STUDIES ON PATHOGENIC AND NON-PATHOGENIC SMALL FREE-LIVING AMOEBAE AND THE BEARING OF NUCLEAR DIVISION ON THE CLASSIFICATION OF THE ORDER AMOEBIDA†

By B. N. SINGH and S. R. DAS
Central Drug Research Institute, Lucknow, U.P., India

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[Plates 28 to 30]

CONTENTS

A systematic study of small free-living amoebae has been made under standardized and reproducible cultural conditions, and their pathogenicity has been tested in mice. *Naegleria aerobia*, *Hartmannella culbertsoni* and *H. rhysodes* are pathogenic; *H. castellanii*, *H. astronyxis*, *H. palestinensis*, *H. glebae*, *H. exundans*, *H. vermiformis*, *Schizopyrenus russelli*, *Didascalus thorntoni* and *Tetramitus rostratus* are

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non-pathogenic. Strains of *H. culbertsoni* and *H. rhysodes* are present in Indian soils. A classification of the order Amoebida Kent into families Schizopyrenidae Singh, Hartmannellidae Volkonsky, 1931 emend. Singh, 1952, and Endamoebidae (Calkins), based on nuclear division, is proposed, and the relation of this classification to previously defined families and genera of amoebae and its bearing on phylogeny are discussed. Metronidazole and other anti-amoebic drugs are ineffective against *N. aerobia* and *H. culbertsoni in vitro* and in meningo-encephalitis in mice.

I. Introduction

In the revised classification of the phylum Protozoa by Honigberg *et al.* 1964, the class Rhizopodea von Siebold, 1845, has been divided into subclasses Lobosia Carpenter, 1861, and Filosia Leidy, 1879. The order Amoebida Kent, 1880, has been included in the subclass Lobosia for naked, typically uninucleate amoebae, the majority of which are free-living, but many are parasitic. A system of classification of Amoebida based on sound characters and probable phylogenetic relationships has yet to be developed.

The classification of amoebae on form, locomotion and pseudopodial characters has not been found to be of diagnostic value, since these characters vary with the chemical and physical conditions of the environment. Thus Dobell (1914, p. 171) states that the general appearance of *Amoeba fluvialis* is somewhat like that of a small *proteus* amoeba, the pseudopodia at times are large and flattened, and unlike those of typical *A. proteus*, but at times they resemble those of *limax* amoebae with a single large anterior pseudopodium and slug-like creeping movements, and sometimes those of *A. vespertilio*. He could not separate any of these pseudopodial forms or movements as typical. Minchin (1922, p. 217) says that the characters of the pseudopodia may vary, not only in different phases of the life cycle, but even in the same phase under the influence of different media, and he quotes Verworn as having shown that the *limax* and the star-like *radiosa* forms, which appear so distinct that they were originally regarded as distinct species, can be changed into one another by altering the media. He also states that Doflein obtained similar form-changes in *A. vespertilio*, and had concluded that the body-form and pseudopodial character were inadequate for distinguishing *Amoeba* sp., since they depend on the environment and the nature of the medium.

Notwithstanding these comments, Schaeffer (1926) and Bovee (1953) have based their classification of amoebae on form, locomotion and pseudopodial characters (see Bovee & Jahn 1966, for the earlier literature); their views have received little support from protozoologists, but Page (1967*a*, *b*, 1968) has used them as generic criteria for the genera of *Hartmannella*, *Acanthamoeba*, *Flabellula*, *Rugipes* and *Hyalodiscus*.

Ray & Hayes (1954), for instance, say that in *Hartmannella astronyxis* lobopods, filopods and micropseudopodia may be present in the same individual at the same time. Broad flat lobopods are commonly present during active locomotion in individuals in a liquid environment and in contact with a smooth solid surface; in locomotion on an agar surface pseudopodia tend to be narrow and tapering, even extremely long and attenuated. In individuals suspended in the surface film or floating free, relatively short, narrow, radiating filopods are common; they are often slowly bent and moved about. When the oxygen tension is slightly reduced, the 'limax' shape is normally assumed; in salt concentrations above 0.5 % branched pseudopods are produced. Kudo (1959), in a critical review of Schaeffer's (1926) classification, remarks that a genus must be founded on distinctive diagnostic characters by which it is distinguished from other genera without any difficulty by any competent worker, and therefore rejects such 'ambiguous' characters as shape, locomotion and pseudopodial form as generic criteria.

As the characters of locomotion and pseudopodia are insufficiently stable, Wenyon (1926), Calkins (1933), Kudo (1966) and others have attempted to classify amoebae into families on the basis of such characters as the production of temporary flagella, their parasitic or free-living nature, and the presence of an accessory body (Nebenkörper; see Singh (1952) for the earlier literature). Although these systems are neither sound nor, probably, of any phylogenetic value, they are, unfortunately, still retained in most recent textbooks of protozoology and microbiology. *Entamoeba moshkovskii*, a free-living anaerobic amoeba discovered by Tshalaia (1941, 1947) in Moscow and subsequently by workers in Brazil, Britain, the U.S.A. and elsewhere, closely resembles *E. histolytica* morphologically, and casts doubt on the validity of a family set up for parasitic forms only. Moreover, since some strains of aerobic free-living amoebae belonging to the genera *Hartmannella* and *Naegleria* have been found to be pathogenic to animals and man (Culbertson, Smith & Minner 1958; Culbertson, Smith, Cohen & Minner 1959; Culbertson, Ensminger & Overton, 1965, 1968 *a*, *b*; Butt, Baro & Knorr 1968 *a*, *b*; Calicott *et al.* 1968; Carter 1968; Červa, Zimák & Novák 1969; Dwivedi & Singh 1965) it has become very difficult to decide whether amoebae are parasitic or not. Nor does the creation of such families as Bistadiidae or Dimastigamoebidae, to include only those forms that produce temporary flagella, seem to be justified in a rational classification of amoebae, nor does the possession of Nebenkörper seem to have much evolutionary significance.

It is becoming increasingly evident that amoebae, whatever their size and nucleation, and whether they are parasitic or not, can be divided into families based on the mode of nuclear division. This method has suffered in the past from difficulties in observing the stages of normal nuclear division and from the use of haematoxylin and non-specific aniline dyes to examine dividing nuclei; but the discovery of the Feulgen reaction has made it possible to locate the chromosomal chromatin (DNA) in the resting nucleus of amoebae, and to trace its behaviour during nuclear division.

Singh (1950) devised a culture method for the study of nuclear division in small free-living amoebae; he later (1952) combined this method with the Feulgen reaction to study nuclear division in nine species of free-living amoebae. Unfortunately, not all brands of basic fuchsin give satisfactory Feulgen reactions. Grübler's basic fuchsin gave excellent results with all species of amoebae examined by Singh (1952) and in *Entamoeba invadens* (Narasimhamurti 1964), but B.D.H. material (Singh 1952), Diamant fuchsin (Edward Gurr, London) and fuchsin basic (Hopkins and Williams) (Narasimhamurti 1964) were unsatisfactory.

On the basis of nuclear division demonstrated in this way, Singh (1952) created the family Schizopyrenidae for amoebae whose resting nucleus contains a more or less central Feulgen-negative nucleolus, which divides during mitosis to form 'polar masses'. Since this family was defined, *Heteramoeba clara* Droop, 1962, a free-living amoeba, has been found whose resting nucleus has several Feulgen-negative nucleoli, which during mitosis form 'polar masses'. In consequence the family Schizopyrenidae must be modified to include amoebae having several Feulgen-negative nucleoli.

Singh (1952) also created the family Hartmannellidae for the well-known genus *Hartmannella*. In this family the resting nucleus has either a single Feulgen-negative nucleolus or several such nucleoli; during mitosis the nucleolus or nucleoli disappear, and a spindle develops on which the chromosomes arrange themselves as an equatorial plate, as in higher animals and plants. Amoebae may be uninucleate or multinucleate, and no temporary flagella have been observed.

Because of their parasitic nature, a number of imperfectly studied amoebae have been put in

the family Endamoebidae; generic differentiation has been based on nuclear morphology. As amoebae in some genera of Endamoebidae have a type of nuclear division different from that of Schizopyrenidae and Hartmannellidae, it seemed worthwhile to find out whether Endamoebidae could be defined on the basis of their nuclear division rather than their parasitic nature.

II. Materials and methods

Isolation and culture of amoebae from soil and other sources

Singh (1946, 1955, 1960) has stressed that for the culture of protozoa that feed on bacteria from soil or other sources with a mixed microbial population, it is essential to use a medium, such as non-nutrient agar or silica gel, that discourages the growth of inedible bacteria and toxigenic micro-organisms, other than protozoa present in the inoculum, and to provide a suitable edible bacterium. Singh (1960) has found that *Aerobacter* sp. is eaten by a range of soil amoebae and amoeboid organisms. Since Culbertson *et al.* (1968*b*) and Butt *et al.* (1968*b*) have found that a *Naegleria* sp. from a fatal case of amoebic meningo-encephalitis did not grow in agar to which NaCl and antibiotics had been added, NaCl and antibiotics should be excluded from the medium.

The following method was used for the culture of amoebae from soils (see also Singh 1955). Agar is washed for 3 to 5 days in distilled water changed every half day; agar so treated discourages the growth of soil bacteria. About 15 ml of a 1.5 % (w/v) solution of this agar, sterilized at 103.5 kN/m^2 (15 lbf/in^2) pressure for 15 min, pH 6.6 to 7.0, is poured into a Petri dish and allowed to set. *Aerobacter aerogenes* or *Escherichia coli*, taken from 2 to 3-day-old nutrient agar slope cultures, is spread as a thick suspension on the agar suface in a circular patch *ca.* 2.5 cm in diameter. A fragment of soil is placed in the centre of the patch (the 'bacterial circle') and the plate is covered and incubated at 25 °C or at 37 °C for 5 to 10 days for the development of amoebae. The mixed population of different species of amoebae is then subcultured two or three times on fresh bacterial circles to get rid of other protozoa, fungi and other organisms.

For pure-line cultures of single species of amoebae, a single cyst or trophic form is washed a few times in sterile distilled water from micropipettes, and then transferred to a bacterial circle.

Pathogenicity of amoebae in mice

Albino 10 to 15 g mice, maintained at the Central Drug Research Institute and fed on bread and milk were used. Cultures 2 days old of a mixed population of amoebae from soil, maintained on *Aerobacter aerogenes* were harvested in distilled water, washed a few times in distilled water by centrifugation at 500 rev/min for 5 min to get rid of most of the bacteria, and resuspended in distilled water. The number of amoebae was determined by counting in a haemocytometer. The mice were anaesthetized with ether, and 0.03 ml of the suspension of amoebae, containing about 20 000 trophozoites, was given intranasally to each mouse. For control observations 0.03 ml of distilled water, containing about the same number of *A. aerogenes*, was given to each mouse. Very sick mice with symptoms of meningo-encephalitis were killed, and cultures for amoebae were made from brain and lungs on bacterial circles of *A. aerogenes* on agar plates. Mice surving 14 days were also killed, and cultures were made as above. Brain and lung sections were also examined for amoebae. For further study, pure-line cultures of amoebae were made from single cysts from infected tissues.

Strains of amoebae

The strains of amoebae used in this work were as follows:

(*a*) Nine strains of pathogenic *Hartmannella* isolated from Indian soils; FS (flower bed), GS (garden) and RS (Gomti river bank) were from Lucknow, and B-1–6 from Baroda soils (B-1–3 from different gardens, B-4–6 from cultivated fields).

(*b*) Six strains of *Hartmannella* (five, A 5 and 30, HN 3, 15, and 17 from American soils and one, A-1, a contaminant of a mammalian cell culture) and one strain (HB-1) of *Naegleria* from a human case of amoebic meningo-encephalitis, were obtained from Dr C. G. Culbertson.

(*c*) *Acanthamoeba polyphaga*, *Hartmannella exundans*, *H. vermiformis*, *Vahlkampfia jugosa* and *V. avara* were obtained from Dr F. C. Page, *H. astronyxis* from Dr K. M. G. Adam, and *A. castellanii*, *Mayorella palestinensis* and *Tetramitus rostratus* from Dr W. Balamuth. For *Schizopyrenus russelli*, *Didascalus thorntoni*, *Naegleria gruberi*, *H. rhysodes* (strains 14 and 15) and *H. glebae*, see Singh (1952).

Maintenance of amoebae

All strains of amoebae were maintained in pure-line culture on non-nutrient agar with *Aerobacter aerogenes*; *Hartmannella* A-1 and *Naegleria* HB-1 were grown at 37 °C and the others at 25 °C.

In-vitro *effect of metronidazole against pathogenic free-living amoebae*

About 5000 trophozoites of *Hartmannella* A-1 or *Naegleria* HB-1 from 24- to 48-h-old cultures with *A. aerogenes*, suspended in 0.25 ml water were placed in a number of hollow ground slides. After 30 to 40 min, when amoebae were actively motile and were feeding, a range of dilutions of the drug in 0.25 ml 0.1 % (w/v) agar in distilled water were added to the cultures. For controls, only 0.1 % agar was added to cultures. The cultures were incubated for 24 to 48 h in moist chambers in Petri dishes, and the amoebicidal end-point was determined by microscopy. If there was any doubt, subcultures with *A. aerogenes* were made on agar plates.

To determine the effect of the drug on the flagellate stage of *Naegleria* HB-1, large numbers of amoebae from 1- to 2-day-old cultures on agar plates were placed in distilled water in cavity slides. After 4 to 6 h, when numbers of flagellates were present, a range of concentrations of the drug was added, and the test completed as above.

Effect of anti-amoebic drugs against pathogenic free-living amoebae in mice

The following compounds were tested: emetine HCl (B.D.H., U.K.), chlorhexidine (Hibitane I.C.I., U.K.), found effective in intestinal amoebiasis in rats (Das, Saxena & Singh 1963), metronidazole (Flagyl, May and Baker, U.K.), paromomycin, chlorostrep, camoquin and chloroquin (all from Parke-Davis, U.S.A.), carbarsone (Abbot Laboratories, U.S.A.), entobex (Ciba, Switzerland), emetine bismuth iodide (Burroughs Wellcome, U.K.) and sulphonamide (Naarden, Holland).

Twenty thousand trophozoites from a young culture were injected intranasally into each mouse, and starting one day later the mice were fed with the drug for 5 days. Mice similarly infected but untreated were always kept as controls. All mice were kept for 14 days.

Cysticidal effect of metronidazole

About 10 000 viable sterile cysts of *Hartmannella* A-1, obtained by the method of Singh, Saxena & Iyer (1965) from cultures with *A. aerogenes* only, were suspended in tubes in 0.1 % (w/v) agar containing a range of concentrations of metronidazole. After 48 h they were thoroughly washed in distilled water, and their percentage excystment was determined in aqueous *A. aerogenes* extract by the method of Singh, Mathew & Anand (1958). Cysts were also stained with 0.125 % aqueous eosin solution to assess their viability; dead cysts were stained within a few seconds, whereas living cysts remained unstained up to 10 min.

Production of antisera against Naegleria *HB-1: the immobilization reaction*

Rabbits were bled before use for control sera.

Young and actively multiplying amoebae were washed in sterile distilled water and stored at −20 °C; a series of ten intravenous injections of 250 000 amoebae was given to each rabbit at intervals of 4 days, and the animals were bled by cardiac puncture for antisera 10 days after the last injection.

For the immobilization test, about 10 000 amoebae were suspended in 0.1 ml distilled water in each of a number of cavity slides. After the amoebae had settled down and were adhering to the glass, a range of twofold dilutions of inactivated antiserum in distilled water was added to the suspension. The mixtures were incubated at 37 °C when the amoebae were *Naegleria* HB-1, and at 25 °C when other amoebae were used in the tests. The reaction was recorded as positive when 90 % of the amoebae were immobilized after 20 to 30 min. For parallel normal control tests, inactivated normal serum was used.

Nuclear division in amoebae

Singh's (1950) culture method was used; amoebae were fixed in Carnoy's fixative and stained with iron-haematoxylin by the method of Singh (1952).

Excystment of amoebae

The stages of excystment were studied in hanging-drop preparations.

III. Nuclear division and other characters in small free-living amoebae

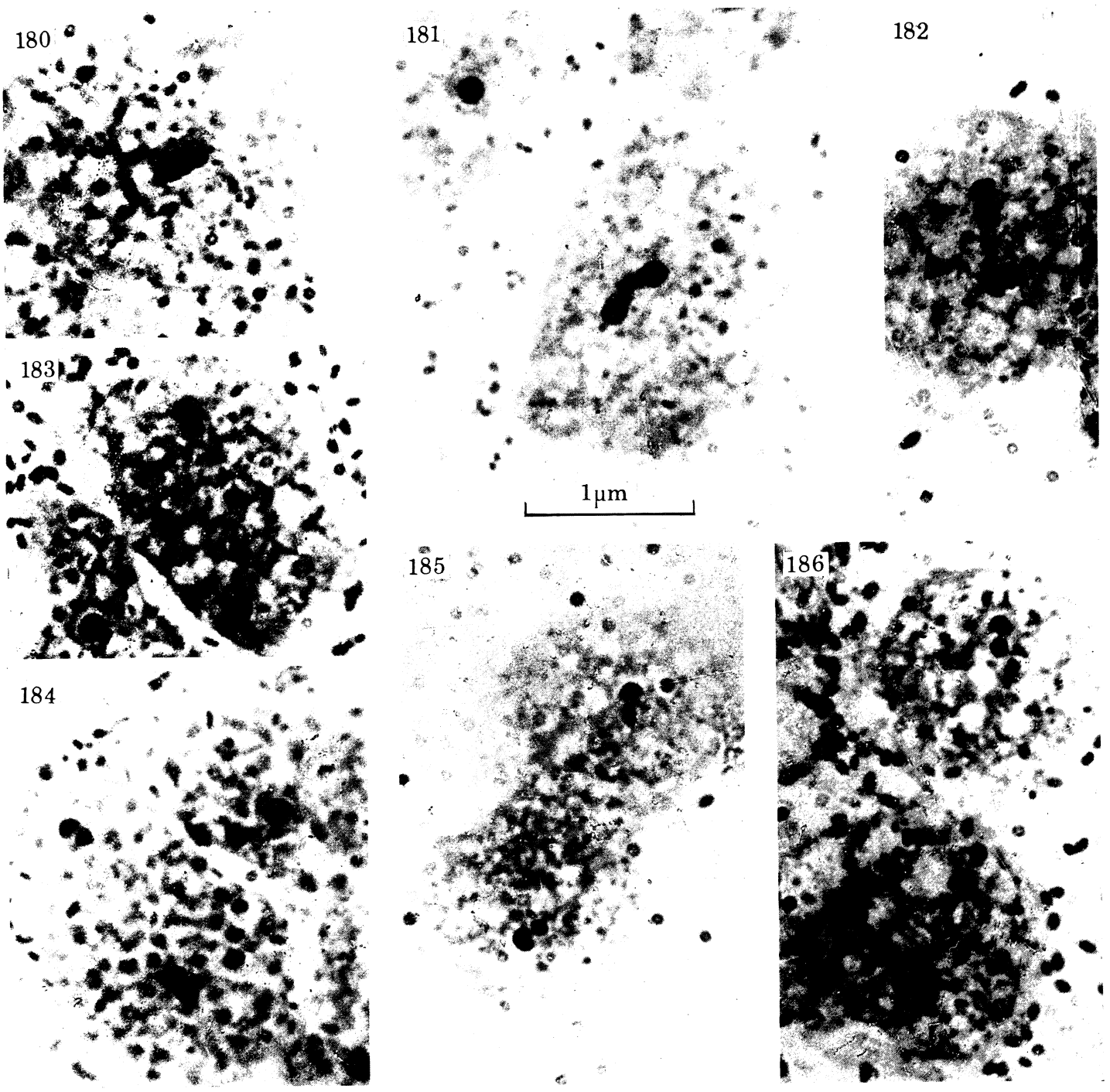
The genus ***Naegleria***

In both species of *Naegleria* here described pseudopodia are formed eruptively and *limax*-like forms are common. The ectoplasm and endoplasm are well defined; there is one contractile vacuole.

(1) ***Naegleria aerobia*** sp. nov.†

This description is based on *Naegleria* strain HB-1; the organism did not grow on non-nutrient agar containing 0.5 % (w/v) NaCl, but 0.1 to 0.2 % NaCl appeared to have no effect on its growth.

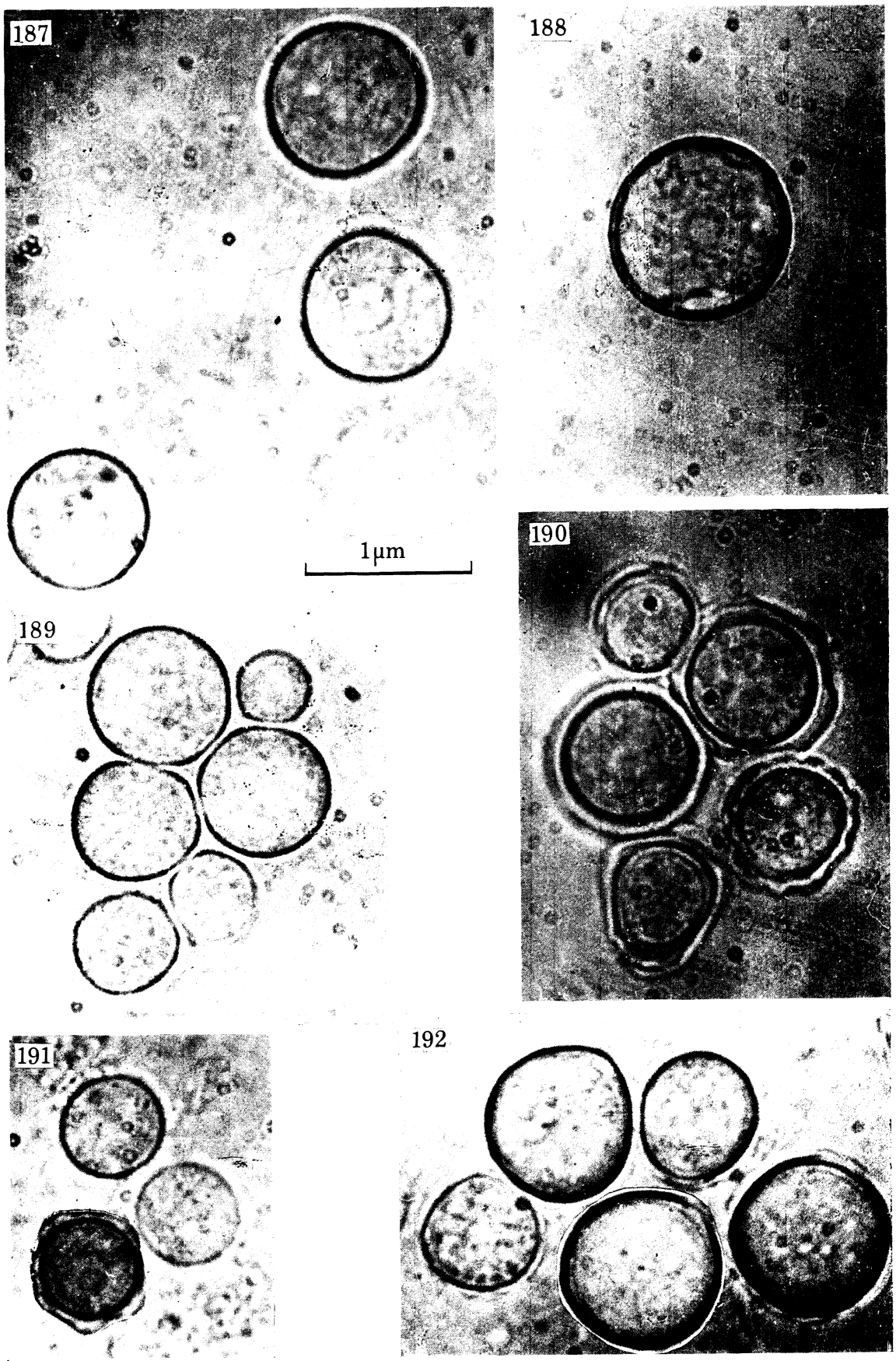
† This species has been created on the basis of the aerobic nature of the organism, in contrast with the anaerobic nature of other parasitic amoebae.



Successive stages in division of *N. aerobia*. Fixed in Carnoy and stained in iron-alum haematoxylin. The scale represents 10 μm and not 1 μm as marked.

FIGURE 180. Metaphase stage.
FIGURE 181. Formation of 'interzonal body'.
FIGURES 182 to 186. Division of 'interzonal body' into two equal halves and its subsequent behaviour during mitosis.

(*Facing p.* 440)



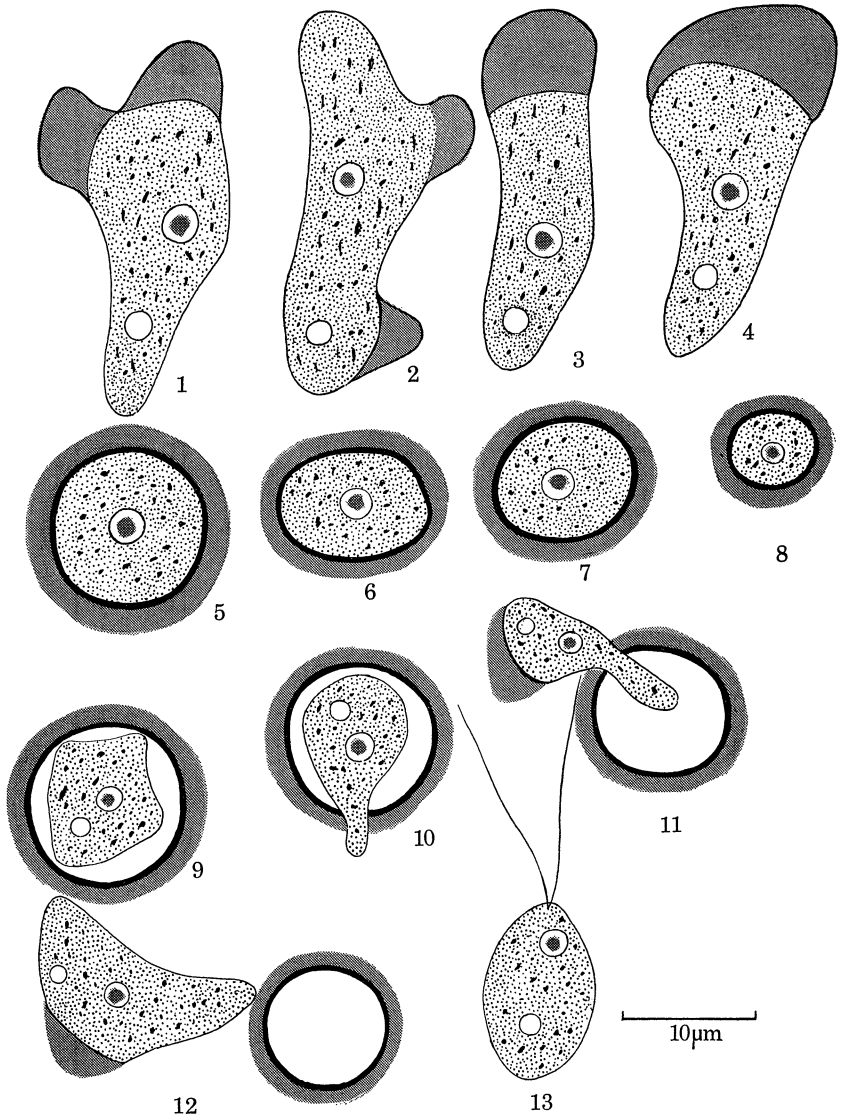
Cysts of amoebae in the living condition. The scale represents 10μm and not 1μm as marked.

FIGURE 187. *Naegleria aerobia* without any pores.
FIGURE 188. *N. gruberi* with pores.
FIGURE 189. *Didascalus thorntoni*.
FIGURE 190. *Schizopyrenus russelli*.
FIGURE 191. *S. jugosa*.
FIGURE 192. *Hartmannella culbertsoni*.

Morphology

The diameter of the amoebae in the rounded condition is *ca.* 10 to 15 μm.

The living cysts are rounded or slightly oval in outline and very variable in size, with a single wall, whose outside consists of a fairly thick transparent gelatinous layer. As Butt, *et al.* (1968*b*) also found, there are no obvious pores in the cyst wall (figures 5 to 8; figure 187, plate 29).



Naegleria aerobia. FIGURES 1 to 13 drawn in the living condition.
FIGURES 14 to 27 fixed in Carnoy and stained with iron–alum haematoxylin.
FIGURES 1 to 4 trophic forms; 5 to 8 cysts; 9 to 12 excystment stages; 13 flagellate stage.

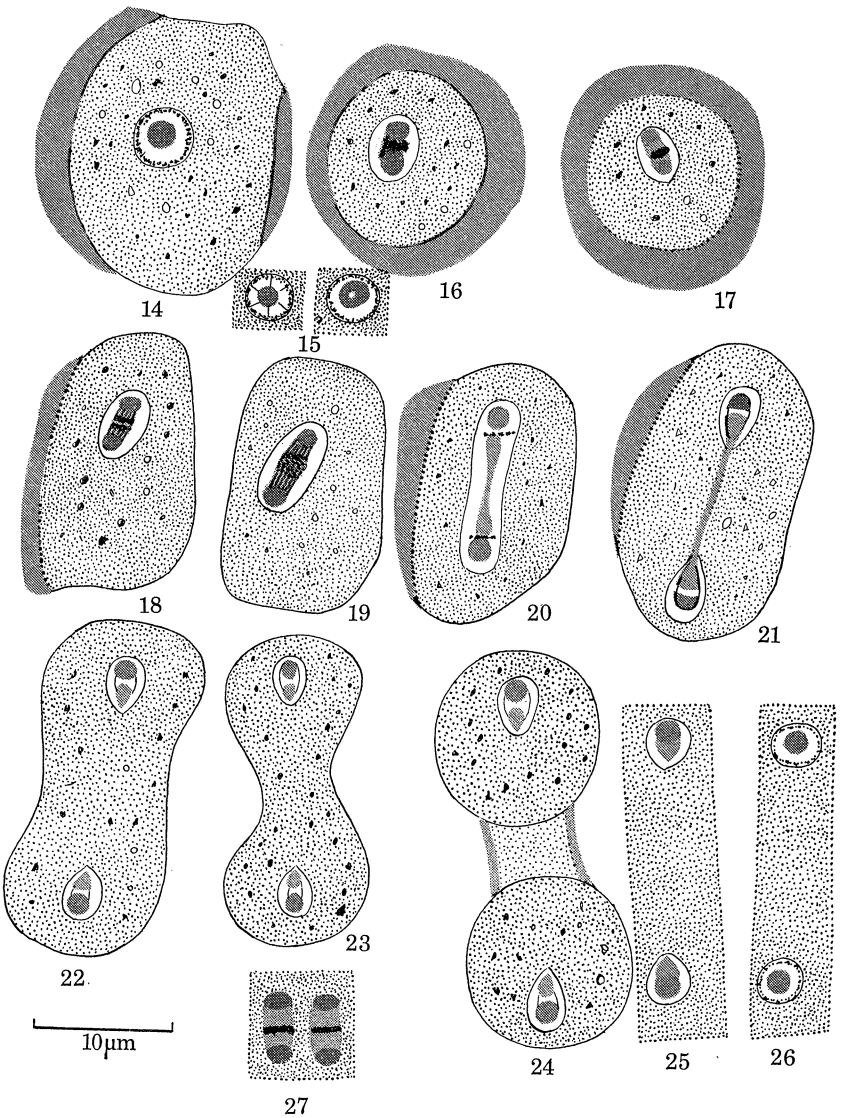
Culbertson *et al.* (1968*b*) observed a pore-like structure in the wall of a minority of cysts. There is a single nucleus, with a nucleolus, but no chromatic granules can be distinguished in unstained cysts.

As there are no preformed pores in the cyst wall, some digestion of the cyst wall probably occurs, to allow passage of the amoeba during excystment (figures 9 to 11). The outline of the cyst wall can be clearly seen after excystment (figure 12).

The flagellate stage is readily produced by incubating large numbers of actively growing amoebae, from 24-h-old cultures from non-nutrient agar plates, in distilled water in cavity slides in moist chambers in Petri dishes at 37 °C; by *ca.* 4 to 6 h, 70 to 80 % of amoebae have changed into the flagellate stage. These have a rigid oval shape, and move actively in water with the aid of two equal flagella, slightly longer than the body, arising from the anterior end. There is a single posterior contractile vacuole and a single nucleus (figure 13).

The resting nucleus

In the living amoeba the resting nucleus consists of a distinct central spherical nucleolus surrounded by a clear zone. Chromatin granules are not distinctly seen (figures 1 to 4). In iron-haematoxylin preparations the nucleolus and chromatin granules near the nuclear membrane are readily demonstrable (figures 14 and 15). Occasionally one or two unstained patches



FIGURES 14, 15. Ordinary individual and the structure of three resting nuclei.
FIGURES 16 to 26. Successive stages in division.
FIGURES 19 to 25. Showing the formation of 'interzonal body' and its division into two equal halves.
FIGURE 27. Two nuclei dividing at the same time in an amoeba.

can be seen inside the nucleolus, and in some nuclei thread-like structures radiate from it (figure 15). The nucleolus stains more deeply than the chromatin granules, and resists decolorization with iron alum longer than the chromatic substance. There is usually one nucleus in each amoeba; two are rare.

Mitotic division

The behaviour of the nuclear structures during nuclear division has been studied in detail in *N. gruberi* by Rafalko (1947), Singh (1952) and Chang (1958). Nuclear division in *N. aerobia* is similar to that in *N. gruberi*. In this present paper the observations are based on iron-haematoxylin-stained preparations, used mainly to trace the origin and behaviour of 'interzonal bodies' during mitosis.

Prophase

The amoebae do not round off during division. At the beginning of nuclear division the nucleus swells and the nucleolus elongates. The chromatin granules lie beside the nucleolus (figure 16); they then begin to fuse and the nucleolus assumes a dumbbell shape and divides into two halves the 'polar masses'.

Metaphase

After polar masses are formed, a spindle connecting them can be seen, though there are no distinct spindle fibres, and a solid mass of chromatic material, without distinguishable chromosomes, occupies the position of the equatorial plate (figure 17; figure 180, plate 28).

Anaphase

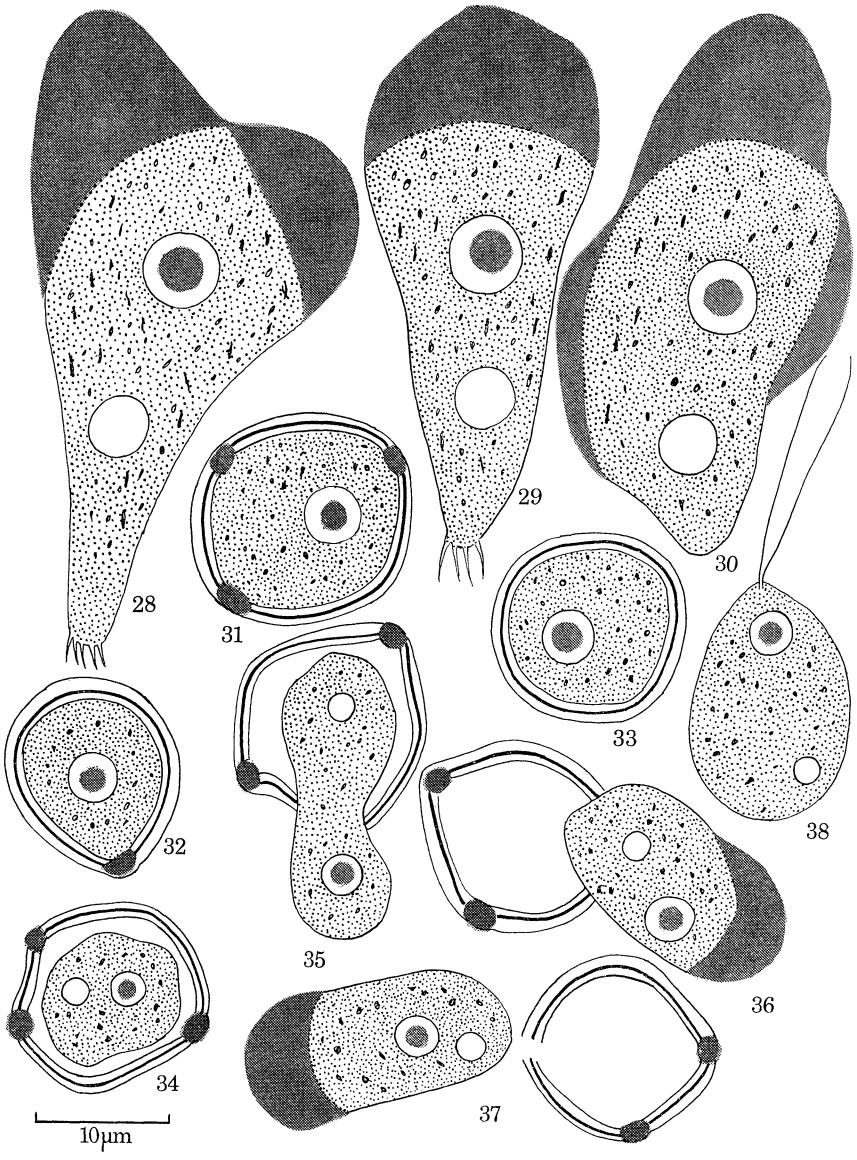
The band of chromatic material divides into two (figure 18), and each half moves towards its pole. When the chromatic material has reached its pole, granular material probably derived from the nucleolus during its division into polar masses (Chang 1958) and shown to be non-chromatic by Rafalko (1947) in *N. gruberi*, is distinctly seen lying half-way between the polar masses. This 'interzonal body' increases in size and divides into two (figure 19 and 20; figures 181 and 182, plate 28). The nuclear membrane persists during division; it becomes elongated and constricts, giving rise to two daughter nuclei (figures 20 and 21).

Telophase

After the nucleus has divided into two, the amoeba becomes elongated and constricts to give rise to two daughter individuals. In the process the thread-like structure joining the two interzonal bodies is ruptured, and each interzonal body fuses with its polar mass to form the nucleolus (figures 22 to 26; figures 183 to 186, plate 28). In a few amoebae two nuclei divide at the same time (figure 27).

Polar caps

Ford (1914) described achromatic caps between the ends of the elongated karyosome and the nuclear membrane during division in a free-living *limax* amoeba. Polar caps were readily demonstrated in *N. gruberi* by Rafalko (1947), Singh (1952) and Chang (1958); they appear to be absent in *N. aerobia*, though Culbertson *et al.* (1968*b*) and Butt *et al.* (1968*b*) found them occasionally in that species. There are no centrioles in *N. aerobia* at any stage of nuclear division, as is also the case in *N. gruberi* (Singh 1952; Chang 1958; Page 1967*a*).



Naegleria gruberi. FIGURES 28 to 38 drawn in the living condition.
FIGURES 28 to 30 trophic forms; 31 to 33 cysts; 34 to 37 excystment stages; 38 flagellate stage.

Immobilization reaction

Inactivated antiserum against *N. aerobia* immobilized *N. aerobia* at a dilution of 1 in 32, and also agglutinated it. Recovery of the amoebae was much slower when they were washed after exposure to higher concentrations of antiserum than after exposure to lower concentrations. Inactivated normal sera had no immobilizing effect. Sera produced against *N. aerobia* had no immobilizing effect on *N. gruberi* even at a dilution of 1 in 2, nor did they effect *Didascalus thorntoni*, *Tetramitus rostratus* or *Schizopyrenus russelli* at that concentration.

Critical comment

The genus *Naegleria* was distinguished by Singh (1952) from other members of the family Schizopyrenidae by its possession of polar masses and interzonal bodies during mitosis, and by

having a temporary flagellate stage. Culbertson *et al.* (1968*b*) and Butt *et al.* (1968*b*) had no difficulty in finding the interzonal body in *N. aerobia*, and placed it in the family Schizopyrenidae. We have examined a few hundred anaphase and telophase stages of *N. aerobia*; an interzonal body or bodies were invariably present.

Pittam (1963) and Page (1967*a*) consider that interzonal bodies are not a constant feature of nuclear division in *N. gruberi*, though they are usually present. Page (1967*a*) takes the view that the occurrence of interzonal bodies is too uncertain and unimportant to justify generic separation, and suggests that the genus *Naegleria* should be defined so as to include all amoebae with promitosis and a temporary biflagellate stage. As Rafalko (1947), Singh (1952) and Chang (1958) all found that interzonal bodies were consistently present in dividing *N. gruberi*, it seems likely that Pittam's (1963) and Page's (1967*a*) failure to find them consistently was due to the use of faulty techniques.

(2) ***Naegleria gruberi*** (Schardinger, 1899)

The morphology, the cystic and the flagellate stages and nuclear division of *N. gruberi* have been described by Rafalko (1947), Singh (1952), Chang (1958), Page (1967*a*) and others.

Morphology

The amoebae (figures 28 to 30) in rounded forms are 15 to 30 μm in diameter.

The great majority of the cysts are pierced by one or more pores plugged with some structureless substance (figures 31 to 33), through one of which the amoeba escapes during excystment; after this the pore can be seen (figures 34 to 37).

Distinguishing characters for N. gruberi *and* N. aerobia

(1) Under similar cultural conditions and with similar food supply, the trophic, cystic and flagellate (figure 38) forms of *N. gruberi* are larger than those of *N. aerobia*.

(2) *N. gruberi* grows best at 24 to 25 °C and can tolerate 0.5 % NaCl incorporated in non-nutrient agar. *N. aerobia* grows best at 37 °C and cannot tolerate 0.5 % NaCl.

(3) *N. gruberi* cysts have one or more pores, through one of which the amoeba escapes during excystment. *N. aerobia* cysts have no pores, there is an outer thick gelatinous layer (cf. figures 187 and 188, plate 29); during excystment some digestion of the cell wall occurs.

(4) *N. gruberi* and *N. aerobia* are serologically distinct.

(5) *N. gruberi* is non-pathogenic, *N. aerobia* pathogenic.

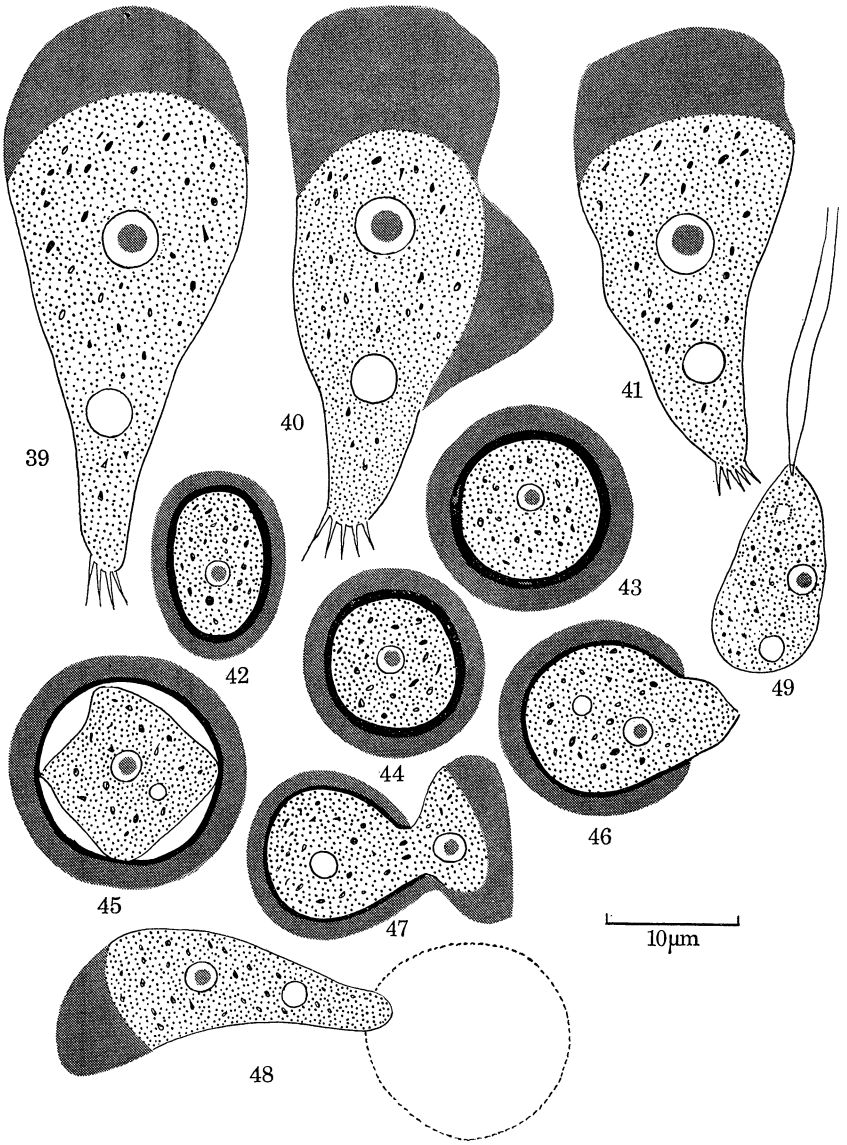
The genus ***Didascalus***

(3) ***Didascalus thorntoni*** Singh, 1952

The morphology, cystic and flagellate stages and nuclear division of *D. thorntoni* have been studied by Singh (1952).

Morphology

The trophic, cystic and flagellated stages and the mode of excystment of *D. thorntoni* are shown in figures 39 to 49, and in figure 189, plate 29. In the rounded state the amoebae are 15 to 20 μm in diameter. The cysts have a single wall with a gelatinous outer layer. During excystment the amoebae gradually detach themselves from the cyst wall, and an active contractile vacuole appears. No preformed pore can be seen in the cyst, and some digestion of the cyst wall appears to occur (figures 45 to 47). The cyst wall is eventually dissolved after emergence of the amoeba (figure 48).



Didascalus thorntoni. FIGURES 39 to 49 drawn in the living condition.
FIGURES 39 to 41 trophic forms; 42 to 44 cysts; 45 to 48 excystment stages; 49 flagellate stage.

Remarks

The genus *Didascalus* was erected by Singh (1952) for amoebae possessing polar masses without interzonal bodies, and having a temporary flagellate stage. According to Page (1967*a*) this genus is a synonym of *Naegleria*; as interzonal bodies are constantly present in both *N. aerobia* and *N. gruberi*, it seems reasonable to separate the genera *Naegleria* and *Didascalus*, both of which have temporary flagella, on the basis of presence or absence of interzonal bodies.

The genus **Schizopyrenus**

In both species of *Schizopyrenus* described here pseudopodia are formed eruptively and *limax*-like forms are common. The ectoplasm and endoplasm are well defined; there is one contractile vacuole.

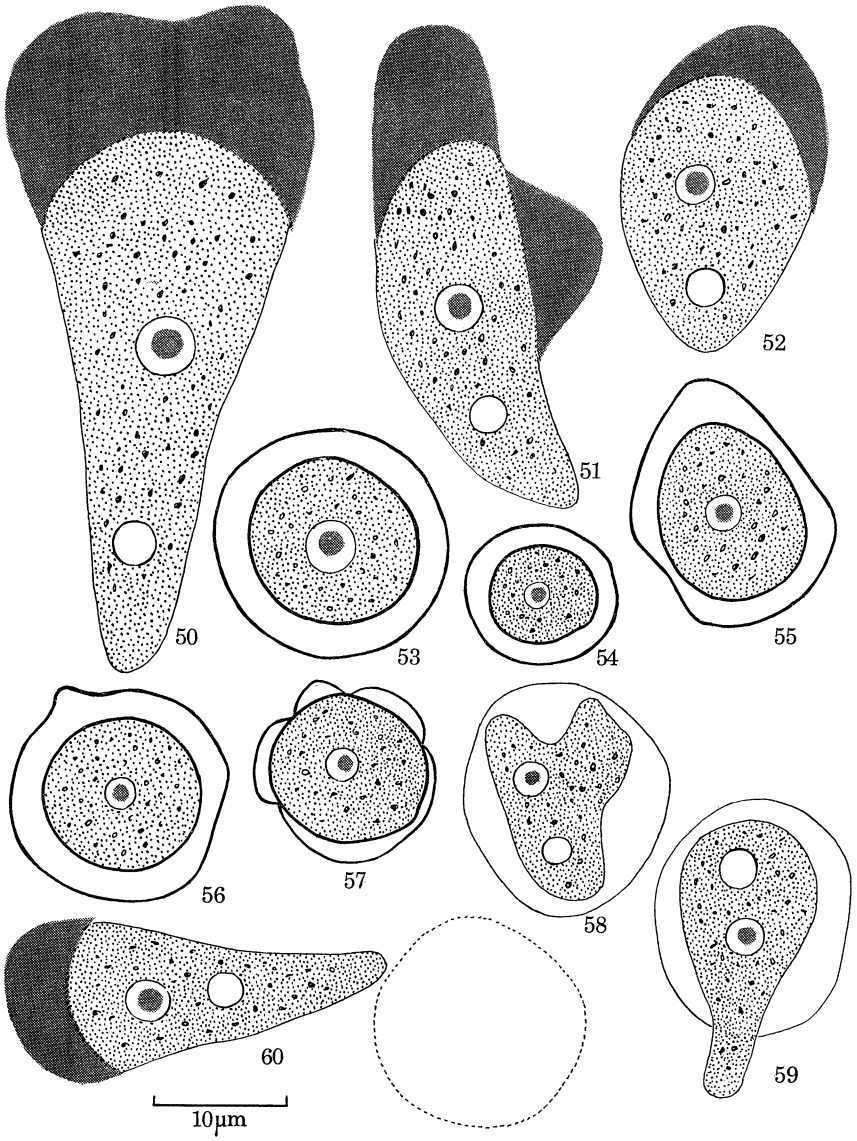
(4) ***Schizopyrenus russelli*** Singh

A brief description of the morphology, cyst character and excystment of *S. russelli* was given by Singh (1952); he points out that the resting nucleus in living amoebae and in stained preparations and the stages of nuclear division are indistinguishable from those of *D. thorntoni*.

Morphology

The amoebae (figures 50 to 52) in rounded forms are *ca.* 15 to 23 μm in diameter.

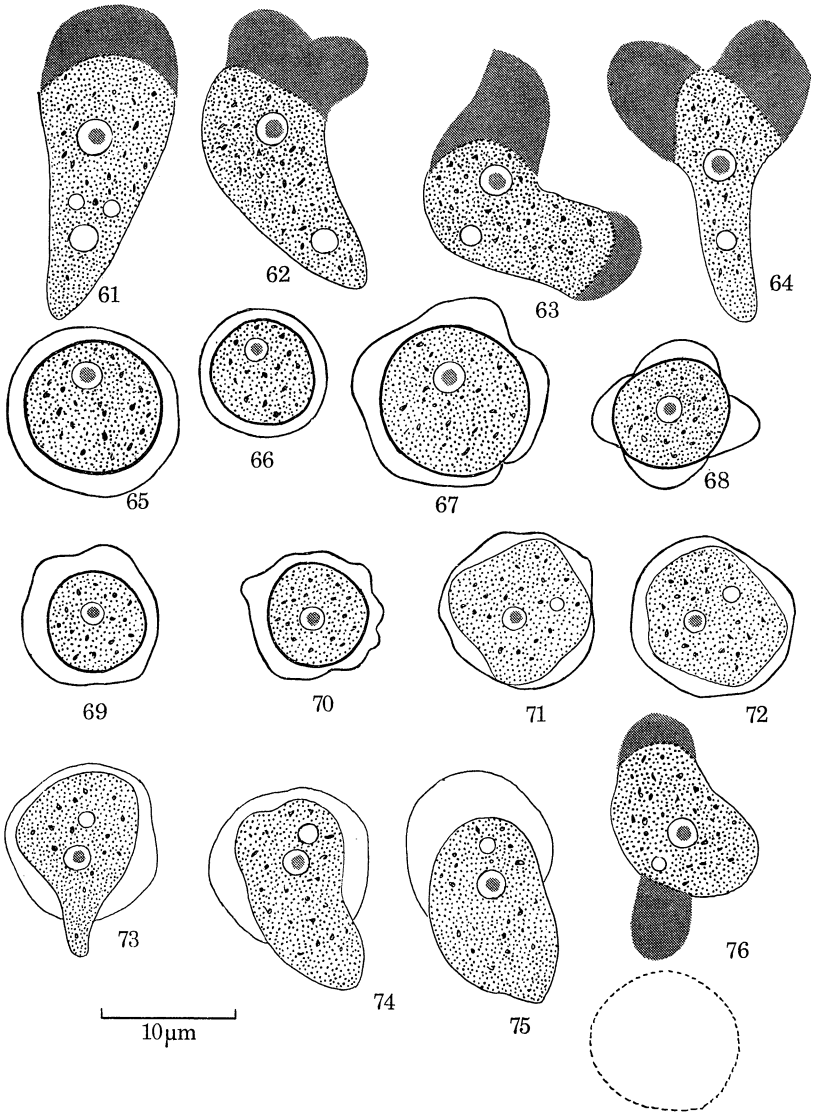
Singh (1952) described the living cysts as spherical, and very variable in size. Each cyst has two definite walls; in some the outer wall is thrown into irregular waves (not figured by Singh (1952). In recent cultures of *S. russelli* with *Aerobacter aerogenes* we have frequently found that the outer wall of the great majority of the cysts has this irregular wavy appearance, and in some of



Schizopyrenus russelli. FIGURES 50 to 60 drawn in the living condition.
FIGURES 50 to 52 trophic forms; 53 to 57 cysts; 58 to 60 excystment stages.

the cysts the inner and outer wall are on contact at several points (figure 55 to 57; figure 190, plate 29). It was thought that the culture was contaminated with another amoeba, but a clone culture made from a single rounded cyst with two walls (figures 53 and 54; figure 190, plate 29) produced cysts of both types. Sometimes the rounded cysts predominate, at other times the irregular wavy forms. Other workers in this Institute have had similar experience with *S. russelli*. The factors responsible for the production of cysts with wavy ectocysts are being studied.

Crump (1950, p. 17) states that 'Amoeba 4' (*S. russelli*) forms a double-walled cyst and excystment takes place in two stages: first the inner wall disappears and a small amoeba moves freely within the outer wall; then the outer wall gives way and the amoeba emerges. If the outer wall remains impenetrable, as often happens in unfavourable circumstances, the amoeba dwindles away and finally dies. Our figure 58 and figure 93*e* of Singh (1952) show that the inner wall first disappears and an amoeba with a single contractile vacuole moves freely inside the outer wall. It then comes out by digestion of the outer wall, which is eventually dissolved (figure 59 and 60).



Schizopyrenus jugosa (*Vahlkampfia jugosa*). FIGURES 61 to 76 drawn in the living condition. FIGURES 61 to 64 trophic forms; 65 to 70 cysts; 71 to 76 excystment stages.

Critical comment

Singh (1952) erected the genus *Schizopyrenus* in the family Schizopyrenidae for amoebae possessing polar masses during nuclear division but having no temporary flagella. Page (1967*a*), in agreement with Calkins (1913), thinks that these amoebae should be included in the genus *Vahlkampfia* (see remarks on earlier systems of classification of amoebae included in the family Schizopyrenidae).

Page (1967*a*) has described *V. jugosa* sp. nov., as a form with a double-walled cyst, frequently with a hilly or mound-like appearance of the ectocyst. The morphology, the characters of the cyst, the nuclear division, and the mode of excystment (figures 61 to 76; figure 191, plate 29), are exactly similar to those of *S. russelli*, though movement of the amoeba within the ectocyst is not clear as in that species.

We have found that in similar cultural conditions and with the same food supply, *V. jugosa* is smaller than *S. russelli* (diameter of rounded forms of *V. jugosa* 8 to 13 μm, of *S. russelli* 15 to 23 μm).

Page (1967*a*) found no uroidal filament in *V. jugosa*, and says that such filaments were shown in one trophozoite of *S. russelli* by Singh (1952). Careful examination of a large number of trophozoites of *S. russelli* has shown that these filaments are absent. In our opinion the presence or absence of uroidal filaments has no diagnostic value (see also Kudo 1959).

We suggest that in accordance with the system proposed by Singh (1952) *V. jugosa* should be transferred to the genus *Schizopyrenus*.

(5) ***Schizopyrenus atopus*** Singh, 1952

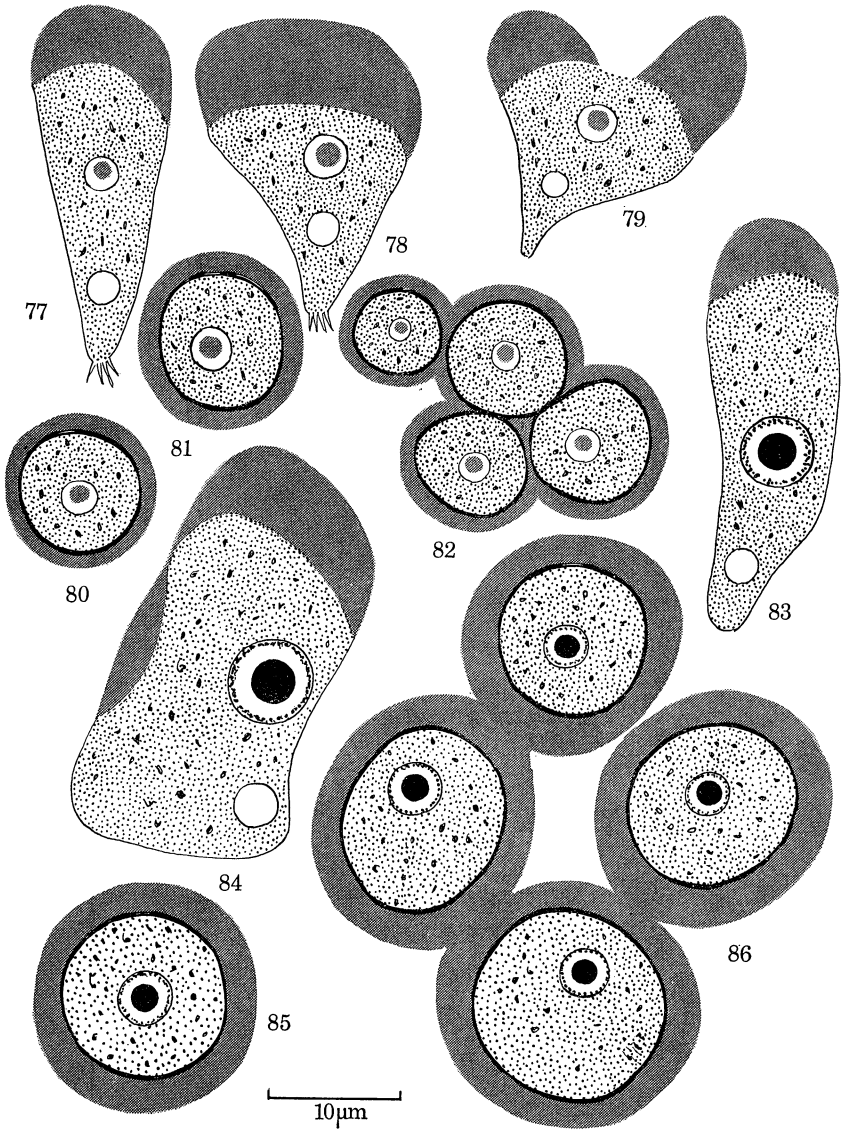
The morphology, cyst character and nuclear division of *S. atopus* are described by Singh (1952). Unfortunately the culture of this amoeba has been lost. In the rounded state the amoebae are *ca.* 15 to 30 μm in diameter. The cysts are usually rounded or spherical, with a single wall; the outside of the cyst consists of a transparent gelatinous layer (figures 85 and 86) as in *D. thorntoni* and *N. aerobia*. The outline of the cyst wall is sometimes irregular, depending on the grouping of amoebae during encystment.

Remarks

Page (1967*a*) has described *Vahlkampfia avara* sp.nov., distinguishing it from other *Vahlkampfia* spp. by the tendency of the gelatinous coating of the cyst to collect debris. It is exactly like *S. atopus* in morphology and cyst character (cf. figures 77 to 82 and 83 to 86), but is smaller in size (diameter of rounded forms, *ca.* 8 to 12 μm). It should therefore be called *Schizopyrenus avara*.

Earlier systems of classification of amoebae included in the family Schizopyrenidae

The earlier literature on the classification of amoebae possessing polar masses during mitosis is so confused that a detailed review is of little value (see Singh 1952, for the earlier literature). Vahlkampf (1905) was the first to observe polar masses during division of a *limax* amoeba. From his figs. 13 and 18 to 21, plate 6, it is certain that interzonal bodies were present. According to Chatton & Lalung-Bonnaire (1912) cysts of *Vahlkampfia* spp. have a perforated wall (see also Kudo 1966). From the nature of nuclear division and of the cyst wall we may conclude that Vahlkampf was dealing with *Naegleria gruberi* or some other *Naegleria* sp. with both amoeboid and flagellate forms. As Alexeieff (1911) pointed out, Vahlkampf's measurements for both



Schizopyrenus avara (*Vahlkampfia avara*). FIGURES 77 to 82 drawn in the living condition.
FIGURES 77 to 79 trophic forms; 80 to 82 cysts.

Schizopyrenus atopus. FIGURES 83 to 86 fixed on Carnoy and stained with iron-alum haematoyxlin.
FIGURES 83 and 84. Ordinary individuals and the structure of the resting nucleus.
FIGURES 85 and 86. Cysts.

amoebae and cysts should be multiplied by 8 or 10 times, thus giving 7.5×15 to 40 μm with nuclei 3 to 5 μm in diameter for amoebae, and cysts 15 μm in diameter.

So far no amoeba with interzonal bodies but no flagellate stage has been reported. Vahlkampf (1905) did not provide the conditions necessary for flagellation to occur, and consequently did not record the temporary flagellate stage. Gläser (1912) figured distinct interzonal bodies in *N. tachypodia*, but did not record the temporary flagellate stage, but Pietschmann (1929) had no difficulty in demonstrating it, though she wrongly placed the organism in the genus *Vahlkampfia*.

Calkins (1913), unaware that Vahlkampf (1905) was dealing with *Naegleria* sp., suggested that amoebae possessing polar masses but no flagellate stage should be included in the genus *Vahlkampfia* and those with both polar masses and a flagellate stage in the genus *Naegleria*. If the

family Vahlkampfidae, based on the type genus *Vahlkampfia* (Jollos 1917; Zulueta 1917) is recognized, as has been done by Page (1967*a*) and others, it will then include only those amoebae that have a temporary flagellate stage; for these amoebae Kudo (1966) has erected the family Naegleridae. It seems more logical to erect a family for amoebae possessing polar masses and to include in it amoebae with and without temporary flagella. If an amoeba is found that possesses polar masses, interzonal bodies and no temporary flagellate stage, the genus *Vahlkampfia* will have to be recognized, and included, along with *Naegleria*, *Didascalus*, *Schizopyrenus* and a few other known genera of amoebae in the family Schizopyrenidae (see p. 467).

The genus **Hartmannella**

In all the species of *Hartmannella* described here, hanging-drop preparations show hyaline, tapering and filamentous projections, which usually develop from the anterior edge of a lobopodium, and are resorbed into the advancing amoeba. The ectoplasm and endoplasm are well defined; there is one contractile vacuole.

(6) ***Hartmannella culbertsoni*** sp. nov.†

The description given here is based on *Hartmannella* A-1 described as a contaminant of a mammalian cell culture by Culbertson *et al.* (1959).

Morphology

The amoebae (figure 87 to 89) in rounded forms are 12 to 25 μm in diameter. They are usually uninucleate, though two nuclei are occasionally found.

Living cysts are rounded or oval and very variable in size (figures 90 to 94; figure 192, plate 29), and have two walls, In some cysts the outer wall is nearly circular, in others it is irregular in outline. The great majority of the cysts are perforated by one or more pores or opercula plugged by a structureless substance as in *N. gruberi*; a few have no pores (figure 91). The inner wall is in contact with the outer at the point of the operculum (figures 90, 92 to 94). The nucleolus is very distinct, but no chromatin granules could be made out. During excystment the amoeba escapes through a pore (figures 95 to 98). The cyst wall outline is still visible after the amoeba has emerged.

Attempts to produce a temporary flagellate stage have completely failed.

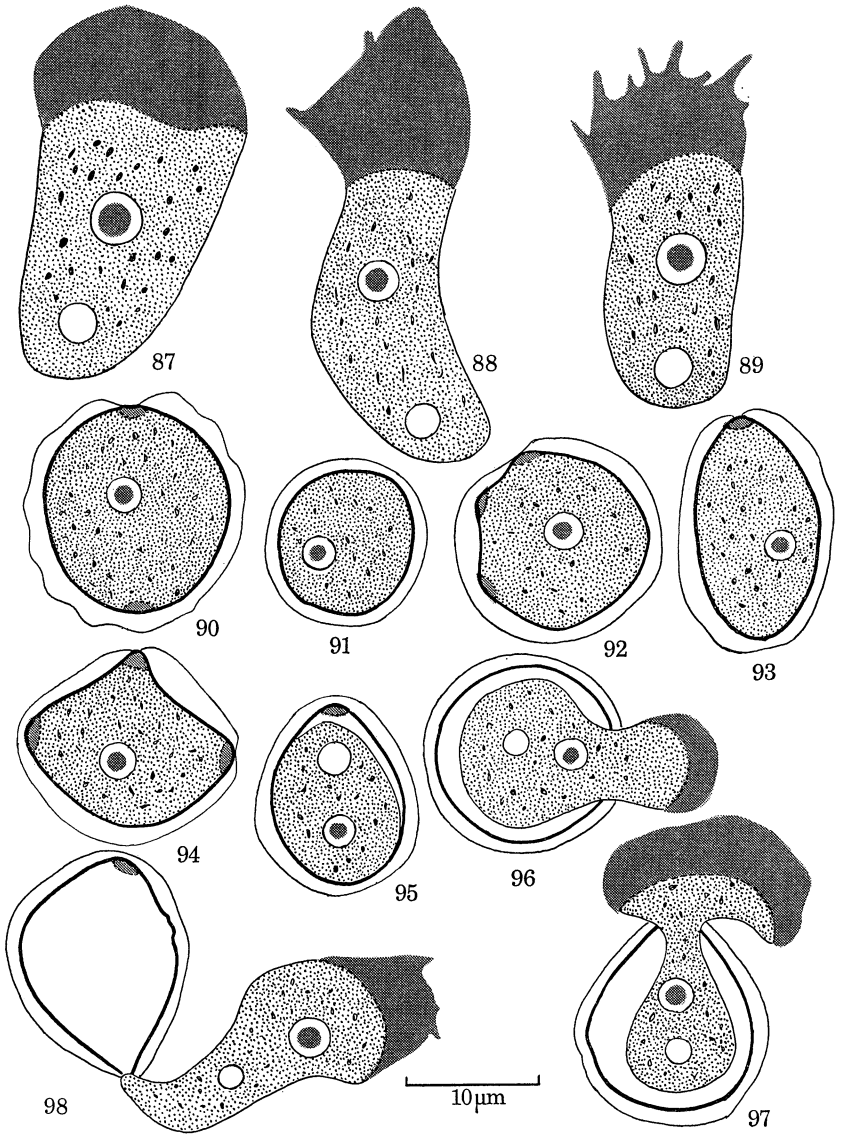
The resting nucleus

In live amoebae the nucleus contains a fairly large nucleolus surrounded by a clear zone (figures 87 to 89); no chromatin granules can be seen. In iron-haematoxylin preparations the nucleolus and the chromatin granules, which are near the nuclear membrane, are distinctly seen, as, in some nuclei, are thread-like structures radiating from the nucleolus (figure 99). The nucleolus stains more deeply than the chromatic granules and resists decolorization with iron alum much longer than the chromatic substance. There is usually one nucleus in each amoeba; two (figure 111) are rare.

Mitosis

The form of nuclear division, as shown in iron-haematoxylin preparations, is similar to that in *H. glebae* and *H. rhysodes* (Singh 1952). The amoebae become rounded and motionless at the beginning of nuclear division.

† This species has been named in honour of Dr C. G. Culbertson, whose pioneer work has changed the concept of amoebiasis, and shown the *Entamoeba histolytica* is not the only amoeba pathogenic to man.



Hartmannella culbertsoni. FIGURES 87 to 98 are drawn in the living condition. FIGURES 99 to 111 fixed in Carnoy and stained with iron-alum haematoxylin. FIGURES 87 to 89 trophic forms; 90 to 94 cysts; 95 to 98 excystment stages.

Prophase

In this stage the chromatic granules move to the centre of the nucleus (figure 100), thus simulating fragmentation of the nucleolus with formation of chromatin granules (Gläser 1912; Dobell 1914). Use of the Feulgen reaction has shown that in amoebae nucleoli never contain chromatin, nor do they give rise to it at any stage (see Singh 1952). The chromatin granules fuse, and the nucleolus gradually disappears, probably giving rise to the spindle.

Metaphase

The fused chromatin granules assume the form of a solid band at the equatorial plate (figure 101); no chromosomes could be distinguished. The spindle is somewhat globular at this stage, and shows no distinct fibres. The chromatic band then divides into two (figure 102), but division of the chromosomes into halves could not be seen.

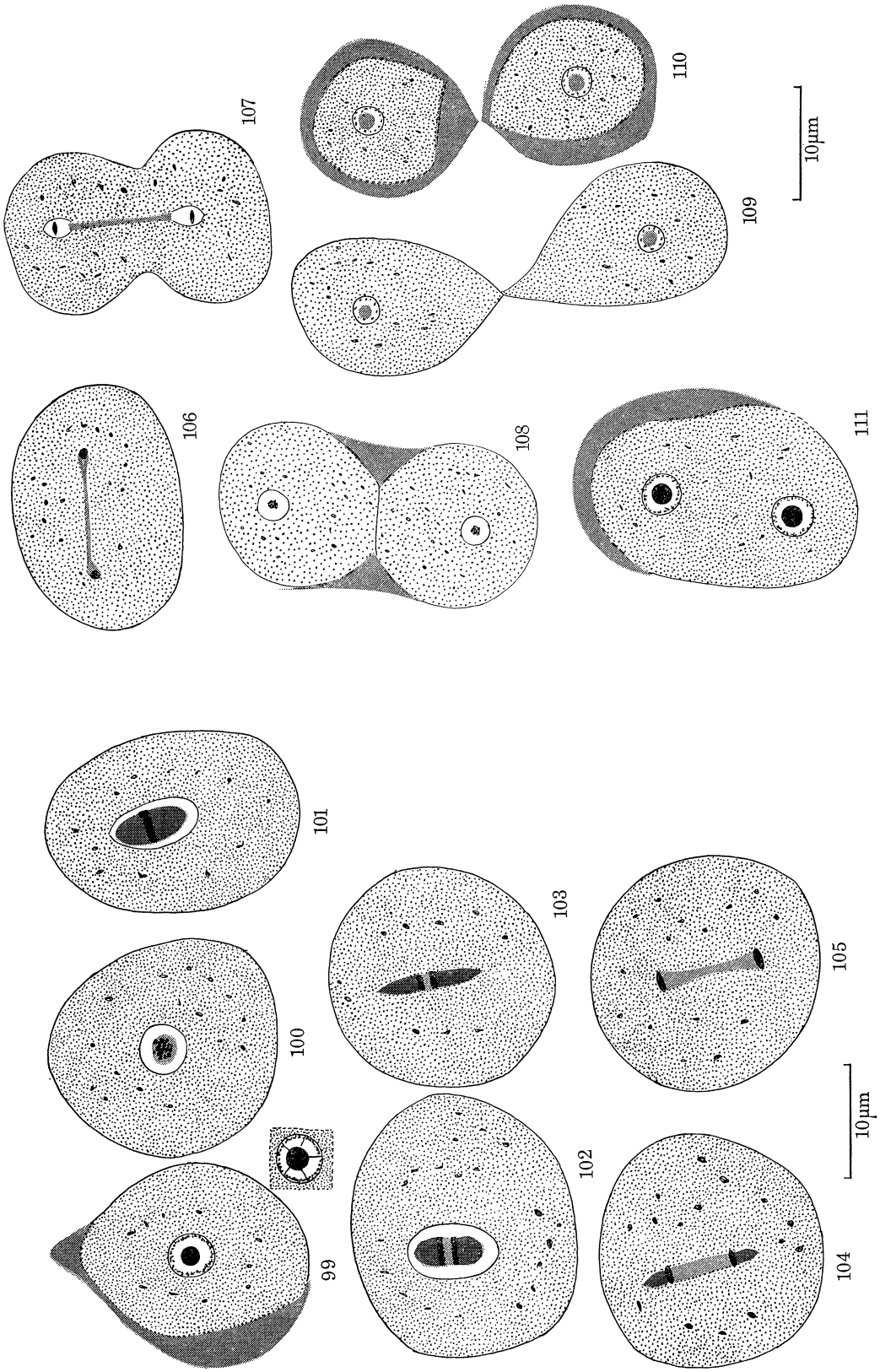


FIGURE 99. Ordinary individual and the structure of two resting nuclei. FIGURES 100 to 110. Successive stages in division. Chromatin granules give rise to chromosomes and the nucleolus disappears. FIGURE 111. Ordinary individual with two resting nuclei.

Anaphase

The nuclear membrane disappears. The globular spindle gradually elongates (figures 103, 104). The parts that lie between the chromosomes and the poles stain more deeply and appear like caps (figures 103, 104); they become smaller and smaller as the chromosomes move to the poles, and they finally disappear. When the chromosome masses reach the poles they are connected by a thread-like structure constricted in the middle (figures 105, 106).

Telophase

A constriction appears in the middle of the elongated amoeba and two daughter cells are produced (figures 107 to 110); a strand of protoplasm connects the daughter cells for a short period. Nuclear membranes appear around each mass of chromatin (figures 107, 108); these masses fragment into granules, and a nucleolus is gradually formed; later on the granular material is organized as in a resting nucleus.

There is no aster or centrosome—intranuclear or extranuclear—at any stage of mitosis.

A detailed examination of 14 pathogenic strains of *Hartmannella* (9 from Indian and 5 from American soils) shows that in their form of nuclear division they closely resemble *H. culbertsoni*. By the form of their cysts, however, they are divisible into two groups: in B-1, B-2, B-3 and B-6 the cysts are identical in appearance with those of *H. culbersoni*; in the remaining 10 (RS, FS, GS, B-4, B-5, A-5, A-30, HN-3, HN-15 and HN-17) they are identical with those of *H. rhysodes* (see figure 193, plate 30 and p. 455).

Adam (1964) found that the Lilly strain (*H. culbertsoni*) grew very poorly on agar plates with *Aerobacter*; few cysts were formed. It encysted well when grown in roller-tube cultures with mammalian cells. She also states that cysts of *H. culbertsoni* are very similar to those of *H. castellanii*, but that older cysts have been found in which further shrinkage has led to the formation of a third cyst wall. In our hands, *H. culbertsoni* grows very well on non-nutrient agar with *A. aerogenes* or *E. coli*, and readily forms cysts in large numbers. These characters have remained unchanged for more than three years. The cysts produced are unlike those of *H. castellanii* or *H. rhysodes* even a month or more after their formation (cf. figure 192, plate 29 and figures 193 and 195, plate 30). We have been unable to make out the third cyst wall shown by Adam (1964, fig. 7).

Remarks

Amoebae that possess a resting nucleus containing a single Feulgen-negative nucleolus, and pass through a mitotic process in which the nucleolus disappears and a spindle with chromosomes on an equatorial plate develops, have been included in the genera *Hartmannella*, *Acanthamoeba*, *Mayorella* and others.

Singh (1952) considered that *Acanthamoeba* was a synonym of *Hartmannella*, and Adam (1964) and Siddiqui & Balamuth (1965) took the same view of *Mayorella*. The differentiation of genera in the Hartmannellidae will be taken up later (p. 468).

Adams (1964) found that the Lilly strain (*H. culbertsoni*) was serologically distinct from *H. rhysodes*, *H. castellanii*, *Hartmannella* (*Acanthamoeba*) Neff, and 17 other strains of hartmannellid amoebae, as on the results of the immobilization reaction; *Hartmannella* Neff was related to *H. rhysodes* and *H. castellanii*. Adam (1964) suggested that the Lilly strain was a distinct species. Culbertson, Ensminger & Overton (1965), who used complement-fixation tests, also found that *H. culbertsoni* showed very little antigenic relationship to other pathogenic strains (A-30 to A-36)

of *Hartmannella*. According to Červa (1965), who also used complement-fixation tests, *H. castellanii*, *Hartmannella* Neff and *H. culbertsoni* are antigenically related; there are, however, significant differences between *H. culbertsoni* and the other two strains. *Hartmannella* Neff appears very similar to, but not identical with, *H. castellanii*. Balamuth & Kawakami (1967), by agar gel diffusion techniques, have shown that *H. culbertsoni* is closely related to, but not identical with, *H. rhysodes*. Pant, Prasad & Singh (1968) have confirmed the findings of Balamuth & Kawakami (1967) by immobilization, agglutination and agglutinin-absorption techniques. Though there is some relationship between *H. culbertsoni*, *H. rhysodes*, *H. castellanii* and *Hartmannella* Neff, *H. culbertsoni* should be regarded as a distinct species, readily distinguishable from the other three, whose cysts are similar, by the characters of its cysts.

(7) ***Hartmannella rhysodes*** Singh, 1952

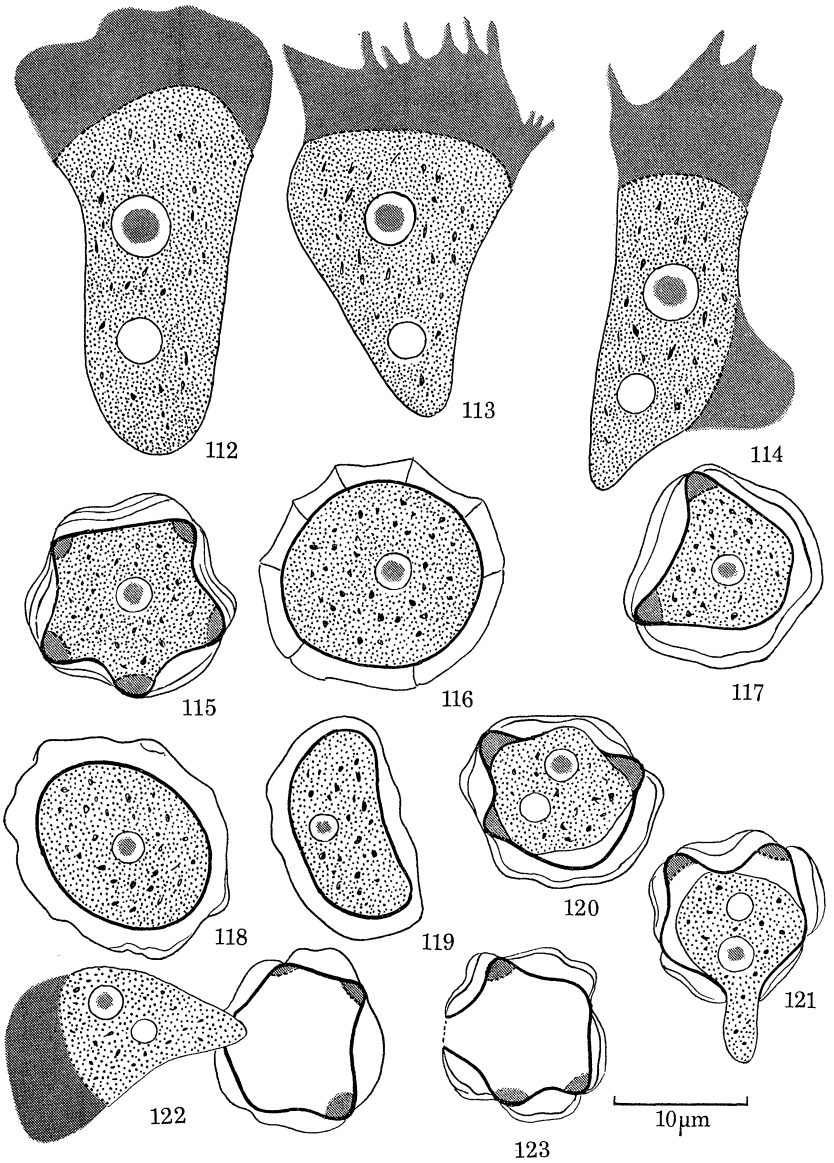
Morphology

A short description of the morphology and cystic characters and a detailed description of the nuclear division of *H. rhysodes* were given by Singh (1952). The amoebae are shown in figures 112 to 114; in the rounded state strains 14 and 15 (table 1) are approximately 15 to 35 μm in

TABLE 1. DIAMETER OF ROUNDED FORM OF HARTMANNELLID AMOEBAE GROWN UNDER SIMILAR CULTURAL CONDITION WITH *A. AEROGENES* AS FOOD SUPPLY

amoeba *H. rhysodes* strains	range of diameter in μm	amoeba *H. culbertsoni* strains	range of diameter in μm
14	15–35	A-1	12–25
15	15–35	B-1	15–25
RS	12–27	B-2	15–25
FS	15–30	B-3	12–27
GS	12–25	B-6	15–25
B-4	12–30	*H. palestinensis*	25–40
B-5	15–30	*H. astronyxis*	25–40
A-5	12–27	*H. glebae*	15–30
A-30	15–30		
HN-3	12–30		
HN-15	12–26		
HN-17	15–30		
H. castellanii	30–45		

diameter. Living cysts are very variable in size and shape (figures 115 to 119; figure 193, plate 30); each consists of two walls, irregular in outline and with a wrinkled appearance. The outer wall has folds and ripples and is often loosely applied to the inner wall, which is irregularly stellate, with truncated rays, or irregularly polyhedral in appearance. The two walls are in contact at points along the inner wall, where pores, plugged with a structureless substance, are present. The nucleolus is very distinct, but no chromatin granules could be made out. During excystment the amoeba emerges from one of the pores (figures 120 to 122), which can still be seen after the amoeba has escaped (figure 123).



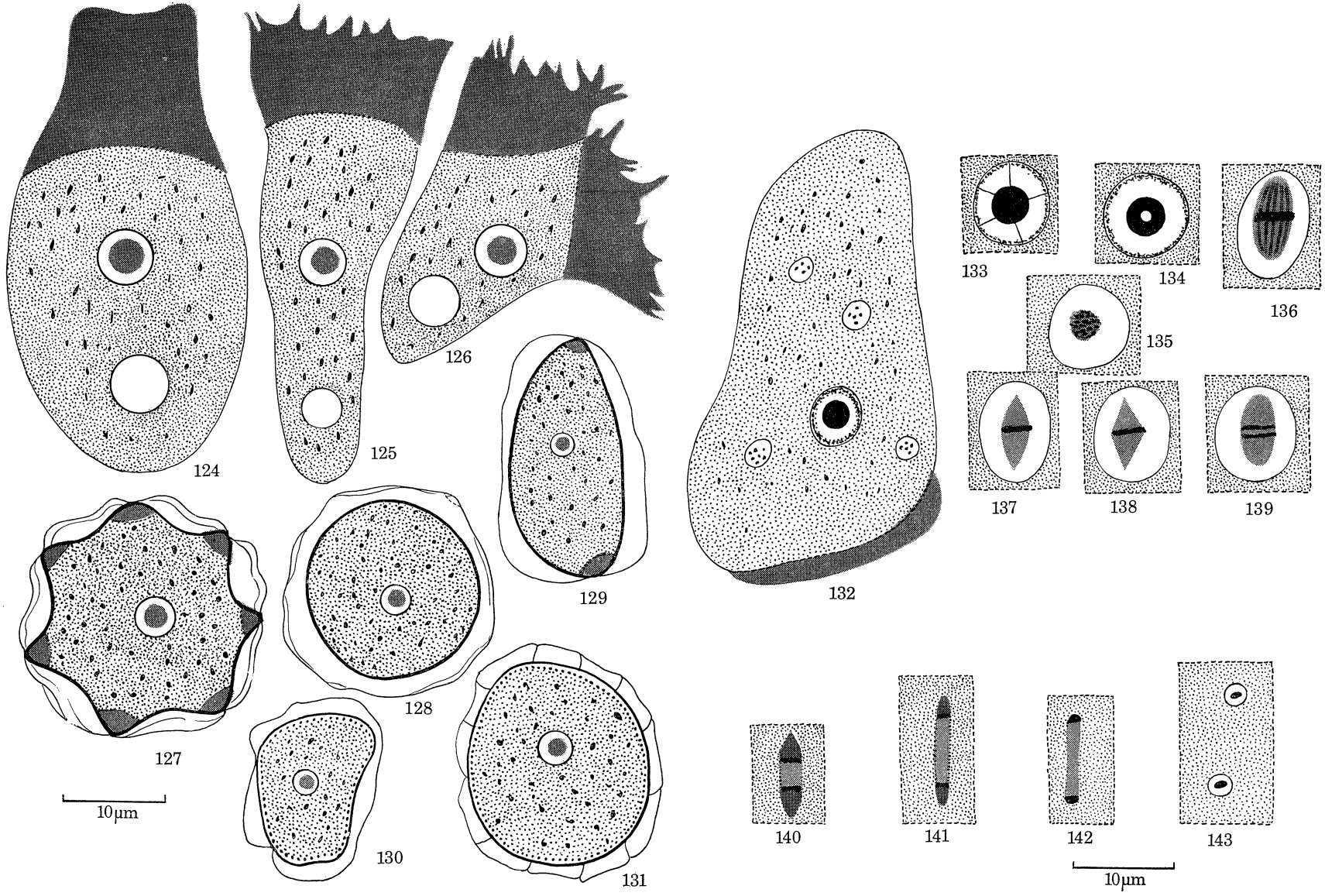
Hartmannella rhysodes. FIGURES 112 to 123 drawn in the living condition.
FIGURES 112 to 114 trophic forms; 115 to 119 cysts; 120 to 123 excystment stages.

(8) ***Hartmannella castellanii*** (Douglas, 1930)

Morphology

The morphological characters of *H. castellanii* (figures 124 to 131; figure 195, plate 30) are similar to those of *H. rhysodes*. In the rounded state *H. castellanii* is larger (diameter *ca.* 30 to 45 μm) than *H. rhysodes* (*ca.* 15 to 30 μm) (table 1); over the last year we have confirmed this under similar conditions of culture and food supply. Page (1967*b*) has also found that when the amoebae are in motion, the extended length of *H. castellanii* and *Hartmannella* Neff is greater than that of *H. rhysodes*.

Mitotic division in *H. castellanii* (illustrated in detail in figures 132 to 143) is similar to that of *H. rhysodes*. Both globular and somewhat pointed spindles are present; the typical conical



Hartmannella castellanii (*Acanthamoeba castellanii*).

FIGURES 124 to 131 drawn in the living condition.
FIGURES 132 to 143 fixed in Carnoy and stained with iron-alum haematoxylin.
FIGURES 124 to 126 trophic forms; 127 to 131 cysts.

FIGURES 132 to 134. Ordinary individual and structure of three resting nuclei.
FIGURES 135 to 143. Successive stages in division. Chromatin granules give rise to chromosomes and the nucleolus disappears.

spindle with pointed ends (Volkonsky 1931) could not be clearly demonstrated. No aster or centrosome was observed (cf. Page 1967*b*).

Remarks

Adam (1964) considers that *H. rhysodes*, *Hartmannella* Neff and *H. glebae* are synonyms of *H. castellanii*. We shall show later that *H. glebae* is distinct from *H. castellanii*. Page (1967*b*) states that, to judge from cyst characters, *Hartmannella* Neff and *H. rhysodes* are indistinguishable from *H. castellanii*. Culbertson, Ensminger & Overton (1965) found that *Hartmannella* Neff and *H. castellanii* are non-pathogenic in mice. We have found also that *H. castellanii* is non-pathogenic, whereas *H. rhysodes* is pathogenic. *H. rhysodes* was the commonest form in Rothamsted soils over a period of ten years (Singh 1952), and it can also be readily isolated from Indian soils. Griffiths & Hughes (1968) found that the replacement medium developed by Band (1963) for *H. rhysodes* was unsatisfactory for *H. castellanii* (Neff strain). Unless it is shown that *H. castellanii* and *H. rhysodes* are serologically indistinguishable, it is better to call the smaller pathogenic amoeba *H. rhysodes* and the larger non-pathogenic one *H. castellannii*. Warhurst & Armstrong (1968) have studied a small amoeba, which they called *H. castellanii* from a mammalian tissue culture, infected with 'Rayan virus'; rounded forms were 12.3 to 27.7 μm in diameter, which, combined with its pathogenic nature, suggests that the amoeba was *H. rhysodes*.

We have been unable to distinguish *H. polyphaga* (*Acanthamoeba polyphaga*) (Puschkarew 1913), described by Page (1967*b*) as a distinct species, from *H. rhysodes* or *H. castellanii* on the basis of its cyst characters (cf. figures 193 to 195, plate 30); it is not pathogenic to mice.

Pussard (1963; 1964*a*, *b*) has treated the location and appearance of centrioles as important taxonomic criteria for separating *Acanthamoeba castellanii* and two new species *A. terricola* and *A. comondoni*; as others (e.g. Singh 1952; Page 1967*a*, *b*; Warhurst & Armstrong 1968) have been unable to find centrioles in *Hartmannella* (*Acanthamoeba*) spp., and Page (1967*b*) considers that *A. terricola* is in all other respects very like *A. polyphaga*, Pussard's findings need confirmation before they can be accepted.

The photograph of the cysts of *Hartmannella* (*Acanthamoeba*) *gigantea*, a salt water form described by Schmoller (1964) looks very much like those of *H. castellanii*.

(9) ***Hartmannella astronyxis*** Ray & Hayes, 1954

Ray & Hayes (1954) described *H. astronyxis*, separating it from other *Hartmannella* spp. on the basis of its regular production of uniform, consistent, distinctive biconvex cysts, with the two walls on contact at a variable number of points sealed by parabolic opercula; the inner wall is star-shaped. Page (1967*b*) states that *H. astronyxis* cysts have ectocysts with inconspicuous, usually circular, folds or ripples, with a stellate endocyst with conical rounded rays. Vickerman (1962), in an electron-microscope study, could not find pores at the points of contact of the ectocyst and endocyst.

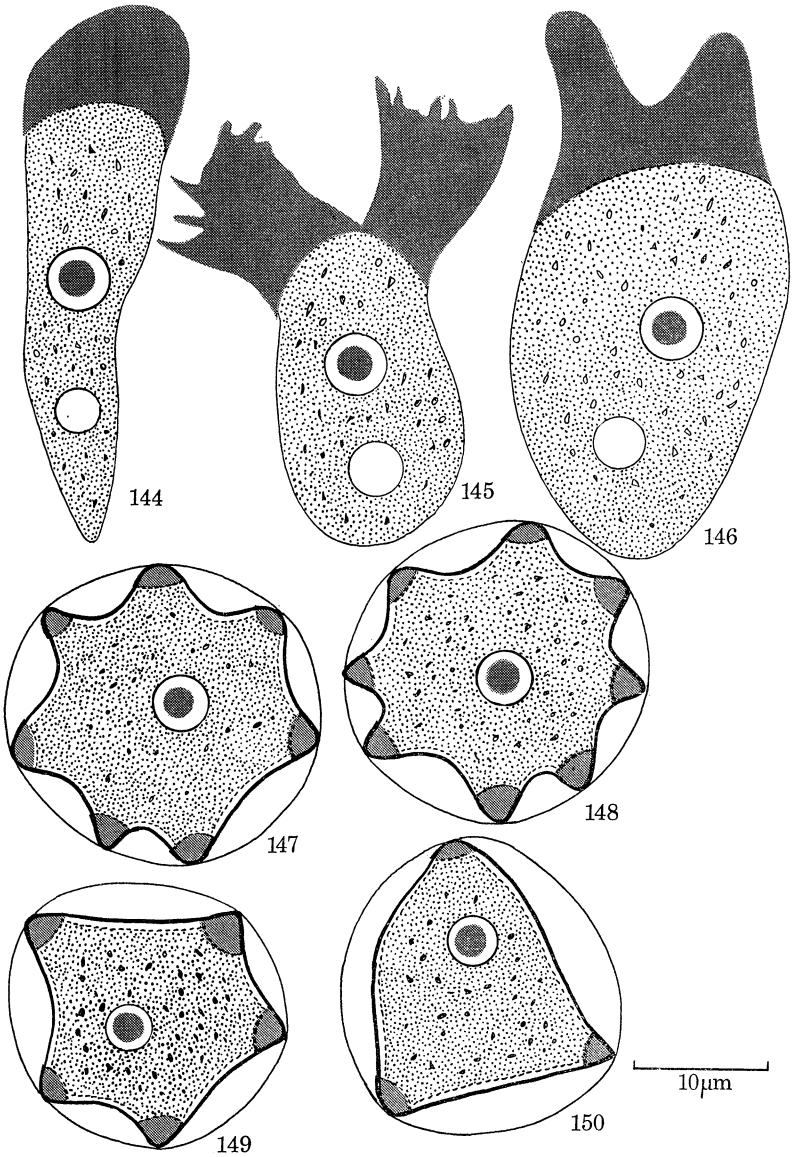
Morphology

In the rounded state the amoebae (figures 144 to 146) are *ca.* 25 to 40 μm in diameter.

The cysts of *H. astronyxis* (Ray & Hayes 1954; Page 1967*b*) are readily produced in culture (figures 147 to 150; figure 196, plate 30), and are quite distinct from those of any other known hartmannellid amoeba. The amoeba is also serologically distinct from all others tested (Adam 1964).

Remarks

Page (1967*b*) has, on the basis of pseudopodial characters, included *H. astronyxis* in the genus *Acanthamoeba*, the validity of which is discussed on p. 464.



Hartmannella astronyxis. FIGURES 144 to 150 drawn in the living condition. FIGURES 144 to 146 trophic forms; 147 to 150 cysts.

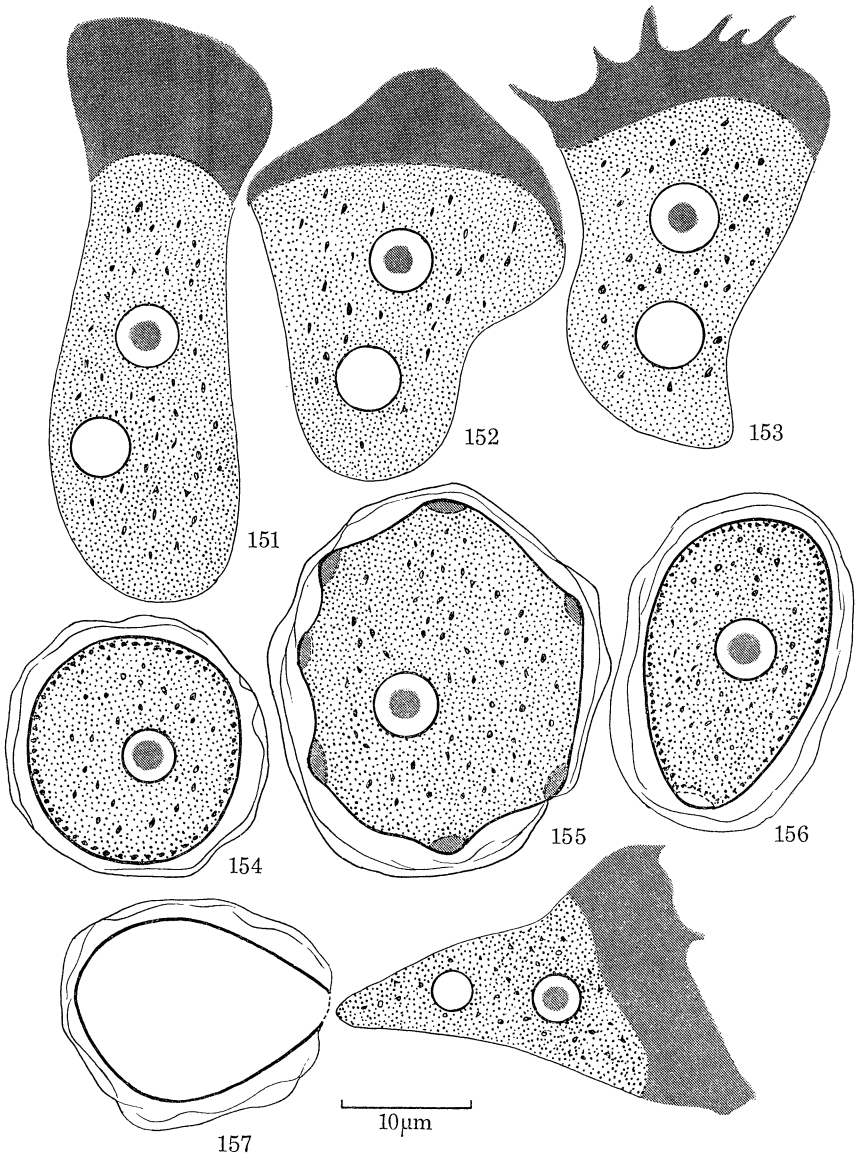
(10) ***Hartmannella palestinensis*** (Reich 1933)

Reich (1933) has described *Mayorella palestinensis* as a new species; as it has the same type of nuclear division as is found in the genus *Hartmannella*, it should be called *H. palestinensis*.

Morphology

In the rounded state the amoebae (figures 151 to 153) are *ca.* 25 to 40 μm in diameter (the figure, 14 to 19 μm, given by Reich (1933) may be due to the use of unsuitable cultural conditions). There is usually one nucleus, occasionally two; Reich figures an amoeba with four.

The living cysts are very variable in size (figures 154 to 156; figure 197, plate 30); of the two walls, the ectocyst is fairly thick and very irregular and wrinkled. The great majority of the cysts, but not all of them (figure 154), appear to be pierced by one or more pores or opercula, plugged with a structureless substance; the inner wall sometimes retains contact with the



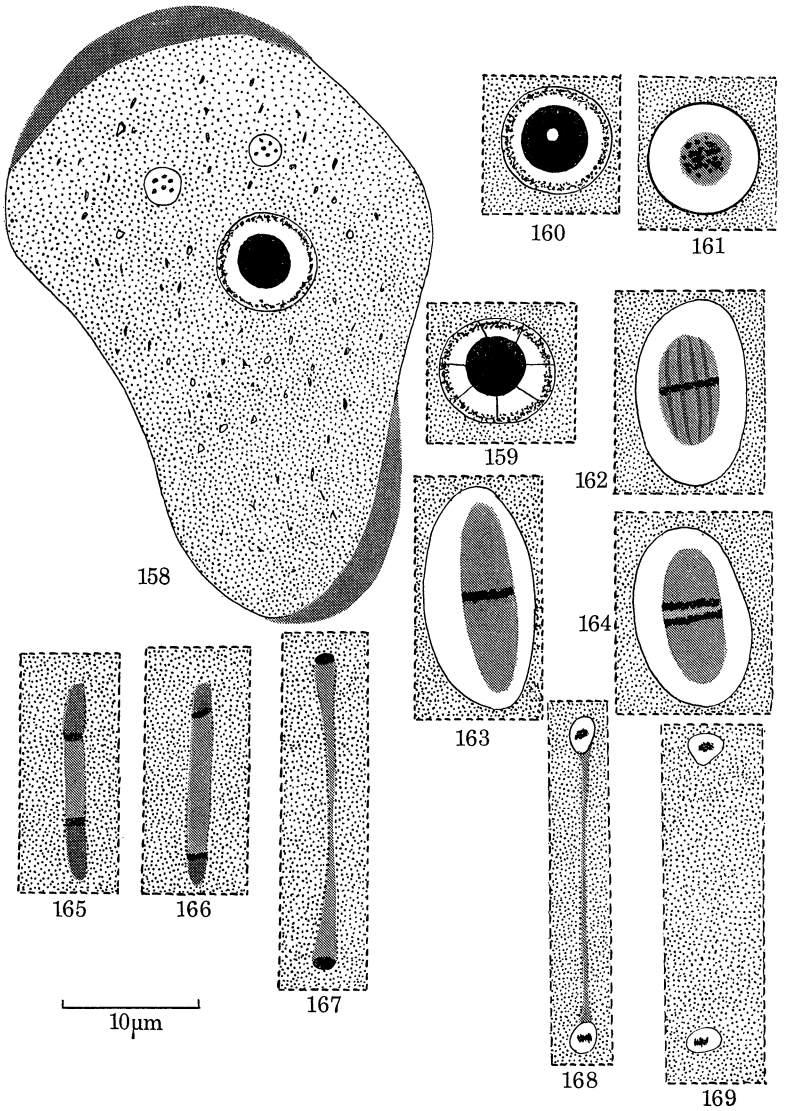
Hartmannella palestinensis. FIGURES 151 to 157 drawn in the living condition.
FIGURES 158 to 169 fixed in Carnoy and stained with iron–alum haematoxylin.
FIGURES 151 to 153 trophic forms; 154 to 156 cysts; 157 empty cyst after the emergence of amoeba.

ectocyst at the points of the opercula (figure 155). The nucleolus is distinct, but no chromatin granules can be made out. There is usually one nucleus, rarely two (cf. Reich (1933), for two or four nuclei in a cyst). The amoeba emerges through an operculum (figure 157); the outline of the cyst wall and the operculum can be seen after the emergence of the amoeba (figure 157).

Efforts to produce a temporary flagellate stage have completely failed.

The resting nucleus

In live amoebae the nucleus contains a fairly large central nucleolus surrounded by a clear zone. In iron-haematoxylin preparations the nucleolus, and chromatin granules situated near the nuclear membrane, are distinctly seen (figure 158). In some nuclei thread-like structures may be seen radiating from the nucleolus (figure 159), which occasionally shows an unstained patch (figure 160).



FIGURES 158 to 160. Ordinary individual and structure of three resting nuclei.

FIGURES 161 to 169. Successive stages in division. Chromatin granules give rise to chromosomes and the nucleolus disappears.

Mitosis

Iron-haematoxylin preparations show that the amoebae round off and become motionless at the beginning of nuclear division; in prophase the chromatin granules move to the centre of the nucleus (figure 161), the nucleolus stains less densely and gradually disappears, probably giving rise to the spindle. The chromatin granules fuse.

Metaphase

The fused chromatin granules assume the shape of a solid band along the equatorial plate (figures 162 and 163) of the globular spindle, which in some cases (figure 162) shows fibres, and divide into two (figure 164). Individual chromosomes could not be distinguished at any stage.

Anaphase

The globular spindle becomes elongated and the nuclear membrane disappears. The parts of the spindle that lie between the chromosomes and the poles stain much more deeply and appear like caps (figures 165, 166); they become smaller as the chromosomes move to the two poles. When the chromosomes reach the poles they are still connected by a centrally constricted filament (figure 167, 168).

Telophase

The amoeba gradually elongates and constricts in the middle; the two daughter amoebae are connected by a strand of cytoplasm before they separate. A nuclear membrane surrounds each mass of chromatic material (figures 168, 169), which then fragments into granules; a nucleolus formed, so restoring the form of the resting nucleus. There is no aster or centrosome at any stage.

Remarks

Adam (1964) found that *H. palestinensis* was serologically unrelated to *H. rhysodes*, *H. culbertsoni* and to one other strain of hartmannellid amoeba, but was related to *H. castellanii*. Siddiqui & Balamuth (1965) found some serological relationship between *H. rhysodes* and *H. palestinensis* by the use of diffusion-precipitation and fluorescent antibody techniques. In our opinion, the cyst characters are adequate to distinguish *H. palestinensis* as a separate species.

(11) ***Hartmannella glebae*** (Dobell, 1914)

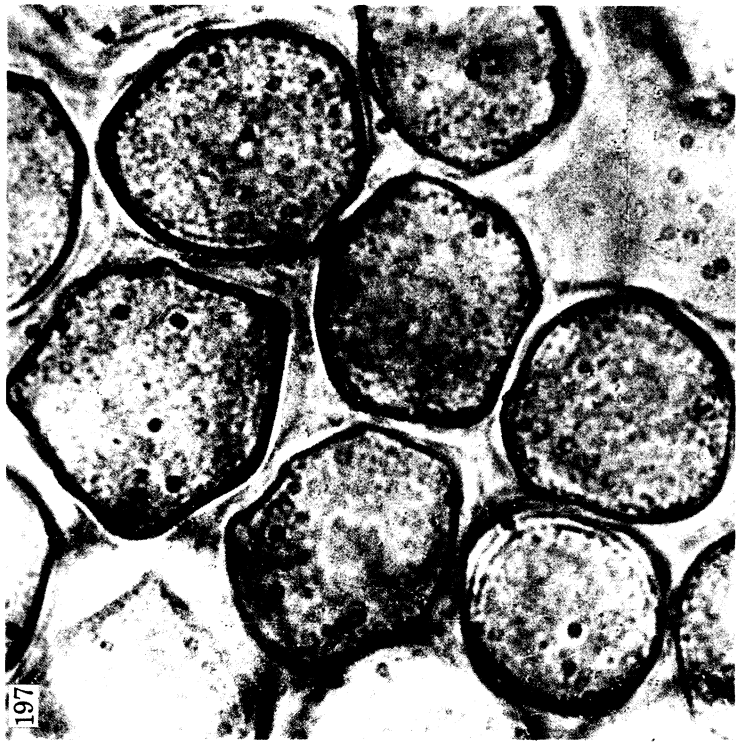
Dobell (1914) isolated *H. glebae* from soil; it has since been studied by several workers. Sandon (1927) recorded it from soils in Kenya, Sudan, Egypt, Palestine, Grenada and Rothamsted; he says (pp. 139, 140) that the diameter of the rounded amoeba is 12 to 20 μm; the pseudopodia are pointed, with the ectoplasm often drawn out into thin web-like sheets between them; the cysts are spherical, diameter 10 to 13 μm, with thick smooth outer walls without pores. Singh (1952) isolated *H. glebae* from Barnfield (plot 4A) complete minerals plus ammonium sulphate soil at Rothamsted at a soil dilution of 1 in 25 600 (w/v).

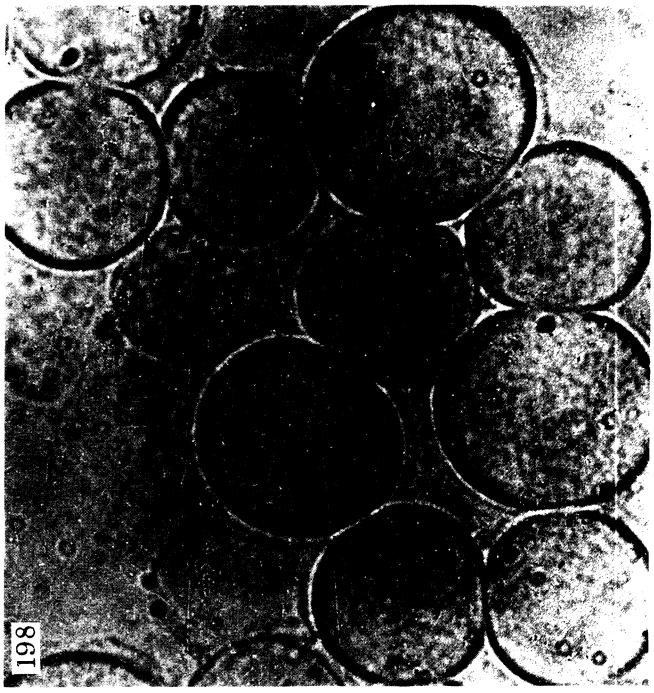
Morphology

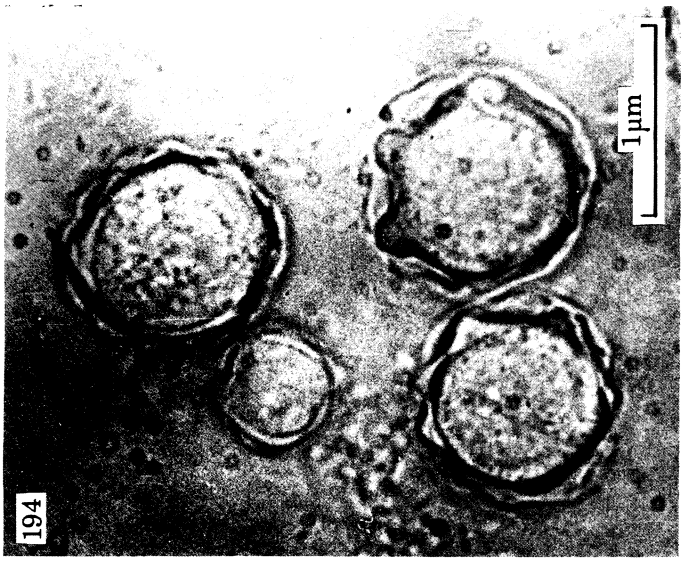
The rounded form of the amoeba (figures 170 to 172) is 15 to 30 μm in diameter; the lower figures given by Dobell (1914) and Sandon (1927) are due to the use of unsuitable cultural conditions. The living cysts are round or spherical with a single, apparently two-layered wall without pores (figures 173, 174). Singh (1952) gives a detailed account of the nuclear division.

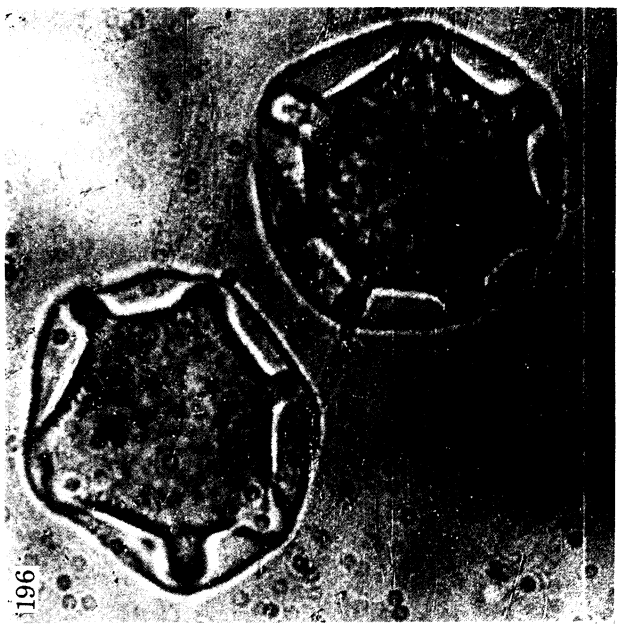
Chang (1959) has isolated *H. glebae* from river and well water and says (p. 398) that it is a relatively large species, average diameter of rounded forms about 25 μm; the cysts have a single wall with no pores.

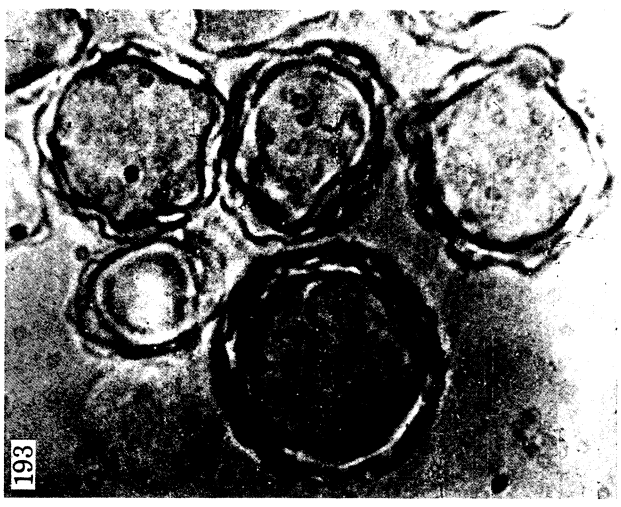
During excystment (figures 175, 176) no preformed pore can be seen (figures 177, 178), so presumably some digestion of the wall occurs. The outline of the cyst wall and the place of emergence of the amoeba can be seen in the empty cyst (figure 179).

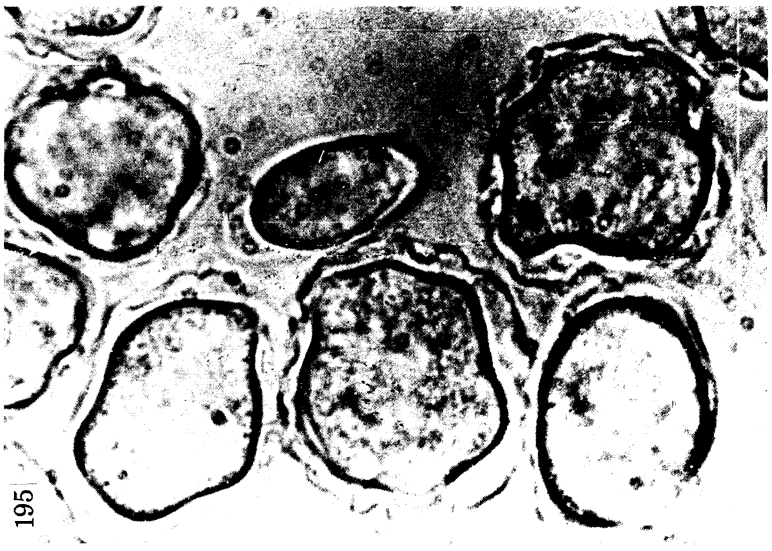












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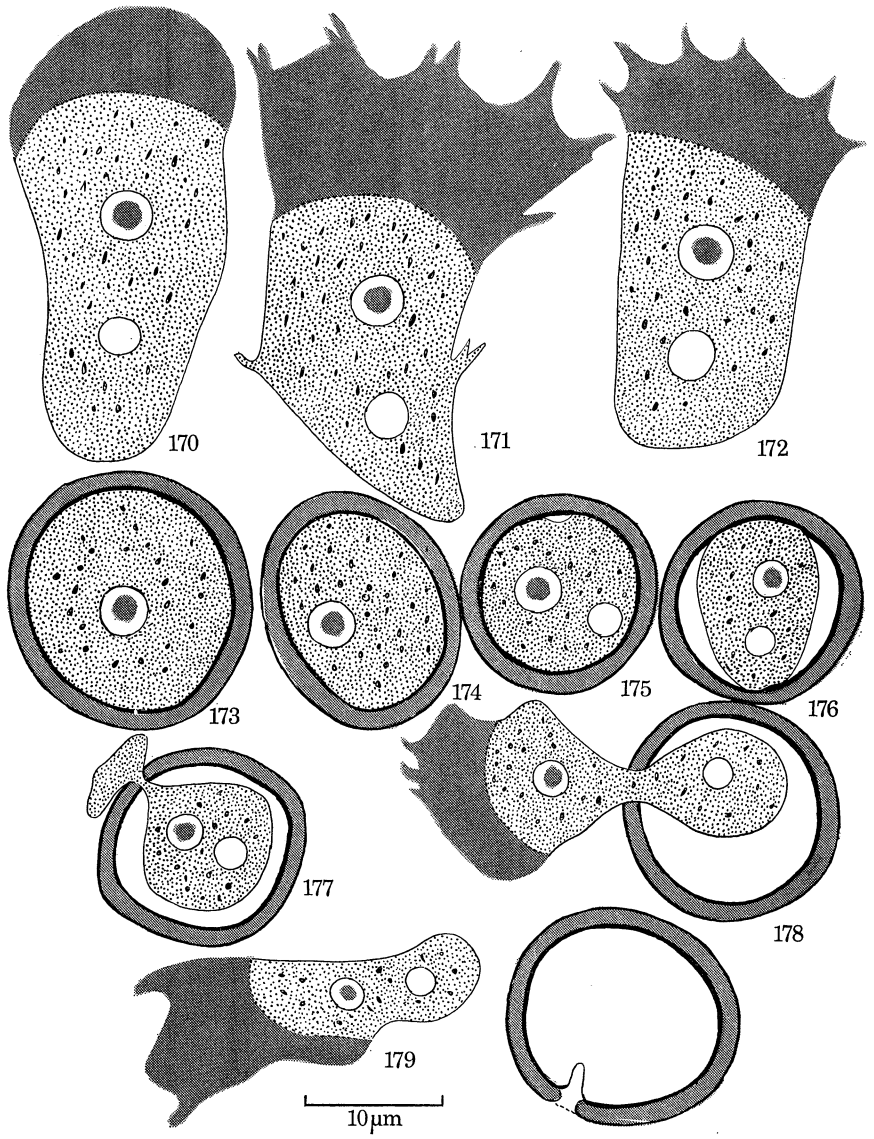

Hartmannella glebae. FIGURES 170 to 179 drawn in the living condition.
FIGURES 170 to 172 trophic forms; 173 and 174 cysts; 175 to 179 excystment stages.

Critical remarks

Singh (1952) made *H. glebae* the type species of the genus *Hartmannella*. Adam (1964), who had never studied *H. glebae*, considered *H. castellanii* Douglas, 1930, as the type species of the genus, and regarded *H. glebae* and *H. rhysodes* as synonyms of that species. The cyst characters of *H. glebae* are so different from those of *H. rhysodes* and *H. castellanii* that we hardly imagine that

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EXPLANATION OF PLATE 30

Cysts of amoebae in the living condition. The scale represents 10 μm and not 1 μm as marked.

FIGURE 193. *Hartmannella rhysodes.*
FIGURE 194. *H. polyphaga* (*Acanthamoeba polyphaga*).
FIGURE 195. *H. castellanii* (*Acanthamoeba castellanii*).
FIGURE 196. *H. astronyxis.*
FIGURE 197. *H. palestinensis* (*Mayorella palestinensis*).
FIGURE 198. *H. glebae.*

anyone who had seen *H. glebae* and the other two species could suppose them to be the same. Moreover, the mode of excystment of *H. glebae* is different from that of the other two species, and it has been shown by Singh & Singh (1966) and Pant *et al.* (1968) that *H. glebae* is serologically distinct from *H. rhysodes*, to judge from the results of immobilization tests.
According to the International Rules of Zoological Nomenclature *H. glebae* has priority over *H. castellanii*, and should be regarded as the type species of the genus *Hartmannella*.

Remarks on inclusion of genera in the family Hartmannellidae

Volkonsky (1931) created a subfamily Hartmannellinae in the family Amoebiadae to include only such amoebae as have a single nucleolus. He placed in it three genera; *Hartmannella* (type *H. glebae* Dobell, 1914) in which the spindle is barrel-shaped or cylindrical and the cyst wall is smooth or lightly folded; *Glaeseria* (type *G. testudinis* Ivanić, 1926), with the same spindle shape, but with division occurring in the cyst; and *Acanthamoeba* (type *A. castellanii* Douglas, 1930) for amoebae with conical, pointed-ended spindle and rough cyst wall.

Singh (1952) suggested that spindle shape was an inadequate character for separating the genera *Hartmannella* and *Acanthamoeba*, and established the genus *Hartmannella* for amoebae having a single Feulgen-negative nucleus and showing true mitosis. (We have shown in the present paper that *H. castellanii* has both globular and somewhat pointed spindles.) He also raised the subfamily Hartmannellinae to the status of a family Hartmannellidae, considering that the form of nuclear division justified this. Ray & Hayes (1954) and Adam (1964) have agreed with Singh.

Page (1967*a*, *b*; 1968), however, considers that the separation of *Hartmannella* and *Acanthamoeba* is both necessary and justified. He (1967*a*) has defined the genus *Hartmannella* as follows (p. 519): 'Amoebae in active locomotion with one or, briefly, more broadly digitiform or hemispherical lobose pseudopods, sometimes with small filamentous projections but never with well-developed conical or tapering psuedopods; most active locomotive form *limax*-like, with length twice breadth or greater. Locomotion not strongly eruptive. Normally uninucleate; nucleus vesicular, usually with a single nucleolus. Nuclear division with the disintegration of nucleolus during prophase and usually dissolution of nuclear membrane in prophase or early metaphase. Cyst may or may not be formed; where known, smooth-walled and rounded. No nuclear division in cyst'.

Page has set up *H. hyalina* Dangeard, 1900, as the type species of the genus *Hartmannella*, according to the International Rules of Zoological Nomenclatures, in spite of the fact that Dangeard's (1900) description, based entirely on fixed preparations, does not refer to locomotion. Sandon (1927), who found *H. hyalina* widespread in soils, describes it as follows (p. 133): 'Active form very like *N. gruberi*, but usually slightly larger: protoplasm rather more fluid and pseudopodia generally formed 'eruptively', i.e. they burst suddenly through the surface of the amoeba and the granular protoplasm flows rapidly into the newly formed process; generally, instead of at once forming a long finger-like pseudopodium the eruption flows round the outside of the original surface of the body which ultimately becomes absorbed. The cysts are spherical; outer wall usually more or less wrinkled; walls not perforated; protoplasmic contents rounded lying quite freely within the wall; nucleus not so visible as in *N. gruberi*.' Kudo (1966) has also figured the cyst of *H. hyalina* as described by Sandon (1927). Obviously such reliance on the International Rules of Zoological Nomenclature to create genera based on imperfectly studied amoebae is of little value.

Page (1967*b*) has defined the genus *Acanthamoeba* (Volkonsky) as follows (pp. 721–2): 'Amoebae in active locomotion with broad, anterior hyaline lobopodium from which are produced singly or 2's or 3's several or many slender, hyaline projections (acanthopodia) which taper to a finely rounded end; acanthopodia may move occasionally and eventually are resorbed by advancing amoeba or remain on the surface until resorbed at posterior end. Length of the amoeba in locomotion may reach 2 or 3 times breadth; outline often highly irregular, especially when not in most active locomotion. Locomotion by flow into anterior hyaline region, not markedly eruptive, slow. Normally uninucleate; nucleus vesicular with a single nucleolus. Nuclear division with disintegration of nucleolus and dissolution of nuclear membrane during late prophase. Feeding by means of large food cup formed by short, blunt pseudopodial projections, usually at posterior end or posterolaterally. Food vacuoles and contractile vacuole prominent. Endoplasm often contains many small, yellowish, refractile globules which are not crystalline. Cyst wall consisting of endocyst, which is more or less polyhedral or stellate, and ectocyst, which may be wrinkled and more or less closely appressed to endocyst or circular in outline and showing only slight ripples. General form of the cyst thickly biconvex or polyhedral, depending on distribution of corners of endocyst; appearance generally more or less mammillated. Endocyst positive for cellulose. Cyst uninucleate, with single oriented layer of small granules around periphery of cytoplasm in most species. Excystment by removal of operculum into interior of cyst from point of contact between endocyst and ectocyst, followed by exit of amoeba thru opening at that point.' Page (1968) (p. 25) says: 'Within the genus *Acanthamoeba*, the cysts, tho very distinctive as a generic characteristics, are somewhat more confusing as a means of distinguishing species.' Page (1967*b*) has made *A. castellanii* the type species.

Adam & Blewett (1968) have recognized the genus *Acanthamoeba* as defined by Page (1967*b*). Richards (1968) has described two new species of small amoebae from freshwater molluscs, and, on the basis of *limax*-like locomotion and smooth spherical cysts, has placed them in the genus *Hartmannella*, without, regrettably, studying their nuclear division.

Page (1967*a*, *b*, 1968) has separated *Hartmannella* from *Acanthamoeba* on the basis of pseudopodial and cyst characters. It may be of interest to point out that *H. glebae* does produce acanthopodia, but the cysts are rounded or spherical with smooth wall and without pores or opercula. The mode of excystment of this amoeba is quite different from that of *A. castellanii*. On the basis of cyst character and mode of excystment, *H. glebae* cannot be included in the genus *Acanthamoeba* as defined by Page (1967*b*). Only on pseudopodial character, which is of no diagnostic value in creating genera or species, as mentioned in the introduction, can *H. glebae* be placed in the genus *Acanthamoeba*. Any one recognizing the genus *Acanthamoeba* will have to recognize *H. glebae* as the type species in accordance with the International Rules of Zoological Nomenclature.

Moreover Page's (1967*a*, *b*, 1968) classification will not accommodate forms like *H. agricola* (Goodey, 1916) or *H. leptocnemus* Singh, 1952, both of which divide by true mitosis. They have neither *limax*-like locomotor forms, nor eruptive pseudopodia nor acanthopodia. In *H. agricola* the distinction between ectoplasm and endoplasm is not at all clear either in the living or the stained amoebae. Both species move slowly. In *H. leptocnemus* the body presents a great variety of sizes and shapes and the pseudopodia are irregular and lobose. The individuals often become elongated and have a characteristic shape. There is no clearly defined ectoplasm and endoplasm even when the amoebae move. The living cysts are rounded or spherical with a double wall

without pores. It is better to retain these forms in the genus *Hartmannella*, as defined by Singh (1952), than to create a third genus for them based on characters of pseudopodia and movement. It seems that the creation of both the genera *Acanthamoeba* and *Hartmannella*, as done by Page (1967*a*, *b*, 1968), is very misleading and is not justified. *Acanthamoeba* is a synonym of *Hartmannella*. At this stage, it is more important to create species in the genus *Hartmannella*, based on sound characters, which can be identified with ease by any competent worker, than to split the genus *Hartmannella* into a number of genera.

As suggested by Volkonsky (1931) the amoebae, like *A. mira* (Gläser 1912) and *H. testudinis* (Ivanić 1926), where nuclear division takes place in the cyst, may be taken out of the genus *Hartmannella* and placed in the genus *Glaeseria*. The mode of nuclear division places the genus *Glaeseria* in the family Hartmannellidae.

Page (1968), using the Feulgen reaction to study their nuclear division, has placed some amoebae in the genera *Flabellula*, *Rugipes* and *Hyalodiscus*, and has, in accordance with Schaeffer's (1926) system of classification, placed them in three families. Page (1968) figures beautiful stages of true mitosis, just as found in the family Hartmannellidae, in *Hyalodiscus actinophorus*. Although the stages of nuclear division are not so well illustrated in the other genera he has described, it is clear that they all show true mitosis, and we feel that all three can easily be accommodated in the family Hartmannellidae. Forms like *Flabellula platypodia* (Gläser 1912) and *F. mira* (Schaeffer 1926) should be transferred to *Hartmannella*.

IV. Suggested classification of amoebae

The suggested classification is based on the nuclear structure, mode of nuclear division and possible phylogenetic relationship. The amoebae fall into three main groups according to the mode of nuclear division. In the first group Feulgen-negative nucleolus or nucleoli give rise to polar masses and the nuclear membrane persists throughout division; in the second group Feulgen-negative granules or nucleoli do not give rise to polar masses and the nuclear membrane remains intact during division; in the third group Feulgen-negative nucleolus or nucleoli and the nuclear membrane disappear. In all three groups the pre-existing Feulgen-positive chromatin gives rise to chromosomes, and at the end of nuclear division, the mass of fused chromosomes disperses and returns to the position seen in a resting nucleus. The nuclear division in amoebae is not of a primitive nature, nor there is much diversity of type. There is no justification for writers of text-books on biology to show an amoebae dividing by amitosis or in some such primitive way, as was thought about a hundred years ago.

These three distinct types of nuclear division seem to provide a logical basis for dividing the order Amoebida Kent into three families, Schizopyrenidae, Endamoebidae and Hartmannellidae. Such a system of classification throws light on the probable trends of evolution of amoebae.

A proper study of the normal stages of nuclear division in amoebae placed in Endamoebidae, using Feulgen reaction, has not been made. The culture method of growing *E. histolytica* and other anaerobic amoebae for the study of their nuclear division, developed by Dubey & Das (1966), will be helpful in determining the normal stages of nuclear division. The review of nuclear division in *Entamoeba* by Neal (1966) clearly shows that the different stages of mitosis are not clearly understood. Neal has pointed out that the nuclear membrane is always intact during division and the type of mitosis appears to be different from others so far described. According to Narasimhamurti (1964), whose work Neal has not quoted, the Feulgen-negative

peripheral granules in *E. invadens* do not seem to take part in mitosis and no centrioles and spindle mechanism are present.

The nuclear division in *Dobellina mesnili*, placed in Endamoebidae by Kudo (1966), has been studied with care by Bishop & Tate (1939), using Feulgen reaction. On the basis of the type of nuclear division, the genus *Dobellina* should be transferred to the family Hartmannellidae.

The definitions of the families Schizopyrenidae and Hartmannellidae, given below, are slightly modified, and the family Endamoebidae has been redefined. No attempt has been made to give a complete list of genera belonging to these families. Some of the genera given are merely as an illustration. As more knowledge of nuclear division in amoebae accumulates, it will be possible to classify them according to the present system of classification.

SCHIZOPYRENIDAE (Singh 1952)

Definition. The resting nucleus contains a more or less central Feulgen-negative nucleolus or several Feulgen-negative nucleoli, which during mitosis form 'polar masses'. Nuclear membrane persists throughout division. 'Interzonal bodies' may be present. Amoebae may have more than one nucleus, and some genera may produce a flagellate stage.

Type Genus: *Schizopyrenus*: Other genera *Naegleria*, *Didascalus*, *Tetramitus*, *Trimastigamoeba*, *Heteramoeba* and *Sappinia*.

Genus ***Schizopyrenus*** Singh, 1952

Definition. Feulgen-negative nucleolus dividing during mitosis to form 'polar masses'. Temporary flagella are not produced.

Type species. Schizopyrenus russelli, Singh, 1952

Genus ***Naegleria*** Alexeieff emend. Singh, 1952

Definition. 'Polar masses' are formed. Feulgen-negative 'interzonal bodies' are present during late stages of nuclear division. Temporary flagella are produced.

The flagellate stage has two flagella and no division takes place in this stage.

Type species. Naegleria gruberi (Schardinger).

Genus ***Didascalus*** Singh, 1952

Definition. 'Polar masses' without 'interzonal bodies' are present during nuclear division. Temporary flagella are produced.

The flagellate stage has two flagella and no division takes place in this stage.

Type species. Didascalus thorntoni Singh, 1952.

Genus ***Tetramitus*** Perty emend. Auctt.

Definition. 'Polar masses' and 'interzonal bodies' are present. Division takes place both in the amoeboid and flagellate stages. The flagellate stage has four flagella.

Type species. Tetramitus rostratus (Perty)

The nuclear division both in the amoeboid and flagellate stages of *T. rotratus* has been studied in detail by Rafalko (1951), using the Feulgen reaction.

Genus ***Trimastigamoeba*** Whitmore emend. Auctt.

Definition. 'Polar masses' are present. Division takes place both in amoeboid and flagellate stages. The flagellate stage has four flagella.

Type species. Trimastigamoeba philippinensis Whitmore.

The morphology of amoeboid and flagellate stages of *T. philippinensis* has been described in detail by Bovee (1959). According to Bovee, division takes place in both the amoeboid and flagellate stages. From the figure 66*b* given by Boeck & Stiles (1923), it seems that 'polar masses' are present during division of amoebae. It would be interesting to study the nuclear division in this amoeba both in the amoeboid and flagellate stages and to find out whether both the 'polar masses' and 'interzonal bodies' are present or only 'polar masses'. The morphology of the flagellate stage is different from the flagellate stage of *T. rostratus*, as pointed out by Bovee (1959).

Genus ***Heteramoeba*** Droop, 1962 emend. Auctt.

Definition. Resting nucleus contains several Feulgen-negative nucleoli. 'Polar masses' are present. Division takes place both in the amoeboid and flagellate stages. Flagellate stage has two flagella.

Type species. Heteramoeba clara Droop, 1962.

Droop (1962) found *H. clara* in brackish supralittoral pools in Scotland. He has shown 'polar masses' during division of the amoeba stage. A detailed study of the nuclear division in *H. clara*, both in the amoeboid and flagellate stages, would be most interesting in order to find out whether both 'polar masses' and 'interzonal bodies' are present or only 'polar masses'. The structure of the resting nucleus in *H. clara* is quite different from amoebae included in other genera of the family Schizopyrenidae.

Genus ***Sappinia*** (Dangeard)

Definition. The amoebae contain two closely associated nuclei. The resting nucleus contains a single nucleolus. The nuclei divide simultaneously with the formation of 'polar masses'.

Type species. Sappinia diploidea (Hartmann & Nägler).

The nuclear division of *S. diploidea* has not been studied by Feulgen reaction. Nägler's (1909) cytological work on *S. diploidea* is very poor, although it sugggests that this amoeba may possess 'polar masses'. Noble (1958) has found this organism in elk faeces. Noble's figure 20 suggests that 'polar masses' are produced.

HARTMANNELLIDAE Volkonsky, 1931 emend. Singh, 1952

Definition. This family was created after the well-known genus *Hartmannella.*

The resting nucleus has either a single Feulgen-negative nucleolus or several nucleoli. During mitosis the nucleolus, or nucleoli, disappear, and a spindle with chromosomes arranged as an equatorial plate resembling that found in higher animals and plants develops. The nuclear membrane disappears during mitosis. Amoebae may be uni- or multi-nucleate; no temporary flagella have been discovered.

Genus ***Hartmannella*** Alexeieff emend. Singh, 1952

Definition. The resting nucleus contains a single Feulgen-negative nucleolus. During mitosis the nucleolus disappears and a spindle with chromosomes arranged as an equatorial plate is formed. No temporary flagella are produced.

Type species. Hartmannella glebae (Dobell).

Genus ***Amoeba*** Ehrenberg emend. Auctt.

Definition. Amoebae uninucleate. Resting nucleus contains several Fuelgen-negative nucleoli. During mitosis the nucleoli disappear and a spindle with chromosomes arranged as an equatorial plate is formed.

Type species. Amoeba proteus (Pallas).

The mitotic division in *A. proteus* has been described by Liesche (1938).

Genus ***Pelomyxa*** Greeff emend. Auctt.

Definition. Amoebae multinucleate; division by plasmotomy. Resting nucleus contains several Feulgen-negative nucleoli. During mitosis the nucleoli disappear and a spindle with chromosomes arranged as an equatorial plate is formed.

Type species. Pelomyxa carolinensis Wilson, 1900.

Kudo (1947) has given an excellent account of nuclear division in *P. carolinensis* by the use of the Feulgen reaction.

Genus ***Dobellina*** Bishop and Tate emend. Auctt.

Definition. Amoebae multinucleate; division by plasmotomy. The resting nucleus contains a single Feulgen-negative nucleolus. During mitosis the nucleolus disappears and a spindle with chromosomes arranged as an equatorial plate is formed.

Type species. Dobellina mesnili (Keilin).

An account of the nuclear division in *D. mesnili*, using the Feulgen reaction, has been given by Bishop & Tate (1939). According to these workers, the Feulgen-positive chromatin in the resting nucleus is arranged mostly on the surface of the nucleolus (karyosome). At metaphase stage, Bishop & Tate (1939) have figured centrosomes and centrodesmose.

ENDAMOEBIDAE Calkins emend. Auctt.

Definition. The resting nucleus contains a Feulgen-positive karyosome or Feulgen-positive chromatin granules. Feulgen-negative granules or nucleoli do not give rise to 'polar masses', and the nuclear membrane is always intact during mitosis. Amoebae may be uni- or multinucleate, and no temporary flagellate stage has been discovered.

Type genus. Entamoeba.

Genus ***Entamoeba*** Casagrandi and Barbagallo emend. Auctt.

Definition. The resting nucleus has a relatively small Feulgen-positive karyosome located in or near the centre and Feulgen-negative peripheral granules lining the nuclear membrane. During mitosis peripheral granules do not give rise to 'polar masses' and the nuclear membrane is always intact. No temporary flagella are produced.

Type species. Entamoeba histolytica (Schaudinn).

Genus ***Endamoeba*** Leidy emend. Auctt.

Definition. The resting nucleus has Feulgen-positive chromatin without a karyosome and Feulgen-negative nucleoli.

Type species: *Endamoeba blattae* (Bütschli).

The nuclear structure and nuclear division of *Hydramoeba hydroxena*, using iron–haematoxylin-stained preparations, has been studied by Reynolds & Threlkeld (1929). The nuclear membrane persists throughout division. The genus *Hydramoeba* Reynolds & Looper and other genera, in which 'polar masses' are not produced and the nuclear membrane persists throughout division, can be included in the family Endamoebidae.

V. Nuclear division and phylogeny

Classification of amoebae based on form, locomotion and pseudopodial characters and on parasitic or free-living nature is of no probable phylogenetic value. Such diverse amoebae like *S. russelli* or *N. aerobia* and *E. histolytica* have eruptive *limax*-type movement. *N. aerobia*, a free-living form, and *E. histolytica*, are both human pathogens. Classification of amoebae based on nuclear division shows that in Schizopyrenidae the Feulgen-negative nucleolus or nucleoli give rise to 'polar masses' during mitosis. The nuclear membrane persists throughout division. A form such as *N. gruberi*, which during division has 'polar masses' and 'interzonal bodies' and can readily produce temporary flagellate stage, may be regarded as a primitive amoeba. During the course of evolution it seems that amoebae lose the power to produce 'interzonal bodies', although they are able to produce temporary flagella, as is the case in *D. thorntoni*. In a form like *S. russelli* no temporary flagellate stage is produced, although the nuclear division of this amoeba is indistinguishable from *D. thorntoni*. Thus the primitive character of temporary flagella production in *S. russelli* is lost, but it retains the family character in having 'polar masses'. In Endamoebidae Feulgen-negative granules or nucleoli do not give rise to 'polar masses' and the nuclear membrane is intact during division. In Hartmannellidae the Feulgen-negative nucleolus or nucleoli and the nuclear membrane disappear during nuclear division, as happens in *H. glebae*, *D. mesnili*, *A. proteus* and *P. carolinensis*. A spindle with chromosomes arranged as an equatorial plate is formed, as in higher animals and plants. Judging from the advanced type of nuclear division in amoebae put in the family Hartmannellidae and the loss of the flagellate stage, it seems that amoebae are evolved from flagellate ancestors and not the flagellates from amoebae.

VI. Pathogenicity of small free-living amoebae

H. rhysodes strain RS was tested for its pathogenicity in four strains of mice (Albino, Parks, Swiss and Calcutta). 5000, 10 000, 20 000 and 100 000 trophozoites per mouse was given to six mice intranasally. All the Albino mice died in 3 to 14 days, showing symptoms of meningo-encephalitis, when the inoculum was from 5000 to 100 000. Large numbers of motile amoebae could be seen in smear preparations made from the brain of mice about to die. Amoebae could also be readily cultured from the brain tissue. Cultures made from lung tissues also revealed the presence of amoebae when the inoculum of trophozoites was high. One to two, out of six, Parks, Swiss and Calcutta strains of mice survived up to 14 days when the inoculum contained 5000 or 10 000 amoebae. The control mice remained healthy up to 14 days. On the basis of these findings, Albino mice were used in further experiments on pathogenicity of different strains of amoebae, and 20 000 trophozoites were inoculated per mouse.

The pathogenicity of strains of *H. culbertsoni*, *H. rhysodes* and *N. aerobia* to mice are given in table 2. *H. culbertsoni* strain A-1 and *N. aerobia* seem to be more pathogenic than the other strains. Smears made from brain tissue of all the mice killed when showing symptoms of meningo-encephalitis, revealed the presence of large numbers of motile amoebae. Amoebae could also be readily cultured from the brain tissue.

Six mice were inoculated with *H. castellanii*, *H. polyphaga* strains 21 and 42 (*A. polyphaga*), *H. astronyxis*, *H. palestinensis*, *H. glebae*, *H. exundans*, *H. vermiformis*, *N. gruberi* two strains (one isolated locally and the other obtained from Dr Balamuth), *D. thorntoni*, *S. russelli* and *T. rostratus* trophozoites. None of the mice showed symptoms of meningo-encephalitis. When the

mice were killed after 14 days, no culture of amoebae could be obtained from the brain tissue. All the non-pathogenic amoebae were tested thrice in mice and they were found to be non-pathogenic.

TABLE 2. PATHOGENICITY OF FREE-LIVING AMOEBAE IN ALBINO MICE

amoeba	no. of mice	death time of mice in days
H. culbertsoni strains		
A-1	6	2, 2, 3, 3, 5, 5
B-1	6	7, 8, 8, 9, 10, 10
B-2	6	8, 8, 9, 9, 10, 10
B-3	6	6, 7, 7, 7, 8, 8
B-6	6	6, 7, 7, 8, 8, 8
H. rhysodes strains		
14	14	7, 7, 8, 8, 9, 10, 10, 12, 12, 12, 14, 14, 14, 14
15	13	7, 8, 8, 8, 8, 9, 9, 10, 10, 10, 12, 12, 13
RS	6	4, 6, 7, 7, 8, 9
FS	6	3, 3, 3, 6, 7, 8
GS	8	3, 3, 3, 7, 8, 9, 12, 12
B-4	6	6, 6, 6, 7, 7, 8
B-5	6	5, 7, 7, 7, 7, 8
A-5	10	3, 3, 4, 6, 6, 6, 6, 10, 10, 12
A-30	6	5, 7, 7, 7, 8, 10
HN-3	7	7, 8, 8, 9, 10, 10, 12
HN-15	8	5, 6, 6, 7, 7, 11, 12, 12
HN-17	8	5, 6, 7, 7, 7, 10, 11, 12
N. aerobia	12	2, 2, 2, 2, 4, 4, 4, 4, 5, 6, 6, 6
control (*A. aerogenes*)	8	S†, S, S, S, S, S, S, S

† S = mice surviving up to 14 days.

VII. EFFECT OF DRUGS ON AMOEBAE

The amoebicidal effect of a drug against *E. histolytica in vitro* was tested by the method of Saxena, Das & Singh (1963) and in experimental intestinal amoebiasis of the rat by the method of Singh, Das & Saxena (1963).

Metronidazole killed *E. histolytica* trophozoites *in vitro* at 1/256 000 (w/v) dilution. It had little or no effect against trophic and flagellate stages of *N. aerobia* and the trophic stage of *H. culbertsoni* at 1/400 (w/v) dilution.

When cysts of *H. culbertsoni* were treated for 48 h with 1/40 and 1/80 dilutions (w/v) of metronidazole, their percentage excystment, after the removal of the drug, was about 65 to 75 %. The control gave 88 % excystment. This shows that metronidazole has little or no cysticidal property. The majority of the treated cysts, like the control cysts, did not take stain with eosin.

In experimental intestinal amoebiasis of the rat infected with a virulent strain of *E. histolytica* (STA), metronidazole was found to be very effective. When ten rats were fed orally with 900, 300 or 100 mg kg^{-1} day^{-1} for 5 days, 2 days after infection, there was complete cure of amoebiasis. At the above doses, it also completely eliminated the flagellates from the caecum of rats.

Six Albino mice were infected intranasally with *N. aerobia* and another six with *H. culbertsoni* and treated with metronidazole. At 100, 300, 500 and 900 mg kg^{-1}, all the mice died, showing symptoms of meningo-encephalitis, as was the case in the control mice. In the case of

H. culbertsoni infection, the treated mice (900 mg kg^{-1} day^{-1}) survived a few days longer than the untreated control mice. They were all dead within 9–11 days.

Chlorhexidine (200 mg kg^{-1} day^{-1}), paromomycin (300 mg kg^{-1} day^{-1}), carbarsone (300 mg kg^{-1} day^{-1}), entobex (300 mg kg^{-1} day^{-1}), emetine bismuth iodide (10 mg kg^{-1} day^{-1}), emetine HCl (4 mg kg^{-1} day^{-1}), chlorostrep (300 mg kg^{-1} day^{-1}), camoquin (300 mg kg^{-1} day^{-1}), chloroquin (300 mg kg^{-1} day^{-1}), and sulphonamide (300 mg kg^{-1} day^{-1}) have been found to be completely ineffective against *H. culbertsoni* infection in mice.

None of the antibiotics and other drugs tried in human meningo-encephalitis cases, caused by free-living amoebae, have been found to be effective. Culbertson, Homes & Overton (1965) found sulphadiazine effective in experimental *H. culbertsoni* infection in mice, although it was completely ineffective against *N. aerobia* infection in mice (Culbertson *et al.* 1968*b*). Sulphadiazine has also been found ineffective against *N. aerobia* in human cases (Carter 1968; Butt *et al.* 1968*b*). Metronidazole has been considered to be the most effective and least toxic of direct-acting drugs for both acute human dysentery and human liver abscess. It is also very effective against *E. histolytica in vitro* (see *Indian Practitioner* 1968). McFadzean, Squires & Whelan (1968) claim that metronidazole is effective against *H. culbertsoni in vitro* and *in vivo* against a lethal nasal infection. In the present work, metronidazole has been found to be ineffective against *H. culbertsoni* both *in vitro* and *in vivo*. Since metronidazole is ineffective against cysts, as is the case with other known antiamoebic drugs, it may have little or no effect in curing chronic amoebiasis due to *E. histolytica*.

VIII. Summary

1. A study of morphology, cystic character and excystment, nuclear division and pathogenicity in mice of small free-living amoebae has been made.

2. *Naegleria aerobia* sp. nov., from a fatal human case of amoebic meningo-encephalitis, has been described. This amoeba differs from *N. gruberi* in cystic character, mode of excystment and antigenicity. *N. aerobia* is pathogenic to mice while *N. gruberi*, *Schizopyrenus russelli*, *Didascalus thorntoni* and *Tetramitus rostratus* are non-pathogenic.

3. *Hartmannella culbertsoni* sp. nov., found as contaminant of mammalian cell culture, and pathogenic to mice and monkeys, has been described. *H. rhysodes* is pathogenic to mice while *H. castellanii*, *H. glebae*, *H. astronyxis*, *H. palestinensis*, *H. exundans* and *H. vermiformis* are non-pathogenic. Strains of pathogenic *Hartmannella*, isolated from Indian and American soils, were found to be either *H. culbertsoni* or *H. rhysodes*.

4. On the basis of the type of nuclear division, the order Amoebida Kent has been divided into families Schizopyrenidae Singh, Hartmannellidae Volkonsky, 1931 emend. Singh, 1952 and Endamoebidae (Calkins). The definition of the former two families has been slightly modified and the latter has been redefined. The relation of the proposed classification to previously defined families and genera of amoebae, and its bearing on phylogeny are discussed.

5. Metronidazole and other antiamoebic drugs have been found to be ineffective against *H. culbertsoni* and *N. aerobia in vitro* and in experimental meningo-encephalitis of mice.

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Addendum

Dr J. L. Griffin, Armed Forces Institute of Pathology, Washington, D.C. 20305, U.S.A., circulated a letter on 26 April 1968 to pathologists, parasitologists, protozoologists and others suggesting that the genera *Acanthamoeba*, *Hartmannella*, *Naegleria* and *Vahlkampfia*, as defined by Page (1967*a*, *b*), might be recognized. According to him the genera *Schizopyrenus* and *Didascalus* (Singh 1952) were derived from the classification of mitotic figures and cannot be evaluated except by a skilled amoeba cytologist. Since this paper was written, Griffin has kindly sent to one of us (B. N. S.) comments of twenty-five persons. Griffin has compiled his and other views under the heading 'Facultative pathogenic amoebae: A survey on nomenclature with a bibliography'. It may be of interest to mention the views expressed by a few protozoologists. R. N. Band (p. 4) says—'Obviously phenotypic characters (morphological and physiological) are the result of the interaction between DNA and the environment. Thus, the use of phenotypic characters, as genetic markers, in taxonomy can be complicated by the environment to varying degrees. Page's approach has been to assume that axenic cultures are not useful in morphological taxonomy since the natural environment is not replicated. I submit that there is no basis for the assumption that bacterized cultures mimic the natural environment any more than do axenic cultures. It also would be rather naive to treat the natural environment as a constant. It is possible to produce smooth or wrinkled cysts and to vary the degree of fine pseudopodial extensions with just one of the hartmannellid amoebae by varying the "*in vitro*" environment'. Band has pointed out that *Mayorella palestinensis*, *Hartmannella rhysodes* and *Acanthamoeba castellanii* (the real one) are physiologically distinguishable. W. Balamuth (p. 8) says—'I strongly agree with Drs Page and Griffin that the classification of amoebae should depend wherever possible upon multiple characters (e.g. pseudopodial morphology and behaviour, nuclear structure and behaviour in mitosis, cyst structure, etc.). Pseudopodial morphology is not adequate to separate genera sharply, and also represents an unstable character when environment conditions are altered. I believe that we shall be greatly aided by introduction of comparative antigenic analysis and study of base-pairing of the nucelic acids of some of these related forms.' S. L. Chang has recognized the family Schizopyrenidae and has included the genera *Schizopyrenus*, *Didascalus* and *Naegleria* in this family. He does not think that the establishment of the genus *Vahlkampfia* is justified until it is proven not to be *Naegleria* or *Didascalus*. Chang did find 'interzonal bodies' during mitosis of the amoeba that caused encephalo-meningitis in man (reported in Florida by Cecil Butt). The same amoeba has been used in this work and has been named *N. aerobia*. Chang says (p. 15)—'The genus *Schizopyrenus* is definitely established. I have encountered several times this amoeba in examintion of well water, industrial wastes (especially

from paper mills), and water stored in cisterns. In all cases, a pink color was imparted to the water. The amoeba fits the description of Singh's for *Schizopyrenus*'. Chang has included the genera *Hartmannella*, *Acanthamoeba* and *Singhella* gen. nov. (named after Singh for *H. leptocnemus*) in the family Hartmannellidae. According to him, the genus *Mayorella* should be omitted; most of the described species belong either to *Hartmannella* or *Acanthamoeba*.

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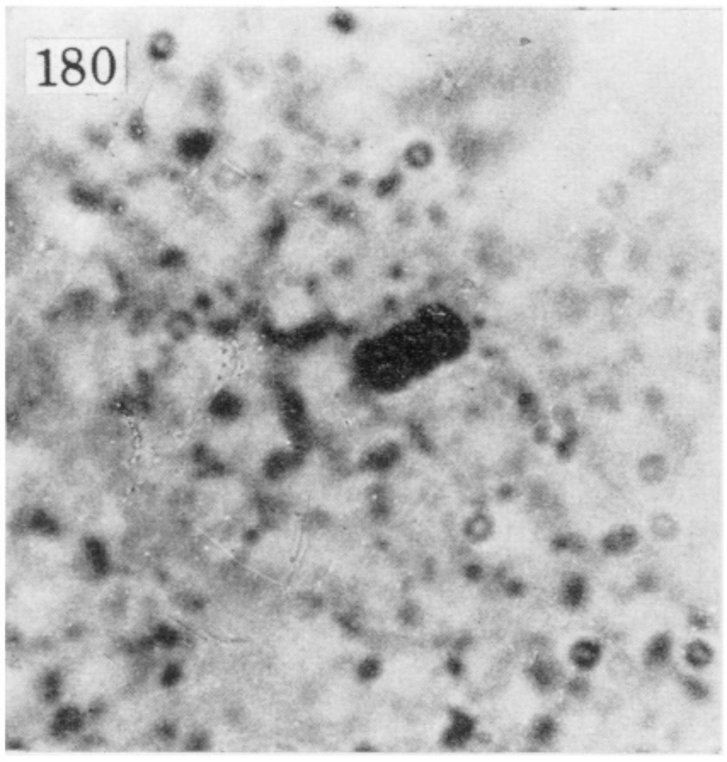
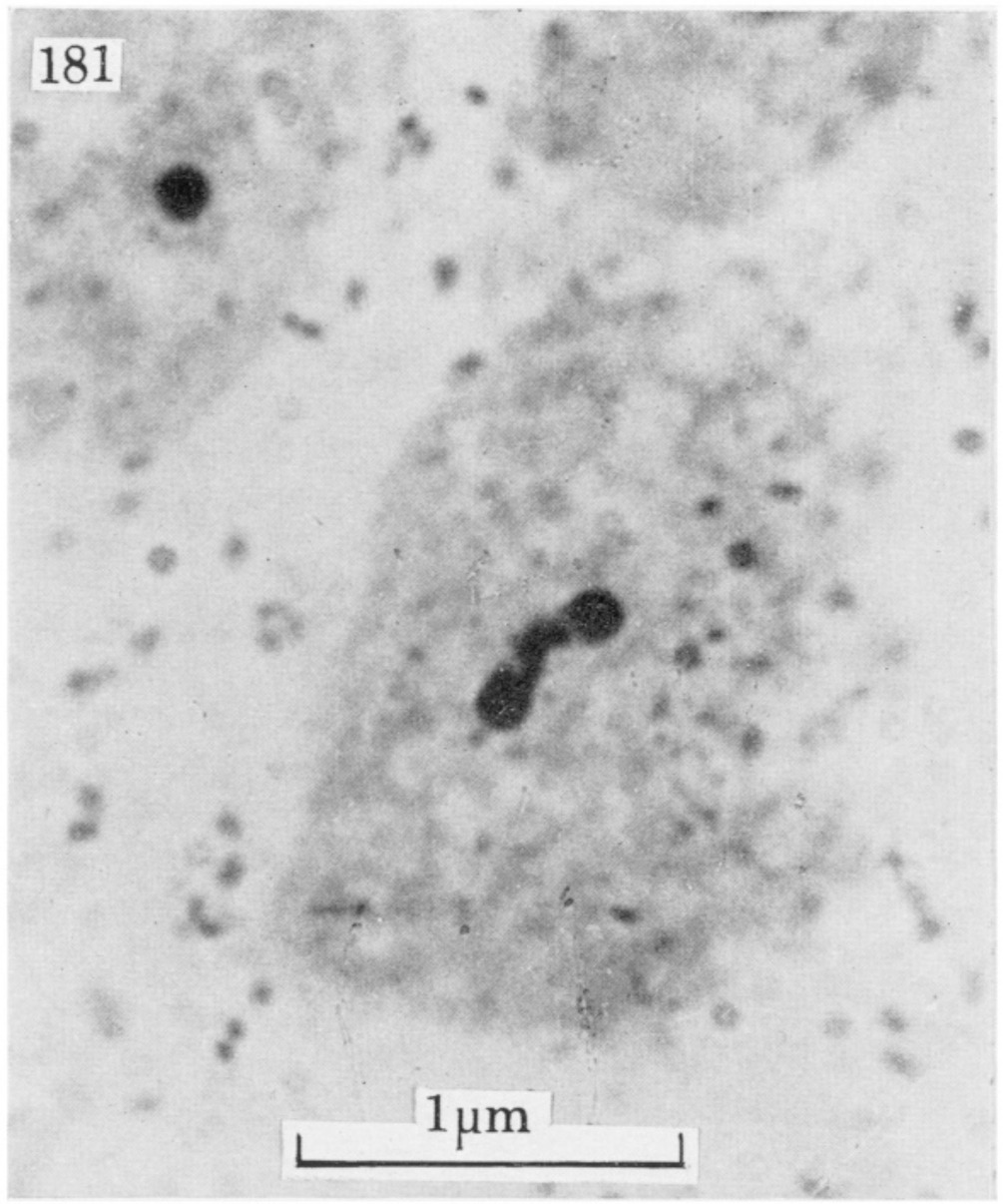
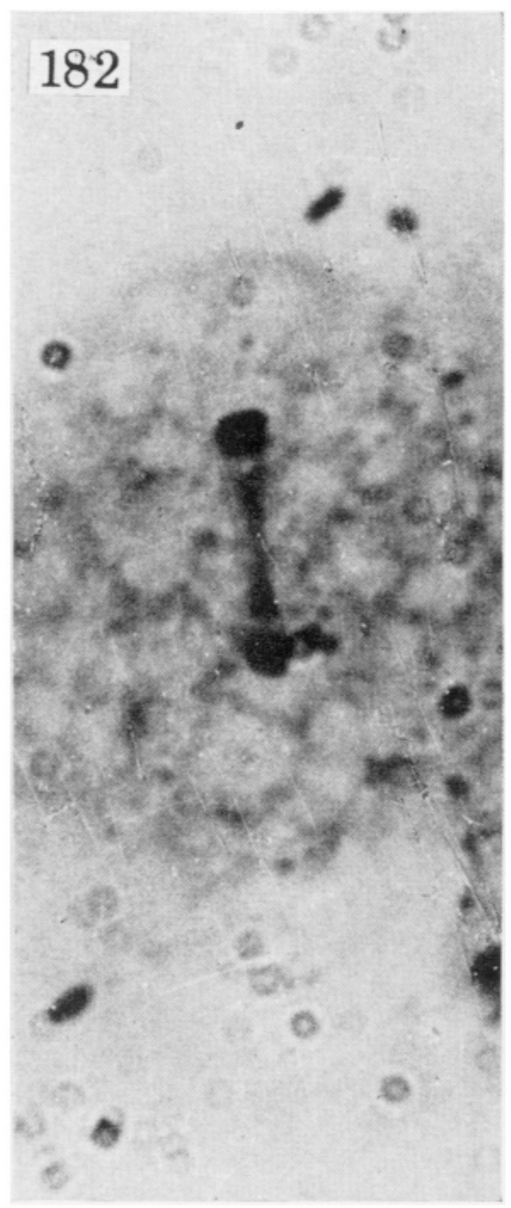
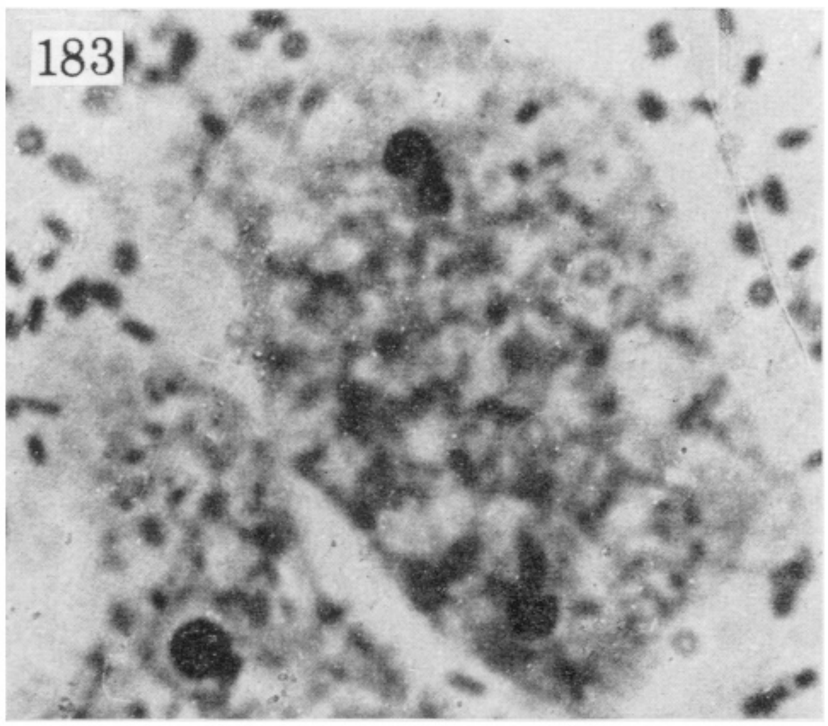
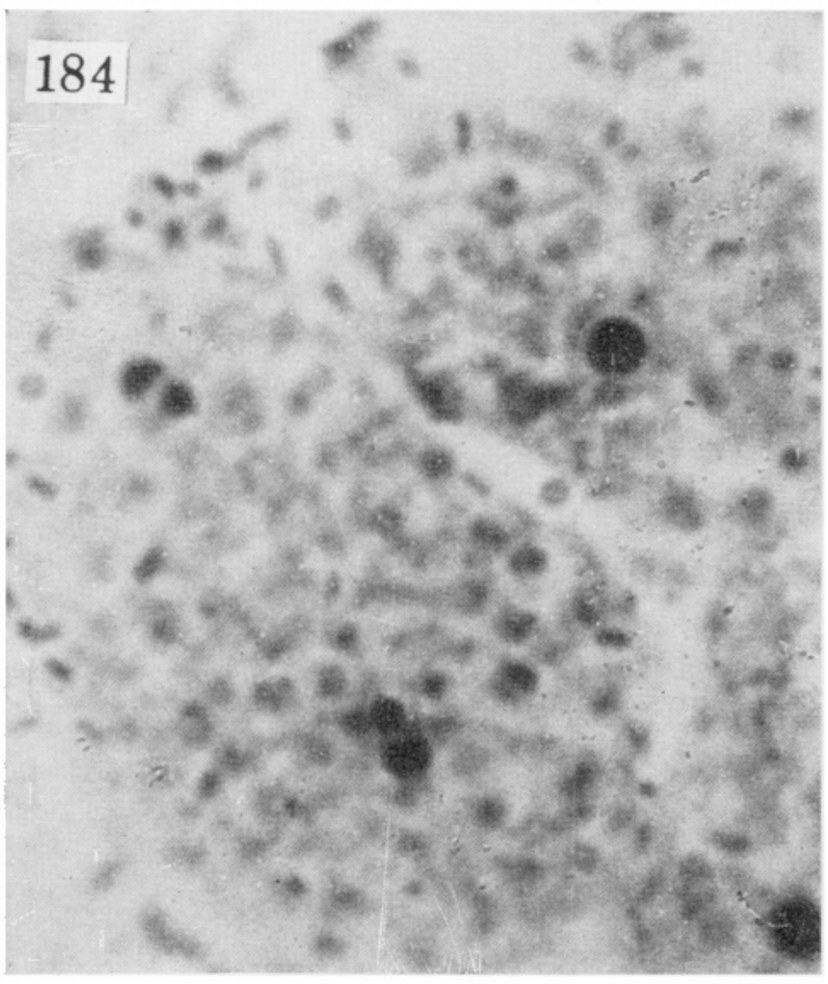
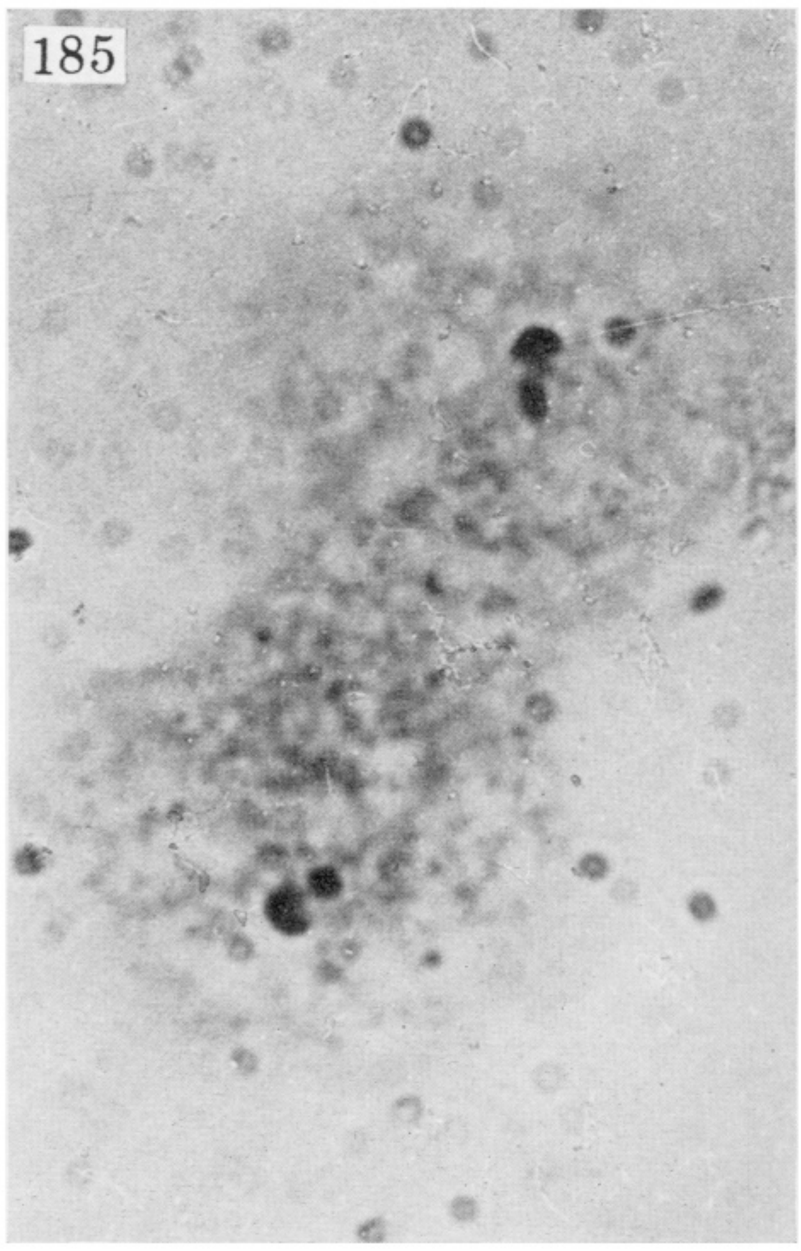


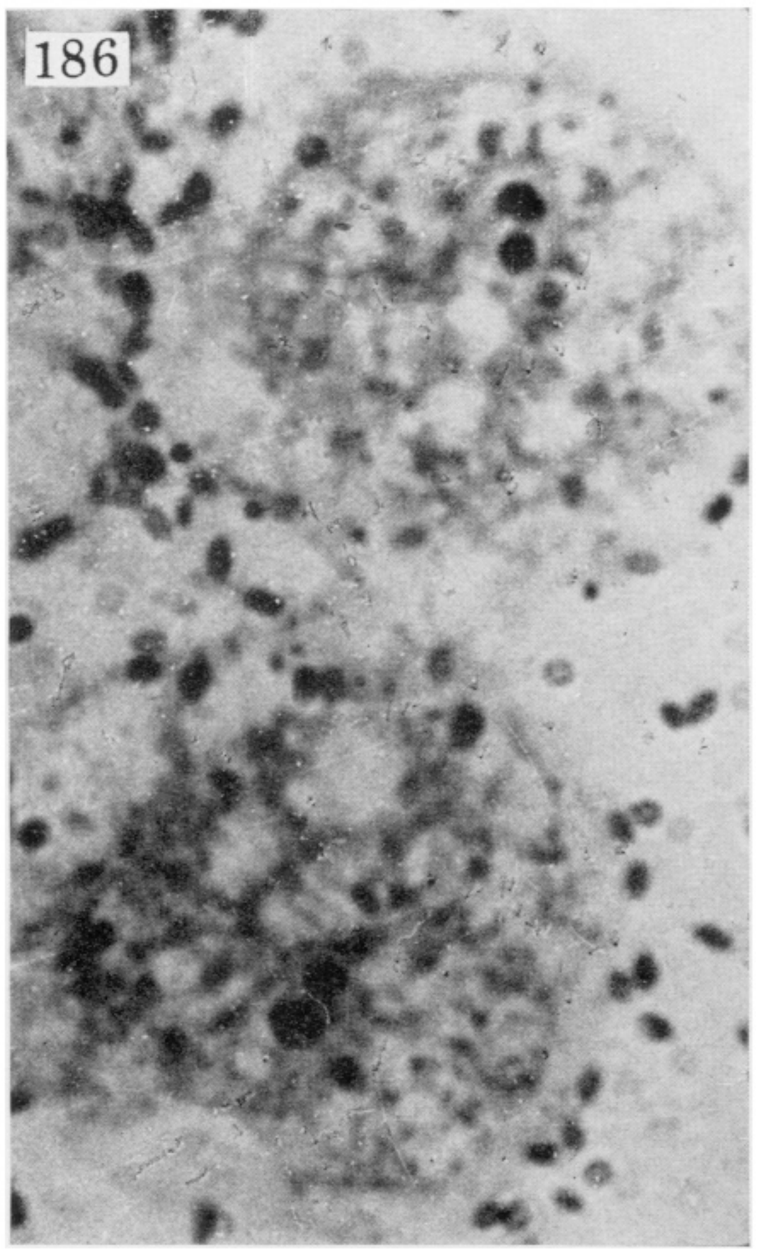






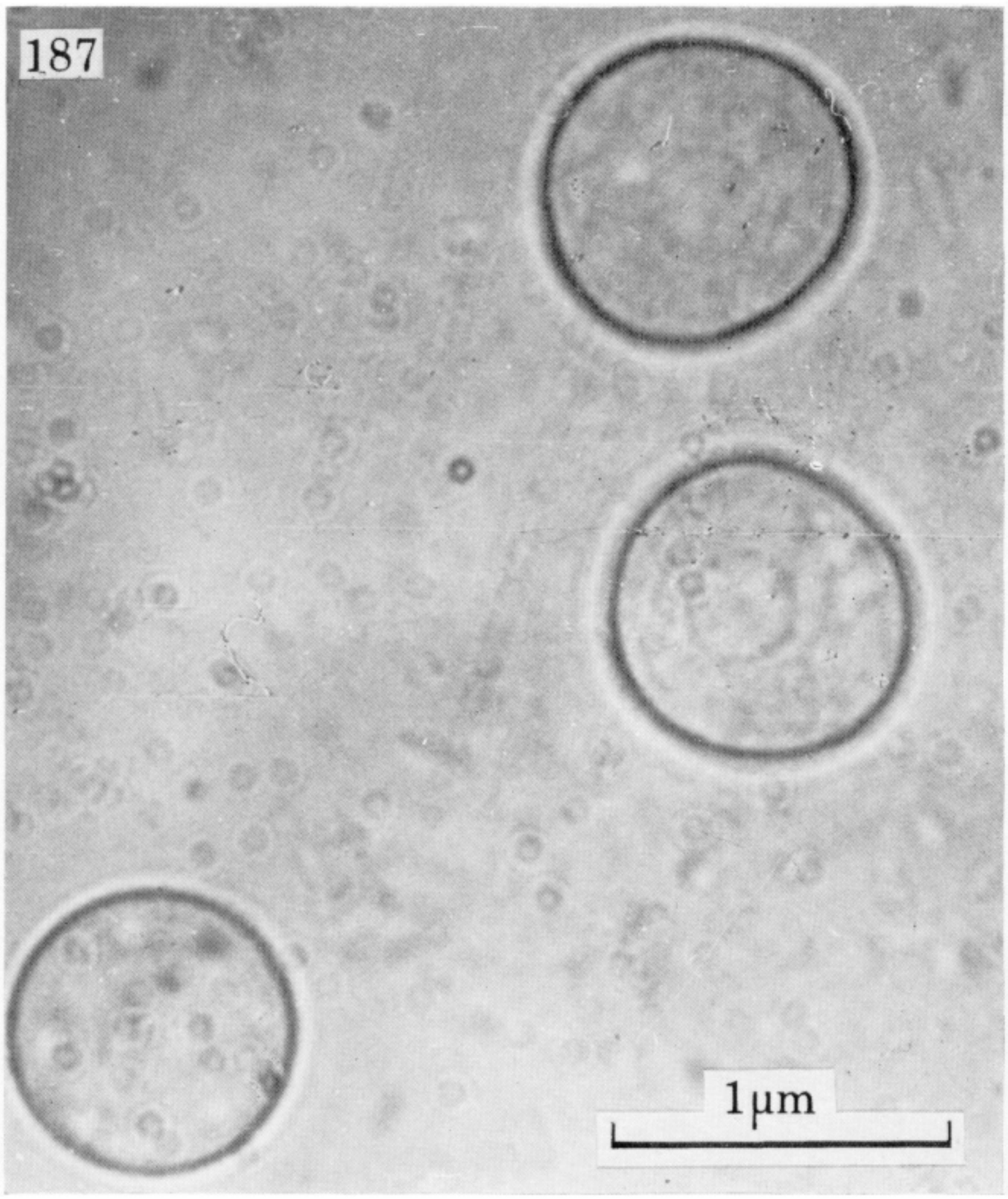


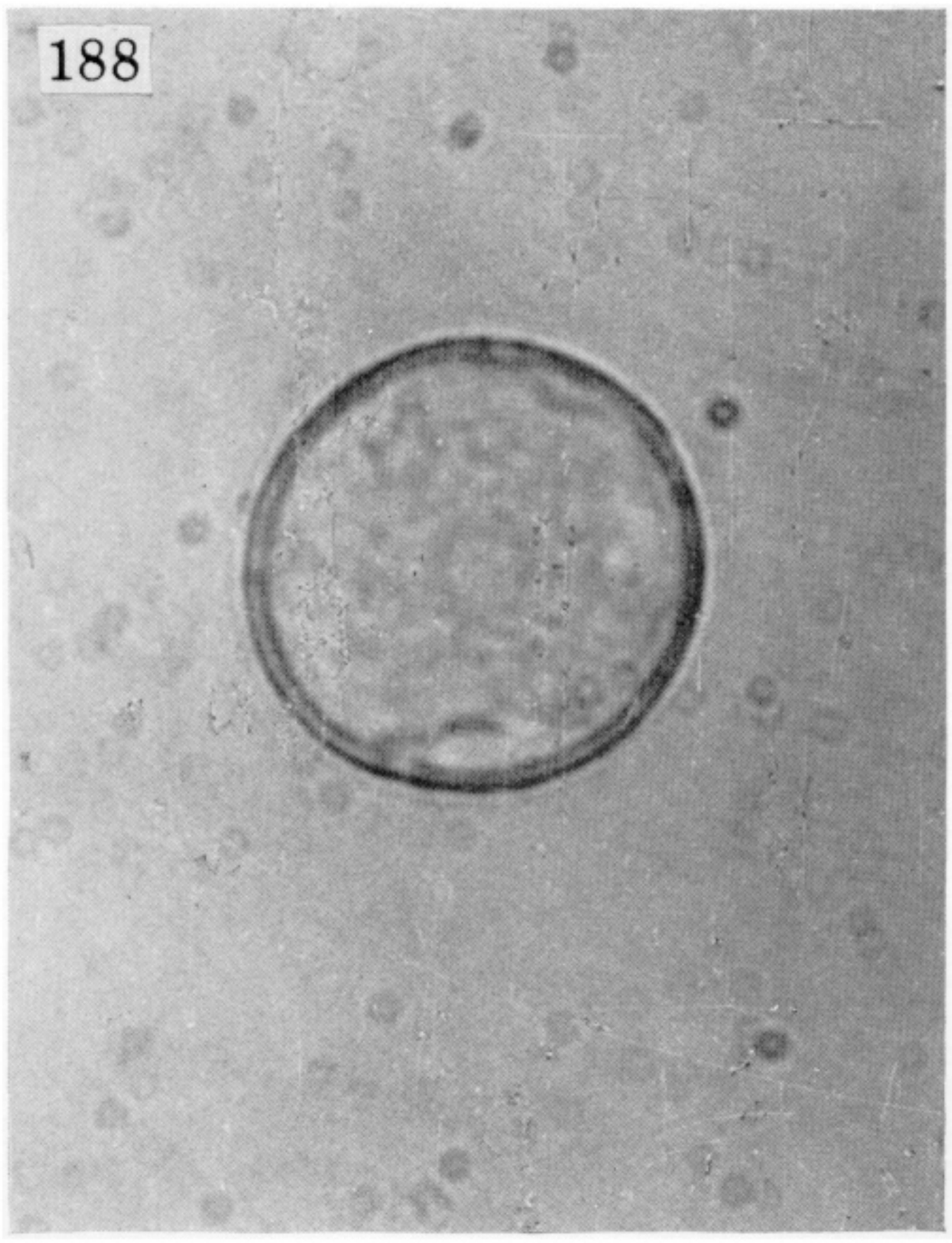


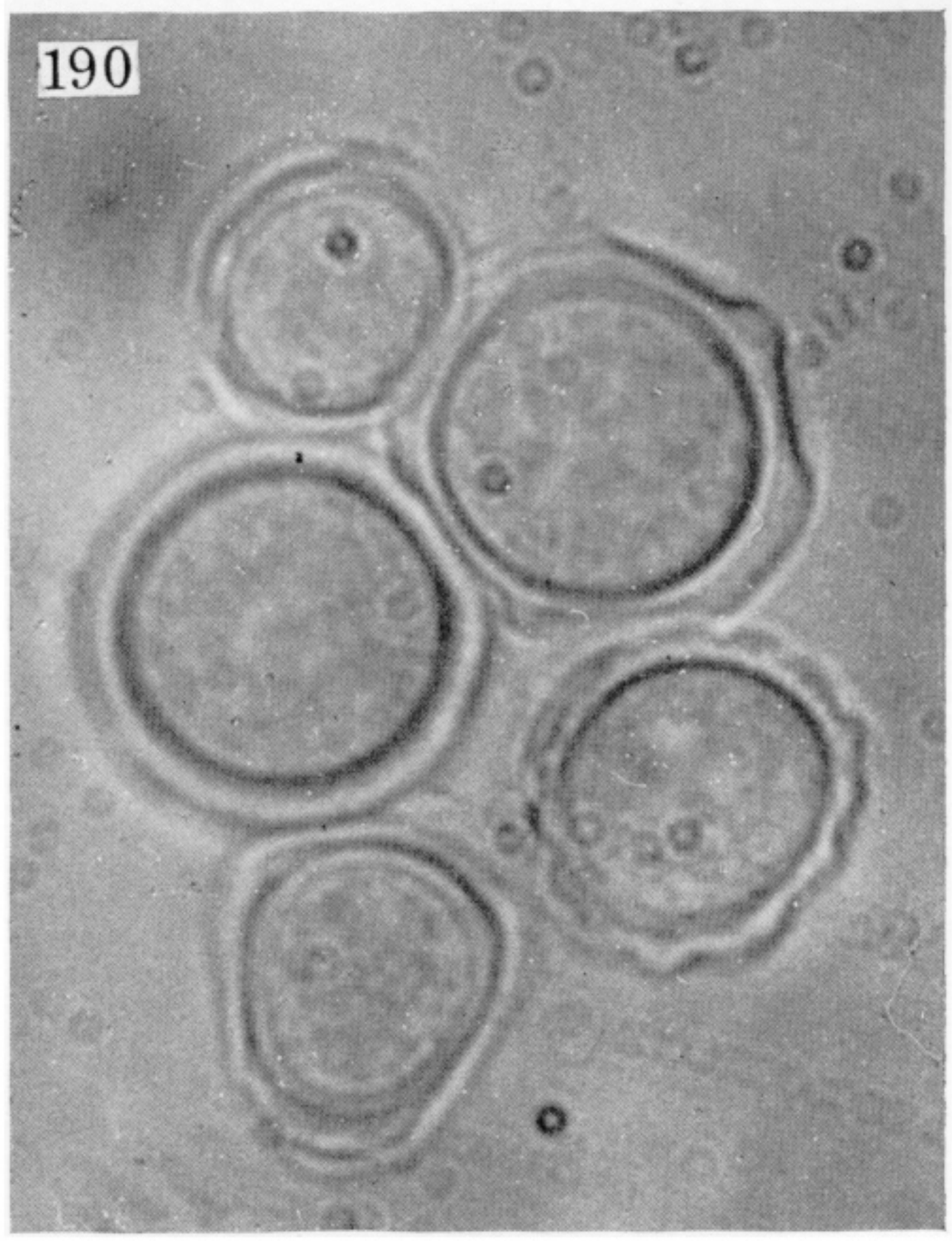


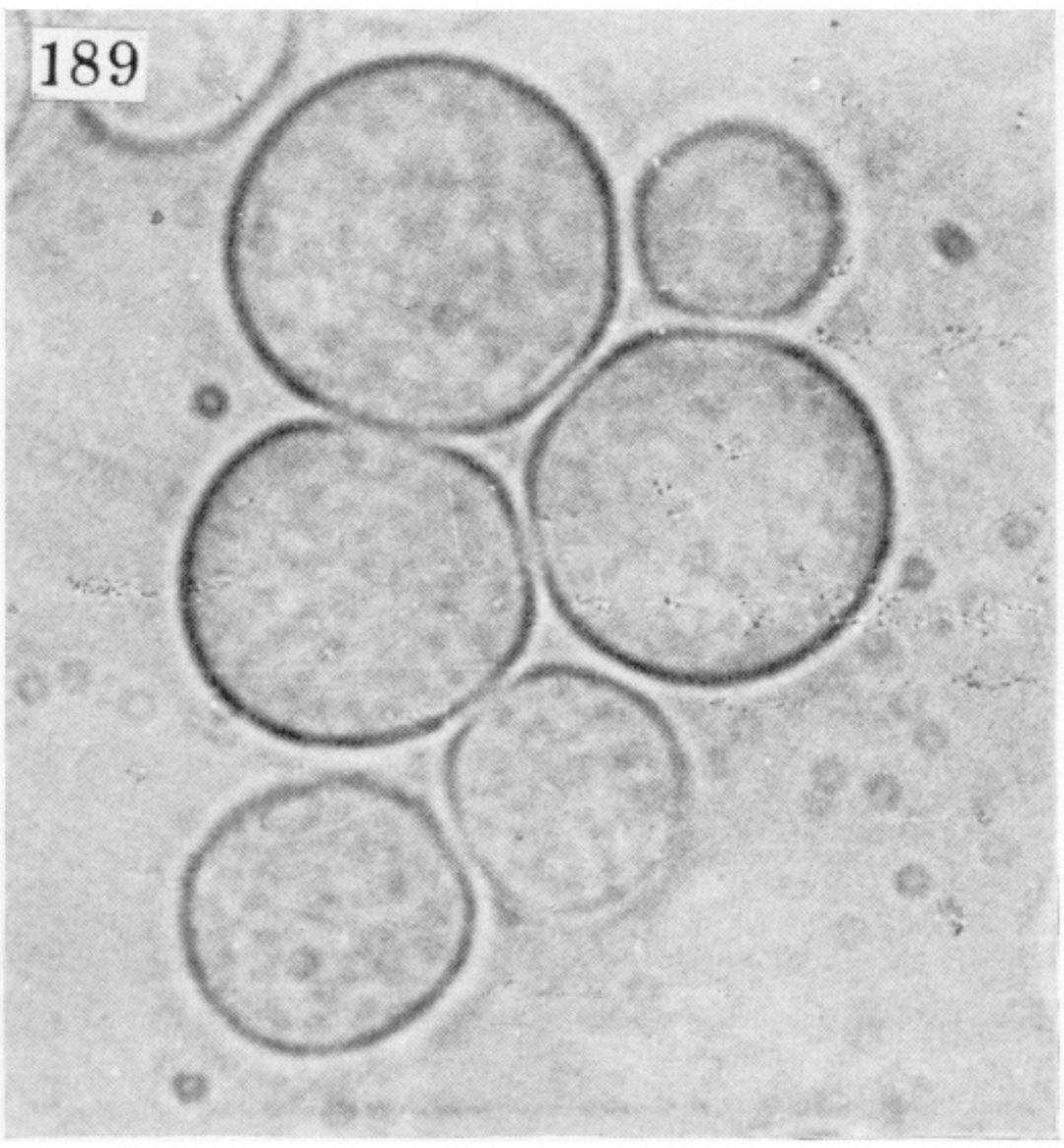
Successive stages in division of *N. aerobia*. Fixed in Carnoy and stained in iron-alum haematoxylin. The scale represents 10 μm and not 1 μm as marked.

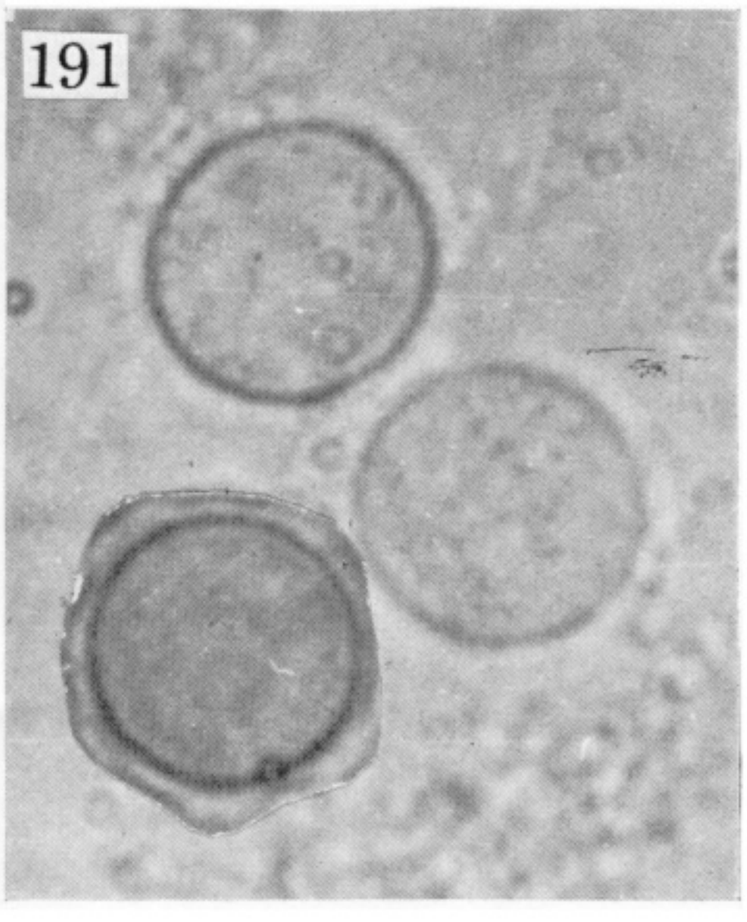
FIGURE 180. Metaphase stage.
FIGURE 181. Formation of 'interzonal body'.
FIGURES 182 to 186. Division of 'interzonal body' into two equal halves and its subsequent behaviour during mitosis.

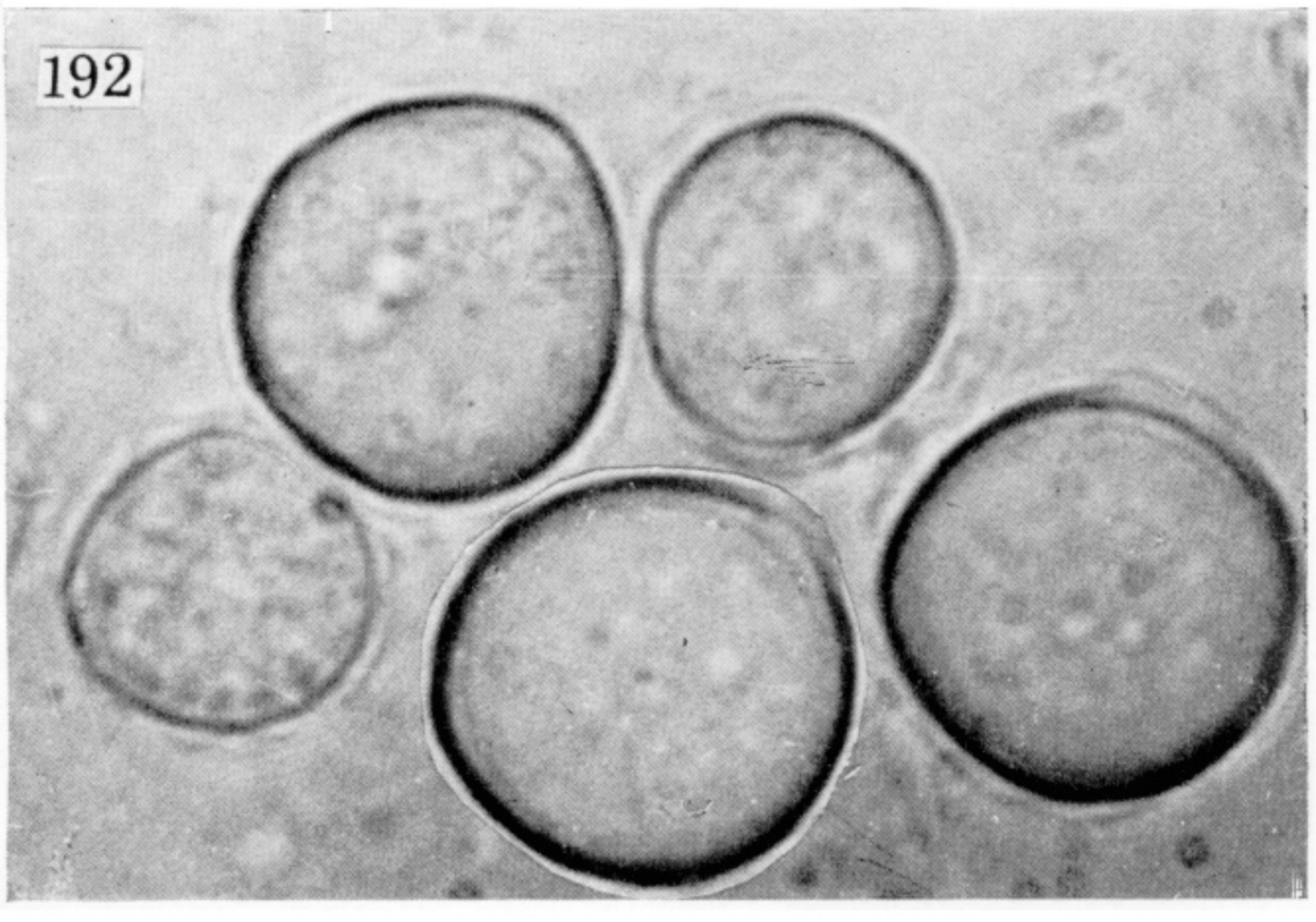












Cysts of amoebae in the living condition. The scale represents 10μm and not 1μm as marked.

FIGURE 187. *Naegleria aerobia* without any pores.
FIGURE 188. *N. gruberi* with pores.
FIGURE 189. *Didascalus thorntoni*.
FIGURE 190. *Schizopyrenus russelli*.
FIGURE 191. *S. jugosa*.
FIGURE 192. *Hartmannella culbertsoni*.

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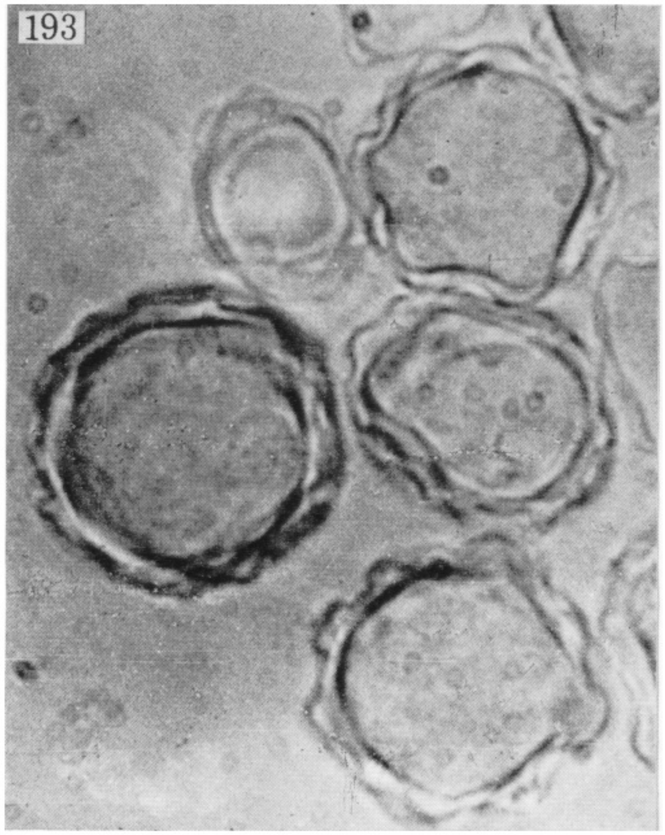

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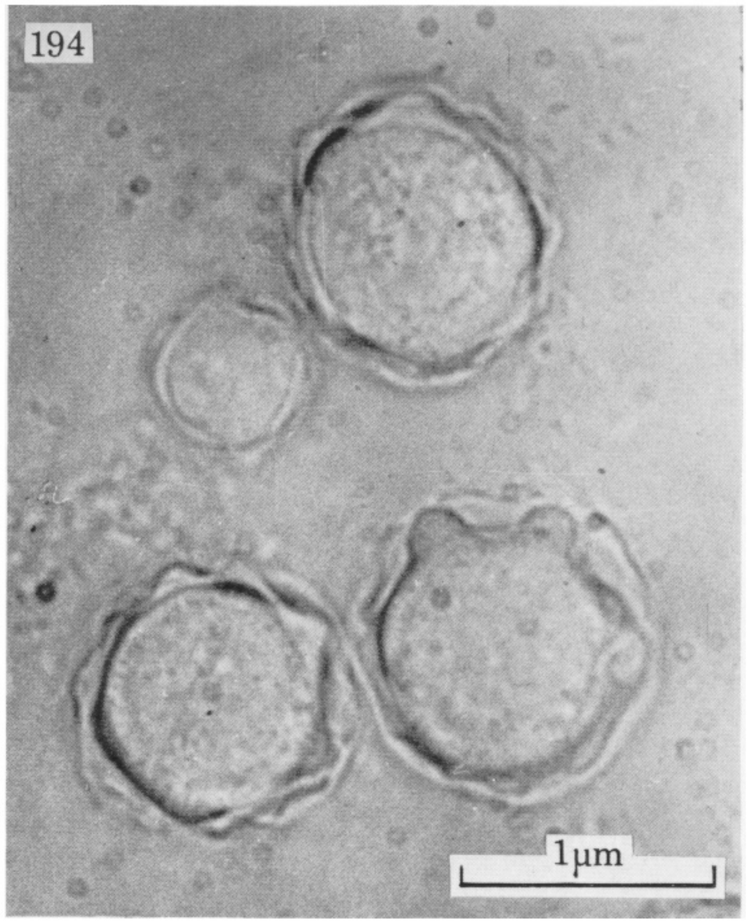

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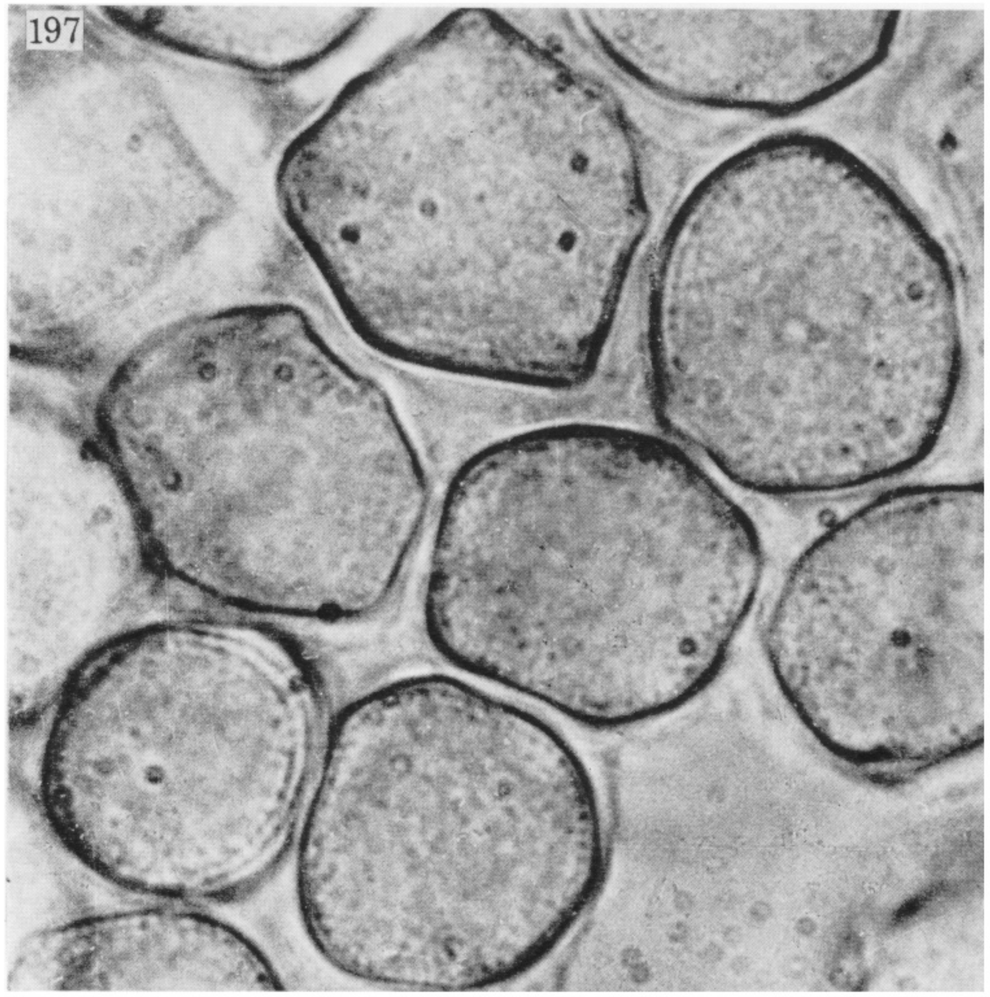

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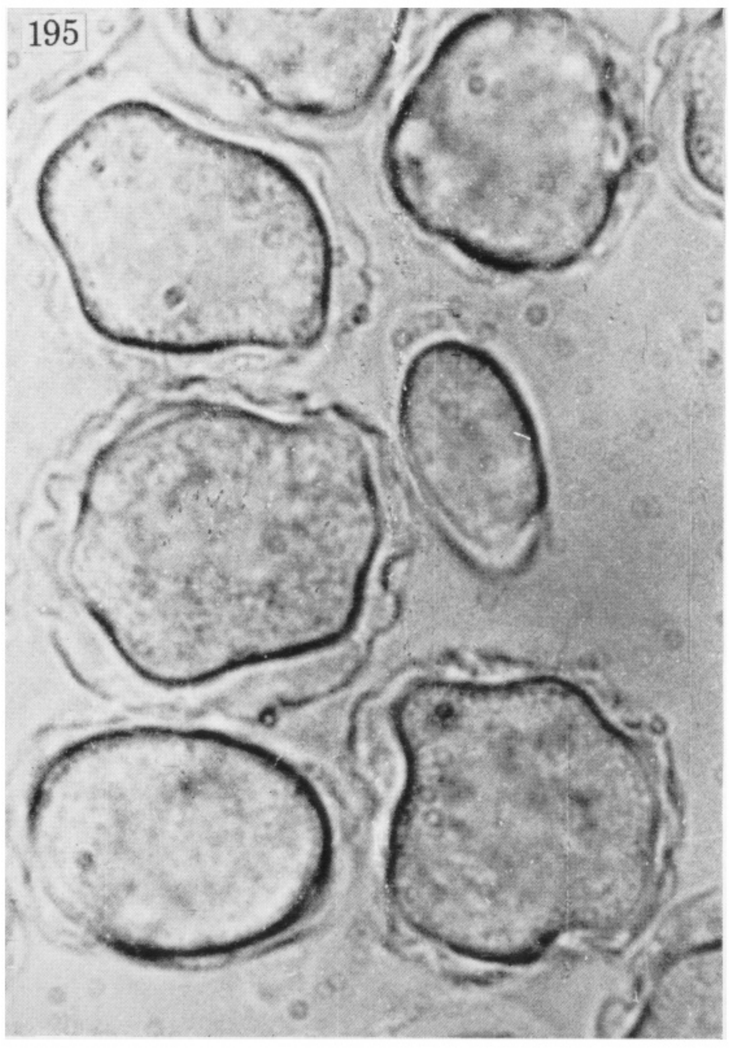

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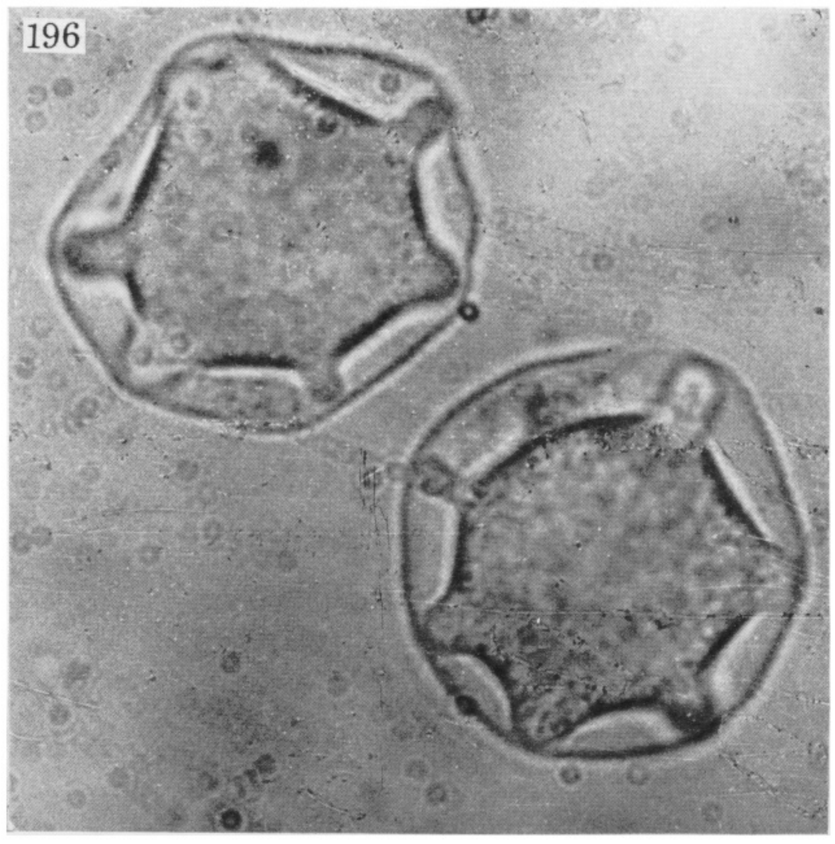

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