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Euhyperamoeba fallax Seravin et Goodkov, 1982 (*Lobosea, Gymnamoebia*) — Multinucleate Marine Limax Amoeba — Morphology, Biological Peculiarities and Systematic Position

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Synopsis. The most full data about external morphology of locomoting form and behaviour, feeding and other biological peculiarities of multinucleate naked lobose marine amoeba *Euhyperamoeba fallax* are presented. The characteristic property of this protozoan is the ability to the spontaneous pseudocopulation, and also the temporary plasmodization (during the "collective feeding"). In this way non-sexual forms of genetical interactions (referred to as agamic genetical interactions) of *E. fallax* individuals become possible. Only two species of true multinucleate naked lobose amoebae (*Gymnamoebia*), which live in the sea water, are reliably known at the present time: *Gruberella flavescens* and *Euhyperamoeba fallax*. The essential differences of *E. fallax* from the other amoebae make us to distinguish it into the independent family *Euhyperamoebidae*. The corresponding applicable diagnoses of the species, genus and family are presented here. Because of the decision a question about systematic position of *E. fallax*, and also because of some other reasons, more exact definition of existant diagnosis of the class *Lobosea* and the order *Amoebida* is made. The new diagnoses of these taxones are placed here. *E. fallax* is included into the order *Amoebida*, the subclass *Gymnamoebia* of the class *Lobosea*.

In the preface for the key to marine amoebae, Bovee and Sawyer (1979) write the following: "The amoebae of the ocean are not yet well known. A great many unknown species remain to be found, described, identified, and classified" (p. 1). This statement remains true until now, though a definite progress has been made in studies of marine naked amoebae (the subclass *Gymnamoebia*) during the last years, especially of the uninucleate ones (see Page 1983). At the same time the multinucleate species belonging to this group of *Protozoa*, are mostly found among the freshwater forms. These are the representatives of the following genera: *Chaos* Linnaeus, 1767; *Pelomyxa* Greeff, 1874; and *Thecochaos* Page, 1981.

The first marine multinucleate amoeba was described by Gruber (1889) under

the name of *Amoeba flavescens*. It has become perfectly clear at present, on basis of all the characteristics which are taken into account by modern systematics of *Gymnamoebia*, that this protozoa cannot be included into the genus *Amoeba*. Therefore Page (1983) is quite justified to distinguish it as a new genus *Gruberella* with the only one species *G. flavescens* (Gruber 1889), described in detail in a special publication (Page 1984).

We have discovered one more marine multinucleate naked amoeba — *Euhyperamoeba fallax* (Seravin et al. 1982), the results of investigation being presented in a series of publications (Goodkov et al. 1982, Seravin et al. 1982, Seravin and Goodkov 1983, Goodkov and Seravin 1984).

In literature there are the descriptions of two more species of the marine limax amoebae characterized by multinuclearity. Those are *Rhizamoeba polyura* (Page 1972) and *Pseudovahlkampfia emersoni* (Sawyer 1980). However, the presence of more than one nucleus in *R. polyura* is occasionally observed only in some individuals, whereas the overwhelming majority of the forms is characterized by uninuclearity (Page 1972). Besides, at present *Rhizamoeba* is excluded from *Gymnamoebia* and included into the family *Leptomyxidae* of the class *Acarpomyxea* (Pussard and Pons 1976, Levine et. al. 1980, Page 1983). As for *P. emersoni*, firstly it is not a a free-living amoeba but the one that inhabits the digestive tract of the crab *Callinectes sapidus*, and secondly, only one stage or form of this organism is characterized by multinuclearity (so far known only under lab culture conditions), whereas its second living form is a typical limax uninucleate amoeba (Sawyer 1980). Thus, until now only two true multinucleate free-living marine naked amoebae are known — *Gruberella flavescens* and *Euhyperamoeba fallax*.

The present report includes the most complete information on biology, morphology, details of ultrastructural organization, as well as other data obtained during the examination of *E. fallax*. The detailed analysis of the systematic position of this *Amoeba* species, is also made.

Materials and Methods

Euhyperamoeba fallax was discovered in algal sediments on the walls of sea aquarium where the samples of natural sea-water with flora and fauna specimens were stored after collecting them from the littoral of the Peter-the-Great Gulf of the Sea of Japan. Through the year 1980 two isolates (100 ml each) containing the scraps of algal sediments, were taken from the aquarium. In both isolates a great amount (hundreds of individuals) of amoebae later named *E. fallax*, was detected. The population of isolated amoebae was cultured in Petri dishes of different diameter. An artificial sea water with a salinity of 32 to 34‰, according to Kinne (1976) in modification of Schubravyi (1983), was used as a culture medium.

At first the amoebae were fed with different pro- and eukaryotic immobile photosynthesizing organisms contained in scraps of algal sediments. Later a monoculture of trichomic cyanobacterium *Oscillatoria phycophytica* Fremy was separated from the initial isolates and used as main food.

The dishes with cultures were kept at room temperature (17–22°C) and natural light.

Observation and photography of living amoebae were performed with an inverted microscope "MicroStar" directly in culture vessels. More fine details of the structure and external morphology were examined with an interference contrast of a microscope "Polyvar".

The fixed material was studied on the total preparations stained with Heidenhain's iron haematoxylin, or with gallocyanine. The short-lasting preparations of protozoa stained with silver nitrate, were also used.

Besides, the amoebae were fixed for 30 min in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.2) brought with sucrose to proper osmolarity. This was followed by washing, dehydratation in alcohols, and embedding in Epon. The prepared blocks were cut on a LKB-3 ultratome.

The semithin sections (1 μm) were stained in blue toluidine and examined under a light microscope. The ultrathin sections were contrasted with uranyl acetate and lead citrate according to Reynolds, and then examined with an EMV-100L and a Hitachi HU-IIE electron microscope.

Results and Discussion

Locomotion and Morphology of *Euhyperamoeba fallax*

Locomotion and external morphology of the naked amoebae are so closely related that they should be discussed in the same section.

E. fallax is a highly mobile lobose amoeba of the limax type (Pl. I 1). The dimensions of its monopodial form are as follows: 92 to 230 μm long (the mean length 191 μm); 17 to 42 μm broad (the mean breadth 31 μm), the mean ratio of the body length to its breadth (L : B) being about 6.0.

Locomotion. The active translocation of *E. fallax* on the substrate is accomplished exclusively by its monopodial form and always involves a continuous, uniform flow of the cytoplasm. Retardation or full stop of locomotion under certain inner or outer circumstances, is usually accompanied by the loss of monopodiality, when the shape of amoeba's body becomes irregular or practically round. *E. fallax* comes out of its low-mobile state mostly through formation of one large pseudopodium (Pl. I 2). Sometimes, however, two or even three pseudopodia could be formed before the amoeba starts to move intensively, though amoeba again becomes monopodial at the beginning of locomotion, i.e., truly polipodial forms are never observed in moving *E. fallax*.

Change in direction of protozoa movement occurs due to a small lateral swelling in the anterior part of the body, a bulk of granular cytoplasm rushing quickly to that place. For a short period *E. fallax* becomes bipodial (Pl. I 3). A new pseudopodium starts to dominate in size and then it remains as the only one, and the amoeba becomes monopodial again. Besides, the direction of locomotion on the substrate can be changed through a change in orientation, when the anterior end of the body turns aside, and the protozoan bends and starts to move in a new direction.

Hyaline Cap. *E. fallax* can move non-stop for a long time, keeping a characteristic body shape. In this period the hyaline cap at the anterior end of the body is practically absent. Only in rare cases a hardly noticeable zone of transparent hyaloplasm can be seen as a thin crescent at the frontal edge of the moving organism.

The hyaline cap is well expressed just at the moments of essential changes of *E. fallax* body shape. So, when amoeba comes out of the low-mobile or round-shape state, a zone of hyaline cytoplasm is usually distinct in the first large forming pseudopodium. It appears also at the tip of a new pseudopodium when amoeba starts to change direction of its locomotion. It should be noted that in the last case a hyaline cap appears sometimes not only in the new pseudopodium but also in the old one.

Uroid and Uroidal Structures. In the medium with small amounts of food, when majority of *E. fallax* move actively on the substrate having a typical monopodial form, it seems at first that all the amoebae look very similar and differ only in size. However, careful observations show that it is not quite so: the structure of the posterior body end (uroid) is different in separate individuals. Three groups of *E. fallax* can be distinguished in the same culture on basis of morphological features.

First group — the most numerous one — has strongly developed and differentiated uroidal structures. There is a bulbous swelling of the posterior end of amoebae which is separated from the rest of the body by a quite pronounced constricting ring (Pl. I 1). and is thickly covered by numerous short transparent filamentous protrusions (Pl. I 4). In a rapidly moving protozoa this filamentous-bulbous uroid is slightly elevated above the substrate's surface and is localized either at the very end or displaced somewhat laterally. Long-term observations show that in process of amoeba's locomotion the filamentous-bulbous uroid is retained even i fthe protozoan changes frequently the direction of its movement forming new pseudopodia.

Amoebae of the second group lack the bulbous swelling, although the rounded caudal part of their body carries a low number of trailing over the substrate, transparent, and rather thin protrusions of different length. As the protozoan moves, the protrusions are gradually retracted into the main body and disappear completely. In such a case the amoeba can already be attributed to the third group which includes *E. fallax* having a typical monopodial form, but their rounded posterior body end does not carry any noticeable uroidal structures.

A long-lasting loss of monopodiality connected with cessation of translational movement and with transition into a low-mobile or rounded state, results in resorption of uroidal structures. It was interesting to elucidate the conditions of restoration of these structures as well as the cause of difference in external morphology of the posterior body end in all three differentiated groups of *E. fallax*.

Observations have shown that the formation of filamentous-bulbous appendage at the posterior body end begins when amoeba comes out of the low-mobile state and resumes locomotion on the substrate. The bulk of protozoan's contents rushes into the single forming pseudopodium (Pl. I 2). A large amount of granular cytoplasm is soon found to be included in this pseudopodium (Fig. 1 A, a—d), and a substantial part of plasmalemma surrounding just recently the immobile amoeba and staying outside the zone of active events, starts to deflate and wrinkle (Fig. 1 A, d, e). During the continuous growth of pseudopodium and final formation of the monopodial body of *E. fallax*, this zone of plasmalemma rounds up completely into

a wrinkled node filled with hyaline cytoplasm. During the further condensation of the node, the transparent filamentous protrusions begin to emerge from it (Fig. 1 A, f). It should be noted that the final stages of formation of the filamentous-bulbous uroid occur just after removing of the posterior body end from the substrate.

As a result of special observations it has been found that bright illumination affects very strongly the behaviour of *E. fallax*. The monopodial forms change

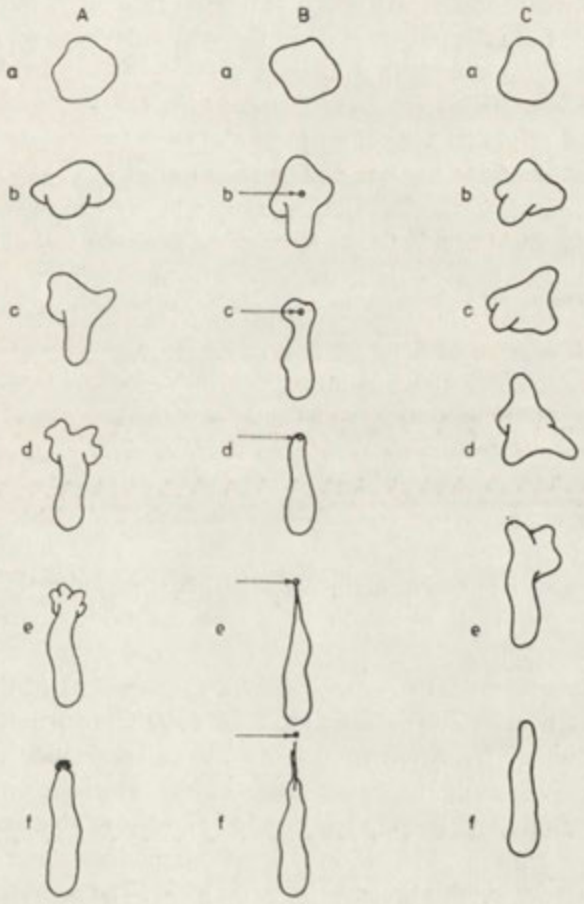

Fig. 1. "Nature" of three groups of *Euhyperamoeba fallax* individuals differing in external morphology of their caudal body region (uroid). A – Scheme of formation of filamentous-bulbous uroid in the case of amoeba's quick transition from the low mobile state to intensive locomotion on the substrate (a – rounded shape, b–e – consecutive stages of formation of the leading pseudopodium and uroidal structures, f – the arisen monopodial individual with a filamentous-bulbous appendix), B – Formation of transitory structures of adhesive nature (a–e – transition from rounded state to locomotion on the substrate with formation of a single leading pseudopodium and extension of the cell region attached to the substrate, f – detachment of the posterior body end from the substrate with formation of adhesive filaments). Arrow indicates zone of cell attachment to the substrate. C – "Nature" of monopodial individuals without distinct structures at the posterior body end (a–d – transition from low mobile state to locomotion on the substrate with transitory extension and retraction of several pseudopodia, e – formation of the leading pseudopodium, f – monopodial *E. fallax* without uroidal structures)

abruptly the direction of their movement away from the illuminated area. When the brightness of light increases, the feeding or rounded amoebae form a leading pseudopodium and turn to locomotion activity trying to avoid the irritating factor. All the amoebae which were forced by light effect to turn from the immobile or low-mobile state to intensive movement, carry the specific filamentousbulbous uroid at the posterior body end. However, bright light has no effect on the formation of the uroidal structures in the third group of monopodial *E. fallax*. It occurs, that not bright light itself induces the origin of uroidal structures, but a quick passage of the protozoans from the passive state to the active moving over the substrate (for example, as a result of change in illumination).

Formation of uroidal structures characteristic for the second distinguished group, occurs in result of sticking of protozoan's plasmalemma parts to the substrate surface. In consequence of the translational movement of organism its main mass begins to move ahead, while the attached zone remains still in its place (Fig 1 B, a—c). Zone of attachment turns finally to be at the posterior end of protozoan (Fig. 1 B, d). During further translocation this part of amoeba loses the granular cytoplasm, becomes transparent, stretches considerably, and gets thin (Fig. 1 B, e). When such *E. fallax* removes its posterior end from the substrate, one, two, or several strongly stretched, sticky filaments remain at this end (Fig. 1 B, f). The formations of this type are used to be called the uroidal structures of adhesive type or adhesive uroidal filaments.

Uroidal structures of adhesive type occurring in *E. fallax* can appear either in amoebae coming out of the low-mobile state or in the active monopodial individuals. Firm sticking of the surface of amoeboid protozoa to the substrate and further formation of different adhesive filaments take place periodically in many species (which in the same time do not form other types of uroidal structures) and has a spontaneous character.

In contrast to the uroidal structures specific for amoebae of the second group, the formation of filamentous-bulbous uroid in *E. fallax* depends significantly on the rate of organism's transition from immobile or low-mobile state to intensive locomotive activity. Starting the translocation on the substrate, protozoa can sometimes emerge not only one leading pseudopodium, as it is observed in amoebae of the first and second groups, but two or three pseudopodia at once (Fig. 1 C, a—d), any of which are later able to become the leading one. The organism seems to be in intermediate state, and can remain in it for rather long time, enlarging or diminishing the size of any pseudopodium, and retracting completely some pseudopodia or extending the new ones. Nevertheless, *E. fallax* starts finally an active translocation on the substrate, attaining a characteristic monopodial form of the body; however, in this case the uroidal structures are not formed and the posterior end of protozoan does not carry any evident formations (Fig. 1 C, e, f). This is one of the reasons of origin of the third group of amoebae. Another reason was spoken about earlier: *E. fallax*, having no uroidal structures, originate also from amoebae of the second group in result of retraction of not numerous adhesive filaments.

Nuclear Apparatus. It is well seen in all the examined samples that *E. fallax* is a multinucleate amoeba. The number of nuclei amounts several dozens. The precise calculation is rather difficult in each given case because of the abundant inclusions inside the cytoplasm, the digestive vacuoles of different size being among them. The greatest number of nuclei which we have managed to count in a rather large individual, amounts to 87. In semithin sections (1 μm) the maximal nuclear number is 4 (Pl. I 5); however, it is quite clear that only an insignificant number of nuclei can get into such a section.

The nuclei are spherical (or slightly oval in very rare cases), of a vesicular type, with only one large central nucleolus, usually a round one. Diameter of nucleus is 5 to 6 μm, and the diameter of nucleolus is 2.0 to 2.5 μm (Pl. II 6).

Some Details of Ultrastructural Organization. In the first place it should be noted that although we have not studied in detail the pattern of nuclear division in *E. fallax*, a certain information on this process has been obtained. Thus, we succeeded in observing the nuclei at the stage of mitotic reorganization of their structure (Pl. II 7). On basis of the available data it can be concluded that mitosis involves intranuclear formation of spindle's microtubes, disintegration of nucleolus (see Pl. II 7). and preservation of nuclear membrane (a closed mitosis). Besides, the presence of normal interphase nuclei, as well as the nuclei at different stages of division in the same individual, testifies that the mitoses are not synchronous in the cell.

All the numerous mitochondria of *E. fallax*, without exception, are completely surrounded by the cysterns of rough endoplasmic reticulum which adjoin very closely the external mitochondrial membrane (Pl. II 8). The internal structure of mitochondria seems to be not enough clear at first sight. Cristae look flattened in some places, and in other places they look like strongly swollen sacs. Matrix has an irregular electron density (Pl. II 8). It is interesting that highly similar peculiarities of mitochondrial organization, including the surrounding cysterns of endoplasmic reticulum, are observed in a number of other amoebae species (such as representatives of the genera *Vahlkampfia*, *Heteramoeba*, *Gruberella* et al.), studied by different authors using the different methods of fixation (see Page and Blanton 1985).

Biological Peculiarities of *Euhyperamoeba fallax*

Feeding. *E. fallax* feeds on photosynthetic organisms, i.e., it is a phytophagous amoeba, as a matter of fact. That's why it can be easily cultured on unicellular eukaryotic algae and large cyanobacteria (*Oscillatoria phycophytica* in particular). It is quite possible that to a certain extent *E. fallax* is capable to absorb also some colourless bacteria. However, we did not succeed in observing how these amoebae capture and swallow the colourless eukaryotic organisms. Moreover, when *E. fallax* eats away all oscillatoria in culture medium, a number of amoebae begins to drop in spite of the presence of other potential food organisms (bacteria, colourless flagellates, etc.), what can finally result in complete desolation of amoebae. If we add

cyanobacteria to such a failing culture, *E. fallax* start the intensive feeding and breeding. This indicates that the deterioration of amoebae's state was caused by lack of proper food and not by other reasons.

E. fallax moving on the substrate crawls over small algae and absorb them practically without a change in body shape. The amoeba flows around the groups of algae from all the sides. At that time protozoan becomes temporarily shapeless and stops to move on the substrate. After finishing of the including of captured food into digestive vacuoles the normal movement resumes and the monopodial body shape is restored.

The absorption of long elastic trichomic cyanobacteria is usually carried out by amoeba in the following way. Protozoan crawls over the lying oscillatoria which, as a rule, exceeds in length the body size of *E. fallax*. At that time amoeba loses the monopodial shape (the translational movement on the substrate is ceased) and spreads over a section of filament of prokaryotic organism. The over-crawling can be started from the end of trichome (at that time amoeba often looks "threaded" on cyanobacterium's filament as well as from any other place of its full length (Pl. III 9, Fig. 2 A–C). Then the protozoan begins to pull the filament gradually but rather rapidly, coiling it in a sort of spiral inside the body (Fig. 2 B) until the whole trichome is packed as a tight structure inside the amoeba's cytoplasm. A similar process was described in detail by Rhumbler (1898) in a freshwater *Amoeba verrucosa*, which also swallowed cyanobacteria of the genus *Oscillatoria*.

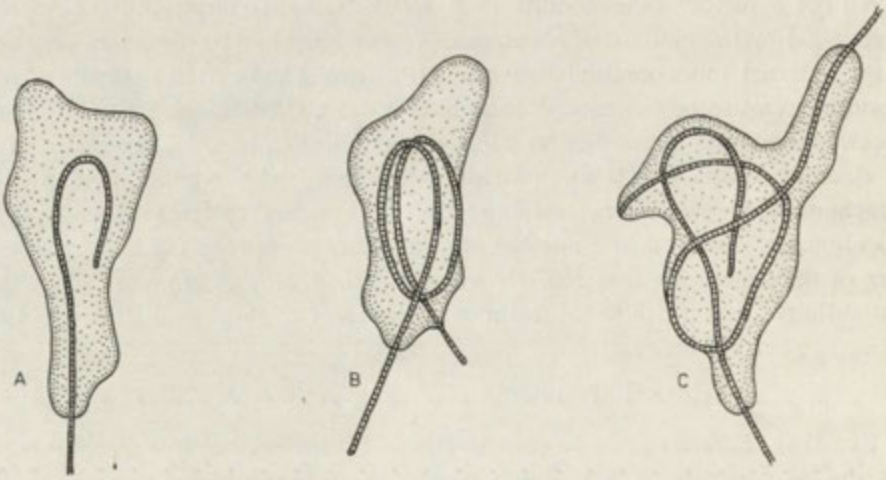

Fig. 2. Pictures observed during feeding of *E. fallax* on trichomic cyanobacteria. A – One of the initial moments of capturing the oscillatoria during overcrawling of amoeba from the filament's end, B – Spiral-like coiling of a single trichome inside amoeba's body. C – Amoeba capturing two filaments of oscillatoria at once

It is natural that the digestive vacuole containing a spring-like oscillatory filament (or a part of it) should be fixed in some way. It is well seen in the semithin sections (Pl. I 5) that in *E. fallax* these vacuoles are encircled not by a single membrane but by a whole complex of concentric cytoplasmic membranes.

Inside the amoeba the coiled filament of cyanobacterium undergoes a rapid pri-

mary destruction and falls into separate pieces soon after the capture (sometimes even before the capture is completed). At that time the initial large digestive vacuole gradually splits into a number of far more small vacuoles which soon are uniformly distributed through the whole cytoplasm of the protozoan.

An important peculiarity of *E. fallax* is a "collective feeding" which is most brightly manifested during the eating of clusters of trichomic cyanobacteria. In this case a complete fusion of many amoebae with each other occurs (sometimes up to 100 individuals or more). As a result, the flattened, shapeless, net or branched plasmodia are formed (Pl. III 10 and 11), which capture a great number of oscillatoria filaments at once (Goodkov et al. 1982, Seravin and Goodkov 1983). When the swallowed filaments are destroyed in result of digestion and a great number of small digestive vacuoles is formed, a gradual disintegration of plasmodium into common monopodial amoebae — deplasmodization, begins (Pl. III 12).

Soon after the adding of fresh portion of oscillatoria to the *E. fallax* cultures, the small, medium, and large plasmodia of this amoeba appear at the bottom of Petri dish. There is no doubt that under the natural conditions the protozoa of this species are also capable for plasmodization as well as for "collective" feeding so easily reproducable under the laboratory conditions.

Transitory plasmodia of *E. fallax*, in contrast to stable plasmodia of *Eumycetozoa*, are not capable of translocation on the substrate and do not form the spores. They disintegrate into separate independent amoebae easily and rapidly after finishing the "collective" feeding, and do not represent themselves either main phase or even obligatory stage of the protozoa's life cycle. In other words, there is no phylogenetic connection between the transitory plasmodia of *E. fallax* and the plasmodia of other groups of *Protozoa*, i.e., they have appeared independently during the evolution.

Formation of Aggregates. *E. fallax* are capable of forming the transitory dense clusters (Pl. III 13). Such aggregates can consist of several individuals up to dozens of protozoa, while the amoebae mostly adjoin each other closely with their surfaces. Each of such a cluster is a dynamic formation, where some individuals, mainly in the periphery of aggregate, can move changing their localization and penetrating the cracks and spaces between other individuals of *E. fallax*. In rare cases they can crawl over each other for that purpose, although as aggregate as a whole consists of one layer of amoebae. The significance of the formation of such groups in *E. fallax* is not known for the present. It should be only noted that, in spite of the long-lasting close contact between many individuals, there is no evidence for the formation of cytoplasmic bridges among them or for other forms of morphological interaction.

Spontaneous Binary and Plural Fusions of *E. fallax* Individuals. Up to recently, the phenomenon of complete fusion of the vegetative individuals with each other was not known among *Amoebida* which are the agamic organisms. The phenomenon was discovered in *E. fallax* (Goodkov et al. 1982, Seravin et al. 1982, Seravin and Goodkov 1983). During their locomotion on the substrate the amoebae usually behave themselves as independent individuals. At casual

contacts the protozoa separate quickly in different directions. In rare cases an amoeba can just crawl over another amoeba.

However, in cultures of *E. fallax* some individuals periodically start to seek each other actively and to make contacts. In contrast to casual collisions, in this case the protozoa do not intend to separate at once in different directions but, on the contrary, they can drift in one direction for a long time, drawn together by the lateral body surfaces. Only after a significant time interval the contact gets broken and the organisms separate, but later they can often return to each other. A case was registered when two protozoa met and parted again three times in a minute. In a culture where the contacts between pairs of amoebae are observed, their spontaneous fusions with each other soon take place.

After longterm observations of *E. fallax* behaviour it has become perfectly clear that the readiness for fusion in a majority of individuals in the culture is synchronized and periodical, what seems to depend on some external and internal factors. However, no special investigations of this question were carried out.

The process of fusion of two mobile *E. fallax* (binary fusion) can be conditionally divided into several stages. First of all, the cells get close to each other and come actively into a long-lasting contact. There are several forms of such contact, but most frequently the amoebae at first touch each other at certain angle by lateral regions of their frontal body ends (Fig. 3 a, b), and then, moving together, they get parallel to one another, staying tightly adjoined by the lateral body surfaces. It is a lateral contact (Fig. 3 c). It can last for a minute or more, although it is often finished in only 10 to 15 s. At this stage both amoebae still retain steadily their monopodial form.

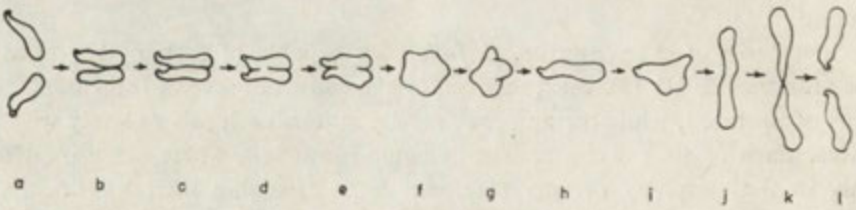

Fig. 3. Cycle "binary fusion — separation" in *E. fallax* (a, b — approach and entry into contact of two monopodial individuals, c — close contact by lateral surfaces, d — appearance of cytoplasmic bridge, e, f — gradual widening of cytoplasmic bridge and complete fusion of cytoplasm of both partners, g, h — transition of the joined organism to locomotion on the substrate with attaining a typical monopodial body form, i, j, k, l — consecutive moments of separation of the fusion product into two independent amoebae)

The next stage begins from the fusion of membranes of the contacting partners in a small region of the zone of close contact between the cells. In result, a narrow cytoplasmic bridge is formed between the individuals (Fig. 3 d), their translational movement being slowed down and soon, as a rule, stopped at all. Cytoplasmic bridge begins to widen simultaneously in the direction of the anterior as well as the posterior cell end, including the whole zone of close contact between the amoebae (Fig. 3

e, f). At that moment the intensive, usually opposite streams of granular cytoplasm from one individual into another, are well seen. In this way a complete fusion of cytoplasm of both *E. fallax* occurs. In Plate IV 14 (a–f) the photos of the consecutive stages of fusion of a pair of specimens are presented.

Fusion of amoebae can occur also at other forms of their contact. Thus, amoebae can touch each other with their frontal body ends (frontal contact), or one protozoan comes into a long-lasting contact of its frontal end with the lateral surface of another protozoan (frontal-lateral contact). Sometimes at lateral contact, when two amoebae stay in immediate proximity to each other but their surfaces are still not in contact, they form small protrusions directed to meet each other. The protrusions fuse forming a cytoplasmic bridge. All the following events occur as in other cases.

In result of the complete fusion of cytoplasm of two amoebae, a joined organism arises. At the beginning it has no definite form (Fig. 3 f), but soon, starting an active locomotion on the substrate, it takes a monopodial form (Fig. 3 g, h). Since the body size of this protozoan species varies significantly, the joined organism does not differ in outward appearance from the ordinary amoebae in the culture. Due to the different large and small inclusions (granules and coloured vacuoles) in the cytoplasm of *E. fallax*, the direction and velocity of cytoplasmic streaming can be easily determined. Therefore, one could clearly see how mixing of the contents of the initial amoebae begins during the locomotion. From time to time the joined amoeba can strongly slow down the locomotion or stop it, losing its monopodial form. At that time a rapid eruptive wave of transparent hyaloplasm runs from amoeba's anterior end to the posterior one or, on the contrary, from the posterior to the anterior end (Pl. III 15) which is followed by a considerable part of granuloplasm, forming counter-flows and turbulences on its way (Goodkov et al. 1982, Seravin and Goodkov 1983, Goodkov and Seravin 1984). As a result of such process, an additional mixing of the whole contents of joined cytoplasm of the protozoa occurs, including all the organelles. After that the amoeba resumes locomotion and regains the monopodial form. The process of such mixing can be repeated several (2 to 5) times usually in a time interval from 2 to 20 min, as counted from the moment of their complete fusion.

The new organism formed in that way, however, happens to be not stable. After a certain period of active movement, separation disjunction of fusion product into independent individuals, occurs. At that time two large pseudopodia are firstly formed, which grow rapidly in opposite directions (Fig. 3 i, j), and soon turn into two new amoebae connected with each other only by a narrow cytoplasmic bridge (Fig. 3 k). The contents of "hybrid" amoeba is distributed rather uniformly. This is distinctly seen in the cases when one of the fused protozoa contained a large number of green-coloured digestive vacuoles while the other one was practically deprived of them. During separation of the joined amoeba arisen from them, two new individuals are formed, which contain approximately equal number of such vacuoles. When the thin bridge connecting the forming new individuals breaks, the process of separation comes to its end (Fig. 3 l).

The separation of "hybrid" amoeba into two independent organisms usually begins in 10 to 60 min after the fusion of partners. It is true, however, that in one case two new amoebae were already formed in 3 min after fusion. Unsuccessful attempts of separation, when protozoa fused again at different stages of their partition, were registered even in one minute after the formation of joined organism.

The examined binary fusion of *E. fallax* can be observed often enough in the culture of these protozoa (we observed it more than 200 times). Far more seldom not two but three actively moving protozoa can get into contact resulting in complete fusion of amoebae, and the almost simultaneous fusion of four individuals was registered only once. However, in all cases the fusion product moves actively on the substrate, maintaining a body shape typical for *E. fallax*, while the processes of mixing of cytoplasmic contents are held inside the product. As a results of disjunction of the joined organism, as many individuals are actually formed as initially took part in fusion. Plate V 16 (a—h) shows a series of successive stages of fusion process and subsequent disjunction of three amoebae.

We are talking about separation and not about division of a joined protozoan for two reasons. Firstly, separation into two new individuals can occur in 3 to 10 min after fusion, i.e., almost immediately after the formation of joined organism, when nuclear mitosis could not yet be accomplished as well as the synthesis of any new substances in essential amounts. Secondly, an increase in number of individuals characteristic for the process of division, is not obsreved here, since as many amoebae appear in result of separation as initially took part in a fusion, though these ones are completely different amoebae because of the mixing of cytoplasm.

Analysis of literature (Seravin and Goodkov 1984 a) shows that agamic fusions of protozoa individuals are rather wide-spread among certain *Rhizopoda*, for example, in the classes *Filosea*, *Acarpomixea*, *Heliozoea*, to say nothing of *Eumycetozoea* and *Plasmodiophorea*. Despite the specificity of this phenomenon in representatives of different systematic groups, the common features are also well expressed. Three[1] basic types and 6 forms of agamic fusions of protozoa can be distinguished (Seravin and Goodkow 1984 a).

I Type — pseudocopulation (binary and plural) — has got its name since it imitates the copulation of gametes (especially at hologamia when the gametes do not practically differ in outward appearance from the vegetative cells). Two (binary pseudocopulation) or several (plural pseudocopulation) vegetative cells fuse together. As a results protozoan is formed which differs in no way from the initial individuals in outward appearance (except the dimensions) and locomotion pattern. The fusion is transitory. After a certain period of time, disjunction (separation) of the joined organism occurs giving the same number of individuals that took part in fusion.

II Type — plasmodization (transitory or stable). In this case, as a result

[1] The fourth distinguished type of agamic cell fusion (Seravin and Goodkov 1984 b)— congregation — still has not been discovered among the representatives of the mentioned *Protozoa* taxons, therefore we do not consider it in the present paper.

of fusion of many individuals, a multinucleate plasmodium is formed which differs in appearance as well as in locomotion pattern (if it is just capable of translocation) from the initial individuals. The transitory plasmodia soon disintegrate into single individuals (deplasmodization occurs). Stable plasmodia give rise to fruiting bodies (sporangia, sorocarps, etc.) in which the spores are formed.

III Type – pseudoconjugation (binary and plural): partial (by means of cytoplasmic bridges) transitory fusion of protozoa with retention of morphological individuality of the partners and the following separation of the same individuals. It is found in *Heliozoea*, testate amoebae, and others.

As we have shown, binary and plural pseudocopulation takes place in *E. fallax*, as well as transitory plasmodization (at "collective" feeding).

Transitory fusion of *E. fallax* multinucleate individuals with their subsequent separation, has the important genetic consequences (Seravin and Goodkov 1984 b). After separation of the joined organism in two (or several) amoebae, the totally new amoebae arise (not those which initially took part in fusion). Each new amoeba carries away not only the mixed protoplasm but also the "mixed" nuclear set (heterokaryosis arises). Thus, the transitory fusion of *E. fallax* individuals is a peculiar agamic form of the hereditary material exchange, a parasexual process which we named paracopulation (Seravin et al. 1982, Seravin and Goodkov 1983). It was shown later (Seravin and Goodkov 1984 b) that paracopulation was a particular case of pseudocopulation (when the last is accompanied by nuclei exchange between the temporarily fused vegetative individuals).

Experimental Fusions of *E. fallax* Individuals and Their Fragments. It was interesting to elucidate the possibility of getting the fusion of *E. fallax* individuals by bringing them in contact mechanically at those time periods when the natural spontaneous fusions of amoebae are not observed in the culture (in present paper we deliberately do not consider the possible methods of chemical treatment of the protozoa cells in order to attain their fusion). For that purpose 25 attempts were made to bring two of *E. fallax* individuals in contact by means of glass needles. In 23 cases the complete fusion of amoebae was obtained. In two experiments the amoebae were traumatized by needles and disintegrated. However, the destruction is not an obstacle for the fusion process if amoeba is in a limited space. For example, an *E. fallax* individual was accidentally squashed on glass slide by pressure of cover glass. The formed fragments of different size rounded up at once except the largest fragment which, starting to move, "gathered" quickly the immobile parts of squashed amoeba fusing consecutively with each of them. After all, a highly mobile monopodial individual was formed. As a matter of fact, just this observation has suggested the idea of experiments on fusion of artificially isolated fragments of *E. fallax*. The amoebae were cut and their isolated parts were brought in contact by glass needle. Twenty experiments were done altogether. The cut-off parts were of different size from 1/4 to 1/2 of initial individual. In all the cases the same results were obtained: at contact (by glass needles) the fragments of amoebae fused inevitably.

It is interesting that the early investigators have already mentioned the ability of some *Rhizopoda* to restore the integrity of their organisms under natural and experimental conditions by means of fusion of isolated or artificially cut-off body parts (pseudopodia) with the bulk of the cell (only in the case of not too long period after the moment of isolation). This was observed in a number of specimens of the class *Granuloreticulosea* as well as in some testate amoebae (the subclass *Testacealobosia*) belonging to the class *Lobosea* (Verworn 1897, Jensen 1901, Kepner and Reynolds 1923, Reynolds 1924). Moreover, using a mechanical irritation by glass needle one can attain the full fusion of two or even several *Myxotheca arenilega* (the class *Granuloreticulosea*) brought in contact with each other, for example, by their reticulopodia (Schwab and Schwab-Stey 1980).

Nevertheless, there is still no convincing evidence of the ability to fuse (even under the experimental conditions) of the whole individuals as well as their fragments with parental organisms among the specimens of the subclass *Gymnamoebia*. We do not consider here the studies of Daniels and others (Daniels 1962, Daniels et al. 1969, and others) who obtained in artificial way not only a fusion of parental individuals with their fragments and intact amoebae with each other, but also a fusion of intact amoebae with fragments of heterological individuals, and even the different species of amoebae with each other, since the microsurgical methods were used in their experiments. Therefore, we set up a number of experiments similar with those performed on *E. fallax*.

We used the following species from the order *Amoebida*: *Amoeba proteus*, *A. lescherae*, *A. discoides*, *Polychaos dubia*, and *Chaos carolinensis*, as well as *Pelomyxa palustris* from the order *Pelobiontida*. Fifteen experiments on fusion of intact individuals and 10 experiments on fusion of isolated parts were performed with the representatives of each species. In all the cases the negative results were obtained.

Thus, it can be concluded that *E. fallax* possesses certain specific morphological-biochemical properties of plasmalemma, which are absent in many — if not in all other — naked amoebae. Just because of that, *E. fallax* are capable for spontaneous or permanent (under conditions of simple experiments) fusions.

Systematic Position of *Euhyperamoeba fallax*

As it has been shown, *E. fallax* is a lobose amoeba of a limax type. Of course, the presence of transitory lobose, limax-like form in amoebae is in itself not important enough criterion for determination of their systematic position in case when such organisms possess another basic living form. Thus, for example, the genus *Rhizamoeba* described by Page (1972), was at first included by him into the family *Hartmannellidae* (the order *Amoebida*, the class *Lobosea*) on basis of its ability to take a limax-like form during active movement, and was afterwards removed to the class *Acarpomyxea* (Pussard and Pons 1976, Levine et al. 1980; Page 1983) since these protozoa in the whole have quite different appearance, different char-

acter of translocation on the substrate, and a number of other features more resembling those of the specimens from the family *Leptomyxidae*. Moreover, Pussard and Pons (1976) note the ability of some other typical *Leptomyxidae* to take limax-like form at certain changes of external conditions.

Euhyperamoeba fallax represents a totally different case: the lobose, limax-like form is its basic form and the only possible type of mobile stage; even strictly transitory plasmodia associated with feeding pattern of this protozoan differ in outward structure and organization from those of the specimens of the *Acarpomyxea* class, and, besides, they are not capable for locomotion on the substrate.

Thus, it is obvious that the class *Lobosea* seems to be the only one close to this organism. However, at present the diagnosis of this taxon includes the following thesis: the cell is usually uninucleate; multinucleate forms are different from the flattened or much-branched plasmodia (Page 1976, Levine et al. 1980). Page (1976) interprets it as follows: in contrast to the true plasmodia, the multinucleate lobose amoebae are formed not through the fusion of individuals but due to division of nuclei inside the cytoplasm of the organism. It should be at once noted that the formation of plasmodia in mixomycetes can also occur through consecutive multiple division of the firstly single nucleus inside the diploid amoeboid (Olive 1975).

Besides, as shown above, *E. fallax* is able to form the transitory multinucleate plasmodia by the way of fusion of a great number of single individuals, and those plasmodia often have rather flattened and branched form.

Thus, from the purely formal point of view, *E. fallax* cannot be included into the class *Lobosea* and it should be distinguished as an independent class being a part of the superclass *Rhizopoda*. However, this should not be done since the basic living form of that protozoan is a typical lobose amoeba. The existing diagnosis of the class *Lobosea* should be just precised. This ought to be done also because in some specimens of the testate amoebae (the subclass *Testacealobosia*) a pseudocopulation is observed (see: Seravin and Goodkov 1984 a).

Diagnosis of the Class *Lobosea* (Carpenter 1861) – Lobose pseudopodia, if more or less filose, are secondary, formed from the broad hyaline zone (lobe), and not anostomozing. The amoebae are mostly uninucleate. Multinucleate forms arise through consecutive division of the nucleus. A species is known which can form transitory plasmodia through fusion of individuals. Some species are characterized by pseudoconjugation and pseudocopulation. There are no sorocarps, sporangia, or similar fruiting bodies.

For analogous reasons, *E. fallax* should be included in the subclass *Gymnamoebia*. Moreover, it could be quite possibly included in the order *Amoebida*, if one precises the diagnosis used for this taxon at present (Page 1976, Levine et al. 1980) by the following addition: "A species is known which is able to form transitory plasmodia through fusion of individuals; it is also characterized by pseudocopulation". There is no doubt that, due to the extensive studies, the other species and genera related

to *E. fallax*, will be found. By number of characters this amoeba resembles the specimens of the family *Hartmannellidae*: true limax type, continuous (not eruptive) movement, nuclei of vesicular type. However, there are also the important differences: multinuclearity of *E. fallax*, its ability for "collective" feeding and formation of transitory plasmodia, pseudocopulation, etc. — all this provides a severe difference between the given amoeba and all the other *Amoebida*. Therefore, the isolation of the family *Euhyperamoebidae* seems quite indispensable.

Diagnosis of the Family *Euhyperamoebidae* (Seravin et Goodkov 1982) — limax, multinucleate, with non-eruptive movement, able to form transitory plasmodia through fusion of single individuals; pseudocopulation present.

Diagnosis of the Genus *Euhyperamoeba* (Seravin et Goodkov, 1982) — stable hyaline cap is usually absent in monopodial forms (or sometimes present as a narrow edging at the frontal tip of the body); filamentous-bulbous uroid is characteristic for locomotive individuals.

The only known species — *E. fallax* — is a marine one.

Diagnosis of the Species *Euhyperamoeba fallax* (Seravin et Goodkov 1982) — multinucleate amoeba, body length 92 to 230 μm (mean value 141 μm), breadth 17 to 42 μm (mean value 31 μm), mean ratio of body length to its breadth (L : B) about 6.0; spherical nuclei with the only one large central nucleolus, diameter of nucleus 5 to 6 μm, diameter of nucleolus 2.0 to 2.5 μm; feed on photosynthesizing pro- and eukaryotic microorganisms; cysts unknown.

Discovered in isolates from the Peter-the-Great Gulf of the Sea of Japan.

As was already said at the beginning of the paper, besides *E. fallax*, only one more marine multinucleate naked amoeba is known at present — *Gruberella flavescens*. Besides the multinuclearity and resemblance in the habitat, *E. fallax* and *G. flavescens* have more of the similar features of organization. Nevertheless, Page (1983) compared the essential characteristics of both of them, and concluded that they are different species. He was quite right to emphasize the important differences of locomotive patterns of these protozoa (uniform in *E. fallax* and eruptive in *G. flavescens*), in organization of uroidal structures (filamentous-bulbous uroid in *E. fallax* and collopodium in *G. flavescens*), etc. However, in a more recent publication (Page 1984) the author has started to develop an opposite point of view, considering the available data as insufficient for answering a question whether *E. fallax* and *G. flavescens* are the same species or not. In a special work on analysis of similarities and differences between these protozoa (Seravin and Goodkov 1985) it is shown that they are not only the independent, well-distinguishable species, but also the genera. Moreover, in connection with this a question was put whether *G. flavescens* should be included in the family *Euhyperamoebidae*, or ought it to be isolated in an independent family *Gruberellidae* within the limits of the subclass *Gymnamoebia* (Seravin and Goodkov, 1985).

Indeed, Page and Blanton (1985) later validated the isolation of a monotypical family *Gruberellidae* for *G. flavescens*, to our mind quite reasonably placing it close

to the family *Vahlkampfiidae* inside the same order *Schizopyrenida*. However, the isolation of *Schizopyrenida* from the class *Lobosea* and inclusion of them (together with *Acrasidae*) in a new class *Heterolobosea*, is a quite disputable question.

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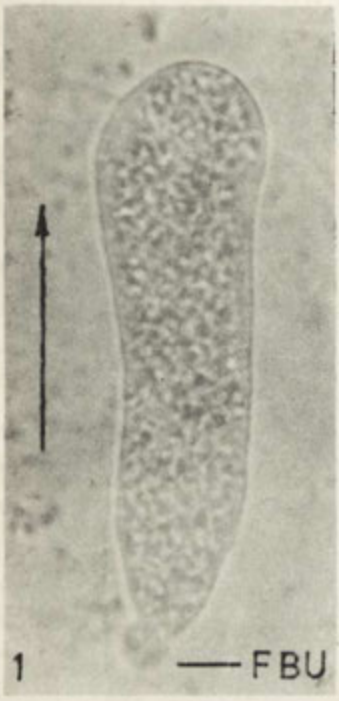
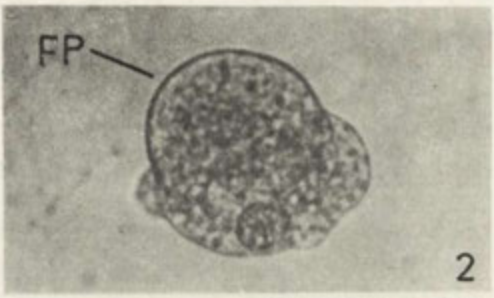
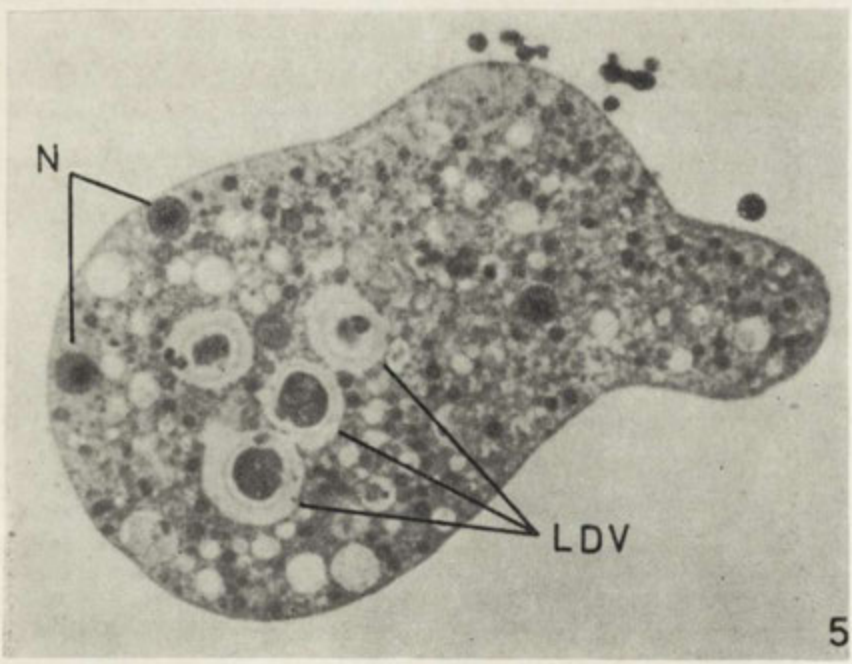
EXPLANATION OF PLATES I–V

1: Monopodial *Euhyperamoeba fallax* moving on the substrate (arrow indicates the direction of movement, FBU – filamentous-bulbous uroid)
2: Beginning of the formation of a single leading pseudopodium at transition from the rounded state to locomotion on the substrate (FP – forming pseudopodium)
3: Bipodial *E. fallax* – change in the direction of movement due to the formation of a new leading pseudopodium
4: Filamentous-bulbous uroid (interference contrast)
5: Semithin section of *E. fallax* (N – nuclei, LDV – large digestive vacuoles)
6: Nucleus of *E. fallax*
7: Nucleus at a presumably early stage of division (MT – spindle's microtubes, NM – nucleolus' material)
8: Mitochondria of *E. fallax* surrounded by cysterns of endoplasmatic reticulum
9: Feeding of *E. fallax:* capturing of a trichome (arrow) *Oscillatoria phycophytica*
10: Beginning of the plasmodium formation at the "collective" feeding of trichomic cyanobacteria
11: Large plasmodium at the moment when the captured food included in more or less small digestive vacuoles has been distributed over the whole cytoplasm
12: Process of disintegration of the plasmodium into independent amoebae (deplasmodization)
13: Aggregation of a small number of *E. fallax* individuals
14: Photos of consecutive stages (a–f) of fusion of two *E. fallax* individuals, starting from the moment of their approach (a, b) and entry into contact (c), up to the moment of complete fusion of their cytoplasm (f), arrow indicates the site of cytoplasmatic bridge formation between the amoebae
15: Moment of passing of the lateral wave of transparent hyaloplasm (arrow) directed from the anterior cell end to the posterior one (interference contrast)
16: Photos of consecutive stages of the cycle "fusion – separation" of three *E. fallax* individuals (a – approach of amoebae, b – contact between two amoebae, c – fusion of two contacting individuals, d – product of fusion of two amoebae with the third one, e – formation of cytoplasmic bridge and fusion with the third amoeba, f – product of fusion of three individuals after complete unification of their cytoplasm, g – beginning of the separation process: formation of two oppositely directed pseudopodia, h – final separation of three new amoebae)







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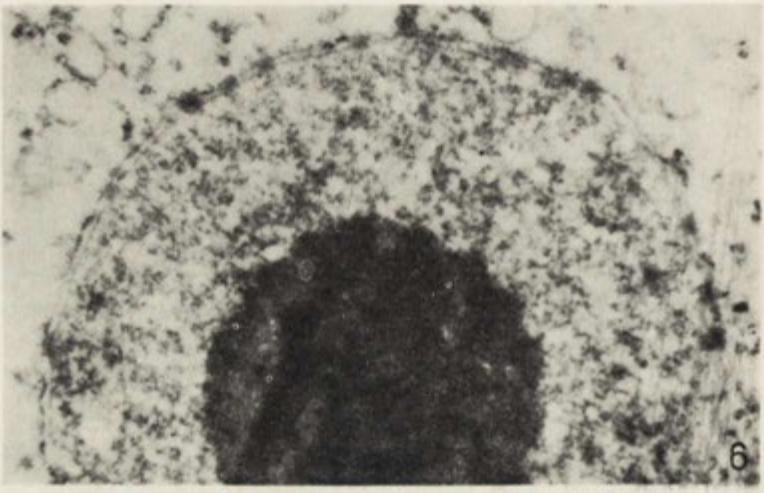

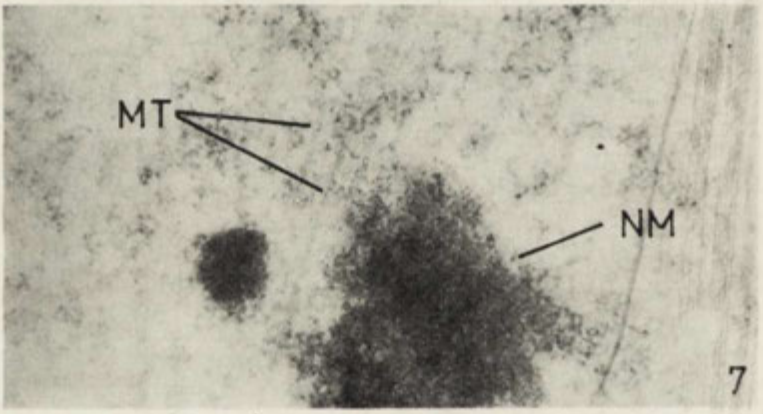

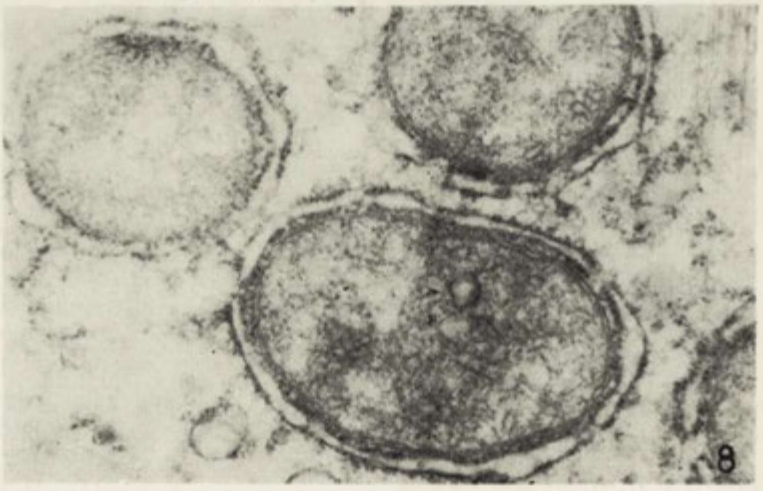

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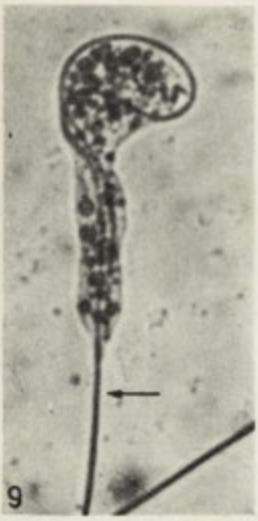

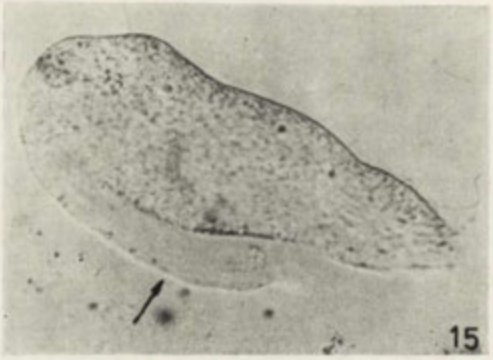

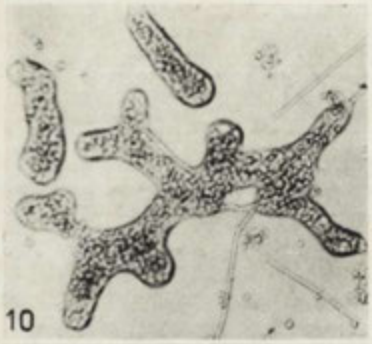

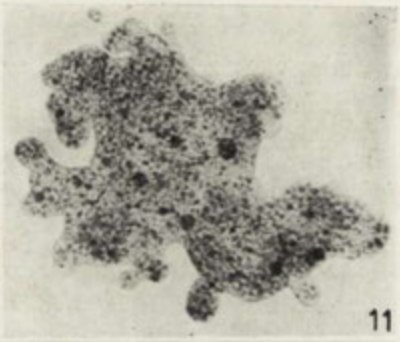

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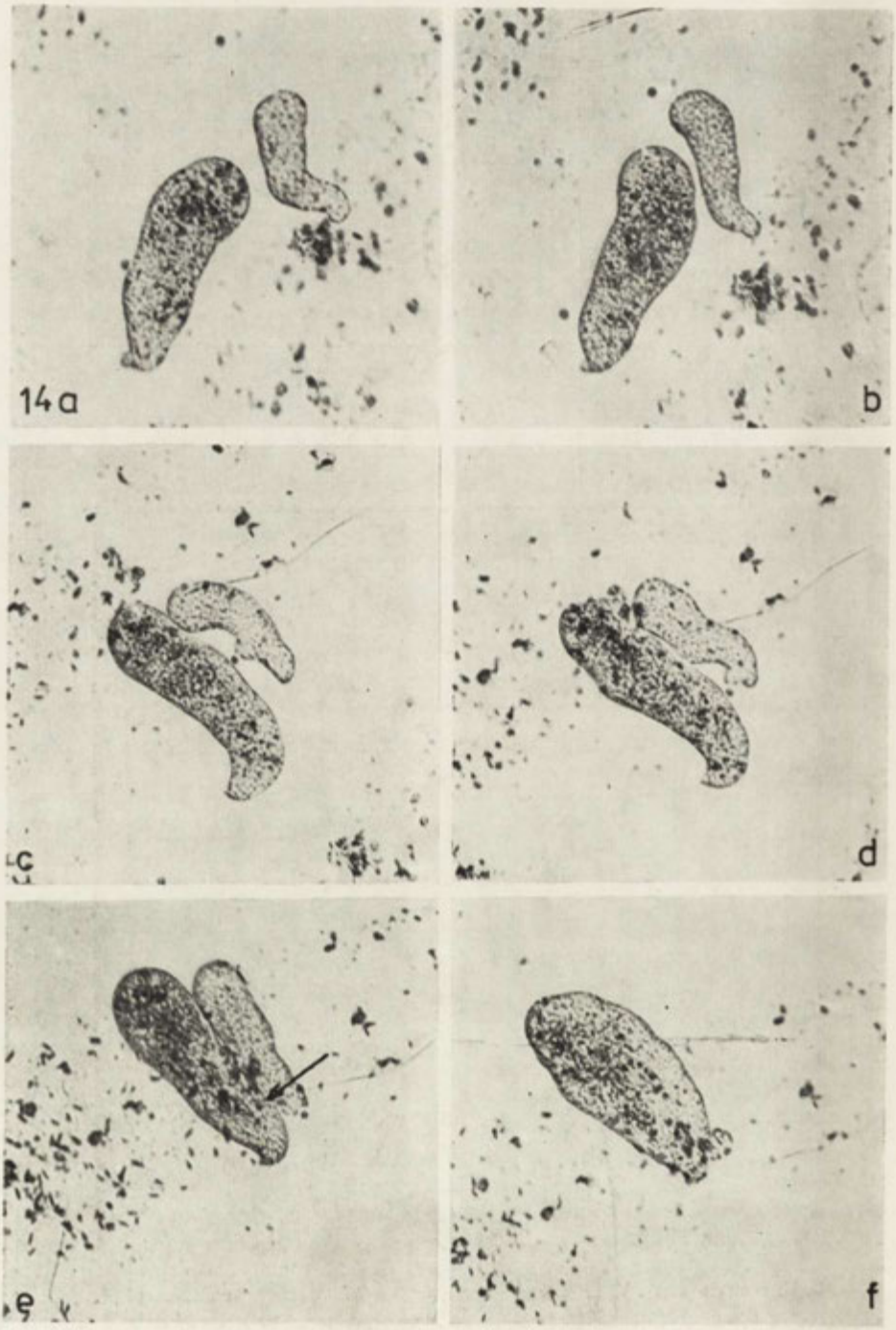

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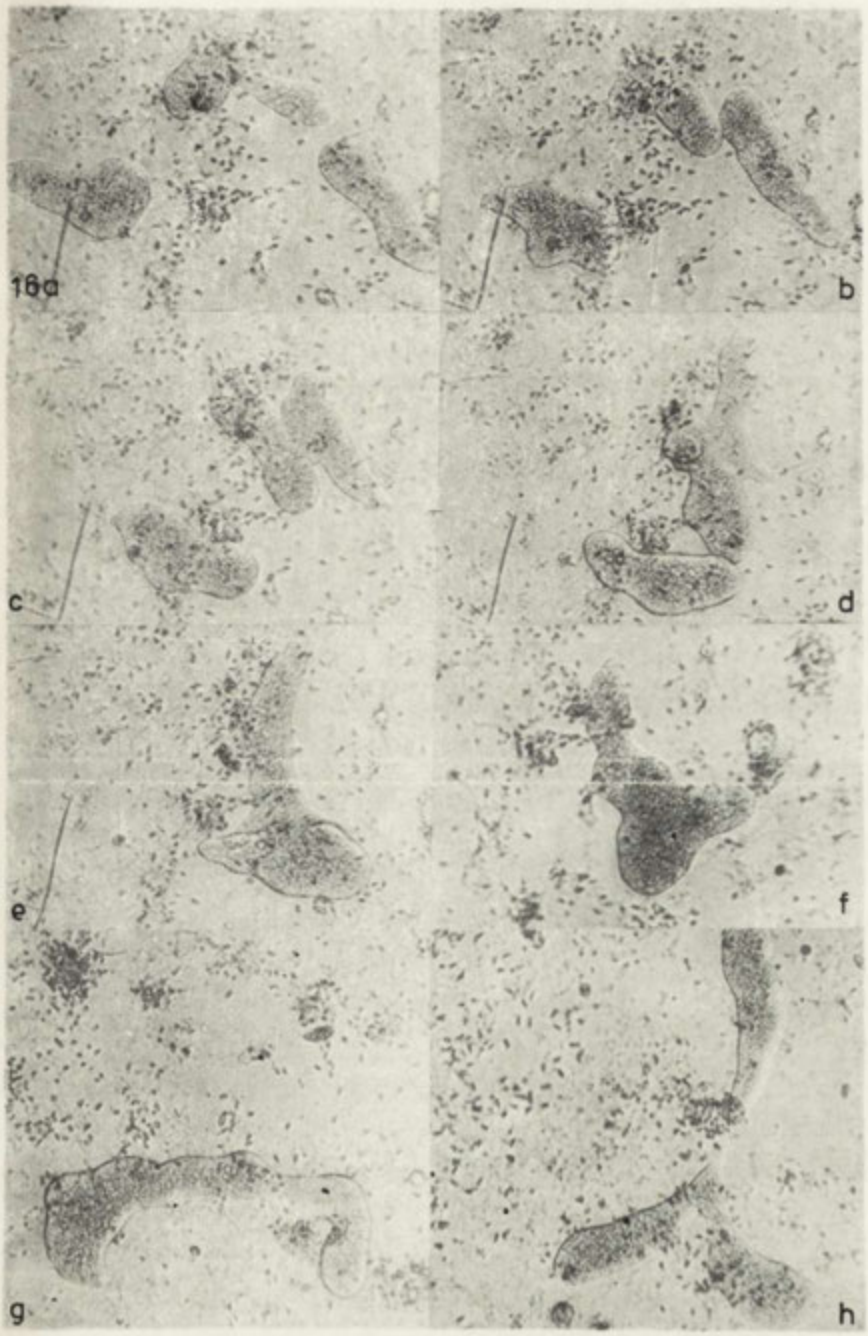

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Ultrastructure of Trophozoites and Cysts of the Amoeba *Malamoeba locustae* King et Taylor, 1936, Parasitizing the Locust *Locusta migratoria* R. et F.

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Synopsis. Ultrastructure of vegetative stages and cysts of the amoeba *Malamoeba locustae* King et Taylor, 1936, parasitizing in malpighic tubes of the locust *Locusta migratoria* R. et F. was investigated. Changes in the ultrastructure of malpighic tubes of the host after the parasite invasion were followed. It was found that, in addition to common organelles, the trophozoites usually contain a single large vacuole with residues of the host tissue. Vegetative cells with two nuclei were only rarely observed. During the encystation of the parasite its cytoplasm contained large amounts of lipoid granules; the nucleus was reduced and the cell was surrounded by a thick cyst wall. Fine vesicles were sometimes observed on the surface of encysted stages, persisting also in fully mature cysts.

The significance of amoebae living as parasites on insects as potential pathogens used for the biological control against pests has recently increased. Purrini (1980) found that the amoeba *Malamoeba scolyti* infected 30% of the bark beetles *Dryocoetes autographus* and 14% of the bark beetles *Hylurgops palliatus* out of a large collection of individuals investigated (a total of more than 1200 bark beetles). In both species of bark beetles the amoebae invaded exclusively the malpighic tubes, leading to a serious disease resulting in the host's death. It has been previously assumed (e.g., Hanrahan 1975) that the diseases caused by amoebae are not serious and are only little manifested in the pest population. At present, it is generally accepted that it is the availability of the parasite in the area where the host is disseminated that is involved, rather than the low pathogenicity of the amoebae.

About 20 species of amoebae invading various arthropods are known. Only a single species — *Malamoeba locustae* King et Taylor, 1936, is a parasite in the locusts *Locusta migratoria* R. et F. This amoeba is rare in the nature (about 0.3%), however, it is rather frequent in laboratory breeds and can destruct them (Weiser 1966). The ultrastructure of the amoeba *M. locustae* has been studied in several communications (Hanrahan 1975, Harry and Finlayson 1976, Papillon and

Cassier 1978, Žižka 1978, Hunt and Charnley 1981 and Žižkà 1985). In the last three papers only the surface of the cysts has been studied in a scanning electron microscope.

Material and Methods

Spontaneously infected locusts *L. migratoria* derived from the breeds of the Institute of Entomology of the Czechoslovak Academy of Sciences in Prague were used to study trophozoites and cysts of the amoeba *M. locustae*. Small pieces of malpighic tubes of the infected individuals were fixed with glutaraldehyde (SERVA Feinbiochemica) and OsO_4 (BDH) in cacodylate buffer according to Sabatini et al. (1962). The fixed material was washed with the buffer solution, embedded in 2% agar (DIFCO), dehydrated with the alcohol series (30, 50, 70, 96 and 100% ethanol) and via propyleneoxide transferred to Durcupan ACM (FLUKA). The material was polymerized in gelatin capsules at 70°C and cut with a glass knife in the ultramicrotome LKB Ultrotom III. The sections were stained with a saturated uranyl acetate solution and lead citrate according to Reynolds (1963) and observed in the electron microscope TESLA BS 613.

Results

Adhering trophozoites (vegetative stages of amoeba), roughly spherical (7—9 μm) or elongated (up to 4×10 μm) can be found on the epithelium of malpighic tubes of the locusts (Pl. I 1, 2). The invaded tubes of the epithelium of malpighic tubes degenerate, loose microvilli and flatten. The tube swells later in many places and inflammatory nodules are formed. These later become melanized and are characteristic of this parasite. The tubes sometimes rupture and the parasite is released to the insect coelom. The disease usually terminates in death of the host in spasm.

On its surface the cytoplasmic membrane is smooth or slightly undulated, mostly monolayered (Pl. I 3). Below it there is a thin layer of ectoplasm, which is sometimes distinctly separated from the lighter endoplasm. In addition to endoplasmic reticulum, the cytoplasm contains several spherical mitochondria of a tubular type (3—4 on single ultrathin section), several fine alimentary vacuoles and usually one large vacuole (up to 4 μm) containing residues of the phagocytized host tissue. Each trophozoite is equipped with a single large nucleus (3—3.5 μm) in diameter with a sizable, excentrically localized nucleolus of up to 1 μm in diameter (Pl. I 3). Vegetative cells of amoebae with two nuclei were rarely observed in the lumen of malpighic tubes (Pl. II 4).

After the termination of the growth and division, the trophozoites become rounded and their cytoplasm becomes electron denser, in its surface layer in particular. Several fine alimentary vacuoles can be seen in younger cells (Pl. II 5). On the surface in these stages, in a close proximity to the cytoplasmic membrane, fine vesicles are sometimes formed (Pl. II 5, 6) that persist even in fully mature cysts (Pl. II 8). Higher numbers of reserve lipidic granules of 0.7—0.9 μm in diameter are observed in the cytoplasm (Pl. II 7). A thick, originally layered wall is excreted around the

cell and the cytoplasm turns darker. A mature cyst (9.7—10.2×6—6.5 μm) contains a single nucleus which is slightly smaller (2.2—2.4 μm) than that of vegetative cells. It is enveloped by a thick, light cyst wall which is about 0.4 μm thick (Pl. II 8).

Discussion

In our laboratory breeds of the locust *L. migratoria* it was found that malpighic tubes of all individuals (100%) are invaded by the amoeba *M. locustae*, in agreement with the results of Harry and Finlayson (1975). The infected malpighic tubes have reduced microvilli of brush epithelium, the wall of the tubes is reduced and ruptures and inflammatory regenerative nodules that become melanized later are formed. It is a serious disease terminating in death of the host is spasm. In addition, Papillon and Cassier (1978) found changes in the content of glycogen in the cells of the adipose tissue of *Schistocerca gregaria*. On the other hand, Hanrahan (1975) assumes that the disease is not so serious and that rather a mechanical damage resulting in life reduction and decreased fertility of the locusts is involved. In honey bees, *Malpighamoeba mellificae* causes sometimes a serious damage of malpighic tubes, leading to the flattening of epithelial cells and the reduction of microvilli (Schwantes and Eichelberg 1984, Liu 1985), similarly to the situation observed in our material. The disease of the beetles *Dryocoetes autographus* and *Hylurgops palliatus* invaded by the amoeba *Malamoeba scolyti* is also serious (Purrini 1980, Purrini and Žižka 1983).

In our material, trophozoites were oval to elongated without protrusions, equipped with the cytoplasmic membrane on their surface. On the other hand, Hanrahan (1975) found protrusions on the surface of trophozoites. Trophozoites of *M. mellificae* (Liu 1985), *M. scolyti* (Purrini and Žižka 1983) and *Entamoeba histolytica* (Luschbauch and Pittman 1979) are also equipped with pseudopodia that are sometimes even branched, but never have amaoebostomes similar to those in *Naegleria fowleri* (John et al. 1985).

In *M. locustae* we could discriminate a thin layer of darker ectoplasm from endoplasm below the cytoplasmic membrane and the boundary observed was sometimes very sharp. On the other hand, Harry and Finlayson (1976) in *M. locustae*, living parasitically in *S. gregaria*, could not detect any differences in the morphology of the cytoplasm. The cytoplasm of *M. locustae* contains several mitochondria with short tubules as confirmed by Hanrahan (1975), Harry and Finlayson (1976) and Papillon and Cassier (1978). On the other hand, in intestinal amoebae (e.g., *E. histolytica*) mitochondria were not detected (Ludvík and Shipstone 1970, Feria-Velasco and Treviño 1972). The cytoplasm of *M. locustae* also contains several alimentary vacuoles of various size (in our material up to 4 μm), similarly to other amoebae. On the other hand, we could not detect hellicoidal, cylindrical and subpelicular bodies desribed by many authors (Ludvík and Shipstone 1970, Feria-Velasco and Treviño 1972 and others). We could neither detect a secondary nucleus found in *Paramoeba perniciosa* (Perkins and Castagna 1971).

In our material, the nuclei of trophozoites of *M. locustae* measured 3—3.5 μm. Other authors (Hanrahan 1975, Papillon and Cassier 1978) observed smaller nuclei, similar to those in *M. scolyti* (Purrini and Žižka 1983). Two nuclei in a single trophozoite could sometimes be observed (Hanrahan 1975, this study). We did not find any intranuclear bodies, as compared with Ludvík and Shipstone (1970) and Feria-Velasco and Treviño (1972).

During encystation the cytoplasm becomes denser, the number of lipidic granules increases and a bulky cyst wall is formed around the cyst. Similar changes were observed by Hanrahan (1975), Harry and Finlayson (1976), Papillon and Cassier (1978), Schwantes and Eichelberg (1984). At the beginning the wall is multilayered, the layers fuse later, but in *Iodamoeba bütschlii* 4 layers can still be discriminated (Zaman 1972). In a scanning electron microscope, the surface of cysts is smooth (Žižka 1978, 1985 and Hunt and Charnley 1981), but on sections in a transmission electron microscope a layer of surficial fine vesicles formed already at the beginning of encystation could sometimes be detected. Changes occurring during the preparation of amoebae might also be involved. Cysts of *M. mellificae* have a smooth but sometimes also a wrinkled surface (Liu 1985), whereas those of *M. scolyti* are often equipped with protrusions, apparently residues of envelope membranes (Purrini and Žižka 1983). Differences in thickness of the cyst wall in *M. locustae* are also observed. Thus, for instance, in our material the wall is only 0.4 μm thick, but Papillon and Cassier (1978) described a three times thicker wall (1—1.5 μm). In the middle of the cyst there is usually a nucleus which is, according to our measurements, by 1/4 smaller than in the trophozoites. Other authors (Hanrahan 1975, Papillon and Cassier 1978) did not detect this size difference, although in the paper by the latter authors a small difference could be observed.

The ultrastructure of trophozoites and cysts of *M. locustae* resembles that of *M. mellificae* and *M. scolyti*, although branched pseudopodia in vegetative cells and protrusions of the cyst wall could not be detected. The differences are rather quantitative than qualitative (e.g., size of nuclei). The layer of fine vesicles on the surface of the parasite, formed sometimes during encystation and persisting even in mature cysts, was detected for the first time.

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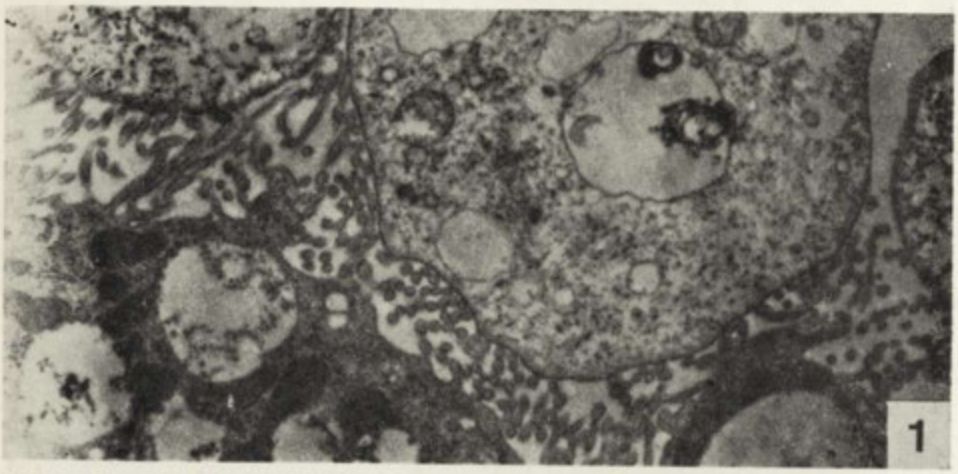
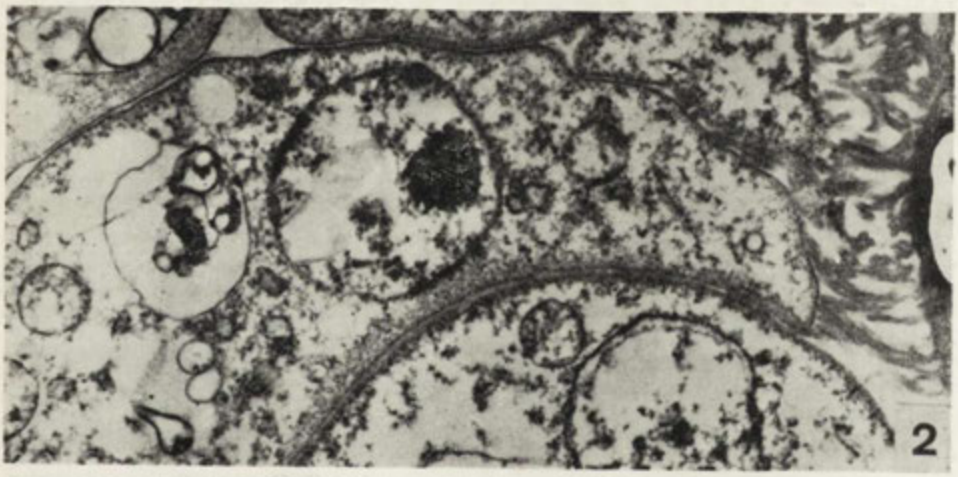
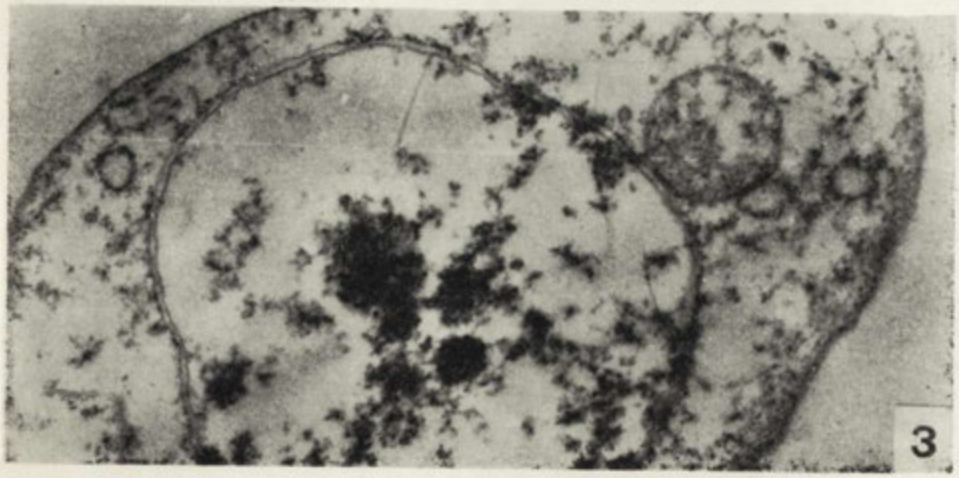
EXPLANATION OF PLATES I–II

Amoeba *Malamoeba locustae* King et Taylor, 1936, parasitizing malpighic tubes of the locust *Locusta migratoria* R. et F.

1: Spherical trophozoites adhering to the epithelium of malpighic tube. ×9060
2: Elongated trophozoite adhering to the epithelium of malpighic tube. ×10 420
3: A detail of the trophozoite nucleus. ×20 200
4: Trophozoite with two nuclei. ×9060
5: Beginning of encystation. The cell becomes rounded, cytoplasm becomes electron denser. A layer of fine vesicles also persisting in mature cysts is formed on surface. Alimentary vacuoles are also visible. ×9060
6: Cytoplasm of the encysting cell is electron denser. Alimentary vacuoles disappeared and higher numbers of lipidic granules are observed. ×9060
7: The immature cyst is surrounded by a thin cyst wall and cytoplasm is already highly electron dense. ×9060
8: The mature cyst is surrounded by a thick cyst wall. Lipidic granules and nucleus are clearly seen. ×9060







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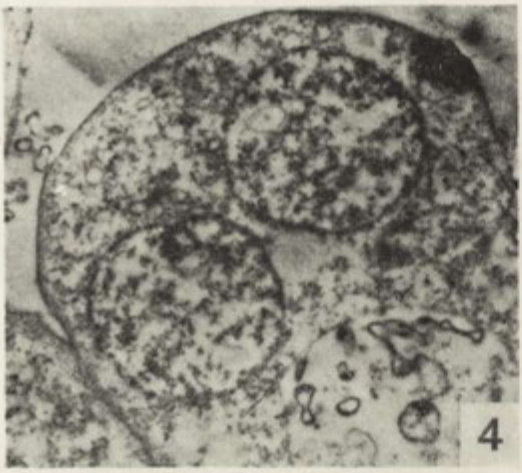

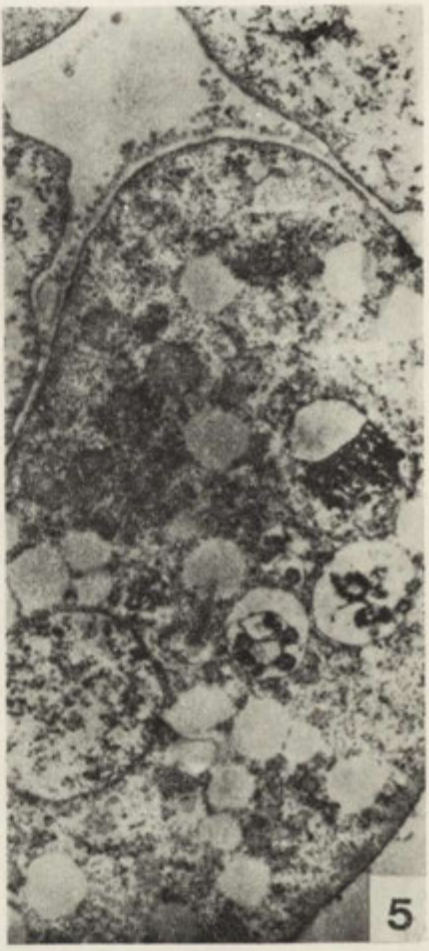

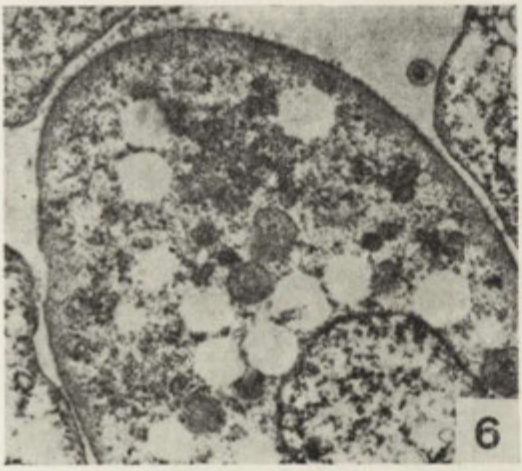

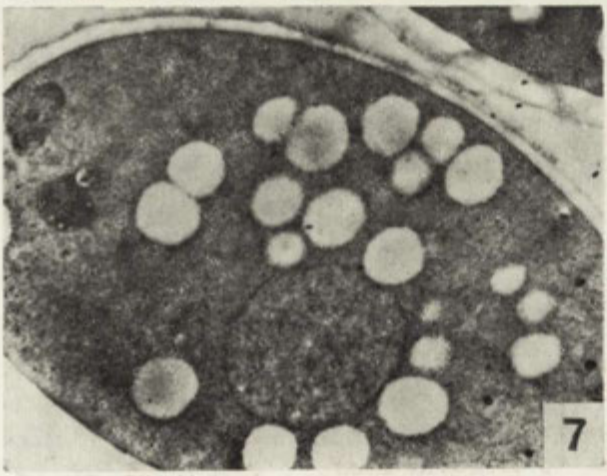

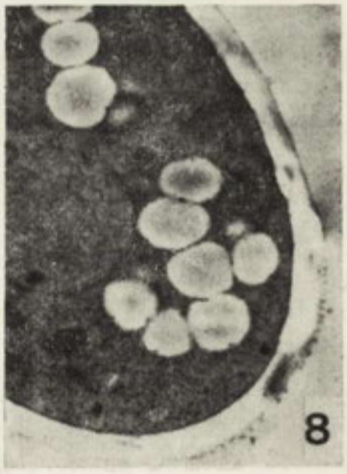

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L'étude sur la morphologie et stomatogenèse de *Spathidium muscicola* Kahl, 1930

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Synopsis. Les auteurs décrivent la morphologie et stomatogenèse de *Spathidium muscicola* Kahl, 1930 en comparant leurs resultats avec ceux obtenus par les autres chercheurs. Ils prouvent que chez cette espèce la cinétie circumorale, dont parle Foissner, n'existe pas.

Le genre *Spathidium* contient environ 100 espèces décrites surtout par Kahl (1930), qui les observait seulement *in vivo*. Elles sont difficiles à distinguer à cause de la variabilité de leurs caractères. En consequence on a parfois décrit la même espèce sous les noms differents. L'introduction du protargol dans les techniques d'imprégnation a rendu possible de révéler les structures qui resistaient au nitrate d'argent. Il est devenu évident que ce groupe des ciliés a besoin d'une revision générale, basée sur les techniques modernes. Le premier travail moderne accompli par Fryd-Versavel et al. (1975) concerne quatre espèces de *Spathidium*. Foissner (1984) en s'appuyant sur l'existence de la soi-disante cinétie circumorale a isolé de *Spathidium* plusieurs genres nouveaux.

Notre travail qui est une contribution à la connaissance de ce groupe des ciliés présente l'étude de l'espèce *Spathidium muscicola* Kahl, 1930.

Matériel et méthodes

Le cilié a été trouvé dans la mousse provenant de Bieszczady (sud-est de la Pologne). On le cultivait pendant quelques semaines, en le nourissant de petits ciliés comme *Tetrahymena*. Après l'avoir observé *in vivo* on a fait des préparations imprégnées au protargol d'après la méthode de Wilbert (1975).

Morphologie et stomatogenèse

Le corps allongé et latéralement aplati est legèrement aminci au-dessous de l'ouverture buccale (Pl. I 1). Les individus étudiés sur les préparations mesuraient de 105 à 192 μm de long (moyenne 142.5 μm) et 30 à 76 μm de large (moyenne

45.47 μm). Le nombre des cinéties est peu variable: 17 à 23 (en moyenne 20.2). Tableau 1 présente des données plus détaillées. La vacuole contractile est située au bout du corps. Le macronoyau en forme d'un ruban est accompagnée de plusieurs micronoyaux. Le cytoplasme contient beaucoup de toxicystes qui s'y forment. Dans la region antérieur existe une "structure en brosse", composée de 3 cinéties avec des cinétosomes plus serrés.

Tableau 1
Données morphométriques de *Spathidium muscicola*

Caractère	$\bar{x}$	M	SD	CV	MIN	MAX	N
Longueur en μm	142.50	138.84	20.99	0.15	105	192	34
Largeur minimale en μm	45.47	44.46	10.49	0.23	30	76	34
Largeur maximale en μm	18.99	18.64	3.93	0.21	12	31	34
Longuer du bourrelet buccal en μm	31.34	31.20	3.77	0.12	23	41	33
Nombre des cinéties	20.20	20.00	1.81	0.09	17	23	30
Nombre des cinétosomes dans une cinétie	65.43	66.00	11.73	0.18	44	94	23
Nombre des cinéties dans la brosse	3.00	3.00	0.00	0.00	3	3	32
Nombre des pairs des cinétosomes dans la deuxième cinétie de la brosse	28.28	27.50	3.74	0.13	22	37	18
Longuer des toxicystes en μm	9.60	9.92	1.57	0.16	5	13	33

Abrèviations: CV – coefficient de variation, SD – ecart type, M – médiane, MAX – maximum, MIN – minimum, N – nombre de spécimen, X – moyenne arithmétique

L'ensemble des caractères correspond à la déscription de *Spathidium muscicola* Kahl (1930). Buitkamp (1977) décrit la même forme d'une savanne de la Côte d'Ivoire. Il attire l'attention sur le parcours des cinéties somatiques dont le bouts ne terminent pas devant l'ouverture buccale mais ils se courbent et courrent paralèllement à la fente buccale. D'après Buitkamp *S. muscicola* serait identique avec *S. atypicum* Wenzel (1953) et *S. alpinum* Gellert (1955). Dragesco (1970) a trouvé au Cameroun une forme de *S. muscicola* dont le macronoyau était composé de quatre fragments mais tous les autres caractères s'accordaient avec la déscription de Kahl (1930). Foissner (1981) décrit une forme de cette espèce dont les cinéties sont plus nombreuses: 27.

Le debut de la stomatogenèse se manifeste par la multiplication des cinétosomes dans la partie équatoriale du corps (Pl. II 4, Fig. 1 a). Les cinétosomes deviennent ici plus serrés. A l'étape suivant les cinéties se rompent (Pl. II 5, Fig. 1 b) et leurs bouts se courbent (Pl. II 6, Fig. 1 c, d) jusqu'ils atteignent la cinétie précedante (Pl. II 7, Fig. 1 e). Sur le côté gauche du corps les bouts des cinéties s'inflechissent à 90° en formant une ligne legèrement dentelée où chaque cinétie est facile à distinguer (Pl. I 3). Sur le côté droite chaque cinétie se courbe doucement avant d'atteindre

l'équateur du corps et ensuite se dirige obliquement, (à la fin parallèlement à l'équateur) vers la cinétie précedante (Pl. I 2). En consequence les bouts des cinéties forment chez l'individu adulte une ligne qui entoure la bouche.

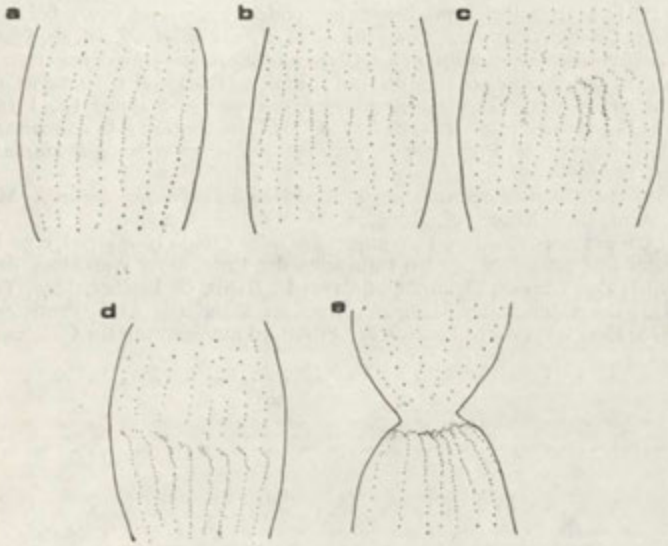

Fig. 1. Stomatogenèse de *Spathidium muscicola*. a — multiplication des cinétosomes dans les parties équatoriales des cinéties, b – rupture des cinéties, c–e — inflechissement des cinéties

Discussion

Les resultats que nous avons obtenus s'entendent bien avec les observations faites sur une espèce indéterminée de *Spathidium* par Fryd-Versavel et al. (1975). Chez cette espéce aussi au cours de la stomatogenèse les cinéties somatiques se courbent et leurs bouts entourent la bouche. Buitkamp (1977) présente d'excellents dessins de *S. muscicola*, qui ne laissent aucun doute que la ciliature entourant la bouche est constituée par les bouts des cinéties somatiques. Chez cette espèce alors "la cinétie circumorale" dont parle Foissner (1984) n'existe pas. Si elle existait elle devrait se manifester au cours de la stomatogenèse. Dragesco et Dragesco-Kerneis (1979) s'occupent aussi de ce problème: "Chez *Spathidium muscorum* l'illusion de la cinétie circumorale est encore plus grande, car les cinéties sont moins nombreuses et les cinétosomes peu serrés... Du côté droit, l'inclinaison des cinéties étant plus faible, l'illusion d'une cinétie peribuccale indépendante est encore plus forte...". Malheureusement l'auteur n'a pas étudié la stomatogenèse de l'espèce qu'il décrit. Pour élucider la question de la „cinétie circumorale" ainsi que d'autres problémes que pose le genre *Spathidium* la continuation des recherches sur la morphologie et stomatogenèse de toutes les autres espèces semble necessaire.

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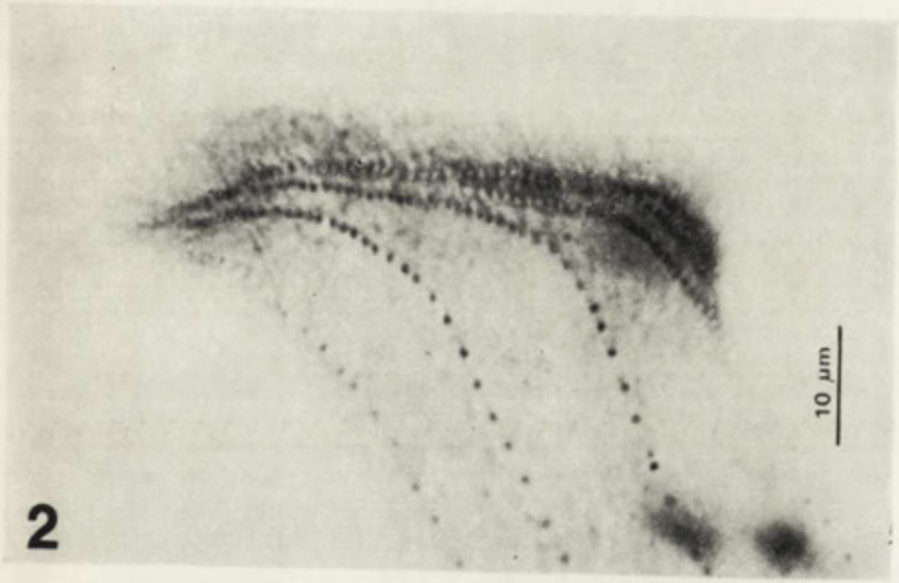
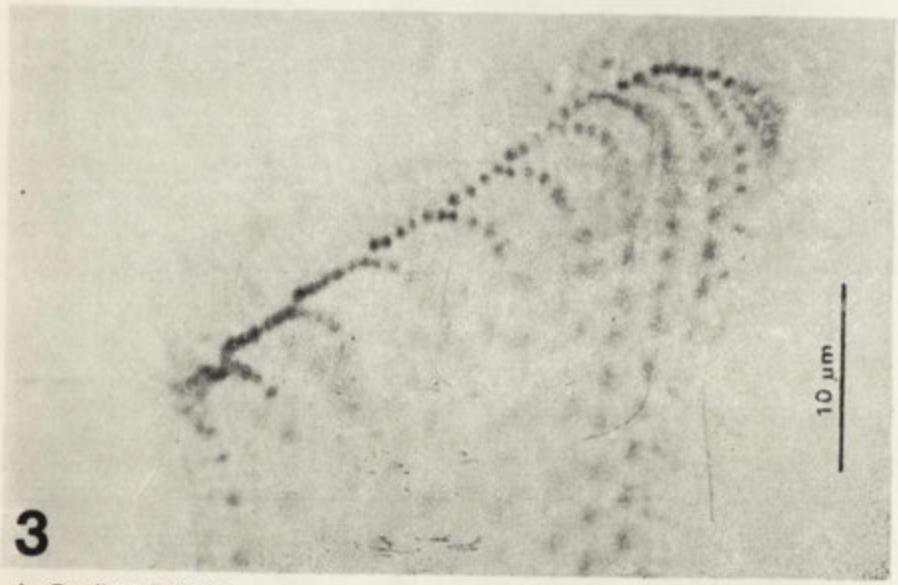
EXPLICATION DES PLANCHES I–II

1: *Spathidium muscicola*. Aspect général, animal impregné au proteinate d'argent. Ma – macronoyau, Tx – toxicystes
2: Partie anterieure du corps – le côté droit
3: Partie anterieure du corps – le côte gauche
4: Stomatogenèse chez *Spathidium muscicola*. Multiplication de cinétosomes dans la partie équatoriale
5: Rupture des cinéties
6–7: Inflechissement des cinéties





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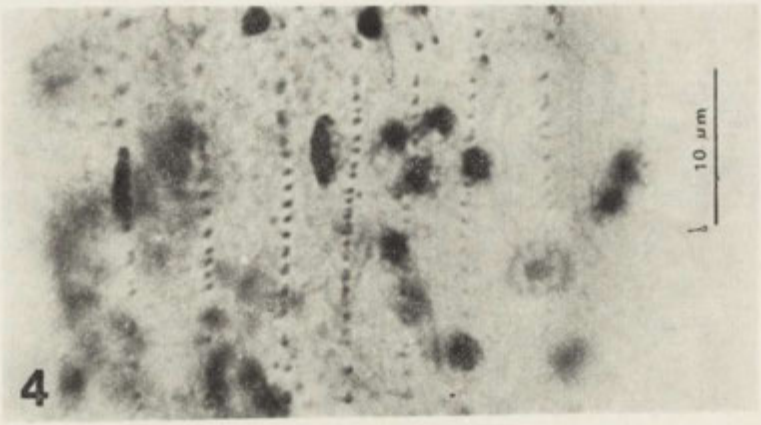

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The Ethogram of *Euplotes crassus* (*Ciliata, Hypotrichida*). II. The Paralyzed Mutant

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Synopsis. The behaviour of a "paralyzed" mutant of *Euplotes crassus* has been studied qualitatively and quantitatively, in an attempt to individualize the behavioural parameters affected by this puntiform mutation. The outcome of this effort is represented by an integrated series of data which, beyond describing a complex phenomenon, such as the behaviour actually is, as objectively as possible, enable us to compare them with the similar ones already available for the wild type. The ethogram of the P-mutant proved to differ from that of the wild euplotes in the quantitative parameters rather than in the qualitative elements: on this basis it seems likely that the P-mutation affects the motor apparatus rather physiologically *sensu lato* than morphologically. Several working hypotheses are also put forward, in an attempt of indicating tentative solutions to different problems which arose from this round of experimental work.

An almost complete lack of descriptive reports about the behaviour of *Protozoa* (Van Houten et al. 1981), suggested the need to analyze qualitatively and quantitatively the motility of ciliates: Ricci (1981, 1982) described the complex of motor patterns of *Oxytricha bifaria* following the concept of "ethogram" given by Eibl-Eibesfeldt (1967), in the wake of the "action system" indicated by Jennings since 1906. Thus the motility of *Protozoa* became suitable for scientific, quantitative investigations (Ricci 1986), and this new attitude led to draw the ethograms of *Litonotus lamella* (Ricci et al. in press) and of *Euplotes crassus* (Ricci et al. in press).

The present paper deals with the ethogram of a particular behavioural mutant of *E. crassus*, the so-called "paralyzed" mutant, kindly provided by Dr. Miceli, who isolated it, after noticing its strongly reduced velocity and very poor motor activity. These strains, P1 and P2, have been subjected to a cross-breeding analysis and the results indicate that the differences between "mutant" and "normal" strains have a simple genetic basis, which follows the Mendelian rules (Miceli in preparation).

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The qualitative and quantitative analysis of their motility was undertaken, not only with the obvious purpose of describing it *per se*, but also in an attempt of comparing the ethogram of the wild type with that of the mutants: this would allow a clear evaluation of the differences occurring between the elements of the wild and mutant motor behaviour, thus facilitating the experimental handling of the whole matter and representing an achievement of a certain relevance in the study of behavioural mutants (Kung 1976).

With all this in mind, the motility of the "paralyzed" mutant of *E. crassus* was studied and the ethogram eventually drawn.

Materials and Methods

Two mutant strains P1 and P2 were used throughout our study: "P", for "paralyzed", indicates the behavioural trait characterizing the mutants themselves. They belong to different mating types, in order to obtain the conjugating pairs, when needed. The experimental populations were grown according to the already standardized culturing techniques: the cells, kept in 1000 cc Erlenmeyer flasks, were daily fed on the green alga *Dunaliella tertiolecta*, under a cycle of 12 h of light and 12 h of darkness. The cells were collected from the flasks, washed free from food and then mildly starved, for about 24 h if conjugation was to be induced, different populations were similarly starved. When properly starved, the cells, either unmixed or mixed, were put between a slide and a coverslip, according to a well established protocol: this preparation allows the proper recording of the motor behaviour, both by the already standardized photographic time-exposure-dark-field technique (Dryl 1958, Ricci 1981) and by the microscopic TV-colour technique (Ricci 1981), provided that several experimental parameters are properly adjusted: (a) the density of the population, which, for *E. crassus*, was found to be optimal when ranging around 50 cells/cm^2; (b) the thickness of the experimental chamber, namely the distance between the slide and coverslip, which was of 2 mm; (c) the time of exposure for the photographic recording, which was of 5" for *E. crassus*. The rationals of these technical choices have been already reported in the first paper about the ethogram of the wild type of *E. crassus* (Ricci et al. in press), in which also the analytic study of the tracks, both on photographs and on TV screen, was thoroughly described.

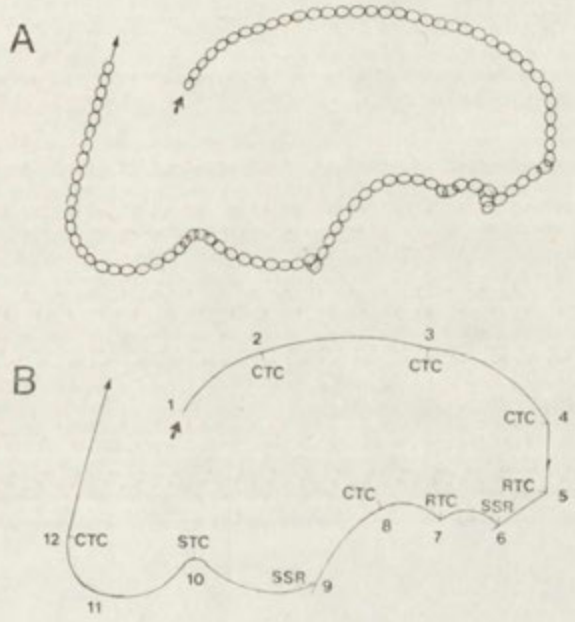
The experimental tools used in this study are the following: (a) the Wild M 420 stereomicroscope and (b) the Leitz Orthoplan optical microscope, both coupled with either (c) their specific dark-field apparatuses or (d) a Hitachi colour TV camera and a Sony Betamax C-7 colour videotape-recorder.

Results

A — Single, P-mutant *Euplotes crassus*. I. The creeping

The track of a mutant moving on the substrate is described by a piecewise linear (Fig. 1 A, B), formed by rightward arcs (48.3%), segments (32.8%) and leftward arcs (18.9%). Every element is geometrically perfect ($p < 0.01$) and the whole picture of the different measures is given in Fig. 1, C. The rightward arcs ($n = 115$) have a length of 336 μm ± 180 μm, a width of 45°±29°, and a radius of 546 μm±

345 μm, while the leftward arcs (n = 55) have a length of 262 μm±112 μm, a width of 38°±17°, and a radius of 487 μm ± 312 μm. Thus, generally speaking, the creeping of these mutants is characterized by quantitative parameters widely variable around their means, as indicated by the large standard deviations: in turn, such a variability seems to depend rather upon intercellular than intracellular differences, in as much as one single *Euplotes* tends to move preferentially along elements rather constant in their geometric elements (length, width, radius).



C

	rightward arcs 48.3%	segments 32.8%	leftward arcs 18.9%
number	115	162	55
length (μm)	336±180	255±164	262±112
width (°)	45±29	—	38±17
radius (μm)	546±345	—	487±312

Fig. 1. A piecewise linear describing the creeping of a P-mutant of *Euplotes crassus* (A) and its interpretation (B) in terms of geometric elements (rightward arcs, segments, leftward arcs) and of changes of trajectory (CTC, STC, RTC, SSR). The panel C gives the quantitative values of different elements

The geometric elements just described form the piecewise linear and are joined together by five kinds of change of trajectory, which have been called Continuous Trajectory Change (= CTC, 61%), Smooth Trajectory Change (= STC, 3%), Rough Trajectory Change (= RTS, 28%), normal Side Stepping Reaction (= SSR, 5%), wide Side Stepping Reaction (= SSRw, 3%), according to Ricci (1981).

The CTC (Fig. 2, upper part) is clearly distinguishable for the lack of any particular geometric "point", along the track: the cell passes from one element of its pathway to the next, without any discontinuity. The more rare STC (Fig. 2, central part), on the contrary, is well characterized by an interruption, usually occurring between two successive rightward arcs: it is recognizable as a short arc with a small radius and bent to the opposite side of the track (usually, leftwards): while performing it, the euplotes moves at a reduced velocity. The RTC (Fig. 2, lower part) represents

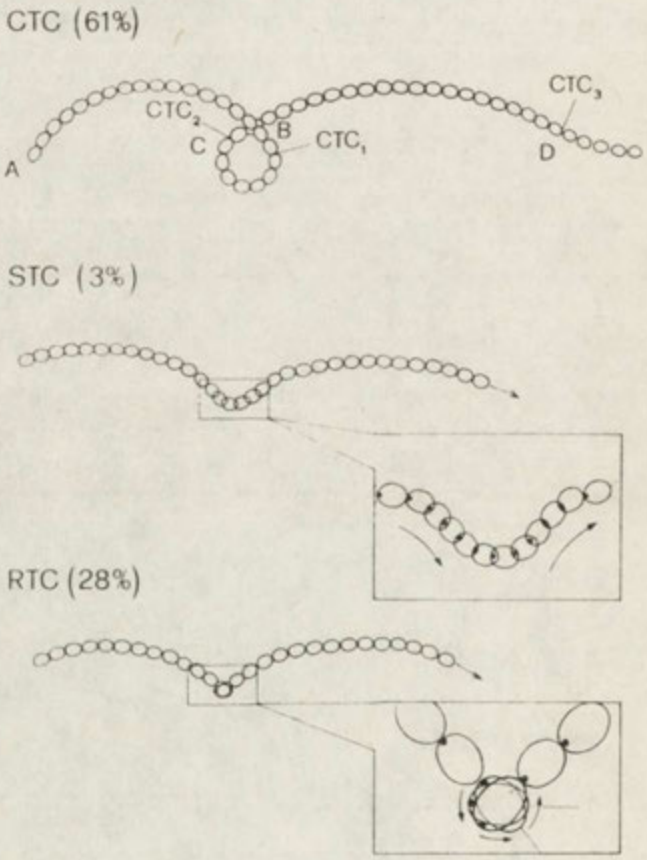


Fig. 2. The principal kinds of interruption (and their relative frequencies) along the creeping track of a P-mutant: the CTC, the STC and the RTC; for both the STC and the RTC, a panel is given on the right, describing the reaction itself at higher magnification and more particularly: the black spots label the posterior end of the cells

a well defined reaction, characterized by a sharp interruption between an arc and the successive element of the piecewise linear: at the level of this point, the cell stops for about 1/2" and turns toward its left rotating around a point, which lies posteriorly, at about 1/2 of its length.

The SSR (Fig. 3, upper part) is a rather exceptional event for the "P" mutants, being performed in not more than 5% of the changes of trajectory: however, in

spite of such a rare occurrence, both the backward jerk ($s = 108\ \mu m \pm 55\ \mu m$; $n = 8$) and the clockwise rotation ($\alpha° = 86° \pm 25°$, $n = 8$), namely the two geometric traits characterizing the SSR and following the sudden stop, are quite constant in their quantitative expression.

The fifth kind of reaction, by which the P mutant of *Euplotes crassus* passes from one trait of its trajectory to the next, has been called "wide SSR" (Fig. 3, lower part). This reaction differs from the normal SSR, for two different reasons: the first is a quantitative one, because the cell rotates clockwise at an angle far wider than that of the SSR (~134°); the second is a qualitative one, bacause its general pattern is a new one, consisting of two successive elements: the first is the backward jerk ($s = 263\ \mu m \pm 70\ \mu m$, $n = 7$) which lies at $\sim 40° \pm 12°$, ($n = 7$), on the left of the initial track (Fig. 3, lower part), while the second is the clockwise rotation ($\alpha° = 94° \pm 21°$, $n = 7$) occurring at the end of the backward movement (Fig. 3, lower part).

The time elapsing between two successive spontaneous interruptions of the tracks of the P mutants was measured as a parameter likely mirroring the changes possibly undergone by the electrical state of the cell membrane as a consequence of the P-mutation: it was found that the average frequency according to which one of the different kinds of trajectory change occurs is $0.78\ s \pm 0.41\ s$ ($n = 342$),

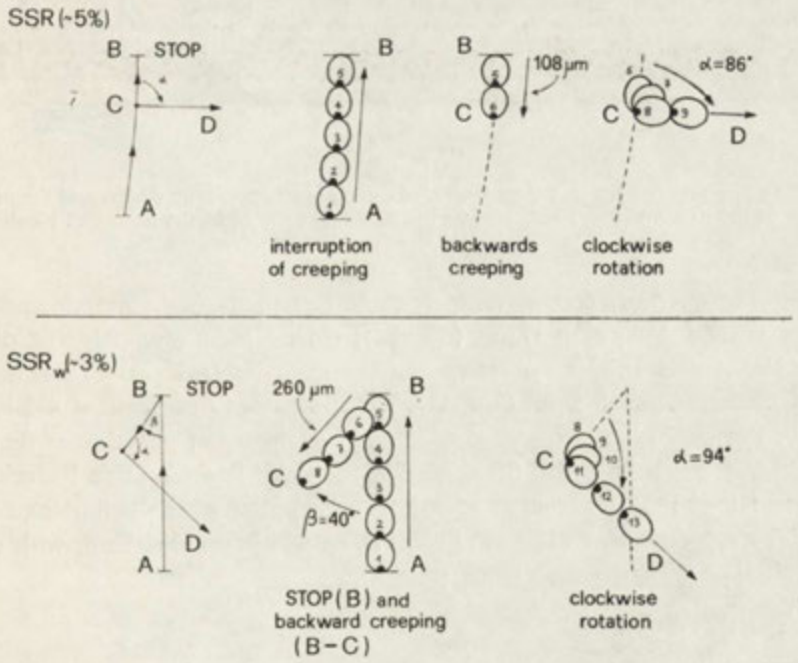


Fig. 3. The SSR (upper part): on the left, the general pattern is schematically drawn, while the three principal elements of the reaction are given separately on the right; the wide SSR (SSRw) (lower part) is represented on the left, while the succession of specific reactions occurring during it, is given on the right

corresponding to uninterrupted traits on the average as long as 286 μm ± 152 μm (n = 342). Another typical trait of the creeping behaviour of the P mutants is the strong tendency of the cells to lie quite motionless on the substrate; when it happens, the cell quits creeping and remains still for as long as 2–3 s. Both the frequent interruptions and the relatively long stops affect the average velocity ($\bar{x}$ = 367 μm/s ± 119 μm/s, n = 34), which is far lower than that of the wild *Euplotes*.

II. The Swimming

First of all one has to be aware that the P mutants swim only very rarely; however, when they start swimming they move along a perfect helicoid, bent to the left, keeping their ventral surface towards the central, straight axis of symmetry. The helicoid, in turn, is characterized by two elements (Fig. 4): (a) its pitch, which is

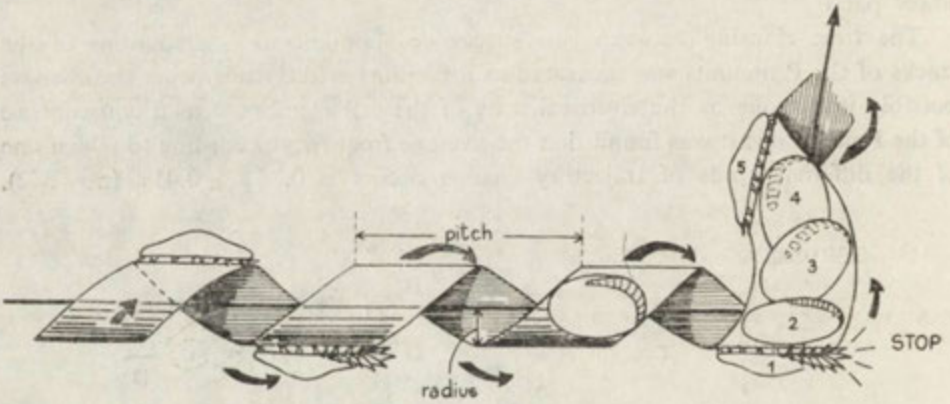


Fig. 4. The swimming pattern of a P-mutant of *E. crassus* is schematically drawn and the pitch and the radius of the movement are indicated in the figure. On the right the Stop and Reorientation Reaction (SRR) is schematically drawn

the distance between two successive, corresponding points of the swimming pathway: it ranges around 138 μm ± 37 μm (n = 84); (b) its radius, namely the distance between the central axis of symmetry and the most internal part of the helicoid: it is as large as 84 μm ± 5 μm (n = 12). The velocity of these cells is 413 μm/s ± 138 μm/s. The only kind of reaction mediating the change of direction of the swimming pathway has been called SRR, namely Stop and Reorientation Reaction (according to Ricci 1981): it consists of a short interruption of the swimming activity, followed by a rotation of 81° ± 25° (n = 17), in whichever direction, with respect to the initial axis of the movement (Fig. 4).

III. The Starvation

To evaluate the effects of starvation on the P mutants, two different stocks of cells were starved for 4 and 11 days, respectively, and their behaviour analyzed from 3 different points of view: (a) the percentage of still cells and the percentage of

the cells lying sideways on the substrate; (b) the average length of the uninterrupted elements of the track; (c) the mean creeping velocity.

For the 4-day-starved cells it was found that 44% of the cells lie motionless on the substrate and 1% lie sideways; the remaining 55% creep along very short segments and arcs ($s = 150\ \mu m \pm 191\ \mu m$; $n = 140$) at a velocity of $93\ \mu m/s \pm 109\ \mu m/s$ ($n = 23$).

In 11-day-starved populations, the motionless euplotes are as many as about 75% of the whole population, while 14% lie on the substrate sideways. The remaining 11% creep along uninterrupted traits as long as $252\ \mu m \pm 78\ \mu m$ ($n = 13$), at a velocity of $213\ \mu m/s \pm 57\ \mu m/s$ ($n = 20$).

B — The Mating Behaviour

The mating behaviour is shown by cells belonging to two different mating types of the P mutants when they are put together, to interact with each other by direct contacts.

During the first 15′–20′, the clones P1 and P2 show a behaviour which can be described as follows (Fig. 5): (1) the cells creep on the bottom of the vessel; only a small percentage (~1%) still swims; (2) the velocity drops rapidly to almost 0 μm/s, due to the progressive increase of the periods during which the cells remain motionless; (3) more and more frequently the cells perform backward jerks as long as 200–300 μm (Fig. 5 A); (4) they tend to form groups of 5–7 cells which contact

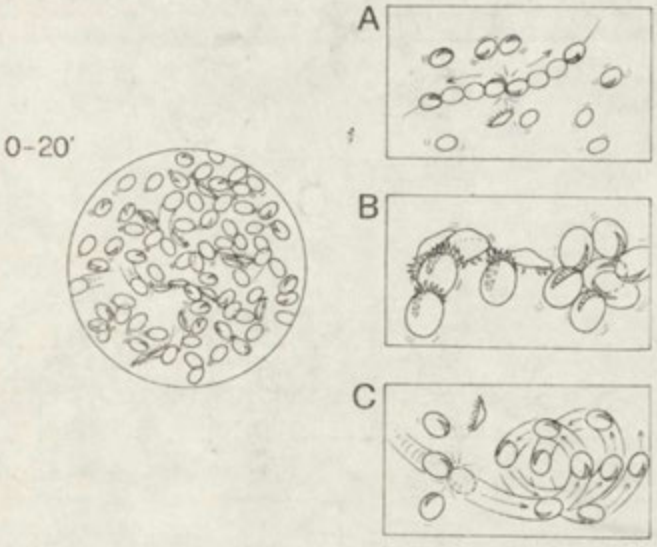


Fig. 5. The very first stages of mating behaviour (0′–20′ from the mixture of different types). In the circle on the left the whole population has been indicated, mostly represented by almost motionless cells, a few cells lie sideways, while other organisms still creep at almost normal velocity. A — the prolonged, backward motion of two cells bumping against each other; B — early, direct contacts among cells belonging to different mating types by which some cluster may be formed: C — the very long (4″–10″) backward creeping of the cell disturbed while lying still on the substrate

each other by their anterior ends (Fig. 5 B); (5) about 60% of the cells are quite still on the substrate; (6) about 10–20% of these cells lie sideways, contacting each other by the ventral surfaces; (7) the still *Euplotes*, if stimulated by other cells bumping against them, may perform striking jerks or casual motions uninterrupted for 5–10 s (Fig. 5 C).

During the second period (20–40 min, from the mixture of the two different types), the population shows several peculiar traits of its behaviour, which can be summarized as follows (Fig. 6): (1) after bumping against each other, no SSR is shown (Fig. 6 A); (2) the cells tend to explore each other by "prolonged contacts", thus evoking the reciprocal response by the partner (Fig. 6 B); (3) the partners, after contacting each other properly, namely, by the respective anterior tips, orient each

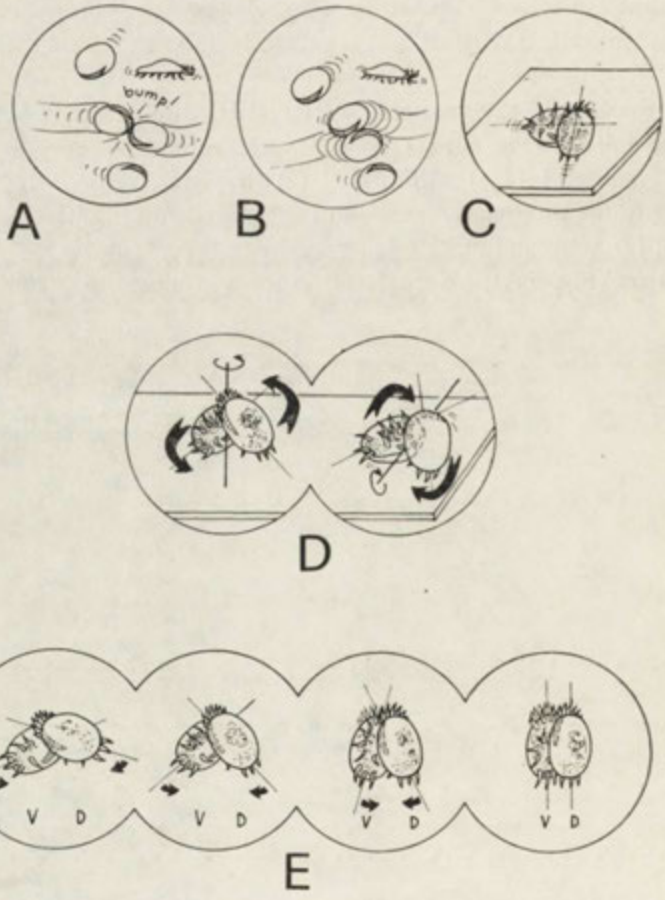


Fig. 6. Between 20′ and 40′ from the mixture of different mating types, the P-mutants quit moving backwards after bumping against each other (A), rather performing the "prolonged contacts" (B), before positioning at about 100–120 degrees, with respect to each other (C) — in D, the "free-dance" above the substrate is represented, to show how poorly the "swimming engines" of the partners work. At the end of the free dance, the partners, united by their ventral surfaces, form angles more and more narrow: from 120° to 0° (E)

other in such a way that their longitudinal axes form an angle of about 100–120 degrees (Fig. 6 C); (4) the "free dance" begins, during which the partners spin around their common longitudinal axis, though remaining very close to the substrate or even still contacting it (Fig. 6 D); (5) if disturbed during the free dance, the partners, only lightly connected to each other, separate without any searching reaction.

The last period preceding the pair formation roughly lasts as long as 35–40 min: it occurs from 40 to 75–80 min after the mixture of the two different mating types. This period is characterized by the following traits: (1) the population shows a weak tendency to form clusters, which are quite different from the normal "mating cloud" (already described for the wild type of *E. crassus*), both for their fairly small dimensions, and for their symmetry: in fact, rather than subspherical, these clusters are flattened and parallel to the substrate, due to the reduced motility generally characterizing the mutants; (2) within these clusters the specific pre-mating steps occur between the potential partners now contacting each other, two by two, by the respective ventral surfaces: the angle formed by their longitudinal axis, from initial values as large as 100–120°, progressively drops to zero in the perfectly formed pair (Fig. 6 E); (3) the outcome of these preconjugant behavioural patterns is rather poor (not more than 5 pairs form, on the average, from an initial population of 100 cells) so that it can be concluded that in the P-mutants the efficiency of the pre-mating behavioural steps leading different cells to the pairing is strongly reduced.

C — The Behaviour of Pairs

The motor behaviour of the pairs formed by partners belonging to the different mating types, P1 and P2, of the mutant *E. crassus* is almost entirely nonexisting. Both the creeping and the swimming can be described only as very rare, very short, definitely casual and random movements: it can be concluded that the pairs of the P mutants are virtualy incapable of any coordinated, efficient motor activity.

Discussion

According to the rationals indicated by Ricci (1986), the study of the motile behaviour of the P ("paralyzed") mutants of *E. crassus* was undertaken in order to draw their ethogram: after accomplishing the task, it can be stated that such an ethogram represents the first example of a study describing qualitatively and quantitatively the motility of a behavioural mutant of a protozoan. Once one thinks, for instance, of the line of research followed by Kung and coworkers (as already mentioned in the Introduction), the convenience of this kind of investigation can be properly evaluated: the exact and precise description of both qualitative elements and quantitative variables, characterizing the motor behaviour of any mutant, leads to figures lending themselves to comparative considerations far more articulated, exhaustive and suggestive than a mere analysis of simple photographs.

Beyond these initial, general considerations, a series of more specific comparisons can be made among the data describing the motor behaviour of the wild type (Ricci et al. in press) and of the P-mutants, respectively.

The striking and fundamental differences occurring between the behaviour of these two different populations are evident even at a first glance, however, once some more specific parameters are compared, the picture, in its general traits, becomes even clearer: (a) the different percentages of rightward arcs, segments and leftward arcs, forming the piecewise linears: 37%, 13%, 50%, respectively, for the wild type, 48%, 33%, 19%, for the P-mutant: (b) the different radii of both the rightward arcs (1568 μm for wild type vs 546 μm for P-mutants) and the leftward arcs (864 μm for wild cells vs 487 μm for P-mutants); (c) the different width of the rightward arcs (63° for the wild type, 45° for the P-mutant) and of the leftward ones (74° for the wild type and 30° for P-mutant); (d) the pitches (425 μm for the wild type vs 138 μm for the P-mutant), the radii (43 μm for the wild type vs 84 μm for the P-mutant) and the velocities of the swimming cells (803 μm/s for the wild type vs 138 μm/s for the P-mutant). On the basis of this collation, one can immediately visualize the general motor pattern typical of either population: however, quite unsolved still remains the problem of the exact level of integration between the nuts and bolts forming the cellular motor machineries, affected by the P-mutation and responsible for such a deep difference between the two ethograms. Indeed, these behavioural differences might, in turn, depend upon fine morphological differences (such as those possibly distinguishing the cirri as to their ciliary composition), or even upon their spatial arrangement and distribution. On the other hand, an ultrastructural difference accounting for the described different behaviour of the two populations cannot be excluded: can any difference occur at the level of the tips of the cilia, or even at the level of the ciliary necklace or, finally, at the level of the cortex? A third element possibly involved in the mechanisms responsible for the different behaviour of the P-mutants is the physiological component: does the state of the cell membrane (number and function of gates and pumps for different ions and so on) affect the membrane potentials, in turn probably responsible for the integration and coordination of the ciliary beat (Machemer 1974, Sleigh 1984)? Thorough studies are presently carried on, in an attempt of giving an answer at least to some of these questions, also in the line of those indicated by Naitoh and Sugino (1984).

Another parameter, strongly differentiating the ethogram of the wild *E. crassus* from that of the P-mutants, is the velocity, which is on the average 849 μm/s for the former and 364 μm/s for the latter. Such a dramatic difference is mainly due to the spontaneous interruptions of creeping, which occur very frequently in the P-mutants, namely every 0.78 s vs 2" in the wild euplotes, and which affect also the length of the average uninterrupted traits of the creeping pathway, another parameter definitely different from that of the wild population.

If the clearcut differences so far discussed cannot but distinguish the behaviour of the P-mutants from that of the wild clones, one has to be aware that also several

similarities seem to occur between the two pools of data describing the two populations (Fig. 6): (a) the relative frequencies of CTC, STC, RTC and SSR are very similar for both wild (69%, 5%, 14%, 12% respectively) and mutant cells (61%, 3%, 28%, 8%); (b) the elements of the SSR: the backward motion (129 μm for the wild type and 108 μm for the P-mutants) and the clockwise rotation (76° for the wild type and 86° for the P-mutants). These data reveal that these reactions are fairly stereotyped for both populations and that they can be ordered according to the sequence CTC→STC→RTC→SSR (where the effectiveness in changing the direction of the trajectory is increasing steadily from the first to the fourth), for both wild and mutant strains. Such a conclusion, as to the very stereotyped reactions by which the ciliates change their trajectories has been already drawn from the comparisons between the ethograms of *Oxytricha bifaria* and *Litonotus lamella* (Ricci et al. in press) and from that between these two ethograms and that of the wild *E. crassus* (Ricci et al. in press).

The data obtained about the geometric characters of the swimming pattern and the velocities showed that the P-mutants have a radius twice as large as that of the wild type and a velocity equal to about half the velocity of the wild cells. Although any clear conclusion about the causes of these differences still seems far from being drawn, at least an indication may be found: as already suggested by Ricci (1981) for *O. bifaria*, it seems likely that the helicoids along which the ciliates unfailingly swim, do not change randomly and continuously but, on the contrary, a basic principle seems to be true among the variables characterizing this kind of movement: the larger the radius, the shorter the pitch, the smaller the velocity, the cause-effect relationships occurring among these three elements being unknown.

The effects of starvation on P populations were studied by monitoring three principal variables (the relative motility of the cells, the uninterrupted spaces and the mean velocity) capable of describing the changes occurring in the motor behaviour of the starving cells.

It was shown that (1) the percentage of still cells jumps from 0% in the normal population, to 44% after a 4-day starvation and to 75% after an 11-day starvation; (2) the percentage of cells lying sideways on the substrate in a 4-day starved population is of ~1% and of ~14% in 11-day starved cells; (3) the cells behaving normally represent 100% in the control population, 55% after a starvation of 4 days and 11% after undergoing a starvation as long as 11 days. Thus, the picture emerging from these data shows a progressive increase of inertness of the population as the starvation goes on. After the 4-day starvation, the velocity is reduced to about one fourth of the normal value and the uninterrupted space to about one half of the normal value: these data confirm what was already found in the wild type of *E. crassus* (Ricci et al. in press), namely that the longer the starvation, the larger the membrane potential instability. More puzzling, on the contrary, is the partial "recovering" shown by the 11-day starved cells in their behaviour: while their velocity increases up to about two thirds of that of controls, the mean uninterrupted space returns to values quite similar to the normal values. A tentative hypothesis could

be put forward to explain this apparent paradox: about 10% of the population is unaffected by the starvation and this percentage is evident when the starvation becomes so severe, as to immobilize the remaining 90%. This could account for the observation made on the cells after a 4-day starvation, according to which both velocity and mean uninterrupted spaces are dramatically reduced; under these conditions about 40% of starvation-sensitive cells would be still active, thus affecting the general values of the population. Such an idea seems somewhat confirmed by the observation that the standard deviations of the data referring to the 4-day starved populations are unusually large, much larger than those of the data describing the 11-day starved populations.

Still unclear remains the meaning of the cells which lie sideways on the substrate, and which are still capable of resuming an almost normal, temporaneous motor activity, once properly stimulated. One can just say that they show a peculiar behaviour, definitely starvation-dependent (cfr. 1% of cells lying sideways after 4 days of starvation vs 14% of similar cells after 11-day starvation) but how and why they behave in that way is still far from being accounted for.

The last aspect of the ethogram of the P-mutants to be considered is the mating behaviour. Two points are to be noticed: (a) the successive steps of the mating dance are quite the same as those in the wild type; (b) the efficiency of the process seems to be very low ($\sim$5 pairs formed from 100 cells) with respect to 40–45 pairs formed by 100 wild euplotes. These two results indicate that the P-mutants behave quite normally before conjugating: they just have "to face" a large number of obstacles along the preconjugant processes so that a very small percentage of cells succeeds in pairing. In turn, this conclusion suggests that (1) the preconjugant cell interactions in *E. crassus* represent a sort of all-or-none process, in as much as either the cells pass through a complete series of steps leading to pair formation and they conjugate successfully, or they interrupt the succession of these steps at whatever level and miss completely the basic targets, such as cell union, trigger of meiosis etc.; (2) the P-mutation seems to affect mainly the motor behaviour of the cells (the more reduced the motility, the smaller the efficiency) rather than the specific, physiological steps following the cell union.

In conclusion it can be stated that in the study of the biology of any protozoan species, the ethogram actually represents that polyhedric tool already suggested by Ricci (1985), and that it will become more and more useful, as it will be drawn more and more completely, with regard to its qualitative elements and to its quantitative variables.

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Antigenic Differences Among *Giardia intestinalis* Isolates from One Geographic Area

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Synopsis. The surface antigenic differences among 20 *G. intestinalis* isolates newly established in culture were studied by indirect immunofluorescence assay. Some isolates were similar to the human Portland-1 *G. intestinalis*, whereas others to the CAT-1 *G. cati* or to the GP-1 *G. caviae*. The remaining *G. intestinalis* human isolates showed unique surface antigens dissimilar to the other groups of strains. The investigation showed that some of human *Giardia* strains were different, although isolated from the population in one geographic location. On the other hand, the results confirmed the opinion that man and cat did not seem to possess their own species of *Giardia*.

An infection with *Giardia intestinalis*, a common intestinal protozoan parasite of man, may produce a variety of clinical manifestations ranging from transient intestinal complaints to fulminant diarrhea and malabsorption. One possible reason for this may be the differences in surface antigens correlated with parasites pathogenicity and varying host's response. Indeed, *G. intestinalis* isolates showed variable virulence (Aggarwal et al. 1983) as well as differences in their surface antigens, DNA banding patterns, and excretory-secretory products (Smith et al. 1982, Nash et al. 1983, 1985, Nash and Keister 1985, Korman et al. 1986). The differentiation of one *Giardia* isolate from another will also be important for epidemiological and chemotaxonomical considerations.

To ascertain the surface antigenic differences among *G. intestinalis* we studied, by indirect immunofluorescence assay, 20 isolates newly established in culture and obtained from patients with different clinical manifestations in one geographic location in Poland.

Material and Methods

Giardia isolates and culture. Twenty *G. intestinalis* axenic isolates established newly at the Department as well as one isolate each of *G. intestinalis* (Portland-1 = P-1), of *G. cati* (cat-1/Portland = CAT-1), and of *G. caviae* (guinea-pig-1/Portland = GP-1) originally isolated by E. A.

Meyer and E. Fortess (Meyer 1970, 1976, Fortess and Meyer 1976) were studied. The methods and source of isolation as well as the origin of the fresh isolates are shown in Table 1 and were described previously (Kasprzak and Majewska 1985). The isolates were maintained in culture with modified BI-S-33 medium supplemented with bile at 37°C in a slant (Keister 1983). For routine maintenance of the culture the trophozoite suspension (1×10^4 to 2×10^5 of cells) was being reinoculated into fresh medium every 3 to 7 days, depending on the growth of *Giardia*.

Antigen preparation. As the antigen, intact *Giardia* trophozoites were used; late log phase viable trophozoites that adhered to glass tubes were harvested by cooling the tubes in ice water for 10 min, followed by centrifugation at 600 g for 5 min. The sediment of trophozoites dislodged from the tube-walls was washed three times by centrifugation in chilled phosphate-buffered saline (PBS) pH 7.2.

Antisera preparation. Hyperimmune antisera to P-1 *G. intestinalis*, CAT-1 *G. cati*, and GP-1 *G. caviae* were produced by weekly injection of male rabbits, weighing 2.5 kg each, with a suspension of about 5×10^6 of intact trophozoites and complete Freund adjuvant (1:1 vol/vol). During the eight-week immunization period the blood was withdrawn for the follow-up antibody formation. The sera were pooled seven days after the last (8th) subcutaneous injection; the animals were anesthetized with Brevinarcon and bled by cardiac puncture.

Indirect immunofluorescence technique. Indirect immunofluorescence assays with formalin-fixed *Giardia* trophozoites were performed. The sediment of trophozoites was suspended in 1% neutral formalin in PBS, centrifuged and washed thrice with PBS. The final pellet was suspended in distilled water to obtain about 30 cells per one microscopic field (250×), and the parasite suspension was dropped into the wells of slides coated with teflon. The prepared slides were

Table 1
List of *G. intestinalis* strains isolated from human patients

Strain	Clinical course	Origin of the strain
HP-10	symptomatic	faeces, cysts
HP-34	symptomatic	faeces, cysts
HP-42	asymptomatic	faeces, cysts
HP-50	asymptomatic	faeces, cysts
HP-53	asymptomatic	faeces, cysts
HP-63	asymptomatic	faeces, cysts
HP-88[1]	symptomatic	duodenal fluid, trophozoites
HP-89[1]	symptomatic	duodenal fluid, trophozoites
HP-92[1]	symptomatic	duodenal fluid, trophozoites
HP-94	symptomatic	duodenal fluid, trophozoites
HP-98	asymptomatic	faeces, cysts
HP-99[2]	asymptomatic	faeces, cysts
HP-100[2]	asymptomatic	faeces, cysts
HP-101[3]	symptomatic	duodenal fluid, trophozoites
HP-102[3]	symptomatic	faeces, cysts
HP-103	symptomatic	faeces, cysts
HP-104	asymptomatic	faeces, cysts
HP-105	symptomatic	faeces, cysts
HP-106	symptomatic	faeces, cysts
HP-107	asymptomatic	faeces, cysts

[1] Strains isolated from the same patient
[2] Strains isolated from the same patient
[3] Strains isolated from the same patient

dried at room temperature and were stored at −20°C. The antisera absorbed with trophozoites of particular cross-reacting *Giardia* species were used. For absorption, the samples of antisera were added to a pellet of washed *Giardia* trophozoites at 37°C and with continuous agitation. After 2 h of incubation the trophozoites were removed from the antisera by centrifugation. The absorption procedure was repeated several times until negative reaction with homologous antisera was obtained.

Results

Hyperimmune antisera prepared in rabbits against *G. intestinalis* P-1, *G. cati* CAT-1, and *G. caviae* GP-1, were used to compare all the 20 newly established *G. intestinalis* isolates as well as the P-1 and the two strains isolated from a cat and a guinea-pig. The reactivity of these isolates with three antisera is shown in Table 2. Five major groups of isolates could be distinguished.

Table 2

IFA results* with cross-absorbed antisera against *G. intestinalis* Portland-1, *G. cati* and *G. caviae* strains

Strain	Antisera					
	G. intestinalis absorbed with		*G. cati* absorbed with		*G. caviae* absorbed with	
	G. cati	*G. caviae*	*G. intestinalis*	*G. caviae*	*G. intestinalis*	*G. cati*
P-1	512	128	—	128	—	32
HP-50	128	128	—	—	128	—
HP-105	128	128	—	—	32	64
HP-104	128	128	—	—	32	128
HP-10	32	64	—	—	32	256
Cat-1	—	128	2048	256	256	—
HP-103	—	—	2048	256	256	—
Pig-1	32	—	1024	—	512	32
HP-101	32	64	1024	—	128	—
HP-102	64	32	1024	32	256	128
HP-99	32	32	2048	—	128	128
HP-100	32	—	2048	—	64	128
HP-98	32	32	2048	32	128	128
HP-53	32	32	128	32	128	128
HP-92	32	—	128	32	128	128
HP-89	32	—	32	—	128	128
HP-34	—	—	64	32	64	128
HP-42	—	—	64	—	64	256
HP-94	—	—	32	64	128	64
HP-63	32	—	—	—	—	32
HP-88	—	—	—	—	64	—
HP-106	32	—	—	—	32	—
HP-107	—	—	—	—	—	—

* Results are expressed as reciprocal titre. The titres given represent the highest dilution giving a 2+ fluorescence (a scale of 1+ to 4+ was employed, with 4+ representing the brightest fluorescence).

— = negative at titre 1:32.

In the first group the trophozoites of four *G. intestinalis* strains (P-1, HP-50, HP-104, HP-105) reacted well with antiserum produced against *G. intestinalis* P-1, weakly with antiserum to *G. caviae*, and did not react with the anti-*G. cati* serum. Trophozoites of the fifth HP-10 strain reacted moderately with antisera to P-1 and GP-1 and did not react with anti- *G. cati* serum, therefore this strain was also included in the first group.

In the second group we found two strains — *G. cati* CAT-1 and *G. intestinalis* HP-103. Both strains are very closely related antigenically; the antiserum to *G. cati* absorbed with *G. intestinalis* reacted equally well with these strains isolated from a cat and from man. There was a lack of reactivity or a weak reactivity with the antiserum to *G. intestinalis*, a moderate reaction with antiserum to *G. caviae* absorbed with *G. intestinalis* trophozoites, and a lack of reactivity to this antiserum absorbed with *G. cati* trophozoites.

The third group comprised six strains — five *G. intestinalis* strains isolated from humans and one *G. caviae* strain isolated from a guinea-pig and they reacted well with the antiserum to *G. cati* absorbed with trophozoites of *G. intestinalis* P-1. Some of the strains also reacted moderately with the antiserum to *G. caviae*.

Six *G. intestinalis* strains included in the fourth group reacted moderately with the antiserum to *G. caviae*, and to some extent with the antiserum to *G. cati*, absorbed with trophozoites of *G. intestinalis* or *G. cati*. All the strains failed to react or reacted in a low degree with the antiserum to *G. intestinalis*.

The reactivity of four *G. intestinalis* strains which formed the fifth group was restricted; the strains did not react — as a rule — with any antiserum produced against *G. intestinalis*, *G. cati* or *G. caviae*, or reacted weakly with some antisera.

With two of the three antisera produced against *G. intestinalis* and *G. cati* the reactivity was highest with homologous antigens. The antiserum against *G. caviae* reacted to a higher degree with trophozoites of *G. cati* than with homologous *G. caviae*. The reactivity of strains isolated from the same patients at different times was similar (strains HP-99 and HP-100, HP-101 and HP-102) with the exception of three isolates (HP-88, HP-89 and HP-92) from one person; the HP-88 strain differed from the other two strains which were very similar.

Discussion

Smith et al. (1982) found that in spite of the overall similarity in antigens of four *G. intestinalis* (*G. lamblia*) strains from widely differing geographic regions, more clear differences in antigens reactive with rabbit anti-*G. intestinalis* serum could be revealed. On the other hand, Korman et al. (1986) found also a significant heterogeneity in *G. intestinalis* (*G. lamblia*) strains from a single region. The results of the present investigations of surface antigens by using immunofluorescence after exposure to homologous and heterologous rabbit antisera showed such differences among many human *Giardia* isolates from one geographic location. Five groups of

Giardia isolates could be distinguished. Some of our *G. intestinalis* isolates were similar to the human Portland-1 *G. intestinalis*, whereas others were identical with the CAT-1 *G. cati* strain or similar to the GP-1 *G. caviae* from guinea-pig. The remaining *G. intestinalis* human isolates did not react with any rabbit anti-Giardia serum and therefore showed unique surface antigens dissimilar to the other groups of strains.

The results of our investigations are similar to the previous findings of other authors. Nash et al. (1985) and Nash and Keister (1985) detected differences of the surface antigens among some human *Giardia* isolates and showed that these differences were reflected in differences in the DNA banding patterns. Besides the wide dissimilarity of isolates from the same host, some strains from man and animals (beaver and cat) were similar in their surface antigens and DNA banding patterns. The authors distinguished three groups of *Giardia* strains which comprised isolates both from humans and animals and concluded that mammals and man did not possess their unique species of *Giardia*. The authors believed, as did Smith et al. (1982) and Korman et al. (1986), that the antigenic differences and dissimilarities in DNA banding patterns among *Giardia* strains might contribute to the variation in host immune response and might be related to different clinical manifestation. We did not observe any correlation between the clinical manifestation in patients and strains reactivity with rabbit antiserum to different *Giardia* species. Therefore the clinical consequences of the antigenic differences observed among *Giardia* isolates from man are unclear.

Although all the antisera reacted highly with the homologous isolates (P-1, CAT-1, GP-1), some factors may be responsible for a higher reactivity of the antiserum to GP-1 with CAT-1 than with a homologous antigen. It is remarkable that the same higher reactivity of antibody to GP-1 with the excretory-secretory products of CAT-1 was found by Nash and Keister (1985). We employed the same antisera with trophozoites of all *Giardia* isolates and found the results, i.e., the similarities and differences in reactivities, reproducible. It is worthy of notice that strains isolated at different periods from the same persons (HP-89 and HP-92; HP-99 and HP-100; HP-101 and HP-102), with one exception (strain HP-88), were similar in their surface antigens. Two of the isolates from the same patient were established by using different material for inoculum preparation; the HP-101 was isolated by inoculation of trophozoites obtained by duodenal aspiration, whereas the HP-102 by inoculation of trophozoites obtained by *in vitro* excystation procedure.

Our investigation showed that some human *Giardia* strains were characterized by different surface antigens, although isolated from a population in one geographic location. On the other hand, the high similarity in the surface antigens of isolates from man and cat which we found by immunofluorescence, confirmed the opinion of other authors that these hosts did not seem to possess their own species of *Giardia* and that cat might serve as a reservoir of *Giardia* potentially infective to humans. However, the results of recent experimental cross-transmission studies concerning the *Giardia* from cat (Belosevic et al. 1984, Kirkpatrick and Farrell 1984,

Kirkpatrick and Green 1985, Woo and Paterson 1986) were contradictory and therefore the zoonotic potential of this parasite remains open to speculation (Kirkpatrick 1986).

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Morphology and Life-cycle of Two New Species of *Leptomonas*, *L. indica* sp. n. and *L. bakeri* sp. n. from Lygaeid Insects

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Synopsis. The morphology and life cycle of two new species of *Leptomonas*, *Leptomonas indica* sp. n. from the crop, mid-gut and hind-gut of the plant bug *Lygaeus hospes* Fabr. and *Leptomonas bakeri* sp. n. from the mid-gut and hind-gut of *Lygaeus militaris* Fabr. are described. *Leptomonas indica* is characterized by tadpole-shaped promastigotes measuring 18.5×2.8 μm. Length:width is 6.6:1, length of flagellum 21.6 μm, body legnth:flagellar length is 0.85:1, nuclear index 1.206, kinetoplast index 1.984, length of flagellar pocket 2.48. *Leptomonas bakeri* is characterized by elongate-fusiform promastigotes. Body measuring 20.8×26 μm, body length:width 7.9:1, length of flagellum 24.3, body length:flagellum length 0.85:1, nuclear index 1.96, kinetoplast index 3.87, length of flagellar pocket 2.49.

While extensive studies were carried out on *Leishmania* and *Trypanosoma* because of their pathogenicity, comparatively little has been done on the lower trypanosomatids, even though they are phylogenetically important. Because of their small size, the species identification has always been difficult and controversial. Species were generally identified on the basis of host-specificity, since the structural differences were found to be insufficient for establishing new species. Hoare and Wallace (1966) identified the insect trypanosomatids based on the relative position of the kinetoplast and axoneme. Both the organelle are too small to be measured under the light microscope. However, Wallace et al. (1983) suggested using the morphological parameters, such as length and width of the body, length of the free flagellum, position of the kinetoplast and the nucleus, ratio of length of the body and length of the flagellum, and the nuclear and kinetoplast indices, for specific identification of lower trypanosomatids.

Fifty eight species of *Leptomonas* Kent have so far been reported from different insect hosts, of which the life-cycles of only 7 species are described in detail. The present communication deals with the morphology and life-cycle of 2 new species of *Leptomonas*, from the gut of 2 species of plant bugs belonging to the genus *Lygaeus* (Order: *Hemiptera*). The flagellates were identified as new species based on the morphological parameters suggested by Wallace et al. (1983).

Material and Methods

Two species of *Lygaeus*, *L. hospes* Fabr. and *L. militaris* Fabr. occurred in moist areas commonly associated with green plants in the vicinity of Visakhapatnam (Andhra Pradesh, India). *L. hospes* was collected from underneath the leaves of cashew nut plantations and on *Calotropes* plants in the University Campus. *L. militaris* was collected from the moist areas with thick grass. They were found to aggregate in large numbers on used and discarded soapnut. Insects were collected during the early hours of the day, brought to the laboratory and maintained in small aquaria tanks with *Calotropes* branches with flowers. Some of the bugs were isolated and maintained in glass-jars with a moist filter paper. Eggs laid by the insects in nature as well as those maintained in the laboratory were isolated and reared separately in the laboratory.

Immediately after collection, the insects were decapitated and various parts of the body were observed under the microscope. Smears of the gut contents were prepared from natural populations as well as from the laboratory reared insects. They were either air-dried, fixed suitably and stained either with Heidenhain's iron haematoxylin or treateda ccording to Feulgen's technique or according to Walcott's (1962) method or according to methyl green pyronin-γ technique.

Leptomonas indica sp. n.

Host: *Lygaeus hospes* Fabr.
Site of infection: Crop, mid-gut and hind-gut
Locality: Andhra University campus, Waltair
Type slides: Authors' collection and Department of Zoology, Andhra University, Waltair.

Diagnosis

Promastigotes tad-pole shaped, body, 18.50×2.80 μm; length:width 6.6:1; length of the flagellum 21.6 μm; body length:flagellar length 0.85:1; nuclear index 1.206; kinetoplast index 1.984; length of the flagellar pocket 2.48 μm; parasitic in the crop, mid-gut and hind-gut of *Lygaeus hospes*.

Eighty to ninety per cent of the adult insects and nymphs were infected. Promastigotes were observed in the lumen of the crop and mid-gut showing the typical flagellary movement. Some of the forms were attached to the mid-gut epithelium often "carpetting" the epithelial cells (Fig. 1 *1*). They appear to be attached with the help of the tip of the flagellum and exhibited a swaying movement. They were 'tad-pole', shaped with a broadly rounded anterior end, and a pointed posterior end with maximum breadth in the anterior third of the body (Fig. 1 *2*). They measured 18.5×2.8 μm. L:W is 6.6:1. A vesicular nucleus with a centrally placed endosome measuring 1.46 μm in diameter was situated in the posterior third of the body. There was a rim of coarse peripheral chromatin granules adherent to the inner wall of the nuclear membrane. Kinetoplast was rounded or transversely elongated. Free flagellum arose from a circular achromatic area, a little in front of the kinetoplast. The flagellar pocket measured 2.48 μm in length. A distinct vacuole measuring 0.5–1.0 μm in diameter was present above the kinetoplast. Nuclear index varied

from 1.26–1.19. Kinetoplast index varied from 1.93–1.52. Details of the morphometric ranges with standard deviation are given in Table 1.

Amastigotes with a deeply stained nucleus and a dot-like kinetoplast, measuring 4.0–6.0×3.0–4.5 μm were observed in the lumen of the mid-gut (Fig. 1 *3*).

Resistant stages, probably of the nature of 'cysts' were observed in the crop region of the nymphs. They were elongately oval with 1–4 deeply stained nuclei measuring 4–7×1.0–2.0 μm.

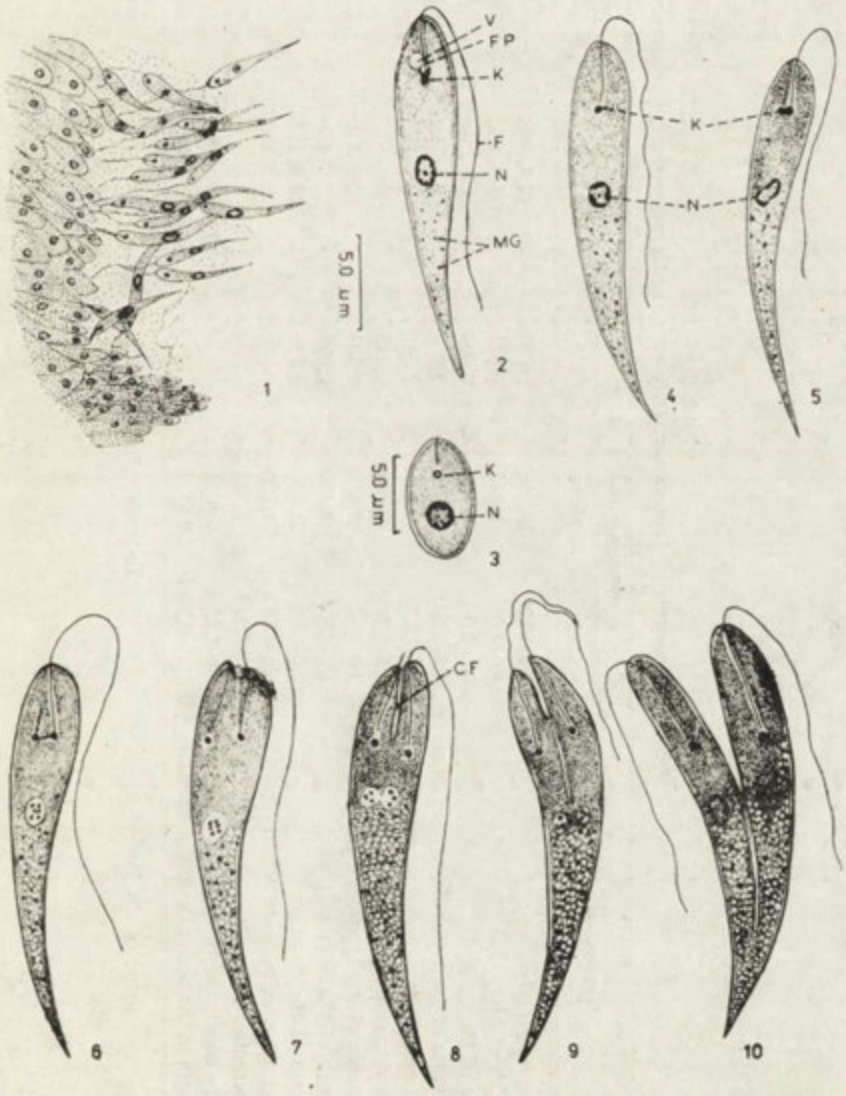

Fig. 1. *Leptomonas indica* sp. n. *1* — Section of the crop showing the promastigotes, *2* — A promastigote stained with Giemsa, *3* — Amastigote stage, *4–10* — Stages in longitudinal binary fission

Table 1
Body measurements of *Leptomonas indica* sp. n. and *Leptomonas bakeri* sp. n. (in μm)

Morphometric factor	*Leptomonas indica* sp. n.			*Leptomonas bakeri* sp. n.		
	Range	Mean	SD	Range	Mean	SD
Length of body	10.0–29.0	18.56	6.27	12.4–26.8	20.836	3.445
Width of body	1.0–4.0	2.812	0.921	1.6–3.6	2.632	0.590
Size of the nucleus	0.8–2.0	1.460	0.404	1.0×1.2–2.0×2.2	1.72×1.853	0.244×0.394
Distance of the nucleus from anterior end	4.6–13.0	8.972	3.0	4.4–9.2	7.036	1.304
Distance of the nucleus from posterior end	5.8–15.5	10.626	2.641	8.0–16.8	13.810	1.264
Distance of the kinetoplast	0.5–1.5	0.768	0.315	0.5–1.0	0.696	0.182
Distance of the kinetoplast from nucleus	3.0–10.2	5.456	2.284	2.0–5.0	3.568	0.797
Length of the flagellar pocket	1.5–3.8	2.480	0.699	2.0–4.0	2.996	0.623
Length of the flagellum	12.0–32.5	21.65	7.139	14.0–28.0	24.300	3.657
Nuclear index NI = (PN/NA)	1.26–1.192	1.206		1.818–1.826	1.962	
Kinetoplast index KI = (PN/KN)	1.93–1.519	1.984		4.0–3.36	3.87	
Body length: body width	10:1–7.25:1	6.60:1		8.0:1.0–7.44:1.0	7.91:1.0	
Length of the body: Length of flagellum	0.83:1–0.90:1	0.85:1		0.88:1.0–0.95:1.0	0.857:1.0	
Shape of the body	'Tad-pole' shaped			fusiform		

In heavily infected adults, the promastigotes were seen in the salivary glands and malphigian tubules also.

Multiplication was by symmetrogenic asexual binary fission. Initiation of division was by the kinetoplast and was followed by karyokinesis and cytokinesis. Stages of mitotic division of the nucleus, showing chromosomes were seen clearly

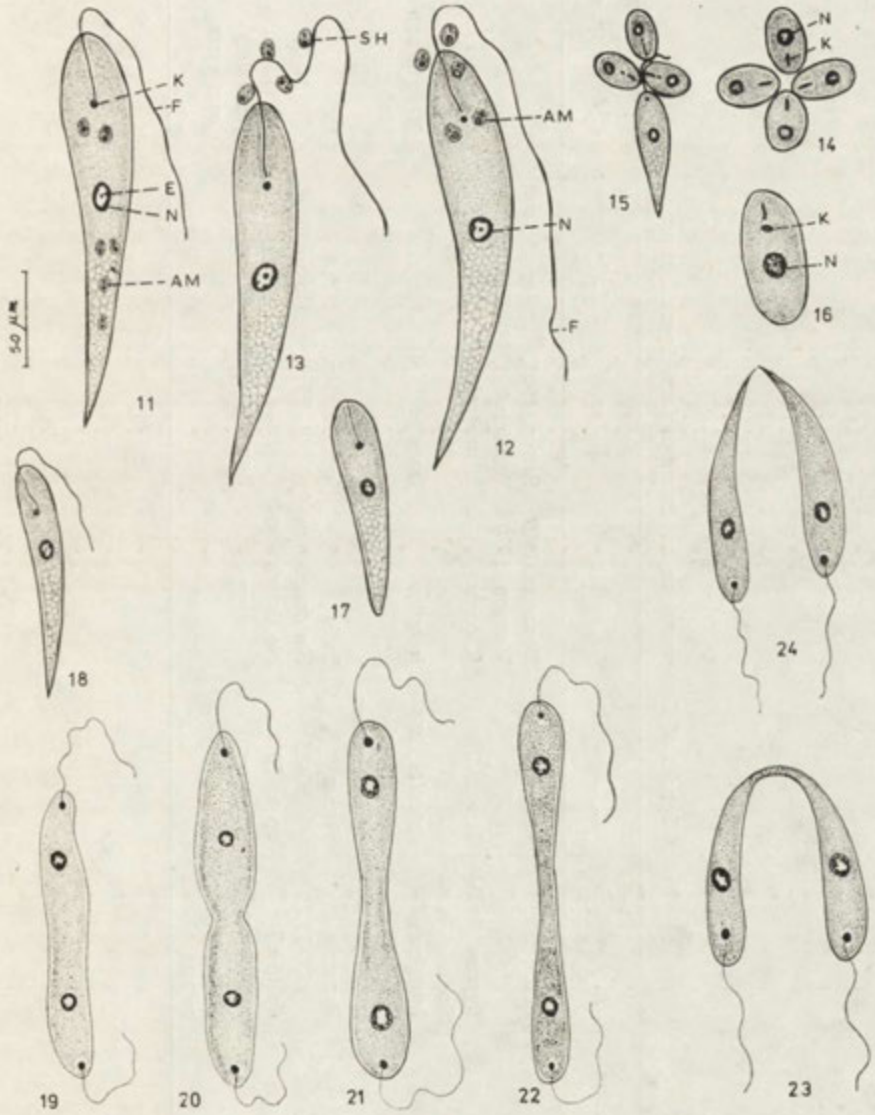

Fig. 2. *Leptomonas indica* sp. n. *11* — Promastigote showing endogenous budding, *12–13* — Promastigotes with "Strap hangers", *14–15* — Stages in the division of amastigotes, *16–18* — Amastigotes transforming into a promastigote, *19–24* — Stages in the transverse binary fission of the promastigotes

Table 2
Species of *Leptomonas* so far described from Lygaeid insects

Specification	*L. familiaris*	*L. f. saxatilis*	*L. inhospes*	*L. lygaei*	*L. lygaeorum*	*L. indica* sp. n.	*L. bakeri* sp. n.
Author	Zotta, 1923	Poisson, 1930	Donovan, 1909, Wenyon, 1926	Patton, 1908, Woodcock, 1914	Noguchi and Tilden, 1926		
Host	*Lygaeus familiaris*	*L. saxatilis*	*L. hospes*	*L. militaris*	*L. kalmii*	*L. hospes*	*L. militaris*
Location	Intestine	Intestine, haemocoel and salivary glands	Intestine	Intestine	Intestine	Mid-gut and hind-gut	Mid-gut and hind-gut
Locality	Rumania	France	India	India, Sudan	U.S.A.	India	India
Length (μm)	18.0–33.75	30–35	No description	20–25	Up to 19	10–29	12.4–26.8
Flagellum (μm)	up to 67	up to 70	No description	—	—	12–31	14.0–28.0
	Small rounded forms in rectum	—	—	Rounded cysts	—	Elongately oval cysts	Crescent-shaped bodies

in the smears stained according to Feulgen's technique. Owing to the very small size the number of chromosomes could not be counted with definiteness. The flagellum of the parent was retained by one of the daughter individuals, while the other developed a new flagellum which synchronised with nuclear division. Cytokinesis was indicated as a break in the continuity of the cytoplasm at the anterior end when the nucleus was in the late anaphase stage. The cytoplasmic furrow gradually deepened and extended towards the posterior end to form two daughter promastigotes (Fig. 1 *4–10*).

"Strap hanger" formation was observed occasionally. They were formed by the division of the kinetoplast and nucleus probably by a process of internal budding. They seem to glide forward along the parent flagellum to be set free ultimately at the tip of the flagellum (Fig. 2 *11–13*). This process of budding was observed more in the posterior region than at anterior end (Fig. 2 *11*).

Sometimes, even the amastigote stages were seen undergoing division and appeared in groups of four with their pointed ends directed to a central point (Fig. 2 *14*). One of them may first develop into a promastigote while the other three remained in the amastigote stage, with the result the three amastigotes appeared like "strap-hangers" attached to the flagellum of the promastigotes (Fig. 2 *15*). These amastigotes later got separated and developed a new flagellum (Fig. 2 *16–18*). Occasionally the promastigotes were seen undergoing binary fission along the transverse plane as described by Simpson (1968) in the promastigotes of *Leishmania tarentolae* (Fig. 2 *19–24*).

Systematic position

Of the 58 species of *Leptomonas* Kent so far reported from insect hosts, 5 species, *L. familiaris* Zotta (1923) from *Lygaeus familiaris*, *L. f. saxatilis* Poisson (1930) from *Lygaeus saxatalis*, *L. inhospes* (Donovan 1909) Wenyon (1926) from *Lygaeus hospes*, *L. lygaei* Woodcock (1914) from *Lygaeus militaris* and *L. lygaeorum* Noguchi and Tilden (1926) from *Lygaeus kalmi* are described from hemipterans belonging to the genus *Lygaeus*. Among them only 2 species, *L. inhospes* and *L. lygaei* are described from Indian sub-continent. A comparison of the morphometrics of the present form with others shows that it does not resemble anyone of the above mentioned species (Table 2). *L. inhospes* is incompletely described and as Wallace (1966) remarks "The figures show a *Leptomonas* that is indistinguishable from those described from other Lygaeids" and hence a comparison is not possible. The present form differs from *L. lygaei* Woodcock, in having a characteristic 'tad-pole' shaped body measuring 10–29 μm in length, while the promastigotes of *L. lygaei* are elongated measuring 20–25 μm. They also differ in having spherical 'cysts', while in the present form 'cysts' are oval in shape.

In view of these differences in the size and shape and since it is from a different locality and from a different species of host, it is considered new to science, for which the name *Leptomonas indica* sp. n. is proposed.

Leptomonas bakeri sp. n.

Host: *Lygaeus militaris* Fabr.
Site of infection: Mid-gut and hind-gut
Locality: Krishnanagar, Visakhapatnam, Andhra Pradesh
Type slides: Authors' collection and Department of Zoology, Andhra University, Waltair

Diagnosis

Promastigotes elongate and fusiform; body 20.8×2.6; body length:width is 7.91:1; length of the flagellum 24.3; body length:flagellar length 0.85:1; nuclear index 1.96; kinetoplast index 3.87; length of the flagellar pocket 2.99; parasitic in the mid-gut and hind-gut of *Lygaeus militaris*.

Eighty to eighty five per cent of the adult insects were infected. Smaller trophozoites, presumably the younger forms, were attached to the mid-gut epithelium (Fig. 3 *25*) while larger forms were found free in the lumen. They were elongate, straight and fusiform, with a pointed anterior end and a drawn out posterior end. They measured 20.7×2.6 μm. L:W is 7.91:1. An oval vesicular nucleus measuring 1.7×1.85 μm with a centrally placed endosome and a clear nuclear membrane, was present in the anterior third of the body. Kinetoplast was small and dot-like. Flagellar pocket measured 2.9 μm. Flagellum was anchored in a circular achromatic area in front of the kinetoplast. Nuclear index varied from 1.81–1.82. Kinetoplast index varied from 4.0–3.36 (Fig. 3 *26*). Promastigotes measuring more than 20 μm contained eosinophilic granules in the cytoplasm. Two vacuoles, one in front and the other behind the nucleus were present in the large promastigotes (Fig. 3 *27*). Infection was never found in the salivary glands and the malpighian tubules.

Amastigote stages were spherical or oval in shape measuring 6.4×3.0 μm, with a deeply stained nucleus and an oval kinetoplast. Smears prepared from crop showed various stages of transformation of amastigotes to promastigotes, with the elongation of the kinetoplast and formation of a flagellum (Fig. 3 *28–31*).

Crescent-shaped 'cysts' measuring 9.0–12.5×1.6–2.2 μm enclosing either 2 or 4 spindle-shaped bodies with an oval nucleus and a dot-like kinetoplast were observed in smears prepared from the hind-gut. The nuclear structure remained the same as in the promastigote (Fig. 3 *32–34*).

Multiplication was by symmetrogenic longitudinal binary fission. Preparatory to the division the posterior part of the promastigote showed enlargement and the nucleus was pushed closer to the kinetoplast (Fig. 3 *35*). Cytoplasm could be differentiated into an anterior hyaline region and a posterior coarsely alveolated region containing intraalveolar eosinophilic granules. The nuclear membrane at this stage was incipient and a rim of chromatin material was seen around the endosome. Meanwhile the kinetoplast divided to form the two kinetoplasts. The two daughter nuclei were separated but were still connected by two fine strands even in the telophase

stage. Subsequently a second flagellum was formed. Later cytokinesis extending from the flagellar end to the posterior and resulted in the formation of 2 daughter individuals (Fig. 3 *36–39*).

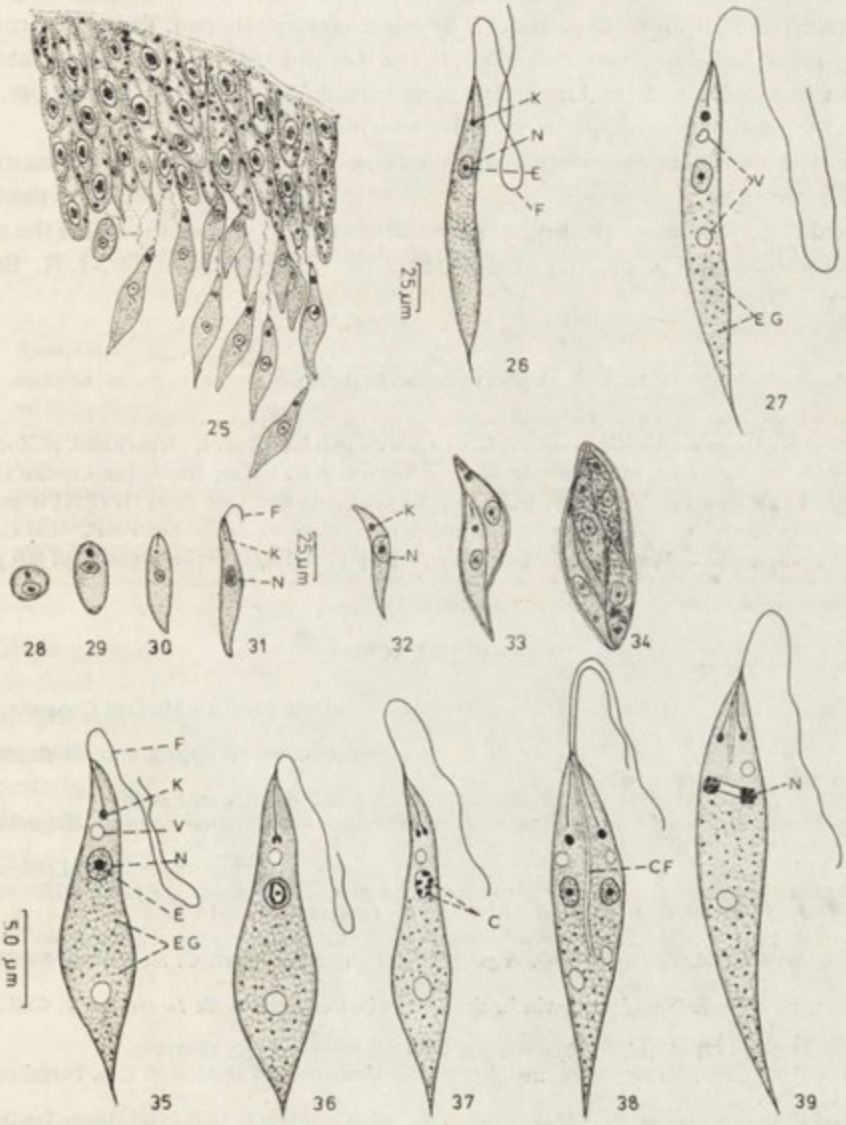

Fig. 3. *Leptomonas bakeri* sp. n. *25* — Section of the mid-gut showing the promastigotes, *26–27* — Promastigotes, *28–32* — Amastigotes changing into promastigote, *33–35* — "Cysts", *36–39* — Stages in the longitudinal binary fission

Key to the lettering: AM — amastigotes, C — chromosomes, CF — cytoplasmic furrow, E — endosome, EG — eosinophilic granules, F — free flagellum, FP — flagellar pocket, K — kinetoplast, MG — metachromatic granules, N — nucleus, SH — "strap hangers", V — vacuole

Systematic position

This form is smaller in size than all the other species described earlier from lygaeid insects (Table 2). *L. lygaei* is the only previous report from *Lygaeus militaris*, but it differs from the present form in size and shape of the body of promastigote as well as resistant stages — "cysts" (Table 2). The promastigotes of the present form have a characteristic fusiform shape and the cysts are crescent-shaped. They differ considerably from *L. indica* described earlier, in the size and shape of the body (Table 1). The nuclear index and the kinetoplast index are different in both the species. The present form shows eosinophilic granules which are absent in *L. indica*.

In view of the morphometric differences in the promastigote and amastigote stages between the two species, and since it is from a different species of the host, collected from a different locality, it is considered new to science for which the name *Leptomonas bakeri* sp. n. after the distinguished protozoologist Dr. J. R. Baker, is suggested.

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Effects of Malathion and Endosulfan on the Growth of *Paramecium aurelia*

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Synopsis. Cultures of *Paramecium aurelia* were exposed to malathion and endosulfan and the toxic effects were assessed up to 5 days. The ciliates survived up to 100 ppm of malathion but were lysed at 5 ppm of endosulfan (94% pure). The cell numbers were comparable to controls at 0.1 and 1 ppm of endosulfan and malathion respectively. At higher concentrations the insecticides significantly reduced the density of population. The toxicity was considerably lower in stabilized cultures. Both the isomers of endosulfan had similar effects and the ciliates survived at 5 ppm of 97.6% pure insecticide.

Ciliate protozoans are among the most common microorganisms at the base of aquatic food chains and are more important as nutrient regenerators, particularly of nitrogen and phosphorus. Studies on the toxicity of pesticides in these organisms are essential for accurately evaluating the potential environmental effects of these compounds. DDT was earlier reported to reduce the growth of *Tetrahymena pyriformis* and *Stylonychia notophora* (Lal and Saxena 1979, 1980). In India, after the ban of endrin and restricted use of DDT, endosulfan is being extensively used in agriculture while malathion is favoured for mosquito control. The present study is an attempt to describe the effects of malathion and endosulfan on the growth of a common freshwater ciliate protozoan, *Paramecium aurelia*.

Materials and Methods

Cultures of *Paramecium aurelia*, collected from freshwater ponds around Delhi, were maintained in sterilized hay infusion at 28±1°C. The cultures were enriched on alternate days with Horlicks' malted milk. Subculturing was done once in a week. Stock solutions of malathion (95% pure from Cyanamid India Ltd., Valsad) and endosulfan (94% pure from Bharat Pulverising Mills Ltd., Bombay) were prepared in acetone.

For toxicity studies, Paramecia (around 175 organisms) were transferred to 15 ml test tubes

containing 5 ml sterilized hay infusion, so that the initial inoculum was 35 organisms per ml. The cultures were immediately treated with malathion and endosulfan. Acetone controls were kept simultaneously. Care was taken to restrict acetone levels in treated cultures to 0.1% or below at which the solvent was found to be harmless. The cultures were fixed with equal volume of neutral formalin at regular intervals of 24 h for 5 days and cell numbers were counted in multidepression sockets (Pyrex, U.S.A.) using binocular microscope. In addition to the growth studies as described above, experiments were set up to see whether the ciliate recovers quickly from the toxic effects when higher inoculum ensued. For this stabilized cultures were exposed to the insecticides. Further, fresh cultures were also exposed to 97.6% pure endosulfan and its isomers, obtained from Environmental Protection Agency, Research Triangle Park, U.S.A., to see the difference, if any, in the toxicity levels. Observations were made only after 5 days of treatment.

Results

The growth of *Paramecium aurelia* was not altered in the presence of 1 ppm malathion and 0.1 ppm endosulfan (Table 1). The ciliate recovered from initial toxic effects at 10 and 20 ppm of malathion but the cell population was significantly ($P < 0.001$) reduced at 50 and 100 ppm throughout the incubation period. Endosulfan inhibited the growth by 60.56, 61.26 and 89.49% on day 1 at 0.5, 1.0 and 2.5. ppm respectively. The adverse effect was appreciably reduced when the ciliates entered into log phase and the population returned to control levels at 0.5 ppm. This recovery was ephemeral in nature and the inhibition again rose to 29.75, 47.68 and 66.94% on day 5 when the experiment was terminated.

Stabilized cultures of *Paramecium* were reduced by 2.95, 11.59, 18.25, 50.39 and 60.69% by malathion at 1 to 100 ppm after 5 days of treatment. The corresponding percent inhibition with endosulfan were 3.53, 2.88, 5.77 and 43.27 at 0.1, 0.5, 1.0 and 2.5 ppm respectively. The cultures survived even at 5 ppm of 97.6% pure

Table 1
Effect of malathion and endosulfan on the growth of *Paramecium aurelia*

Concentration in ppm		Cell number/ml				
		Day 1	2	3	4	5
Control		284±28	480±71	670±30	610±10	600±61
Malathion	1	272±56	440±42	580±56	600±16	530±37
	10	240±37	412±47	406±50[b]	447±50[b]	480±92
	20	206±5[b]	320±28[a]	360±28[c]	413±25[c]	455±94
	50	152±34[c]	250±50[c]	252±65[c]	253±57[c]	208±34[c]
	100	40±10[c]	40±10[c]	40±12[c]	90±10[c]	165±25[c]
Endosulfan	0.1	272±40	480±42	578±108	570±86	526±82
	0.5	112±33[b]	340±85[a]	542±26[b]	600±90	422±16[b]
	1.0	110±20[c]	306±70[a]	503±83[a]	340±28[c]	314±89[b]
	2.5	30±10[c]	120±20[c]	350±9[c]	313±52[c]	200±20[c]

a, b and c: significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$ levels respectively.

endosulfan. The toxicity was reduced by 7 to 10% at 1 and 2.5 ppm as compared to 94% pure insecticide. Both the isomers of endosulfan exerted similar effects on the cell population. The morphology of *Paramecium* was not affected at the concentrations used but lysis of cells occurred at 5 ppm of 94% pure endosulfan.

Discussion

In the present study the growth of *Paramecium aurelia* was adversely affected by malathion only at higher concentrations of 50 and 100 ppm. The cell numbers in 1 ppm treated cultures were comparable to controls. Moore (1970) reported a negligible effect of malathion on *Euglena gracilis* at 0.3 ppm while parathion at 1 ppm was found to be non-toxic to *Paramecium bursaria* and *P. multimicronucleatum* (Gregory et al. 1969). Poorman (1973) even reported stimulation of *E. gracilis* by malathion and parathion at 10 ppm or less. Thus, organophosphorus insecticides like malathion appear safe and if used at recommended doses, may not be deterimental to ciliate protozoans like *Paramecium*. On the contrary, endosulfan was found to be highly toxic as *Paramecium* could survive up to a concentration of 2.5 ppm only. The ciliate population was significantly reduced at 0.5 ppm, a concentration close to the usual field application levels. *Tetrahymena pyriformis* and *Stylonychia notophora* were reported to survive at 100 ppm of DDT (Lal and Saxena 1979, 1980). Dive et al. (1980) reported toxicity of many pesticides only at 10 ppm or above towards *Colpidium campylum*. Endosulfan was observed to exert similar toxic effects on *Tetrahymena* and blue-green algae in our laboratory. This high toxicity may be attributed to the chlorine atoms present in endosulfan besides its structural stability. Mirex, another highly chlorinated insecticide also significantly reduced the population of *Tetrahymena* at 0.09 and 0.9 μg/l (Cooley et al. 1972). The mode of action and the target sites were not identified in this study. However, DDT interference in oxidative phosphorylation in algae (Butler 1977) and in the synthesis of DNA, RNA and proteins in protozoans (Lal and Saxena 1980) was earlier documented. Geike and Parasher (1976) attributed the Hexachlorobenzene inhibition of *Tetrahymena* to the restriction of nutrient uptake while French and Roberts (1976) reported alterations in the fine structure of plasma membrane, thereby affecting various biochemical processes.

The adverse effects were found to be considerably lower in this study when stabilized cultures of *Paramecium* were exposed to the insecticides. The cell number was reduced by 66.94% at 2.5 ppm of endosulfan in fresh cultures while it was only to the extent of 43.27% in stabilized cultures at the same concentration. Thus the degree of inhibition appears not to depend on the concentration of insecticides but was related to the ratio of insecticide to the number of organisms. This is in agreement with the observation of Subbaraj and Bose (1983) who found that the inhibition of cell number, pigment content or photosynthesis in the alga *Chlorella* decreased with increased number of cells. Further, *Paramecium* survived at 5 ppm

of 97.6% pure endosulfan while 100% mortality occurred with 94% pure insecticide. This clearly shows that impurities present in the pesticides are also responsible to a certain extent in enhancing the toxic nature.

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The Influence of Non-Protein-Nitrogen on the Growth of Rumen Ciliate *Entodinium caudatum in vitro*

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Synopsis. The growth of *Entodinium caudatum* in the medium containing non-protein-nitrogen (NPN) was examined. The maintenance of ciliates on urea as a sole nitrogen source was possible when the culture medium was supplemented with powdered hay. Urea as a supplement to protein of Leguminosae plant seeds increased protozoa concentration by 12–71%, but the daily gain of ciliate number per mg N added was similar or lower than in control cultures, i.e., without the urea supplement. The protozoa counts in the medium supplemented with urea in the ratio 0.014–0.224 mgN/ml/day was not higher than 6.5×10^3/ml and showed a negative correlation with the urea level. The ciliate number in cultures receiving corresponding doses of wheat gluten varied from 10.3×10^3 to 28.0×10^3/ml. The daily gain of ciliates per mg of urea or wheat gluten-N varied from 0.70×10^3 to 18.2×10^3 and from 7.4×10^3 to 39.9×10^3 respectively. Urea supplied in the dose of 360 and 480 μg/ml/day showed a toxic effect on rumen ciliates. A correlation was also found between bacteria and ciliate number.

Non-protein-nitrogen (NPN) is commonly used for the supplementation of ruminant diet and Pilgrim with coworkers showed as early as 1970 the incorporation of ammonium sulfate-N into both bacteria and ciliate matter. Later Grędziński (1975) cultured ciliates from the genus *Entodinium* on the medium consisting of rumen fluid, urea and barley flour, reaching a population density about 10^3cells/ml. However, there is a scarcity of comparative data concerning the effect of NPN on protozoa growth. In the presented experiments we have made an attempt to estimate the influence of urea as a sole or supplementary N source, on the survival and development of the population of rumen ciliate *Entodinium caudatum*.

Material and Methods

The ciliates originated from the rumen of sheep. The population of *Entodinium caudatum* was obtained by picking up the protozoa showing typical features of this species (Dogiel 1927) from mixed cultures consisting of *Entodinium caudatum*, *Entodinium exiguum*, *Diploplastron affine*

and *Polyplastron multivesiculatum*. Fifty to a hundred cells were picked and introduced to the Erlenmeyer flask containing culture medium (40 ml) with a well developed bacteria population. The culture medium consisted of "*caudatum* type" salt solution (see below), ground hay, soya protein and barley flour. The protozoa were cultured by the routine method. When the population reached a density of about 10^3 cells/ml, it was transferred into a continuous culture apparatus (Michałowski 1980) and cultured for the duration of the experiments described in this paper. The samples of this material were used for inoculations of experimental cultures.

All cultures were maintained on the "*caudatum* type" salt solution (Coleman et al. 1972) consisting of (g/l): K_2HPO_4 – 6.3; KH_2PO_4 – 5.0; NaCl – 0.60; $MgCl_2 \times 7\,H_2O$ – 0.09; $CaCl_2 \times 6H_2O$ – 0.09; and CH_3COONa – 0.75.

Experiment 1. The possibility of maintaining the ciliates on a medium containing non-protein-nitrogen as a sole nitrogen source was studied. Ammonium carbonate, ammonium sulfate or urea (Polskie Odczynniki Chemiczne, Gliwice) was supplied in the dose of 25 μg/ml/day. Pectins, cellulose powder (Koch Light Lab.) and pure barley starch were given alone or mixed in equal proportions and were offered as energetic component of the medium. They were supplied in the dose of 0.75 mg/ml/day. The control cultures received a diet consisting of ground hay, soya protein and barley flour at the ratio (w/w) of 1:1:1. The daily ration of this mixture was 0.75 mg/ml/day. Every 4 days the culture medium was supplemented with rumen fluid in the ratio of 10% of the volume of culture medium. It was added to fresh salt solution immediately before introducing the protozoa (see cultivation methods). The rumen fluid was taken from the rumen of sheep 4 h after feeding, filtered through filter paper and sterilized twice in the Koch apparatus. Pure barley starch was obtained according to Whelan (1955). In the next culture set different doses of urea, from 1.8 to 240 μg/ml, were used as N source.

Experiment 2. The survival of ciliates in the medium containing urea and supplemented with powdered hay was examined. The daily ration of urea was 30, 60, 90, 120, 180 and 240 μg/ml culture medium. Both the mixture of carbohydrates (pectins, cellulose powder and barley starch) and the powdered hay were supplied in the dose of 0.75 mg/ml/day. The control cultures received only hay and the mixture of carbohydrates. The experiment lasted 30 days.

Experiment 3. The effect of urea addition to the medium containing plant protein on the protozoa number was examined. Urea was supplied in the ratio of 45 μg/ml/day and provided 50% N of the medium. Other components of the diet were: powdered hay (1 mg/ml/day), flour from the seeds of bean, pea, field pea, vetch and lupin and pure starch from these seeds. The flour was given in a dose providing 12.5 μg of protein/ml/day and pure starch up to the total content of this constituent of 0.25 mg/ml/day. The control cultures were run without urea. The protein content of the flour used was determined according to Lowry et al. (1951) and starch according to Hascid and Neufeld (1964). Pure starch from the used seeds was obtained according to Whelan (1955).

Experiment 4. The growth of *Entodinium caudatum* in the medium containing urea or wheat gluten as N source was studied. Urea was supplied in the ratio of 30, 90, 240, 360 and 480 μg and wheat gluten respectively of 75, 225, 600, 925 and 1252 μg/ml/day. The N content of the corresponding doses of urea and wheat gluten was 0.56, 1.68, 4.48, 6.72 and 8.96 mg per 40 ml of the culture volume. Other components of the diet, i.e., hay and carbohydrate mixture, were supplied in the same dose as in experiment 2. The control cultures received only hay and carbohydrate mixture. The experiment lasted 30 days. The doses of wheat gluten were calculated on the basis of its amino-acid analysis. The samples of protein were hydrolysed in 6 N HCl at 107°C for 16 h and analysed in Durrum atumatic amino acid analyser. Ammonia-N and amino acid-N were then added to each other and used for these calculations.

Each culture was initiated by introducing 20 ml of the suspension of protozoa into a flask containing 20 ml of the appropriate medium. Three cultures were run on each kind of medium. The cultivation was performed by the routine method used in this laboratory (Michałowski 1975). The food was given every day. Every fourth day a half of the culture volume (20 ml) was transferred into another flask containing 20 ml of fresh medium.

The samples for protozoa and bacteria counts as well as for ammonia estimation were taken every fourth day from the material remaining after culture transfer. The ciliates were counted in the samples of 0.1 ml volume and bacteria – in Thoma counting chamber. Ammonia was estimated by the colorimetric method of Chaney and Marbach (1962). All analyses were three times repeated.

The obtained results were statistically analysed by calculating mean values with their standard deviations. The differences between the mean values were analysed statistically using Student *t*-test. The correlation between obtained results was also examined. All statistical calculations were performed according to Ruszczyc (1970).

Results

The ciliates disappeared gradually from the cultures receiving pure carbohydrates and either urea, ammonium carbonate or ammonium sulfate. The longest survival of protozoa (20 days) was noted in the cultures receiving a mixture of all three carbohydrates and NPN. In the medium containing single carbohydrates the ciliates survived no longer than 8 days. The protozoa also disappeared gradually from the cultures receiving carbohydrate mixture and different doses of urea. They survived up to the 16th day in a medium supplemented with 1.2 and 2.4 mg urea/day i.e. 30 and 60 μg/ml/day. In the control cultures the ciliates were cultured over a year in a concentration above 10^3/ml.

The enrichment of culture medium with ground hay provided appropriate conditions for the survival of protozoa, except these containing the smallest dose of urea where protozoa disappeared after 24 days of cultivation. A permanent decline of the ciliate number in these cultures was observed. This concerns also the control cultures in which protozoa died after 12 days of cultivation. The mean values characterizing the ciliates and bacteria in other cultures of this experiment are presented in Table 1. A significant difference was found only between mean counts of thread-like colonies of bacteria in the medium supplemented with 60 and 240 μg urea/ml/day.

The concentration of *Entodinium caudatum* in the cultures on proteins from Leguminosae seeds varied from 0.1×10^3/ml to 11.1×10^3/ml in relation to the day, the kind of protein and the urea supply. The mean values are presented in Table 2. The urea supplement caused an increase in ciliate concentration by 12–71%. The

Table 1

The number of *Entodinium caudatum* ($\times10^3$/ml), single-cell bacteria ($\times10^7$/ml) and thread-like colonies of bacteria ($\times10^4$/ml) in cultures supplemented with different doses of urea

Microorganisms	Urea doses (μg/ml/day)				
	60	90	120	180	240
Ciliates	4.1±2.10	6.1±3.71	5.9±2.08	6.3±1.46	3.0±2.01
Single-cell bacteria	9.2±1.60	10.4±2.20	10.4±2.21	10.5±2.34	10.9±1.73
Thread-like colonies	27.0±6.44	22.7±6.15	17.0±6.02	21.2±6.30	14.4±5.22

Table 2

The concentration ($\times 10^3$/ml) and gain of cells ($\times 10^3$/mg N/day) of *Entodinium caudatum* in cultures on proteins from Leguminosae seeds (control) supplemented with urea (experimental)

Protein from:	Concentration		Daily gain	
	control	experimental	control	experimental
Bean	2.1±0.93	3.6±1.63	3.1±2.91	3.2±1.86
Pea	1.8±1.08	2.8±1.51	2.6±2.21	2.3±1.38
Field pea	2.5±1.38	3.0±1.21	3.1±1.82	2.5±1.23
Vetch	2.2±2.67	3.2±1.46	2.5±3.95	2.6±1.19
Lupin	3.2±1.76	3.6±1.46	4.0±1.43	2.8±1.43

increment was the highest in the cultures on bean proteins and the lowest in these on lupin. The daily gain of ciliate cells in the cultures with urea was, however, similar or lower than in the control cultures. Significant differences were not found due to high day-to-day variations in both the protozoa number and their daily gain. They were especially high in the cultures on vetch proteins.

The number of protozoa decreased with the increase of urea dose ($P < 0.01$) and was 2 to 13 times lower ($P < 0.01$) than on the corresponding doses of wheat gluten (Table 3). They died within 12–16 days in the cultures supplemented with the two highest doses of urea. The mean concentration of ciliates in the cultures supplemented with 925 μg wheat gluten/ml/day was 24.1×10^3/ml and in these on 1225 μg of wheat gluten — 22.6×10^3/ml. Ammonia concentration in these cultures, measured every fourth day, was 140±49.4 and 138.6±41.0 mg NH_3-N/l, while in those supplemented with two highest urea doses — 144.2±47.6 and 107.8±36.8 mg of NH_3-N/l respectively. The number of bacteria in the cultures supplemented

Table 3

The number of *Entodinium caudatum* ($\times 10^3$/ml) single-bacteria cells ($\times 10^7$/ml) and thread-like colonies of bacteria ($\times 10^4$/ml) as well as daily gain of ciliate cells ($\times 10^3$/mg of urea or wheat gluten-N) and ammonia concentration (mg NH_3-N/l) in cultures supplemented with corresponding doses of urea (A) or wheat gluten (B)

Item	Urea or wheat gluten-N in daily ration (mg)					
	0.56		1.68		4.48	
	A	B	A	B	A	B
Ciliates	6.5±2.61	14.4± 6.41	5.1± 1.41	10.3± 3,76	2.2± 1.41	28.0±10.20
Single-bacteria cells	6.5±3.21	16.4± 7.07	4.6± 2.10	12.2± 7.49	3.4± 1.72	33.3±14.41
Thread-like colonies	7.1±1.36	16.7± 8.18	4.1± 1.71	8.2± 3.52	2.0± 0.85	29.2±11.16
Daily gain of ciliates	18.2±6.31	39.9±13.12	4.4± 0.98	7.3± 3.49	0.7± 0.41	9.1± 3.70
Ammonia concentration	11.6±2.66	8.0± 3.50	44.8±21.28	43.1±24.36	123.2±61.86	78.4±34.90

with urea was also lower than in these receiving wheat gluten. A significant difference, however, was found only between the bacteria on the highest urea and wheat gluten doses ($P < 0.01$). The correlation between bacteria and protozoa number was found in both kinds of cultures ($P < 0.01$). The data concerning the daily gain of protozoa cells per mg of urea or wheat gluten N offered, as well as the ammonia concentration, are also given in Table 3.

Discussion

The rumen ciliates require different factors for their growth. The lack or insufficiency of some of these factors was probably the cause of a gradual decline of *Entodinium caudatum* in the cultures reared in the medium composed of salt solution (Coleman et al. 1972), purified carbohydrates and either urea or ammonium sulfate or ammonium carbonate. The obtained data suggest that neither bacteria nor rumen fluid added every fourth day in the ratio of 10% of culture medium volume were not able to provide these factors and that they occurred probably in hay. It is well known that hay may contain components like choline or sitosterol and such substances seem to be the growth factors for rumen ciliates. (Hino and Kametaka 1975, Broad and Dowson 1976). On the other hand, hay was also a sole source of protein. *Entodinium caudatum*, however, is not cellulolytic (Hungate 1966) and these protozoa were not observed to contain any green plant particles in their endoplasmic sacs. Hence hay protein could be inaccessible or only partially accessible for these ciliates, since the cellulose walls of plant cells surrounded the protoplasts containing protein. It was perhaps the cause whey the addition of nitrogen source other than hay was necessery in the present experiments. The obtained results suggested that such supplementation could be made with urea if supplied in appropriate dose. That dose in the present experiments was 60–240 μg/ml/day.

Urea added to the medium containing Leguminosae plant proteins increased the population density, but the daily gain of ciliates per mg N added tended to be higher in the control cultures. Both the concentration and daily gain of protozoa cells in the cultures supplemented with urea was also lower than in those receiving corresponding doses of wheat gluten. The obtained results showed that the medium with protein made better conditions for the growth of *Entodinium cuadatum* than the one with urea. It is well known that ciliates *Entodinium caudatum* are not ureolytic (Abou Akkada and Howard 1962) and thus they could utilize the urea N for the growth processes only by the uptake and digestion of bacteria which were able to grow in the medium with urea as a sole N source. In such conditions the density of ciliate population was usually lower than when there was a possibility of direct utilization of dietary N by ciliates (Muszyński et al. 1985). The lower bacteria number in the cultures supplemented with urea (Table 3) may be a consequence of an increased bacteria ingestion by ciliates due to the lack of accessible protein. But it cannot be excluded that the composition of the medium made impossible the

growth of some bacteria strains which needed N sources other than urea. Thus the low protozoa concentration in such cultures could also be a consequences of the restricted growth of bacteria.

In these experiments there was a distinct negative correlation between the urea dose and ciliate number (Table 3). Moreover, the two highest doses caused a rapid death of the cultures. It seems that the reasons of such phenomenon are not related to the ammonia level, because its concentration in the medium with corresponding doses of wheat gluten was similar. Notwithstanding, the protozoa grew well in such medium and their concentration was over 20×10^3/ml. Perhaps urea has a toxic or inhibitory effect if it is supplied in high doses. This problem needs, however, further investigation.

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The Volatile Fatty Acids Production by Ciliate Protozoa in the Rumen of Sheep

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Synopsis. The total microbial and protozoal production of volatile fatty acids (VFA) in the rumen of three sheep was measured using "zero-time" method. The animals were fed 300 g hay and 350 g concentrate every 12 h.

The protozoal production rate of VFA varied from 6.9 to 32.0, while total microbial from 18.9 to 70.7 μmol/g of rumen content/h. The production reached a maximum about 1 h after giving the food and was at its minimum immediately before feeding. A significant correlation was found between the production and concentration of volatile fatty acids in the rumen ($P < 0.01$).

All ciliates in the rumen produced 0.83–1.07 mol of VFA within 12 h, while the total microbial production was 2.32–2.91 mol. About 70–80% of butyrate, 29–41% of acetate and only 9–12% of propionate in the rumen was of protozoal origin.

There was no relationship between the total number of ciliates and the protozoal production of VFA, while the correlation between this production and both protozoal DM and protein was significant ($P < 0.001$).

A significant correlation was also found between protozoal production of volatile fatty acids and the number, DM and protein middle-sized ($P < 0.01$) and large ($P < 0.05$) protozoa.

The rumen microorganisms are either obligatory or facultative anaerobes which release to the rumen liquid H_2, CO_2 and volatile fatty acids (VFA) as the end products of their carbohydrate metabolism.

The production of volatile fatty acids in the rumen was examined both in sheep (Leng and Leonard 1965, Weston and Hogan 1968, Van der Walt and Briel 1976, Van der Walt 1977, 1978 nad others) and in cattle (Whitelaw et al. 1970, Chaturevedi et al. 1973, Rogers and Davis 1982). The production measured was a result of activity of both bacteria and protozoa. The protozoa formed a great part of microbial biomass in the rumen and 30–50% of the metabolic activity was of protozoal origin (Hungate 1966). These data suggest that the contribution of the ciliates to the formation of certain metabolites, including VFA, is high. The studies of Nuzback et al. (1983) on the proportions of bacterial and protozoal

ATP in the rumen provided an indirect evidence supporting such assumption. A direct examination, however, was not undertaken.

The aim of the present investigation was to try to determine the contribution of protozoa to the volatile fatty acids production in the rumen.

Material and Methods

The experiments were performed on rumen microorganisms of three 1 year old sheep weighing 30–40 kg. The animals were equipped with permanent rumen fistulae and large abomasum fistulae, which were used in another experiments (Michałowski et al. 1986). The sheep were kept in separate boxes and fed 300 g of hay (95 g CP/kg) and 350 g of concentrate (185 g CP/kg) every 12 h. The daily dose of digestible energy was approximately 14.7 MJ. Water was available all the time.

The "zero-time" method of Carroll and Hungate (1954) was used for the determination of VFA production. The total microbial and protozoal production was measured. The portions of rumen content, about 200 g, were taken immediately before feeding and then 30 min, 1, 2, 3, 5, 8 and 12 h after giving the food. The content taken was precisely mixed and four samples (20 g each) were then weighed. Two of them were used for the determination of total microbial production, while two others – to determine the production by ciliates. The rest of rumen content was returned to the rumen. All the samples were incubated for 30 min in glass tubes sealed with rubber stoppers and placed in a water bath at 38°C. The mixing of the tube content and anaerobic conditions were provided by the constant flow of CO_2, of about 50–60 cm^3/min.

The estimation of protozoal production of VFA was carried out simultaneously with the determination of total production. The samples of rumen content for determination the protozoal production were centrifuged five times at 1000 rpm for 2 min and washed with warm "*caudatum* type" salt solution (Coleman et al. 1972) to separate the ciliates from bacteria. After the last washing the pellet containing protozoa was resuspended in the bacteria-free rumen fluid warmed to 38°C. The volume of the added rumen fluid was the same as the volume of the fluid removed from the tubes after the first centrifugation of the samples (see above). The rumen fluid used for resuspending the protzoa was taken from the rumen of the same animals 1–2 days before the fermentation experiments. Portions of rumen fluid of about 100 ml were taken from the rumen at the same time-points as the incubation began. They were centrifuged at 36 000 g for 30 min. The deposit was discharged and supernatant was stored at −20°C. It was warmed to 38°C immediately before incubation and chloramphenicol (50 μg/ml) was added for preventing any bacterial growth. Six fermentation experiments were performed on the sheep No. 2 and three experiments on sheep No. 4 and No. 5.

The VFA production was calculated on the basis of an increment in acid concentration during the incubation period. The samples for volatile fatty acids determination (2 g) were taken from incubation tubes immediately before beginning of incubation and at 15 and 30 min of incubation period. They were introduced into plastic tubes immersed in a water-ice bath and fixed with 25% HCl according to Ranft (1973). The tubes were tightly sealed and kept in a refrigerator for at least 30 min and then centrifuged at 36 000 g for 30 min. The pellet was discharged and the supernatant was used for VFA estimation.

The samples for protozoa counts were taken from each tube after incubation. The samples of rumen content for the determination of the number of ciliates were also taken. All samples were fixed with 4% formaldehyde solution.

The separation and quantitative determination of particular acids was performed using gaschromatograph according to Ranft (1973). Any sample was three times analysed.

The ciliates were counted under light microscope using Fuchs-Rosenthal counting chamber. Every sample was six times counted.

Both the total microbial and the protozoal production of volatile fatty acids were calculated on the basis of the production of acetate, propionate and butyrate. Isobutyrate, valerate and isovalerate were not used for these calculations because of their high measure error. The concentration of these three acids did not exceed, however, 2% of the total volatile fatty acids in the rumen. For the calculation of production in the whole 12 h period, the production at subsequent time-points was plotted against time and the curve was drawn by the interpolation. The area between the coordinates of the plot and the production curve was measured using a planimeter. The resulting integer was equal to a 12 h production per 1 g of rumen content. Finally this value was multiplied by an average weight of rumen content. The data related to the weight of rumen content of three sheep were published elsewhere (Michałowski et al. 1986).

The DM content of the protozoa was estimated by multiplying DM of a single ciliate cell by the number of ciliates. The mean content of dry matter in the single cell of small, middle-sized and large protozoa was 4.8±1.10, 47.2±16.52 and 401.8±83.4 ng respectively. The protozoal protein was calculated by multiplying their N by factor 6.25. The N content of small, middle-sized and large ciliates was 9.6±1.14, 9.4±0.87 and 8.6±0.68% of their DM respectively. The procedure of the protozoal groups separation and estimation of their DM and N contents will be described in another paper.

Mean values with their standard deviations were calculated from the obtained results. The mean values were also used for correlation coefficient calculations. The correlations between production and concentration of volatile fatty acids in the rumen, between protozoal production of VFA and ciliate number, protozoal DM and protozoal protein were calculated. The relationships found to be significant were described using the regression equations. The *t* test was used for the comparison of mean values. All statistical calculations were made according to Ruszczyc (1970).

Results

The ciliate fauna of the sheep consisted of many species and entodinia were the most numerous (Table 1). The concentration of ciliates of the particular species from the genus *Entodinium* was not determined. The small ciliates i.e., *Entodinium*

Table 1
Characteristics of protozoal population, protozoal DM and protozoal N in the rumen of three sheep during fermentation experiments

Item	Sheep No. 2	Sheep No. 4	Sheep No. 5
Total protozoa counts ($\times 10^3$/g)	1320.0±518.73	685.5±63.91	1000.4±29.78
Entodinium sp. (%)	86.2±11.22	93.1± 0.81	92.1± 0.93
Diplodinium sp. (%)	8.9± 4.21	4.7± 0.68	5.9± 0.35
Ophryoscolex caudatus (%)	0.4± 0.13	0.2± 0.07	0.0
Dasytricha ruminantium (%)	4.0± 3.47	1.3± 0.09	1.8± 0.29
Isotricha sp. (%)	0.5± 0.05	0.7± 0.02	0.4± 0.10
Protozoal DM mg/g rumen content	15.6± 0.88	8.1± 0.71	10.1± 1.42
small protozoa (%)	37.3± 17.90	39.0± 2.47	45.1± 5.35
middle-sized protozoa (%)	28.1± 2.56	18.2± 2.91	26.2± 1.44
large protozoa (%)	34.6± 14.90	42.8± 2.20	28.6± 6.82
Protozoal N mg/g rumen content	1.4± 0.08	0.7± 0.06	1.0± 0.17
small protozoa (%)	38.6± 18.05	41.1± 2.20	45.6± 6.87
middle-sized protozoa (%)	28.8± 3.03	18.5± 2.21	26.2± 2.48
large protozoa (%)	32.6± 15.6	40.4± 2.15	28.2± 9.40

exiguum, *Entodinium nanellum*, *Entodinium simplex* and *Entodinium minimum* prevailed, however, quite frequently over other entodinia. The *Diplodinium* group consisted of *Anoplodinium denticulatum*, *Diploplastron affine*, *Ostracodinium triloricatum* and *Polyplastron multivesiculatum* with a visible preponderance of the second, while the concentration of *Polyplastron multivesiculatum* was only 0.2–0.4% of the total protozoa counts.

The small ciliates i.e., *Dasytricha ruminantium* and entodinia formed over 90% of the total protozoa counts, but their mass (DM) was about the mass of large ciliates, i.e., *Polyplastron multivesiculatum*, *Ophryoscolex caudatus* and *Isotricha* sp., whose number never exceeded 1.5% of the total counts. The similar proportions were found for the participation of particular groups in protozoal N (Table 1).

The rate of volatile fatty acids production by the protozoa varied from 6.9 to 32.0 and the total microbial production — from 18.9 to 70.7 μmol/g of rumen content/h depending on the animal and the time after feeding. Mean values are shown in Fig. 1. The increase in VFA production (total microbial and protozoal) was

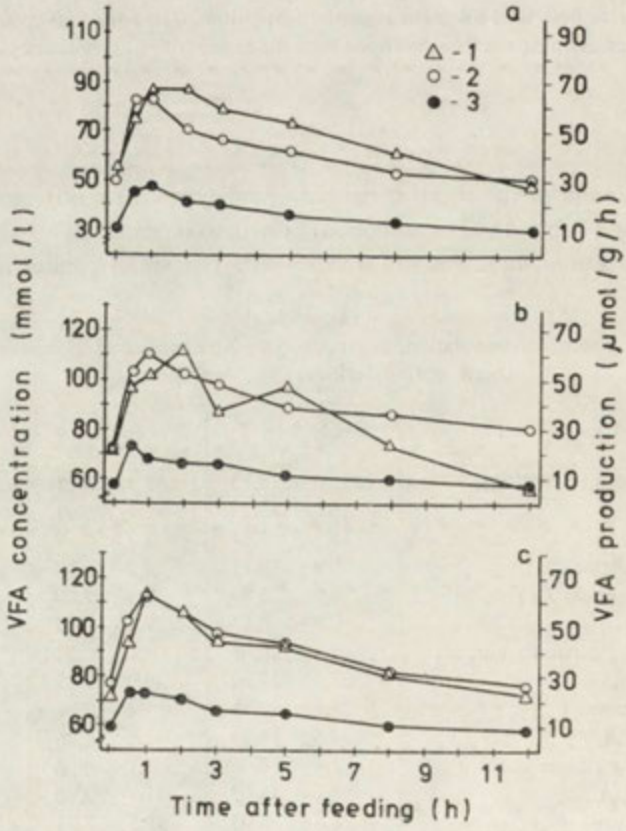

Fig. 1. Changes in the concentration of volatile fatty acids (1), total microbial (2) and protozoal (3) production rate of VFA in the rumen of sheep No. 2 (a), sheep No. 4 (b) and sheep No. 5 (c)

observed within 1 to 2 h after feeding ($P < 0.01$) and it was followed by a gradual decrease. These changes were accompanied by the changes in volatile fatty concentration. A significant correlation ($P < 0.01$) between the production and concentration of VFA in the rumen was found (Fig. 2).

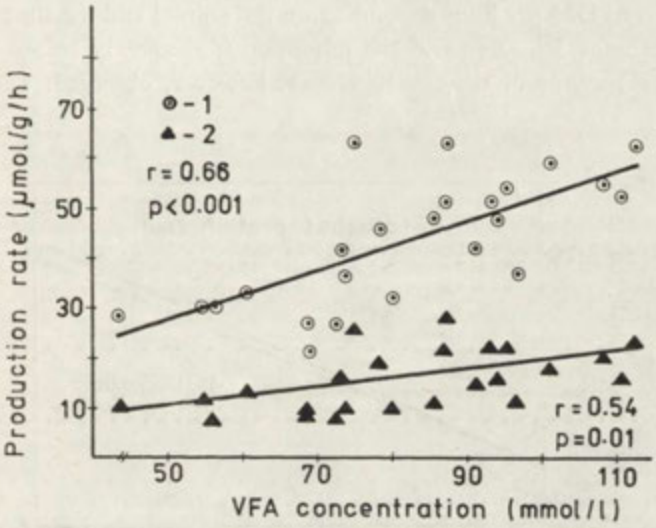

Fig. 2. Straight-line relationship between concentration of volatile fatty acids and total microbial (1) and protozoal (2) production rate of VFA in the rumen of sheep

The ciliates from the whole rumen contents produced from 0.75 to 1.17 moles of VFA. The protozoal volatile fatty acids formed 30–46% of the total acids produced in the rumen. The mean results, with the proportions of individual acids, are presented in Table 2. The total microbial production of acetate was significantly higher than the production of propionate and butyrate ($P < 0.001$), but a significant difference between the production of the last two acids was not found ($P > 0.05$). In contrast, the protozoal production of acetate was higher than the production of butyrate only in the case of sheep No. 2. The protozoal production of propionate was significantly lower than that of acetate and butyrate ($P < 0.001$).

Table 2

Protozoal and total microbial production of volatile fatty acids (mol/12 h) and molar proportion of particular acids in the total production (%)

Item	Sheep No. 2		Sheep No. 4		Sheep No. 5	
	Protozoal	Microbial	Protozoal	Microbial	Protozoal	Microbial
Total production	1.07±0.10	2.59±0.13	0.94±0.07	2.91±0.23	0.83±0.07	2.32±0.03
Acetic acid	56.3 ±5.06	56.8 ±4.98	51.3 ±8.56	57.3 ±4.15	51.1 ±5.96	58.2 ±4.93
Propionic acid	6.4 ±1.92	22.3 ±4.26	6.6 ±1.57	23.1 ±3.01	7.1 ±1.65	21.0 ±3.97
Butyric acid	40.0 ±5.02	20.9± 3.05	42.1 ±8.69	19.6 ±3.20	41.8 ±6.29	20.8 ±3.93

The propionate of protozoal origin formed only 9–12% of propionic acid produced in the rumen in contrast to 70–80% butyrate and 29–41% acetate.

There was no correlation between the protozoal production of volatile fatty acids and total number of ciliates (Table 3). The significant correlation was, however, found between this production and protozoal DM, as well as protozoal N content in the rumen (Fig. 3, Table 3). About 0.75–2.6 mmol of VFA was produced per 1 g protozoal DM per hour depending on the animal and the time after feeding, with the maximum 3 h after and the minimum immediately before feeding ($P < 0.01$). Large variations of these values were, however, observed. There were also

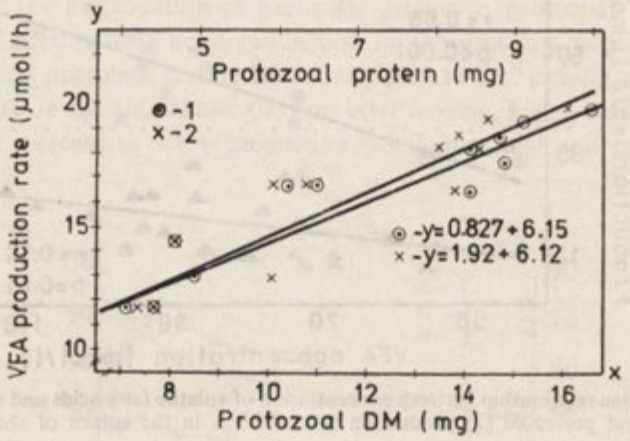

Fig. 3. Straight-line correlation between protozoal production of volatile fatty acids and protozoal DM (1) as well as protozoal protein level (2) in the rumen content of sheep

Table 3

The probability of correlation between protozoal production of volatile fatty acids and some protozoal parameters in the rumen content

Correlated parameters	Correlation coefficient (r)	Degrees of freedom	The level of statistical significance
Total protozoa counts vs. VFA production	0.40	10	n.s.
Small protozoa counts vs. „ „	0.35	10	n.s.
Middle-sized protozoa counts vs. „ „	0.89	10	0.01
Large protozoa counts vs. „ „	0.62	10	0.05
Total protozoal DM vs. „ „	0.94	10	0.001
Small protozoa DM vs. „ „	0.40	10	n.s.
Middle-sized protozoa DM vs. „ „	0.90	10	0.01
Large protozoa DM vs. „ „	0.63	10	0.05
Total protozoal protein vs. „ „	0.92	10	0.001
Small protozoa protein vs. „ „	0.35	10	n.s.
Middle-sized protozoa protein vs. „ „	0.90	10	0.01
Large protozoa protein vs. „ „	0.63	10	0.05

Table 4

Protozoal production rate of volatile fatty acids at different time after feeding (hour) expressed as mmol VFA/g protozoal DM (A) and as mmol VFA/g protozoal protein/h (B)

Time after feeding	Sheep No. 2		Sheep No. 4		Sheep No. 5	
	A	B	A	B	A	B
Before	0.80±0.21	1.15±0.14	0.85±0.41	1.50±0.50	0.86±0.07	1.45±0.11
0.5	1.69±0.42	2.58±0.50	2.09±0.60	3.64±1.03	2.09±0.54	3.58±0.92
1	1.72±0.63	2.57±0.53	2.46±0.26	4.01±0.51	1.88±0.36	3.20±0.58
2	1.71±0.62	2.48±0.43	1.91±0.81	3.38±2.98	1.82±0.41	3.09±0.69
3	1.83±0.62	2.82±1.01	2.61±0.47	4.04±2.04	2.40±0.34	4.08±0.63
5	1.49±0.36	2.25±0.60	2.09±0.31	3.51±0.51	2.24±1.38	3.74±0.77
8	1.16±0.32	1.69±0.26	1.68±0.48	2.80±0.41	1.30±0.03	2.18±0.03
12	0.96±0.32	1.45±0.34	1.00±0.14	1.71±0.17	0.75±0.07	1.27±0.12

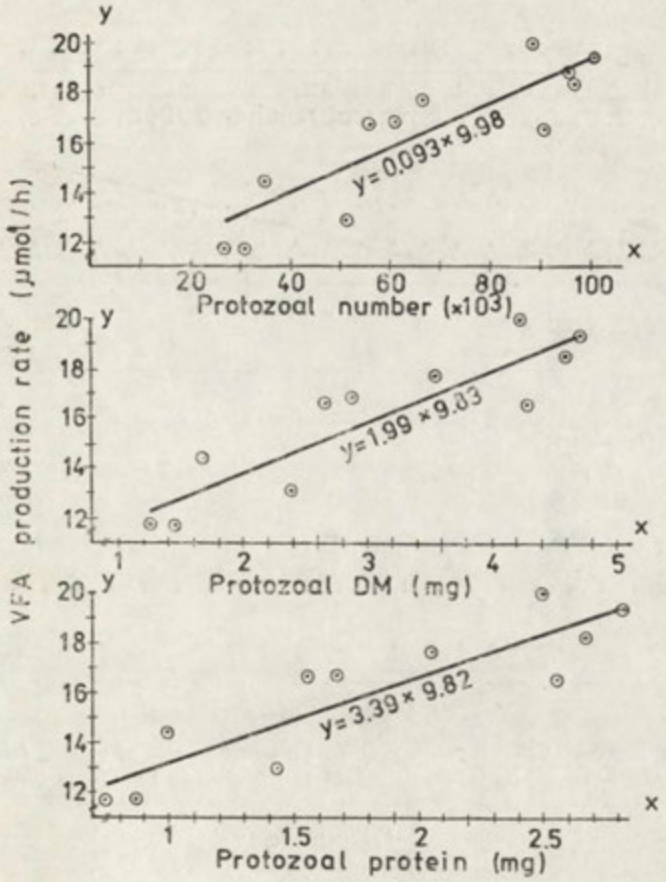

Fig. 4. Straight-line correlation between protozoal production rate of volatile fatty acids and the number of "middle-sized" protozoa, their DM and protein in the rumen content

large variations in production expressed in mmol of VFA per 1 g of ciliate protein per hour (Table 4).

No correlation was found between the protozoal production of volatile fatty acids and the number of small protozoa forming over 90% of total protozoa counts, as well as between this production and DM and N of these ciliates. The positive correlation between protozoal production of VFA and number, DM and N of middle-sized (Fig. 4) as well as of large ciliates (Fig. 5) was found. In the case of the last group the correlation coefficient was significant only at 5% of probability level.

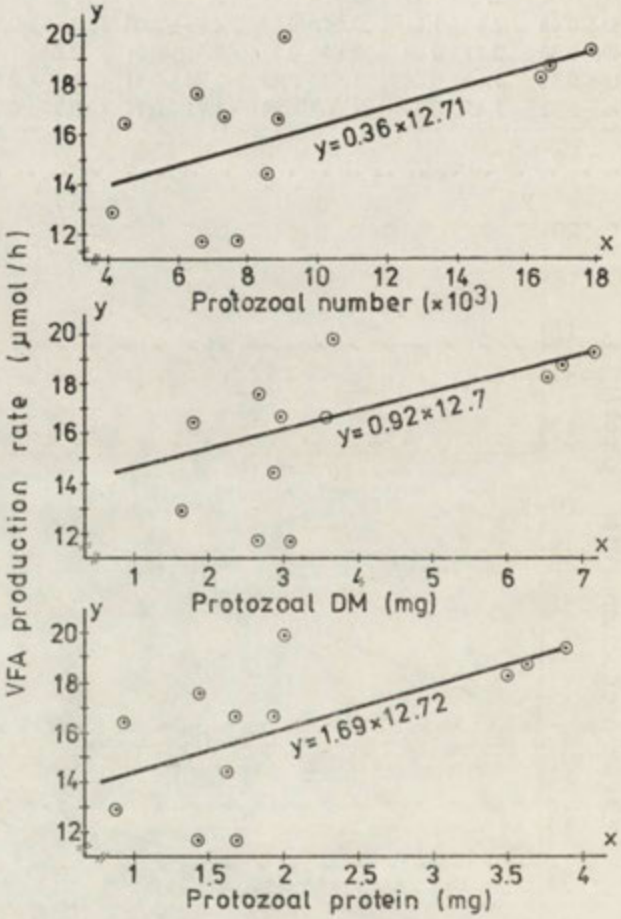

Fig. 5. Straight-line correlation between protozoal production rate of volatile fatty acids and the number of "large" protozoa, their DM and protein in the rumen content

Discussion

The results presented here provide some information as to the quantitative and qualitative production of volatile fatty acids by the ciliates in the rumen. The experiments were carried out using the "zero-time" method of Carroll and Hungate

(1954). Recently the izotopic method have been preferred. For example, Van der Walt and Briel (1976), Van der Walt (1977, 1978) used such method. The total microbial production of VFA in these experiments varied in the majority of cases between 2 and 4 mol/12 h. Similar results were found in the presented studies. The relationship between the concentration of VFA in the rumen fluid and their production rate was also similar. For the calculation of VFA production in the whole rumen, the average weight of rumen content was used. Such method of calculation was proposed by Morant et al. (1978) for non-steady-state conditions as the method giving results comparable to the true values.

The protozoa produced mainly acetate and butyrate. These findings are in good agreement with results cencerning the carbohydrate metabolism of certain species of rumen ciliates (Abou Akkada and Howard 1960, Williams et al. 1961, Howard 1963, Van Hoven and Prins 1977, Prins and Van Hoven 1977). However, only traces of propionate (if any) were found in the mentioned experiments, while in this experiment propionic acid formed about 6–7% of the total of volatile fatty acids produced by ciliates. It cannot be excluded that a part of this propionate may originate from internal bacteria as well as the bacteria attached to plant particles and (or) the bacteria attached to protozoal cuticle, despite the washing procedure and the use of antibiotic. On the other hand, however, Mah and Hungate (1965) showed that propionate produced by *Ophryoscolex purkynjei* formed about 5% of the total of volatile fatty acids produced by this ciliate.

The protozoal production of VFA was not correlated with the total number of the ciliates, but with their biomass and protein. Small protozoa formed over 90% of the total protozoa counts, but middle-sized and large ciliates gave a majority of the protozoal biomass and protozoal N (Table 1). From the group of small protozoa, entodinia utilize starch as a glucose source, while *Dasytricha ruminantium* is able to ferment soluble sugars only (Hungate 1966). The protozoa from the last two groups except *Isotricha* sp. readily ingest and utilize plant fragments as well as starch granules. They also possess more active glycolytic enzymes than entodinia (Williams et al. 1984). Therefore it seems plausible that the middle-sized and large ciliates determined the protozoal pool of volatile fatty acids in the rumen.

The protozoa produced above 70% of butyric acid in the rumen. This result suggests that butyrate in the rumen depends on the presence and abundance of protozoa population. This suggestion is in agreement with the results of Whitelaw et al. (1972) and Hinkson et al. (1976), who found a higher proportion of butyric acid in faunated animals as compared to ciliate-free. On the contrary, however, Van Nevel et al. (1985) observed the fall in the proportion of propionate and the increase of butyrate after the defaunation of sheep rumen. This shows that the molar proportion of volatile fatty acids depends on many factors.

Protozoal VFA production rate varied from 1.42 to 1.83 mmol/g protozoal DM/h or from 2.12 to 3.15 mmol/g protozoal protein/h (Table 4). It was found in parallel studies performed on the same animals, that the bacterial DM varied from 16 to 24 mg/g of rumen content and N formed about 8.4% of bacterial DM (in

preparation). On the basis of these results and the data presented in Fig. 1 it was calculated that bacterial production rate was 1.1–1.6 mmol VFA/g bacterial DM/h or 2.0–2.95 mmol/ VFA/g bacterial protein/h. Thus both groups of microorganisms produced VFA practically with the same intensity. This suggestion, however, related only to the total production.

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Advances in Protozoological Research

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