PL ISSN 0065-1583

POLISH ACADEMY OF SCIENCES
NENCKI INSTITUTE OF EXPERIMENTAL BIOLOGY

ACTA PROTOZOOLOGICA

VOLUME 27

Number 2

PAŃSTWOWE WYDAWNICTWO NAUKOWE
WARSZAWA 1988 WROCŁAW

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POLISH ACADEMY OF SCIENCES
NENCKI INSTITUTE OF EXPERIMENTAL BIOLOGY

ACTA PROTOZOOLOGICA
International Journal of Protozoology

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ACTA PROTOZOOLOGICA appears quarterly. The indexes of the previous volume will appear in No. 1 of the next volume.

Indexed in Current Contents and in Protozoological Abstracts.

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ACTA PROTOZOOLOGICA
VOL. 27, No. 2, pp. 83–96 (1988)

Polarity of the Motor Functions in *Amoeba proteus*. I. Locomotory Behaviour

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Received on 20 November 1987

Synopsis. The axial polarity of body shape and external morphology of amoeba is tightly related to the direction of movement. Reorientation of locomotion necessarily involves restructuration of cell polarity. At the ultrastructural level the polarity is not determined by the distribution of contractile proteins: F-actin cortical network is evenly developed along the lateral body walls of amoeba; thick filaments of highly polymerized myosin prevail in the tail region, but there is no gradient of oligomeric myosin which is sufficient for contraction. The motor polarity is therefore not determined by the contractile cortex as such, but by the dynamics of membrane-cortex links. It is experimentally demonstrated that local breaking of these links creates a new front and reorientates cell polarity and movement. In the natural fronts the cortex is regularly detached from the membrane what makes its contraction mechanically inefficient, and creates a low pressure spot. The new pressure gradient leads to new motor polarity. It is postulated that anaesthetics, which are potent attractants, and probably other positive stimuli, locally destabilize the membrane-cortex links by the depletion of membrane-bound Ca. Experimental data indicate that a relaxation factor is produced by the cell nucleus of amoeba and distributed across the cell by cytoplasmic streaming. It amplifies and stabilizes the polarizing effects of external stimuli and thus helps to perpetuating the former polarity and direction of movement. So, the nucleus is neither needed by amoeba to contract nor to establish the stimuli-induced polarity, but is required to maintain the polarity stable until the cell is reorientated by a new stimulus.

Amoeba proteus is the representative of those motile cells which, in contrast to the ciliated or flagellated cells, have never developed any permanent motor organelles. The pseudopodia extended by amoebae are ephemeral structures and the internal composition of their cytoplasmic layers is also incessantly renewed. Nevertheless, *A. proteus* may keep for long periods of time a clear functional polarity reflec-

This paper was supported by the Research Project CPBP 04.01 of the Polish Academy of Science.

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ted in the external morphology (Pl. I 1). The anterior parts of advancing pseudopodia are always smooth, while the posterior pole, the uroid is strongly corrugated (Czarska and Grębecki 1966, Haberey et al. 1969, Stockem et al. 1969). This morphologically expressed differentiation of the frontal zone and tail region, which begins already at the moment of separation of both daughter cells after cytokinesis (Goldacre and Lorch 1950, Rappaport and Rappaport 1986), is in the adult migrating amoeba held up by the streaming of cytoplasm which flows always in the direction of cell movement. Any deviation of locomotion is accompanied by respective changes in the direction of intracellular flow and in body shape. The complete reversal of locomotion by 180° is followed by unwrinkling the contour of the former uroid which is transformed into the smooth front, whereas the formerly smooth frontal pseudopodia fold and corrugate owing to the outflow of the cytoplasm and eventually form the new uroid.

All these observations put in evidence the tight relation existing between the body shape of an amoeboid cell and its locomotion. This relation is also clearly demonstrated by the lack of locomotory capacities in those cells of *A. proteus* which have lost their morphological polarity, that is do not display any more the former typical axial symmetry. All known forms of this amoeba which morphologically present the radial symmetry, such as the highly and uniformly folded mitotic, pinocytotic and postpinocytotic rosettes, as well as smooth spheres, which sometimes arise spontaneously in cultures or are experimentally produced (e.g., Korohoda and Stockem 1975 a, Grębecki and Kwiatkowska in press), are never capable to locomote. The progressive movement is gradually recovered as they tend to elongate (Seravin 1966 a), that is re-establish the axial symmetry and polarity.

There is no doubt therefore, that the factor or factors deciding about the polarity of the cell of *A. proteus* play the essential role in the organized amoeboid movement. At the first sight it seems promising to make an attempt of relating the morphological polarity of the migrating cell with the intracellular distribution of the molecular system responsible for generating the motive force.

It is now generally accepted that all locomotory and non-locomotory movements manifested by amoebae are due to active contraction which is based, as in the muscle cells, on the interaction of actin and myosin. It should be noticed, however, that in the amoeboid cells most probably the highly polymerized actin microfilaments may interact even with the oligomeric myosin (cf. Albanesi et al. 1985). In most motile cells, and among them in *Amoeba proteus*, the microfilamentous actin in association with other proteins involved in contraction, is peripherally distributed in the form of membrane-attached network, called the cell cortex. The contraction of the actomyosin system, as well as its depolymerization-repolymerization cycle related to the reversible solation and gelation of the respective cytoplasmic layers, create such differences of the body wall resistance and the internal hydrostatic pressure, that promote the cytoplasmic streaming resulting in cell translocation (see the reviews by Taylor and Condeelis 1979, Grębecki 1986 a, Stoc-

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kem and Kłopocka, in press). In many motile cells the actin-binding proteins which link the cortical F-actin to the cell membrane were identified (for example in *Acanthamoeba* by Korn and Wright 1973, Pollard and Korn 1973, in *Dictyostelium* by Spudich 1974, in fibroblasts by Gruenstein et al. 1975, in ascites tumor cells by Carothers-Carraway et al. 1985). In *Amoeba proteus* the nature of these proteins was not yet determined, but the existence of cortex-membrane linkage has been demonstrated by electron microscopy (cf. Stockem and Kłopocka in press).

Motor Polarity and the Distribution of the Cortical Proteins

The experiments *in vitro* on motile extracts of the cytoplasm of *A. proteus* allowed Taylor (Hellewel and Taylor 1979, Taylor and Fechheimer 1982) to formulate the hypothesis of contraction and solation coupling in the amoeboid cells. According to this concept, the cortex represents a mosaic of microdomains of two different types. Some of them are stabilized by the abundance of actin-binding proteins which restrict the mobility of filaments and play therefore rather a structural cytoskeletal role. In other microdomains, more loose filaments are free to slide one past another, that is the structure may contract. It means that in the cortex of amoeba the gradual contraction in the loose microdomains must be accompanied by gradual solation in the stabilized ones, in order to produce and sustain any steady movement. Solation enables movement of the stabilized microdomains and translocation of their components, making them contractile on their turn. The solation-contraction coupling hypothesis relates the differences between the successive body regions of moving amoeba to the local interactions of the cortical proteins, which change the physical state and properties of the peripheral cell layer; it does not necessitate any gradiental distribution of the F-actin or myosine pool.

Several attempts to evaluate the expectable axial differentiation of abundance of the actin filaments, relative to the morphological polarity of moving amoeba, led to equivocal conclusions. In general, the different methods produced discordant results. Stockem for example, initially postulated the existence of F-actin gradient expressed by thickness of the cortical layer of *A. proteus* changing from 0.1 μm at the front to 0.5 μm in the rear, as estimated from EM pictures (Stockem et al. 1982). Later on, however, the application of immunofluorescent techniques led the same author to the conclusion that F-actin layer is thick, stable and uniform along the lateral, intermediate body walls, but faint as well at the anterior as at the posterior pole (Stockem et al. 1983 a, b). On the other hand, the similar immunofluorescent methods applied *in vivo* by Taylor et al. (1980 b) demonstrated actin filaments to form bundles in the frontal and tail regions, but not in the intermediate body parts of amoeba. It seems that so discrepant results and conclusions give no reason to think that the anterio-posterior morphological and functional differentiation in amoeba might depend on the distribution of F-actin in its cortex.

The distribution of actin in cytoplasm of *A. proteus* has been also studied by

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Opas (1981) on the glycerinated models of amoebae with two different techniques: fluorescein labeled heavy meromyosin and specific anti-actin antibody. Both used techniques showed clearly that the distribution of actin in fixed amoebae was uniform.

As far as the distribution of myosin in the amoeboid cells is concerned, the results seem to be less ambiguous. The concentration of thick filaments in the uroid of *Amoeba villosa* has been already reported by Bhowmick (1967). Even earlier similar aggregations of thick filaments were seen around the wounds, in the damaged cells of *A. proteus* (Nachmias 1964) and *A. villosa* (Bhowmick and Wohlfarth-Bottermann 1965). The myosin polymers are also seen in electron microscope near the cytokinetic furrow experimentally induced in *A. proteus* by spermin (Gawlitta et al. 1981). Stockem et al. (1982, 1983 a, b) demonstrated that in migrating *A. proteus* the distribution of myosin polymers seen in the electron and fluorescence microscope is restricted to the uroid and retracted pseudopodia. In the phagocytizing specimens of *Chaos carolinensis* and *Amoeba proteus*, the thick filaments were also observed beneath the membrane adhering to the captured prey (Jeon and Jeon 1982).

There is no doubt that the polymeric myosin seen as thick filaments is asymmetrically distributed within the cell of amoeba. Should this statement be extended to the total myosin and causally related to the motor polarity of migrating amoebae? It is now largely admitted that in the non-muscle motile cells a high degree of myosin polymerization is not needed for contraction. Therefore, not the distribution of thick filaments identifiable by EM, but distribution of total myosin should be relevant. Any polar or gradiental distribution of total myosin has never been observed nor postulated in *A. proteus*. On the contrary, already Guindon and Couillard (1964) demonstrated by cytochemical methods that the myosin-type ATPase activity is evenly manifested across the whole cell of this amoeba. It should be added that the uniform distribution of both interacting proteins, actin and myosin, is also postulated for the motile tissue cells by Fujiwara and Pollard (1976). In general, the significance of the intracellular localization of highly polymerized thick filaments for the localization of contraction sites and expression of motor polarity appears doubtful. It is rather plausible that as well the F-actin as the oligomeric myosin are evenly distributed along the peripheral layer of *A. proteus*, which accounts for the well known potential contractility of every body region of amoeba.

Distribution of Cortex-membrane Links and the Origin of Fronts

In the past the problem of the motor polarity of amoeba was most commonly associated to the question of site at which the motive force is generated. Now, we have good reasons to start from other principles. On one hand, the factors determining the localization of new fronts control the locomotion, and therefore they control the cell polarity. Moreover, it should be kept in mind that in the amoeboid cells the contraction may exert the mechanical effects only when the cortical actin

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is tightly associated with the cell membrane (as in the muscle fibres actin must be bound to the Z lines). Therefore, the molecular and ultrastructural differentiation which decides upon the polarity of locomoting amoebae may be not attributed to the conformation of the contractile cortex as such, but to an uneven distribution of the cortex-membrane linkage.

The cell periphery may shrink and wrinkle everywhere, where the cortex is attached to the membrane, and therefore, retracts and folds the cell surface during its own contraction. If the membrane-cortex links are locally broken, the detached sheet of actin filaments may contract as usually, but the cell membrane will not obey in that area the tensions developed by the contracting cortex; on the contrary, it will be locally distended outward and produce a blister. The cortex of amoebae, like the cytoskeleton of other cells, functions as stabilizer of the conformation of cell surface; at the moment of its disengagement from the membrane, the lipid bilayer follows its own natural tendency to produce spherical surface. That was very well demonstrated by the experiment of Elgsaeter et al. (1976) on the erythrocytes: local aggregation of their cytoskeleton, and its local detachment from the membrane induced by pH changes, were followed by the production of small membrane vesicles, which afterwards separated from the cell surface. Exactly the same phenomenon of membrane destabilization and vesiculation was observed in *A. proteus* exposed to ethanol (Korohoda and Stockem 1975 b), to a slight increase of temperature (Grębecki 1986 b), or to the cytochalazin B (Pl. II 3).

The space left free between the locally detached cortex and the hemispherically bulging membrane is gradually increasing, due to its filling with the hyaloplasm filtered through the contracting actin network. That place initiates the development of a new pseudopodium and consequently, becomes the front of the migrating cell, because the filtration of hyaloplasm through the detached cortex provokes a local drop of the intracellular hydrostatic pressure. In that way the pressure gradient is built up in the cell and it determines the direction of endoplasmic flow and cell movement. Such course of events is postulated by the generalized cortical contraction theory of amoeboid movement (Grębecki 1982). If it is so, the polar assymetry of shape of a moving amoeba (which in fact reflects the uneven ratio of membrane vs. cortex pool along the cell — Grębecka and Kłopocka 1985) is controlled by the topography of membrane-cortex contacts within the cell of amoeba.

The best description *in vivo* of membrane lifting and bulging when the new front is initiated in *A. proteus*, was given by Kalisz and Korohoda (1976). The basic fact that in the frontal zones the microfilamentous cortex is detached from the cell membrane and retracted behind the hyaline cap was first established by EM independently by us (Grębecka and Hrebenda 1979) and by Wehland et al. (1979). In that way the discussion between earlier authors about the existence and nature of the so-called plasmagel sheet in that place, has been resolved. Further progress in its study was made mainly by immunofluorescence techniques (Gawlitta et al. 1980, Stockem et al. 1983 a, Hoffman et al. 1984).

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However, the idea that a local break of contacts between the membrane and the cortex creates the low pressure spot, and thus polarizes the cell of amoeba, is earlier than the discovery of cortex detachment in the hyaline fronts of normal polytactic form of *A. proteus*. It came from the study of monotactic forms of this species in which the extremely strong polarity depends on the presence of very prominent frontal cap (Seravin 1966 b), which is not hyaline but vacuolar in nature (Korohoda and Stockem 1976). The present concept was formulated after the demonstration how this type of cap is formed by fusion of cytoplasmic vacuoles and how immediately it polarizes the cell shape and movement, when it arises either spontaneously (Grębecka 1978 a) or under experimental conditions (Grębecka 1978 b). The polarity induced by breaking the cortex by the vacuoles pressed against the inner side of the cell membrane is so strong that the monotactic amoebae fail to react to external stimuli of normal, effective intensity. The monotactic amoebae were also produced artificially by Goldacre (1961), by injecting a droplet of paraffin oil under the membrane. Such amoebae and the polarity changes provoked by competition of multiple oil caps in the same cell, were studied in consequence (Grębecka 1977 b). It was demonstrated by EM that really the microfilamentous layer is completely broken around such vesicular oil caps (Hrebenda and Grębecka 1978). Study of the mechanism of supranormal polarity of monotactic forms allowed us to understand the meaning of the detachment of cortical microfilamentous layer in the hyaline caps of normal amoebae, when it was subsequently revealed.

Retraction of the contractile cortex from the membrane in the normal hyaline fronts of amoeba is followed by its reconstruction under the membrane from the G-actin which may penetrate across the detached old microfilamentous network (Gawlitta et al. 1980). It was demonstrated *in vivo* in the non-locomotive heat-pretreated amoebae, called the hyalospheres, that as a result the consecutive cortical sheets serially detach from the membrane and move across the hyaloplasm inward (Grębecki and Kwiatkowska, in press).

It becomes therefore important, for understanding the origin of motor polarity of amoebae, to learn what provokes the local detachment of the cortical layer from the cell membrane. It may be helpful to refer to the explanation proposed by Nicolson and Poste (1977) for the local effects of anaesthetics on the membrane-cortex contacts in the motile tissue cells. It is suggested that anaesthetics have a high affinity to the Ca-binding sites in the membrane and can displace this ion, indispensable to maintaining stable cortex-membrane links. In normal locomoting polytactic amoebae the explorative character of migration (Pl. I 2) clearly indicates that the formation of the succesive new fronts is influenced by the environmental stimuli. It may be imagined that the positive stimuli (attractants) exert their effects, like the anaesthetics, by depletion of membrane-bound calcium, which results in local destabilization of membrane-cortex links and the initiation of new fronts of locomotion. Some experimental data seem to justify such a hypothesis: (1) Just the anaesthetics are commonly known as potent chemoattractants for amoeboid cells. For

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example Korohoda (1977) by local application of them by a micropipette provoked the formation of fronts and the organized movement in the normally motionless enucleated fragments of *Amoeba proteus*. (2) The detachment of cortex in the normal fronts and its detachment by anaesthetics are morphologically identical. (3) As demonstrated by Taylor et al. (1980 a), the aequorin luminescence which indicates the presence of free Ca, has a pulsating character at the front of locomoting amoeba. These calcium pulses might be related to the cyclic detachment and retraction of successive cortical sheets. Moreover, the extracellular current detected close to the cell surface of moving amoeba by vibrating probe (Nuccitelli et al. 1977) oscillates in correlation with the frontal activity and is carried by Ca^{2+} movements.

It can be stated in general that the amoeboid locomotion depends not only on the motor efficiency of the membrane-bound cortex beneath the major part of cell surface, but moreover requires the arisal and perpetuation of other zones, in which the cyclic detachment and reconstruction of this submembraneous layer maintains the cell polarity and steers cell locomotion. These zones create the system of hydrostatic gradients, that promote the endoplasmic flow, coordinate the activity of different pseudopodia, and as a final result are responsible of the organized locomotory movement.

The view that transformation of the contraction force into the cytoplasmic flow and cell movement is mediated by the hydrostatic pressure gradients, was generally admitted until the demonstration by Allen et al. (1971) that very high negative pressure externally applied by a relatively large micropipette to the surface of one of the pseudopodia of *Chaos carolinensis* is incapable to reverse the cytoplasmic streaming in this amoeba. It was, however, demonstrated later (Grębecka 1980) that the walls of pseudopodium to which the sucking force was externally applied do actively contract and, therefore, the negative pressure is not transmitted into the cell interior. On the contrary, even the pressure by one order of magnitudes lower is sufficient to control the flow and provoke durable reversal of cell polarity, if it is applied by a thin micropipette directly to the cell interior (Pl. II 4). Moreover, the direction of flow and the whole cell polarity could be reversed by simple perforation of the peripheral cell layers at the posterior body pole, that is by the difference between the intracellular pressure and the external atmospheric pressure (Grębecka 1981). It seems therefore that application of the classical hydrodynamic model to the present concept of arisal and perpetuation of the polarity of locomoting amoebae is still valid.

Motor Polarity and Cell Nucleus

An attempt should be made to apply the present concept of the motor polarity of amoeba to the puzzling question of the role of cell nucleus in amoeboid locomotion. It is known from a hundred years (cf. the review by Lorch 1973), that after

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removal of the nucleus amoeba stops and becomes more or less spherical in shape (Pl. II 5). The motor apparatus of such enucleated cell seems not to be damaged and is ready to perform work. It may be presumed, therefore, that the nucleus (or rather a factor emanating from it) does not influence the generation of the motive force, but is involved in the anterio-posterior polarization of the cell. It should be also kept in mind that amoeba may be immobilized without affecting its nucleus, and on the other hand, the motionless enucleated individuals can transiently recover the capability of locomoting, either spontaneously (Clark 1942, 1943, 1944, Lorch and Danielli 1950) or under experimental treatment (Jeon 1968).

The anuclear fragments of *A. proteus* were successfully stimulated to perform directional movements, by local application of benzene and some other anaesthetics to limited areas on their surface (Korohoda 1977). In similar experiments of Grębecki et al. (1978) the anuclear fragments, enucleated cells and polynuclear mutants of *A. proteus* moved toward the shaded zone, when a light-shade difference has been established across them. In both cases the authors concluded that the nucleus is needed for maintaining the motor polarization of amoeba.

More recently this question has been approached again by Swanson and Taylor (1982) in the study of chemotactic response of the amoeboid stages of *Dictyostelium discoideum* to cAMP. The authors make a clear distinction between two phases of the reaction. "The first observable response to stimulation is generated locally, indicating that the sensation of the attractant is probably a local event ..." (what apparently corresponds to the first step of response to anaesthetics and photic stimuli). The second phase that is the "actual movement of the cell in the gradient ... seems to require coordination of the cytoplasm". This coordination is probably depending on the nucleus, because" ... only the half-cell with the nucleus can move toward the microneedle; the enucleated fragment is paralyzed."

However, as it was quoted above, the anuclear fragments and enucleated cells of *Amoeba proteus* behaved differently after exposure to the anaesthetics or to the light-shade difference: they manifested coordinated locomotion toward the attractants. Is it the expression of difference between the two species of amoebae, or between the applied stimuli? The effect of cAMP was consequently tested on *A. proteus* (Grębecka, unpublished). Only a transient response manifested by extension of new pseudopodia toward the attractant was produced, but no durable effect on the directional locomotion could be observed (Pl. II 6). It seems therefore that cAMP is less efficient as cell polarizing stimulus (or may be it acts in different manner on the cell), and probably for that reason in *Dictyostelium* it could not provoke coordinated directional migration in the absence of the nucleus.

The experiments on *Amoeba proteus* clearly indicate that the presence of nucleus is not necessary for inducing the motor polarity and cell locomotion. Position of the front and, consequently, the direction of movement are not determined by the nucleus, but by a localized external stimulus. The nucleus seems to cooperate at the later stage, when the stimulus already has initiated these morphological changes

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within the cell by which the motor polarity is expressed, that is, the functional relaxation of frontal zone due to the break of cortex-membrane links. Two independent experimental studies (Korohoda 1977 and Grębecka 1977 a) led to the same conclusion that the hypothetical agent emanating from the nucleus plays the role of a relaxation factor. Therefore, when the nuclear factor is carried forward by the endoplasmic streaming, it should stabilize the position of the front, cell polarity, and the direction of locomotion. At the beginning, when the stimulus is still acting, it will amplify its effects, and later, sustain the same polarity axis and direction of cell movement, even after interruption of the initiating stimulus. It may be defined therefore as a perpetuation factor of amoeboid movement.

The observation that the nuclear factor may exert its polarizing effects because it is distributed across the cell by the cytoplasmic streaming, resulted from a comparative study of the anterior and posterior, nucleated and anuclear fragments of *A. proteus* (Grębecka 1977 a). They were obtained by cutting amoebae during normal locomotion and after an experimentally produced reversal of streaming. The relaxing effects were invariably observed only in those anuclear fragments which, before the operation, were situated downstream in reference to the nucleus. The upstream anuclear fragments were always apolar and immobilized in the contracted state.

It seems attractive (though as yet purely speculative) to suggest, as a working hypothesis, that the nuclear factor perpetuating cell polarity and locomotion exerts its effects, like the anaesthetics and probably other positive stimuli do, by restricting in the front the membrane-bound Ca required for the stability of membrane-cortex links (Fig. 1). If so, the nuclear factor would deplete membrane calcium and perpetuate the detachment of successive cortical sheets from the frontal membrane as long as the cytoplasm flows in the same direction. But, when a sufficiently strong stimulus initiates the formation of a new front in another region of cell periphery, the nucleus will obviously stabilize again the new direction of cell locomotion.

The present concept of the role of nucleus in supporting the cell polarity and the directional migration of amoebae, may help to understand some puzzling aspects of the behaviour of enucleated cells or cell fragments of *A. proteus*. For example, a few days after enucleation amoebae may spontaneously recover the motility for brief periods of time (Clark 1942, Lorch and Danielli 1950). It seems probable that this phenomenon is due to sporadic action of uncontrolled external stimuli, similar to the experimentally demonstrated effects of anaesthetics or light-shade difference.

The role of the cell nucleus in maintaining motor polarity of amoeba and stabilizing the directional character of movement, does not exclude the possible role of other factors or gradients developed along the amoeboid cell. Among them the hypothesis of calcium gradients is often put forwards, as the mechanism regulating not only the contraction-relaxation, but also the actin polymerization-depolymerization and cytoplasm solation-gelation cycles. Moreover, there are some other motor phenomena known in amoebae, which are produced by the same contractile apparatus and are also gradientally manifested, although they are induced by uni-

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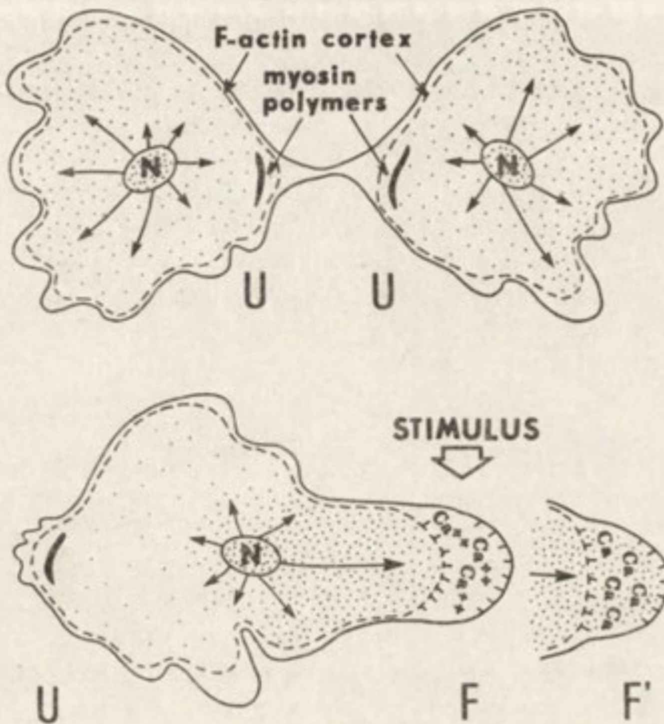

Fig. 1. Scheme summarizing the hypothesis of the role of nucleus in maintaining the polarity of amoeboid cell. Top: Before the separation of two daughter cells of *A. proteus* the remnants of the contractile ring determine the position of their posterior body poles; the anterior pole is not yet clearly established. Bottom: A strong external stimulus initiates the formation of strictly localized anterior pole and completes the polarization of the cell; the perpetuation factor of nuclear origin is carried by the cytoplasmic streaming to the frontal tip, it depletes there the Ca involved in the membrane-cortex association, and thus it maintains the former cell polarity even in the absence of stimulus.
U — uroid, F — front, dotted areas show the postulated distribution and transport of the nucelar perpetuation factor

form, non-directional external factors and are independent of the presence of the nucleus. Such features characterize the pinocytosis and (partially) capping, which will be discussed in the second part of this review.

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EXPLANATION OF PLATES I–I

1: Typical locomoting polytactic specimen of *Amoeba proteus*. Note the difference in shape and conformation of cell surface between the frontal zones (F) and the posterior, uroidal region (U)

2: Tracks of locomoting polytactic amoebae produced by 15 min intermittent exposure in the dark field. Note the ramification of path which demonstrates the simultaneous translational and explorative character of pseudopodial movements

3: Membrane vesiculation in an amoeba exposed to the cytochalasin B

4: A cell of *Amoeba proteus* moving to the right, with the micropipette inserted inside its uroidal region (a). Application of the negative pressure through the micropipette reverses the streaming direction and cell polarity, and then the new polarity is maintained even after withdrawal of the micropipette from the cell (b)

5: *Amoeba* stops moving and rounds up after removal of the nucleus. The extruded nucleus (N) is seen outside the cell

6: Local application of cAMP diffusing from the micropipette to the lateral surface of *Amoeba proteus* (a) induces a transient formation of new fronts and extension of pseudopodia toward the stimulus (b–c), but no effective locomotion

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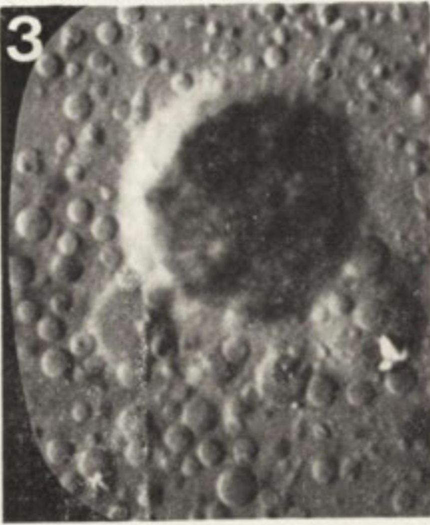

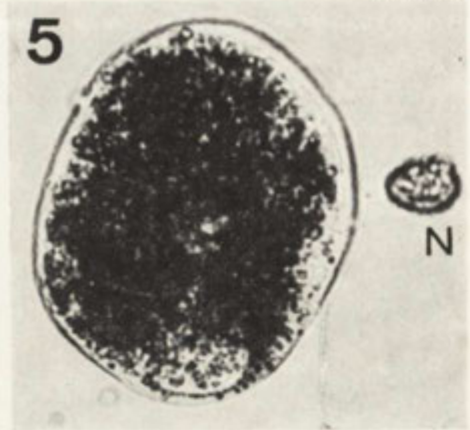

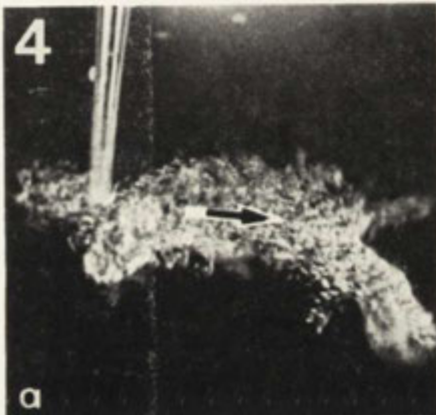

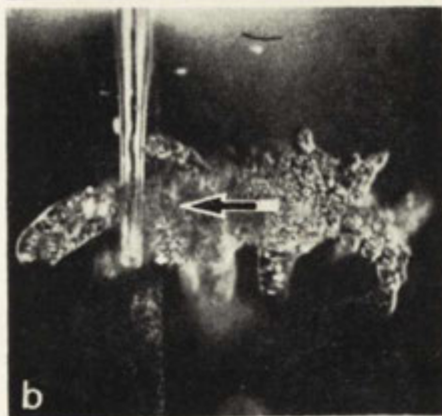

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ACTA PROTOZOOLOGICA
Vol. 27, No. 2, pp. 97–106 (1988)

Locomotory Response of *Amoeba proteus* to H^+ and a Protonophore (FCCP)

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Received on 26th October 1987

Synopsis. Exposure of amoebae to 10 μm solution of FCCP (carbonylcyanide-p-trifluoromethoxyphenylhydrazone) at pH 6.0 results in the complete cessation of movement of virtually all cells in the sample. Administration of the same protonophore in the original medium (pH 7.0) or at pH 8.0 has no clear effects. FCCP at pH 6.0 promptly stops any further advance of the fronts of amoebae, while the tails only gradually slow down, apparently as a consequence of the inhibition of fronts. Without the protonophore, the shifts of extracellular pH from 7.0 to 6.0 or (in a lesser extent) to 8.0 elicit a temporary disturbance of the motor polarity, which is most often followed (especially on the acid side) by the full and durable reorientation of the cell, i.e., a phobic response analogous to the avoiding reaction known in other Protists.

The early studies of the role of pH in the locomotion and behaviour of the proteus-type amoebae were reviewed, among others by Zimmerman (1959) and Jahn and Bovee (1973). The intracellular pH of different amoebae was determined by Needham and Needham (1925) and Chambers (1928) within the range between 6.9 and 7.6. The most recent study by Heiple and Taylor (1980) establishes its value in *Amoeba proteus* at 6.75. The dependence of the locomotion rate of *A. proteus* on the external pH is characterized by a bimodal curve with two maxima, at 5.9 and 7.5 (Pitts and Mast 1933). The long incubation of *Amoeba proteus* or *Chaos carolinensis* in the acid media results in the reduction of the number of pseudopodia and a tendency to the monopodial mode of locomotion (Mast 1928, Käppner 1961). More recently, Braatz-Schade et al. (1973) described the effects of prolonged cultivation of *A. proteus* in the media of different pH and attempted to correlate the changes in the cell shape and the rate of locomotion with the minor differencies in their membrane potential value. Taylor et al. (1971) studied the role

This study was supported by the Research Project CPBP 04.01 of the Polish Academy of Science.

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of pH on the contraction–relaxation phenomena and sol–gel transformation in the amoeba cytoplasm *in vitro* and found that the shift of pH to the basic side promotes the formation and stabilization of actomyosin gel in the extracts.

The objective of the present experiments was to test, how the motor responses of amoebae to the physiologically and ecologically fully tolerable shifts of pH, within the 6.0–8.0 range, are influenced by the external application of the FCCP (carbonylcyanide-p-trifluoromethoxyphenylhydrazone), which is known as a potent H^+ ionophore.

Material and Methods

Amoeba proteus was cultured in the standard Pringsheim medium (0.11 mM Na_2HPO_4, 0.34 mM KCl, 0.73 mM $Ca(No_3)_2$, 80 μM $MgSO_4$, 4 μM $Fe_2(SO_4)_3$, pH 7.0) and fed once a week on *Tetrahymena pyriformis*. Between the 3rd and 7th day after feeding the cells were collected for experiments. They were sucked out of the bottom of the culture dish with a Pasteur pipette and concentrated by re-sedimentation in a Godet chamber. Then, approximately 100 specimens were transferred to the test chamber (20×8×0.2 mm in size), in which medium could be rapidly exchanged.

As experimental solutions the buffered media were used (0.68 mM NaCl, 0.34 mG KCl, 0.73 mM $Ca(NO_3)_2$, 80 μM $MgSO_4$, 4 μm $Fe_2(SO_4)_3$, 0.5 mM HEPES adjusted by 0.5 M NaOH to pH 6.0, 7.0 or 8.0). These media were applied to amoebae with or without addition of the FCCP. The FCCP, made by Fluka, was initially dissolved in ethanol and then diluted with the Pringsheim solution or the appropriate buffer medium. The final solutions contained 10 μM of the ionophore and 0.01% ethanol. Any physiological effects of ethanol on the cell shape and locomotion of amoebae begin in the concentrations at least 10 times higher (see e.g., Hülsmann et al. 1976).

Amoebae transferred to the test chamber filled with the standard Pringsheim medium were left for 15 min to allow them to re-attach to the glass and to start normal locomotion. Then, the test chamber was perfused with 250 μl of an experimental solution, which typically took 20 to 30 s. Only very few cells detached from the bottom during the procedure of exchanging the medium.

The locomotion of amoebae was observed in the Biolar microscope with 10× objective lens, and usually 3–10 individuals within the field of view. Their behaviour was filmed at the speed of 1 frame per second with a Bolex H16 Reflex camera driven by a combined Bolex and Robot time-lapse equipment. The filming was started 2 min before the exchange of the medium and followed for 5 min or more after that. Each experiment was repeated at least 5 times with new samples of amoebae. The cinematographic records were analyzed frame-by-frame with the LW International Photooptical Data Analyzer.

Results

As indicated by the Fig. 1, the Pringsheim culture medium in the test chamber was replaced by one of the five different experimental solutions: FCCP alone dissolved in the Pringsheim solution (or in some supplementary tests, in the buffer medium at pH 7.0), the pH 6.0 buffer with and without FCCP, and the pH 8.0 buffer also with or without the protonophore.

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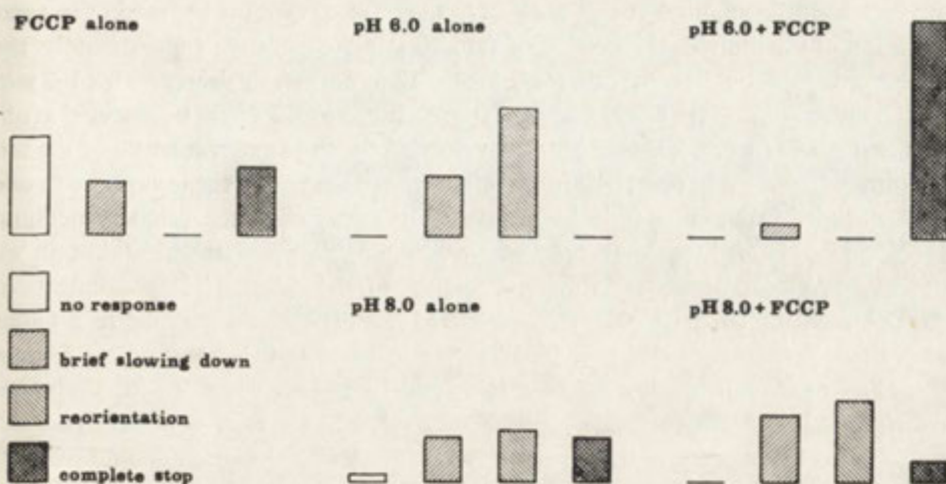

Fig. 1. Influence of five experimental media differing by H^+ concentration and the presence or absence of the protonophore (FCCP), expressed by the relative frequency of four well differentiated types of the motor behaviour of amoebae. In each experiment the behaviour of 20–30 cells was evaluated from the film records

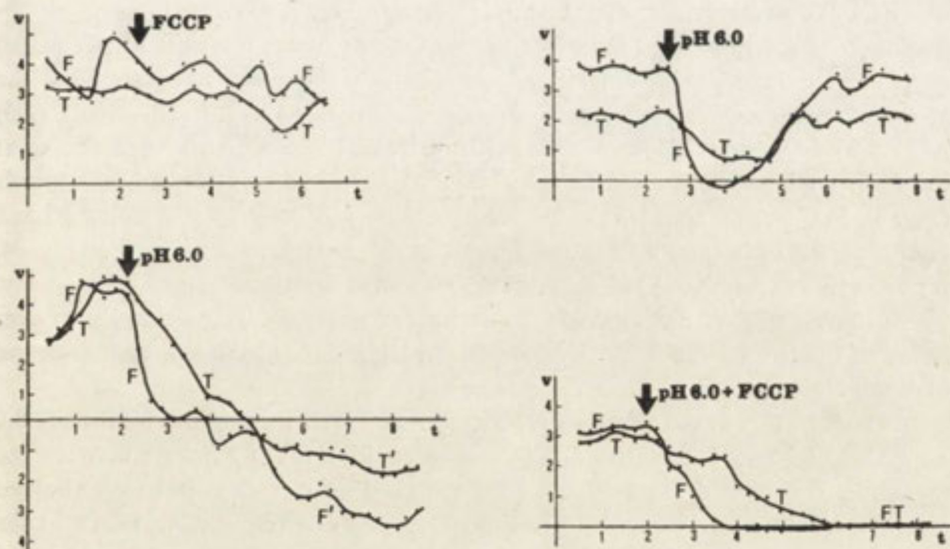

Fig. 2. Velocity of the frontal progression (F) and tail withdrawal (T) recorded in the individual amoebae 2 min before the exchange of the medium and 4–6 min after that. The time axis (t) is calibrated in min and the velocity axis (v) in μm/s. The four graphs are representative examples of the lack of any reaction (in FCCP in the original medium), the brief slowing down or reorientation (both at pH 6.0), and the complete cessation of locomotion (in FCCP at pH 6.0). Note that in the third graph the reorientation of cell polarity is indicated by the velocity values becoming negative and by F (original front) becoming T' (new tail), while T (original tail) becomes F' (new front)

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Effects of the addition of FCCP alone to the original medium are subject to great variation of the individual reactions of amoebae. In 30% of cells approximately, the movement stops, but in some others the locomotion rate merely decreases for 1–2 min and in the major group of amoebae no response to the FCCP could be detected at all. The first graph in Fig. 2 shows how the velocity of frontal extension and of the tail withdrawal changed in time, before and after the application of the ionophore to one of such individuals, apparently insensitive to its presence in the original medium. It should be stressed that the group of non-reacting individuals was absent in all other types of experiments involving a change of the external H^+ concentration.

The situation becomes completely different if FCCP is administered in a buffer at pH 6.0. Practically 100% individuals stop moving after the exchange of medium (Fig. 1). As shown by the last graph in Fig. 2, the frontal activity of amoeba is immediately affected and it completely ceases within the 2nd min of treatment (the tail needs much longer time to be arrested). No recovery of locomotion could be observed within the time limits of this experiment. The fact that the effects of FCCP on the locomotion, which are uncertain in the original medium, become prompt and unequivocal with a slight increase of the extracellular H^+ concentration, directly proves that the mechanism of action depends on the protonophore capacity of this compound.

The need to test in a control experiment the influence of the pH 6.0 buffer without FCCP became evident. The results, as seen in Fig. 1, were again distinctly different from the two preceding experiments. On one hand, the shift of pH to 6.0 never exerted any immobilizing effects on the amoebae, but on the other hand the group of non-reacting cells also disappeared. The majority of individuals (2/3) reacted by full reorientation of their motor axis, that is new fronts were arising at the former tails while the old fronts were withdrawn and transformed into uroids (Fig. 4). In the remaining 1/3 the former direction of locomotion was re-established after a period of back and forth oscillations of the endoplasmic streaming (Fig. 3). It means that in fact 100% individuals were subject to the alterations of cell polarity, which in over 60% of them led to the durable reorientation. The examples of evolution of the frontal and uroidal velocities in both groups are shown by the second and third graph in Fig. 2.

As seen in Fig. 1, the effects of replacing the Pringsheim medium by the pH 8.0 buffer may be very differentiated. There is an equilibrium of numbers between the immobilized cells and those which, after the stage of disturbed polarity, adopted either the former or the reverse direction. The results of introducing FCCP at the pH 8.0 also appear not clear. It seems plausible that the protonophore, when applied from the outside, cannot significantly modify the effects of a decreased extracellular H^+ concentration.

The three characteristic types of the locomotory response encountered in the present study (slowing down, reorientation and the cessation of movement) were analyzed stage-by-stage and the typical examples are presented in Figs. 3, 4 and 5.

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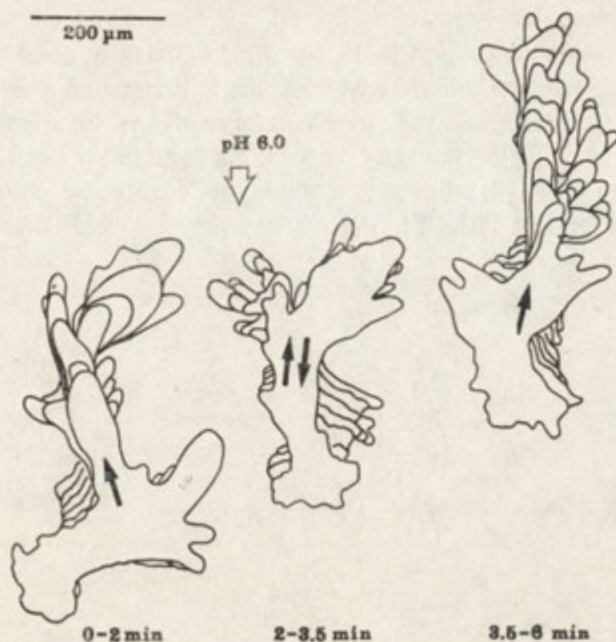

Fig. 3. Contour analysis of the frontal progression in an individual which, after a period of normal locomotion in the Pringsheim medium (0–2 min), reacted to the increase of H^+ concentration by oscillations of the direction of cytoplasmic streaming (2–3.5 min) and eventually recovered its former motor polarity (3.5–6 min). The contour changes in Figs. 3, 4 and 5 were redrawn from the film records at the intervals of 15 s

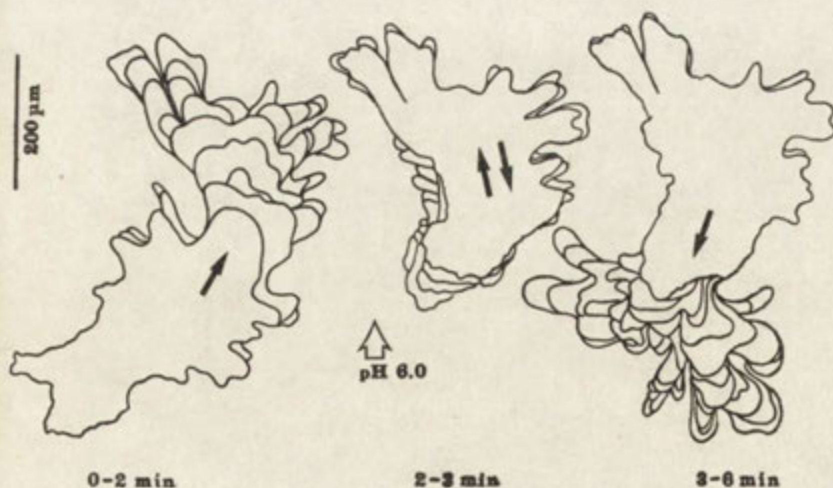

Fig. 4. Contour analysis of the frontal progression in another specimen, in which the temporary streaming oscillations elicited by the increased H^+ concentration resulted in the full reversal of motor polarity

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The separate contour analysis of the frontal progression and tail withdrawal, in the course of cells' immobilization by FCCP at pH 6.0, clearly demonstrates (Fig.5) that the fronts are arrested immediately, while the tail retraction is followed as long as it is still possible to evacuate the endoplasm forwards without any frontal activity (exactly as after blocking the fronts by the localized light, in the experiment of Grębecka and Grębecki 1981). In the two other types of response, the slowing down (as in Fig. 3) and reorientation (as in Fig. 4), the time difference between the reaction

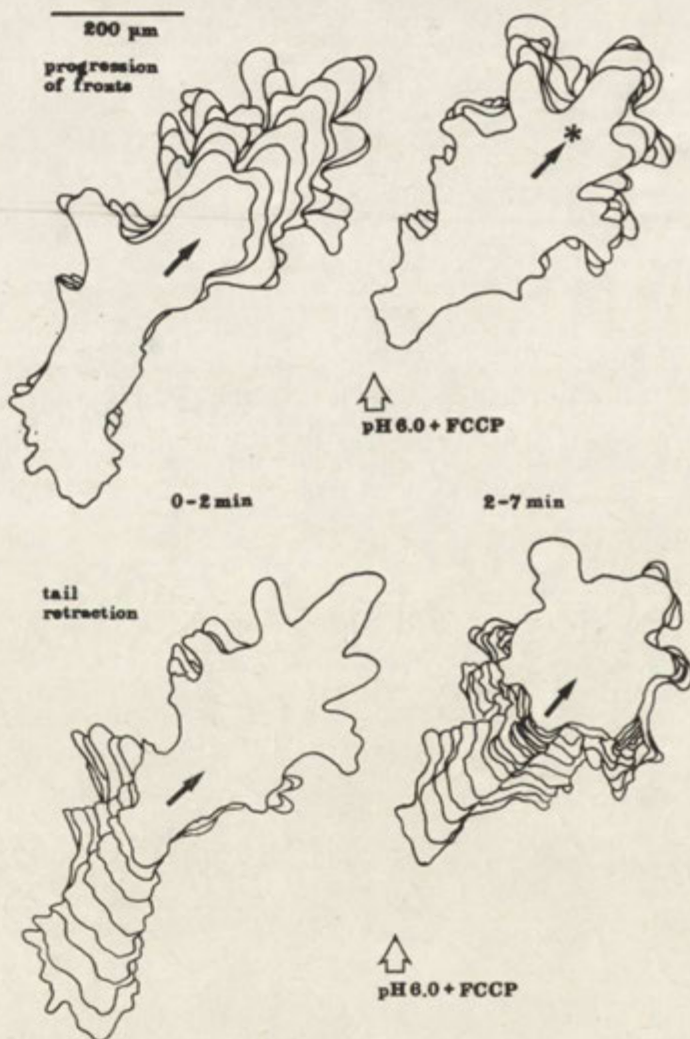

Fig. 5. Contour analysis of the frontal progression matched with the tail retraction of the same amoeba, 2 min before and 5 min after its exposure to FCCP at pH 6.0. Note that after administration of the test solution the cytoplasm still flows steadily in the former direction and the tail retracts (arrows), though the front stops very quickly (asterisk)

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of the fronts and the tails may be read out from the respective graphs in Fig. 2. In these two cases as well the reaction of the front distinctly precedes any change in the activity of the tail.

It was found that the original motor behaviour of amoebae may be always re-established by rinsing them in the standard Pringsheim medium. Amoebae may also gradually recover with time, when still kept in the experimental solution without washing. Such experiments were run in the most drastic situation, that is with amoebae immobilized by FCCP at pH 6.0. About 100 specimens were taken per sample and their behaviour was photographically documented. By comparing the position of the cells on the serial still pictures they could be divided in four estimative groups: the non-motile ones and manifesting a slow, moderate or fast motion. The histograms shown in Fig. 6 demonstrate that, after the period of complete immobilization, the share of slowly or moderately locomoting cells begins to rise again in the 15th min and the distribution of the groups approaches the normal in the 25th min of the experiment.

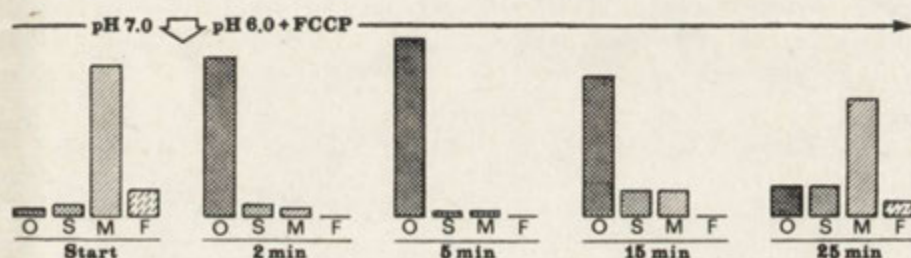

Fig. 6. Relative frequency of the individuals manifesting no appreciable movements (O), slow locomotion rates less than 1 μm/s (S), moderate velocity between 1 and 3 μm/s (M) and fast movement over 4 μm/s (F), in the original Pringsheim medium at pH 7.0 and at different stages after the introduction of FCCP at pH 6.0. Note the gradual recovery of the motor capacities by amoebae immobilized at the first stages of the experiment

Discussion

Since FCCP is known as a potent uncoupler of the oxidative phosphorylation (Heytler and Prichard 1962), there is a tendency to attribute its multiple physiological effects to the depletion of ATP. Such interpretation of the present results may be ruled out because of the immediate character of the changes in the locomotory behaviour of amoebae. For the same reason the inhibition by the FCCP of the transport of membrane proteins between the endoplasmic reticulum, Golgi apparatus and the cell surface (Tartakoff et al. 1978, Kääriainen et al. 1980, Glickman et al. 1983, Antoine and Jouanne 1986), should not be taken into consideration. It appears that the immediate motor reactions of amoebae to the FCCP are merely due to the protonophore capacity of this compound (see e.g., McLaughlin and Dilger 1980).

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Therefore, the sudden cessation of movement in the presence of an increased H^+ concentration and FCCP in the medium, may be explained either by the movement of H^+ into the cell down the electrochemical gradient, or alternatively, by hampering the active extrusion of H^+ from the cell, if a proton pump is present in *Amoeba proteus* (as it was demonstrated for example in another amoeboid motile system, *Physarum polycephalum*, by Matveeva et al. 1979). In both cases, with or without the pump, the changes in the flux of protons across the cell membrane should influence the membrane potential, and thus might provoke the prompt motor reactions of amoebae.

It is most important for the mechanism of the locomotory behaviour that such prompt reaction to FCCP at pH 6.0 is manifested only by the fronts of amoebae, which imeediately stop any further progression, while the tail regions seem not to be affected but are only mechanically inhibited later, by the gradual exhaustion of further possibilities of the outflow of endoplasm. The higher sensitivity of the fronts of locomotion to the external stimuli has been demonstrated in the response of *A. proteus* to light (Grębecki 1981), and was considered as one of the manifestations of the steering role of the fronts in the amoeboid movement (Grębecki et al. 1981).

The highest sensitivity of the fronts to the increase of external H^+ concentration, with and without the protonophore, cannot be accounted for by any gradient of the intracellular pH. The old (e.g., Pantin 1923) and more recent (see Taylor and Condeelis 1979) concepts postulating the existence of such a gradient in large amoebae, were experimentally disproved (Heiple and Taylor 1980). On the other hand, the higher sensitivity of fronts to various stimuli that presumably provoke the transmembrane ion fluxes, might be related to the role membrane potential is playing in the amoeboid movement. The interrelation between the streaming, amoeboid locomotion and membrane potential has been demonstrated by the double chamber technique (Kamiya 1964), microelectrodes (Bingley 1966) and vibrating probe (Nuccitelli et al. 1977).

The reversal of direction of the endoplasmic flow and of the whole motor polarity of the cell, was the second striking phenomenon manifested in the present experiments. That was the most typical response of amoebae to an increase of the external H^+ concentration (without the protonophore), but in a lesser extent it followed too a decrease of H^+ in the medium. Again, it seems not to be a specific reaction to the change of pH, but a rather universal response to various stimuli. It was encountered as a premortal phenomenon during the fixation of amoebae in glutaraldehyde (Grębecka et al. 1987), provoked by strong illumination (Grębecki, unpublished), by the thermic and mechanical shocks (Grębecka and Kaczanowski, unpublished). In all these situations, as in the experiments reported here, the directional response of the cell, orienting it rearward, is elicited by an omnipresent non-directional stimulus. It might be therefore defined as a general phobic response (like the ciliary reversal in ciliates), which under natural conditions might lead to the avoidance of the environmental change. Its physiological mechanism is not yet experimentally

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demonstrated. In general, the view that the locomotion of *A. proteus* is principally unaffected within the pH range 6.0–8.0 (see the introduction) proved to be only partially true. As a matter of fact, the response is brief and very soon the mode and rate of locomotion look normal again. Nevertheless, the earlier authors missed the momentary disturbance of the motor polarity of amoeba, which is often followed (especially on the acid side) by its full reorientation and has the meaning of an avoiding reaction.

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ACTA PROTOZOOLOGICA
Vol. 27, 2, pp. 107–115 (1988)

The Influence of Liposomes Containing Cholesterol on Phagocytic Activity and Proliferation of *Tetrahymena* Cells

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Received on 9th October 1987

Synopsis. The ability to form a digestive vacuole (dv) was investigated in *Tetrahymena pyriformis* cells after 3 and 24 h starvation and than incubation in nutrient suspensions (2% proteose peptone, yolk and liposomes). As control served the ciliates kept in sterile Pringsheim solution. Observation of the phagocytosis rate induced by the 5 min exposure to the latex suspension demonstrated that the rate of dv formation decreased distinctly after 2 h incubation in cholesterol rich liposomes. The rate was not affected if the cells were exposed to the other nutrients. It was, moreover, demonstrated that the duration of starvation has an important influence on cell phagocytic activity. With the prolongation of starvation period, the rate of dv formation markedly decreased. The application of labelled thymidine and measurement of the density of cell population in control and tested suspensions showed no correlation between the phagocytic activity reduction of cliates subjected to the liposomes enriched in cholesterol and the stage of the cell cycle. The observed phenomenon may be explained by the rather specific influence of cholesterol, which being taken up and incorporated into the cells, causes changes in the physico-chemical parameters of the cell membrane, thus possibly leading to disturbances in some their physiological processes.

The ciliates *Tetrahymena pyriformis* are generally considered as a convenient model organism for the cell biology investigation. By taking advantage of the rapid growth of these cells and their ability to incorporate the lipid membrane precursor, one can modify the cell membrane of *Tetrahymena* by way of incubation with sterols (Thompson 1967, Nozawa and Thompson 1971), their composition and interrelation with the particular lipids, thus leading to the changes in the functioning of the cell (Thompson et al. 1972, Onimaru et al. 1980, Conner et al. 1982, Fabczak 1986).

Changes in the fluidity and microviscosity of the cell membrane as a result of an increase of cholesterol concentration have been noted both *in vivo* and *in vitro* by many investigators. By altering in this way the cell membrane, modifications in its

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functioning were obtained in the form of changes in the permeability to cations and anions, reduction of activity of certain enzymes associated with the lipid bilayer of the membrane and changes in electrophysiological parameters (Deuticke and Ruska 1976, Cooper et al. 1975, Wiley and Cooper 1975, Shinozawa et al. 1979, Shapiro and Barchi 1981, Ortega and Mas-Oliva 1986, Zyzek et al. 1983).

It was noted that the rate of dv formation decreased if lipid vesicles (liposomes) enriched in cholesterol were used to feed *Tetrahymena* cells (Fabczak 1986). This is probably caused by disturbances in the functioning of the cell membrane, evoked by the exchange of terahymanol (permanent component of the cell membrane in *Tetrahymena*) for cholesterol contained in the liposomes (Conner 1968). The liposomes are taken up by the ciliates mainly by means of phagocytosis (Fabczak 1987).

Cameron and Jeter (1970) achieved a synchronization of the cell cycle of *Tetrahymena* by starvation and refeeding. If we presume that the liposome suspension may be treated by the cells as food, than the experimental conditions performed by Fabczak (1986) may be considered similar to those described by Cameron and Jeter (1970). In this connection the present paper is an extension and supplementation of earlier results reported by Fabczak (1986). The aim of this paper is to prove that cholesterol incorporated into cell membrane of *Tetrahymena* is chiefly responsible for phagocytosis rate depression. To rule out the possibility that the stage of cell cycle in *Tetrahymena* may disturb the phagocytosis rate, the influence of some selected nutrient media, DNA synthesis and cell proliferation was compared.

Materials and Methods

Tetrahymena pyriformis, strain GL, was cultured at 28°C in 2% proteose peptone medium enriched by 0.1% yeast extract (both from Difco). The cells were harvested in a logarithmic phase by centrifugation at 800 g and washed twice in Pringsheim solution. Collecting and washing procedures were carried out in aseptic conditions. The washed cells were resuspended and then starved in sterile Pringsheim solution for two different period time (i.e., 3 or 24 h at 20°C).

Phagocytosis Assay. The starved cells (about 2×10^5 cells per ml) were divided into four groups and exposed to: (a) Pringsheim solution (control), (b) proteose peptone plus 0.1% yeast extract, (c) liposome suspension (10 μg per ml). The molar ratio of cholesterol (Chol) to egg lecithin (PC) was 1. Liposomes were prepared according to the method of Batzri and Korn (1973) and modified by Fabczak (1986), (d) yolk suspension (10 μg per ml). Samples of the control and tested cells from different nutrient media (i.e., peptone, yolk and liposome suspension) were pulsed with 0.1% latex suspension (from Polyscience) during 5 min and 30 min time intervals, and then fixed in 2.5% glutardialdehyde solution in cacodylate buffer. The number of digestive vacuoles formed was counted under Zeiss microscope in < 50 cells in each sample.

Ciliate Proliferation. Parallel with the previous experiments the observation of the rate of the divisions of the ciliates were performed. For this the proportion of dividing individuals was determined among the cells starved initially for 3 or 24 h time period and then placed in the tested

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nutrient media for a period of 24 h. The cell density in the particular samples was determined with the use of Levy counting chamber.

DNA Estimation. Estimation of DNA amount was performed according to method of Bollum (1959). Cells starved for 24 h were incubated in ^{14}C-thymidine to estimate the DNA synthesis. The radiolabelled material (12 μCi per ml) was added directly to the 0.5 ml suspension of cells for 30 min exposure at temperature of 28°C. Then the samples were treated with 0.5 ml trichloroacetic acid (TCA) and incubated for 2 h at temperature of 0°C. DNA precipitated by TCA was filtered through Synpor membrane (0.1 μm of filter pore dia.) and washed five times in 5% TCA and later twice in 96% ethanol. The samples were then dried and resuspended in 5 ml of toluene-based scintillation coctail and labelled thymidine was determined in Beckman counter. The concentrations of cell protein were estimated according to method of Lowry (Lowry et al. 1951).

Results

The rate of latex-induced phagocytosis in cells of *Tetrahymena* incubated in "edible" suspensions (peptone, yolk and liposomes) is much higher than in the control cells (Table 1 and 2). For instance, cells starved for 24 h period, after 30 min incubation in 2% proteose peptone suspension, form a mean number of 3.5 food vacuoles per cell, whereas in the controls only 1.4 dv per cell are formed. If one compares the values obtained in both starvation periods (Table 1 and 2), it is clear that the

Table 1

The rate of phagocytic activity of *Tetrahymena pyriformis* incubated in different nutrients after 24 h starvation

	Duration of exposure (in min) to the media with different suspension						
	0	30	60	90	120	150	180
Peptone	2.8	3.5	3.5	3.6	3.4	3.6	3.5
Yolk	2.1	2.5	2.2	2.3	2.6	2.5	2.5
Liposomes	1.8	2.0	2.4	1.7	1.7	1.5	1.0
Control	1.5	1.4	1.4	1.5	1.5	1.6	1.4

The data in columns represent mean values from five experiments (≤ 50 cells). Time of phagocytosis induction was 5 min. In control, ciliates were incubated in Pringsheim solution

Table 2

The rate of *Tetrahymena pyriformis* phagocytosis in various nutrient media after 3 h starvation

	Duration of exposure (in min) to the media of different suspensions						
	0	30	60	90	120	150	180
Peptone	5.0	4.7	4.6	4.4	4.2	4.3	4.1
Yolk	4.1	3.9	3.6	3.4	3.6	3.5	3.4
Liposomes	2.9	3.0	3.1	2.6	1.8	1.7	1.5
Control	2.6	2.8	2.4	2.2	2.0	2.1	2.0

Exposure to latex suspension was 5 min. Columns include the data as mean values of five experiments (≤ 50 cell). Control cells were incubated in Pringsheim solution

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duration of starvation has a distinct influence on the phagocytic activity of the ciliates. The process of dv formation is much more efficient in the case short-lasting starvation (3h) than when this treatment is prolonged. Moreover, an analysis of histograms (Fig. 1 and 2) of the phagocytic rate distribution in the particular cells shows that the ciliates which do not form vacuoles represent a large fraction of cells which were starved during 24 h. This phenomenon is hardly noted after a short-lasting cell starvation (Fig. 2).

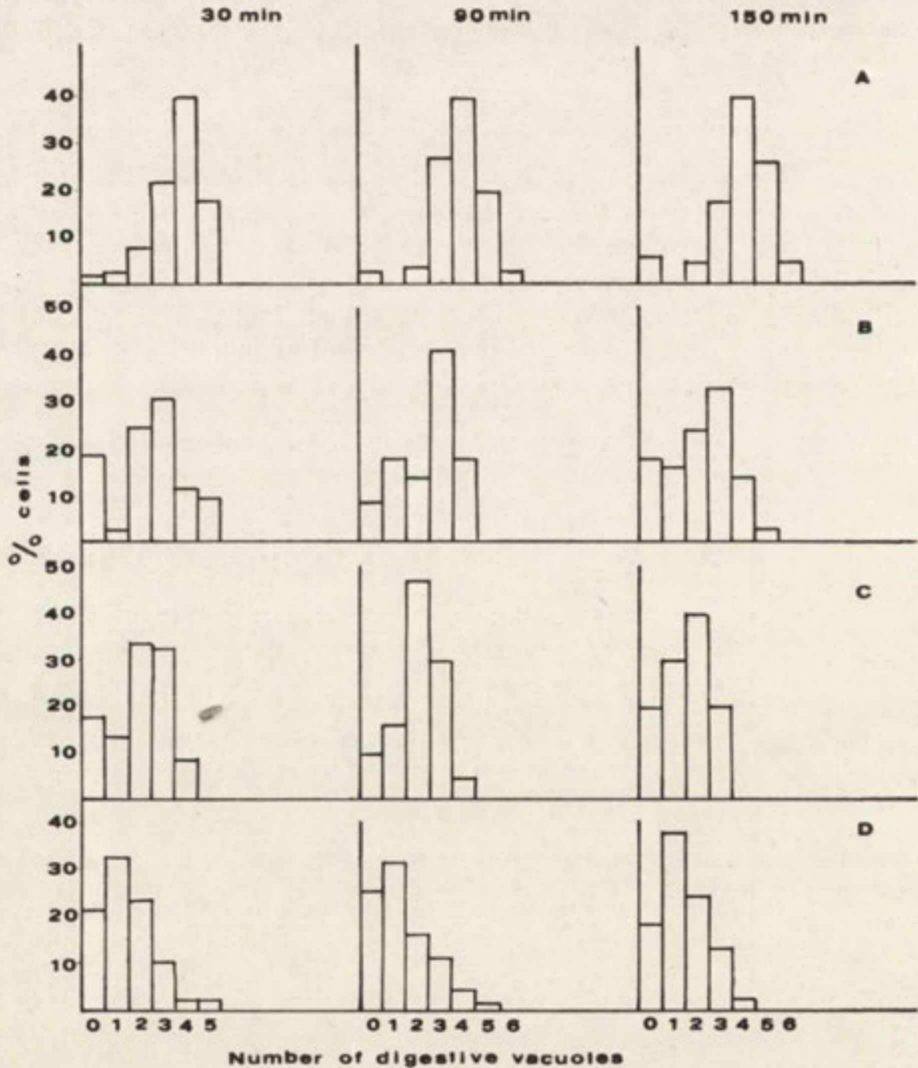

Fig. 1. Distribution of digestive vacuoles formed by *Tetrahymena pyriformis* during exposure to different food media: peptone (A), yolk (B), liposomes (C) and control (D). Exposure to latex particles and starvation time durations were 5 min and 24 h, respectively

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Tetrahymena cells exposed to the liposome suspension does not show in the first 60 min any major difference in the rate of dv formation as compared with other ciliates in "edible" suspensions. The prolongation of exposure affects the dv formation, so that after 150–180 min of continuous treatment with liposomes, the rate values are equal or even lower than those of the control. This is true in the cells starved for 3 and 24 h period. (Table 1 and 2).

The percentage of dividing cells in the population starved for 3 h and then exposed to the nutrient media or Pringsheim solution (control) are shown in Table 3. After continuous feeding for 2 h, about 1% of cells are in the course of division (division furrow is visible). Table 3 presents data only for cells subjected to the short

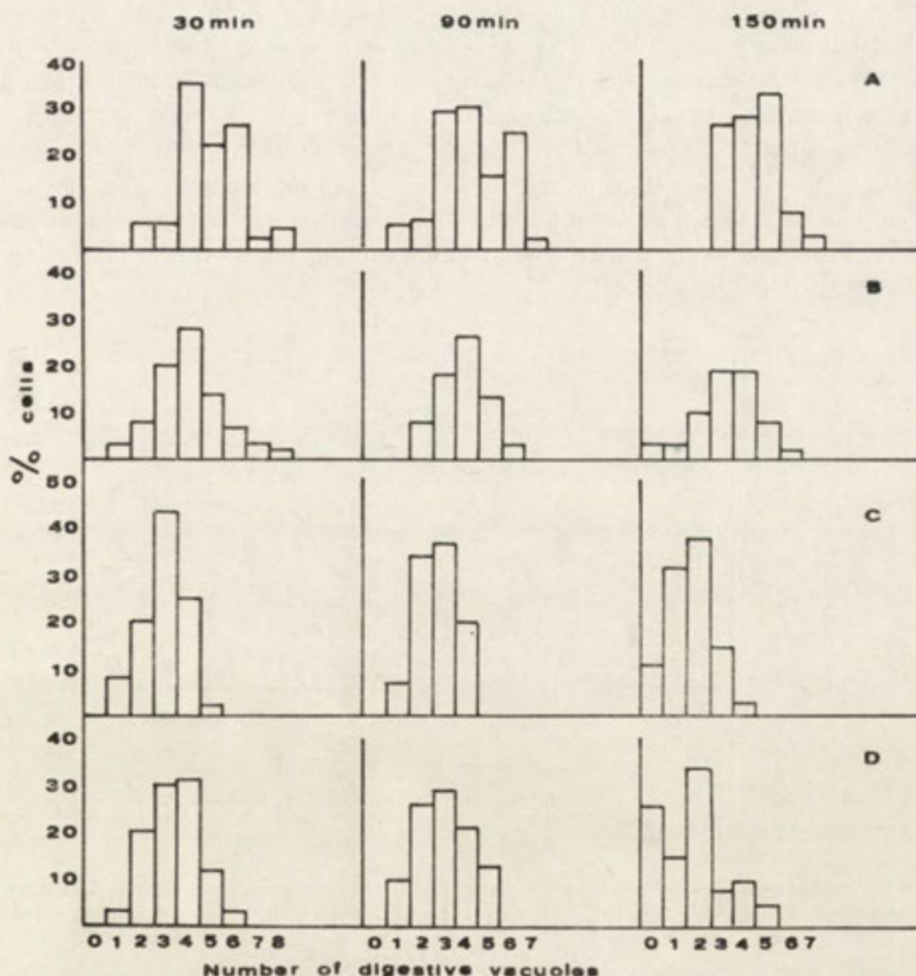

Fig. 2. Distribution of digestive vacuole formation in *Tetrahymena pyriformis* cells during incubation in peptone (A), yolk (B), liposomes (C) and control (D) suspensions. Exposure to latex particles and starvation time durations were 5 min and 24 h, respectively

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Table 3

Tetrahymena cells exposed to various nutrient suspensions during 2 or 4 h. Starvation time was 3 h

	Nutrient suspensions			
	Peptone	Yolk	Liposomes	Control
2 h	1%	0.3%	1.0%	–
4 h	9%	6%	7.0%	2.0%

The results of five experiments are expressed as percentage of dividing cells in the samples of 50 cells in each experiment

starvation, since in samples starved for 24 h no dividing cells were found. This does not indicate an inhibition of mitosis in this group of cells, since cell density calculated after period of 24 h of continuous feeding showed a doubling of the number of *Tetrahymena* cells. The cells subjected to 3 h of starvation and then fed for 24 h undergo about 2 divisions.

In the nutrient suspensions, 120 min after the start of incubation in ^{14}C-thymidine, DNA synthesis begins (Fig. 3). This process occurs most rapidly and efficiently in cells exposed to 2% peptone. Cells in yolk and liposome suspensions synthesize DNA slower and in a smaller quantity. In the controls the process of increasing of DNA pool is hardly noticeable.

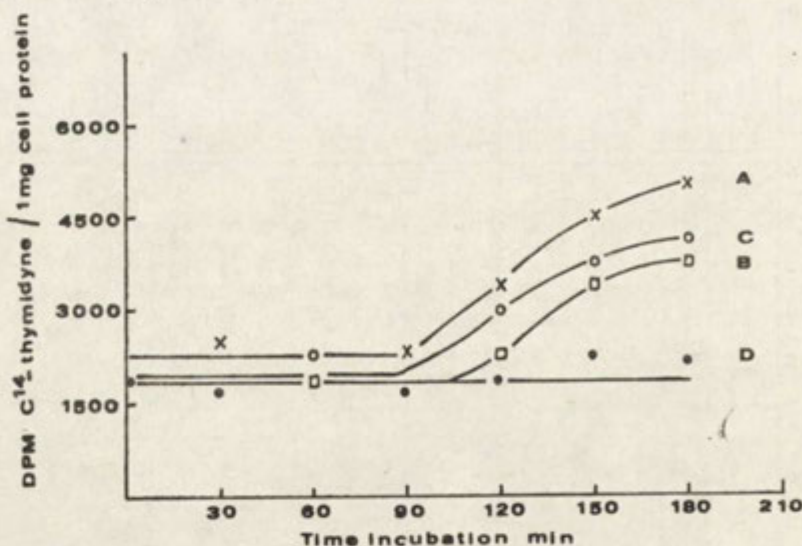

Fig. 3. Amount of ^{14}C-thymidine incorporated in DNA of *Tetrahymena* cells during incubation in suspensions: peptone (A), yolk (B), liposomes (C) and control (D). Cells were starved for 24 h

Discussion

Phagocytosis is a common form of food uptake, not only among protozoans, but in cells of higher organisms also. Ciliates, however, are a convenient material for investigation of such processes, because the specimens are easily grown under labo-

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ratory conditions and relatively simple approach might be utilized in the analysis one. Although phagocytosis has been studied in protozoan organisms for many years, the details are not elucidated to this day. It was ascertained that the cell membrane is the structure playing a very decisive role in the course of phagocytosis. On the one hand, membrane is a barrier separating the cytoplasm from the environment, but on the other hand, it mediates introduction of food in liquid (pinocytosis) or solid (phagocytosis) form.

Previously it has been demonstrated that the incubation of cells of *Tetrahymena* in cholesterol contained liposome suspensions results in a considerable reduction of the rate of food vacuole formation process (Fabczak 1986). It was suggested that those changes are connected with the influence of cholesterol on protozoan cell membrane function. The investigations carried out on the vacuole formation in *Tetrahymena* by Chapman-Andresen and Nilsson (1968) and Nilsson (1979) have showed that the cell phagocytic activities are dependent greatly on a stage of cell cycle. The highest rate of food ingestion is seen just prior to the cell division, but no vacuoles are formed during the period of cell division. To preclude possibility that the depression of vacuole formation, observed in cells exposed to cholesterol enriched liposomes, occurs on account of the division of *Tetryhamena* cells the influence of selected nutrient media on phagocytic activity and ^{14}C-thymidine incorporation into the cells were performed. The results presented in Table 1 and 2 show that during incubation of *Tetrahymena* in "edible" media (yolk, peptone) the phagocytic activity levels are much higher than in control and almost constant within the applied incubation time, whereas the ^{14}C-thymidine incorporation has tendency to rise. Considering the fact that the experimental conditions of the measurements approached nearly the conditions of cell synchronization (Cameron and Jeter 1970), and above-mentioned data it is reasonable to presume that observed phagocytic activity inhibition by cholesterol contained liposomes is due to cholesterol action on cell membrane rather than the division of *Tetrahymena* cells. The similar phenomenon has been observed in monocyte-macrophage like line cells (cholesterol auxotroph mutant) (Esfahani et al. 1986). The authors suggested that the fall of the endocytosis (pinocytosis) activity level was caused by a drastic decrease of the cholesterol to phospholipid ratio followed by the alteration of membrane fluidity. Conner et al. (1968, 1971) demonstrated that the presence of sterols (cholesterol and ergosterol) in the cell medium not only inhibits tetrahymanol synthesis in cells of *Tetrahymena*, but these lipids may substitute it in the protozoan membrane. Nozawa et al. (1975) noted that, for instance, ergosterol is cumulated by *Tetrahymena* cells in high quantities. One may presume that a similar phenomenon takes place in the case of cholesterol.

If we compare the results in Table 1 and 2, a wide disproportion is seen between the rates of digestive vacuole formation in cells starved for 3 and 24 h initially and then exposed to phagocytosis induction by latex. Such phenomenon was described by Riketts (1971, 1972). According to author, *Tetrahymena* cells are capable to distinguish nutritive particles from non-nutritive (undigestible) ones, but the essen-

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tial factor influencing the rate of phagocytic activity is the degree of cell starvation. *Paramecium* cells behave similarly to cells of *Tetrahymena* (Wasik et al. 1987). This observation is difficult to explain, possibly the "curious alteration in membrane lipid composition" which occurs during a prolonged starvation of cells of *Tetrahymena* is the one of the cases (Thompson et al. 1972). The author reported that the molar ratio of tetrahymanol to phospholipid in the whole lipid pool rose to 270% of normal value during the starvation period of 93 h.

On the basis of above-presented data, it may be suggested that an alteration of the amount of cholesterol molecules in the cell membrane of protozoan ciliate *Tetrahymena* can much influence the cell membrane function (phagocytic activity) as a result of an alteration of membrane properties (fluidity) what frequently has been demonstrated in mammalian and model membranes.

ACKNOWLEDGEMENTS

The author is grateful to Mrs. Bożena Groszyńska for her helpful technical assistance.

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ACTA PROTOZOOLOGICA
Vol. 27, No. 2, pp. 117–123 (1988)

The Resting and Action Membrane Potentials of Ciliate *Blepharisma japonicum*

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Received on 23rd October 1987

Synopsis. The electrophysiological studies were performed on a fresh water ciliates *Blepharisma japonicum* by means of glass microelectrode and standard electronic recording techniques. In Pringsheim's external solutions the resting membrane potential was −41 mV +/−1.7 (SEM). It was found that it depends on the concentrations of extracellular potassium and sodium ions. When the concentration of both ions was increased in Pringsheim's medium the depolarization of the cell membrane potential was observed. Suprathreshold intracellular current injections induced action membrane potentials associated with ciliary reversal. For this type of stimulations the voltage-current relationship was plotted. The resting and action potential parameters of the cell membrane of *Blepharisma* were essentialy similar to those of other ciliates.

Ciliate protozoan cells have long been known as an excellent material for biological research, because the specimens are easily grown under laboratory conditions and relatively simple approach might be utilized in the analysis of behavior of a single cell organism as compared with metazoa, where cell behavior reflects rather complex mechanisms. Recently, some electrical characteristics of the cell membrane and the possible causal relationship between cell behavior and changes in membrane potential were investigated by several research groups on various ciliate cells and well documented data were obtained (Kinosita et al. 1964, Machemer and de Peyer 1977, Byrne and Byrne 1978, Doughty and Dryl 1981, Wood 1982). Much less is known the mechanism of ion movement between the protozoan cell interior and its environment, which is of essential importance for cell membrane electrogenesis and for the maintenance of normal cell behavior as well. The existence of such or similar ionic regulation across the cellular membrane in protozoa as those encountered in the nerve and muscle cells has been postulated, but obtained experimental data on this subject are incomplete and often contradictory (Carter 1957, Dun-

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ham and Child 1961, Andrus and Giese 1963, Conner 1967, Hilden 1970 Wood 1982, Connolly and Kirkut 1984). This study was designed to investigate the basic electrophysiological properties of the ciliate *Blepharisma japonicum* and to compare them with those of other ciliate specimens.

Materials and Methods

Ciliates and Culture Conditions. Stock cultures of the cells of *Blepharisma japonicum* were maintained in Pringsheim's medium at pH 7,2 in semidarkness at room temperature (19–21°C). The food source for the protozoans were *Tetrahymena pyriformis* from axenic cultures. The cells chosen for experiments were transferred to a fresh culture medium without nutritional component for a few hours, and after the change of the medium, they were placed in the experimental chamber.

Measurement Procedure. The cell membrane potentials were measured in a designed teflon chamber mounted on a stereoscopic microscope stage. The chamber had a volume of about 0.8 ml and was perfused with the tested solutions by a peristaltic pump at a rate of 3.0 ml/min continuously, or by an automatic micropipette. The bath solution temperature in the chamber was continuously controlled by a feedback Peltier's device and monitored by an additional solid state thermistor (Philips Co., W. Germany), calibrated against a precise digital quartz thermometer (type PTK-1, Zopan, Poland). All experiments were carried out at constant temperature of 16°C, which reduced the cell swimming velocity and facilitated cell microelectrode puncturing. At the beginning of each measuring procedure the cells chosen for the experiment were adapted for half an hour in the experimental chamber to the new environment (i.e., bath temperature, medium with changed of K^+ or Na^+ concentrations), and one cell was penetrated by two glass microelectrodes with the aid of an horizontal mechanical micromanipulator (type KM-1, USSR).

Recording Equipment. Conventional glass micropipettes were drawn from pyrex tubes (type 1B150F-4, W.-P. Instr., USA) with an horizontal electric puller. Each micropipette was filled with 1 M KCl solution. Before recordings, the filled glass micropipette was put with the aid of a micromanipulator in the chamber and its tip was gently broken by touching to the chamber bottom to obtain the micropipette outer tip diameter of 0.5 to 1.5 μm, i.e., the microelectrode resistance of 30 to 50 Mohms. The microelectrode resistance was usually checked after cell penetration as well, and the measured membrane potential data were taken into account when no change in resistance occurred. A liquid junction potential for employed glass microelectrodes was usually lass than 3 to 5 mV. The voltage recording microelectrode was connected to the electronic recording system through nonpolarizable Ag-$AgCl_2$ wire. The bath solution was coupled with the circuit via Ag-$AgCl_2$-agar half cell. The recorded fast and slow electrical transients delivered by microelectrodes were displayed on an oscilloscope (type 5103N, Tektronix Inc., USA) or on a digital panel voltmeter type V-650, Meratronik, Poland).

Results

Resting Membrane Potential. The glass microelectrodes before use were first kept outside the cell in the tested solution for some time to equilibrate and reduce the tip junction potential of a voltage recording microelectrode to zero. The ciliate cell was penetrated usually through the cell membrane in the middle part of the cell body by two glass microelectrodes, voltage and current, simultaneously.

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The correctness of microelectrode penetration was monitored occasionally by passing the current across the cell membrane with the current microelectrode. In standard conditions (i.e., Pringsheim's medium, bath temperature of 16°C) the membrane potential for nonstimulated *Blepharisma* cell was −41 mV +/−3.7(SEM) (n = 23).

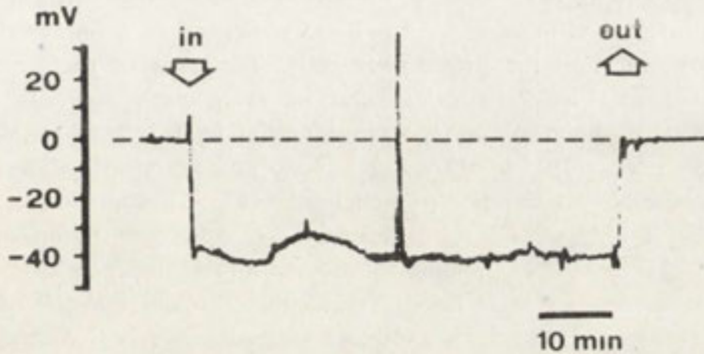

Fig. 1. Oscilloscope trace of membrane potential measurement in *Blepharisma japonicum*. Occasionally spontaneous rapid and slow depolarizations were seen. Arrows mark the insertion (in) and withdrawal (out) of voltage microelectrode

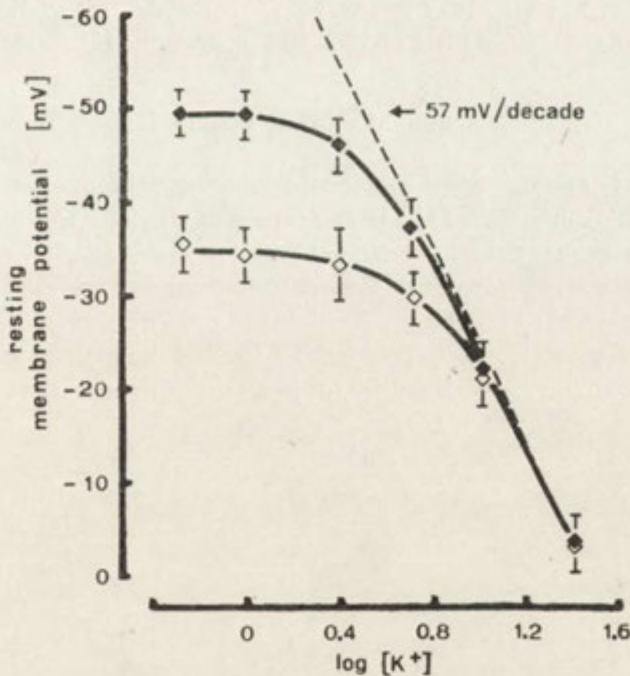

Fig. 2. The relation between extracellular potassium concentration and resting membrane potential in standard Pringsheim's solutions (open rhombes) and when sodium was reduced by substitution with TRIS-HCI buffer or choline chloride (closed rhombes). The dotted line represents the predicted K^+-concentration-potential relationship by Nernst equation. Each point is the mean (+/− SEM) of the number of punctured cells

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Figure 1 illustrates a typical electrophysiological experiment where cell resting membrane potential was measured over a period of half an hour. The various cells show a high dependence of a transmembrane potential on external potassium concentration, in a manner suggesting that a passive diffusion of potassium ions to the outside of the cell produces a rise of the cell membrane potential. For this reason, the resting internal membrane potential was measured as a function of extracellular K^+ concentration for *Blepharisma* cells. The data of such measurements are presented in Fig. 2, showing that in Pringheim's solution the evident depolarization of the membrane potential occurs as K^+ increases, however the relationship between potential and K^+ concentration clearly differs from that predicted by the Nernst equation (dotted line in Fig. 2), and, in fact, marginally approximates it only at high K^+ concentrations. At concentration of potassium below 2.5 mM membrane potential becomes almost independent. These data suggest that ions other than K^+ are permeable through the cell membrane in *Blepharisma* as well. However, when the experiment was repeated using TRIS-HCl buffer or choline chloride solutions instead of NaCl present in standard medium (thereby decreasing the bulk Na^+), the potential-K^+-concentration relationship became more linear and more closely approximated the Nernst equation for potassium ions, but still this linearity was very poor below 2.5 mM of K^+. The X-intercept of the line gave an estimate of 28.2 mM of cytosolic K^+ concentration.

Action Membrane Potential

The *Blepharisma* cells bathed in standard Pringsheim's solution produced spikes (action potential) in nongraded manner when depolarized to approximately −33 mV, and sometimes the cells generated spikes spontaneously. The rapid reversal of ciliary beating was correlated with both stimulated and spontaneous potentials. At the normal resting membrane potential, the action potentials had amplitudes of 59 to 71 mV above the resting potential value and duration of the spike at half amplitude was 97.2 ms+/−5.9(SEM). An injection of inward current into cell cy-

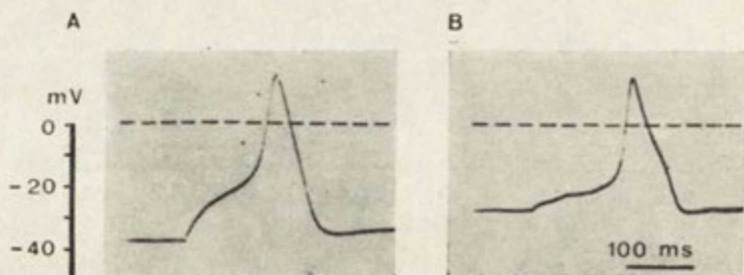

Fig. 3. Oscilloscope traces of action potential measurements from the cells of *Blepharisma japonicum*. The potentials were evoked by intracellular current injection (A) or generated spontaneously by the cell (B)

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toplasm produced a passive RC response of the membrane with following gradual decrease in the potential (Fig. 3). To determine the membrane input impedance for *Blepharisma* cells, the squares pulse and ascending ramp analyses were utilized (Fatt and Katz 1951, Starzak et al. 1977). The voltage responses of the membrane at the end of the long current pulses of various intensities or with current rump stimulation are plotted in Fig. 4. The membrane impedance of *Blepharisma* cells determined from standard I–V curves was in the range of 28 to 40 Mohms and displayed a distinct outward going rectification.

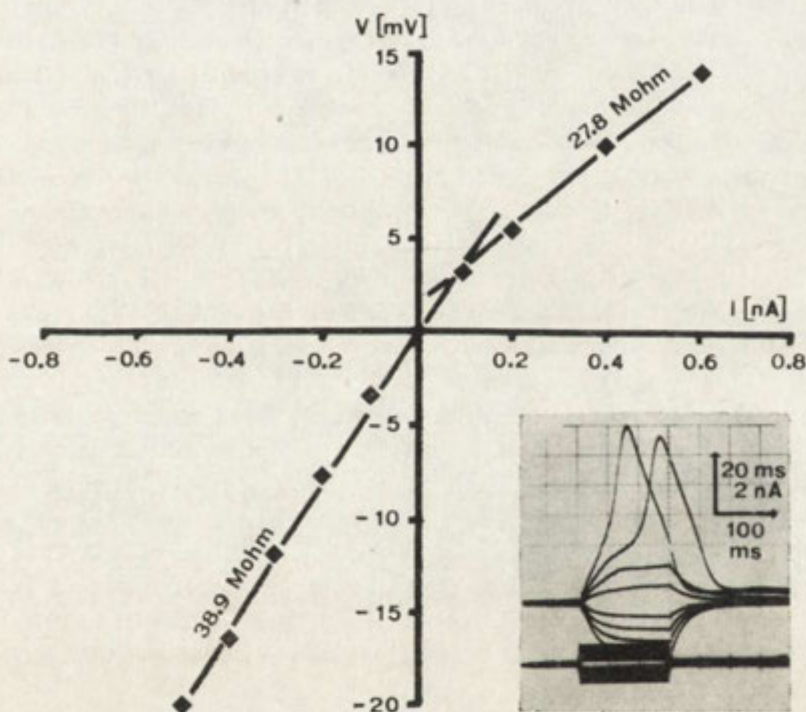

Fig. 4. The current-voltage relationship for the cell membrane of *Blepharisma*. Part of the data of current injection and voltage recording is included as an inset: lower traces-current record, upper traces-voltage responses

Discussion

The resting membrane potential of *Blepharisma* cells, as in case of other ciliates, inside negative, responds to the changes of the potassium concentration in a medium, hence it is clear that the cell membrane is permeable to those ions (Fig. 2, Akita 1941, Wood 1982, Connolly and Kirkut 1984). Considering the level of cytosolic concentration of potassium in *Blepharisma* measured by spectrophotometry by Hil-

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den (1970), the electrochemical equilibrium K^+ potential has to be much higher (about —103 mV) than the observed factual membrane potential value (Fig. 1 and 2). On the other hand, the potential-K^+-concentration analysis for the line X-intercept in Fig. 2 gives the value of 28.2 mM of K^+ in the cell cytoplasm much lower than the amount estimated by spectrophotometry (Hilden 1970). The reasons of discrepancy between the above mentioned data for the *Blepharisma* cell might be the following: (a) the cell potassium activity is greatly diminished, (b) the most of the cytoplasmic potassium is bound, or (c) the cell membrane potential is maintained by a specific active ion transport. The first case does not seem likely, since it would require that the activity of potassium varies almost instantaneously with the external potassium level changes in order to account for the observed linear relationship between the membrane potential and the higher external K^+ concentration. The second possibility may occur in the *Blepharisma* çell, since it has been observed in amoeba cells that cytosolic potassium is not entirely exchangeable and occurs in the cytoplasm partially as a bound fraction (Klein 1959, 1964). From that point of view the data obtained by means of flame spectrophotometry are incomplete, as a direct ion activity measurement is impossible. No definite information about the specific mechanism of ionic regulation in *Blepharisma* cells is not yet available. It seems likely that at least sodium ions are actively transported against an electrochemical gradient, and in the absence of any reliable results on the intracellular level of potassium and potassium fluxes across the membrane, it is unwise to exclude an active uptake of K^+ ions (Hilden 1970). Similarly, the existence of active potassium transport was put forward by Glynn (1959) to explain discrepancy between K^+ concentration within the red cells and the magnitude of the transmembrane potential. The failure of the *Blepharisma* cell membrane to behave as true potassium electrode has been found in various metazoan cells that have been investigated. Thus the relationship of membrane potential to external K^+ potassium of the squid axon is not consistent with the Nernst equation at lower K^+ concentrations (Hodgkin 1958). In frog muscle cells the membrane potential was appreciably lower than the K^+ equilibrium value (Tobias 1950, Adrian 1956).

As in other ciliate protozoa, a depolarizing electrical stimulus causes the change of membrane potential in *Blepharisma*; the cell cytoplasm become less negative (Fig. 3). If the stimulus is strong enough to cause ciliary reversal, the slow initial rise in membrane potential is followed by a very rapid one to a peak potential. When inward current was injected into the cell, passive membrane responses were observed (inset in Fig. 4). The input membrane resistance of *Blepharisma* cell was approximately 40 Mohms (Fig. 4), which is in the same range as that for other ciliates (Machemer and de Peyer 1977, Connolly ad Kirkut 1984). The ionic bases of the action potential generation in *Blepharisma* was not investigated. It will be the object of the next paper.

The conclusions to be made from this preliminary study is that the resting membrane and action potential characteristics of *Blepharisma* cells are generally similar

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to those of other ciliates. The study indicates that the cells of *Blepharisma* would be a viable and usefull physiological preparation for conventional or ion-selective electrophysiological investigations.

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ACTA PROTOZOOLOGICA
Vol. 27, No. 2, pp. 125–134 (1988)

Selective Precipitation of Calcium Ions by N,N-naphthaloylhydroxylamine — a Cytochemical Approach

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Received on 30 October 1987

Synopsis. The process of precipitation of cellular important inorganic cations (Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}) by N,N-naphthaloylhydroxylamine (NHA) in test tubes was examined. NHA is capable to form complexes exclusively with calcium ions as was demonstrated by means of ion-selective microelectrodes and radioisotope techniques. No interaction of NHA with Mg^{2+}, K^{+}, Na^{+} was detected. The formed Ca-NHA complex was very stable; being insoluble in 100 mM EGTA solution. High stability of Ca-NHA complexes and high selectivity of NHA to precipitate only Ca^{2+} ions indicate on the unique properties of NHA as Ca-capturing reagent. The NHA procedure was used for visualization of cellular calcium under electron microscope.

Concentration of calcium ions in cytoplasm of animal cells is in a range of 0.1–5.0 μM (Carafoli and Crompton 1978, Connor 1986). Such low level of free calcium ions in cytoplasm is maintained by different mechanisms. Part of cellular calcium is stored in a non-ionic form and through its mobilization the concentration of calcium ions within the cells can be regulated (Coleman et al. 1972, Cavero and Spedding 1983, Langer 1984). Therefore, much attention is focused on localization of calcium at subcellular level.

Different cytochemical methods are used for localization and visualization of calcium ions within the cells (Caswell 1979). The cellular calcium is usually precipitated by capture agents and in result the insoluble, electron-dense precipitates appear, which are detectable under electron microscope. At present time potassium pyroantimonate and ammonium oxalate are often used as Ca-capturing agents for demonstration of calcium in biological samples (Constantin et al. 1965, Podolsky et al. 1970, Slocum and Roux 1982, Wick and Hepler 1982, Borgers et al. 1984). Oxalate as a Ca-capturing agent shows relatively high specificity but low sensitivity for calcium ions (Caswell 1979). Contrary to oxalate, potassium pyroantimonate although sensitive, is not a Ca-selective capture agent, since it precipitates

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in various degrees a number of other cellular cations such as Na^{+} and Mg^{2+} (Lane and Martin 1969, Shina et al. 1970, Torack and LaValle 1970, Klein et al. 1972).

Calcium ions can also be precipitated by N,N-naphthaloylhydroxylamine (NHA). This calcium-capturing agent was firstly used by Beck (1951) for quantitative determination of calcium in solutions. Later, the agent was applied in cytochemistry by Voight (1957) for demonstration of cellular calcium under light microscope. Zechmeister (1979) and Przełęcka et al. (1986) used it for demonstration of cellular calcium under electron microscope. However, the selectivity of this reagent towards calcium ions vs. magnesium ones was not checked.

In this study we examine the selectivity of NHA to precipitate calcium, magnesium, sodium and potassium ions. For this purpose two independent techniques, radioisotope and ion-selective microelectrode, were used. The NHA-evoked precipitates *in vitro* and within the cells are demonstrated.

Materials and Methods

Acanthamoeba Culture

Acanthamoeba castellanii (Neff strain) was cultured axenically in optimal growth medium as described earlier (Sobota et al. 1984). The 7-day old culture were used for the experiments.

Localization of Cellular Calcium by NHA

Acanthamoeba cells were fixed in 2.5% glutaraldehyde containing 5 mM NHA as Ca-precipitating agent, 120 mM NaCl, and 50 mM collidine buffer, pH 7.4, for 2 h at 20°C. Then the cells were pelleted by centrifugation (600 g, 2 min) and washed twice with a solution containing 120 mM NaCl, 50 mM collidine buffer, and 5 mM NHA. The samples were postfixed with 1% osmium tetroxide containing NaCl-collidine-NHA solution. Dehydration of the samples was carried out in a graded ethanol (30, 70, 100%) supplemented with 5 mM NHA (except 100% ethanol). The control samples were prepared without NHA in all the used solutions.

Radioisotope Study of Precipitation of [^{45}Ca]$CaCl_2$ by NHA

N,N-naphthaloylhydroxylamine (Fluka) was dissolved in deionized water and a 50 mM stock solution was freshly prepared before each set of experiments. The formation of Ca-NHA precipitates was carried out in plastic tubes according to procedure described earlier (Sobota et al. 1987). Briefly, the radioactive labelled calcium ions [^{45}Ca]$CaCl_2$ in range of 5 μM–1 mM, were mixed with 5 mM NHA solution containing 2% glutaraldehyde and 100 mM collidine buffer, pH 7.4, and incubated for 1 h at 20°C without agitation. The total volume of incubation medium was 1 ml. After incubation the precipitates were pelleted by centrifugation (11 000 g, 10 min). The formed [^{45}Ca]Ca-NHA precipitates occurred to be incompletely soluble in acids (6N H_2SO_4, 2N HCl) as well as in 100 mM EGTA, therefore for calculation of the amount of precipitated calcium ions the resting $^{45}Ca^{2+}$ radioactivity of supernatant was substracted from the initial total radioactivity before precipitation. For that the corresponding supernatants were collected, dried in scintillation vials, and after addition of liquid scintillator, were counted in Beckman liquid counter.

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Measurements of Ca^{2+} and Mg^{2+}-ion Activities with Ion-selective Microelectrode

To determine the activities of calcium and magnesium ions the ion-selective and reference microelectrodes were pulled from cleaned glass Kwik-Fill cappilaries (1B150F, W-P Instr., USA) in standard microelectrode pullers to tip sizes of about 2 to 5 μm in diameter. The reference microelectrode was filled with 1 M KCl solution from the stem end of capillary. Ca^{2+}-ion-selective microelectrode were filled with 0.1 M $CaCl_2$ and ion exchanger (IE-202, W-P Instr., USA), whereas Mg^{2+}-ion selective microelectrodes were filled with 0.1 M $MgCl_2$ and ion exchanger (IE-120, W-P Instr., USA). The internal surface of the glass capillaries was siliconized and filled with ion-exchanger as it has been described elsewhere (Walker 1971, Fabczak 1983).

Measurements of ionic electrochemical gradients in tested solutions as electrical signals appeared at the microelectrode outputs were performed with high input impedence differential amplifier. The signals were displayed on a storage slow-sweep dual beam oscilloscope (5103N, Tetronix Inc., USA) or on the digital voltmeter (V560, Meratronic, Poland) for precise voltage readings.

The determination of Ca^{2+} and Mg^{2+}-ion activities was done in a chamber of 0.8 ml volume with perfusing capability at rate of about 1.5 ml/min. During experiments the tested solutions were changed without interrupting fluid flow by means of a six-way tap mounted near the chamber.

Results

Precipitation of Calcium by NHA

Addition of NHA to solution containing calcium ions evoked the appearance of orange-pink precipitate. Figure 1 shows that calcium ions over a range of 5 μM to 1 mM was completely precipitated by 5 mM NHA. The precipitation at lower concentration of calcium ions was not examined since the formed tiny precipitates may be lost during their separation by centrifugation. However, the possibility to precipitate calcium ions at such a low concentration as 5 μM would be very important for cytochemical and cell biology studies of cellular free calcium ions.

The precipitation of calcium ions dependend on concentration of NHA added to the media. The 1 mM Ca^{2+} was completely precipitated by 3 mM NHA (Fig. 2). Addition of 2 mM $MgCl_2$ to 1 mM solution of $CaCl_2$ had no influence on precipitation of calcium ions by NHA (Fig. 2). On the other hand, if the 1 mM $CaCl_2$ was supplemented with 2 mM $SrCl_2$ (calcium analogue), the precipitation of calcium ions by NHA was significantly reduced. No changed in precipitation of calcium ions by NHA was observed in the presence of 100 mM NaCl or 100 mM KCl (data not presented).

The selective capturing of calcium ions by NHA vs. magnesium ions was also demonstrated in experiments with ion-selective microelectrode. Table 1 presents the measurements of subsequent changes in Ca^{2+} ion activity under different conditions. Addition of 5 mM NHA to a solution containing 0.1 mM $CaCl_2$ evoked dramatic changes in content of free calcium ions in the solution: the concentration of Ca^{2+} ions decreased below 10^{-7} M. The similar changes of calcium ions concentration was observed when 0.1 mM calcium solution was supplemented with 5 mM EGTA, a well-known calcium-chelating agent.

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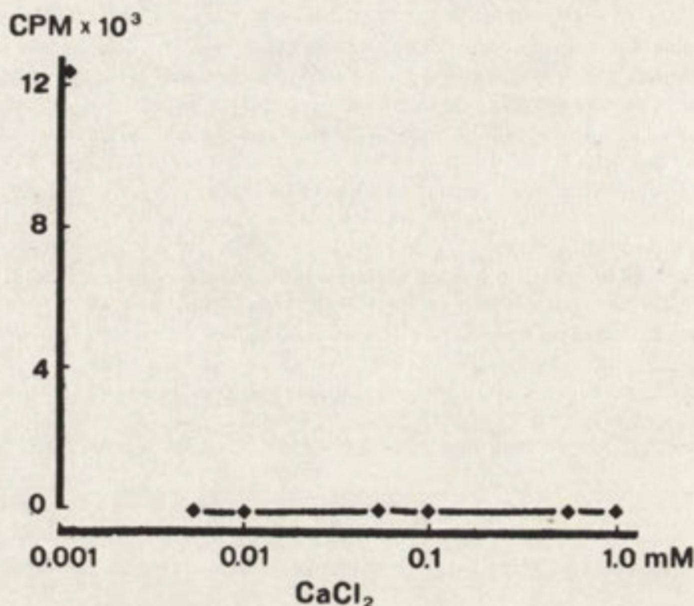

Fig. 1. Precipitation of calcium ions by 5 mM NHA. The examined calcium solutions (5 μM to 1 mM) were supplemented with ${}^{4}CaCl_2$ (13200 cpm) and 5 mM NHA. After 1 h incubation the formed precipitates were pelleted. The remaining ${}^{45}Ca^{2+}$-radioactivity of supernatants was counted and substracted from initial radioactivity, indicating the level of non-precipitated calcium ions

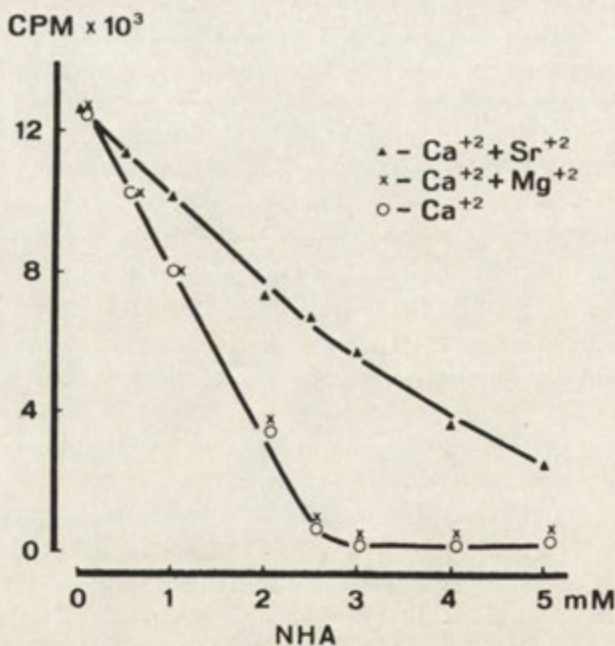

Fig. 2. Precipitation of calcium ions by different concentrations of NHA in the presence of Mg^{2+} or Sr^{2+}-ions

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Table 1

Calcium ions activities (αCa^{2+}) in different solutions as measured by means of Ca^{2+}-selective microelectrodes. The electrode was calibrated by perfusing the experimental chamber with 0.5, 5.0 and 50 mM $CaCl_2$ solution

Tested solution	Electrode potential E^{2+}_{Ca} (mV)	Ca^{2+}-activity α^{2+}_{Ca} (mM)
H_2O	−70	0.006
0.5 mM $CaCl_2$	−13	0.5
0.5 mM $CaCl_2$+5 mM NHA	−125	< 0.00001
H_2O	−70	0.006
0.5 mM $CaCl_2$+5 mM EGTA	−115	< 0.00001
H_2O	−70	0.006
0.5 mM $CaCl_2$	−12	0.5
5.0 mM $CaCl_2$	+19	5.0
50.0 mM $CaCl_2$	+51	50.0

Activity of magnesium ions in the presence of NHA was measured with Mg^{2+}-ion selective microelectrode. As shown in Table 2, the activity of Mg^{2+} ions was not changed in the presence of NHA.

In result, the radioisotope analysis and, independently, ion-selective measurements of the activity of Ca^{2+} and Mg^{2+} ions in the presence of NHA clearly demonstrated that NHA was a selective Ca-capturing agent which practically did not interact with Mg^{2+}, Na^{+}, and K^{+} ions in solution.

Table 2

Magnesium ions activities (αMg^{2+}) in different solutions as measured by means of Mg^{2+}-selective microelectrode. The electrode was calibrated by perfusing the experimental chamber with 0.5, 5.0 and 50.0 mM $MgCCl_2$ solution

Tested solution	Electrode potential E^{+2}_{Mg} (mV)	Mg^{2+}-activity αCa^{2+}(mM)
H_2O	−78	0.005
2 mM $MgCl_2$	−4	2.0
2 mM $MgCl_2$+5 mM NHA	−2	2.05
H_2O	−77	0.005
0.5 mM $MgCl_2$	−20	0.5
5.0 mM $MgCl_2$	+10	5.0
50.0 mM $MgCl_2$	+45	50.0

Stability of Ca-NHA Precipitates

During the handling of Ca-NHA precipitates it was found that these complexes were very stable. The Ca-NHA precipitates could not be completely dissolved in 6N H_2SO_4, 4N HCl, and 2N CH_3COOH. Also an incubation of Ca-NHA precipitates with strong Ca-chelating agents, such as 100 mM EGTA and 100 mM so-

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dium citrate, had no effect. The dissolving of the precipitate was observed only on the fifth day of incubation with 100 mM EGTA. Insolubility of Ca-NHA precipitate in the above mentioned solutions indicated its high stability and suggested that this complex was much more stable than the Ca-EGTA one.

The stability of Ca-NHA precipitate was examined also under the conditions similar to these used during preparation of biological material for electron microscopy. Therefore, the precipitates labelled with $^{45}Ca^{2+}$ consecutively treated with 2% glutaraldehyde, collidine washing solution, 1% osmium tetroxide, and series of ethanol. Amounts of the dissolved Ca-NHA in all the solutions were measured as radioactivity level of the supernatants. When the precipitates were treated with solutions containing 5 mM NHA, no measurable amounts of $^{45}Ca^{2+}$ were detected in the supernatants. When the precipitates were treated with the solutions deprived in NHA, about 7% of Ca-NHA complexes were dissolved. These results suggested that washing of Ca-NHA precipitates in the presence of NHA allowed to keep all the precipitated calcium completely insoluble.

Electron Microscopy of Ca-NHA Complexes

The morphology of Ca-NHA precipitates obtained in test tubes by mixing of 0.1 mM $CaCl_2$ with 5 mM NHA is shown in Fig. 3. The large, uniform, cuboidal form crystals were observed. However, when intact *Acanthamoeba* cells containing high amount of calcium (about 1–2 mmoles per 1000 g of wet weight — Sobota

Fig. 3. Electron micrograph of Ca–NHA precipitate. Uniform, cuboidal crystals were formed in test tube by mixing of 0.1 mM $CaCl_2$ with 5 mM NHA. Magnification — 20 000×

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et al. 1984), were fixed with glutaraldehyde supplemented with 5 mM NHA, no characteristic Ca-NHA crystals were observed inside the cells. In this case only very tiny electron-dense material was hardly distinguished in cytoplasm (Pl. I A); while mitochondria, lipid droplets, and plasma membrane (Pl. I AB) exhibited much higher electron densities than it was observed for control samples (Pl. I C). Lipid droplets were "dark" and had distinguished electron-dense grain; plasma membrane was sharply delineated with tiny electron-densities in submembrane vicinity. The observed dark regions most probably represented Ca-NHA precipitates in their amorphous forms.

Discussion

In cytochemistry for visualization of calcium in biological material pyroantimonate is commonly used as precipitating agent. However, this Ca-capturing compound is considered as not very useful for selective precipitation of calcium ions. Beside calcium, pyroantimonate forms insoluble complexes with sodium and magnesium ions as it was demonstrated by means of atomic absorption analysis as well as by X-ray microanalysis (Klein et al. 1972; Lane and Martin 1969).

We have analysed the selectivity of N,N-naphtaloylhydroxylamine to form precipitates with cellular inorganic cations. As shown above, NHA is capable to interact exclusively with calcium ions and its analogues. No interaction of NHA with Mg^{2+}, Na^{+}, and K^{+} occurs as demonstrated by means of ion-selective microelectrodes and radioisotope techniques. The formed Ca-NHA precipitates are very stable, being insoluble in 100 mM EGTA. Such high stability of Ca-NHA complex and high selectivity of NHA to precipitate only calcium ions vs. magnesium, sodium and potassium ions indicate the unique properties of NHA as Ca-capturing agent.

The selectivity of NHA towards calcium was earlier indicated by Zechmeister (1979) who demonstrated that calcium was the main element of NHA-evoked precipitates in muscle tissues. In Zechmeister's procedure the muscle tissue was prefixed with glutaraldehyde devoided of NHA; NHA was added only after washing the tissue. However, during fixation of cells and tissues with glutaraldehyde without capturing agents a rapid loss of many water-soluble cations (Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}) from the samples was often observed (Shoenberg et al. 1973, Van Iren et al. 1979). These observation suggest that calcium detected by Zechmeister in NHA-treated samples after their prefixation represented probably only the tighly bound form of this cation in the cells.

Our previous study (Sobota et al. 1987) has shown that practically no loss of cellular calcium occurred during fixation of cells with glutaraldehyde in the presence of NHA.

Lack of distinguished precipitates (big grains) in *Acanthamoeba* cells treated with NHA, when no loss of cellular calcium occurs, suggests that this reagent may preci-

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pitate cellular calcium as microprecipitates without their aggregation. Such non-aggregated Ca–NHA complexes may reflect the physiological distribution of calcium inside the cells. However, the non-aggregated Ca–NHA complexes are hardly visible under electron microscope. Therefore, for their better visualization additional techniques like X-ray microanalysis or autoradiography should be applied.

The presented N,N-naphthaloylhydroxylamine procedure for imobilization and visualization of cellular calcium is much simpler, possesses high sensitivity and selectivity, and is much simpler than the conventionally used pyroantimonate technique (compare with: Slocum and Roux 1982, Wick and Hepler 1982). Therefore, the NHA technique could be valuable in any ultrastructural studies on subcellular localization of calcium.

ACKNOWLEDGEMENT

The excellent technical assistance of Mrs K. Mrozińska is gratefully acknowledged.
This work was supported by grant No. C.P.B.P. 04.01. from the Polish Academy of Sciences.

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EXPLANATION OF PLATE I

Precipitation of cellular calcium in *Acanthamoeba* by N,N-naphthaloylhydroxylamine

A, B: The cells were fixed with glutaraldehyde containing 5 mM NHA. Numerous tiny, electron-dense material in cytoplasm was distinguished. Mitochondria and plasma membrane exhibit much higher electron densities than it was observed for control sample (C)
B: Lipid droplets of high electron densities were observed. Numerous micrograins inside the droplets were visible
C: Control sample fixed without NHA

Magnifications: A — 28 000×; B — 44 000×; C — 24 000× A, B, C — unstained sections

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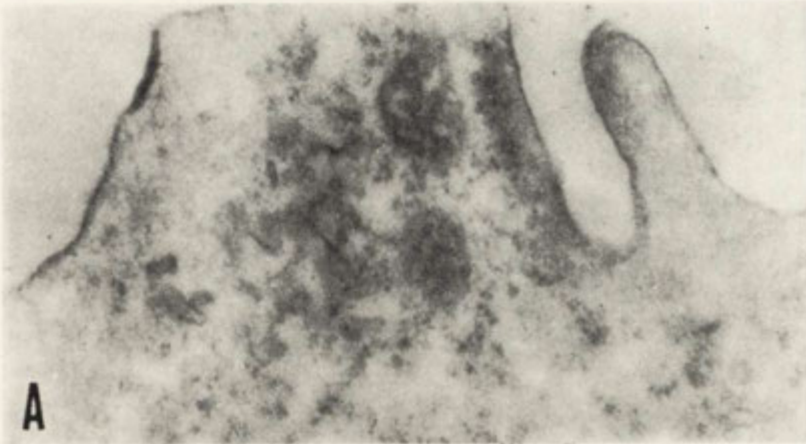

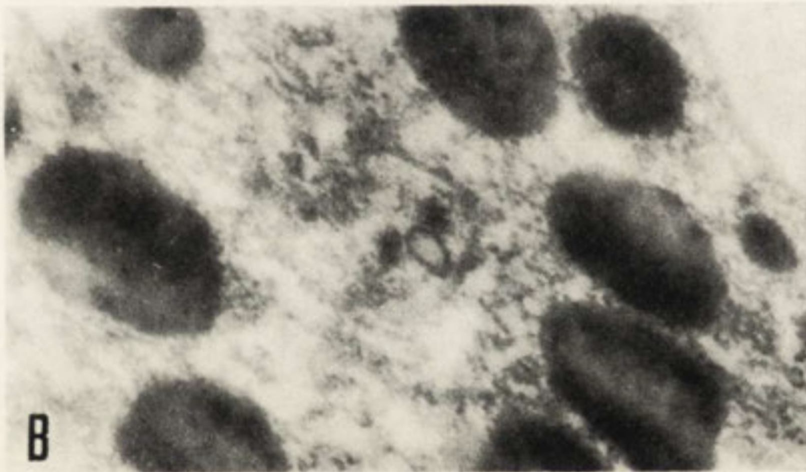

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ACTA PROTOZOOLOGICA
Vol. 27, No. 2, pp. 135–140 (1988)

Visualization of Binding Sites of Calcium and its Analogue — Cadmium in *Acanthamoeba* Cells

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Received on 20th October 1987

Synopsis. In the subplasmalemmal region of trophozoites of *Acanthamoeba castellanii*, exposed to excess of cadmium supplied jointly with the glutaraldehyde, there appear electron-dense deposits, in size and distribution undistinguishable from calcium deposits appearing in similar conditions. However, when both Cd^{2+} and Ca^{2+} are present in the reactive mixture, the electron-dense deposits appear also in cell interior in the form of larger aggregates mainly internalized in vacuolar compartment. Basing on the physico-chemical properties of cadmium ions, it is supposed that Cd^{2+} alone can easily replace Ca^{2+} in the subplasmalemmal capturing microregions, but in the presence of calcium ions cadmium cannot displace calcium from Ca^{2+} binding sites. In such case cadmium ions presumably enter the cell interior, where they may be bound by metallothioneins, ligands with nitrogen and sulphur donors, which show preference for the binding of cadmium ions.

Various cells, when exposed to the excess calcium ions inflowing together with glutaraldehyde, possess the ability to bind the metal in their subplasmalemmal region. This is conspicuous, among others, in cells active in pino- and endocytosis, like e.g., in insect intestinal cells (Oschman and Wall 1972) and insect vitellogenic oocytes (Przełęcka and Sobota 1976). The trophozoites of *Acanthamoeba castellanii*, in natural conditions living in soil moisture, are characterized by continuous pinocytosis and a very rapid membrane recycling (Bowers and Olszewski 1972). Perhaps this is the reason why in trophozoites of *Acanthamoeba* the phenomenon of subplasmalemmal calcium binding appears with great clarity. Both the size and the abundance of the appearing electron dense Ca-dependent deposites (CaDD) make them easy for observation (Sobota et al. 1977). By chemical analysis of the trichloroacetic extracts of cells containing CaDD and by partial experimental destruction of the latter, it was demonstrated that in *Acanthamoeba* calcium is deposited

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within a fibrillar network formed by subplasmalemmal cytoskeleton fragments, by their pyro- and orthophosphate terminals (Sobota 1985). It may be not excluded that under the influence of glutaraldehyde in other cells as well, similar microstructures are organized and are engaged in the formation of CaDD, appearing as a result of the described reaction. It was observed that some other divalent cations, like Mg^{2+}, Sr^{2+}, Ba^{2+} may induce formation of the deposits. However, the authors underlined, that the interpreting of positive results requires caution because of possible calcium contamination in the reagents they have used (Oschman and Wall 1972). The question arose whether cadmium, an analogue of calcium, may be captured in the cell within the same region. The problem seems to be the more interesting, that cadmium is a known environmental and professional hazardous element. The way in which the cell reacts to the pollution by this element may be of some importance. The present paper reports experimental results dealing with the problem.

Material and Methods

The experiments were performed on trophozoites of a small free living soil amoeba, *Acanthamoeba castellanii* (Neff strain), cultivated in an artificial medium as previously described (Sobota et al. 1977). The experiments were made on amoebae from 6 to 7-day old cultures. The cells, after being removed from the culture medium and washed twice with mild centrifugation with 150 mM NaCl were fixed in 2.5% glutaraldehyde buffered with 50 mM collidin, pH 7.4 supplied with either 10 mM $CaCl_2$, or with 10 mM $CdCl_2$, or with the two cations together, each one in 5 mM concentration. Subsequently, the samples after rinsing were postfixed with osmium tetroxide buffered as above and containing the corresponding cation (s). All the rinsing fluids also contained the cation(s) used in the given experiment, according to the procedure of Oschman and Wall (1972). After Epon embedding, ultrathin sections of the samples were examined in JEM 100 B (JEOL) electron microscope, always with and without routine contrasting with lead and uranyl salts.

Control samples were fixed and processed as above, but without the addition of the cations to the reactive mixtures.

Results and Discussion

The experiments were made in order to check whether cadmium, which is a calcium analogue, may be captured in the subplasmalemmal cytoskeletal region, when supplied to the cell jointly with the fixing agent. Plate I 1–3 shows that the answer is highly positive. Cadmium dependent deposits, in the form of electron dense granules (CdDD) were formed in the same region in which CaDD could be observed by us previously (Sobota et al. 1977). They were placed at the cytoplasmic site of the plasma membrane and were not conspicuous in any other cell compartment. Their diameters ranged from a dozen to well over a hundred nm (in average 70 nm) and they were distributed along the plasma membrane profile at distances amount-

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ing from several to over 150 nm. These values do not allow to discern them from CaDD, which suggests that in *Acanthamoeba* both calcium and cadmium may be captured in similar microregions.

The model of the reaction used here is widely applied in cytochemistry. One of the example may be the well known ruthenium red (RR) staining introduced by Luft (1971). The basis of the RR reaction consist in treating the negatively charged cell surface residues with a low molecular weight, positively charged RR, showing a high affinity to the surface residues. The fixing agent present in the reaction medium links the appearing complexes to other cell membrane constituents, which prevent their shifting from the primary reaction site. In the case of the reaction used by us in this paper, it seems that the subplasmalemmal cytoskeletal fragments form such a barrier for the inflowing cation. Thus the inflowing cadmium ions can be captured within this barrier, which prevents their further penetration. This interpretation fully accords with the just mentioned results presented by Sobota (1985), concerning the subplasmalemmal binding of calcium. The author suggests that calcium is deposited in the form ortho- or pyrophosphate salts, which indicates that the binding is done by oxygen donor ligands.

Quite unexpected results were obtained when the cells were exposed to glutaraldehyde supplied with both Ca^{2+} and Cd^{2+} jointly. In this case the electron dense deposits appeared not only in the subplasmalemmal region, but also in cell interior, enclosed mainly in vesicles (Pl. II 4, 5). Some of the latter could be easily identified as secondary lysosomes, because they contained also fragments of partially disintegrated cell structures (Pl. III 6,7). The close contact between those vacuoles which is frequently observed, as well as their shape, suggests that they have been fixed just before fusion leading to the formation of larger digestive vacuoles (Pl. III, 6).

It must be added that the deposits appearing in the vesicles are much larger than those observed in the subplasmalemmal region, their diameter amounting even up to 200 nm, whereas that of the former usually does not exceed 100 nm. We have never observed such pictures by inducing the formation of CaDD (Sobota et al. 1977). When the formation of CaDD was examined in *Acanthamoeba* trophozoites undergoing encystation, or in just formed cysts, the CaDD appeared inside mitochondria, but not in the vacuolar compartment (Sobota and Przełęcka 1981). The interpretation of the described phenomenon is rather difficult. Apparently, when both Ca^{2+} and its analogue Cd^{2+} are present in the reactive mixture, the first subplasmallemmal barrier is unsatisfactory for stabilizing and preverting their further diffusion. Clearly, the non-trapped cations penetrate easily the cell, jointly with the infusing glutaraldehyde, reaching other cell constituents capable to capture them. However, it remains an open question whether these cations are Ca^{2+} or Cd^{2+}, or a mixture of both.

The observation of amoeba in the shape characteristic for its locomotor activity, which has been exposed to the reactive mixture supplied with both kinds of cations, also reveals two distinctly different compartments where the electron dense deposits

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are found (Pl. IV, 8, 9). At the plasmalemma of the frontal part, where the extended pseudopodia are visible, the deposits are very delicate and scarce. Contrariwise, the subplasmalemmal region of the rounded distal part of cell body is distinctly stuffed with electron opaque precipitate. Such distribution of calcium deposits, marking the distribution of its binding sites, was observed in *Amoeba proteus* by Stockem and Klein (1979). Like always when both Ca^{2+} and Cd^{2+} binding sites are followed, in our material — besides these subplasmalemmal deposits — laıger deposits are seen agglomerated within the vacuolar compartment. The controles were totally deprived of any precipitate.

Returning to the main question, which of the two applied cations are captured and in which organelles, the following supposition based on their physico-chemical properties seems to be reasonable. If the first subplasmalemmal Ca-capturing barrier yields oxygen donor ligands, in the absence of calcium, cadmium is able to replace these sites. But, in the presence calcium, cadmium cannot easily displace the latter from its oxygen donors binding sites. On the other hand, even a minority of nitrogen and sulphur donors gives preference to Cd^{2+} binding (Martin 1984). Thus, it may be supposed that when both kinds of cations, in equal molarity, are supplied to the cell together with glutaraldehyde, calcium can be linked at the subplasmalemmal region, whereas cadmium ions diffusing further the cell interior can be linked by nitrogen and sulphur donors, which may be proteins. It is known that in the vertebrates a specific class of low molecular weight proteins-metallothionein — is engaged in protecting the cell against heavy metal poisoning (Shaikh and Smith 1977). The existence of a similar group of cytosolic, soluble and metal chelating proteins was reported also in representants of blue green alge and flagellates (McLean et al. 1972), and in *Amoeba proteus* (Ord and Al-Atia 1979). Most likely they exist in *Acanthamoeba castellanii* as well. It may be supposed that the delicate precipitate discernible in the cytoplasm of the cells treated jointly with Ca^{2+} and Cd^{2+}, is cadmium metallothionein precipitate, which may be internalized in the vacuolar compartment. The chemical character of mentioned proteins ensures their preference of being ligands for Cd^{2+} and not Ca^{2+}. The final solution of this question requires, however further study.

ACKNOWLEDGEMENTS

The authors are very indebted to mgr Maria Zwierzyk and Mrs Kazimiera Mrozińska for their excellent technical assistance.

This work was supported by grant no C.P.B.P. 04.01 from the Polish Academy of Science.

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EXPLANATION OF PLATES I–IV

1–3: Trophozoites of *Acanthamoeba castellanii* exposed to excess of cadmium. Electron dense deposits are seen in the subplasmalemmal region. 1 and 2 — sections contrasted with Ur and Pb salts, 3 — uncontrasted section
4–5: Trophozoites of *Acanthamoeba castellanii* exposed to excess of both cadmium and calcium supplied jointly. Delicate electron dense deposits are seen at the cytoplasmic site of the plasma membrane, large granular deposits are seen in the cell interior, mainly enclosed in vacuoles (arrows)
6, 7: Trophozoites of *Acanthamoeba castellanii* exposed to excess of both cadmium and calcium supplied jointly. Electron dense deposits are seen at the plasma membrane and in the vacuoles
7: High power micrograph of the cell shown on phot. 6, presenting fragment of a vacuole including the electron dense deposits and disintegrated cell structures
8, 9: Trophozoites of *Acanthamoeba castellanii* fixed in the moment of movement exposed to the excess of cadmium and calcium supplied jointly. Small-size electron dense deposits are seen at the plasma membrane and the large ones internalized in vacuoles
9: High power micrograph of a fragment of the cell shown on phot. 8

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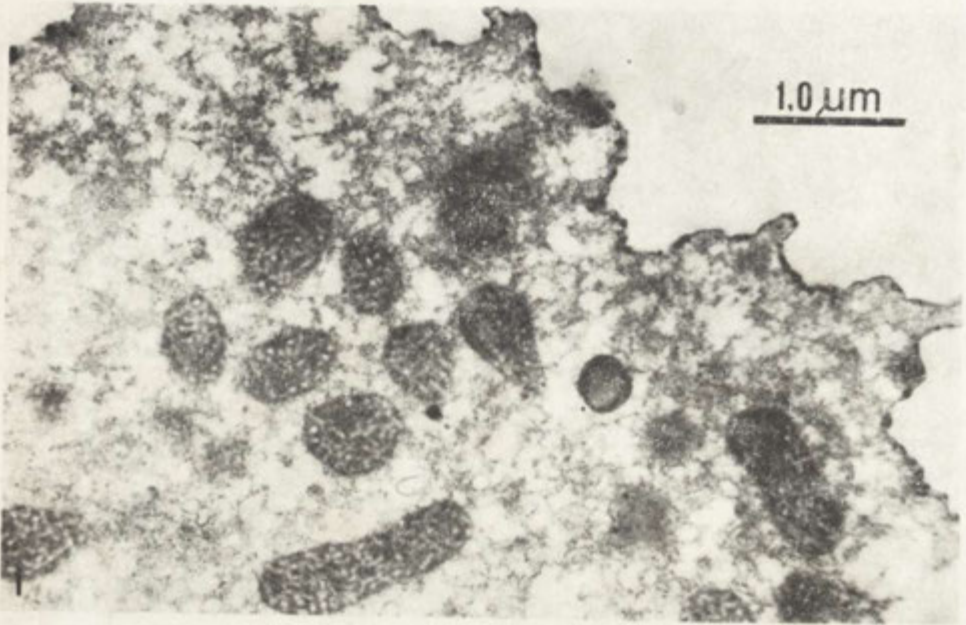

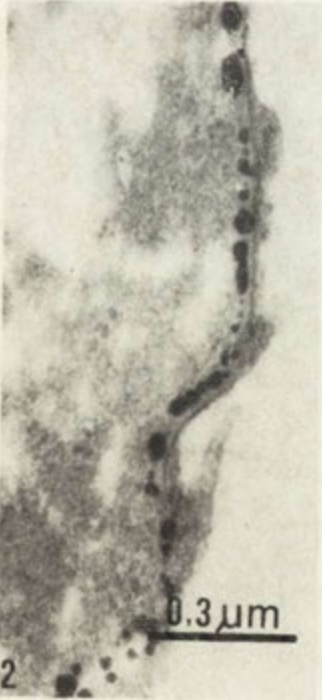

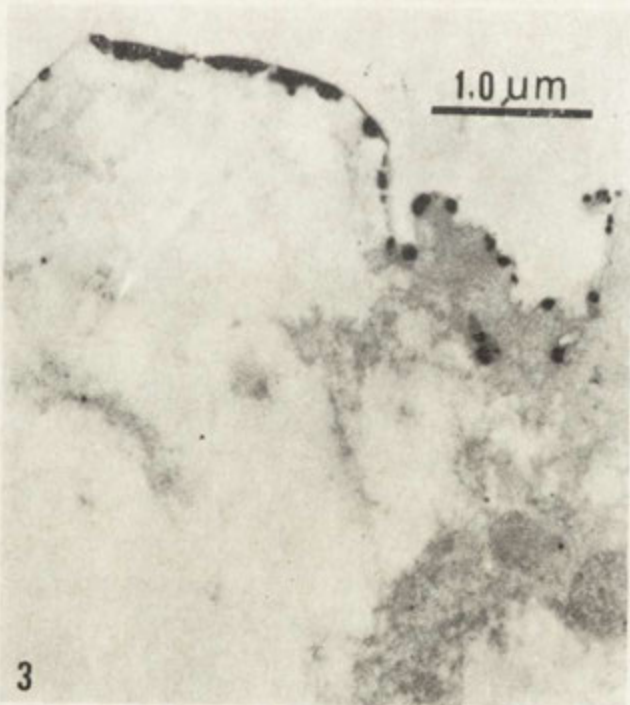

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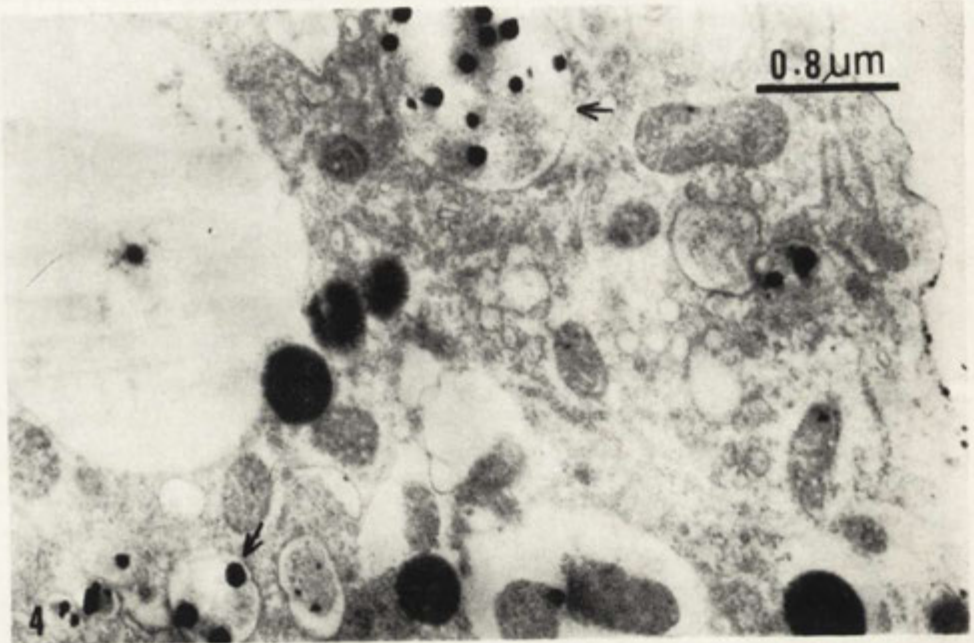

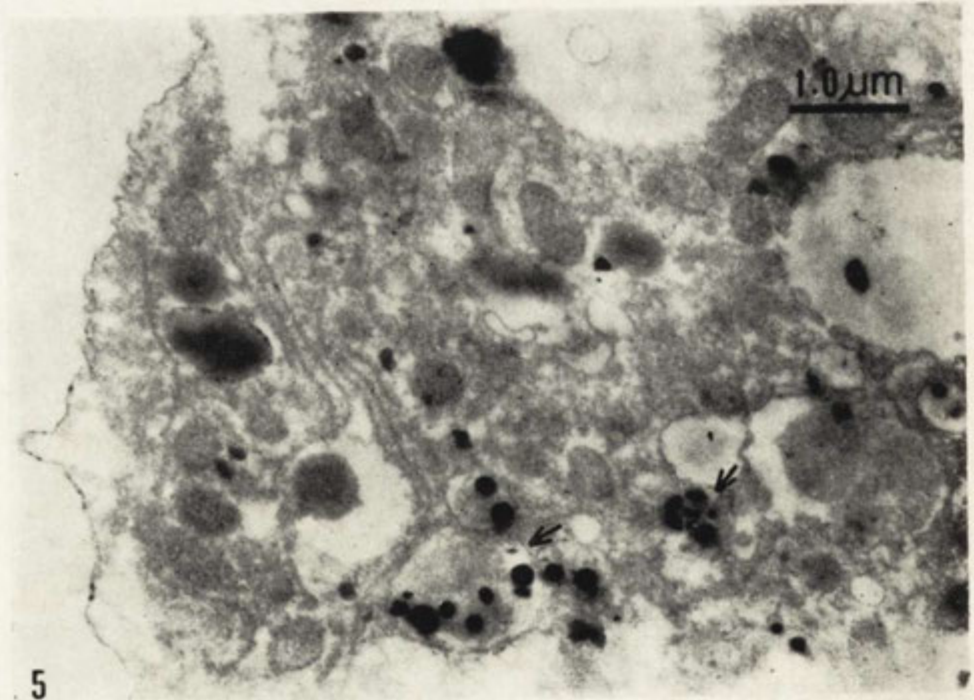

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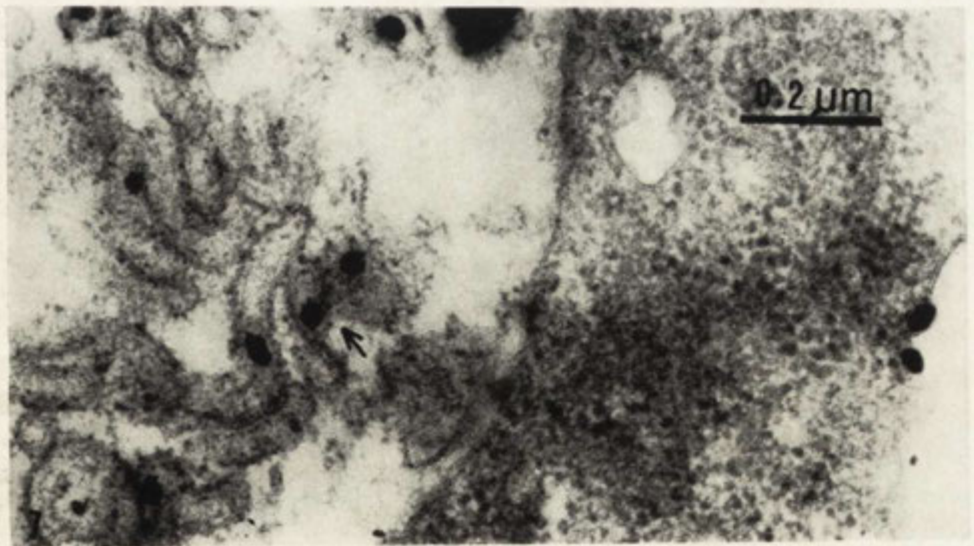

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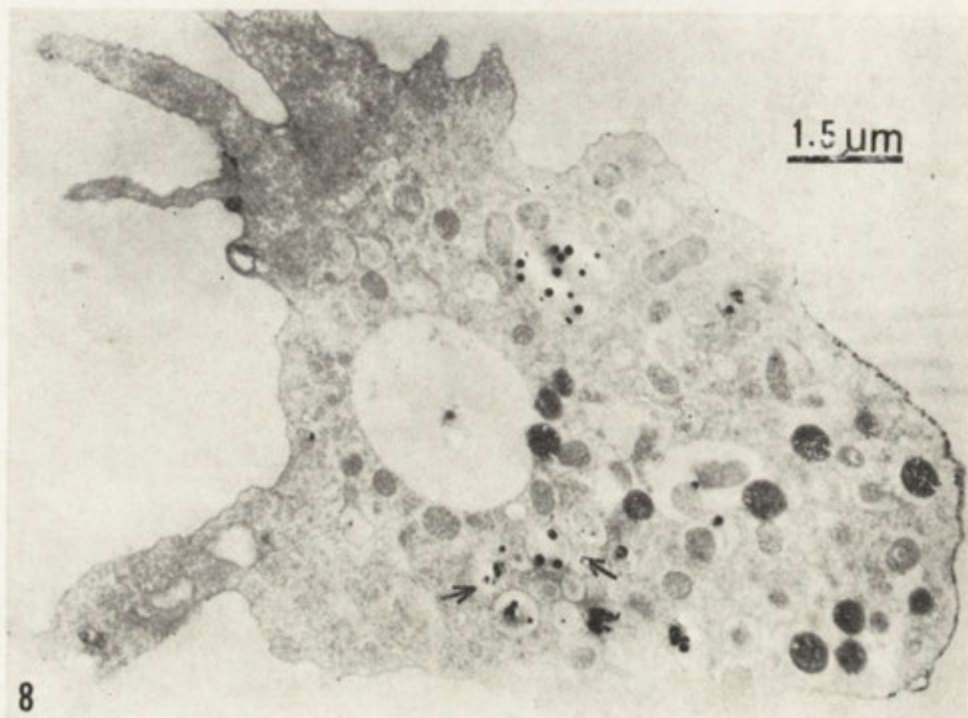

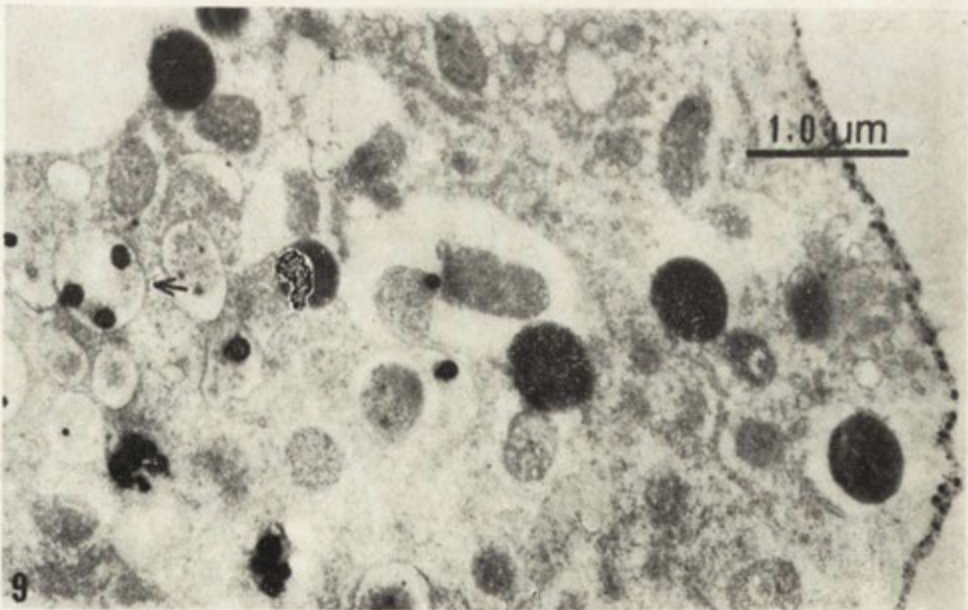

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ACTA PROTOZOOLOGICA
Vol. 27, No. 2, pp. 141–159 (1988)

Effects of Lead and Cadmium in a Community of Protozoans

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Received on 25th May and *revised on* 6th October 1987

Synopsis. We have studied the isolated effect of two metals, cadmium and lead, on a natural community of protozoans. The obtained results, concerning the dynamics of the species and individuals of both ciliates and non-ciliates and their survival, are described.

The toxic effects of lead are very well known and their many aspects have been studied before, especially with the protozoans as test objects (Fernandez-Leborans et al. 1985, Cairns et al. 1975, Fernandez-Leborans and Antonio-García 1986). The stability of lead is very high and in higher animals it can remain for years, although without obvious metabolic changes. Most of the retained lead is found in the skeleton. It can impede the biosynthesis of haemoglobin and it also affects the membrane permeability in kidney, liver and brain cells. As a result, functional disturbances may occur. Other effects happen in the pulmonar tissue (Bingham et al. 1968, Forstner and Wittmann 1983, Corpas-Vazquez et al. 1982).

The zinc, mercury and cadmium group is very important in polluted waters, because these three elements can give relatively stable combinations with SH groups. The stability of such complexes increases in the order: Zn — Cd — Hg. The influence and conservation of cadmium specially concerns many molecules that are an essential part of organisms, human organism included. For instance, cadmium and mercury compete and displace zinc in metalloenzymes that normally contain zinc, form irreversible compounds in active sites, thus deteriorating normal metabolism (Főrstner and Wittman 1983). The effect of zinc and mercury on some definite communities of protozoans was described by us in earlier works (Fernandez-Leborans et al. 1983, Antonio-García et al. 1983, Fernandez-Leborans and Antonio-García 1986).

The microorganisms are important for an assay of the presence and bioasimilation of metals. In this aspect, the protozoa have a privileged place, because of their

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way of feeding, their capacity to ingest complexes that include metals, and their great value in the overall aquatic biomass. They can be used to study water purification and can be employed for water characterization.

Materials and Methods

The populations employed in this work were obtained directly from samples from Manzanares river (La Pedriza, Madrid). The samples were prepared for analysis as previously described (Fernandez-Leborans and Antonio-Garcia 1986). The metals were added to the enriched medium (after a previous checking of their absence from the environment) in the form of acetate salts, to the following concentrations: Pbl, 50 μg/l; Pb2, 100 μg/l; Pb3, 500 μg/l; Pb4, 1000 μg/l; Cdl, 10 μg/l; Cd2, 50 μg/l; Cd3, 100 μg/l; Cd4, 500 μg/l. The ciliate species have been determined using the silver carbonate technique (Fernandez-Leborans and Castro de Zaldumbide 1986). Data were obtained about: (1) Parameters of pH and temperature, (2) biotic parameters: number of individuals

Table 1

Temperatures measured in different fractions during the experiment (C – control)

Measurement No.	Fractions								
	C	Pbl	Pb2	Pb3	Pb4	Cdl	Cd2	Cd3	Cd4
I	15.5	16.5	17.4	18.8	19.3	15.6	15.9	15.8	17.7
II	16.8	16.5	17.4	18.8	19.3	15.6	15.9	15.8	17.7
III	19.4	19.4	19.9	20	19.5	19.5	20	19.6	20
IV	21.5	17.8	18.1	18.2	19.7	18.8	19.5	19.3	19.8
V	11.2	13	14.5	16.6	15.4	13.4	14.9	13.5	15.9
VI	17.6	18.7	17.5	17.8	18.5	18.1	17.6	17.8	18.2
VII	17.6	16.7	17.8	16.1	17.1	16.1	16.6	16.4	16.2
VIII	11	7.8	9.1	8.3	9.7	9	10.6	10.2	12.2
IX	16.5	13.1	13	14.6	16.5	13.4	14.1	14.3	15.3
X	9.7	11.5	10.5	12.4	14.2	11.6	13.1	13.1	15.3
XI	17.5	16.4	12.9	13.3	14.1	16.7	14.6	14	14.5
XII	16	16.3	16.5	16.9	16.5	16.6	16.6	16.9	17.1

of ciliated protozoa, number of ciliate species, number of individuals of different ciliate species, number of individuals of other protozoa. These data have been calculated for every fraction, including the control, every three days (in all, 14 measurements). On the other hand, the survival percentage of all protozoans and ciliates in every fraction has been calculated in respect of different concentrations of the employed metals. The temperature at which the samples were kept oscillated, depending on the weather, between 7.8°C and 20°C, and the differences which occurred between different fractions on the same day were small (Table 1).

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Observations

pH changes. Among the fractions with lead, the most important differences with respect to the control, occurred in Pb3 (500 μg/l) and Pb4 (1000 μg/l). Between the measurements VI and VII, the pH of the fractions with Pb3 decreased below the control. Until measurement VII, the control values were below pH 6.7, but in measurement X they reached 6.8, whereas the Pb3 value was only 6.62. The pH of fractions with Pb4 remained over the values of the control all along the experiment, and measurement IX showed the greatest difference with regard to the control (Fig. 1 and 2).

In the fractions with cadmium, the main differences with regard to the control

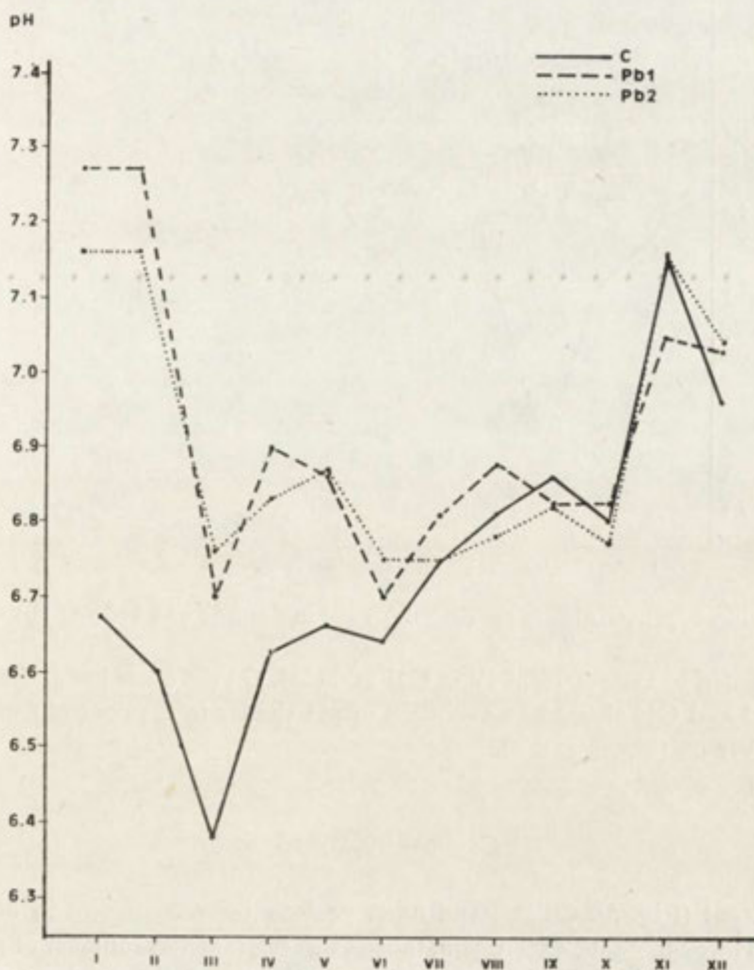

Fig. 1. pH variation in the control (C) and in fractions with Pbl and Pb2

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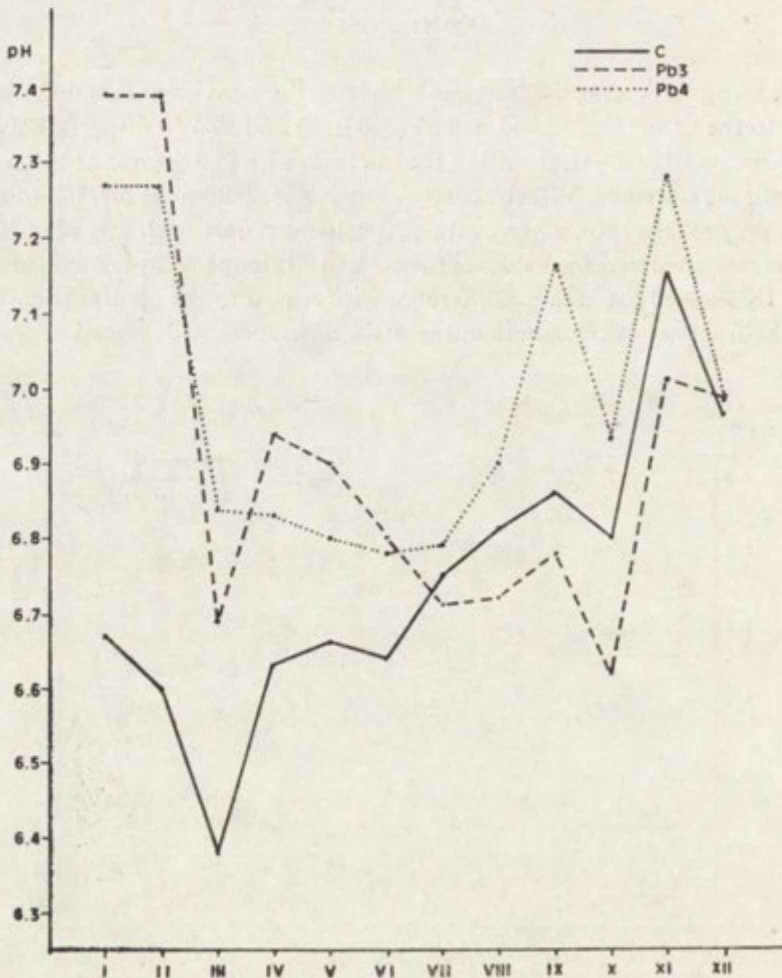

Fig. 2. pH variation in the control (C) and in fractions with Pb3 and Pb4

corresponded to measurement IV in Cd1 and Cd2. On the other hand, the fractions with Cd3 and Cd4 showed an evolution of pH similar to the control from measurement VII on (Fig. 3 and 4).

Community Dynamics

Control (Fig. 5). Ciliates and other protozoans were present in the control fractions throughout the experiment, though three parameters (number of protozoan cells, number of ciliate species and number of ciliate cells) did not always follow the same dynamics. A list of the species found is given in Table 2.

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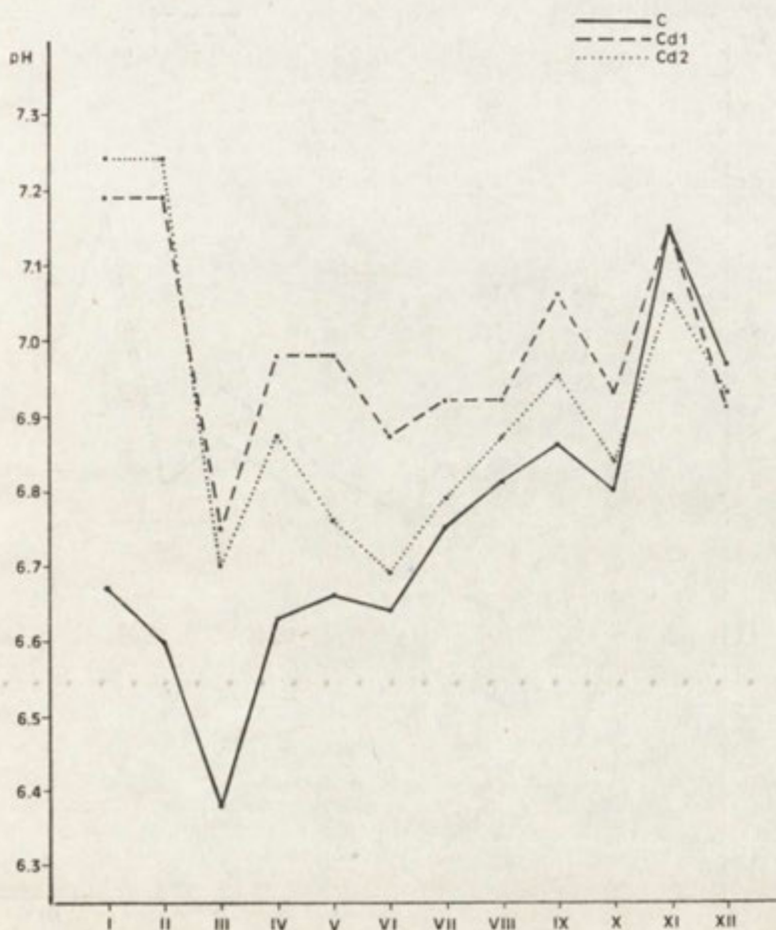

Fig. 3. pH variation in the control (C) and in fractions with Cd1 and Cd2

On measurement III, an increase of the protozoan total number (up to 255) was observed, but the number of ciliate species decreased to only two (*Coleps hirtus* and *Keronopsis pulchra*). Generally, until measurement V the number of protozoan cells was much higher than the number of ciliate cells, the non-ciliated protozoans (flagellates, amebae, etc.) thus dominated over the ciliates. On measurement VI, both values decreased, but later, the maximum number of individuals (217) and species (10) of ciliates was reached on measurement IX. The most numerous at that time were *Coleps hirtus* (26/10 ml) *Dileptus mucronatus* (28/10 ml) and *Rhagadostoma nudicaudatum* (26/10 ml). Measurement X gave the maximum number of protozoans (318 cells/10 ml of which 104 were non-ciliates: 72 flagellate and 32 gymnameabe, see Fig. 5).

Pb1 (Fig. 6). Three days after the addition of the metal, in measurement II, one

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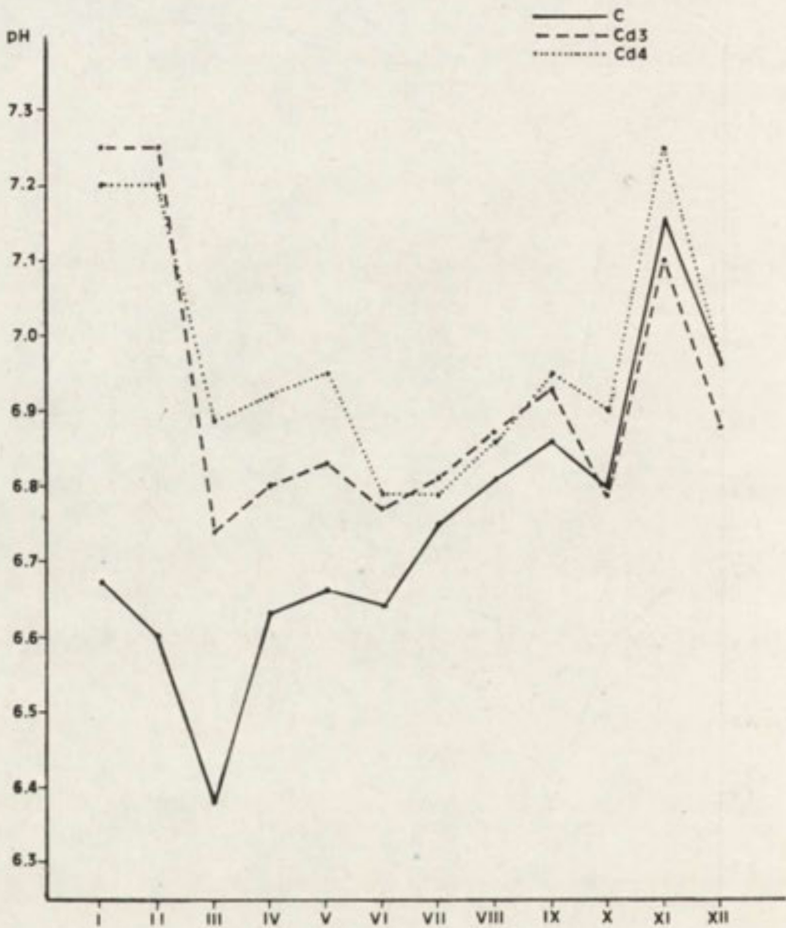

Fig. 4. pH variation in the control (C) and in fractions with Cd3 and Cd4

could see a remarkable decrease of the parameters, especially of the number of non-ciliated protozoans (in the control: in all 157, flagellates 124, thecamebae 25 and gymnamebae 8; in Pb1: in all 99, flagellates 79 and thecamebae 20). During the rest of the experiment, the total number of protozoan cells remained well over the number of ciliates, which means that neither the non-ciliated protozoans nor the ciliates disappeared following the treatment (Fig. 6).

Pb2 (Fig. 7). Until measurement VIII, the evolution of the population was more or less similar to that of the fraction with Pb1. But from that day on, there was a very important development of non-ciliated protozoans with respect to the ciliates. This increase, which took nine days, was caused by the number of flagellates, which increased from 72/10 ml in measurement VIII to 248/10 ml in measurement XI. Next, the flagellates almost disappeared and showed only a little reestablishment on measurement XIII (61/10 ml). The number of ciliates changed less (the maximum was in

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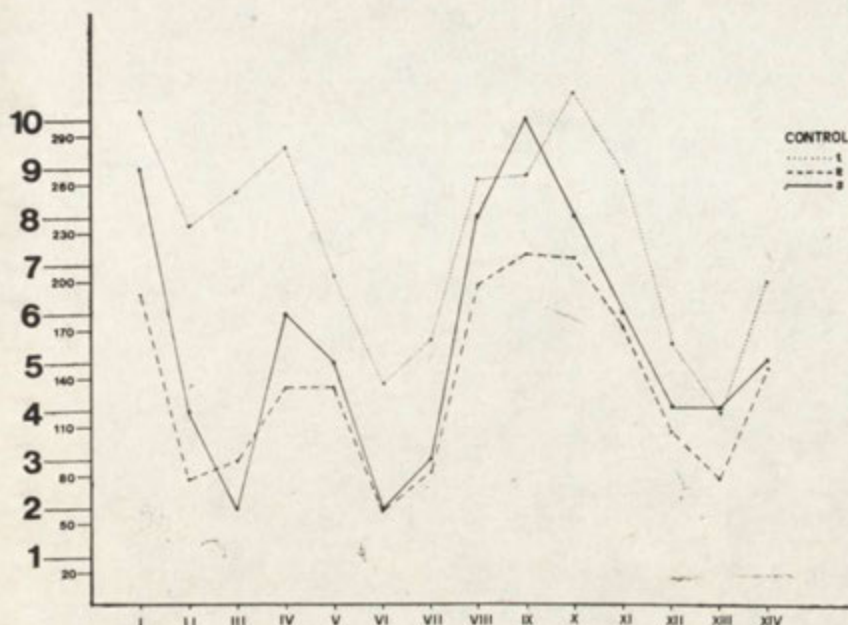

Fig. 5. *1* — number of protozoa; *2* — number of ciliated protozoa; *3* — number of species of ciliated protozoa. Outer scale number of species, inner scale number of cells per 10 ml. Variation in the control

Table 2

List of species of ciliates found in the study

1. *Urocentrum turbo*
2. *Colpidium colpoda*
3. *Coleps hirtus*
4. *Stentor roeseli*
5. *Paramecium caudatum*
6. *Keronopsis pulchra*
7. *Glaucoma scintillans*
8. *Paramecium bursaria*
9. *Brachonella spiralis*
10. *Amphileptus claparedei*
11. *Trochilioides fimbriatus*
12. *Holophrya matritensis*
13. *Paruroleptus musculus*
14. *Colpidium campylum*
15. *Litonotus lamella*
16. *Chlamydonella polonica*
17. *Holosticha muscicola*
18. *Dileptus mucronatus*
19. *Spirostomum ambiguum*
20. *Aspidisca cicada*
21. *Tachysoma perisincirra*
22. *Rhagadostoma nudicaudatum*
23. *Strongylidium muscorum*
24. *Histriculus similis*
25. *Cristigera minor*
26. *Opisthonecta bivacuolata*
27. *Balladyna parvula*
28. *Spirostomum teres*
29. *Vorticella campanula*
30. *Prorodon ovum*
31. *Halteria grandinella*
32. *Lacrymaria olor*
33. *Amphisiella oscensis*
34. *Oxytricha siseris*
35. *Pleurotricha lanceolata*
36. *Uronema marinum*
37. *Loxophyllum meleagris*
38. *Histiobalantium natans*
39. *Enchelydium virens*
40. *Lagynophrya acuminata*
41. *Prorodon hispanicus*

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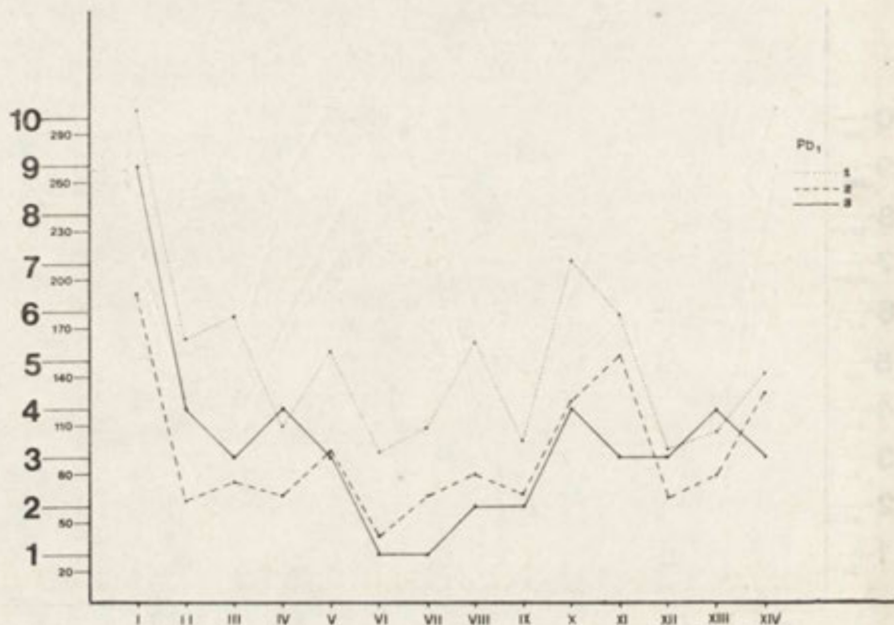

Fig. 6. *1* — number of protozoa; *2* — number of ciliated protozoa; *3* — number of species of ciliated protozoa. Outer scale number of species, inner scale number of cells per 10 ml. Variation in the fraction with Pb1

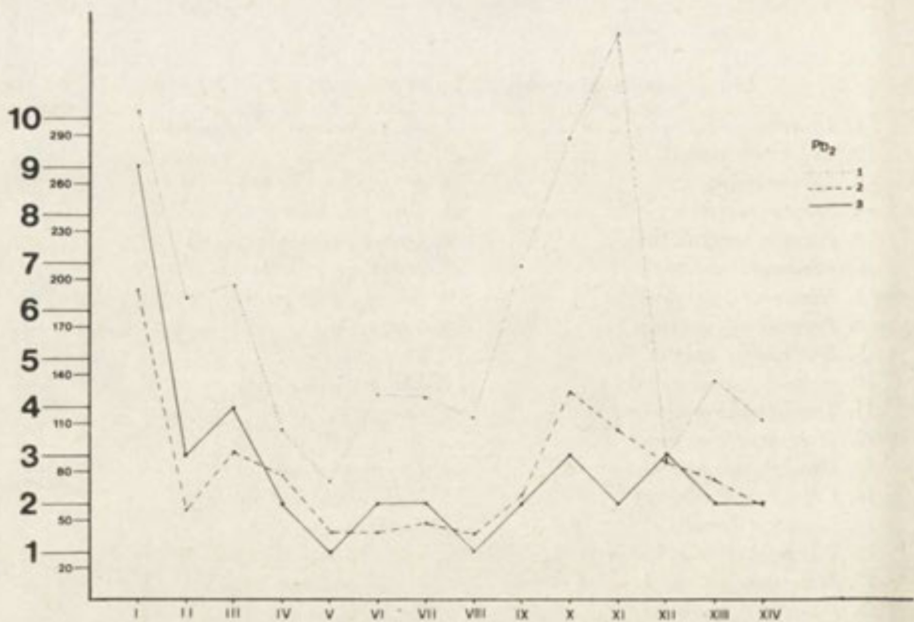

Fig. 7. *1* — number of protozoa; *2* — number of ciliated protozoa; *3* — number of species of ciliated protozoa. Outer scale number of species, inner scale number of cells per 10 ml. Variation in the fraction with Pb2

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measurement X: 130/10 ml, and from that day, it decreased until the end of the experiment). The dominating ciliate species was *Coleps hirtus*, which appeared in every measurement, and it was accompanied, in general, by two or three other species of which the most frequent was *Glaucoma scintillans* (Fig. 7).

Pb3 (Fig. 8). Unlike to the above fractions of lesser concentration, a clear lethal effect of the pollutant was observed here, since neither ciliates nor non-ciliated protozoa were observed starting from the XIth day of measurement. The non-ciliated protozoa disappeared in measurement X, and the ciliates in measurement XI. Only the flagellates showed a little quantitative increase between the measurement days VI (16 ind/10 ml) and VII (42 ind/10 ml) (Fig. 8).

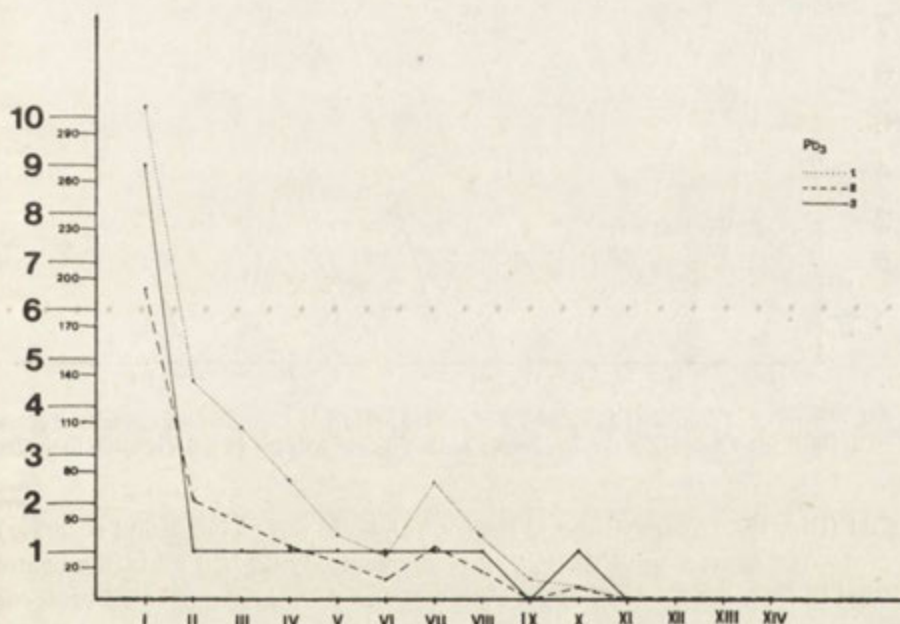

Fig. 8. *1* — number of protozoa; *2* — number of ciliated protozoa; *3* — number of species of ciliated protozoa. Outer scale number of species, inner scale number of cells per 10 ml. Variation in the fraction with Pb3

Pb4 (Fig. 9). The disappearance of the ciliated happened quicker than in the Pb3 fractions: in measurement VIII, there were no vegetative ciliate forms. Besides, the dominant species, *Coleps hirtus*, dissappeared already at measurement VII. The number of non-ciliated forms decreased mainly also by the VIIth measurement day. The number of flagellates changed from 92/10 ml on the VIIth measure day to 26/10 ml on the VIIIth day. However, from the measure day VIII there was an important development of flagellates, whose number increased from 26 to 220 cells/10 ml. The ciliates, namely, *Aspidisca cicada*, re-appeared in the Xth measurement

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(54 ind/10 ml). Three more ciliate species joined it on measurement day XI: two hypotrichs, *Paruroleptus musculus* (32/10 ml) and *Holosticha muscicola* (43/10 ml) and a hapterid, *Lacrymaria olor* (27/10 ml) (Fig. 9).

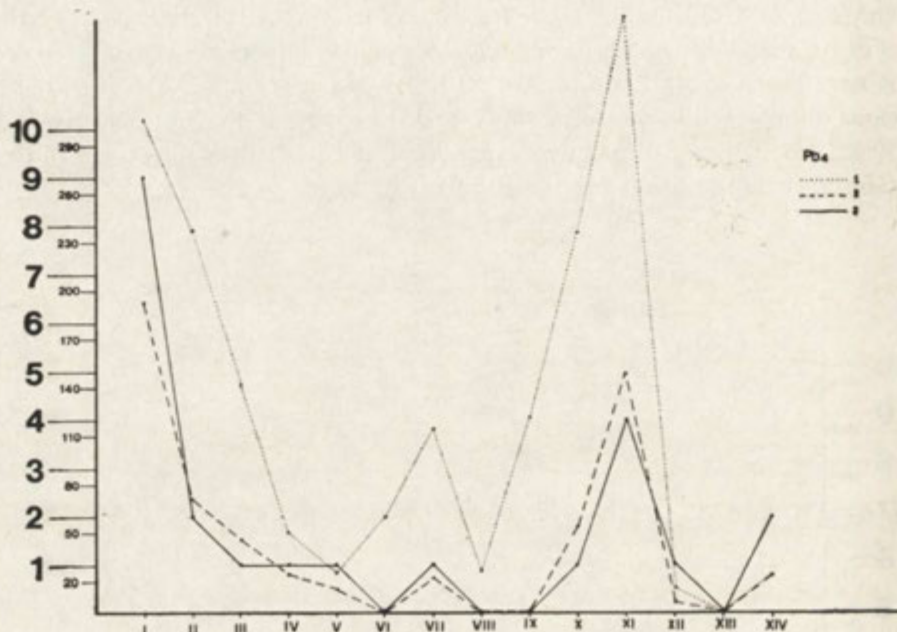

Fig. 9. *1* — number of protozoa; *2* — number of ciliated protozoa; *3* — number of species of ciliated protozoa. Outer scale number of species, inner scale number of cells per 10 ml. Variation in the fraction with Pb4

Cd1 (Fig. 10). In comparison to the control, there was an important decrease of the protozoan number until the IVth measurement day (282/10 ml in the control, 51/10 ml in Cd1, the IV day). From IVth measure day, the non-ciliated protozoan number strongly re-increased, mainly on account of the flagellates and gymnamebae (98 and 28/10 ml respectively on the Vth measurement). The ciliates, represented only by *Coleps hirtus* in measurement III and IV, disappeared in the Vth measurement, inversely to the non-ciliates. However, they re-appeared again, the biggest ciliate species number occurring at the XIth measurement (30 days after metal addition), with the prostomatid *Coleps hirtus* (86/10 ml), the hymenostomatid *Glaucoma scintillans* (48/10 ml), and two hypotrichs, *Paruroleptus musculus* (21/10 ml) and *Aspidisca cicada* (53/10 ml) (Fig. 10).

Cd2 (Fig. 11). A decrease of the number of species and individuals was observed especially in the IVth measurement. Only two species of ciliates (*Coleps hirtus* and *Stentor roeseli*) were preserved of the six which occurred at the IInd measurement. The number of both individuals and species of ciliated protozoa dropped less between the first and second measurements in this fraction than in the rest of the frac-

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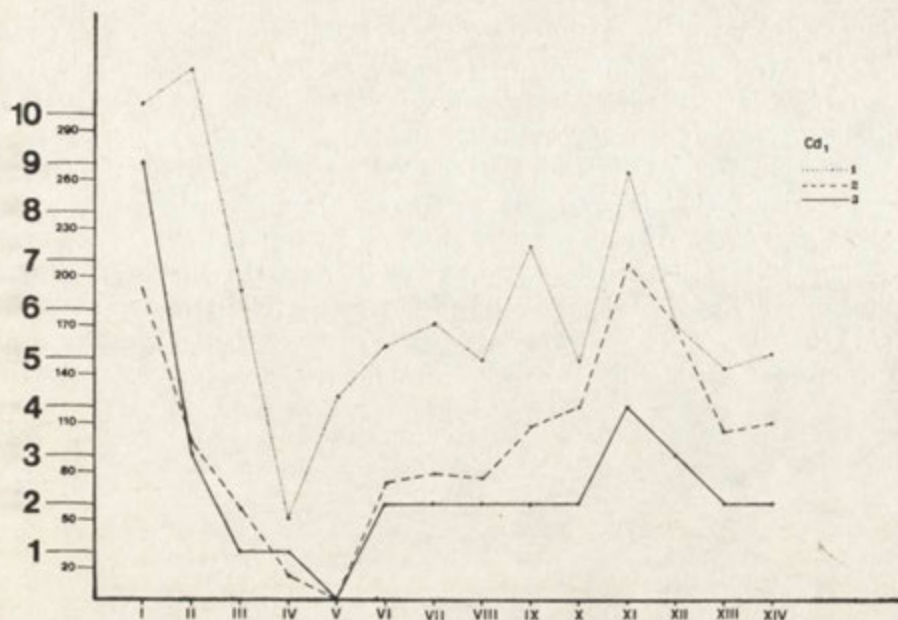

Fig. 10. *1* — number of protozoa; *2* — number of ciliated protozoa; *3* — number of species of ciliated protozoa. Outer scale number of species, inner scale number of cells per 10 ml. Variation in the fraction with Cd1

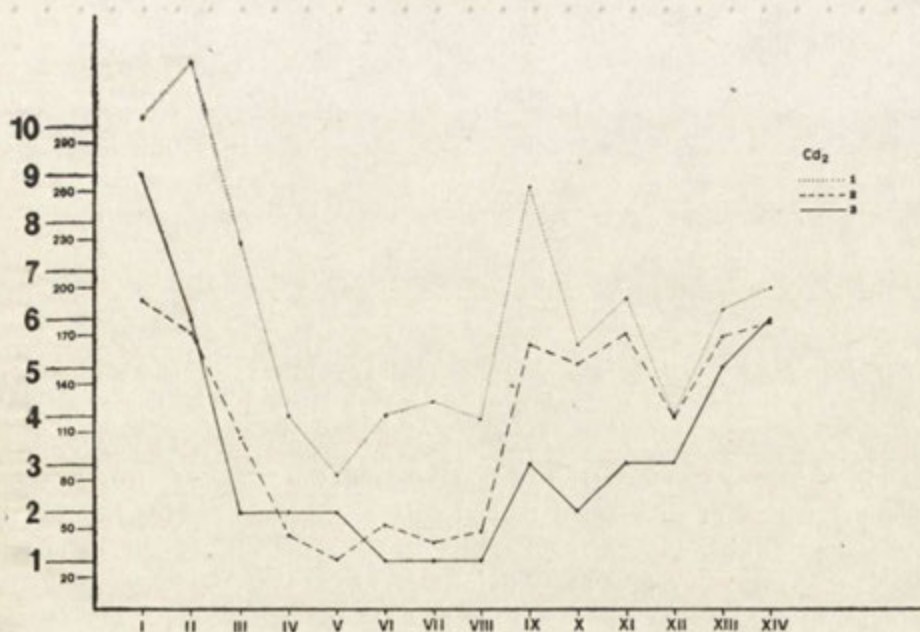

Fig. 11. *1* — number of protozoa; *2* — number of ciliated protozoa; *3* — number of species of ciliated protozoa. Outer scale number of species, inner scale number of cells per 10 ml. Variation in the fraction with Cd2

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tions with cadmium. On measurement day II, there were six species of ciliates: *Coleps hirtus* (75/10 ml) *Stentor roeseli* (12/10 ml), *Paramecium caudatum* (26/10 ml), *Litonotus lamella* (15/10 ml), *Chlamydonella polonica* (26/10 ml) and *Urocentrum turbo* (18/10 ml). This high density probably permitted the bioelimination of the greater part of the pollutant. At the IIIrd, IVth and Vth measurements, only two indicator species remained, (*Coleps hirtus* and *Stentor roeseli*), which later became reduced to one, *Coleps hirtus*. After the number of ciliate species had been reduced to one, the number of flagellates increased, and six days later also the number of individuals and the diversity of ciliated species began to increase (Fig. 11).

Cd3 (Fig. 12). There was an initial decrease of the number of species and individuals of ciliates and, at the IInd measurement, there were only *Coleps hirtus* (85/

Fig. 12. *1* — number of protozoa; *2* — number of ciliated protozoa; *3* — number of species of ciliated protozoa. Outer scale number of species, inner scale number of cells per 10 ml. Variation in the fraction with Cd3

/10 ml), *Holosticha muscicola* (19/10 ml) and *Paramecium bursaria* (22/10 ml). Later, while the number of non-ciliated protozoa decreased (from 286 ind/10 ml at the IInd measurement to 181 ind/10 ml at the Vth measurement), the number of individuals and species of ciliates began to increase, and at the Vth measurement there were five species, of which three did not occur in the initial composition (*Rhagadostoma nudicaudatum*, 19/10 ml; *Strongylidium muscorum*, 12/10 ml and *Litonotus lamella*, 22/10 ml). At the VIth measurement, a great decrease was observed on the population in general, as it also occurs in the fractions with Cd2. But in the case of

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Cd3, the decrease of both the number of non-ciliated protozoa and that of the ciliates was bigger (Cd2: ciliates 52/10 ml, protozoans 120/10 ml; Cd3: ciliates 40/10 ml, protozoans 82/10 ml). Later, the population got restored but was composed, in a part, of other species, such as *Cristigera minor* (12/10 ml) and *Holophrya matritensis* (28/10 ml) (Fig. 12).

Cd4 (Fig. 13). The effect on the ciliates of Cd4 was greater than in the rest of the fractions. However, the non-ciliated protozoa underwent an important development from the measure day V, which coincides with the disappearance of the ciliate

Fig. 13. *1* — number of protozoa; *2* — number of ciliated protozoa; *3* — number of species of ciliated protozoa. Outer scale number of species, inner scale number of cells per 10 ml. Variation in the fraction with Cd4

vegetative forms. The most resistant species in other fractions, *Coleps hirtus* (12/10 ml in the IVth measurement) did not appear at the Vth and subsequent measurements. At measurement VIII, *Spirostomum teres* was found (15/10 ml) and, since the XIth measurement, there were, with variations depending on the sampling, a maximum of five species in the XIIIth measurement (*Rhagadostoma nudicaudatum*, 26/10 ml; *Histiobalantium natans*, 17/10 ml; *Enchelydium virens*, 21/10 ml; *Lagynophrya acuminata*, 31/10 ml; *Colpidium colpoda*, 34/10 ml). The development of the non-ciliated protozoa depended mostly on the flagellates, together with the sporadic appearance of thecamebae and gymnamebae (*Arcella*, 12/10 ml — II measurement, gymnamebae, 87/10 ml — VII measurement; gymnamebae, 31/10 ml — XI measurement (Fig. 13).

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Survival

Ciliated Protozoa (Table 3). The addition of the pollutant determines a more rapid effect on the population in the case of lead. Thus, three days after lead addition, any lead fraction shows less than 50% survival of ciliates, while in the fractions with cadmium the values are higher than 50, except in the Cd4 (20.83%). Survival rates below 50% occur in all cadmium fractions 12 days after metal addition. In the case of lead, the fractions with Pb3 and Pb4 are the ones which show the lowest non-zero rates of survival (Table 3, Fig. 14).

Table 3

Percent of survival of ciliated protozoa in different fractions and at different measurements (a measurement every 3 days)

Measurements No.	Fractions							
	Pb1	Pb2	Pb3	Pb4	Cd1	Cd2	Cd3	Cd4
II	33.33	29.68	32.29	36.45	51.04	89.58	65.62	20.83
III	39.06	47.39	24.99	23.95	29.68	55.72	56.24	13.02
IV	34.89	40.62	16.66	12.49	7.81	23.95	45.31	6.24
V	48.95	21.87	12.49	7.81	0	16.14	53.64	0
VI	21.35	21.87	6.24	0	38.02	27.08	20.83	0
VII	34.89	24.99	16.14	11.45	41.14	21.35	90.10	0
VIII	41.66	21.35	9.37	0	39.58	24.99	81.24	7.81
IX	35.41	34.37	0	0	56.77	85.41	69.79	0
X	64.58	67.70	4.16	28.12	62.49	79.16	69.79	0
XI	79.68	55.20	0	77.60	108.33	89.06	101.56	35.93
XII	33.85	44.79	0	3.12	88.54	61.97	66.66	44.79
XIII	41.14	39.58	0	0	54.68	88.02	66.66	67.18
XIV	67.70	30.72	0	11.45	57.29	92.70	113.54	14.06

As to cadmium, it has to be noted that the lowest non-zero rates of survival appear in fractions with Cd1 and Cd4 (Table 3). In the ones with Cd2 and Cd3, the rapid re-increase of the number of species and individuals and the succession of species probably depend on an important capacity of bioelimination of cadmium by the ciliates, which leads to a permanent reestablishment of the ciliates. However, an obvious difference between fractions with Cd2 and Cd3 is observed with respect to survival: low rates of survival appear earlier in fractions with Cd2 (16.14%, Vth measurement) than in fractions with Cd3 (20.83%, VI measurement). In the case of Cd1, the lowest non-zero value of survival (7.81%) appears after nine days of treatment and in the Cd4, there are two minima: 6.24% after nine days of treatment, due specially to *Coleps hirtus*, and 7.81% after 21 days, due to *Spirostomum teres* (Fig. 15). Perhaps the re-appearance of some protozoa is simply the result of disappearance of the metal from the sample.

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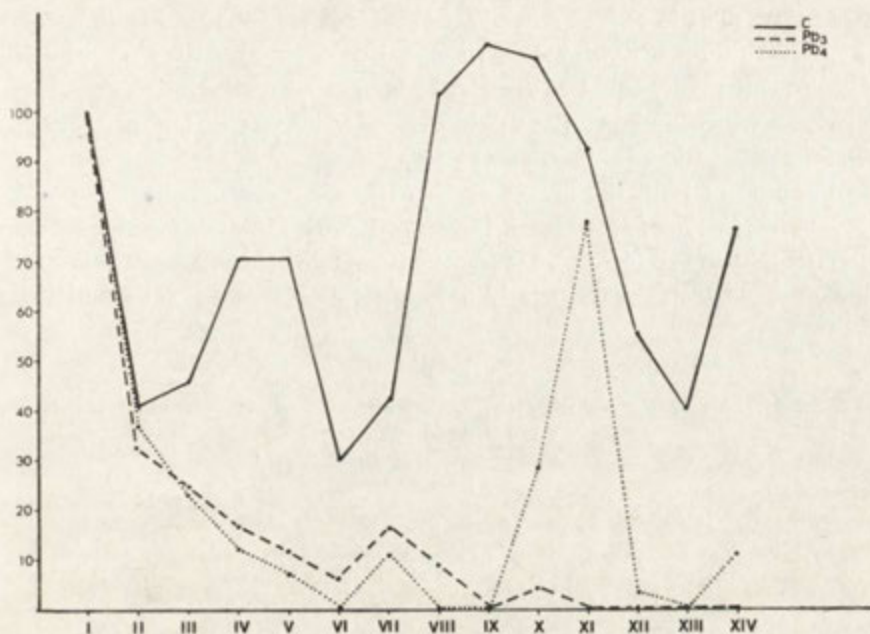

Fig. 14. Survival (%) in the control (C) and in fractions with Pb3 and Pb4 of the ciliated protozoa, during the treatment

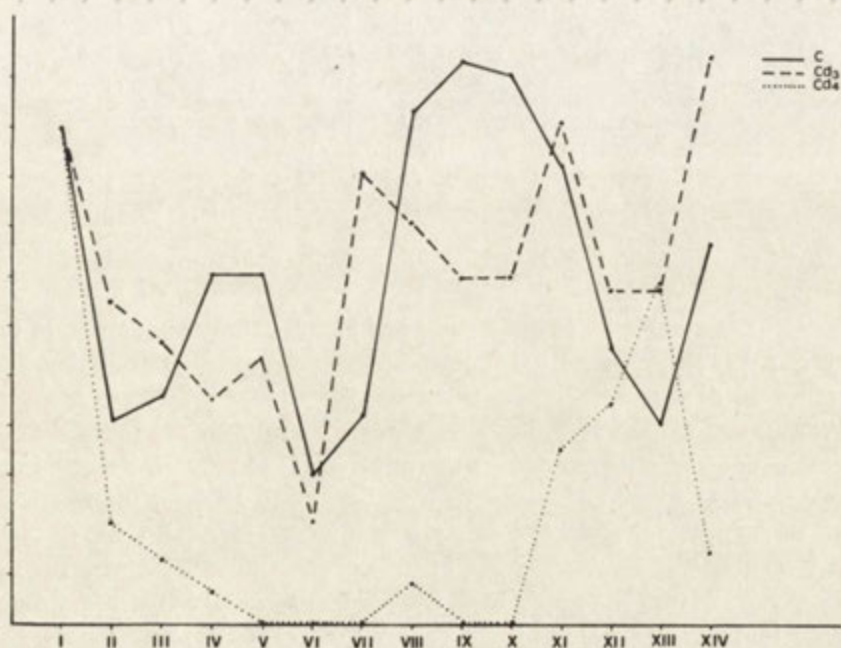

Fig. 15. Survival (%) in the control (C) and in fractions with Cd3 and Cd4 of the ciliated protozoa, during the treatment

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Protozoa (Table 4). The non-ciliated protozoa tend to increase, in general, the total survival values of protozoans. In global terms, the survival rates inferior or equal to 50% (lethality equal or superior to L50) occur earlier in fractions with lead than in those with cadmium (after 9 days — in all fractions with lead; after 12 days — in all fractions with cadmium, except Cd3).

In the case of lead, the lowest non-zero values occurs in fractions with Pb3 (2.61% after 27 days of treatment). In fractions with Pb4 the lowest value appears 6 days later than in Pb3 (4.90%). This fact contrasts with the minimum value obtained for the ciliates, which occurs after 12 days of treatment, and is caused by the great

Table 4

Percent of survival of all protozoa in different fractions and at different measurements (a measurement every 3 days)

Measurements No.	Fractions							
	Pb1	Pb2	Pb3	Pb4	Cd1	Cd2	Cd3	Cd4
II	53.26	61.43	44.44	78.10	107.19	111.11	93.46	44.77
III	57.84	64.05	34.96	46.40	62.42	74.51	72.55	38.56
IV	35.62	34.64	24.18	16.33	16.66	39.21	50.65	33.98
V	50.98	24.18	13.07	8.16	41.17	27.12	59.15	36.60
VI	30.39	41.83	9.15	19.60	51.63	39.21	26.79	68.63
VII	35.62	41.17	23.85	37.25	55.88	42.15	73.53	106.86
VIII	52.61	36.92	13.07	8.49	15.68	38.56	79.41	73.53
IX	32.68	68.30	3.92	39.54	71.57	85.62	90.52	113.07
X	68.95	94.11	2.61	77.12	48.36	53.59	56.21	79.41
XI	58.17	115.68	0	120.59	86.27	63.07	88.56	116.99
XII	30.72	28.10	0	4.90	55.55	38.88	41.83	48.36
XIII	34.31	44.77	0	0	46.73	60.45	57.51	65.68
XIV	46.08	36.27	0	7.19	49.67	62.03	84.96	39.54

development of the flagellates between 24 and 33 days after the addition of the pollutant. For that reason, the L100 is obtained quicker in Pb3 (after 30 days) than in Pb4 (after 36 days).

With respect to cadmium, the lowest values of survival are reached earlier in fractions with a high concentration of this metal (Cd4: 33.9% after 9 days). Fractions with Cd2 and Cd3 reach their minima between 12 and 15 days, and fractions with Cd1 show their minimum not earlier than after 21 days (15.68%). These facts suggest a closer relation to the concentration of the metal, and contrasts with the survival of the ciliates. In the case of fractions with cadmium we can talk about a possible compensation effect of the non-ciliated protozoa.

Species Dynamics and Discussion. We have not found *Colpidium campylum* in the lead fractions. According to Dive and Leclerc (1977), the DMA

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(minimal active dose) of lead is 400 µg/l, although we observed earlier (Fernandez-Leborans et al. 1983) that this ciliate cannot tolerate more than 50 µg/l, and after 12 days cannot support even 10 µg/l. With cadmium, Dive and Lecler (1977) demonstrated that DMA is 160 µg/l. However, we have found *Colpidium campylum* in the fractions with 100 µg/l and 500 µg/l at the end of treatment (at 30 and 36 days in 100 µg/l, at 30 days in 500 µg/l). It can be apply with some differences for *Colpidium colpoda*. This ciliate does not survive lead treatment, such was observed before (Fernandez-Leborans et al. 1985). In the fractions with cadmium only *Colpidium colpoda* was found at the end of treatment (at 36 and 39 days in Cd4 fractions: 500 µg/l), which probably coincides with the community recuperation and the pollutant bioelimination.

Prorodon ovum, as well as *Amphileptus claparedei*, do not survive even at minimal concentrations of both metals, and they were found only in the control. This fact agrees with previous observations (Fernandez-Leborans et al. 1985).

According to our earlier data, *Litonotus lamella* does not survive any lead addition (Fernandez-Leborans et al. 1985) and the same occurs with zinc and mercury (Fernandez-Leborans et al. 1983); any species tolerates the joint action of lead and zinc (Fernandez-Leborans and Antonio-García 1986). According to our present data, it was found only in the Pb2 fractions (100 µg/l) after 18 days and in the Pb1 fractions (50 µg/l) after 27 days. As to cadmium, this ciliate supports the concentrations of 50 µg/l for only 9 days.

With respect to survival in the presence of cadmium, Cairns and Pratt (1985) confirm that the percent of survival of a protozoan community at 21 days is 37.0% for 10 µg/l fractions and 10.0% for 500 µg/l fractions. Curiously, these data are very similar to our data referring to ciliates only (21 days: 10 µg/l — 39.58%; 500 µg/l — 7.81%). However, there are remarkable differences if we consider our data for the total protozoans (ciliates and non-ciliates): 21 days: 10 µg/l — 15.68%; 500 µg/l — 73.53%. In relation to lead, we observed (Fernandez-Leborans and Antonio-García 1985) that the addition of 500 µg/l reduced the protozoan population to 75% in 18 days. In the present study the reduction of the population is comparatively more rapid, especially in the case of ciliated protozoa (6 days, survival: 24.99%) but also in the case of all protozoa (9 days, survival: 24.18%).

Conclusions

The absence in literature of data comparable with those obtained in this study does not permit to relate significantly the observations presented here with those of other authors. It is so because in other more or less similar works one or several aspects are different, namely:

(a) Cultures of one or several species of protozoa (ciliates in most of the cases) are used, and not natural communities.

(b) The abundance of individuals of each species is not measured.

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(c) The taxonomic composition of the community is not comparable.

(d) The effects on the dynamics of the population, including survival, are not considered.

It is appropriate to point out several facts observed in this study:

(1) The behaviour of the community with regard to each metal is different and, in general terms, the effect is greater with lead than with cadmium.

(2) On the other hand, in reference to each metal, the community to which lead has been added, shows effects which are related more or less directly to the concentration of the metal. This phenomenon is shown clearly in ciliated protozoa (more concentration — less survival). However, in the community to which cadmium is added, the effects are not so clearly related to metal concentration, and the results depend on the taxonomic composition of the community. It is deduced that global data about the number of species and the number of individuals are not sufficient to explain the changes in the community dynamics.

(3) From our point of view, it is very important to have in mind the relation between ciliated and non-ciliated protozoa, as this has its effect on survival, as well as to explain some steady states observed during the treatment. It is also appropriate to bring out the influence of the composition of non-ciliated protozoa (especially flagellates, dinoflagellates, heliozoa, gymnamoebae and thecamoebae) on different responses to pollutants.

We can consider possible trophic causes of fluctuations of the individual numbers. The number of predators can and will decrease when they do not have enough food. If the food is sensitive to pollution, the predator will also suffer, though indirectly. On the other hand, if the predator is inhibited by metals, its number will decrease or it will disappear. This can create favorable conditions for the development of its prey, for instance, flagellates. That can be the cause of a rapid growth of flagellates in some fractions, which coincides with or follows the decrease or disappearance of ciliates. Then, with abundant food, the remaining predators can have a peak of multiplication in their turn, and so on. It is a possible explanation of the periodic fluctuations of the number of protozoans.

(4) As far as lethaly is concerned, our data concerning the ciliates give an idea of the effects in relation to some states of the community during the treatment. Data referring to all protozoa propose a clear explanation about the relations with different concentrations of the metals used and their effects.

ACKNOWLEDGEMENTS

The authors thank Dr. Igor B. Raikov for his assistance in the elaboration of this work.

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ACTA PROTOZOOLOGICA
Vol. 27, No. 2, pp. 161–164 (1988)

Centropyxis moldavica sp. n. (*Rhizopoda, Testacea*), eine neue Art der Bodentestaceen aus der Tschechoslowakei

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Received on 29 July, *revised on* 20 October 1987

Synopsis. Centropyxis moldavica sp. n. wurde aus Bodenproben des Naturschutzgebietes Český Jílovec (Südböhmen) beschrieben. *Centropyxis moldavica* sp. n. kommt in sehr feuchten Substraten (besonders Fichtenabfall und Boden unter *Marchantia* sp.) vor.

Im Verlauf der Bearbeitung der Boden- und Moosproben aus den Naturschutzgebieten Český Jílovec und Vyšenské kopce in Süd-Böhmen wurden 83 Testaceenarten gefunden (Balík, in press). Zwischen den gefundenen Testaceen wurden interessante Schalen von neuer Art der Gattung *Centropyxis* Stein gefunden. Die neue Art wird in der vorliegenden Arbeit beschrieben.

Centropyxis moldavica sp. n.
(Abb. 1 a–j und Taf. I)

Beschriebung der Schalen: Pseudostompartie von unten elliptisch oder breit oval, Hinterende dreieckig. Vorderende beim Seitenblick schwach bombiert. Schalen am Hinterende mit auffalenden laibigen oder elliptischen Seitenflächen.

Schalen aus organischer Kittmasse mit dünn zerstreuten Mineralpartikeln bedeckt, die auf den ovallen Seitenflächen fehlen. Schalen hell- bis dunkelbraun, Seitenflächen immer heller. Pseudostom elliptisch mit unregelmässig lappigem Rand.

Plasmakörper: Plasma farblos bis gelblich, innen ein Kern und 2–3 Nährungsvakuolen. In Nährungsvakuolen kommen oft die Nährungsreste (wie Pilzhyphen, Algen, Detritus, usw.) vor. Pseudopodien immer farblos, fingerförmig mit rundlichen Spitzen, 2–5 zugleich.

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Dimensionen: Schalenlänge 57–62 µm, Schalenbreite 47–51 µm, Schalenhöhe 16–2 µm, Länge der Seitenflächen 30–32 µm, Höhe der Seitenflächen 9–15 µm, Pseudostombreite 15–17 µm, Pseudostomlänge 7–10 µm. Genauere biometrische Charakteristiken sieh Tabelle 1.

Tabelle 1

Biometrische Charakteristik von *Centropyxis moldavica* sp. n., alle Messungen in µm

Merkmal	$\bar{x}$	M	s	$s_{\bar{x}}$	V	Min	Max	n
Schalenlänge	59,2	59,25	1,59	0,46	2,69	57,0	62,0	25
Schalenbreite	49,3	49,5	1,35	0,39	2,73	47,0	51,0	25
Schalenhöhe	19,0	18,75	2,31	0,67	12,16	16,0	22,0	25
Länge der Seitenfläche	30,9	31,0	0,73	0,21	2,36	30,0	32,0	25
Höhe der Seitenfläche	12,4	12,75	2,08	0,60	16,77	9,0	15,0	25
Pseudostombreite	16,0	16,25	0,77	0,22	4,82	15,0	17,0	25
Pseudostomlänge	8,7	9,0	1,62	0,47	18,62	7,0	10,0	25

$\bar{x}$ — arithmetisches Mittel, M — Median, s — Standardabweichung, $s_{\bar{x}}$ — Standardfehler des Mittelwertes, V — Variationskoeffizient, Min — Minimum, Max — Maximum, n — Anzahl der untersuchten Individuen

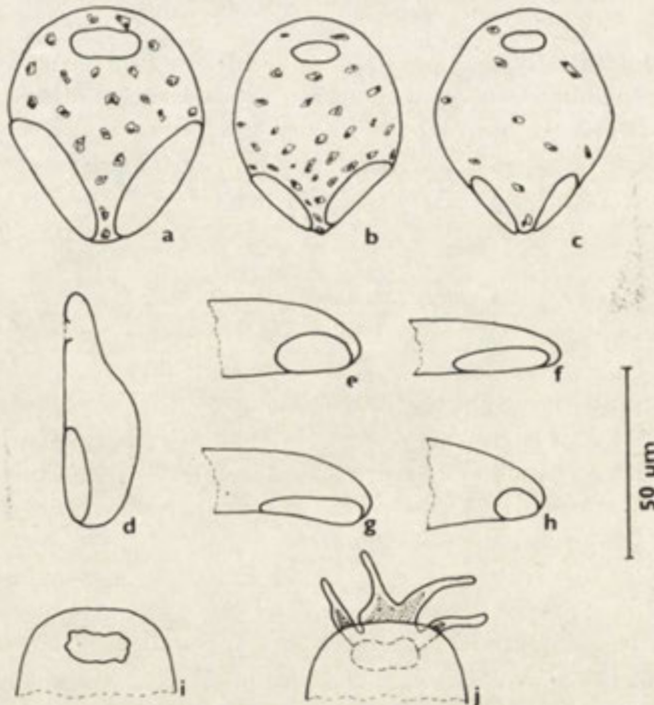

Abb. 1. *Centropyxis moldavica* sp. n., a–c — Schale von unten, d — Schale von Seite, e–h — verschiedene Formen der Seitenflächen i — Pseudostom, j — Pseudopodienemission

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Locus typicus: Tschechoslowakei, Süd-Böhmen, Český Jílovec, etwa 20 m vorn linken Ufer des Flusses Vltava (Moldau), im sehr feuchten Boden unter *Marchantia* sp. und Fichtenstreu im Fichtenwald, 9.7.1984 und 28.5.1986, insgesamt 25 Exemplare, leg. V. Balík.

Ökologie: Diese Art lebt in sehr feuchten Böden und Fichtenstreu in Fichtenwäldern.

Verwandschaftsbeziehungen: Die neue Art ähnelt *Centropyxis oomorpha* Schönborn et al. (1983). Die Schalen sind aber kleiner und zeichnen sich durch die laibigen Seitenflächen und durch das dreieckige Hinterende aus. *C. oomorpha* ist eine typische Art für die Buchenwälder, *C. moldavica* sp. n. wurden dagegen in einem Fichtenwald gefunden und bevorzugt sehr feuchten Substraten.

Typenmaterial: Holotypus- und drei Paratypuspräparate in der Sammlung des Autors, Institut für Bodenbiologie der Tschechoslowakischen Akademie der Wissenschaften, České Budějovice.

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LEGENDE ZUR TAFEL I

Pseudostompartie der Schale, ein Detail der Oberfläche der Schale von *Centropyxis moldavica* sp. n., Vergrösserung 1.900 mal (REM — Tesla)

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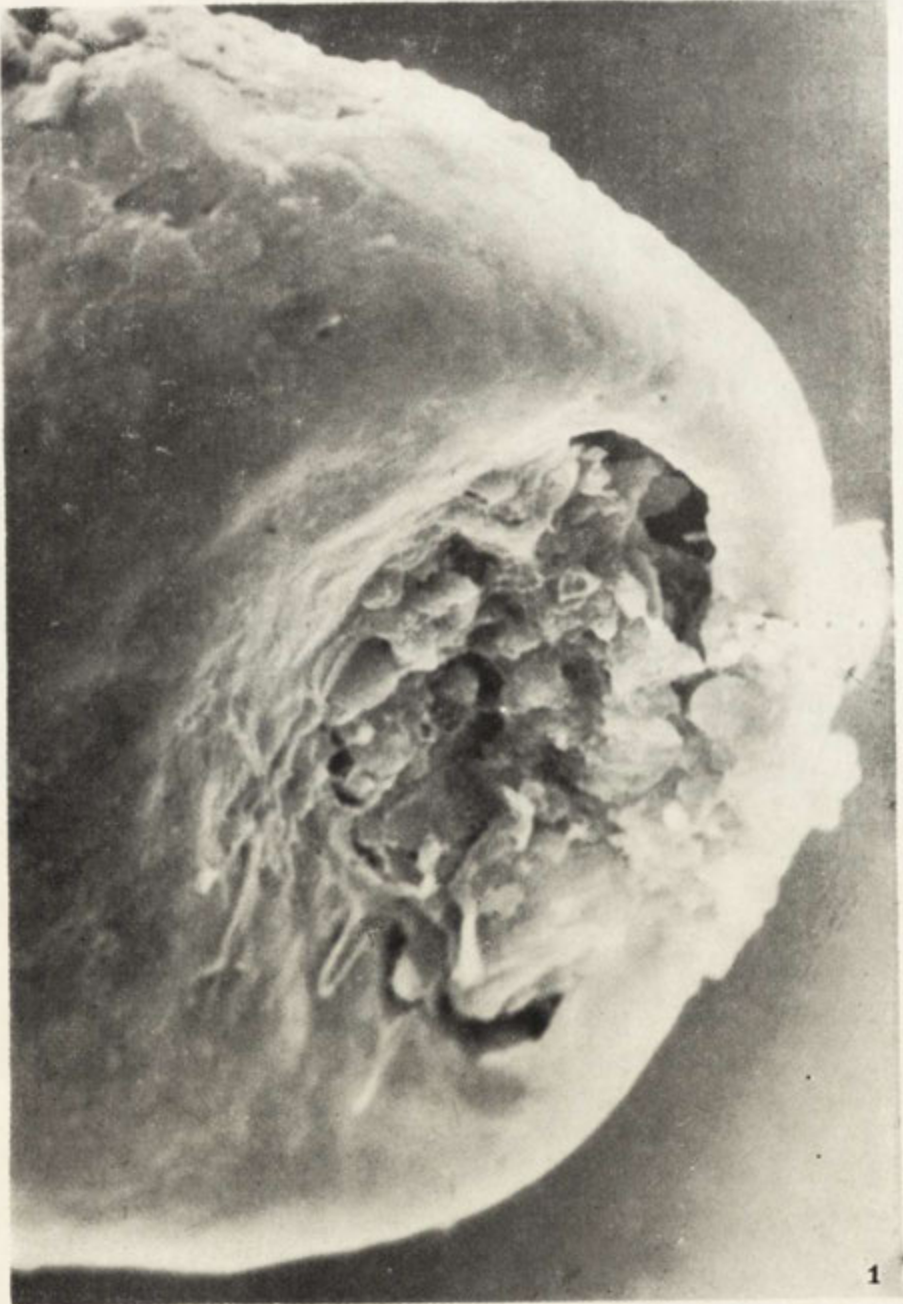

V. Balik

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ACTA PROTOZOOLOGICA
Vol. 27, No. 2, pp. 165–170 (1988)

Anisolobus indicus sp. n. of Septate Gregarines (*Apicomplexa*: *Sporozoea*) from the Beetle, *Coccinella septempunctata* (*Coleoptera*: *Coccinellidae*)

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Received on 10 July 1987, *revised on* 4 November 1987

Synopsis. This paper deals with the morphology and life history of a new species of septate gregarines (*Apicomplexa*:*Sporozoea*) in the gut of the beetle, *Coccinella septempunctata* L. The gregarine possesses an interesting organ of attachment and has ratio of LP:TL = 1:4.0–5.0 (1:6.8) and WP:WD = 1:1.0–1.8 (1:1.4). The gregarine is named *Anisolobus indicus* sp. n.

The genus *Anisolobus* was established by Vincent (1924) for a cephaline gregarine obtained from the gut of a small beetle, *Dacne rufifrons* Fabr. (*Coleoptera*), which closely resembled the genus *Gregarina* in many of its important characters, but was strictly different from the latter in the presence of peculiarly shaped sucker-like protomerite and the absence of a true epimerite. Vincent (1924) designated *Aniso-*

Table 1

A chronological list of different species of *Anisolobus* from insects of the world

Anisolobus sp.	Host(s)	Author(s)	Country
Anisolobus dacnecola	*Dacne rufifrons* Fabr. (Fam. *Dacnidae*)	Vincent (1924)	Great Britain
A. bulliardi	*Autispyris planicollis* Marsh. (Fam. *Curculionidae*)	Théodoridès and Jolivet (1959)	Africa
A. aleocharae (Ruchalles)	*Aleochara intricata* Maunh. (Fam. *Staphylinidae*)	Geus (1959)	France
A. gymnopholi	*Gymnopholus marquarti* Brown. (Fam. *Curculionidae*)	Théodoridès et al. (1972)	New-Guinea
A. desportis	*Cratopus frappieri* Deyr. (Fam. *Curculionidae*)	Théodoridès and Jolivet (1981)	Island of the Reunion

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lobus daenecola as the type species of the genus. Subsequently, Théodoridès and Jolivet (1959), Geus (1969), Théodoridès et al. (1972) and Théodoridès and Jolivet (1981) described four more species under this genus from different parts of the world (Table 1).

The present form infecting the lady-bird beetle, *Coccinella septempunctata* L. collected from the cabbage plants *Brassica camprestris*, does not resemble any other previously described species of the genus *Anisolobus* and hence it is described here as *Anisobolus indicus* sp. n.

Material and Methods

The host insects were collected at Bidhan Chandra Agricultural University Campus at Kalyani, West Bengal. Thin smear preparations of their gut contents were fixed in Schaudinn's fluid and subsequently stained with Heidenhain's haematoxylin. Cysts were collected from the faecal pellets of the host insects and cultured in moist chamber for sporulation (Sprague 1941). The following abbreviations have been used:

TL — Total length;
LP — Length of protomerite; LD — Length of deutomerite;
LN — Length of nucleus; WP — Width of protomerite;
WD — Width of deutomerite; WN — Width of nucleus.

The ratios used in this paper are those of length of protomerite to total length (LP:TL) and the width of protomerite to width of deutomerite (WP: WD).

Observations

Anisolobus indicus sp. n.

Host: *Coccinella septempunctata* L. from *Brassica camprestris*.

Incidence: 17 out of 79 hosts (21.5%) examined are infected with this gregarine, during the months of December, January and February of 1981–1982 and in subsequent years.

Sporadin: The parasite does not possess a true epimerite, but a sucker-like organ is formed at the anterior end of protomerite which acts as the organ of attachment (Fig. 1 *1* and *2*). The fully grown sporadin moves freely in gut lumen of the host insect. Protomerite is elongated, cylindrical, measuring 117.5 μm in length in the average. Deutomerite is cylindrical with a rounded posterior extremity. Cytoplasm is almost uniformly granulated throughout the protomerite and deutomerite. Nucleus is spherical to elliptical in shape and variable in position. Details of measurements of individual parts of the body are shown in Table 2.

Association: Regular bioassociative forms (Fig. 1 *3* to *5*) are found in the smear preparations. The primite and the satellite are morphologically different. The protometrite of the primite retains its sucker-like structure, while that of the satellite shows no sign of such a structure. The protomerite of the satellite is smaller in size with a cup-shaped concavity in which the deutomerite of the primite fits firmly.

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Table 2

Showing details of measurements (in μm) of the different parts of 20 specimens of *Anisolobus indicus* sp. n.

Sl. No.	TL	LP	LP:TL	LD	LN	WP	WD	WP: WD	WN
(1)	124.8	8.3	1:15.0	116.5	14.6	41.6	49.9	1:1.2	12.5
(2)	120.6	20.8	1:5.8	99.8	18.7	27.0	45.8	1:1.7	16.6
(3)	104.0	10.4	1:10.0	93.6	14.6	37.4	49.9	1:1.3	16.6
(4)	108.2	25.0	1:4.3	83.2	14.6	37.4	62.4	1:1.6	12.5
(5)	116.5	12.5	1:9.3	104.0	12.5	41.6	49.9	1:1.2	10.4
(6)	99.8	20.8	1:4.8	79.0	12.5	35.4	49.4	1:1.4	10.4
(7)	108.2	14.6	1:7.4	93.6	12.5	37.4	45.8	1:1.2	12.5
(8)	87.4	12.5	1:7.0	74.9	12.5	37.4	45.1	1:1.4	10.4
(9)	91.5	22.9	1:4.0	68.6	12.5	33.3	58.2	1:1.7	10.4
(10)	99.8	12.5	1:8.3	87.4	10.4	29.1	52.0	1:1.8	8.3
(11)	91.5	18.7	1:4.9	72.8	16.6	29.1	41.6	1:1.4	12.5
(12)	95.7	16.6	1:5.8	79.0	8.3	31.2	37.4	1:1.2	12.5
(13)	153.9	18.7	1:8.2	135.2	16.6	33.3	54.1	1:1.6	10.4
(14)	133.1	20.8	1:6.4	112.3	16.6	25.0	41.6	1:1.7	12.5
(15)	124.8	18.7	1:6.7	106.1	14.6	41.6	54.1	1:1.3	10.4
(16)	91.5	20.8	1:4.4	70.0	12.5	20.8	29.1	1:1.4	10.4
(17)	83.2	14.6	1:5.7	68.6	10.4	20.8	25.0	1:1.2	10.4
(18)	95.7	20.8	1:4.6	74.9	12.5	29.1	45.8	1:1.6	12.5
(19)	116.5	18.7	1:6.2	93.6	14.6	37.4	37.4	1:1.0	12.5
(20)	112.3	14.6	1:7.7	97.8	12.5	22.9	29.1	1:1.3	10.4

Average of TL = 117.5; LP = 18.9; LD = 90.6; LN = 13.5; WP = 32.4; WD = 45.7; WN = 11.8; LP:TL = 1:6.8 WP:WD = 1:1.4

Gametocyst and spore: Cysts (Fig. 1 *6* and *7*) are milky-white, more or less spherical, isogamous and measure 132.8 μm to 149.4 μm in length and 99.6 μm to 116.2 μm in width. The constriction separating two gametocytes disappears at about 12 h of development. Two sporoducts appear at about 60 h of development in the moist chamber, each measuring 58.1 μm×16.6 μm. The barrel-shaped spores are released in long chains which are 5.8 μm×3.3 μm in the average dimensions. Eight sporozoites are formed, arranged in two rows, four on each side of the spore (Fig. 1 *8* and *9*).

Material

(a) Holotype: On slide No. C 1/1, prepared from the midgut contents of the insect, *Coccinella septempunctata*. L. collected from Kalyani, West Bengal, India by S. Ray on 20 December, 1981.

(b) Paratypes: Many on the above-numbered slide and on other slides; other particulars are the same as for the holotype material.

Holotype and paratypes have presently been deposited to the Department of Zoology, University of Kalyani.

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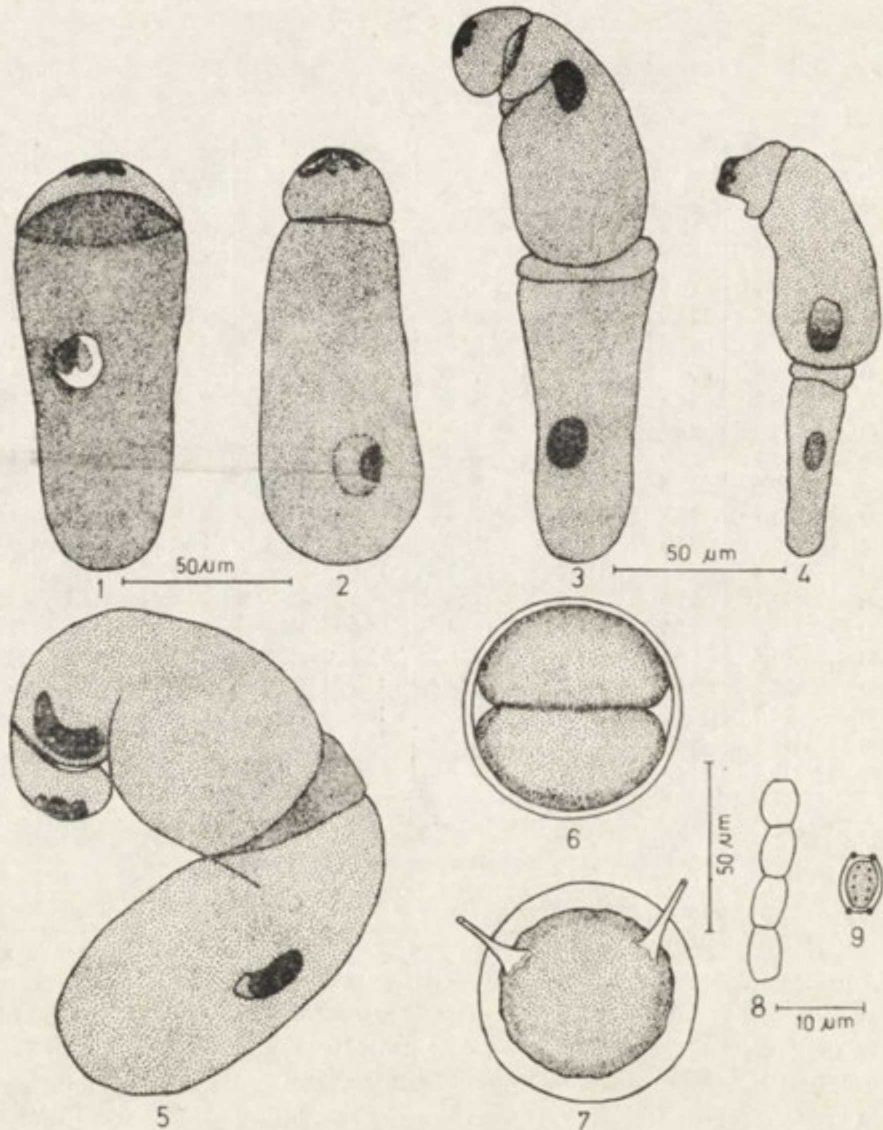

Fig. 1 *1–9*. Camera lucida drawings of *Anisolobus indicus*. *1–2* — Mature sporadins with sucker-like epimerite, *3–4* — Sporadins in syzygy, *5* — Gametocytes prior to cyst formation, *6* — An early gametocyst with two equal gametocytes, *7* — A gametocyst with two sporoducts, *8* — Spores in chain immediately after liberation, *9* — A barrel-shaped spore after formation of eight sporozoites

Discussion

According to Vincent (1924) the genus *Anisolobus* closely resembles the genus *Gregarina* in three important characters, namely, the association of the sporadins in couples, the dehiscence of the cysts by means of sporoducts and the barrel-shaped

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spores. But the peculiarly shaped sucker-like protomerite and the absence of true epimerite form a clear wall between these two genera. Literature available reveals that there are only five species of *Anisolobus* known till date. None of these are known from any Indian hosts. Moreover, the structure of the protomerite in the present form is distinctly different from the other species of *Anisolobus*. However, the sucker-like protomerite, association of sporadins in couples, dehiscence of the cysts by means of sporoducts and the barrel-shaped spores justify beyond doubt its inclusion under the genus *Anisolobus*. Since there are variations in the shape of the protomerite, measurements, number of sporoducts and geographical location of the host, it is given a separate specific status and designated as *Anisolobus indicus* sp. n.

A thorough search into the literature reveals that until now no record has been made of any gregarine species from *Coccinella septempunctata* in India (Haldar et al. 1985). It is, however, interesting to find that a number of species of *Gregarina* have earlier been reported in this host from various parts of the world (Watson 1915, Foerster 1938 a, b, Lipa 1967, Geus 1969). Obviously, a question arises

Table 3

Comparative characters of the type species and the present form of the genus *Anisolobus* Vincent

Characters	*Anisolobus dacnecola* Vincent	*Anisolobus indicus* sp. n.
(1) Epimerite	True epimerite absent	True epimerite absent
(2) Organ of attachment	Expanded, sucker-like in young forms, but irregular lobes are found at maturity	Sucker-like structure all throughout
(3) Sporadin	Large, intensely granular, nucleus often masked, associates at a very young stage	Large, solitary forms with elongated, cylindrical protomerite; 117.5 μm long; uniformly granular, nucleus spherical to elliptical
(4) Association	Early association, protomerite of primite varies from that of satellite by possessing its sucker-like structure, total length of association varies between 100–300 μm, and breadth 20–50 μm	Biassociative, protomerite of primite retains its sucker-like structure, while that of satellite possesses no such structure, but a cup-shaped, concavity in which the deutomerite of primite fits firmly
(5) Gametocyst	Ellipsoidal, surrounded by a thick transparent ectocyst, evacuates at early stage; measures 130–150 μm × 80–90 μm in dimension; envelop	Milky-white, more or less spherical, isogamous and measures 132.8–149.4 μm × 99.6–116.2 μm in dimension; no ectocyst
(6) Sporoduct	Six or eight sporoducts are developed which are about 40 to 50 μm in length	Two sporoducts appear at about 60 h each measuring 58.1 × 16.6 μm
(7) Spore	Barrel-shaped, measure 6 μm × 4 μm, extruded in long chains	Barrel shaped, released in long chains, 5.8 μm × 3.3 μm in average

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as to why *C. septempunctata* is free from any *Gregarina* species in India, where there is a good gregarine fauna in insects. In a number of recent papers, the senior author and his collaborators have discussed the role of climatic factors and food among other things to be reasons for gregarine infection in insects (Ghose et al. 1986 a, b, c, 1987). It may thus be tentatively inferred that climatic and other variations have induced changes in the structure of the gregarine, justifying its inclusion under the genus *Anisolobus*. In fact, the two genera, *Anisolobus* and *Gregarina*, differ very slightly in their morphological details. It is possible that the particular host has evaded the observations of earlier workers in India, although research on septate gregarines was initiated in the early twenties of this century in this country.

The comparison of characters of the type species and the present form are summarized in Table 3.

ACKNOWLEDGEMENTS

The senior author is thankful to the Indian National Science Academy, New Delhi and Council of Scientific and Industrial Research, New Delhi, for financial assistance.

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Note added in the proof. After the manuscript was sent for publication, we have come across with a reference in which Mandal et. al (1986) described *Gregarina dasguptai* sp. n. from *Coccinella septempunctata* of a tea garden in Darjeeling, India (Mandal, D., Rai, M., Pradhan, B., Gurung, D., Sharma, P. and Mandal, T. 1986, Arch. Protistenkd., 131; 299—302).

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ACTA PROTOZOOLOGICA
Vol. 27, No. 2, pp. 171–176 (1988)

On a New Myxosporean Parasite (*Myxozoa*), *Lomosporus indicus* gen. et sp. n. from the Freshwater Fish, *Labeo calbasu* (Hamilton)

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Received on 25 August 1987, *revised on* 23 November 1987

Synopsis. This paper describes *Lomosporus indicus* gen. et sp. n. from the gills and operculum of freshwater fish *Labeo calbasu*. The new genus is characterized by a single spherical polar capsule at the anterior end of the spore present at right angle to the longitudinal axis of the spore and opens on the lateral side. Spores broad and round anteriorly and narrow posteriorly. A new key to the genera of family *Myxobolidae* Thélohan, 1892 has been formulated.

While examining the freshwater fishes for the infection of myxozoan parasites we came across a new parasite *Lomosporus indicus* gen. et sp. n. Spherical, milky-white coloured cysts of this parasite were found in the operculum and gill arch of *Labeo calbasu* (Hamilton). Out of total of 54 *Labeo calbasu* dissected, five were found infected with this parasite.

Material and Methods

Gills, fins, scales and all the internal organs of fish were examined for myxosporean parasites. Fresh preparations in normal saline with or without Lugol's solution as intravitam stain were studied. Polar filaments of the spores were extruded by treating the spores with 5–10% KOH or saturated solution of urea. Air dried smears were fixed in acetone-free methyl alcohol and stained with Giemsa. Some of the smears were wet fixed in hot Schaudinn's fixative (50°C) and stained with Heidenhain's iron haematoxylin. All the measurements are in microns (μm) unless otherwise stated.

Observations

Lomosporus indicus gen. et sp. n.

(Fig. 1, 2, Pl. I 3–6)

Cyst: Spherical, milky-white in colour, present in the operculum (Fig. 1) and in gill arch (Fig. 2) of *Labeo calbasu*; 0.190–0.323×0.190–0.285 mm in size.

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Spore: Egg-shaped, with rounded anterior as well as posterior extremity; anterior end broader than posterior end (Fig. 1, Pl. I 5). Valves symmetrical; sutural line straight. Polar capsule one, spherical situated at the anterior end at right angle to the longitudinal axis of the spore, opening laterally near anterior end (Fig. 1, Pl. I 5) Polar filament very thick, extrudes laterally near anterior extremity (Fig. 2); single

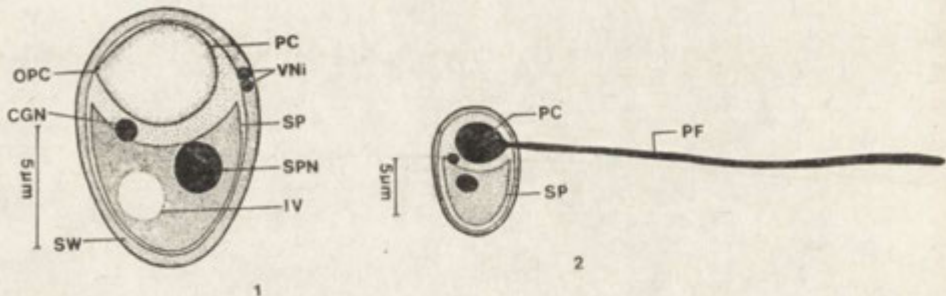

Fig. 1. *Lomosporus indicus* gen. et sp. n. A spore in valvular view (iron haematoxylin)

Fig. 2. *Lomosporus indicus* gen. et sp. n. A spore with polar filament extruded, note polar filament extrude laterally near anterior end (Giemsa)

rounded nucleus of capsulogenic cell present near polar capsule (Fig. 1, Pl. I 6). Sporoplasm occupies postcapsular space (Fig. 1, Pl. I 5) contains rounded, one (fused) or two sporoplasmic nuclei and a spherical iodinophilous vacuole at its centre (Fig. 1, Pl. I 6). Two valvular nuclei present near anterior end of the spore (Fig. 1, Pl. I 6).

Measurements:

Spore
length 10–11 (10.5, 0.5), breadth 6–6.5 (6.25, 0.25)
Polar capsule
length 4–4.5 (4.05, 0.15), breadth 4–4.5 (4.15, 0.229)
Polar filament 31–44
Iodinophilous vacuole (diameter) 1.6–1.8
(Figures within parentheses indicate mean and standard deviation of ten specimens)

Host: *Labeo calbasu* (Hamilton)
Site of infection: Gills and operculum
Locality: Harike and Ropar, Punjab, India.
Dates of collection: 11 September, 1985, 8 October 1985 and 18 February 1986
Holotype: On slide number 728 obtained from gills of *Labeo calbasu* collected at Harike (Punjab) on 18 February 1986, deposited in the Museum of Department of Zoology, Panjab University Chandigarh, India.
Paratype: On slide number 728 as well as in tubes number 39 and 40 obtained from gills and operculum of *L. calbasu* collected at Ropar and Harike.

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Discussion

In having the characters, viz. spores flattened parallel to the straight sutural line, in possessing one polar capsule and an iodinophilous vacuole in their spores, the specimens have been assigned to the family *Myxobolidae* Thélohan, 1892. There are eight genera, so far known, included under the family *Myxobolidae*. The genera are: *Myxobolus* Bütschli, 1882; *Henneguya* Thélohan, 1892; *Thelohanellus* Kudo, 1933; *Unicauda* Davis, 1944; *Neohenneguya* Tripathi, 1952; *Trigonosporous* Hoshina, 1952; *Phlogospora* Qadri, 1962 and *Dicauda* Hoffman and Walkar, 1978. Of these only two genera, *Phlogospora* and *Thelohanellus* possess a single polar capsule in their spores, like the present specimens, rest all of them possess two polar capsules in their spores. *Phlogospora* possesses bifurcated caudal process fitted to the posterior end of the spore. Only the genus *Thelohanellus* comes close to the present genus. But in *Thelohanellus* spores are pyriform, tear-shaped or elliptical in valvular view, being narrow anteriorly and broad in the middle or at the posterior end, whereas in the present genus spores are egg-shaped, broad anteriorly and narrow posteriorly. Moreover, in *Thelohanellus* polar capsule is placed medially, parallel to the longitudinal axis of the spore and opens at the anterior attenuated end but in the spores of the present genus, polar capsule is at right angle to the longitudinal axis of the spore and opens on the lateral side of the spore near broad anterior end.

From the foregoing discussion it is evident that the present genus is new to science and has been named *Lomosporus* in honour of Professor Jiři Lom, eminent Protozoologist from Institute of Parasitology, Czechoslovak Academy of Science, Prague, Czechoslovakia.

The type species, viz. *Lomosporus indicus* is named after the country, India, from where the parasite has been recovered.

Lomosporus gen. n.

Diagnosis: *Myxobolidae*: Spores egg-shaped in valvular view, broad and round anteriorly and narrow posteriorly. A single spherical polar capsule at the anterior end, present at right angle to the longitudinal axis of the spore and opens on the lateral side near anterior end of the spore. Histozoic in freshwater fish.

Type species: *Lomosporus indicus* sp. n.

Key to genera of family *Myxobolidae* Thélohan, 1892

1. Polar capsule one 2
   Polar capsules two 4

2 (1) Without any caudal process at the posterior end 3
   With an independent bifurcated caudal process fitted to the posterior end of the spore along with a distinct boundary . . . .
   . . . . *Phlogospora* Qadri, 1962

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3 (1) Spores pyriform, tear-shaped or ellipsoid in valvular view; polar capsule placed medially along the longitudinal axis of the spore and opens at the anterior attenuated end . . . . *Thelohanellus* Kudo, 1933
Spores egg-shaped in valvular view, broad and rounded anteriorly and narrow posteriorly; polar capsule at right angle to the longitudinal axis of the spore and opens on the lateral side of the spore near the anterior end . . . . *Lomosporus* gen. n.
4 (1) Spores with caudal processes 5
Spores without caudal processes . . . . *Myxobolus* Bütschli, 1882
5 (4) Caudal processes extensions of the shell valves, composed of the same material as the shell valves 6
Caudal processes are not extensions of the shell valves, composed of different material from that of the shell valves 8
6 (5) Caudal processes present only at the posterior side of the spore 7
Caudal processes present at both anterior and posterior end of the spore . . . . *Neohenneguya* Tripathi, 1952
7 (6) Spores triangular; caudal processes long, filamentous, arise from the lateral faces of the shell valve on the posterior side, each pair connected by a filament . . . . *Trigonosporous* Hoshina, 1952
Spores round, ellipsoid or spindle-shaped in valvular view; each valve continues as a caudal projection at the posterior pole of the spore both of which can be sometimes apposed for the part or whole of their length . . . . *Henneguya* Thélohan, 1892
8 (5) Single caudal appendage near posterior end . . . . *Unicauda* Davis, 1944
Two caudal appendages, extending in opposite directions, adhering to the shell valves along a distinct boundary on the lateral sides . . . . *Dicauda* Hoffman and Walker, 1978

ACKNOWLEDGEMENTS

We are thankful to the authorities of U.S.PL-480 for the financial assistance and Chairman of the Department of Zoology, Panjab University, Chandigarh for laboratory facilities.

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EXPLANATION OF PLATE I

Lomosporus indicus gen. et sp. n.

3: Operculum of *Labeo calbasu* showing cysts (arrows)
4: Gill of *Labeo calbasu* showing cysts (arrows)
5: Fresh, unstained spores in valvular view
6: Spores stained with iron haematoxylin showing sporoplasmic (a), capsulogenous (b) and valvular nuclei (c)

Key to lettering: CGN — Capsulogenous nucleus, IV — Iodinophilous vacuole, OPC — Opening of the polar capsule, PC — Polar capsule, PF — Polar filament, SP — Sporoplasm, SPN — Sporoplasmic nucleus, SW — Spore wall, VNI — Valvular nuclei

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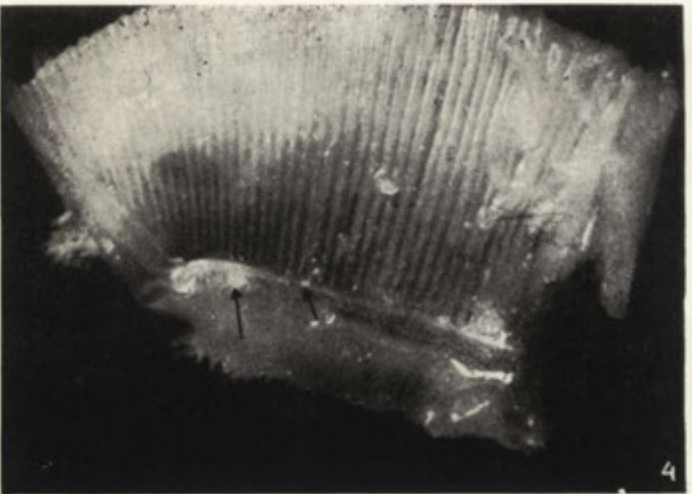

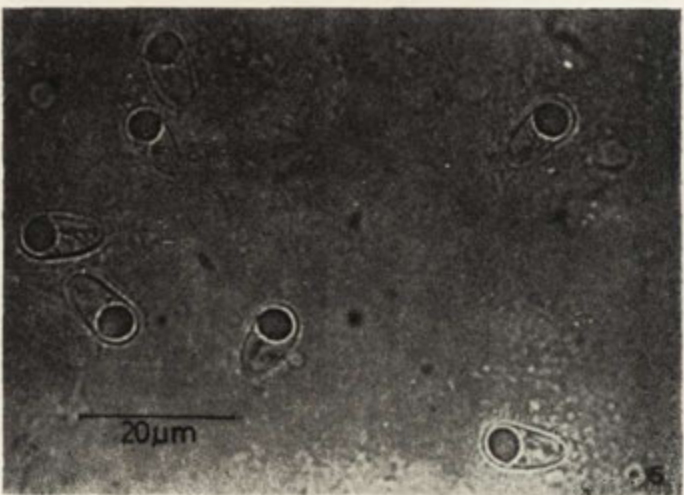

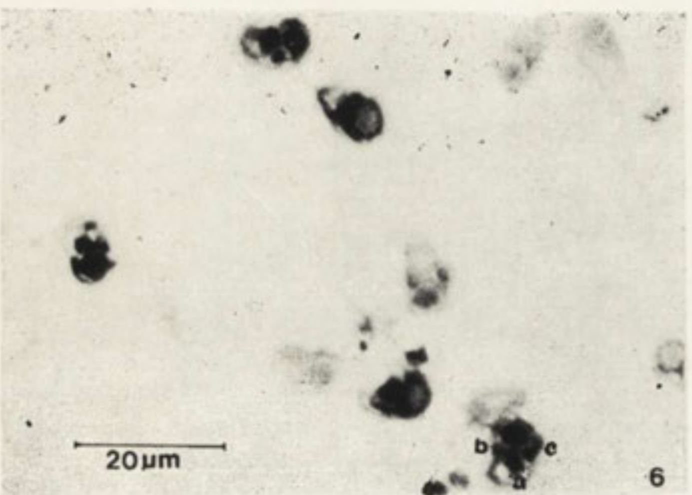

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