Comparative Morphology of Three Pathogenic Strains of *Naegleria gruberi*

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Abstract. Comparative morphological studies have been carried out with *Naegleria gruberi* amoebae of the Czech strain Vítek, the American strain HB-1 and of amoebae found in histological mounts of material from an outbreak of amoebic meningoencephalitis in Ústí nad Labem. A considerable difference was found in the size of the trophozoites of the American and Czech strains growing under axenic conditions or in animal tissues. A typical feature of the Czech strain is the formation of cytoplasmic granules or crystals and the frequent appearance of cysts. Phagocytosis of erythrocytes in the tissue was demonstrated with both isolates. Special features of the strain from the Ústí epidemics are intranuclear corpuscles and invasion of Purkynje's cells. All strains were identifiable as *Naegleria gruberi*.

The first pathogenic strain of the amoeba *Naegleria* sp. was isolated on the European continent in June 1968 (ČERVA et al. 1969a). This strain grew repeatedly from the cerebrospinal fluid of an 11 year—old boy who died shortly afterwards from amoebic meningoencephalitis confirmed by pathological examination (ČERVA et al. 1969b). This paper presents the results of a morphological study of the new isolate and a comparison of some of its properties with other pathogenic strains.

MATERIAL AND METHODS

Strains. The Vítek strain was isolated in Northern Bohemia in 1969 (ČERVA et al. 1969). The HB-1 strain was isolated in the U.S.A. in 1967 (BUTT et al. 1968, CULBERTSON et al. 1968) and obtained by the kindness of Dr. Culbertson from the Lilly Laboratories, U.S.A. The Ústí strain has been studied only in histological specimens from patients who died during the epidemics of amoebic meningoencephalitis in Ústí nad Labem (ČERVA et al. 1968).

Cultivation. The Vítek strain was isolated and further passaged in the laboratory in petri dishes on 1 % Difco Bacto-Agar which had been coated with a suspension of a thermally killed culture of *Aerobacter aerogenes* (CULBERTSON et al. 1965). The petri dishes were placed upside down in plastic bags, hermetically closed and incubated at 37 °C. The axenic cultivation was carried out on 2 % Bacto Casitone (Difco) in distilled water with 10 % fresh horse serum (ČERVA 1969). Slide and cover-slip cultures were prepared in petri dishes filled with this liquid medium.

Staining. The various kinds of material were stained with trichrome after Masson, iron haemato-xylin after Heidenhain, with Giemsa, the PAS reaction, the Feulgen reaction and with Giemsa-Robinow.

RESULTS

Size and shape of the amoebae. The shape of both isolates of *Naegleriae* is mostly oval when cultivated on agar plates with bacterial suspension. The ratio of length to width is about 4 : 3 (Plate I, Figs. 1, 2). Differences in size of the individual specimens are minimal. The average size of the Vítek strain is about $22.5 \times 15.5 \,\mu$, the extreme values range from 12—30 μ. The mean values of the American strain HB-1 are $25.7 \times 17 \,\mu$, the extreme values range from 12—34 μ. The outlines of the *Naegleriae* moving on the surface of the agar (without coverslip) are remarkably smooth and rounded. A relatively small pulsating vacuole could be seen in only 1/3rd of the specimens. The relatively distinct nucleus was visible at low magnification already.

The reproduction rate of amoebae of the Vítek strain increases under axenic conditions. Although smaller, the amoebae are more differentiated in size (mean value 16 μ, extreme values 8—26 μ). Under these conditions the size of the HB-1 strain differs remarkably more from that of the Czech strain than when cultivated on agar plates (Plate I, Figs. 3, 4) (mean value 26 μ, extreme values 17—42 μ).

In histological preparations of the original human material, the average size of the amoebae of the Vítek strain was 8.3 μ at the nucleus level; it ranged from 6.5—10 μ. The size of the same strain obtained from experimentally infected guinea pigs and measured in histological slides, was only 7.1 μ (4.3—8.6 μ), while that of the HB-1 strain, measured under identical conditions, was 9.6 μ (8.2—12.0 μ). The average size of the amoebae identified in the material of the epidemic outbreak of meningoencephalitis at Ústí n/L. was 7.4 μ (5.2—9.1 μ) and hence resembling that of the Vítek strain.

Locomotion. The locomotion is identical with that of amoebae of the genus *Naegleria* described by PAGE (1967). It is effectuated by the successive eruption of broad, smooth pseudopodia which ultimately join forming the hyaline anterior portion of the body. No differences in the movement of the various strains have been observed.

Cytoplasm. The cytoplasm of amoebae of the strains Vítek and HB-1 cultivated on agar plates in association with bacteria, contains numerous vacuoles of varying size. Some of the amoebae had 2—3 extremely large vacuoles surpassing greatly the size of the nucleus.

The cytoplasm of amoebae growing under axenic conditions also contains numerous vacuoles; these, however, are more uniform in size and never larger than the nucleus. A typical feature of the strain Vítek cultivated under these conditions is the formation of numerous cytoplasmic granules or crystals (PAGE 1967) which are either spherical or rod-shaped, size 0.25—0.75 μ; they stain black with iron

haematoxylin and red with trichrome. They do not stain with the PAS reaction. Similar granules have not been observed in the HB-1 strain (Plate II, Figs. 1, 2).

We have been unable to differentiate with certainty the pulsating vacuole in any of the stained preparations. By contrast to amoebae of the genus *Acanthamoeba*, the pulsating vacuole of *Naegleriae* is most indistinct, it is in fact impossible to differentiate it from the other vacuoles in the cytoplasm. Neither in viable amoebae nor in stained preparations of both pathogenic strains did we observe formations which could have been considered to be centrosomes.

In our description of histological slides of amoebic meningoencephalitis from Ústí we maintained that the cytoplasm of the amoebae did not contain phago-cytozed cellular elements. In slides stained with Masson's trichrome we observed in the grayish-green cytoplasm of the amoebae some indistinctly bordered formations only, which stained feebly red. Staining with iron haematoxylin after Heiden-hain, however, revealed formations which stained contrastly black and resembled phagocytozed erythrocytes. Although this suggestion seems highly probable, the identification of these formations is far from being final. The relatively late fixation of the human section material may have been responsible for the advanced stage of digestion of the phagocytozed cells. In both strains (Vítek and HB-1) phago-cytosis of erythrocytes could be observed in the brains of experimentally infected mice and guinea pigs, fixed immediately after the death of the animals. Erythro-phagocytosis occurs in areas of advanced destruction of the tissue with extensive haemorrhages. Phagocytosis of the erythrocytes is more common in the HB-1 strain perhaps because the ratio of size between the amoebae and the erythrocytes is more favourable than that in the smaller Vítek strain.

The flagellate stage. After the first passages of the strain Vítek, in liquid media without bacteria, the flagellate stages are formed very readily. A high percentage of flagellates was present even in 5 day-old cultures. After longlasting cultivation in liquid media their number began to decline.

The shape of the body of the flagellate stages of the strain Vítek may be spherical, piriform or elongate elliptic. Their size ranges from 5.3—17.0 μ. The body is bi-flagellate with two independent basal bodies. The length of the flagellum is about the same as that of the body. Typical features of the cytoplasm of these flagellates are the minute spherical vacuoles measuring about 0.5 μ in diameter (Plate IV, Figs. 1, 2).

The cyst. The Czech strain Vítek forms cysts within the first days of growth on the agar plates. The cysts are distributed over the surface of the agar in numerous typical groups. The cysts are very conspicuous for their high grade of refraction caused by their flat, lenticular shape (Plate III, Fig. 1). Their outline appears circular to oval; when in naturally formed groups, it may be slightly deformed but never into sharp angles. The edges of the cysts are generally slightly undulated in vertical direction. Hence, there seem to be minute irregularities in the curve of the cyst when viewed from above. The membrane of the cyst is about 0.5 μ thick and appears unilayered in the light microscope (Plate III, Fig. 2). Ostioles were visible

only in a few cysts. Mature cysts of this strain were not found in axenic culture.

The size of the cysts ranges from 7.0—12.5 μ (mean value about 9.0 μ), these values being also considerably lower than those found in other known strains of the genus *Naegleria*. We were unable to compare the morphology of these cysts with that of the HB-1 strain. When cultivating the American strain under identical conditions, cyst production could not be provoked and when an occasional cyst did occur it seemed immature and unsuitable for detailed microscopical studies.

In the phase contrast, fine granules can be seen in the contents of the cyst. These are concentrated mainly round the nucleus and responsible for the fact that both the nucleus and nucleolus are well-visible when studied under higher magnification in the phase contrast (Plate III, Fig. 2). In none of the two strains investigated did we observe cyst formation either in human tissue or in experimentally infected animals.

The nucleus. The nucleus of the Vítek strain measures about 3 μ, that of the HB-1 strain 4 μ. The size of the nucleolus is an unimportant determining feature because it varies considerably during the ontogenetical development of the amoeba. The nucleus is spherical or slightly elongate in shape. Generally, the nucleolus is situated in the centre, but in specimens of the Vítek strain its location may often be slightly excentric. The outlines of the karyoplasm stained with Heidenhain's iron haematoxylin or trichrome are only feebly accentuated by a circular accumulation of a small amount of chromatine. The most suitable reaction for demonstrating nuclear chromatine is the staining method recommended by Giemsa-Robinow. The dividing spindle is very elongate in both strains, in the HB-1 strain it often attains a length of 15 μ (Plate IV, Figs. 3, 4).

In the histological material from the epidemic outbreak of meningoencephalitis in Ústí we observed several stages of nuclear division of the amoebae. In spite of the coarse fixation with formaline used routinely for these mounts, the dividing stages with a preserved nuclear membrane and polar masses confirm the fact that the pathogenic agent belongs to the family Vahlkampfiidae, of which the genus *Naegleria* is a member (Plate V, Figs. 1, 2, 3).

We should like to draw attention to a special feature observed in the Ústí strain. In preparations stained with iron haematoxylin we found frequently in the karyoplasm of resting nuclei, in addition to the nucleoli, black stained corpuscles of only 0.2—0.3 μ (Plate V, Figs. 4, 5, 6) in size. Similar formations could not be demonstrated with any staining method in the two pathogenic strains under consideration. Neither has their presence in the species *Naegleria gruberi* been confirmed by any available reference in the literature. In our opinion the artificial origin of these corpuscles is out of question. They may be intranuclear centrosomes, but there is nothing to support our hypothesis because this strain has not been isolated in culture.

The intracellular parasitism of *Naegleriae*. The penetration of *Naegleriae* into Purkynje's cells presents a very special incidence of intracellular parasitism. This phenomenon has been observed repeatedly in the material from the Ústí epidemics.

In our earlier description of the histological material (ČERVA and NOVÁK 1968) we drew attention to the special affinity of the amoebae to the granular layer of the cerebellum. While invading the granular layer the amoebae pass actively into the cytoplasm of Purkynje's cells. It has been possible to observed the various phases of this phenomenon up to the complete destruction of the attacked cells (Plate VI, Figs. 1—4). In one instance we succeeded in demonstrating the stage of amoebic division inside the cell of the host.

Until the present, we have not been able to reproduce this phenomenon in mice or guinea pigs infected with the strains Vítek and HB-1. It seems that the different anatomical organisation of the rodent's brain is responsible for this failure. These small animals die before the amoebae can pass into the tissue of the cerebellum, because they are unable to survive the enormous primary onslought of parasitic infestation in the frontal parts of the brain starting soon after the intranasal inoculation.

DISCUSSION

We feel that a detailed morphological comparison of pathogenic amoebae isolated from human cases of protozoan meningoencephalitis in the U.S.A. and in Europe and their correct identification as an identical species is necessary in order to obtain comprehensive knowledge of the new pathogenic agent. In the case under consideration the amoebae have been isolated from two well-established endemic areas of occurrence of amoebic meningoencephalitis, one situated in the south-eastern coast of the U.S.A., the other in central Europe.

The isolates differ mainly in the measurements of the vegetative stages cultivated under axenic conditions and in the tissues of experimentally infected animals. Almost no differences have been observed in isolates cultivated on agar plates and nourished with a dead bacterial suspension. This indicates the different reaction of these strains to changes of the system of nutrition (from corpuscular to osmotic). These differences, indicated by the size of the trophozoites, may influence the pathogenetic properties of both strains. Another confirmation of the different metabolic processes in both strains growing under axenic conditions may be the formation of conspicuous intraplasmatic granules or crystals in the Vítek strain.

Phagocytosis of erythrocytes is typical of the dysenteric forms of *Entamoeba histolytica*. By contrast, the pathogenic strain A 1 of *Acanthamoeba castellanii* previously used as an experimental model of the pathogenic amoebae type limax, tends to phagocytoze nuclear cells—chicken erythrocytes or leucocytes (CHI et al. 1959, ČERVA 1967). Hence, phagocytosis of erythrocytes by the *Naegleriae* is an important feature. By no means, however, do the erythrocytes in the tissues represent the main source of nutrition of the *Naegleriae*. This has never been observed to occur in the principal sites of amoebic growth but only in necrotizing tissue.

Intracellular parasitism demonstrated so far only in the Ústí strain seems to be of considerable theoretical importance. Among parasitic amoebae this phenomenon is quite unique. Whether this phenomenon is of practical importance in the pathogenesis of amoebic meningoencephalitis remains to be investigated. There is, however, some indication that the cerebellum of man is attacked at a later stage of disease, i.e. at a time when the typical symptoms of damage to the function of the cerebellum are overshadowed by deep unconsciousness.

The measurements of the Czech isolate—strain Vítek—are within the lower range of sizes given for the species *Naegleria gruberi*; they remain, however, within the range of its variability.

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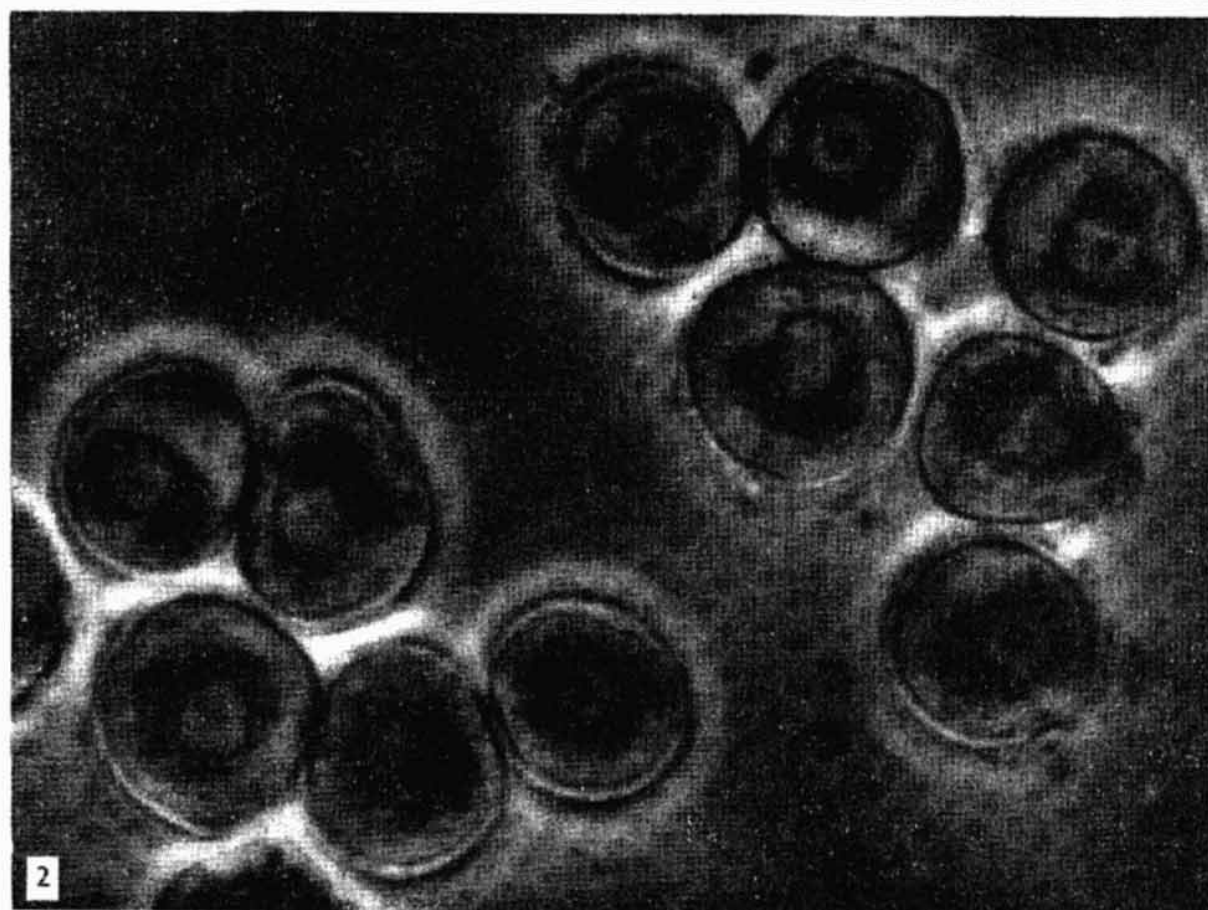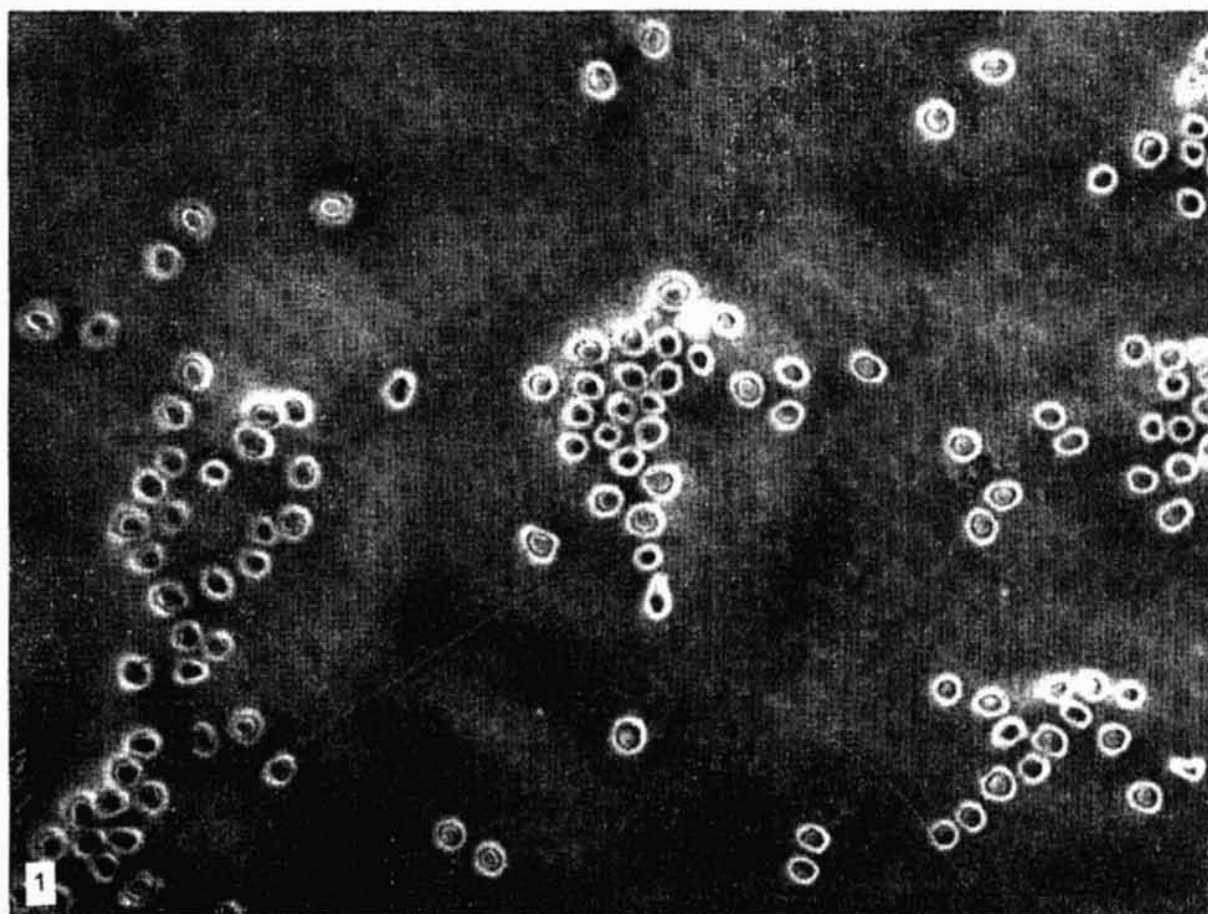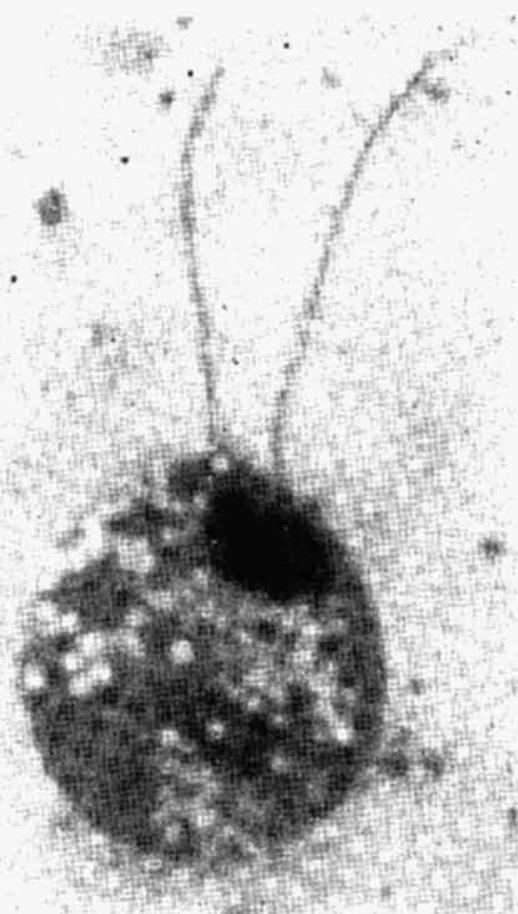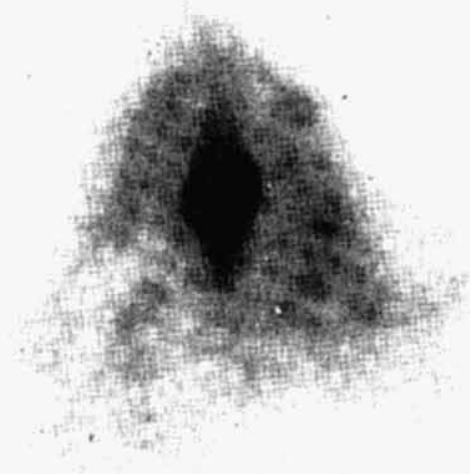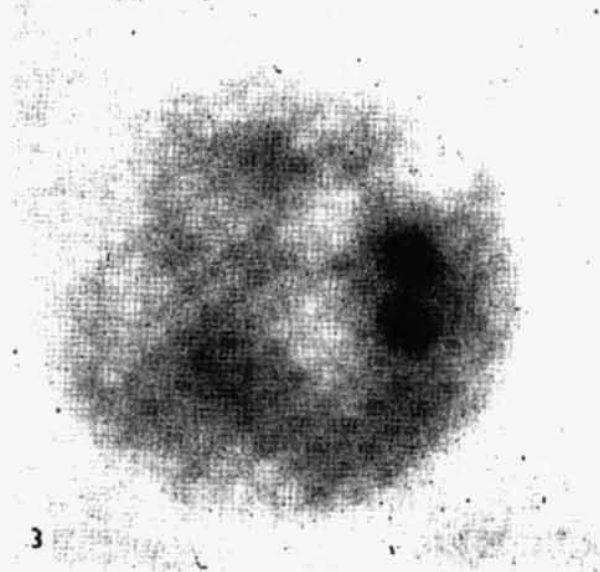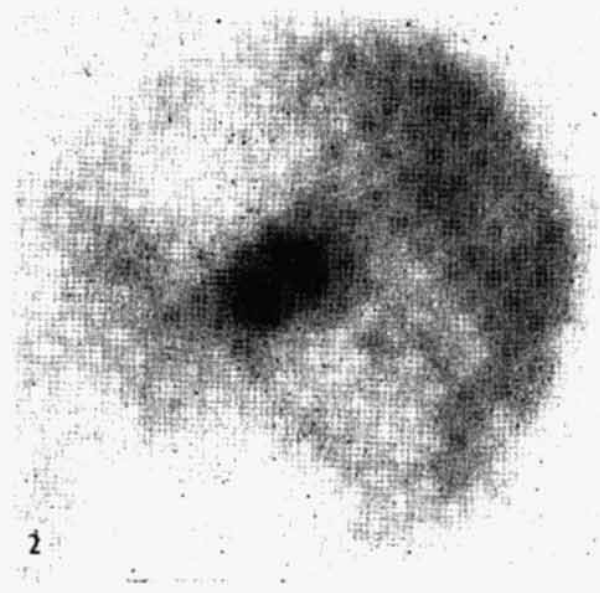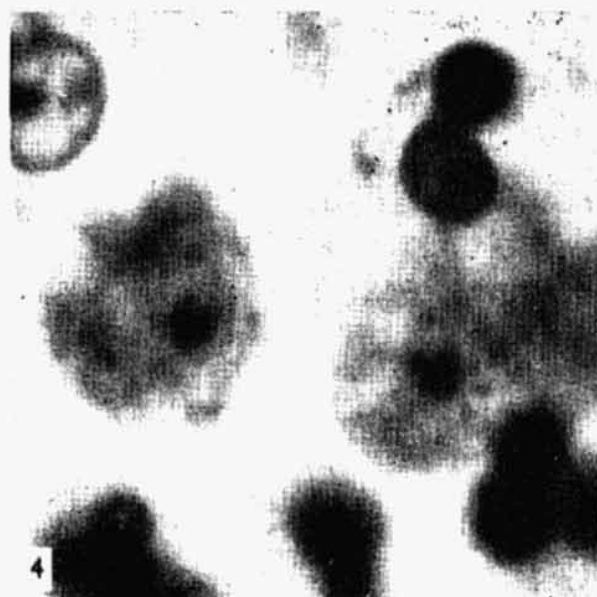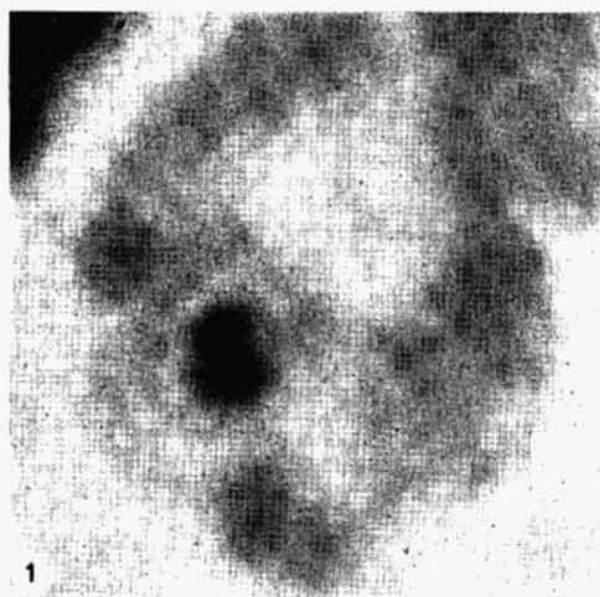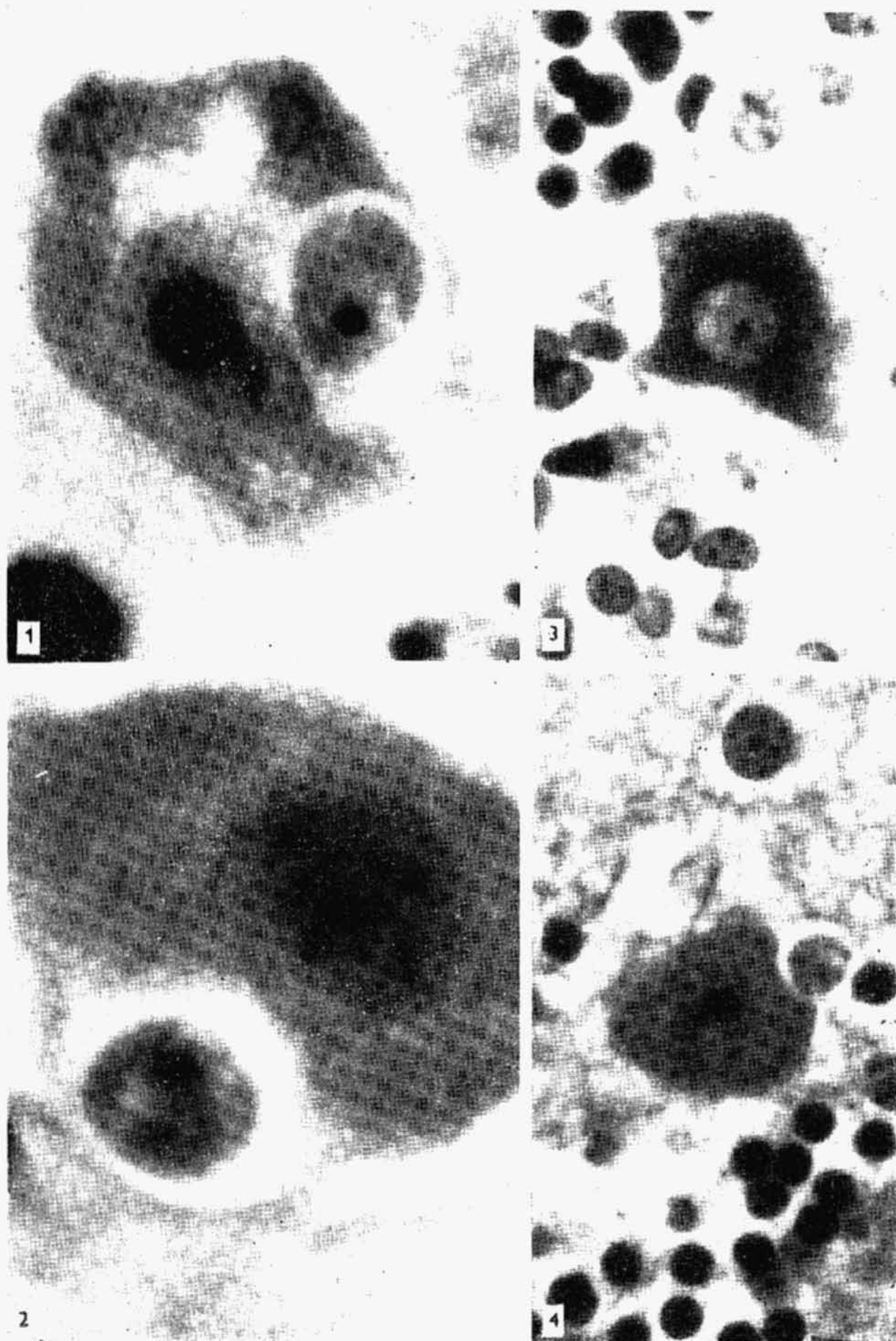L. Č., Vojenský ústav hygieny, epidemiologie a mikrobiologie, Praha-Střešovice, ČSSR
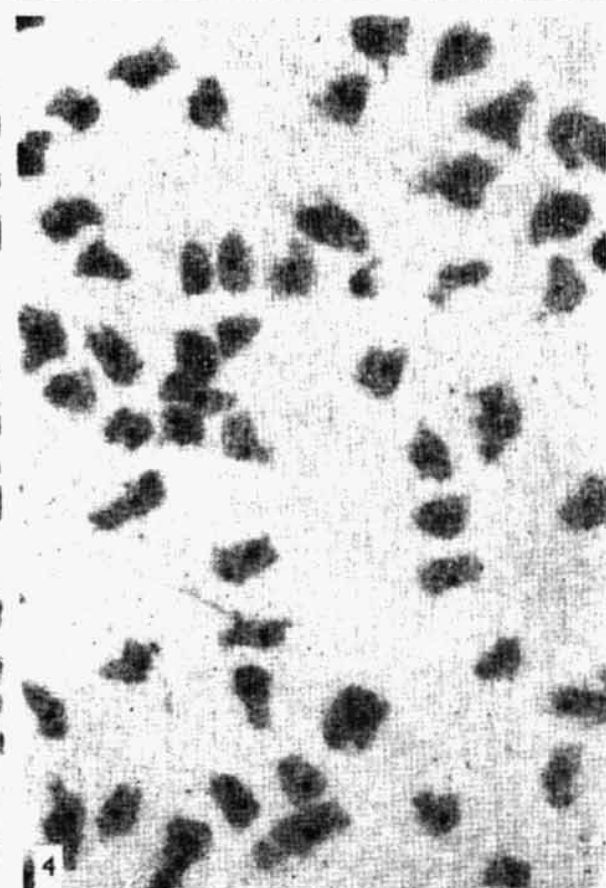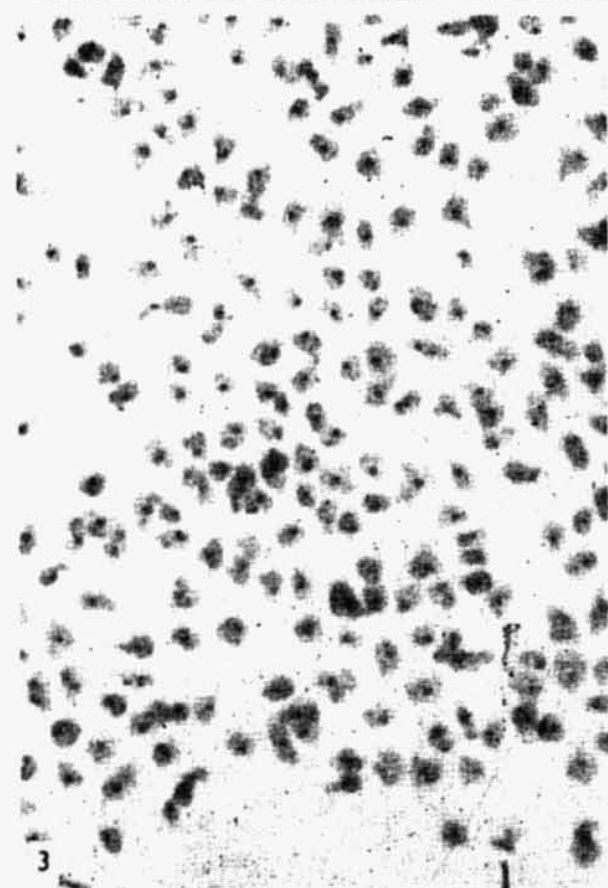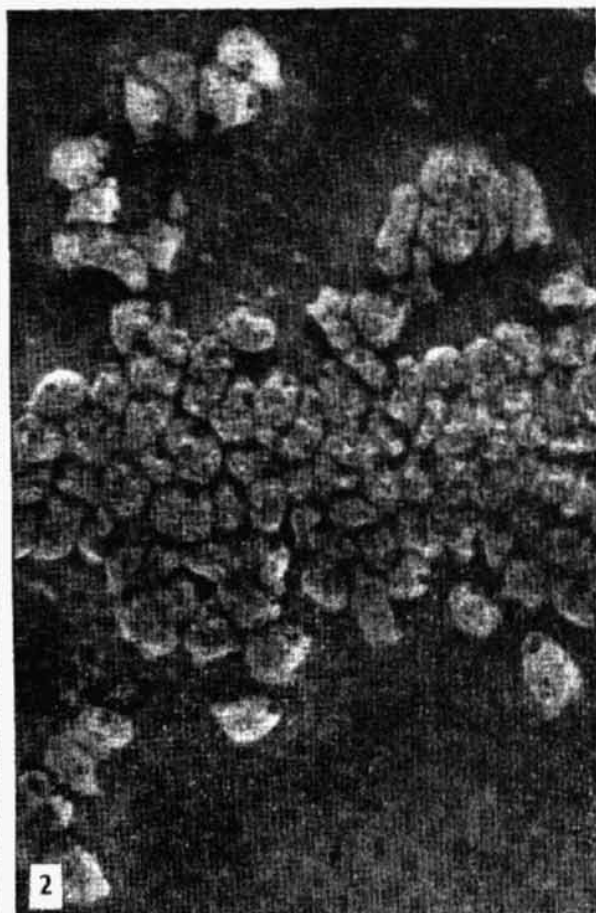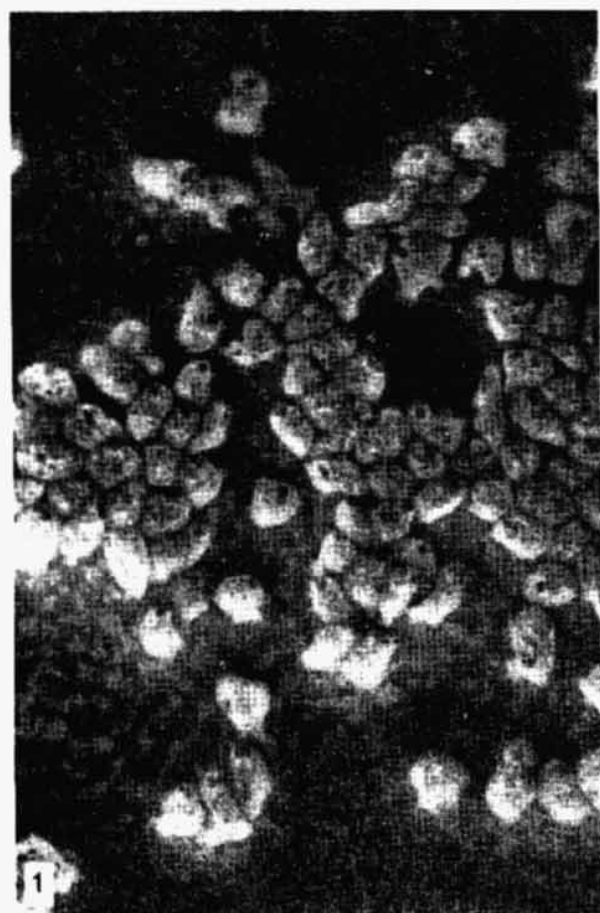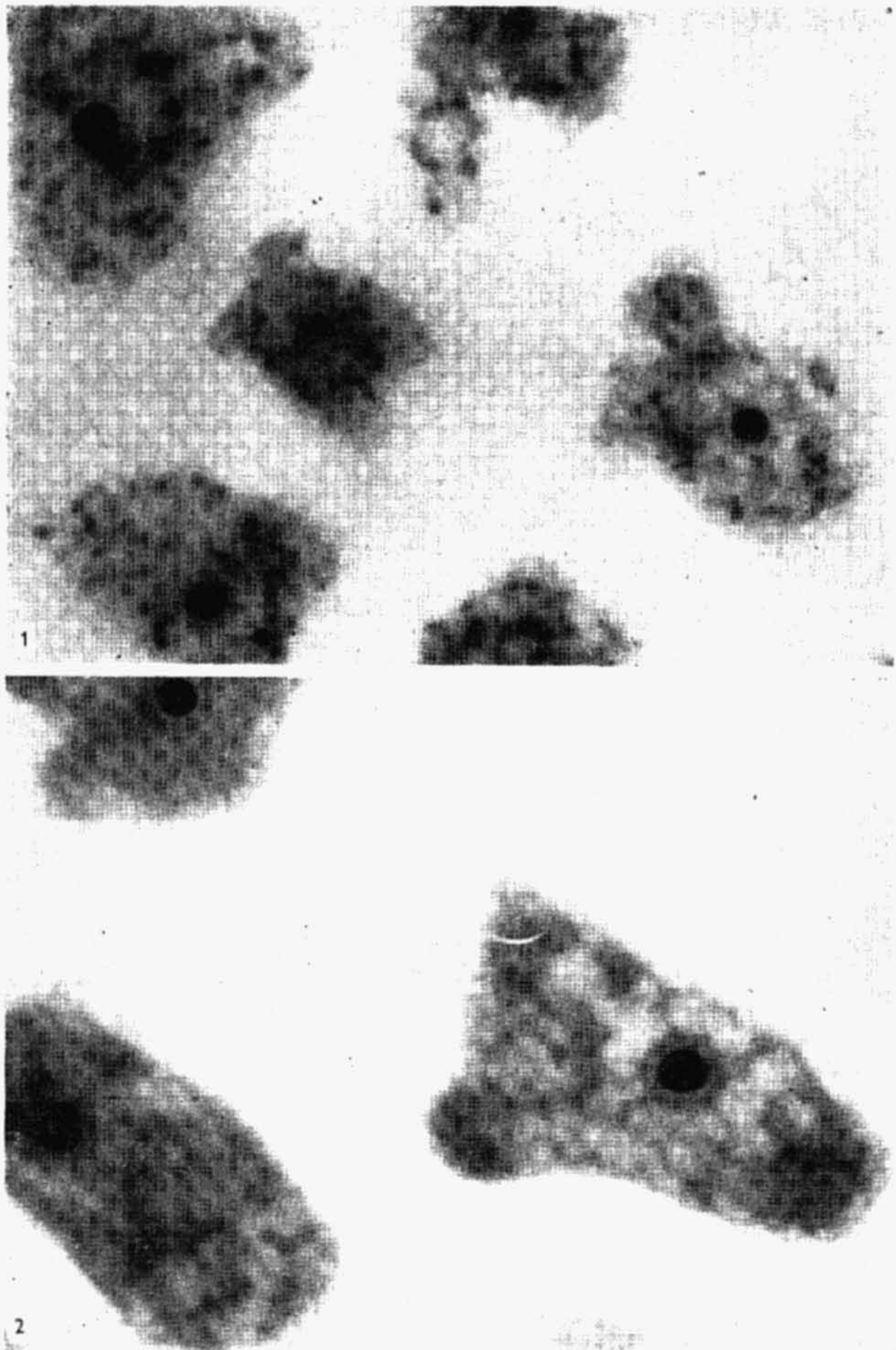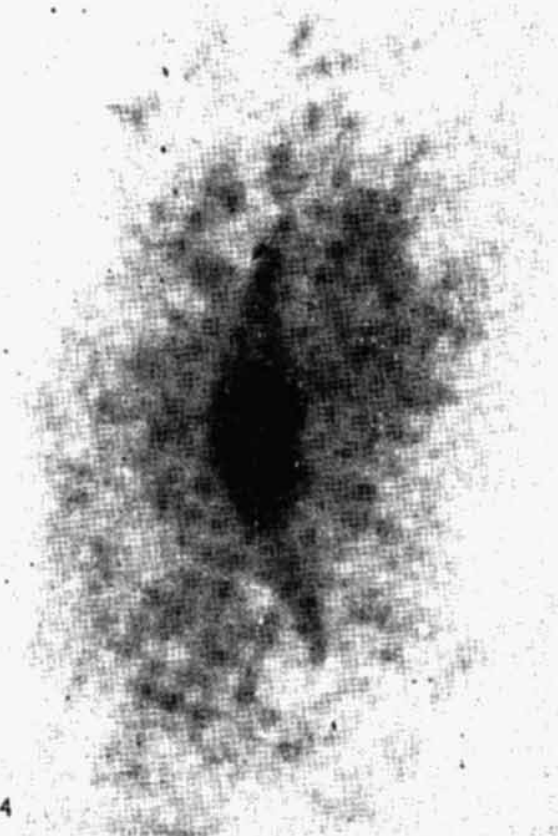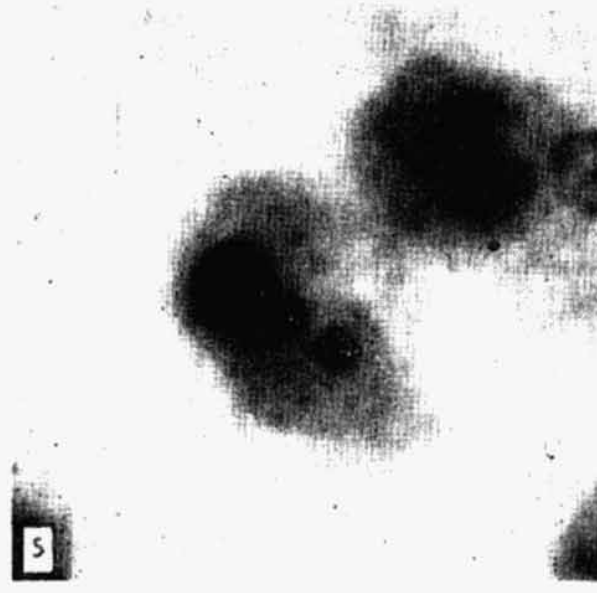
EXPLANATIONS TO THE PLATES

Plate I

Figs. 1, 2. Zones of growth of the pathogenic strains of *Naegleria gruberi* on the agar plates. Phase contrast (× 300). Fig. 1. Vítek strain. Fig. 2. IIB-1 strain.

Figs. 3, 4. Slide cultures of pathogenic strains of *Naegleria gruberi* cultivated under axenic conditions. Giemsa-Robinow (× 200). Fig. 3. Vítek strain. Fig. 4. HB-1 strain.

Plate II Slide cultures of pathogenic strains of *Naegleria gruberi* cultivated under axenic conditions. Iron haematoxyline (× 2,000).

Fig. 1. Vítek strain with the typical black stained cytoplasmic granules.

Fig. 2. HB-1 strain with a clear cytoplasm.

Plate III

Fig. 1. Natural groups of cysts typical of *Naegleria gruberi* on agar plates. Vítek strain. Phase contrast (× 300).

Fig. 2. Cysts of the Vítek strain on agar plates. The relatively thick membrane appears to be uni-layered, the ostioles are indistinct. The nucleus is surrounded by plasmatic granules. Phase contrast (× 2,000).

Plate IV

Figs. 1, 2. Flagellate stages of the Vítek strain. Dry smears. Giemsa (× 3,000).

Fig. 3. Nuclear division of Vítek strain. Giemsa-Robinow (× 3,000).

Fig. 4. Nuclear division of IIB-1 strain. Giemsa-Robinow (× 3,000).

Plate V

Figs. 1, 2, 3. Various stages of nuclear division of the Ústí strain. Trichrome (× 6,700).

Figs. 4, 5, 6. Intranuclear corpuscles of the Ústí strain. Iron haematoxylin (× 3,000).

Plate VI

Figs. 1, 2. Purkynje's cells attacked by *Naegleriae* of the Ústí strain. Trichrome (× 3,000).

Fig. 3. *Naegleria* replacing the nucleus inside a Purkynje's cell. Trichrome (× 150).

Fig. 4. Amoeba when entering a Purkynje's cell. Trichrome (× 150).