particles up to 100 nm in diameter, suggestive of a virus, and polyhedral in shape, have been detected in the cytoplasm of three strains of Naegleria fowleri. Similar particles were not observed in Naegleria gruberi or the Acanthamoeba strain examined. For the first time, 5-7 nm and 17-19 nm diameter microfilaments morphologically similar to F-actin and myosin respectively have been observed in the cytoplasm of several strains of Naegleria.

Amoebae and cysts of a pathogenic strain of Acanthamoeba were examined under the transmission electron microscope. The ultrastructure of the plasma membrane, food vacuoles, lipid bodies, Golgi apparatus, microtubules and the nucleus were similar to those in published reports of other Acanthamoeba. The Acanthamoeba mitochondria differed from those of Naegleria, as they were elongated, slightly dumbbell-shaped, or spherical with conspicuous intracristal granules. In all the amoebae examined no structure suggestive of a surface-active lysosome was found.

B. INTRODUCTION

The first published electron micrograph of Naegleria gruberi was by Willmer (460) in 1956, and the first detailed comparative study of the ultrastructure of Naegleria, Acanthamoeba and other limax amoebae was that of Vickermann (423) in 1962. Since those early reports, the knowledge of the fine structure of Naegleria has increased, beginning with Schuster's

significant contributions on the amoeboid and flagellate stages (367) and the cyst stage (368). His further work on the ultrastructure of the cyst (371,379) and of mitosis (370) in Naegleria are landmark studies. Finally, Schuster & Dunnebacke (138-140, 372-377) have published a definitive series of papers on the study of virus-like particles in Naegleria. Other workers (126,127,169,175,378,383) have concentrated their attention on specific ultrastructural problems in Naegleria gruberi. To date, the only ultrastructural study of Naegleria jadini is that of Carosi et al.(43).

The first published electron micrographs of Naegleria fowleri were those of those of the 1966 and 1969 strains reported by Carter (46) in 1970. Maitra et al.(262) described the HB-1 strain, Martinez et al.(273, 277) portrayed the Lee and CJ-1 strains, while Visvesvara et al.(427,428) also investigated the HB-1 strain of Naegleria fowleri. J.B. Jadin (205) has reported on the fine structure of the 0359 strain of Naegleria fowleri. Martinez et al.(271) and Rondanelli et al.(358 have examined two strains of Naegleria fowleri.

The above reports represent all data available to date from transmission electron microscope studies of the seven strains of Naegleria fowleri, and a report is often illustrated by a single electron micrograph. It was decided to undertake a detailed study of the ultrastructure of as many strains of pathogenic and non-pathogenic Naegleria as possible, and to compare the fine structure of these organisms with a pathogenic strain of Acanthamoeba. Although Acanthamoeba trophozoites and cysts have been used for various cell physiological studies involving transmission electron microscopy, these have been specific studies rather than a survey of the fine structure of the whole organism. Vickermann (423), and Bowers & Korn (30,31) have given detailed reports on the ultrastructure of Acanthamoeba castellanii amoebae and cysts. Martinez et al.(274) described the fine structure of pathogenic Acanthamoeba castellanii and Acanthamoeba polyphaga in mouse brain. Maitra et al.(263) reported on the ultrastructure of Hartmannella (=Acanthamoeba) culbertsoni in mouse brain and under different cultural conditions. Dunnebacke & Williams (141) have investigated the fine structure of Acanthamoeba sp. pathogenic to tissue culture cells.

All strains of amoebae examined were scrutinised for specific pathogenic ultrastructural features such as surface-active lysosomes similar to those of Entamoeba histolytica (143,144,344,345,347,356,357) or the virus-like particles of Entamoeba histolytica (122,123,201,202,281,282). A portion of the findings reported in this chapter has formed the first

direct ultrastructural comparison of a large number of strains of Naegleria fowleri and Naegleria gruberi (253). Some of the results have been used for an ultrastructural comparison of Naegleria fowleri, Naegleria gruberi and Acanthamoeba (255), and for a report on the microfilaments in Naegleria (254).

C. MATERIALS

Organisms	Sources are recorded on p.146-147.
Media	Described on p.148-149.
Chemicals	Described on p.149-150.
Apparatus	Hitachi HU-11E-1; Phillips EM 300; Siemens 101 transmission electron microscopes and ancillary equipment.

D. METHODS

D.1 Production, harvesting and preparation of amoebae and cysts

For the production and harvesting of amoebae to the stage involving centrifugation to remove bacteria, the procedure outlined on p.39 was followed.

D.2 Preparation for electron microscopy

The motile amoebae or cysts were fixed in 1-2% glutaraldehyde buffered with 0,1 M Na cacodylate buffer, pH 7,2 at 4°C for one hour. Two more washes in cacodylate buffer at 4°C were carried out. Secondary fixation occurred in 1% OsO in cacodylate buffer for one hour. This and subsequent steps were done at 25°C. The cells were dehydrated through an alcohol series to absolute alcohol with 20 minutes per step, and then embedded in Araldite. Grey or silver sections were cut on an LKB Ultrotome III ultramicrotome or on a LKB Huxley ultramicrotome. Electron microscope grids were stained with uranyl acetate followed by lead citrate. During the course of the investigation it was found that minor variations of the above procedure such as using acetone instead of ethanol, or substituting Spurr's resin for Araldite resin did not significantly affect the ultrastructure of the cells examined.

Viewing and photography were done on Hitachi HU-11E-1, Phillips EM 300 or Siemens 101 transmission electron microscopes operating at 60 or 80 Kv. Precise measurements were taken from contact prints of the original negatives, or from photographic enlargements of the negatives.

E. RESULTS

Comparative transmission electron microscope studies of six strains of Naegleria gruberi, 13 strains of Naegleria fowleri and a pathogenic strain of Acanthamoeba have revealed similarities as well as striking ultrastructural differences between the virulent and avirulent amoebae. A selection from over 650 transmission electron micrographs representative of the fine structural details of all the strains of amoebae examined is shown in Figs 6.1 - 6.40, p.58-72.

E.1 Naegleria fowleri and Naegleria gruberi

E.1.i Whole amoebae

The typical limax shape of Naegleria amoebae with a single blunt pseudopod and slender body shape was demonstrated by the Naegleria fowleri strain, HB-1 (Fig. 6.1). Naegleria amoebae were also capable of assuming a more compact, rounded shape (Figs 6.2 - 6.6) and the terminal uroid process was clearly seen in Naegleria fowleri (Fig. 6.3) and Naegleria gruberi (Fig. 6.6)

E.1.ii Nucleus

In all strains of Naegleria amoebae examined, the nucleus and nucleolus were prominent features (Figs 6.1 - 6.6). Figure 6.8 illustrates the interphase/prophase nucleus of a Naegleria gruberi, Bristol strain amoeba. The centrally located nucleolus consisted of electron-opaque, 20 nm diameter particles, which formed areas of varying density. Nucleoplasm, presumed site of the chromosomal chromatin, was of a homogeneous appearance. The nuclear membrane was formed of a double unit membrane, each layer about 10 nm thick, separated by an electron-transparent space 25 nm wide. Nuclear pores consisting of a central granule surrounded by eight peripheral subannuli were clearly shown in a Naegleria fowleri, Oram strain amoeba (Fig. 6.11). Figures 6.9 and 6.10 illustrate microtubules 25 nm in diameter in the nucleoplasm of a Naegleria fowleri, Oram strain amoeba.

E.1.iii Ectoplasm and endoplasm

Under the electron microscope, endoplasm was not as easily differentiated from ectoplasm as it was under the light microscope, except that the ectoplasm was usually free of inclusions (Figs 6.1,6.2,6.5).

E.1.iv Plasma membrane

Naegleria amoebae were bounded by a plasma membrane about 10 nm wide and exhibited a unit membrane structure of two electron-opaque layers 2-3 nm thick, sandwiching an electron-transparent layer (Fig. 6.28). No external structures or fringes were detected on the plasma membranes of any of the amoebae examined.

E.1.v Endoplasmic reticulum

The rough endoplasmic reticulum (r.e.r.) consisted of ribosome-covered tubules or vesicles 20-25 nm wide, as was illustrated in the cytoplasm of Naegleria gruberi, Bristol strain (Fig. 6.19). Similar structures were observed in the other strains of Naegleria gruberi examined. The r.e.r. tended to be in close association with oval or round mitochondria (Figs 6.19-6.21), and was found also in close proximity to the nuclear membrane (Fig. 6.8). In the strains of Naegleria fowleri examined (Figs 6.1-6.4, 6.11-6.18), the r.e.r. was much less obvious than in Naegleria gruberi. In all amoebae examined ribosomes studded the nuclear envelope (Fig.6.8) and were found free in the cytoplasm, either singly or as polysomes (Fig. 6.19).

E.1.vi Mitochondria

Marked differences occurred in the shape and structure of the mitochondria of virulent and avirulent Naegleria amoebae. In the Naegleria gruberi amoebae examined only cylindrical or oval mitochondria 1,0-1,5 µm long by 0,5 µm wide were seen, as was illustrated for the SA-2, Bristol and NB-1 strains (Figs 6.5-6.7, 6.19-6.21). All strains of the Naegleria fowleri examined under the electron microscope possessed numerous prominent cup-shaped or dumbbell-shaped mitochondria 1-2 µm long by up to 0,5 µm wide. Often all or most of the mitochondria were of this type, with only an occasional oval or cylindrical mitochondrion present, as was seen in the HB-1, Oram and G.J. strains (Figs 6.1, 6.2, 6.4). Higher magnifications of dumbbell-shaped or cup-shaped mitochondria were seen in Figs 6.16-6.18.

In all the Naegleria mitochondria observed, two membranes separated a space of 18-20 nm wide and enclosed the mitochondrion, the inner membrane gave rise to 20-30 nm wide tabular, oblique and transverse cristae. A new finding was that the mitochondria of pathogenic Naegleria fowleri when seen in transverse section displayed a honeycomb-like pattern of cristae. This configuration was seen in strain 0359 (Fig.6.4), and in cytoplasmic details of strains 0359, MW4U, G.J., PA-14, McMahon and Vitek (Figs 6.11-6.15, 6.30). Occasionally Naegleria fowleri mitochondria may exhibit both a cup-shape or dumbbell-shape as well as a honeycomb cristal pattern, as is seen in strain G.J. (Fig. 6.16). These patterns have not previously been reported for Naegleria gruberi. Intramitochondrial bodies were not detected in any of the Naegleria strains examined in this study.

E.1.vii Food and contractile vacuoles

In all the Naegleria amoebae examined, food vacuoles up to 3 µm in diameter, bounded by a 10 nm wide unit membrane, were seen (Figs 6.1-6.7).

Amoebae freshly subcultured on Fulton's A medium often had numerous large, empty food vacuoles (Fig. 6.2). When amoebae were fed on bacteria the food vacuoles displayed complex, laminated structures composed of 9-10 nm wide unit membranes (Figs 6.22-6.25). No indication was seen of any link between these membranes and the membrane bounding the food vacuole. Fusing of smaller vacuoles into a larger one was a common occurrence (Fig. 6.25). Under the electron microscope, contractile vacuoles up to 1,5 µm in diameter were identified. The contents of both food and contractile vacuoles can be discharged at any point on the surface but usually it is at the uroid that collapse at systole occurs.

E.1.viii Microfilaments

Thin and thick microfilaments morphologically similar to actin and myosin respectively were present in the cytoplasm of Naegleria fowleri amoebae, strains G.J., Oram and Vitek (Figs 6.26-6.28). Although not illustrated, three other strains of Naegleria fowleri, HB-1, McMahon, and PA-14 also displayed similar microfilaments. A relaxed, non-motile amoeba of the G.J. strain was illustrated in Fig 6.26. Numerous thin microfilaments 5-7 nm in diameter and up to 256 nm long were randomly scattered through the cytoplasm and were concentrated near (and possibly attached to) the plasma membrane. A distinct, collaterally aggregated bundle of thin microfilaments measuring 1640 nm long and 180 nm wide was situated in the cytoplasm near the plasma membrane of a motile Oram strain amoeba (Fig. 6.28). Individual thin microfilaments in the bundle were 6 nm wide and up to 390 nm long, and were regularly spaced 17 nm apart. Similar bundles of thin microfilaments in close proximity to the plasma membrane were seen in longitudinal and transverse section in the other strains of Naegleria examined. The cytoplasm of the Vitek strain (Fig. 6.27) displayed both thin and thick microfilaments. The thin microfilaments were 5-7 nm wide and up to 395 nm long. The thick microfilaments were short thick rods measuring 17-19 nm in diameter and ranged between 152 and 190 nm in length, with a mean length of 176 nm.

E.1.ix Virus-like particles

Polyhedral, electron-opaque particles up to 100 nm in diameter and suggestive of a virus were detected in the cytoplasm of the NH-1, Oram and Vitek strains of Naegleria fowleri (Figs 6.29-6.32). The Oram strain displayed these bodies in abundance. Figures 6.29 and 6.32 illustrat clusters and single examples of "empty" and "full" (electron-dense) polyhedral virus-like particles up to 95 nm in diameter. Similar virus-like particles were not detected in any of the Naegleria gruberi

strains examined under the electron microscope.

Unusual osmophilic dense bodies up to 900 nm long by 170 nm wide, with 6–8 nm wide structures attached, were detected in the Naegleria fowleri Oram strain (Figs 6.33-6.35). These were tentatively identified as being mycoplasma (personal communication, Feb.1976, Dr.T. Hickson, State Vaccine Institute, Pinelands, Cape Town).

E.1.x Other cytoplasmic inclusions

Small, electron-dense granules, probably glycogen, were found scattered through the cytoplasm of the amoebae. A most conspicuous feature in the cytoplasm was the presence of electron-dense, non-membrane-bound globules which were probably lipid and measured up to 1 µm in diameter (Figs 6.3,6.18,6.32). No structure suggestive of a surface active lysosome or Golgi apparatus was found in any of the amoebae examined in this study.

E.2 ACANTHAMOEBA, strain APG

E.2.i Whole amoebae

The typical shape of Acanthamoeba with numerous conspicuous acanthopodia was demonstrated by an Acanthamoeba, strain APG amoeba (Fig. 6.36).

E.2.ii Nucleus

The prominent nucleus (Figs 6.36,6.37) had a centrally located nucleolus composed of granular, 11 nm diameter particles and an electron-dense nucleolonema which anastomosed and branched to form an irregular tridimensional network. Membranes of the nuclear envelope were 25–30 nm apart. Ribosomes studded the outermost element of the nuclear envelope and 90 nm diameter nuclear pores were detected, although not as easily seen as were those in Naegleria amoebae. Granular particles 7 nm in diameter filled the space between the nuclear envelope and the nucleolus. Chromosomes were not detected in the Acanthamoeba cells examined.

E.2.iii Ectoplasm and endoplasm

Under the transmission electron microscope the endoplasm and ectoplasm were not always as easily differentiated as under the light microscope. The ectoplasm was however usually free of the larger cell inclusions (Figs 6.36,6.37).

E.2.iv Plasma membrane

Acanthamoeba, strain APG was bounded by a plasma membrane 9–10 nm wide whi exhibited a unit membrane structure of two electron-dense layers sandwiching an electron-transparent layer. No external structures or fringes were seen on the plasma membrane of any of the amoebae examined.

E.2.v Endoplasmic reticulum

In the amoebae examined, the rough endoplasmic reticulum was seen to be composed of numerous straight or curved, 50–60 nm wide tubules with enlarged ends, or of 60–70 nm wide microvesicles (Figs 6.36–6.39). Smooth endoplasmic reticulum was difficult to differentiate from numerous small vesicular elements in the cytoplasm.

E.2.vi Mitochondria

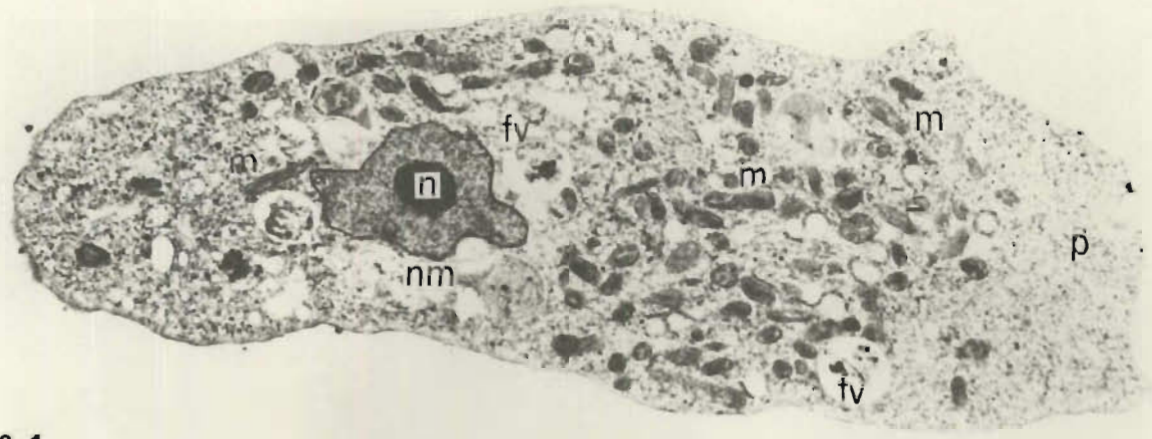
The mitochondria observed were of two types; compact spherical or oval forms, 0,5–1 µm x 0,5–1,5 µm, and elongate, slightly dumbbell-shaped forms measuring 0,3–0,5 µm x 1–1,5 µm (Figs 6.36–6.39). Both types of mitochondria had prominent branching, anastomosing tubular cristae up to 60 nm wide, which arose from the inner mitochondrial membrane. Inner and outer mitochondrial membranes were separated from each other by a space of 15–20 nm. The mitochondrial matrix is electron-dense and appears to be composed of gramular material slightly smaller than cytoplasmic ribosomes (Figs 6.38–6.39). Often these mitochondria contained electron-dense, amorphous granules up to 120 nm in diameter. These gramules lay in an enlargement of cristal tubules (Fig. 6.38).

E.2.vii Food and contractile vacuoles

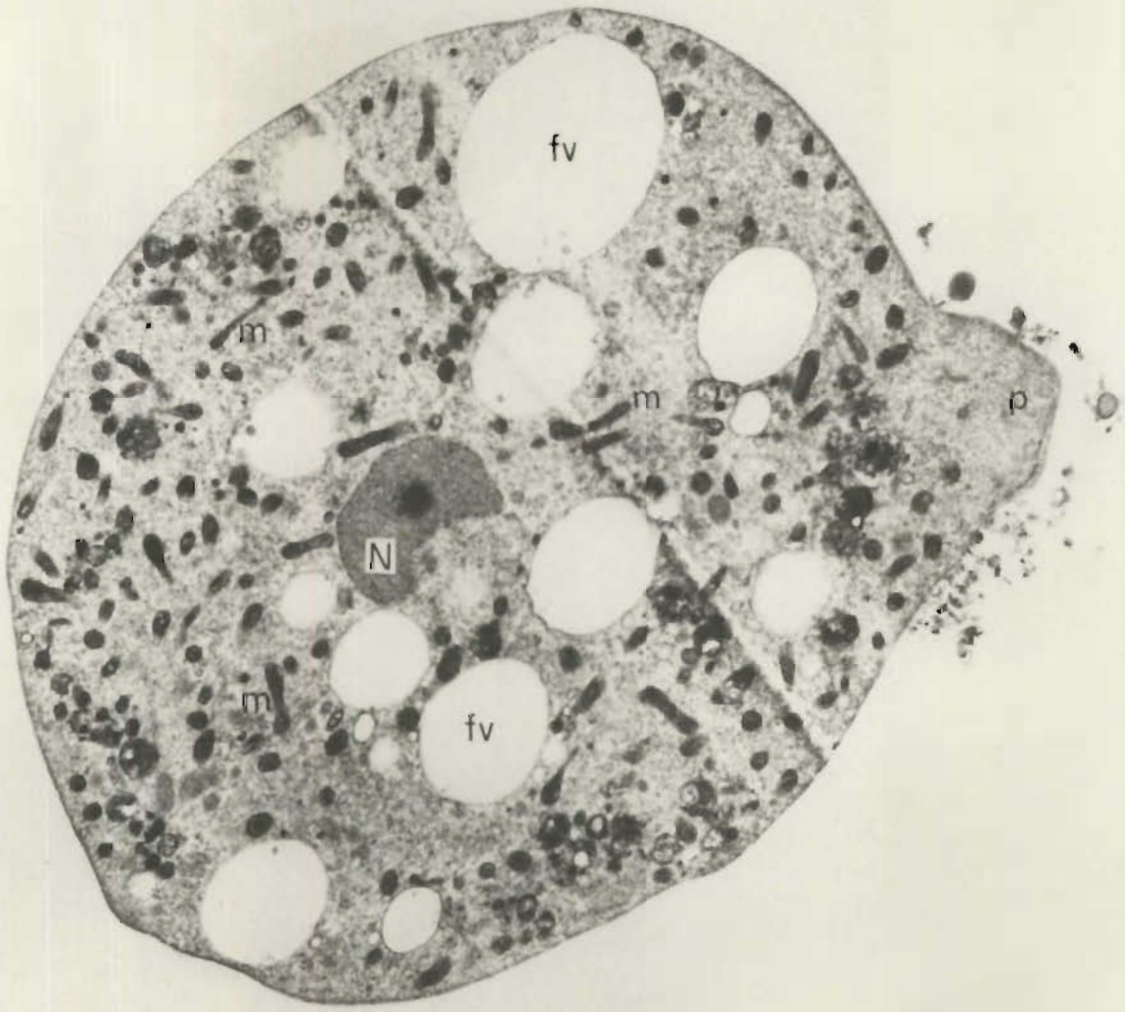
Numerous vacuoles were observed in amoebae of the APG strain. Both food vacuoles (with conspicuous particulate matter) and contractile vacuoles (without particulate matter) were bounded by a 9–10 nm wide unit membrane of two electron-dense layers sandwiching an electron-transparent space. Since the APG strain of Acanthamoeba was grown in axenic medium, no convoluted, laminated food vacuoles were seen. The contractile vacuole system when seen under the transmission electron microscope, was composed of a vacuole up to 2 µm in diameter, and a number of smaller vacuoles emptying into the larger one. Associated with the contractile vacuole system was a membranous network of tubules or vesicles - the spongiome, as illustrated in the upper right hand corner of Fig. 6.39. No permanent pore for vacuolar discharge was found.

E.2.viii Other cytoplasmic inclusions

Non-membrane bound spherical lipid-like bodies measuring 0,6 µm in diameter were found scattered throughout the cytoplasm (Figs 6.36–6.37). Granules 20–30 nm wide were occasionally seen, and were probably glycogen. Golgi apparatus (Fig. 6.37) was comprised of a series of lamellae separated by a 15–20 nm space, or of microvesicles. It was not easy to detect, due to the difficulty in distinguishing it from the numerous small vesicular elements of the cytoplasm. Microtubules, 25 nm in diameter



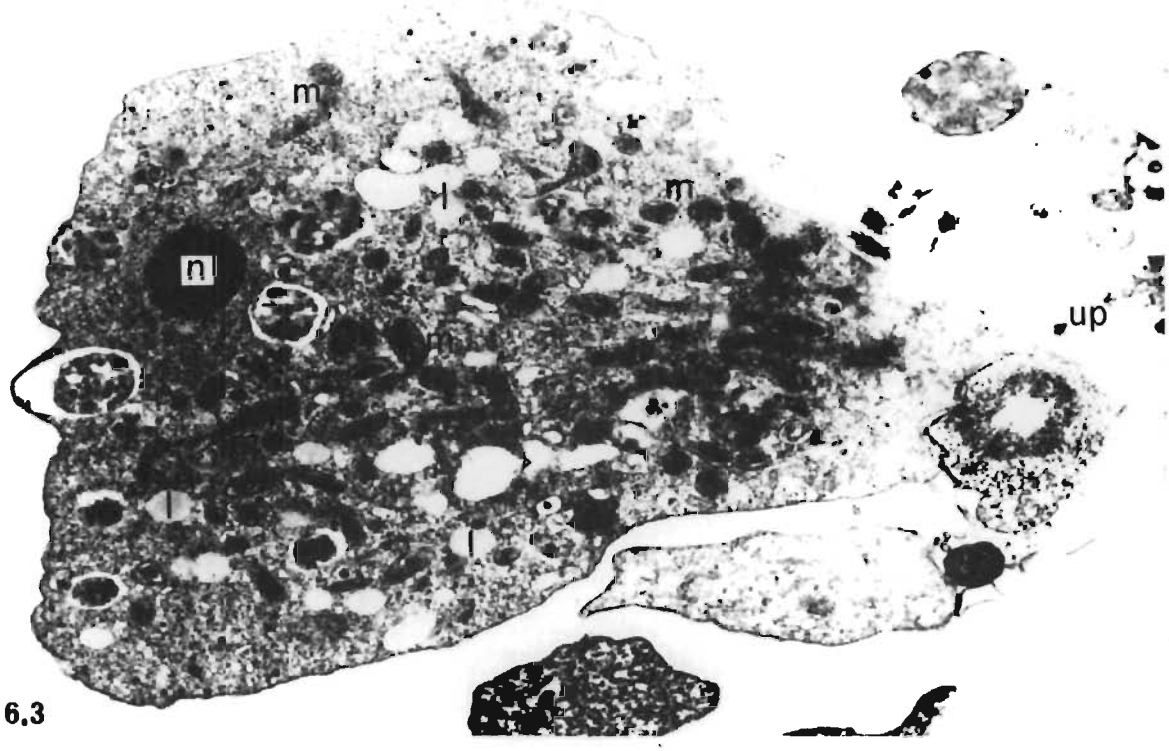
6.1



6.2

Fig. 6.1 Naegleria fowleri, HB-1 amoeba with an advancing pseudopod (p) exhibits typical limax shape. A prominent nucleus with nuclear membrane (nm) and nucleolus (n), food vacuoles (fv), and elongated mitochondria (m) are present. X7 700.

Fig. 6.2 An amoeba of Naegleria fowleri, Oram strain with an erupting pseudopod (p). A nucleus (N), conspicuous empty food vacuoles (fv) and numerous elongated mitochondria (m), are seen. X6 000.



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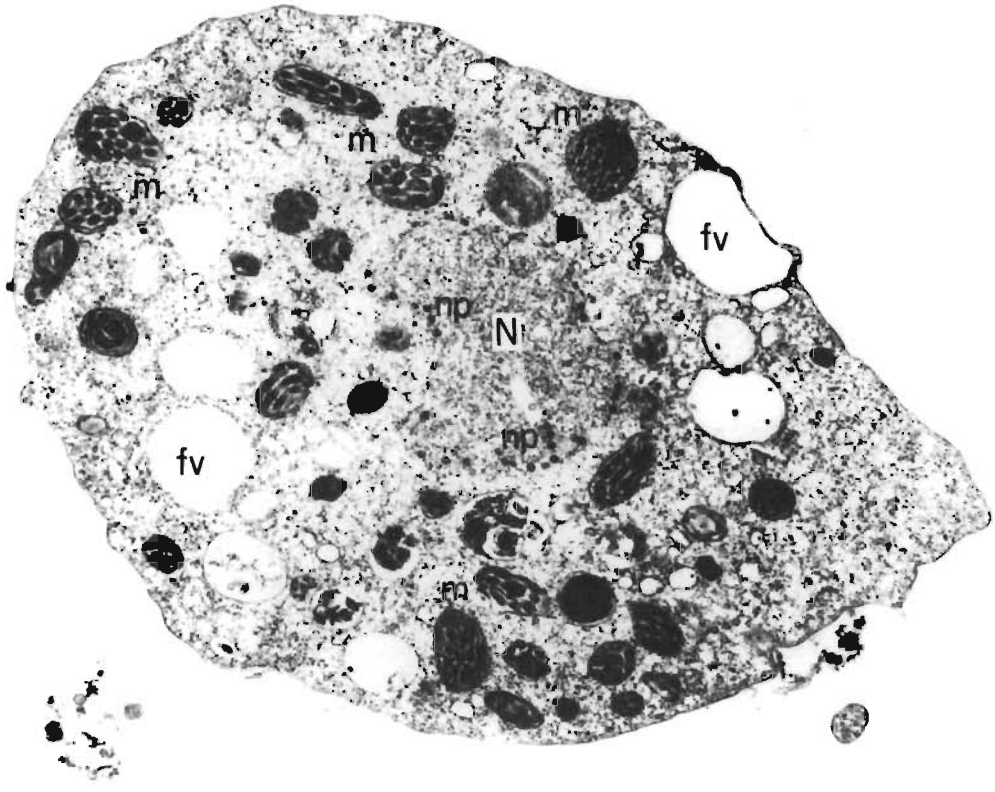
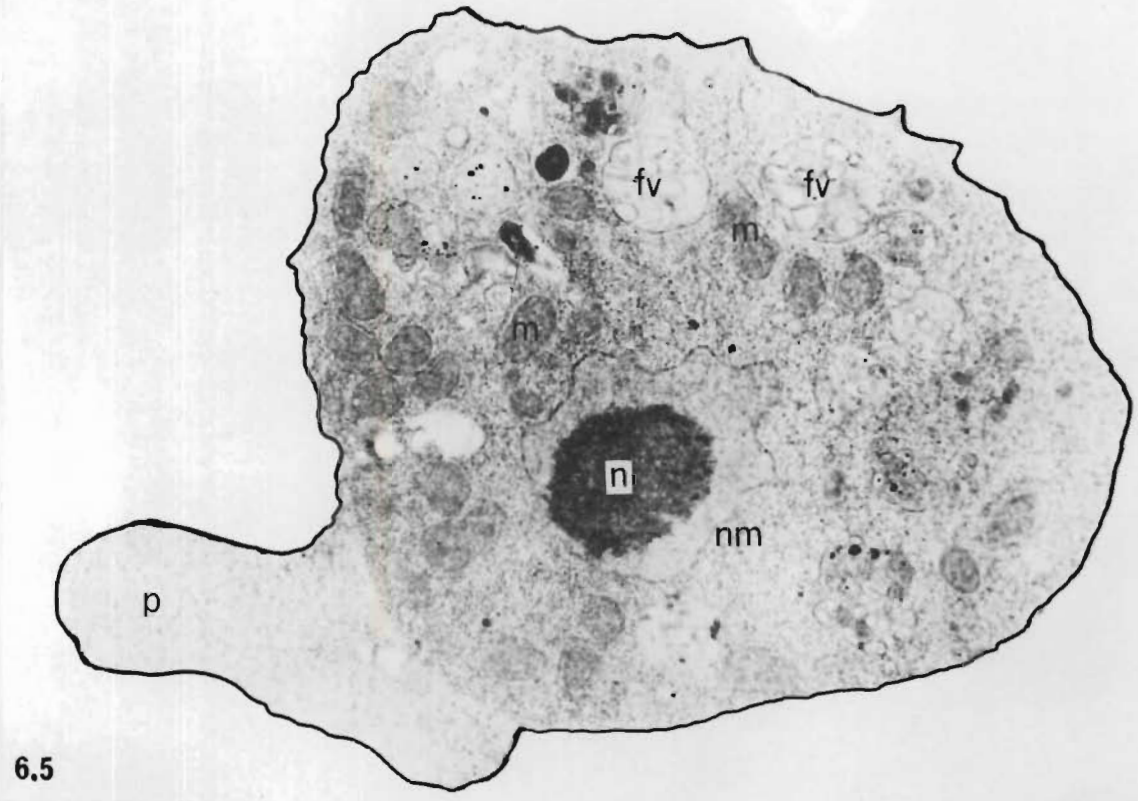


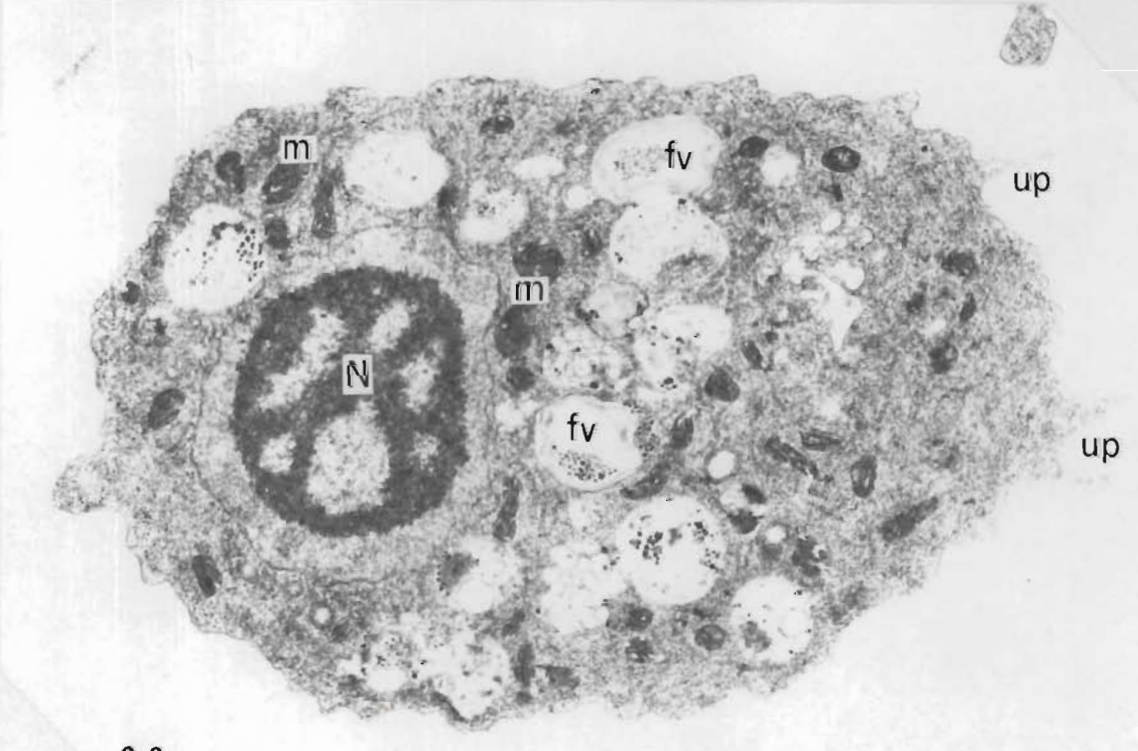
6.4

Fig. 6.3 Naegleria fowleri, G.J. amoeba with a conspicuous nucleolus (n), elongated mitochondria (m), lipid globules (l) and a prominent uroid process (up). X12 500.

Fig. 6.4 An amoeba of the Naegleria fowleri, 0359 strain with empty food vacuoles (fv), and a nucleus (N) with well defined nuclear pores (np). The prominent mitochondria (m) have hexagonal arrays of cristae when seen in transverse section. X10 000.



6.5



6.6

Fig. 6.5 A Naegleria gruberi, Bristol strain amoeba displays an advancing pseudopod (p). A prominent nucleus with a conspicuous nucleolus (n) and nuclear membrane (nm), food vacuoles (fv) and oval mitochondria (m) are seen. X9 750.

Fig. 6.6 An amoeba of the Naegleria gruberi, SA-2 strain with a prominent nucleus (N) in late prophase. A well defined uroid process (up), mitochondria (m) and food vacuoles (fv) are present. X10 975.

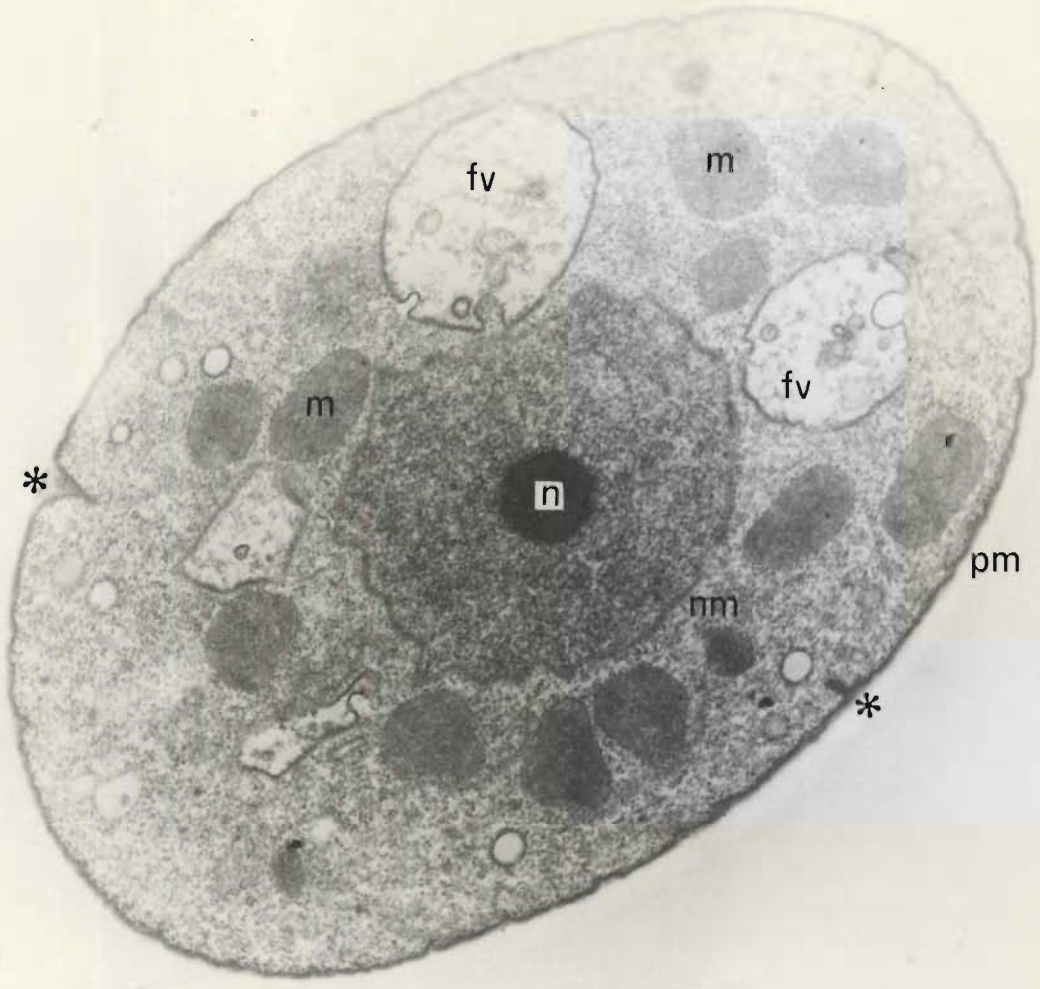
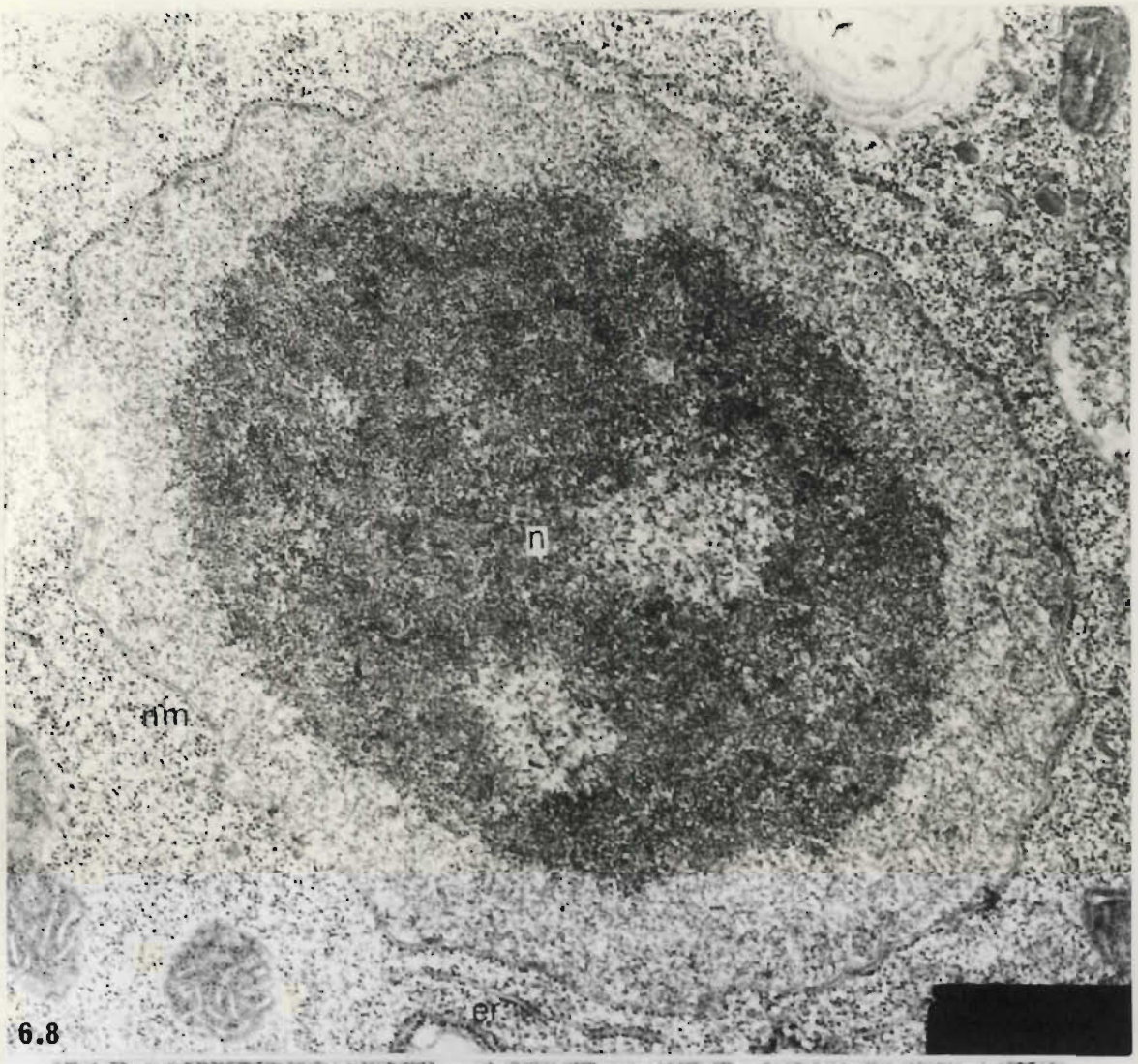
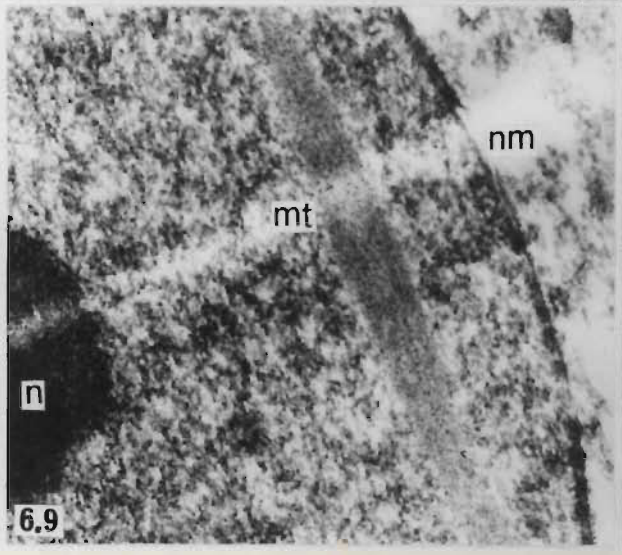
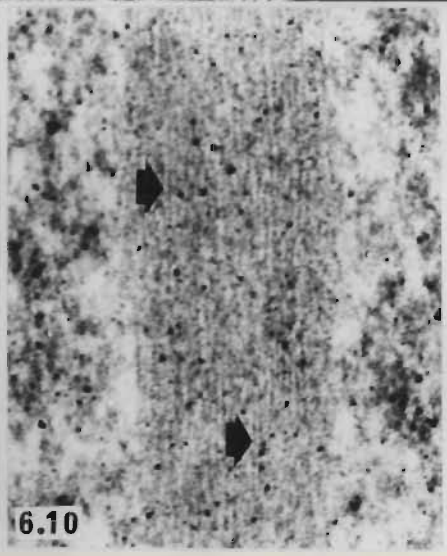


Fig. 6.7 A Naegleria gruberi, NB-1 amoeba in the early stages of encystment. Food vacuoles (fv), a nucleus with a well defined nuclear membrane (nm) and nucleolus (n) and numerous, compact oval mitochondria (m) are present. The amoeba has assumed the typical round or oval shape of the cyst and the asterisks indicate infoldings in the thickening plasma membrane (pm). X18 400.







er=endoplasmic retic., n=nucleolus, nm=nuclear membrane, mt=microtubule

Fig. 6.8 Late prophase nucleus of a Naegleria gruberi, SA-2 amoeba. A conspicuous nucleolus is made up of 20 nm diameter granules forming areas of varying density. X37 800.

Fig. 6.9 Detail of an Oram strain, Naegleria fowleri amoeba nucleus with an aggregation of 25 nm diameter nuclear microtubules. X27 500.

Fig. 6.10 Detail of Fig. 6.9. Arrows mark individual microtubules. X103 000.

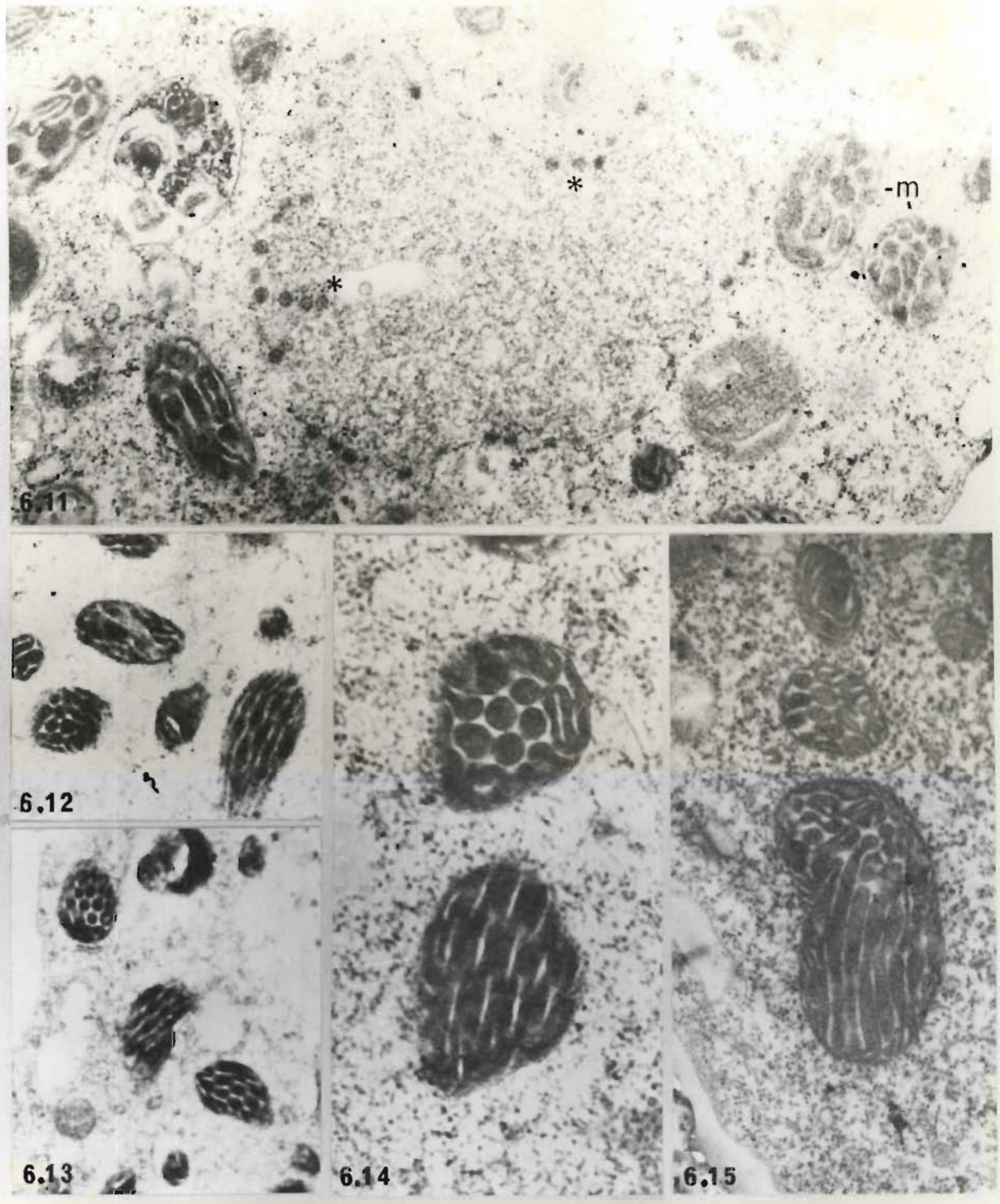


Fig. 6.11 Cytoplasmic detail of a Naegleria fowleri, 0359 amoeba (Fig. 6.4). Asterisks indicate nuclear pores. Transverse sections of mitochondria (m) display a "honeycomb" arrangement of cristae. X27 000.

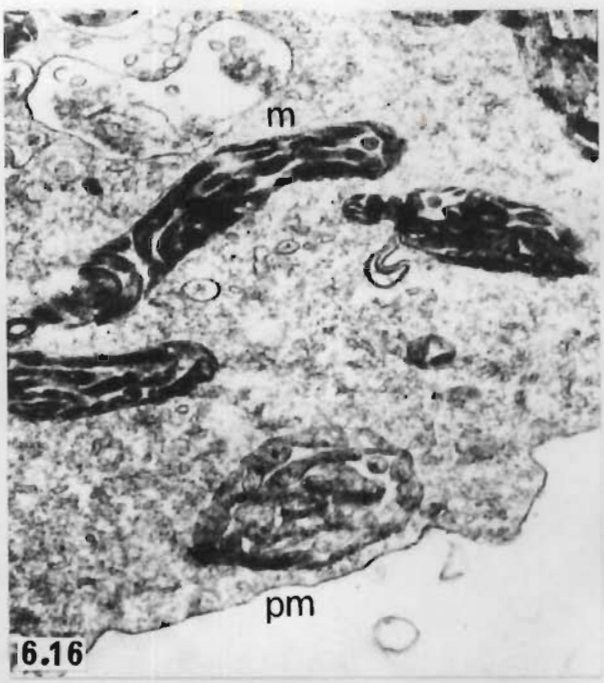
Cytoplasmic details of various strains of Naegleria fowleri amoebae showing transverse and longitudinal sections of mitochondria.

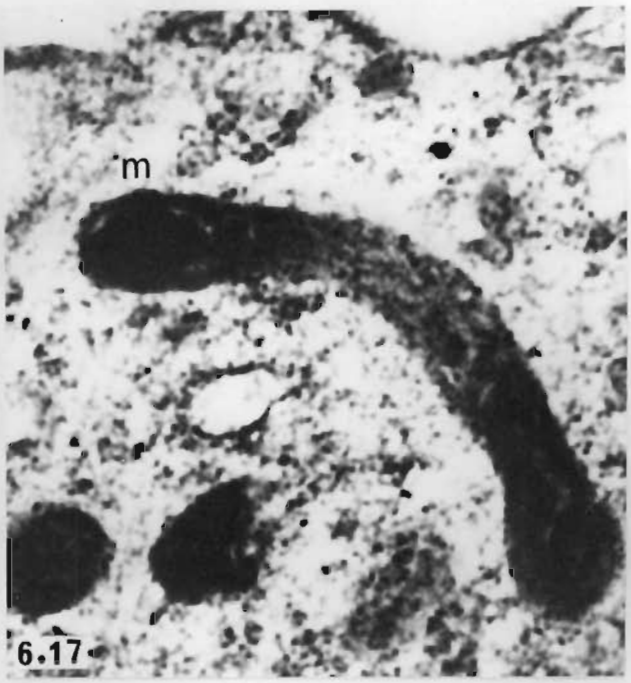
Fig. 6.12 Naegleria fowleri, strain MW4U. X23 500.

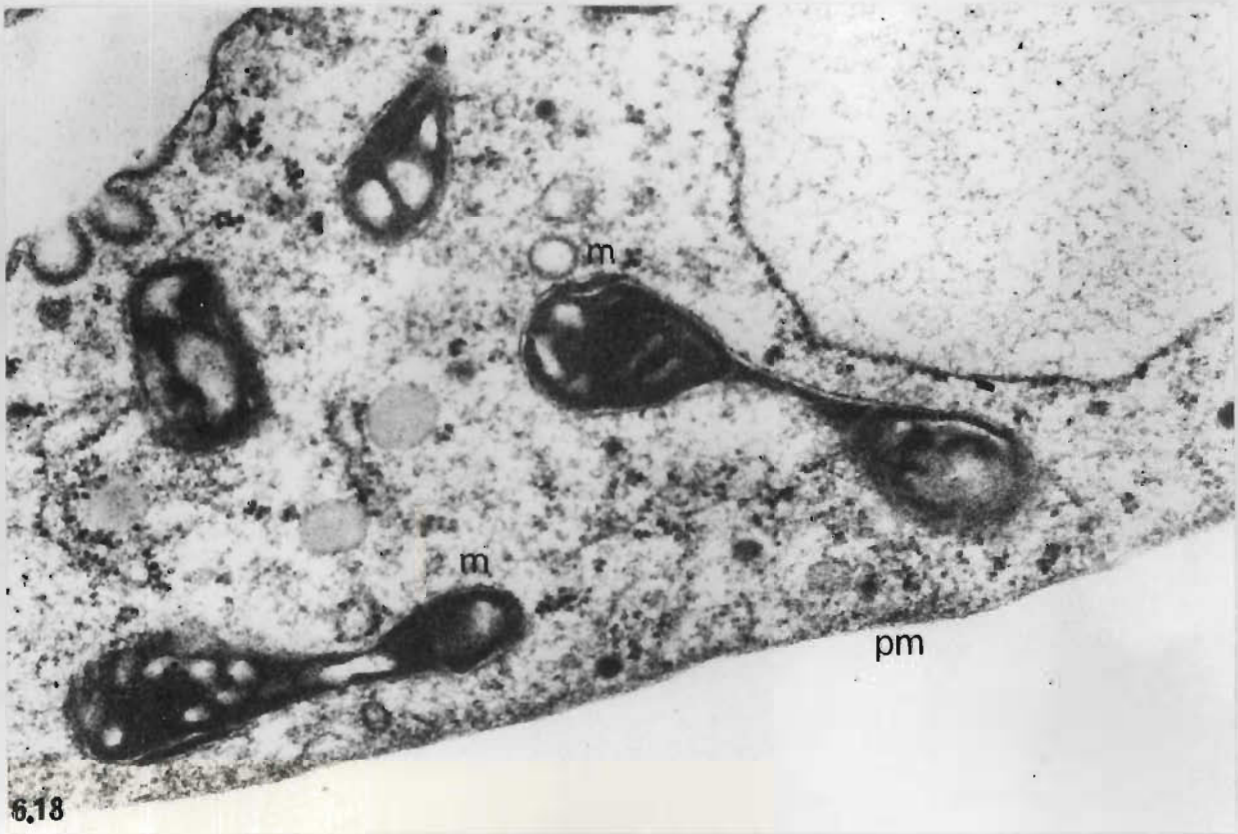
Fig. 6.13 Naegleria fowleri, strain G.J. X23 250.

Fig. 6.14 Naegleria fowleri, strain PA-14. X46 400.

Fig. 6.15 Naegleria fowleri, strain McMahon. X27 000.





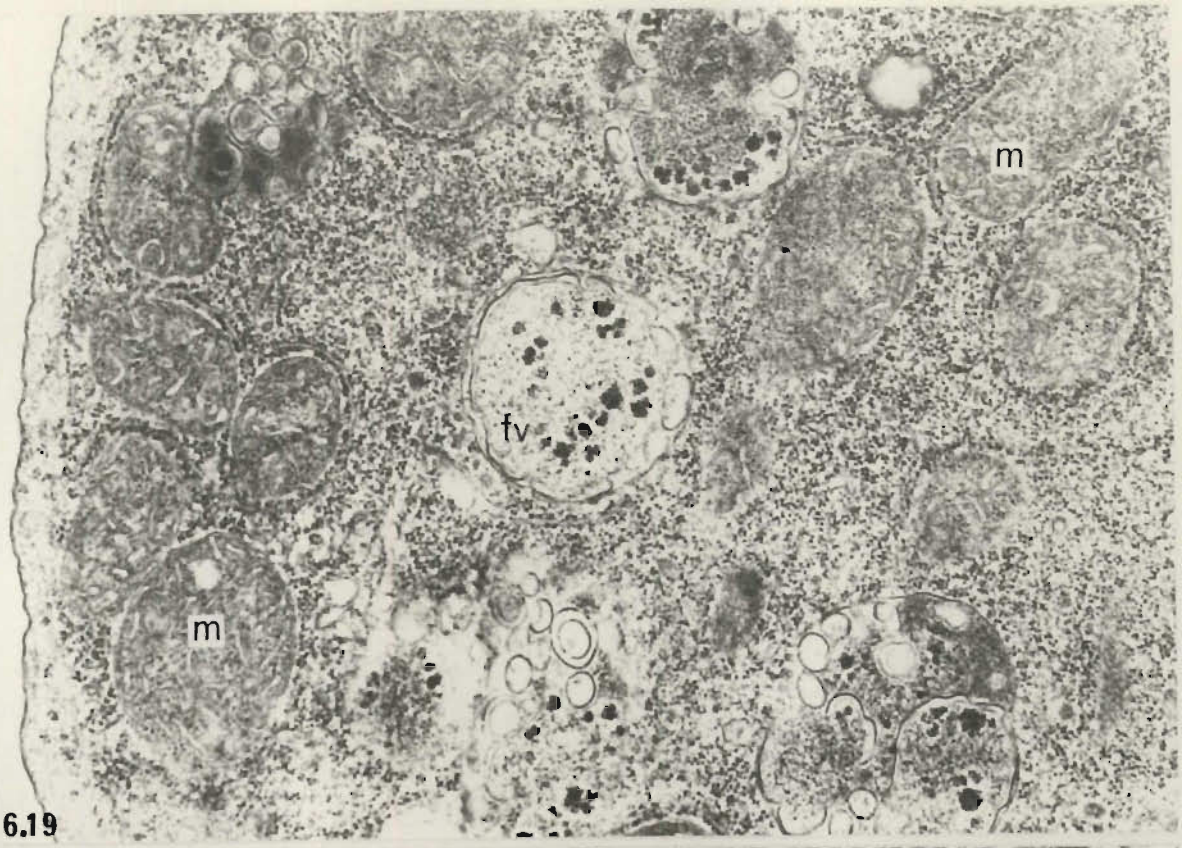


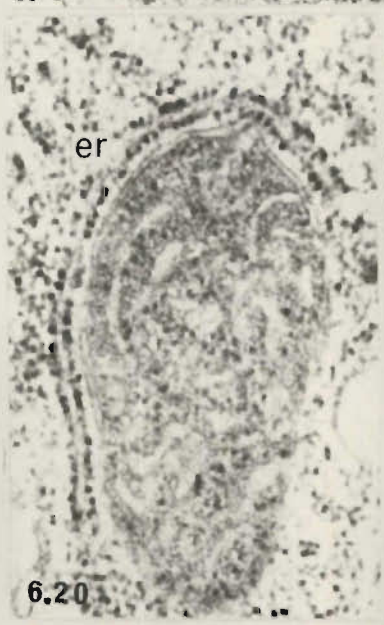
m=mitochondria, pm=plasma membrane

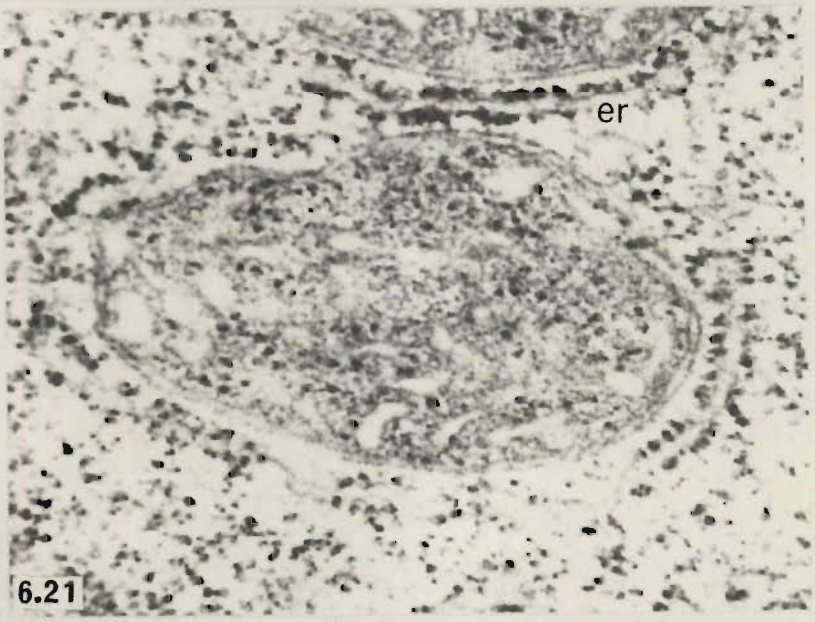
Fig. 6.16 Cytoplasmic detail of a Naegleria fowleri, G.J. amoeba showing elongated, cup-shaped mitochondria. X24 500.

Fig. 6.17 A cup-shaped mitochondrion of an amoeba of Naegleria fowleri, Oram strain. X32 650.

Fig. 6.18 Detail of Naegleria fowleri, Oram strain amoeba cytoplasm with prominent dumb-bell shaped mitochondria. X45 000.





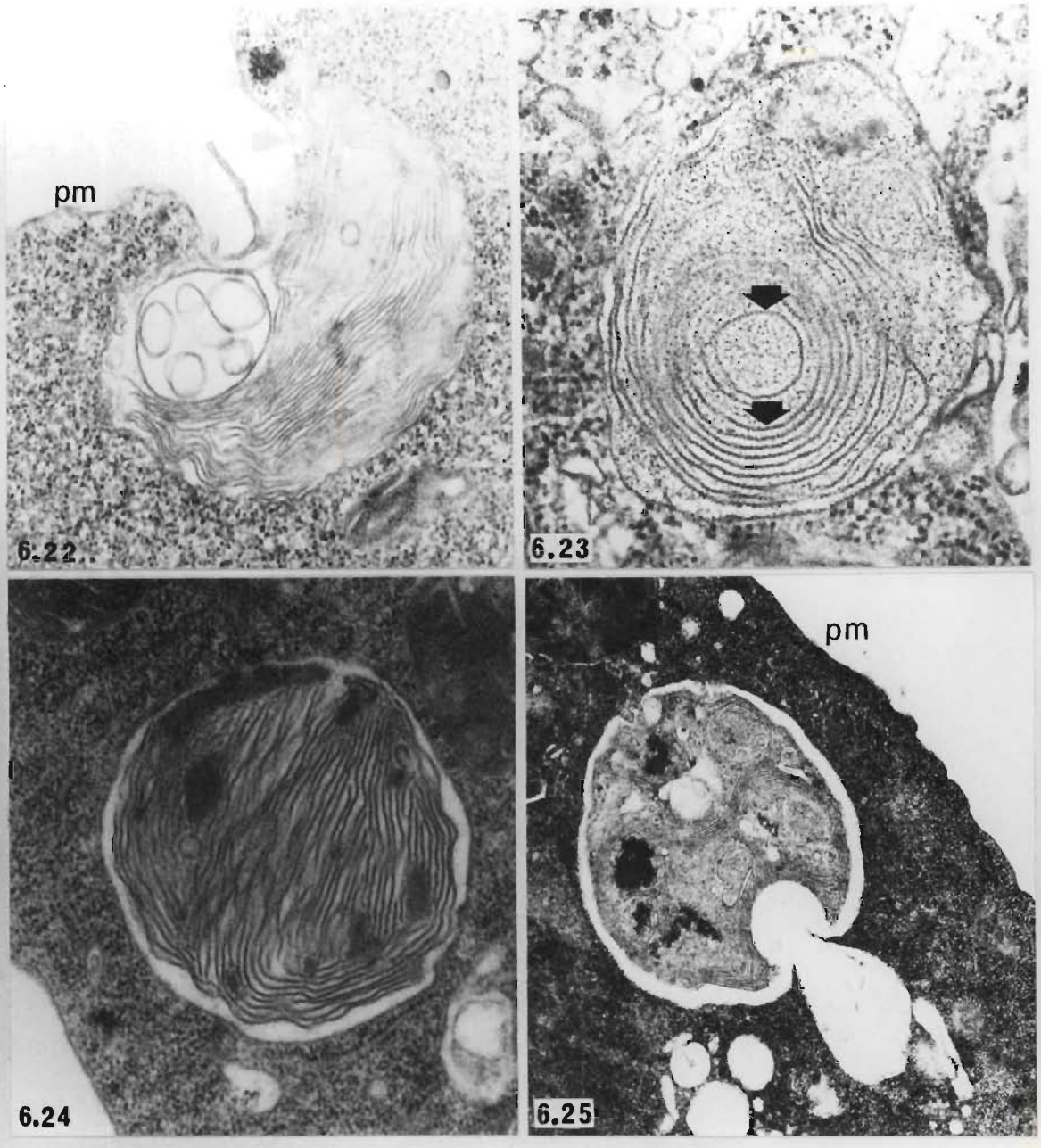


er=endoplasmic reticulum, fv=food vacuole, m=mitochondria

Fig. 6.19 A Naegleria gruberi, Bristol strain amoeba shows numerous oval or cylindrical mitochondria. X35 500.

Fig. 6.20 Cytoplasmic detail of a Naegleria gruberi, SA-2 strain amoeba displays a typical mitochondrion with closely applied endoplasmic reticulum. X70 000.

Fig. 6.21 A Naegleria gruberi, NEG amoeba mitochondrion. X109 000.



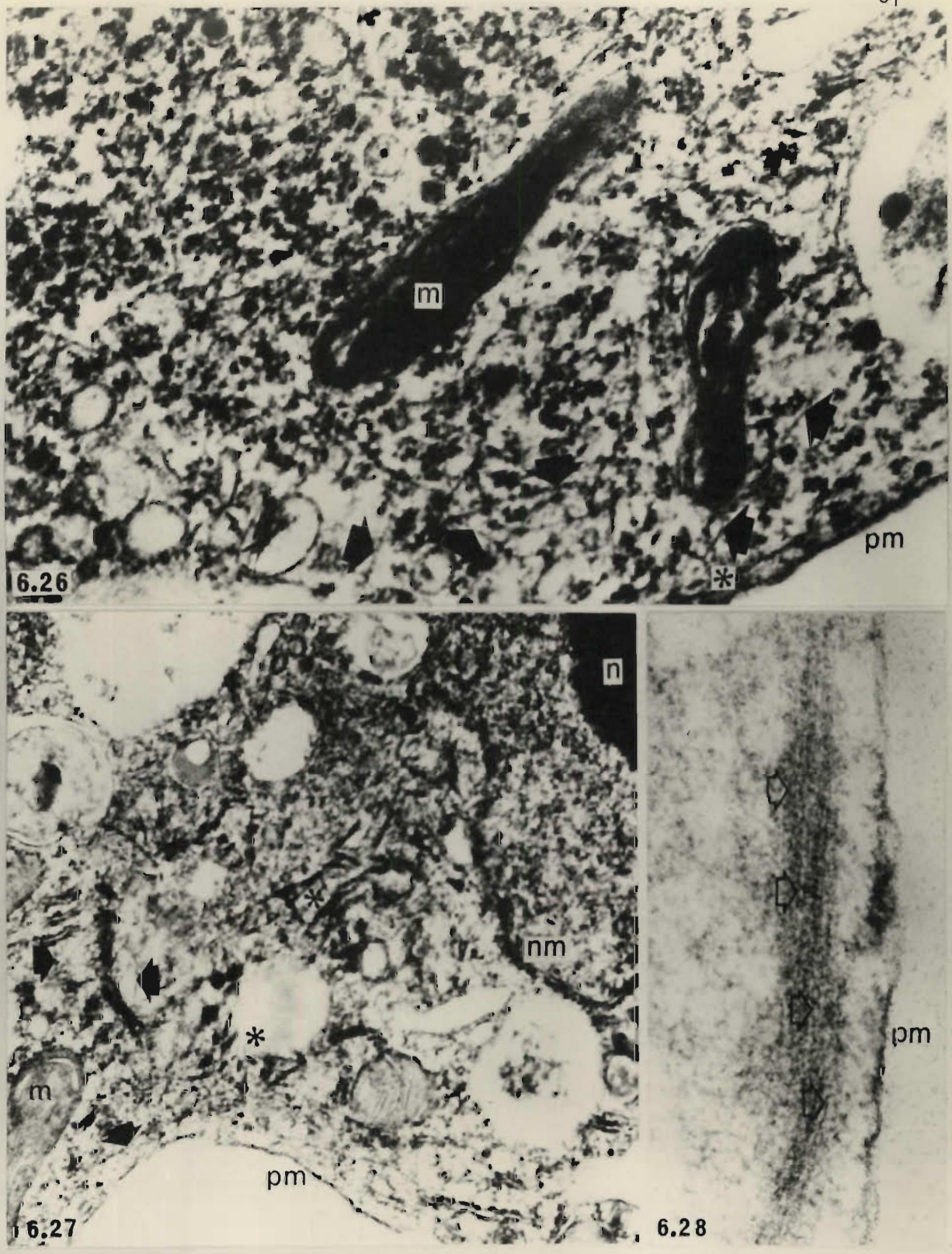
pm=plasma membrane

Fig. 6.22 A Naegleria gruberi, SA-2 amoeba food vacuole about to eject into the external medium. X45 500.

Fig. 6.23 A complex, laminated food vacuole deep in the cytoplasm of a Naegleria gruberi, Bristol strain amoeba. The arrows point to the unit membrane structure of the food vacuoles: 2 electron opaque layers sandwiching an electron transparent space. X73 500.

Fig. 6.24 A particularly complex food vacuole of a Naegleria gruberi, NB-1 amoeba. X40 000.

Fig. 6.25 A Naegleria fowleri, 1969 strain amoeba. A large food vacuole is fusing with a smaller, empty vacuole. X22 300.

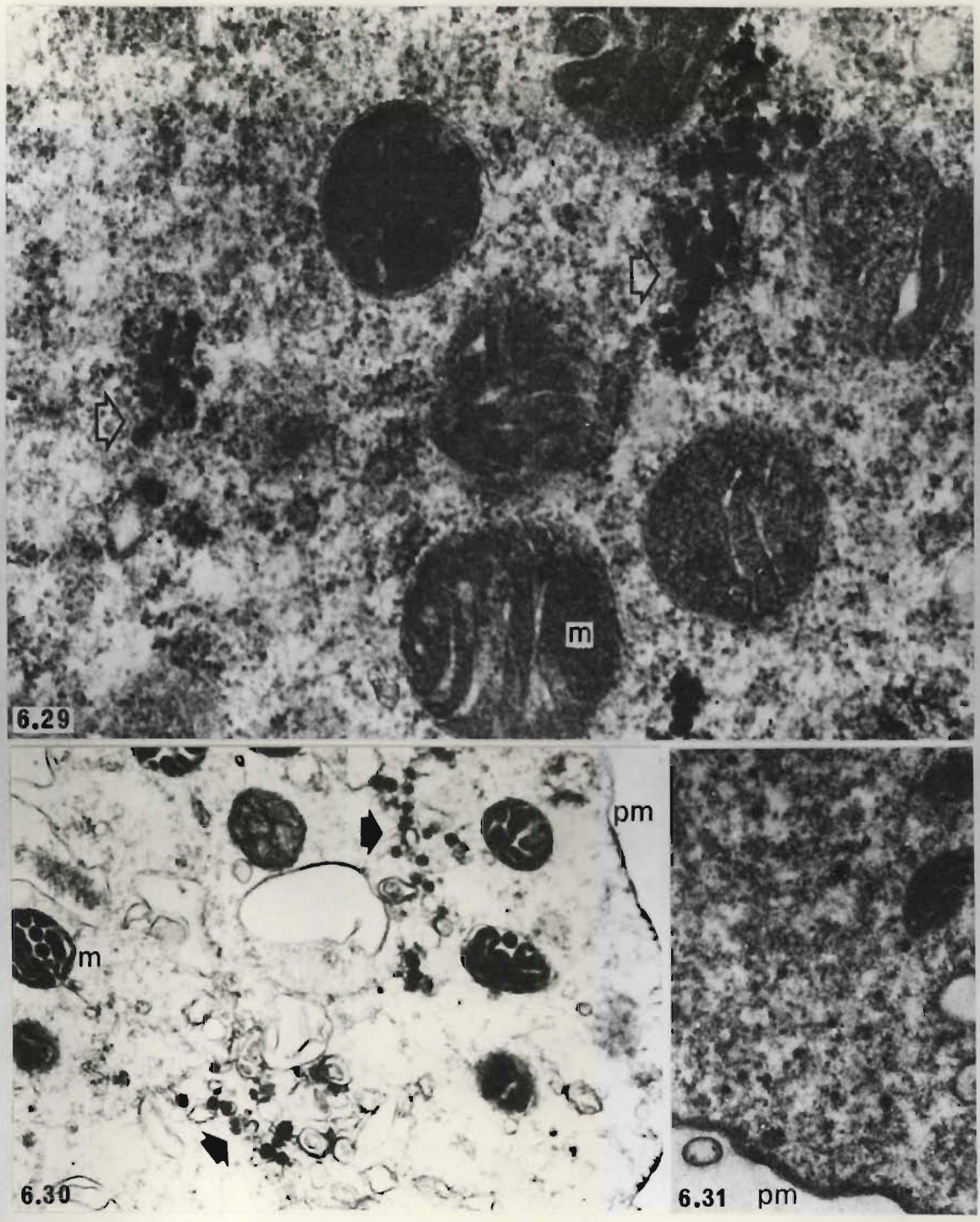


m=mitochondria, n=nucleolus, nm=nuclear membrane, pm=plasma membrane

Fig. 6.26 A Naegleria fowleri, G.J. amoeba with many 5-7 nm diameter microfilaments (arrows) randomly distributed in the cytoplasm. Asterisk indicates microfilaments in close proximity (and possibly attached) to the plasma membrane, X84 500.

Fig. 6.27 Amoeba of Naegleria fowleri, Vitek strain. Individual 5-7 nm dia. microfilaments (arrows) and clusters of 17-19 nm dia. microfilaments (asterisks) are present. X39 200.

Fig. 6.28 Naegleria fowleri, Oram strain. A bundle of colaterally aggregated 5-7 nm dia. microfilaments is in close proximity to the plasma membrane. Arrows mark individual microfilaments. X61 250.

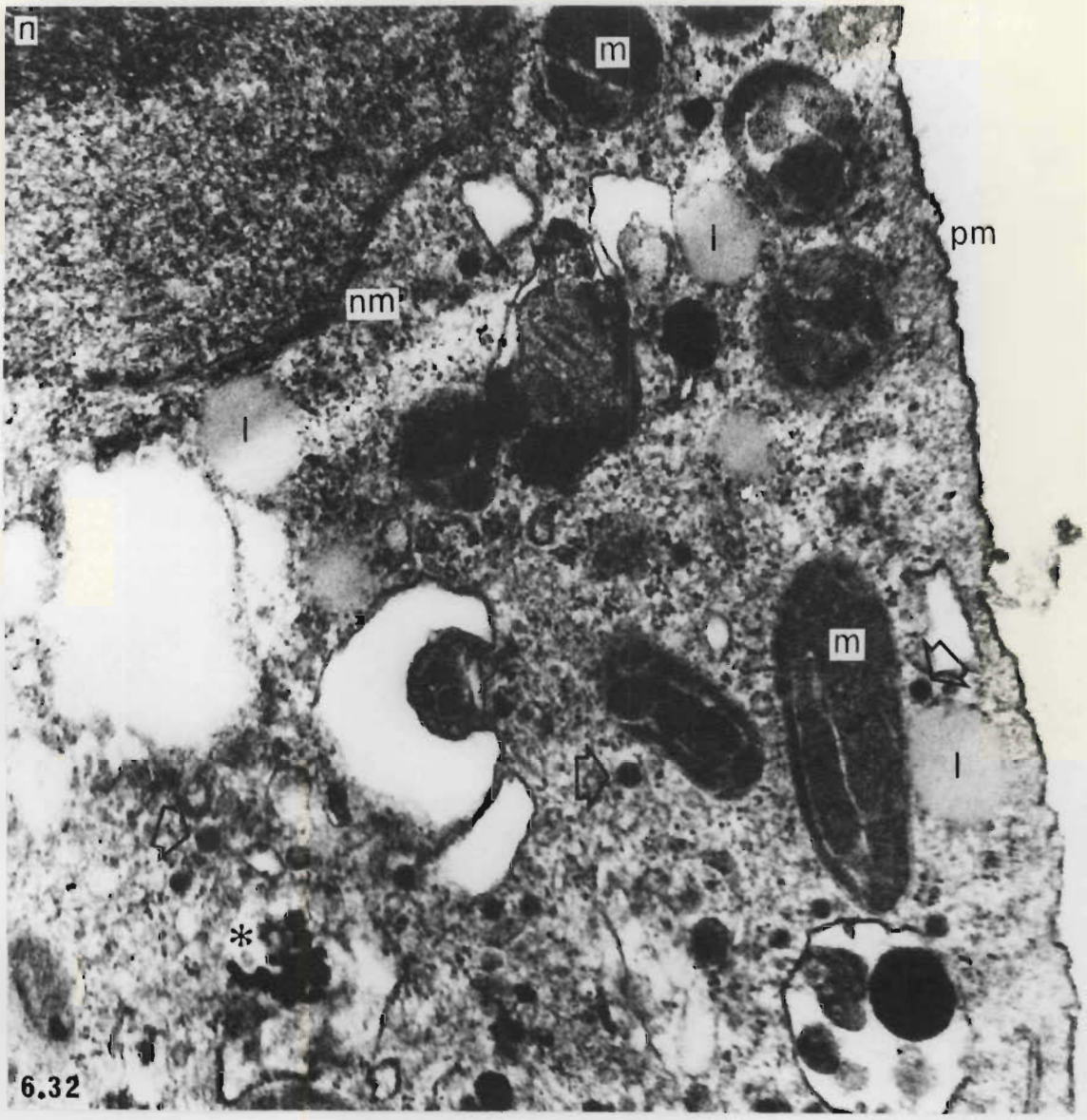


m=mitochondria, pm=plasma membrane

Fig. 6.29 Arrows mark clusters of angular, osmophilic bodies 75-88 nm in diameter prominent in the cytoplasm of a Naegleria fowleri, Oram strain amoeba. X55 600.

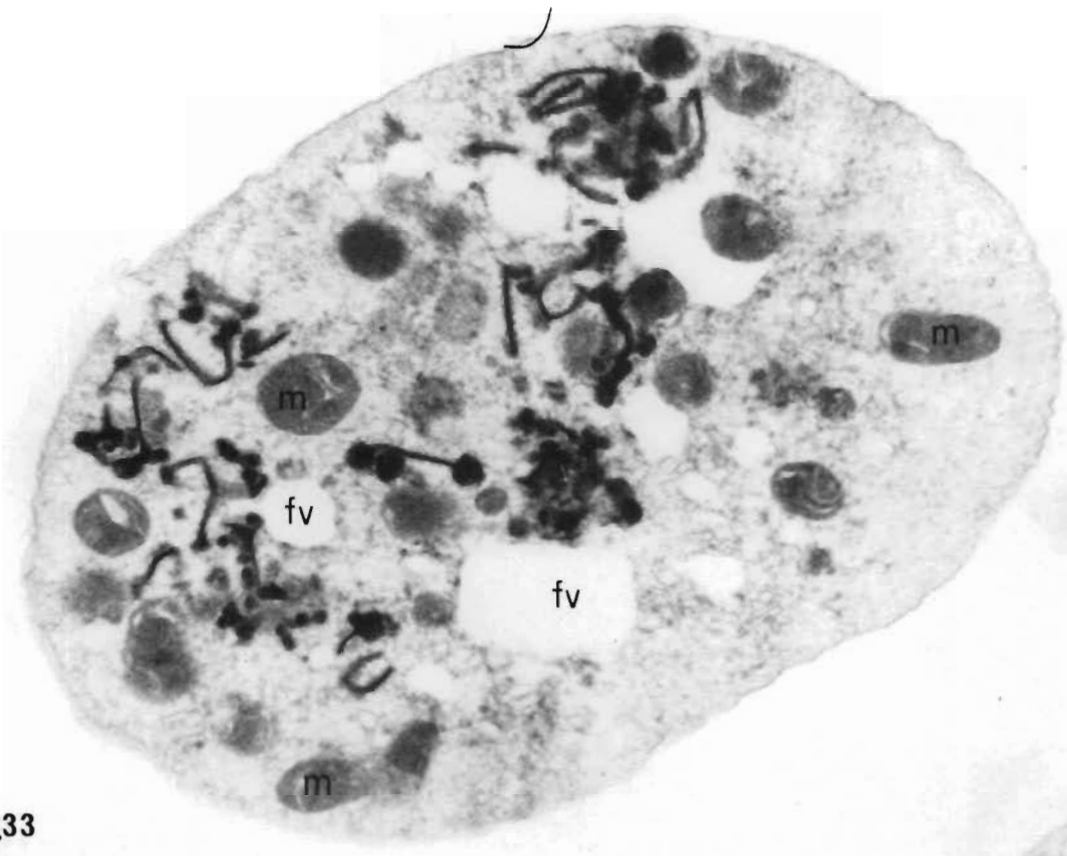
Fig. 6.30 Naegleria fowleri, Vitek strain amoeba. Arrows point to groups of angular, 80-92 nm diameter osmophilic bodies. X23 200.

Fig. 6.31 Naegleria fowleri, NH-1 strain amoeba with angular, osmophilic bodies 76-85 nm in diameter scattered in the cytoplasm. X25 200.

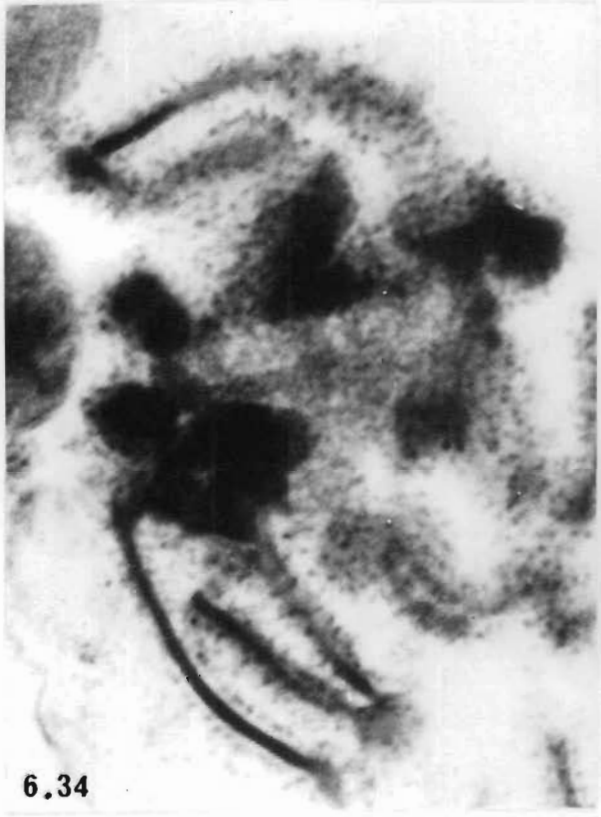


l=lipid body, m=mitochondria, n=nucleolus, nm=nuclear membrane, pm=plasma membrane

Fig. 6.32 Detail of Naegleria fowleri, Oram strain amoeba. The cytoplasm has numerous osmophilic bodies 85-95 nm in diameter (arrows). These are angular and appear to be pentagonal or hexagonal. The asterisk indicates a cluster of these bodies. X30 000.



6.33



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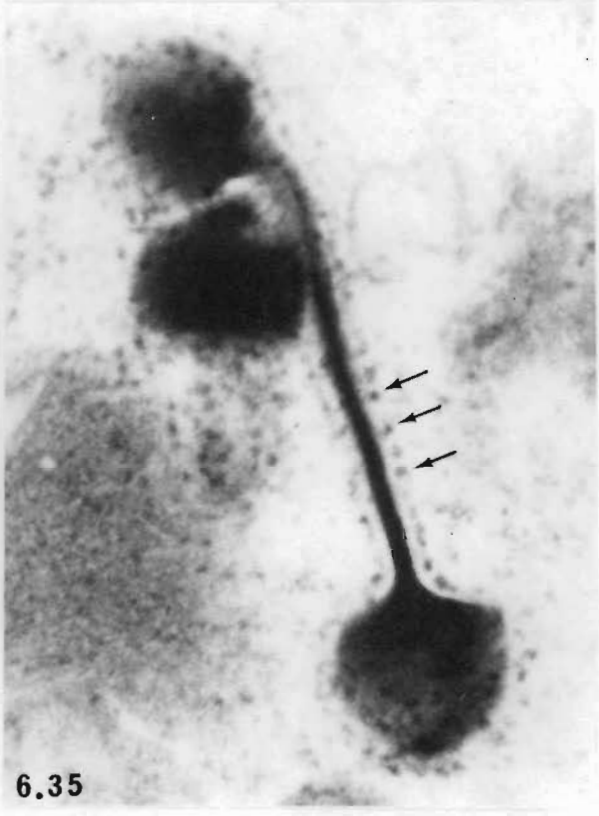
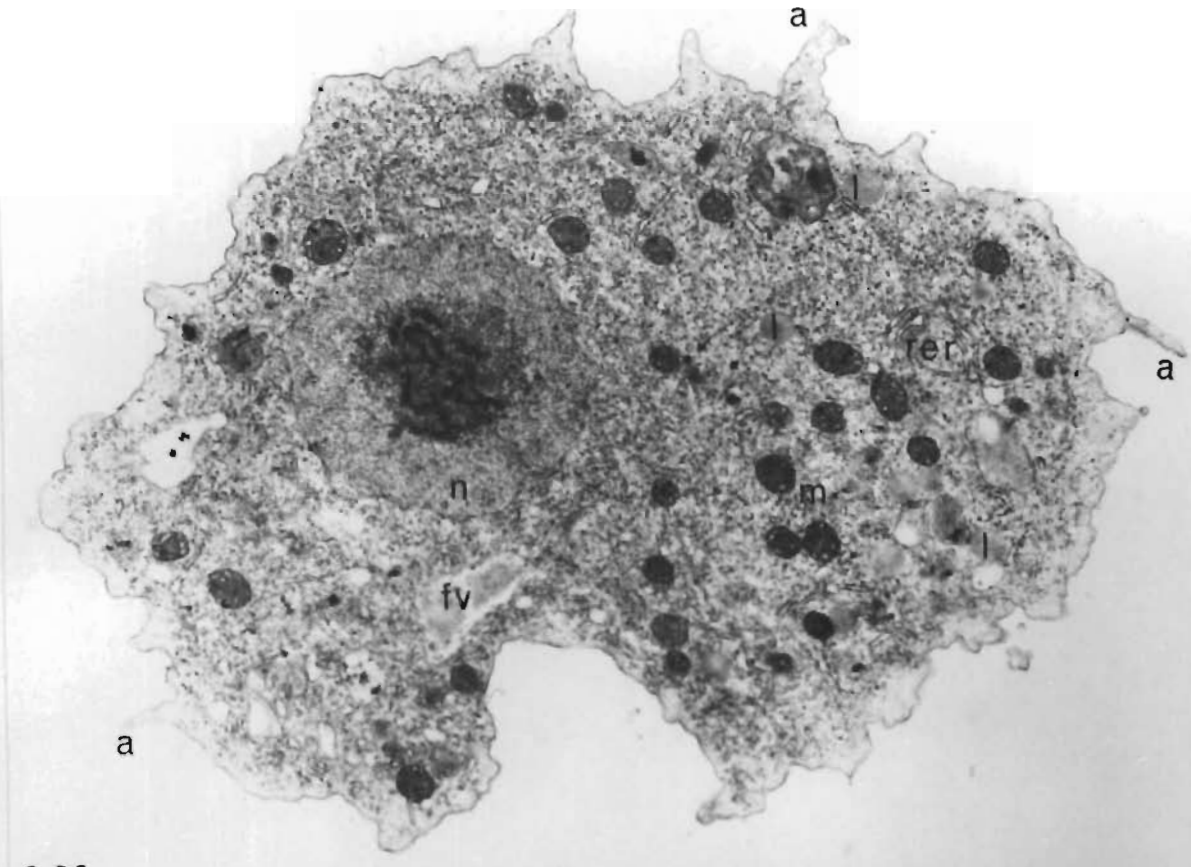


6.35

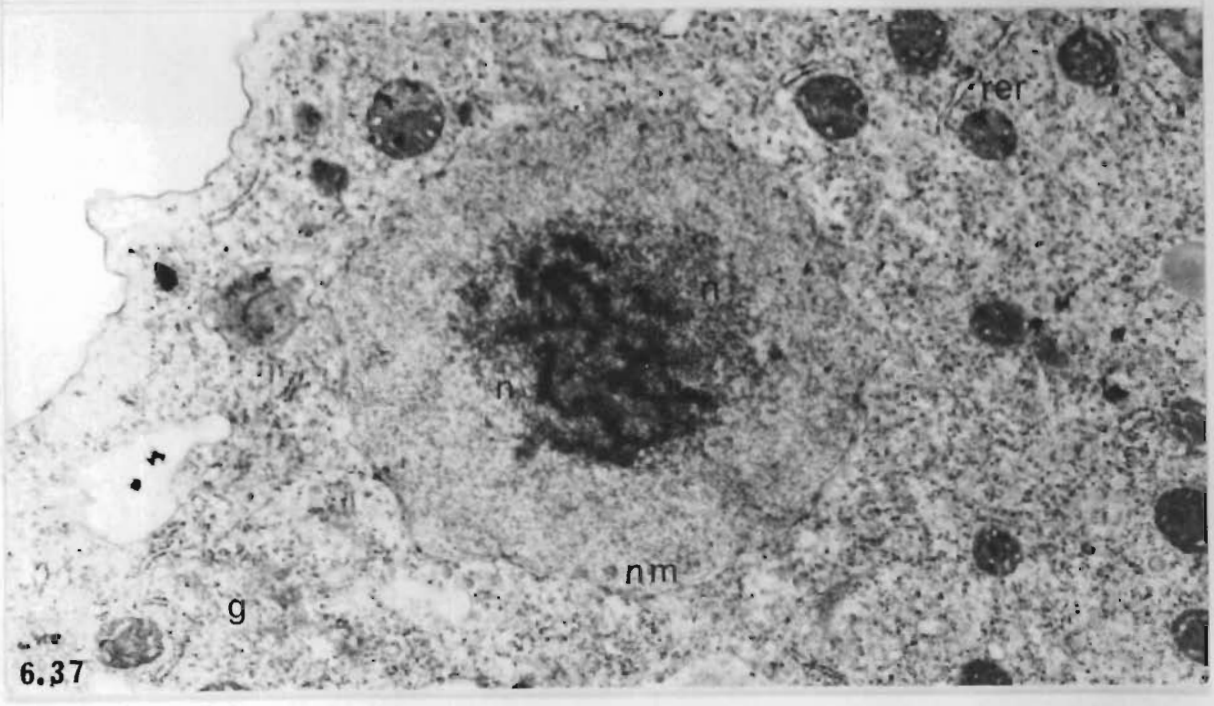
Fig. 6.33 Oram strain, Naegleria fowleri amoeba with unusual osmophilic bodies up to 900 nm long.
fv = food vacuoles, m = mitochondria. X20 250.

Fig. 6.34 Detail of Fig.6.33. Osmophilic bodies appear to have smaller structures attached. X78 300.

Fig. 6.35 Detail of Fig.6.33. 6-8 nm diameter structures (arrows) stud an osmophilic body. X143 500.



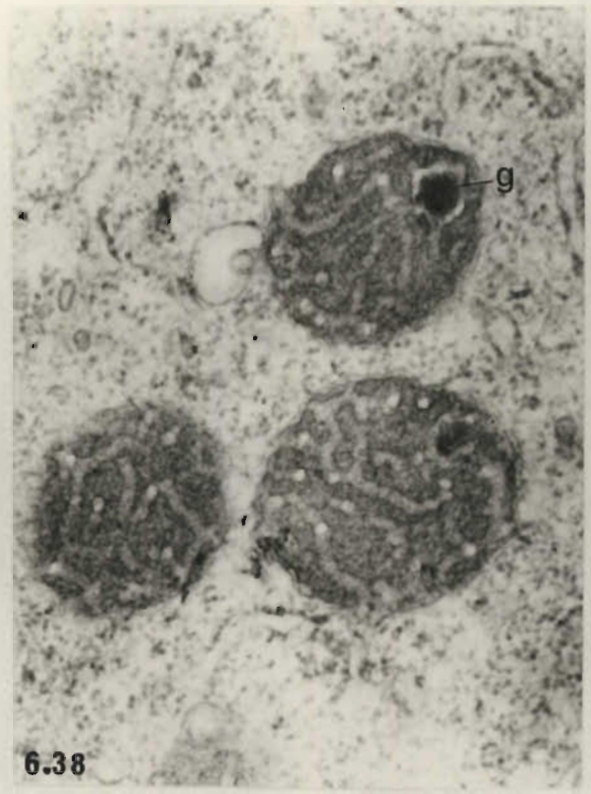
6.36

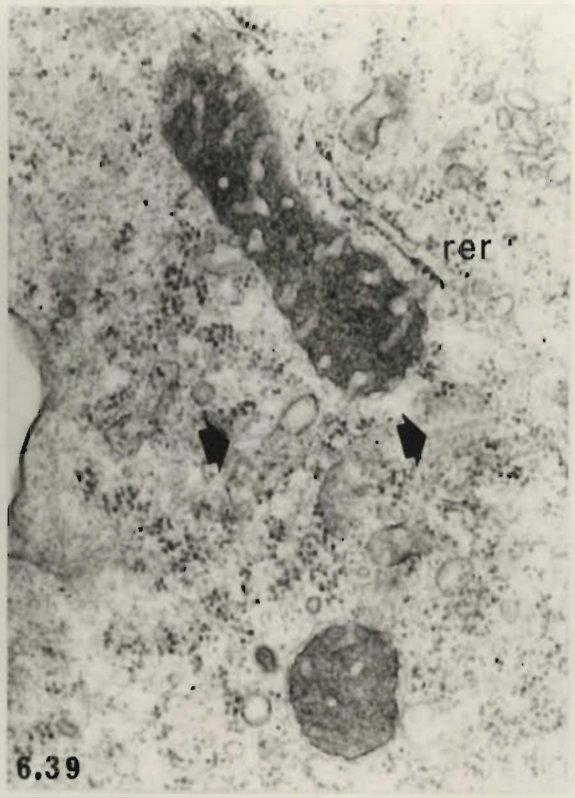


6.37

Fig. 6.36 Acanthamoeba, strain APG amoeba with a prominent nucleus (n), mitochondria (m), acanthopodia (a), lipid bodies (l), rough endoplasmic reticulum (rer) and a food vacuole (fv). X10 000.

Fig. 6.37 Detail of figure 6.36. The nucleolus is composed of granular 11 nm diameter particles and a nucleolonema of densely staining fibres. The region between the nuclear membrane (nm) and the nucleolus (n) is composed of granular particles 7 nm in diameter. A Golgi complex (g) and rough endoplasmic reticulum (rer) are visible. X17 750.





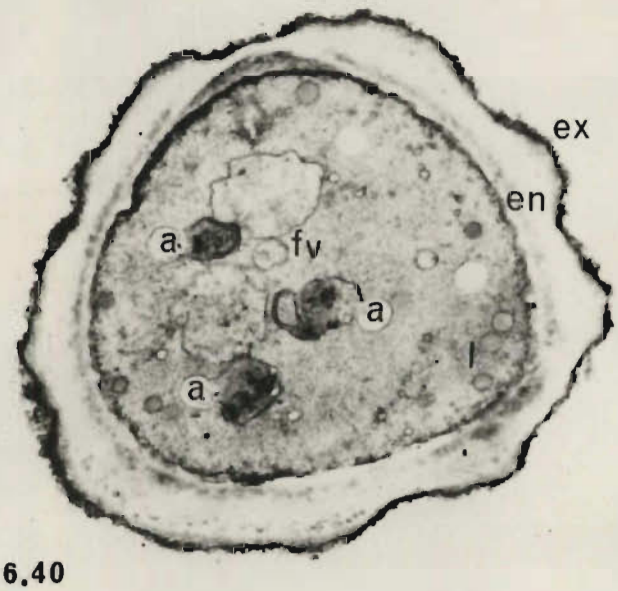


Fig. 6.38 Cytoplasmic detail of an Acanthamoeba, strain APG amoeba showing 3 spherical mitochondria with anastomosing tubular cristae. An electron dense intracristal granule (g) is present. X41 000.

Fig. 6.39 Polysomes, ribosomes, rough endoplasmic reticulum (rer), an elongated mitochondrion and microtubules (arrows) are present in the cytoplasm of an Acanthamoeba, strain APG amoeba. X39 200.

Fig. 6.40 An Acanthamoeba, strain APG cyst. The dense cytoplasm contains autolysosomes (a), food vacuoles (fv), and lipid bodies (l). Material is caught between the endocyst (en) and the exocyst (ex). X7 500.

and up to 400 nm long (Fig. 6.39) and occasional 5-7 nm wide microfilaments were seen scattered throughout the cytoplasm. No structure suggestive of a surface-active lysosome or a virus-like particle was detected in any of the Acanthamoeba strain APG amoebae examined.

E.2.ix Cysts

Acanthamoeba strain APG cysts, 10-16 µm in diameter, were examined under the transmission electron microscope (Fig.6.40). The cysts were characterised by a prominent double-walled envelope comprised of a fibrous, electron-dense convoluted exocyst and a less electron-dense, round or polyhedral endocyst. Material, most probably cytoplasmic debris, was found caught up between the two cyst layers. The cytoplasm of the cyst was electron-dense, and conspicuous lipid-like bodies bounded by an electron-dense line were present. Autolysosomes were a prominent component of the cytoplasm of encysting amoebae, and were readily identified as vacuoles containing substances of glycogen, lipid, or mitochondrial origin. Other features of the cyst such as nuclei, mitochondria, food and contractile vacuoles and ostioles were also observed.

F. DISCUSSION

Results presented in this chapter of the ultrastructural investigations of six strains of non-pathogenic Naegleria gruberi, 13 strains of pathogenic Naegleria fowleri and a pathogenic strain of Acanthamoeba are in good agreement with the findings of other workers (30,31,46,169,205,216, 262,263,271,273,274,277,359,367,422,423,427). Additional fine structural features such as the existence of thin and thick microfilaments, differences in mitochondrial morphology and the presence of virus-like particles have come to light.

F.1 Nuclei

Observations on the nuclei of Naegleria were similar to those of other investigators (175,370). There was no indication of chromosomes, but Schuster (370) suggested that under the electron microscope the chromosomes may have a density similar to that of the nucleoplasm, making detection difficult. Nuclear pores in Naegleria were similar to those depicted for Pelomyxa carolinensis by Daniels (102). When dividing nuclei were encountered they followed the same sequence as figured by Schuster (370).

F.2 Microfilaments

It is now generally accepted that amoeboid motion is based on the presence and contractility of actin-like and myosin-like protein complexes in the cytoplasm of amoebae (204,216). Thin and thick microfilaments exhibiting the morphological characteristics of actin and myosin respectively have been demonstrated in the limax amoeba, Acanthamoeba (336), the Amoeba-Chaos group (85,224,294,295,334,353,354,413), the shelled amoeba Difflugia (145,468), Entamoeba (289,290) and Thecamoeba (192). The thin microfilaments of Acanthamoeba (446) and Amoeba (292), are biochemically similar to actin filaments. Nachmias (296) produced thick microfilaments from purified Physarum myosin that were identical in size and shape to those found in prepared sections of Physarum amoebae.

The results presented in this chapter demonstrated for the first time the existence of thin and thick microfilaments in situ in the cytoplasm of Naegleria amoebae. Although Visvesvara & Callaway (427) observed "microfibers" in the HB-1 strain of Naegleria fowleri, they gave no details or interpretation.

The microfilaments are distinct structures and cannot be mistaken for any other cell structure such as the endoplasmic reticulum. Microfilaments, particularly thick microfilaments, are labile - glutaraldehyde being an essential fixative in their preparation for the electron microscope (295). Prolonged uranyl acetate staining makes it easier to see amoeboid microfilaments under the electron microscope (289). A combination of extended contact with glutaraldehyde and double staining with uranyl acetate in pellet and in section has preserved and enhanced the Naegleria microfilaments, which are similar in size and shape to those found in other amoebae. Possibly, the thin 5-7 nm wide microfilaments in Naegleria are membrane-bound as they are in Acanthamoeba (335) and in Chaos (85). Bundles of collaterally-aggregated thin microfilaments positioned near the plasma membrane occur in Naegleria as well as in a variety of other amoebae (85,145,192,289,294,295,334,468). The thick, 17-19 nm diameter microfilaments of Naegleria, like those of Amoeba proteus (334), are randomly orientated and do not appear to be attached to the plasma membrane.

The above observations are necessary prerequisites for theories of amoeboid motion based on modifications of the sliding filament model of muscular contraction (204,354). Durham (142) proposed that in relaxed amoebae actin-like filaments are attached to the inner surface of the plasma membrane, and are in random array with associated myosin-like

filaments which are free in the cytoplasm. On contraction, these filaments would undergo synaeresis and a compact array just under the plasma membrane would result. Rinaldi & Hrebenda (353), using EDTA in their electron microscope fixation procedure, have shown overlapping arrays of thin and thick microfilaments in close proximity to the plasma membrane of Amoeba proteus. Huxley (204) proposes that amoeboid motion is a result of active shearing between cytoplasmic filaments (myosin-like) and membrane-attached filaments (actin-like). Rinaldi et al.(354) proposed a sliding filament theory of amoeboid motion based on actin and myosin filaments free in the endoplasm, but organised and closely associated in the ectoplasm. These microfilaments provide the structural basis for contractility of the ectoplasmic tube.

I have isolated a protein complex similar to smooth muscle actomyosin and Physarum plasmodial myosin B from Naegleria amoebae (248). This protein, which comprises 0,7 per cent of the total cell protein, has solubility, enzymatic ATPase and superprecipitation properties similar to those of other actomyosins (256). Dissociation of the Naegleria actomyosin into its constituent actin and myosin was accomplished by Sepharose 4B elution in the presence of ATP and Mg^{+2} ion. Microfilaments were produced in vitro from ATP-dissociated extracts of Naegleria actomyosin by the addition of 2,5 mM EDTA or EGTA (248). Thus it is suggested that the actin-like and myosin-like proteins in Naegleria are organised in the form of thin and thick microfilaments respectively, and interaction between these microfilaments is the mechanical means of accomplishing amoeboid motion in Naegleria. Fulton & Fuller (176) have suggested that motility in Naegleria amoebae depends on actin-like microfilaments while motility in Naegleria flagellates depends on microtubules.

F.3 Mitochondria

Marked differences occur in the shape and structure of the mitochondria of virulent and avirulent Naegleria amoebae. In the strains of Naegleria gruberi examined, only cylindrical or oval mitochondria were seen. All the strains of Naegleria fowleri, in addition to these mitochondrial phenotypes, had prominent cup-shaped and dumbbell-shaped mitochondria. Martinez et al.(271,273,277) found oval, elongate, dumbbell-shaped or cup-shaped mitochondria in three strains of Naegleria fowleri. Carter (46) reported that the 1966 and 1969 strains of Naegleria fowleri possess cup-shaped and dumbbell-shaped mitochondria 1 µm long by 0,25 µm wide. The HB-1 strain of Naegleria fowleri has cup-shaped and dumbbell-shaped mitochindria as reported by

Maitra et al.(262) and Visvesvara & Callaway (427). J.B.Jadin (205) has described cup-shaped and dumbbell-shaped mitochondria in Naegleria fowleri strain 0359.

Honeycomb-like cristal patterns were observed in some strains of Naegleria fowleri but not in Naegleria gruberi or the APG strain of Acanthamoeba. The appearance of these cristae is puzzling, as only five of the 13 Naegleria fowleri strains possessed them. There are two alternatives - firstly that two or more mitochondrial phenotypes can exist in the cytoplasm of Naegleria fowleri, or secondly, that a physiological process has altered some of the mitochondria. As for the first alternative, the protozoans Paramecium (240) and Euplotes (228) possess several types of mitochondria in a common cytoplasm, and Flickinger (162) has reported on two mitochondrial phenotypes in Amoeba. The second alternative appears less likely, as the ultrastructure of all Naegleria fowleri amoebae was identical except for these differences in the mitochondria. Daniels & Breyer (104) found that in the giant amoeba Pelomyxa carolinensis mitochondrial tubules lie at random in the matrix. When, however, these amoebae are starved the mitochondrial tubules enlarge and are aligned in a parallel, zig-zag pattern. Fawcett (160) stated that in cardiac-muscle mitochondria the parallel zig-zag, tubular arrangement of the cristae may be modified. If adjacent cristae are out of register so that angularities touch, that is, a concavity of one tubule is not opposite a convexity of another tubule, cristae could fuse to produce a honeycomb pattern. A similar explanation may account for the observed results in Naegleria fowleri amoebae, that is, a physiological process has altered some of the mitochondria. All of the cells were cultured and prepared for the electron microscope under identical conditions. It is possible that the mitochondria of some strains of Naegleria fowleri are more susceptible to physiological stress than those of the other amoebae examined, and alterations in the architecture of the mitochondria may have resulted.

Schuster & Rechthand (378) in their recent study of the effects of Amphotericin B on the ultrastructure of Naegleria have stated that Naegleria fowleri mitochondria were much more sensitive to drug damage than were the mitochondria of Naegleria gruberi. They conclude that under the influence of Amphotericin B the Naegleria fowleri mitochondria swell, and the cristae which were usually obscured by the dense mitochondrial matrix became prominent. The supporting electron micrographs of Amphotericin B-damaged mitochondria are strikingly similar to the

honeycomb-like cristal patterns seen in Naegleria fowleri amoebae, strains 0359, MW4U, G.J., PA-14 and McMahon.

Vickermann (424,425) has observed with Trypanosoma that the stage in the life cycle of the organism may determine the structure of the mitochondria. Presumably the greater surface area offered by the cup-shaped and dumbbell-shaped mitochondria in Naegleria fowleri is an adaptation to facilitate the diffusion of substances into or out of the mitochondria, resulting in increased metabolic activity.

Mitochondria of the APG strain of Acanthamoeba are organised differently from those of Naegleria. The cristae of Acanthamoeba are distinct tubules, rather than the flattened saccules found in Naegleria. Intracristal granules present in the mitochondria of the APG strain may be involved in osmotic regulation, or may organically bind Ca^{2+} and other divalent ions (160). Similar intramitochondrial granules have been reported for Acanthamoeba castellanii by Bowers & Korn (30) and by Vickermann (422,423).

F.4 Virus-like particles (v.l.p.s)

In 1960, Miller & Swartzwelder (291) observed 40 nm diameter virus-like particles (v.l.p.s) in bacteria-grown cultures of Entamoeba histolytica. Subsequently, Diamond et al.(122,123,201,202,281,282) have identified and characterised two different viruses in Entamoeba histolytica. One virus is filamentous and found mostly in the nucleus, the other is a polyhedral, 70-75 nm diameter virus and is confined to the cytoplasm. In 1969 Schuster (369) reported finding a 100 nm diameter polyhedral v.l.p. in the nucleus and cytoplasm of the EG strain of Naegleria gruberi. The introduction of these v.l.p.s appeared to coincide with the use of chicken embryo extract as a culture medium supplement. The transferral of axenically grown EG amoebae into monobacterial culture triggered off the appearance of the v.l.p.s. Later work by Dunnebacke & Schuster (138-140,372-377) extended these initial observations. The passage of v.l.p.s from their origin in the nucleus into the cytoplasm of the cell is accomplished via tubules at the pores of the nuclear envelope. The cytoplasm of infected EG amoebae contains bacteria-like bodies 0,5 µm x 2 µm, containing v.l.p.s. Spheres 900 nm in diameter containing v.l.p.s eject into the external medium and probably represent the vehicle of transmission of the v.l.p.s. Freeze-thawed and filtered cell-free lysates of infected EG cultures were capable of lysing chicken embryo fibroblast cultures within three to six days.

In an attempt to determine if these v.l.p.s are the causative agents of primary amoebic meningoencephalitis, Schuster & Dunnebacke (375) inoculated EG_s (a sub-strain of EG) amoebae and cell-free millipore filtered lysates of EG_s amoebae intranasally into mice. They obtained erratic results, as some of the mice died while others did not. Ultrastructural examination of mouse brain tissue revealed no v.l.p.s. Lysates from such brain tissue could however produce a cytopathogenic effect in chick fibroblast cultures. Inoculation of mice with the Naegleria fowleri strain MB 41 (=HB-1) which did not contain v.l.p.s, caused most of the mice to die. Ultrastructural studies of the amoebae found in the mouse brain tissue revealed no v.l.p.s. EG_b, another substrain of the EG isolate, also revealed no v.l.p.s under the electron microscope. Both substrains were capable of producing a pronounced cytopathogenic effect in chick fibroblast culture (138). Other ultrastructural studies of mice infected with pathogenic Naegleria fowleri have revealed no particles suggestive of a virus (262,273,277,427). Thus, the cytopathogenic effect is not always directly linked to the presence of v.l.p.s in Naegleria amoebae. Schuster & Dunnebacke (140,375) found that four strains of Naegleria fowleri and five strains of Naegleria gruberi exhibited a cytopathogenic effect on chicken embryo cells and of these, examination by electron microscope revealed v.l.p.s in only the EG_s amoebae. Under similar conditions, Acanthamoeba culbertsoni and Polysphondylium pallidum which are virulent to mice, and free-living amoebae Acanthamoeba astronyxis and Acanthamoeba castellanii did not visibly affect chicken embryo cells. Schuster & Dunnebacke (140,375) concluded that the cytopathogenic effect observed was restricted to amoebae of the genus Naegleria, and was not associated with the presence of v.l.p.s which are present in both free-living and pathogenic strains of Naegleria and may be related to the scappie agent. Another possibility is that the infectious agent could be a viroid - an infectious, low molecular weight RNA implicated in several diseases (279), or a lytic enzyme as suggested by Chang (74).

Polyhedral, electron-opaque particles up to 95 nm in diameter and suggestive of a virus were detected in Oram, NH-1, and Vitek strains of Naegleria fowleri amoebae. None of the Naegleria gruberi strains or the APG strain of Acanthamoeba displayed such particles under the electron microscope. These particles are remarkably similar to the cytoplasmic icosahedral, 70-80 nm diameter v.l.p.s of Entamoeba histolytica (122,123, 201,202,281,282), Entamoeba moshkovskii (42) and the polyhedral v.l.p.s,

100 nm in diameter, found in the EG_S substrain of Naegleria gruberi (369,373,376,377).

Unusual osmophilic bodies up to 900 nm long with attached 6-8 nm wide structures were detected in the Oram strain of Naegleria fowleri. These structures were tentatively identified as mycoplasma. On viewing these particular electron micrographs, Prof. F.Schuster of Brooklyn College, New York, suggested that the osmophilic bodies are "probably of viral origin" (personal communication, March, 1976). Dunnebacke & Schuster (138) have described mycoplasma in the EG strain of Naegleria gruberi, but they present evidence that the mycoplasma were not infectious agents. Proctor & Gregory (343) have reported electron-dense bodies 200-300 nm long by 140-150 nm wide in the cytoplasm of pathogenic Entamoeba histolytica.

It is highly probable that the particles suggestive of a virus found in the Oram, NH-1 and Vitek strains of Naegleria fowleri amoebae are not the pathogenic agents responsible for primary amoebic meningoencephalitis. The virus-like particles may have arisen by chance infection in culturing the amoebae. Such v.l.p.s could easily pass through the millipore filters routinely used to sterilize the commercial calf or foetal calf serum used as a culture supplement for axenic growth of the amoebae. It would appear that the mechanism of pathogenesis in Naegleria is not associated with surface-active lysosomes or virus particles, but is due to some other mechanisms - possibly a lytic enzyme.

G. CONCLUSION

The ultrastructural comparison of amoebae of pathogenic Naegleria fowleri, non-pathogenic Naegleria gruberi and the APG strain of Acanthamoeba has shown both comparable and divergent characteristics. Examination of the fine structure of the nuclei, mitochondria, Golgi apparatus and other cell features has made it possible to differentiate Naegleria fowleri, Naegleria gruberi and Acanthamoeba. It is possible to determine the taxonomic status of any particular amoeba using ultrastructural considerations. Although this would be laborious, it would not be as onerous as identification based solely on an examination of mitotic figures! The information in this chapter provides additional support for the establishment of pathogenic Naegleria fowleri as a discrete and separate species from the non-pathogenic Naegleria gruberi.

CHAPTER 7

STUDIES ON THE INTERACTIONS OF LIMAX AMOEBAE WITH SELECTED BACTERIA

A. ABSTRACT

Distinct feeding preferences were evident between Naegleria fowleri and Naegleria gruberi amoebae when they were tested on a variety of gram-negative and gram-positive bacteria on two different agar media. Generally, the gram-negative bacteria were more acceptable as a food source and good growth of all Naegleria strains was obtained with Bordetella bronchiseptica, Escherichia coli and Klebsiella aerogenes. Sarcina lutea and Xanthomonas campestris were toxic to all the Naegleria strains, while Bacillus cereus and Staphylococcus albus supported the growth of Naegleria fowleri and Naegleria gruberi. Naegleria fowleri amoebae grew more slowly than Naegleria gruberi amoebae at 30°C, but at 43°C Naegleria fowleri strains grew well, while the Naegleria gruberi strains did not grow at all.

Four Pseudomonas strains were toxic to cysts and motile amoebae and prevented growth and encystment of amoebae. Three additional Pseudomonas strains allowed very slow amoeboid growth and sometimes the large, occasionally multinucleated amoebae produced multinucleated cysts. Pseudomonas cell suspensions and cell residues present at the initiation of the amoeba-to-flagellate transformation completely inhibited this well-defined morphogenic process. If Pseudomonas extracts were added to flagellates, reversion to amoebae or lysis rapidly occurred.

Cell-free filtrates of all seven Pseudomonas strains had no detectable toxic effect on excystment or amoeboid growth of Naegleria sp., Acanthamoeba culbertsoni and Tetramitus rostratus. Encysting amoebae tended to produce larger than normal cysts. The amoeba-to-flagellate transformation of Naegleria was slightly accelerated. An unknown factor in the cell-free filtrate blocked cytokinesis but not karyokinesis in approximately 17 percent of a test population, and produced multinucleated amoebae. Pyocyanin extracted from these strains of Pseudomonas, even when applied in excess, had no observable toxic effect on Naegleria other than the production of larger than normal cysts.

B. INTRODUCTION

Amoebae and bacteria have a variety of interactions. J.B.Jadin (207,208) has presented evidence that the limax amoebae Acanthamoeba castellanii, Acanthamoeba culbertsoni and Naegleria fowleri may act as vectors for pathogenic mycobacteria and Mycobacterium leprae. Phillips et al.(330-332) have shown that the bacterial associates of Entamoeba

histolytica markedly affect the virulence of the amoebae in germ-free guinea pigs. Drozański (131) has isolated a soil bacterium that is parasitic to 11 strains of limax amoebae, including the amoebo-flagellates Naegleria gruberi and Tetramitus rostratus. Paramoeba eihardi and Amoeba discoides can be parasitised by bacteria which are capable of causing the death of the amoebae (184,225). Various workers (11,103,105,223) have reported that the cytoplasm of some of the large free-living amoebae may contain several different species of symbiotic bacteria.

Cutler et al.(100) in studies at the Rothamsted Experimental Station in England, found large fluctuations in the numbers of Naegleria and other limax amoebae when test plots were sampled for 365 consecutive days. These fluctuations could not be linked to environmental conditions, and when more bacteria of certain species appeared, there were fewer amoebae. Sandon (361) in his world-wide study of soil protozoa noted that the two ubiquitous amoebae, Hartmannella hyalina and Naegleria gruberi were found co-existing in only 13 of 148 soil samples. He also reported that in one experimental field Naegleria was the most common soil amoeba with very few Hartmannella, while in a neighbouring test plot the situation was reversed. Sandon offered no explanation, but possibly the different amoebae have distinct bacterial preferences and a shift in the bacterial composition of the soil could affect the amoeboid population. Singh (384,386,389) found that over half of 159 species of bacteria were suitable as food for small soil amoebae. He also proposed (385,390) that the production of toxic pigments and exo-toxins by bacteria is a protective mechanism against attack by other organ-isms. Chang (71,73,74), and Groscop & Brent (189) reported on the edibility of a variety of bacteria to pathogenic and non-pathogenic Naegleria and other amoebae. Recently, Anderson & Jamieson (9) have studied the growth of Naegleria fowleri and Naegleria gruberi on ten species of bacteria. Bacteria have been shown to affect dramatically the excystment as well as the growth of limax amoebae (14,87,130,395). Singh (388,390) found that the multinucleated amoeboid organism Leptomyxa reticulata lost the ability to encyst when grown on certain species of bacteria.

Pseudomonas aeruginosa is a widespread plant and animal pathogen (118) and it is also toxic to Naegleria (9,71,73,189,387). Diffusible water-soluble pigments in Pseudomonas and Xanthomonas are important characteristics for identification. Most strains of Pseudomonas aeruginosa produce the blue-green pigment pyocyanin and the yellow-green pigment fluorescein (200), while Xanthomonas produces the yellow pigment carotenoid (118,407). The final colour produced by a pigment-forming strain of Pseudomonas

aeruginosa in liquid or solid media depends on the type of media used, on growth factors and on inorganic ions (180,181). Many of the pigments of Pseudomonas are phenazines, and these may be involved in electron transfer (118).

This chapter confirms prior observations and provides new quantitative and qualitative data on the relationships between limax amoebae and selected bacteria. A portion of these results has been published (250).

C. MATERIALS

Organisms	Sources are recorded on p.146-147.
Media	Described on p.148-149.
Chemicals	Described on p.149-150.
Apparatus	Shaking water bath. Spectronic 20 spectrophotometer.

D. METHODS

D.1 Bacteria

The Pseudomonas strains used were kept as stocks at 5°C on King A agar medium and subcultured every two to three weeks. For log-phase growth, King A medium was used and incubated at 37°C for 16-18 hours with agitation. Other species of bacteria were kept as stocks on Difco Penassay broth agar slants at 22-25°C and subcultured every two to three weeks. Log-phase growth occurred at 25-30°C in 16-20 hours in liquid Penassay broth. Cell concentrations of the Pseudomonas strains were determined with a Spectronic 20 spectrophotometer set at 650 µm. All Pseudomonas strains gave good reference curves which were used to check the state of growth of the test bacterial cultures.

D.2 Amoebae

Stock cultures of the SA-1, NEG and NB-1 strains of Naegleria gruberi, and the strains of Acanthamoeba culbertsoni and Tetramitus rostratus were grown on NM or PA agar with Klebsiella aerogenes as a bacterial associate. Naegleria fowleri strains G.J., Oram, PA-17 and PA-90 were grown axenically in Fulton's A medium.

For the calculation of growth rates and the toxic effects of Pseudomonas aeruginosa on amoebae and cysts, bacterial-streak agar plates were prepared. NM or PA agar plates less than 14 days old were used and three bacterial streaks 0,5 cm wide applied with a sterile loop to the surface of the agar. Log-phase cultures of Pseudomonas aeruginosa were filtered through a sterile 0,2 µm millipore filter. Klebsiella aerogenes was added as a food source to Pseudomonas cell residues and cell-free filtrates.

A sterile loopful of cysts or amoebae was touched to one end of each of the bacterial streaks, the agar plates were inverted to retard dehydration, stored at 30°C and examined as often as possible over the next few weeks. Each series of experiments was done three times. To remove any contaminating amoebae from the cyst preparations, the cell suspensions were treated briefly with 2mg/ml digitonin to lyse the amoebae (256) while leaving the cysts undamaged.

For the production of cysts and flagellates, the procedures outlined on pages 38 and 39 were followed.

E. RESULTS

E.1 Choice of bacterial food by Naegleria

The ability of amoebae to migrate along a bacterial streak was used as a criterion for growth. As the amoebae grew, the translucent or opaque growth of bacteria on agar gave way to the almost transparent growth of the amoebae after the bacteria had been eaten. In general, gram-negative bacteria were a better food source than were gram-positive bacteria.

The feeding reactions of three strains of Naegleria fowleri and three strains of Naegleria gruberi to six strains of bacteria on PA and NM agar are summarised in Table 7.1 on the following page.

Failure of either Naegleria species to grow on Staphylococcus albus on PA agar, and Bacillus cereus on NM agar, is attributed to a density-dependent factor. Staphylococcus albus on PA agar produced a very thinly populated bacterial streak which was incapable of supporting an amoeba population large enough to form a growth front. All the strains of amoebae were viable, as under the light microscope amoebae could be seen on the sterile agar adjacent to the bacterial streak. The inability of Staphylococcus to support a Naegleria population cannot be due to toxicity because the same bacterium on NM agar produced a bacterial streak capable of supporting Naegleria growth along the entire length.

In the case of Bacillus cereus on NM medium it is suggested that the bacterial population was so dense as to prevent the amoebae from migrating along the streak. The bacteria flourished so well that the streaks grew from 0,5 cm to 1,5 cm in width and although an initial growth front was observed 0,5 cm away from the point of inoculation, the bacteria recolonised this area rapidly. On PA agar a much thinner streak of Bacillus cereus allowed complete growth of the three Naegleria gruberi strains, and partial growth of the three Naegleria fowleri strains.

TABLE 7.1

FEEDING REACTIONS OF 6 STRAINS OF *NAEGLERIA* TO 6 SPECIES OF BACTERIA ON PA AND NM AGAR AT 30°C

BACTERIA	*NAEGLERIA* STRAIN		GROWTH ON PA	GROWTH ON NM
Staphylococcus albus	*N.gruberi*	SA-1	D	A
		SA-3		
		NB-1		
	N.fowleri	G.J.		B
		Oram		
		PA-90		
Bacillus cereus	*N.gruberi*	SA-1	A	D
		SA-3		
		NB-1		
	N.fowleri	G.J.	C	
		Oram		
		PA-90		
Sarcina lutea	*N.gruberi*	SA-1	C	C
		SA-3		
		NB-1		
	N.fowleri	G.J.		
		Oram		
		PA-90		
Bordetella bronchiseptica	*N.gruberi*	SA-1	A	A
		SA-3		
		NB-1		
	N.fowleri	G.J.	B	C
		Oram		
		PA-90		
Escherichia coli & *Klebsiella aerogenes*	*N.gruberi*	SA-1	A	A
		SA-3		
		NB-1		
	N.fowleri	G.J.	B	B
		Oram		
		PA-90		

A = Bacteria that were totally eaten within five days
B = Bacteria that were totally eaten in five days or more
C = Bacteria that were partially eaten over a small area, after which the amoebae died or encysted
D = Inedible bacteria, no growth

All the strains of Naegleria tested were incapable of using Sarcina lutea grown on PA or NM agar as a food source. Typically, the growth front advanced 1,5 to 2,5 cm along the bacterial streak in slow, erratic steps, ending with death or encystment of the amoebae. Another common pigmented bacterium, Xanthomonas campestris was tested and found to be toxic to strains of Naegleria gruberi and Naegleria fowleri.

All the remaining data presented in Table 7.1 on the preceding page indicate that Naegleria fowleri and Naegleria gruberi amoebae feed differently. For each of the remaining bacteria/agar combinations, the three Naegleria fowleri strains exhibited a different feeding response from the three Naegleria gruberi strains. This difference is illustrated by Bacillus cereus on PA agar and Bordetella bronchiseptica on NM agar where the Naegleria gruberi strains migrated rapidly across the bacterial streaks, but the Naegleria fowleri strains consumed only part of the streak. A density-dependent factor may have operated in the case of Bordetella bronchiseptica with Naegleria fowleri on PA and NM media. Typically, the amoebae ate 1,0 to 3,0 cm along the bacterial streak producing a central wedge of bacteria which then migrated back slowly into the cleared area. A possible reason for this unusual growth front is that feeding was facilitated along the margins of the streak where bacterial density was low and this, in turn, facilitated feeding in the resulting central wedge. The wedges were never entirely consumed. The three Naegleria gruberi strains consumed the entire bacterial streak.

The results for Naegleria fowleri on PA agar with Bacillus cereus are difficult to interpret as the growth front was always poorly developed. The bacteria colonised the area surrounding the streak and accurate measurements were difficult to obtain. It was often doubtful whether the amoebae had eaten along the streak which had been recolonised by the bacteria, or whether the trophozoites were unable to penetrate the streak. The rest of the data was concerned with the total consumption of the bacteria at rates which were relatively different in Naegleria fowleri and Naegleria gruberi associated with the same bacterium and medium.

A second series of experiments attempted to obtain quantitative as well as qualitative data on the growth rates of Naegleria fowleri and Naegleria gruberi on selected bacteria. A preliminary examination of the time/distance results obtained in the first series of experiments revealed that after the lag period was taken into account, the rates of migration of the amoebae along the bacterial streaks were uniform for

those bacteria that could be entirely consumed. These results were consistent, and could be plotted as a linear graph. Theoretically, the different slopes of these graphs could represent different rates of migration of the various amoebae, and could be of taxonomic use. These results are summarized in Table 7.2 where the advancement of the growth fronts of the three strains of Naegleria fowleri and three strains of Naegleria gruberi on three species of bacteria at 30°C are given for PA and NM media. Only one Naegleria strain and one bacterial strain were

TABLE 7.2

GROWTH KINETICS OF 6 STRAINS OF NAEGLERIA AMOEBAE ON 3 SPECIES OF BACTERIA ON PA AND NM AGAR AT 30°C

BACTERIA	NAEGLERIA STRAIN	GROWTH RATE[a] mm/hr		LAG PERIOD[b] hr	
		PA	NM	PA	NM
Staphylococcus albus	SA-1	C	3,4±0,4	C	14±2
	SA-3		3,5±0,6		18±3
	NB-1		3,4±0,2		15±3
	G.J.		0,6±0,1		91±7
	Oram		0,8±0,2		16±2
	PA-90		0,9±0,2		93±7
Klebsiella aerogenes	SA-1	10,6±0,5	11,9±0,8	12±2	17±2
	SA-3	9,2±0,4	9,2±0,7	13±3	16±2
	NB-1	9,4±0,7	10,6±0,4	13±2	17±1
	G.J.	1,7±0,4	0,6±0,3	32±4	95±9
	Oram	2,0±0,1	0,6±0,4	24±3	81±8
	PA-90	1,9±0,5	0,9±0,2	20±3	81±8
Escherichia coli	SA-1	8,0±0,5	9,1±0,5	7±2	10±2
	SA-3	7,4±0,6	8,4±0,7	6±2	15±2
	NB-1	7,9±0,9	9,2±0,3	11±3	11±1
	G.J.	1,3±0,3	0,9±0,3	32±5	85±4
	Oram	1,6±0,5	1,0±0,3	27±5	85±3
	PA-90	2,0±0,2	1,0±0,3	36±6	80±5

a = Advancement of growth front in mm/hr.
b = Lag period = The time in hours from inoculation of the amoebae to the first visual evidence of the formation of a growth front.
C = Inedible bacteria, no growth

used in each petri dish, with three streaks of log-phase bacteria 0,5 cm wide positioned equidistant from each other on either PA or NM agar. For all combinations of Naegleria strain, bacteria, and medium type, the results were consistent in that the Naegleria fowleri strains grew much more

slowly than did the Naegleria gruberi strains at 30°C. An example is that of Klebsiella aerogenes on NM medium, where the Naegleria gruberi strains grew more than ten times as quickly as Naegleria fowleri. With Klebsiella aerogenes and Escherichia coli as bacterial associates, Naegleria fowleri grew better on PA agar, while Naegleria gruberi grew slightly better on NM agar. Lag period, or the time in hours from inoculation of amoebae to the first visual evidence of a growth front, was measured. Under experimental conditions, the lag period for Naegleria fowleri was up to 8,5 times as long as that for Naegleria gruberi.

A third series of experiments was conducted with these six strains of Naegleria grown with Klebsiella aerogenes on PA and NM agar at an elevated temperature of 43°C. The results are recorded in Tables 7.3 and 7.4. None of the three strains of Naegleria gruberi were capable of growing at 43°C.

TABLE 7.3

GROWTH OF NAEGLERIA FOWLERI AND NAEGLERIA GRUBERI ON KLEBSIELLA AEROGENES ON PA AND NM AGAR AT 43°C

NAEGLERIA	Strain	PA MEDIUM growth rate mm/hr	PA MEDIUM lag period hr	NM MEDIUM growth rate mm/hr	NM MEDIUM lag period hr
N.gruberi	SA-1 SA-3 NB-1	no growth		no growth	
N.fowleri	G.J.	2,85±0,3	13±1	2,0±0,5	25±3
	Oram	2,65±0,2	14±1	2,3±0,1	18±2
	PA-90	2,55±0,3	15±2	2,2±0,2	21±2

TABLE 7.4

COMPARISON OF GROWTH AT 43°C WITH GROWTH AT 30°C OF NAEGLERIA FOWLERI ON KLEBSIELLA AEROGENES

NAEGLERIA FOWLERI Strain	PA MEDIUM AT 43°C growth rate % increase	PA MEDIUM AT 43°C lag period % decrease	NM MEDIUM AT 43°C growth rate % increase	NM MEDIUM AT 43°C lag period % decrease
G.J.	+68	-59	+334	-62
Oram	+27	-37	+365	-75
PA-90	+40	-30	+225	-73

The Naegleria fowleri strains were not only viable at 43°C, but produced feeding rates on NM agar which were even faster than the calculated rates on PA agar at 30°C. The percentage difference in feeding rates at 43°C when compared with feeding at 30°C, as seen in Table 7.4 on the preceding page, reveal that there was a substantial increase in growth of up to 334% and a decrease of up to 73% in the lag time. This seems to indicate that the generation time for Naegleria fowleri is much shorter at 43°C than at 30°C.

E.2 Toxicity of Pseudomonas aeruginosa to Naegleria amoebae, cysts and flagellates

The effect of cell suspensions of seven strains of Pseudomonas aeruginosa on the growth of three strains of Naegleria gruberi is illustrated in Table 7.5. The control bacteria, Klebsiella aerogenes allowed rapid and complete excystment, growth and normal encystment within 72 hours along the bacterial streak. Pseudomonas strains 5,9,24 and 41 completely prevented excystment of the viable Naegleria gruberi strains tested. Pseudomonas strains 20,21 and 31 allowed very poor growth of amoebae that were larger than normal and produced angular, abnormally shaped cysts.

TABLE 7.5

THE EFFECTS OF 7 STRAINS OF PSEUDOMONAS AERUGINOSA ON THE EXCYSTMENT OF 3 STRAINS OF NAEGLERIA GRUBERI ON NM AGAR AT 25°C

BACTERIA		NAEGLERIA GRUBERI STRAIN		
		NEG	NB-1	SA-1
Klebsiella aerogenes		A	A	A
Pseudomonas aeruginosa	5	B	B	B
	9	B	B	B
	20	C	C	C
	21	C	C	C,D
	24	B	B	B
	31	C,D	C,D	B
	41	B	B	B

A= Complete growth along bacterial streak, encystment within 72 hours
B= No excystment
C= Very slow growth, very large amoebae
D= Irregularly shaped cysts

That Pseudomonas aeruginosa can affect Naegleria growth has been confirmed in a second series of experiments, as is seen in Table 7.6 on the following page. A portion of the log-phase cultures of each Pseudomonas strain tested was filtered through a 0,2 µm millipore filter. Cell

suspensions, cell-free filtrates and cell residues of each Pseudomonas strain were tested with three Naegleria gruberi strains. A definite pattern emerges - the same inhibition seen in Table 7.5 on the preceding page was repeated by both the cell suspensions and the cell residues. The cell-free filtrates with Klebsiella aerogenes as a food source permitted complete and rapid growth along the streaks, but produced very large amoebae and cysts up to 50 µm in diameter. When these multinucleated cysts, with up to 16 nuclei per cyst, were washed in distilled water and placed on fresh Klebsiella streaks, the resulting Naegleria amoebae and cysts were normal in all respects. This is a clear indication that the increase in size and number of nuclei in amoebae and cysts is not hereditary. It is apparent that the cell residue after millipore filtering contained the toxic agent(s) preventing excystment and growth of Naegleria amoebae.

TABLE 7.6

THE EFFECTS OF VARIOUS FRACTIONS OF PSEUDOMONAS AERUGINOSA ON THE GROWTH AND ENCYSTMENT OF 3 STRAINS OF NAEGLERIA GRUBERI ON NM AGAR AT 25°C

BACTERIA		NAEGLERIA GRUBERI STRAINS NEG, NB-1, SA-1		
		1	2	3
Klebsiella aerogenes		A		
Pseudomonas aeruginosa	5	B	B	A,D
	9	B	B	A,D
	20	C	C	A,D
	21	C	C	A,D
	24	B	B	A,D
	31	C	C	A,D
	41	B	B	A,D

A = Complete growth along bacterial streak, excystment within 72 hours
B = No growth
C = Very slow growth by amoebae, 10-14 days to migrate along entire streak
D = Multinucleated amoebae and cysts

1 = Suspension of bacterial cells
2 = Cell residue (after millipore filtering) and Klebsiella aerogenes
3 = Cell-free filtrate and Klebsiella aerogenes

Cell-free filtrates of all seven Pseudomonas strains had no detectable affect on Naegleria excystment, and on the growth and encystment of Acanthamoebae culbertsoni and Tetramitus rostratus. An unknown factor in the cell-free filtrates produced multinucleated cells and blocked cytokinesis

but not karyokinesis in approximately 17% of the Naegleria amoebae. The specific nature of this factor, which also induced production of Naegleria cysts larger than usual, is not known, except that cell-free filtrates tested five weeks after being stored at 22-25°C were still capable of causing the same effect.

Pyocyanin, even in a five-fold concentration, prepared by the method of Liu et al.(260), had no effect on the growth of Naegleria sp., Acanthamoeba culbertsoni and Tetramitus rostratus amoebae. The fraction that contained all other dialysable substances other than pyocyanin (fraction Ib by Liu's procedure (260)) induced binucleated cysts in about 20% of the Naegleria population, and had no effect on the growth and encystment of Acanthamoeba culbertsoni and Tetramitus rostratus.

Figure 7.1 is a representative graph of the effect of Pseudomonas aeruginosa strain 41 on the amoeba-to-flagellate transformation in the NEG strain of Naegleria gruberi. Log-phase Pseudomonas strain 41 made up

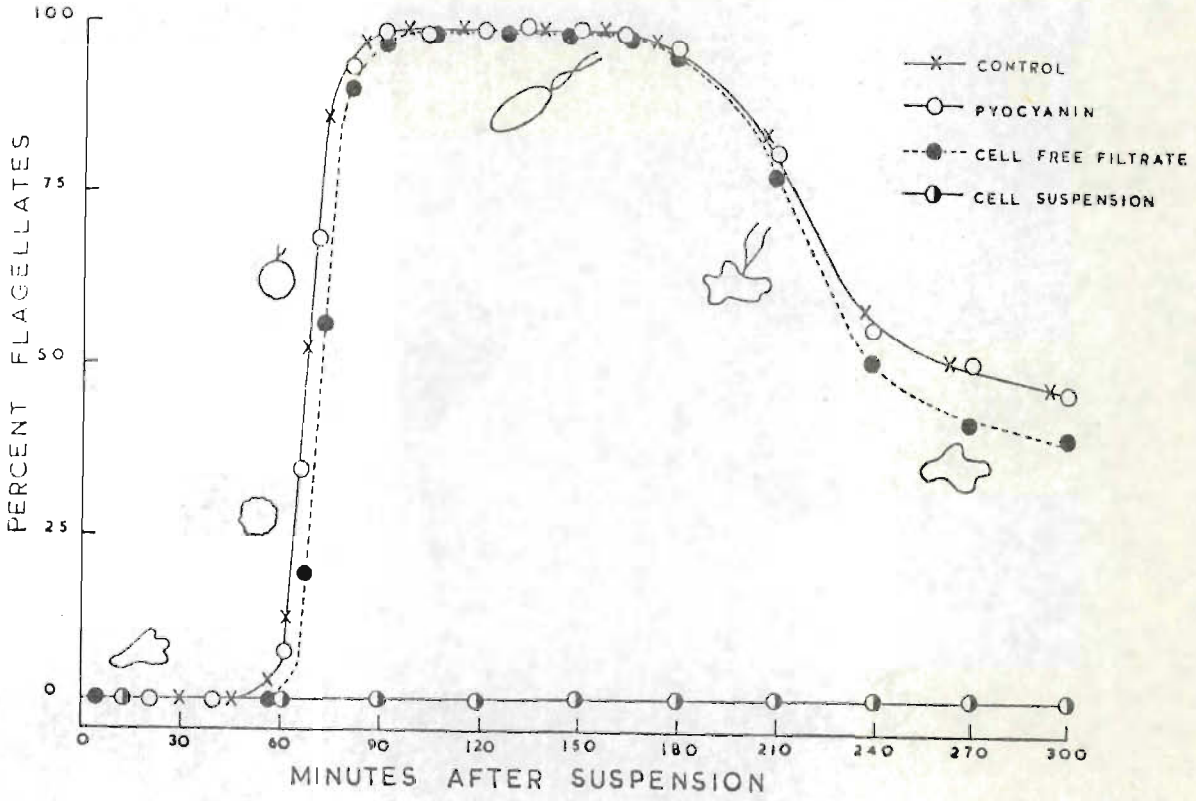


Figure 7.1 Effect of various fractions of Pseudomonas aeruginosa strain 41 on the amoeba-to-flagellate transformation of the NEG strain of Naegleria gruberi at 25°C. The abscissa refers to time after the amoebae have been first exposed to Tris-HCl buffer, pH 7,2. The ordinate refers to the percentage of the population that are flagellates. The small figures illustrate the typical shape of the cells as they transform and revert.

8% of the final volume of the test solution. The control flask with no Pseudomonas added gave the expected transformation curve and a T_{50} value (when 50% of the cells were flagellated) of 72 minutes at 25°C. The log-phase culture of Pseudomonas aeruginosa strain 41 completely inhibited the amoeba-to-flagellate transformation in Naegleria. When the Pseudomonas cell suspension was added to a control flask at 120 minutes after the start of transformation, the flagellated cells immediately reverted back to amoebae. Concentrations of Pseudomonas from 8% to 20% gradually increased the number of flagellates that would lyse instead of revert back to amoebae. Concentrations of Pseudomonas greater than 20% caused immediate lysis of all the flagellated cells and amoebae present. Pyocyanin, even when applied in concentrations five times that of the cell suspensions, had no effect on the transformation process. The cell-free filtrate had the effect of delaying the onset of transformation by 8-10 minutes, and of slightly increasing the rapidity of flagellate-to-amoeba reversion. Identical results were obtained with Pseudomonas aeruginosa strains 5,20,21 and 24 on the NEG, NB-1 and SA-1 strains of Naegleria gruberi.

F. DISCUSSION

The results presented in this chapter are in good agreement with the findings of others (9,71,74,189,389) and present new information on limax amoebae/bacteria interactions.

F.1 Choice of bacterial food by Naegleria

Distinct feeding preferences were exhibited by Naegleria fowleri and Naegleria gruberi when presented with a choice of bacteria. Klebsiella aerogenes and Escherichia coli supported the growth of Naegleria fowleri and Naegleria gruberi on PA and NM agar. Bordetella bronchiseptica was an excellent food bacteria and on PA agar gave good growth of both Naegleria fowleri and Naegleria gruberi. Chang (74) found that when grown on Bordetella bronchiseptica, the non-pathogenic Naegleria formed plaques devoid of any residue, while the pathogenic Naegleria plaques always had a characteristic central residue. Similar results are reported in this chapter. The lytic activity of Naegleria may have been due to N-acetylmuramidase, as has been reported for other amoebae (23,234). Upadhyay (418) found that an extract of Hartmannella glebae similar to egg-white lysozyme, could lyse many gram-positive bacteria.

Naegleria gruberi strains always had a faster growth rate (up to ten-fold) than any of the Naegleria fowleri strains tested under the same

conditions. Naegleria gruberi amoebae also had a shorter lag period (up to 8,5 times as rapid) than those of Naegleria fowleri. There are a number of possible reasons for these findings. Inoculation of all the bacterial streaks was done from stock plates where the viable cysts greatly outnumbered the trophozoites. Naegleria gruberi cyst plates always had a far higher density of cysts per plate than those of Naegleria fowleri. This probably resulted in a disproportionate number of cysts per inoculation. Carter (46) reported that Naegleria fowleri strains had a large proportion of non-viable cysts. Lastly, Naegleria gruberi has a generation time as short as 1,7 hours (169) which is much shorter than that of Naegleria fowleri. Increase in lag period when NM agar is used indicates that there is a direct relationship between density of bacteria and size of the population of amoebae feeding upon it. If the bacterial population increases, the amoebae must also increase to a higher density before a growth front appears. Naegleria strains with the fastest feeding rates had the shortest lag period. My suggestion is that those strains which migrated the fastest along the bacterial streaks reached a population density capable of producing a growth front more rapidly than other strains. This is indicative of a faster generation time under test conditions.

F.2 Temperature and growth

The results presented in this chapter indicate that Naegleria gruberi was unable to survive at 43°C while Naegleria fowleri thrived at this temperature. In 1972, Griffin (189) reported that in his study of the temperature tolerance of nine strains of Naegleria fowleri and of three strains of Naegleria gruberi, the latter could not grow at temperatures higher than 37°C, while the Naegleria fowleri strains could grow at temperatures of 44°C or higher. Similar results have been reported by Chang (77). The initial work on growth of Naegleria strains at 43°C, presented here, was done in 1971 before the publication of the excellent report by Griffin (189).

F.3 Toxicity of Pseudomonas aeruginosa to Naegleria amoebae, cysts and flagellates

It was found that the cell suspensions and the cell residues after millipore filtration of all seven Pseudomonas aeruginosa strains tested affected the growth, excystment, encystment, cell division and the amoeba-to-flagellate transformation in Naegleria.

In their study of the toxicity of various fractions of Pseudomonas, Liu et al.(260) found that the loosely bound slime layer adhering to the bacterial cell showed considerable toxicity for mice. They report that

"the so-called toxicity of Pseudomonas aeruginosa was primarily due to the slime on the surface". The slime was a good antigen and the anti-serum produced with slime agglutinated the bacterial cells and protected mice from infection. This protection appeared to be type-specific and was effective only against strains of Pseudomonas which possessed the same serological type of slime. Liu et al.(260) also found that cell suspensions washed free of slime at a concentration of about 2 billion cells per ml lacked toxicity toward mice. Elrod & Braun (149) found that the cells of Pseudomonas were not toxic. Chang (73) reported that thoroughly washed cells of Pseudomonas were edible by Hartmannella agricola, Hartmannella glebae and Naegleria gruberi while the unwashed cells were not. Although the toxicity of the slime fraction of Pseudomonas was not tested in my experiments, this fraction would appear to be a probable cause of the toxic effects observed on Naegleria.

Pyocyanin extracted from these strains of Pseudomonas had no observable effect on excystment, cell division and amoeba-to-flagellate transformation in Naegleria, although some of the encysting amoebae produced larger than normal cysts. Liu et al.(260) stated that "one of the most remarkable findings of this study was that the cells and the pyocyanin of Pseudomonas aeruginosa, which perhaps have been the two best known fractions of this species, were the least important fractions in the pathogenesis of this organism". Cruickshank & Lowbury (86) reported that pyocyanin was toxic to skin cells and leucocytes cultured in vitro, but it was not toxic when injected into test animals. Groscop & Brent (189) found that non-pigmented Pseudomonas aeruginosa was toxic to five different species of small free-living amoebae, so substances other than pyocyanin must have been responsible for toxicity. Singh (385) stated that the toxicity of the crude pyocyanin extract is due to substances other than pyocyanin. Undoubtedly the observed toxicity of Pseudomonas to Naegleria and other limax amoebae is due to a number of factors. Slime is most probably responsible for the death of the organisms. Fractions such as haemolysin, extracellular enzymes and dialysable substances other than pyocyanin may be responsible for the observed phenomena of multinucleated amoebae and cysts, and the retarded growth rate of the amoebae. All prior investigations to my knowledge have dealt only with the effects of Pseudomonas on the growth of limax amoebae.

G. CONCLUSION

The clear-cut differences in the growth of Naegleria fowleri and Naegleria gruberi on various food bacteria on PA or NM agar substrates are

a basic difference in the biology of these amoebae. The fact that Naegleria fowleri will grow at 43°C, while Naegleria gruberi will not, is yet another pronounced difference between the two species of Naegleria. Both of these considerations are significant for taxonomy and will be of use in the further differentiation of pathogenic from non-pathogenic Naegleria.

The work presented in this chapter confirms and extends earlier observations and supplies new information on the toxicity of Pseudomonas to excystment, encystment and the amoeba-to-flagellate transformation in Naegleria. It is apparent that the question of antibiosis of Pseudomonas aeruginosa is more complex than formerly thought. Further work should be performed to obtain information about the identity and mode of action of all the antibiotic substances. For example, a detailed investigation of the production of multinucleated amoebae and cysts would yield valuable information on the basic biological processes of encystment and karyokinesis.

CHAPTER 8

STUDIES ON THE MOTILITY AND TRANSFORMATION OF *NAEGLERIA FOWLERI* AND *NAEGLERIA GRUBERI*

A. ABSTRACT

Differences between *Naegleria fowleri* and *Naegleria gruberi* were noted in the amoeba-to-flagellate transformation at both 25°C and 43°C. Such inhibitors of motility and of metabolic processes as caffeine, cytochalasin B, EDTA, EGTA, and PCMB were shown to affect amoeboid motility and transformation in *Naegleria*. Colchicine had no effect on either *Naegleria* amoebae or flagellates.

For the first time, La^{3+} ion, a competitive inhibitor of Ca^{2+} ion was shown to inhibit amoeboid motion, transformation and flagellar beating in *Naegleria*. Phase-contrast and electron microscope observations suggest that the site of La^{3+} ion action is on the plasmalemma.

B. INTRODUCTION

Study of amoeboflagellates has aroused interest in the causative agents that favour transformation from amoeba-to-flagellate. This phenomenon has been examined in *Adelphamoeba galeacystis*, *Physarum polycephalum* and *Tetramitus rostratus* by various workers (17,34,35,170,303,311,437). How the amoeboflagellates evolved is unknown, but Margulis (266) suggests that they arose from a symbiotic ingestion of spirochetes by primitive amoebae in Precambrian times.

The protozoan *Naegleria gruberi* exists in three morphologically distinct states in its life cycle - as an amoeba, as a flagellate and as a cyst. Environmental factors may cause an amoeba to cease its vigorous cytoplasmic streaming and to transform into a rigid fusiform cell with functional flagella (72,333,349,368,460). In turn, the flagellate is capable of absorbing the flagellar axonemes into its cytoplasm and reverting to an actively motile amoeba. Reversion in *Naegleria* flagellates may be induced by mechanical (349,450) and thermal (72,126,169) factors and by deuterium oxide (341).

Naegleria remains the organism of choice for studies of transformation, as populations will transform synchronously and it is hardy in laboratory manipulation.

Numerous investigators have used physical (299,302,414,415) and chemical (300,304,310,340,435,472) means to gain knowledge of transformation in *Naegleria*. In the last decade Fulton *et al.* (127,168,171,172,174-6,178, 403,438-440) have made real progress in the understanding of amoeba-to-

flagellate transformation at the molecular level. The flagellar actin-like material, tubulin, is synthetised de novo during transformation (235,242-4). Precursors of the flagellar apparatus (rhizoplasts, basal bodies and associated structures) have not been detected in extensive electron microscope studies of Naegleria amoebae (Chapter 6 of this thesis, 175,367). This de novo synthesis of flagella provides an ideal model of cytodifferentiation, and has been used in this chapter to examine the effects of various inhibitors on cell motility and transformation. The compounds tested were: caffeine which causes rigor by release of intracellular Ca^{2+} ion in non-muscle systems, colchicine which prevents the formation of microtubules, cytochalasin B, an inhibitor of microfilament formation, EDTA and EGTA which bind Ca^{2+} ion and Mg^{2+} ion, and finally, PCMB a sulphydryl inhibitor.

Lanthanum ion is electron-dense and easily seen under the electron microscope. Many workers have incorporated La^{3+} ions in their fixation protocols in order to observe ultrastructural detail. Concomitantly, La^{3+} ion is a competitive inhibitor of Ca^{2+} ion in smooth muscle. A light and electron microscope study was conducted to study the effect of La^{3+} ion on amoeboid motion, on amoeba-to-flagellate transformation and on flagellar beating in Naegleria. There are only two reports in the literature of La^{3+} ion inhibition of amoeboid motion (195,227) and none on its effects on amoeba-to-flagellate transformation and flagellar beating.

For the first time, quantitative information has been obtained on the amoeba-to-flagellate transformation of Naegleria fowleri and Naegleria gruberi at 25°C and 43°C. These data are of taxonomic value as the two species of Naegleria exhibit quite distinct differences in transformation.

A portion of the results presented in this chapter has been published (251).

C. MATERIALS

Organisms	Sources are recorded on p.146-147.
Media	Described on p.148-149.
Chemicals	Described on p.149-150.
Apparatus	Siemens 101 transmission electron microscope and ancillary equipment.

D. METHODS

D.1 Preparation of amoebae and flagellates

Growth and transformation of the amoebae were accomplished as outlined on p.39.

D.2 Inhibitors

These chemicals were made up in glass-distilled water and were tested in the following concentrations: caffeine (10^{-1} - 10^{-3}M); colchicine (10^{-2} - 10^{-3}M); cytochalasin B (10 - 100 µg/ml); EDTA and EGTA (10^{-1} - 10^{-2}M); PCMB (10^{-2} - 10^{-4}M); and lanthanum nitrate (10^{-1} - 10^{-8}M).

D.3 Inhibition of amoeboid motility

Solutions of inhibitors were placed in separate depressions in a ceramic spotting tile. Viable amoebae were transferred from a growth plate to the solutions of inhibitor on the tile. Amoebae were frequently examined under phase-contrast for up to two hours until cytoplasmic streaming, formation of pseudopodia and other signs of motility ceased.

D.4 Inhibition of transformation

For quantitative transformation kinetics, amoebae were suspended in inhibitor solutions or in Tris-HCl buffer, pH 7,2, as a control, as outlined on p.39. Samples were taken at specific time intervals in the ratio of four drops of cell suspension to one drop of Lugol's iodine. During transformation, cultures were monitored under phase-contrast. A minimum of 100 cells per sample was scored for the presence of flagella, independent of cell shape. Each transformation experiment was done at least three times. Qualitative examination of the effects of inhibitors on fully formed flagellates was also done.

D.5 Electron microscope preparations

Preparations were made as outlined on p.52 of this thesis but modifications were necessary for La^{3+} ion-treated amoebae. Lanthanum nitrate in cacodylate buffer was added to the washed and resuspended amoebae 10 minutes before the cacodylate buffered glutaraldehyde was added. Lanthanum nitrate was also added to the O_sO_4 post-fixing step. Except for the omission of La^{3+} ion, control amoebae were treated in exactly the same way.

E. RESULTS

E.1 Temperature and transformation

Differences in the transforming ability of Naegleria fowleri and Naegleria gruberi amoebae at 25°C and at 43°C are shown in Table 8.1. At 25°C more Naegleria gruberi amoebae transformed to flagellates and in a shorter time than did Naegleria fowleri amoebae. At 43°C Naegleria fowleri amoebae transformed more rapidly and to a greater extent than

they did at 25°C, while Naegleria gruberi amoebae did not transform at all. Differences in transforming ability at 43°C have been useful in differentiating Naegleria fowleri from Naegleria gruberi (Chapter 2).

TABLE 8.1

TRANSFORMATION OF NAEGLERIA GRUBERI AND NAEGLERIA FOWLERI AT 25°C AND AT 43°C

	PERCENTAGE OF CELLS WITH FLAGELLA			
	25°C		43°C	
Minutes after suspension	N. fowleri Oram strain	N. gruberi SA-1 strain	N. fowleri Oram strain	N. gruberi SA-1 strain
60	0	0	0	0
70	0	40	21	0
80	14	71	50	0
90	36	89	78	0
120	59	94	82	0
150	50	94	75	0
180	28	66	42	0
210	17	45	34	0
240	4	36	17	0
T_{50}min	96	71	80	0

T_{50}min = Time in minutes when 50% of the cell population were flagellates

E.2 Inhibition of amoeboid motility

The effects of inhibitors on the motility of Naegleria gruberi are shown in Table 8.2 on the following page. Caffeine, cytochalasin B, EDTA and La^{3+} ion all affected the morphology and motility of Naegleria amoebae in some degree, and the most obvious effects were cessation of motility, rounding up of the cell and membrane blebbing. Higher concentrations of caffeine and La^{3+} ion caused cell lysis. In the concentrations tested, colchicine and PCMB did not affect the amoebae.

TABLE 8.2

EFFECT OF SPECIFIC INHIBITORS ON *NAEGLERIA GRUBERI*, STRAIN EG AMOEBAE AT 25°C

INHIBITOR	CONCENTRATION (M)	EFFECT ON MORPHOLOGY	EFFECT ON MOTILITY
Caffeine	10^{-1}	Lysis.	-
	10^{-2}	Cytoplasm separated from membrane. Large vacuoles formed.	Rounded up
	10^{-3}	Normal morphology.	Mobile
Colchicine	10^{-2}	None.	None
	10^{-3}	None.	None
EDTA	10^{-2}	Cell rounds up. Blebs on membrane.	Immobile
	10^{-3}	Cell rounds up.	Immobile
EGTA	10^{-2}	Cell rounds up. Blebs on membrane.	Immobile
	10^{-3}	Cell rounds up.	Immobile
La^{3+}	10^{-3}	Large vacuoles formed. Cell lysis.	Rounded up
	10^{-4}	Large vacuoles formed. Membrane and cytoplasm disrupted.	Immobile
	10^{-5}	Blebs on membrane.	Movement very slow and irregular
	10^{-6}	Cytoplasm disrupted. Endoplasm and ectoplasm not differentiated.	
	10^{-7}	Endoplasm and ectoplasm not differentiated.	Mobile
PCMB	10^{-2}	None.	None
	10^{-3}	None.	None
Cytochalasin B	10-100 µg/ml	Cell rounds up.	Immobile

E.3 Inhibition of transformation

The effects of various inhibitors on the amoeba-to-flagellate transformation of *Naegleria gruberi* are summarized in Table 8.3 on the following page. Transformation was reduced to less than 10% when amoebae were suspended in EDTA, EGTA or PCMB solutions, with essentially no transformation at the higher concentrations of the inhibitors tested. Transformation

was totally inhibited by 10^{-1}M caffeine. In the concentrations tested, colchicine and cytochalasin B had no appreciable effect on transformation.

TABLE 8.3

EFFECTS OF SPECIFIC INHIBITORS ON TRANSFORMATION IN *NAEGLERIA GRUBERI*, STRAIN EG AT 25°C

INHIBITOR	CONCENTRATION (M)	METABOLIC EFFECT OF INHIBITOR	T_{50} (min)	MAX. % F
Caffeine	10^{-1}	Caused release of intracellular calcium store, and rigor.	-	0
	10^{-2}		-	5
	10^{-3}		-	30
Colchicine	10^{-2}	Inhibited microtubule formation.	72	96
	10^{-3}		78	92
EDTA	10^{-1}	Bound Ca^{2+} and Mg^{2+}.	-	5
	10^{-2}		-	32
EGTA	10^{-1}	Bound Ca^{2+}.	-	8
	10^{-2}		-	35
PCMB	10^{-2}	Sulphydryl inhibitor.	-	16
	10^{-3}		-	20
	10^{-4}		-	25
Cytochalasin B	100 µg/ml	Disrupted contractile microfilaments.	80	89
Control			72	96

T_{50}min = Time in minutes when 50% of the cell population were flagellates.
Max. % F = Maximum percentage of flagellates counted.

Lanthanum ion, even in concentrations as low as 10^{-8}M, inhibited the transformation of *Naegleria gruberi* amoebae. At a concentration of 10^{-3}M La^{3+} ion, complete inhibition of transformation was observed (Table 8.4 on the following page).

The effects of La^{3+} ion on amoebae which had already transformed was qualitatively examined. Flagellates were suspended in a drop of 10^{-4}M La^{3+} ion and within 3 minutes the flagella ceased beating, the cells rounded up and became immobile and blebs appeared on the plasmalemma.

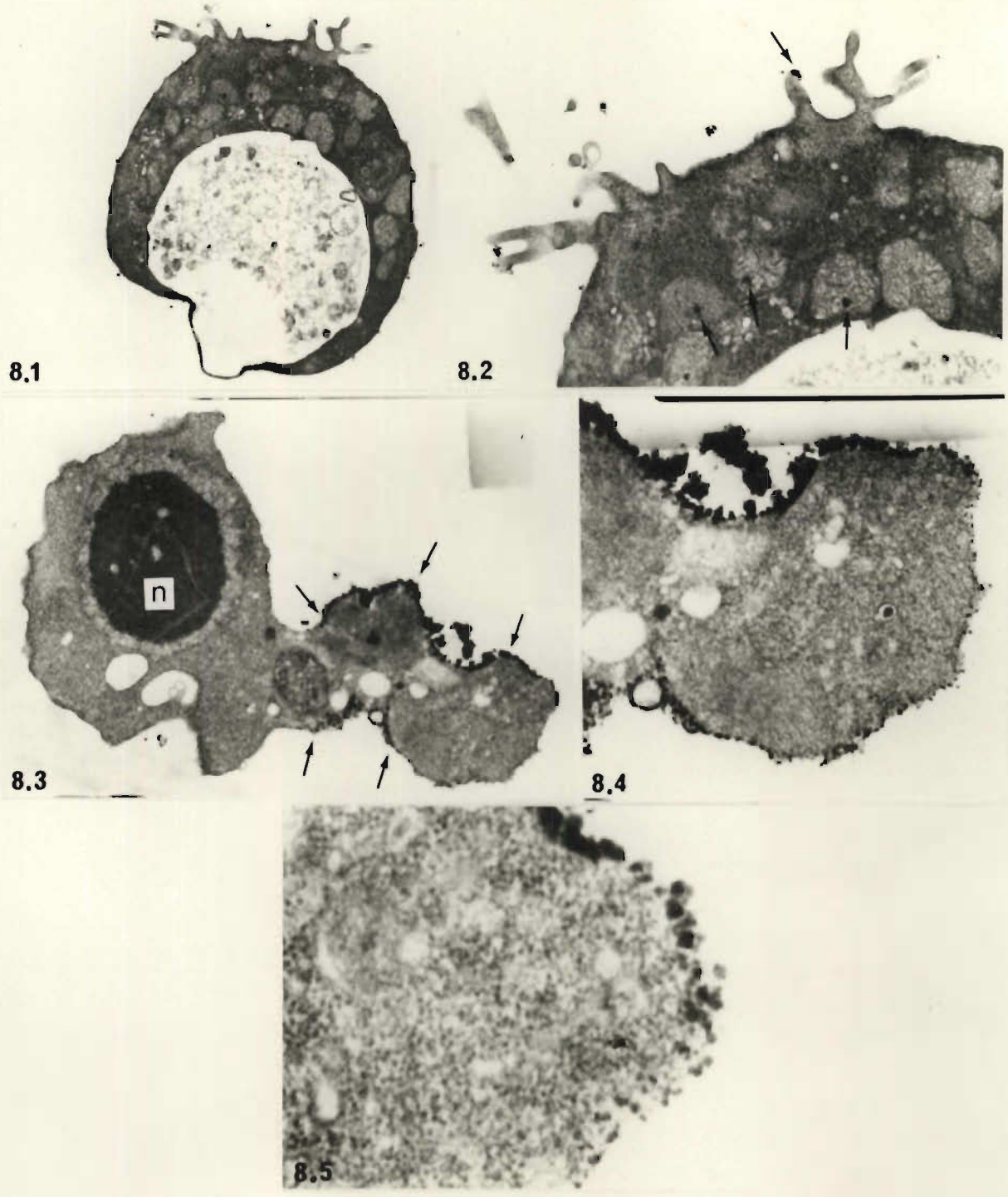


Fig. 8.1 Naegleria gruberi, strain EG amoeba treated with 10^{-3} M La^{+3}. Note the large vacuole and atypical pseudopodia. X4 000.

Fig. 8.2 Detail of Fig. 8.1. Arrows indicate electron dense inclusions on the plasmalemma and in the mitochondria. X15 000.

Fig. 8.3 A motile N. gruberi, strain EG amoeba treated with 10^{-4} M La^{+3}. Arrows indicate a preferential electron dense deposit of La^{+3} on the advancing pseudopod. The remainder of the plasmalemma is free of any such deposits. n = nucleolus. X13 000.

Fig. 8.4 Detail of Fig.8.3. La^{+3} deposits on plasmalemma. X45 000.

Fig. 8.5 Detail of Fig. 8.4. X80 000.

TABLE 8.4

EFFECT OF La^{3+} ion ON TRANSFORMATION IN NAEGLERIA GRUBERI, STRAIN EG AT 25°C

La^{3+} ion CONCENTRATION (M)	PERCENTAGE OF FLAGELLATES OBSERVED AFTER 60 min	70 min	80 min	90 min	120 min	150 min	180 min	210 min	240 min	Max. % F
10^{-3}	0	0	0	0	0	0	0	0	0	0
10^{-4}	0	0	1	2	3	2	3	0	0	3
10^{-5}	0	0	4	3	4	12	9	4	0	12
10^{-6}	0	1	9	6	10	21	16	4	1	21
10^{-7}	0	6	7	8	14	22	14	3	1	22
10^{-8}	0	7	9	11	21	38	21	8	1	38
Control	0	34	82	88	94	93	70	49	21	94

Max. % F = Maximum percentage of flagellates observed.

Figures 8.1–8.5 on p.101 illustrate the effects of La^{3+} ion when applied to Naegleria gruberi amoebae as observed with the electron microscope. When amoebae were exposed to 10^{-3}M La^{3+} ion, large vacuoles, atypical pseudopodia, and electron-dense granules on the plasmalemma and in the mitochondria were observed (Figs 8.1,8.2). When 10^{-4}M La^{3+} ion was applied to the amoebae, the La^{3+} ion deposits appeared to be preferentially absorbed onto the advancing pseudopod (Figs 8.3–8.5). These deposits were not uniformly distributed but appeared as distinct granules on the pseudopod, while the rest of the plasmalemma was free of such granules. None of the above observations were seen in the control amoebae.

F. DISCUSSION

In general, the results presented in this chapter have confirmed and extended published reports of other workers (169,435,472). However, new findings are apparent such as the quantitative differences in the transformation of Naegleria fowleri and Naegleria gruberi at both 25°C and 43°C. Inhibitors such as caffeine, cytochalasin B, EDTA, EGTA, and La^{3+} ion affected amoeboid motion and transformation in Naegleria.

F.1 Temperature and transformation

Naegleria fowleri amoebae transformed at 43°C while Naegleria gruberi amoebae did not. The quantitative results presented in Table 8.1 confirm and extend prior qualitative observations by Griffin (187).

Differences in the transforming ability of Naegleria fowleri and Naegleria gruberi at 43°C are an important taxonomic consideration and have been useful in detecting potentially pathogenic Naegleria (Chapter 2 of this thesis, 187).

F.2 Inhibition of amoeboid motility

Inhibition of movement was observed in Naegleria amoebae exposed to 10^{-2} - 10^{-3}M caffeine. A higher concentration of caffeine, 10^{-1}M, caused cell lysis. Amoebae treated with 10^{-2} - 10^{-3}M solutions of the Ca^{2+}ion and Mg^{2+}ion chelators EDTA and EGTA, rounded up and blebs formed on the plasmalemma. This indicates the role of Ca^{2+}ion in Naegleria membrane stability. Similar blebbing effects were observed with La^{3+}ion-treated amoebae where presumably all the superficial Ca^{2+}ion sites were blocked. Neither the microtubule inhibitor colchicine nor the sulphydryl inhibitor PCMB affected amoeboid motility, a finding similar to the report by Yuyama (472).

Cytochalasin B, a recognised disrupting agent of contractile microfilaments, completely inhibited amoeboid motion at a concentration of 100 µg/ml. This inhibition was temporary, as removal of the drug caused cytoplasmic streaming in the amoebae to recommence precipitously. Inhibition of amoeboid motility by cytochalasin B has been reported for Entamoeba histolytica (288) and other cells (48,49,247). Cytochalasin B at a concentration of 10 µg/ml blocked cytokinesis but not karyokinesis in Naegleria, as has been reported for other cells (48,49).

F.3 Lanthanum ion inhibition of amoeboid motility

Calcium plays a key role as a regulating ion controlling a wide variety of cellular functions, particularly motility. The use of La^{3+}ion as a partial antagonist of Ca^{2+}ion has provided much valuable information on Ca^{2+}ion binding sites in muscle (195). La^{3+}ion has been shown to inhibit the motility of Naegleria amoebae and flagellates. Under the electron microscope, the electron-dense La^{3+}ion appeared deposited as distinct granules in the plasmalemma, possibly as a result of competition between La^{3+}ion and Ca^{2+}ion for negatively charged membrane sites. These sites may be channels for Ca^{2+}ion influx as has been suggested for Amoeba proteus (227). La^{3+}ion at high concentrations (10^{-3} - 10^{-4}M) may completely disrupt the ion flux across the plasmalemma and lyse the cell.

Durham (142) proposed that amoeboid movement involving actin-like and myosin-like microfilaments is controlled by a Ca^{2+}ion flow across the plasma membrane. Blocking of Ca^{2+}ion sites on the Naegleria plasmalemma

by La^{3+} ion probably destroyed the Ca^{2+} ion sensitivity of actin-like microfilaments, the presence of which has been demonstrated for Naegleria (Chapter 6 of this thesis, 248,254,256). Durham (142) also postulated that pseudopod extension was due to two factors - relaxation of the actomyosin network just beneath the plasmalemma, and local depletion of Ca^{2+} ion. This concept is supported by the observation that the heaviest deposits of La^{3+} ion were in the base and central section of the advancing pseudopod. Portions of the plasmalemma not involved in pseudopod formation had no La^{3+} ion deposits. Rinaldi & Hrebenda (353) found that thick and thin microfilaments under the plasmalemma did not extend all the way into the advancing pseudopod of Amoeba proteus.

An interesting observation was the presence of electron-dense granules in the mitochondria of Naegleria treated with La^{3+} ion. While similar granules were observed in Acanthamoeba mitochondria where they may serve as Ca^{2+} ion stores, they were not seen in the Naegleria amoebae used as a control (Chapter 6).

F.4 Lanthanum ion inhibition of transformation

The amoeba-to-flagellate transformation in Naegleria is strongly inhibited by La^{3+} ion. In smooth muscle, La^{3+} ion has been shown to act as a Ca^{2+} ion antagonist, acting by disrupting the ion balance and affecting the structural composition of the plasmalemma. How La^{3+} ion disrupts transformation is not known, but Perkins & Jahn (326) suggested that a control for transformation was the ionic ratio in the cell. Fulton (169) demonstrated that the flagellar membrane is continuous with the plasmalemma and thus it may be the flagellar membrane which is sensitive to La^{3+} ion. Occupation of superficial Ca^{2+} ion binding sites on the plasmalemma by La^{3+} ion may cause the release of Ca^{2+} ion into the cell, disrupting the microtubules of the flagellar axoneme.

F.5 Other inhibitors of transformation

Caffeine totally inhibited transformation at a concentration of 10^{-1} - 10^{-2}M, and only 30% of the amoebae transformed at 10^{-3}M. Inhibition of transformation may be due to the release of intracellular Ca^{2+} ion which could prevent microtubule assembly. The Ca^{2+} ion and Mg^{2+} ion chelators, EDTA and EGTA in the concentration range 10^{-1} - 10^{-2}M, inhibited transformation, most likely by disrupting the ionic balance within the cell. PCMB, a sulphydryl inhibitor, partially inhibited transformation in the concentration range 10^{-2} - 10^{-4}M, confirming the report of Yuyama (472). Colchicine and cytochalasin B did not appreciably affect transformation. Inhibitors such as EDTA and EGTA inhibited both

amoeboid motion and transformation in Naegleria. Colchicine had no effect on these processes.

F.6 Membrane sensitivity

The flagellates, but not the amoebae, of Naegleria were inhibited by 10^{-3}M caffeine, 10^{-7}M La^{3+} ion and 10^{-3}M PCMB. Cytochalasin B inhibited motility in the amoebae but not in the flagellates of Naegleria. This implies a difference in membrane sensitivity between the two phenotypes. These observations are extremely interesting, as Willmer (463) has suggested that the amoeboid and flagellated stages of Naegleria maintain their ionic equilibria by different means, and that the membrane is to some extent polarised in opposite directions in the two forms. Willmer also suggests that the flagellate form has an inwardly directed cation pump. Other investigators (163,229,242,324,327), using a variety of techniques, add supporting evidence for this concept.

G. CONCLUSION

Differences in the transforming ability of Naegleria fowleri and Naegleria gruberi at 43°C are useful criteria for the taxonomy of the genus. The data presented in this chapter have formed a modest beginning to a new avenue of research into the study of motility. A detailed examination of membranes, particularly of the cation binding sites, will yield valuable and fundamental new information on the basic biological processes of amoeboid motion, amoeba-to-flagellate transformation and flagellar beating in Naegleria.

CHAPTER 9

PATHOGENICITY OF LIMAX AMOEBAE

A. ABSTRACT

A brief review of the history of primary amoebic meningoencephalitis, the effects of chemical agents on the amoebae, experimental animal pathogenicity and virulence is given. Material from two suspected cases of primary amoebic meningoencephalitis was examined and cultured but no limax amoebae were found.

B. INTRODUCTION

Primary amoebic meningoencephalitis is a disease involving the human brain and meninges which is caused by small free-living limax amoebae of the genera Acanthamoeba and Naegleria. This disease is quite distinct from infection of the brain by Entamoeba histolytica (261), which is usually secondary to disease elsewhere in the body. That organs other than the brain may be involved was shown by Markowitz et al.(268) who found that seven of sixteen cases of the disease had focal or diffuse myocarditis. The cases of this disease reported in the literature appear to be of two types: (1) a rapid, acute form with sudden onset and a swift, invariably fatal conclusion within a week caused by Naegleria, and (2) a chronic form with an unobtrusive beginning and a prolonged duration of up to several months caused by Acanthamoeba. The portal of entry for Naegleria has been shown to be through the nasal mucosa, via the cribriform plate. Infection follows swimming in, or other exposure to, contaminated water (46,90). There is no apparent connection between swimming and the meningoencephalitis caused by Acanthamoeba.

Recognition of the causative limax amoebae in suspected cases of primary amoebic meningoencephalitis is essential as the disease closely resembles bacterial, cryptococcal and viral meningitis (5). Culbertson (92) has found that specific indirect immunoenzymatic (peroxidase) staining is useful in the identification of potentially pathogenic amoebae in formalin-fixed paraffin tissue sections. This technique will allow re-examination of histological material at a later date.

Excellent reviews of the clinical, pathogenic and historical aspects of primary amoebic meningoencephalitis have been given by Carter (47),

Chang (74) and Duma (133). Chang (76) has recently reviewed the comparative aetiology and epidemiology of this disease. In 1974 Willaert (453) published a select bibliography and a tabular survey of 84 definite and possible cases.

B.1 History and distribution

Probably the first reported cases of human primary amoebic meningoencephalitis were those of Derrick (120) in 1948 and Kernohan et al.(237) in 1960. The first definitive reports of the responsible organisms were those of Fowler & Carter (164) in 1965 and Butt (36) in 1966. Since these early reports, pathogenic Naegleria have been found or suspected in cases of this disease in the following countries: Australia (6,7,44-47,164), Belgium (198,210,212,419,420), Britain (12), Czechoslovakia (62,63,66,67,69), India (22,320), New Zealand (264,305), Uganda (190), the United States of America (36,37,40, 41,88-91,95,128,134,136,137,405) and Venezuela (32,33). Pathogenic hartmannellid amoebae have been isolated in Britain (188), the United States of America (41,215,323,355,360) and Zambia (24). Retrospective study of autopsy material has uncovered more cases of primary amoebic meningoencephalitis, one dating back to 1909 (128,412).

B.2 Effects of chemical agents

The drugs penicillin, sulphadiazine, chloramphenicol, oxytetracycline hydrochloride, streptomycin, methotrexate, emetine, quinine and metronidazole were ineffective on pathogenic Naegleria amoebae when administered at therapeutic levels in man (44,46). Amphotericin B was found to be highly amoebicidal in vitro and in vivo when tested on mice (45). Other investigators (74,107,112,132,133,205,265,339,433) have confirmed and extended these observations.

Carter (47) reported a case of human primary amoebic meningoencephalitis where the administration of amphotericin B probably saved the patient's life. Amphotericin B binds preferentially to, and attacks cell membranes so that cell lysis occurs (239). Schuster & Rechthand (378) examined the effects of amphotericin B on the ultrastructure of Naegleria fowleri and Naegleria gruberi. They found that amphotericin B was toxic in the range of 0,25 - 1,0 µg/ml and produced dramatic blebbing of the plasma membrane and damaged other organelles in Naegleria. Since amphotericin B has dangerous side effects, other drugs are being sought for the treatment of this disease.

Jamieson (218,221) has found that clotrimazole tested in vitro against 18 strains of Naegleria was an active amoebicide in the concentration

range of 0,125 - 0,250 µg/ml, but the drug was ineffective in protecting mice against pathogenic Naegleria. In vitro, the dyes brilliant and malachite green are effective in destroying Naegleria amoebae (60), and 5-fluorocytosine is an effective agent against Acanthamoeba (410). The quaternary ammonium compound Deciquam 222 was found to be highly amoebicidal both in vitro and in vivo against trophozoites and cysts of Naegleria, Acanthamoeba and Entamoeba (111). Experimental chemotherapy has indicated that sulpha drugs, particularly sulphadiazine, are effective in the protection of mice infected with pathogenic Acanthamoeba (97). In vivo, sulpha drugs have little effect on Naegleria amoeba (89), and amphotericin B is not very effective against Hartmannella trophozoites (133). The in vitro studies of Visvesvara & Balamuth (426) have shown that amphotericin B at a concentration of 1 µg/ml or higher was lethal to Naegleria, while Acanthamoeba was not affected by a concentration of 50 µg/ml of the drug. Carter (47) stated that amphotericin B should be administered in suspected cases of Naegleria meningoencephalitis, while sulphadiazine is the drug of choice for the treatment of suspected hartmannellid meningoencephalitis.

B.3 Pathogenicity in experimental animals

Natural animal infections by limax amoebae have been described in Chapter 2 of this thesis. Various investigators (46,51,52,57,74,76,88, 98,99,226,270,272-275,277,337,428,470,471) have studied induced primary amoebic meningoencephalitis caused by inoculation of pathogenic Acanthamoeba and Naegleria into mice, rats, guinea pigs, rabbits and monkeys. In both natural and experimentally induced primary amoebic meningoencephalitis the amoebae invade intranasally, disrupt the olfactory mucosa, enter the submucosal plexus and pass through the cribriform plate to the central nervous system (269,273,329). Electron microscope studies by Visvesvara et al.(427,428) of Naegleria-infected mice revealed that the amoebae caused extensive damage by active phagocytosis of brain tissue, neurons and glial cells and destroyed extensive areas of the olfactory lobes and superficial regions of the cerebrum. The olfactory epithelium of mice appears to be unusually susceptible to amoebic invasion, and at present these animals provide the best experimental model (133,275).

Only Naegleria trophozoites are infective, although Das (110) claimed that Naegleria flagellates were pathogenic in mice. Intranasal or intracerebral inoculation of pathogenic Naegleria cysts was found to be avirulent in mice (46,47,78,90).

Diffley et al.(125) recorded a delayed-type hypersensitivity in guinea pigs when they were injected subcutaneously with Naegleria fowleri Culbertson et al.(96) found that subcutaneous injection of axenically grown Naegleria fowleri into guinea pigs resulted in debilitation and death by visceral damage. Invasion from the site of injection was via the lymphoid tissue and was without involvement of the nervous system. Acanthamoeba culbertsoni tested under these conditions produced local abscess formation. Culbertson et al.(96) also suggest that human infections other than primary amoebic meningoencephalitis may be caused by Naegleria and Acanthamoeba. Bovee et al.(28,466) found that subcutaneous injection of Acanthamoeba castellanii in mice and rats caused tissue damage. Phagocytosis of erythrocytes by Acanthamoeba has been observed in vitro (82,348).

Wong et al.(469-471) found that in monkeys intranasal or intravenous inoculation of Naegleria fowleri or Acanthamoeba culbertsoni did not produce disease, while 50% of the animals which received intrathecal inoculation of either amoeba developed fatal primary amoebic meningoencephalitis. Pathogenicity appeared to be influenced by amoeba virulence, cultural conditions, growth phase, inoculum size and the age and other physiological factors of the host. Similar or complementary findings on the variables of limax amoeba pathogenicity have been reported by Cerva (52,57) and Duma et al.(135).

B.4 Virulence

Culbertson (90) reported that the A-1 strain of Acanthamoeba culbertsoni was as virulent in 1971 as when it was first isolated in 1958. Between 2 to 50 amoebae of this strain injected intranasally, or 1 to 5 amoebae injected intracerebrally, were lethal to a mouse (51,90). Stevens & O'Dell (409) found that the virulence of the A-1 strain was very low, but increased when passaged through mouse brain.

Some of the pathogenic Naegleria strains are extremely virulent, as Carter (46) reported that only 39 Naegleria fowleri amoebae injected intranasally formed a lethal dose for a mouse. Culbertson et al.(95) found that 2 of 10 mice developed fatal amoebic "rhinencephalitis" when inoculated with 50 pathogenic Naegleria trophozoites. Phillips (329) noted that 18 to 31 amoebae of the CJ strain of Naegleria fowleri inoculated intranasally into guinea pigs caused primary amoebic meningoencephalitis in 31 of 33 test animals within nine days.

B.5 Virulence and other organisms

The apparent variation in virulence of Entamoeba histolytica has been attributed to differences in the associated bacterial flora (330-332), although Diamond et al.(124) and others (196,467) have found variation in virulence of axenically grown amoebae. Elsdon-Dew (150-152) postulates a genetic transformation due to invasion of the amoebae by some virus-like particle, similar to that which occurs in Clostridium botulinum (147,148).

Naegleria strains may lose their virulence in axenic culture (A.Jamieson, personal communication, Dec., 1974), but as these enter through the nose the associated bacteria may well play a part in determining virulence, although their role has still to be ascertained. Viruses have been demonstrated in Entamoeba histolytica (122), but their significance is uncertain. Extensive electron microscope studies of virulent limax amoebae have failed to reveal any virus particles involved in pathogenicity (Chapter 6 of this thesis).

B.6 Virulence and enzymes

In vivo pathogenic limax amoebae may secrete an enzyme or toxin capable of destroying or lysing surrounding tissue (88,94,215,273,427). Acanthamoeba castellanii is cytopathic to tissue culture cells (13,443) and has a toxin capable of killing chick embryos at 37°C (285). Elson et al.(153) have demonstrated a lipogenic toxin for Acanthamoeba rhysodes which made mammalian cells in culture round up and fill with fat droplets. Visvesvara & Balamuth (426) found that pathogenic Acanthamoeba liberate a phospholipase enzyme. They also reported that these amoebae were often seen surrounded by a clear zone in the midst of tissue culture cells which suggested proteolytic action. Chang (74) reported the isolation from pathogenic Naegleria of an enzymatic protein capable of cytopathic action on tissue culture cells. Virulent and avirulent Entamoeba histolytica have different surface properties (278), and pathogenicity may be due to a surface active lysosome. However, detailed scanning and transmission electron microscope studies of the surface properties of virulent limax amoebae have failed to reveal any such structures (Chapters 5 and 6 of this thesis).

C. MATERIALS

Organisms Sources are recorded on p.146-147.

Media Described on p.148-149.

Chemicals Described on p.149-150.

D. METHODS

D.1 Examination of two suspected cases of primary amoebic meningoencephalitis

Case 1: On 3rd March, 1972 I was notified by the staff of Grey's Hospital, Pietermaritzburg, Natal, South Africa, of a suspected case of primary amoebic meningoencephalitis in a 60 year old male. Amphotericin B was administered, but the patient died.

I was present at the autopsy and collected samples of cerebrospinal fluid, blood, heart, lung, cribriform plate, brain, kidney, spleen and liver. These specimens were cultured in Fulton's A medium and on PA or NM agar at 25°C and 37°C with Klebsiella aerogenes as a food source.

Case 2: On 6th July, 1976 I was notified by the staff of the Red Cross War Memorial Childrens' Hospital, Cape Town, South Africa, of a suspected case of the disease in a 6 month old female infant.

Cerebrospinal fluid samples were cultivated in Fulton's A medium and on PA and NM agar with Klebsiella aerogenes at 25°C and 37°C. Treatment was instigated for bacterial meningitis and the patient was subsequently released.

E. RESULTS

Case 1: Autopsy material cultured over a nine-day period gave no growth of limax amoebae. Bacterial growth was present in the samples of brain, cribriform plate, cerebrospinal fluid and lung. Growth of mould occurred with samples of the cribriform plate, olfactory bulb and lung. This mould was identified as belonging to the genus Alternaria by a staff member of the Department of Plant Pathology and Microbiology, University of Natal, Pietermaritzburg. Initially it was thought that the mould was an Aspergillus, and, although rare, aspergillotic meningoencephalitis has been reported (179). The histology of the lesions suggested a tuberculosis aetiology (R.Elsdon-Dew, personal communication).

Case 2: No limax amoebae were isolated from the samples cultivated over a period of ten days.

F. CONCLUSION

No amoebae were isolated from either of the cases examined. This demonstrates the ease with which amoebic meningoencephalitis may be confused with other forms of the disease. Isolation of the causative limax amoebae from patients is the only certain way of identifying primary amoebic meningoencephalitis. Continued awareness of this potentially fatal disease is essential as the causative agents have been isolated from the environment (Chapter 2).

CHAPTER 10

COMMENTS ON THE TAXONOMY OF LIMAX AMOEBAE

A. ABSTRACT

The taxonomy of limax amoebae is reconsidered. Results of work done for this thesis provide suggested amendments to Chang's classification of small, free-living amoebae.

B. CLASSIFICATION

"Rien n'est plus difficile, en effet, que de determine une amibe". Dangeard's quotation made in 1900 and cited by Page (312) is even more true today. Classification of Acanthamoeba Hartmannella, Naegleria and other limax amoebae is confusing, frustrating and, at times, seemingly impossible. There are three schemes of taxonomy currently in use in the literature. Singh & Das (109,389-392) have classified limax amoebae on the basis of mitosis. Page (312-315,317) placed increased reliance on other morphological features. The classification scheme of Chang (74) is a compromise between the other two schemes. Keys for the classification of limax amoebae have been presented by Chang (74) and by Page (317).

B.1 Naegleria gruberi Schardinger, 1899

In 1899 Schardinger (365) provided the first description of Amoeba gruberi. Alexeieff (3) in 1912 proposed the generic name, Naegleria, for small, free-living amoebae with a prominent nucleus and nucleolus dividing to form polar masses during mitosis. At almost the same time Chatton & Lalung-Bonnaire (80) advanced the generic name Vahlkampfia for the same protozoan as Alexeieff's Naegleria. Some controversy has reigned, but in 1913 Calkins (39) distinguished between Vahlkampfia and Naegleria. Amoebae of both genera divide with polar masses in the nucleus but only Naegleria has the ability to transform into flagellates. Wilson (465) and subsequent workers (312,349,390) have recognised Naegleria as the generic name for Amoeba gruberi. The following features are regarded as characteristic of Naegleria gruberi (74,312,392):

B.1.i Rounded amoebae 10-30 µm in diameter. Pseudopodia usually lobose. One or more contractile vacuoles. Nucleus and nucleolus prominent.

B.1.ii Amoebae reproduce by binary fission characterised by intranuclear karyokinesis. Nuclear membrane and nucleolus persistent. Polar masses formed from the nucleolus. Interzonal body present at anaphase/telophase.

B.1.iii Amoebae capable of temporary transformation into flagellates. Flagellates usually spindle-shaped; generally two flagella anteriorly directed.

B.1.iv Cysts 10-20 µm in diameter; reticulate, double-walled; two or more plugged pores for excystment.

B.1.v Amoebae free-living. Not known to be pathogenic

B.2 Naegleria fowleri Carter, 1970

In 1970 Carter (46) was the first to name Naegleria fowleri, a few months before Singh & Das (392) proposed the name Naegleria aerobia. In 1971 Chang (74) advanced the name Naegleria invades for the same amoeba. Following the Rule of Priority, Naegleria fowleri takes precedence of Naegleria aerobia and Naegleria invades which are junior synonyms. Naegleria fowleri has the following characteristics according to Carter (44,46,47) and others (186,392):

B.2.i Rounded amoebae 5-15 µm in diameter. Pseudopodia usually lobose. One or more contractile vacuoles. Nucleus and nucleolus prominent.

B.2.ii Amoebae cannot tolerate 0,5% NaCl in the growth medium.

B.2.iii Amoebae grow at 46°C.

B.2.iv Amoebae reproduce by binary fission.

B.2.v Amoebae capable of temporary transformation into flagellates.

B.2.vi Cysts 5-15 µm in diameter; single-walled; excystment via rupture of the cyst wall.

B.2.vii Amoebae pathogenic to man and other animals.

B.3 Naegleria jadini Willaert & Le Ray, 1973

In 1973 Willaert & Le Ray (459) described a third species of Naegleria - Naegleria jadini, which was distinguished from Naegleria fowleri and Naegleria gruberi by two-thirds of its antigenic structure. Naegleria jadini is differentiated from Naegleria gruberi by the absence of obvious pores in the cyst wall, and from Naegleria fowleri by its inability to grow at 37°C or higher temperatures.

B.4 Other Naegleria species

Willaert (personal communication, Jan.,1975) has found a fourth Naegleria species antigenically different from the other three species. In 1935 McCullough (284) reported isolating another species, Naegleria bistadialis, which in the flagellated state has two unequal flagella. Recent extensive sampling by a number of investigators (169,312,317,361) has failed to re-isolate this species.

B.5 Other limax amoebae

The diagnostic features of Acanthamoeba, Hartmannella and other limax amoebae have been described in detail (74,169,312,313,317,392).

B.6 Immunology

Immunological techniques such as precipitin (16,381), complement fixation (50,59,88,93,236), immobilisation (1,246,321,396), agglutination (6,8,9,321,363,421), indirect fluorescent antibody (65,116,245, 381,406,421) and immunoelectrophoresis (426,429,431,432,455,456,458,459) have been used to study limax amoebae.

Willaert et al.(456,458) in an immunoelectrophoretic study of nine strains of pathogenic Naegleria isolated in Belgium, Czechoslovakia, Australia, New Zealand and the U.S.A., found that all the strains had complete antigenic identity - indicating that geographically different isolates of pathogenic Naegleria are members of the same species. De Jonckheere et al.(116) used the indirect fluorescent antibody technique to evaluate 21 strains of pathogenic Naegleria, most of which were isolated from cases of primary amoebic meningoencephalitis. All these strains were identified as Naegleria fowleri. Various workers (65,116, 429,455,457) have shown that Naegleria fowleri and Naegleria gruberi are serologically distinct. Naegleria has also been reported to be antigenically different from Acanthamoeba, Hartmannella, Entamoeba and Schizopyrenus (16,91,396,421,426,431,432).

Visvesvara & Balamuth (426) noted differences in the antigenic composition of Acanthamoeba and Hartmannella and concluded that they are distinct genera.

C. DISCUSSION

Singh & Das (392) organise their classification scheme on the structure and pattern of nuclear division. A different approach is taken by Page (312,313) who combines nuclear with other parameters. Chang's (74) approach is essentially a compromise between the other schemes of classification and has been most useful in this study. The difficulty with Singh's method of classification is that the very fine differences in mitotic division on which the classification rests are not readily identifiable. Finding the appropriate pattern of nuclear division is not easy (Chapter 3) and the practical use of this system in clinical situations is very restricted.

Page (312) has stressed easily identifiable features such as the morphology of limax amoebae in his classification scheme and reported that the interzonal body, an essential element in Singh's scheme, is not always

present. The dispute over the elusiveness of the interzonal body has been resolved as Page (317) has recently recognised the interzonal body as a constant feature of the nuclear division cycle in Naegleria, as have other investigators (37,74,95,349,392).

The lack of consensus on classification has led to confusion on the taxonomy of pathogenic limax amoebae and the many synonyms which have been created in referring to them. Some authors use the generic name Acanthamoeba (426) while others use Hartmannella (90) for the same protozoan. Sawyer & Griffin (362) proposed the erection of the family Acanthamoebidae and and the separation of the genera Acanthamoeba and Hartmannella within this family.

A study of the DNA base composition of limax amoebae could be a useful taxonomic tool. Adam & Blewett (2) have begun such studies on the DNA of various strains of Acanthamoeba. Another useful technique may be that of disc electrophoresis (232).

Results here presented have provided new information and extended established observations on the similarity of various strains of pathogenic Naegleria. The eighteen pathogenic Naegleria isolates examined all belong to the same species - Naegleria fowleri, which is quite distinct from non-pathogenic Naegleria gruberi and other limax amoebae. The following taxonomic parameters for the classification of Naegleria fowleri and Naegleria gruberi have emerged from my research:

The amoebae and flagellates of Naegleria fowleri and Naegleria gruberi are morphologically similar (Chapters 3,4,5).

There are ultrastructural differences between Naegleria fowleri and Naegleria gruberi (Chapter 6).

The two species of Naegleria have distinct bacterial feeding preferences, growth rates and temperature tolerances (Chapter 7).

The quantitative aspects of the amoeba-to-flagellate transformation in the two species are different (Chapter 8).

These features have been used as a basis for suggested amendments to Chang's classification of small, free-living amoebae (Table 10.1 on the following page

TABLE 10.1

CHANG'S CLASSIFICATION OF LIMAX AMOEBAE MODIFIED TO INCLUDE *NAEGLERIA FOWLERI* AND *NAEGLERIA JADINI*

ORDER: Amoebida

SUPERFAMILY: Amoebaceae

FAMILY: Schizopyrenidae. Active limax form common. Transient flagellates may be present. Nucleolus is origin of polar masses. Polar caps and interzonal bodies may be present.

GENUS: *Naegleria*. Cysts single or double-walled. Flagellates transient; readily formed. Polar caps and interzonal bodies present in mitosis.

SPECIES: *Naegleria gruberi* Schardinger, 1899.
Limax amoebae mean measurement 22,4 µm x 6,9 µm. Distinct endoplasm, ectoplasm, nucleus, nucleolus and one or more contractile vacuoles. Polar caps, interzonal bodies present in mitosis. Under the transmission electron microscope, mitochondria round or oval. At 30°C better growth on the bacteria *Staphylococcus albus*, *Klebsiella aerogenes* and *Escherichia coli* than on *Naegleria fowleri*. At 25°C amoebae transform into flagellates more easily than *Naegleria fowleri*. No growth or amoeba-to-flagellate transformation at 43°C. Cysts round to oval; double-walled; excystment via preformed exit pores. Not known to be pathogenic.

SPECIES: *Naegleria fowleri* Carter, 1970.
Limax amoebae mean measurement 14,2 µm x 5,9 µm. Distinct endoplasm, ectoplasm, nucleus, nucleolus and one or more contractile vacuoles. Polar caps, interzonal bodies present in mitosis. Under the transmission electron microscope, mitochondria cup-shaped or dumbbell-shaped. At 30°C does not grow as well on the bacteria *Staphylococcus albus*, *Klebsiella aerogenes* and *Escherichia coli* as *Naegleria gruberi*. At 25°C amoebae do not transform into flagella as easily as *Naegleria gruberi*. Growth and amoeba-to-flagellate transformation at 43°C. Cysts round to oval; single-walled; excystment by rupture. Pathogenic.

(continued overleaf....)

SPECIES: Naegleria jadini Willaert & Le Ray, 1973.

Limax amoebae mean measurement 29,8 µm x 9,8 µm. Polar caps, interzonal bodies present in mitosis. Antigenically different from Naegleria gruberi and Naegleria fowleri. Cysts single-walled; excystment via inconspicuous exit pores. Non-pathogenic.

CHAPTER 11

CONCLUSION

The primary aim of this thesis was to compare and contrast selected aspects of the biology of a number of strains of limax amoebae, particularly those of the genus Naegleria. Eighteen strains of Naegleria pathogenic to man or other animals have identical features and belong to the same species - Naegleria fowleri. These amoebae were distinct in many respects from the non-pathogenic Naegleria gruberi and other limax amoebae.

In general, results presented in this thesis have substantiated and extended the work of other investigators, but a number of new features have emerged, namely:

The isolation of potentially pathogenic limax amoebae from South Africa (Chapter 2).

A time-lapse microcinematographic study of cell division and transformation in Naegleria (Chapter 4).

Temperature-shock induction of cytokinesis in Naegleria amoebae (Chapter 4).

Detailed study of virulent and avirulent limax amoebae by scanning and transmission electron microscopy (Chapters 5,6).

The examination of feeding preferences and other interactions between limax amoebae and selected bacteria (Chapter 7).

Quantitative aspects of amoeboid motion and the amoeba-to-flagellate transformation in virulent and avirulent Naegleria (Chapter 8).

New criteria for the classification of Naegleria have been proposed (Chapter 10).

The secondary aim of this thesis was to investigate motility in Naegleria. Actin-like and myosin-like microfilaments instrumental in amoeboid motion were found in Naegleria (Chapter 6). New information on the role of Ca^{2+} ion and microfilaments has been obtained by the use of inhibitors such as caffeine and cytochalasin B (Chapter 8). La^{3+} ion which competes with Ca^{2+} ion, was shown to inhibit Naegleria motility - electron microscope studies suggest that Ca^{2+} ion binding-sites on the plasmalemma are involved.

Naegleria provides a useful system for the study of motility at the single cell level as entire populations of these amoebae may be induced to transform into flagellates (Chapter 8). It is hoped that the pilot studies here presented will provide a base for continued research on the basic phenomena of amoeboid motion, amoeba-to-flagellate transformation and flagellar beating.

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Appendix A

ORGANISMS

Bacteria			Source
Bacillus cereus			6
Bordetella bronchiseptica			7
Klebsiella aerogenes			2
Escherichia coli			4
Pseudomonas aeruginosa, strains 5,9,20,21,24,31,41			5
Sarcina lutea			6
Staphylococcus albus			6
Xanthomonas campestris			5
Amoebae			
Acanthamoeba, strain APG.		p.a.m. Texas, April, 1974.	7
Acanthamoeba castellanii, strain 1501/2			4
Acanthamoeba culbertsoni, strain A-1			4
Acanthamoeba rhysodes, strain 1534/4			4
Naegleria gruberi			
strain	EG		8
	NB-1		2
	NEG		2
	SA-1	Soil isolate. Sivunguvungu Pan, Natal, South Africa, February, 1971.	
	SA-2	Isolated from Umbilo River, Durban, South Africa, June, 1971.	
	SA-3	Isolated from outdoor aquarium, Cape Town, South Africa, February, 1973.	
	27		1
	1815/1S		4
Naegleria fowleri			
strain	K-71	Isolated from pipeline water, Australia, February, 1972.	1
	HB-1	p.a.m. Florida. Isolated July, 1966.	1
	G.J.	p.a.m. Florida. Isolated July, 1973.	7
	McMahon	p.a.m. Australia.	1
	MW4U	Isolated from pipeline water, Australia, March, 1972.	1
	N.f.1966	p.a.m. Australia.	4
	N.f.1969	p.a.m. Australia.	4
	NH-1	p.a.m. New Zealand. Isolated April, 1972.	1
	Northcott	p.a.m. Australia. Isolated February 1971.	1
	Oram	p.a.m. Australia. Isolated October, 1971.	1
	PA-14	Isolated from pipeline water, Australia, February, 1972.	1
	PA-17	Isolated from pipeline water, Australia, February, 1972.	1
	PA-90	Isolated from pipeline water, Australia, February, 1972.	1
	Q838	p.a.m. Belgium.	1
	R.L.	p.a.m. Florida. Isolated October, 1975.	7
	Vitek	p.a.m. Czechoslovakia. Isolated June 1968.	1
	161A	Nasal swab isolate.	7
	0359	p.a.m. Belgium. Isolated November 1970	1

Amoebae (continued)	Source
Naegleria jadini, strain 0400. Isolated from a swimming pool. Belgium, July, 1971.	3
Tetramitus rostratus, strain 1581/1	4
Schizopyremus erythaenusa	7
Singhella leptocnemus	7

Abbreviation: p.a.m. = from a case of human primary amoebic meningoencephalitis.

SOURCES

1. Ms. A.Jamieson, Amoebic Research Unit, Adelaide, Australia.
2. Dr. C.Fulton, Brandeis University, Waltham, Mass., U.S.A.
3. Dr. E.Willaert, Institut de Médecine Tropicale Prince Léopold, Antwerp, Belgium.
4. Dr. J.Griffin, Armed Forces Institute of Pathology, Washinton, D.C., U.S.A.
5. Dr. J.Joubert, Department of Plant Pathology and Microbiology, University of Natal, Pietermaritzburg, South Africa.
6. Ms. J.Lomberg, Department of Microbiology, University of Cape Town, South Africa.
7. Dr. S.L.Chang, U.S.Environmental Protection Agency, Cincinnati, Ohio, U.S.A.
8. Prof. F.Schuster, City University of New York, Brooklyn, N.Y., U.S.A.

Appendix B

GROWTH MEDIA AND REAGENTS

MEDIA

Difco Penassay broth: 1,75g Difco Penassay broth (antibiotic medium 3) was added to 100ml glass distilled water, dispensed in cotton plugged test tubes and autoclaved. Fresh medium was made up every 4 - 6 weeks.

Fulton's A medium: Modified from Fulton (169).

PO_4

KH_2PO_4	1,7g
$Na_2HPO_4 \cdot 7\ H_2O$	3,4g
Glass distilled water to	50ml

Autoclave

DEX

Dextrose	13,5g
Glass distilled water to	50ml

Autoclave

HL-5

Difco proteose-peptone	3,0g
Difco yeast extract	1,5g
Glass distilled water to	195ml

Dissolve; Distribute 65ml per screw-capped bottle; Autoclave.
When cool, add to each bottle: PO_4 2ml
DEX 2ml

Prepared HL-5 medium was stored at room temperature and used within a month.

For axenic cultivation of amoebae, 6,5ml of prepared HL-5 medium and 0,7ml of sterile foetal calf or calf serum was added to 2,8ml glass distilled water in a sterile cotton plugged, 50ml flask.

King A medium:

Difco bacto-peptone	20g
Glycerol	10g
K_2SO_4	10g
$MgCl_2$	1,4g
Glass distilled water to	1,0 l

Adjust pH to 7,2 and autoclave.

MEDIA (continued)

NM (nutrient agar) medium:

Difco bacto-peptone	2,0g
Dextrose	2,0g
K_2HPO_4	1,5g
KH_2PO_4	1,0g
Difco bacto agar	20g
Glass distilled water to	1 l

Autoclave; pour into sterile petri dishes.

PA (plain agar) medium:

Difco bacto agar	20g
Glass distilled water to	1 l

Autoclave; pour into sterile petri dishes.

REAGENTS

Lugol's iodine:

Iodine	4g
Potassium iodide	6g
Glass distilled water to	100ml

Stored in a tightly stoppered bottle at room temperature.

2 mM Tris-HCl buffer, pH 7,2:

Tris	6,05g
Glass distilled water to	500ml

pH to 7,2 with 1N HCl
This 0,1M stock solution was stored at 4°C and diluted as required.

0,1 M Na cacodylate buffer, pH 7,2:

Na cacodylate	80g
Glass distilled water to	250ml

pH to 7,2 with 1N HCl
This 0,1M stock solution was stored at 4°C and diluted as required.

Araldite resin:

Araldite CY212	10,0ml
Dodecenyl succinic anhydride	10,0ml
Dibutyl phthalate	0,6ml
Epoxy accelerator C2204	0,4ml

All chemicals were added in succession and stirred thoroughly. Chemicals were Electron Microscope grade and were obtained from Polaron Equipment, Watford, England.

REAGENTS (continued)

Spurr's resin:

Nonenylsuccinic anhydride	26g
Diglycidyl ether of polypropylene glycol	6g
Vinylcyclohexane dioxide	10g
Dimethyaminoethanol	0,4g

All chemicals were added in succession and stirred thoroughly. Chemicals were EM grade and were obtained from Taab Laboratories, Reading, England.

Autoclaving: This was done at 15lb/in^2 for 20 minutes.

Sources of material: General laboratory chemicals were obtained from E. Merck, A.G., Darmstadt, Germany or British Drug Houses Ltd., Poole, England, and were of analytical grade. Some of the uncommon reagents are listed below.

REAGENT	SOURCE
Caffeine	SIGMA Laboratories, St Louis, U.S.A.
Colchicine	SIGMA Laboratories, St Louis, U.S.A.
Cytochalasin B	I.C.I. Laboratories, Aderley Park, England
Digitonin	SIGMA Laboratories, St Louis, U.S.A.
EDTA	SIGMA Laboratories, St Louis, U.S.A.
EGTA	SIGMA Laboratories, St Louis, U.S.A.
Eosin	Edward Gurr, Buckinghamshire, England
Foetal calf serum	Grand Island Biological Co., Grand Island, U.S.A.
Glutaraldehyde	Taab Laboratories, Reading, England
Haematoxylin	Edward Gurr, Buckinghamshire, England
Lanthanum nitrate	Taab Laboratories, Reading, England
Osmium tetroxide	Fluka, A.G., Switzerland
PCMB	SIGMA Laboratories, St Louis, U.S.A.

Bacto agar, bacto peptone, penassay broth, proteose peptone and yeast extract were obtained from Difco Laboratories, Detroit, U.S.A.

Appendix C

Publications arising from this thesis

1. LASTOVICA, A.J. 1971.
Isolation, purification and characterization of a strain of the amoebo-flagellate, Naegleria gruberi.
S. Afr. J. Sci. 67: Supp. 39.

2. LASTOVICA, A.J., & ELSDON-DEW, R. 1971.
Primary amoebic meningoencephalitis caused by Naegleria (sp.).
S. Afr. J. Sci. 67: 464-466.

3. LASTOVICA, A.J. 1972.
Toxicity of Pseudomonas aeruginosa on the amoebo-flagellate Naegleria gruberi.
Studia Microbiol. 1: 13.

4. LASTOVICA, A.J. 1973.
Intracellular contractility in Naegleria gruberi.
S. Afr. J. Sci. 69: 154-155.

5. LASTOVICA, A.J. 1974.
Scanning electron microscopy of pathogenic and non-pathogenic Naegleria cysts.
Int. J. Parasit. 4: 139-142.

6. LASTOVICA, A.J., & WILLIAMS, E.D.F. 1974.
Amoeba-to-flagellate transformation and subsequent reversion in Naegleria fowleri and Naegleria gruberi.
S. Afr. J. Sci. 70: 380.

7. LASTOVICA, A.J. 1975.
Ultrastructure of pathogenic and non-pathogenic Naegleria amoebae.
Trans. R. Soc. Trop. Med. Hyg. 69: 286-287.

8. LASTOVICA, A.J. 1976.
Microfilaments in Naegleria fowleri amoebae.
Z. Parasit. 50: 245-250.

9. LASTOVICA, A.J. 1976.
Comparative studies on free-living and pathogenic Naegleria and Acanthamoeba, in: Proc. Int. Symp. Medicine in a Tropical Environment, Pretoria, July 19-23, 1976. Cape Town, Balkema. IN PRESS.

10. LASTOVICA, A.J. 1977.
Studies of motility in Naegleria fowleri and Naegleria gruberi, in: Proc. Vth Int. Cong. Protozoology, New York City, June 26-July 2, 1977. IN PRESS.

Four additional publications in preparation.

South African Journal of Science

67(6): Supp. 39 (1971).

LASTOVICA, A. J.: Isolation, purification, and characterization of a strain of the amoebo-flagellate, *Naegleria gruberi*.
Sections D and E 9.00 a.m. Wednesday

Naegleria gruberi, a small soil amoeba, is almost universally distributed. A South African strain of *Naegleria* was isolated and purified by selective use of the bacterium, *Aerobacter aerogenes* on nutrient agar. *Naegleria* can exist in three morphologically distinct states in its life cycle, as cyst, amoeba or flagellated cell. The uninucleate cysts are spherical, and their smooth, thick walls contain several plugs inserted in pores. Excystment of the amoeboid form occurs via these exit pores. Locomotion in the uninucleate amoeba while monopodal, is usually sinuous. Reproduction by binary fission occurs only in the amoeboid form. A distinctive characteristic of *Naegleria* is the ability of the amoeboid cell to transform to the flagellated cell when subjected to an aqueous environment. the flagellates are fusiform, usually with two flagella, very rarely one, or more than two flagella. This transformation process is temporary, as the flagellate will revert back to the amoeboid form again. Reproducible, synchronous transformation of whole populations of amoeboid to flagellated cells has been accomplished *in vitro*. The kinetics and parameters of the transformation process are discussed. The value of *Naegleria* as a model system for development studies at the single cell level is emphasized. Recently *Naegleria* has been found to be pathogenic, causing meningoencephalitis. Epidemics have occurred in Czechoslovakia, and other parts of the world, resulting from swimming in fresh water with a high *Naegleria* titre.

PRIMARY AMOEBIC MENINGOENCEPHALITIS CAUSED BY NAEGLERIA (sp.)

A. J. LASTOVICA

DEPARTMENT OF ZOOLOGY, UNIVERSITY OF NATAL, PIETERMARITZBURG.

R. ELSDON-DEW

AMOEBIASIS RESEARCH UNIT,* INSTITUTE FOR PARASITOLOGY, DURBAN.

FREE-LIVING amoebae of the genus *Naegleria* are widely distributed in soil and fresh water. Fowler and Carter (1966) were the first to report that *Naegleria* is pathogenic in man, causing Primary Amoebic Meningoencephalitis. This disease is the direct invasion of of the Central Nervous System by amoebae rather than indirect dissemination from other infected areas of the body. *Naegleria* invades the nasal mucosa, and reaches the brain *via* the olfactory nerve filaments. Bathing in freshwater lakes, ponds, or heated swimming pools is the most likely source of infection.

Over forty fatalities including several epidemics due to Primary Amoebic Meningoencephalitis have been documented from various parts of the world. In Czechoslovakia, Červa, Novák, and Culbertson (1968) stated that sixteen deaths had occurred in the period 1962-1965, and all infections were traced back to swimming in the same indoor swimming pool. Callicott and co-workers (1968) linked six fatalities with swimming in two fresh water lakes in Virginia. The pathology of six Australian cases was described in detail by Carter (1969). Butt (1966) documented three cases in Florida. Additional cases of this disease have been reported by Butt and co-workers (1968), Callicott (1968), and Duma and co-workers (1969). All of these cases occurred during the hottest months of the year in very localized geographical regions in the respective countries. Possibly, optimal growth for the pathagen occurs during this period.

Undoubtedly, Primary Amoebic Meningoencephalitis is wide-spread and additional cases must have been overlooked.

Amoebae as causative agents must be considered in every case of purulent meningitis where bacteria are not implicated. During routine microscopic examination of cerebrospinal fluid, amoebae may be mistaken for degenerate macrophages or virus inclusions (Armstrong & Pereira 1967). Clinical detection of *Naegleria* may be accomplished by utilizing the procedures of Carter (1968), and Culbertson, Ensminger, and Overton (1968). Fresh, unstained cerebrospinal fluid preparations should be examined at 25-37°C. Care must be taken to avoid freezing the specimens, as the trophozoites will be destroyed (Carter, (1968): Culbertson *et al.*, (1966.) Re-examination of old histological specimens may prove valuable, as Symmers (1969) has uncovered evidence that Primary Amoebic Meningoencephalitis may have occurred in England in 1909, and in Ireland in 1937. Although *Naegleria* can exist in three morphologically distinct states in its life cycle, as cyst, amoeba or flagellated cell, only the amoeboid form has been detected in humans. The pathogenic amoeba is most probably *Naegleria gruberi* although Carter (1970) has described a new species, *Naegleria fowleri* isolated from Australian cases of Primary Amoebic Meningoencephalus.

Some *Naegleria* are extremely virulent, since Carter reported that as few as 39 amoebae injected intranasally, killed 1 of 5 mice in 11 days. Culbertson, Ensminger, and Overton

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(1968) reported that 2 out of 10 mice developed fatal amoebic "rhinencephalitis" in 8-10 days when inoculated intranasally with 50 amoebae. In humans the progress of the disease is rapid, death occurred in all but one of the reported cases within two weeks of exposure.

Carter (1968) has reported that the drugs emetine, sulfadiazine, penicillin, chloramphenicol, streptomycin, and methotrexate were totally ineffective in combating *Naegleria* in humans. These drugs plus oxytetracycline, hydrochloride, metronidazole and quinine were also described by Carter (1969) as having no amoebicidal effect on *Naegleria* in vitro. Chemotherapy is at present restricted to amphotericin B which is the sole effective agent in the treatment of Primary Amoebic Meningoencephalitis caused by *Naegleria*. Amphotericin B has a proved amoebicidal effect *in vitro*, and markedly protects mice from *Naegleria* infection (Carter, (1969): Culbertson *et al.*, (1968.) Symmers (1969) reported that amphotericin B was used successfully in England. This drug reduced the number of *Naegleria* amoebae from the cerebrospinal fluids of two children with symptons of upper respiratory infection and incipient meningitis. One child recovered completely: his brother died.

While other soil amoebae particularly members of the genus *Hartmanella* have been proved to be pathogenic in animals (10), they appear to be much less of a human health hazard than are the *Naegleria*.

As the organism has been discovered in this country (Lastovica, 1971) physicians and others should consider the possibility of *Naegleria* being the infective agent in cases of so called aseptic meningitis, especially where there is a history of bathing in open fresh water. In such cases, meticulous examination at 25° – 37°C of wet preparations of the purulent fluid should be made, and, where facilities exist, culture on a lawn of *Aerobacter* carried out.

SUMMARY

The finding of the amoebo-flagellate *Naegleria* spp. in local waters has called attention to the possibility of this organism being a cause of meningoencephalitis.

OPSOMMING

Die ontolekking van die amoebo-flagellate *Naegleria* spp. in plaaslike waters het aandag op die inroutlikheid dat hierdie organisme 'n oorsaak van meningoencephalitis is.

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LASTOVICA, A. J. (in the press) Isolation, purification, and characterization of a strain of the amoebo-flagellate, Naegleria gruberi. *S.A.J. Science.*

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BOOK REVIEWS

PHYSICS

MASS SPECTROMETRY AND ION-MOLECULE REACTIONS by P. F. KNEWSTUBB. *Cambridge: The University Press, 1969. vii + 134 pp. Price 40/- (Cloth); 15/- (Paper).*

This is a delightful book to read. There is evident throughout the sense of excitement which invariably comes through when the author has thought about his themes and believes in them and is actively engaged with them, and now wants to tell the world about it. As a book which forms part of a series aimed at senior undergraduates or others of similar accomplishment, it is ideal. It is a survey of the scope of mass spectrometry, written at just the right level for its intended audience. It is not a specialist monograph of which a number of good examples already exist. Nor is it concerned solely with the use of mass spectrometry in structure determination, which is an area already well-covered in books at the undergraduate level. There is, in other words, a real gap in the literature which this book, with its coverage and level of treatment, fills very well.

The chapter titles adequately indicate the scope of the book. Chapter 1: Reactions between ions and molecules, Chapter 2: Preparation and reaction of an ion sample, Chapter 3: Types of mass spectrometer, Chapter 4: The use of mass spectrometry in problems of analysis, Chapter 5: The interpretation and prediction of mass spectra.

This book must be in all academic libraries serving undergraduate and postgraduate students. The admirably inexpensive paper edition should attract a number of personal purchases. I hope so.

J. D. BRADLEY

BIOCHEMISTRY

THE BIOCHEMISTRY OF VIRUSES edited by H. B. LEVY. *New York and London: Marcel Dekker. 1969. xiv + 612 pp. illus. n.p.*

There has been considerable overlap between the fields of molecular biology and virology. Much research in molecular biology has been carried out with viruses, while basic problems in virology have been tackled using the techniques of molecular biology. It was therefore felt that it would be useful for research workers if the state of knowledge in the field of common interest could be authoritatively reviewed. The contributors were directed to address themselves to an audience only slightly less knowledgeable than themselves. The first two chapters deal with the chemical composition of animal viruses and their architecture, and these are followed by chapters in which most of the major groups of animal viruses are considered. The final chapters are devoted to the bacteriophages and the biochemistry of interferon. A uniformly high standard has been maintained throughout, and this book can be recommended as a reference source.

R. W. CHARLTON

CHEMISTRY

ELECTRON SPIN RESONANCE IN CHEMISTRY by P. B. AYSCOUGH. *London: Methuen & Co., Ltd., 1967. xvii + 443 pp. Many figs. and tables, Price 84/-.*

Electron Spin Resonance becomes steadily more available as a tool for chemical use with the development of reliable spectrometers, together with computerization of their operation and data analysis. Dr Ayscough's book provides a very convenient source of both theoretical introduction to the subject and review of the experimental data, and as such it can be recommended to the seriously interested chemist, willing to work towards obtaining a good understanding of ESR.

The book commences with a brief Introduction, to get into the "swing" of things, but this is not always very successful and the reader must be well prepared by prior reading if he is to make much of it. However, the next chapters, on g-factors, nuclear hyperfine structure and relaxation processes and line widths treat the material well and thorougly, except that the arguments are kept at a qualitative and physical level rather than going through complex mathematical arguments. The clarity is hampered by a rather greater than fair share of errors here, not all typographical, and some unfortunate confusion of signs.

The fifth chapter, perhaps a little belatedly, deals with experimental aspects, to lead into the remaining chapters (rather more than half the book) which review the behaviour of free radicals in the many different experimental situations; the treatment covers the ESR of both inorganic and organic systems, in solution and in the solid state. The final chapters examine "Paramagnetic Species in Biological Systems", a topic of steadily frowing importance.

L. GLASSER

Studia Microbiologica 1(1): 13 (1972).

TOXICITY OF *PSEUDOMONAS AERUGINOSA* ON THE AMOEBO-FLAGELLATE *NAEGLERI GRUBERI*

A. J. LASTOVICA, Zoology Department, University of Natal, Pietermaritzburg

ALTHOUGH both the amoebo-flagellate *Naegleria gruberi* and the bacterium *Pseudomonas aeruginosa* are ubiquitous, well investigated soil organisms, there is essentially no detailed information on their interactions. An investigation of the toxic properties of *Pseudomonas* on *Naegleria* was undertaken with seven strains of *Pseudomonas* and three strains of *Naegleria.*

It was found that the cell suspensions of all seven *Pseudomonas* strains affected excystment, encystment, cell division and the amoeba to flagellate transformation of *Naegleria.* Four strains of *Pseudomonas* were toxic to cysts and motile amoebae, preventing excystment and growth of the amoebae along bacterial streaks on nutrient agar. While the three additional strains of *Pseudomonas* allowed very slow growth, the large, sometimes multi-nucleated amoebae produced irregularly shaped, occasionally multinucleated cysts. Cell suspensions of *Pseudomonas* present at the initiation of the amoeba to flagellate transformation completely inhibited this well-defined morphogenic process. If cell suspensions of *Pseudomonas* were added to a population of flagellates, reversion to amoebae or cell lysis rapidly occurred.

Cell-free filtrates of all seven *Pseudomonas* strains had no detectable toxic effect on *Naegleria* excystment or the amoeba to flagellate transformation. However, encysting amoebae tended to produce larger than normal cysts. An as yet unidentified factor in the cell-free filtrate blocked cytokinesis but not karyokinesis in approximately four percent of the population, producing multinucleated amoebae. This is of interest, as *Naegleria* is almost without exception mononucleate. Pyocyanin extracted from these strains of *Pseudomonas* had no observable toxic effect on excystment, cell division and the amoeba to flagellate transformation, but did cause some amoebae to produce larger cysts.

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Intracellular Contractility in *Naegleria gruberi*

Albert J. Lastovica
Department of Zoology, University of Cape Town

The ubiquitous protozoan *Naegleria gruberi* exists in three morphogically distinct states in its life cycle: as an amoeba, a flagellate, and as a cyst. Environmental factors are capable of causing the amoeba to cease vigorous cytoplasmic streaming and to transform into a rigid fusiform cell with functional flagella. In turn, the flagellate is capable of absorbing the flagellar axonemes into its cytoplasm and reverting to an actively motile amoeba.

The cytoplasm of *Naegleria* amoebae contains a protein complex strikingly similar to smooth muscle actomyosin and slime mould plasmodial myosin B, as judged on the basis of a combination of various muscle biochemical criteria. The *Naegleria* protein precipitates in 0.05 M KCl, but is completely soluble in 0.5 M KCl. It shows a large and reversible drop in viscosity on the addition of ATP in 0.5 M KCl. The protein displays Ca^{2+} activated and Mg^{2+} inhibited ATPase activity and undergoes a strong and rapid ATP-induced super-precipitation reaction. While there is no biologically active actomyosin-like protein in the dormant cyst, the protein comprises 0.04% of the wet weight and 0.7% of the protein of the amoebae.

This actomyosin-like protein is instrumental in the process of amoeboid motion, being organized into a labile network of microfilaments in the amoeba cytoplasm. *In vitro*, colateral bundles of microfilaments are produced from the purified amoeba actomyosin-like extracts by the addition of the chelating agents EDTA and EGTA to a final concentration of 2.5 mM. Cytochalasin B, a recognized disrupting agent of contractile microfilaments completely inhibits amoeboid motion at a concentration of 100 μg/ml while having no effect on flagellar activity. This inhibition is temporary, as removal of the drug causes cytoplasmic streaming in the amoeba to recommence precipitously.

It would be of great interest to investigate the possibility that elements of the amoeba actomyosin-like protein could be utilized for the production of the flagellar axoneme during the amoeba-to-flagellate transformation. The flagellate-to-amoeba reversion might involve utilization of the flagellar contractile elements in the formation of amoeboid microfilaments.

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SCANNING ELECTRON MICROSCOPY OF PATHOGENIC AND NON-PATHOGENIC *NAEGLERIA* CYSTS

A. J. LASTOVICA

Department of Zoology, University of Cape Town, Rondebosch, C.P., South Africa

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Abstract—LASTOVICA A. J. 1974. Scanning electron microscopy of pathogenic and non-pathogenic *Naegleria* cysts. *International Journal for Parasitology* **4**: 139–142. Cysts of 4 strains of non-pathogenic *Naegleria gruberi* and 5 strains of pathogenic *Naegleria fowleri* were examined in the scanning electron microscope. Excystment of the *Naegleria gruberi* amoebae occurred via preformed exit pores in the cyst wall. Similar structures were not found in the cysts of *Naegleria fowleri*, and excystment occurred by rupture of the cyst wall. The sequence of cyst wall rupture is illustrated for one of the pathogenic strains.

INDEX KEY WORDS: *Naegleria gruberi*; *Naegleria fowleri*; scanning electron microscopy; amoeba excystment; exit pores.

INTRODUCTION

AMOEBAE of the genus *Naegleria* are capable of causing fatal primary amoebic meningoencephalitis in man and experimental animals (Carter, 1970, 1972; Culbertson, 1971). Anderson & Jamieson have recently isolated 10 strains of *Naelgeria* from soil, tap water and a rain puddle, all of which are pathogenic to mice (Anderson & Jamieson, 1972 a,b; Jamieson & Anderson, 1973; personal communication, March, 1973). Nearly 60 documented cases of primary amoebic meningoencephalitis in humans have been reported to date, as reviewed by Carter (1972). The ability to distinguish pathogenic from non-pathogenic *Naegleria* is of particular importance, as *Naegleria* is a widely distributed soil and fresh water organism (Fulton, 1970).

Accumulating evidence with respect to: cultural fastidiousness and ultrastructure (Carter, 1970, 1972), serology (Anderson & Jamieson, 1972b) and temperature tolerance (Griffin, 1972), definitely indicate that the *Naegleria* pathogens are a distinct species rather than a pathogenic strain of *Naegleria gruberi*. Carter (1970) was the first to name the pathogen *Naegleria fowleri*, and the other published names, *Naegleria aerobia* (Singh & Das, 1970) and *Naegleria invades* (Chang, 1971) should be considered to be junior nonvalid synonyms.

This report is the first scanning electron microscope investigation of virulent and avirulent *Naegleria*. It supplies information on cyst morphology and excystment in these amoebae and provides additional support for the consideration of *Naegleria fowleri* as a species separate from *Naegleria gruberi*.

MATERIALS AND METHODS

Strains

Naegleria gruberi, strains NB-1 and NEG, and the bacterium, *Klebsiella aerogenes* were obtained from Dr. C. Fulton, Brandeis University, Waltham, Massachusetts. *Naegleria fowleri*, strains 1966 and 1969 were received from Dr. J. Griffin, Armed Forces Institute of Pathology, Washington, D.C., while the Northcott and Oram strains were obtained from Dr. K. Anderson and Miss A. Jamieson, Amoebic Research Unit, Adelaide, Australia. Dr. S. L. Chang, U.S. Environmental Protection Agency, Cincinnati, Ohio, kindly supplied strain 161A. Two South African strains of *Naegleria gruberi* were investigated. Strain SA-1 was isolated from the soil of the Sivunguvungu pan of Northern Zululand (Lastovica, 1971) while strain SA-2 originally came from the Umbilo river, Natal. These strains are designated *Naegleria gruberi* on the basis of diagnostic features characteristic of the species: intranuclear karyokinesis, cysts with preformed exit pores, and the production of temporary flagellates.

All four strains of *Naegleria gruberi* were found to be non-pathogenic to mice (Fulton, 1970; Lastovica, unpublished results). *Naegleria fowleri*, strains 1966, 1969, Oram and Northcott were isolated from human cases of primary amoebic meningoencephalitis. Of these, only the patient Oram survived the disease (personal communication, Anderson & Jamieson, March, 1973). Strain 161A was originally isolated from the nasal swab of a healthy 7-year-old boy by Shumaker and associates (1971). Dr. S. L. Chang (personal communication November, 1972) has found that this *Naegleria* strain exhibits a pronounced cytopathogenic effect in tissue culture and is a virulent mouse pathogen.

Production, harvesting and preparation of cysts

All amoebae were grown in association with *Klebsiella aerogenes* on autoclaved agar. Strains of *Naegleria*

gruberi were grown on NM agar (Fulton, 1970), at 34°C, while the five pathogenic *Naegleria fowleri* strains were grown on 1·5% Difco Bacto agar at 37°C. Approximately 10^5 cysts and 0·1 ml of a 16–20 h liquid Difco Penassay broth culture of *Klebsiella aerogenes* grown at 34°C was spread over the surface of the agar in 85-mm dia. petri dishes, and incubated at 34 or 37°C. The *Naegleria gruberi* amoebae excysted, grew to stationary phase, and encysted within 72 h. However, the *Naegleria fowleri* strains took up to 11 days for complete encystment to occur. Petri dishes of encysted *Naegleria gruberi* were stored at 34°C for 8–10 days after preparation, while the encysted *Naegleria fowleri* were stored at 37°C and used 20–24 days after preparation. Usually, one petri dish of *Naegleria gruberi* cysts was adequate for a preparation for the scanning electron microscope, but as *Naegleria fowleri* cysts were sparser, routinely three petri dishes of each strain were used for sample preparation.

For harvesting, 15 ml of a 20 mM Tris-HCl buffer solution, pH 7·2 was added to the petri dishes and the cysts removed with a bent glass rod. The buffer solution and all other subsequent solutions were used at 25°C. Cysts suspensions were centrifuged for 115 sec at 500 *g* in a swinging bucket rotor. The supernatant fluid was discarded and the cysts washed twice more with buffer. Cysts were fixed with 3% glutaraldehyde in buffer for 60 min. After centrifugation, the cysts were washed twice more with buffer. Next, they were taken through a graded ethanol series to absolute ethanol, with 15–20 min per step. The absolute ethanol was changed twice more before the cyst suspension was pipetted directly onto the specimen stubs. These were air dried overnight in a desiccator and surface coated by vacuum evaporation of a gold palladium alloy.

Scanning electron microscopy

All specimens were examined and photographed with a HITACHI Scanscope SSM 2 operating at 10 or 20 kV. Before and during preparation, cysts were routinely examined under phase contrast microscopy for any evidence of artifact formation or cyst distortion. Several preparations of each strain were examined under the scanning electron microscope. At each viewing, at least 200 individual cysts of each preparation were examined critically for shape, surface texture and the presence or absence of preformed exit pores or wall ruptures.

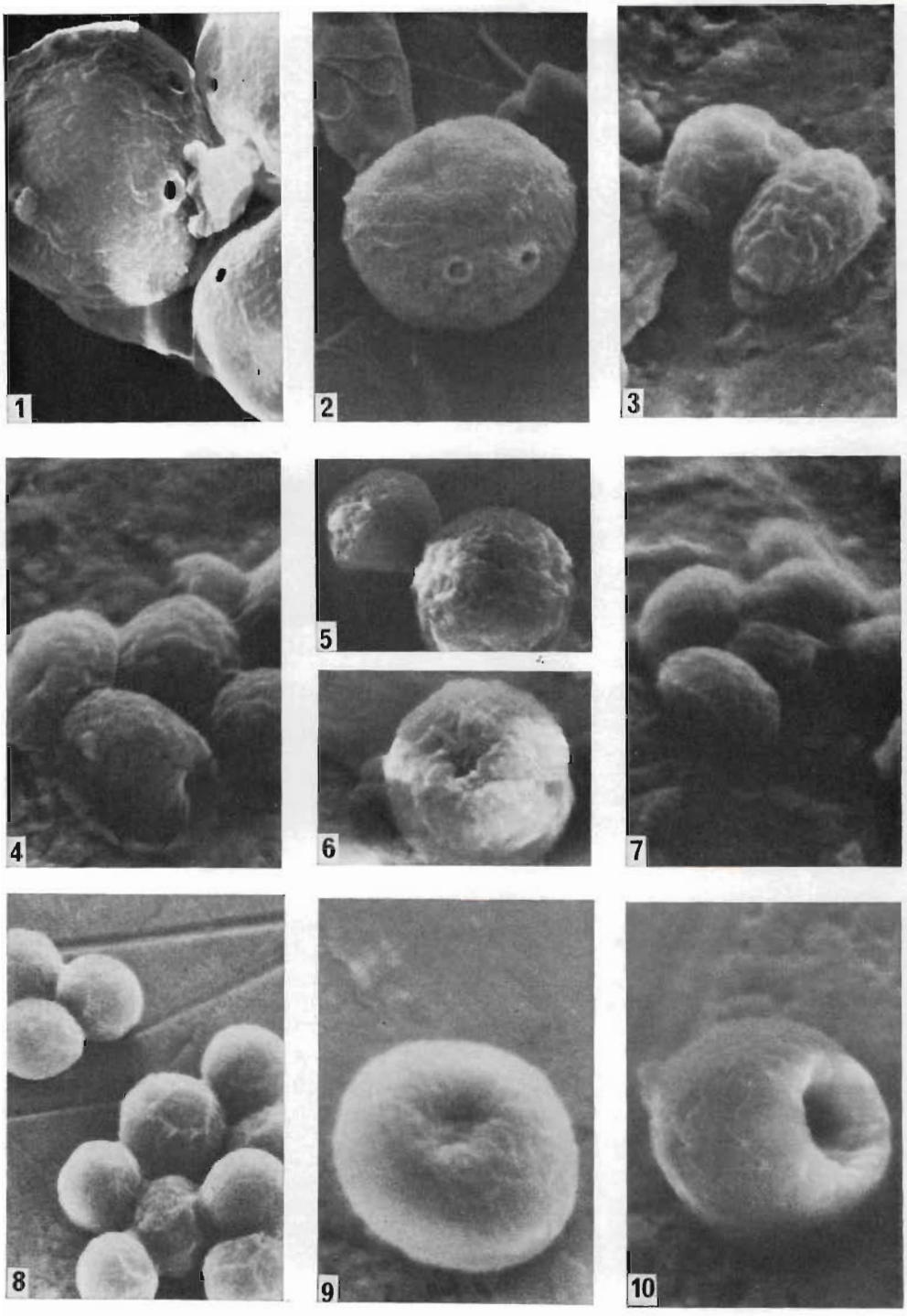
RESULTS

Naegleria gruberi cysts measured before preparation for the scanning electron microscope were between 8 and 19 μm dia. The cysts of all four strains of *Naegleria gruberi* are spherical to slightly oval. Under the light microscope cyst surface texture appears smooth, while under the scanning electron microscope, surface features appear smooth to finely reticulated. All strains of *Naegleria gruberi* showed definite preformed exit pores piercing the cyst wall, with up to five pores per cyst being visible under the scanning electron microscope. Both Schuster (1963) and Fulton (1970) reported that *Naegleria gruberi* cysts have preformed exit pores. In strain NEG (Fig. 1), the excysted amoebae have left the three empty cyst casings behind, clearly indicating the perforations in the cyst walls. A cyst of strain NB-1 (Fig. 2) exhibits a plugged exit pore. These polysaccaride containing plugs are probably dissolved by the excysting amoebae before emergence, as reported by Schuster (1963). The pore size has been estimated by Schuster (1963) to be 0·6 μm at max dia. While not illustrated, identical results were obtained for the two South African strains of *Naegleria gruberi*, SA-1 and SA-2.

Cyst size of the pathogenic *Naegleria fowleri* strains measured before preparation for the scanning electron microscope varied from 4 to 11 μm dia. These five strains exhibited a more varied cyst morphology with respect to shape and surface texture than the strains of *Naegleria gruberi* examined. Usually the cysts were spherical, but oval cysts were occasionally seen, similar to those reported by Červa (1970). The surface textures varied from smooth or finely reticulate for strain 161A (Figs. 8–10), to ridged or coarsely reticulate for the other four strains (Figs. 3–7). Singh & Das (1970) have observed that the cysts of pathogenic *Naegleria* are coated "in a fairly thick gelatinous layer". Under phase contrast microscopy some of the pathogenic cysts were observed to have a similar coating. Possibly the rough surface texture of the cysts under the scanning electron microscope may be due to varying amounts of this material adhering to the cyst walls.

In no case was any structure resembling an exit pore or associated plug seen in any of the pathogenic cysts. For each of the pathogenic strains, at least some of the cysts exhibited rupture of the cyst wall. Examples of this can be seen for *Naegleria fowleri*, strains 1966 (Fig. 6) Oram (Fig. 4) and 161A (Fig. 10). The ruptures are prominent and usually 2 or more μm across. The sequence of cyst rupture is illustrated for strain 161A in Figs. 8–10. The smooth, structureless cysts (Fig. 8) become invaginated (Fig. 9) and develop large ruptures in the cyst walls (Fig. 10).

FIG. 1. Three cysts of *Naegleria gruberi*, strain NEG, after excystment. The exit pores are especially prominent in the walls of the empty cysts (×3000).
FIG. 2. A cyst of *Naegleria gruberi*, strain NB-1, with a plugged exit pore (×2275).
FIG. 3. Two cysts of *Naegleria fowleri*, Northcott strain, showing a ridged surface texture (×5000).
FIG. 4. A group of *Naegleria fowleri*, Oram strain cysts. The two cysts in the foreground exhibit wall ruptures (×4800).
FIG. 5. Two cysts of *Naegleria fowleri*, strain 1966 (×2500).
FIG. 6. An empty cyst of *Naegleria fowleri*, strain 1966, showing rupture of the cyst wall (×3350).
FIG. 7. Cysts of *Naegleria fowleri*, strain 1969 (×4350).
FIGS. 8–10. Cysts of *Naegleria fowleri*, strain 161A, illustrating the excystment sequence.
FIG. 8. A group of poreless cysts (×2875).
FIG. 9. An invaginating cyst (×4700).
FIG. 10. A fully ruptured cyst (×4950).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DISCUSSION

Strain 161A was originally reported to be *Naegleria gruberi* on the basis of its mitotic characteristics and the production of a temporary flagellated state (Shumaker *et al.*, 1971). Rather, this strain should now be considered to be *Naegleria fowleri* not only on the above characteristics but also on the basis of pathogenicity, and excystment by cyst rupture rather than by preformed exit pores.

The presence or absence of preformed exit pores provides a valuable taxonomic feature for the differentiation of *Naegleria fowleri* from *Naegleria gruberi*, as these pores are an established diagnostic feature of *Naegleria gruberi* (Fulton, 1970). The exit pores of *Naegleria gruberi* are visible in the light microscope (Fulton, 1970; Singh & Das, 1970) and the transmission electron microscope (Schuster, 1963). Similar observations on *Naegleria fowleri* have indicated a lack of any such structures. Carter (1970) stated that the excystment of *Naegleria fowleri* occurred by rupture of the cyst wall and that pores and associated plugs were not seen. Singh & Das (1970) also reported a lack of preformed pores in the cyst wall of pathogenic *Naegleria*, and that some digestion of the cyst wall took place to allow excystment of the amoebae.

Similarly, a study of the surface texture is taxonomically useful, as the pathogenic *Naegleria* cysts tend to exhibit a rough surface texture, most probably due to the presence of a thick, gelatinous outer layer, as suggested by Singh & Das (1970).

The scanning electron microscopic evidence produced in this report has confirmed and extended prior light microscopic observations. Marked differences in cyst morphology and excystment provides yet another aspect for the consideration of *Naegleria gruberi* and *Naegleria fowleri* as two distinct species. Continuing research in progress utilizing both the transmission electron microscope and the scanning electron microscope will provide further information on ultrastructural differences in the amoeboid and flagellate stages of pathogenic and non-pathogenic *Naegleria*.

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be a very useful tool in assessing the incidence and type of infection of wheat stem rust. Carbohydrates specific to this rust fungus, such as arabinitol, mannitol and mannose-containing polymers, could be determined and high levels of these in the infected host would indicate a susceptible reaction. This study is at an early stage, however.

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Amoeba to Flagellate Transformation and Subsequent Reversion in *Naegleria fowleri* and *Naegleria gruberi*

A. J. Lastovica* and E. D. F. Williams†
**Department of Zoology, University of Cape Town;*
†*Electron Microscopy Unit, University of Cape Town.*

Pathogenic *Naegleria fowleri* and non-pathogenic *Naegleria gruberi* are common soil and fresh water protozoans that exist as cysts, amoebae or flagellated cells during their life cycle[1–3]. Environmental changes are capable of causing *Naegleria* amoebae to cease active streaming and transform into rigid, ovoid cells with functional flagella[4]. This morphological change is temporary, as the flagellates are capable of reverting back into motile amoebae. Ultrastructural studies have not detected elements of the flagellar apparatus or their precursors in amoeboid cells. It appears that the basal body, rhizoplast, and associated structures are produced *de novo* during the transformation process.

The amoeba to flagellate transformation may be rigorously controlled in the laboratory, and routinely, up to 100% of an amoeboid population will transform in glass-distilled water or dilute buffer. Detailed time course studies and frame by frame analysis of time lapse microcinematographic films have outlined the major events of transformation, which is identical in both *Naegleria fowleri* and *Naegleria gruberi*. Initially, depending on experimental conditions, a lag period of up to 80 min occurs after the amoebae have been removed from their growth medium and suspended in transformation medium. Cells cease amoeboid movement, the cytoplasm seems to "gel", and the amoebae become spherical. Flagella protrude and elongate until they are about the same length as the diameter of the rounded amoebae. Concomitantly, the cells twitch, and rapidly rotate for several minutes until the fully formed flagellates swim off. Most probably, stabilisation of cell shape occurs by formation of a microtubule cytoskeleton. Flagellates have not been observed to feed or divide; these activities are limited to the amoeboid state. Most flagellates possess 2 flagella, but up to 8 flagella per cell have been seen. Experimentally, it has been found that temperature shifts during transformation increase the average number of flagella per cell.

Cells are active flagellates for at least an hour, and then revert to amoebae, but with less synchrony than found in transformation. Reversion is characterised by a loss of rigidity in cell shape and by a "paralysis" of the flagella. The inert flagella are drawn into the cytoplasm of the now motile amoebae. Individual cells may repeat the process of transformation and reversion several times before encystment.

Continuing studies will investigate the origin and fate of the amoeboid actomyosin-like protein[5] and its role in transformation and reversion.

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^{15}N-^{14}N Study of the Role of the Leaf in the Nitrogen Nutrition of the Seed of *Datura stramonium* L.

O. A. M. Lewis
Department of Botany, University of Cape Town.

^{15}N-Nitrate feeding via the transpiration stream and simultaneous feeding of ^{14}C via photosynthesis to a leaf-fruit system in *D. stramonium* indicate that glutamine is the prime recipient of photosynthetically reduced nitrogen in the leaf. Analysis of midvein, petiole and seed indicate that glutamine supplies the seed with most of the reduced nitrogen required for amino acid synthesis. Carbon and nitrogen assimilation in the leaf do not appear to be directly related in that serine-aspartate and not glutamine receive the heaviest initial ^{14}C label.

^{14}C-Assimilation Studies on the Poplar and Pelargonium Rust Infections

S. M. Roberts and D. T. Mitchell
Department of Botany, University of Cape Town.

It is well known that rust fungi alter the distribution of host metabolites but the demonstration of this phenomenon has been considered in only a few examples. There is an indication that there are two types of rust infections; in one no alteration of carbon distribution occurs (in the case of stripe rust of wheat)[1], and in the other assimilates are attracted to the infected leaves as has been demonstrated in bean rust[2]. The occurrence of two types of infection is attributed to differences between monocotyledonous and dicotyledonous plants in possible routes of translocation[3]. There is a real need to investigate this further and to assess critically ^{14}C-assimilation of different rust infections under defined conditions.

In this study two rust infections (pelargonium and poplar rust) have been used and $^{14}CO_2$ has been fed to both infected and uninfected shoots. Some of the shoots had the lower halves of the lamina as well as the petiole and stem completely covered with aluminium foil. The covered portions should act as "sinks" for metabolites and should then be in competition with the pustule regions of the infected leaves. Shoots were first exposed

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ULTRASTRUCTURE OF PATHOGENIC AND NON-PATHOGENIC *NAEGLERIA* AMOEBAE

SIR,—Amoebae of the genus *Naegleria* are the causative agents in fatal human primary amoebic meningoencephalitis (CARTER, 1972). Differentiation of pathogenic from non-pathogenic *Naegleria* is of critical importance, and evidence with respect to cyst morphology (LASTOVICA, 1974), temperature tolerance (GRIFFIN, 1972) and serology (ANDERSON and JAMIESON, 1972) indicates that the pathogenic *Naegleria* are a separate species rather than a pathogenic strain of non-pathogenic *Naegleria*. This report is the first comparative electron microscopic study of a number of strains of both virulent and avirulent *Naegleria* amoebae. Striking similarities as well as sharply defined ultrastructural differences have been revealed between the pathogenic and non-pathogenic amoebae.

Naegleria fowleri, strains HB-1, McMahon, MW4U, N.f.'69, NH-1, Oram, 0359, Q838, and Vitek were obtained from Miss A. Jamieson, Institute for Medical and Veterinary Science, Adelaide, Australia. Strain MW4U was originally isolated from pipeline water, while all the other strains were isolated from cases of human primary amoebic meningoencephalitis. All 9 strains of *N. fowleri* have proved to be pathogenic to mice (Jamieson, A., personal communication). *N. gruberi*, strains NB-1, NEG and the bacterium *Klebsiella aerogenes* were obtained from Dr. C. Fulton, Brandeis University, Waltham, Mass., U.S.A. Two South African strains of *N. gruberi* were investigated. Strain SA-1 was isolated from the soil of the Sivunguvungu pan of Northern Zululand, while strain SA-2 originally came from the Umbilo river, Natal. All 4 strains of *N. gruberi* have proved to be non-pathogenic to mice (FULTON, 1970; LASTOVICA, 1974). The strains of *N. gruberi*, and the N.f.'69 strain of *N. fowleri* were grown in association with *K. aerogenes* on agar, while all the other strains were grown axenically in Fulton's A media (FULTON, 1970). For electron microscopy, the motile amoebae were fixed in 2% glutaraldehyde buffered with 0·1 M Na cacodylate buffer, pH 7·2, postfixed in 1% OsO_4 buffered with the same cacodylate buffer, dehydrated through an alcohol series, embedded in Araldite, and stained with uranyl acetate followed by lead citrate. Viewing and photography were done on either a Phillips EM 300 or a Siemens 101 electron microscope.

Detailed examination of over 500 electron micrographs showed certain ultrastructural features are common to all amoebae examined. The plasma membrane of *Naegleria* amoebae is 10 nm. wide and exhibits a unit membrane structure of 2 electron opaque layers sandwiching an electron transparent layer. Endoplasm is not always readily differentiated from ectoplasm, except the latter is usually free of inclusions. The inconspicuous, poorly organized, rough endoplasmic reticulum consists of ribosome covered elongated tubules or vesicles, 20 to 25 nm. wide. Ribosomes stud the outermost element of the nuclear membrane and are abundant in the cytoplasm. Food vacuoles are recognized by their complex, laminated, whorled structure, and, with the contractile vacuoles, are surrounded by a 10 nm. unit membrane. While non-membrane bound lipid globules are present, no golgi apparatus has been recognized. The prominent nucleus contains a conspicuous electron opaque nucleolus which consists of dense 20 nm. granular particles. The nuclear membrane is formed of double 10 nm. unit membranes separating an electron transparent space of 25 nm., while nuclear pores of 80 nm. diameter are present.

Marked differences occur in the shape and structure of the mitochondria of pathogenic and non-pathogenic amoebae. In the non-pathogenic *N. gruberi* examined, only cylindrical and oval mitochondria were observed, while all the pathogenic *N. fowleri* examined possess prominent cup-shaped or dumbbell-shaped mitochondria. The greater surface area offered by cup-shaped or dumbbell-shaped mitochondria in the pathogens may be required for increased metabolic activity. Two membranes separate a space of 18 nm. and enclose the mitochondria; the inner membrane gives rise to 20 to 30 nm. tubular, oblique and transverse cristae. Very frequently, cristae of pathogenic *Naegleria* display a "honeycomb" profile when the mitochondria are seen in transverse section. Similar cristal profiles have not been observed in non-pathogenic *Naegleria*. Intramitochondrial bodies have not been detected in any of the amoebae examined.

Electron opaque particles 100 nm. in diameter, suggestive of a virus, have been detected in the cytoplasm of several strains of the pathogenic amoebae. MAITRA et al. (1974) reported similar particles in a pathogenic strain of *Naegleria*. Such particles have not been observed in the non-pathogenic amoebae. Further research will be required to elucidate the specific nature of these particles and their possible role in pathogenesis.

Ultrastructural differences provide additional support from the establishment of the pathogens as a discrete and separate species from the non-pathogenic *Naegleria*. An examination in progress of the fine structure of the flagellate and cyst stages of the *Naegleria* life cycle will provide further data of taxonomic value.

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A. J. LASTOVICA,
Department of Zoology,
University of Cape Town,
Rondebosch 7700,
South Africa.

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Microfilaments in *Naegleria fowleri* Amoebae

A. J. Lastovica

Department of Zoology, University of Cape Town

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Summary. Examination by electron microscopy has revealed 2 types of microfilament in the cytoplasm of 3 strains of axenically grown *Naegleria fowleri* amoebae. Thin, actin-like microfilaments 5–7 nm in diameter are randomly oriented in the nonmotile amoebae, and are concentrated near the plasma membrane. In the actively motile amoebae these microfilaments aggregate to form colateral bundles in close proximity to the plasma membrane. Thick, myosin-like microfilaments 17–19 nm in diameter also occur in the amoebae cytoplasm. The significance of these 2 kinds of microfilament in amoeboid motion is discussed.

Introduction

It is now generally accepted that amoeboid motion is based on the presence and contractility of actin-like and myosin-like protein complexes in the cytoplasm of amoebae (Huxley, 1973; Jahn and Bovee, 1969). Thin and thick microfilaments exhibiting the morphological characteristics of actin and myosin respectively, have been demonstrated in a variety of amoebae: from the limax amoeba *Acanthamoeba* (Pollard and Korn, 1973); from the *Amoeba-Chaos* group (Comly, 1973; Nachmias, 1964, 1968; Pollard and Ito, 1970; Rinaldi and Hrebenda, 1975; Rinaldi et al., 1975; Taylor et al., 1973); in the shelled amoeba *Difflugia* (Eckert and McGee-Russell, 1973; Wohlman and Allen, 1968); in *Entamoeba* (Michel and Schupp, 1974, 1975); and in *Thecamoeba* (Haberey, 1973).

Biochemical characterization of thin microfilaments as being actin-like has been demonstrated for *Acanthamoeba* (Weihing and Korn, 1971), and for *Amoeba* (Morgan, 1971). Nachmias (1972) produced thick microfilaments from purified *Physarum* myosin that were identical in size and shape to those found in fixed sections of *Physarum* amoebae.

This report is the first demonstration of thin and thick microfilaments in situ in *Naegleria fowleri* amoebae.

Materials and Methods

Naegleria fowleri, Oram and Vĭtek strains were obtained from Ms. A. Jamieson, Institute for Medical and Veterinary Science, Adelaide, Australia. Strain G. J. of *Naegleria fowleri* was kindly supplied by Dr. S. L. Chang, U.S. Environmental Protection Agency, Cincinnati, Ohio. All 3 strains of *Naegleria fowleri* were originally isolated from fatal cases of human primary amoebic meningoencephalitis, and were maintained axenically at 37° C in Fulton's A medium (Fulton, 1970).

For electron microscopy, the motile amoebae were fixed in 1% glutaraldehyde buffered with 0,1 M sodium cacodylate buffer, pH 7.2 at 4° C overnight. Three washes in cacodylate buffer at 4° C were carried out. Secondary fixation occurred in 1% OsO_4 buffered with cacodylyte buffer for 1 hour. This and subsequent steps of the preparation were done at 22–23° C.

Next, the samples were stained with 2% uranyl acetate in 10% acetone for 30 minutes. The amoebae were dehydrated through an acetone series to absolute acetone and embedded in Araldite. Grey or silver sections were cut on an LKB Ultratome III ultramicrotome. Electron microscope sections were stained with uranyl acetate followed by lead citrate. Viewing and photography were done on a Siemens 101 electron microscope. Precise measurements were taken from contact prints of the original negatives, or from appropriate enlargements.

Results

Thin and thick microfilaments morphologically similar to actin and myosin respectively, are present in the cytoplasm of *Naegleria fowleri* amoebae, strains G. J., Oram and Vĭtek. Although not illustrated, 3 other strains of *Naegleria fowleri*, HB-1, McMahon and PA-14 also displayed similar microfilaments. A relaxed, non-motile amoeba of the G. J. strain is illustrated in Figs. 1 and 2. Numerous thin microfilaments 5–7 nm in diameter and up to 256 nm long are randomly scattered through the cytoplasm and are concentrated near (and possibly attached to) the plasma membrane. A distinct, colaterally aggregated bundle of thin microfilaments measuring 1640 nm long and 180 nm wide is situated in the cytoplasm near the plasma membrane of a motile Oram strain amoeba (Fig. 3). Individual thin microfilaments in the bundle are 6 nm wide and up to 390 nm long, and are regularly spaced 17 nm apart. Similar bundles of thin microfilaments in close proximity to the plasma membrane have been seen in longitudinal and transverse section in the other strains of *Naegleria fowleri* amoebae examined.

The cytoplasm of the Vĭtek strain (Fig. 4) displays both thin and thick microfilaments. The thin microfilaments are 5–7 nm wide and up to 395 nm long. The thick microfilaments are short, thick rods measuring 17–19 nm in diameter and ranging from 152 to 190 nm in length, with an average of 176 nm. The appearance of the thick microfilaments doses not in any way suggest that they are an aggregation of the thin microfilaments, or any other cellular structure, but rather they appear to be a distinct type of microfilament.

Discussion

The results presented in this report are the first to demonstrate the existence of thin and thick microfilaments in situ in the cytoplasm of *Naegleria fowleri* amoebae. These microfilaments are distinct structures and cannot be mistaken for any other cell structure such as the endoplasmic reticulum. Microfilaments, particularly thick microfilaments are labile and glutaraldehyde is an essential fixative in their preparation for the electron microscope (Nachmias, 1968). Prolonged uranyl acetate staining enhances amoeboid microfilaments for visualization under the electron microscope (Michel and Schupp, 1974). A combination of extended contact with glutaraldehyde and double staining with uranyl acetate in pellet and in section has preserved and enhanced *Naegleria* microfilaments. *Naegleria* microfilaments are similar in size and shape to those found in other amoebae (see: Introduction for references). Possibly, the thin microfilaments of *Naegleria* are membrane bound as they are in *Acanthamoeba* (Pollard and Korn, 1972) and in *Chaos* (Comly, 1973). Bundles of colaterally aggregated thin microfilaments positioned near the plasma membrane occur in *Naegleria* as well as in a variety of other amoebae (Comly, 1973; Eckert and McGee-Russell, 1973; Haberey,

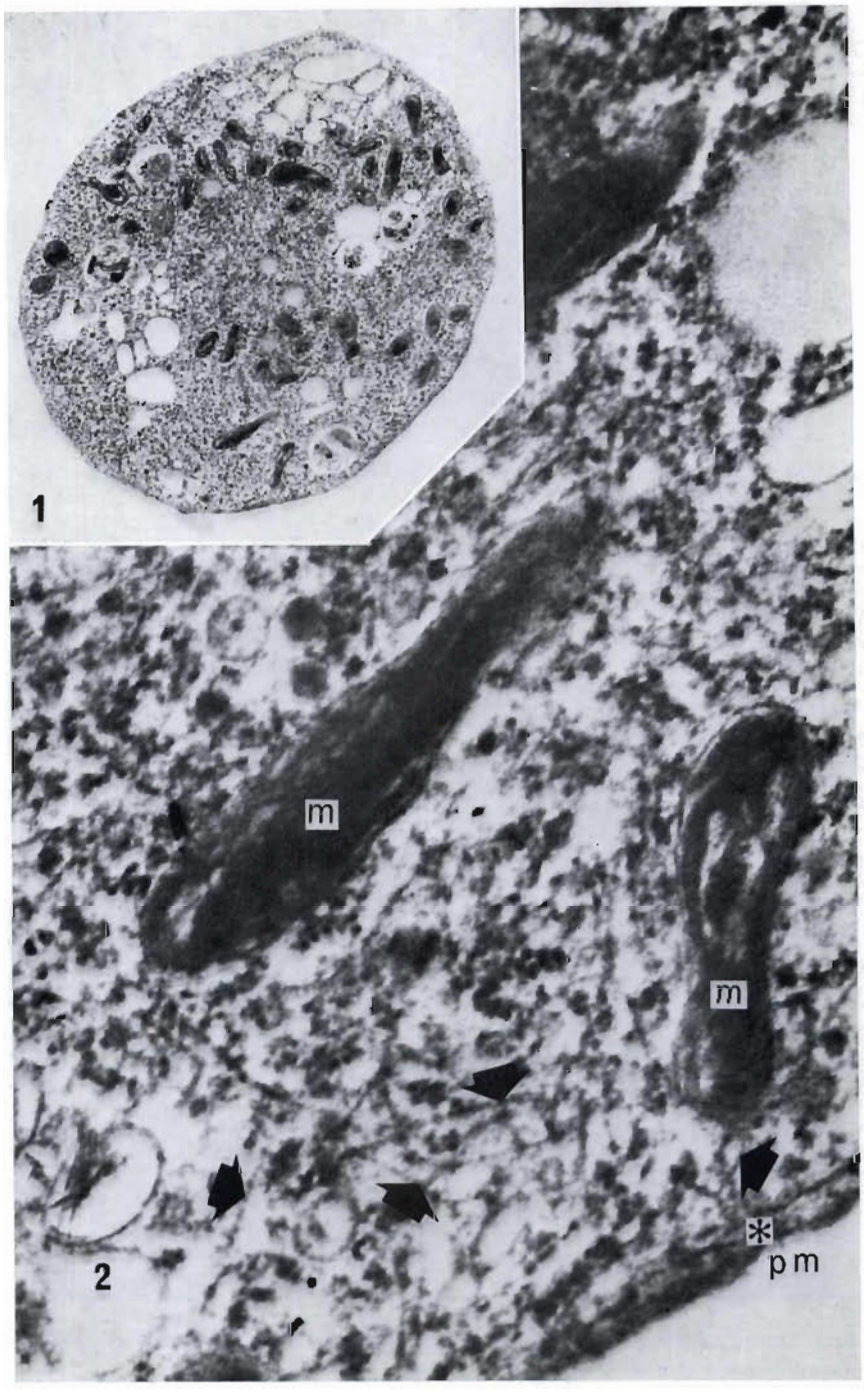
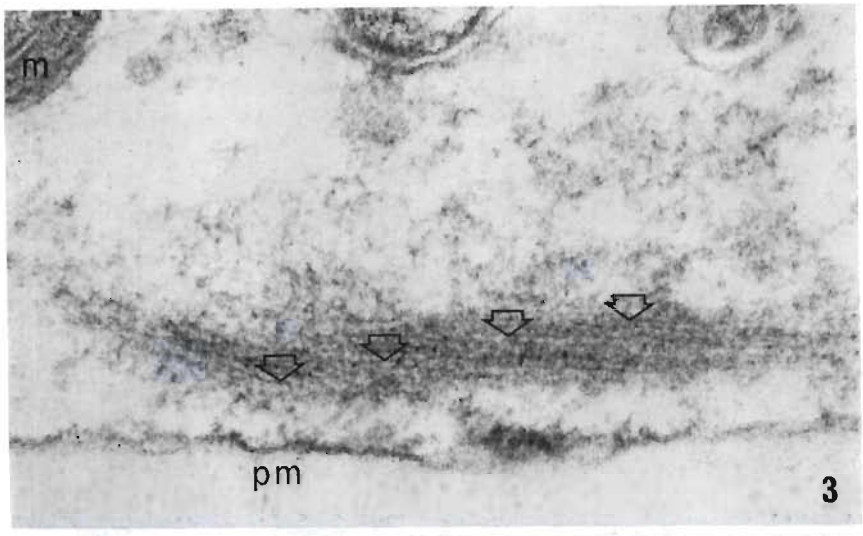


Fig. 1. *Naegleria fowleri*: Non-motile amoeba, G. J. strain. ×10,750

Fig. 2. *N. fowleri*: Detail of Fig. 1. Arrows mark some of the numerous 5–7 nm wide thin microfilaments randomly distributed in the cytoplasm. Asterisk indicates thin microfilaments in close proximity (and possibly attached) to the plasma membrane. ×103,200 *m* mitochondria, *pm* plasma membrane



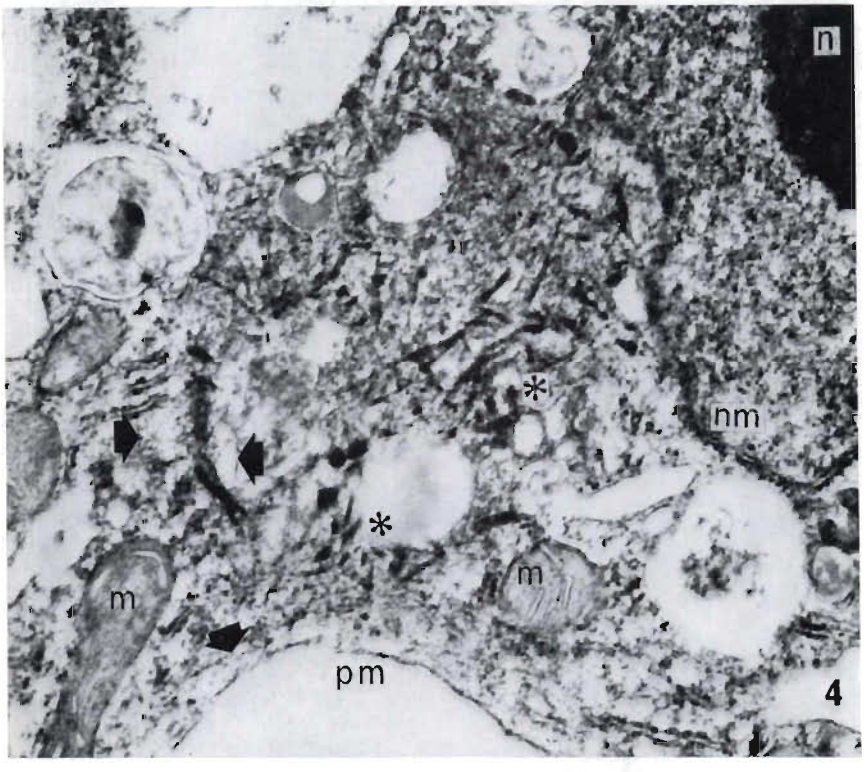


Fig. 3. *N. fowleri*: Detail of Oram strain amoeba cytoplasm. A bundle of colaterally aggregated thin microfilaments is seen in close proximity to the plasma membrane. Arrows mark individual microfilaments. ×61,250

Fig. 4. *N. fowleri*: Detail of Vĭtek strain amoeba cytoplasm. Individual thin microfilaments are marked by arrows while clusters of 17–19 nm thick microfilaments are indicated by asteriks. ×39,250, *m* mitochondria, *n* nucleolus, *nm* nuclear menbrane, *pm* plasma membrane

1973; Nachmias, 1964, 1968; Pollard and Ito, 1970; Michel and Schupp, 1974; Wohlman and Allen, 1968). The thick microfilaments of *Naegleria* are randomly oriented and do not appear to be attached to the plasma membrane, similar to those of *Amoeba proteus* (Pollard and Ito, 1970). The above observations are necessary prerequisites for theories of amoeboid motion based on modifications of the sliding filament model of muscular contraction (Huxley, 1973; Rinaldi et al., 1975). Durham (1974) proposed that in relaxed amoebae actin-like filaments are attached to the inner surface of the plasma membrane and are in random array with associated myosin-like filaments free in the cytoplasm. On contraction, these filaments would undergo synaeresis and a compact array just under the plasma membrane would result.

Rinaldi and Hrebenda (1975) using EGTA in their electron microscopic fixation procedure have shown overlapping arrays of thin and thick microfilaments in close proximity to the plasma membrane of *Amoeba proteus*. Huxley (1973) proposes that amoeboid motion is a result of active shearing between cytoplasmic filaments (myosin-like) and membrane attached filaments (actin-like). Recently, Rinaldi et al. (1975) have proposed a sliding filament theory of amoeboid motion based on actin and myosin filaments free in the endoplasm, while these microfilaments are organized and closely associated in the ectoplasm, and provide the structural basis for contractility of the ectoplasmic tube.

A protein complex similar to smooth muscle actomyosin and plasmodial myosin B has been isolated from *Naegleria* amoebae. This protein, which comprised 0,7% of the total cell protein, has solubility, ATPase and superprecipitation properties similar to other actomyosins (Lastovica and Dingle, 1971). Dissociation of the *Naegleria* actomyosin into its constituent actin and myosin has been accomplished by Sepharose 4B elution in the presence of ATP and Mg^{+2} ion. Microfilaments have been produced in vitro from ATP dissociated extracts of *Naegleria* actomyosin by the addition of 2,5 mM EDTA or EGTA (Lastovica, 1970).

It is suggested that the actin and myosin in *Naegleria* amoebae is organized in the form of thin and thick microfilaments respectively, and interaction between these microfilaments is the mechanical means of accomplishing amoeboid motion in *Naegleria*.

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Dr. A. J. Lastovica
Department of Zoology
University of Cape Town
Rondebosch, 7700
Cape Town, South Africa